UNIVERZA V LJUBLJANI
VETERINARSKA FAKULTETA
Podiplomski študij biomedicine

Darja Pavlin

UČINKI ELEKTRIČNO POSREDOVANEGA VNOSA PLAZMIDNE DNA, KI NOSI ZAPIS ZA INTERLEVKIN-12, PRI ZDRAVLJENJU INDUCIRANIH TUMORJEV MIŠI IN SPONTANIH TUMORJEV PSOV

Doktorska disertacija

EFFECTS OF ELECTRICALLY-ASSISTED DELIVERY OF GENE ENCODING INTERLEUKIN-12 IN THE TREATMENT OF INDUCED MURINE TUMORS AND SPONTANEOUSLY OCCURING CANINE TUMORS

Ph. D. Thesis

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Darja Pavlin, dr. vet. med.

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Delo je bilo opravljeno na:
- Kliniki za kirurgijo in male živali Veterinarske fakultete Univerze v Ljubljani, predstojnik: prof. dr. Jože Kogovšek, dr. vet. med.
- Onkološkem inštitutu Ljubljana, Oddelku za eksperimentalno onkologijo, vodja: prof. dr. Gregor Serša, univ. dipl. biol.

Javni zagovor je bil opravljen v Ljubljani, dne _____

Mentorica: prof. dr. Nataša Tozon, dr. vet. med.
Somentorica: prof. dr. Azra Pogačnik, dr. vet. med

Člani strokovne komisije za oceno in zagovor:
Predsednik: prof. dr. Zlatko Pavlica, dr. vet. med.
Član: prof. dr. Gregor Serša, univ. dipl. biol.
Članica: prof. dr. Maja Čemažar, univ. dipl. biol.

KAZALO VSEBINE

KRATICE IN OKRAJŠAVE

ada-SCID kombinirana imunska pomanjkljivost zaradi pomanjkanja adenozin deaminaze (*ang*. severe combined immunodeficiency due to adenosine deaminase deficiency)

ALT alanin aminotransferaza

AP alkalna fosfataza

B16F1 celična linija mišjega melanoma

CK kreatin kinaza

DNA deoksiribonukleinska kislina (*ang*. deoxyribonucleic acid)

ECT elektrokemoterapija (*ang.* electrochemotherapy)

EGT elektrogenska terapija

GFP zeleni fluorescirajoči protein (*ang.* green fluorescent protein)

GHRH sproščevalni hormon rastnega hormona, somatoliberin (*ang.* growth hormone releasing hormone)

GM-CSF granulocitno makrofagne kolonije – spodbujajoči dejavnik (*ang. g*ranulocyte-macrophage colony-stimulating factor)

hIL-12 humani interlevkin-12

IFN interferon

IL interlevkin

LPB celična linija mišjega fibrosarkoma

MCT mastocitom (*ang.* mast cell tumor)

SA-1 celična linija mišjega fibrosarkoma

TNF dejavnik tumorske nekroze (*ang.* tumor necrosis factor)

TVT prenosljivi genitalni tumor (*ang.* transmissible veneral tumor)

IZVLEČEK

Ključne besede: Novotvorbe-zdravljenje; genska terapija; elektroporacija-metode; interlevkin-12-genetika; melanom-zdravljenje; fibrosarkom-zdravljenje; mastocitom-zdravljenje; plazmidi-genetika; miši; psi.

Izhodišča. Na predkliničnem nivoju se je izkazalo, da ima genska terapija s terapevtskim genom, ki kodira nastanek interlevkina-12 (IL-12), protitumorski učinek na različnih tumorskih modelih. Eden izmed možnih načinov vnosa terapevtskega gena v zdravljen organizem je tudi elektrogenska terapija (EGT). Izvedemo jo z direktnim vbrizganjem gole plazmidne DNA v tarčno tkivo, čemur sledi elektroporacija tkiva z ustreznimi električnimi pulzi, ki omogočijo prehod DNA skozi celično membrano in posledično izražanje kodiranega transgena.

Metode. V prvem delu raziskav smo določili najustreznejše parametre za učinkovit električno posredovani vnos plazmidne DNA v tumorje miši in skeletno mišičnino miši in psov. V ta namen smo uporabili dva reporterska plazmida, pCMV-Luc, ki nosi zapis za luciferazo, in pEGFP-N1, ki nosi zapis za zeleni fluorescirajoči protein (GFP, ang. *green fluorescent protein*). S pomočjo obeh reporterskih genov smo določili najoptimalnejši časovni razmik med intratumoralno aplikacijo reporterskega plazmida in aplikacijo električnih pulzov na induciranih mišjih tumorjih B16F1 melanomu in LPB fibrosarkomu. Pri optimizaciji protokola za transfekcijo skeletne mišičnine pa smo z uporabo plazmida pEGFP-N1 določili najprimernješo kombinacijo parametrov električnega polja, časovnega razmika med aplikacijo plazmida in odmerka plazmida za uspešno transfekcijo mišice *m. tibialis cranialis* pri miših.

Isti reporterski plazmid smo uporabili tudi za optimizacijo električno posredovanega vnosa DNA v skeletno mišičnino psov pri transfekciji mišice *m. semitendinosus*.

Najprej smo izvedli EGT s terapevtskim plazmidom, ki kodira nastanek IL-12. Pri miših smo uporabili plazmid pORF-mIL-12 za primerjavo protitumorske učinkovitosti intratumoralne in peritumoralne EGT pri fibrosarkomih SA-1 s pomočjo testa zaostanka rasti.

Pri psih s kožnimi mastocitomi smo ugotavljali učinkovitost in morebitne stranske učinke intratumoralne EGT s plazmidom pORF-hIL-12, ki kodira nastanek humanega IL-12.

Rezultati. Najučinkovitejšo transfekcijo smo dosegli, če smo elektroporacijo tumorskega tkiva pri miših izvedli 5–15 minut po vbrizganju reporterskega plazmida. Ugotovili smo, da je za električno posredovani vnos plazmidne DNA v mišičnino najoptimalnejša uporaba kombinacije enega visokonapetostnega (600 V/cm, 100 μs) in štirih nizkonapetostnih pulzov (4 x 80 V/cm, 100 ms, 1 Hz), apliciranih neposredno (manj kot 5 s) po intramuskularnem vbrizganju plazmidne DNA. Ta protokol se je izkazal za najučinkovitejšega pri transfekciji mišičnine pri miših in psih.

Ugotovili smo, da je EGT z *IL-12* zelo učinkovita metoda zdravljenja induciranih SA-1 fibrosarkomov pri miših, s katero dosežemo popolno ozdravitev preko 90 % tumorjev, zdravljenih z intratumoralno EGT in 16 % tumorjev, zdravljenih s peritumoralno EGT. Poleg tega imata oba načina aplikacije tudi protitumorski učinek na oddaljene, nezdravljene tumorske nodule. Dokazali smo, da intra- in peritumoralna EGT pri miših omogoči lokalno izražanje in sistemsko izločanje tako IL-12 kot interferona-γ (IFN-γ).

Intramuskularna EGT s humanim *IL-12* pri psih izzove sistemsko izločanje humanega IL-12 in proizvodnjo pasjega IFN-γ brez kakršnihkoli stranskih učinkov. Intratumoralna EGT pa omogoča lokalno izražanje in sistemsko izločanje IL-12, ki povzroči histološko zaznavne strukturne spremembe tumorskega tkiva z zmanjšanjem števila malignih celic in infiltracijo tkiva z vnetnimi celicami ter posledično zmanjšanjem velikosti tumorja.

Zaključki. Ugotovili smo, da je elektrogenska terapija z *IL-12* učinkovita in varna metoda zdravljenja tumorjev, ki izkazuje tako lokalne kot sistemske protitumorske učinke, tako na zdravljene kot oddaljene nezdravljene tumorje.

ABSTRACT

Key words: Neoplasms-therapy; gene therapy; electroporation-methods; interleukin-12-genetics; melanoma-therapy; fibrosarcoma-therapy; mast-cell sarcoma-therapy; plasmids-genetics; mice; dogs.

Background. The results of preclinical studies show that gene therapy with therapeutic gene encoding interleukin-12 (IL-12) displays pronounced antitumor effects in various tumor models. Electrogene therapy (EGT) is one of the possible delivery methods, suitable also for *in vivo* gene delivery. It employs direct injection of plasmid DNA containing the therapeutic gene into the target tissue, followed by local delivery of electric pulses, which enhance intracellular uptake and expression of the encoded transgene.

Methods. Optimal parameters for successful electrically-assisted DNA delivery into murine tumors and murine and canine skeletal muscle were first determined. Plasmids encoding two different reporter genes were used for this purpose: pCMV-Luc, encoding luciferase and pEGFP-N1, encoding green fluorescent protein. Using these two reporter genes, the optimal time lag between intratumoral application of the plasmid and local delivery of the electric pulses was determined in murine B16F1 melanoma and LPB fibrosarcoma. Optimization of an electroporation protocol for efficient transfection of skeletal muscle in mice was performed using pEGFP-N1, determining optimal electric pulses parameters, the time lag between plasmid injection and delivery of electric pulses and the dose of plasmid on *m. tibialis cranialis* muscle. The same plasmid was also used for optimization of the electroporation protocol for efficient transfection of canine muscle *m. semitendinosus*. In dogs, five different electroporation protocols were compared.

Using the parameters determined in the first part of the work, EGT with plasmid encoding IL-12 was performed on mice with induced tumors and dogs with spontaneously occurring tumors. In mice, plasmid pORF-mIL-12 was used for comparison of the antitumor effect of intratumoral and peritumoral EGT on SA-1 fibrosarcoma. In dogs, a clinical study was performed in order to determine the efficiency and possible side effects of intratumoral EGT with plasmid pORF-hIL-12 in patients with cutaneous mast cell tumors.

Results. The highest transfection efficiency was achieved when electroporation of tumor nodules was performed 5 to 15 minutes after intratumoral injection of plasmid DNA. Studying the effect of different parameters on transfection efficiency in skeletal muscle, it was determined that the highest transfection was achieved using a combination of one high-voltage pulse (600 V/cm, 100 μs) and four

low-voltage pulses (4 x 80 V/cm, 100 ms, 1 Hz), delivered immediately (less than 5 s) after intramuscular injection of plasmid DNA.

The study demonstrated that EGT with *IL-12*, applied either intratumorally or peritumorally, is an effective therapeutic approach, with local as well as systemic effects in the treatment of SA-1 fibrosarcoma. It resulted in a significant percentage of tumor curability (over 90% after intratumoral and 16% after peritumoral delivery), the induction of long-term antitumor immunity and even elicited a systemic antitumor effect, demonstrated by delayed growth of untreated tumors growing at a distant site. The antitumor effectiveness of *IL-12* EGT arises from high intratumoral and systemic secretion of biologically active IL-12, which induces the production of IFN-γ, without noticeable side effects.

Intramuscular EGT with *IL-12* in dogs resulted in the systemic release of hIL-12 and the production of IFN-γ, without any side effects. Intratumoral EGT with *IL-12* elicited good local antitumor effects in treated animals, with systemic transgene release without any noticeable side effects. The local antitumor effects of this therapy were demonstrated by a significant reduction in tumor size (mean 52% of the initial value) and a change in the histological structure, with a reduction in the number of malignant mast cells, coupled with inflammatory cell infiltration of treated tumors. Systemic effects included the systemic release of IL-12 and the induction of the patient's IFN-γ response.

Conclusions. The study demonstrated that EGT with *IL-12* is an efficient and safe method for the treatment of tumors, eliciting both local and systemic antitumor effects.

1 UVOD

1.1 IZHODIŠČA RAZISKAVE

V zadnjih nekaj desetletjih je intenzivno raziskovalno delo na področju molekularne biologije močno okrepilo razumevanje molekularnih osnov karcinogeneze, kar je vodilo v bistveno izboljšanje zdravljenja rakavih obolenj. Kljub velikemu napredku pa nekatere vrste raka še vedno ostajajo slabo odzivne na splošno uveljavljene metode zdravljenja. Zaradi tega je velik delež raziskovalnega dela na področju onkologije usmerjen v odkrivanje novih načinov zdravljenja raka.

Ena od novih oblik zdravljenja je gensko zdravljenje, ki obljublja možnost učinkovitega in visoko specifičnega zdravljenja rakavih obolenj. Dosedanje predklinične in klinične študije na področju genske terapije raka in tumorske imunoterapije kažejo, da lahko genska terapija samostojno ali v povezavi s klasičnimi metodami zdravljenja rakavih obolenj bistveno izboljša uspeh zdravljenja onkoloških bolnikov. Izraz *genska terapija* označuje vsak klinično terapevtski poseg, pri katerem se rekombinantni gen vstavi v človeške ali živalske somatske celice z namenom, da bi nadomestili določeno beljakovino v organizmu ali vplivali na patološki proces oziroma bolezen (1). Končni cilj takega zdravljenja je doseči ozdravitev ali izboljšati klinično stanje bolnika. Genska terapija obljublja veliko in zaradi širokih možnosti uporabe poskušajo raziskovalci najti čim bolj učinkovit način vnosa terapevtskih genov v različna tkiva, med drugim različne epitelije in endotelije, živčno tkivo, organe, kot so jetra, srce, ledvice, vezi in možgani in različne tumorje (2).

Pri genski terapiji sta se razvila dva pristopa k zdravljenju: *ex vivo* genska terapija, ki predstavlja izolacijo in gensko preoblikovanje bolnikovih specifičnih celic, ki jih po obdelavi zopet vrnemo v organizem in *in vivo* pristop, ki pomeni neposredni vnos genov v tkivo bolnika s pomočjo določenih vektorjev (1).

Postopek genske terapije na ljudeh je bil prvič izveden leta 1990, in sicer za zdravljenje genetsko pogojene bolezni ada-SCID (kombinirana imunska pomanjkljivost zaradi pomanjkanja adenozin deaminaze, *ang*. severe combined immunodeficiency due to adenosine deaminase deficiency) (3). Leto kasneje pa je bila izvedena prva klinična študija z uporabo genske terapije pri zdravljenju raka (melanoma) (4). Do sedaj je bilo na ljudeh izvedeno že preko 1400 kliničnih študij z različnimi indikacijami, večina od njih (65 %) pri zdravljenju raka (5).

Glavna prepreka, ki zaenkrat onemogoča širšo uporabo genske terapije v klinični praksi, je še vedno relativna neučinkovitost vnosa genskega materiala v celice. Protokol, ki je primeren za gensko terapijo *in vivo* mora biti namreč učinkovit, varen in ponovljiv. Idealni vnosni sistemi bi tako morali zagotavljati stabilno in dolgotrajno izražanje vnešenih genov v ciljnem tkivu, ki bi ga bilo mogoče uravnavati, hkrati pa terapija ne bi smela imeti stranskih učinkov na organizem, kot so na primer vpliv na genom gostitelja, toksičnost za organizem ali kancerogena transformacija celic (1, 6). Hkrati pa je zaželeno, da bi bila proizvodnja takega vektorja mogoča v velikih količinah, lahko izvedljiva in poceni. Zaradi tega je velik del raziskav na tem področju trenutno usmerjen v razvoj učinkovitih in varnih vektorjev in tehnik za vnos genov v tkivo *in vivo*.

Vektorske sisteme za vnos genskega materiala v celice *in vivo* lahko razdelimo v dve glavni skupini: virusne vektorje za gensko transdukcijo in nevirusne tehnike za gensko transfekcijo. Vsaka od tehnik ima svoje prednosti in pomanjkljivosti. Idealnega sistema, ki bi bil hkrati učinkovit in varen za pacienta in ki bi omogočal dolgotrajno in stabilno izražanje genov, pa zaenkrat še niso odkrili.

Pri raziskavah učinkovitosti genske terapije raka rekombinantni virusi še vedno predstavljajo večino genskih vnosnih sistemov. Njihova glavna prednost je večja učinkovitost transdukcije v primerjavi z nevirusnimi metodami. Virusi, ki se najpogosteje uporabljajo pri genski terapiji, so pridobljeni z gensko modifikacijo predvsem retro- in adenovirusov, poleg teh pa se lahko uporabljajo tudi prilagojeni herpes-, pox-, parvo- in bacculovirusi. Vendar pa ima genska terapija, ki temelji na virusnem načinu vnosa, tudi negativne lastnosti, zaradi katerih je lahko klinična uporabnost takega vnosa precej vprašljiva. To sta zlasti spodbujanje pacientovega imunskega sistema, ki lahko bistveno zmanjša učinkovitost ponavljajočih se aplikacij, in pa sistemska toksičnost določenih virusnih vektorjev (7, 8).

Zato so se kot alternativa virusnemu načinu vnosa DNA razvile številne nevirusne metode, ki za razliko od virusnih tehnik zaenkrat omogočajo manj učinkovito transfekcijo celic, vendar pa so njihove glavne prednosti v tem, da niso patogene in imunogene, lahko se uporabljajo v več zaporednih aplikacijah in imajo bistveno večjo kapaciteto za vključitev tuje DNA, priprava potrebnih količin vektorjev pa je enostavnejša (9). Nevirusne tehnike transfekcije lahko razdelimo v dve glavni skupini: vnos gole (plazmidne) DNA s pomočjo fizikalnih metod (elektroporacija, biobalistični način, vnos s pomočjo ultrazvoka) in kemične metode (polipleksi (kompleksi kationskih polimerov in DNA), lipopleksi (kompleksi liposomov in DNA) in transdukcijski proteini).

Eden najpreprostejših in najvarnejših nevirusnih načinov vnosa je neposredna sistemska (v krvni obtok) ali lokalna (npr. v tumor ali mišico) aplikacija gole plazmidne DNA brez uporabe kakršnekoli druge prenašalne molekule (9, 10). Izražanje vnešenih genov je pri tej tehniki sicer zelo nizko, vendar še vedno dovolj dobro za uporabo pri genskih vakcinah (1). Slaba uspešnost transfekcije tkiva po sistemski aplikaciji je posledica hitre razgradnje z nukleazami in odstranitve s fagocitozo s strani makrofagov (9). Zaradi slabe učinkovitosti neposrednega vnosa so raziskovalci začeli iskati nove metode, ki bi predvsem izboljšale nivo transfekcije. Mednje spadajo elektroporacija, biobalistika, uporaba ultrazvoka in hidrodinamična aplikacija.

1.2 ELEKTRIČNO POSREDOVANI VNOS DNA V TKIVA

Elektroporacija ali *elektropermeabilizacija* je metoda, pri kateri z uporabo kontroliranih električnih pulzov, ki sami po sebi nimajo negativnega vpliva na preživetje celic, dosežemo prehodno prepustnost celične membrane (11). Elektroporacijo celice dosežemo, ko vsiljeni transmembranski potencial, ki nastane pod vplivom zunanjega električnega polja, doseže kritično vrednost in preseže določen prag, kar vodi v zvečano prepustnost celične membrane. Zaradi tega lahko v celično notranjost vstopijo molekule, za katere je celična membrana sicer neprepustna. Na ta način lahko v celice vnesemo različne molekule, kot so ioni, barve, radioizotopi, različna zdravila, RNA, DNA, oligonukleotidi, peptidi in proteini. Ta strukturna sprememba celice je lahko le začasna in reverzibilna, če uporabimo natančno določene električne pulze, lahko pa povzroči uničenje celice, če vsiljeni transmembranski potencial preide preko t. i. ireverzibilne pragovne vrednosti (12).

Eno od pomembnejših področij uporabe elektroporacije, ki se že uporablja v humani in veterinarski klinični praksi, je elektrokemoterapija (13, 14, 15, 16, 17, 18, 19, 20, 21). Ta nova metoda zdravljenja onkoloških pacientov predstavlja kombinacijo intratumorske ali sistemske kemoterapije in lokalne aplikacije električnih pulzov na tumor. Na ta način zvečamo učinkovitost določenih kemoterapevtikov, ki le stežka ali pa sploh ne vstopajo v celico. Zdravili, ki sta se do sedaj izkazali primerni za uporabo pri postopku elektrokemoterapije, sta cisplatin in bleomicin, saj jima v kombinacji z elektroporacijo zvečamo protitumorsko učinkovitost v redu velikosti od nekaj desetkrat do nekaj tisočkrat (22, 23). Učinkovitost elektrokemoterapije je bila potrjena pri zdravljenju različnih vrst kožnih in podkožnih tumorjev in je že vpeljana v klinično prakso kot ena od standardnih metod zdravljenja novotvorb tudi na Onkološkem inštitutu Ljubljana in Kliniki za kirurgijo in male živali Veterinarske fakultete Univerze v Ljubljani.

Drugo široko področje uporabe elektroporacije je vnos genskega materiala v celico, za kar se uporablja izraz *električno posredovani vnos DNA* ali *genska elektrotransfekcija* oziroma *elektrogenska terapija (EGT)*, kadar s pomočjo elektroporacije v tkivo vnašamo katerega od terapevtskih genov (6, 24, 25, 26). S to metodo lahko vnesemo DNA v vsako prokariontsko in evkariontsko celico. V *in vitro* pogojih se ta metoda uporablja že od zgodnjih 80. letih prejšnjega stoletja (11). V začetku 90. let prejšnjega stoletja pa se je začela uporabljati tudi *in vivo* in od takrat so uspeli vnesti tujo DNA v številna različna tkiva (27).

EGT je kombinacija aplikacije gole plazmidne DNA v tarčno tkivo, ki ji sledi elektroporacija tarčnega tkiva z ustreznimi električnimi pulzi, ki omogočijo prehod DNA skozi celično membrano. Poskusi z električno posredovano transfekcijo različnih tkiv z uporabo različnih reporterskih in terapevtskih genov, ki so bili do sedaj opravljeni na poskusnih živalih, so dokazali, da je ta metoda *in vivo* preprosto izvedljiva, ni imunogena, toksična in ne povzroča insercijske mutagenosti ter omogoča primerno izražanje vnešenega gena, s čimer dosežemo terapevtski učinek (6, 10, 28).

Učinkovitost električno posredovane transfekcije *in vivo* je odvisna od fizioloških lastnosti tkiv. Poleg tega na učinkovitost elektroporacije vplivajo še drugi fiziološki mehanizmi, kot na primer prekrvitev tkiva in delovanje imunskega sistema. Glede na to, da je končni namen transfekcije s katerokoli metodo učinkovita transkripcija in translacija vnešenega gena, ki vodi v izražanje tuje DNA v celici, mora celica, v katero vnašamo DNA, ostati po aplikaciji električnega polja čim bolj zdrava in sposobna preživetja (29). Torej sam postopek ne sme nepovratno poškodovati struktur celice, da bodo procesi, potrebni za začetek izražanja vnešenega gena, v njej lahko stekli. Zato je za uspešno transfekcijo posamezne vrste tkiva potrebno upoštevati ustrezne električne pulze (njihovo število, amplituda, frekvenca, čas trajanja) za določeno vrsto tkiva, uporabiti ustrezne oblike elektrod, interval med aplikacijo plazmidne DNA v tkivo in električnimi pulzi, koncentracijo in odmerek plazmidne DNA, itd. Vsem protokolom elektrogenske terapije je skupno, da morajo biti molekule DNA prisotne v tkivu pred oziroma med aplikacijo električnih pulzov, kar se razlikuje od principov za vnos manjših molekul v celico s pomočjo elektroporacije, kjer lahko molekule apliciramo v tkivo tudi določen čas po aplikaciji električnih pulzov (26, 30, 31).

Ugotovljeno je bilo, da se DNA med aplikacijo električnega pulza sprva usidra na celično membrano, sam proces prehoda molekul v citoplazmo pa poteka šele v minuti po koncu aplikacije električnega polja (32). V prvi fazi nastanka elektropor po aplikaciji električnega polja naj bi se zaradi interakcije med DNA in lipidnimi molekulami celične membrane ustvarilo veliko število majhnih por (premera manj kot 2 nm), ki pa so premajhne za vstop DNA v celico (31, 32). Te pore naj bi predstavljale

podlago za nastanek večjih por, premera približno 100 nm, ki se tvorijo v drugi fazi procesa (32, 33). Šele te pore naj bi omogočile negativno nabitim molekulam DNA vstop v celico s pomočjo elektroforeze. Domneva se, da igra DNA ob vstopu v celice med procesom elektroporacije aktivno vlogo pri nastanku por v celični membrani (31, 32).

Sprva se je električno posredovani vnos DNA v tkiva izvajal s protokolom elektroporacije, ki je bil enak protokolu za elektrokemoterapijo, torej z aplikacijo večjega števila kratkotrajnih pulzov (dolžina pulza 100 μs) z visoko amplitudo (1000–1500 V/cm). Kmalu se je izkazalo, da ta protokol ne daje optimalnih rezultatov (26). Bistveno bolj učinkovito transfekcijo dosežemo z uporabo dolgotrajnih električnih pulzov (reda velikosti milisekund) z nizko amplitudo (reda velikosti nekaj 100 V/cm), ki imajo bistveno boljši elektroforetski učinek na molekule DNA v primerjavi s kratkotrajnimi in intenzivnimi električnimi pulzi, ki se uporabljajo pri elektrokemoterapiji (26). Kot najuspešnejšo metodo pa se v zadnjem času opisuje kombinacijo kratkotrajnega visokonapetostnega pulza, katerega cilj je zvečanje prepustnosti membrane tarčnih celic (odprtje elektropor), ki mu v nekaj sekundah sledijo dalj časa trajajoči nizkonapetostni pulzi, ki dovoljujejo elektroforetični prehod plazmida oziroma gole DNA v notranjost celice preko destabilizirane celične membrane (34, 35).

S pomočjo elektroporacije so do sedaj uspeli vnesti plazmidno DNA v številne organe in tkiva: v kožo, jetra, pljuča, roženico, možgane, žile, sečni mehur, vezi, hrustanec, mišičnino in tumorje (2, 36, 37). Med njimi se zdijo za klinično uporabo pri zdravljenju tumorjev najprimernješi skeletna mišičnina, koža in tumorji.

1.2.1 Električno posredovani vnos DNA v skeletno mišičnino

Leta 1990 je bilo objavljeno prvo znanstveno poročilo o uspešnem vnosu genskega materiala v skeletno mišičnino (38). V zadnjih tridesetih letih se je tako nakopičila velika količina dokazov, da je skeletna mišičnina primeren del telesa za električno posredovani vnos DNA, še posebej, kadar želimo doseči dolgotrajno izražanje vnešenega gena. Skeletna mišičnina je namreč obsežno, dobro dostopno in dobro ožiljeno tkivo z visoko sposobnostjo regeneracije; za mišične celice pa je značilno, da so dolgožive in imajo visoko zmožnost tvorbe različnih proizvodov vnešenih genov (39, 40).

Vnos genskega materiala v mišice se lahko uporablja za lokalno zdravljenje različnih obolenj mišic (npr. Duchennova mišična distrofija) (41), za vakcinacijo (36) in sistemsko izločanje različnih terapevtskih beljakovin, kot na primer eritropoetina, α1-antitripsina, faktorjev strjevanja krvi, citokinov, monoklonalnih protiteles itd (42, 43). *In vivo* raziskave so potrdile, da vnos genskega materiala v mišične celice dejansko lahko povzroči sistemsko izločanje tolikšnih količin terapevtskih

beljakovin, da z njimi dosežemo terapevtski učinek pri živalskih modelih različnih bolezni, na primer diabetesa mellitusa (44) ali hiperholesterolemije (45). Glavni slabosti neposredne aplikacije gole DNA v mišično tkivo, ki omejujeta njeno širšo uporabo za učinkovito gensko terapijo, sta velika individualna variabilnost v izražanju vnešenega gena in nizka stopnja transfekcije, še posebej v primerjavi z virusnimi načini vnosa (26).

Ena od možnih metod za izboljšanje učinkovitosti transfekcije mišičnine je uporaba elektroporacije. Ugotovljeno je bilo, da lahko električno posredovani vnos DNA v mišice bistveno, tudi do 2000-krat, izboljša izražanje in hkrati zmanjša variabilnost izražanja vnešenih genov v primerjavi z neposrednim vnosom gole DNA brez uporabe električnih pulzov (24, 26, 46). Med različnimi tkivi, v katere je bila do sedaj na ta način vnešena DNA, je bila najboljša transfekcija dosežena ravno v mišičnem tkivu. Posebej pomembno pa je dejstvo, da je zaradi dolge življenjske dobe mišičnih celic, ki se ne delijo in zaradi tega ne pride do izgubljanja plazmidne DNA, možno doseči dolgotrajno izražanje vnešenega gena, ki traja tudi do enega leta (26).

Učinkovitost transfekcije tkiva in s tem uspešnost genske terapije je odvisna od številnih fizikalnih in bioloških dejavnikov. Pri elektrogenski terapiji sodijo mednje predvsem parametri električnih pulzov, lastnosti plazmida (njegova velikost, sestava, koncentracija, volumen in odmerek) ter lastnosti tarčnega tkiva (na kar vpliva starost in spol živali, sestava medceličnine) (2, 47, 48). Zato je za učinkovito elektrogensko terapijo nujna optimizacija protokola elektroporacije. Kljub obširnemu delu na tem področju pa še vedno ni doseženo soglasje glede tega, kakšen naj bi bil optimalen protokol za transfekcijo skeletne mišičnine s pomočjo električno posredovanega vnosa DNA.

Prvi poskusi optimizacije učinkovitosti transfekcije mišičnega tkiva z električno posredovanim vnosom DNA so bili usmerjeni predvsem v optimizacijo parametrov električnih pulzov, to je njihove oblike, frekvence, amplitude, dolžine trajanja in uporabljenega števila pulzov. Na podlagi rezultatov teh raziskav sta se razvila dva različna protokola elektroporacije.

Sprva so se za transfekcijo skeletne mišičnine uporabljali le nizkonapetostni pulzi amplitude 100-200 V/cm in trajanja 20-50 ms (24, 26). Mir s sodelavci je ugotovil, da z uporabo osmih pulzov dolžine po 20 ms in amplitude 200 V/cm s frekvenco 1 Hz doseže značilno zvečanje ekspresije luciferaze v primerjavi z intramuskularno aplikacijo gole DNA brez uporabe električnih pulzov (26). Novejše raziskave pa so potrdile, da je za transfekcijo skeletne mišičnine kombinacija visoko- in nizkonapetostnih pulzov bistveno učinkovitejša (omogoča boljše izražanje vnešenih genov) in varnejša (povzroči manjšo okvaro tarčnih celic zaradi uporabe manjše električne energije) v primerjavi z

drugimi protokoli. Najučinkovitejša je kombinacija enega visokonapetostnega pulza z amplitudo 600-800 V/cm in dolžino trajanja 100 μs, ki mu sledi eden ali več nizkonapetostnih pulzov z amplitudo 80-100 V/cm, ki trajajo v redu velikosti nekaj deset do nekaj sto ms (34, 35, 49, 50, 51).

Učinkovitost električno posredovanega vnosa DNA v skeletno mišičnino je bila do sedaj potrjena na različnih poskusnih živalih, miših, podganah in kuncih (26), govedu (52, 53), drobnici (52, 54), prašičih (55, 56, 57), opicah (26, 58), ribah (59) in psih (60, 61, 62, 63).

Ena izmed omenjenih raziskav (61) je posebej pomembna, ker dokazuje, da tudi pri psih z različnimi novotvorbami, ki imajo zaradi bolezni lahko močno oslabel imunski sistem, pride do zadostnega izražanja terapevtske učinkovitosti vnešenega plazmida. V vseh raziskavah, izvedenih na psih, so za električno posredovani vnos plazmidne DNA v skeletno mišičnino uporabili le nizkonapetostne pulze. Za gensko elektrotransfekcijo plazmida, ki nosi zapis za GHRH (*growth hormone releasing hormone*, somatoliberin) so uporabili dva podobna protokola: 6 električnih pulzov po 60 ms, 100 V/cm in 5 električnih pulzov po 52 ms, 100 V/cm (61, 62). Za gensko elektrotransfekcijo plazmida, ki nosi zapis za humani koagulacijski faktor IX, pa so uporabili protokol s 6 električnimi pulzi trajanja 60 ms in amplitudo 200 V/cm (60).

1.2.2 Električno posredovani vnos DNA v tumorje

Električno posredovani vnos terapevtskih genov v tumorje omogoča intratumoralno proizvodnjo visokih koncentracij produktov vnešenih genov, s čimer omogočimo zadostno lokalno terapevtsko koncentracijo brez sistemskega vnosa visokih odmerkov terapevtskih genov ali beljakovin. To je predvsem pomembno za citokine, pri katerih so visoke sistemske koncentracije lahko močno toksične (64). EGT z neposrednim intratumoralnim vnosom DNA se lahko uporablja kot samostojno zdravljenje ali pa v povezavi z drugimi načini zdravljenja tumorjev, na primer z elektrokemoterapijo.

Prva publikacija, v kateri je opisan električno posredovani vnos DNA v tumorske celice *in vivo*, objavljena leta 1998, je določila parametre elektroporacije, ki bi bili lahko uporabljeni pri vnašanju proteina ali plazmidne DNA v tumorje malignega melanoma pri miših (25). Prva študija, ki je potrdila učinkovitost elektrogenske terapije za zdravljenje tumorjev, je bila prav tako izvedena na mišjem modelu malignega melanoma leta 1999 (65).

Do sedaj so s pomočjo elektroporacije vnesli različne terapevtske gene v številne eksperimentalno povzročene tumorje, poleg malignega melanoma tudi v različne karcinome (ploščatocelični, hepatocelularni, ledvični, kolorektalni, in karcinom prostate, pankreasa, mlečne žleze), različne

sarkome in limfom (50). Terapevtski geni, ki so se izkazali za učinkovite, so predvsem geni, ki nosijo zapis za nastanek citokinov (npr. interlevkin (IL)-2, IL-12, IL-18, interferon (IFN)-α), poleg njih pa tudi drugi geni, na primer gen, ki kodira izražanje timidin kinaze herpes simplex virusa, gen, ki kodira nastanek proteina p53, antiangiogeni geni in geni, ki izzovejo apoptozo celic (50).

Nivo transfekcije, ki ga dosežemo pri električno posredovanem vnosu DNA neposredno v tumorje, je nižji v primerjavi z drugimi tarčnimi tkivi (6, 25, 66). Kljub temu pa na ta način lahko dosežemo dobre protitumorske učinke neposredno na zdravljenem tumorju, kar se kaže z zaustavitvijo rasti, delno ali popolno regresijo nodula. Poleg tega pa lahko z intratumoralno EGT zavremo angiogenezo in s tem vplivamo na metastaziranje (67, 68) ali pa celo zagotovimo dolgotrajno protitumorsko imunost pri zdravljeni živali (69, 70, 71).

Pri električno posredovanemu vnosu DNA v tumorje so v literaturi trenutno opisani trije sklopi protokolov za uspešno elektrotransfekcijo. Nekatere od raziskovalnih skupin uporabljajo aplikacijo t. i. pulzov za elektrokemoterapijo, torej kratkotrajnih (100 μ) visokonapetostnih pulzov (1000-1500 V/cm) (69, 70, 71). Drugi sklop zajema uporabo nekaj ms do nekaj deset ms dolgih pulzov z amplitudo nekaj sto V/cm, ki se je izkazal za primernejšega (6, 25, 66, 72). V ta sklop spada "klasični" protokol za EGT tumorjev z aplikacijo osmih pulzov dolžine 5 ms, amplitude 600 V/cm in frekvenco 1 Hz. V zadnjem času pa se tudi pri tumorjih raziskuje učinkovitost kombinacije enega visokonapetostnega pulza v povezavi z več nizkonapetostnimi pulzi, podobno kot pri elektrotransfekciji skeletne mišičnine (72).

Leta 2008 je Cutrera s sodelavci (73) objavil študijo o učinkih elektrokemo-genske terapije (istočasna aplikacija kemoterapevtika in terapevtskega plazmida) pri zdravljenju tumorjev vratu in glave neznane histološke klasifikacije pri psih. Kot kemoterapevtik je uporabil bleomicin, injiciran intravensko, za gensko terapijo pa plazmid, ki kodira IL-12, v kombinaciji z elektroporacijo. Ugotovil je protitumorski vpliv te vrste zdravljenja na primarne tumorje in kostne metastaze. Druga objava (74) opisuje vpliv intratumoralne EGT s plazmidoma, ki kodirata IL-6 in IL-15, na TVT pri psih (*ang.* transmissible veneral tumor, prenosljivi genitalni tumor). Odgovor tumorjev na terapijo je zajemal močno zvečanje izražanja antigena poglavitnega histokompatibilnostnega kompleksa na tumorskih celicah in masovno infiltracijo tumorskega tkiva s CD8+ T-limfociti ter regresijo zdravljenih nodulov. Na istem tumorskem modelu je bila izvedena tudi raziskava, v kateri so z EGT s humanim *IL-12* dosegli lokalno regresijo zdravljenih tumorjev ter sistemske protitumorske učinke na oddaljene nezdravljene tumorje s sistemsko zaznavnim izločanjem humanega IL-12 (75).

1.2.3 Električno posredovani vnos DNA v kožo

Z električno posredovanim vnosom DNA v kožo lahko dosežemo kombinacijo tako lokalne intradermalne proizvodnje transgenov kot sistemsko izločanje kodiranega transgena v krvni obtok (76, 77). Prednost uporabe kože kot tarčnega tkiva za imunogensko zdravljenje je njena dobra dostopnost, ki omogoča hkratno transfekcijo velike količine tkiva in prisotnost velikega števila imunokompetentnih celic, ki so odločilne za razvoj imunskega odgovora organizma po vnosu terapevtskega gena (77, 78, 79). Ena od omejitev uporabe kože kot tarčnega tkiva za gensko zdravljenje je kratka življenjska doba kožnih celic, ki onemogoča dolgotrajnejše izražanje transgenov (42, 80). Zato je primernejša za indikacije, kjer dolgotrajno izražanje ni potrebno oz. zaželeno, na primer za vakcinacijo (56).

Terapevtski gen, ki kodira IL-12, je že bil uspešno vnešen v kožo, pri čemer je prišlo do sistemskega izločanja tako IL-12 kot indukcije interferonskega odgovora (81).

1.3 PROTITUMORSKA UČINKOVITOST EGT Z *IL-12*

IL-12 je proinflamatorni citokin velikosti 70 kDa, ki ga sestavljata dve podenoti, p35 in p40 (82, 83). V organizmu ga proizvajajo antigen-predstavitvene celice, to so monociti, makrofagi, dendritične celice, eozinofilci, mastociti vezivnega tkiva in B-celice (84, 85). Ima enega najmočnejših protitumorskih učinkov med številnimi citokini, protitumorski učinek izkazujejo namreč tudi npr. IL-2, IL-4, IL-7, IL-8, TNF-α, GM-CSF (86). Učinki IL-12, ki omogočajo protitumorsko delovanje, izhajajo iz njegovih različnih vplivov na imunski odziv organizma ter angiogenezo. Te učinke lahko razdelimo v tri sklope: sprožitev proizvodnje IFN-γ s strani celic ubijalk in T-limfocitov, kar povratno spodbuja proizvodnjo IL-12 (87, 88), antiangiogeni učinki (89) in regulacija imunskega odziva s pomočjo tvorbe Th1 in citotoksičnih limfocitov (90).

Zaradi teh lastnosti se je IL-12 izkazal kot učinkovit citokin pri imunski terapiji določenih rakavih obolenj. Prvi poskusi z uporabo IL-12 pri zdravljenju raka so bili usmerjeni v uporabo rekombinantnega proteina IL-12 (89, 91, 92). Tak način aplikacije je v vsakdanji klinični praksi precej nepraktičen, v prvi vrsti zaradi kratke razpolovne dobe, zaradi katere so za zagotovitev ustrezne sistemske koncentracije in dosego kliničnega učinka potrebne pogoste ponavljajoče aplikacije IL-12. Tako visoki odmerki pa so se izkazali za potencialno toksične tako pri poskusnih živalih na predkliničnem nivoju (93, 94, 95) kot pri prvih kliničnih študijah na ljudeh (96, 97). Stranski učinki zajemajo povišano telesno temperaturo, glavobol, slabost in bruhanje, nevtropenijo, limfopenijo,

anemijo in trombocitopenijo (98). V humanih kliničnih študijah faze II je prišlo po zdravljenju s rekombinantnim proteinom IL-12 celo do smrtnih primerov (96). Predpostavlja se, da so ti stranski učinki posledica toksičnega delovanja IFN-γ, ki se tvori pod vplivom IL-12 (99, 100). Zaradi tega se je raziskovanje usmerilo v iskanje novih strategij vnosa IL-12 v organizem, s katerimi bi izboljšali klinično učinkovitost in zmanjšali pojavnost stranskih učinkov. Ena izmed možnih alternativ je tako vnos terapevtskega gena, ki kodira nastanek IL-12, namesto uporabe rekombinantnega proteina. *In vivo* so do sedaj uporabili različne virusne in nevirusne metode vnosa terapevtskega gena za IL-12. Na podlagi pozitivnih rezultatov predkliničnih raziskav je genska terapija z *IL-12* že prešla tudi v nekaj kliničnih študij tako v humani kot veterinarski medicini (101, 102, 103, 104, 105, 106, 107).

V veterinarski medicini so do sedaj objavljeni rezultati treh kliničnih študij genske terapije z *IL-12*, bodisi z uporabo virusnega vektorja (106) ali pa z nevirusnim načinom vnosa (75, 104). Virusni vnos terapevtskega gena za IL-12 so uporabili v klinični študiji, ki je bila izvedena na mačkah s spontanimi fibrosarkomi (106). V tej študiji so kot vektor uporabili adenovirus, ki je nosil zapis za mačji IL-12 s toplotno inducibilnim promotorjem. Genski konstrukt so vbrizgali neposredno v tumorske nodule, ki so jih 24 ur pozneje ogreli na 40–44°C z namenom aktivacije specifičnega promotorja, s čimer so dosegli omejitev izražanja terapevtskega gena le lokalno v tumorju. Gensko terapijo so kombinirali še z radioterapijo. Ker je šlo za klinično študijo faze I, so ugotavljali le možnost izvedbe, maksimalni varni odmerek genskega konstrukta in potencialno toksičnost te terapije. Pri uporabljenih nizkih odmerkih so opazili le blažje stranske učinke, najpogosteje nekajdnevno prehodno levkopenijo. Pri visokih odmerkih pa so opazili hujše stranske učinke, ki so zajemali neješčnost, slabo počutje, pljučni edem, hepatotoksične učinke zdravljenja (aktivacija jetrnih encimov, zvečane serumske koncentracije bilirubina) in odstopanja v hematoloških parametrih (levkopenija, trombocitopenija, anemija) (106).

V drugih dveh študijah pa so uporabili nevirusne metode vnosa, bodisi z direktnim intratumoralnim vbrizganjem terapevtskega plazmida (104) ali pa z elektrogensko terapijo (75). Leta 2001 je Heinzerlingova s sodelavci objavila izsledke raziskave o učinkovitosti neposrednega intratumoralnega vbrizganja plazmida, ki nosi zapis za humani IL-12, v kožne melanome konjev (104). Ugotovili so, da je omenjeni plazmid biološko aktiven tudi pri konjih, saj so s terapijo pri vseh zdravljenih tumorjih dosegli zaustavitev rasti ali zmanjšanje volumna nodulov pri 11 od 12 zdravljenih tumorjev, od katerih je pri enem prišlo tudi do popolne regresije. Volumen tumorjev je bil najmanjši 10. dan po vbrizganju plazmida, učinek je trajal povprečno 30 dni, nato pa so tumorji začeli ponovno rasti. Stranskih učinkov zdravljenja ni bilo.

Raziskava iz leta 2009 pa je bila izvedena na induciranih TVT psov, pri katerih so izvedli intratumoralno EGT s plazmidom, ki nosi zapis za humani IL-12 (75). S to terapijo so dosegli značilno zmanjšanje velikosti nodulov s popolno regresijo pri 12 od 16 zdravljenih tumorjih. Ugotovili so, da EGT z *IL-12* poleg lokalnega protitumorskega učinka izzove tudi močan sistemski učinek, viden kot zastoj rasti oddaljenih nezdravljenih nodulih in razvoj odpornosti na ponoven izrast iste vrste tumorjev, brez tokičnih vplivov na zdravljene živali (75).

Med nevirusnimi metodami vnosa terapevtskega gena, ki kodira nastanek IL-12, se električno posredovani vnos plazmida pogosto uporablja na različnih živalskih modelih tumorjev. *IL-12* je bil eden prvih citokinov, uporabljenih v EGT. Protitumorska učinkovitost EGT z *IL-12* je bila do sedaj dokazana na številnih tumorskih modelih: melanomu (70, 71, 108, 109, 110, 111, 112), limfomu (108) in različnih karcinomih, npr. ploščatoceličnem karcinomu (113, 114, 115, 116), hepatocelularnem karcinomu (117, 118, 119), kolorektalnem adenokarcinomu (120, 121) in karcinomu ledvičnih celic (120). Terapevtski plazmid je bil vnešen bodisi v mišico ali pa neposredno v tumor. EGT z *IL-12* se je izkazala za učinkovito tudi v kombinaciji z drugimi citokini, npr. IL-18 (111, 122) ali drugimi protitumorskimi geni (npr. gen, ki kodira herpes simplex timidin kinazo) (121) oziroma v kombinaciji s kemoterapevtiki kot elektrokemo-genska terapija (73, 112, 116, 123).

Najbolj očiten učinek EGT z *IL-12* je upočasnitev rasti tumorjev ali popolna regresija tumorskih nodulov, ki je izrazitejša pri intratumoralni aplikaciji, v primerjavi z intramuskularno (70, 115). Po intratumoralni aplikaciji terapevtskega gena so pri melanomu dosegli popolno dolgotrajno regresijo tumorjev pri 50–80 % zdravljenih živali, odvisno od števila zaporednih ponovitev terapije (70, 71). Pri karcinomu pa je bil tak učinek dosežen pri 40 % zdravljenih živali (114). Z intramuskularno EGT z *IL-12* pri melanomu niso dosegli popolne regresije tumorjev (70), pri limfomu pri 50 % (108), pri ploščatoceličnem karcinomu pa pri 40 % zdravljenih tumorjev (113).

Poleg zaviranja rasti tumorjev so z EGT z *IL-12* dosegli tudi dolgotrajno protitumorsko zaščito, zmanjšanje števila pljučnih metastaz in posledično podaljšanje življenjske dobe zdravljenih živali v primerjavi s kontrolnimi skupinami (70, 108, 114, 117, 124).

Pomemben vidik EGT z *IL-12* je njena morebitna toksičnost, še posebej v luči znanih stranskih učinkov rekombinantnega proteina IL-12. Heller s sodelavci je izvedel obširno raziskavo o morebitnih stranskih učinkih intratumoralne EGT na melanomih (109). Pri živalih je spremljal številne hematološke in biokemijske parametre v različnih časovnih intervalih po izvedeni terapiji. Živali, ki so bile zdravljene z EGT z *IL-12*, niso imele nikakršnih odstopanj v krvnih parametrih v primerjavi z

nezdravljenimi. Edino odstopanje, ki so ga opazili, so bile histopatološko ugotovljene vnetne spremembe na ledvicah 16 in 30 dni po izvedeni terapiji (109).

Na podlagi rezultatov omenjenih raziskav, s katerimi je bila potrjena protitumorska učinkovitost EGT z *IL-12* na predkliničnem nivoju, so izvedli tudi že prvo humano klinično študijo o učinku intratumoralne EGT z *IL-12* na 24 pacientih z metastatskim melanomom, ki so imeli vsaj dva kožna ali podkožna tumorska nodula (107). Dosegli so lokalni klinični odgovor na zdravljenih tumorjih, poleg tega pa pri 53 % pacientov tudi sistemski protitumorski učinek na nezdravljenih nodulih drugje po telesu. EGT je imela le blažje stranske učinke, kot najhujšega omenjajo kratkotrajno bolečino in krvavitev na mestu elektroporacije, odstopanj v hematoloških parametrih niso ugotovili(107).

2 NAMEN RAZISKAVE IN HIPOTEZE

Namen doktorskega dela je bil določiti protitumorski učinek EGT z *IL-12* pri zdravljenju različnih induciranih tumorjev miši in spontanih tumorjev psov. Končni namen dela pa je bil uvesti to terapijo v veterinarsko klinično prakso za zdravljenje različnih tumorjev psov.

Naša **hipoteza** je bila, da je *električno posredovani vnos plazmidne DNA v tumorje in mišice učinkovita metoda transfekcije, ki vodi do izražanja vnešenega gena in da je EGT s plazmidno DNA, ki nosi zapis za IL-2, izvedljiva, varna in učinkovita pri zdravljenju različnih induciranih tumorjev miših in spontanih tumorjev psov.*

Za potrditev hipoteze smo si zadali naslednje **specifične cilje**:

1. Določiti optimalni časovni razmik med vbrizganjem plazmidne DNA in aplikacijo električnih pulzov za uspešen električno posredovani vnos plazmidne DNA v inducirane tumorje miši.

2. Določiti najustreznejše parametre električnih pulzov, odmerek plazmidne DNA in optimalni časovni razmik med vbrizganjem plazmidne DNA in aplikacijo električnih pulzov za uspešen električno posredovani vnos plazmidne DNA v skeletno mišičnino miši.

3. Določiti protitumorski učinek EGT z *IL-12* na inducirane tumorje miši z lokalno intra- in peritumoralno aplikacijo plazmidne DNA.

4. Določiti protitumorski učinek EGT z *IL-12* na inducirane tumorje miši z aplikacijo plazmidne DNA v skeletno mišičnino miši.

5. Določiti najustreznejše parametre električnih pulzov za uspešen električno posredovani vnos plazmidne DNA v skeletno mišičnino psov.

6. Določiti protitumorski učinek EGT z *IL-12* na spontane tumorje psov z lokalno aplikacijo plazmidne DNA v tumorje psov.

3 IZVIRNI ZNANSTVENI ČLANKI

3.1 SEQUENCE AND TIME DEPENDENCE OF TRANSFECTION EFFICIENCY OF ELECTRICALLY-ASSISTED GENE DELIVERY TO TUMORS IN MICE

VPLIV ČASOVNEGA RAZMIKA MED VBRIZGANJEM PLAZMIDNE DNA IN APLIKACIJO ELEKTRIČNIH PULZOV NA UČINKOVITOST ELEKTROTRANSFEKCIJE TUMORJEV PRI MIŠIH

Čemažar M[1], Pavlin D[2], Kranjc S[1], Grošel A[1], Mesojednik S[1], Serša G[1]

[1]Onkološki inštitut Ljubljana, Zaloška 2, 1000 Ljubljana, Slovenija

[2]Univerza v Ljubljani, Veterinarska fakulteta, Cesta v Mestni log 47, 1000 Ljubljana, Slovenija

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Abstract

Electrically-assisted gene delivery is a non-viral gene delivery technique, using application of square wave electric pulses to facilitate uptake of plasmid DNA into the cells. Feasibility and effectiveness of this method in *vivo* was already demonstrated, elaborating on pulse parameters and plasmid construction. However, there were no studies performed on sequencing and timing of plasmid DNA injection into the tumors and application of electric pulses. For this purpose we measured luciferase expression in two tumor models (LPB fibrosarcoma, B16F1 melanoma) after electrically-assisted gene delivery at varying time intervals between the pCMV-Luc plasmid injection and electroporation. Expression of luciferase was determined by measurement of its activity using luminometer.

The results demonstrated that pCMV-Luc plasmid has to be injected before the application of electric pulses, since no measurable expression was detected in the tumors when pCMV-Luc plasmid was injected after electroporation of tumors. In both tumor models the highest transfection efficiency was obtained when pCMV-Luc plasmid was injected not less than *5* minutes but also not more than *30* minutes before the application of electric pulses. The results also demonstrated variability in the transfection efficiency depending on the tumor model. High expression was obtained in B16F1 tumor model *(~5500* pg luc/mg tumor) and lower in LPB fibrosarcoma *(~200* pg luc/mg tumor).

In conclusion, our results demonstrate that regardless of the susceptibility of the tumors to electrically-assisted gene delivery, the best timing for pCMV-Luc plasmid is between 30 to 5 minutes prior to the application of electric pulses to the tumors.

Izvleček

Električno posredovani vnos DNA je ena od nevirusnih tehnik vnosa genskega materiala v celice, pri kateri z uporabo kontroliranih električnih pulzov izboljšamo vnos DNA v tarčne celice. Metoda se je že izkazala kot izvedljiva in učinkovita v pogojih *in vivo*. Številne dosedanje raziskave so se osredotočile na določanje najprimernejših parametrov električnih pulzov in lastnostih plazmida za najuspešnejšo transfekcijo tarčnega tkiva. Vendar pa do sedaj še ni bila izvedena raziskava o pomenu časovnega razmika med vbrizganjem plazmidne DNA v tumor in aplikacijo električnih pulzov. V ta namen smo v naši raziskavi določali izražanje luciferaze pri električno posredovanem vnosu reporterskega plazmida pCMV-Luc v dva tumorska modela (LPB fibrosarkom in B16F1 melanom). Reporterski plazmid smo v tumorske nodule vbrizgali v različnih časovnih razmikih pred in po aplikaciji električnih pulzov. Izražanje luciferaze smo določili z merjenjem njene aktivnosti s pomočjo luminometra.

Ugotovili smo, da do izražanja luciferaze pride le, če plazmid pCMV-Luc vbrizgamo pred aplikacijo električnih pulzov. Pri obeh tumorskih modelih smo najvišjo transfekcijo dosegli z intratumoralnim vbrizganjem reporterskega plazmida vsaj 5 in ne več kot 30 minut pred aplikacijo električnih pulzov. Rezultati naše raziskave kažejo tudi, da se nivo izražanja reporterskega gena med različnimi tumorskimi modeli razlikuje. V B16F1 melanomu smo dosegli višje izražanje luciferaze (~5500 pg luc/mg tumorja), kot v LPB fibrosarkomu (~200 pg luc/mg tumorja).

