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Transfection of human macrophages by lipoplexes via the combined use of transferrin and pH-sensitive peptides

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ORIGINAL ARTICLE

Transferrin lipoplex-mediated suicide gene therapy of oral squamous cell carcinoma in an immunocompetent murine model and mechanisms involved in the antitumoral response

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Suicide gene therapy has been used for the treatment of a variety of cancers. We reported previously the *in vitro* efficacy of the Herpes Simplex Virus Thymidine kinase (HSV-tk)/ganciclovir (GCV) system to mediate cytotoxicity in oral squamous cancer cells, using transferrin (Tf)-lipoplexes, prepared from cationic liposomes composed of 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP) and cholesterol. In the present study, we evaluated the antitumoral efficacy mediated by this lipoplex formulation in two suicide gene therapy strategies, HSV-tk/GCV and cytosine deaminase (CD)/5-fluorocytosine (5-FC), using a syngeneic, orthotopic murine model for head and neck squamous cell carcinoma. The cellular and molecular events associated with the antitumoral response elicited by both the therapeutic approaches were investigated by analyzing tumor cell death, tumor-infiltrating immune cells and tumor cytokine microenvironment. Significant tumor reduction was achieved upon intratumoral delivery of HSV-tk or CD genes mediated by Tf-lipoplexes, followed by intraperitoneal injection of GCV or 5-FC, respectively. Enhanced apoptosis, the recruitment of NK cells, CD4 and CD8 T-lymphocytes and an increase in the levels of several cytokines/chemokines were observed within the tumors. These observations suggest that suicide gene therapy with lipoplexes modifies the tumor microenvironment, and leads to the recruitment of immune effector cells that can act as adjuvants in reducing the tumor size.
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Keywords: suicide gene therapy; orthotopic murine model for HNSCC; Tf-lipoplexes; tumor cell apoptosis; activation of the immune system

Introduction

Head and neck squamous cell carcinoma (HNSCC) represents the sixth most common cancer in developed countries.[1] Despite recent advances in early detection and diagnosis, current treatments of oral cancer are unsatisfactory and the 5-year survival rate has not improved over the last two decades.[2,3] Strategies for cancer gene therapy include selective prodrug activation by 'suicide' genes, inhibition of activated oncogenes by antisense oligonucleotides, ribozymes or siRNAs, inhibition of angiogenesis and transfer of tumor suppressor and cytokine genes.[4,5]

The prodrug/suicide gene approach involves the delivery of a 'suicide' gene into cells, rendering them sensitive to a specific prodrug.[6] Among the different suicide gene therapy strategies, the Herpes Simplex Virus thymidine kinase (HSV-*tk*)/ganciclovir (GCV) and the bacterial cytosine deaminase (CD)/5-fluorocytosine (5-FC) are the most commonly used.[7] The delivery of the HSV-tk-expressing plasmid to target cells results in the expression of viral thymidine kinase, which selectively phosphorylates GCV. Monophosphorylated GCV is further phosphorylated by endogenous cellular kinases into an active triphosphate purine analog compound. This product is incorporated into cellular DNA, causing chain termination and cell death.[8,9] On the other hand, the CD gene encodes an enzyme which, when expressed in mammalian cells, is capable of converting the non-toxic antifungal agent 5-FC into the widely used chemotherapeutic drug

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5-fluorouracil. 5-FU is then processed to either 5-fluorouracil triphosphate, which is incorporated into RNA and interferes with RNA processing, or 5-fluoro-2-deoxyuridine-5-monophosphate, which irreversibly inhibits thymidylate synthase and interferes with DNA synthesis.[10]

One of the major limitations of gene therapy is the effective delivery of therapeutic genes into target cells. Although viral vectors have proven to be highly efficient in mediating gene expression, which resulted in their use in the majority of clinical trials, safety concerns with the use of viruses has dampened the enthusiasm for their application in the clinic. Therefore, research in gene therapy has been focused on the development of suitable non-viral carriers. Among such non-viral vectors, cationic liposome-DNA complexes (lipoplexes) emerged as promising systems because of their low toxicity and immunogenicity, lack of pathogenicity and versatility.[11,12] An increasing number of clinical trials using cationic liposomes have been reported, illustrating their potential as viable alternatives to viral vectors in gene therapy.[13]

A significant disadvantage of cationic liposomes is their low transfection efficiency as compared with viral vectors.[12,14,15] However, this drawback may be overcome by using suicide gene therapy, because with such an approach transfected tumor cells (effector cells) appear to be capable of inducing death of neighboring non-transfected cells, a phenomenon called local bystander effect. This effect was shown to result in extensive cytotoxicity and complete tumor eradication.[16] In this regard, suicide gene therapy appears as a strategy of choice for the use of non-viral vectors as the low gene transfer efficiency can be compensated by the local bystander effect. Moreover, the disadvantage of a transient gene expression mediated by cationic liposomes can be surpassed, because transgene long-term expression is not absolutely required, as it can be relayed by immune system activation.[17]

Aiming at a successful application of lipoplexes in gene therapy, several strategies have been explored which incorporate components from biological systems that have naturally evolved the capacity to surpass certain cellular barriers. These include association of targeting ligands,[18–20] fusogenic proteins[21,22] or peptides[23,24] and NLS[25,26] peptides. In this regard, we and others have demonstrated that association of human transferrin to cationic liposome/DNA complexes enhances transfection in a large variety of cells, including dividing[18,19,27] and non-dividing cells.[28–30]

As demonstrated in our recent work,[27] Tf-lipoplexes containing 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP)/cholesterol mediated the highest levels of transfection in oral cancer cells in culture, HSC-3 and SCC-7, when prepared at the 3/2 lipid/DNA (±) charge ratio. We have also reported that delivery of HSV-tk-expressing plasmid to these cells mediated by Tf-lipoplexes followed by ganciclovir treatment resulted in essentially 100% cytotoxicity. Cell death was shown to occur mainly by an apoptotic process and the observed cytotoxicity was attributed to the bystander effect taking place through diffusion of the toxic agent into neighboring cells through gap junctions. Moreover, our *in vivo* preliminary results indicated that Tf-lipoplexes containing DOTAP/cholesterol, prepared at the 3/2 charge ratio, were advantageous over naked plasmid DNA in reducing tumor growth upon intratumoral administration of the HSV-tk-expressing plasmid followed by intraperitoneal (i.p.) injection of ganciclovir in a syngeneic orthotopic murine model for HNSCC.[27]

In the present study, we explored different transfection conditions and further evaluated the potential of this lipoplex formulation in the suicide gene therapy approaches, HSV-tk/GCV and CD/5-FC, using the same animal model. In addition, the cellular and molecular events associated with the antitumoral response induced by both the therapeutic approaches were investigated by analyzing tumor cell death, tumor-infiltrating immune cells and tumor cytokine microenvironment.

