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Transfection of oral cancer cells mediated by transferrin-associated lipoplexes: Mechanisms of cell death induced by herpes simplex virus thymidine kinase/ganciclovir therapy

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Abstract

The Herpes Simplex Virus thymidine kinase (HSV-tk) suicide gene/ganciclovir (GCV) approach has been used for the treatment of a variety of cancers. The purpose of the present study was to evaluate the cytotoxic effect of ganciclovir in oral squamous cancer cells, previously transfected with HSV-tk gene delivered by transferrin-associated complexes (Tf-lipoplexes), as well as to investigate the mechanisms involved in the bystander effect and in the process of cell death. The delivery of HSV-tk gene to the oral cancer cells, HSC-3 and SCC-7, mediated by Tf-lipoplexes followed by ganciclovir treatment resulted in essentially 100% cytotoxicity, the observed toxic effect being dependent both on GCV dose and incubation time. Cell death was shown to occur mainly by an apoptotic process. Different experimental approaches demonstrated that the observed cytotoxicity was mainly due to diffusion of the toxic agent into neighbouring, non-transfected cells, via gap junctions. Preliminary in vivo studies in a murine model for oral squamous cell carcinoma have shown a significant inhibition of tumor growth upon injection of Tf-lipoplexes carrying HSV-tk followed by intraperitonal injection of GCV, as compared to controls.

Keywords: HSV-tk/GCV suicide gene therapy; Oral carcinoma; Transferrin-associated lipoplexes; Bystander effect; Apoptosis

1. Introduction

Head and neck squamous cell carcinoma is a cancer with a high rate of morbidity and mortality due to insufficient early diagnosis and treatment. In fact, despite recent advances, the present therapeutic approaches such as surgery, radiation and chemotherapy have significant limitations both in terms of efficacy and ability to prolong survival in patients with advanced cancer disease [1–3].

The potential of gene therapy to constitute a viable molecular-based therapeutic approach has been demonstrated by the promising results achieved in several pre-clinical studies [4,5]. 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, transfer of tumor suppressor genes and cytokine gene transfer [6,7]. Prodrug/suicide gene approach involves the delivery of a suicide gene into cells, rendering them sensitive to a specific prodrug [8]. Delivery of the Herpes Simplex Virus thymidine kinase gene (HSV-tk) to target cells results in expression of viral thymidine kinase, which selectively phosphorylates ganciclovir (GCV). Monophosphorylated GCV is further phosphorylated by endogenous cellular kinases into an active triphosphate purine analog compound. This product is

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incorporated into cellular DNA, causing chain termination and cell death [9,10]. Although low transfection efficiency is usually observed, the transfected tumor cells (effector cells) appear to be capable of inducing the death of neighbouring untransfected cells, a phenomenon called bystander effect [11]. This effect was shown to result in extensive cytotoxicity and complete tumor eradication [12]. The mode by which this effect occurs remains to be fully understood and different mechanisms have been proposed, including the formation of apoptotic vesicles containing the toxic metabolite, which are internalized by neighbouring cells, the diffusion of the toxic metabolite into non-transfected cells through gap junctions and the activation of immune system in vivo [11–13].

One of the major limitations of gene therapy is the effective delivery of therapeutic genes into target cells. The drawbacks associated with the use of viral vectors, namely those related with safety problems, have prompted investigators to develop alternative methods for gene delivery, cationic lipid-based systems (lipoplexes) being the most representative. Cationic liposomes have several attractive features for gene transfer: they are very easy to prepare, when compared to viral vectors, and can be administered by several routes and are usually non-toxic and non-immunogenic at therapeutic doses; in addition, they can be linked to proteins, fusogenic peptides or associated with NLS sequences aiming at promoting the intracellular delivery and nuclear transfer of therapeutic genes, respectively [14–16]. However, some disadvantages including limited efficiency of delivery and gene expression (as compared to viral vectors), toxicity at higher concentrations, potentially adverse interactions with negatively charged macromolecules present in serum and on cell surfaces, and impaired ability to reach tissues beyond the vasculature unless directly injected into the tissue, represent restrictions to their wide successful application. We and others have demonstrated that association of fusogenic peptides or proteins, including albumin or transferrin, to lipoplexes results in a significant enhancement of transfection both in vitro and in vivo [14,17–20].

