

# Surface-shielded polycation-based systems targeting reporter and therapeutic genes to distant tumors

## Review Article

Ralf Kircheis<sup>1\*</sup>, Lionel Wightman<sup>1</sup>, Malgorzata Kursa<sup>1</sup>, Birgit Smrekar<sup>1</sup>, Elinborg Ostermann<sup>1</sup>, and Ernst Wagner<sup>1,2</sup>

<sup>1</sup>Boehringer Ingelheim Austria, Dr. Boehringer Gasse 5-11, A-1121 Vienna, Austria

<sup>2</sup>Current address: Pharmaceutical Biology - Biotechnology, Ludwig-Maximilians- Universität München, Butenandtstr. 5-13, D-81377 Munich, Germany

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\*Correspondence: Ralf Kircheis, MD, Ph.D., Cancer Vaccines & Gene Therapy, Boehringer Ingelheim Austria GmbH Dr. Boehringer-Gasse 5-11, A-1121 Vienna, Austria Tel.: +43-(1)-80105 2790, Fax: +43-(1)-80105 2683; e-mail: ralf.kircheis@vie.boehringer-ingelheim.com

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**Abbreviations:** polyethylene glycol, (PEG); polyethylenimine, (PEI); transferrin, (Tf); transferrin-polyethylenimine, (Tf-PEI); tumor necrosis factor, (TNF )

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## Summary

We have developed surface-shielded transferrin-polyethylenimine (Tf-PEI) - based gene delivery systems which are able to target gene expression to distant tumors after systemic application in murine models. For systemic in vivo application the biophysical parameters of transfection complexes, such as particle size, stability, surface charge, and modification with targeting ligand, were found to be critical for DNA biodistribution, toxicity, and gene transfer efficacy. Two major mechanisms may contribute to the tumor-specific targeting: active targeting via receptor-mediated cell binding and passive targeting via shielding of the surface charge of the complexes. Shielding reduces plasma protein and erythrocyte binding, resulting in prolonged blood circulation and extravasation of DNA complexes in areas of vascular leakiness of the tumor tissue. Shielding of surface charges can be achieved by coating polycation/DNA complexes with either polyethylene glycol (PEG) or by incorporating Tf ligand at high densities. Systemic application of surface-shielded transferrin-polyethylenimine-based DNA complexes coding for tumor necrosis factor (TNF ) localized gene expression to distant tumors, resulting in pronounced hemorrhagic tumor necrosis and inhibition of tumor growth. TNF activity was confined to the tumor without systemic TNF-related toxicity.

## I. Introduction

Gene therapy has become an attractive concept for a broad variety of biomedical applications. The potential of gene therapy for directing the expression of therapeutic genes to the target cells makes it particularly attractive for treatment of cancer. Multiple levels of target specificity are attainable: by exploiting specific delivery mechanisms to target the tumor (biochemical or physical targeting), specific intracellular characteristics of the target cells (e.g. preferential targeting of proliferating cells), controlled tissue-specific expression (transcriptional targeting), and

utilizing biological amplification mechanisms (e.g. during transcription / translation, bystander effects, triggering immune effector mechanism). However, the lack of effective and target-specific vectors is a major bottleneck for somatic gene therapy to date. Non-viral vectors are increasingly being utilized as gene delivery vehicles because of advantages such as stability, low cost, and high flexibility regarding the size of the transgene delivered. However, major limitations for non-viral gene delivery vectors include unspecific binding to non-target tissues, inefficient uptake into the target cells, limited release from

endosomes, and inefficient import into the nucleus of target cells.

Recently we have developed surface-shielded transferrin-polyethylenimine (Tf-PEI) - based gene delivery systems which are able to target gene expression to distant tumors after systemic application in murine models (Kircheis et al, 1999, 2001a,b). For target specificity cell-binding ligands, such as transferrin, EGF, or antibodies, can be coupled to polyethylenimine (PEI) (Kircheis et al, 1997; Zanta et al, 1997; Erbacher et al, 1999; Blessing et al, 2001), resulting in vectors that combine the intrinsic activities of PEI (Boussif et al, 1995) with specific receptor-mediated uptake mechanism (Wagner et al, 1994). Furthermore, DNA biodistribution and gene transfer efficacy after systemic application *in vivo* is determined by the biophysical parameters of transfection complexes, such as particle size, stability and surface charge (Kircheis et al, 1999, 2001a). Shielding of the transfection complexes from unspecific interaction combined with active targeting mechanisms resulted in specific uptake in tumors. Shielding the surface charge of transfection complexes was achieved by coating polycation/DNA complexes with polyethylene glycol (PEG) (Kircheis et al, 1999; Ogris et al, 1999) or by incorporating Tf ligand at high densities (Kircheis et al, 2001a).