Materials and methods

Chemicals

The cationic lipid DOTAP, and cholesterol (Chol), were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Iron-saturated human transferrin was obtained from Sigma (St Louis, MO, USA). The plasmid pCMVtk encodes the therapeutic gene thymidine kinase and was obtained from the National Gene Vector Laboratory at the University of Michigan (Ann Arbor, MI, USA). The plasmid pCMVSPORT-LacZ (Gibco BRL, Gaithersburg, MD, USA) encodes the reporter gene β-galactosidase and was used to determine transfection efficiency. The pCDβgeo plasmid encodes the therapeutic gene cytosine deaminase gene under the control of the CMV promoter.[31] Plasmids were produced using the Qiagen endo-free plasmid giga kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. GCV was a gift from the University Hospital of Coimbra. 5-FC was obtained from Roche (Fontenay Sous Bois, France).

Cells and animal model

SCC-7 murine oral squamous cell carcinoma cells (a gift of Dr B O'Malley and Dr D Li) were maintained at 37 °C, under 5% CO_2, in Dulbecco's modified Eagle's medium-high glucose (DMEM-HG) (Sigma) supplemented with 10% ($v\,v^{-1}$) heat-inactivated fetal bovine serum (FBS) (Sigma), penicillin ($100\,U\,ml^{-1}$), streptomycin ($100\,\mu g\,ml^{-1}$) and sodium bicarbonate ($1.6\,g\,l^{-1}$). For generation of oral tumors, SCC-7 cells were trypsinized, washed and resuspended in cold phosphate-buffered saline (PBS) (0.14 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4), to obtain a cell density of 2×10^6 cells ml^{-1}.

Experiments were performed using a syngeneic orthotopic murine model for HNSCC developed by O'Malley *et al*.[32,33] All the surgical procedures and the care given to the animals were in accordance with institutional guidelines. Six- to eight-week-old female C3H/HeOuJ mice (Charles River Laboratories, Barcelona, Spain) were anesthetized upon intramuscular injection of

a mixture of chlorpromazine (2 mg kg^{-1}) and ketamine (100 mg kg^{-1}). The tumor model was established by injecting 1×10^5 SCC-7 cells in a volume of 50 µl directly into the floor of the mouth, through the neck skin, using a 30 gauge needle.

Cationic liposomes and lipoplexes

Small unilamellar cationic liposomes were prepared by extrusion of multilamellar liposomes (MLV) composed of a 1:1 (mole ratio) mixture of DOTAP and cholesterol (Chol). Briefly, lipids (Avanti Polar Lipids, Alabaster, AL, USA) dissolved in $CHCl_3$ were mixed at the desired molar ratio and dried under vacuum in a rotatory evaporator. The dried lipid film was hydrated with deionized water to a final lipid concentration of 20 mM and the resulting multilamellar liposomes were then sonicated for 3 min, and extruded, 21 times, through two stacked polycarbonate filters of 50 nm pore diameter using a Liposofast device (Avestin, Toronto, Canada). Transferrin-associated lipoplexes (Tf-lipoplexes) were prepared by pre-incubating the liposomes (the amount required for the 3/2 lipid/DNA (±) charge ratio) with human transferrin (32 µg µg^{-1} of DNA) (Sigma) for 15 min, followed by a further 15 min incubation with plasmid DNA solution containing 40 µg of pCMVtk, pCDβgeo or pCMV.SPORT-βgal, at room temperature. Under these conditions, the added amount of plasmid DNA (40 µg) is completely complexed with transferrin-associated liposomes.

Transfection efficiency

Transfection efficiency was evaluated by scoring the percentage of tumor cells expressing β-galactosidase using X-gal as a colorogenic substrate.[19] For this purpose, 15 µm cryosections of tumors treated with PBS or Tf-lipoplexes were fixed for 15 min with 4% (v v^{-1}) paraformaldehyde in 4% sucrose and then washed two times with PBS for 5 min and stained with a solution containing X-gal (1 mg ml^{-1}). The samples were incubated at 37 °C for 24 h and analyzed under a phase contrast microscope (Leica DMIL, Leica Microsystems, Bannockburn, IL, USA) and photographed with a digital camera (Canon Powershot S-40, Canon, Tokyo, Japan). Alternatively, β-galactosidase expression was evaluated by immunohistochemical analysis. Six µm cryosections of tumors were sequentially incubated with 1% Triton (3×15 min), 10% FBS (30 min) and the monoclonal anti-β-gal antibody (1:5000, Molecular Probes) (30 min) at room temperature. Afterwards, slices were washed with 1% Triton (3×15 min), incubated for 1 h with a biotinylated secondary antibody (1:200, Amersham, Uppsala, Sweden), washed with 1% Triton (3×15 min) and then incubated for 1 h with the streptavidin–alkaline phosphatase complex (1:200, Sigma). After washing three times with TBS, and once with Tris-HCl, pH 9.5, the histochemical localization of β-gal was visualized with 5-bromo-4-chloro-3-indolyl-phosphate (Sigma) (3.4 ml ml^{-1} from a 50 mg ml^{-1} stock solution in 100% dimethylformamide) and nitroblue tetrazolium (Sigma) (4.5 ng ml^{-1} from a 70 mg ml^{-1} stock solution in 70% dimethylformamide), in staining buffer (15.8 g l^{-1} Tris-HCl, 5.84 g l^{-1} NaCl, 10.16 g l^{-1} $MgCl_2.6H_2O$, pH 9.5). Incubation was stopped by washing the sections with deionized water, which were subsequently dehydrated in graded ethanol and cleared in toluene. The slices were then mounted with Entellan (Merck) in cover slips and examined under a light microscope (Leica) and photographed with a digital camera (Canon).