Since malignant oral squamous cells overexpress transferrin receptors as compared to non-malignant cells [21,22], we investigated whether treatment of tongue cancer cells with GCV would induce significant cell death following delivery of HSV-tk gene by Tf-lipoplexes. The effect of critical parameters, such as size and lipid/DNA stoichiometry of the complexes on their biological activity, was assessed in order to establish the most efficient transfection conditions for these cells. Studies aiming at gaining insights into the mechanisms involved in the observed bystander effect as well as in the process of cell death were also performed. Moreover, to evaluate the in vivo potential of Tf-lipoplexes in HSV-tk/GCV therapy of oral cancer, exploratory work was carried out in a syngeneic orthotopic murine model for squamous cell carcinoma of the head and neck.

To our knowledge, this is the first systematic study addressing the use of Tf- lipoplexes for the in vitro and in vivo application of suicide gene therapy approaches aiming at treating oral squamous cell carcinoma and understanding the underlying mechanisms.

2. Materials and methods

2.1. Chemicals

The cationic lipid 1,2-dioleoyl-3-(trimethylammonium) propane (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 pCMVluc plasmid (VR-1216) was kindly provided by Dr. P. Felgner (Vical, San Diego, CA, USA) and the plasmid pCMVtk was kindly provided by Dr. N. Düzgünes (University of the Pacific, San Francisco, CA, USA). Alamar Blue dye was purchased from Trek diagnostics systems (Ohio, USA). α-glycyrrhetinic acid (AGA) and dibutyryl adenosine 3′,5′-cyclic monophosphate (db-cAMP) were obtained from Sigma (St. Louis, MO, USA). Ganciclovir (GCV) was a gift from the University Hospital of Coimbra.

2.2. Cells

HSC-3 (human squamous cells) and SCC-7 (murine squamous cells) cells, kindly provided by Dr. N. Düzgünes (University of the Pacific, San Francisco, CA, USA), were maintained at 37 °C, under 5% CO_2, in Dulbecco's modified Eagle's medium-high glucose (DMEM-HG) (Sigma, St. Louis, MO, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA), penicillin (100 U/ml), streptomycin (100 μg/ml) and sodium bicarbonate (1.6 g/l). For transfection, 1×10^5 HSC-3 or SCC-7 cells were seeded in 1 ml of DMEM medium in 48-well culture plates 24 h before transfection and used at 70–80% confluence.

2.3. Cationic liposomes and lipoplexes

Small unilamellar cationic liposomes (SUV) were prepared by extrusion of multilamellar liposomes (MLV) composed of a 1:1 (mole ratio) mixture of 1,2-dioleoyl-3-(trimethylammonium) propane (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 deionised water to a final lipid concentration of 6 mM and the resulting MLV 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). The resulting liposomes (SUV) were then diluted five times with deionised water and filter-sterilised utilising 0.22 mm pore-diameter filters (Schleicher and Schuell BioScience, Germany). For the in vitro studies, complexes were prepared by sequentially mixing 100 μl of a HEPES-buffered saline solution (HBS) (100 mM NaCl, 20 mM HEPES, pH 7.4), with or without human transferrin (320 μg/ml) (Sigma, St.Louis, MO, USA), with liposomes (volume was dependent on the desired lipid/DNA (+/−) charge ratio) and incubated for 15 min at room temperature. One hundred microliters of HBS containing 1 μg of pCMVluc or 1 μg of pCMVtk were added to the mixture and further incubated for 15 min at room temperature. For the in vivo studies, complexes were prepared by sequentially mixing 20 μl of a HBS solution containing human transferrin (Sigma, St.Louis, MO, USA) (128 μg/ml), with liposomes and incubated for 15 min at room temperature. Forty microliters of HBS containing 40 μg of pCMVtk were added to the mixture and further incubated for 15 min at room temperature.

Freshly prepared cationic liposome/DNA complexes, associated or not with human transferrin, were characterized with respect to their size and zeta potential using a Coulter N4 plus and a Coulter DELSA 440 (Coulter Corporation, Miami, FL), respectively as described previously [23].