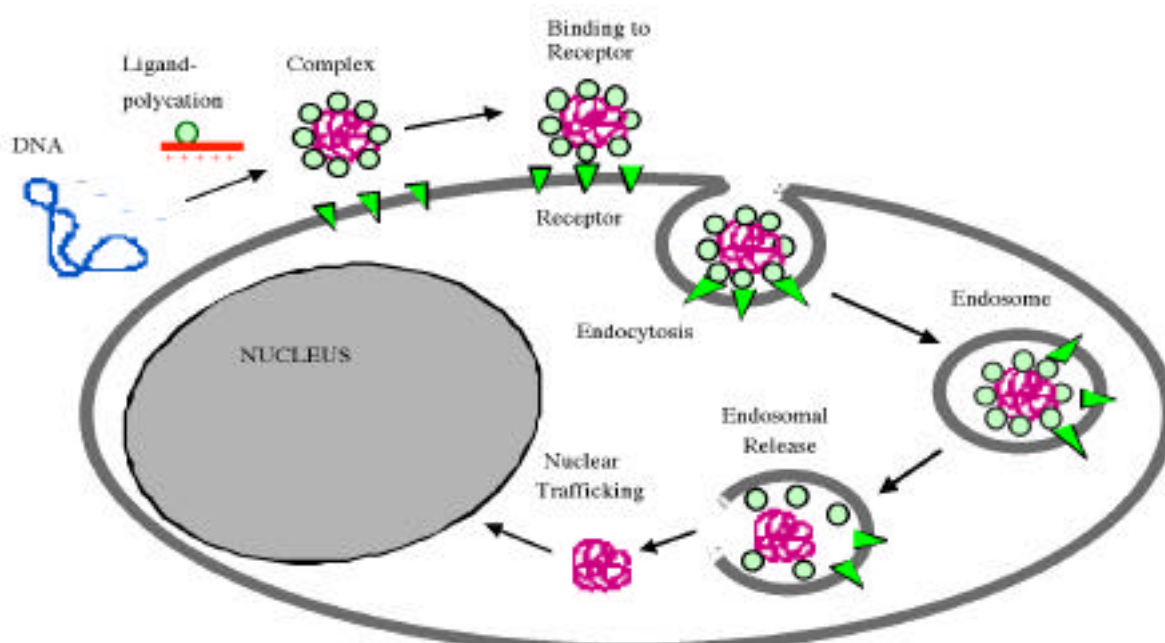
Surface-shielded transferrin-polyethylenimine-based gene delivery systems were used in syngeneic murine tumor models to deliver a therapeutic gene, coding for tumor necrosis factor (TNF). TNF is a highly potent pleiotropic cytokine, and is well known for its ability to

induce hemorrhagic tumor necrosis and tumor regression (Old, 1985). However, the clinical application of TNF is hampered by its high systemic toxicity (Beutler et al, 1985). In contrast, systemic application of surface-shielded Tf-PEI complexes with the TNF gene resulted in pronounced hemorrhagic tumor necrosis and inhibition of tumor growth without systemic TNF-related toxicity due to the localization of the activity of the cytokine to the tumor. These data indicate that targeted gene delivery to tumors may be an attractive strategy applicable to highly active, yet, toxic molecules in cancer treatment.

## II. Results

### A. Ligand-polycation based receptor-mediated gene transfer

Condensation of DNA by electrostatic interactions with polycations is being used to protect DNA from degradation by nucleases, resulting in formation of compact particles that can be taken up by the cells via natural processes such as adsorptive endocytosis or phagocytosis. Among the synthetic vectors, PEI shows particularly promising efficacy in transfection in cell culture as well as in a variety of applications *in vivo* (Boussif et al, 1995; Abdallah et al, 1996; Goula et al, 1998). Beside its DNA condensing activity PEI has an intrinsic endosomolytic activity mediating, by a 'proton sponge mechanism', the escape of DNA from the endosomal department (Boussif et al, 1995; Kichler et al, 2001).