Na podlagi rezultatov naše raziskave lahko torej zaključimo, da je ne glede na dovzetnost tumorjev na električno posredovani vnos DNA najoptimalnejši čas za injiciranje pCMV-Luc 30 do 5 minut pred aplikacijo električnih pulzov.

3.2 THE EFFECT OF THE HISTOLOGICAL PROPERTIES OF TUMORS ON TRANSFECTION EFFICIENCY OF ELECTRICALLY-ASSISTED GENE DELIVERY TO SOLID TUMORS IN MICE

VPLIV HISTOLOŠKIH LASTNOSTI TUMORJEV NA UČINKOVITOST TRANSFEKCIJE ELEKTRIČNO POSREDOVANEGA VNOSA DNA V SOLIDNE TUMORJE

Mesojednik S[1], Pavlin D[2], Serša G[1], Cör A[3], Kranjc S[1], Grošel A[1], Tevž G[1], Čemažar M[1]

[1]Onkološki inštitut Ljubljana, Zaloška 2, 1000 Ljubljana, Slovenija
[2]Univerza v Ljubljani, Veterinarska fakulteta, Gerbičeva 60, 1000 Ljubljana, Slovenija
[3]Univerza v Ljubljani, Medicinska fakulteta, Korytkova 2, 1000 Ljubljana, Slovenija

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Abstract

Uniform DNA distribution in tumors is a prerequisite step for high transfection efficiency in solid tumors. In order to improve the transfection efficiency of electrically-assisted gene delivery to solid tumors in vivo, we explored how tumor histological properties affected transfection efficiency. In four different tumor types (B16F1, EAT, SA-1, LPB), proteoglycan and collagen content was morphometrically analyzed, and cell size and cell density were determined in paraffin-embedded tumor sections under a transmission microscope. To demonstrate the influence of the histological properties of solid tumors on electrically-assisted gene delivery, the correlation between histological properties and transfection efficiency with regard to the time interval between DNA injection and electroporation was determined.

Our data demonstrate that soft tumors with larger spherical cells, low proteoglycan and collagen content, and low cell density are more effectively transfected (B16F1, EAT) than rigid tumors with high proteoglycan and collagen content, small spindle-shaped cells and high cell density (LPB and SA-1). Furthermore, an optimal time interval for increased transfection exists only in soft tumors, this being in the range of 5 to 15 min.

Therefore, knowledge about the histology of tumors is important in planning electrogene therapy with respect to the time interval between DNA injection and electroporation.

Izvleček

Prvi pogoj za visoko transfekcijo solidnih tumorjev je enakomerna porazdelitev DNA v tumorskem tkivu. V raziskavi smo preučili učinek histoloških lastnosti tumorjev na učinkovitost transfekcije električno posredovanega vnosa DNA, z namenom izboljšati učinkovitost te metode genskega vnosa v solidne tumorje. V štirih različnih tipih tumorjev (B16F1, EAT, SA-1 in LPB) smo morfometrično analizirali vsebnost proteoglikanov in kolagena ter določili velikost in gostoto celic na parafinskih preparatih tumorjev s pomočjo presevnega mikroskopa. Določili smo tudi korelacijo med histološkimi lastnostmi tkiva in učinkovitostjo transfekcije glede na časovni razmik med vbrizganjem DNA in elektroporacijo.

Rezultati naše raziskave kažejo, da boljšo transfekcijo dosežemo v tumorjih z velikimi okroglimi celicami, manjšo količino proteoglikanov in kolagena (B16F1, EAT) v primerjavi z gostimi tumorji iz vretenastih celic, ki vsebujejo veliko proteoglikanov in kolagena (LPB in SA-1). Poleg tega smo

ugotovili, da optimalni časovni razmik med vbrizganjem DNA in elektroporacijo, ki omogoča boljšo transfekcijo, velja samo za mehkejše tumorje.

Na podlagi tega lahko zaključimo, da je za načrtovanje elektrogenske terapije nujno poznavanje histoloških lastnosti tumorjev.

3.3 GENE ELECTROTRANSFER INTO MURINE SKELETAL MUSCLE: A SYSTEMATIC ANALYSIS OF PARAMETERS FOR LONG-TERM GENE EXPRESSION

GENSKI ELEKTROTRANSFER V SKELETNO MIŠIČNINO MIŠI: SISTEMSKA ANALIZA PARAMETROV ZA DOLGOTRAJNO IZRAŽANJE TRANSGENOV

Tevž G[1], Pavlin D[2], Kamenšek U[1], Kranjc S[1], Mesojednik S[1], Cör A[3], Serša G[1], Čemažar M[1]

[1]Onkološki inštitut Ljubljana, Zaloška 2, 1000 Ljubljana, Slovenija
[2]Univerza v Ljubljani, Veterinarska fakulteta, Gerbičeva 60, 1000 Ljubljana, Slovenija
[3]Univerza v Ljubljani, Medicinska fakulteta, Korytkova 2, 1000 Ljubljana, Slovenija

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Abstract

Skeletal muscle is an attractive target tissue for delivery of therapeutic genes, since it is well vascularized, easily accessible, and has a high capacity for protein synthesis. For efficient transfection in skeletal muscle, several protocols have been described, including delivery of low voltage electric pulses and a combination of high and low voltage electric pulses. The aim of this study was to determine the influence of different parameters of electrotransfection on short-term and long-term transfection efficiency in murine skeletal muscle, and to evaluate histological changes in the treated tissue. Different parameters of electric pulses, different time lags between plasmid DNA injection and application of electric pulses, and different doses of plasmid DNA were tested for electrotransfection of *tibialis cranialis* muscle of C57Bl/6 mice using DNA plasmid encoding green fluorescent protein (GFP). Transfection efficiency was assessed on frozen tissue sections 1 week after electrotransfection using a fluorescence microscope and also noninvasively, followed by an *in vivo* imaging system using a fluorescence stereo microscope over a period of several months. Histological changes in muscle were evaluated immediately or several months after electrotransfection by determining infiltration of inflammatory mononuclear cells and presence of necrotic muscle fibers.

The most efficient electrotransfection into skeletal muscle of C57Bl/6 mice in our experiments was achieved when 1 high voltage (HV) and 4 low voltage (LV) electric pulses were applied 5 seconds after the injection of 30 μg of plasmid DNA. This protocol resulted in the highest short-term as well as long-term transfection. The fluorescence intensity of the transfected area declined after 2–3 weeks, but GFP fluorescence was still detectable 18 months after electrotransfection. Extensive inflammatory mononuclear cell infiltration was observed immediately after the electrotransfection procedure using the described parameters, but no necrosis or late tissue damage was observed.

This study showed that electric pulse parameters, time lag between the injection of DNA and application of electric pulses, and dose of plasmid DNA affected the duration of transgene expression in murine skeletal muscle. Therefore, transgene expression in muscle can be controlled by appropriate selection of electrotransfection protocol.

Izvleček

Skeletna mišičnina je primerno tarčno tkivo za gensko terapijo, saj gre za dobro dostopno in dobro ožiljeno tkivo z visoko sposobnostjo tvorbe beljakovin. Za učinkovito električno posredovano transfekcijo skeletne mišičnine se uporablja nekaj različnih protokolov elektroporacije, bodisi uporaba le nizkonapetostnih ali kombinacije visoko- in nizkonapetostnih električnih pulzov. Namen naše

raziskave je bil določiti vpliv različnih parametrov na učinkovitost kratkotrajnega in dolgotrajnega izražanja transgenov pri elektrotransfekciji skeletne mišičnine miši in oceniti histološke spremembe v izpostavljenem tkivu. S pomočjo reporterskega plazmida, ki nosi zapis za zeleni fluorescirajoči protein, smo primerjali učinkovitost različnih parametrov električnih pulzov, različnih časovnih razmikov med vbrizganjem plazmidne DNA in aplikacijo električnih pulzov in različnih odmerkov plazmidne DNA pri elektrotransfekciji mišice *tibialis cranialis* miši seva C57Bl/6. Učinkovitost trasfekcije smo določali invazivno na zmrzlih rezih mišic en teden po izvedeni elektrotransfekciji s pomočjo fluorescentnega mikroskopa. Poleg tega smo v časovnem obdobju več mesecev po izvedeni elektrotransfekciji pri živalih s pomočjo fluorescentnega stereomikroskopa tudi *in vivo* neinvazivno spremljali izražanje reporterskega gena. Histološke spremembe tretiranih mišic smo ugotavljali takoj in nekaj mesecev po elektrotransfekciji na podlagi določanja infiltracije vnetnih mononuklearnih celic in prisotnosti nekroze mišičnih vlaken.

Najuspešnejšo kratko- in dolgotrajno transfekcijo skeletne mišičnine pri miših seva C67Bl/6 smo dosegli z uporabo enega visokonapetostnega in štirih nizkonapetostnih pulzov, ki smo jih aplicirali 5 sekund po injiciranju 30 μg plazmidne DNA. Intenzivnost fluorescence transfeciranega področja se je sicer zmanjšala po 2–3 tednih, vendar pa je ostala zaznavna še 18 mesecev po izvedeni elektrotransfekciji. Takoj po elektrotransfekciji smo v preparatih zaznali obsežno vnetno infiltracijo mišičnine, vendar ni prišlo do nekroze tkiva.

Rezultati te raziskave kažejo, da parametri električnih pulzov, časovni razmik med injiciranjem plazmida in aplikacijo električnih pulzov in odmerek plazmidne DNA vplivajo na dolžino izražanja vnešenega transgena v skeletni mišičnini miši. Torej lahko izražanje transgena nadzorujemo z ustreznim izborom protokola elektrotransfekcije.

3.4 LOCAL AND SYSTEMIC ANTITUMOR EFFECT OF INTRATUMORAL AND PERITUMORAL *IL-12* ELECTROGENE THERAPY ON MURINE SARCOMA

LOKALNI IN SISTEMSKI PROTITUMORSKI UČINEK ELEKTROGENSKE TERAPIJE Z *IL-12* NA MIŠJEM SARKOMU

Pavlin D[1], Čemažar M[2], Kamenšek U[2], Tozon N[1], Pogačnik A[1], Serša G[2]

[1]Univerza v Ljubljani, Veterinarska fakulteta, Gerbičeva 60, 1000 Ljubljana, Slovenija
[2]Onkološki inštitut Ljubljana, Zaloška 2, 1000 Ljubljana, Slovenija

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Abstract

Soft tissue sarcomas pose a challenge for successful treatment with conventional therapeutic methods, therefore newer therapeutic approaches are considered.

In this study, we evaluated the antitumor effect of *IL-12* electrogene therapy (EGT) on murine SA-1 fibrosarcoma. The Therapeutic plasmid was injected either intratumorally into subcutaneous SA-1 nodules or intradermally into the peritumoral region. We achieved a remarkable local and systemic antitumor effect with both approaches after single plasmid DNA application, with significant intratumoral and systemic production of IL-12 and IFN-γ. Intratumoral *IL-12* EGT resulted in over 90% complete response rate of the treated tumors with 60% of cured mice being resistant to challenge with SA-1 tumor cells. Peritumoral EGT resulted in a lower complete response rate (16%), with significant growth delay of remaining tumors. Both therapies also resulted in significant inhibition of growth of untreated tumors, growing simultaneously at a distant site.

These data suggest that *IL-12* EGT may be useful in the treatment of soft tissue sarcomas, exerting a local and systemic antitumor effect.

Izvleček

Sarkomi mehkih tkiv predstavljajo velik izziv za uspešno zdravljenje s pomočjo uveljavljenih metod onkološkega zdravljenja, zato se na tem tipu tumorjev preizkuša vedno več novejših terapevtskih pristopov.

V naši raziskavi smo ovrednotili protitumorski učinek EGT z *IL-12* na mišjem modelu SA-1 fibrosarkoma. Terapevtski plazmid smo vbrizgali bodisi intratumoralno v podkožne nodule SA-1 ali intradermalno v peritumoralno področje. Z obema pristopoma smo dosegli izreden protitumorski učinek po enkratnem vbrizganju terapevtskega plazmida s signifikantno intratumoralno proizvodnjo in sistemskim izločanjem IL-12 in IFN-γ. Z intratumoralno EGT smo dosegli preko 90-odstotni popolni odgovor zdravljenih tumorjev in razvoj dolgotrajne protitumorske imunosti pri 60 % ozdravljenih živali. S peritumoralno EGT smo dosegli 16-odstotni popolni odgovor zdravljenih tumorjev s statistično značilnim zastojem rasti preostalih neozdravljenih nodulov. Z obema metodama zdravljenja smo dosegli tudi statistično značilen zastoj rasti oddaljenih nezdravljenih tumorjev.

Rezultati naše raziskave torej kažejo, da bi EGT z *IL-12* lahko bila uporabna nova metoda zdravljenja sarkomov mehkih tkiv, s katero dosežemo tako lokalni kot sistemski protitumorski učinek.

3.5 CONTROLLED SYSTEMIC RELEASE OF IL-12 AFTER GENE ELECTROTRANSFER TO MUSCLE FOR CANCER GENE THERAPY ALONE OR IN COMBINATION WITH IONIZING RADIATION IN MURINE SARCOMAS

KOMBINIRANO ZDRAVLJENJE MIŠJIH SARKOMOV Z OBSEVANJEM IN URAVNANIM SISTEMSKIM SPROŠČANJEM IL-12 PO GENSKEM ELEKTROTRANSFERJU *IL-12* V MIŠICO

Tevž G[1], Kranjc S[1], Čemažar M[1], Kamenšek U[1], Cör A[2], Kržan M[3], Vidic S[1], Pavlin D[4], Serša G[1]

[1]Onkološki inštitut Ljubljana, Zaloška 2, 1000 Ljubljana, Slovenija
[2]Univerza na Primorskem, Visoka šola za zdravstvo Izola, Polje 42, 6310, Izola, Slovenija
[3]Univerza v Ljubljani, Medicinska fakulteta, Korytkova 2, 1000 Ljubljana, Slovenija
[4]Univerza v Ljubljani, Veterinarska fakulteta, Gerbičeva 60, 1000 Ljubljana, Slovenija

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Abstract

Background. The aim of our study was to evaluate the antitumor effectiveness of systemic IL-12 gene therapy in murine sarcoma models, and to evaluate its interaction with irradiation of tumors and metastases. To avoid toxic side effects of IL-12 gene therapy, the objective was to achieve controlled release of IL-12 after intramuscular gene electrotransfer.

Methods. Gene electrotransfer of the plasmid pORF-mIL12 was performed into the *tibialis cranialis* in A/J and C57BL/6 mice. Systemic release of the IL-12 was monitored in the serum of mice after two sets of intramuscular IL-12 gene electrotransfer of two different doses of plasmid DNA. The antitumor effectiveness of IL-12 gene electrotransfer alone or in combination with local tumor or lung irradiation with X-rays was evaluated on subcutaneous SA-1 and LPB tumors as well as on lung metastases.

Results. A synergistic antitumor effect of intramuscular gene electrotransfer combined with local tumor irradiation was observed due to the systemic distribution of IL-12. The gene electrotransfer resulted in up to 28% of complete responses of tumors. In combination with local tumor irradiation, the curability was increased up to 100%. The same effect was observed for lung metastases where a potentiating factor of 1.3-fold was determined. The amount of circulating IL-12 was controlled by the number of repeats of gene electrotransfer and by the amount of the injected plasmid.

Conclusions. This study demonstrates the feasibility of the combined treatment by IL-12 gene electrotransfer with local tumor or lung metastases irradiation on sarcoma tumors for translation into the clinics.

Izvleček

Namen. Namen naše raziskave je bil določiti protitumorski učinek sistemske genske terapije z *IL-12* pri mišjih sarkomih in ovrednotiti medsebojni vpliv te vrste zdravljenja v kombinaciji z obsevanjem tumorjev in metastaz. V raziskavi smo prav tako želeli doseči nadzorovano izločanje IL-12 pri intramuskularni elektrogenski terapiji, da bi se izognili toksičnim stranskim učinkom IL-12.

Metode. Izvedli smo genski elektrotransfer plazmida pORF-mIL12 v mišico *tibialis cranialis* pri miših linij A/J in C57BL/6. Določili smo sistemsko izločanje IL-12 v serumu živali po dveh ponovitvah intramuskularne EGT z *IL-12*, pri katerih smo uporabili dva različna odmerka plazmidne DNA. Protitumorski učinek samostojne elektrogenske terapije z *IL-12* ali v kombinaciji z obsevanjem smo določali na podkožnih SA-1 in LPB tumorjih in njihovih pljučnih metastazah.

Rezultati. Zaradi sistemskega izločanja IL-12 smo z intramuskularnim genskim elektrotransferjem v kombinaciji z lokalnim obsevanjem dosegli sinergistični protitumorski učinek. Z intramuskularno elektrogensko terapijo smo dosegli 28-odstotni popolni odgovor zdravljenih tumorjev. V kombinaciji z lokalnim obsevanjem pa se je ozdravljivost živali povišala do 100 %. Podoben učinek na izboljšanje zdravljenja smo opazili tudi pri pljučnih metastazah. Količino cirkulirajočega IL-12 smo lahko nadzorovali s številom ponovitev elektrotransferja in s količino vbrizganega plazmida.

Zaključki. Rezultati študije dokazujejo izvedljivost kombiniranega zdravljenja z elektrotransfekcijo gena *IL-12* in obsevanjem podkožnih tumorjev ali pljučnih metastaz pri sarkomih in možnost translacije v klinično uporabo.

3.6 EFFICIENT ELECTROTRANSFECTION INTO CANINE MUSCLE

UČINKOVITA ELETROTRANSFEKCIJA MIŠICE PRI PSIH

Pavlin D[1], Tozon N[1], Serša G[2], Pogačnik A[1], Čemažar M[2]

[1]Univerza v Ljubljani, Veterinarska fakulteta, Gerbičeva 60, 1000 Ljubljana, Slovenija
[2]Onkološki inštitut Ljubljana, Zaloška 2, 1000 Ljubljana, Slovenija

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Abstract

Two different types of electroporation protocols have been developed for efficient electrotransfer of plasmid DNA into skeletal muscle of experimental animals. At first, only low voltage electric pulses have been used, but lately, a combination of high and low voltage pulses has been suggested as more efficient. Up to date, in dogs, this type of electroporation protocol has never been used for muscle targeted plasmid DNA electrotransfection.

In this study, we used two different DNA plasmids, one encoding green fluorescent protein and one encoding human interleukin-12. Five different electroporation protocols were evaluated. Three of them featured different combinations of high and low voltage pulses, and two were performed with delivery of low voltage pulses only. Our study shows that combination of 1 high voltage pulse (600 V/cm, 100 μs), followed by 4 low voltage pulses (80 V/cm, 100 ms, 1 Hz) yielded in the same transfection efficiency as the standard trains of low voltage pulses. However, this protocol is performed quicker and thus more suitable for potential use in clinical practice. In addition, it yielded in detectable systemic expression of human interleukin-12. Electrotransfer of either of the plasmids was associated with only mild and transitory local side effects, without clinically detectable systemic side effects.

The results indicate that electrotransfection is a feasible, effective and safe method for muscle targeted gene therapy in dogs, which could have potential for clinical applications in veterinary medicine of small animals.

Izvleček

Za učinkovito transfekcijo skeletne mišičnine poskusnih živali sta bila razvita dva različna tipa protokola za izvedbo elektroporacije. Sprva so se uporabljali le nizkonapetostni električni pulzi, v zadnjem času pa se je izkazalo, da je za transfekcijo mišičnine učinkovitejša kombinacija visoko- in nizkonapetostnih električnih pulzov. Ta kombinacija do sedaj še ni bila nikoli uporabljena za transfekcijo skeletne mišičnine pri psih. V naši raziskavi smo uporabili dva različna DNA plazmida, in sicer plazmid, ki nosi zapis za zeleni fluorescirajoči protein in plazmid, ki nosi zapis za humani IL-12. Med seboj smo primerjali pet različnih protokolov elektroporacije. Pri treh smo uporabili kombinacijo enega viskonapetostnega pulza in različnega števila nizkonapetostnih pulzov, pri dveh pa smo uporabili le nizkonapetostne električne pulze.

V naši raziskavi smo dosegli najboljšo transfekcijo z dvema protokoloma, z uporabo strandardnega nizkonapetostnega protokola (8 pulzov, 200 V/cm, 20 ms, 1 Hz) in s kombinacijo enega visokonapetostnega pulza (600 V/cm, 100 μs) in štirih nizkonapetostnih pulzov (80 V/cm, 100 ms, 1 Hz). Vendar pa se drugi protokol izvede hitreje od prvega in je zato primernejši za klinično uporabo. Poleg tega smo s tem protokolom dosegli tudi sistemsko izražanje humanega IL-12 pri psih po elektrotransfekciji skeletne mišičnine. Električno posredovani vnos obeh plazmidov je povzročil le blage in prehodne lokalne stranske učinke brez klinično zaznavnih sistemskih stranskih učinkov.

Rezultati naše raziskave torej kažejo, da je elektrotransfekcija izvedljiva, učinkovita in varna metoda genskega vnosa v skeletno mišičnino psov in ima zato potencial za klinično uporabo v veterinarski medicini.

3.7 ELECTROGENE THERAPY WITH *IL-12* IN CANINE MAST CELL TUMORS

ELEKTROGENSKA TERAPIJA Z *IL-12* PRI MASTOCITOMIH PSOV

Pavlin D[1], Čemažar M[2,3], Cör A[3], Serša G[2], Pogačnik A[1], Tozon N[1]

[1]Univerza v Ljubljani, Veterinarska fakulteta, Gerbičeva 60, 1000 Ljubljana, Slovenija

[2]Onkološki inštitut Ljubljana, Zaloška 2, 1000 Ljubljana, Slovenija

[3]Univerza na Primorskem, Visoka šola za zdravstvo Izola, Polje 42, 6310, Izola, Slovenija

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Abstract

Background. Mast cell tumors (MCT) are the most common malignant cutaneous tumors in dogs with extremely variable biological behaviour. Different treatment approaches can be used in canine cutaneous MCT, with surgical excision being the treatment of choice. In this study, electrogene therapy (EGT) as a new therapeutic approach to canine MCTs, was established.

Materials and methods. Eight dogs with a total of eleven cutaneous MCTs were treated with intratumoral EGT using DNA plasmid encoding human IL-12. The local response to the therapy was evaluated by repeated measurements of tumor size and histological examination of treated tumors. A possible systemic response was assessed by determination of IL-12 and interferon- γ (IFN-γ) in patients' sera. The occurence of side effects was monitored with weekly clinical examinations of treated animals and by performing basic bloodwork, consisting of the complete bloodcount and determination of selected biochemistry parameters.

Results. Intratumoral EGT with *IL-12* elicits significant reduction of treated tumors' size, ranging from 15% to 83% (median 50%) of the initial tumor volume. Additionally, a change in the histological structure of treated nodules was seen. There was a reduction in number of malignant mast cells and inflammatory cell infiltration of treated tumors. Systemic release of IL-12 in four patients was detected, without any noticeable local or systemic side effects.

Conclusions. These data suggest that intratumoral EGT with plasmid encoding IL-12 may be useful in the treatment of canine MCTs, exerting a local antitumor effect.

Izvleček

Izhodišča. Mastocitomi so eden najpogostejših malignih kožnih tumorjev pri psih. Zanje je značilno zelo raznoliko biološko obnašanje, zaradi česar je lahko določanje kliničnega stadija bolezni in odločanje o ustrezni vrsti zdravljenja zelo težko. Kožne mastocitome pri psih zdravimo na različne načine, vendar je metoda izbora še vedno kirurška terapija. V naši raziskavi smo za zdravljenje mastocitomov uporabili nov terapevtski pristop, intratumoralno elektrogensko terapijo (EGT).

Materiali in metode. V raziskavo smo vključili osem psov s skupno enajstimi kožnimi noduli, ki smo jih zdravili z intratumoralno EGT s plazmidom, ki nosi zapis za humani IL-12. Lokalne učinke EGT smo določali z merjenjem velikosti tumorjev v različnih časovnih obdobjih po terapiji in histološkim pregledom vzorcev zdravljenih tumorjev. Sistemski odgovor na EGT smo ugotavljali z določanjem

IL-12 in IFN- γ v serumu živali. Morebitne stranske učinke zdravljenja smo nadzorovali z določanjem osnovne krvne slike in izbranih biokemičnih vrednosti v serumu zdravljenih živali.

Rezultati. Na zdravljenih psih je intratumoralna EGT z *IL-12* izzvala dober lokalni protitumorski učinek s statistično značilnim zmanjšanjem velikosti zdravljenih tumorjev v razponu od 15 do 83 % začetnega volumna nodulov. Poleg tega smo ugotovili spremembe v histološki zgradbi zdravljenih tumorjev, ki so se kazale kot zmanjšanje števila mastocitov in vnetna infiltracija nodulov. Pri zdravljenih psih smo tudi dosegli sistemsko izločanje IL-12 in IFN-γ brez stranskih učinkov.

Zaključki. Rezultati naše raziskave nakazujejo, da bi bila EGT s plazmidom, ki nosi zapis za humani IL-12, lahko uspešna metoda zdravljenja mastocitomov pri psih, saj izzove tako lokalni protitumorski učinek.

4 DISKUSIJA

Prvi del raziskav v okviru doktorskega dela smo posvetili izbiri najprimernejših parametrov za električno posredovani vnos DNA v tumorje in skeletno mišičnino sprva miši in nato psov. Pri optimizaciji protokola za vnos plazmidne DNA v tumorje miši smo se osredotočili na določitev najoptimalnejšega časovnega razmika med intratumoralno aplikacijo plazmidne DNA in aplikacijo električnih pulzov. Najučinkovitejšo transfekcijo smo dosegli, če smo elektroporacijo tumorskega tkiva izvedli 5–15 minut po vbrizganju reporterskega plazmida. S sistemsko analizo vpliva različnih parametrov na učinkovitost transfekcije skeletne mišičnine smo ugotovili, da je najoptimalnejši protokol za električno posredovani vnos plazmidne DNA v mišičnino uporaba kombinacije enega visokonapetostnega in štirih nizkonapetostnih pulzov, apliciranih neposredno po intramuskularnem vbrizganju plazmidne DNA. Ta protokol se je izkazal za najučinkovitejšega pri transfekciji mišičnine tako pri miših kot pri psih.

Na podlagi dobljenih rezultatov smo delo nadaljevali z ugotavljanjem protitumorske učinkovitosti EGT z *IL-12* na mišjih SA-1 fibrosarkomih. Ugotovili smo, da je EGT z *IL-12* zelo učinkovita metoda zdravljenja teh tumorjev z lokalnim in sistemskim protitumorskim delovanjem. Nazadnje smo izvedli še klinično raziskavo o protitumorskem učinku EGT s humanim *IL-12* pri psih z različnimi spontanimi tumorji. Ugotovili smo, da intramuskularna EGT pri psih lahko izzove sistemsko izločanje hIL-12 in proizvodnjo IFN-γ brez kakršnihkoli stranskih učinkov. Intratumoralna EGT pa omogoča lokalno izražanje in sistemsko izločanje transgena, ki povzroči histološko zaznavne strukturne spremembe tumorskega tkiva z zmanjšanjem števila malignih celic in infiltracijo tkiva z vnetnimi celicami ter posledično zmanjšanjem velikosti tumorja.

4.1 PROTITUMORSKA UČINKOVITOST EGT Z *IL-12* PRI ZDRAVLJENJU INDUCIRANIH TUMORJEV MIŠI

4.1.1 Določitev najustreznejšega časovnega razmika med vbrizganjem plazmidne DNA v tumor in aplikacijo električnih pulzov pri miših

Za razvoj ustreznega protokola za električno posredovani vnos DNA v tarčno tkivo, ki bi bil uporaben v klinični praksi za učinkovito gensko terapijo, je najprej potrebno določiti najustreznejše parametre protokola, s katerimi dosežemo optimalno izražanje vnešenega gena. Optimalni protokol bi moral biti čim manj invaziven, čimbolj varen za organizem in bi moral omogočiti kontrolirano izražanje vnešenih genov, s katerim bi dosegli terapevtske koncentracije želenega proteina.

Ugotovljeno je bilo, da na učinkovitost genske elektrotransfekcije vplivajo številni dejavniki. Med najpomembnejše spadajo parametri apliciranih električnih pulzov: oblika električnega pulza, trajanje posameznega pulza, število pulzov, frekvenca aplikacije pulzov in časovni razmik med vbrizganjem plazmidne DNA in aplikacijo električnih pulzov. Poleg tega pa na učinkovitost genske elektrotransfekcije vplivajo še oblika uporabljenih elektrod, lastnosti tarčnega tkiva, kot so oblika in lastnosti celic, histološka zgradba tkiva in sestava medcelične, lastnosti plazmida, kot so velikost in zgradba plazmida ter koncentracija plazmidne raztopine ter način aplikacije raztopine DNA. Zato se optimalni parametri med različnimi tarčnimi tkivi, na primer tumorji, mišičnino, kožo, lahko zelo razlikujejo. Zaradi tega smo prvi del raziskav usmerili v optimizacijo protokola za učinkovit električno posredovani vnos DNA v tumorje miši in skeletno mišičnino miši in psov.

Večina do sedaj objavljenih raziskav, v katerih so določali najustreznejše parametre za učinkovito elektrotransfekcijo tumorskega tkiva, se je osredotočila predvsem na parametre električnih pulzov, koncentracijo vbrizgane DNA in strukturo plazmida (6, 69, 66, 72, 125, 126). Vpliv časovnega razmika med vbrizganjem plazmidne DNA in aplikacijo električnih pulzov so preučili le v manjšem številu raziskav o učinkovitosti elektrotransfekcije skeletne mišičnine (127, 128, 129). V večini objavljenih raziskav na tumorjih pa temu parametru niso posvečali posebne pozornosti. Genska elektrotransfekcija tumorskih nodulov se je izvajala tako, da je razmik med intratumoralno aplikacijo plazmidne DNA in električnih pulzov minimalen (nekaj sekund do nekaj minut) (6, 25, 50, 66, 130).

Zaradi procesov, ki se v tkivu odvijajo po vbrizganju plazmidne DNA, smo predpostavljali, da časovni interval lahko pomembno vpliva na učinkovitost transfekcije. Rezultati naše raziskave so pokazali, da je učinkovitost elektrotransfekcije tumorskega tkiva odvisna od časovnega intervala med intratumoralnim vbrizganjem DNA in aplikacijo električnih pulzov. Vbrizgana gola DNA je v tkivu namreč podvržena razgradnji z v medcelični prisotnimi DNAzami. Iz tega bi lahko sklepali, da je pogoj za učinkovito transfekcijo čimprejšnji vnos plazmida v celice, da bi imeli izvencelični encimi čim manjšo možnost razgradnje DNA. Po drugi strani pa je molekulam DNA potrebno omogočiti, da se porazdelijo po tkivu in pridejo v stik s površino čim večjega števila celic pred aplikacijo električnih pulzov, za kar je potreben določen čas (131, 132). Z ustreznim časovnim razmikom med vbrizganjem DNA in aplikacijo električnih pulzov bi tako uravnotežili ta dva nasprotujoča si procesa.

Rezultati naše raziskave kažejo, da pri elektrotransfekciji tumorskega tkiva DNA dejansko potrebuje čas, da se porazdeli po tarčnem tkivu pred aplikacijo električnih pulzov. Na dveh tumorskih modelih, melanomu B16F1 in fibrosarkomu LPB, smo ugotovili najboljše izražanje reporterskega gena pri

skupinah, kjer smo električne pulze aplicirali 5 - 15 minut po intratumoralnem vbrizganju DNA. Ta časovni razmik je omogočil dovolj časa, da se je plazmidna DNA optimalno porazdelila v tumorskem tkivu. Po drugi strani pa se masivna razgradnja DNA z DNAzami še ni začela. Zato lahko sklepamo, da se ta verjetno začne približno pol do eno uro po vbrizganju DNA, saj smo pri obeh tumorskih modelih v tem času zaznali izrazit padec v nivoju izražanja reporterskega gena.

Z raziskavo smo tudi potrdili, da je za učinkovito *in vivo* elektrotransfekcijo nujna prisotnost DNA v tkivu pred oziroma med aplikacijo električnih pulzov, saj lahko le tako prestopi permeabilizirano celično membrano (26, 32, 46, 127). V poskusnih skupinah, kjer smo DNA vbrizgali v tumor šele po aplikaciji električnih pulzov, namreč ni prišlo do izražanja reporterskega gena.

Razlike v učinkovitosti transfekcije med različnimi tumorskimi modeli so bile že opisane (6, 66). Pripisujejo jih razlikam v celičnih in tkivnih lastnostih različnih tumorjev, predvsem gostoti celic, velikosti celic, prevodnosti tumorskega tkiva, lastnostim medcelične, itd. Rezultati naše raziskave te ugotovitve potrjujejo. Na mišjem melanomu B16F1 smo dosegli boljše izražanje luciferaze v primerjavi z fibrosarkomom LPB. Melanomi so po histološki zgradbi manj celični in imajo več medcelične v primerjavi z bolj kompaktnimi fibrosarkomi. To verjetno omogoča lažjo in hitrejšo porazdelitev molekul DNA po tumorskem tkivu, kar je razvidno tudi iz primerjave v izražanju reporterskega gena pri poskusnih skupinah, kjer smo plazmidno DNA vbrizgali 30 min pred elektroporacijo. Pri melanomskem tumorskem modelu je izražanje gena za luciferazo v tem časovnem intervalu značilno padlo, ker se je DNA zaradi hitrejše biodistribucije verjetno že izločila iz tkiva. Pri fibrosarkomu pa smo opazili tak drastičen padec izražanja gena za luciferazo verjetno zaradi počasnejše porazdelitve molekul pri skupinah, kjer smo DNA vbrizgali vsaj eno uro pred elektroporacijo.

4.1.2 Določitev najustreznejših parametrov za električno posredovani vnos plazmidne DNA v skeletno mišičnino miši

Mir s sodelavci je leta 1999 objavil eno prvih obsežnejših raziskav, s katero je poskušal določiti najustreznejše parametre za električno posredovani vnos DNA v skeletno mišičnino pri različnih vrstah poskusnih živali (miših, podganah, kuncih in opicah) (26). Ugotovili so, da podobno, kot to velja za tumorje, tudi na učinkovitost električno posredovanega vnosa plazmidne DNA v skeletno mišičnino vplivajo številni dejavniki. V naši raziskavi smo na miših ugotavljali vpliv različnih električnih pulzov, odmerka plazmida in časovni razmik med intramuskularno aplikacijo DNA in električnimi pulzi.

Med najpomembnejše dejavnike, ki vplivajo na učinkovitost elektrotransfekcije mišičnine, sodijo parametri uporabljenih električnih pulzov. Ti morajo zagotoviti električno polje ustrezne jakosti, s katerim dosežemo prehodno prepustnost celične membrane, vendar jakost električnega polja ne sme biti previsoka, da ne povzročimo trajnih poškodb tkiva. Za mišična vlakna je ugotovljeno, da je najvišja jakost električnega polja, ki še ne povzroči nepovratne elektropermeabilizacije, 600-800 V/cm (12, 133). Mir s sodelavci je ugotovil, da naj bi bil optimalni protokol aplikacija 8 električnih pulzov z amplitudo 200 V/cm, dolžino trajanja 20 ms in frekvenco 1 Hz (26). Ti pulzi so se kmalu uveljavili kot nekakšen standardni protokol za gensko elektrotransfekcijo mišice. Nekoliko kasneje se je začela uporabljati kombinacija visokonapetostnih in nizkonapetostnih pulzov. Raziskave (34, 49) so namreč pokazale, da imajo električni pulzi pri genskem elektrotransferju dve vlogi: elektropermeabilizacijo celične membrane, ki jo povzroči kratkotrajen visokonapetostni pulz (600-800 V/cm; 100 μs), temu pa sledijo nizkonapetostni pulzi (ponavadi pod 100 V/cm, dolžine nekaj 10 do 100 ms), ki omogočijo elektroforezo molekul DNA skozi destabilizirano celično membrano.

Na podlagi teh podatkov smo se pri določanju optimalnih parametrov električnih pulzov za gensko elektrotransfekcijo skeletne mišičnine osredotočili na primerjavo učinkovitosti petih različnih protokolov za elektroporacijo s pomočjo reporterskega gena, ki nosi zapis za zeleni fluorescirajoči protein. Pri dveh izmed protokolov smo uporabili le nizkonapetostne pulze; med njima je bil protokol, identičen Mirovemu standardnemu protokolu (8 električnih pulzov, 200 V/cm, 20 ms, 1 Hz), drugi pa je bil izbran zato, ker je že bil opisan kot uspešen za gensko elektrotransfekcijo mišičnine pri psih (6 električnih pulzov, 100 V/cm, 60 ms, 1 Hz) (61, 62, 63). Omenjena protokola smo primerjali s tremi, pri katerih smo uporabili kombinacijo enega viskonapetostnega pulza (600 V/cm, 100μs) in različnega števila nizkonapetostnih pulzov (1 x 400 ms; 4 x 100 ms; 8 x 50 ms; vsi 80 V/cm).

Rezultati naše raziskave kažejo, da kombinacija visoko- in nizkonapetostnih pulzov omogoča višji nivo izražanja transgena, ki traja dlje časa, v primerjavi z uporabo samo nizkonapetostnih pulzov, kar je sicer v nasprotju z drugimi študijami, kjer niso ugotovili značilnih razlik v učinkovistosti transfekcije z uporabo različnih protokolov elektroporacije mišičnine oz. je kombinacija visoko- in nizkonapetnostnih pulzov vodila v nekoliko slabše izražanje transgenov v primerjavi z Mirovim standardnim protokolom (49, 128).

Naslednji parameter protokola, katerega vpliv na učinkovitost elektrotransfekcije smo analizirali, je bil časovni razmik od vbrizganja plazmidne DNA do aplikacije električnih pulzov. Podatki o tem so si precej nasprotujoči. Nekateri raziskovalci priporočajo aplikacijo električnih pulzov takoj po

vbrizganju DNA, drugi pa ugotavljajo, da je lahko časovni razmik od vbrizganja DNA do elektroporacije tudi do 3 ure (127, 128, 129). V naši raziskavi smo primerjali časovne intervale od 5 sekund do 2 uri in ugotovili, da z vbrizganjem DNA tik pred aplikacijo električnih pulzov (< 5s) dosežemo statististično značilno boljšo učinkovitost transfekcije v primerjavi z uporabo daljših časovnih razmikov. To je v skladu z ugotovitvami nekaterih drugih raziskav (129), kjer so potrdili najučinkovitejšo transfekcijo pri uporabi časovnega razmika nič sekund. Učinkovitost transfekcije je z daljšanjem časovnega razmika hitro upadala, saj smo pri razmiku ene minute dosegli že približno 50 % slabše izražanje reporterskega plazmida. Iz tega lahko torej sklepamo, da je razgradnja in izločanje plazmidne DNA iz mišičnega tkiva bistveno hitrejši proces kot v tumorskem tkivu, kjer je prišlo do drastičnega zmanjšanja transfekcije šele po 30 - 60 minutah. Glede na to lahko rečemo, da se večina DNA v mišici razgradi oz. odplavi iz tkiva v času ene minute po vbrizganju.

Tudi odmerek oziroma koncentracija uporabljene plazmidne DNA naj bi imela na učinkovitost transfekcije mišice velik vpliv (129). Predhodno je bilo že večkrat potrjeno, da večja količina DNA, ki je na voljo za vstop v celice, zagotavlja višjo učinkovitost transfekcije (58, 134, 135, 136), pri čemer naj bi imela višji vpliv koncentracija DNA od njene absolutne skupne količine (129). V naši raziskavi smo uporabili enak volumen raztopine (20 µl) z različno koncentracijo in s tem različnim kumulativnim odmerkom. Nivo kratkotrajnega izražanja transgena je bil očitno odvisen od odmerka vbrizgane DNA, saj smo najvišjo učinkovitost transfekcije dosegli z navečjim odmerkom plazmida (30 µg). Za večmesečno izražanje transgena pa je bilo potrebno vbrizgati vsaj 10 µg plazmidne DNA, saj smo z nižjimi odmerki dosegli le kratkotrajno nekajtedensko izražanje transgena. Zanimivo je dejstvo, da smo z uporabo 5 µg plazmida kratkotrajno dosegli le nekoliko slabšo transfekcijo v primerjavi z višjimi odmerki. Ta učinek je pri nizkem odmerku izzvenel v obdobju enega tedna po transfekciji, medtem ko so višji odmerki omogočili bistveno daljše izražanje transgena. To bi lahko imelo klinično uporabno vrednost v primerih, ko želimo gensko zdravljenje časovno omejiti le na kratek čas, ker bi dolgotrajno izražanje terapevtskega gena lahko imelo preveč neželenih stranskih učinkov.

Na koncu smo na miših preučili še vpliv elektrotransferja na poškodbe mišičnih vlaken. Tkivo na elektrotransfer DNA odreagira z nekrozo in infiltracijo vnetnih mononuklearnih celic (46, 137). V naši raziskavi smo ugotovili, da elektrotransfer plazmida, ki nosi zapis za GFP, nekroze mišičnih vlaken sicer ne povzroči, pride pa do infiltracije vnetnih celic v mišično tkivo, predvsem limfocitov in plazmatk. Ta je bila prisotna pri vseh poskusnih skupinah, ki smo jim vbrizgali plazmidno DNA, vendar je bila najočitnejša po elektrotransferju. Za razliko od tega pa pri poskusnih skupinah, ki smo jim aplicirali le električne pulze brez DNA, tega pojava nismo zasledili. Torej lahko sklepamo, da

infiltracija vnetnih celic ni posledica uporabe električnih pulzov, pač pa reakcijo izzove vbrizgana plazmidna DNA, kar se ujema z dognanji v nekaterih drugih podobnih raziskavah (138).

4.1.3 Protitumorska učinkovitost EGT z *IL-12* pri zdravljenju mišjega tumorskega modela sarkoma mehkih tkiv

Sarkomi mehkih tkiv predstavljajo v humani medicini okrog 1 % vseh malignih tumorjev pri odraslih in približno 15 % pri otrocih (139). V veterinarski medicini pa med vsemi kožnimi in podkožnimi tumorji zajemajo približno 7 % pri psih in 15 % pri mačkah (140). Kljub relativno majhni incidenci predstavljajo velik problem, saj so izredno lokalno invazivni. Zaradi tega je kirurško zdravljenje z lokalno odstranitvijo tumorskega tkiva s širokimi varnostnimi robovi še vedno glavna metoda zdravljenja teh vrst tumorjev tako v humani kot v veterinarski medicini. V humani medicini je petletno preživetje pacienta približno 35–65 %, pri čemer je velikost tumorskega nodula najpomembnejši prognostični dejavnik (139, 141). Druge klasične metode zdravljenja, kot sta kemoterapija in radioterapija, so bistveno manj učinkovite. Kljub agresivnem kirurškem zdravljenju so lokalne ponovitve pogoste, kar izredno poslabša dolgoročno prognozo pacienta. Zato se išče nove metode zdravljenja, s katerimi bi lahko izboljšali izid terapije in preživetje pacientov.

Za gensko terapijo z *IL-12* je že bilo ugotovljeno, da je uspešna metoda zdravljanje različnih vrst sarkomov, predvsem z uporabo različnih adenovirusnih vektorjev (106, 142, 143, 144, 145). Rezultati teh raziskav kažejo, da je virusni vnos gena za IL-12 lahko učinkovit pri zaviranju rasti sarkomov in podaljšanju preživetja zdravljenih poskusnih živali. Čeprav virusni vektorji praviloma zagotavljajo visok nivo transdukcije tarčnega tkiva in s tem dober protitumorski učinek, ima njihova uporaba tudi številne slabosti, ki postavlja pod vprašaj varnost njihove uporabe v klinični praksi. Gre predvsem za stimulacijo pacientovega imunskega sistema, potencialno toksičnost sistemske aplikacije in insercijsko mutagenezo transduciranih celic (146, 147, 148). Ena od možnosti za zmanjšanje sistemske toksičnosti virusnega vnosa gena za IL-12 je omejitev izražanja terapevtskega gena le na določeno omejeno tarčno tkivo (npr. tumorski nodul) z neposrednim intratumoralnim vnosom virusnih vektorjev. Na ta način lahko delno omejimo toksičnost zdravjenja zaradi bistveno manjše sistemske proizvodnje IL-12 in posledično manjše stimulacije proizvodnje IFN-γ (145). Vendar pa ta pristop lahko še vedno izzove določene stranske učinke, verjetno zaradi uhajanja virusnih partiklov v sistemsko cirkulacijo in posledično okužbo parenhimskih organov in s tem stimulacijo imunskega sistema (110, 149). Zato predstavljajo nevirusni vektorji varnejšo alternativo virusnemu vnosu terapevtskih genov. Njihove prednosti so predvsem v tem, da niso imunogeni in kužni, so manj toksični in proizvodnja ustreznih količin je v primerjavi z virusnimi vektorji lažja in cenejša (9). Od nevirusnih tehnik vnosa gena za IL-

12 so se na sarkomih izkazali za potencialno učinkovite: neposredna aplikacija gole DNA brez uporabe drugih fizikalnih ali kemičnih metod vnosa, uporaba biobalistične metode (t.i. "*gene gun*") in vnos s pomočjo polietileniminskega vektorja (142, 150, 151, 152, 153). Maloštevilne preizkušene nevirusne tehnike vnosa terapevtskega gena so se izkazale za nekoliko manj učinkovite na sarkomu v primerjavi z adenovirusnimi vektorji.

EGT kot metoda vnosa terapevtskega gena na sarkomih do sedaj še ni bila opisana. Ugotovljeno je namreč bilo, da je uspešnost transfekcije s pomočjo elektroporacije lahko celo primerljiva z virusnimi vektorji, kar ji daje veliko prednost pred drugimi nevirusnimi metodami, ki imajo praviloma dosti slabšo transfekcijo (126). Prav tako v objavljeni literaturi ni mogoče najti primerjave protitumorske učinkovitosti med intra- in peritumoralnim vnosom terapevtskega gena s pomočjo EGT. Zaradi tega smo se odločili, da preizkusimo protitumorsko delovanje EGT z *IL-12* na sarkomih in primerjamo lokalno in sistemsko protitumorsko delovanje obeh tehnik vnosa terapevtskega gena.

Predhodne raziskave so pokazale, da elektrotransfekcija kože lahko vodi do lokalnega ali sistemskega izločanja vnešenega transgena (78, 154, 155). Intradermalni električno posredovani vnos gena za IL-12, je izzval 10-kratno zvečanje sistemske koncentracije IFN-γ v primerjavi s kontrolnimi skupinami (81). Kako je s sproščanjem IL-12 v sistemski krvni obtok po intratumoralnem vnosu plazmida, ni povsem jasno, saj nekateri raziskovalci navajajo, da so IL-12 v serumu zaznali (115, 121), drugi pa niso dosegli sistemskega izločanja citokina (70, 109, 110). Na podlagi teh rezultatov smo predvidevali, da bi z intradermalnim vnosom terapevtskega plazmida tik ob tumorju lahko dosegli tako sistemsko kot učinkovito lokalno intratumoralno izražanje transgena.

V naši raziskavi smo dosegli močan lokalni protitumorski učinek s statistično značilnim zastojem rasti zdravljenih tumorjev. Intratumoralni vnos je omogočil preko 90 % ozdravitev živali z dolgotrajnim izkoreninjenjem tumorskih nodulov, kar je primerljiv rezultat z učinkovitostjo adenovirusne genske terapije z *IL-12* na sarkomih, kjer so dosegli do največ 70–80 % popolni odgovor zdravljenih živali (142, 143, 145). Peritumoralni vnos je bil učinkovit pri 16 % zdravljenih živali. Lokalni učinek je tako primerljiv z rezultati nevirusnih tehnik zdravljenja sarkomov z genom za IL-12 (151, 152, 153). Poleg tega je bil lokalni protitumorski učinek v naši raziskavi statistično značilno boljši tudi v primerjavi z drugimi tumorskimi modeli, na katerih so preverjali protitumorski učinek EGT z *IL-12*. Z istim odmerkom plazmida so na melanomskem tumorskem modelu dosegli popoln odgovor pri 47 % zdravljenih živali (70). Boljšo učikovitost (60–80 %) so dosegli šele z 2 do 3-kratno ponovitvijo aplikacije EGT ali z dodatnim intramuskularnim vnosom terapevtskega plazmida (71). Pri karcinomu

je bil protitumorski učinek še slabši v primerjavi z našimi rezultati, saj so z dvema zaporednima aplikacijama intratumoralne EGT dosegli le 40 % ozdravitev (114).

Doseženi lokalni protitumorski učinek je posledica zelo visoke intratumoralne koncentracije IL-12 in IFN-γ, ki smo jo ugotovili v zdravljenih tumorjih. Koncentracija IL-12 v tumorjih je dosegla vrednost 53.6 ± 13.1 ng/mg tumorskega tkiva po intratumoralni in 22.5 ± 8.7 ng/mg tumorskega tkiva po peritumoralni EGT. Koncentracije IFN-γ so bile nekoliko nižje, in sicer 11.6 ± 2.1 ng/mg tumorskega tkiva po intratumoralni in 6,7 ± 1,6 po peritumoralni EGT. V raziskavah o protitumorski učinkovitosti genskega zdravljenja sarkomov z *IL-12* so le redko določali intratumoralno koncentracijo kateregakoli od omenjenih citokinov. Edini primerljivi podatek je naveden v študiji, ki so jo objavili Jia in sodelavci leta 2006. V tej raziskavi so izvedli dva zaporedna vnosa terapevtskega gena s pomočjo adenovirusnega vektorja neposredno v tumorske nodule. Dva dni po drugi terapiji so določili intratumoralno koncentracijo IL-12, ki je znašala 40 pg/mg tumorskega tkiva in IFN-γ, ki ni presegla 400 pg/mg tumorskega tkiva (150). V naši raziskavi smo 5 dni po enkratni EGT ugotovili statistično značilno višje intratumoralne koncentracije IL-12 in IFN-γ v primerjavi s kontrolnimi skupinami tako pri intra- kot peritumoralni metodi EGT. Koncentracije obeh citokinov smo izražali v nanogramih na mg tumorskega tkiva, torej v redu velikosti 100 do 1000-krat več kot poročajo Jia in sodelavci (150).

Nekoliko več podatkov je v literaturi dostopnih za dosežene intratumoralne vrednosti teh dveh citokinov pri EGT na drugih tumorskih modelih. Lucas s sodelavci je ugotovil, da intratumoralna koncentracija tako IL-12, kot IFN-γ pri melanomih doseže najvišjo vrednost 5 dni po enkratno izvedeni intratumoralni EGT (70). V tej raziskavi so z istim odmerkom plazmida, kot je bil uporabljan pri naši študiji, dosegli koncentracije 3 pg/mg tumorskega tkiva za IL-12 in 8,16 pg/mg tumorskega tkiva za IFN-γ, kar je bistveno nižje od naših rezultatov. Intratumoralno izražanje obeh citokinov po EGT z genom za IL-12 so določali še v drugih raziskavah na karcinomu poroženevajočih celic, hepatocelularnem karcinomu in melanomu (110, 114, 117), vendar so koncentracijo citokinov izražali v razmerju na količino beljakovin v lizatu tumorja, zaradi česar rezultate težje primerjamo z našimi. Rezultati teh raziskav kažejo, da so z EGT dosegli približno 10 do 50-kratno zvečanje intratumoralne koncentracije IL-12 oz. 3 do 10-kratno zvečanje IFN-γ v primerjavi s kontrolnimi skupinami. V naši raziskavi je bilo to zvečanje približno 300-kratno za IL-12 in približno 10-kratno za IFN-γ pri obeh načinih izvedbe EGT.

V raziskavi smo prav tako dokazali sistemski protitumorski učinek zdravljenja. Intra- in peritumoralna EGT z *IL-12* je namreč omogočila razvoj dolgotrajne odpornosti na ponovno rast tumorjev, kar smo dokazali s ponovno podkožno aplikacijo iste vrste tumorskih celic živalim 100 dni po ozdravitvi.

Ugotovili smo, da je po intratumoralno izvedeni EGT bilo preko 60 % živali odpornih na ponovno rast sarkomov.

Druga oblika sistemskega delovanja EGT pa se je pokazala kot vpliv zdravljenja primarnega tumorja na upočasnitev rasti oddaljenih nezdravljenih nodulov, rastočih na kontralateralnem boku živali, kar je verjetno tudi posledica antiangiogenega delovanja cirkulirajočega sistemskega IL-12 (70). Zaostanek v rasti nezdravljenih tumorskih nodulov ni bil statistično značilno različen med intra- in peritumoralno poskusno skupino, kar je najverjetneje posledica dejstva, da so bile sistemske koncentracije obeh citokinov statistično enake pri obeh načinih izvedbe.

Primer oddaljenih vplivov protitumorskega zdravljenja z gensko terapijo z *IL-12* je bil v predkliničnih raziskavah prikazan pri zdravljenju Ewingovega sarkoma z adenovirusnim vektorjem (145) in pri intratumoralni in intramuskularni EGT melanomov (71). Tudi v prvi humani klinični študiji o učinku intratumoralne EGT z *IL-12* pri zdravljenju malignega melanoma je bil ugotovljen sistemski protitumorski učinek na nezdravljenih nodulih drugje po telesu (107). Med omenjenima predkliničnima raziskavama in našo obstajata dve pomembni razliki v izvedbi poskusnega protokola. V naši raziskavi smo oba tumorska nodula inducirali sočasno in sta rastla sočasno, s čimer smo želeli simulirati pogosto klinično situacijo, ko pri pacientu najdemo in zdravimo več tumorskih nodulov hkrati. V drugih dveh raziskavah pa so nezdravljene tumorje inducirali šele več dni potem, ko so živali že imele eno ali več zdravljenj. Torej je bil pri nas uporabljen terapevtski pristop k zdravljenju že izraščenih tumorskih nodulov, za razliko od profilaktičnega pristopa, uporabljenega v ostalih raziskavah. Druga pomembna razlika je v tem, da so naše poskusne živali prejele le enkratno EGT, medtem ko so genski vnos terapevtskega gena v drugih omenjenih raziskavah ponovili večkrat (npr. 5-kratna terapija pri Ewingovem sarkomu) (145).