2.4. Transfection activity

Transfection activity mediated by the complexes was evaluated as described previously [18]. Cells were rinsed twice with serum-free medium and then covered with 0.3 ml of DMEM-HG (without serum, unless indicated otherwise) before lipid-based complexes were added. The complexes were added gently to cells in a volume of 0.2 ml per well. After incubation for 4 h (in 5% CO_2 at 37 °C), the medium was replaced with DMEM-HG containing 10% FBS, and the cells were further incubated under different experimental conditions. Forty-

eight hours after transfection cells were washed twice with phosphate-buffered saline solution (PBS) (0.14 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) and 100 μl of lysis buffer (1 mM DTT, 1 mM EDTA, 25 mM Tris–phosphate at pH 7.8, 8 mM $MgCl_2$, 15% glycerol, 1% (v/v) Triton X-100) were added to each well. The level of gene expression in the lysates was evaluated by measuring light production by luciferase in a Mediators PhL luminometer (Mediators Diagnostika, Vienna, Austria) and using a standard curve for luciferase activity. The protein content of the lysates was measured by the Dc Protein Assay reagent (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard. The data were expressed as nanograms of luciferase per milligram of total cell protein.

2.5. Cytotoxicity studies

Following transfection, cell viability was quantified by a modified Alamar Blue assay (Trek diagnostics systems, Ohio, USA) under the different experimental conditions. Five hundred microliters of 10% (v/v) Alamar Blue in DMEM were added to cells. After 2 h incubation at 37 °C, 200 μl of supernatant were collected from each well and transferred to 96-well plates. The absorbance at 570 nm and 600 nm was measured using a Mediators PhL luminometer (Mediators Diagnostika, Vienna, Austria). Cell viability (as a percentage of control) was calculated according to the formula $(A_{570}-A_{600})$ of treated cells $\times 100/(A_{570}-A_{600})$ of control cells [18]. For assessing the cytotoxic effect of ganciclovir, following transfection with lipid/HSV-tk plasmid DNA complexes, the culture medium was replaced every day by a fresh solution containing 10% FBS and ganciclovir at different concentrations.

2.6. Modulation of gap junctional intercellular communication

For the experiments on the effect of modulation of gap junctions on connexin expression, HSC-3 or SCC-7 cells were incubated with α-glycyrrhetinic acid (AGA) (50 μM) or dibutyryl adenosine 3′,5′-cyclic monophosphate (db-cAMP) (500 μM) for 24 h. Cells were trypsinized, centrifuged at 300×*g* for 5 min and then permeabilized using a Fix and Perm® kit (Invitrogen, California, USA). Cells were washed with PBS and incubated for 15 min with primary antibodies: mouse anti-connexin 43 (Santa Cruz Biotechnology, California, USA) or anti-dephosphorylated connexin 43 (Invitrogen, California, USA). Cells were then washed with PBS and incubated with anti-mouse secondary antibody conjugated with FITC (Dako, Glostrup, Denmark) for 20 min. Cells were further washed with PBS, resuspended in the same buffer and analysed in a FACSCalibur (Becton Dickinson) flow cytometer equipped with an argon ion laser emitting at 488 nm.

For the experiments on the effect of the modulation of gap junctions on HSV-tk/GCV therapy, 1×10^5 HSC-3 or SCC-7 cells were seeded in 48-well culture plates (1 ml of medium) and treated with AGA (25 or 50 μM). In parallel experiments, cells were treated with db-cAMP (250 or 500 μM) in the presence of cAMP phosphodiesterase 4 selective inhibitor, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (10 μM) (Biomol international L.P., PA, USA). When cells reached 70–80% confluence, they were transfected with Tf-lipoplexes containing HSV-tk, as described above. After 4 h of transfection, the medium was replaced with DMEM-HG containing 10% FBS, GCV (at different concentrations depending on the GJIC modulator tested) and the drugs, this mixture being freshly prepared and added every day to the cells.