**Figure 1.** Ligand-polycation based receptor-mediated gene transfer

To combine the high gene transfer efficacy of PEI/DNA complexes with the target-specific mechanism of receptor-mediated uptake, we have incorporated cell-binding ligands (Kircheis et al, 1997; Blessing et al, 2001), such as transferrin (Tf) or EGF into the complex by chemical coupling to PEI.

Binding of the Tf ligand-coated DNA complexes to the Tf receptor on the target cells, followed by endocytosis into vesicles, escape of the DNA from the endosomal compartment, and nuclear entry are critical steps for efficient transfection (**Figure 1**). The efficacy of these intracellular steps is influenced by the cell-binding ligand, the type of the polycationic carrier, and the physical characteristics such as the size of the transfection complex. Large particles (from several hundred nm up to  $\mu\text{m}$ ) generated at physiological salt concentrations were found to have higher transfection efficacy compared to small sized complexes ( $\sim 50\text{nm}$ ) formed in salt-free buffers (Ogris et al, 1998). Particle size is also dependent on the DNA (and polycation) concentration during complex formation and on polycation to DNA ratio. Compact particles of small size are usually obtained at higher polycation/DNA ratios (i.e. N/P ratios), resulting in complexes with a strong net positive charge, i.e. high zeta-potential. At neutrality polycation/DNA complexes have the tendency for particle aggregation. The requirement to have excessive positive charge for efficient DNA complexation, however, can cause major problems particularly for *in vivo* applications.

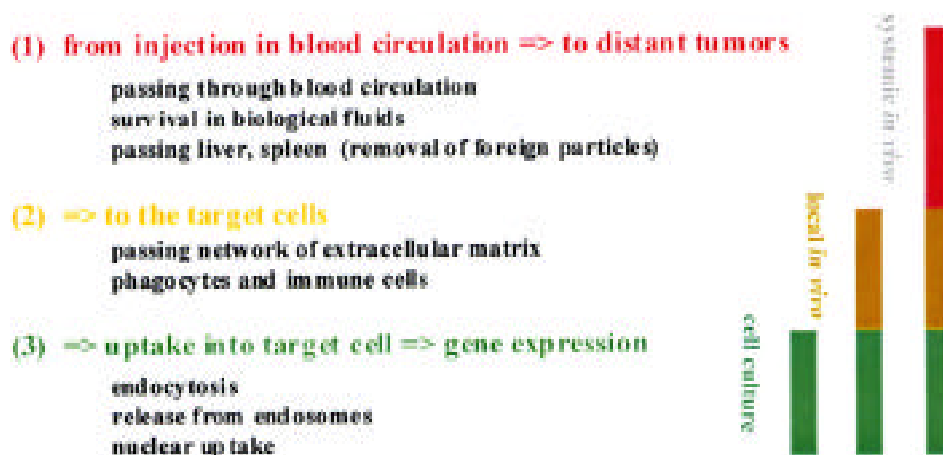
### B. Systemic application *in vivo*

Compared to cell culture applications gene delivery *in vivo* has to overcome a variety of additional problems. Passing blood circulation and organs such as liver and

spleen which are specialized in removal of foreign particles pose obstacles particularly for systemic application of transfection complexes. Furthermore, the particles have to be small enough to leave the vascular system and to diffuse through the tissues to reach their target (**Figure 2**).

Another major problem are unspecific interactions with blood components, extracellular matrix and non-target cells. Positively charged polycation/DNA complexes were found to aggregate in physiological salt, to interact with components of the coagulation and complement systems, and to cause aggregation of erythrocytes which can result in occlusion of capillaries e.g. in the lungs, leading to lung embolism (Plank et al, 1996; Ogris et al, 1999; Kircheis and Wagner, 2000). Furthermore, recognition and uptake by cells of the reticuloendothelial system will cause rapid removal from the circulation (**Figure 3**).

We have shown that unspecific interaction with plasma components or erythrocytes can be prevented by shielding the surface of transfection particles by covalent modification with PEG without affecting the target-specific transferrin ligand - receptor interactions (Kircheis et al, 1999; Ogris et al, 1999) (**Figure 4**). More recently we have employed an alternative strategy for masking the surface charge of DNA complexes. It was found that transferrin in the complex not only can serve as a cell-binding ligand but also mediate efficient shielding of the surface charge. In fact, incorporation of Tf at higher densities into the complex was shown to shield the positive surface charge of PEI/DNA complexes formed with low molecular weight PEIs (e.g. 25kDa, 22kDa) also in the absence of PEGylation (Kircheis et al, 2001a).