Pomembna se nam v naši raziskavi zdi ugotovitev, da smo z enkratno intra- in peritumoralno EGT dosegli statistično značilno višje sistemske koncentracije tako IL-12, kot IFN-γ v primerjavi s kontrolnimi skupinami. Serumska koncentracija IL-12 je v naši raziskavi dosegla vrednost 16,4 ± 8,8 pg/ml po intratumoralni in 19,7 ± 6,9 po peritumoralni EGT. Tudi pri merjenju IFN-γ smo zaznali podobno situacijo, in sicer smo s peritumoralno EGT dosegli nekoliko višje serumske vrednosti IFN-γ (86,1 ± 21,6 pg/ml) kot z intratumoralnim vnosom terapevtskega gena (66,3 ± 15,2 pg/ml). Za razliko od teh rezultatov pa pri intratumoralnem zdravljenju sarkomov z virusnim načinom vnosa *IL-12* na MethA fibrosarkomu ni prišlo do sistemskega izločanja nobenega od omenjenih citokinov (143). Pri MCA205 fibrosarkomu je Gambotto s sodelavci v primerjavi z našo raziskavo dosegel bistveno višje serumske koncentracije tako IL-12 (približno 8 ng/ml) kot IFN-γ (približno 4 ng/ml) (142). Morda je

ravno v tem tudi razlog za boljšo dolgotrajno imunost ozdravljenih živali na ponovno izraščanje tumorjev po naknadni aplikaciji tumorskih celic ozdravljenim živalim. V omenjeni raziskavi (142) avtorji namreč poročajo o 100 % imunosti v primerjavi z rezultati naše raziskave, kjer je bilo odpornih 61 % ozdravljenih živali.

Nasprotujoči si podatki o sistemskem izločanju IL-12 in IFN-γ pri lokalni genski terapiji obstajajo tudi pri uporabi EGT kot genski vnosni metodi. V več raziskavh na melanomskem tumorskem modelu namreč niso dosegli zaznavnih sistemskih koncentracij citokinov, tudi ne s ponavljajočimi se EGT (70, 109, 110). Po drugi strani pa so na različnih karcinomih dosegli serumske koncentracije, podobne našim (115, 121), vendar le po večkratnih izvedbah EGT. Eden od možnih razlogov za bistveno boljše lokalno in sistemsko izločanje transgena v primerjavi z omenjenimi raziskavami je razlika v uporabljenem protokolu za električno posredovani vnos plazmidne DNA v tumorje. Nekatere študije (70, 109) so bile izvedene z uporabo kratkotrajnih visokonapetostnih električnih pulzov (okrog 100 μs; 1300-1500 V/cm), kar označujemo kot t. i. *ECT pulze* (električni pulzi, ki se uporabljajo pri elektrokemoterapiji). V naši raziskavi so bili uporabljeni t. i. *EGT pulzi*, za katere je značilno dolgo trajanje (reda velikosti nekaj deset do sto milisekund) z nizko amplitudo (100-600 V/cm). EGT pulzi namreč omogočajo bistveno boljšo elektrotransfekcijo tkiva v primerjavi z uporabo ECT pulzov za električno posredovani vnos plazmidne DNA v tumorje, kot je bilo dokazano na SA-1 sarkomu in B16F1 melanomu s pomočjo dveh različnih reporterskih plazmidov (72). Iz tega lahko sklepamo, da je bistveno boljša protitumorska učinkovitost naše EGT, izvedene s pomočjo tega terapevtskega protokola, posledica boljše transfekcije tkiva zaradi uporabe optimalnih pogojev elektroporacije tarčnega tkiva.

4.3 PROTITUMORSKA UČINKOVITOST EGT Z *IL-12* PRI ZDRAVLJENJU SPONTANIH TUMORJEV PSOV

4.3.1 Intramuskularna EGT z *IL-12*

Podatke o vplivu različnih dejavnikov na učinkovitost eletrotransfekcije mišičnega tkiva, pridobljene na raziskavah na miših, smo uporabili za učinkovit električno posredovani vnos plazmidne DNA v skeletno mišičnino poskusnih psov.

Med seboj smo primerjali učinkovitost istih petih protokolov električnih pulzov kot pri miših in prišli do podobnih rezultatov. Z uporabo reporterskega gena za GFP smo dosegli najvišjo transfekcijo bodisi z uporabo strandardnega nizkonapetostnega protokola (8 pulzov, 200 V/cm, 20 ms, 1 Hz) ali s

kombinacijo enega visokonapetostnega pulza (600 V/cm, 100 μs) in štirih nizkonapetostnih pulzov (80 V/cm, 100 ms, 1 Hz). Pomembne razlike v izražanju reporterskega gena med tema dvema protokoloma nismo ugotovili. Pri obeh najučinkovitejših protokolih je bilo izražanje transgena enako tako 2 kot 7 dni po izvedeni elektrotransfekciji. Glede na objavljene rezultate drugih raziskav na psih izražanje trasgena v skeletni mišici psov traja bistveno dlje; Tone s sodelavci je zaznal dovolj visok nivo transgenih proizvodov, ki so omogočali biološke učinke še vsaj 6 mesecev po enkratni aplikaciji plazmida (63). Zaradi tega lahko predpostavljamo, da je izražanje transgena v mišici psov dejansko bistveno daljše od sedmih dni, kot smo to zaznali v naši raziskavi, vendar smo zaradi etičnih razlogov poskus zasnovali tako, da smo predvideli le dve mišični biopsiji pri poskusnih živalih, zaradi česar nismo mogli preverjati izražanja transgena dlje kot en teden.

Pri poskusnih psih smo tudi nadzorovali možne stranske učinke postopka. Lokalne stranske učinke smo preverjali z rednimi fizikalnimi pregledi mesta na nogi, kjer smo izvedli električno posredovani vnos plazmida. Sistemske stranske učinke smo spremljali z rednimi kliničnimi pregledi živali in določanjem hematoloških in biokemijskih parametrov v krvnih vzorcih, odvzetih pred posegom in v različnih časovnih intervalih po posegu.

Lokalni stranski učinki so zajemali pojav blage otekline na mestu elektroporacije, ki pa ni vplivala na lokomotorno funkcijo živali. Oteklino lahko delno pripisujemo tudi poškodbi tkiv zaradi invazivne izvedbe elektroporacije (incizija kože in fascije, s čimer smo omogočili lažji dostop do mišice). Do podobnih zaključkov so prišli tudi drugi avtorji, kjer pri veliko živalih opisujejo le prehodne blažje stranske učinke na mestu elektroporacije mišice in brez kakršnihkoli trajnih poškodb kože ali mišice (61, 62).

Elektrotransfekcija reporterskega plazmida, ki nosi zapis za GFP, v naši raziskavi ni povzročila nikakršnih sistemskh stranskih učinkov. Hematološki in biokemijski parametri v odvzetih krvnih vzorcih so ostali znotraj referenčnih vrednosti skozi vse obdobje opazovanja. Prisotnost kliničnih poškodb skeletnih mišičnih vlaken smo ugotavljali z določanjem aktivnosti encima kreatin kinaze (CK) v serumu živali, ki je zelo občutljiv kazalec poškodbe mišičnine (156). Fewell s sodelavci je spremljal aktivnost CK pri psih po izvedenem intramuskularnem električno posredovanem vnosu plazmida, ki nosi zapis za humani koagulacijski faktor IX (60). Ugotovil je prehodno zvečanje serumske aktivnosti CK, ki pa je bilo odvisno od količine vbrizganega plazmida in števila mest elektroporacije mišičnine. Živali, ki so jim plazmid vbrizgali na več mestih in posledično izvedli elektroporacijo večje količine mišičnine, so imele značilno višjo aktivnost CK. Pri naših poskusnih živalih nismo zaznali nikakršnega značilnega zvečanja aktivnosti CK v serumu, saj smo plazmid

vbrizgali na največ dveh mestih, v primerjavi s 6 in 12 mesti vnosa v Fewellovi raziskavi. Iz tega lahko sklepamo, da elektroporacija skeletne mišičnine pod našimi pogoji ne povzroča klinično pomembne mišične poškodbe, kljub temu da je bila elektrotransfekcija skeletne mišičnine učinkovita.

Edini negativni učinek električno posredovanega vnosa plazmida v mišičnino pri psih so bile izrazite mišične kontrakcije med aplikacijo električnih pulzov. Mišične kontrakcije so glavni vir bolečine pri ljudeh med postopkom elektrokemoterapije (157). Rutinski nadzor vitalnih funkcij pacientov med anestezijo je pokazal zvečano frekvenco srčnega utripa in dihanja med samo aplikacijo električnih pulzov, kar je sicer normalni odziv na bolečinski dražljaj tudi pri ustrezno anestezirani živali. Iz tega lahko sklepamo, da je sam postopek elektroporacije mišičnine pri psu boleč, zaradi česar je nujno, da se izvaja v splošni anesteziji in z ustrezno analgezijo.

Na podlagi teh rezultatov smo izvedli še preliminarno študijo o izvedljivosti, učinkovitosti in morebitni toksičnosti električno posredovanega vnosa plazmida, ki nosi zapis za humani IL-12, na poskusnih psih. Ta gen predhodno namreč še ni bil uporabljen za gensko zdravljenje psov. Zanj smo se odločili zaradi tega, ker imata pasji in humani IL-12 približno 90-odstotno homolognost aminokislinskega zaporedja (158). Ugotovljeno je bilo, da je humani IL-12 v *in vitro* pogojih biološko aktiven, saj aktivira proliferacijo in imunski odgovor mononuklearnih levkocitov v periferni krvi pri psih (159), zaradi česar smo predpostavljali, da bi podobne imunološke učinke lahko izzvali tudi *in vivo*.

Za električno posredovani vnos plazmida smo uporabili dva protokola, ki sta se na psih izkazala kot najuspešnejša pri transfekciji mišičnine psov, in sicer uporaba kombinacije enega visokonapetostnega pulza in štirih nizkonapetostnih ter Mirovega standardnega protokola za elektrotransfekcijo skeletne mišičnine (8 pulzov, 200 V/cm, 20 ms, 1 Hz).

Na šestih psih smo preizkusili tri različne odmerke plazmida, 100 μg, 500 μg in 1 mg. Vsakega od odmerkov smo vbrizgali dvema poskusnima živalima, pri eni od njiju smo elektroporacijo izvedli z Mirovim protokolom, pri drugi pa s kombinacijo visoko- in nizkonapetostnih pulzov. Humani IL-12 smo zaznali v serumu le ene živali, in sicer tiste, ki je prejela 1 mg terapevtskega plazmida in pri kateri smo uporabili protokol s kombinacijo enega visokonapetostnega in 4 nizkonapetostnih pulzov. Humani IL-12 smo zaznali v serumu, odvzetem 7 dni po izvedeni EGT, v koncentraciji 19 pg/ml. Čeprav naj bi bil humani IL-12 biološko aktiven tudi pri psih in naj bi izzval proizvodnjo IFN-γ, interferona pri tej živali nismo zaznali v nobenem krvnem vzorcu, odvzetem 7 ali več dni po izvedeni EGT. Možno je, da je bila sistemska koncentracija IL-12 prenizka, da bi izzvala biološki odziv

organizma s tvorbo IFN-γ. Zato bi bilo potrebno bodisi zvečati enkratni odmerek ali pa izvesti več zaporednih terapij z istim odmerkom.

Sistemska aplikacija rekombinantnega proteina IL-12 je povezana s številnimi resnimi stranskimi učinki, saj se je IL-12 izkazal za hepato- in nefrotoksičnega (96). Aplikacija visokih odmerkov IL-12 pa je povezana z začasno imunosupresijo, kar pa pri imunoterapiji še posebej ni zaželeno (96). Toksičnost EGT z *IL-12* so ocenjevali pri mišjem modelu melanoma (109). Intratumoralna terapija je imela izrazit lokalni protitumorski učinek brez sistemsko zaznavnih koncentracij IL-12. Edini stranski učinek, ki so ga ugotovili, je bilo blago fokalno vnetje ledvičnega tkiva 30 dni po izvedeni terapiji, brez hematoloških ali biokemičnih sprememb, ki bi nakazovale na zmanjšano ledvično funkcijo. Kljub temu pa se pri vsaki terapiji z IL-12 priporoča hematološki in biokemijski nadzor ledvične funkcije.

Pri naših poskusnih živalih smo morebitno toksičnost zdravljenja nadzorovali z določanjem hematoloških (hemogram z diferencialno belo krvno sliko) in biokemijskih parametrov (urea, kreatinin, AP, ALT, CK) v krvnih vzorcih, odvzetih v različnih časovnih intervalih po izvedeni EGT. Vsi parametri so ves čas opazovanja ostali v mejah referenčnih vrednosti, z le posameznimi individualnimi odstopanji, ki pa klinično niso bila pomembna oz. jih ne moremo povezovati s samim postopkom, kot je blaga hemokoncentracija zaradi dehidracije ipd. Klinična slika živali se v opazovanem obdobju ni spremenila. Pri živalih nismo opazili nikakršnih odstopanj v količini zaužite vode ali hrane oz. v splošnem stanju. Torej sama EGT z *IL-12* ni povzročila nobenih klinično pomembnih stranskih učinkov.

Glede na dobljene rezultate bi bilo v prihodnosti smiselno predklinično raziskavo na poskusnih psih nadgraditi s klinično raziskavo na psih z različnimi novotvorbami, da bi določili, ali sistemsko izločanje humanega IL-12 po intramuskularni EGT pri psih zadostuje za lokalni in sistemski protitumorski učinek.

4.3.2 Intratumoralna EGT z *IL-12*

Učinke in varnost izvedbe intratumoralne EGT z *IL-12* smo izvedli v obliki klinične študije na psih, ki so bili na Kliniko za kirurgijo in male živali Veterinarske fakultete Univerze v Ljubljani sprejeti z namenom zdravljenja spontano nastalih kožnih mastocitomov (MCT). Za zdravljenje MCT smo se odočili zaradi njihovih specifičnih lastnosti. V prvi vrsti zaradi pogostosti pojavljanja, saj gre za najpogostejše maligne tumorje psov, ki predstavljajo približno 21 % vseh kožnih tumorjev pri psih (160, 161). Mastocitomi imajo zelo raznoliko biološko obnašanje: od tumorjev z zelo nizko stopnjo malignosti do zelo invazivnih tumorjev z visokim metastatskim potencialom, zaradi česar je odločanje

za najustreznejšo vrsto zdravljenja lahko zelo težko in so možnosti zdravljenja omejene. Prognostični dejavniki mastocitomov namreč zajemajo številne lastnosti MCT, od histološkega gradusa in klinične stopnje bolezni, do lokacije nodula, hitrosti rasti, stopnje celične proliferacije, Ki67 indeksa in velikosti, ter lastnosti pacienta, predvsem pasme, starosti in spola živali (162, 163). Poleg tega se za mastocitome predpostavlja, da imajo imunološko etiologijo, kar nakazuje na možnost, da bi bila zanje lahko posebej primerna oblika zdravljenja imunoterapija.

Metode zdravljenja MCT so odvisne od prognostičnih dejavnikov in klinične stopnje tumorja. Možna je radikalna kirurška odstranitev nodula s širokim varnostnim robom, kar pri dobro diferenciranih MCT vodi v odlično prognozo (160, 161). Slabo diferencirani in anaplastični tumorji pa imajo slabšo prognozo s krajšim preživetjem. Pri teh tipih tumorjev je kirurško terapijo potrebno nadgraditi še z drugo vrsto terapije, npr. radio- ali kemoterapijo, ki je edina izbira tudi pri živalih, kjer kirurško zdravljenje ni mogoče oz. pri napredovali bolezni (161, 164). Kljub invazivni terapiji je prognoza slabo diferenciranih mastocitomov relativno slaba. Zato bi bilo za to vrsto tumorjev dobrodošla še kakšna druga učinkovita vrsta zdravljenja, ki bi bodisi izboljšala prognozo preživetja ali zmanjšala potrebo po radikalni kirurški odstranitvi tumorja, ki lahko pomeni v primeru, ko se tumorski nodul nahaja na okončini, tudi amputacijo okončine.

Intratumoralna EGT se je na predkliničnem nivoju izkazala kot zelo uspešna metoda lokalnega zdravljenja različnih tumorjev z možnostjo sistemskega protitumorskega učinka. Leta 2008 so bili objavljeni rezultati prve klinične študije na pacientih z metastatskim melanomom (107). Rezultati te raziskave so pokazali, da z intratumoralno EGT z *IL-12* dosežemo tako lokalni kot sistemski protitumorski učinek, ki se kaže kot regresija zdravljenih tumorjev in zmanjšanje nezdravljenih oddaljenih tumorskih nodulov.

V veterinarski medicini so to vrsto genske terapije do sedaj uporabili pri treh raziskavah na psih: s plazmidi za *hIL-12* in s kombinacijo *IL-6* in *IL-15* so transfecirali inducirani TVT (74, 75), z elektrokemo-gensko terapijo, pri kateri so uporabili kombinacijo ECT z bleomicinom in EGT z *IL-12*, pa so transfecirali tumorje vratu in glave (73). Pri vse treh študijah so dosegli dober protitumorski učinek z regresijo zdravljenih nodulov, poleg tega pa so dokazali tudi vpliv na oddaljene metastaze oz. nezdravljene tumorje (73, 75) ter razvoj dolgotrajne protitumorske zaščite (75) in sistemsko izločanje IL-12 (75).

Klinično študijo smo izvedli na enajstih tumorskih nodulih pri osmih psih, ki so ustrezali vključitvenim kriterijem za klinično študijo. Z intratumoralno EGT z *IL-12* na MCT smo dosegli statistično značilno zmanjšanje velikosti zdravljenih nodulov. Zmanjšanje volumna tumorjev sicer ni

bilo tako izrazito v primerjavi z rezultati predklinične študije na sarkomskem tumorskem modelu oziroma v primerjavi z rezultati drugih raziskav na melanomu in karcinomu (70, 71, 115). V dosedanjih raziskavah se je izkazalo, da je stopnja protitumorskega učinka lokalne EGT odvisna tudi od histološkega tipa tumorja. Pri določenih tipih tumorjev lahko namreč dosežemo le kratkotrajno upočasnitev rasti zdravljenih nodulov, pri drugih pa popolno dolgotrajno izkoreninjenje.

Podatki o lokalni protitumorski učinkovitosti intratumoralne genske terapije z *IL-12* na mastocitomu so zelo skopi, saj je trenutno objavljena le ena raziskava (165). V njej so mišje P815 mastocitome zdravili z biobalistično metodo vnosa terapevtskih genov intradermalno v neposredno okolico tumorjev. S šestimi zaporednimi terapijami so dosegli upočasnitev rasti tumorjev oz. zmanjšanje njihovega volumna za približno 60 % tri tedne po izvedeni terapiji, vendar pri nobenem od zdravljenih živali niso uspeli doseči popolne regresije. V isti raziskavi so na bolj imunogenem MethA fibrosarkomu z istim načinom zdravljenja dosegli bistveno boljše protitumorske učinke, med drugim tudi številne popolne odgovore na terapijo.

V naši raziskavi so se zdravljeni mastocitomi zmanjšali za približno 52 % v času 1-2 tedna po izvedeni terapiji, kar je primerljivo z rezultati zdravljenja mišjega modela mastocitoma z biobalistično metodo. Možno je, da bi s povečevanjem števila ponovitev terapije pri psih dosegli še večjo redukcijo tumorjev, saj je na mišjem modelu bilo za značilno zmanjšanje volumna tumorjev potrebno izvesti šest zaporednih ponovitev terapij (165). Rezultate lahko primerjamo tudi z rezultati raziskave, kjer so plazmid za IL-12 vbrizgali neposredno v nodule melanomov pri konjih (104). Tu so s tremi zaporednimi aplikacijami 250 μg terapevtskega plazmida dosegli zmanjšanje 11 od 12 zdravljenih tumorskih nodulov, med njimi celo popolno regresijo enega tumorja. V tej raziskavi so dosegli povprečno zmanjšanje volumna za 69 %, vendar pa je bil ta učinek le kratkega trajanja, saj so 30 dni po izvedeni terapiji zdravljeni tumorji začeli zopet rasti. Pri naših pacientih smo na večini zdravljenih nodulov en do dva tedna po zadnji EGT izvedli citoreduktivno terapijo (bodisi kirurško odstranitev nodula ali ECT), zato ne moremo predvidevati nadaljnje dinamike rasti teh tumorjev. Štirje od nodulov, pri katerih pa citoreduktivne terapije nismo izvedli, so ostali enake velikosti in se niso povečali na izhodiščno velikost v 12, 36 in 44 mesecih opazovanja.

Histološka analiza zdravljenih tumorjev je pokazala očitne morfološke spremembe. Z EGT smo v zdravljenih tumorjih dosegli zmanjšanje števila tkivnih bazofilcev (za katere se je v klinični veterinarski medicini uveljavil izraz mastociti). Poleg tega smo v zdravljenih tumorjih ugotovili še masovno infiltracijo tumorskega tkiva z limfociti in plazmatkami ter degranulacijo preostalih mastocitov. Ti rezultati so v skladu z rezultati podobnih raziskav, kjer je bilo ugotovljeno, da je intra- in peritumoralna limfocitna infiltracija glavna histološka sprememba v tumorjih, zdravljenih z

intratumoralno gensko terapijo (70, 104, 107, 108, 166). Pomen T limfocitov za protitumorski učinek intratumoralne EGT z *IL-12* je bil potrjen z ugotovitvijo, da s to terapijo dosežemo protitumorski učinek le pri imunokompetentnih živalih, za razliko od atimičnih miši brez T limfocitov (70, 108). Imunološki odgovor zdravljenih tumorjev v naši raziskavi je še nadaljnji pokazatelj, da je humani IL-12 dejansko biološko aktiven pri psih tudi *in vivo*, in ne le na *in vitro* nivoju.

Predhodno objavljene raziskave o učinku EGT na psih so sicer potrdile, da je sistemsko izločanje transgenega produkta možno, vendar je bila večina teh raziskav izvedenih z intramuskularno EGT (60, 61, 62, 63). Vendar pa so z dvema od zadnjih objavljenih raziskav na psih (74, 75) ugotovili, da je sistemsko izločanje transgenov pri psih možno tudi po lokalni intratumoralni EGT. Primerljive rezultate smo dosegli tudi v naši raziskavi. Podobno kot pri predhodni raziskavi na miših s sarkomskim tumorskim modelom smo tudi pri psih z intratumoralno EGT uspeli doseči sistemsko izločanje humanega IL-12 in pasjega IFN-γ. IL-12 in/ali IFN-γ smo zaznali pri štirih pacientih v vzorcih, odvzetih v različnih časovnih obdobjih po izvedeni terapiji (7 do 28 dni). Serumske koncentracije humanega IL-12 so se gibale med 1 in 12,2 pg/ml, IFN-γ pa smo zaznali v koncentracijah med 123 in 388.1 pg/ml.

Pri lokalni genski terapiji s plazmidom za IL-12 je priporočljivo, da dosežemo poleg lokalne intratumoralne proizvodnje IL-12 tudi njegovo sistemsko izločanje in delovanje. Lokalno intratumoralno izražanje IL-12 je pomembnejše za lokalni protitumorski učinek, ki vodi v regresijo tumorjev (70, 108, 145). Po drugi strani pa je cirkulirajoči IL-12 odgovoren za sistemske protitumorske učinke, npr. učinek na oddaljene nezdravljene tumorje in preprečevanje metastaziranja (70, 108, 145, 124), kar smo dosegli tudi v naših predkliničnih raziskavah na sarkomskem tumorskem modelu. Ta učinek naj bi bil posledica antiangiogenega delovanja IL-12 (70, 86). Sistemsko izražanje oz. izločanje transgenega proizvoda se ponavadi doseže s sistemsko gensko terapijo, to je npr. s transfekcijo skeletne mišičnine, kože ali jeter. Pri transfekciji teh tkiv je sistemsko izločanje kodiranega transgena pričakovano, za razliko od transfekcije tumorskega tkiva, ki ima povsem druge lastnosti. Zato je detekcija sistemskega izločanja terapevtskega proteina po lokalni intratumoralni EGT zanesljivo dodatna prednost za klinično uporabo te genske terapije. Na ta način namreč lokalno protitumorsko delovanje razširimo še na sistemski nivo z možnostjo protimetastatskega delovanja, učinka na nezdravljene oddaljene nodule in indukcije protitumorske imunosti, s čimer bi lahko podaljšali preživetje zdravljenih pacientov.

Pomembna ugotovitev naše raziskave je, da intratumoralna EGT z *IL-12* na mastocitomih psov ne izzove klinično pomembnejših stranskih učinkov. Pri terapiji smo bili pozorni na dve vrsti možnih stranskih učinkov, ki bi bili bodisi posledica izločanja histamina iz granul v mastocitomih ali pa

posledica toksičnega delovanja IL-12. Znano je namreč, da mehanska manipulacija mastocitomov lahko privede do degranulacije mastocitov in posledičnega izločanja histamina. Ta je odgovoren bodisi za lokalne stranske učinke, ki se kažejo kot peritumoralni edem in eritem, lahko pa izzove tudi resnejše sistemske stranske učinke, npr. gastrointestinalne razjede ali klinične znake, ki ogrožajo življenje, med drugim hipotenzijo, različne aritmije in bronhospazem. Pri zdravljenih pacientih nismo zaznali nobenega od kliničnih znakov, ki bi jih lahko pripisali izločanju histamina, čeprav se mehanski manipulaciji tumorjev nismo mogli povsem izogniti, saj smo pri izvedbi EGT uporabljali invazivne igelne elektrode, ki se jih vstavi intratumoralno.

Druga skupina možnih kliničnih znakov stranskih učinkov je povezana s toksičnim delovanjem IL-12. V naši raziskavi smo pacientom odvzeli kri v rednih časovnih razmakih in preverjali parametre jetrne in ledvične funkcije, vendar klinično pomembnih odstopanj v omenjenih parametrih nismo zaznali. Prav tako ni prišlo do sprememb v kliničnem stanju zdravljenih živali oz. sprememb v apetitu, količini zaužite vode ali splošnem obnašanju.

Rezultati naše raziskave torej kažejo, da je intratumoralna EGT z *IL-12* na mastocitomih psov izvedljiva, preprosta in varna terapevtska metoda, s katero dosežemo tako lokalno izražanje kot sistemsko izločanje IL-12.

5 SKLEPI

- Ugotovili smo, da je transfekcija tumorskega tkiva najučinkovitejša, če elektroporacijo tumorjev izvedemo 5–15 minut po intratumoralnem vbrizganju plazmidne DNA in da je nivo izražanja reporterskega gena med tumorskimi modeli različen.

- Najboljše izražanje reporterskega gena za GFP v skeletni mišičnini miši smo dosegli z uporabo kombinacije enega visokonapetostnega pulza (600 V/cm, 100 μs) in štirih nizkonapetostnih pulzov (4 x 80 V/cm, 100 ms, 1 Hz), apliciranih manj kot 5 sekund po intramuskularni aplikaciji 30 mikrogramov plazmida.

- Ugotovili smo, da intramuskularno vbrizgana DNA povzroči infiltracijo skeletne mišičnine z vnetnimi mononuklearnimi celicami brez nekroze mišičnih vlaken.

- Izkazalo se je, da je intratumoralna in peritumoralna EGT z *IL-12* zelo učinkovita metoda zdravljenja sarkomov mehkih tkiv pri miših, z izrednim lokalnim protitumorskim učinkom na primarne zdravljene tumorje in sistemskim protitumorskim učinkom na oddaljene nezdravljene nodule.

- Potrdili smo, da pri intratumoralni in peritumoralni EGT z *IL-12* pride do visokega lokalnega intratumoralnega izražanja in sistemskega izločanja IL-12 in IFN-γ.

- Ugotovili smo, da sta za uspešno transfekcijo skeletne mišičnine pri psih primerna dva protokola elektroporacije: bodisi uporaba 8 nizkonapetostnih električnih pulzov (8 x 200 V/cm, 20 ms, 1 Hz) ali pa kombinacija enega visokonapetostnega pulza (600 V/cm, 100 μs) in štirih nizkonapetostnih pulzov (4 x 80 V/cm, 100 ms, 1 Hz). Oba povzročata le blage lokalne stranske učinke brez zaznavnih sistemskih stranskih učinkov.

- Z intramuskularno aplikacijo 1 mg plazmida, ki nosi zapis za *hIL-12*, in uporabo kombinacije enega visoko- in štirih nizkonapetostnih pulzov smo pri psih dosegli sistemsko izločanje humanega IL-12.

- Ugotovili smo, da z intratumoralno EGT z *IL-12* dosežemo protitumorski učinek na kožnih mastocitomih psov, ki se je izkazal kot statistično značilno zmanjšanje velikosti zdravljenih tumorjev s spremembo v njihovi histološki zgradbi.

- Potrdili smo, da pride pri psih po intratumoralni EGT z *IL-12* do sistemskega izločanja humanega IL-12 iz transfeciranih tumorjev in indukcije interferonskega odgovora.

6 POVZETEK

Elektroporacija ali elektropermeabilizacija je metoda, pri kateri z uporabo kontroliranih električnih pulzov, ki sami po sebi nimajo negativnega vpliva na preživetje celic, dosežemo prehodno prepustnost celične membrane in na ta način lahko v celice vnesemo različne molekule. Za vnos genskega materiala v celico s pomočjo elektroporacije se uporablja izraz električno posredovani vnos DNA ali genska elektrotransfekcija oziroma elektrogenska terapija, kadar s pomočjo elektroporacije v tkivo vnašamo terapevtske gene. Ta se izvede z aplikacijo gole plazmidne DNA v tarčno tkivo, čemur sledi elektroporacija tarčnega tkiva z ustreznimi električnimi pulzi, ki omogočijo prehod DNA skozi celično membrano.

IL-12 je proinflamatorni citokin z različnimi vplivi na imunski sistem organizma, med drugim sproži proizvodnjo IFN-γ, ima antiangiogene učinke in regulira imunski odziv organizma s pomočjo tvorbe Th1 in citotoksičnih T limfocitov. Zaradi teh lastnosti so bile izvedene številne študije o učinku genske terapije s terapevtskim genom, ki nosi zapis za IL-12, na različnih tumorskih modelih, ki so pokazale, da ima IL-12 izrazit protitumorski učinek.

Namen doktorskega dela je bil določiti protitumorski učinek elektrogenske terapije s plazmidom, ki nosi zapis za interlevkin-12, pri zdravljenju različnih induciranih tumorjev miši ter izvesti klinično študijo o učinkih te vrste genske terapije na spontane tumorje psov. Končni namen dela pa je bil uvesti to terapijo v klinično prakso zdravljenja različnih tumorjev psov.

Na učinkovitost električno posredovanega vnosa DNA v tkivo vplivajo številni dejavniki, na primer parametri apliciranih električnih pulzov, razmik med vbrizganjem plazmida in aplikacijo električnih pulzov, lastnosti tarčnega tkiva in uporabljenega plazmida, način aplikacije DNA itd. Zato smo v prvem delu raziskav s pomočjo reporterskih genov za luciferazo in GFP izvedli optimizacijo protokola električno posredovanega vnosa DNA v tumorje in v ta namen določili naoptimalnejši časovni razmik med intratumoralnim vbrizganjem plazmidne DNA in aplikacijo električnih pulzov ter izvedli sistemsko analizo vpliva različnih parametrov na učinkovitost elektrotransfekcije skeletne mišičnine pri miših in psih.

Ugotovili smo, da je transfekcija tumorskega tkiva najučinkovitejša, če elektroporacijo tumorjev izvedemo 5–15 minut po intratumoralnem vbrizganju plazmidne DNA. Gola DNA je po vbrizganju v tarčno tkivo namreč podvržena dvema procesoma: encimski razgradnji z DNAzami, zaradi česar bi bilo za uspešnejšo transfekcijo primerneje, da bi plazmid čim prej vstopil v celice, po drugi strani pa

se mora plazmid pred elektroporacijo dobro razporediti po tkivu in priti v stik s čim večjim številom tarčnih celic, da bi omogočili transfekcijo čim večje količine tkiva. Omenjeni razmik je očitno ustrezen kompromis med tema dvema nasprotujočima si procesoma, ki omogoča dobro razporeditev plazmida po tkivu, predno se začne masovna razgradnja molekul DNA. Ugotovili smo tudi, da je nivo izražanja reporterskega gena med različnimi tumorskimi modeli različen, kar pripisujemo razlikam v celičnih in tkivnih lastnostih tumorjev.

Za določitev najustreznejšega protokola za elektrotransfekcijo skeletne mišičnine pri miših smo primerjali učikovitost petih različnih protokolov elektroporacije, pri dveh izmed njih smo uporabili samo nizkonapetostne pulze (8 električnih pulzov z 200 V/cm, 20 ms, 1 Hz oz. 6 električnih pulzov z 100 V/cm, 60 ms, 1 Hz), ker sta bila to protokola, ki sta bila v dostopni literaturi najpogosteje uporabljena za električno posredovani vnos v skeletno mišičnino. Primerjali smo ju s tremi protokoli, kjer smo uporabili kombinacijo enega visokonapetostnega pulza (600 V/cm, 100 μs) in enega (1 x 80 V/cm, 400 ms), dveh (2 x 80 V/cm, 200 ms, 1 Hz) ali štirih (4 x 80 V/cm, 100 ms, 1 Hz) nizkonapetostnih pulzov. Poleg tega smo ugotavljali vpliv odmerka apliciranega plazmida in časovnega razmika med aplikacijo plazmida in električnih pulzov na učinkovitost transfekcije mišičnine. Najboljše izražanje transgena, ki je trajalo 1,5 leta, smo dosegli z uporabo kombinacije enega visokonapetostnega pulza in štirih nizkonapetostnih pulzov, apliciranih manj kot 5 sekund po intramuskularni aplikaciji 30 μg plazmida. Preverjali smo tudi negativne učinke EGT na mišična vlakna in ugotovili, da intramuskularno vbrizgana DNA povzroči infiltracijo skeletne mišičnine z vnetnimi mononuklearnimi celicami.

Rezultate, ki smo jih pridobili v prvem delu raziskav, smo uporabili za ugotavljanje protitumorske učinkovitosti EGT z *IL-12* pri zdravljenju induciranih tumorjev miši. Primerjali smo učinkovitost intratumoralne in peritumoralne EGT z *IL-12* na mišji SA-1 fibrosarkom. V predhodnih predkliničnih študijah na sarkomih mehkih tkiv se je genska terapija z *IL-12* sicer že izkazala za učinkovito, vendar EGT kot vnosni sistem za terapevtski gen na sarkomih do sedaj še ni bil uporabljen. EGT z *IL-12* se je izkazala kot zelo učinkovita metoda zdravljenja sarkomov, saj smo z obema načinoma aplikacije dosegli izreden protitumorski učinek s statistično značilnim zastojem rasti zdravljenih tumorjev. Z intratumoralno EGT smo dosegli ozdravitev pri preko 90 % in s peritumoralno EGT pri 16 % zdravljenih živali. Lokalni protitumorski učinek je bil posledica izredno visoke intratumoralne koncentracije IL-12 in IFN-γ v zdravljenih tumorjih.

Poleg lokalnega smo dosegli tudi sistemski protitumorski učinek, in sicer razvoj dolgotrajne odpornosti na ponovno rast tumorjev pri preko 60 % živali, ozdravljenih z intratumoralno EGT. Druga

oblika sistemskega protitumorskega delovanja pa se je izkazala kot vpliv zdravljenja primarnega tumorja na upočasnitev rasti oddaljenih nezdravljenih tumorjev. To smo dosegli tako z intra- kot s peritumoralno EGT, brez statistično značilnih razlik med obema načinoma aplikacije. Predpostavljamo, da je to delovanje tudi posledica antiangiogenega delovanja cirkulirajočega sistemskega IL-12, ki smo ga zaznali v serumu zdravljenih živali.

Pri psih smo s pomočjo reporterskega gena za GFP primerjali učinkovitost istih petih protokolov elektroporacije kot pri predklinični raziskavi na miših, in ugotovili, da sta se pri psih izkazala za najboljša dva: standardni nizkonapetostni protokol (8 električnih pulzov z 200 V/cm, 20 ms, 1 Hz) in kombinacija enega visokonapetostnega in štirih nizkonapetostnih pulzov. Oba protokola sta povzročila le blage lokalne stranske učinke, vidne kot blažja oteklina tkiva na mestu aplikacije plazmida, ki pa je izzvenela v nekaj dneh. Sistemskih stranskih učinkov, ki bi bili vidni kot odstopanja v kliničnem stanju oziroma v merjenih hematoloških in biokemijskih parametrih, nismo zaznali. Na podlagi teh rezultatov smo izvedli še preliminarno raziskavo o učinkih električno posredovanega vnosa plazmida, ki nosi zapis za humani IL-12 pri psih, da bi ugotovili izvedljivost, učinkovitost izražanja in morebitno toksičnost postopka, saj do sedaj omenjeni gen še ni bil uporabljen za gensko zdravljenje psov. Pri tem smo ugotovili, da z intramuskularno aplikacijo 1 mg plazmida in uporabo kombinacije enega visokonapetostnega in štirih nizkonapetostnih pulzov dosežemo sistemsko izločanje humanega IL-12 pri psih brez kakršnihkoli stranskih učinkov.

Učinke intratumoralne EGT z *IL-12* smo ugotavljali na enajstih kožnih mastocitomih pri osmih pacientih.. Ugotovili smo, da z intratumoralno EGT z *IL-12* dosežemo lokalni in sistemski protitumorski učinek. Lokalni učinki te oblike zdravljenja so zajemali statistično značilno 52-odstotno zmanjšanje volumnov in spremembe v histološki zgradbi zdravljenih tumorjev. Histološka analiza nodulov je pokazala očitne spremembe morfologije tumorjev po izvedeni EGT z zmanjšanjem števila mastocitov, masovno infiltracijo tumorskega tkiva z limfociti in plazmatkami ter degranulacijo preostalih mastocitov. Sistemski učinki zdravljenja pa so se izkazali kot sistemsko izločanje humanega IL-12 iz transfeciranih tumorjev in indukcija interferonskega odgovora pri zdravljenih živalih.

Pomembna ugotovitev tega dela raziskave je tudi, da intratumoralna EGT z *IL-12* na mastocitomih psov ne izzove klinično pomembnih stranskih učinkov. Znano je namreč, da mehanska manipulacija mastocitomov lahko privede do degranulacije mastocitov in posledičnega izločanja histamina, ki v skrajnem primeru lahko povzroči celo življenjsko ogrožujoče klinične znake. Intratumoralna EGT ni povzročila klinično zaznavne histaminske reakcije, kljub uporabi invazivnih intratumoralnih elektrod.

Prav tako smo na tem tumorskem modelu potrdili, da cirkulirajoči humani IL-12, ki se sprosti v krvni obrok po EGT, ne izzove nobenih stranskih učinkov, ki jih povezujemo s toksičnim delovanjem IL-12.

Rezultati naše raziskave torej kažejo, da je intratumoralna EGT z *IL-12* na mastocitomih psov izvedljiva, preprosta in varna terapevtska metoda, s katero dosežemo tako lokalno izražanje kot sistemsko izločanje humanega IL-12.

7 SUMMARY

Electroporation or electropermeabilization is a method for the delivery of various molecules into cells by transiently increasing the permeability of the cell membrane using the application of a controlled external electric field to the cells without effecting their viability. Electroporation-based DNA delivery into tissue is called electrically-assisted gene delivery, or electrogene therapy when therapeutic genes are used. It is performed by the application of naked plasmid DNA into target tissue, followed by electroporation using appropriate electric pulses, which allow cellular uptake of the DNA across the cell membrane.

IL-12 is a proinflammatory cytokine, displaying a wide range of immunomodulatory effects, including induction of Th-1 and cytotoxic lymphocytes, triggering an IFN-γ response and antiangiogenic effects. A number of studies on a variety of tumor models have already been performed using therapeutic gene encoding IL-12, showing a pronounced antitumor effect of IL-12 in *in vivo* settings.

The aim of this work was to determine the antitumor effect of electrogene therapy using plasmid encoding IL-12 in the treatment of various induced tumors in mice and to perform a clinical study defining the effects of this type of therapy in spontaneously occurring tumors in dogs. The end purpose of the work was to introduce *IL-12* electrogene therapy into veterinary clinical practice for the treatment of oncologic patients.

Studies have shown that the transfection efficiency of electrically-assisted gene delivery into tissue depends on various factors, including the parameters of applied electric pulses, the time lag between the application of the plasmid and delivery of the electric pulses, target tissue properties, as well as the method of DNA injection etc. Optimization of a protocol for electrically-assisted DNA delivery was therefore performed in the first part of the work, using reporter genes encoding luciferase and GFP. For this purpose, the optimal time lag between intratumoral injection of plasmid DNA and the delivery of electric pulses was determined and an analysis of the effect of various parameters on the efficiency of electrotransfection of murine and canine skeletal muscle was performed.

It was established that the highest transfection efficiency in tumors is obtained if electroporation of the tumor nodule is performed 5 - 15 minutes after intratumoral application of the plasmid DNA. Naked DNA is subjected to two different processes in tissue. The first is enzymatic degradation with DNAses, which can be prevented by the rapid uptake of DNA intracellularly, thus increasing transfection efficiency. On the other hand, the DNA must be evenly distributed throughout the tissue before delivery

of electric pulses to allow contact with as many cells as possible, in order to achieve transfection of a large portion of the target tissue. This time interval appears to be a suitable compromise between these two opposing processes, allowing good distribution of plasmid throughout the target tissue before mass degeneration of the DNA molecules begins. In addition, it was demonstrated that the level of transficiency depends on the tumor model, which is possibly due to the specific cellular and tissue properties of the tumor tissue.

Optimization of the electroporation protocol for efficient transfection of skeletal muscle in mice was performed by comparing five different electroporation protocols. Two of them were selected based on the fact that they were the most commonly used in published reports on electroporation of muscle and they utilized only low-voltge pulses (8 pulses with 200 V/cm, 20 ms, 1 Hz and 6 pulses with 100 V/cm, 60 ms, 1 Hz). They were compared to three different protocols in which a combination of one high-voltage pulse (600 V/cm, 100 μs) and different numbers of low voltage pulses (1 x 80 V/cm, 400 ms or 2 x 80 V/cm, 200 ms, 1 Hz or 4 x 80 V/cm, 100 ms, 1 Hz) was used. Additionally, the effect on transfection efficiency of the plasmid dose and the time lag between plasmid application and electric pulse delivery was determined. The highest transfection efficiency, which lasted 1.5 years, was achieved using one high-voltage and four low-voltage pulses, delivered less than 5 seconds after intramuscular injection of 30 micrograms of plasmid. In this part of the study, the side effects of EGT on myofibres were also studied, demonstrating that intramuscularly injected plasmid DNA causes infiltration of inflammatory mononuclear cells into the treated muscle.

Using the parameters determined in the first part of the study, the antitumor effect of EGT with plasmid encoding IL-12 on induced murine tumors was investigated, comparing intratumoral and peritumoral EGT on SA-1 fibrosarcoma. Previous preclinical studies have already demonstrated the antitumor efficiency of *IL-12* gene therapy on soft tissue sarcomas, but EGT as a therapeutic gene delivery system has never been used on this tumor model. In this study, *IL-12* EGT proved to bea very efficient treatment modality of sarcoma, achieving a statistically significant growth delay in tumors treated by either intratumoral or peritumoral EGT application. Intratumoral *IL-12* EGT resulted in over 90% and peritumoral EGT resulted in 16% complete response rate of the treated tumors. This local antitumor effect was the result of an exceptionally high intratumoral concentration of IL-12 and IFN-γ in treated tumors.

In this experiment, in addition to the local effect, a systemic antitumor effect was also achieved, demonstrated by the induction of long-term antitumor resistance in over 60 % of animal, cured with intratumoral EGT. Furthermore, both intra- and peritumoral EGT delivery resulted in a significant

growth delay of untreated tumors, growing at distant sites. The mechanism responsible for this observed effect is probably the antiangiogenic effect of IL-12, which was released into systemic circulation.

In dogs, the reporter gene encoding GFP was used to compare the efficiency of the same five electroporation protocols as in the preclinical study on mice. The results of the study demonstrated that the highest transfection efficiency in canine muscle can be achieved with two different electroporation protocols - either the standard low-voltage protocol applying 8 electric pulses of 200 V/cm, 20 ms, 1 Hz or a combination of one high- and four low-voltage pulses. Both protocols resulted in only mild local side effects, seen as mild transitory tissue swelling at the site of plasmid injection, without any clinically detectable systemic side effects.

Based on these results, a preliminary study on the effect of electrically-assisted delivery of gene encoding human IL-12 in dogs was conducted, in order to determine the feasibility of this method on dogs, as well as the transfection efficiency and possible toxicity, since this therapeutic gene has never been used in dogs. It was determined that intramuscular injection of 1 mg of therapeutic plasmid resulted in systemic expression of human IL-12, without any side effects in the treated patients.

The antitumor effect of intratumoral *IL-12* EGT in dogs was determined on eleven cutaneous mast cell tumors in eight patients. We established that intratumoral *IL-12* EGT elicits a local and systemic antitumor effect in this tumor type. The local antitumor effect comprised a reduction in the size of the treated tumor and a change in the histological structure of treated nodules, seen as a reduction in the number of malignant mast cells and inflammatory cell infiltration with plasma cells and lymphocytes and degranulation of the remaining mast cells. In addition, systemic effects were achieved, including systemic release of hIL-12 from transfected tumors and induction of an IFN-γ response in treated animals.

An important finding of this part of the research was that intratumoral *IL-12* EGT in canine mast cell tumors did not elicit any clinically detectable adverse side effects. One of the major concerns of any mechanical manipulation of mast cell tumors is degranulation of mast cells, causing histamine release from the granules, which, in the worst case scenario, can even lead to life-threatening systemic signs. Despite using invasive needle electrodes inserted intratumorally, EGT did not cause any clinically detectable adverse histamine reaction. Furthermone, it was demonstrated that systemically released IL-12 in dogs does not cause any side effects that could be linked to a potential systemic toxicity of IL-12.

The results of this study show that intratumoral *IL-12* EGT on canine mast cell tumors is a feasible, simple and safe treatment option, eliciting both local expression and systemic release of IL-12.

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10 PRILOGE

PRILOGA A Objavljeni in v objavo sprejeti znanstveni članki

Sequence and Time Dependence of Transfection Efficiency of Electrically-Assisted Gene Delivery to Tumors in Mice

Maja Cemazar[1,*], Darja Pavlin[2], Simona Kranjc[1], Alenka Grosel[1], Suzana Mesojednik[1] and Gregor Sersa[1]

[1]*Institute of Oncology Ljubljana, Zaloska 2, 1000 Ljubljana, Slovenia,* [2]*University of Ljubljana, Veterinary Faculty, Cesta v Mestni log 47, 1000 Ljubljana, Slovenia*

Abstract: Electrically-assisted gene delivery is a non-viral gene delivery technique, using application of square wave electric pulses to facilitate uptake of plasmid DNA into the cells. Feasibility and effectiveness of this method in *vivo* was already demonstrated, elaborating on pulse parameters and plasmid construction. However, there were no studies performed on sequencing and timing of plasmid DNA injection into the tumors and application of electric pulses. For this purpose we measured luciferase expression in two tumor models (LPB fibrosarcoma, B16F1 melanoma) after electrically-assisted gene delivery at varying time intervals between the pCMV-Luc plasmid injection and electroporation. Expression of luciferase was determined by measurement of its activity using luminometer.

The results demonstrated that pCMV-Luc plasmid has to be injected before the application of electric pulses, since no measurable expression was detected in the tumors when pCMV-Luc plasmid was injected after electroporation of tumors. In both tumor models the highest transfection efficiency was obtained when pCMV-Luc plasmid was injected not less than *5* minutes but also not more than *30* minutes before the application of electric pulses. The results also demonstrated variability in the transfection efficiency depending on the tumor model. High expression was obtained in B16F1 tumor model *(~5500* pg luc/mg tumor) and lower in LPB fibrosarcoma *(~200* pg luc/mg tumor).

In conclusion, our results demonstrate that regardless of the susceptibility of the tumors to electrically-assisted gene delivery, the best timing for pCMV-Luc plasmid is between 30 to 5 minutes prior to the application of electric pulses to the tumors.

Keywords: Electroporation, delivery system, tumors, mice, luciferase.

INTRODUCTION

Gene therapy holds great promise for cancer treatment, however for routine clinical use many limitations have to be overcome. One of the major limitations still remains efficient and targeted transfection of the gene of interest. Transfection of cells with exogenous DNA can be achieved using different viral and non-viral techniques. The main advantage of viral vectors, among which adeno-, adeno-associated, retro- and herpes viruses are most frequently used, is their high transfection efficacy. On the other hand, some serious disadvantages pertinent to specific viral vectors, such as stimulation of the patient's immune system, toxicity of systemic application and insertional mutagenesis, raised concerns about safe clinical use of these vectors [1-3].

As an alternative to delivery of DNA with viral vectors, different non-viral gene delivery techniques have been developed, including injection of naked plasmid DNA, protein-DNA complexes, liposomes and their analogues, gene gun technique, and electroporation [2, 4]. All these techniques use naked plasmid DNA, which is noninfectious and non-immunogenic, with low toxicity and besides that a large amount of endotoxin-free DNA is relatively easy to produce [1, 2, 4, 5]. However, the major drawback of these techniques lies in low *in vivo* transfection efficiency, compared to viral methods. Therefore, it is crucial to improve and optimize these methods in order to augment transfection efficiency of non-viral gene delivery techniques to make them more applicable for use in clinical gene therapy.

One of the most promising non-viral gene delivery techniques is electroporation, which is already well established as *in virro* physical method for increased delivery of a range of different molecules, such as DNA and chemotherapeutic drugs, into different types of cells [4]. This method has already reached clinical testing as electrochemotherapy, which utilizes electroporation to locally enhance delivery of chemotherapeutic drugs into tumors of different histologies [6, 7]. Electroporation as *in vivo* gene delivery system has also been reported. It has been applied for efficient delivery of plasmid DNA (reporter and therapeutic genes) into different tissues, such as tumors, skeletal muscle, skin and liver. It was demonstrated that electrically-assisted gene delivery can greatly enhance transfection efficacy compared to direct injection of naked plasmid DNA alone [8 - 12].

Several different diseases were treated by electrogene therapy, including cancer, muscle disorders, such as muscle dystrophy, blood disorders and arthritis [12]. Furthermore,

*Address correspondence to this author at the Department of Experimental Oncology, Institute of Oncology, Zaloska 2, SI-1000 Ljubljana, Slovenia; Tel: +386 1 5879 544; E-mail: mcemazar@onko-i.si

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skeletal muscle could be used as a pool of the various molecules that expressed after electrically-assisted gene delivery and a shed into the blood stream where they act as therapeutic proteins. So far, reports were mainly on diabetes, anemia (erythropoietin) and DNA vaccines [13-16]. Since the obvious target of DNA vaccination is muscle or skin, electrogene therapy holds great promise in this field.

Studies showed that transfection efficiency of ***in vivo*** electrically-assisted gene delivery depends on various factors, including DNA and target tissue properties as well as parameters of applied electric pulses [12, 17, 18, 19]. Despite extensive research, all parameters for optimal transfection in tumors by electroporation are still not determined. Many different electroporation protocols (*i.e.* frequency, amplitude, duration and number of electric pulses used) have been proposed, yet several studies showed that the use of square wave electric pulses with lower amplitude and longer duration resulted in better transfection efficiency of tumors than the use of electric pulses with higher amplitude and shorter duration or exponential pulses [8, 10, 11, 17, 18, 20]. Besides parameters of electric pulses, the time interval between the plasmid DNA injection and application of electric pulses to the tissue of interest may play important role in achieving efficient transfection. In most of the studies the time interval between DNA injection and application of the electric pulses to the tumors was kept to minimum (from few seconds to 5 minutes) [8, 10, 12, 17, 18]. However, Cappelletti *et al.* have reported that intramuscular injection of plasmid DNA 30 minutes prior to application of electric pulses yielded better transfection of skeletal muscle compared to longer or shorter time interval [21]. In another study on electrically-assisted gene delivery to muscle, 20 seconds, 5 minutes, 1, 2, 3 and 6 hours intervals were tested. It was determined that there was no difference between transfection efficiency at the selected time intervals [22]. To our knowledge, there is only one report on electrically-assisted gene delivery to solid tumors (B16 melanoma) that tested two different time intervals, namely 1 and 10 minutes and there was no transfection detected at the 10 minutes interval [8].

Therefore, the aim of the present study was to determine optimal sequencing and timing of intratumoral plasmid DNA injection and application of electric pulses for efficient electrically-assisted gene delivery. For this purpose we used two different murine tumor models, LPB fibrosarcoma and B16F1 melanoma, and plasmid DNA encoding reporter gene luciferase (pCMV-Luc plasmid), which was injected intratumorally at different time points prior to and following application of electric pulses to the tumors and the level of its expression determined.

MATERIALS AND METHODS

Experimental Animals, Cell Lines and Tumor Models

Animal studies were carried out according to the guidelines of the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (permission #: 323-02-170/2004/2), and in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Bethesda MD).

In the experiments female C57Bl/6 mice, purchased from the Institute of Pathology, Faculty of Medicine, University of Ljubljana, Slovenia were used. At the beginning of the experiments, animals were 10-12 weeks old. Mice were kept in a conventional animal colony at constant room temperature (21°C) and natural daylnight light cycle. Food and water was provided *ad libitum*. Animals were subjected to an adaptation period of 7-10 days before experiments.