2.7. Morphological analysis

Following transfection, HSC-3 cells, treated or not with GCV, were trypsinized and centrifuged at 200×*g* for 5 min. 0.3×10^6 cells were then resuspended in 30 μl of FBS and placed on a slide for microscopic analysis. The cells were stained upon incubation for 5 min with May–Grünwald solution (0.3% v/v in methanol) (Sigma, St. Louis, MO, USA), diluted in distilled water at 1:1 (v/v) ratio and then stained with Giemsa solution (0.75% w/v in glycerol/methanol 1:1) (Sigma, St. Louis, MO, USA) previously diluted in distilled water (8×). After rinsed with distilled water, the cells were left to dry at room temperature. Cell morphology was analysed by light microscopy using a Leitz Dialux 20 microscope associated with a Canon Powershot S40 digital camera.

2.8. Mechanistic studies of cell death

Different approaches were used to evaluate the contribution of apoptotic or necrotic processes to cell death induced by GCV treatment of HSV-tk transfected HSC-3 cells. Chromatin condensation was analysed by fluorescence microscopy (Nikon Diaphot with xenon lamp Nikon XPS-100 and Triple filter Omega FX 64) using the probes SYTO-13 (Molecular Probes) and propidium iodide (PI). SYTO-13 labels RNA and DNA in live cells with an UV-excited green emission and PI is excluded from viable cells, with an UV-excited red emission. Cells (5×10^4) were incubated for 3 min in a 20 μl solution of sodium medium: 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 5.6 mM glucose, 20 mM HEPES (pH 7.4), containing 4 ìM SYTO-13 and 4 μg/ml PI.

Quantification of the relative contribution of apoptosis or necrosis to cell death was performed by flow cytometry [24]. For that purpose, HSC-3 cells were stained with Annexin V (AV), labelled with the fluorescent probe fluorescein isothiocyanate (FITC), and PI. Following transfection, the cells treated or not with GCV for different times, were trypsinized, centrifuged at 300×*g* for 5 min and incubated for 10 min at 4 °C with 440 μL Annexin buffer containing 5 μl FITC-labeled AV (Kit from Immunotech SA, Marseille, France) and 5 μl PI. Cells were then washed with phosphate buffer (PBS), resuspended in the same buffer and analysed in a FACSCalibur (Becton Dickinson) flow cytometer equipped with an argon ion laser emitting at 488 nm. The fluorescence of AV-FITC and PI was evaluated at 525 and 610 nm, respectively. The results were expressed as percentage of viable, apoptotic and necrotic cells.

2.9. In vivo studies

In vivo experiments were performed using a syngeneic orthotopic murine model for squamous cell carcinoma of the head and neck developed by O'Malley et al [25,26]. Six to eight week old female C3H/HeJ mice were anesthetized upon intramuscular injection of a mixture of chlorpromazine (2 mg/kg) and ketamine (100 mg/kg). The tumor model was established by injecting 1×10^5 SCC-7 cells directly into the floor of the mouse. Five days after cell implantation, the mice were anesthetized as before, and the developed tumors were injected with 40 μg of naked DNA (in PBS) or 40 μg of DNA complexed with transferrin-associated lipoplexes. PBS alone was also injected into control animals. Four hours after, mice were injected with ganciclovir (25 mg/kg) intraperitoneally twice a day during 8 days and sacrificed 13 days following tumor implantation. The tumors were measured in 3 dimensions everyday with callipers.

2.10. Statistical analysis

Data were analysed 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).

3. Results

3.1. Effect of lipid/DNA (+/−) charge ratio and association of transferrin on the transfection activity of oral squamous cancer cells

We evaluated the effect of lipid/DNA charge ratio and association of transferrin on the transfection activity of cationic liposome/DNA complexes, containing DOTAP/Chol, in HSC-3 cells. For plain lipoplexes, the highest biological activity was observed when they were prepared at the 2/1 (+/−) charge ratio (at which they exhibit a small mean diameter and a positive surface charge) (Fig. 1). An enhancement of transfection was observed upon association of transferrin to the lipoplexes for almost all charge ratios, this being more significant at the 1/1 and 3/2 charge ratios. For the Tf-lipoplexes the highest values of

