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RESEARCH ARTICLE

Polycation-Based DNA Complexes for Tumor-Targeted Gene Delivery *in vivo*

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Abstract

Background Efficient and target-specific *in vivo* gene delivery is a major challenge in gene therapy. Compared to cell culture application, *in vivo* gene delivery faces a variety of additional obstacles such as anatomical size constraints, interactions with biological fluids and extracellular matrix, and binding to a broad variety of non-target cell types.

Methods Polycation-based vectors, including adenovirus-enhanced transferrinfection (AVET) and transferrin-polyethylenimine (Tf-PEI), were tested for gene delivery into subcutaneously growing tumors after local and systemic application. DNA biodistribution and reporter gene expression was measured in the major organs and in the tumor.

Results Gene transfer after intratumoral application was 10–100 fold more efficient with Tf-PEI/DNA or AVET complexes in comparison to naked DNA. Targeted gene delivery into subcutaneously growing tumors after systemic application was achieved using electroneutral AVET complexes and sterically stabilized PEGylated Tf-PEI/DNA complexes, whereas application of positively charged polycation/DNA complexes resulted in predominant gene expression in the lungs and was associated by considerable toxicity.

Conclusion For systemic application, the physical and colloidal parameters of the transfection complexes, such as particle size, stability, and surface charge, determine DNA biodistribution, toxicity, and transfection efficacy. By controlling these parameters, DNA biodistribution and gene expression can be targeted to different organs. Copyright © 1999 John Wiley & Sons, Ltd.

Keywords gene therapy; tumor targeting; polyethylenimine; *in vivo* gene transfer

Introduction

A variety of non-viral vectors effective for gene transfer in cell culture have been developed [1–5]. However, efficient and target-specific *in vivo* gene delivery remains a major challenge. Compared to cell culture application, *in vivo* gene delivery faces a variety of additional obstacles such as anatomical size constraints, interactions with biological fluids and extracellular matrix, and binding to a broad variety of non-target cell types. Particle size, charge, and stability of the transfection complexes may become important factors determining circulation time, biodistribution and transfection efficacy *in vivo* [6–10]. For systemic applications, transfection complexes should be soluble, small enough to pass physiological barriers [11], specific for binding to the target cells but inert against both body fluids [12] and unspecific interactions with tissues and cells, such as the reticuloendothelial system (RES) [13,14]. In the present study we compared several vectors including adenovirus-

enhanced transferrinfection (AVET) [3] and transferrin-polyethylenimine (Tf-PEI) [15] for tumor-targeted gene delivery *in vivo* after local and systemic application.

Materials and methods

Chemicals, expression vectors, and cells

Polyethylenimine (PEI), molecular weight 800 kDa, was obtained from Fluka, Buchs (50% w/v). Synthesis of transferrin-PEI (800 kDa) conjugates has been described previously [15]. Methoxy-succinimidyl-proprionat-PEG (M-SPA-PEG, m.w. 5000 Da) was obtained from Shearwater Polymers, Inc., USA and dissolved just before use in DMSO at a concentration of 10 mg/ml. The plasmids pCMVL coding for the *Photinus pyralis* luciferase gene and pCMVβ coding for β-galactosidase, were purified as described [15]. The murine M-3 melanoma cell line (ATCC CCL 53.1) was cultured in HAM'sF10 medium/15% horse serum/5% FCS. Murine Neuro2A neuroblastoma cells (ATCC CCL 131) were cultured in RPMI 1640 medium /10% FCS.

Preparation of transfection complexes

Standard (Tf)PEI/DNA complexes: were prepared as described previously [15]. Briefly, indicated amounts of plasmid DNA were complexed with PEI (800 kDa) or Tf-PEI (molar ratio transferrin/PEI = 4/1) at indicated N/P ratios (N/P - molar ratio PEI nitrogen to DNA phosphate, e.g. N/P = 6: 7.5 µg (Tf-)PEI and 10 µg DNA). Complexes were mixed at a DNA concentration of 200 µg/ml in 0.3 × HBS or 0.5 × HBS (i.e. 75 mM NaCl, 10 mM HEPES pH = 7.4). These salt concentrations were chosen to generate medium-size particles. Small particles formed in water/glucose were found to have a lower *in vivo* [16] and *in vitro* [17] transfection efficacy. To ensure iso-osmolarity, glucose was added to a final concentration of 3.5% or 2.5% (w/v), respectively.

PEGylated TfPEI/DNA complexes: plasmid DNA (25 µg/ml) was complexed with Tf-PEI at indicated N/P ratios in 0.3 × HBS or 0.5 × HBS. After 30 min, MSPA-PEG was added to the complexes at a final PEG/PEI ratio of 10/1 (w/w) and incubated for 1 h. Glucose was added to a final concentration of 3.5% or 2.5% (w/v), respectively. The PEGylated TfPEI/DNA complexes were concentrated to a final DNA concentration of 200 µg/ml on VIVA-spin-4000 microconcentrators (Vivascience, Lincoln, U.K.) as described in detail in [16].

AVET complexes: were prepared as described previously [3] with the difference that complexes were mixed in water. Briefly, 5×10^{11} particles/200 µl of E4-defective, biotinylated and psoralen/UV-inactivated adenovirus (dl1014) were mixed with 10 µg/200 µl streptavidin-polylysine. After 30 min incubation 200 µg/200 µl plasmid DNA were added and mixed thoroughly. After further 30 min 200 µg/300 µl of transferrin-polylysine were added, mixed thoroughly and incubated again for 30 min. Finally, glucose was added to give a final concentration of 5% (w/v).