**Figure 2.** Multiple barriers for polycation/DNA complexes for targeted gene expression following systemic *in vivo* application

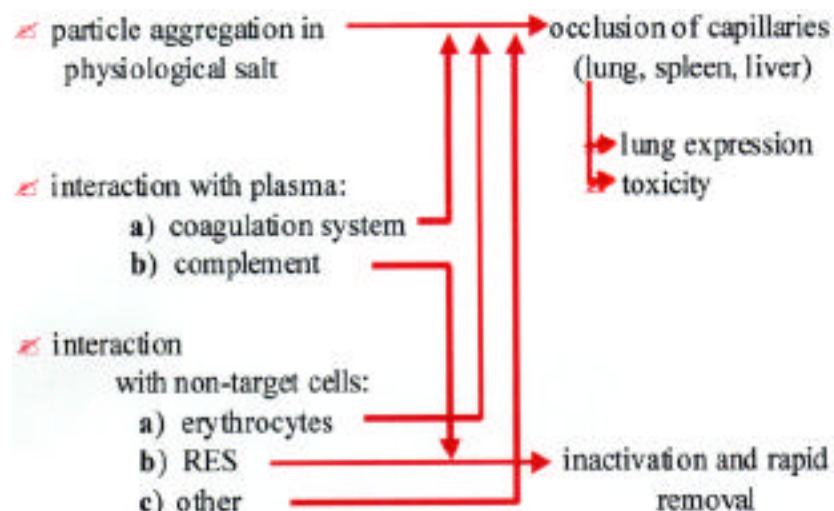


Figure 3. Unspecific interactions of positively charged polycation/DNA complexes

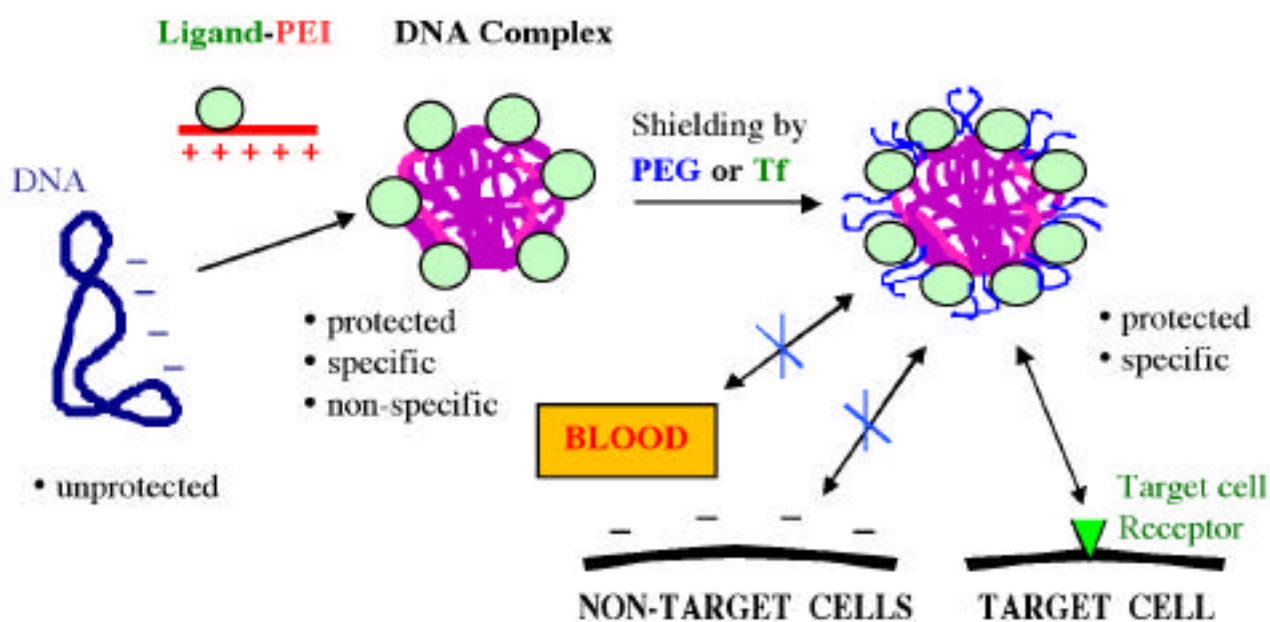


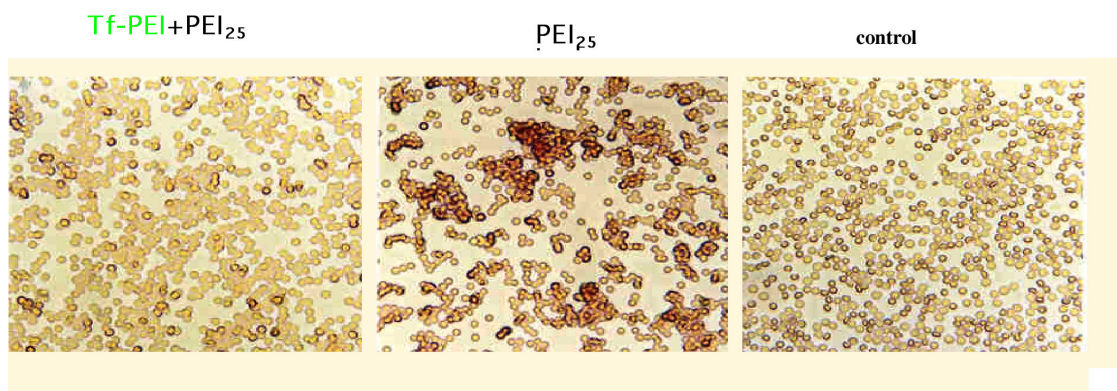
Figure 4. Shielding transfection complexes from non-specific interactions and incorporation of targeting mechanisms DNA is condensed into compact positively charged particles by excessive polycation. Incorporation of cell-binding ligands provides the possibility for specific binding to the target cells. At the same time also non-specific interactions with blood components and non-target cells are possible. Non-specific interactions can be blocked by shielding the surface of the transfection complexes either by covalent coupling of polyethylene glycol (PEG) or by incorporating the ligand transferrin at sufficiently high densities.

Shielding of the surface charge of PEI/DNA complexes, generating particles with a near neutral zeta-potential, blocks the aggregation of erythrocyte. In contrast, non-shielded complexes induce massive erythrocyte aggregation (**Figure 5**). Systemic application of non-shielded luciferase reporter gene complexes into tumor-bearing mice resulted in high gene expression in the lungs, lower expression in other organs, such as the heart and liver, but was often accompanied by considerable toxicity. Particularly when high molecular weight PEI800/DNA complexes were used approximately half of the animals died with signs of lung embolism (Kircheis et al, 1999). Complexes using the lower molecular weight PEIs (e.g. 25kDa or 22kDa) showed generally lower toxicity, particularly when the linear PEI22 was used (Goula et al, 1998; Wightman et al, 2001). With all PEIs lung expression was prominent with varying expression levels in other major organs or the tumor. In contrast Tf-PEI/DNA complexes shielded either by PEG or high density Tf resulted in preferable reporter gene expression in the tumor. Furthermore, expression in the lungs or in the other organs was dramatically reduced (**Figure 6**). Shielding of the transfection complexes from unspecific interactions was shown to lead to longer circulation times in the blood (Ogris et al, 1999) resulting in extravasation in areas of higher vascular permeability such as tumors (passive targeting) (Gerlowski and Jain, 1986; Kircheis and