Antitumoral activity

Five days after implantation of SCC-7 cells into the floor of the six- to eight-week-old female C3H/HeOuJ mice, the animals were anesthetized as described above, and the developed tumors were injected with 40 µg of plasmid DNA (pCMV.SPORT-Bgal, pCMVtk or pCDβgeo) in 50 µl of PBS, either naked or complexed with Tf-associated liposomes at a 3/2 (±) charge ratio (182 nmol of cationic lipid). The same volume of PBS was also injected into control animals. Four hours after, mice were injected daily through i.p. administration with GCV (50 mg kg^{-1}) or 5-FC (100 mg kg^{-1}) for the animals treated with pCMVtk or pCDβgeo, respectively. The mean tumor volume at the beginning of treatment was around 36 mm^3. Animals were observed daily with special attention to tumor size, which was measured in three dimensions with calipers.

TUNEL assay

DNA fragmentation as an index of apoptosis was detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) using a DeadEnd Colorimetric TUNEL Assay kit (Promega), according to the manufacturer's instructions. Briefly, 8 days after administration of the different treatments, animals were killed, tumors were removed. Six µm cryosections of tumors were fixed for 15 min in 4% (v v^{-1}) paraformaldehyde and then washed two times with PBS for 5 min. Sections were incubated with proteinase K (20 µg ml^{-1}) for 20 min at room temperature and further washed two times in PBS. Slices were further incubated for 10 min with equilibration buffer (200 mM potassium cacodylate (pH 6.6), 25 mM Tris-HCl (pH 6.6), 0.2 mM DTT, 0.25 mg ml^{-1} bovine serum albumin, 2.5 mM $CoCl_2$) prior to incubation for 1 h at 37 °C in a humidified chamber with dTdT reaction mixture prepared according to the manufacturer's instructions (biotinylated nucleotides, equilibration buffer and rTdT enzyme). The reaction was stopped by immersing the slices into a saline citrate solution (0.3 M NaCl, 0.67 M sodium citrate, pH 7.2). Endogenous peroxidases were blocked with 0.3% H_2O_2 solution for 5 min before incubation with streptavidin horseradish peroxidase (HRP) for 30 min at room temperature. For visualization, the sections were treated with 0.05% diaminobenzidin solution containing 0.01% H_2O_2. Samples were mounted with Dako medium (DAKO, Glostrup, Denmark) and TUNEL-positive apoptotic nuclei were observed by light microscopy under a Zeiss Axioscope microscope (Zeiss, Jena, Germany) and identified by the presence of dark brown staining.

Tumoral infiltration of T and NK cells

Six μm cryostat tumor sections were fixed with acetone, blocked using 10% FBS and incubated for 1 h with an FITC-labeled rat anti-mouse mAb against CD3 protein (Pharmingen, BD Biosciences, San José, USA), PE-labeled rat anti-mouse mAb against CD8 protein (Abd serotec, Oxford, UK) and Alexa Fluor® 647-labelled rat anti-mouse mAb against CD94 protein (NK cells) (Abd serotec). Unbound antibody was removed by washing with PBS. Sections were then observed by fluorescence microscopy using a Zeiss axioskop2 microscope and the number of immunostained cells was counted under a microscopic field at $\times 400$ magnification.

Detection of cytokines and chemokines in tumors

The presence of cytokines and chemokines in tumors was detected by FlowCytomix (Bender Medsystems, Austria), an ELISA-based flow cytometry assay, according to the manufacturer's instructions. Briefly, 8 days after administration of the different treatments, animals were killed, tumors were removed and homogenized in lysis buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl, 2 mM EDTA; 0.1% Triton X-100; 2 mM DTT, 0.1 mM PMSF and $1\,\mu g\,ml^{-1}$ of a protease inhibitor cocktail (Sigma)). The protein content of the lysates was measured by the Dc Protein Assay reagent (Biorad, Hercules, CA, USA) using bovine serum albumin as the standard and samples were diluted to obtain a protein concentration of $1\,mg\,ml^{-1}$. IL-10, IL-2, TNF-α, IFN-γ and RANTES standard solutions were prepared according to the manufacturer's instructions. The standard solutions and samples were directly added to cytometry tubes and incubated for 2 h in the dark at room temperature, with antibodies for each molecule associated to beads or biotin. These mixtures were then washed two times with assay buffer from the FlowCytomix kit by centrifugation at 200 *g* for 5 min and subsequently incubated, for 1 h in the dark at room temperature, with streptavidin-PE and finally washed two times with assay buffer from the FlowCytomix kit by centrifugation at 200 g for 5 min. IL-10, IL-2, TNF-α, IFN-γ and RANTES expression levels in tumor tissue were measured using a FACSCalibur System (Becton Dickinson), according to the manufacturer's instructions. Data were expressed as cytokine/chemokine concentration in $pg\,ml^{-1}$.

Statistical analysis

Data were analyzed using the Prism software (version 4.0). Statistically significant differences ($P<0.001$) between the experimental groups were determined by one-way analysis of variance (ANOVA) for cytokine and chemokine detection experiments and by two-way ANOVA for antitumoral activity experiments.