In this study, two different murine tumor cell lines were used: LPB fibrosarcoma, which is a clonal derivate of TBL.C12, a methylcholanthrene-induced C57Bl/6 mouse sarcoma tumor, and B16F1 melanoma (CLR6323; American Type Culture Collection, Manassas, VA). Cells were routinely maintained in Eagle minimal essential medium (EMEM; Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS; Sigma Chemical Co., St. Louis, MO, USA) and antibiotics in humidified atmosphere at 37°C, containing 5% CO_2. For tumor induction $1.3x10^6$ viable LPB and $1.0x10^6$ B16F1 cells/0.1 ml were prepared in EMEM supplemented with 2% FCS.

Solid LPB fibrosarcoma and B16F1 melanoma tumors were initiated by subcutaneous injection of cell suspension in the shaved right flank of mice. When the tumors reached approximately 6 mm in diameter the animals were randomly divided into experimental groups and subjected to specific experimental protocols. Experimental groups consisted of 6 animals.

Protocol of Electrically-Assisted Gene Delivery

To determine the optimal sequencing and timing of the plasmid DNA injection and application of electric pulses for efficient electrically-assisted gene delivery *in vivo*, plasmid DNA encoding luciferase (pCMV-Luc plasmid) was intratumorally injected at varying time points before and after the application of electric pulses on two different murine tumor models, and its expression measured.

The pCMV-Luc plasmid (generously provided by Dr. L.M. Mir) encoding reporter gene luciferase was prepared using the Quiagen Endo-Free Maxi kit (Quiagen, Hilden, Germany), according to manufacturer's instructions. Electrically-assisted gene delivery was performed by intratumoral injection of pCMV-Luc plasmid prior to or after application of electric pulses to the tumor. In the first experiments on LPB tumors pCMV-Luc plasmid (50 µg in 50 µl of water) was injected at different time points before and after (from 2 hours before to 30 minutes after) application of electric pulses to the tumors, which were delivered through two parallel stainless steel plate electrodes with 6 mm distance between them. The electrodes were placed percutaneously at the opposite margins of the tumor and eight square-wave electric pulses were delivered to the tumor in two sets of 4 pulses in perpendicular directions at frequency of 1 Hz, amplitude over distance ratio 600 V/cm and 5 ms duration. Good contact between electrodes and the overlying skin was assured by hair clipping and by use of a conductive gel. Electric pulses were generated by an electroporator Jouan GHT 1287 (Jouan, St. Herblain, France). The procedure was well tolerated by the animals and did not require general anesthesia. In subsequent experiments on both tumor models pCMV-Luc plasmid was injected only prior to application of

electric pulses to the tumors. Control groups received only intratumoral injection of pCMV-Luc plasmid without application of electric pulses.

Assessment of Transfection Efficacy

Tumors were excised 48 hours post-transfection, weighed, immediately frozen in liquid nitrogen and stored at -80°C until further procedures. Thawed tumors were homogenized in 1 ml of Cell Culture Lysis Reagent (Promega, Madison, WI) using sonificator. Thereafter the samples were centrifuged at 12000 x g for 10 minutes and the supernatant stored at -80°C. Luciferase activity was measured in thawed supernatants using TD-20120 luminometer (Turner design, Maude Avenue, Sunnyvale, CA). Photoemission was measured during 10-second period, starting 3 seconds after the addition of 100 µl Luciferase Assay Substrate (Promega, Madison, WI) to 20 µl of tumor cell lysate supernatant. Luciferase activity was quantified as relative light units and then converted to pg luciferase/mg tumor tissue, using the pre-prepared calibration curve of known luciferase quantities.

Statistical Analysis

All data were first tested for normality using the Kolmogorov-Smirnov normality test. In the case of luciferase concentration in B16F1 tumors the data were normally distributed, so the results were presented as arithmetic mean of luciferase concentration and standard error of the mean for each experimental group of animals. Significance tests were carried out using analysis of variance (ANOVA). Values of $P<0.05$ were considered as significant. In the case of luciferase concentrations in LPB tumors the data were not normally distributed, so the Kruskal Wallis ANOVA on Ranks was used, followed by Dunn's test for comparison (SigmaStat, Systat Software, Inc., Richmond, CA).

RESULTS

Transfection efficiency of electrically-assisted gene delivery using pCMV-Luc plasmid was dependent on the time interval between the pCMV-Luc plasmid injection and electroporation of tumors. In both tumor models the highest transfection efficiencies were obtained when pCMV-Luc plasmid was injected not less than 5 and not more than 30 minutes before electroporation of tumors (Fig. **1,2**). However, the amount of luciferase was higher in B16F1 tumors than in LPB tumors at all tested time points (Fig. **1,2**).

Specifically, in LPB tumors the pCMV-Luc plasmid was injected before and after the application of electric pulses to undoubtedly demonstrate that DNA needs to be present during the application of electric pulses for electrically-assisted gene delivery (Fig. 1). Injection of pCMV-Luc plasmid after the application of electric pulses did not result in measurable luciferase expression. Luciferase concentration was measurable only in those tumors that were injected with pCMV-Luc plasmid from 1 hour to 30 seconds before electroporation of tumors. However, the highest level of luciferase concentration was obtained when the pCMV-Luc plasmid was injected 5 to 30 minutes before application of electric pulses.

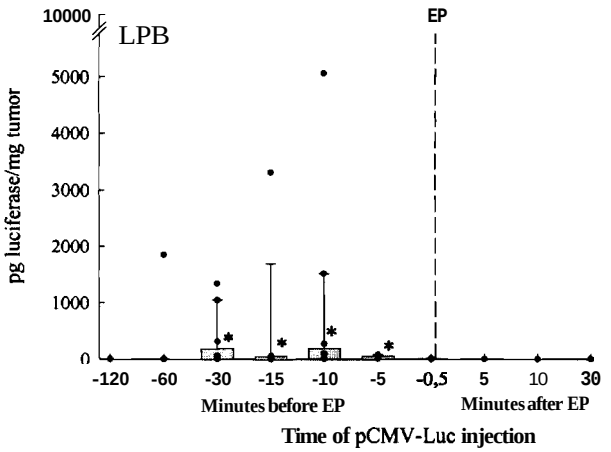

Fig. (1). Luciferase expression in LPB tumors. LPB tumors were exposed to electric pulses prior to or after intratumoral injection of 50 µg PCMV-Luc plasmid. Tumors were excised 48-h post-transfection and luciferase concentration determined using luminometer. Bars represent median of data pooled from 6 mice, error bars represent 25 and 75 percentile. Points revresent individual data. Levels of expression that are statistically significant different from control group (injected with plasmid DNA only) are labeled with asterisk.

The results in LPB tumors demonstrated that no expression of luciferase was obtained if electroporation preceded pCMV-Luc plasmid injection. Therefore, in another histologically different tumor model B16F1 melanoma, luciferase expression was measured only when pCMV-Luc plasmid was injected prior to electroporation of tumors (Fig. 2). A significant increase in luciferase expression was observed in B16F1 tumors, which were injected with pCMV-Luc plasmid 5 to 15 minutes before electroporation. In contrast to LPB fibrosarcoma, where at 30 minutes interval transfection efficiency was still in the range of 5-15 minutes time interval, in B16F1 tumors transfection efficiency at this time point was much lower compared to shorter time intervals.

DISCUSSION

Results of this study show that for efficient electrically-assisted gene delivery to solid tumors, the optimal sequencing and timing of plasmid DNA injection in relation to application of electric pulses is important. We demonstrate that the best time interval for efficient gene expression is injection of plasmid DNA not less than 5 minutes and not more than 30 minutes prior to the application of electric pulses to the tumors.

Delivery of genetic material to tumors still represents major obstacle for efficient gene therapy of cancer. Delivery of DNA to tumors and transfection of cells in tumors can be achieved using different viral vectors and a number of non-viral techniques, among which electrically-assisted gene delivery recently gained much attention as a feasible method of *in vivo* gene delivery, especially into skeletal muscles [12].

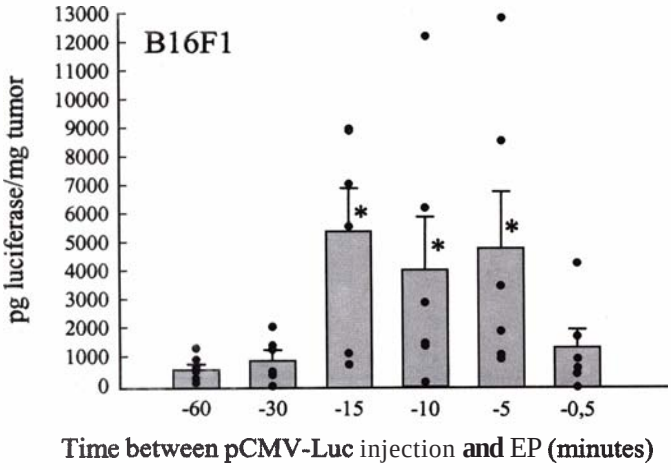

Fig. (2). Luciferase expression in B16F1 tumors. B16F1 tumors were exposed to electric pulses only after intratumoral injection of 50 μg PCMV-Luc plasmid. Tumors were excised 48-h post-transfection and luciferase concentration determined using luminometer. Bars represent arithmetic means of data pooled from 6 mice, error bars represent standard error of the mean. Points represent individual data. Levels of expression that are statistically significant different from control group (injected with plasmid DNA only) are labeled with asterisk.

So far, most of the work on optimization of electrically-assisted gene delivery was focused on determination of suitable electric pulse parameters for efficient transfection, whilst preserving tissue function and viability. Most of these studies were based on testing of different electroporation parameters [17, 18, 23]. Mathematical modeling, which could help to predict the suitable choice of electric pulse parameters for effective transfection is very difficult, due to the parameters that determine tissue characteristics, such as non spherical shape of the cells, as well as its non-homogenous composition (presence of different types of cells and blood vessels) [24]. Another important aspect is construction of plasmid DNA, especially with regard to the appropriate choice of promoters specific for induction of expression in the tissue of interest and for the duration of expression of specific therapeutic genes [25].

Distribution and degradation of plasmid DNA in tissue is another topic that has to be taken into account when using electrically-assisted gene delivery method. So far, this topic has been addressed only in the case of electrically-assisted gene delivery to the muscle. It was demonstrated that degradation of a large amount of injected DNA starts already 5 minutes after injection, but the level of transfection efficiency was not changed when testing intervals from 20 seconds to 3 hours, suggesting that injected DNA is proportionally partitioned between at least G o compartments:-one, in which DNA is quickly degraded and cleared and the other "electrotransferable" pool of DNA [22]. In addition, it was demonstrated that DNA is distributed over the large portion of muscle already 5 minutes after radiolabelled plasmid DNA injection with or without application of electric pulses [22]. In another study in muscle, significantly higher expression was obtained when plasmid DNA was injected 30 minutes before electroporation, compared to shorter intervals (10 and 5 minutes) [21]. Similar experiments were not done for solid tumors. In only one study it was indicated that the time interval should not be longer than 10 minutes [8]. In the present study we demonstrated on two tumor models that significant level of transgene expression is obtained only in a narrow time window of plasmid DNA injection before the application of electric pulses. In general, we can conclude that for efficient gene expression, DNA should not be injected more that 30 minutes and not less than 5 minutes before electroporation. Based on the known facts of DNA degradation in muscle, we can presume that degradation of DNA in tumors is a quicker process compared to muscle [22]. Furthermore, presuming the analogy of DNA degradation in muscle and tumors, and therefore existence of electrotransferable pool of DNA, based on our results, it is apparent that the degradation and clearance of DNA from this pool in the tumors is a quicker process.

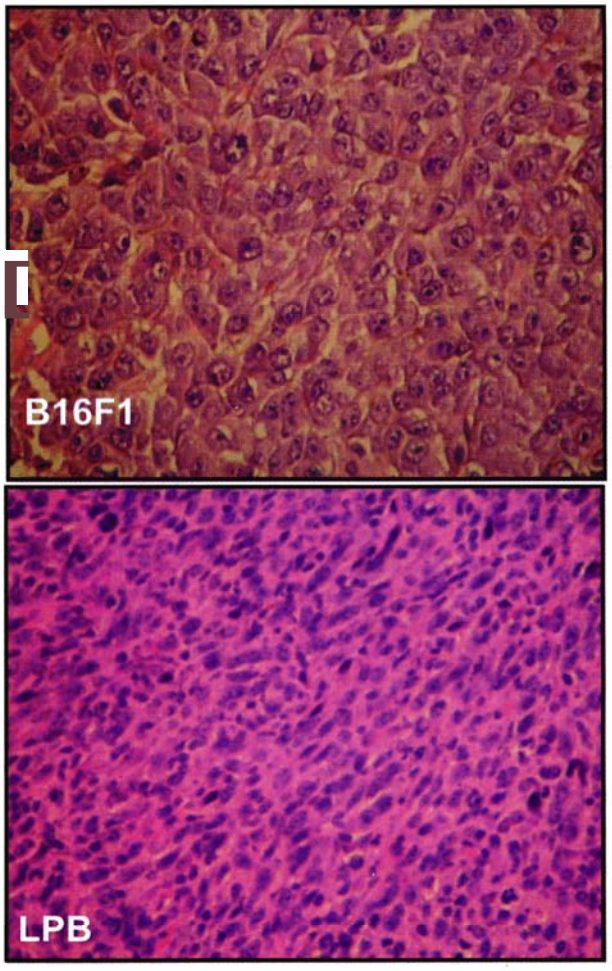

Fig. (3). Representative images of B16F1 and LPB tumors. Note the difference in cell density between the two tumor models. Tissue sections were stained with H&E according to the standard method (x20).

In addition, comparison of results between the two tumor models used showed that electrically-assisted gene delivery

to B16F1 melanoma yielded higher transfection efficiency compared to LPB fibrosarcoma. Specific properties of the tumor tissues. such as extracellular matrix of the tumor, tissue architecture, necrosis, tissue conductivity, tumor cell size and ability of tumor cell to express the transgene can influence transfection efficiency [17, 18]. The observed discrepancy in level of transfection is most probably due to the specific differences of the two tumor models used in this study. Histology of the tumor models showed that B16F1 melanoma tumors are less cellular with more extracellular space, therefore **DNA** distribution throughout the tumor should be easier and quicker process, which in turn should result in high transfection efficiency in contrast to more cellular and more compact LPB fibrosarcoma where distribution of **DNA** should be **a** slower process (Fig. **3).** In support to this presumption are the data obtained at 30 minutes interval. In less cellular B16F1 melanoma **DNA** was probably already cleared from the tumors 30 minutes after injection and therefore the transfection efficiency was at the level of **DNA** injection only, while in more cellular LPB tumors, where distribution of **DNA** should be slower process, at 30 minutes time interval the transfection efficiency was still significantly higher compared to **DNA** injection only. As already demonstrated cell density has impact on transfection efficiency of electrically-assisted gene delivery. Transfection efficiency was higher in tumors with lower cell density (*e.g.* B16 melanoma) compared to those with higher (*e.g.* SaF sarcoma) [17]. This observation can also be supported by the fact that modulation of extracellular space by pretreatment of tumors with hyaluronidase and/or collagenase leads to better transfection efficiancy [26, our unpublished data]. In addition, electric field intensity necessary for transfection is also likely to differ between the tumor models due to their different histological properties.

Furthermore, our results on electrically-assisted gene delivery to solid tumors for the first time confirm the data obtained at the single-cell level *in vitro* and in muscles *in vivo,* that also in tumors *in vivo* **DNA** has to be present during application of electric pulses to obtain an increase of transfection efficiency compared to the injection of plasmid **DNA** alone [21, 27, 28]. Specifically, no increase in expression of reporter gene was observed when the pCMV-Luc plasmid was injected after the application of electric pulses compared to injection of pCMV-Luc plasmid alone.

CONCLUSION

In conclusion, the results of this study show that for efficient electrically-assisted gene delivery to solid tumors, the optimal timing of plasmid **DNA** injection is between 5 up to 30 minutes prior to the application of electric pulses to the tumors. Further studies are needed to fully elaborate on the faith (distribution, degradation and clearance) of plasmid **DNA** following intratumoral injection in order to improve electrically-assisted gene delivery to tumors.

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ORIGINAL ARTICLE

The effect of the histological properties of tumors on transfection efficiency of electrically assisted gene delivery to solid tumors in mice

S Mesojednik[1], D Pavlin[2], G Sersa[1], A Coer[3], S Kranjc[1], A Grosel[1], G Tevz[1] and M Cemazar[1]
[1]Department of Experimental Oncology, Institute of Oncology Ljubljana, Zaloska 2, Ljubljana, Slovenia; [2]University of Ljubljana, Veterinary Faculty, Cesta v Mestni log 47, Ljubljana, Slovenia and [3]University of Ljubljana, Medical Faculty, Korytkova 2, Ljubljana, Slovenia

Uniform DNA distribution in tumors is a prerequisite step for high transfection efficiency in solid tumors. To improve the transfection efficiency of electrically assisted gene delivery to solid tumors in vivo, *we explored how tumor histological properties affected transfection efficiency. In four different tumor types (B16F1, EAT, SA-1 and LPB), proteoglycan and collagen content was morphometrically analyzed, and cell size and cell density were determined in paraffin-embedded tumor sections under a transmission microscope. To demonstrate the influence of the histological properties of solid tumors on electrically assisted gene delivery, the correlation between histological properties and transfection efficiency with regard to the time interval between DNA injection and electroporation was determined. Our data demonstrate that soft tumors with larger spherical cells, low proteoglycan and collagen content, and low cell density are more effectively transfected (B16F1 and EAT) than rigid tumors with high proteoglycan and collagen content, small spindle-shaped cells and high cell density (LPB and SA-1). Furthermore, an optimal time interval for increased transfection exists only in soft tumors, this being in the range of 5–15 min. Therefore, knowledge about the histology of tumors is important in planning electrogene therapy with respect to the time interval between DNA injection and electroporation.*
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Keywords: *electrotransfer; solid tumors; plasmid DNA; luciferase; GFP; extracellular matrix*

Introduction

The potential for using genetic technology to treat human diseases (i.e., gene therapy) is receiving considerable attention at present; it is significantly promising as an adjuvant therapeutic mode for cancer treatment. However, many challenging obstacles have also arisen from the use of genetic materials as therapeutic agents. One important challenge in cancer gene therapy is to identify the fragilities of cancers as unique biological systems and to develop a delivery system that is specific, safe and efficient. Different delivery systems have been developed; in general these are divided into viral and non-viral vectors. The latter are favored over the former, due to their simplicity and lack of specific immune response stimulation. However, they are limited by their lower gene transfer efficiency compared with viral vectors. Therefore different non-viral gene delivery methods have been developed, including the injection of naked DNA, protein–DNA complexes, liposomes and their analogs, and delivery by 'gene gun' or electroporation, as well as various combinations of these methods.[1] Plasmid DNA injection and subsequent electroporation of tumor tissue is one of the most promising non-viral delivery systems for use in clinical gene therapy for cancer.[2,3] It is a physical method of gene delivery that facilitates the uptake of DNA into target cells by increasing the transmembrane potential above a certain threshold.

Although many studies employing non-viral gene delivery systems have been shown to efficiently deliver DNA to tumor cells *in vitro*,[4,5] only a minority of them were capable of achieving sufficient *in vivo* gene transfer to tumor tissue.[6–15] Moreover, studies on different tumor types using the same delivery system have shown different transfection efficiencies.[6–10] There is a substantial amount of literature explaining variable transfection efficiencies *in vitro* as well as *in vivo* due to variations in optimal experimental conditions, therapeutic genes, plasmid DNA backbone and mice age,[16–18] whereas the results of only a few studies performed on tumors *in vivo* and on tumor spheroids have drawn attention to tumor microenvironment and to the problems related to transport of DNA through the tumor tissue.[19–21] Therefore, as compared with methodological factors, specific tumor properties may have equal or greater influence on transfection efficiency in tumors. Further supporting this hypothesis are preclinical and clinical studies on anticancer drug penetration through tumor tissue.[22–26]

Correspondence: Professor M Cemazar, Department of Experimental Oncology, Institute of Oncology Ljubljana, Zaloska 2, Ljubljana SI-1000, Slovenia.
E-mail: mcemazar@onko-i.si
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Limited penetration of anticancer drugs through tumor tissue has been proposed as a potential cause of resistance of solid tumors to anticancer drugs.[22,24,26] These kinds of studies lead to novel treatment strategies that might improve the cell kill by enhancing drug penetration through the tumor tissue.

Compared to anticancer drugs, diffusion, distribution, degradation and uptake of DNA into tumor cells in tumor tissue has been investigated to a much smaller extent. There are a few *in vivo* reports showing that delivery of macromolecules such as genes or antibodies to cells in tumors is affected by the amount of DNases, which degrade exogenous DNA, by necrosis and macrophage infiltration,[19] as well as by increased interstitial fluid pressure[27] and the amount of extracellular matrix components, such as collagen[28,29] and hyaluronan.[30] On the other hand, *in vitro* studies have exposed the effect of the shape, size and orientation of cells in tissue and cell density.[31,32]

Therefore, an important challenge for efficient gene therapy in solid tumors is to identify the structural and physiological properties of the tumor tissue. Better understanding of tissue barriers might be useful for the prediction of transgene expression patterns in certain tumor types *in vivo*, as well as for further optimization of gene delivery systems.

Therefore, the aim of our study was to determine the effect of the histological properties of solid tumors on the transfection efficiency of electrically assisted gene delivery. The emphasis was on microscopic analysis of tumor tissue, determining cell size, cell density and the amount of extracellular matrix components, proteoglycans and collagen. To demonstrate the influence of the histological properties of solid tumors on electrically assisted gene delivery, the correlation between histological properties and transfection efficiency with regard to the time interval between DNA injection and electroporation was determined.

Results

Histological properties of tumors

On the macroscopic level, morphological differences were found between B16F1, EAT, SA-1 and LPB tumors. The B16F1 melanoma tumors appeared very soft and pulpous. SA-1 and LPB fibrosarcomas were solid and rigid, whereas EAT mammary carcinoma tumors fell between melanoma and fibrosarcoma tumors as regards tissue consistency. The histological properties of these tumors were examined by the determination of cell density, cell size, and amount of extracellular matrix components, proteoglycans and collagen. For each tumor type, 18 microscopic fields, obtained from six tumor samples and three fields of view per tumor section, were analyzed. Tumor sections were stained with hematoxylin-eosin (H&E) for the determination of cell density and cell size. Periodic acid-Schiff (PAS) staining was performed to visualize the proteoglycan content, and Masson's trichrome staining to visualize the collagen content (Figure 1).

Both cell density and cell size differed statistically significantly between tumor types. The highest cell density was found in LPB and SA-1 fibrosarcomas, somewhat lower in EAT mammary carcinoma, and the lowest in B16F1 melanoma. A statistically significant negative relationship existed between cell size and cell density. B16F1 melanoma was composed of the biggest

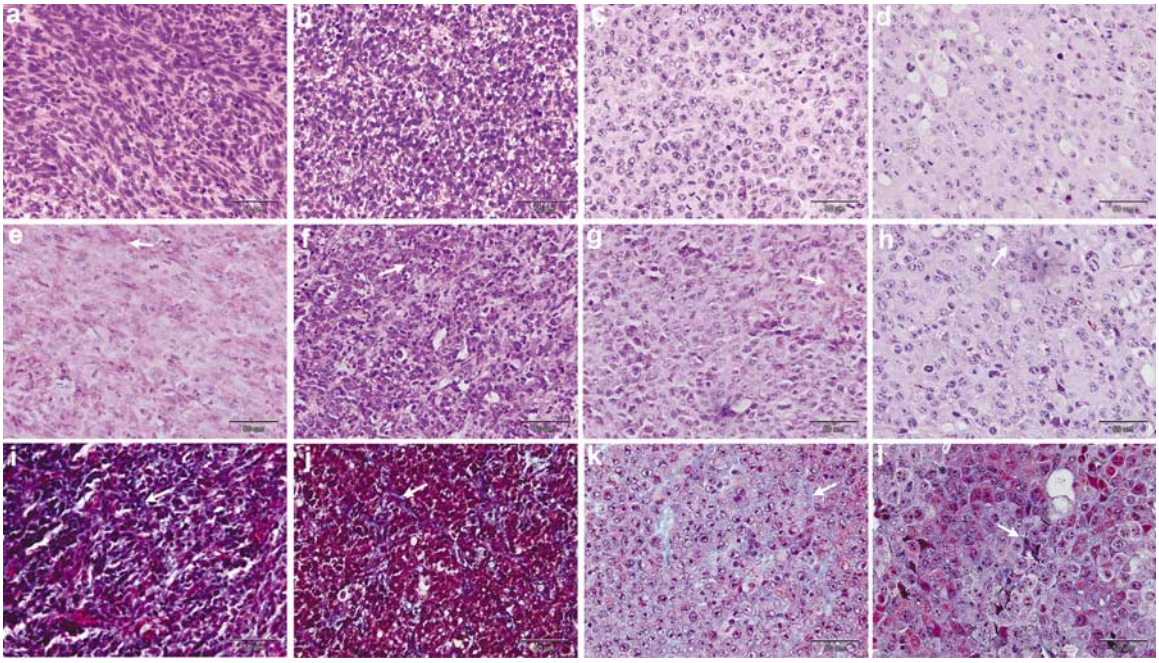

Figure 1 Differences in histological properties between four tumors. LPB fibrosarcoma (**a**, **e** and **i**), SA-1 fibrosarcoma (**b**, **f** and **j**), EAT mammary carcinoma (**c**, **g** and **k**) and B16F1 melanoma (**d**, **h** and **l**) sections were prepared as described in Materials and Methods. Cell density and cell size of H&E-stained sections (**a**–**d**), dark violet-colored proteoglycans of PAS-stained sections (**e**–**h**) and blue-colored collagen of Masson's trichrome-stained sections (**i**–**l**) were evaluated under a transmission microscope equipped with a CCD camera. Bars are 50 μm. Arrows indicate proteoglycans and collagen. H&E, hematoxylin-eosin.

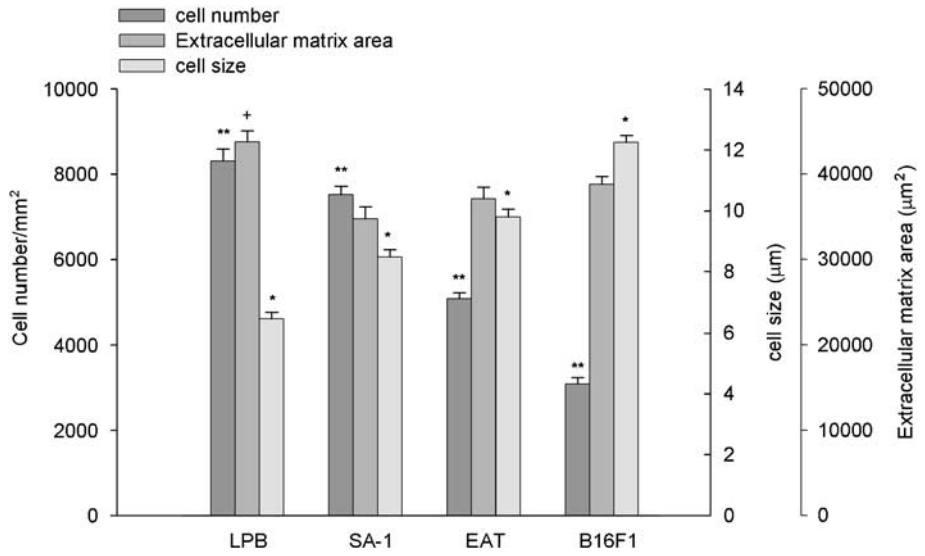

Figure 2 Tissue cell density, cell size and extracellular matrix area in four different tumors. LPB fibrosarcoma, SA-1 fibrosarcoma, EAT mammary carcinoma and B16F1 melanoma tumor sections were prepared as described in Materials and methods. H&E-stained sections were used to analyze cell density, cell size and extracellular matrix area by using image analysis. Bars are mean values of six tumors per tumor type, calculated from the mean values of three fields of view of each tumor section. *P, **$P<0.05$ significantly different between tumor types; $^{+}P<0.05$ vs SA-1, EAT and B16F1 tumors. H&E, hematoxylin-eosin.

cells and LPB of the smallest. Furthermore, differences in cell shape were also observed. LPB and SA-1 fibrosarcoma cells have a more spindle-like shape, whereas EAT mammary carcinoma and B16F1 melanoma cells are more spherical. In contrast to cell size, the fraction of extracellular matrix did not correlate with cell density. In LPB tumors there was significantly more extracellular matrix than in the other three tumor types. In B16F1 melanoma, higher values of extracellular matrix area were obtained, as was expected on the basis of proteoglycan and collagen content. These could be partly due to the presence of adipocytes, which occupied ~2% of the measured test area and were included in the calculation of the extracellular matrix area. In addition, the presence of fluids such as blood plasma or serum could also contribute to this observation (Figures 1 and 2).

To analyze differences in proteoglycan and collagen content between the four tumor types, the areal density of proteoglycans and collagen was determined in 18 fields of view per tumor type using the M-100 multipurpose stereological test system. Areal densities of proteoglycans and collagen were normalized to the extracellular matrix area. The PAS-stained sections showed that LPB, SA-1 and EAT tumors had high proteoglycan content, whereas in B16F1 proteoglycans were observed only in trace amounts (Figures 1e–h and 3). The mean fractional value of proteoglycans (0.20) did not differ between LPB, SA-1 and EAT tumors. In the Masson's trichrome-stained sections, the collagen content was at approximately the same level in LPB, SA-1 and EAT (~0.20). In the LPB and SA-1 fibrosarcomas, intense staining for collagen with apparently well-organized collagen lattices was observed, whereas collagen organization was not evident in EAT mammary carcinoma. Furthermore, only traces of collagen were found in B16F1 melanoma, and the mean fractional value was statistically significantly lower compared to the other three tumor types (Figures 1i–l and 3).

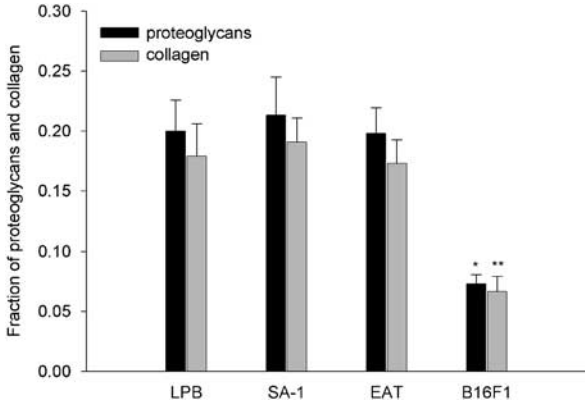

Figure 3 The content of proteoglycans and collagen in four tumors. LPB fibrosarcoma, SA-1 fibrosarcoma, EAT mammary carcinoma and B16F1 melanoma tumor sections were prepared as described in Materials and methods. PAS- (proteoglycans) or Masson's trichrome- (collagen) stained sections were morphometrically analyzed using the M-100 multipurpose test system. Represented are mean values of six tumors per tumor type, calculated from the mean values of three fields of view of each tumor section. *$P<0.05$ vs proteoglycan content in LPB, SA-1 and EAT tumors; **$P<0.05$ vs collagen content in LPB, SA-1 and EAT tumors.

In addition, tumor samples were checked for the presence of necrosis and immune cells. Neither necrosis nor infiltration of immune cells was observed in histological samples from any tumor type.

Transfection efficacy of electrically assisted gene delivery in solid tumors

The importance of the time interval between DNA injection and subsequent electroporation of tumors for effective electrically assisted gene delivery was already

demonstrated in our previous study, which indicated that it is tumor type dependent.[33] Namely, in tumors with higher cell density and high proteoglycan and collagen content, the penetration of plasmid DNA should be slower; thus, longer time intervals between DNA injection and electroporation are needed for achieving optimal transfection efficiency. Therefore, to confirm the effect of histological properties on DNA distribution in different tumors and consequently on the transfection efficiency of electrogene transfer, different time intervals were tested between DNA injection and the subsequent electroporation of tumors.

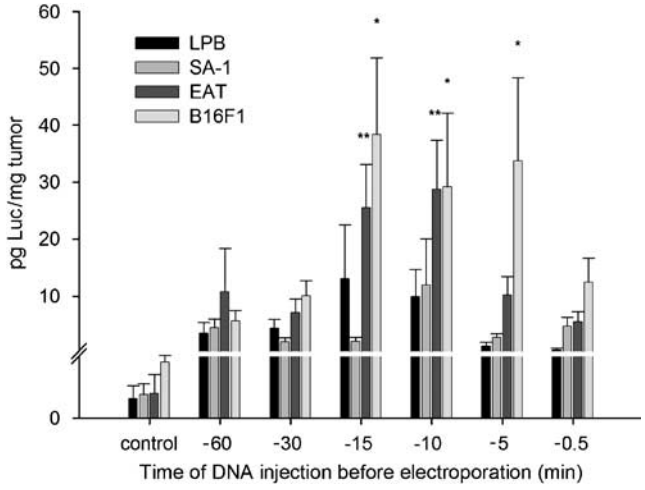

Figure 4 *In vivo* expression of luciferase reporter gene (pg Luc/mg tumor) in four different tumors (LPB, SA-1, EAT and B16F1) at different time intervals of plasmid DNA injection before electroporation of tumors. Represented are mean values, calculated from 18 to 24 animals per group and their standard errors. $^{*}P<0.05$ vs control group, 60, 30 and 0.5 min groups in B16F1 melanoma; $^{**}P<0.05$ vs control group and 60, 30, 5, 0.5 min groups in EAT mammary carcinoma.

Solid tumors were treated with intratumoral injection of plasmid pCMVLuc or pEGFP-N1 and then electroporated at different time intervals (0.5, 5, 10, 15, 30 and 60 min) after plasmid DNA injection. The levels of luciferase and green fluorescent protein (GFP) expression were significantly higher in B16F1 melanoma and EAT mammary carcinoma tumors, compared to both LPB and SA-1 fibrosarcoma tumors, at any time interval tested (Figures 4 and 5).

The optimal time interval between DNA injection and electroporation for achieving the highest transfection efficiency varied depending on the tumor type. Significantly increased luciferase activity was detected in B16F1 melanoma if plasmid DNA was injected between 5 and 15 min before electroporation. Similar results were obtained in the case of EAT mammary carcinoma, with significantly improved transfection detected at the time intervals 10 and 15 min. In LPB and SA-1 tumors, the highest luciferase activity was obtained when plasmid DNA was injected 10 or 15 min before electroporation of tumors. However, statistical analysis showed no significant difference between any of the time intervals tested for LPB and SA-1 tumors (Figure 4). The large error bars in the figure demonstrate high tumor heterogeneity and variability.[10] In addition, error bars in the groups treated with application of electric pulses were smaller than error bars in the control group.[34]

The results on GFP expression confirmed the results on luciferase activity in tumors, demonstrating that the highest GFP expression was obtained in B16F1 melanoma and much lower in both fibrosarcomas (Figure 5).

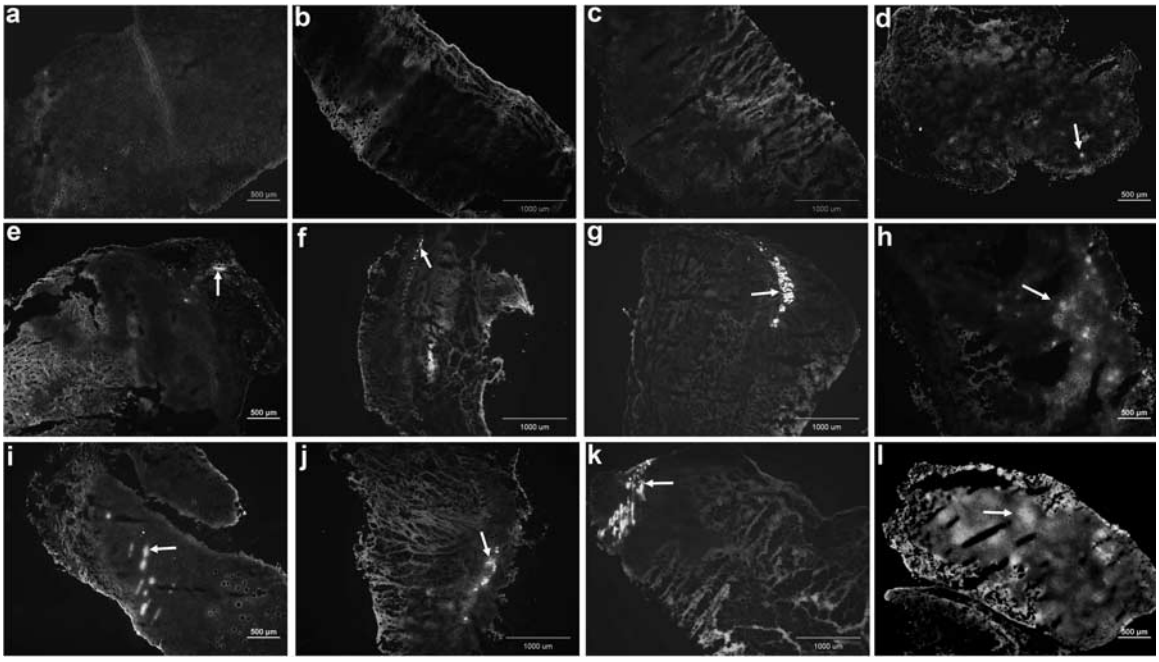

Figure 5 *In vivo* expression and spatial distribution of GFP reporter gene in four different tumors in control groups (**a**–**d**), at 60 min (**e**–**h**) and 10 min (**i**–**l**) time intervals between plasmid DNA injection and electroporation. LPB fibrosarcoma (**a**, **e** and **i**), SA-1 fibrosarcoma (**b**, **f** and **j**), EAT mammary carcinoma (**c**, **g** and **k**) and B16F1 melanoma (**d**, **h** and **l**) tumors were cut in 20-μm-thick cryosections and analyzed under a fluorescence microscope. Bars: 500 μm (**a**, **d**, **e**, **h**, **i** and **l**), 1 mm (**b**, **c**, **f**, **g**, **j** and **k**). Arrows indicate transfected area.

Increased levels of GFP expression were observed in all four tumor types at 5–15 min time intervals between DNA injection and subsequent electroporation. At these time intervals, GFP was observed both at the periphery and in the center of tumor sections. Outside the optimal time window, where lower transfection efficiencies were obtained, GFP was detected only at the periphery of tumor sections (Figure 5). Specifically, in B16F1 melanoma, where the highest transfection efficiency was obtained, GFP was distributed throughout the tumor section (Figure 5). These results indicate that an optimal time interval between DNA injection and electroporation of tumors for achieving pronounced transfection efficiencies exists for soft, low cell density tumor types with larger cells (B16F1 and EAT), as compared to rigid, high cell density tumor types with smaller cells (LPB and SA-1), where transfection efficiency was not dependent on the time interval between DNA injection and electroporation. Furthermore, Pearson correlation statistics showed a significant relationship ($P<0.05$) between the cell number or cell size and transfection efficiencies at time intervals from 0.5 to 10 min, as well as between proteoglycan and collagen content and transfection efficiencies at the 5 min time interval among the four tumor types.

Discussion

In this study, we have shown that the histological properties of tumors, such as cell density, cell size, and proteoglycan and collagen content, influence the transfection efficiency of electrically assisted gene delivery to tumors. The examined histological properties correlated statistically significantly with transfection efficiency at shorter time intervals between DNA injection and electroporation (0.5–10 min). We demonstrated that in tumors with large cells and low cell density, there exists a narrow optimal time interval between DNA injection and electroporation, during which pronounced transfection efficiency occurs. In contrast, for tumors with small cell size and high cell density, there is no optimal time interval and transfection occurs regardless of time between DNA injection and electroporation of tumors. However, the transfection efficiency in high cell density tumors with small cell size (LPB and SA-1) was much lower compared to tumors with low cell density and larger cells (B16F1 and EAT). In addition, we showed that proteoglycan and collagen content, which affects DNA distribution in tumors, might have an effect on transfection efficiency as demonstrated by the high correlation coefficient at the 5 min time interval, but this effect was less pronounced compared to other histological properties.

The effects of tumor cell size, cell shape and cell density on the electropermeabilization of cells and consequently on the transfection efficiency of electrically assisted gene delivery have already been studied by mathematical modeling and *in vitro* cell electroporation experiments.[21,31,32,35,36] Theoretical models and *in vitro* experimental results showed that permeabilization is not only a function of electric field intensity and cell size, but also of cell shape and orientation, as well as cell density and cell organization within a specific model of multicellular structure. It was demonstrated that with increased cell size, a lower field strength is required for membrane electroporation and subsequent DNA uptake.[36] Dependence on cell orientation (cell shape) is more pronounced for elongated cells (spindle-like cells), whereas it is negligible for spherical cells.[31,35] The results of our study confirmed the *in vitro* results and extended them to solid tumors *in vivo*. Utilization of the same electric pulse parameters resulted in much higher transfection efficiencies in B16F1 melanoma tumors, having the largest and the most spherical cells, and EAT mammary carcinoma tumors with slightly smaller spherical cells, as compared to LPB or SA-1 fibrosarcoma tumors with smaller cell size and spindle-like cell shape.

Furthermore, mathematical models, as well as study on tumor spheroids suggested that a multicellular environment decreased the effects of electroporation on cell membrane permeability, and these effects varied inversely with the number of cells around a given cell. Therefore, a multicellular environment resulted in a reduced uptake of molecules into the cells.[21,32] These results support the results of our present and previous *in vivo* studies on solid tumors, where cell density correlated with transfection efficiency.[7,33] Low levels of luciferase or GFP expression were found in high cell density LPB and SA-1 fibrosarcomas, whereas high levels were found in the low cell density B16F1 melanoma and EAT mammary carcinoma.

Another factor that might affect DNA distribution in tumors and eventually the uptake of DNA into tumor cells by determining the transport properties of plasmid DNA injected into tumors is the composition, structure and area of the extracellular matrix in tumors.[2] As demonstrated for anticancer drugs, as well as for high molecular weight immunoglobulins, the extracellular matrix represents a so-called 'physiological resistance' to treatment.[22–26,28–30] Especially in the case of immunotherapy and gene therapy, where high molecular weight molecules have to reach tumor cells, the effectiveness of treatment can be seriously reduced owing to the failure of molecules to penetrate the extracellular matrix of tumors. In the case of electrically assisted gene delivery, physiological resistance can be reduced in part by inducing an electric field in tumors, which can facilitate DNA distribution. Zaharoff *et al*.[20] showed that the average plasmid DNA movements via a pulsed electric field were 4.2-fold farther in B16F1 melanoma than 4T1 sarcoma. Plasmid DNA mobility was correlated with tumor collagen content, which was approximately eight times greater in 4T1 than in B16F1 tumors. This is in agreement with the results of our study, where histological analysis of tumors showed two to three times greater proteoglycan and collagen content in fibrosarcomas than in B16F1 melanoma. Differences in extracellular matrix composition translated into differences in transfection efficiency, being the highest in B16F1 melanoma with the lowest proteoglycan and collagen content. However, other histological factors might play a role in hindering DNA diffusion through tumor tissue, such as proteins involved in cell-to-cell contacts, as well as other proteins of the extracellular matrix. To evaluate further and confirm the effect of the composition of the extracellular matrix on mobility and distribution of DNA in tumors, different time intervals between DNA injection and electroporation of tumors were tested to

demonstrate that DNA needs a longer time to distribute in high proteoglycan and collagen content tumors, compared to low proteoglycan and collagen content tumors. Although Zaharoff *et al*.[20] showed in tumor slices *ex vivo* that free diffusion of plasmid DNA is a very slow process and is facilitated by applying an external electric field, we demonstrated that free diffusion, at least in tumors with low collagen content, plays a role in achieving transgene expression. Namely, we found that B16F1 melanoma, with a low proteoglycan and collagen content, has higher transfection efficiencies achieved at shorter time intervals than the other three tumor types. These three tumor types should have the same DNA mobility, but due to differences in cell size, cell shape and cell density, electrically assisted gene delivery in EAT mammary carcinoma yielded better transfection efficiency. LPB and SA-1 fibrosarcomas with high proteoglycan and collagen content, high cell density, spindle-like cell shape and small cell size resulted in low transfection efficiencies at all tested time intervals between DNA injection and electroporation. To fully demonstrate different mobility and distribution of plasmid DNA in different tumor types, further studies using fluorescently labeled DNA should be performed.

In our study, when using the melanoma (B16F1) or carcinoma (EAT) tumor types with the largest cell size and lowest proteoglycan and collagen content, an increased level of luciferase expression was obtained only in a narrow time window ($\sim$5–15 min) between plasmid DNA injection and the application of electric pulses. As already indicated in our previous study, the results of this study also support the notion that the degradation of DNA in tumors compared to muscle tissue is a quicker process from both compartments of DNA in tissue – the one that is quickly degraded and cleared out, and the other electrotransferable one.[33] However, in the case of fibrosarcoma tumors there was no significant increase in the level of luciferase expression; this can be explained by poor distribution of DNA through compact tumor tissue, high proteoglycan and collagen content, and a too-low external electric field, resulting in a low proportion of permeabilized cells, predominantly in the middle plane of the tumor. Differences in time-dependent transfection efficiencies were also reflected in the expression level and spatial distribution of GFP. In LPB and SA-1 tumors, GFP was detected only at the periphery of the tumor sections, with the exception of the 10 min time interval, where GFP was visible in the middle of the tumor sections. In melanoma (B16F1) and carcinoma (EAT) tumors, higher transfection efficiencies were obtained and GFP was distributed throughout the whole tumor sections. However, if DNA was injected outside the optimal time window, lower transfection levels were obtained and GFP was detected only at the periphery of the tumor sections, as in LPB and SA-1 tumors. One might speculate that the effect of the time interval on transfection efficiency could be due to pressure of injection or needle penetration. However, in a recent study Andre *et al*.[37] showed that there is no statistically significant difference in transfection efficiency of tumors when using slow or fast injection (low or high pressure). This indicates that the influence of time interval on transfection efficiency cannot be linked to the damage caused by needle penetration or pressure of injection, but is rather due in large part to plasmid DNA diffusion.

Finally, the unique biological properties of different tumor types, fibrosarcoma, mammary carcinoma and melanoma may also be responsible for differences in transfection efficiency. It is well known that tumors are biologically heterogeneous systems with differences in growth rate and architecture, antigenicity, immunogenicity, morphology, histology, genotype and biochemical properties (such as the presence of DNases in tumors). Differences regarding tumor type sensitivity to chemotherapeutic agents and ability to express transgenes has also been well documented; the reason for this phenomenon might lie in the variable biological properties.[38] Furthermore, plasmid DNA characteristics can also affect transgene expression. The main properties of plasmid DNA that can influence transgene expression are volume, size of the injected plasmid DNA, type of promoter (constitutive vs inducible, tissue specific), and presence of CpGs.[2,39,40] In our case, cytomegalovirus (CMV) promoter was used in all experiments. Although CMV promoter is a strong promoter, we cannot rule out that the relative level of reporter expression may depend on cell type transcription in response to the CMV promoter. However, this should not affect the time course of transfection within each tumor type.

So far the major determinants of tumor heterogeneity responsible for different levels of electrogene transfection efficiency have not been well defined. In this study as well as in our previous one, we propose that electrogene transfection efficiency is at least in part dependent on tumor type. Namely, we found that the highest transfection efficiency was obtained in melanoma (B16F1), followed by carcinomas (EAT and T24), with the lowest transfection efficiency obtained in sarcomas or carcinosarcoma (LPB, SA-1, SaF and P22).[7,33] Exactly which biological properties of cells in solid tumors contribute to differences in transfection efficiency (besides the already known physical parameters of applied electric pulses and histological properties of tissues) are as yet unknown, but molecular biology techniques such as genomics and proteomics could provide an answer to this question.

Collectively, the results of our study point out the importance of the histological properties of tumors for effective electrogene therapy. Specifically, this is important for tumors that possess unique characteristics, attributed in part to an embryonic-like stage of their development with an extensive synthesis of extracellular matrix, leading to substantial differences in composition and assembly compared to the host tissue. Tumors with larger spherical cells, low cell density and low proteoglycan and collagen content are more effectively transfected than high proteoglycan and collagen content tumors with small spindle-shaped cells of high density. Furthermore, in those tumors that had larger, spherical cells, an optimal time interval for transfection does exist. Regardless, knowledge about the histology of tumors can assist in planning electrogene therapy in a clinical setting,[3] with respect to the time interval between DNA injection and electroporation, as well as the selection of electrical parameters to obtain sufficient electric field distribution in tumors.

Materials and methods

Experimental animals, cell lines and tumor types

Animal studies were carried out according to the guidelines of the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (permission No.: 323-02-632/2005/6), and in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA).

In the experiments female C57Bl/6, CBA and A/J mice purchased from the Institute of Pathology (Medical Faculty, University of Ljubljana, Slovenia) were used. At the beginning of the experiments, animals were 10–12 weeks old. Mice were kept in a conventional animal colony at a constant room temperature (21°C) and a natural day/night light cycle. Food and water were provided *ad libitum*. Animals were subjected to an adaptation period of 7–10 days before experiments.

In this study, four different murine tumor cell lines were used: SA-1 fibrosarcoma syngeneic to A/J mice, LPB fibrosarcoma (a clonal derivate of TBL.C12) and B16F1 melanoma (CLR6323; American Type Culture Collection, Manassas, VA, USA) syngeneic to C57B1/6 mice, and EAT mammary carcinoma syngeneic to CBA mice. LPB and B16F1 cells were routinely maintained in Eagle's minimal essential medium (Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% fetal calf serum (Sigma) and antibiotics in a humidified atmosphere at 37°C, containing 5% CO_2. SA-1 and EAT cells were obtained by peritoneal lavage of ascitic tumor-bearing donor mice with 3–5 ml 0.9% NaCl solution. Ascitic tumors were induced with 5.0×10^5 viable SA-1 cells or EAT cells injected intraperitoneally and harvested 4–5 days later. Owing to the different generation time of selected tumor cells, different amounts of cells (1.3×10^6 LPB, 5.0×10^5 SA-1, 3.0×10^6 EAT and 1.0×10^6 B16F1 cells in 0.1 ml of 0.9% NaCl) were injected subcutaneously to obtain tumors of 6 mm in diameter in approximately 8–12 days. During this period of time, no tumor necrosis was observed. After tumors reached the desired size (approximately 6 mm in diameter), the animals were randomly divided into experimental groups and subjected to specific experimental protocols.

Determination of the composition and structure of tumor interstitial matrix, cell size, cell density, necrosis and immune cell infiltration

Histological analysis for proteoglycan and collagen content, cell density and cell size was performed on subcutaneous tumors of 6 mm in diameter. Tumors were excised, cut into two pieces along the largest diameter, and fixed in buffered formalin. The tumors were then embedded in paraffin, cut into 5 μm sections and stained with Masson's trichrome for collagen, PAS for proteoglycans, and H&E for cell density estimation. Tumor slides were observed by transmission microscopy with a $\times 60$ objective, and images were taken by CCD camera DP70 (Olympus, Hamburg, Germany). In H&E-stained tumor sections, tumor necrosis, macrophage and lymphocyte infiltration, as well as the number and size of cells in the microscopic fields, were determined for each tumor type by using DP Soft (Olympus). Three fields were scored per section and six tumors were used per tumor type. Cell density was determined as average number of cells per field and expressed as number of cells per mm^2, cell size was determined by measuring cell diameter of cells in the field. The longest diameter of the cells was used to determine the cell size. To determine proteoglycan and collagen content in the tumors, the M-100 multipurpose test system was used. Areal density of proteoglycans and collagen was estimated in each tumor slide.

Plasmids

Plasmids pEGFP-N1 (encoding GFP; Clontech, Basingstoke, UK) and pCMVLuc (encoding luciferase, kind gift from Dr Lluis M Mir, Institute Gustave Roussy, France) were prepared using the EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions and diluted in H_2O to a concentration of 1 μg/μl.

Tumor transfection protocol

Electrically assisted gene delivery was performed by intratumoral injection of plasmid DNA (50 μl/tumor) with subsequent electroporation of the tumor. The procedure involving intratumoral injection lasted $\sim$10–15 s, and included both injection of the plasmid and retention of the needle in the tumor for few seconds to prevent leakage of the solution. Injections were made using an insulin syringe with a very thin needle of 28 G. All the injections were made by one person to avoid interpersonal variability, and the speed of injection was controlled manually. Electric pulses were delivered through two parallel stainless steel electrodes with 6 mm distance between them. Eight square-wave electric pulses were delivered in two sets of four pulses in perpendicular directions at a frequency of 1 Hz, amplitude over distance ratio 600 V/cm and 5 ms duration. This amplitude over distance ratio was chosen on the basis of *in vitro* as well as *in vivo* results, demonstrating that at this amplitude a plateau of transgene expression level was obtained in different tumor types.[6,7,10] Furthermore, higher amplitudes could also lead to irreversible electrical injuries. Good contact between electrodes and the overlying skin was assured by hair clipping and use of a conductive gel. Electric pulses were generated by electroporator Jouan GHT 1287 (Jouan, St Herblain, France). Electrodes were placed percutaneously at opposite margins of the tumor. Experimental groups differed in the time interval between plasmid DNA injection and application of electric pulses (from 1 h to 0.5 min). Control groups received only intratumoral injection of plasmid DNA without application of electric pulses. Tumors were excised 48 h post-transfection, as the level of gene expression reaches a plateau between 24 and 48 h, which has already been determined in *in vitro* as well as *in vivo* studies in different tumor cell types.[4,41] Groups consisted of six tumors per group if treated with pCMVLuc or three tumors per group if treated with pEGFP-N1. Experiments were repeated two to three times.