Intratumoral application

A/J mice or DBA/2 mice (female, 12 weeks) were injected subcutaneously with 1×10^6 Neuro2A neuroblastoma cells or 4×10^5 M3 melanoma cells, respectively. After two weeks, when the tumors had reached 6–8 mm in size, transfection complexes containing 20 µg DNA (pCMVL) complexed with 12 µg PEI or Tf-PEI (N/P = 4.8) were injected into the subcutaneously growing tumors (100 µl/tumor). After 24 h mice were sacrificed by cervical dislocation. Tumors were resected and homogenized in 250 mM TRIS-buffer, pH 7.5, using an IKA-Homogenizer, frozen in liquid nitrogen, and stored at −80°C. Luciferase activity in the tissue lysate was measured using a Lumat LB9501/16 instrument, Berthold, Bad Wildbad, Germany as described previously [15]. Luciferase background (300–400 RLU) was substracted from each value and transfection efficacy is expressed as RLU per tumor. One million RLU correspond to 2 ng luciferase. The average tumors used in this study correspond to approximately 8–9 mg protein extract as determined by a standard BioRad protein assay.

Determination of biodistribution of transfection complexes after systemic application

A/J mice were injected subcutaneously with 2×10^6 Neuro2a cells. After two weeks, when tumors had reached approximately 10–13 mm in size, transfection complexes were applied. AVET complexes (containing 50 µg DNA/250 µl), PEGylated TfPEI/DNA complexes (50 µg DNA/250 µl, N/P = 6), or standard TfPEI/DNA complexes (25 µg DNA/250 µl, N/P = 6) were injected into the tail vein. At 24 h after application animals were sacrified and the indicated tissues were resected, frozen in liquid nitrogen, and stored at −80°C.

Isolation of DNA was performed according to the QIAamp® Tissue Kit protocol (Qiagen Cat. No. 29304). Southern blots were hybridised with a probe generated from plasmid pCMVL and washed as described in the DIG Labeling and Detection Kit (Boehringer Mannheim, Cat. No. 1585614). Immunological detection was done with Vistra ECF Substrate (Amersham RPN5785) that can be detected in a Phosphor Imager (Molecular Dynamics).

Determination of gene expression after systemic application

AVET complexes (50 µgDNA/250 µl), PEG/DNA/TfPEI complexes (50–80 µg DNA/250–300 µl; N/P = 4.8, 6, or 7.2), standard DNA/TfPEI complexes (50–80 µg DNA/250–300 µl; N/P = 6), or naked DNA (80 µg/250 ml)

were injected into the tail vein of Neuro2a tumor-bearing A/J mice. Animals were sacrified at indicated time points. The indicated tissues were resected and homogenized, and luciferase activity was determined as described.

For immunohistological staining tumors were resected and snap-frozen in liquid nitrogen. Cryosections (thickness 7–10 μm) were prepared by means of a microtom (at −20°C).

β-Galactosidase assay and immunofluorescence staining

Microslides were stained for β-gal activity [15]. After 2 h incubation at 37°C, the slides were washed, counterstained with eosin, and embedded in DAKO® Faramount mounting medium.

For immunofluorescence staining acetone-fixed slides were blocked with PBS/1% BSA for 10 min, 10% goat serum for 15 min, and incubated with the primary antibody: a) anti-Luciferase (Firefly) [rabbit] polyclonal antibody, Europe Research Products, Cambridge U.K., RPCR2029RX, 1:200 diluted, incubation 1.5 h and anti-mouse macrophage F4/80 [rat], Serotec, Oxford, U.K., clone: Cl:A3-1 (F4/80), diluted 1:50, incubation 1 h or b) anti-β-gal [mouse] mAb, Promega, 1:200 diluted, incubation 1 h and anti-murine CD31 [rat] mAb-FITC labeled, Pharmingen, diluted 1:50, incubation 1 h.

After washing three times, slides were incubated with the secondary antibodies: a) anti-rabbit IgG FITC [goat], Rockland, Gilbertsville PA, 611-102-122, diluted 1:200, incubation 45 min, and anti-rat $F(ab)_2$ Texas red [goat], Cedarlane, Ontario, Canada, diluted 1:300, incubation 45 min, or b) anti-mouse IgG [goat] mAb-Cy3 labeled, Jackson, Nr. 115-165-068, incubation 40 min. Slides were washed three times, mounted with DAKO® Fluorescent mounting medium, and evaluated under a Zeiss Axioskop II fluorescence microscope equipped with a Hamamatsu CCD camera.

Results

Gene delivery after intratumoral application

Transfection complexes of PEI/DNA, Tf-PEI/DNA, naked DNA, or AVET complexes were injected directly into subcutaneously growing Neuro2a tumors. Transfection efficacy was estimated by using an expression plasmid coding for the luciferase reportergene (pCMVL). Naked DNA, which is known to have potential for gene delivery *in vivo* [18,19], was used for comparison and resulted in significant gene expression (Figure 1A). Tf-PEI/DNA and AVET showed a ten-fold higher efficacy. Intratumoral application into M-3 murine melanoma resulted in approximately 100-fold higher efficiency (data not shown).

To assess the distribution of gene expression in the tumor, luciferase expression was visualized by immunohistofluorescence. A non-uniform distribution of luciferase-expressing cells within the tumor was found (Figure 1B). Histology indicated that the transfected cells were tumor cells. Double-staining against luciferase and the macrophage marker F4/80 revealed infiltration of macrophages into transfected areas (Figure 1C).