Results

In vivo *transfection efficiency of Tf-lipoplexes*

We have previously demonstrated the *in vitro* efficacy of the HSV-*tk*/GCV suicide gene therapy in oral squamous cancer cells using Tf-lipoplexes, prepared from cationic liposomes composed of DOTAP:Chol (1:1, mole ratio) and associated with transferrin.[27] We reported that transfection of human oral HSC-3 and murine oral SCC-7 cells was optimal when Tf-lipoplexes were prepared at a 3/2 (±) lipid/DNA charge ratio. Therefore, this formulation was selected for the present work. Before testing the antitumoral effect of these strategies in this animal model, we evaluated the efficiency of Tf-lipoplexes to mediate *in vivo* transfection of SCC-7 cells. Figure 1 shows the levels of β-galactosidase gene expression evaluated using X-gal as a colorigenic substrate (a) or by immunohistochemistry (b). Tf-lipoplexes were able to transfect SCC-7 cells *in vivo* when directly injected into tumors, although at a relatively low extent (approximately 10% transfection).

Antitumoral effect of HSV-tk/GCV suicide gene therapy

To investigate whether intratumoral delivery of the HSV-tk gene mediated by Tf-lipoplexes would result in tumor cell killing and size reduction upon GCV treatment, mice with an established tumor in the floor of the mouth were submitted to different treatments. As illustrated in Figure 2a, injection of DOTAP/Chol-Tf-tk was significantly more effective in delaying tumor progression than PBS or naked pCMVtk injections. At day 7 after treatment, a significant reduction in tumor size was observed in pCMVtk-treated animals when gene delivery was mediated by Tf-lipoplexes ($P<0.001$) as compared with PBS-treated mice or mice that received naked pCMVtk. However, a decrease in tumor size ($P<0.05$) was also observed upon intratumoral administration of DOTAP/Chol-Tf-β-gal or pCMVtk in the absence of GCV treatment (data not shown), although much less pronounced than that achieved upon injection of DOTAP/Chol-Tf-tk followed by GCV treatment.

Aiming at improving the therapeutic response in terms of tumor size reduction and ultimately its complete eradication, we tested the effect of repeated intratumoral administrations of Tf-lipoplexes containing 40 μg pCMVtk followed by GCV treatment for 14 days after the first gene delivery (Figure 2b). As observed, at day 14 after the first treatment, the tumor size was reduced by 3- to 5-fold in pCMVtk-treated animals when gene delivery was mediated by Tf-lipoplexes as compared with PBS-treated mice (control) and mice that have received naked pCMVtk ($P<0.001$). Figure 2b also shows that increasing the number of Tf-lipoplex injections did not improve the therapeutic effect. In fact, a single lipoplex injection was as effective as two or three injections.

It is important to emphasize that a significant increase in the survival of the mice treated with Tf-lipoplexes (carrying the therapeutic gene followed by GCV treatment) was observed when compared with that of controls (PBS- or GCV-treated animals). In fact, all GCV-treated mice died before the thirteenth day of treatment, and only one PBS-treated mouse survived during the whole treatment. On the other hand, all mice that received pCMVtk delivered by Tf-lipoplexes followed by GCV injection survived until the end of the treatment.

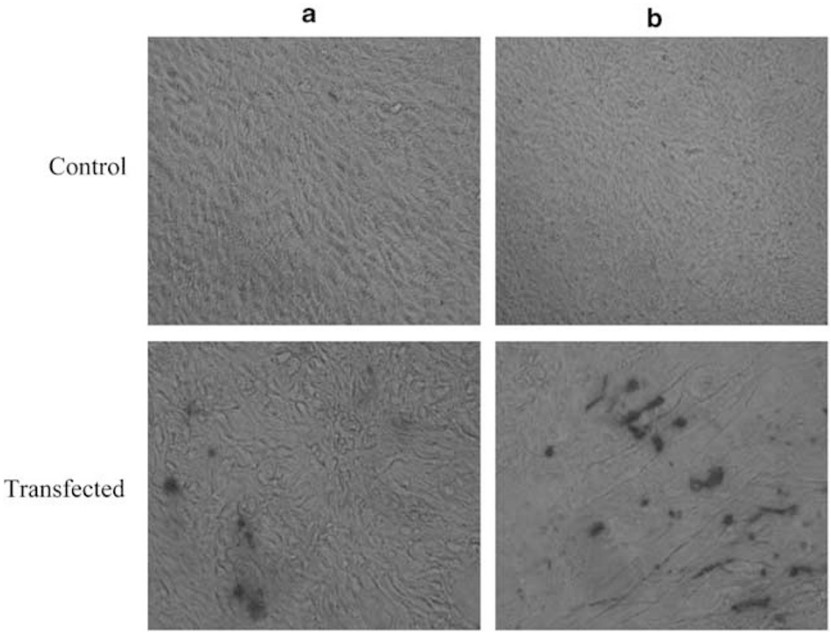

Figure 1 *In vivo* transfection efficiency of Tf-lipoplexes. SCC-7 cells were injected into the anterior tongue of the syngeneic mice C3H/HeOuJ. After 5 days, animals with established tumors were injected intratumorally with 50 μl of PBS (control) or lipoplexes prepared from transferrin-associated liposomes composed of DOTAP/Chol complexed with pCMV-SPORT-LacZ (40 μg) at a 3/2 (±) lipid/DNA charge ratio (transfected). The levels of β-galactosidase gene expression were evaluated by X-gal coloration (**a**) or immunohistochemistry (**b**), as described in 'Materials and methods'.

Comparison of the antitumoral effect mediated by HSV-tk/GCV and CD/5-FC therapies

The CD/5-FC suicide gene therapy has been widely reported to be effective in several tumor animal models.[34–37] Therefore, we investigated the therapeutic potential of CD expression mediated by Tf-lipoplexes followed by 5-FC treatment in tumors established in the syngeneic mice C3H/HeOuJ. The results illustrated in Figure 3 show that CD gene delivery was also highly effective in reducing tumor size when mediated by Tf-lipoplexes. There were no significant differences between the tumor sizes of CD/5-FC-treated animals and those treated with HSV-*tk*/GCV.