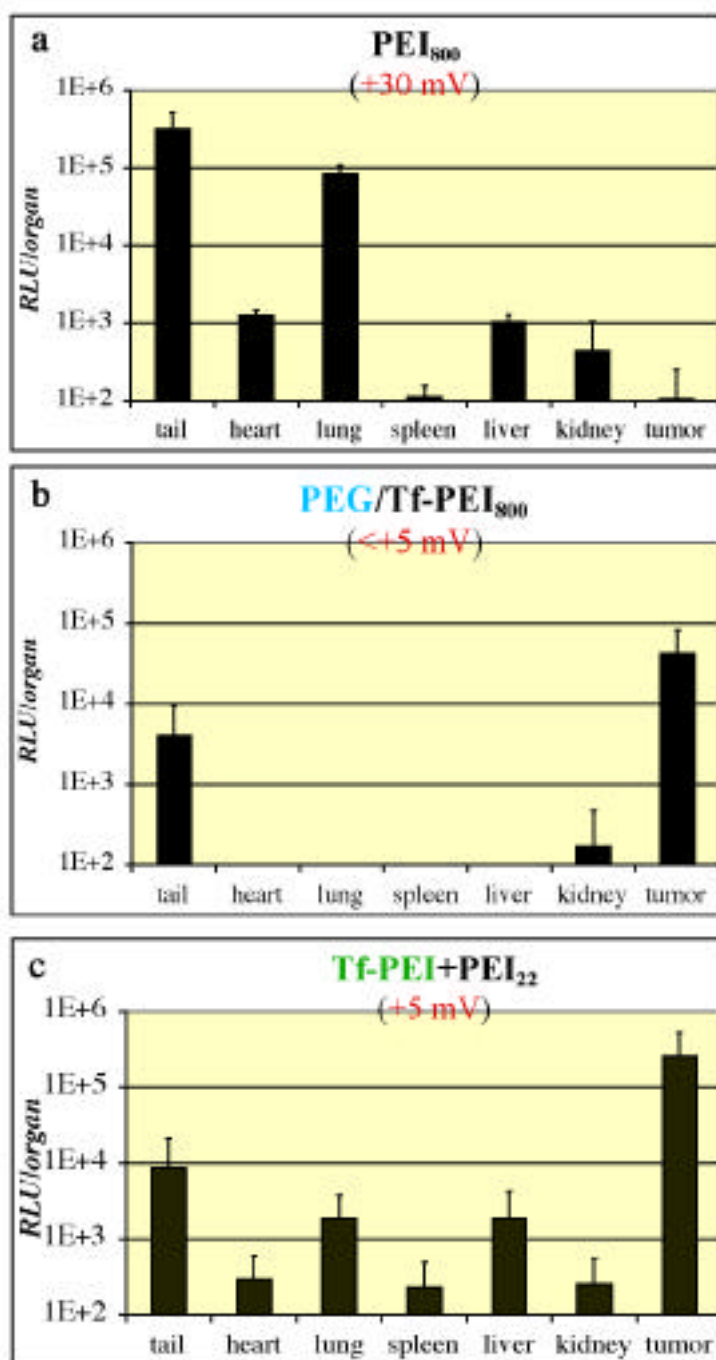
Wagner, 2000). Further studies on the biodistribution of transfection complexes showed a significant accumulation of shielded transfection complex in the tumor compared to non-shielded complexes (Kircheis et al, 1999; 2001b). Beside the passive targeting obviously also active targeting mechanisms such as enhanced uptake by Tf-receptor expressing (Wagner et al, 1994; Kircheis et al, 1997) and highly proliferating tumor cells (Brunner et al, 2000) are contributing to the preferential gene expression in the tumor.

### C. Tumor-targeted gene delivery of TNF

We were particularly interested in applying this tumor-targeted gene delivery system for delivering a highly active effector molecule TNF. TNF is a cytokine with pronounced antitumor activity (Old, 1985). It is known to act on a broad variety of cells, particularly damaging the vascular system of the tumor. The problem with conventional TNF protein therapy has been its high systemic toxicity (Beutler et al, 1985). Since both, antitumor activity and systemic toxicity seem to share common pathophysiological mechanisms, the only possibility to separate the antitumor activity from its systemic toxicity is to localize TNF activity to the tumor.



**Figure 5.** Shielding of the surface charge of transfection complexes blocks aggregation of erythrocytes. Non-shielded positively charged polycation/DNA complexes induce aggregation of erythrocytes. Shielding of the surface charge by incorporation of the ligand transferrin at high densities in the complex blocks the aggregation of erythrocytes.



**Figure 6. Reporter gene expression after systemic gene delivery into tumor-bearing mice.** Positively charged PEI/DNA complexes (a), or high density transferrin incorporation (b), or charge-shielded Tf-PEI/DNA complexes after PEGylation (c) were injected into the tail vein of A/J mice bearing subcutaneously growing Neuro2a tumors. Zeta potential of the complexes was measured using a Malvern Zetasizer and is shown in mV. Gene expression in the major organs and tumor was measured by luciferase assay 24 h after application. Mean values  $\pm$  SEM are shown.