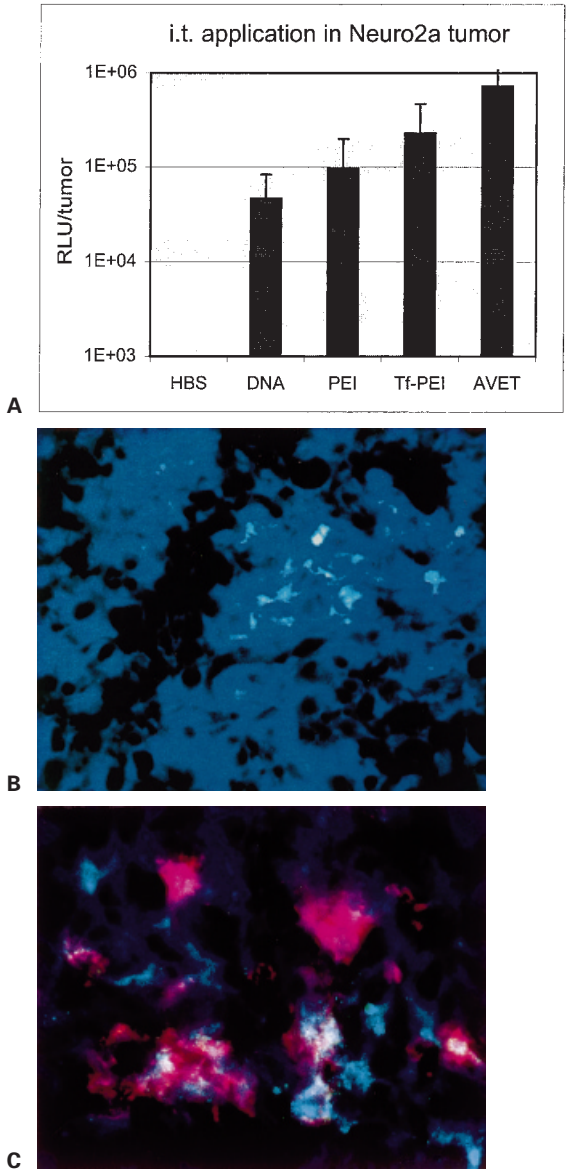

Figure 1. Gene expression after intratumoral application. Transfection complexes containing luciferase expression plasmid pCMVL: PEI/DNA, Tf-PEI/DNA, AVET complexes, or naked DNA were injected into subcutaneously growing Neuro2a neuroblastoma in A/J mice. Transgene expression at 24 h after application was evaluated by luciferase assay (A) or by histoimmunofluorescence staining (B,C). A: Luciferase activity represents mean ± SEM of $n \geq 3$ animals for each group. B,C: Application of Tf-PEI/DNA (B) or AVET complexes (C). Staining for luciferase with [rabbit] anti-luciferase Ab and [goat] anti-rabbit IgG FITC (B) or double-staining for luciferase and for mouse macrophages with [rat] anti-F4/80 Ab and [goat] anti-rat F(ab')2 Texas Red (C). Magnification ×40 (B) and ×300 (C). The fluorescence images are superimposed on the autofluorescence image (dark-blue background) resulting in light blue (luciferase) or pink staining (macrophages)

Gene delivery after systemic application

The vectors were tested for systemic application in tumor-bearing mice. Transfection complexes were injected into the tail vein of mice with subcutaneously growing Neuro2a tumors two weeks after tumor cell injection. At this time the tumors have established vascularization. Luciferase activity in different organs was measured after 48 h (Figure 2). With naked DNA, considerable expression was found only in the tail, ie the injection site, but not in distant organs (Figure 2A). No systemic toxicity was observed after injection of naked DNA.

Systemic application of PEI/DNA and Tf-PEI/DNA complexes was hampered by particle aggregation at the required DNA concentration ($\geqslant$50 μg/250 μl). This problem was overcome to a large extent [17] by preparing complexes at higher positive charge (N/P$\geqslant$6) and lower ionic strength (0.3–0.5 × HBS). Dynamic laser light scattering (LLS) revealed average particle sizes of 330 ± 50 nm which theoretically should not pose problems to systemic application. However, application of PEI/DNA or Tf-PEI/DNA complexes resulted in severe acute toxicity with 50% of the mice dying (2/4 or 6/13, respectively) with clinical signs of acute lung embolism. Quantification of the distribution of plasmid DNA one h after injection of these complexes revealed high accumulation in the liver, lung, spleen, and kidney (data not shown). Gene expression in the surviving animals showed considerable expression in the lung and at the injection site (Figure 2B,C). Some very low expression was found in various other organs including the heart, liver, kidney, illeum, and, in the case of Tf-PEI/DNA, also in the tumor.

In contrast to Tf-PEI/DNA complexes, systemic application of AVET transfection complexes showed no lethality. And, notably, most of the gene expression was found in the tumor. In contrast, only marginal expression was found in the major organs (Figure 2D). No expression was found in brain or bone marrow (data not shown).