Tumor cell apoptosis mediated by HSV-tk/GCV and CD/5-FC therapies

CD/5-FC and HSV-*tk*/GCV suicide gene therapy systems have been reported to induce tumor cell death through apoptosis in various tumor models.[35,38–40] We have shown previously that human oral cancer cells in culture transfected with Tf-lipoplexes containing pCMVtk gene followed by GCV treatment died mainly by an apoptotic process.[27] In the present work, we investigated whether the tumor regression observed in the HNSCC murine model upon application of the different treatments was associated with tumor cell apoptosis. TUNEL staining has been used extensively to identify cells with nuclear DNA fragmentation and, therefore, this assay was selected for these studies. In the animals injected with PBS, cells were essentially negative for TUNEL staining (Figure 4). Although a few apoptotic cells could be detected in the animals injected intratumorally with naked pCMVtk followed by GCV treatment, or Tf-lipoplexes carrying pCMVtk but not treated with GCV, the number of apoptotic cells was very low, indicating the low toxicity of the lipoplexes. In contrast, a large number of apoptotic cells were observed in animals injected with Tf-lipoplexes carrying pCMVtk and treated with GCV. Similar results were obtained in parallel experiments using CD/5-FC suicide gene therapy (data not shown).

Tumor infiltration of NK and T cells mediated by HSV-tk/GCV and CD/5-FC therapies

As T-lymphocytes and natural killer cells (NK cells) contribute to the antitumoral responses induced by the immune system,[41] we evaluated the tumoral infiltration of T-lymphocytes (CD3- and CD4-positive cells) and NK cells following application of the two suicide strategies.

Figure 5 illustrates the results obtained for the extent of tumoral infiltration of T-lymphocytes and NK cells, as assessed by immunohistochemistry 8 days after treatment. An increased infiltration of NK cells, CD3- and CD4-T cells was observed in tumors from animals injected with Tf-lipoplexes carrying the suicide genes HSV-tk or CD and treated with GCV (DOTAP/Chol-Tf-tk) or 5-FC (data not shown), respectively, compared with intratumoral injection of naked DNA followed by drug treatment. Tumors from animals injected with control complexes containing the reporter gene β-galactosidase (DOTAP/Chol-Tf-β-gal) presented a more extensive cell infiltration than those from PBS-treated mice (control), but less extensive than that observed in tumors from

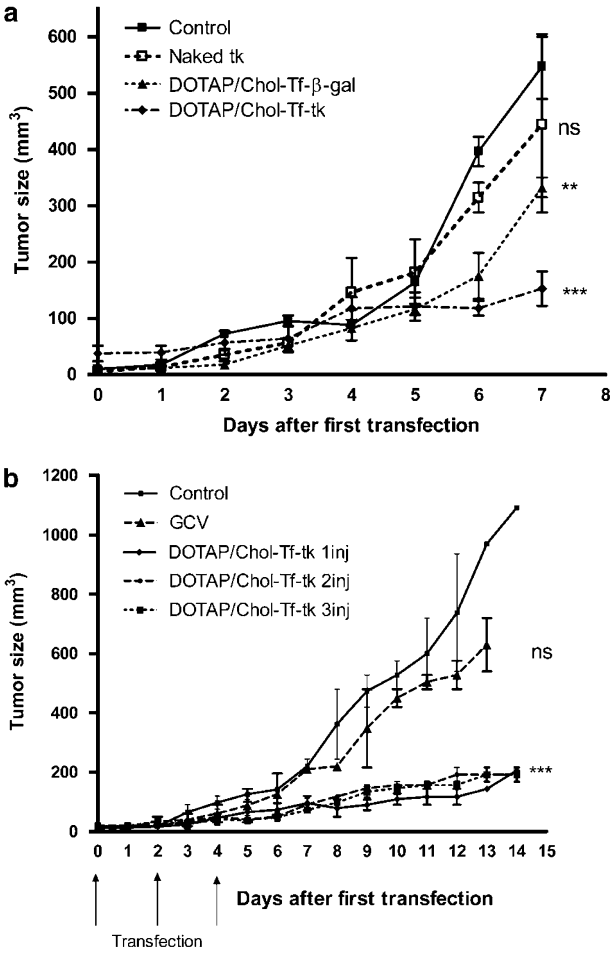

Figure 2 Antitumoral effect of HSV-tk expression followed by treatment with GCV in an orthotopic murine model for oral carcinoma. Induction of the mouse tumor was performed as described in the legend to Figure 1. (**a**) Five days after SCC-7 cell implantation, mice with established tumors were submitted to intratumoral injection with 40 μg of naked DNA (naked tk) or Tf-lipoplexes prepared from DOTAP/Chol liposomes at a 3/2 (±) lipid/DNA charge ratio (DOTAP/Chol-Tf-tk). 'Control' represents mice that only received PBS. DOTAP/Chol-Tf-β-gal represents mice that were injected with Tf-lipoplexes containing pCMV-SPORT-LacZ and then treated daily with 50 mg kg^{-1} GCV for 8 days. (**b**) Mice were further treated with 40 μg of Tf-lipoplexes, prepared from DOTAP/Chol liposomes at a 3/2 (±) lipid/DNA charge ratio, at days 2 and 4, and then injected daily with 50 mg kg^{-1} GCV for 14 days after the first DNA administration. DOTAP/Chol-Tf-tk 1inj, DOTAP/Chol-Tf-tk 2inj and DOTAP/Chol-Tf-tk 3inj correspond to mice that received one, two or three injections of Tf-lipoplexes, respectively. 'Control' represents mice that only received PBS, and 'GCV' those that only received GCV treatment. The tumor size was measured every day. Results represent the tumor size after treatment (mean ± s.d.) of different experimental groups ($n=10$). Statistical significance between treated and control groups was determined by one-way ANOVA analysis (***$P<0.001$; **$P<0.005$; not significant (ns) $P>0.05$).

animals injected with Tf-lipoplexes carrying the suicide gene. No significant differences were found in the extent of cell infiltration between control groups (PBS-treated