Surface-shielded transfection complexes containing an expression plasmid coding for murine TNF were repeatedly applied systemically into the tail vein of BALB/c mice bearing subcutaneously growing MethA fibrosarcoma on their flank. After a few days the majority of the animals developed pronounced hemorrhagic tumor necrosis, which is one of the hallmarks of the antitumor activity of TNF (Figure 7). Moreover, hemorrhagic necrosis was focused specifically to the tumor, and no systemic toxicity was seen.

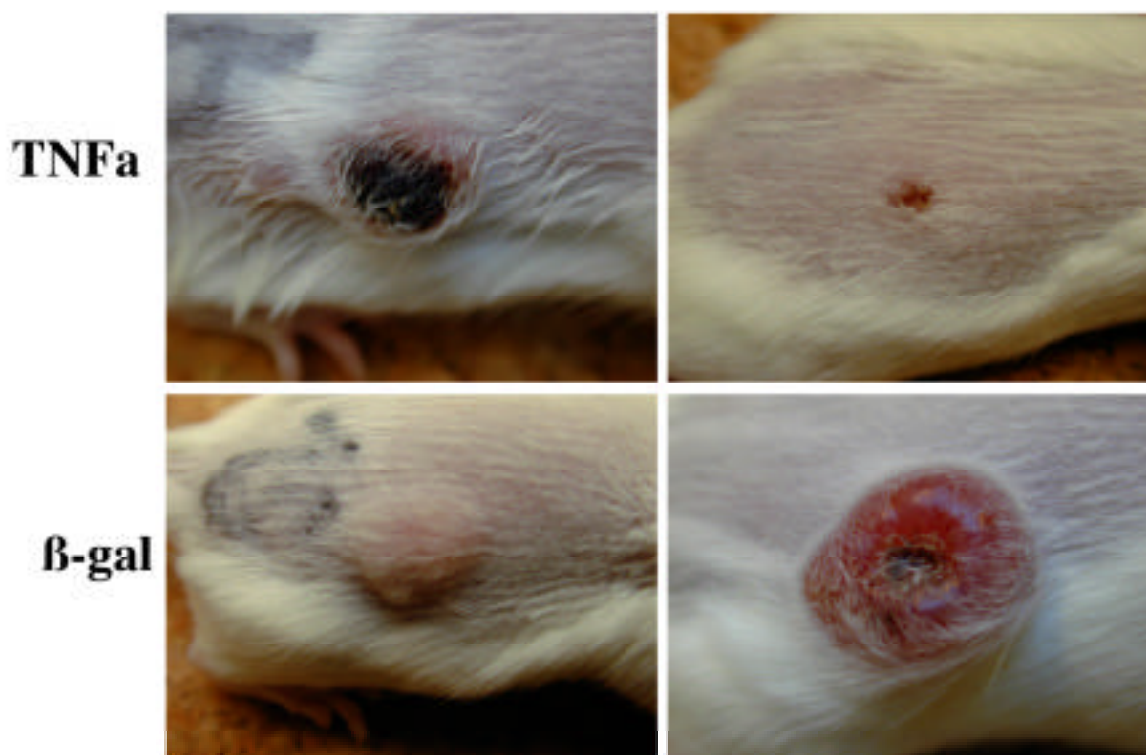
Induction of hemorrhagic tumor necrosis was associated by visible inhibition of tumor growth. In approximately 60% of the TNF treated animals finally a complete tumor regression was seen (Figure 7). These animals were also protected from subsequent tumor rechallenge. In untreated animals or control animals, which received similar transfection complexes containing

the  $\beta$ -galactosidase gene, no hemorrhagic necrosis and only occasionally spontaneous tumor regressions were observed. TNF-specific induction of hemorrhagic tumor necrosis was also demonstrated in another tumor model, the Neuro2a neuroblastoma. Surface-shielded transfection complexes coding for TNF were systemically applied into Neuro2a bearing A/J mice, resulting in significant TNF gene expression (as measured by ELISA) within the tumor, without detectable serum levels (data not shown). After one week of treatment 85% of the TNF treated animals developed hemorrhagic tumor necrosis while in animals without treatment or treated with similar transfection complexes containing the  $\beta$ -galactosidase reporter gene or the non-expressing pSP65 plasmid tumor necrosis was found only in 5%, 16%, or 12%, respectively. Induction of hemorrhagic tumor necrosis