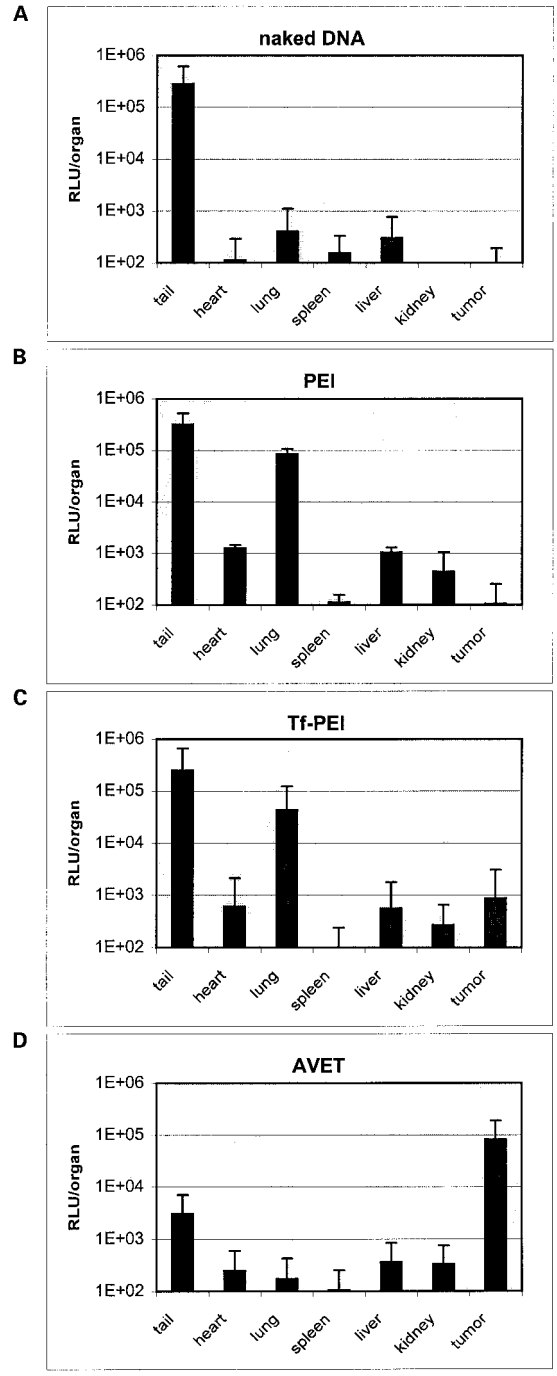

Figure 2. Gene expression after systemic gene delivery into tumor-bearing mice. Transfection complexes: naked DNA (pCMVL) (A), PEI/DNA (N/P = 6) (B), Tf-PEI/DNA (N/P = 6) (C), or AVET transfection complexes (D) were injected into the tail vein of Neuro2a tumor-bearing A/J mice. Transgene expression at 48 h after application was measured by luciferase assay. Luciferase activity represents mean ± SEM of $n = 6$, 2, 7, and 7 animals in (A), (B), (C), and (D), respectively. Note, there was high toxicity in (B) and (C) with 2/4 and 6/13 animals dying, respectively. Luciferase background levels of untreated animals are below 100 RLU/organ (not shown)

Measurement of particle size and surface charge of transfection complexes

Dynamic laser light scattering showed similar particle sizes for PEI/DNA, Tf-PEI/ DNA, and AVET complexes of approximately 300–400 nm. Zeta-potential measurement revealed a strong positive surface charge of +30 mV and +20 mV for PEI/DNA and Tf-PEI/DNA complexes (both at N/P = 6), respectively, whereas AVET complexes were found to be nearly neutral (see insert in Figure 3). The comparison of these data with the *in vivo* results suggested that the positive surface charge of the (Tf)-PEI/DNA complexes could be responsible for the toxicity. We hypothesized that the neutral charge of AVET particles is crucial for the improved behaviour of this system *in vivo*. For the (Tf)-PEI/DNA system, however, complexes formed at neutral charge were not stable in size. Even when prepared in water, these complexes were found to grow after coming into contact with physiological salt concentrations [16,17].