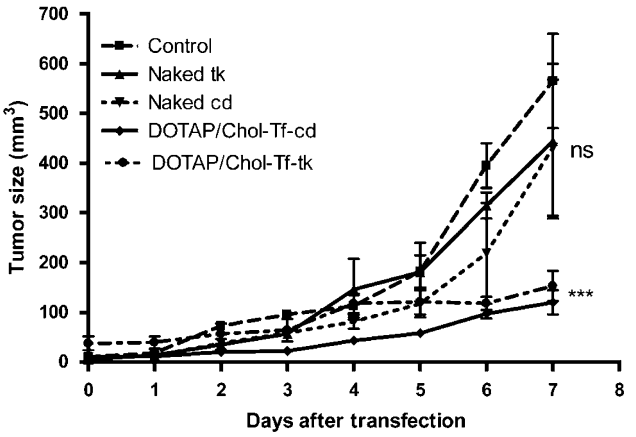

Figure 3 Comparison of the antitumoral effect mediated by HSV-tk/GCV and CD/5-fluorocytosine (5-FC) therapies. Five days after SCC-7 cell implantation, animals with established tumors were treated with 40 μg of tk or CD genes, naked or complexed with Tf-liposomes at a 3/2 (±) lipid/DNA charge ratio, and then injected daily i.p. with 50 mg kg^{-1} GCV or 100 mg kg^{-1} 5-FC, respectively. The tumor size was measured every day. 'Control' represents mice that only received PBS. Results represent the tumor size after treatment (mean ± s.d.) of different experimental groups ($n=10$). Statistical significance between experimental groups and control group was determined by one-way ANOVA analysis (***$P<0.001$; not significant (ns) $P>0.05$).

mice) and those treated with naked DNA. CD3-T lymphocytes include both cytotoxic CD8- and helper CD4-T cells. In the animal groups injected with Tf-lipoplexes containing the therapeutic or reporter genes, extensive tumoral infiltration of CD3-T cells was observed, in contrast with that of CD4-T cells. This result suggests that the observed tumoral lymphocyte infiltration involves mainly CD8+ cytotoxic T cells.

Activation of cytokine and chemokine expression mediated by suicide gene therapy

To further understand the mechanisms involved in the observed antitumoral effect, we analyzed the tumor microenvironment by investigating the levels of cytokines and chemokines (IL-2, IL-10, TNF-α, INF-γ and RANTES) using the FlowCytomix assay. Three tumors in each experimental group (experimental groups involved injections with Tf-lipoplexes carrying β-galactosidase (DOTAP/Chol-Tf-β-gal) or HSV-tk followed by treatment with GCV (DOTAP/Chol-Tf-tk) and with naked tk (naked tk) or PBS (control) were individually analyzed and the expression levels of each cytokine are presented in Figure 6. The cytokine IL-2 was overexpressed in the DOTAP/Chol-Tf-tk group ($P<0.05$) as compared with control, naked tk or DOTAP/Chol-Tf-β-gal groups, which did not exhibit differences in the IL-2 levels. On the other hand, a reduction of IL-10 expression was observed in all treated animal groups compared with the control group (PBS-treated mice), this decrease being more extensive in the group injected with Tf-lipoplexes carrying pCMVtk, followed by GCV treatment (data not shown).

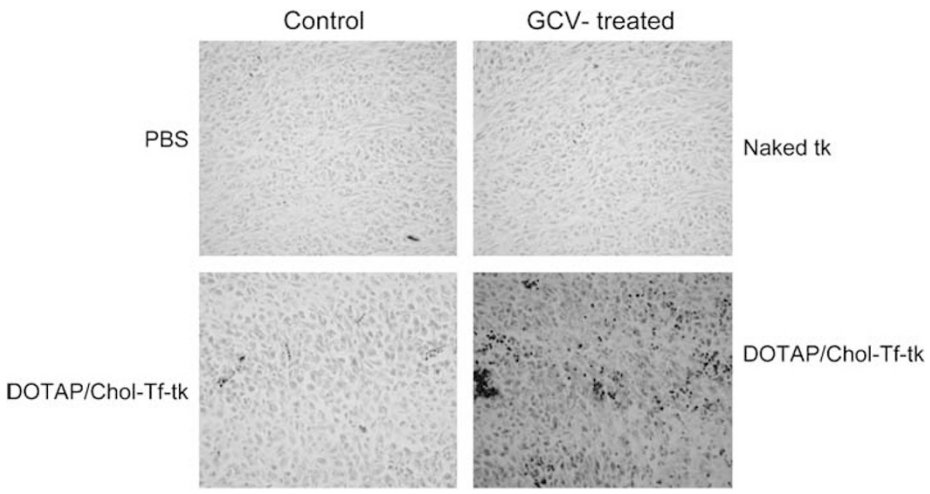

Figure 4 Detection of apoptotic tumor cells in mice treated with HSV-tk/GCV therapy using the TUNEL assay. Induction of the tumor and treatment were performed as described in the legends to Figures 1 and 2, respectively. Eight days after application of the different treatments, animals were killed, tumors were removed and 6 μm cryostat sections were obtained. DNA fragmentation as an index of apoptosis was detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL assay). TUNEL-positive nuclei of apoptotic cells were identified by the presence of dark brown staining. Slices were counterstained with nuclear fast red and observed by light microscopy. Original magnifications for all panels were $\times 400$.