Transfection efficiency assessment

Tumors treated with plasmid pCMVLuc were weighed, immediately frozen in liquid nitrogen and stored at −80°C until further procedures. Thawed tumors were homogenized in 1 ml of Glo Lysis Reagent (Promega,

Madison, WI, USA) using sonificator UP200 H (Dr Hielscher, Teltow, Germany). Thereafter the samples were centrifuged at 10000 *g* for 10 min and the supernatant stored at −80°C. Luciferase activity was measured in thawed supernatants using Genios luminometer (Tecan, Zurich, Switzerland). Photoemission was measured during a 5 s period after 5 min incubation at room temperature of 100 μl tumor cell lysate and 100 μl Luciferase Assay Substrate (Promega). Luciferase activity was quantified as relative light units and then converted to pg luciferase/mg tumor tissue, using the pre-prepared calibration curve of known quantities of luciferase (Promega).

Tumors treated with plasmid pEGFP-N1 were embedded in Tissue-Tek OCT Compound (Miles Inc., Elkhart, IN, USA) and stored at −20°C for subsequent processing. Cryosections of 20 μm at different depths of tumor (24 sections per tumor) were cut for analysis. Transfection efficiency and spatial distribution of GFP was estimated in frozen tumor sections using fluorescence microscope BX51 equipped with a DP70 CCD camera (Olympus).

Statistical analysis

The data were tested for normality of distribution using the Kolmogorov–Smirnov test. Differences between experimental groups were statistically evaluated by one-way analysis of variance followed by the Holm–Sidak test for multiple comparisons. Correlation between histological properties and transfection efficiency with regard to the time interval between DNA injection and electroporation was determined by Pearson's correlation statistics. A *P*-value of less than 0.05 was considered to be statistically significant. Statistical analysis was done using Sigma Stat (SPSS Inc., Chicago, IN, USA) software.

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Gene Electrotransfer into Murine Skeletal Muscle: A Systematic Analysis of Parameters for Long-term Gene Expression

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Gregor Tevz,B.Sc.[1]
Darja Pavlin, D.V.M.[2]
Urska Kamensek[1]
Simona Kranjc, Ph.D.[1]
Suzana Mesojednik[1]
Andrej Coer, Ph.D.[3]
Gregor Sersa, Ph.D.[1]
Maja Cemazar, Ph.D.[1,*]

[1]Institute of Oncology Ljubljana
Department of Experimental Oncology
Zaloska 2, SI-1000 Ljubljana, Slovenia
[2]University of Ljubljana
Veterinary Faculty
Gerbiceva 60, SI-1000 Ljubljana, Slovenia
[3]University of Ljubljana
Faculty of Medicine
Korytkova 2, SI-1000 Ljubljana, Slovenia

Skeletal muscle is an attractive target tissue for delivery of therapeutic genes, since it is well vascularized, easily accessible, and has a high capacity for protein synthesis. For efficient transfection in skeletal muscle, several protocols have been described, including delivery of low voltage electric pulses and a combination of high and low voltage electric pulses. The aim of this study was to determine the influence of different parameters of electrotransfection on short-term and long-term transfection efficiency in murine skeletal muscle, and to evaluate histological changes in the treated tissue. Different parameters of electric pulses, different time lags between plasmid DNA injection and application of electric pulses, and different doses of plasmid DNA were tested for electrotransfection of *tibialis cranialis* muscle of C57Bl/6 mice using DNA plasmid encoding green fluorescent protein (GFP). Transfection efficiency was assessed on frozen tissue sections one week after electrotransfection using a fluorescence microscope and also noninvasively, followed by an *in vivo* imaging system using a fluorescence stereo microscope over a period of several months. Histological changes in muscle were evaluated immediately or several months after electrotransfection by determining infiltration of inflammatory mononuclear cells and presence of necrotic muscle fibers. The most efficient electrotransfection into skeletal muscle of C57Bl/6 mice in our experiments was achieved when one high voltage (HV) and four low voltage (LV) electric pulses were applied 5 seconds after the injection of 30 µg of plasmid DNA. This protocol resulted in the highest short-term as well as long-term transfection. The fluorescence intensity of the transfected area declined after 2-3 weeks, but GFP fluorescence was still detectable 18 months after electrotransfection. Extensive inflammatory mononuclear cell infiltration was observed immediately after the electrotransfection procedure using the described parameters, but no necrosis or late tissue damage was observed. This study showed that electric pulse parameters, time lag between the injection of DNA and application of electric pulses, and dose of plasmid DNA affected the duration of transgene expression in murine skeletal muscle. Therefore, transgene expression in muscle can be controlled by appropriate selection of electrotransfection protocol.

Key words: Electroporation; Electrotransfection; Skeletal muscle; Plasmid DNA; Green fluorescent protein; Inflammatory mononuclear cells infiltration; Long-term gene expression; and Transfection efficiency.

Introduction

The first report on using skeletal muscle as a target for the introduction of different transgenes was published in 1990 (1). Ever since, a wide range of evidence

[*]Corresponding Author:
Maja Cemazar, Ph.D.
Email: mcemazar@onko-i.si

Abbreviations: EP, Electroporation; LV, Low voltage electric pulses; HV, High voltage electric pulses; GFP, Green fluorescent protein; EGT, Gene electrotransfer.

has been presented showing that skeletal muscle is a promising target for gene manipulation due to its specific properties, including the high capacity of protein synthesis and post-mitotic status of muscle fibers, which enables long-lasting transgene expression leading to either local intramuscular secretion or systemic shedding of transgene products (2).

The easiest introduction of foreign transgenes into skeletal muscle is by injection of naked plasmid DNA, which results in sustained transgene expression (1, 3, 4). The potential of this gene delivery technique is limited not only by low transfection efficiency, compared to viral vectors, but also by highly variable inter-individual levels of gene expression. In order to increase the efficiency of such gene delivery to skeletal muscle, different methods are being developed, one of them being *in vivo* electroporation. Electroporation is a method for delivery of various molecules into cells by transiently increasing the permeability of the cell membrane using the application of a controlled external electric field to the cells or tissues (5, 6).

Skeletal muscle electrotransfection combines injection of plasmid DNA into skeletal muscle with subsequent electroporation of tissue. DNA electrotransfer dramatically increases gene expression in skeletal muscle, compared to simple injection of plasmid DNA, even up to 2000 times, and strongly reduces inter-individual variability of transgene expression (7-9). DNA electrotransfer is of special interest for possible use in clinical gene therapy, since it is an efficient non-viral strategy for gene delivery and also because of its safety, ease of use and low costs (10-12).

A number of published reports have concentrated on optimization of electric pulse protocol and other parameters that were believed to influence the efficiency of electrotransfection of skeletal muscle. Different protocols of square wave electric pulses were previously described as resulting in efficient electrotransfection of murine skeletal muscle. At first, only low voltage electric pulses with long duration (100-200 V/cm, 20-50 μs) were used (7-9). More recent studies have shown that transfection can be achieved by using the combination of 1 HV short duration pulse (600-800 V/cm, 100 μs) followed by a different number of LV long duration electric pulses (80-100 V/cm, duration in the range of tens to hundreds of milliseconds) (13-15).

Previous work has shown that the duration of the time lag between injection of plasmid DNA and delivery of the electric pulses is important for the efficient transfection of skeletal muscle (16-18). Other variables potentially important for the outcome of gene therapy include the method of injection, electrode geometry, plasmid properties (*e.g.*, size and composition of plasmid, concentration, volume, and dose of injected plasmid), and tissue properties (sex and age of experimental animals, composition of extracellular matrix) (11, 17, 19, 20). Reports on optimal conditions regarding all these parameters are highly variable among different authors, without reaching a firm agreement on optimal protocol for the most efficient electrotransfection of skeletal muscle.

Muscle tissue damage has been described after electrotransfection as the infiltration of inflammatory mononuclear cells and muscle fiber necrosis. The latter was shown to decrease transfection efficiency by reducing the number of muscle fibers capable of transgene expression (19). On the contrary, muscle regeneration after injury has been shown to increase transfection efficiency (21). Moreover, muscle fiber regeneration from transfected myogenic satellite cells could be the underlying mechanism of prolonged transgene expression (22).

The influence of different parameters of electric pulses on long-term gene expression has not been thoroughly studied. A few studies have demonstrated that long-term transfection can be achieved, but with contradictory results (23-25). Therefore, the aim of this study was to determine the influence of different electrotransfection parameters on short-term and long-term transfection efficiency in murine skeletal muscle, and to evaluate histological changes in the treated tissue. Specifically, we compared the short- and long-term transfection efficiency of different protocols of electric pulses, of different time lags between the injection of plasmid DNA and application of electric pulses, and of different doses of plasmid DNA. Furthermore, we evaluated histological changes in muscle tissue after electrotransfection.

Materials and Methods

Experimental Animals

In all experiments, female C57Bl/6 mice obtained from Institute of Pathology, Faculty of Medicine, University of Ljubljana, Slovenia, were used. At the beginning of the experiments, the animals were 10-12 weeks old. Mice were housed and maintained in a conventional animal colony under standard laboratory conditions at constant room temperature (21 ºC) and natural day/night light cycle. Food and water was provided *ad libitum*. Animals were subjected to an adaptation period of 7-10 days before the experiments. All procedures on animals were performed in accordance with the official guidelines of the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (permission No.: 323-02-632/2005/6). Each experimental group consisted of 3-6 animals.

Plasmid

Plasmid pEGFP-N1 (Clontech Laboratories, Inc., Mountain View, CA, USA), encoding green fluorescent protein (GFP), was prepared using the Quiagen Endo-Free Plasmid Mega

Kit (Quiagen, Hilden, Germany) according to manufacturer's instructions, and diluted to five different concentrations from 0.05 to 1.5 mg/ml. Plasmid DNA concentration and purity was determined spectrophotometrically and by gel electrophoresis.

Gene Electrotransfer

Our study was divided into three consecutive sets of experiments. In the first part, different parameters of electric pulses were tested for electrotransfection of murine skeletal muscle (Table I, Electric Pulse Protocol). In the second part, the optimal time lag between plasmid DNA injection and application of electric pulses was determined (Table I, Time Lag), and in the third part the optimal dose of plasmid DNA (Table I, Dose Of Plasmid DNA). All experimental animals were anaesthetized with intraperitoneal injection of a combination of acepromazine (Promace, Fort Dodge Animal Health, IA, USA; 0.05 mg/mouse), ketamine hydrochloride (Bioketan, Vetoquinol, Paris, France; 2.5 mg/mouse), and xylazine hydrochloride (Rompun 2%, Bayer AG, Leverkusen, Germany; 0.25 mg/mouse) 10 minutes before the transfection procedure. Plasmid was injected into the right *m. tibialis cranialis* with a thin needle of 26 G. The injection volume was 0.02 ml in all experiments. The leg was placed between the plate electrodes, which consisted of two flat parallel stainless steel electrodes of dimensions 20 mm × 10 mm with rounded corners and 6 mm distance between them (IGEA s.r.l., Carpi, Italy); electric pulses were generated by electric pulse generator Cliniporator™ (IGEA s.r.l., Carpi, Italy). Good contact between the electrodes and the overlying skin was assured by hair removal and use of a conductive gel. The control group received only plasmid injection without application of electric pulses.

Table I
Parameters of electrotransfection in three separate consecutive experiments.

Electric parameters	pEGFP-N1 dose/20 µl	Time lag
I Electric Pulse Protocol		
1 HV (600 V/cm, 100 µs) + 1 LV (80 V/cm, 400 ms, 1Hz)	20 µg	10 min
1 HV (600 V/cm, 100 µs) + 4 LV (80 V/cm, 100 ms, 1Hz)	20 µg	10 min
1 HV (600 V/cm, 100 µs) + 8 LV (80 V/cm, 50 ms, 2Hz)	20 µg	10 min
6 LV (100 V/cm, 60 ms, 1Hz)	20 µg	10 min
8 LV (200 V/cm, 20 ms, 1Hz)	20 µg	10 min
II Time Lag		
1 HV + 4 LV	20 µg	**5 s**
1 HV + 4 LV	20 µg	**1 min**
1 HV + 4 LV	20 µg	**3 min**
1 HV + 4 LV	20 µg	**5 min**
1 HV + 4 LV	20 µg	**10 min**
1 HV + 4 LV	20 µg	**20 min**
1 HV + 4 LV	20 µg	**30 min**
1 HV + 4 LV	20 µg	**60 min**
1 HV + 4 LV	20 µg	**120 min**
III Dose Of Plasmid DNA		
1 HV + 4 LV	**1 µg**	5 s
1 HV + 4 LV	**5 µg**	5 s
1 HV + 4 LV	**10 µg**	5 s
1 HV + 4 LV	**20 µg**	5 s
1 HV + 4 LV	**30 µg**	5 s

Non-invasive Optical Imaging of Gene Expression

Transfection efficiency was assessed by two different methods: fluorescence microscopy of frozen tissue sections of transfected muscles and non-invasive stereo microscopy fluorescence imaging of GFP expression in muscle cells.

One week after the electrotransfection procedure, each experimental group was randomly divided into two equal groups. One half of the animals were euthanized and the transfected *m. tibialis cranialis* of each mouse was removed, quick-frozen in liquid nitrogen and stored at -70 ºC. The transfection efficiency of electrically-assisted gene delivery was assessed on 24 frozen tissue sections (20 µm) that were cut at different depths of *m. tibialis cranialis*, using a fluorescence microscope (Olympus BX51, Olympus, Hamburg, Germany) equipped with a cooled digital color camera for recording the images (Olympus).

In the other half of the animals, transfection efficiency was followed *in vivo* using a fluorescence stereo microscope, which enabled non-invasive follow-up of the duration of GFP expression. At each observation under the fluorescence stereo microscope, animals were anaesthetized as mentioned before and the hair over the transfected muscle was removed with hair removal cream (Vitaskin, Krka, d.d., Novo mesto, Slovenia). GFP fluorescence was observed transcutaneously at regular time intervals, as follows: every seven days during the first four weeks after electrotransfection, every 14 days up to the 24th week, and every 28 days thereafter. Each time, digital images of the *m. cranialis tibialis* were recorded using a Leica MS5 fluorescence stereo microscope (Leica Microsystems GmbH, Letzler, Germany) equipped with a Nikon E4500 digital color camera (Nikon Corporation, Tokyo, Japan).

The images of frozen muscle sections, as well as transcutaneous images of muscles expressing GFP were analyzed using the ImageJ software tool (National Institute of Mental Health, Research Services Branch, Bethesda, MD, USA). Transfection efficiency was determined as the normalized area of transfection on images of frozen muscle sections and as the normalized fluo-

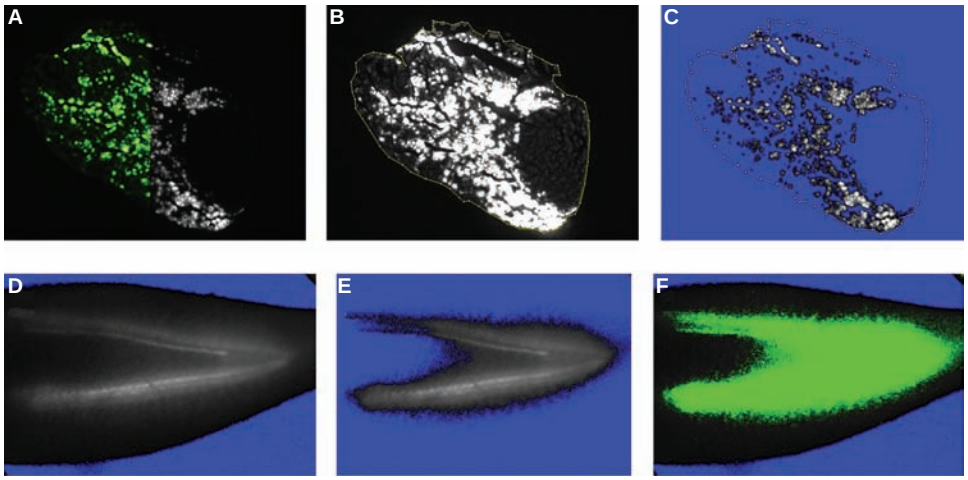

Figure 1: Transfection efficiency determination. **A**. *m. tibialis cranialis* tissue section transfected with pEGFP-N1. **B**. Area of whole muscle section was measured within the selection. **C**. A pixel intensity threshold value was determined, which separated the transfected from the non-transfected region. Only the area of transfection was visible and measured. Selection shows the edge of whole muscle section. **D**. Stereo microscopic image of mouse leg. Transfected muscle fibers were seen through skin. **E**. Mean fluorescence intensity was measured in the transfected region and **F**. non-transfected region.

rescence intensity on images of transcutaneous *in vivo* observations. Threshold value of pixel intensity was determined for each image of muscle sections in order to separate the transfected area from the non-transfected one. Area of transfection was limited to the pixels with values higher than the determined threshold. That area was then normalized to the area of the whole muscle section (Figure 1A, B, C). Mean fluorescence intensity was determined on images of transcutaneous *in vivo* observation. Longitudinal transfected muscle fibers were easily separated from the background fluorescence. Mean fluorescence intensity of the transfected muscle fibers was normalized to the mean fluorescence intensity of the non-transfected region (Figure 1D, E, F).

Assessment of Histological Changes of the Muscle tibialis cranialis

In order to evaluate histological changes caused by electrotransfection protocol resulting in high transfection efficiency, mice were treated with the selected electrotransfection protocol (1HV + 4LV, 5 s time lag, 30 μg DNA). Treated muscles were removed at different time intervals after the procedure to assess early tissue damage (up to two days), as well as late tissue damage (several months).

In order to discriminate between the contributions of the plasmid DNA and the vehicle in which the DNA was dissolved to the occurrence of muscle damage, a new control group was included, receiving phosphate buffer saline (PBS) instead of distilled water.

The harvested muscles were fixed in formaldehyde and embedded in paraffin blocks that were cut into tissue sections of 10 μm. Ten sections per muscle were cut. Tissue sections were differentially stained with hematoxylin-eosin dye. The extent of histological change was determined as a fraction of the area with inflammatory mononuclear cell infiltration with respect to the whole muscle area. In addition, muscles were inspected for the presence of necrosis. Images of muscle sections were captured by digital color camera (Olympus) and analyzed with the ImageJ software tool.

Statistical Analysis

The data were first tested for normality of distribution using the Kolmogorov-Smirnov normality test. Significance tests were carried out using one-way analysis of variance (ANOVA), followed by the Holm-Sidak test for multiple comparisons. Values of $p<0.05$ were considered to be statistically significant. Statistical analysis was performed using Sigma Stat, SPSS Inc., Chicago, IL, USA software.

Results

Protocol of Electric Pulses

Several different electric pulse protocols were tested and the transfection efficiency was determined as listed in Table I. Ten minutes before the application of the electric pulses, pEGFP-N1 was injected into the skeletal muscle. The combination of HV and LV electric pulses resulted in statistically significantly ($p<0.05$) larger areas of transfection on frozen tissue sections of *tibialis cranialis* muscles than the LV electric pulses alone one week post-transfection. The best electric pulse protocol proved to be 1HV+4LV, with 10% of the area transfected ($p<0.05$). LV electric pulses (8LV and 6LV) alone resulted in a transfected area of 4% for 8LV and 2% for 6LV electric pulses (Figure 2A).

Non-invasive transcutaneous observation confirmed the superior transfection efficiency of 1HV+4LV pulses, since the fluorescence intensity of the transfected area showed the highest values throughout the observation period (Figure 2B). The peak of transfection, determined by the normalized fluorescence intensity of the transfected area, was achieved the first week after electrotransfection and was significantly higher in

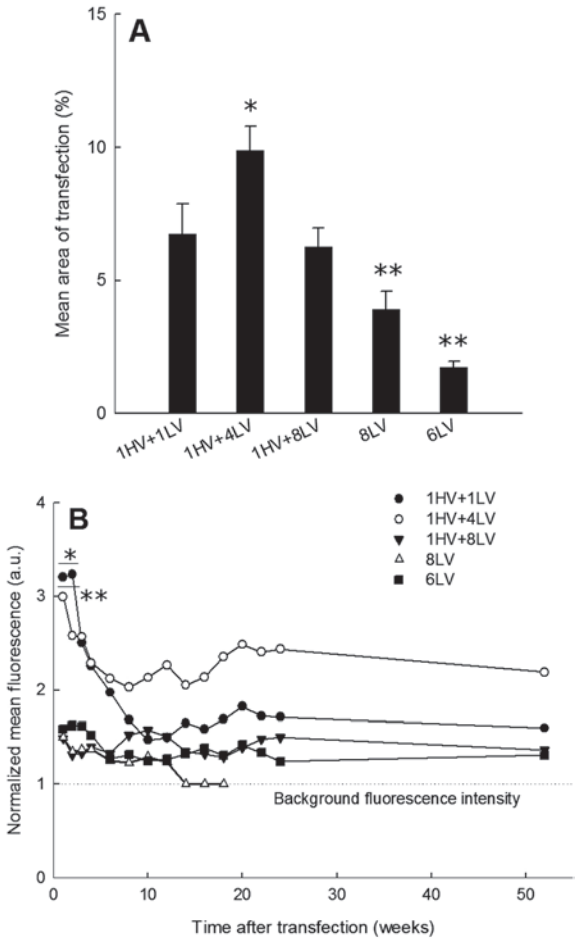

Figure 2: Transfection efficiency and duration of plasmid expression in *m. tibialis cranialis* with different electric pulse protocols. **A**. 20 μg of pEGFP-N1 was injected into *tibialis cranialis* muscle and different sets of electric pulses were applied 10 min later [HV = 600 V/cm, 100 μs, LV = 80-200 V/cm, 20-400 ms, 1-2 Hz (see Table 1)]. Muscle tissue was removed one week after gene transfer and cut into 20 μm frozen tissue sections, which were inspected for area of transfection. *p<0.05 vs. other electric pulse protocols; Error bars represent SEM. **p<0.05 vs. combinations of 1HV+different numbers of LV electric pulses (n=3). **B**. Mice of the same group were non-invasively observed transcutaneously. Mean fluorescence intensity of transfected area was normalized to mean fluorescence intensity of the non-transfected area. Error bars are not presented to prevent figure overloading. 1HV+1LV and 1HV+4LV at first two weeks after the electrotransfection were statistically significantly different from other electric pulse protocols. *p<0.05 vs. 1HV+8LV, 8LV and 6LV; **p<0.05 vs. 1HV+8LV, 6LV and 8LV (n=3).

the 1HV+4LV and 1HV+1LV groups (p<0.05), compared to other electric pulse protocols. Using these two electric pulse protocols, the mean fluorescence intensity of the transfected area reached 3 times higher values than of the non-transfected area in the first week; it decreased in the following weeks to a level of 2.3 for 1HV+4LV and to 1.6 for 1HV+1LV, where it stayed until the end of the observation period (more than 18 months, data not shown). Other electric pulse protocols resulted in lower fluorescence intensities of the transfected area and were constant throughout the observation period.

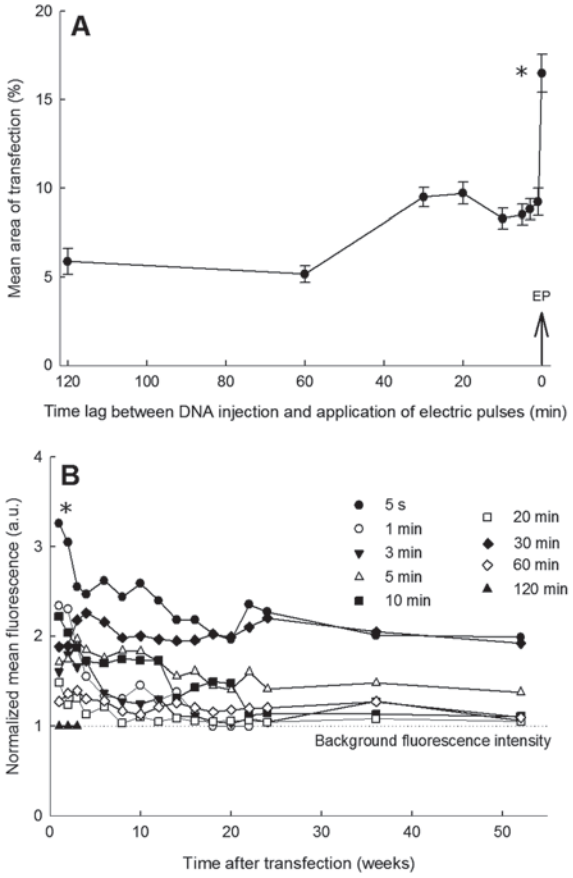

Figure 3: Transfection efficiency and duration of plasmid expression in *m. tibialis cranialis* with different time lags between plasmid DNA injection and application of electric pulses. **A**. 20 μg of pEGFP-N1 was injected into *tibialis cranialis* muscle and 1HV+4LV electric pulses were applied after different time lags from 5 s to 2 h. Muscle tissue was removed one week after gene transfer and cut into 20 μm frozen tissue sections, which were inspected for area of transfection. Error bars represent SEM. *p<0.05 vs. other time lags (n=3). **B**. Mice of the same group were non-invasively observed transcutaneously for as long as 18 months. Mean fluorescence intensity of transfected area was normalized to mean fluorescence intensity of the non-transfected area. Error bars are not presented to prevent figure overloading. 5 s at first week after the electrotransfection was statistically significantly different from other time lags.*p<0.05 vs. other time lags (n=3).

Time Interval Between Injection of Plasmid DNA and Application of Electric Pulses

Different time lags between the injection of plasmid DNA and the application of electric pulses were tested, from 5 s to 2 h with a dose of 20 μg pEGFP-N1 and 1HV+4LV electric pulse protocol. The analysis of frozen muscle sections one week post-transfection showed that the largest area of transfection was obtained when electric pulses followed plasmid injection immediately (5 s). It occupied ~16% of the whole tissue section and was significantly larger compared to the areas obtained at other time lags (p<0.05). Longer duration of the time lag from 1 min to 30 min resulted in ~10% transfected area, and 1 h or 2 h time lag resulted in only 6% transfected area

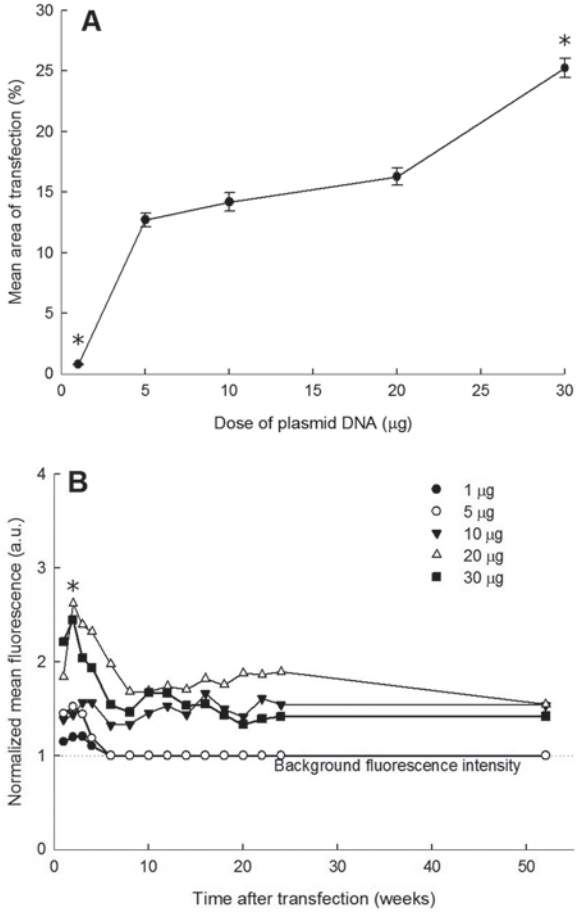

Figure 4: Transfection efficiency and duration of plasmid expression in *m. tibialis cranialis* with different doses of plasmid DNA. Different doses from 1 to 30 μg of pEGFP-N1 were injected into *tibialis cranialis* muscle and 1HV+4LV electric pulses were applied 5 s post-injection. **A**. Error bars represent SEM. *p<0.05 vs. other plasmid DNA doses (n=3). **B**. Mice of the same group were non-invasively observed transcutaneously for as long as 18 months. Mean fluorescence intensity of transfected area was normalized to mean fluorescence intensity of the non-transfected area. Error bars are not presented to prevent figure overloading. 30 μg and 20 μg at two weeks after the electrotransfection were statistically significantly different from other doses of plasmid DNA. *p<0.05 vs. other plasmid DNA doses (n=3).

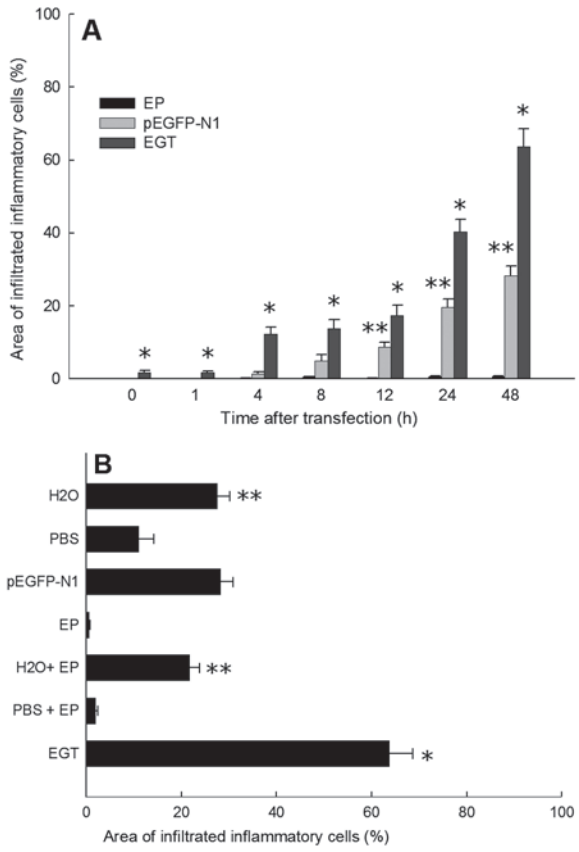

Figure 5: Area of inflammatory mononuclear cell infiltration in *m. tibialis cranialis* in the first 48 h after electrotransfection. Muscles were treated either by injection of plasmid DNA alone (pEGFP-N1), electric pulses alone (1HV+4LV; EP) or a combination of both (EGT). EPs were applied immediately after plasmid injection (20 μg). **A**. Inflammatory mononuclear cell infiltration was determined on hematoxylin-eosin stained tissue sections as a fraction of whole tissue section. *p<0.05 vs. EP or pEGFP-N1; +p<0.05 vs. EP. **B**. The controls showed the contributions of individual components of electrotransfection. 20 μl H_20, PBS or plasmid were injected into *tibialis cranialis* muscle alone or in combination with 1HV+4LV electric pulses applied immediately after injection. *p<0.05 vs. other groups; +p<0.05 vs. PBS, PBS (1HV+4LV) and 1HV+4LV.

(Figure 3A). No correlation between time lag and expression level was obtained for the time lags from 1 min to 2 h.

The time lag of 5 s showed superior transfection efficiency also in non-invasive transcutaneous muscle observation during the first 15 weeks post-transfection. The peak of normalized fluorescence intensity (for 5 s time lag) was achieved the first week after electrotransfection and was significantly ($p<0.05$) higher than fluorescence intensity observed at any other time lag used. During the first week the fluorescence intensity of the transfected area was more than 3 times higher than that of the non-transfected area, and by the end of the third week it decreased to 2.5; it reached 2.0 by the end of the ninth month after electrotransfer. Thirty minutes' time lag resulted in sustained transfection throughout the observation period, and resulted in transfection comparable to 5 s lag after 15 weeks post-transfection. Other time lags, except 120 min, resulted in low fluorescence intensity in the first few weeks (about half the value of 5 s time lag); in the following weeks intensity was further reduced and almost reached the values of non-transfected areas at ~15 weeks post-transfection (Figure 3B). Similar to the results for frozen muscle sections, no correlation was found between expression level at different time lags, except for the time lag 5 s at the first week post-transfection.

Dose of Plasmid DNA

Different doses of plasmid DNA ranging from 1 μg to 30 μg were injected into the *tibialis cranialis* muscle of the mice 5 s prior to the application of electric pulses (1HV + 4LV).

One week after transfection, a dose of 1 μg plasmid DNA resulted in an area of transfection less than 1%. When 5 μg or 10 μg of plasmid DNA were used, 12-14% of the whole muscle area was transfected. A minor increase in transfected area was observed when 20 μg of plasmid DNA was used for electrotransfection (16%). The area of transfection was the largest when 30 μg of pEGFP-N1 was used for electrotransfection; this reached 25% of the whole muscle section. It was statistically significantly larger than the areas of lower doses ($p<0.05$) (Figure 4A).

Non-invasive transcutaneous observation confirmed the dose dependency of transfection efficiency in the muscle. The normalized fluorescence intensity of the transfected area at the highest doses was significantly higher for the first four weeks compared to other plasmid DNA doses used ($p<0.05$). Thereafter the fluorescence intensity of the highest doses declined until it reached the same level as the 10 μg plasmid DNA dose, and then remained at this level until the end of the observation period (18 months, data not shown). Fluorescence of transfected muscle fibers in groups with plasmid DNA doses of 1 μg and 5 μg decreased to the background fluorescence intensity levels at 6 weeks post-electrotransfection (Figure 4B).

Infiltration of Inflammatory Mononuclear Cells into the Treated Muscle

Infiltration of inflammatory mononuclear cells during the first two days after the procedure was found in the treated muscle using an electrotransfection protocol that yielded high GFP expression (1HV+4LV, 20 μg pEGFP-N1 and 5 s time lag). Inflammatory infiltrate was diffusely distributed in the endomysium around the muscle fibers. Lymphocytes and plasma cells predominated among inflammatory cells. The percentage of muscle section area with inflammatory infiltrate was evaluated; the results showed no significant increase in the area of inflammatory mononuclear cell infiltration after the application of electric pulses only (Figures 5A, 6C). Injection of plasmid DNA resulted in infiltration of inflammatory mononuclear cells, which became detectable 8 h after injection (Figure 5A). The area of infiltration increased from ~5% at the 8 h time interval to about 30% at 48 h after injection (Figures 5A, 6B). Infiltration of inflammatory mononuclear cells was observed even earlier in the electrotransfection group (Figure 5A). The area of infiltration was significantly larger in this group compared to plasmid DNA injection or application of electric pulses at all time intervals tested ($p<0.05$). It was significantly increased already at 4 h after electrotransfection (~15%) compared to other groups, and further increased to above 60% at 48 h after the procedure (Figures 5A, 6D).

In order to discern whether the inflammation was due to the DNA or the vehicle in which the DNA was dissolved, two different vehicles were compared: PBS and distilled water (Figure 5B). The area of infiltration was less than 10% when PBS was injected into the muscle alone or combined with application of electric pulses. Injection of distilled water alone, without DNA, resulted in an area of infiltration of inflammatory mononuclear cells comparable to plasmid DNA injection. The area of infiltration was in both cases increased when the injection was combined with application of electric pulses, but to a significantly greater extent in the case of plasmid DNA injection combined with application of electric pulses ($p<0.05$) (Figure 5B).

The samples were checked also for the presence of necrosis. No necrosis of muscle fibers was observed 48 h after the procedure. To assess late histological changes, muscles from the treated mice were harvested several months after electrotransfection (6-12 months post-transfection). The muscle tissue had a normal appearance and no necrosis or muscle regeneration was observed (Figure 6E).

Discussion

The results of our study demonstrate that the level and duration of expression of transgene in mouse muscle can be regulated by appropriate selection of electric pulse parameters,

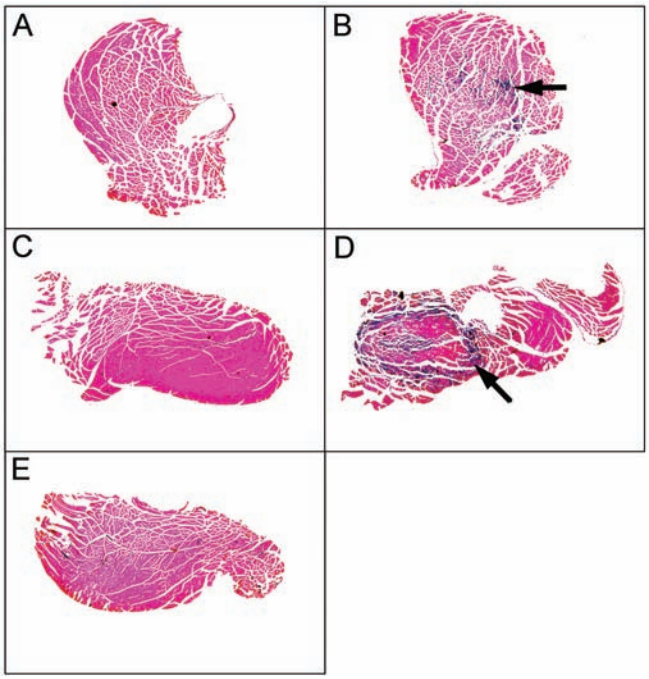

Figure 6: Infiltration of inflammatory mononuclear cells. Muscles were removed 48 h after procedure (A-D), fixed in formaldehyde and embedded in paraffin blocks that were cut into tissue sections of 10 μm and stained with hematoxylin-eosin dye. Arrows show area of infiltration of inflammatory mononuclear cells. **A**. Control; **B**. injection of pEGFP-N1 plasmid; **C**. application of 1HV+4LV electric pulses; **D**. injection of pEGFP-N1 and application of 1HV+4LV electric pulses; **E**. histology changes assessed 12 months after the procedure (injection of plasmid DNA and application of 1HV+4LV electric pulses).

dose of plasmid DNA and time lag between DNA injection and application of electric pulses. We determined that the most efficient electrotransfection protocol tested in our study for short-term expression was 1HV+4LV electric pulses applied immediately (5 s) after the injection of 30 μg of plasmid DNA into the muscle. In addition, we demonstrated that the combination of 1HV+4LV resulted in longer transgene expression compared to other groups, and that the dose of injected DNA, if above 10 μg/muscle, had no influence on the duration of transgene expression. Furthermore, the time lag between the plasmid DNA injection and the application of electric pulses is important for short-term transgene expression, but to a lesser extent for long-term expression.

Compared to other studies, short-term GFP expression levels in our study were lower. The reason for this discrepancy is most probably due to the different methods of measuring gene expression: namely, transfection efficiency in those studies was determined as a percentage of transfected muscle fibers in the traverse muscle sections and reached 50-80% (26, 27). In our study transfection efficiency was determined as a percentage of the area of transfected muscle, which yielded lower percentages of transfection efficiency.

Electric pulse protocol is one of the most important parameters of electrotransfection. The application of electric pulses should result in a sufficient electric field to permeabilize the cell membrane, but should not be too high in order to avoid irreversible cell damage. The electric pulse parameters used in different studies varied substantially. Electric pulses were either unipolar square wave LV millisecond pulses, HV microsecond pulses, trains of 1,000 square wave bipolar pulses, microsecond pulses, or lately the combination of HV and LV pulses has also been used (7-9, 13-17, 19, 20). The HV pulse should exceed the electric field permeabilization threshold, which proved to be 540 V/cm at 100 μs for skeletal muscle (10). Several LV electric pulses of long duration (>50 ms), which follow the HV pulse, are supposed to contribute to the electrophoretic mobility of DNA towards and into the cells (14). The results of our study demonstrate that a combination of HV+LV pulses resulted in higher and longer-lasting expression of reporter gene compared to the application of LV pulses alone. Only a few studies so far have compared the transfection efficiency of a combination of HV+LV pulses with other electroporation protocols. In contrast to the results of our study, the results of other studies did not demonstrate a significant difference in transfection efficiency between the two protocols used (13, 18). The reason for the obtained discrepancy lies most probably in the very low dose of plasmid used in the studies of Bureau *et al*. (3 μg/muscle) compared to the dose in our study (20 μg/muscle). Such a low dose of plasmid DNA might result in a relatively low expression, and therefore significant differences in levels of transgene expression were not identified.

Several studies have already addressed the influence of time lag between the injection of plasmid DNA and the application of electric pulses on short-term transfection efficiency, however, without firm conclusions (16-18). Bureau *et al*. described no significant difference in transfection efficiency if the gene for reporter protein luciferase was injected in murine *tibialis cranialis* muscle immediately or 3 h before the application of electric pulses (18). Moreover, they hypothesized that plasmid DNA is distributed between two compartments very rapidly (within 5 min) after intramuscular injection. In the first compartment, plasmid DNA is rapidly cleared and degraded, but in the second one it is protected from cleavage by DNAases for a longer time; that's why this compartment represents the electrotransferable pool of plasmid DNA (18). In another study, significantly higher expression was obtained when plasmid DNA was injected 30 min before electroporation, compared to shorter intervals (10 and 5 min and 5 s) (16). Furthermore, in the study of Wang *et al*., only 0, 30, and 60 s time lag intervals were tested, showing that the 0 s interval led to the highest transfection level (17). In our study we observed 50% better transfection efficiency when we applied electric pulses immediately after the injection of plasmid DNA (at 5 s following plasmid DNA injection) compared to any other time lag from 1 min to 2 h. This is in agreement with the results of the study of Wang *et al*., demonstrating that indeed, application of electric pulses must be performed immediately after plasmid DNA injection. In most of the studies, the authors state that electroporation was performed immediately post-injection, but this interval can be as long as 1 min, which can result in lower transfection efficiency. Based on our results and the results of the study by Bureau *et al*., we can speculate that clearance and degradation of injected DNA is a very fast process, which is completed within 1 min, and that ~50% of injected DNA is lost in this way.

The interesting result in our study was that the 30 min interval yielded the same levels of reporter gene expression as the 5 s time interval at longer post-transfection times. The levels of transgene expression were higher during the first 15 weeks following electrotransfer for the 5 s time interval. The time interval 30 min between plasmid DNA injection and application of electric pulses resulted in a steady level of transgene expression throughout the observation period (more than 1.5 years). The expression of reporter gene after electrotransfer utilizing all other time intervals resulted in a minimal transfection efficiency that declined close to background levels within 10-20 weeks post-transfection. The decline of transgene expression could be for several reasons. GFP expression from pEGFP-N1 is regulated by CMV promoter, which was shown to be methylated after gene transfer to the host cells. The methylation was observed to result in transcriptional silencing (28). Another study indicated that the immunogenicity of a transgene could also be a reason

for the decline of transgene expression, since transgene-expressing muscle fibers might be destroyed by immune cytolytic destruction (29). However, further studies are clearly needed to fully clarify the influence of the time lag between DNA injection and application of electric pulses on transfection efficiency, especially over long time periods.

Another parameter that has a great impact on transfection efficiency is the dose of plasmid DNA. As already demonstrated by many authors, increasing the dose of plasmid DNA used for electrotransfection results in an increase of transgene expression (27, 30–32). The concentration of injected DNA was shown to be even more important than the cumulative dose (17). In our study, the increase of concentration was assured with the constant volume of injected plasmid DNA at each dose. Short-term transgene expression showed a dose-dependent increase in transfection efficiency. Long-term observation of transgene expression, on the other hand, showed that doses lower than 5 μg of plasmid DNA resulted in complete arrest of transgene expression by the end of the fifth week after electrotransfection. Transgene expression was observed for an additional 17 months in a dose-independent manner when doses higher than 10 μg/muscle were used for electrotransfection. This difference is important, since it indicates that using 5 μg of plasmid DNA results in undiminished transgene expression 1 week after electrotransfection, but in shorter lasting transgene expression compared to higher doses. This is crucial for some applications of gene therapy, where it is preferable to restrict transgene expression to only a short period of time after electrotransfection. Short-term expression is suitable for certain kinds of applications, such as gene therapy of cancer, where prolonged expression of therapeutic genes can lead to undesirable side effects after the tumor is eradicated.

Muscle damage after electrotransfer is an important topic with high clinical relevance. In order to translate the electrotransfer of genetic material into clinical practice, the safety of this method must be demonstrated. Electric pulse parameters used for electrotransfer have to be carefully selected in order to induce permeabilization of the cell membrane, allowing transfer of the molecules into the cells without causing cell death. Recently, an algorithm that monitors *in vivo* cell electroporation and is able to adjust electric field strength in real time has been developed, implemented in a prototype device, and tested. The algorithm has proved to be useful for ensuring that excessive voltages to muscles, which lead to intense muscle damage and consequently lower expression of transgenes, are not applied (33). Besides muscle necrosis, infiltration of inflammatory mononuclear cells is a common tissue reaction to the electrotransfer of DNA. The results of a vast number of studies have demonstrated that muscles are heavily populated with inflammatory mononuclear cells (34-37). Muscle fiber necrosis was reported at excessive electric field intensities or electric pulse duration (8, 38). In a recent study, expression profile was investigated and histological changes evaluated after electrotransfection of skeletal muscle using HV and LV electric pulses. A faint striation pattern was observed in a few muscle fibers 48 h after the treatment, but there was no significant change in the expression of proteins involved in inflammatory responses or muscle regeneration, indicating limited muscle damage and regeneration (39). No necrotic muscle fibers were observed at the 48 h interval after electrotransfection using HV+LV electric pulses. Nor was there any tissue damage or necrotic muscle fibers several months after electrotransfection, which showed that muscle cells tolerate the expression of GFP. Furthermore, the infiltration of inflammatory mononuclear cells that was observed after electrotransfer is desirable, as it can enhance the immune response to DNA vaccines introduced by electroporation. The infiltration of inflammatory mononuclear cells was the highest after electrotransfer of plasmid DNA. Application of electric pulses did not result in infiltration, while injection of plasmid DNA alone resulted in ~30% infiltration, but 50% less compared to combined treatment. In order to discern whether the inflammation was due to the plasmid DNA or the vehicle in which the DNA was dissolved, two different vehicles were compared: PBS and distilled water. Our results show that the same level of infiltration was achieved when distilled water or DNA dissolved in water was injected into the mouse muscle. The same level of infiltration was also achieved when injection of water was followed by application of electric pulses. Therefore, the significant increase in mononuclear cell infiltration following a combination of plasmid DNA injection and electroporation can be attributed to the intracellular presence of DNA and the expression of reporter genes. This is further supported by the fact that the infiltration of mononuclear cells started to increase at 4 h post-transfection, when the translation of the transferred gene is already underway (40), and was further increasing at longer time points.

There are many factors that influence the efficiency and duration of transgene expression in muscle, such as pretreatment of muscle with hyaluronidase, selection of appropriate promoters in plasmid DNA, electric pulse parameters, dose and size of the plasmid DNA, and also the age and strain of the animals. In our study we demonstrated that the level as well as duration of expression can be achieved and controlled by appropriate selection of electric pulse protocol, time lag, and plasmid dose. We also showed that higher long-term duration of transgene expression (>1.5 years) can be achieved by the use of 1HV+4LV pulses compared to 8 LV pulses, extending their use in the therapeutic fields where long-term sustained secretion of therapeutic protein into the blood stream is needed, such as in the treatment of monogenetic diseases.

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Local and systemic antitumor effect of intratumoral and peritumoral IL-12 electrogene therapy on murine sarcoma

Darja Pavlin,[1] Maja Cemazar,[2] Urska Kamensek,[2] Natasa Tozon,[1] Azra Pogacnik[1] and Gregor Sersa[2,*]

[1]Veterinary Faculty Ljubljana; University of Ljubljana; Ljubljana, Slovenia; [2]Department of Experimental Oncology; Institute of Oncology Ljubljana; Ljubljana, Slovenia

Key words: electroporation, electrogene therapy, sarcoma, IL-12, tumor, skin

Soft tissue sarcomas pose a challenge for successful treatment with conventional therapeutic methods, therefore newer therapeutic approaches are considered. In this study, we evaluated the antitumor effect of IL-12 electrogene therapy (EGT) on murine SA-1 fibrosarcoma. The therapeutic plasmid was injected either intratumorally into subcutaneous SA-1 nodules or intradermally into the peritumoral region. We achieved a remarkable local and systemic antitumor effect with both approaches after single plasmid DNA application, with significant intratumoral and systemic production of IL-12 and IFNγ. Intratumoral IL-12 EGT resulted in over 90% complete response rate of the treated tumors with 60% of cured mice being resistant to challenge with SA-1 tumor cells. Peritumoral EGT resulted in a lower complete response rate (16%), with significant growth delay of remaining tumors. Both therapies also resulted in significant inhibition of growth of untreated tumors, growing simultaneously at a distant site. These data suggest that IL-12 EGT may be useful in the treatment of soft tissue sarcomas, exerting a local and systemic antitumor effect.

Introduction

Soft tissue sarcomas are a heterogenous group of mesenchymal tumors, originating from connective tissues (e.g., muscle, fibrous, adipose, neurovascular). In human medicine, they represent approximately 1% of malignancies in adults and 15% of pediatric malignancies.[1] Local tumor control is the most important aspect of tumor management, with surgical resection being the cornerstone of therapy. However, local recurrence after conservative surgical excision is common and can be as high as 65%. This warrants radical resection with recommended margins of at least 2–3 cm around the tumor mass and one fascial layer deep. Such aggressive treatment is warranted because long-term survival strongly correlates with permanent local control with the first treatment. Other treatment options include radiotherapy and chemotherapy, which can be combined with surgical techniques for larger lesions, since large tumors (>5 cm in diameter) respond poorly to nonsurgical treatment.[1,2] In humans with metastatic disease, chemotherapy produces poor response rates of 20%, which does not have an impact on overall survival.[1,2] Overall, 5 y survival rate is approximately 35–65%, with size of the tumor being the most important prognostic factor.[1,3] Due to a poor response rate and limited treatment options, newer therapeutic approaches are considered, either as primary or adjuvant therapy to conventional methods in order to improve the clinical outcome of cancer treatment.

One of the newer therapeutic modalities, which have already been successfully evaluated in sarcoma tumor models, is gene therapy with interleukin-12 (IL-12), utilizing both viral and nonviral gene delivery. Viral delivery was instituted using adenoviral vectors, which were delivered primarily intratumorally, for example into different types of fibrosarcoma[4-6] and Ewing's sarcoma.[7] Adenoviral constructs expressing IL-12 were also delivered intranasally for treatment of osteosarcoma lung metastases.[8] Published reports suggest that viral IL-12 gene therapy can be efficient in inhibition of growth of treated tumors and prolonging survival of treated animals.[5,7,8] However, despite being highly efficient, possible systemic toxicity and stimulation of the patient's immune system raise concerns about their safe clinical use. Therefore, as a safer alternative method of gene delivery, different nonviral techniques are being investigated. Among them only a few were evaluated for antitumor efficiency in sarcoma, including use of naked plasmid DNA,[9,10] gene gun[11] and polyethylimine DNA vector.[12-14] Although they resulted in regression of tumor growth, induction of long-lasting antitumor immunity and eradication of induced lung metastases, they elicited a poorer therapeutic effect compared to viral IL-12 delivery.

One of the approaches to nonviral gene delivery, which can dramatically improve the transfection efficiency of plasmid DNA, is the use of electroporation for gene electrotransfer or electrogene therapy (EGT).[15-18] It is performed by direct injection of the therapeutic gene into the target tissue, followed by application

*Correspondence to: Gregor Sersa; Email: gsersa@onko-i.si
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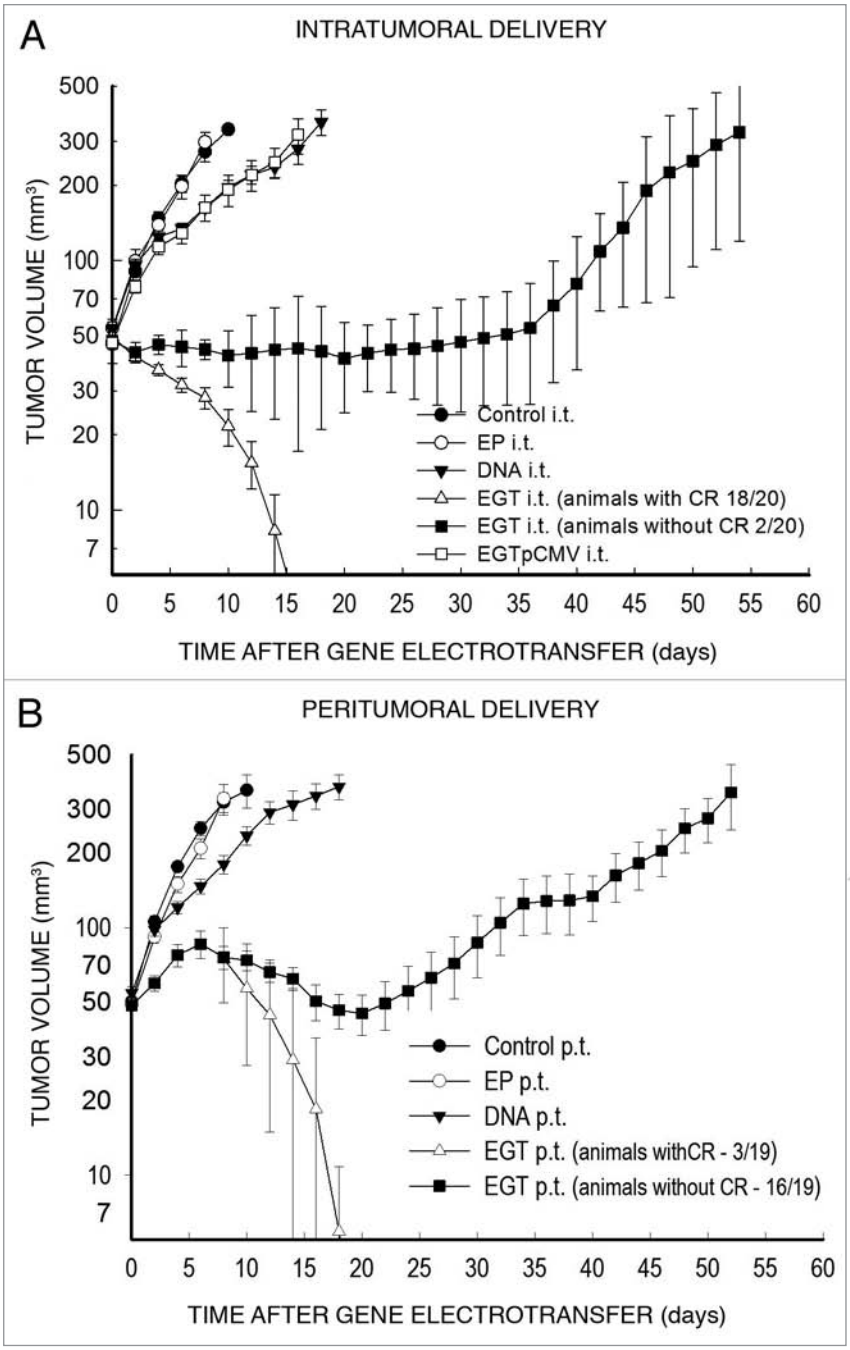

Figure 1. Antitumor effectiveness of electrogene therapy (EGT). (A) Intratumoral EGT on subcutaneous SA-1 tumors resulted in a high level of complete responses (18/20 tumors) by day 20 after initiation of therapy, with significant inhibition of tumor growth in the remaining two tumors. (B) Peritumoral EGT on subcutaneous SA-1 tumors resulted in a lower complete response rate (3/19 tumors), with the remaining 16/19 showing significant delay in tumor growth.

of controlled electric pulses which facilitate intracellular uptake of DNA molecules. EGT, using a plasmid encoding IL-12, has already been utilized in a number of different tumor models both at the preclinical level[19-21] and in clinical trials.[22] Tumor models which favorably responded to IL-12 EGT were melanoma,[23-28] lymphoma[27] and a variety of different carcinomas.[29-37]

To date, there is no published data regarding the effect of intratumoral and peritumoral EGT with the plasmid encoding IL-12 in a sarcoma tumor model. Therefore, the aim of this study was to evaluate antitumor efficacy of EGT with IL-12 for treatment of murine sarcoma. Antitumor effectiveness was determined after the therapeutic gene was injected either intratumorally or intradermally in the peritumoral region of established subcutaneous murine SA-1 tumors, followed by application of electric pulses. IL-12 and IFNγ concentrations in serum and in tumor tissue were followed and, in addition, the antitumor effectiveness on distant untreated tumors was evaluated.