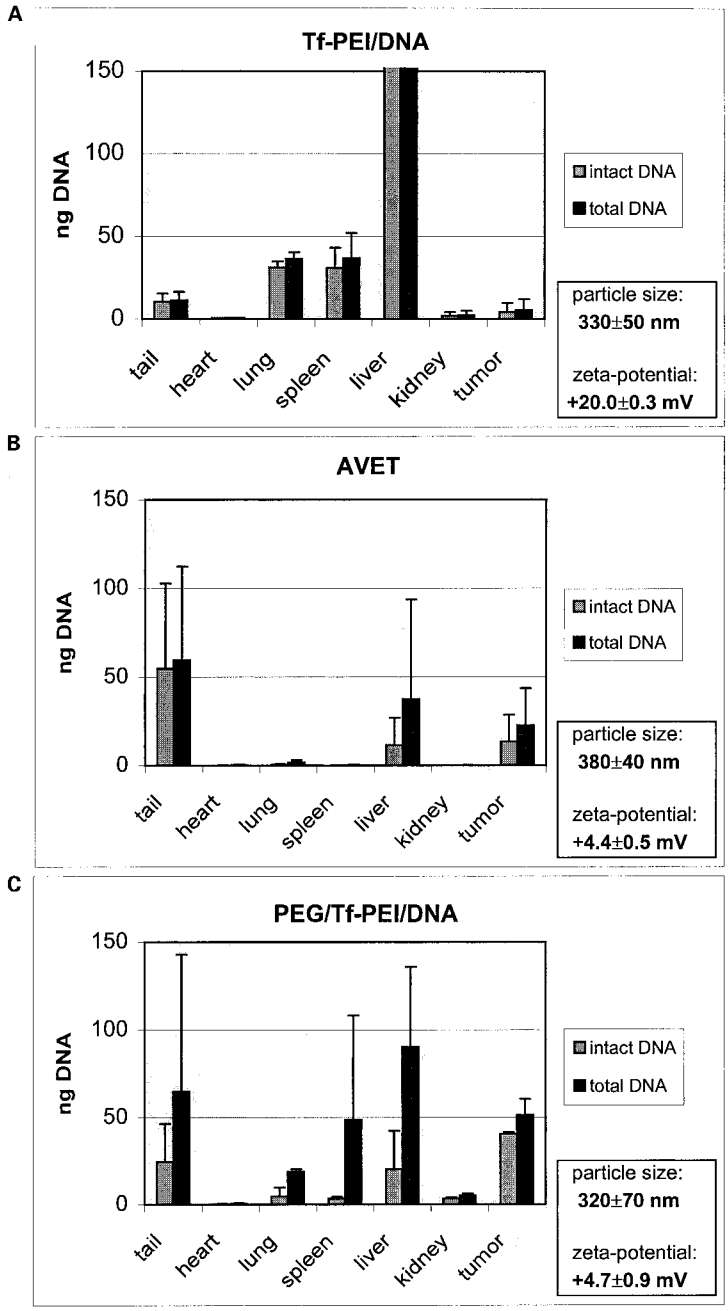

Figure 3. Physical parameters of transfection complexes and tissue distribution of plasmid DNA after systemic gene delivery. Transfection complexes: standard Tf-PEI/DNA (A), AVET complexes (B), or PEG/Tf-PEI/DNA complexes (C) were injected into the tail vein of Neuro2a tumor-bearing mice. DNA was isolated from tissues after 24 h. Amounts of intact and total plasmid DNA (mean ± SEM) in different organs are shown.Particle size of the transfection complexes (inserts) was determined by quasi-elastic (dynamic) laser light scattering as described [17] and zeta-potential (mean ± SEM) was measured using a ZetaPALS analyser (Brookhaven)

Steric stabilization of transfection complexes by PEGylation

Recently we showed that coating positively-charged Tf-PEI/DNA complexes with a shield of polyethylene glycol (PEG) stabilizes the complexes against salt-induced aggregation and interaction with plasma components [16]. Moreover, we found that after PEGylation DNA complexes can be concentrated on microconcentrators. This method makes it possible to prepare transfection complexes at low DNA concentration (where the formation of aggregates is low) and concentrate the PEGylated complexes up to the DNA concentration required. This method is particularly useful when complexes are prepared at low N/P ratios.

PEGylated Tf-PEI/DNA complexes with an average diameter of 320 nm and zeta-potential of +4.7 mV, unmodified Tf-PEI/DNA complexes, or AVET complexes were injected into the tail vein of tumor-bearing mice. The DNA biodistribution after 24 h was quantified (Figure 3). With unmodified Tf-PEI/DNA complexes, where severe toxicity was observed, high amounts of DNA

were found in the liver, lung, and spleen (Figure 3A). This pattern is in good agreement with other work [7,8,20,22] reporting on systemic application of DNA lipoplexes. PEGylation of the complexes changed the DNA distribution pattern resulting in decreased amounts in the lung, spleen and liver, and considerable levels of intact DNA in the tumor (Figure 3C). AVET complexes showed a DNA distribution pattern similar to the PEGylated complexes (Figure 3B).

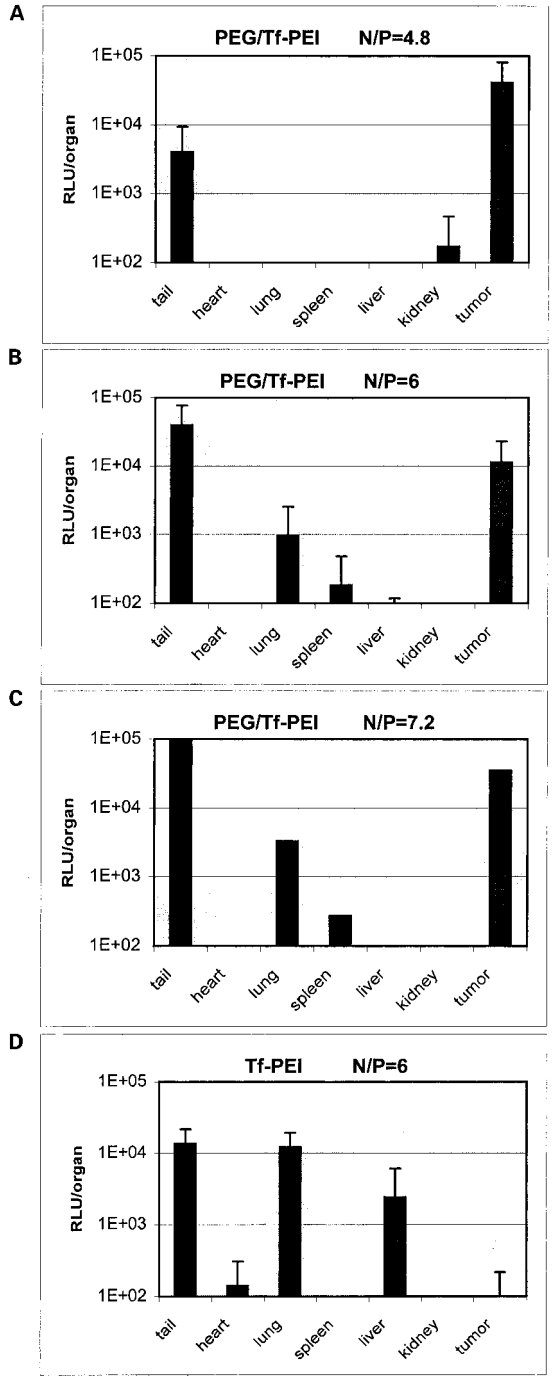

Figure 4. The effect of the N/P ratio of PEG/Tf-PEI/DNA complexes on systemic gene delivery. PEG/Tf-PEI/DNA complexes at different N/P ratios (A,B,C), or standard Tf-PEI/DNA (D) complexes were injected into the tail vein of Neuro2a tumor-bearing mice. Luciferase activity at 72 h after application is shown and represents mean ± SEM of $n = 3$, 6, or 4 animals in (A), (B), or (D), respectively. Note, there was high toxicity in (C) and (D) with 3/4 and 4/8 animals dying respectively