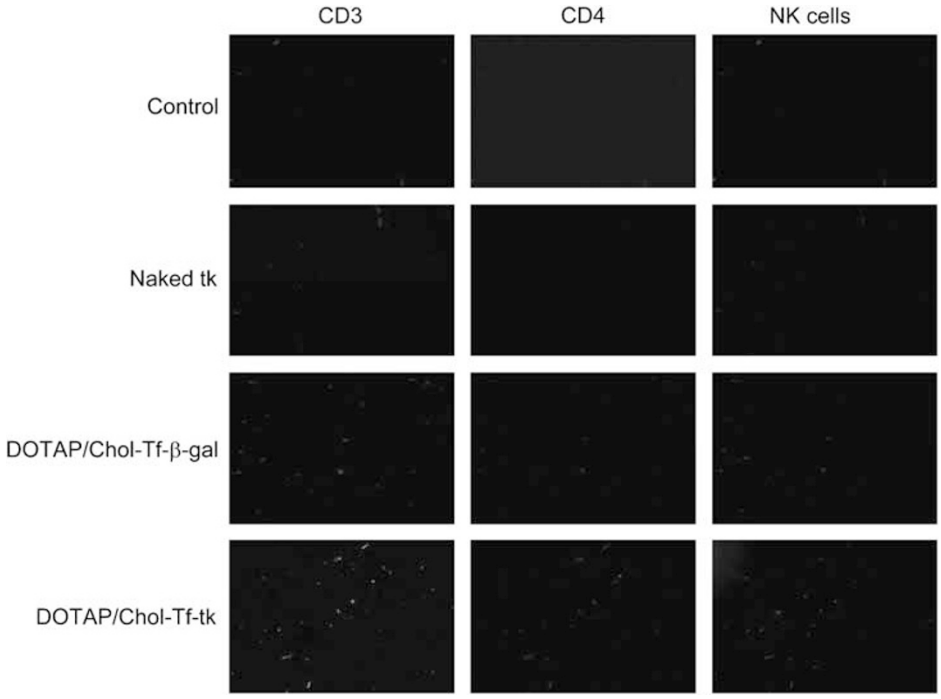

Figure 5 Detection of T-lymphocytes and NK cells in tumors from mice treated with suicide gene therapy. Representative images of tumoral infiltration of T-lymphocytes and NK cells were obtained by fluorescence microscopy. Induction of the tumor and treatment were performed as described in the legends to Figures 1 and 2. Eight days after treatment, animals were killed, tumors were removed and 6 μm cryostat sections were incubated with a rat anti-mouse FITC-labeled mAb against CD3 protein, a rat anti-mouse PE-labeled mAb against CD8 protein and a rat anti-mouse Alexa Fluor 647-labelled mAb against NK cells. Original magnifications for all panels were $\times 400$.

Regarding the INF-γ expression, a small but significant increase was observed in DOTAP/Chol-Tf-tk and DOTAP/Chol-Tf-β-gal groups ($P<0.01$) as compared with control or naked tk groups. Furthermore, intratumoral administration of Tf-lipoplexes carrying pCMVtk followed by GCV treatment induced a fourfold increase in the expression of RANTES ($P<0.001$) over that observed for the control or naked tk groups. The levels of TNF-α were

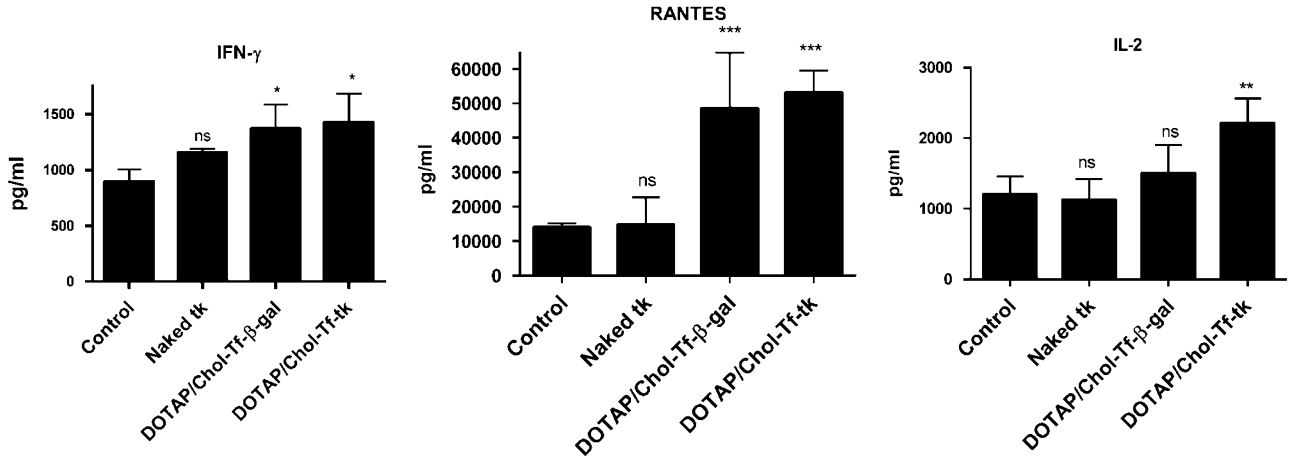

Figure 6 Detection of cytokines and chemokines in tumors from mice treated with HSV-tk/GCV therapy using the FlowCytomix assay. The presence of the cytokines IL-2, INF-γ and the chemokine RANTES was detected in tumor homogenates by FlowCytomix, an ELISA-based flow cytometry assay. Induction of tumor and treatment were performed as described in the legends to Figures 1 and 2. Eight days after application of the different treatments, animals were killed; tumors were removed and homogenized in lysis buffer. Three tumors per condition were analyzed and the results are expressed as pmol ml^{-1}. Statistical significance between treated groups and control groups was determined by one-way ANOVA analysis (***$P<0.001$; **$P<0.005$; *$P<0.01$; not significant (ns) $P>0.05$).

too low to be detected, independently of the tested condition.