Results

Antitumor effectiveness of intratumoral and peritumoral EGT with IL-12. The local antitumor effect of EGT with IL-12 on subcutaneous murine sarcoma was determined, utilizing either intratumoral or peritumoral injection of the therapeutic plasmid, followed by electroporation of the injected tissue. EGT with IL-12, applied either intratumorally or peritumorally, was very effective; growth of tumors was significantly suppressed (**Fig. 1**). Doubling time and growth delay in the intratumoral EGT group could not be determined since most of the tumors completely regressed and only two slowly grew again after the treatment (**Fig. 1A**). Doubling time of the peritumoral EGT group was 32.74 ± 3.34 days (for tumors, which did not reach a complete response) with growth delay of 30.9 d (**Fig. 1B**). Control groups, receiving only application of water, and experimental groups, receiving either application of electric pulses alone, plasmid DNA, or EGT with control plasmid pCMV, showed progressive tumor growth with similar doubling times in all groups, ranging from 1.84 ± 0.18 to 3.2 ± 0.41 d. The differences in doubling times of these groups were not statistically significant.

A complete response to therapy was achieved only in the experimental groups receiving EGT. Intratumoral EGT led to complete disappearance of subcutaneous nodules in 90% (18/20) of treated animals, all of them remaining tumor-free at the end of the 100 d observation period (**Fig. 2**). The first complete response occurred on day 9 after initiation of therapy and the number of cured animals rapidly progressed until day 20. Peritumoral EGT led to complete response in 15.6% of animals (3/19), with the first complete tumor disappearance occurring on day 14, and the remaining two by day 20 (**Fig. 2**).

Intratumoral and systemic secretion of IL-12 and IFNγ. Local intratumoral and systemic secretion of IL-12 and its induction of the IFNγ response were determined in tumor tissues and serum samples collected from each experimental group 5 d after therapy. The results showed that both intratumoral and peritumoral EGT with IL-12 led to local and systemic secretion of high intratumoral and serum concentrations of both IL-12 and IFNγ.

IL-12 was detected in the serum of all experimental groups, with significantly elevated concentrations ($p < 0.05$) in both groups receiving EGT. Intratumoral EGT resulted in an IL-12 concentration of 16.4 ± 8.8 pg/mL and peritumoral EGT resulted in a concentration of 19.7 ± 6.9 pg/mL (**Fig. 3**). Similarly, serum concentrations of IFNγ were the highest in two groups receiving intratumoral EGT (66.3 ± 15.2 pg/mL) and peritumoral (86.1 ± 21.6 pg/mL) EGT. The difference in serum concentrations of both cytokines between the intratumoral and peritumoral EGT group was not statistically significant ($p > 0.05$). In the experimental groups receiving only injection of DNA or application of electric pulses, serum concentrations of IL-12 did not exceed 1.03 pg/mL and serum concentrations of IFNγ were less than 1.55 pg/mL (**Fig. 3**). The differences in concentration for both cytokines between all these groups were not statistically significant ($p > 0.05$).

Intratumoral concentrations of both cytokines were detected in all experimental groups. However, significantly elevated levels ($p < 0.05$) were detected in both experimental groups receiving EGT (**Fig. 4**). IL-12 concentrations reached 53.6 ± 13.1 ng/mg of tumor tissue after intratumoral EGT and 22.5 ± 8.7 ng/mg of tumor tissue after peritumoral EGT. Intratumoral levels of IFNγ were similar in both of these groups, with a concentration of 11.6 ± 2.1 ng/mg of tumor tissue after intratumoral EGT and 6.7 ± 1.6 ng/mg of tumor tissue after peritumoral EGT. The differences in concentration of either cytokine were not statistically significant between these two experimental groups. IL-12 in other experimental groups did not exceed 173 pg/mg of tumor tissue and the concentration of IFNγ was typically in the range of 1 ng/mg of tumor tissue (**Fig. 4**).

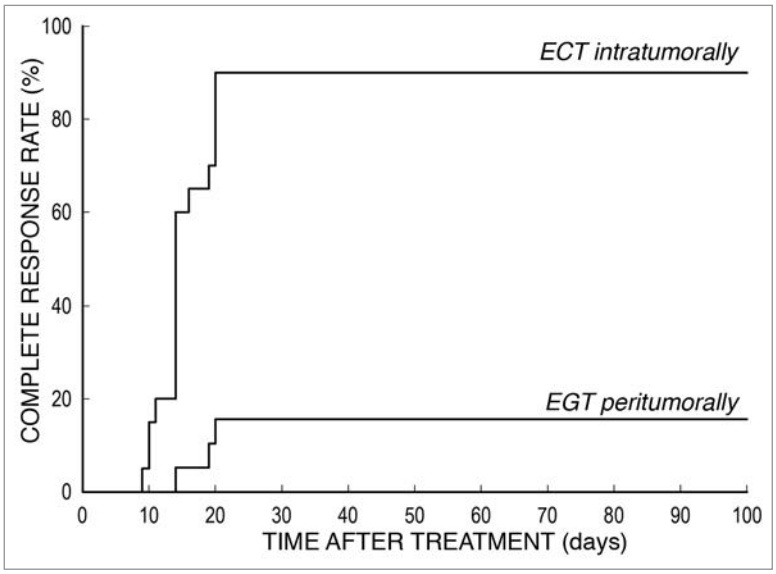

Figure 2. Complete response rate achieved in intratumoral and peritumoral EGT group.

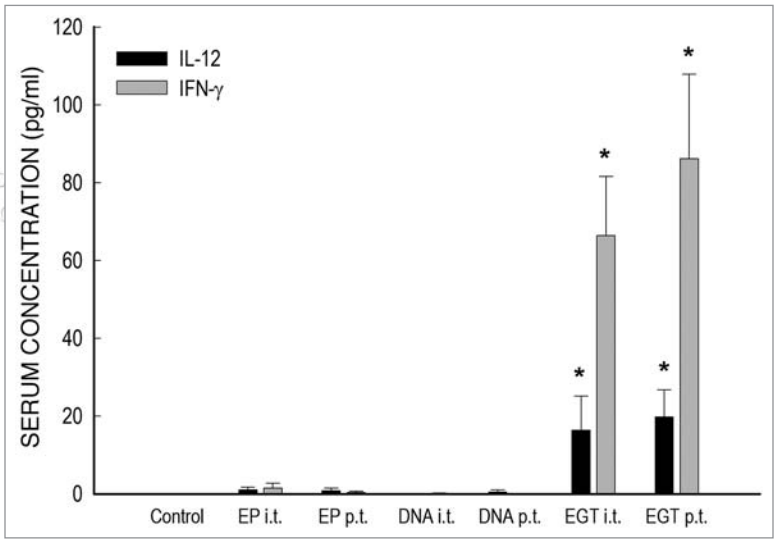

Figure 3. Serum concentrations of IL-12 and IFNγ after EGT. Significantly elevated levels of both cytokines were detected in mice after intratumoral (i.t.) and peri tumoral (p.t.) EGT (*). The difference in serum cytokine concentrations between intratumoral and peritumoral EGT was not significant.

Side effects of the procedure. Animals were weighed and their general health status was followed on a regular basis in order to evaluate possible systemic side effects of EGT with IL-12. All animals died due to euthanasia when their tumor nodule reached approximately 350 mm^3 and no deaths from other causes occurred. No significant weight loss was observed in any of the experimental groups (data not shown). In the intratumoral and peritumoral EGT group, animals with a complete response to therapy lived for approximately 120 d until challenged with second injection of SA-1 tumor cells, and were euthanized around 30–40 d thereafter, when new tumors reached volume of approximately 350 mm^3. These animals therefore survived altogether over 150 d. At the end of experiment, they had an approximately 20% increase in body weight, compared to day 0, and they all were in very good general condition.

Resistance to challenge. In the experimental group receiving intratumoral EGT in which tumors completely responded to therapy, animals were challenged with 5 x 10^5 tumor cells injected subcutaneously on the opposite flank one hundred days after complete disappearance of primary tumor nodules, without any additional therapy. Of the 18 animals challenged, 11 (61%) were resistant to tumor regrowth (**Fig. 5**). In the experimental group receiving peritumoral EGT, only three animals with complete regression of tumors survived 100 d, and all three

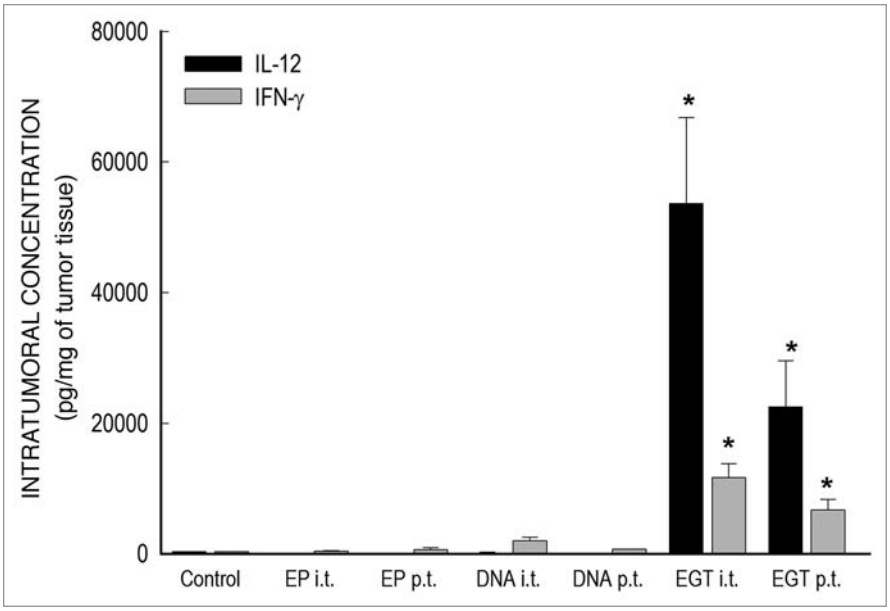

Figure 4. Intratumoral concentrations of IL-12 and IFNγ after EGT. Significantly elevated levels of both cytokines were detected in tumors after intratumoral (i.t.) EGT and peritumoral (p.t.) EGT (*p < 0.05). The difference in intratumoral cytokine concentrations between intratumoral and peritumoral EGT was not significant.

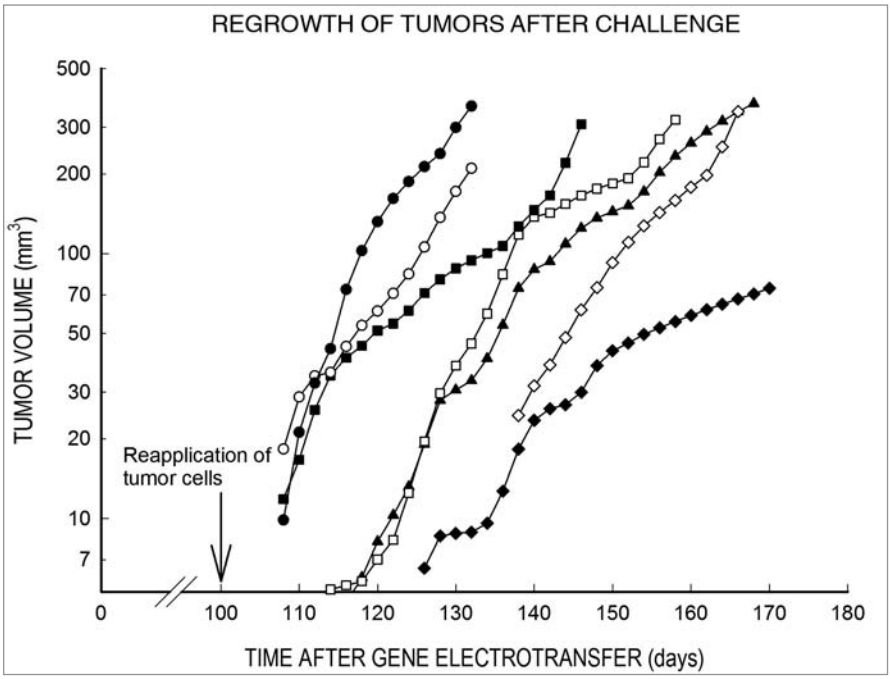

Figure 5. The growth curves of tumors in animals that were challenged with tumor cells injected 100 d after the initial treatment. Each line represents growth of specific tumor that appeared after challenge. 11/18 animals were resistant to challenge with tumor cells.

were resistant to challenge with application of tumor cells. This result suggests the development of an immune response memory following treatment of the initial subcutaneous tumor.

Effect of EGT on untreated subcutaneous tumors growing at a distant site. A possible systemic antitumor effect of intratumoral and peritumoral EGT with IL-12 on growth of untreated tumors at a distant site was also evaluated. Treated tumors in all experimental groups responded to therapy in a similar fashion as treated tumors in the first part of the study, with similar doubling times and complete response rates. In this part of the study we even achieved 100% complete response rate in tumor nodules treated with intratumoral EGT.

Untreated tumors in both experimental groups receiving EGT showed statistically significant inhibition of growth (**Fig. 6**). This effect in untreated tumors was less pronounced and of shorter duration compared to treated nodules. Intact tumors in both EGT groups exhibited only delayed growth, and no complete response to therapy was reached. Untreated tumors in the experimental group receiving intratumoral EGT had a tripling time 22.0 ± 3.9 d and growth delay of 18.06 d (**Table 2**). Untreated tumors in the experimental group receiving peritumoral EGT had a tripling time 14.69 ± 3.6 d with a growth delay of 10.74 d. The difference in tripling times in these two groups was not statistically significant. Other groups did not show any growth delay for untreated tumors, with tripling times ranging from 3.32 ± 0.62 – 3.95 ± 0.61 d (**Table 2**). The differences in growth of treated and untreated tumors in these groups were not statistically significant (**Table 2**).

Discussion

Our study demonstrates that IL-12 EGT, applied either intratumorally or peritumorally, is an effective therapeutic approach with local as well as systemic effects in the treatment of sarcoma tumors. It results in a significant percentage of tumor curability, induction of long-term antitumor immunity and even elicits a systemic antitumor effect, demonstrated by delayed growth of untreated tumors growing at a distant site. The antitumor effectiveness of IL-12 EGT arises from high intratumoral and systemic secretion of biologically active IL-12 which induces production of IFNγ, without noticeable side effects.

One of the approaches to gene therapy, which already demonstrated an antitumor effect in sarcoma, is delivery of the therapeutic gene encoding IL-12, using either adenoviral or a few nonviral vectors. However, to date, there is no published data determining the efficacy of EGT on subcutaneous sarcoma

and none of the studies compared the effectiveness of the intra- and peritumoral approach in any type of tumor model. The main advantage of EGT, compared to viral vectors, is its nonexistent toxicity, low cost and simplicity of large scale vector (plasmid DNA) preparation. Furthermore, transfection efficiency close to those of viral vectors can be achieved, which is its main advantage compared to other nonviral gene delivery methods.[38]

The goal of our study was to evaluate the antitumor effectiveness of IL-12 EGT, applied either intratumorally or intradermally into the peritumoral region in the treatment of sarcoma tumors. The majority of research on the antitumor effect of intratumoral EGT with IL-12 was done on a melanoma tumor model and a number of different carcinomas, even progressing to human clinical trials.[22] Comparison of therapeutic efficiency of intratumoral versus intradermal EGT was carried out based on the fact that intradermal gene electrotransfer can result in either local or systemic transgene expression.[39-41] Successful intradermal IL-12 gene electrotransfer has already been achieved, resulting in as high as a 10-fold increase of systemic concentrations of IFNγ, compared to control groups,[42] whereas data on possible systemic production of IL-12 after intratumoral delivery is ambiguous. Based on these facts we assumed that with intradermal EGT positioned into the peritumoral region, we will combine highly efficient production of IL-12 both at the systemic and local intratumoral level.

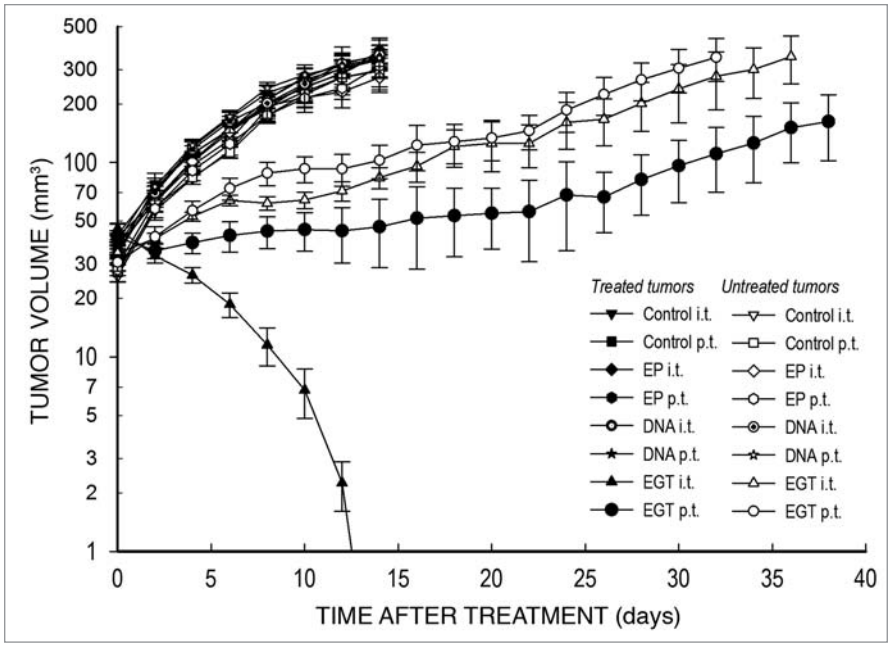

Figure 6. Comparison of growth of treated and untreated tumor nodules. Growth of treated tumors was similar to tumor nodules in the first part of the study (Fig. 1). Untreated tumors in experimental groups receiving EGT on contralateral tumor nodules, showed significant inhibition of growth after both intratumoral (i.t.) and peritumoral (p.t.) application.

Two important goals of successful antitumor gene therapy are long-term eradication of established tumors and possible generation of a systemic immune response and resistance to development of new tumors. They were both accomplished in our study using single intra- or peritumoral application of EGT with IL-12. We achieved a remarkable local antitumor effect on treated sarcomas, with significant inhibition of growth and long-term complete response rates reaching 90–100% of tumors treated with intratumoral EGT and approximately 16% in tumors treated with peritumoral EGT. This direct antitumor effect was better compared to all published reports on the effectiveness of IL-12 gene therapy on sarcomas, employing either viral or nonviral therapeutic gene delivery. Even though viral gene therapy is believed to be superior to nonviral in terms of transfection efficiency,[39] intratumoral IL-12 EGT produced higher complete response rates compared to adenoviral intratumoral delivery of the same therapeutic gene in sarcomas, where up to 70–80% complete response rates were reached.[4,5,7] Furthermore, in our experiment, this antitumor

Table 1. Details of the experimental protocol

	Intratumoral application		Peritumoral application	
	Experimental protocol	**No. of animals**	**Experimental protocol**	**No. of animals**
Control	50 µl of distilled water	14	2 x 25 µl of distilled water	11
EP*	50 µl of distilled water EP delivered after 10 min	12	2 x 25 µl of distilled water EP delivered immediately	12
DNA	50 µl of plasmid DNA	12	2 x 25 µl of plasmid DNA	12
EGT**	50 µl of plasmid DNA EP delivered after 10 min	20	2 x 25 µl of plasmid DNA EP delivered immediately	19
EGT pCMV**	50 µl of pCMV control plasmid EP delivered after 10 min	10		

*EP = Electric pulses; **EGT = Electrogene therapy.

Table 2. Comparison of tripling times (TT) and growth delay (GD) in experimental groups, receiving treatment and groups of untreated contralateral tumors

		Treated tumors		Untreated tumors		P
Experimental group		**TT (days)**	**GD (days)**	**TT (days)**	**GD (days)**	
Control	i.tu.	4.61 ± 0.6		3.94 ± 0.49		>0.05
	p.tu.	4.57 ± 0.14		3.95 ± 0.61		>0.05
EP	i.tu.	4.59 ± 0.12		3.86 ± 0.36		>0.05
	p.tu.	4.8 ± 0.34	0.23	3.87 ± 0.54		>0.05
DNA	i.tu.	5.3 ± 0.74	0.69	3.85 ± 0.48		>0.05
	p.tu.	4.23 ± 0.21		3.32 ± 0.62		>0.05
EGT	i.tu.	N/A	N/A	22.0 ± 3.9	18.06	N/A
	p.tu.	35.86 ± 7.57	31.29	14.69 ± 3.6	10.74	0.029

p value refers to significance in difference of TT between treated and untreated tumors in each experimental group.

effect was achieved with just a single intratumoral EGT, whereas in some instances of viral delivery, multiple consecutive applications were needed.[7]

Nonviral delivery methods, which were employed in similar experiments on sarcomas, include intramuscular and intravenous injections of plasmid DNA alone and intramuscular bioballistic gene delivery.[9-11] Even though significant growth delay of tumor nodules was achieved using these techniques, long-term complete responses in animals were either not achieved or they were low, reaching up to 40% of treated animals after intramuscular IL-12 gene delivery using a gene gun in rat sarcoma.[11] These studies produced a comparable direct antitumor effect to peritumoral delivery in our study and drastically lower efficiency compared to intratumoral EGT. It has already been established that use of naked plasmid DNA alone or bioballistic gene delivery (gene gun) yield lower transfection efficiency compared to use of electrotransfection. Electroporation significantly increases transfection efficiency in different tissues, even up to 2,000-fold compared to application of naked plasmid DNA alone.[17,18] Additionally, one of the major disadvantages of the gene gun technique is limitation of gene transfection only to superficial tissues.[39] Therefore, in the case of intramuscular bioballistic delivery of IL-12, surgical exposure of skeletal muscle had to be performed,[11] which makes this technique an invasive procedure, compared to the noninvasive nature of intra- and peritumoral EGT.

Our therapeutic approach produced a better local antitumor effect not only compared to the published viral and nonviral delivery of IL-12 in sarcoma, but also in comparison to intratumoral IL-12 EGT employed in other tumor models. For example, single intratumoral EGT with the same dose of IL-12 plasmid resulted in 47% of complete responses in melanoma,[25] where increased complete response rates (60–80%) were achieved only after increasing the number of intratumoral applications to two or three or addition of intramuscular gene delivery.[26] In carcinoma, the therapeutic effect was even lower, since only a 40% complete response rate was reached after two consecutive applications of the therapeutic plasmid.[31]

This clinical effect on treated tumors in our study was a result of high concentrations of both IL-12 and IFNγ expressed intratumorally after both intratumoral and peritumoral EGT. Data on intratumoral cytokine production after local gene delivery into the sarcoma tumor model are sparse. Jia and colleagues report that intratumoral adenoviral IL-12 delivery resulted in intratumoral production of IL-12 at around 40 pg/mg of tumor tissue and the intratumoral IFNγ concentration did not exceed 400 pg/mg of tumor tissue 2 d after two consecutive therapies.[7] Another approach to local delivery of the IL-12 gene in sarcoma was tested by Duan and colleagues, who utilized intranasal application of a polyethylenimine vector for intrapulmonary gene therapy.[14] In that study, gene therapy administered twice weekly for six consecutive weeks produced intrapulmonary IL-12 concentrations of around 400 pg/mg of lung tissue. Lower intratumoral cytokine concentrations were achieved with intratumoral IL-12-based EGT in other tumor models, e.g., melanoma,[25] where peak intratumoral levels of both measured cytokines did not exceed 10 pg/mg of tumor tissue. Compared to these studies, intratumoral concentrations of IL-12 and IFNγ after IL-12 EGT in our experiment were significantly higher, since at day 5 after therapy, both intratumoral and peritumoral delivery techniques resulted in cytokine levels even as high as 53.6 ng IL-12 per mg of tumor tissue.

Systemic effect of intramuscular EGT with IL-12 was already demonstrated in several tumor models.[25-27,43,44] Systemic effects on distant tumors include inhibition of tumor growth, antimetastatic effect and induction of long-term systemic immunity to regrowth of new nodules. However, the direct antitumor effect on established tumors after intramuscular EGT delivery is generally less pronounced, compared to intratumoral therapeutic gene application.[25,45] Results of our study show that both intra- and peritumoral EGT with IL-12 also exert similar systemic antitumor effects in sarcoma tumor model, along with remarkable direct antitumor effect. Treatment induced long-term resistance to tumor regrowth with both delivery methods. Additionally, single IL-12 EGT contributed to short-term inhibition of growth of the untreated sarcoma tumor nodules growing at a distant site. With simultaneous induction of two tumor nodules, we tried to simulate a frequent clinical situation in which patients are presented with coincident multiple tumor nodules disseminated in different parts of the body. Our results are in accordance with a few of the published studies, which took into consideration the effect of therapy of the primary tumor on growth of distant untreated tumors. In sarcoma, a similar systemic effect

was achieved utilizing intratumoral adenoviral IL-12 gene therapy in Ewing's sarcoma.[7] The effect of EGT with IL-12 on untreated tumors was also demonstrated in melanoma.[26] The mechanism responsible for the observed effect is most probably anti-angiogenic.[25] However, an important difference between these and the presented experiment was that in other studies, the effect was achieved after multiple repetitive gene therapy applications (e.g., five treatments in Ewing's sarcoma), compared to our single intratumoral or peritumoral treatment. Furthermore, these studies are not completely comparable to ours, since none of them investigated the effect of therapy on simultaneously growing tumor nodules. In these studies, secondary tumor nodules were induced 3–9 d after induction of primary tumor nodules with at least one session of gene therapy having been already delivered before induction of secondary tumors. Therefore, this antitumor therapy was employed as a prophylactic approach rather than a therapeutic one, as it was in our study.

The systemic effect demonstrated on untreated tumors of both intratumoral and peritumoral EGT delivery was not statistically different, resulting in similar growth delay of untreated tumors, regardless of the location of injection of the therapeutic plasmid. This may be due to the fact that in both intra- and peritumoral delivery, similar serum levels of both measured cytokines were detected, without statistically significant differences in their concentrations.

In our study, significant systemic elevations of both IL-12 and IFNγ were detected 5 d after EGT with a single application of the therapeutic plasmid. Reports on systemic secretion of either IL-12 or IFNγ after local intratumoral gene delivery are contradictory. In sarcoma, intratumoral adenoviral delivery of theIL-12 gene did not result in systemically measurable levels of both cytokines in all published reports. For example, single intratumoral IL-12 viral delivery into Meth-A fibrosarcoma did not produce any systemically detected cytokine expression.[5] On the other hand, very high serum concentrations of both cytokines were achieved with gene therapy of MCA205 fibrosarcoma,[4] with the IL-12 concentration reaching up to 8 ng/ml of serum on day 2 and rapidly dropping to only 0.1 ng/ml by day 6 after intratumoral application of the adenoviral construct. The serum concentration of IFNγ peaked 2 d after therapy at 4.1 ng/ml and declined to 2.0 ng/ml by day 6. Compared to these results, serum concentrations of both measured cytokines in our study were significantly lower. IL-12 concentrations five days after EGT were approximately 20 pg/ml after either of the delivery techniques, whereas IFNγ did not exceed 100 pg/ml. It is possible that this marked difference in serum concentrations of both cytokines between adenoviral IL-12 delivery and EGT is responsible for better long-lasting immunity, as demonstrated by resistance to challenge with inoculation of the same tumor cells after complete response was achieved (100% of challenged animals were resistant in the study by Gambotto and colleagues, compared to 61% of resistant animals in our study).

Even though serum cytokine levels in our experiment were lower compared to those after adenoviral IL-12 delivery, they are comparable to systemic levels achieved with intramuscular bioballistic delivery in a rat sarcoma model.[11] This nonviral technique produced serum concentrations of both IL-12 and IFNγ around 100 pg/ml on day 10, which regressed to 40 pg/ml around day 28 after therapy.

Systemic production of IL-12 and IFNγ after IL-12 EGT employed in other tumor models are similarly ambiguous. For example, in melanoma, no significant systemic cytokine levels could be detected,[24,25] even after multiple consecutive plasmid applications.[28] On the other hand, systemic expression of IL-12 similar to ours was achieved in different carcinomas,[32,34] but only after multiple applications of gene therapy. One of the possible reasons for such high local and systemic transgene expression, as well as the better antitumor effect achieved in our study, is the difference in electroporation protocols which were used for transfection of tumor nodules.

In conclusion, our study indicates that EGT with IL-12 may offer an effective new approach to therapy of sarcoma, especially in cases with recurring tumor nodules, where other therapeutic options are limited. In smaller tumor nodules, intratumoral EGT could elicit very good local tumor control, providing long-term anti-tumor immunity and an effect on distant nodules. Similar treatment was already successfully utilized in human clinical trial for treatment of melanoma.[22] In larger sarcoma lesions, where conventional therapeutic procedures are limited and effective intratumoral gene electrotransfer is not feasible, peritumoral EGT could provide a useful therapeutic option. With this approach, both a systemic and local antitumor effect could be achieved, which could be enhanced by application of concomitant cytotoxic therapies.

Materials and Methods

Experimental animals. In the present experiments, male A/J mice, purchased at the Institute of Pathology, Faculty of Medicine, University of Ljubljana, Slovenia, were used. At the beginning of the experiments, animals were 10–12 w old. Mice were kept in an animal colony under SPF conditions at constant room temperature (21°C) and 12 h light cycle. Food and water was provided ad libitum. Animals were subjected to an adaptation period of 7–10 d before experiments. All procedures on animals were performed in accordance with the official guidelines of the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (permission No. 323-02-632/2005/6) and in compliance with EU Directive 86/609/EEC.

Tumor induction. Tumors were induced by subcutaneous injection into the right flank of 5 x 10^5/0.1 ml of SA-1 fibrosarcoma tumor cells, which were syngeneic to A/J mice. Cells were prepared from the ascitic form of the tumor. When tumors reached an approximate volume of 40–50 mm^3, mice were randomly divided into experimental groups and therapy was instituted, which constituted day 0 of our study. Tumors were measured in three perpendicular directions (a, b, c) every 2–3 d using a digital caliper. Tumor volume was calculated using the formula: $V = a \times b \times c \times \pi/6$. Doubling time (DT) or tripling time (TT) for each tumor was determined as the time when tumors reached double or triple the volume on day 0, respectively, and was expressed in days. Growth delay (GD) for each experimental

group was determined as the difference between DT or TT of the experimental group and DT or TT of its control group which received only water.

In order to evaluate the effect of therapy on distant untreated tumors, two tumors were induced simultaneously on opposite flanks; animals received additional injection of 3 x 10^5/0.1 ml of SA-1 tumor cells into the left flank. When the right nodule reached an approximate volume of 40 mm^3, mice were randomly divided into experimental groups and only the primary tumor nodule on the right side underwent treatment whilst the secondary nodule on the left side was left intact. Both tumors were measured using a digital caliper as described above.

When challenged, mice were subcutaneously injected with 5 x 10^5/0.1 ml of SA-1 tumor cells 100 d after complete regression of primary tumors. Tumor cells were injected into the left flank and, if they appeared, they were measured as described above.

Plasmid DNA. The plasmids encoding murine IL-12 (pORF-mIL-12, InvivoGen, Toulouse, France) and pCMV Neo-Bam vector (pCMV) were prepared using the Qiagen Maxi Endo-Free kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions and diluted to a concentration of 1 mg/ml. pCMV was gift from B. Vogelstein (John Hopkins University, Baltimore, MO, USA). The characterization of the plasmid has been described previously.[46] Purified plasmid DNA was subjected to quality control and quantity determinations using agarose gel electrophoresis and spectrophotometry.

Tumor treatment. Animals were divided into nine experimental groups and tumors received treatment according to **Table 1**. We included one experimental group of animals, which received intratumoral EGT with control plasmid pCMV, since it was previously shown that EGT with vector plasmid can exert antitumor effect.[47] No effect of this therapy on growth of treated tumors was detected in our study, therefore we didn't perform additional experiment with peritumoral application in relation to implementation of 3R's animal protection principles.

Animals, which received intratumoral treatment, were in jected with 50 μl of either water or plasmid DNA intratumorally. Animals which received peritumoral treatment were injected with 2 x 25 μl of distilled water or plasmid DNA intradermally in the peritumoral region on contralateral sides of the tumor nodule. Electric pulses were delivered using the electric pulse generator Jouan GHT 1287 (Jouan, St. Herblain, France), using plate electrodes with dimensions of 20 mm x 10 mm with rounded corners. The distance between the electrodes was 6 mm for intratumoral EP delivery and 4 mm for peritumoral EP delivery. Eight square-wave electric pulses were applied in two sets of four pulses in perpendicular directions at amplitude of 600 V/cm, 5 ms duration and frequency of 1 Hz. In experimental groups receiving intratumoral therapy, the lag between intratumoral injection of either distilled water or plasmid DNA and application of electric pulses was 10 min. In experimental groups receiving peritumoral therapy, electric pulses were delivered immediately after intradermal injection.

Six additional animals in each experimental group underwent the same treatment independently. In these animals, blood and tumor tissue was collected 5 d after initiation of therapy for determination of intratumoral and serum concentrations of IL-12 and IFNγ.

Evaluation of possible side effects of therapy. All animals were monitored for possible systemic side effects with a physical examination every two days from the start of the experiment. This included monitoring of each animal's body weight and evaluation of general health status with observation of the animal's appetite, locomotion, coat and general appearance.

IL-12 and IFNγ determination. Blood was collected from the intraorbital sinus into a blood collection tube (Vacuette serum tube with gel, Greiner Bio-One International AG, Kremsmünster, Austria) and stored at 4°C for 20 min until coagulated. Serum was extracted from blood samples by centrifugation at 2,500 rpm for 5 min and immediately stored at -80°C until analysis. Tumors were removed, immediately weighed and snap frozen in liquid nitrogen. Frozen samples were mechanically macerated. Each sample was diluted with 500 μl of PBS containing protease inhibitors (Protease Inhibitor Cocktail, PMSF and Sodium Orthovanadate, all Santa Cruz Biotechnology, Inc., Heidelberg, Germany, 10 μl of each per ml of PBS), thoroughly mixed and centrifuged for 10 min at 3,000 rpm. The supernatant was separated from the sediment and stored at -80°C until analysis. Both sets of samples were analyzed using ELISA kits (R&D Systems, Minneapolis, MN, USA) for detection of IL-12 and IFNγ. Concentrations of both measured cytokines were calculated as pg of cytokine per ml of serum or ng of cytokine per mg of tumor tissue.

Statistical analysis. Statistical analysis was performed using SigmaStat software (Systat Software, Inc., Richmond, CA). All data was first tested for normality with the Kolmorogov-Smirnov normality test. In the case of normal distribution of data, significance tests were carried out using analysis of variance (ANOVA) and two-tailed Student's t-test. When data was not normally distributed, the Kruskal Wallis ANOVA on ranks and Mann-Whithey rank sum tests were performed. Values of $p < 0.05$ were considered significant.

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RESEARCH ARTICLE

Controlled systemic release of interleukin-12 after gene electrotransfer to muscle for cancer gene therapy alone or in combination with ionizing radiation in murine sarcomas

Gregor Tevz[1]
Simona Kranjc[1]
Maja Cemazar[1]
Urska Kamensek[1]
Andrej Coer[2]
Mojca Krzan[3]
Suzana Vidic[1]
Darja Pavlin[4]
Gregor Sersa[1]*

[1]*Institute of Oncology Ljubljana, Department of Experimental Oncology, Ljubljana, Slovenia*

[2]*University of Primorska, College of Health Care Isola, Izola, Slovenia*

[3]*University of Ljubljana, Faculty of Medicine, Ljubljana, Slovenia*

[4]*University of Ljubljana, Veterinary Faculty, Ljubljana, Slovenia*

*Correspondence to: Gregor Sersa, Department of Experimental Oncology, Institute of Oncology Ljubljana, Zaloska cesta 2, SI-1000 Ljubljana, Slovenia.
E-mail: gsersa@onko-i.si

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Abstract

Background The present study aimed to evaluate the antitumor effectiveness of systemic *interleukin (IL)-12* gene therapy in murine sarcoma models, and to evaluate its interaction with the irradiation of tumors and metastases. To avoid toxic side-effects of *IL-12* gene therapy, the objective was to achieve the controlled release of IL-12 after intramuscular gene electrotransfer.

Methods Gene electrotransfer of the plasmid pORF-mIL12 was performed into the *tibialis cranialis* in A/J and C57BL/6 mice. Systemic release of the IL-12 was monitored in the serum of mice after carrying out two sets of intramuscular *IL-12* gene electrotransfer of two different doses of plasmid DNA. The antitumor effectiveness of *IL-12* gene electrotransfer alone or in combination with local tumor or lung irradiation with X-rays, was evaluated on subcutaneous SA-1 and LPB tumors, as well as on lung metastases.

Results A synergistic antitumor effect of intramuscular gene electrotransfer combined with local tumor irradiation was observed as a result of the systemic distribution of IL-12. The gene electrotransfer resulted in up to 28% of complete responses of tumors. In combination with local tumor irradiation, the curability was increased by up to 100%. The same effect was observed for lung metastases, where a potentiating factor of 1.3-fold was determined. The amount of circulating IL-12 was controlled by the number of repeats of gene electrotransfer and by the amount of the injected plasmid.

Conclusions The present study demonstrates the feasibility of treatment by *IL-12* gene electrotransfer combined with local tumor or lung metastases irradiation on sarcoma tumors for translation into the clinical setting.

Keywords gene electrotransfer; interleukin-12; irradiation; mice; sarcoma

Introduction

Gene therapy is receiving much attention as a local and systemic treatment for a variety of cancers. However, because of its limited impact on bulky local disease, its clinical applicability is foreseen in combination with other established cytotoxic treatment modalities (i.e. radiotherapy). The radiosensitizing role of different cytokines has already been examined, in particular, interleukin (IL)-12 has proven to be promising [1].

The discovery of IL-12 and its potent immunostimulatory effect on natural killer (NK) cells of the innate immune system, as well as on cytotoxic

T lymphocytes of the adaptive immune system, showed great promise in the treatment of malignant disease [2–4]. In addition, Voest *et al*. [5] discovered potent anti-angiogenic properties of IL-12. The antitumor and antimetastatic activities of IL-12 were demonstrated in preclinical tumor models [6–9]. Preclinical studies encouraged clinical trials, where the safety and antitumor efficacy of recombinant human IL-12 (rhIL-12) were examined [10–17]. However, the first reports of phase I and II clinical trials demonstrated the toxic side-effects of rhIL-12 at doses barely resulting in any antitumor effectiveness [18]. In the most effective study, treatment with rhIL-12 resulted in a partial or complete response in 56% of patients with cutaneous T-cell lymphoma [19].

Gene transfer introduced an advanced route of administration and improved action of IL-12. Recent studies have evaluated antitumor effectiveness and the safety of intratumoral *IL-12* gene therapy. The gene encoding IL-12 was transferred to tumors in the form of a naked DNA injection alone [20] or in combination with electroporation [21–25], or packed in adenoviral [26–30] or other viral vectors [31,32]. Clinical studies were also initiated and first results showed that intratumoral IL-12 is a safe treatment with some beneficial clinical effect [33–38].

Systemic *IL-12* gene therapy has been studied with caution as a result of the previously described toxicity of systemic administration of rhIL-12. The systemic release of IL-12 after transfection of skeletal muscle or skin resulted in a good antitumor and an especially good antimetastatic effect [23, 24,39–42].

Cytokines have been shown to sensitize different tumors to local tumor irradiation [43]. Radiosensitization of tumors was investigated by recombinant IL-12 systemic injection, as well as by intratumoral *IL-12* gene therapy, with good local and systemic effects [1,44–47]. However, none of the studies investigated intramuscular *IL-12* gene electrotransfer with controlled systemic release of the transgene and its radiopotentiating effect.

Therefore, in the present study, transfection of muscle tissue with the gene encoding IL-12 was tested using different doses of plasmid DNA and a different number of repeats of *IL-12* gene electrotransfer, aiming to achieve the controlled systemic release of IL-12 without side-effects. The antitumor effectiveness of systemic gene therapy on solid subcutaneous tumors and experimental metastases was studied alone or in combination with irradiation, aiming to explore the radiosensitizing effect of the combined treatment for possible clinical application.

Materials and methods

Cell lines, tumors and animals

Murine sarcoma LPB [48] and fibrosarcoma SA-1 (Jackson Laboratory, Bar Harbor, ME, USA) cells were used in the experiments. For the *in vitro* studies, the cells were cultured in Advanced MEM (Gibco, Grand Island, NY, USA) supplemented with 2% fetal calf serum (Sigma-Aldrich, St Louis, MO, USA) in a humidified atmosphere at 37 °C with 5% CO_2. LPB cells are syngeneic to C57BL/6 and SA-1 to A/J mice. Solid LPB subcutaneous tumors were induced on the rear dorsum of C57BL/6 mice by injection of 1.3×10^6 viable tumor cells in Advanced MEM prepared from cell culture *in vitro*. SA-1 tumor cells were obtained from the ascitic form of the SA-1 tumors in A/J mice, serially transplanted every 7 days. Solid SA-1 subcutaneous tumors were initiated on the rear dorsum by an injection of 5×10^5 SA-1 cells in 0.1 ml of 0.9% NaCl solution. Lung metastases were induced by tail vein injection of 5×10^5 SA-1 or 1×10^6 LPB cells in 0.3 ml of 0.9% NaCl solution. Female C57BL/6 and A/J mice were obtained from the Institute of Pathology, Faculty of Medicine, University of Ljubljana (Ljubljana, Slovenia). Mice were housed and maintained in an animal colony under SPF laboratory conditions at constant room temperature (21 °C) and under a 12 : 12 h light/dark cycle. Food and water were provided *ad libitum*. Animals were subjected to an adaptation period of 7–10 days before the experiments. At the beginning of the experiments, the animals were 10–12 weeks old. Each experimental group consisted of 6–12 animals. All procedures on animals were performed in accordance with the official guidelines of the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (permission no. 323-02-632/2005/6).

Plasmid

The plasmid encoding murine IL-12 (pORF-mIL12, Invivogen, Toulouse, France), was prepared using the Qiagen Endo-Free Plasmid Mega Kit (Quiagen, Hilden, Germany) according to the manufacturer's instructions, and diluted to a final concentration of 1 μg/μl or 0.25 μg/μl. The control treatment delivering only backbone plasmid DNA without the IL-12 insert was not performed, because the plasmid DNA was delivered into distant tissue and no local effect of DNA molecule to the tumor could be expected.

In vitro radiosensitization assay

Radiosensitization of SA-1 and LPB cells to recombinant murine IL-12 (rmIL-12), obtained from R&D Systems (Minneapolis, MN, USA), was determined by a colony-forming assay. For irradiation of cells *in vitro*, a Darpac 2000 X-ray unit (Gulmay Medical Ltd, Shepperton, UK), operated at 220 kV, 10 mA, and with 0.55 mm Cu and 1.8 mm Al filtration, was used. Cells were irradiated in Petri dishes (Corning Life Sciences BV, Schiphol-Rijk, NL) at a dose rate 2 Gy/min with graded doses (2–8 Gy) and thereafter incubated for 8–14 days at 37 °C in

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a humidified atmosphere with 5% CO_2 until colonies of more than 50 cells were formed. The colonies were stained with crystal violet dye (Sigma-Aldrich) and counted. The radiosensitization by two different concentrations of rmIL-12 (10 pg/ml and 1 ng/ml) were examined. Survival of cells irradiated in combination with rmIL-12 was normalized to the effect of rmIL-12 to determine the interaction between the treatments. All data were pooled from three independent experiments performed in triplicate.

Intramuscular gene electrotransfer

All mice were anaesthetized with anaesthesia apparatus and 3% isoflurane (1 l/min O_2, 1 l/min N_2O). Plasmid pORF-mIL12 was injected with a 29-gauge needle into muscle *tibialis cranialis*. The leg was placed between plate electrodes of dimensions 20 × 10 mm with rounded corners and a 6 mm distance between them (IGEA srl, Carpi, Italy) and square-wave electric pulses (EPs) 1 × 600 V/cm (100 μs) + 4 × 80 V/cm (100 ms) were generated by the EP generator Cliniporator™ (IGEA srl). Good contact between the electrodes and the overlying skin was assured by hair removal and the use of a conductive gel (Kameleon, Ltd, Maribor, Slovenia) [49].

Determination of intramuscular *IL-12* gene electrotransfer efficiency

IL-12 and interferon (IFN)-γ serum concentrations were determined after single or quadruple intramuscular *IL-12* gene electrotransfer to nontumor bearing mice. Two different doses of plasmid pORF-mIL12 (5 μg and 20 μg) were used for single or quadruple gene electrotransfer into the muscles of A/J and C57BL/6 mice. Quadruple gene electrotransfer was performed alternating right and left muscles *tibialis cranialis* with a time lag of 48 h. To determine IL-12 and IFN-γ protein production *in vivo*, blood was collected on days 4, 6, 8, 10, 14 and 28 after the first gene electrotransfer and placed into a 1.5-ml microfuge tube. Samples were incubated at room temperature for 2 h and were then centrifuged for 15 min at 2000 g. Serum was drawn from the samples, placed into fresh microfuge tubes, and stored at −80 °C. Enzyme-linked immunosorbent assay (ELISA) was performed for determination of the serum concentration of IL-12 and IFN-γ in A/J and C57BL/6 mice. ELISA kits were purchased from R&D Systems and were used in accordance with the manufacturer's instructions. The area under the concentration versus time curve (AUC) was calculated by use of the trapezoidal rule from the first (t_0) to the last measurable time point (t_{last}) using Prism software, version 4.0 (GraphPad Software Inc., San Diego, CA, USA). Control values were used as a baseline.

Assessment of side-effects of intramuscular *IL-12* gene electrotransfer

Infiltration of immune cells to treated muscle and the change in animal body weight were determined in treated animals. Single gene electrotransfer-treated muscles *tibialis cranialis* were removed from C57BL/6 mice on days 4, 6, 8, 10, 14 and 28 after gene electrotransfer, fixed in buffered formalin and embedded in paraffin. Tissue sections (5 μm thick) were cut from paraffin blocks and stained with hematoxylin and eosin (H&E). Immune cell infiltration in the treated muscle was analysed and the area of infiltration was determined on microscopic images by ImageJ software tool (National Institute of Mental Health, Research Services Branch, Bethesda, MD, USA).

Antitumor effectiveness of *IL-12* gene electrotransfer alone or combined with irradiation

Subcutaneous tumors SA-1 and LPB were treated by triple intramuscular gene electrotransfer, starting when the tumor volume reached 50 mm^3. Each gene electrotransfer was performed alternating right and left muscles *tibialis cranialis* with a time lag of 48 h and a cumulative dose of 60 μg (3 × 20 μg) plasmid pORF-mIL12. Tumors were irradiated with Darpac X-ray unit 24 h after the first gene electrotransfer at a dose rate 2.1 Gy/min with a single dose of 10 Gy. Mice were placed in a holder for six mice on the X-ray unit with the apertures for irradiation of the tumors; the rest of the body was protected by a lead block. Mice were restrained in the holders, but not anaesthetized. To ensure uniform dose distribution through the tumor volume, the tumors were exposed to irradiation by two opposing treatment fields through each of which 50% of the dose was delivered [50].

The tumor volume was determined by measuring three orthogonal tumor diameters (e_1, e_2 and e_3) with a Vernier caliper. The volume was calculated by the formula $V = \pi \times e_1 \times e_2 \times e_3/6$. Tumor regression and regrowth was followed until the tumors grew up to 300 mm^3, and then the animals were sacrificed. Animals with tumors in regression after therapy were examined for the presence of the tumor at 4–5-day intervals up to 100 days. The animals were considered cured if they were tumor-free at day 100. Because the tumors of treated mice started to regress after they had already reached the tripling volume, tumor growth delay was calculated from quadrupling time (QT) by subtraction of tumor QT of the nontreated group. The radiosensitizing effect was determined by the analysis of SA-1 and LPB tumor growth quadrupling time. The synergistic effect of a combination of two treatments with an independent mechanism of action was calculated using a formula that takes into account the statistical variability inherent in biological systems [51].

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SA-1- and LPB-induced lung metastases were treated by intramuscular *IL-12* gene electrotransfer using 20 μg of plasmid pORF-mIL12, which was delivered at four different time points 24 h before (prophylactic treatment) or 24, 72 and 120 h after the injection of SA-1 or LPB cells into the tail vein of mice, as a single treatment. In addition, quadruple gene electrotransfer was also performed at each of these time points. In control groups 1HV + 4LV EPs alone were applied as treatment in four repeats or plasmid pORF-mIL12 was injected (pORF-mIL12) at a cumulative dose of 80 μg (4 × 20 μg) or no treatment performed (Control). For the determination of the radiosensitizing effect of IL-12 on induced lung metastases, intramuscular *IL-12* gene electrotransfer was performed as prophylactic treatment 24 h before the injection of SA-1 cells with 5 μg of plasmid pORF-mIL12 or it was performed with 20 μg of plasmid pORF-mIL12, 24 h after the injection of cells. Lung metastases were irradiated with Darpac X-ray unit 48 h after the injection of cells with a single dose of 4 Gy in the case of prophylactic treatment and with a graded dose of radiation from 0 to 4 Gy (dose rate 1.38 Gy/min) when gene electrotransfer was performed after the injection of cells. Mice were restrained and only the lung region was exposed to the radiation beam. The rest of the body was protected by a lead cover.

Mice were sacrificed 8 days (SA-1 metastases) or 14 days (LPB metastases) after the injection of cells into the tail vein. Lungs were removed and dissected to five lobules and placed in Bouin's fixative for at least 1 h. The number of lung metastases was determined under a stereomicroscope. The number of lung metastases in each experimental group was divided by the number of lung metastases in the control group. In the combined treatment by gene electrotransfer and radiotherapy, the survival fraction curve was fitted to the linear quadratic model regression curve and the potentiating factor of a combined treatment was determined.

Histology of subcutaneous tumors

Mice bearing SA-1 and LPB tumors were treated by *IL-12* gene electrotransfer three times as described above. Tumors were removed from the treated and untreated mice 8 days after *IL-12* gene electrotransfer and fixated in formaldehyde. They were embedded in paraffin blocks and cut into tissue sections of 10 μm. Tissue sections were differentially stained with H&E dye. Infiltration of immune cells was inspected by microscopy.

Statistical analysis

The data were tested for normality of distribution using the Kolmogorov–Smirnov test. Differences between experimental groups were statistically evaluated by one-way analysis of variance (ANOVA) followed by the Holm–Sidak test for multiple comparison. $p < 0.05$ was considered statistically significant. Statistical analysis was performed using SigmaStat (Systat Software Inc., London, UK).

Results

Level of expression of IL-12 after intramuscular *IL-12* gene electrotransfer

Intramuscular *IL-12* gene electrotransfer resulted in elevated serum IL-12 levels during the 28 days observation time, with the highest concentration between days 4 and 14, depending on the dose of the plasmid used and the number of the repeats of pORF-mIL12 transfection in both strains of mice. IFN-γ serum levels were elevated with an approximate 4-day time delay with regard to IL-12 levels, and returned close to pretreatment values within 28 days. However, a significant difference in maximum concentration of IL-12 and IFN-γ between the two strains of mice was observed; IL-12 serum concentration was three-fold higher in C57BL/6 compared to A/J mice, which was also reflected in a five-fold higher maximum concentration of IFN-γ serum concentration. The highest serum concentration of IL-12 and IFN-γ was observed after quadruple *IL-12* gene electrotransfer with 20 μg of pORF-mIL12. The maximum serum concentration of IL-12 was 1.9 ng/ml in C57BL/6 mice and 0.6 ng/ml in A/J mice and the maximum IFN-γ serum concentration was 1.9 ng/ml in C57BL/6 mice and 0.4 ng/ml in A/J mice (Figure 1A).