In further experiments, the luciferase expression as a function of the N/P ratio of the PEG/Tf-PEI/DNA complexes was studied (Figure 4). At lower ratios of N/P = 4.8 or 6, high gene expression was found in the tumor. Expression in the lung was significantly decreased, and the application was well tolerated by the animals with no toxicity observed (Figure 4A,B). With an increase in the N/P ratio the gene expression in the tail and in the lung increased while the expression in the tumor did not change (Figure 4B,C). At a high ratio of N/P = 7.2 application of the complexes was accompanied by high toxicity (Figure 4C). Compared to PEG/Tf-PEI/DNA complexes, PEG/PEI/DNA complexes had a slightly higher zeta-potential (at similar N/P ratios) [16]. Data on gene expression after systemic application of PEG/PEI/DNA complexes indicated a generally similar expression pattern with significantly decreased lung expression, however, expression in the tumor was approximately five-fold lower than the average tumor expression found with PEG/Tf-PEI/DNA complexes (data not shown). A control group injected with standard Tf-PEI/DNA complexes (N/P = 6) showed high toxicity and gene expression in the tail and lung (Figure 4D).

The results indicate that up to a certain extent the positive charge of the complexes can be shielded by PEGylation, resulting in a preferential gene expression in the tumor and good tolerability.

Kinetics of gene expression

Following systemic application of PEG/Tf-PEI/DNA and AVET complexes, luciferase expression was found for several days. With PEG/Tf-PEI/DNA complexes gene expression was maximal at days two and three (Figure 5A). Low expression was found at day four (data not shown). With AVET complexes expression was already high at 24 h after application gradually decreasing through day four (Figure 5B). Repeated applications of AVET complexes on two or three consecutive days (days one-two or one-two-three) were well tolerated and resulted in longer-lasting high gene expression (Figure 5C).

Histological distribution of gene expression in the tumor

To assess the spacial distribution of gene expression in the tumor, animals were injected i.v. with AVET complexes coding either for β-galactosidase (Figure 6A) or luciferase (Figure 6B,C). Expression analysis showed patches within the tumor with strong transgene activity. Only a few patches were found per section, however, and they usually consisted of multiple positive-staining cells. Frequently, patches of positive staining cells were found near to lacunas in the tumor mass which sometimes resembled primitive blood vessels (Figure 6A). However,

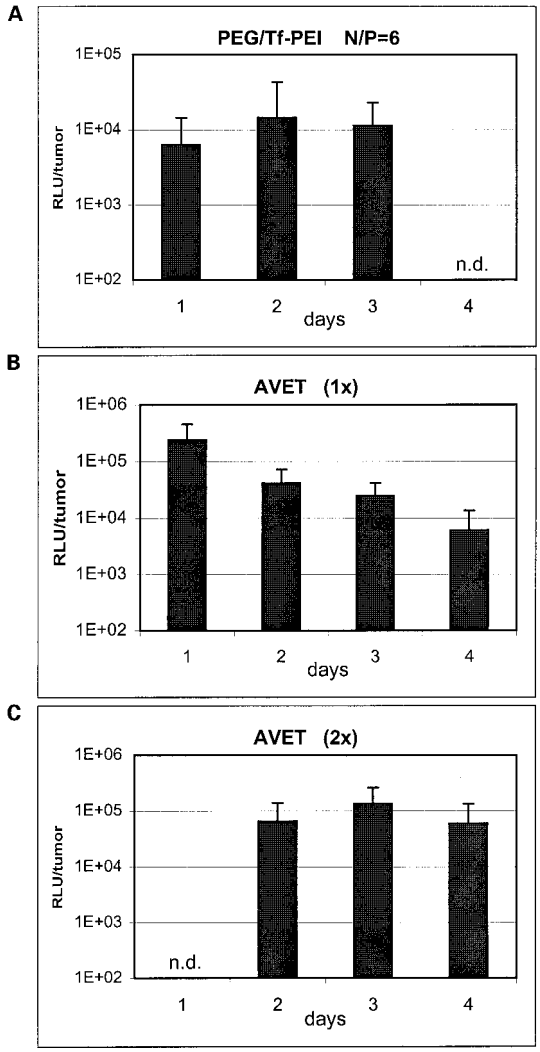

Figure 5. Kinetics of gene expression in the tumor after systemic application. PEG/Tf-PEI/DNA complexes (A), or AVET complexes (B,C) were injected into the tail vein of Neuro2a tumor-bearing mice. Complexes were applied once (A,B) or repeated at two consecutive days (C). Luciferase expression at indicated time points represents mean ± SEM of $n \geqslant 3$ animals for each time point of each group. N.d: not done

none of the positive staining cells was found to express the mouse endothelial marker CD31 as determined by immunohistochemical double-staining (data not shown). Macrophages were visualized via the F4/80 marker (Texas Red labeled) revealing infiltration of transfected areas by macrophages. In most cases the luciferase positive cells (FITC labeled) were F4/80 negative (Figure 6B). Gene expression was found equally distributed throughout the cytoplasm. These results together with the cell morphology indicated that the gene expressing cells are tumor cells. However, some cells were found with distinct luciferase-positive granulas (Figure 6C). These cells were found to be F4/80 positive indicating an uptake of the gene product or of parts of transfected cells by macrophages.