Discussion

Head and neck cancer is a devastating disease and new therapeutic modalities are needed. Numerous studies have been reported regarding the *in vivo* application of viral gene therapy for head and neck cancer,[42–47] but safety concerns regarding the use of viral vectors has dampened the enthusiasm for their use in the clinic. Although viral vectors are efficient in gene delivery, immune responses against them are likely to inhibit repeated administrations. Given the limitations and pitfalls of viral gene therapy, non-viral vectors have been studied as an alternative for gene delivery *in vivo*. However, there have been only a few *in vivo* applications of non-viral vectors to deliver therapeutic genes to head and neck tumors, most likely because of their low transfection activity.[48,49]

In this work we have demonstrated that Tf-lipoplexes can mediate transfection of SCC-7 cells *in vivo*, although at a relatively low extent (Figure 1). Taking into account this low transfection efficiency, the significant therapeutic effect observed (Figures 2 and 3) upon intratumoral injection of pCMVtk or pCD plasmids in Tf-lipoplexes followed by GCV or 5-FC treatment, respectively, may be attributed to the bystander effect. This effect is partly because of a transfer of the toxic metabolites (phosphorylated GCV and 5-FU), through gap junctions and/or by phagocytosis of apoptotic vesicles to non-transfected cells, thereby causing cell death. In this regard, our results (Figure 4) show that CD/5-FC and HSV-tk/GCV suicide gene therapy systems induce tumor cell death through apoptosis, in agreement with other reports.[35,38–40]

The bystander effect has been reported to act differently in various tumor models.[17,35] In the murine model used in the present work both suicide gene therapy approaches showed the same therapeutic effect in terms of reducing tumor size. It is interesting to note that in a prostate tumor model, application of HSV-tk/GCV also resulted in the same efficiency as that of CD/5-FC therapy, despite the low levels of connexins and the consequent poor transfer of the toxic metabolites.[50] On the other hand, in a human colorectal tumor mouse model, the CD/5-FC system proved to be superior to HSV-tk/GCV therapy, which was explained by the potential of the CD/5-FC therapy to offer an extracellular route for the bystander effect, namely the export of 5-FU (assumed to be freely diffusible as a small, uncharged molecule) and uptake by neighboring cells in a process that does not require cell–cell contact.[51]

The strong therapeutic response promoted by the bystander effect may also be attributed to the activation of the immune system. The use of an immunocompetent murine model, such as that used in the present work, offers the possibility of studying the contribution of NK and CD8-T cells and immunomodulators to the tumor regression induced by the suicide gene therapy.[52–55] In this context, our results show that delivery of the suicide genes mediated by Tf-lipoplexes results in an increase of tumoral infiltration of NK and T cells, mainly cytotoxic CD8-T cells, in the treated animals when compared with controls (Figure 5). This increase may be partly responsible for the observed antitumoral activity. Nevertheless, some infiltrations of NK and T cells as well as a slight increase in the expression of cytokines and chemokines were also observed in animals treated with Tf-lipoplexes carrying the reporter gene. This is probably due to the immunostimulation provoked by cytosine-phosphate-guanine (CpG) motifs present in the DNA and by the

lipoplexes *per se*. Several studies have demonstrated that cationic liposome/DNA complexes activate the immune system. For example, i.p. injection of lipoplexes into immunocompetent mice resulted in infiltration of inflammatory cells, secretion of INF-γ, and increased NK activity within the peritoneal cavity.[56] It has also been reported that the CpG motifs present in the DNA are responsible for the immunostimulatory effect, since the methylation or elimination of these motifs resulted in a reduction of cytokine production.[57–59] These findings may explain the delay in the tumor growth observed when mice were injected with Tf-lipoplexes carrying the β-galactosidase gene (Figure 2a)

The microenvironment of the tumors was also altered in treated animal groups. A significant increase in IL-2 expression was observed upon intratumoral administration of Tf-lipoplexes carrying the therapeutic gene following drug treatment (Figure 6). IL-2 is normally produced during an immune response. The binding of a tumoral antigen by the T cell receptor can stimulate the secretion of IL-2 which induces proliferation and differentiation of NK, T and B cells and consequently an antitumoral effect.[60]

An increase of the inflammatory cytokine IFN-γ and the chemokine RANTES was also observed in animal groups injected with Tf-lipoplexes carrying both the therapeutic and reporter genes. IFN-γ is secreted by NK cells and T-lymphocytes, mainly cytotoxic CD8-T cells.[61] Therefore, our observation of an increased expression of IFN-γ likely reflects the tumoral infiltration of these cells in animals treated with Tf-lipoplexes. Furthermore, IFN-γ is involved in the production of chemokines by a variety of tumor cells, which act as chemoattractants for lymphocytes, inhibiting the differentiation and proliferation of endothelial cells and angiogenesis.[62,63] Among these, the inflammatory and chemostatic chemokine RANTES, with the help of particular cytokines (that is, IL-2 and IFN-γ), has been shown to play an important role in the recruitment, proliferation and activation of effector T and NK cells, thus stimulating cytolysis.[64] Although the same levels for INF-γ and RANTES have been observed in animal groups injected with Tf-lipoplexes carrying the therapeutic or reporter genes, the difference observed in the extent of tumoral infiltration of NK and T cells, and particularly in tumor regression, for the two groups may be explained by the selective cell death induced by the suicide gene therapy. The decreased expression of IL-10 observed in the animal group treated with Tf-lipoplexes carrying the therapeutic gene followed by drug treatment, compared with that found in the other experimental groups, may also contribute to the observed difference in tumor size. IL-10 is an immunosuppressive and anti-inflammatory cytokine which can both inhibit the synthesis of pro-inflammatory cytokines and suppress the antigen presentation capacity of antigen-presenting cells.[64,65] Therefore, the decrease in the levels of IL-10 observed in the treated animals may allow increased levels of IL-2 and infiltration of inflammatory cells, thus contributing to the pronounced tumor regression in these animals.

Overall, our results provide evidence that suicide gene therapy mediated by Tf-lipoplexes induces tumor cell apoptosis in the syngeneic orthotopic murine model for head and neck squamous cell carcinoma. Activation of resident NK and T cells modifies the tumor microenvironment and leads to the recruitment of immune effector cells that can act as an adjuvant to the suicide gene strategy. These findings will be important in the future design of strategies for efficient treatment of human head and neck squamous cell carcinoma, namely by combining suicide gene therapy strategies and immunotherapy through delivery (or co-delivery) of immunomodulator genes using Tf-lipoplexes.

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