Quadruple *IL-12* gene electrotransfer of a lower dose of pORF-mIL12 (5 μg) plasmid resulted in a significantly lower IL-12 and IFN-γ serum concentration compared to quadruple electrotransfer of a higher dose of pORF-mIL12 plasmid (20 μg) in both strains of mice, indicating that, with the number of repeats of *IL-12* gene electrotransfer and by selecting different doses of the plasmid, the systemic distribution of the transgene product can be controlled and adjusted to the desired quantity (Figures 1Aa and 1Ac). This statement is supported by the total IL-12 production which was estimated by determination of the AUC. Single *IL-12* gene electrotransfer resulted in a lower total amount of IL-12 and IFN-γ than quadruple transfection, whereby lower doses of the transfected pORF-mIL12 plasmid resulted in a lower total amount than higher dose of plasmid (Figure 1). The total amount of IL-12 produced in C57BL/6 was up to two-fold higher than the total amount of IL-12 produced in A/J, whereas the total amount of IFN-γ was up to five-fold higher in the C57BL/6 mice compared to the A/J mice ($p < 0.05$) (Figure 1B).

Side-effects of intramuscular *IL-12* gene electrotransfer

Infiltration of immune cells into the treated muscle tissue and the loss of animal body weight after *IL-12* gene

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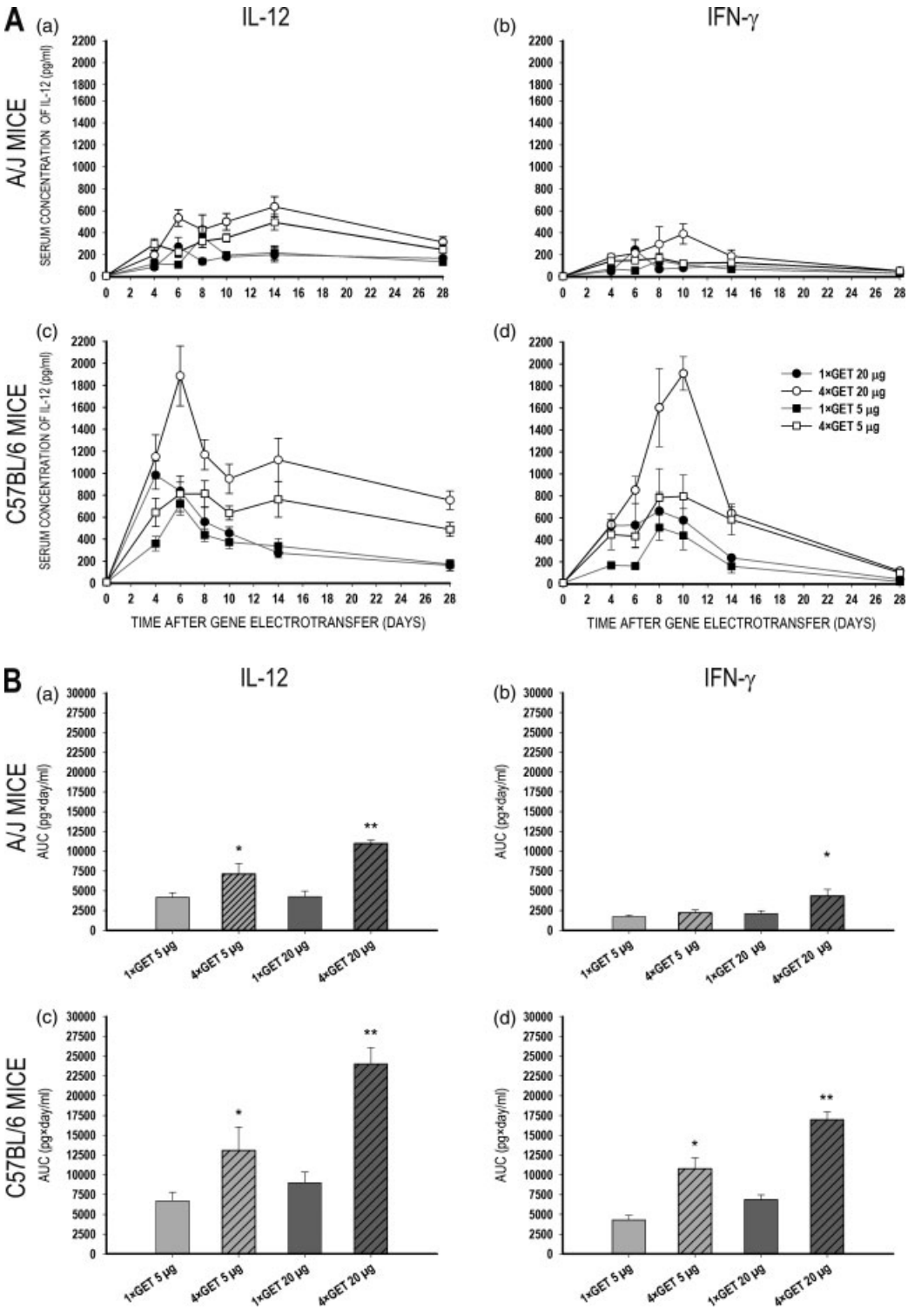

Figure 1. Serum concentration and AUC of IL-12 and IFN-γ after intramuscular *IL-12* gene electrotransfer. Two different doses of plasmid pORF-mIL12 (5 μg and 20 μg) were used for single (1 × GET) or quadruple (4 × GET) gene electrotransfer in mouse muscle *tibialis cranialis*. (A) Serum concentration of IL-12 and IFN-γ in A/J and C57BL/6 mice (a, b, c, d). (B) (a) AUC of IL-12 in A/J mice; *$p < 0.05$ versus 1 × GET 5, 20 μg, 4 × GET 20 μg; **$p < 0.05$ versus 1 × GET 5, 20 μg, 4 × GET 5 μg. (b) AUC of IFN-γ in A/J mice; *$p < 0.05$ versus 1 × GET 5, 20 μg, 4 × GET 5 μg. (c) AUC of IL-12 in C57BL/6 mice; *$p < 0.05$ versus 1 × GET 5, 20 μg, 4 × GET 20 μg; **$p < 0.05$ versus 1 × GET 5, 20 μg, 4 × GET 5 μg. (d) AUC of IFN-γ in C57BL/6 mice; *$p < 0.05$ versus 1 × GET 5, 20 μg, 4 × GET 20 μg; **$p < 0.05$ versus 1 × GET 5, 20 μg, 4 × GET 5 μg. Serum concentration of IL-12 in the control group was 10 pg/ml in C57BL/6 mice and 9 pg/ml in A/J mice, whereas serum concentration of IFN-γ in the control group was 9 pg/ml in C57BL/6 and 4 pg/ml in A/J mice. The results represent the average of serum concentration measured in four to six mice; error bars indicate the SE

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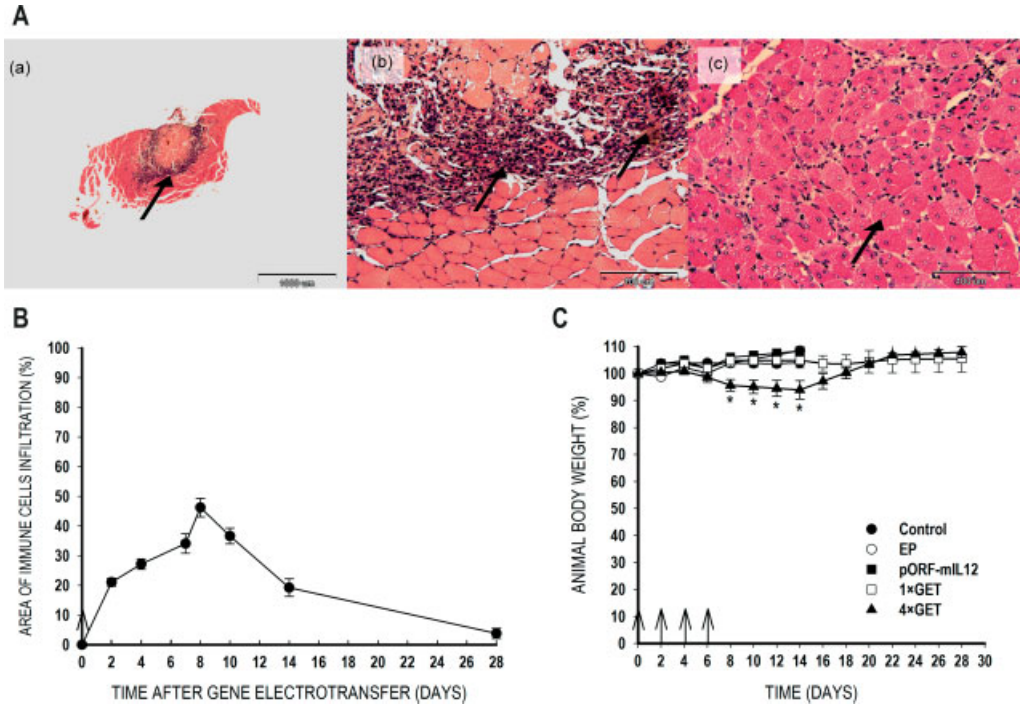

Figure 2. Side-effects of *IL-12* gene electrotransfer. (A) (a) H&E stained muscle cross section shows immune cells infiltration into treated muscle tissue after single gene electrotransfer (× 40 magnification). (b) × 100 magnification. The arrows show the infiltration of immune cells. (c) Muscle tissue regeneration 14 days after *IL-12* gene electrotransfer. The arrow shows central nuclei, characteristic for regenerated muscle fibers (× 400 magnification). (B) Area of immune cells infiltration into treated muscle *tibialis cranialis*. Results represents the average area of immune cells infiltration determined in six mice; error bars indicate the SE. (C) Loss of animal body weight after single (1 × GET) or quadruple (4 × GET) *IL-12* gene electrotransfer; EP, quadruple application of EPs alone; pORF-mIL12, quadruple intramuscular injection of plasmid pORF-mIL12. The results represent the average loss of weight compared to the starting weight determined in six mice; error bars indicate the SE. *$p < 0.05$ versus control or all other groups

electrotransfer were determined in C57BL/6 mice, the strain of mice where the highest serum concentrations of IL-12 and IFN-γ were observed (Figure 2). In the area of immune cell infiltration, regenerated muscle fibers were observed. Immune cells infiltration increased until a peak was achieved at day 8 and then started to decrease (Figure 2B). Only 3% of the whole tissue section was populated by immune cells at the end of observation 28 days after *IL-12* gene electrotransfer. Extensive muscle regeneration was also observed, starting on day 8 after gene electrotransfer. The regeneration was visible by the increased number of muscle fibers with central nuclei (Figure 2A).

In the experiments with *IL-12* gene electrotransfer, none of the mice died as a result of IL-12 systemic toxicity. However, a decrease in animal body weight was observed in C57BL/6 mice, namely in the group that received the highest number of repeats of *IL-12* gene electrotransfer and the highest dose of plasmid (four times; 20 μg pORF-mIL12). Mice in this group lost 14% of body weight in comparison to mice in the control group ($p < 0.05$). Mice started to regain body weight after 14 days. There was no change in body weight, when single *IL-12* gene electrotransfer was performed (1 × GET) or when EPs alone were delivered or when plasmid pORF-mIL12 (pORF-mIL12) alone was injected (Figure 2C).

Antitumor effectiveness of intramuscular *IL-12* gene electrotransfer on subcutaneous tumors

Antitumor effectiveness of *IL-12* gene electrotransfer to muscle was demonstrated in established subcutaneous SA-1 and LPB tumors growing distantly from the transfection site; the growth of the tumors was delayed and also some complete responses of the tumors were observed (Figures 3A and 3B). The tumors started to regress after 4 days, when the last *IL-12* gene electrotransfer was performed. Intramuscular *IL-12* gene electrotransfer was performed only three times, to enable evaluation of radiosensitization at the level of tumor growth delay. The response of SA-1 tumors to triple *IL-12* gene electrotransfer was more pronounced than that of the LPB tumors. The majority of SA-1 tumors responded with significant tumor growth delay (27.3 ± 2.6 days) and the remainder (28%) responded with complete tumor eradication after *IL-12* gene electrotransfer. LPB tumors also responded with significant tumor growth delay (13.0 ± 1.3 days) and the remainder (14%) responded with complete tumor eradication after *IL-12* gene electrotransfer. Tumor regrowth with slower growth kinetics was observed in mice that responded with tumor growth delay, whereas the mice with tumor complete

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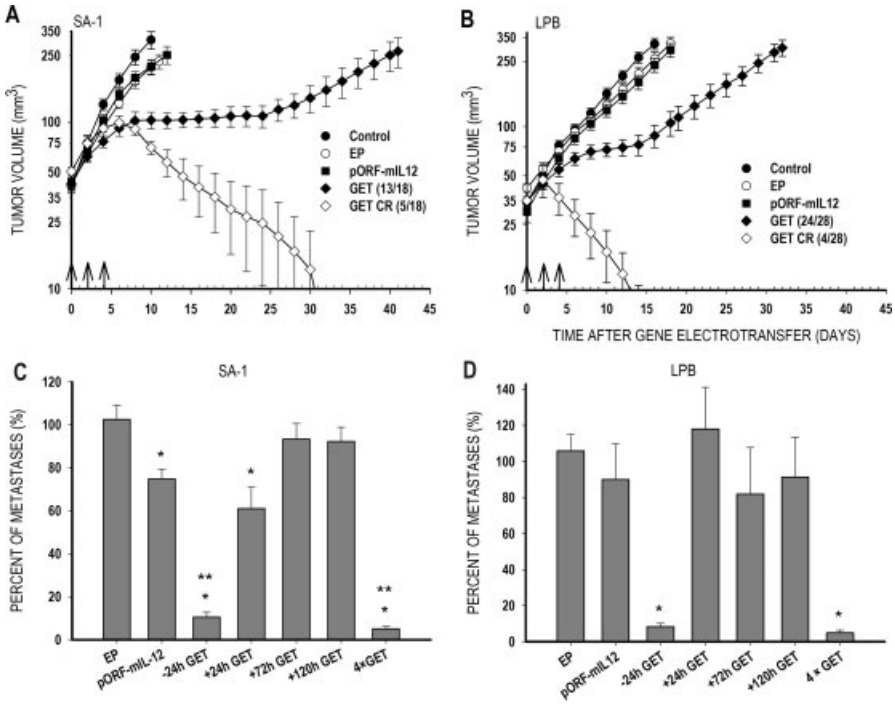

Figure 3. Antitumor and antimetastatic effect of intramuscular *IL-12* gene electrotransfer on SA-1 and LPB tumors and induced lung metastases. The curves represent the growth of (A) SA-1 and (B) LPB subcutaneous tumors and are an average tumor volume of at least nine mice; error bars indicate the SE. Arrows represent the schedule of *IL-12* gene electrotransfer. The dose of plasmid pORF-mIL12 was 20 μg. EP, application of Eps; GET CR, gene electrotransfer with tumor complete response. (C) Inhibition of growth of SA-1 lung metastases; *$p < 0.001$ versus Control, EP, +72 h, +120 h GET; **$p < 0.001$ versus Control, EP, pORF-mIL12, +24 h, +72 h, +120 h GET. (D) Inhibition of growth of LPB lung metastases; *$p < 0.001$ versus control, EP, pORF-mIL12, +24 h, +72 h, +120 h GET. Bars represent mean percentage of metastases in each group normalized to the number of metastases in the control group; error bars indicate the SE. *IL-12* gene electrotransfer was performed at different times before or after the injection of cells

responses were tumor-free for 100 days. The effect of application of EPs alone or intramuscular injection of plasmid pORF-mIL12 alone (pORF-mIL12) had no effect on tumor growth on either of the models.

Histology of tumors after intramuscular *IL-12* gene electrotransfer

The histology of SA-1 and LPB tumors was different; nontreated SA-1 tumors presented extensive tumor necrosis, whereas LPB tumors did not have necrotic areas. Systemic *IL-12* gene therapy did not influence the histology of the tumors at day 8 after gene electrotransfer. No significant increase in lymphocyte infiltration or necrotization of the tumors was observed. A slight reduction in tumor size 8 days after the beginning of systemic *IL-12* gene therapy was observed (Figure 4).

Antitumor effectiveness of intramuscular *IL-12* gene electrotransfer on induced lung metastases

Intramuscular *IL-12* gene electrotransfer inhibited the growth of induced SA-1 and LPB lung metastases. Single *IL-12* gene electrotransfer was performed at four different time points (−24 h, +24 h, +72 h, +120 h with respect to induction of metastases) to discriminate the prophylactic effect from the effect on established lung metastases with variable size. The prophylactic treatment reduced the number of SA-1 metastases by 90% and LPB metastases by 92%. The antitumor effect was less efficient on already established lung metastases. A significant ($p < 0.05$) 40% reduction of SA-1 lung metastases after gene electrotransfer was observed, when performed 24 h after the injection of cancer cells. Any later gene electrotransfer (+72 h, +120 h) resulted in a number of lung metastases that was not statistically different from the control, and therefore showed no antimetastatic effect. By contrast to SA-1 lung metastases, no inhibition of growth of LPB metastases was observed if *IL-12* gene electrotransfer was performed after the injection of LPB cells. Intramuscular injection of plasmid pORF-mIL12 alone (pORF-mIL12) had a moderate antimetastatic effect on SA-1 metastases and no effect on LPB metastases. Application of EPs had no significant effect on growth of induced SA-1 or LPB lung metastases.

Quadruple *IL-12* gene electrotransfer with a 48-h time lag between each gene electrotransfer resulted in the most efficient inhibition of growth of SA-1 or LPB lung metastases (95%), but there was no statistically significant

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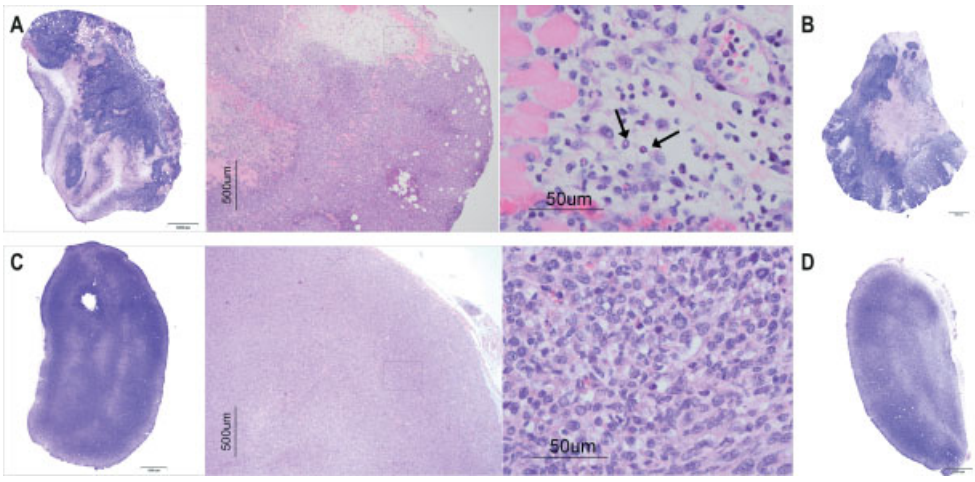

Figure 4. Histology of SA-1 and LPB tumors. (A) (from left to right) H&E stained tissue section of nontreated SA-1 subcutaneous tumor (× 40 magnification), × 100 magnification, × 1000 magnification (the arrows show infiltrating immune cells). (B) H&E stained tissue section of SA-1 subcutaneous tumor 8 days after *IL-12* gene electrotransfer. (C) (from left to right) H&E stained tissue section of nontreated LPB subcutaneous tumor (× 40 magnification), × 100 magnification, × 1000 magnification. (D) H&E stained tissue section of LPB subcutaneous tumor 8 days after *IL-12* gene electrotransfer

difference between prophylactic and quadruple gene electrotransfer (Figures 3C and 3D).

In vitro radiosensitization of SA-1 and LPB cells with rmIL-12

When the cells were irradiated with graded doses of X-rays (2–8 Gy) in combination with two different concentrations of rmIL-12 protein (10 pg/ml and 100 ng/ml), the same response as with irradiation of cells alone was observed, meaning no radiosensitization in SA-1 and LPB cells *in vitro* with rmIL-12 was observed (Figure 5A).

Radiosensitizing effect of intramuscular *IL-12* gene electrotransfer on SA-1 and LPB subcutaneous tumors

The radiosensitizing effect of *IL-12* gene electrotransfer was observed in SA-1 and LPB subcutaneous tumors in combination with tumor irradiation. Subcutaneous tumors (50 mm^3) were irradiated with 10 Gy 24 h after the first intramuscular *IL-12* gene electrotransfer, and two consecutive treatments were performed thereafter.

SA-1 tumors were more radioresistant than LPB tumors. A significant SA-1 tumor growth delay of 8.1 ± 1.7 days was determined on SA-1 tumors without tumor cures, whereas, on LPB tumors, a tumour growth delay was 22.2 ± 4.0 days and 60% of tumors were cured. Triple *IL-12* gene electrotransfer resulted in 28% of SA-1 tumor cures and the growth of the rest of the tumors was delayed by 27.3 ± 2.6 days. The same treatment was performed in LPB tumors, where 14% of the tumors responded with complete eradication, but the majority of tumors (86%) were only delayed in tumor growth by 13.0 ± 1.3 days. A combination of *IL-12* gene electrotransfer and SA-1 tumor irradiation resulted in 44% of tumor cures and 56% of tumors were delayed in growth by 49.0 ± 7.7 days. Treatment of LPB tumors combined with irradiation resulted in 100% tumor cures (Figure 5B).

The combined treatment of SA-1 and LPB tumors was better than the additive effect of separate treatments considering tumor growth delay and the percentage of cures. The synergistic effect of a combination of two treatments was confirmed on SA-1 tumors. Only mice without tumor complete responses were used in the calculation and tumor quadrupling time was implemented as a measure of the antitumor effect. The synergistic effect of a combined treatment of LPB tumors could therefore not be calculated, but could be assumed because the therapy resulted in a 100% complete response.

Gene therapy by intramuscular *IL-12* gene electrotransfer (3×20 µg of plasmid pORF-mIL12), irradiation of tumors, or combined treatment had only little effect on animal body weight (6% loss). In addition, the skin reaction in the irradiated field was no more pronounced in the combined gene and irradiation treatment (data not shown).

Radiosensitizing effect of intramuscular *IL-12* gene electrotransfer on SA-1- and LPB-induced lung metastases

Prophylactic intramuscular *IL-12* gene electrotransfer with 5 µg of pORF-mIL12 combined with 4 Gy of irradiation resulted in a radiosensitizing effect on lung metastases (Figures 5C and 5D). *IL-12* gene electrotransfer alone resulted in a 5.3-fold reduction of the number of lung metastases established after the injection of SA-1 cells, irradiation of lung metastases by 4 Gy alone resulted in a 3.3-fold reduction, but the combined treatment resulted in a 198.3-fold reduction compared to

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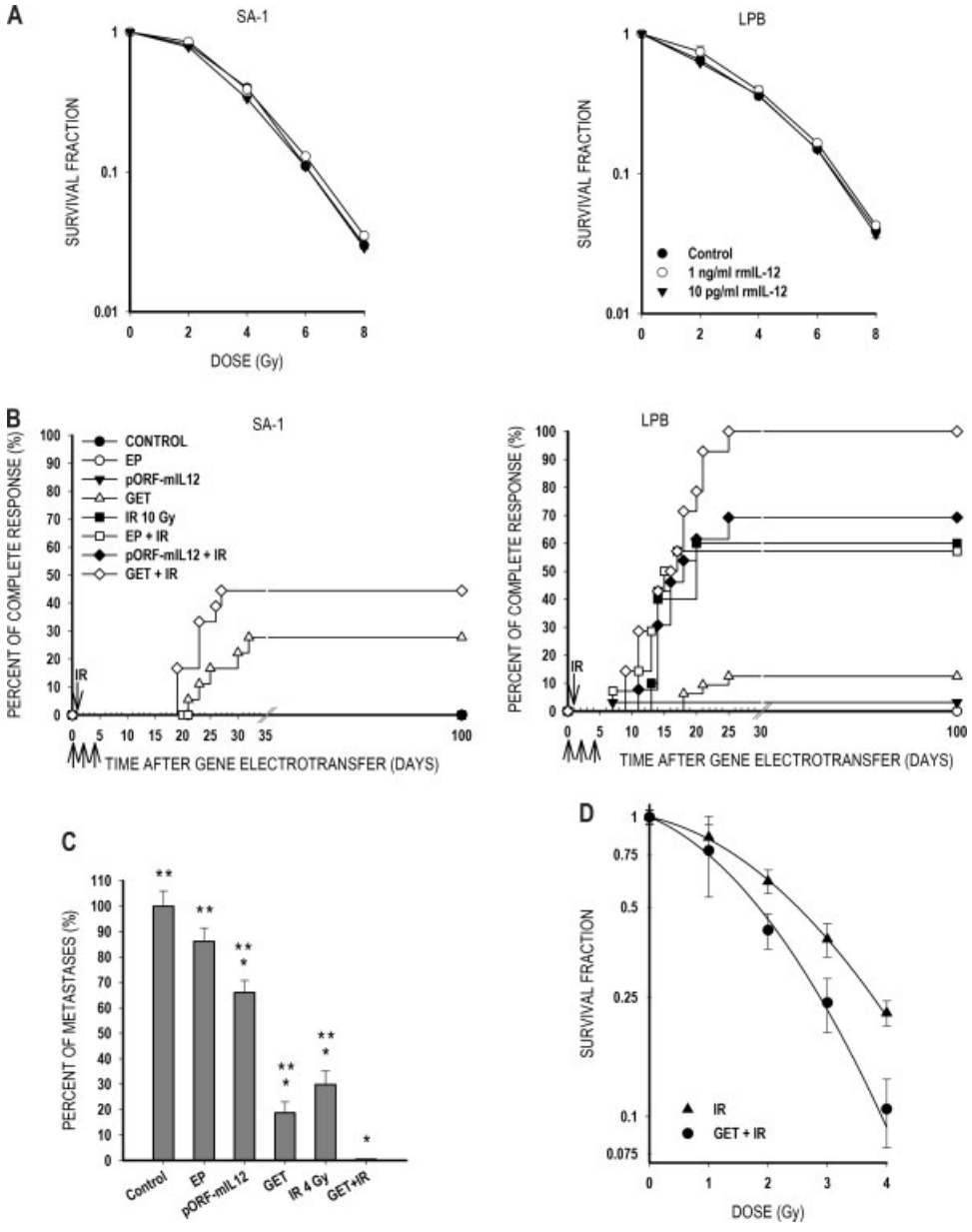

Figure 5. Radiosensitization of SA-1 and LPB cells by *IL-12*. (A) SA-1 cells or LPB cells were chronically exposed to 10 pg/ml (physiological serum concentrations) or 1 ng/ml (serum concentration after single *IL-12* gene electrotransfer) of rmIL-12 and irradiated with graded doses of radiation. The results represent survival fraction; error bars indicate the SE. (B) Percentage of complete responses after treatment with intramuscular *IL-12* GET in SA-1 and LPB-bearing mice; application of EPs alone, intramuscular injection of pORF-mIL12 (Plasmid), irradiation of subcutaneous tumors with a dose of 10 Gy (IR) and a combination of GET, EP, Plasmid with IR. The arrows in bold represent the schedule of *IL-12* gene electrotransfer and the arrow above the *x*-axis shows the time of tumor irradiation. (C) Inhibition of growth of SA-1 induced lung metastases after prophylactic *IL-12* gene electrotransfer (5 μg of plasmid pORF-mIL12); *$p < 0.05$ versus control and EP; **$p < 0.05$ versus GET + IR. (D) Survival fraction of SA-1 induced lung metastases after irradiation (IR) or irradiation combined with intramuscular *IL-12* gene electrotransfer (24 h after the injection of SA-1 cells) (GET + IR). Regression curve of a linear-quadratic model was fitted to the data

the untreated control. The synergistic effect of the combined treatment was determined. Intramuscular injection of plasmid pORF-mIL12 alone showed a moderate antimetastatic effect and application of EPs alone showed no antimetastatic effect. The radiosensitizing effect was also confirmed when intramuscular *IL-12* gene electrotransfer was performed after the injection of SA-1 cells into the tail vein and combined with graded irradiation doses. At all irradiation doses tested, the combined treatment with intramuscular *IL-12* gene electrotransfer decreased the fraction of lung metastases. In combined treatment, a 1.3-fold lower dose was needed compared to irradiation alone (Figure 5D).

Discussion

The present study showed that intramuscular *IL-12* gene electrotransfer resulted in significant growth delay and even complete responses of SA-1 and LPB sarcoma

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solid tumors and greatly inhibited the initiation and growth of induced lung metastases. Efficient expression of the *IL-12* gene and systemic distribution of its product was determined after intramuscular *IL-12* gene electrotransfer. Using single or quadruple *IL-12* gene electrotransfer of two different doses of plasmid DNA, we could achieve different levels and duration of serum IL-12 concentrations. Treatment by gene electrotransfer was further improved with radiotherapy; the combined treatment had a synergistic antitumor effect on both solid tumors and lung metastases.

The toxicity associated with systemic administration of rIL-12 was the major obstacle for further progression of rIL-12 into clinical practice [18]. Therefore, controlled and sustained levels of IL-12 in blood are of importance to its success in the clinic. In the present study, we examined the dynamics of systemic distribution of IL-12 after single or quadruple treatment by *IL-12* gene electrotransfer, using two different doses of plasmid DNA. We clearly showed that the concentration of IL-12 increased in the first 6 days and rapidly decreased thereafter, which is in agreement with another study where increased concentrations of IL-12 after intramuscular *IL-12* gene electrotransfer were determined from day 5 to day 17 [52]. However, the maximum concentration of IL-12 determined in the present study in C57BL/6 mice was approximately ten-fold higher than in the previously mentioned study. Another observation of the present study was that the serum levels of IL-12 were mouse strain-dependent because they were three-fold higher in C57BL/6 than in A/J mice. The differences in transgene expression between different mouse strains after intramuscular gene electrotransfer were already described; the expression level of the transgene was 20–40-fold higher in C3H than in C57BL/6 mice [53]. The underlying mechanisms responsible for the differences in transgene expression are not yet explained and need further investigation. However, the relative increase as a result of the different doses and number of treatments were similar regardless of the strain of mice. Furthermore, we demonstrated that single or quadruple treatment by intramuscular *IL-12* gene electrotransfer of two different doses of plasmid DNA resulted in different levels of systemic distribution of the transgene product, indicating that the levels can be controlled and adjusted to the desired quantity. Namely, we showed that the total quantity of IL-12 (AUC) produced in 1 month was similar after single treatment by *IL-12* gene electrotransfer using two different doses. However, quadruple gene electrotransfer resulted in a significant difference in the total amount of IL-12 depending on the dose of plasmid DNA used. The same pattern, but with a few days delay, was also demonstrated for IFN-γ. The delay of the peak concentration of IFN-γ is a result of the IL-12-dependent stimulation of proliferation of immune cells, mostly NK and CD8+ cells, which produce IFN-γ [54]. In clinical trials, the levels of IFN-γ were a clear indicator of therapeutic as well as side-effects of IL-12 therapy [10]. Therefore, the appropriate selection of *IL-12* gene doses and the number of intramuscular gene electrotransfer can lead to good antitumor effectiveness without undesirable side-effects.

Based on pharmacological data, systemic gene therapy by *IL-12* gene electrotransfer using triple electrotransfer with 20 μg of plasmid DNA was evaluated in subcutaneous SA-1 and LPB tumors. The results demonstrated good antitumor effectiveness. The tumors were significantly reduced in growth or completely eradicated (27% SA-1 and 13% LPB). A significant reduction of subcutaneous tumors of squamous cell carcinoma without complete responses was determined with triple gene electrotransfer using 10 μg of IL-12 expression plasmid [39]. In addition, double intramuscular gene electrotransfer of 50 μg of IL-12 plasmid did not result in B16.F10 melanoma tumor regression [23]. These data further support the importance of a properly designed gene therapy protocol, which should be based on pharmacological data.

The antimetastatic effect of intramuscular *IL-12* gene electrotransfer was observed on both sarcoma tumor models, SA-1 and LPB. Moreover, the results of our study showed that an increased level of IL-12 at the time of initiation of metastases (prophylactic treatment) greatly reduced the number of metastases (by 90%). Because lung metastases were induced only 24 h after the gene electrotransfer when the maximum serum concentration of IL-12 was not yet achieved, these results showed that only moderately elevated serum concentrations of IL-12 at the time of injection of cancer cells (blood circulating metastases) were already sufficient for a good antimetastatic effect. In other studies, where the antitumor effect of systemic *IL-12* gene therapy on lung metastases was determined, prophylactic treatment was not examined. After double *IL-12* gene electrotransfer performed on the same days as injection of B16.F1 melanoma tumor cells and 3 days thereafter, only 37.5% of mice developed lung metastases [24]. In a similar study, single intramuscular *IL-12* gene electrotransfer with 100 μg of plasmid performed 3 days after the injection of tumor cells resulted in 87% reduction of the number of induced melanoma B16.F1 metastases and 80% reduction of colon adenocarcinoma CT-26 metastases [40]. Systemic IL-12 treatment of orthotopic hepatocellular carcinoma by electrosonoporation 7 days after the injection of tumor cells prevented the formation of spontaneous lung metastases in 90% of treated mice. In the control group, 80% of mice developed lung metastases [42]. In the present study, single treatment of established lung metastases was not so effective. The reduction of metastases was determined only on SA-1 metastases when treatment was performed 24 h after the injection of tumor cells. However, quadrupling treatment, which included prophylactic treatment and three repeats after the injection of tumor cells, was very effective, resulting in almost complete inhibition of formation of metastases in both tumor models.

Current treatment strategies for cancer treatment include a combination of different treatments, one of them being combination of radiotherapy with immune

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therapies. Radiosensitization of tumors by recombinant cytokines was demonstrated in many previous studies [43,55] and, more recently, combinations of radiotherapy with gene therapy using different cytokines were examined [56]. The radiosensitizing effect of systemic treatment by rIL-12 was first examined in animals bearing Lewis lung carcinoma. Treatment with rIL-12 in combination with fractionated radiation therapy resulted in synergistic interaction [1]. The subsequent study by Teicher *et al*. [57] with recombinant murine IL-12 determined that, for efficient treatment, rIL-12 should be administered prior to or during the radiation therapy. The first clinical studies of safety of recombinant IL-12 treatment showed systemic toxicity, which was a major drawback for further research [10,18]. Although research in the field of cancer treatment with IL-12 was re-established by gene therapy, the present study is the first to examine the antitumor effectiveness of systemic *IL-12* gene therapy in combination with radiotherapy. In the present study, the radiosensitizing effect of systemic gene therapy by intramuscular *IL-12* gene electrotransfer was shown in SA-1 and LPB subcutaneous tumors and SA-1-induced lung metastases. A synergistic effect was clearly demonstrated on both subcutaneous tumor models and SA-1-induced lung metastases. Systemic *IL-12* gene therapy potentiated the antitumor effectiveness of radiotherapy in lung metastases by a factor of 1.3, widening the use of IL-12 electrogene therapy for the treatment of disseminating disease. So far, other studies have combined radiotherapy only with local intratumoral IL-12 administration using viral or nonviral vectors [44–47,58]. The results of these studies demonstrated an increased antitumor effectiveness of radiotherapy combined with *IL-12* gene therapy in mammary, melanoma, fibrosarcoma, metastatic prostate and head and neck squamous cell carcinoma tumors.

The treatment with rIL-12 of cancer patients was stopped because of intolerable side-effects. A high IFN-γ serum concentration was shown to be responsible for the toxicity of rIL-12 in clinical studies [18]. Toxic side-effects were observed when the serum concentration of IFN-γ increased above 6 ng/ml (up to 25 ng/ml); however, the patients tolerated the treatment well when the serum concentration of IFN-γ was below 6 ng/ml. In the same study, a preclinical evaluation showed no severe toxicity in C3H/Hej mice, when the serum levels of IFN-γ were approximately 300 ng/ml [18]. In the present study, the highest IFN-γ serum concentration in mice was approximately 2 ng/ml and only moderate side-effects were observed. Although the toxicity and antitumor effectiveness of IL-12 can vary between different strains of mice and between different species, the comparison of serum levels of IFN-γ between patients and mice can provide some orientation about the toxicity. Therefore, we can presume that toxicity of gene therapy in clinical setting would be tolerable because we obtained an approximately 100 $\times$ lower concentration of IL-12 compared to the above mentioned study. In the present study, the body weight of mice was reduced by 14% at the highest serum concentration of IL-12 and IFN-γ, which was a significant reduction, but still tolerable. Mice regained their body weight after the serum levels of IFN-γ declined and all mice survived the treatment. Suboptimal weight and even mortality was observed in another study, where double intramuscular *IL-12* gene electrotransfer with 50 μg of plasmid encoding IL-12 was performed on young (less than 6 weeks old) mice [40], stressing the importance of careful selection of the plasmid DNA dose and electrotransfer repeats. Local side-effects in muscle included muscle fiber degeneration and infiltration of immune cells. Immune cell infiltration was transient and dissolved in 1 month. The infiltration was not a specific response to produced IL-12 because it was previously observed after intramuscular gene electrotransfer of different transgenes [59,60]. In our previous study, the transfection with plasmid encoding green fluorescent protein using the same parameters of gene electrotransfer resulted in an even higher area of immune cell infiltration [60]. Another comprehensive study looking at the toxicity of *IL-12* gene electrotransfer showed no treatment-specific toxicity; however, the treatment was performed locally into tumors and no significant increases in serum IL-12 levels were detected [61].

In conclusion, we demonstrated that careful selection of parameters for systemic *IL-12* gene therapy based on pharmacological data results in pronounced antitumor effectiveness in solid subcutaneous tumors and induced lung metastases. In addition, systemic *IL-12* gene therapy had a good radiosensitizing effect without limiting side-effects, which make this therapy a suitable candidate for translation into clinical studies, especially because local *IL-12* gene therapy has already been evaluated in a clinical trial for the treatment of malignant melanoma [33–38,62].

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Efficient Electrotransfection into Canine Muscle

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Darja Pavlin, D.V.M.[1]
Natasa Tozon, D.V.M., Ph.D.[1]
Gregor Sersa, Ph.D.[2]
Azra Pogacnik, D.V.M., Ph.D.[1]
Maja Cemazar, Ph.D.[2,*]

[1]University of Ljubljana
Veterinary Faculty Ljubljana
Gerbiceva 60
SI-1000 Ljubljana, Slovenia
[2]Institute of Oncology Ljubljana
Department of Experimental Oncology
Zaloska 2
SI-1000 Ljubljana, Slovenia

Two different types of electroporation protocols have been developed for efficient electrotransfer of plasmid DNA into skeletal muscle of experimental animals. At first, only low voltage electric pulses have been used, but lately, a combination of high and low voltage pulses has been suggested as more efficient. Up to date, in dogs, this type of electroporation protocol has never been used for muscle targeted plasmid DNA electrotransfection. In this study, we used two different DNA plasmids, one encoding green fluorescent protein and one encoding human interleukin-12. Five different electroporation protocols were evaluated. Three of them featured different combinations of high and low voltage pulses, and two were performed with delivery of low voltage pulses only. Our study shows that combination of 1 high voltage pulse (600 V/cm, 100 µs), followed by 4 low voltage pulses (80 V/cm, 100 ms, 1 Hz) yielded in the same transfection efficiency as the standard trains of low voltage pulses. However, this protocol is performed quicker and, thus, more suitable for potential use in clinical practice. In addition, it yielded in detectable systemic expression of human interleukin-12. Electrotransfer of either of the plasmids was associated with only mild and transitory local side effects, without clinically detectable systemic side effects. The results indicate that electrotransfection is a feasible, effective, and safe method for muscle targeted gene therapy in dogs, which could have potential for clinical applications in veterinary medicine of small animals.

Key words: Electroporation; Electrotransfection; Muscle; Plasmid DNA; Green fluorescent protein; Interleukin-12; Muscle; and Dogs.

Introduction

Gene therapy is a novel approach for treatment or prevention of different genetic and non-genetic diseases. The most straightforward approach to therapeutic gene delivery is direct injection of plasmid DNA into the tissue of interest (1). However, this is a relatively inefficient method of gene transfer with highly variable interindividual transfection efficiency. Preclinical studies show that transfection efficiency of plasmid DNA application alone can be significantly enhanced when it is followed by electroporation (EP) of target tissue (2, 3).

EP is a method for delivery of various molecules into the cells by transiently increasing permeability of cell membrane using application of controlled external electric field to the cells (4). It is already well established as an *in vitro* method for increasing delivery of various molecules (*e.g.*, RNA, DNA, oligonucleotides, dyes, ions, chemotherapeutic drugs, *etc.*) into different types of cells. *In vivo*, it is gaining much interest as a tool for two prospective therapeutic modalities in cancer treatment: electrochemotherapy, which is already used

[*]Corresponding Author:
Maja Cemazar, Ph.D.
Email: mcemazar@onko-i.si

Abbreviations: EP, Electroporation; IL-12, Interleukin-12; LV, Low voltage electric pulses; HV, High voltage electric pulses; GFP, Green fluorescent protein; PBMC, Peripheral blood mononuclear cells; SAP, Serum alkaline phosphatase; CK, Creatinine kinase.

in clinical practice (*i.e.*, application of controlled electric pulses to tumor cells in order to increase uptake and cytotoxicity of chemotherapeutic drugs bleomycin and cisplatin) (5, 6), and electrogene therapy (*i.e.*, combining injection of plasmid DNA, encoding therapeutic genes into target tissue with application of electric pulses) (2, 3), which is already being tested in a number of clinical trials (7). A number of different types of tissues have been successfully transfected *in vivo* using this approach, including tumors (8, 9), skeletal muscle (10, 11), skin (12, 13), and liver (14).

In skeletal muscle EP enhances expression of plasmid DNA up to 2000-times and significantly reduces variability of gene expression, compared to application of plasmid DNA alone, resulting in long-term expression of exogenous DNA, which can last up to 1 year (11). Electrotransfer of plasmid DNA into skeletal muscle has been successfully achieved in different experimental animals: mice, rats and rabbits (10, 11), cattle (15, 16), goats (15), sheep (17), pigs (18, 19), dogs (20, 21), and monkeys (22). There have been two different types of EP protocols developed for this purpose. At first, only low voltage (LV) electric pulses with long duration (100-200 V/cm, 20-50 ms) were successfully used. Lately, it has been shown that a better transfection efficiency can be achieved using combination of one high voltage (HV) electric pulse (600-800 V/cm, 100 μs) followed by different numbers of LV electric pulses (80-100 V/cm, duration in time range of tens to hundreds of milliseconds) (23, 24). It has been hypothesized that the HV pulse first causes permeabilization of cell membrane, followed by electrophoresis of DNA across destabilized cell membrane during the LV pulses (23, 25). However, recent published paper presented evidence that does not support this hypothesis (26); therefore, further research is clearly needed to elucidate this aspect of electrotransfer of plasmid DNA into the cells.

To date there have been only a few reports on electrotransfection of canine skeletal muscle (20, 21, 27, 28). They account of two different plasmids that were efficiently transfected: plasmid encoding growth hormone-releasing hormone and plasmid encoding human coagulation factor IX. Three similar EP protocols were used for transfection of these two plasmids, all employing LV pulses only. None of the published experiments on dogs has yet utilized the combination of high and low voltage pulses.

Proinflammatory cytokine interleukin-12 (IL-12) has been extensively studied as an antitumor agent, since it exhibits a number of activities, which are potentially important in immunotherapy of cancer, including induction of T-cells and triggering of interferon-γ response. Despite having potential in anticancer therapy (29, 30), systemic application of recombinant IL-12 can result in severe toxicity (31). Therefore, a new approach to IL-12 immunotherapy, utilizing gene therapy, has been investigated and successfully employed on preclinical level on different types of tumours (32-36).

Canine and human IL-12 share approximately 90% genetic identity based on amino acid sequence analysis (37). Human IL-12 activates proliferation of canine peripheral blood mononuclear cells (PBMC) in *in vitro* setting and triggers a number of immune responses in canine PBMC (38), which leads to speculation that it could have therapeutic potential in dogs.

The aim of this study was to compare the efficiency of established low voltage EP protocols with EP protocols utilizing combination of HV and LV pulses, which to date have never been used in dogs. For this purpose we used plasmid encoding green fluorescent protein (GFP), injected into *m. semitendinosus* of beagle dogs, followed by delivery of five different EP protocols. Furthermore, the best two of these protocols were evaluated for transfection efficiency of a single intramuscular application of therapeutic gene, encoding human IL-12. Additionally, local and systemic side effects of electrotransfection of both plasmids were assessed.

Materials and Methods

Experimental Animals

In our experiments, one female and five male beagle dogs, aged from 8-10 years and weighing 13-23 kg, were used. The study was approved by the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia with the limitation on the number of animals used in the study. All procedures on animals were performed under general anesthesia. Prior to all experimental procedures, complete blood count with differential white blood cell count was performed in all animals, using automated laser haematology analyser (Technicon H*1, Bayer, Germany) with species-specific software (H*1 Multi-Species V30 Software). Automated chemistry analyser Technicon RA-XT (Bayer, Germany) was used for determination of the following biochemical parameters: blood urea, creatinine, serum alkaline phosphatase (SAP), and creatinine kinase (CK). In addition, alanin aminotransferase was determined only in the second part of experiment, featuring plasmid encoding human IL-12.

Plasmids

In our experiments, two different plasmids were used: pEGFP-N1, encoding GFP (Clontech Laboratories, Inc.; Mountain View, CA, USA) and pORF-hIL-12 (InvivoGen, San Diego, CA, USA), encoding human IL-12. They were both prepared using the Qiagen Maxi Endo-Free kit (Qiagen, Hilden, Germany), according to manufacturer's instructions and diluted to concentration of 1 mg/ml. Purified plasmid DNA was subjected to quality control and quantity determinations, per-

formed by agarose gel electrophoresis and by means of spectrophotometry. Quality control included the ratio 260/280 between 1.7-1.9 and minimum presence of genomic DNA and RNA determined on gel electrophoresis. The purified DNA contained less that 0.1 EU of bacterial endotoxin per μg of DNA according to the manufacture declaration.

Plasmid Transfection Protocol

In the first part of the study, plasmid encoding GFP was electrotransfected into skeletal muscle of experimental animals. Dogs were premedicated with combination of acepromazine (Promace, Fort Dodge Animal Health, Iowa, USA; 0.02 mg/kg of bodyweight) and metadone (Heptanon, Pliva Zagreb, Croatia; 2 mg/kg of bodyweight). Thirty minutes later, general anesthesia was induced using thiopental (Nesdonal, Merial, Lyon, France; 5 mg/kg of bodyweight) and maintained with isoflurane (Forane, Abbott Laboratories LTD, Queensborough, United Kingdom). During the anesthesia the animals were receiving Hartmann's solution (B. Braun Melsungen AG, Melsungen, Germany) at rate 10 ml/kg of bodyweight/hour.

In animals under general anesthesia hair on femoral regions of both legs was clipped and regions were surgically prepared. Incision of the skin and fascia was made in order to expose *m. semitendinosus*, followed by infiltrative intramuscular injection of 150 μg of plasmid encoding GFP, using 1 ml syringe with 22 G needle. Infiltrative injection is an injection where part of tissue is infiltrated with single application. Position of the needle is slightly changed during emptying of syringe, allowing the content of syringe to infiltrate the target tissue more uniformly. A surgical suture was placed on muscle at the exact site of plasmid injection using nonresorbable polifilament material (Sofsilk® 3-0, USSC, Norwalk CT, USA) to allow future identification of exact plasmid application site for correct placement of electrodes and performing biopsies. Electric pulses were applied to muscles 20 minutes after plasmid injection, according to data available in the literature (39, 40). Electric pulses generator Cliniporator™ (IGEA s.r.l., Carpi, Italy) was employed, using needle electrodes N-18-4B (IGEA s.r.l., Carpi, Italy), which consist of two arrays, each composed of four electrodes with 4 mm distance between them. Skin incisions were closed with standard surgical procedure immediately after application of electric pulses and animals were let to recover from anesthesia spontaneously. Postsurgically, analgesia was provided to all dogs with single intravenous application of carprofen (Rimadyl, Pfizer Animal Health, Dundee, United Kingdom; 4mg/kg of bodyweight).

In the first part of the study using GFP, altogether five different EP protocols were used, each applied to two muscles in such order that each dog received two different EP protocols. Three of these protocols (EP 1 - EP 3) utilized combination of 1 HV pulse, followed by different number of LV pulses. Lag between the HV pulse and the first LV pulse in all three protocols was 1 s. The duration of LV pulses was kept in all protocols constant in order to avoid tissue heating due to the longer duration of pulses delivery in the case of protocols EP2 and EP3. Another two protocols were performed by application of LV pulses only: one of them (EP 4) was the standard train of eight identical pulses of 200 V/cm, 20 ms duration, which was long considered as an optimal EP protocol for electrotransfer of DNA into skeletal muscle. The second low voltage EP protocol (EP 5), utilizing six pulses of 100 V/cm, 60 ms, was chosen due to the fact that this is a protocol which has been employed in majority of published experiments using muscle targeted electrotransfer on dogs (24). The control group received only plasmid application without electric pulses. Details of each protocol are provided in Table I.

Approximately one month after the completion of the first part of the study, electrotransfer of plasmid encoding human IL-12 was performed on the same six animals under the same conditions. The animals were divided into two groups, each comprising three dogs. Two different EP protocols were used, one on each experimental group (Table II). Different doses of plasmid DNA were injected infiltratively into the right *m. semitendinosus* muscle of each dog, using part of the muscle, as far as possible from the site, where electrotransfer of plasmid encoding GFP was performed. Plasmid was injected through intact skin, followed by transcutaneous positioning of needle electrodes and delivery of EP protocol 20 minutes after the plasmid injection (Table II). Postprocedure recovery and analgesia was provided in the same manner as in the first experiment.

Assessment of Transfection Efficiency

In case of experiment, featuring plasmid encoding GFP, transfection efficiency was assessed on day 2 and day 7 after the electrotransfection procedure. Dogs were anaesthe-

Table I
GFP fluorescence in muscle biopsies.

Group	Details of EP protocol	Level of fluorescence	
		Day 2	Day 7
EP 1	1 HV (600 V/cm, 100 μs), followed by 1 LV (80 V/cm, 400 ms)	0	0
EP 2	1 HV (600 V/cm, 100 μs), followed by 4 LV (80 V/cm, 100 ms, 1Hz)	++++	++++
EP 3	1 HV (600 V/cm, 100 μs), followed by 8 LV (80 V/cm, 50 ms, 1Hz)	++	0
EP 4	8 LV pulses (200 V/cm, 20 ms, 1Hz)	++++	++++
EP 5	6 LV pulses (100 V/cm, 60 ms, 1Hz)	0	0
Control	Application of plasmid, without electric pulses	0	0

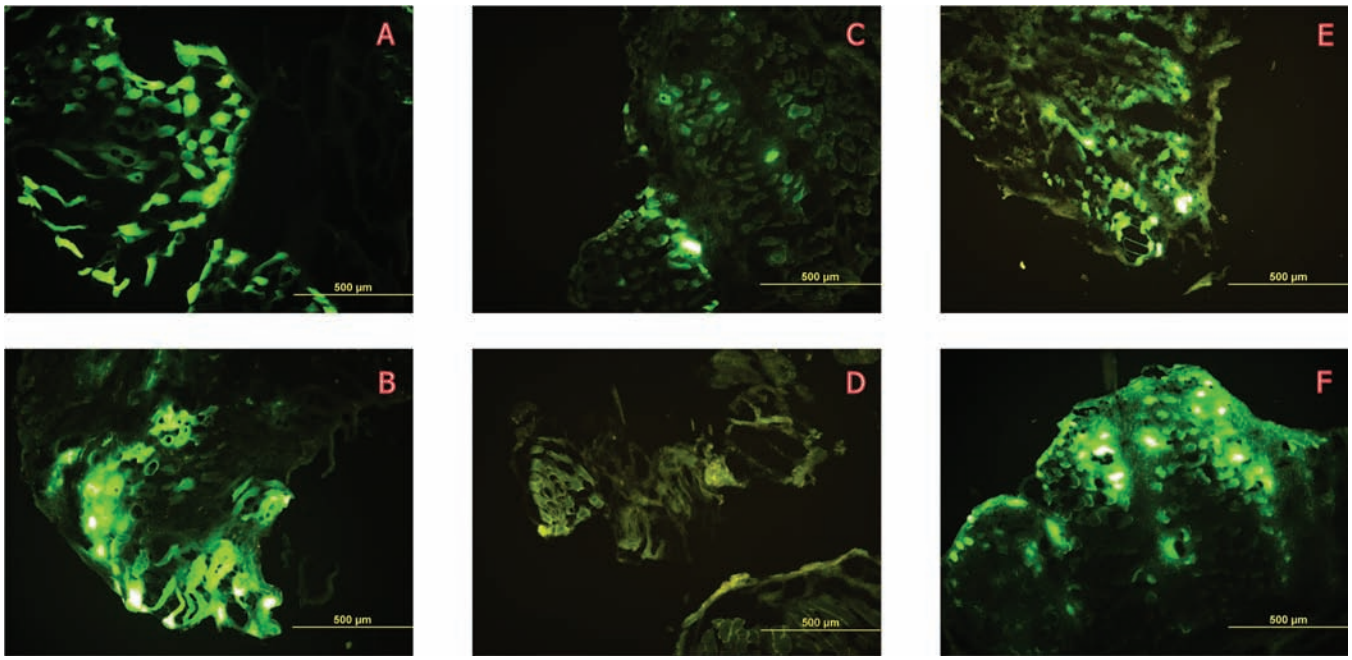

Figure 1: Images of frozen tissue sections of muscle samples, in which GFP fluorescence was detected. Similar level of GFP fluorescence was observed in two sets of muscle samples: in group, where EP 2 (1 HV + 4 LV) was applied (**A** and **B**, which represent samples collected on 2nd and 7th day, respectively) and in group, where EP 4 (8 x LV) was applied (**E** and **F**, which represent samples collected on 2nd and 7th day, respectively). EP 3 (1 HV + 8 LV) yielded in markedly lower degree of transfection, observed only on 2nd day (**C**). In this group, only autofluorescence could be observed in muscle samples, collected seven days after electrotransfection (**D**).

tized using the same protocol as described above and skin sutures were removed. At both time points incision biopsies of transfected muscles were performed, each time removing approximately 0.5 cm × 0.5 cm × 0.5 cm of muscle tissue. Skin incisions were closed with standard surgical procedure immediately after collection of muscle samples and animals received single intravenous application of carprofen.