No positive staining was found in the control animals (i.e. no β-galactosidase activity after injection with pCMVL complexes, no luciferase staining with pCMVβ).

Discussion

Recently several reports have shown successful gene delivery after systemic application of transfection complexes with high gene expression in the lung and lower expression in a variety of organs including the heart, liver, and kidney [6–8,20–24]. The present report describes successful targeting of gene delivery to subcutaneously growing tumors via systemic application using AVET and PEGylated Tf-PEI/DNA transfection complexes. For systemic application the physical and colloidal parameters of the transfection complexes were found to determine biodistribution, transfection efficacy and toxicity. By controlling these parameters, DNA biodistribution and gene expression can be targeted to different organs.

The surface charge of the transfection complexes was found to be most critical for *in vivo* gene delivery. Systemic application of positively charged (Tf)-PEI/ DNA complexes resulted in considerable lung expression, but was also accompanied by severe systemic toxicity with up to 50% of the animals dying from lung embolism. Similarly, lung embolism has been shown after systemic application of cationic lipid/oligonucleotide complexes [9] and high toxicity has been reported for positively charged PEI(22 kDa)/DNA complexes [24].

Obstruction of the lung capillaries is not surprising considering that the lung provides the first capillary bed encountered by the transfection complexes after i.v. injection. The particle size of 300–400 nm of the (Tf)-PEI/ DNA complexes should not cause obstruction of the lung capillaries. Moreover, AVET complexes with similar size, but a nearly neutral charge did not cause lung embolism. On the other hand, the positive charge can be responsible for broad non-specific interaction with plasma components, extracellular matrix, and cell membranes [2,6–10, 22,25]. Positively charged complexes interact with the alternative complement system [12] leading to opsonization and clearance by the RES [7,13,14]. Furthermore, interaction of polycation/DNA complexes with plasma components such as coagulation factors [16] and salt-induced aggregation [16,17] are likely to lead to growth of the particles which finally are retained in the capillaries. This process may be enhanced by the avid binding of the positively charged complexes to cell membranes, e.g. binding to the lung capillary endothelium [6,20,22,26]. Our recent data indicate that binding of complexes to the negative surface of erythrocytes can result in erythrocyte aggregation [16] which could be an additional pathophysiological mechanism for the lung embolism.

Previous reports have demonstrated that coating of liposomes with hydrophilic molecules such as the ganglioside G_{M1} or polyethylene glycol (PEG) suppresses the uptake by the RES ('stealth liposomes') leading to an increased blood circulation time [14,27]. We have adopted this method to stabilize polycation/DNA complexes. PEGylation of Tf-PEI/DNA complexes was found to successfully suppress salt-induced complex aggrega-