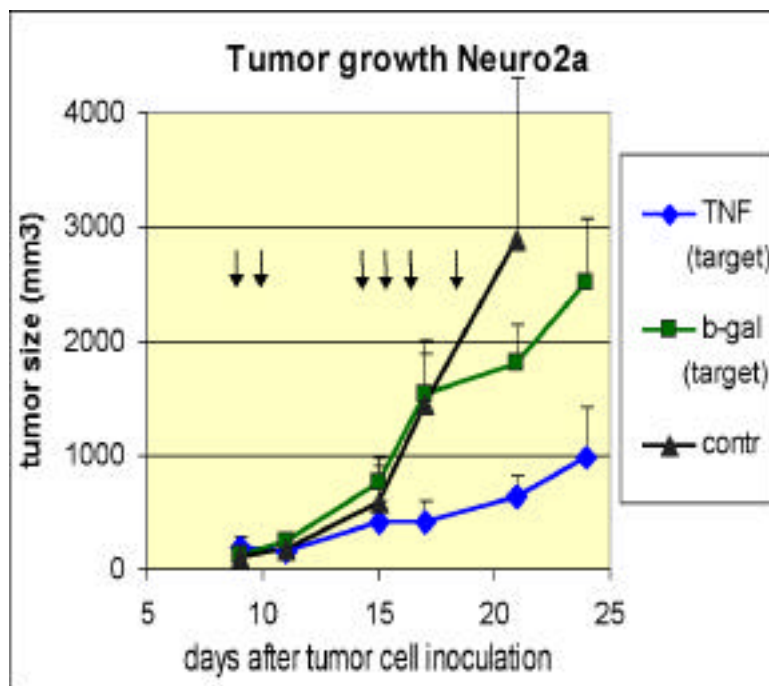
## Tumor-targeted Delivery of TNF $\alpha$ Gene

day 12

day 23



**Figure 7.** Tumor-targeted gene delivery of TNF leads to hemorrhagic tumor necrosis and tumor regression. Surface-shielded transfection complexes containing an expression plasmid coding for murine TNF were repeatedly applied systemically into the tail vein of BALB/c mice bearing subcutaneously growing MethA fibrosarcoma on their flank. 60% of the animals developed pronounced hemorrhagic tumor necrosis (upper left), no systemic toxicity was seen. Induction of hemorrhagic tumor necrosis was associated by inhibition of tumor growth resulting in more than half of the TNF treated animals in complete tumor regression (upper right). Control animals treated with similar transfection complexes containing the  $\beta$ -galactosidase gene did not develop hemorrhagic tumor necrosis (lower panel).



**Figure 8.** TNF specific induction of hemorrhagic tumor necrosis and inhibition in tumor growth in the Neuro2a tumor model. Surface-shielded transfection complexes coding for TNF were systemically applied into Neuro2a bearing A/J mice. After one week of treatment 85% of the TNF treated animals developed hemorrhagic tumor necrosis in contrast to animals treated with similar transfection complexes having the  $\beta$ -galactosidase reporter gene. Induction of hemorrhagic tumor necrosis resulted in a significant inhibition of tumor growth in the TNF treated animals as compared to the control groups. Mean values  $\pm$  SEM of groups of 6 animals are shown.

resulted in a significant inhibition of tumor growth in the TNF treated animals as compared to the control groups (Figure 8).

### III. Conclusions

A therapeutically applicable non-viral gene delivery vector should comprise a number of essential functions including condensation and protection of DNA, uptake into the target cells, and expression of the desired gene at the target site. Incorporation of cell-binding ligands, endosomal release enhancers, and nuclear localization signals can enable specific and efficient gene delivery and expression. For *in vivo* application the physical and colloidal parameters of transfection complexes, such as particle size, surface charge, and stability are critical factors which determine DNA biodistribution and gene expression. Knowing these parameters and their complex interplay will provide the basis for the rational design of gene delivery systems applicable for *in vivo* application. Finally, shielding transfection complexes from unspecific interactions, incorporation of active cell targeting mechanisms, combined with transcriptional targeting by using tissue-specific promoters or hypoxia-responsive

elements (Dachs et al, 1997) should ensure that the gene of interest is only expressed at the desired target site.

The presented data using reporter and therapeutic genes indicate that targeted gene delivery to tumors may be an attractive strategy applicable to highly active, yet toxic molecules such as TNF in cancer treatment.

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