Samples were embedded in Tissue-Tek O.C.T. Compound (Miles Inc., Elkhart, IN, USA) at -20 ºC. Frozen samples were cut into 20 µm thick sections. Transfection efficiency was determined by assessment of green fluorescence using fluorescence microscope (Olympus BX51, Olympus, Hamburg, Germany). Green fluorescence of each sample was evaluated by two independent observers in a blind fashion, with estimation of fluorescence intensity ranging from zero (no visible fluorescence) up to maximum of 5 points.

In case electrotransfection of plasmid encoding human IL-12, transfection efficiency was evaluated with determination of serum concentrations of human IL-12 and canine IFN-γ. Blood samples were collected before and on day 2, 7, 14, and 28 after the procedure. Human IL-12 was determined using Interleukin-12 (p40) (human) ELISA (DRG Instruments GmbH, Marburg, Germany). Canine IFN-γ was determined using Quantikine® Canine IFN-γ Immunoassay (R&D Systems, Inc., Minneapolis, USA).

Assessment of Side Effects

In both parts of our study, animals were under close observation in the first month after the experimental procedure in order to assess possible local and systemic side effects of performed electrotransfection. Clinical examination of each animal was conducted on daily basis as well as assessment of appearance of the area on the leg, where electric pulses were delivered, for any clinical signs, including erythema, edema, pain, secretions, necrosis, *et cetera*. On day 2, 7, 14, and 28 after both electrotransfection procedures, the same hematological and biochemical analyses were performed, as prior to the procedures (described above), in order to assess possible systemic effects of the electrotransfection procedure on the experimental animals.

Results

Electrotransfection of Plasmid Encoding GFP

Transfection Efficiency: The highest level of GFP fluorescence in the muscle was observed in two sets of muscle samples (Table I): samples, taken from the group, where EP 2 (1 HV pulse, followed by 4 LV pulses) was applied and from the group, where EP 4 (8 LV pulses) was applied. In both protocols significant GFP fluorescence was detectable both 2 and 7 days after the transfection (Fig. 1).

Markedly lower degree of transfection was achieved using EP 3 (1 HV pulse, followed by 8 LV pulses) (Fig. 1). In this group, GFP fluorescence was less pronounced compared to EP 2 or EP 4. Furthermore, fluorescence was detectable only in samples, taken at day 2 after electrotransfection. In this group, no GFP fluorescence was observed in muscle samples, taken one week after the procedure.

No GFP fluorescence was detectable either at day 2 or 7 days after electrotransfection on muscle samples, taken from control group and from groups, where EP 1 (1 HV pulse, followed by 1 LV pulse) or EP 5 (6 LV pulses) were used (Table I).

Assessment of Side Effects of the Procedure: Throughout the whole electrotransfer procedure dogs' heart and respiratory rate, ECG, end tide CO_2, noninvasive blood pressure and temperature were routinely monitored for purposes of safe conduct of general anesthesia. During delivery of electric pulses to dogs, increase of dog's heart and respiratory rate were noted for up to 60% (data not shown). This response correlated with delivery of low voltage electric pulses, during which significant muscle contractions of hind legs were also noticed. Heart rate returned to baseline values immediately after intravenous application of analgesic (ketamine hydrochloride, Bioketan, Vetoquinol, Paris, France; 1 mg/kg), additionally demonstrating that the heart rate acceleration was consequence of painful stimulus.

In order to record any possible local side effects, caused by electrotransfection procedure, clinical examination of each experimental animal was performed on daily basis. It was established that the procedure was very well tolerated by the animals. The only observed side-effect was tissue swelling at the site of electroporation, which spontaneously resolved within two days after the procedure, without any signs of impaired locomotory function. Clinically, we couldn't detect any marked difference in severity of observed side effects with regard to different EP protocols used.

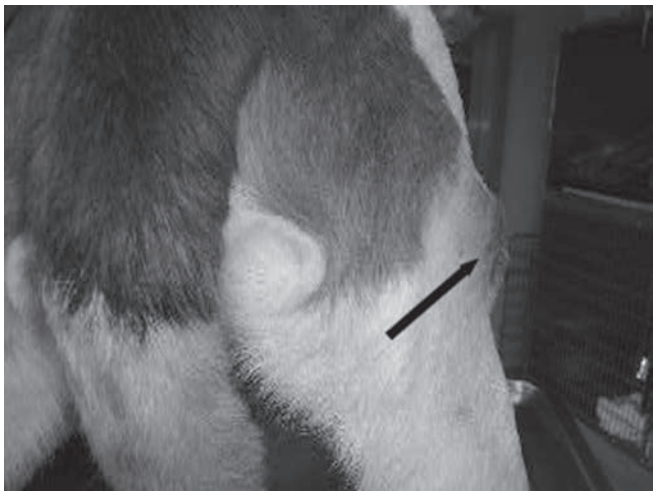

Figure 2: Local side effects of electrotransfection procedure seen as a tissue swelling at the site of injection of plasmid encoding human IL-12 and subsequent transcutaneous application of electric pulses (arrow).

To determine possible systemic side effects, complete blood count with differential white blood cell count and selected biochemistry parameters were analyzed in blood samples, collected before the procedure and 2, 7, 14, and 28 days afterwards. All hematological parameters measured in samples, collected before the procedure, were within reference limits, except one dog with mild thrombocytosis (platelets 564 × 10^9/L; reference values 200-500 × 10^9/L) and three cases of mild relative neutropenia (neutrophils ranging from 57%-59.7%; reference values 60-80%). After the procedure, only mild alterations from reference values were observed, including relative neutropenia on day 28 in one dog that received EP 2 and EP 4 protocols (neutrophils 50%), relative lymphocytosis in two dogs treated with EP 1, EP 4 and untreated (lymphocytes 35.9% in one dog on day 7 and 42% in one dog on day 28; reference values 12-35%), and thrombocytosis in two dogs treated with EP 1, EP 3, EP 4, and untreated (platelet count 544 × 10^9/L in one dog on day 2 and 684 × 10^9/L in one dog on day 28). These alterations were not associated with any clinical signs in animals.

Selected biochemistry parameters of dogs, measured before electrogene transfer demonstrated values of blood urea and CK to be within reference limits (reference values for dogs are presented in Table III), low concentration of creatinine (mean value 50.05 μmol/L), and slightly elevated activity of SAP (mean value 138.08 U/L). Similar biochemistry profile could be seen in dogs after the procedure. Urea and CK remained within reference limits, concentration of creatinine was low at all three post-procedure measures (mean value 49.6 μmol/L at day 2, 77.5 μmol/L at day 7, and 71.4 μmol/L at day 28) and activity of SAP was slightly elevated at day 7 and 28 (mean value 129.5 U/L and 139.4 U/L, respectively). In animals none of these alterations in biochemistry parameters could be detected clinically.

Electrotransfection of Plasmid Encoding Human IL-12

Serum Concentrations of Human IL-12 and Canine IFN-γ: In order to determine serum concentrations of human IL-12 in dogs, blood samples were collected in different time points after the single electrically-assisted intramuscular delivery of plasmid encoding human IL-12. Human IL-12 was detected with ELISA in only one serum sample, which was collected seven days after the electrotransfection procedure, from dog No. 3 (Table II).

Canine IFN-γ was detected at different time points after the electrotransfer procedure in serum samples of three animals (Table II), including dog No. 3, the only animal with detect-

Table II
Details of experimental protocol, using pORF-hIL-12 and detected concentrations of human IL-12 and canine IFN-γ at different time points after the electrotransfer procedure.

No. of animal	Body weight (kg)	Dose of plasmid pORF-hIL-12	EP protocol	Concentration of human IL-12	Concentration of canine IFN-γ
1	13	100 μg/100 μl	EP 2	not deceted	15 pg/ml (day 2) 19.3 pg/ml (day 14) 36.6 pg/ml (day 28)
2	20	500 μg/500 μl	EP 2	not detected	not detected
3	20	1000 μg/1000 μl	EP 2	19 pg/mL (day 7)	23.7 pg/ml (day 2)
4	19	100 μg/100 μl	EP 4	not detected	23.7 pg/ml (day 7) 2.15 pg/ml (day 28)
5	17	500 μg/500 μl	EP 4	not detected	not detected
6	23	1000 μg/1000 μl	EP 4	not detected	not detected

able human IL-12 levels. In this dog, induction of interferone response did not correlate with production of human IL-12, since IFN-γ was detected in serum sample, taken on day 2 and human IL-12 was detected on day 7 after the electrotransfer procedure.

Assessment of Side Effects of the Procedure: In the first month after electrotransfection of therapeutic plasmid, animals didn't show any abnormalities in their clinical status or behavior. Tissue swelling at the site of electroporation (Fig. 2), which was observed, was slightly less pronounced as in the first part of experiment, where we used plasmid encoding GFP.

All hematological parameters measured in samples, collected before the procedure, were within reference limits. The only exception was a dog with clinically nonsignificant thrombocytosis (platelets 684 × 10^9/L). Hematological parameters measured in samples, collected after the procedure, were mainly within reference limits with only two exceptions: mild haemoconcentration on day 28 in dog No. 2 (hemoglobin 187 g/L; reference values 115-180 g/L) and No. 5 (hemoglobin 185 g/L). The only animal, in which human IL-12 was detected (dog No. 3), had all hematology parameters within reference limits at all four measurements after the procedure.

There were some minor abnormalities in biochemistry parameters in blood samples, collected before the procedure, as well as in samples, taken at selected time points after the procedure. However, altered postprocedure values did not have any clinical value, and could not be directly linked to effects of electrotransfection procedure. Typical values of biochemistry parameters are presented in Table III, which shows biochemistry profile of all six dogs on day 7 after electrotransfection of therapeutic plasmid. Measurements on all other time points were similarly uncharacteristic (data not shown).

Discussion

Results of our study suggest, that combination of high and low voltage pulses is more suitable for use in muscle targeted DNA electrotransfer in dogs, than use of standard established trains of low voltage pulses only. Although the best transfection of plasmid encoding GFP was achieved with either combination of one HV pulse (600 V/cm, 100 μs), followed by four LV pulses (80 V/cm, 100 ms, 1Hz) or eight identical LV pulses (each 200 V/cm, 20 ms, 1Hz), the first protocol was shown to be more suitable for potential clinical use. Electrogene therapy, using either of these two plasmids was associated with only mild and transient local side ef-

Table III
Blood biochemistry values of six dogs, measured seven days after receiving single injection of plasmid encoding human IL-12, followed by delivery of two different EP protocols.

	Therapy (dose of therapeutic plasmid; EP protocol)	Urea (mmol/L)	Creatinine (μmol/L)	SAP (U/L)	ALT (U/L)	CK (U/L)
Reference values		3-12.5	60 + (1.2 x bw)	25-117	23-90	< 249
Dog Nr. 1	100 μg/100 μl; EP 2	5.7	55.6	217	34.3	76.5
Dog Nr. 2	500 μg/500 μl; EP 2	7.2	44.8	160.9	69.8	84.1
Dog Nr. 3	1000 μg/1000 μl; EP 2	4.2	54.4	303.5	26.5	71
Dog Nr. 4	100 μg/100 μl; EP 4	8	72.3	49.2	48.8	66.5
Dog Nr. 5	500 μg/500 μl; EP 4	5.6	125.1	51.3	5.5	48.3
Dog Nr. 6	1000 μg/1000 μl; EP 4	6.3	125.1	88.8	56.1	121.1

Abbreviations: SAP, Serum alkaline phosphatase; ALT, Alanin aminotransferase; CK, Creatin kinase; bw, Bodyweight.

fects and did not result in any detectable systemic toxicity.

In the first part of the study, reporter gene encoding GFP was used to evaluate transfection efficiency of five different electroporation protocols, selected according to published reports and recommendations for successful transfection of skeletal muscle using EP in different animal species. Historically, first successful *in vivo* attempts have used only low voltage pulses, according to results of comprehensive study by Mir and colleagues on a number of different animals (11). In that study it was determined that the optimal protocol for electrotransfection of skeletal muscle consists of delivery of eight identical pulses with electric field strength 200 V/cm, duration of 20 ms, and repetition frequency 1 Hz, which became standard optimal EP protocol for electrotransfection of skeletal muscle. In dogs, three different EP protocols have been successfully used for efficient muscle targeted electrotransfection. The highest number of dogs in published experiments received plasmid encoding growth-hormone releasing hormone, either using EP protocol consisting of six pulses of 100 V/cm and 60 ms duration (20, 27), or five pulses of 100 V/cm and 52 ms duration (28). Another plasmid encoding human clotting factor IX, was delivered using six pulses of 200 V/cm, 60 ms duration, and reversed polarity after each pulse (21).

In vitro studies regarding the role of electric pulses in DNA electrotransfer demonstrated that electric pulses could have two roles in successful DNA electrotransfer: electropermeabilization of target cell membrane and electrophoretic effect on DNA molecules. This hypothesis has been investigated by *in vivo* experiments in mice featuring use of two different types of square wave electric pulses for electrotransfection of skeletal muscle (23, 25). It was proposed that the HV pulse (600-800 V/cm) of very short duration (100 μs) causes permeabilization of muscle cells. This field strength was shown to be the highest not to cause irreversible electropermeabilization of muscle cells (41). Subsequent LV pulses (usually less than 100 V/cm, with duration in time range from tens to hundreds of ms) act on DNA molecules, enabling electrophoretic movement of DNA molecules toward and across the cell membrane. However, a recently published study (26) indicates that electrophoretic force may not play as important role in movement of DNA molecules across cell membrane as proposed in these reports. Nevertheless, different combinations of HV and LV pulses were evaluated, and combination of one HV pulse, followed by 4 LV pulses was as efficient as the standard electroporation protocol of 8 identical LV pulses of 200 V/cm, 20 ms, 1 Hz (25).

Our results on dogs confirm and extend the results of previous studies on mice. In addition, to date, the combination of HV and LV pulses has not yet been used in any of the published studies employing electrotransfection of canine skeletal muscle. In our study, the highest transfection efficiency was achieved using either EP 2, *i.e.*, combination of one HV pulse (600 V/cm, 100 μs), followed by four LV pulses (each 80 V/cm, 100 ms, repetition frequency 1 Hz), or using EP 4, which was standard train of eight identical pulses of 200 V/cm, 20 ms duration with repetition frequency of 1 Hz. We didn't detect any marked difference in intensity of fluorescence with both EP protocols. Despite the fact, that both mentioned protocols yielded the same transfection efficiency, there are two major advantages for the use of combination of 1 HV and 4 LV pulses compared to the use of 8 LV pulses only. The first one is that the combination is less harmful to target cells, since the intensity of LV pulses is lower compared to standard train of eight pulses (80 V/cm versus 200 V/cm, respectively) (25). The goal of gene therapy targeted to muscle cells is to produce sufficient levels of therapeutic proteins, which can be achieved only, if target cells remain viable after gene delivery to be able to express transgene products. Therefore, the least possible stress to target cells should be attempted. The second advantage could be important for eventual clinical use of this procedure. In combination, where 1 HV pulse, followed by 4 LV pulses were used, the delivery of electric pulses was concluded faster compared to delivery of eight identical LV pulses (frequency of electric pulse delivery in both protocols was the same, 1 Hz) and caused only four instead of eight painful muscle contractions.

In both protocols, which yielded the highest intensity of GFP fluorescence, expression remained the same on the 2nd and 7th day after gene delivery. According to other reports, transgene expression in skeletal muscle of dogs lasts longer; sufficient levels of transgene products to achieve biological effect after single injection of plasmid in healthy dogs were detected for at least 180 days (28). Therefore, we can speculate that transgene expression of GFP in canine muscle is actually longer than one week, but due to ethical considerations, we designed our experiment in such way as to perform only two muscle biopsies and could not monitor transgene expression beyond that time period.

Based on results of the first part of our study using reporter gene, encoding GFP, we used the best two EP protocols, to achieve electrotransfection of plasmid encoding human IL-12 into canine skeletal muscle. In the second part of the study, injection of therapeutic plasmid as well as placement of electrodes was performed transcutaneously without skin and fascia incisions as in the first experiment. This modification was performed with the intention to develop and evaluate a protocol, which could be in the future used in clinical work for muscle targeted gene delivery in dogs. Transcutaneous application is faster and especially less invasive, which is an important advantage for clinical pattern.

Although low number of samples was used, due to ethical considerations and legal restrictions in designing the experi-

ment, some additional conclusions can be drawn from results of this part of the study, which confirm results, obtained utilizing plasmid encoding GFP. Human IL-12 was detected in serum of a dog, in which the highest dose of plasmid (1 mg diluted in 1 ml) was applied and EP protocol utilizing combination of 1 HV and 4 LV pulses (EP 2) was delivered. The concentration of human IL-12 was low (19 pg/ml), and was detected only in serum sample, collected at seven days after the procedure. To establish whether systemically secreted human IL-12 manifests immunostimulatory effect *in vivo* in dogs, serum levels of canine IFN-γ in blood samples were determined. Canine IFN-γ was detected in low levels (2.15-36.6 pg/ml) in three dogs, but interferon response did not correlate with detection of human IL-12. Although hematology and biochemistry analysis did not show any significant alterations, which could explain induction of IFN-γ production in these three animals, we conclude, that concentration of human IL-12 was probably too low to exert *in vivo* biological effect in the dog and induce unequivocal IFN-γ response. In contrast to our results, in the study of Fewell and colleagues, therapeutic concentrations of human coagulation factor IX in dogs after a single muscle-targeted electrotransfection of therapeutic plasmid were achieved (21). It should be noted that in that study extremely high doses of plasmid were needed to achieve therapeutic response; even up to 3 mg of plasmid per kg of bodyweight. Therefore, it is possible that 1 mg of plasmid encoding human IL-12 per dog, is not a sufficient dose to achieve detectable levels of human IL-12 in beagle dogs and that higher dosage of plasmid should be transfected. Another possible improvement of transgene expression could lie in multiple consecutive repetitions of procedure in order to achieve higher concentrations of human IL-12 in blood, resulting in successful induction of IFN-γ response.

In both parts of our study, all animals were monitored for possible side effects of electrotransfection procedure. Local side effects after delivery of both plasmids included a mild swelling of subcutaneous tissue at the site of electroporation, which didn't cause any impairment of locomotory function. The swelling was slightly more pronounced in experiment with plasmid encoding GFP, which was probably due to tissue damage, caused by incision of skin and fascia. These observations are in agreement with other studies, featuring electrotransfection of muscle in large animals, where only transient local effects were observed (*e.g.*, transitory erythema at the injection point), without any permanent damage to skin or muscle (27, 42). Furthermore, lack of any clinically significant adverse effects on muscle was confirmed by measuring serum activity of CK, which is a very sensitive indicator of muscle damage (43). In the previous studies, featuring electrotransfection of canine skeletal muscle, the activity of CK was measured in only one published paper (21), where transient increase in CK activity was detected, which returned to normal levels by day 7. The increase was clearly dose-dependent, with animals receiving plasmid in more injection sites and, thus, more sites to which electric pulses were delivered, having significantly higher CK activity.

The only negative effect of the electrotransfection procedure in dogs was considerable muscle contractions observed during delivery of electric pulses, which dissipated immediately thereafter. Muscle contractions are reported to be the major cause of pain in human patients, undergoing electrochemotherapy (44, 45). Routine monitoring of animals' vital signs during general anaesthesia revealed increased heart rate without any alterations in ECG and increased respiratory rate during delivery of LV electric pulses, which is the usual response to painful stimulus in an anaesthetised dog, and returned to baseline values immediately after intravenous application of analgesic. During electrochemotherapy pain can be significantly alleviated by raising the treated tumor nodule if possible away from subcutis prior to electric pulses delivery, to avoid painful contractions of subcutaneous muscle. In muscle targeted electrogene therapy this is of course not possible. Therefore, it is mandatory to perform the procedure in animals under adequately deep general anesthesia and to provide sufficient analgesia during delivery of electric pulses.

Our study asserted that electrotransfection of either plasmid did not cause any important systemic side effects. Hematological and biochemical parameters in collected blood samples remained within reference limits throughout the whole observation period with only few clinically nonsignificant and nonspecific alterations. This is a particularly important finding in gene therapy utilizing IL-12. Namely, the systemic administration of recombinant IL-12 is associated with multiple serious adverse effects, including renal and systemic toxicity (46); and high-dose levels were linked to temporary immune suppression, which would not be favorable for effective immunotherapy. Toxicity of electrically-mediated intratumoral delivery of plasmid encoding IL-12 was evaluated in a mouse melanoma model (47). Significant delay in tumor growth was demonstrated without any detectable serum concentrations of IL-12. The only histopathological abnormality, specific to animals, which received plasmid encoding IL-12, was inflammation, associated with the kidney by day 30 after the gene delivery, but without any hematological or biochemistry alterations, contributable to diminished kidney function. In our study, hematological (complete blood count, differential white cell count) and biochemistry parameters (urea, creatinine, SAP, and alanin aminotransferase) in blood samples, collected in different time intervals after delivery of plasmid encoding human IL-12, remained within reference limits, with individual alterations, which were clinically not significant and could not be linked to the performed procedure. Clinical status of all animals remained unaltered and they didn't show any changes in appetency, water intake, and general behavior.

In conclusion, our study shows that electrotransfection is a feasible, effective, and safe method for muscle targeted gene therapy in dogs, which yields in gene expression lasting at least one week. Based on results of our study, we conclude that EP protocol, utilizing one HV pulse (600 V/cm, 100 μs), followed by four LV pulses (80 V/cm, 100 ms, 1 Hz) is more suitable for electrotransfection of canine skeletal muscle than use of EP protocols employing LV pulses only. This EP protocol proved in our study to be more suitable than use of established low voltage protocols, which is an important aspect for eventual clinical use of this gene delivery method, and resulted in comparable level of transfection of muscle cells with plasmid encoding GFP. Our results suggest that this novel approach to gene therapy could have potential for clinical applications in veterinary medicine of small animals.

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research article

Electrogene therapy with interleukin-12 in canine mast cell tumors

Darja Pavlin[1], Maja Cemazar[2,3], Andrej Cör[3], Gregor Sersa[2], Azra Pogacnik[1], Natasa Tozon[1]

[1] University of Ljubljana, Veterinary Faculty Ljubljana, Ljubljana, Slovenia
[2] Institute of Oncology Ljubljana, Department of Experimental Oncology, Ljubljana, Slovenia
[3] University of Primorska, College of Health Care Izola, Izola, Slovenia

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Correspondence to: Prof. dr. Natasa Tozon, University of Ljubljana, Veterinary Faculty Ljubljana, Gerbičeva 60, SI-1000 Ljubljana, Slovenia.
E-mail: natasa.tozon@vf.uni-lj.si

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Background. Mast cell tumors (MCT) are the most common malignant cutaneous tumors in dogs with extremely variable biological behaviour. Different treatment approaches can be used in canine cutaneous MCT, with surgical excision being the treatment of choice. In this study, electrogene therapy (EGT) as a new therapeutic approach to canine MCTs, was established.

Materials and methods. Eight dogs with a total of eleven cutaneous MCTs were treated with intratumoral EGT using DNA plasmid encoding human interleukin-12 (IL-12). The local response to the therapy was evaluated by repeated measurements of tumor size and histological examination of treated tumors. A possible systemic response was assessed by determination of IL-12 and interferon- γ (IFN-γ) in patients' sera. The occurence of side effects was monitored with weekly clinical examinations of treated animals and by performing basic bloodwork, consisting of the complete bloodcount and determination of selected biochemistry parameters.

Results. Intratumoral EGT with *IL-12* elicits significant reduction of treated tumors' size, ranging from 13% to 83% (median 50%) of the initial tumor volume. Additionally, a change in the histological structure of treated nodules was seen. There was a reduction in number of malignant mast cells and inflammatory cell infiltration of treated tumors. Systemic release of IL-12 in four patients was detected, without any noticeable local or systemic side effects.

Conclusions. These data suggest that intratumoral EGT with plasmid encoding IL-12 may be useful in the treatment of canine MCTs, exerting a local antitumor effect.

Key words: electroporation; electrotransfection; electrogene therapy; mast cell tumors; IL-12; IFN-γ

Introduction

Mast cell tumors (MCT) are the most common malignant cutaneous tumors in dogs, accounting for around 21% of all cutaneous tumors.[1] Cutaneous MCT have extremely variable biological behavior, from low-grade tumors to highly invasive lesions with high metastatic potential, which makes proper staging and treatment of MCT very challenging.

Treatment options for canine MCT depend on prognostic factors, primarily the histological grade of the tumor and clinical stage of the disease. The treatment of choice for MCT is wide surgical excision, when possible, which results in excellent prognosis for well-differentiated MCT.[1] Poorly differentiated or anaplastic MCT carry a poor prognosis and in these tumors aggressive surgical treatment should be followed by other treatment modalities, *e.g.* radio- or chemotherapy.[1] In dogs, where it is not possible to perform surgical excision and in cases with advanced stages of disease, systemic chemotherapy is the most appropriate treatment option.[1,2]

One of the newer therapeutic approaches for local tumor control is electrochemotherapy (ECT), which has already been established as a successful treatment option for different histological types of canine tumors, including MCT.[3] It employs intralesional or systemic injection of the chemotherapeutic agent bleomycin or cisplatin, followed by local delivery of electric pulses to the tumor nodule, which significantly increase uptake and cytotox-

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icity of chemotherapeutic drugs.[4-6] The procedure is based on electroporation of the cell membrane, achieving a transient increase in its permeability, thus allowing intracellular uptake of chemotherapeutic drugs from the extracellular space.[7]

The same principle can also be used for intracellular delivery of other molecules, for example plasmid DNA. Combining direct injection of plasmid DNA containing a therapeutic gene into target tissue, together with local delivery of electric pulses is called electrogene therapy (EGT).[8-10] In veterinary medicine, it has already been used for delivery of different transgenes into skeletal muscle[11-13] and intratumorally.[14,15]

IL-12 exhibits a range of biological activities, potentially important in immunotherapy of cancer. These include, for example, activation of natural killer cells, induction of IFN-γ, inhibition of angiogenesis and stimulation of nitric oxide production.[16] Gene therapy using *IL-12* has already shown remarkable antitumor activity in different tumor models at the preclinical level, and has already progressed to a number of clinical trials in both human and veterinary medicine. [17-21]

The aim of our study was to evaluate the local antitumor effect, systemic transgene release and possible side effects of EGT with the therapeutic plasmid encoding human IL-12 in canine MCT. For this purpose, a plasmid encoding human IL-12 was injected intratumorally into spontaneously occurring superficial nodules of MCT in 8 patients, followed by application of electric pulses. Local response to therapy was evaluated by regular measurements of tumor size and histological assessment of excised tumor nodules. Systemic transgene release was determined by measurements of IL-12 and IFN-γ in patients' sera. Possible side effects of the procedure were monitored by regular determination of selected hematology and biochemistry parameters.

Materials and methods

Animals

All animals in this study were referred to the Veterinary Faculty of Ljubljana in February and March 2006 for evaluation of cutaneous or subcutaneous tumor nodules. Eight patients that corresponded to inclusion criteria for the clinical study were included. The study cohort was comprised of 3 intact males and 5 spayed females of 6 different breeds (3 German boxers, and one of each: crossbreed, toy poodle, French bulldog, Siberian husky and bullterrier), their age ranging from 5 to 16 years (Table 1). Inclusion criteria for the study comprised at least one cytologically or histologically confirmed MCT, good general health status of the animal with the basic hematology and biochemistry profile within reference limits and normal renal and cardiovascular function. Animals included in the study were either ones which were planned for surgical excision of the tumor nodule as a part of standard therapeutic procedure and their owners agreed to the EGT procedure prior to surgery, or had recurrent disease in which other conventional therapy methods were already exhausted by previous treatments, or their owners refused any other type of standard treatment at the time of inclusion. Prior to inclusion, written consent for participation in the clinical study for each animal was obtained from their owners. The study was approved by the Ethical Committee at the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (approval No. 323-451/2004-9).

Clinical examination of each animal was performed before the treatment. Fine needle aspiration biopsy of tumor nodules, as well as of local lymph nodes, was taken and cytological examination of samples was performed. In all animals, staging was performed according to modified WHO staging criteria[22] with examination of thoracic radiographs, abdominal ultrasonography and basic bloodwork. Basic bloodwork consisted of a complete blood count with differential white blood cell count, which was performed using an automated laser hematology analyzer (Technicon H*1, Bayer, Germany) with species-specific software (H*1 Multi-Species V30 Software). The automated chemistry analyzer Technicon RA-XT (Bayer, Germany) was used for determination of the following biochemical parameters: blood urea nitrogen (BUN), creatinine, serum alkaline phosphatase (SAP) and alanine aminotransferase (ALT). Additionally, serum concentrations of human IL-12 and canine IFN-γ were determined using ELISA kits (Human IL-12 Quantikine ELISA Kit and Canine IFN-γ Quantikine ELISA Kit, respectively, both R&D Systems, Minneapolis, MN, USA).

A total of 11 tumor nodules were treated with EGT in the 8 patients which were included in the study. Two dogs received only one EGT, four dogs received 2 EGTs, with the second EGT session delivered one week after the first treatment, and one dog received 3 EGT sessions, each one week apart. In the remaining dog, four sessions were performed, each one month apart (Table 2).

In the patients, EGT was performed either as a single therapy (2/8 patients) or it was followed

Table 1. Summary of dogs' characteristics and histories

Pt. No.	Breed	Age (yrs)/ Gender	Body weight (kg)	Duration of clin. signs (months)	Tumor location	Clinical stage	Cytology	Histology	Previous treatment	Response to previous treatment	No. of tumor nodules
1	Toy poodle	16/FS	6	3	back	I	MCT	N/A	no		1
2	Boxer	7/M	42	4	scapula	I	MCT	N/A	no		1
3	Cross-breed	10/FS	16.5	12	hind leg	II	MCT	N/A	no		1
4	Boxer	10/FS	27	6	perineum, back	II	MCT	grade II	no		2
5	Boxer	10/M	35	2	scrotum, scapula	III	MCT	grade II	no		4
6	Bullterrier	5/FS	20	>12	hind leg	II	MCT	grade II	surgery 2x chemotherapy (vincristine, CCNU)	recurrence	2
7	Siberian husky	11/FS	18	< 1	fore leg	III	MCT	grade III	no		1
8	French bull-dog	7/M	12	4	multicentric	III	MCT	grade III	surgery chemotherapy (vincristine)	recurrence PD	>10

FS, spayed female; MCT, mast cell tumor; M, male; CCNU, lomustine; PD, progressive disease

Table 2. Details of EGT treatment and response to therapy

Pt No.	Nodule	Tumor volume before EGT (cm^3)	Tumor volume after EGT[1] (cm^3)	No. of sessions	Dose of pIL12 per EGT session (mg)	Dose of plasmid per body weight (mg/kg)[2]	Post EGT therapy	Follow up after 1st EGT (months)	Response at the end of follow up	
1	1	0.25	0.1	2	0.15/0.15	0.05	/	36	SD	Euth., not related to MCT
2	1	2.3	2.0	1	0.5	0.01	Surgery	24	CR	without recurrence
3	1	3.2	1.6	2	1.0/1.0	0.12	CCNU (4 cycles)	12	SD	stable disease
4	1	0.6	0.3	2	0.5/0.2	0.06	ECT	13	CR	without recurrence
	2	1.2	0.8	2	0.5/0.4		Surgery		CR	without recurrence
5	1	2.9	0.6	2	0.5/0.5	0.03	Surgery	10	CR	without recurrence
6	1	0.03	0.005	4	0.1/0.1/0.1/0.1	0.07	/	44	SD	stable disease
	2	0.27	0.08	4	0.25/0.25/0.25/0.25		/		SD	
7	1	25.4	18.0	3	1.0/0.6/0.6	0.12	Surgery	2	PD	Euth. due to PD
8	1	0.45	0.6	1	0.2	0.025	CCNU (3 cycles)	5	PD	Euth. due to PD
	2	0.03	0.03	1	0.1					

Euth, euthanasia; MCT, mast cell tumor; CR, complete response; CCNU, lomustine; ECT, electrochemotherapy; SD, stable disease; PD, progressive disease
[1] The smallest volume of tumor nodule, achieved 1 week after the last performed EGT session
[2] Cumulative dose of received plasmid per kg of body weight

with one of the following therapies: standard surgical removal of MCT nodules (4/8 patients), chemotherapy (2/8 patients) or ECT (one nodule in a patient with two MCTs) (Table 2). Treated tumors were surgically removed one week after the last EGT session. In patients with systemic chemotherapy, 3 and 4 cycles of CCNU (lomustine, 60-90 mg/m^2, every 3 weeks) were delivered, starting two weeks after the last EGT session. ECT was performed one week after the last EGT session with intratumoral application of cisplatin (Cisplatyl, Aventis, Paris, France) at a dose 1 mg/cm^3 of tumor tissue, followed by application of electric pulses (8 pulses of 100 μs duration at an amplitude of 1300 V/cm and frequency 1 Hz), using the same electric pulse generator as described below.

In animals in which histological samples of tumors were obtained either as part of the diagnostic procedure or with surgical excision after EGT, tumor samples were histologically evaluated. Histological grading was established using Patnaik's histological criteria.[22]

Plasmid preparation

The pORF-hIL-12 plasmid (InvivoGen, Toulouse, France), encoding human IL-12, was selected according to available data indicating that canine and human IL-12 share approximately 90% genetic identity based on amino acid sequence analysis.[23] It has already been shown that human IL-12 activates proliferation of canine peripheral blood mononuclear cells (PBMC) in an *in vitro* setting and triggers a number of immune responses in canine PBMC.[24] The plasmid was prepared using the Qiagen Endo-Free kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions and diluted to a concentration of 1 mg/ml. Purified plasmid DNA was subjected to quality control and quantity determinations, performed by agarose gel electrophoresis and by means of spectrophotometry.

Electrogene therapy procedure

EGT was performed in animals under general anesthesia, which was induced with propofol (Propoven 10 mg/ml, Fresenius Kabi Austria GmbH, Graz, Austria) and maintained with isoflurane (Forane, Abbott Laboratories LTD, Queensborough, United Kingdom). During the anesthesia, animals received Harmann's solution (B. Braun Melsungen AG, Melsungen, Germany) at a rate of 10 ml/kg of body weight/h.

In animals under general anesthesia, hair overlying tumor nodules was removed, carefully avoiding any unnecessary manipulation of tumors which could lead to degranulation of mast cells. Each nodule was measured in three perpendicular directions (a, b, c) and the tumor volume was calculated using the formula: $V = a \times b \times c \times \pi/6$. A sterile solution of plasmid was injected into the tumor with a 1 ml syringe and 22 G needle. The dose of intratumorally injected plasmid ranged from 0.5 to 1 mg/cm^3 of tumor tissue per one EGT session for tumors with volumes ranging from 0.1 cm^3 to 2.5 cm^3. In smaller tumors (< 0.1 cm^3) and in larger tumors (> 2.5 cm^3), an arbitrary dose per tumor nodule was set, being 0.1 mg for smaller and 1 mg for larger tumors (Table 2). Ten minutes after plasmid injection, electric pulses were delivered using the electric pulse generator Cliniporator™ (IGEA s.r.l., Carpi, Italy), using needle electrodes (2 arrays each composed of 4 electrodes with a 4-mm distance between them). One high voltage pulse was delivered (1 x 1200 V/cm, 100 μs), immediately followed by 8 low voltage pulses (8 x 50 ms, 140 V/cm, 2 Hz). After the electrogene procedure, all dogs received single intravenous application of carprofene (Rimadyl, Pfizer Animal Health, Dundee, United Kingdom; 4 mg/kg of bodyweight). When they fully recovered from anesthesia, they were released from hospital. Prior to release into the home environment, animals received Elizabethan collars in order to prevent any wound licking. Furthermore, treated tumor nodules were protected with suitable dressing to prevent any possible contact of humans or animals with the electroporated area.

Evaluation of response to therapy and possible side effects

Animals were examined one, two and four weeks after each EGT session and thereafter monthly. At each examination, a local as well as systemic response to the therapy was determined, along with observation for possible side effects.

The local response to therapy was evaluated with repeated measurements of tumor size as described above and calculation of tumor volumes. Additionally, in animals which underwent surgical excision of tumors, histological examination of tumor samples was performed. The systemic response to the treatment was assessed by determination of IL-12 and IFN-γ in patients' sera.

The possible occurrence of local or systemic side effects was evaluated at each follow- up with clinical examination of the animals and careful as-

sessment of the appearance of the electroporated area for any possible clinical signs, including erythema, oedema, pain, secretions, necrosis, etc. Furthermore, blood samples were taken for the same bloodwork as before the procedure.

Statistical analysis

Statistical analysis was performed using SigmaPlot software (Systat Software, Inc., Richmond CA, USA). To determine the significance of differences in tumor volumes of MCT before and after the treatment, a Friedman repeated Measures Analysis of Variance on Ranks was performed. Values of $P<0.05$ were considered significant.

Results

Response to the therapy

Before EGT, tumor volumes ranged from 0.03 to 25.4 cm^3. Treated nodules reached the smallest volume one to two weeks after the last EGT procedure (Table 2), with their volumes ranging from 0.005 to 18 cm^3, which was statistically significantly smaller compared to the volumes before EGT. One week after the last EGT session and before induction of any other therapeutic procedure, the tumor volume was reduced in 9/11 treated tumors, it had not changed in 1/11 treated tumors and progressed in 1/11 treated tumors. In nodules where reduction of tumor volume was achieved, it ranged from 13% and up to 83% of the initial value (median 50%).

In two patients (#1 and #6) with a total of three tumor nodules, EGT with *IL-12* was not followed by any other treatment (Table 2). In both patients, the tumor nodules reduced in size and treated patients responded to therapy with stable disease throughout the very long observation period (36 and 44 months).

Two patients (#3 and #8) received systemic chemotherapy with CCNU (Table 2). In one of these patients (#3) with stage II disease (regional lymph node involvement), stable disease with reduction in tumor volume by 50% was achieved with regression of detectable mast cell infiltration of lymph nodes and without any signs of distant metastases throughout the 12-month observation period. The second patient, (#8), treated with a combination of EGT and systemic chemotherapy had recurrent stage III disease, unresponsive to any treatment and was euthanized 5 months after EGT due to progress of the disease with systemic clinical signs.

In one patient (#4) with two tumor nodules, EGT in one nodule was followed by surgical removal of the tumor and in the other, due to its location in the perineum, EGT was followed by ECT, achieving a complete response in the treated nodule (Table 2).

In four patients (#2, #4, #5 and #7), surgical removal of 4 grade II and one grade III tumor nodules was performed one week post-EGT (Table 2). After surgery, three had a complete response to therapy without any signs of local recurrence or metastatic disease in over a 1-year observation period. The remaining one (#7), with stage III disease, had progression of clinical signs and was euthanized 2 months after EGT.

Histology of the tumors

All surgically removed tumors underwent histological examination. The control group represented MCT samples taken with a biopsy from the same tumor nodule during the diagnostic workup before inclusion into the clinical study or from untreated tumor nodules which were simultaneously removed in patients with multiple nodules.

Histological analysis of MCT prior to *IL-12* EGT showed nonencapsulated dermal and/or subcutaneous infiltrative growing masses composed of round cells arranged in densely packed cords. Most malignant mast cells were recognized in H&E stained slides by their cytoplasmic light gray-blue granules. Granules stained metachromatically with cationic dyes (toluidine blue staining). Most cells had a single nucleus, however same binucleated mast cells were also found. Among mast cell cords, variable numbers of diffusely distributed or aggregated eosinophils were seen. After the treatment, the distributions of viable malignant mast cells were reduced in comparison to the pretreatment samples (Figure 1A, B). Instead of mast cells in the dermis and subcutis, clusters of leukocyte infiltration were found. Large areas of mostly lymphocytes and plasma cells with eosinophilic cytoplasm and perinuclear halos were seen (Figure 1C, D). No similar infiltrates were found in samples of untreated lesions. Among immune cells, some degranulated mast cells were noticed without prominent neutrophils or eosinophils.

Hematology and systemic release of cytokines

In order to evaluate any possible systemic effects of the therapy, serum concentrations of human IL-12

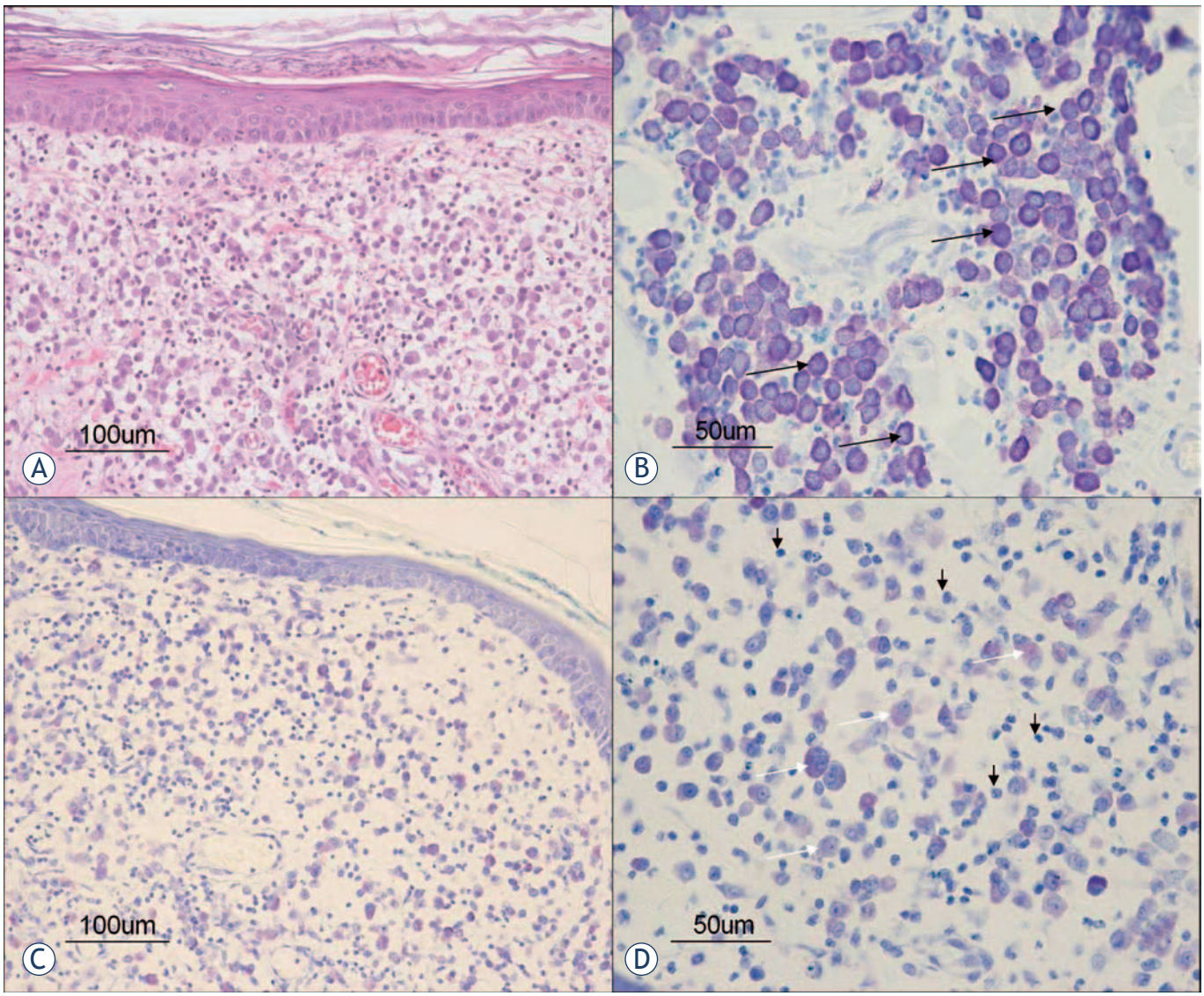

FIGURE 1. Histological pictures of MCTs before (A and B) and after (C and D) EGT. A. Tumor mast cells are loosely arranged in the dermis without epidermal invasion (haematoxylin and eosin staining). B. Tumor mast cells (arrow) have a well granulated metachromatic cytoplasm (toluidine blue staining). C. Decreased number of mast cells in the dermis after the treatement (toluidine blue staining). D. Note the degranulated tumor mast cells with metachromatically weakly stained cytoplasm (white arrows) intermingled with numerous inflammatory cells (black arrows) (toluidine blue staining)

and canine IFN-γ were measured at regular time-points after the procedure.

IL-12 was detected in 5 samples from 3 patients, with serum concentrations ranging from 1 to 12.2 pg/ml. IFN-γ was detected in 5 samples from 2 patients, with concentrations ranging from 123.0 to 815.6 pg/ml. IL-12 and/or IFN-γ were detected in a total of 4 patients. All positive samples were collected after the EGT procedure; in samples, taken before the procedure, neither of the cytokines was detected. The highest systemic concentrations of transgene products were detected in patient #3. In this dog, all 3 post-EGT samples were positive for both IL-12 (2.3 pg/ml to 12.2 pg/ml) and IFN-γ (170 pg/ml to 388.1 pg/ml) (Table 3).

In order to evaluate possible side effects of the procedure, clinical examinations of patients were carried out on a regular basis, with careful examination of the electroporated area and selected bloodwork.

We did not detect any evident side effects, either locally or systemically. All patients, which responded to treatment remained in good clinical condition throughout the observation period, without any additional clinical signs of disease. All analyzed blood parameters remained within

Table 3. Haematology and biochemistry profile of patinet No. 3 with the highest number of serum samples, positive to IL-12 and IFN-γ

		DAY 0	DAY 7	DAY 14	DAY 28	REFERENCE VALUES
Cytokine conc.	IL-12 (pg/ml)	0	2.3	6.1	12.2	N/A
	IFN-γ (pg/ml)	0	388.1	179.6	164.3	N/A
Haematology	WBC (x10^9/l)	4.75	6.14	5.98	6.3	6-17
	RBC (x 10^{12}/l)	5.41	5.38	8.1	7.97	5.5-8.5
	HCT (L/L)	0.38	0.36	0.58	0.57	0.37-0.55
	PLT (x 10^9/l)	287	283	303	224	200-500
Biochemistry panel	BUN (mmol/l)	3.28	4.2	3.94	N/D	2.5-9.6
	Crea (μmol/l)	74.54	75.7	71.23	N/D	44.2-132.6
	SAP (U/l)	47.3	27.5	23.8	47.1	20-156
	ALT (U/l)	21.8	64.0	58.6	217.6	21-102

WBC, white blood cells; RBC, red blood cells; HCT, haematocrit; PLT, platelets; BUN, blood urea nitrogen, Crea, creatinine; SAP, serum alkaline phosphatase; ALT, alanin aminotransferase; N/D, not detected

the normal reference range. The few alterations in bloodwork parameters which occurred were only minimal and clinically irrelevant (for example, mild haemoconcentration in two samples). In two patients, slight elevations of SAP and/or ALT were detected in samples, obtained 1 and 2 months after the procedure, but they were considered side effects of CCNU chemotherapy, which was started 2 weeks after EGT. One of these two patients was euthanized due to progressive disease, unresponsive to all therapies whilst in the other, serum concentrations of both SAP and ALT returned within the reference range after discontinuation of chemotherapy. Details of hematology and the biochemistry profile in patient # 3, in which the best systemic response to treatment with the highest number of serum samples positive to IL-12 and IFN-γ was achieved, are summarized in Table 3.

Discussion

Our study demonstrates that intratumoral EGT with *IL-12* in canine MCT elicits good local antitumor effects in treated animals without any noticeable side effects. Local antitumor effects of this therapy can be seen as significant reduction in tumor size (median reduction of the pretreatment tumor volumes was 50%) and change in histological structure with reduction in the number of malignant mast cells coupled with infiltration of inflammatory cells in treated tumors. We also demonstrated that systemic release of the transgene product is possible after intratumoral EGT.

EGT is a novel treatment in medicine which has already entered the clinical stage of research in human oncology[20], and is also gaining some interest in veterinary medicine.[11-15,25] The results of EGT with various therapeutic genes including plasmid encoding IL-12 in the treatment of tumors are promising.[21] In a recent human clinical study, EGT with *IL-12* plasmid in the treatment of melanoma patients showed local as well as systemic antitumor effects with regression of tumor nodules and with minimal systemic toxicity.[20]

In our study, we treated spontaneously occurring cutaneous nodules of MCT in eight canine patients utilizing locally applied EGT with a therapeutic gene encoding human IL-12. The majority of treated nodules regressed in size after the EGT procedure by 50% (median) around 1-2 weeks after the last EGT session. These results can be compared to published results by Rakhmilevich *et al.* on growth delay of murine P815 mastocytoma[26] after biobalistic *IL-12* gene therapy and Heinzerling *et al.*[17], who utilized a similar approach for treatment of melanoma in horses with direct intratumoral application of plasmid DNA encoding human IL-12 without subsequent electroporation. In the murine mastocytoma model, a 60% reduction in volume was achieved three weeks after therapy. In equine melanomas, the mean reduction in size was 69% of the initial volume. It is possible that in dogs more repetitions of treatment sessions would result in even more pronounced tumor regression, since in the murine MCT model 6 repetitions whilst in horses up to 3 repetitions of treatment were necessary to reach a significant reduction in tumor size.

Histological analysis revealed a noticeable change in tumor morphology after EGT with *IL-12*. Beside reduction in the number of malignant mast cells, the most prominent feature of treated tumors

was diffuse infiltration of tumor tissue with lymphocytes and plasma cells as well as degranulation of remaining mast cells. These histological changes are in accordance with other studies employing intratumoral *IL-12* gene therapy with different delivery systems, where intra- and peritumoral lymphocytic infiltration was found to be a major contribution to histological changes in treated nodules.[17,20,27] The importance of lymphocytic infiltration of treated tumors after intratumoral *IL-12* EGT was shown in a variety of preclinical studies.[27,28] It was established that this mode of therapy does not elicit any antitumor effect in athymic mice deficient in T cells in contrast to immunocompetent mice, indicating the vital role of T lymphocytes in the antitumor activity of local *IL-12* EGT.[27,28] In our study, intratumoral *IL-12* EGT resulted in an immunological response with lymphocytic infiltration of treated tumors, which can be further indication that plasmid encoding human IL-12 is biologically active in dogs *in vivo*, as it was proposed to be in *in vitro* settings.[24]

The importance of the systemic action of IL-12 after local delivery in addition to local intratumoral accumulation of IL-12 has already been shown, demonstrating that circulating IL-12 is responsible for systemic antitumor effects, *e.g.* an antitumor effect on distant untreated tumors and prevention of distant metastases.[29-32] Therefore, systemic release of the transgene product would be extremely favorable in clinical settings, expanding local antitumor therapy into systemic treatment. In our study, systemic release of human IL-12 was detected in only three patients. Even though at the preclinical level there is contradictory data on the possibility of systemic transgene release after intratumoral *IL-12* EGT, in two studies on induced transmissible veneral tumors in dogs[14,15], similar concentrations of human IL-12 as in our three patients were detected. Therefore, further investigations are warranted to determine conditions for achieving systemic effects of intratumoral EGT with *IL-12* in dogs, since some release of IL-12 from treated tumors is clearly possible.

In treated patients we paid attention to two possible groups of adverse side effects. *IL-12*-based intratumoral EGT could lead to degranulation of mast cells, causing histamine release from granules, which is one of the major concerns of any mechanical manipulation of MCT. It can result in either local side effects, demonstrated as peritumoral swelling, edema and erythema, or in systemic clinical signs, for example, gastrointestinal ulceration, or even life-threatening hypotension, arrhythmias and bronchospasm. We did not observe any of these side effects, even though moderate mechanical manipulation of tumors could not be avoided since penetrating needle electrodes, which had to be inserted intratumorally, were used for EGT.

The second group of possible side effects is connected to systemic IL-12 toxicity. It has been shown that systemic administration of recombinant protein IL-12 is associated with multiple serious adverse side effects, including renal and systemic toxicity. High-dose levels were also linked to temporary immune suppression, which would not be favorable for effective immunotherapy.[33] Local intratumoral *IL-12* EGT was associated with significantly less adverse effects, while exhibiting a pronounced antitumor effect, as demonstrated by Heller *et al.* on a mouse melanoma model.[34] Even so, monitoring renal function with selected laboratory parameters (*e.g.* serum concentrations of BUN, creatinine) was recommended in any IL-12-based therapy.[34] In our study, all monitored hematological and biochemical parameters in blood samples remained within reference limits throughout the whole observation period with only few transient clinically nonsignificant and nonspecific alterations, which could be attributed to other factors. The clinical status of all animals that responded to therapy remained unaltered and they didn't show any changes in appetite, water intake and general behavior.

In conclusion, the results of our study demonstrate that intratumoral *IL-12* EGT in canine MCT is a feasible, simple and safe therapeutic procedure, which exerts local transgene expression with systemic release of the encoded protein, making it a promising treatment for canine patients with MCT. However, further refinement for effective use of this method in treatment of MCT is needed, with emphasis on optimization of the treatment protocol, including determination of appropriate dosage of the plasmid used, as well as the best possible number of EGT repetitions and optimal time interval between them.

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