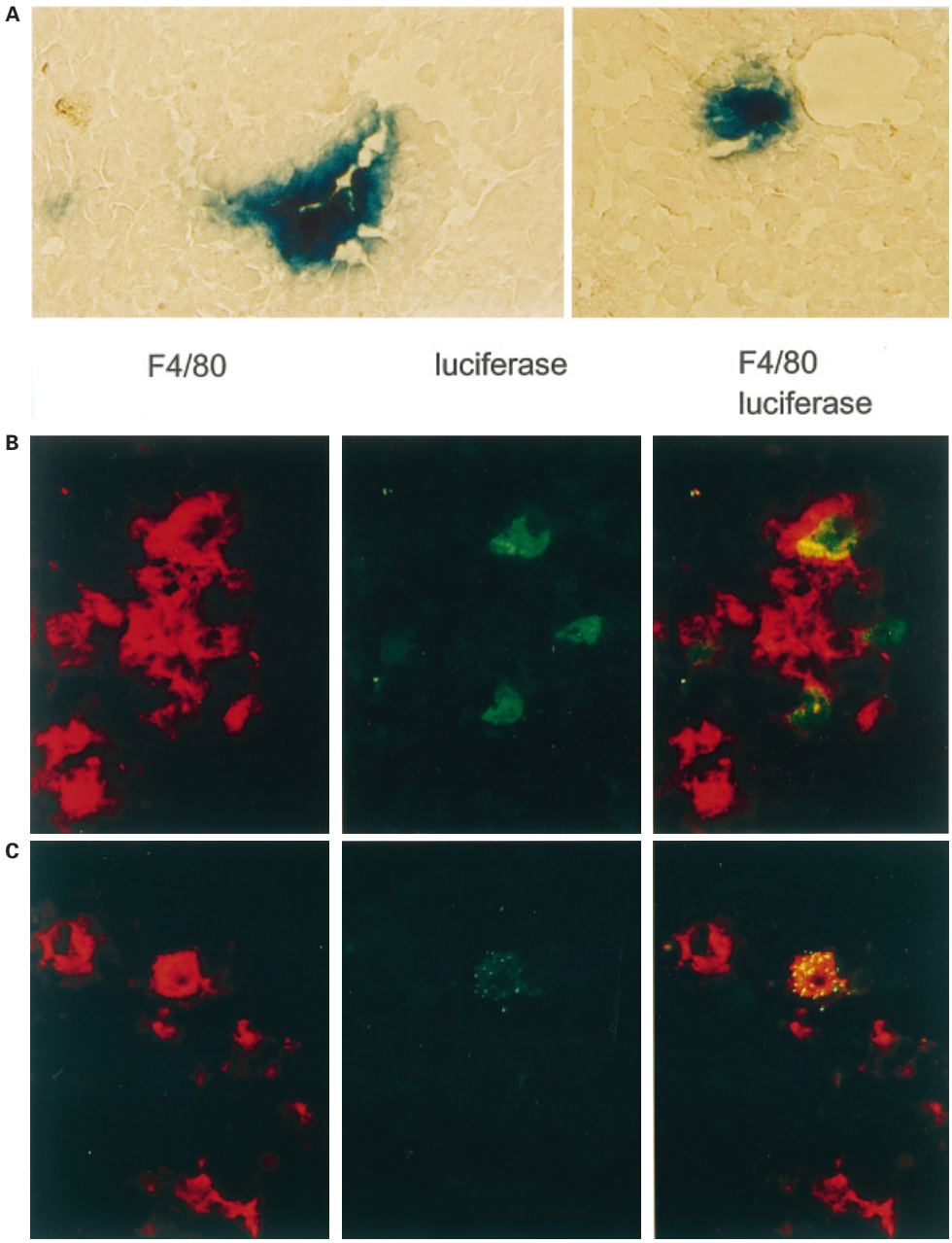

Figure 6. Immunohistological (X-gal) and immunofluorescence staining of the tumors after systemic application of AVET complexes. AVET complexes containing 50 µg pCMVL or pCMV*β* were injected into the tail vein of Neuro2a tumor-bearing mice. A: Gene expression in the tumor at 48 h after systemic application of AVET pCMV*β* complexes. X-gal staining (2 h incubation). (100× magnification) B,C: Gene expression in the tumor at 48 h after systemic application of AVET pCMVL complexes. Staining for macrophages by F4/80 (Texas Red, left), for luciferase (FITC, center), or both (right). (400× magnification)

tion, plasma interactions, and erythrocyte aggregation, resulting in a prolonged blood circulation [16]. As shown here, PEGylation changed the biodistribution of DNA resulting in decreased accumulation in the lung, decreased toxicity, and enhanced accumulation and preferential gene expression in the tumor. Similarly, AVET complexes targeted gene expression to the tumor. Common to both vectors is the nearly neutral surface charge. The targeting of gene delivery to the tumor is remarkable considering the fact that, different from the main organs, the subcutaneously growing tumors are not directly connected to the main blood vessels but rather are supplied via the peripheral vascular system.

Beside accumulation in the tumor, plasmid DNA was also detected in the liver and partially in the spleen. However, no transgene expression was found in these organs. Similarly, high DNA accumulation but low transgene expression in the liver have been reported for lipoplexes [7,8,20,22]. Probably, in organs such as spleen and liver, large or unstable particles are filtered out and taken up by macrophages or Kupffer cells, respectively, without gene expression [7,8,13,14,20,22].

The targeting of transgene expression to the tumor may result from a combination of several mechanisms. Firstly, when a sufficient circulation time of DNA complexes is ensured, 'passive targeting' might be the deciding

mechanism to target gene delivery from the circulation into the tumor. Sterically stabilized liposomes have been shown to passively accumulate in tumors [14]. The basis for this is the leakiness of the tumor vasculature in contrast to the low permeability of normal vasculature [28]. This enables extravasation and passive accumulation of particles in the tumor under the condition of a sufficiently long circulation time. Established vascularization of the tumor seems to be a prerequisite for efficient delivery. We observed that gene delivery was low in small tumors with poor vascularization (data not shown).

In a second step, after the complexes have arrived in the tumor, binding and uptake by the tumor cells is required for effective transfection. Increased binding and uptake of DNA by proliferating cells can play a role here. 'Active targeting' by receptor-mediated endocytosis [3,15] through the transferrin receptor [3,15] although not yet formally proven in the *in vivo* situation, may be involved.

An interesting question is the types of cells in the tumor which are transfected. Recent reports on the systemic application of cationic lipid/DNA complexes have indicated that frequently it is mainly the endothelium which is responsible for DNA uptake and gene expression measured in a variety of organs after systemic application [6,20,22,26,29]. In the present study using electroneutral AVET complexes, the transgene was found to be expressed by tumor cells and not the endothelial cells, although the gene expression was often found in the vicinity of lacunes or blood vessels pointing again to the importance of vascularization.

For future strategies the combination of passive and active targeting with a 'transcriptional targeting' by using tissue specific promotors [19,30] should enable a sufficient level of target specificity which is necessary for the therapeutic use of suicide genes or genes of immunostimulatory cytokines. Furthermore, the differences in the transfection efficacy between the completely synthetic Tf-PEI vector system and the AVET system, with the latter taking advantage of the endosomolytic activity of an inactivated adenovirus, indicate that beside targeting, subsequent steps such as endosomal release and nuclear transport are also important to get therapeutically efficient gene delivery vectors. In addition, different PEI core molecules may be used to further increase transfection efficacy [11,24,31].

Conclusion

Biophysical parameters of transfection complexes, such as particle size, stability, and surface charge, were found to determine DNA biodistribution, toxicity, and transfection efficacy following systemic application. By controlling these parameters, DNA biodistribution and gene expression can be targeted to different organs. Using electroneutral AVET complexes or sterically stabilized PEGylated Tf-PEI/DNA complexes, targeted gene delivery into subcutaneously growing tumors was achieved. Tissue targeting combined with improved control of the subsequent intracellular steps, such as endosomal release and nuclear entry, should finally result in therapeutically efficient gene delivery vectors.

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