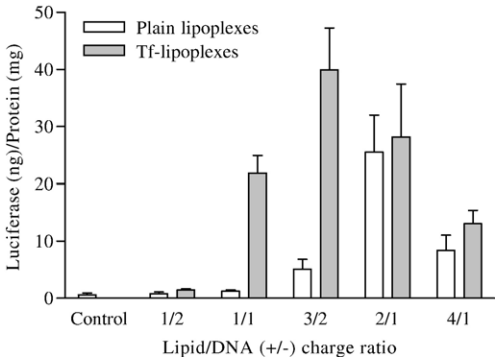

Fig. 1. Effect of associating transferrin to lipoplexes, prepared at different lipid/DNA charge ratios, on luciferase gene expression in HSC-3 cells. Cells were transfected with DOTAP:Chol liposomes, complexed with 1 μg of pCMVluc at the indicated lipid/DNA (+/−) charge ratios in the absence (plain lipoplexes) or presence of 32 μg of transferrin (Tf-lipoplexes) as described in Materials and methods. Data represent the mean ± S.D. of at least three independent experiments performed in triplicate. As expected neutrally charged lipoplexes (e.g. 3/2 (+/−) charge ratio) tend to form large aggregates (mean size ranging from 1500 to 2000 nm) as opposed to lipoplexes prepared at high or low (+/−) charge ratios. Association of transferrin to the lipoplexes results in a decrease of the zeta potential, this being particularly evident for the 2/1 and 4/1 charge ratios. Nevertheless, the tendency of the complexes, prepared at a (+/−) charge ratio close to neutrality, to aggregate is not affected by the presence of this protein.

transgene expression are found for the 3/2 lipid/DNA (+/−) charge ratio, the zeta potential is close to neutrality and large aggregates are formed. Similar results were obtained for SCC-7 cells, in terms of enhancement of the transfection efficiency when lipoplexes were associated with transferrin. The highest transfection activity was also achieved when transferrin-associated lipoplexes were prepared at the 3/2 charge ratio (data not shown). Based on the obtained results, 3/2 (+/−) charge ratio Tf-lipoplexes were selected as the optimal formulation for transfection of both HSC-3 and SCC-7 cells and therefore used for further studies. Using this formulation, results on the duration of transgene expression reveal that the highest values of luciferase gene expression are achieved at 48 h. Nevertheless, the transient nature of transfection was reflected by the significant decrease of gene expression observed at 3 days and its total abolishment at 7 days, following incubation of the complexes with the cells for 4 h (Fig. 2).

3.2. Effect of GCV treatment on the viability of oral squamous cancer cells transfected with HSV-tk

In order to evaluate the cytotoxic effect induced by the HSV-tk/GCV suicide gene approach, HSC-3 and SCC-7 cells were transfected with Tf-lipoplexes (composed of DOTAP:Chol/ DNA) prepared at the 3/2 (+/−) lipid/DNA charge ratio and then incubated for different times with GCV at various concentrations (up to 100 μM). The extent of cytotoxicity was assessed by the Alamar Blue assay. A decrease in cell viability was observed upon increasing GCV concentration, the highest toxicity being obtained at 100 μM GCV for both cell lines (Fig. 3). As illustrated in Fig. 3, GCV does not exert any toxic effect in non-transfected cells. The observed cytotoxic effect was also found

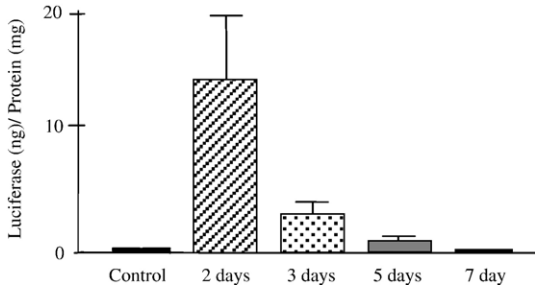

Fig. 2. Duration of transgene expression in HSC-3 cells. Cells were transfected with Tf-lipoplexes composed of DOTAP:Chol/ pCMVluc/transferrin, prepared at the 3/2 (+/−) charge ratio. The levels of luciferase gene expression were evaluated as described in Materials and methods. Data represent the mean ± S.D. of at least three independent experiments performed in triplicate.

to be dependent on the incubation time of GCV with transfected cells, this effect being particularly evident at 100 μM GCV concentration, where essentially 100% cytotoxicity was observed at 7 days incubation for HSC-3 cells and at 5 days for SCC-7 cells. Therefore, SCC-7 cells seem to be more sensitive to GCV than HSC-3 cells, since for the same GCV concentration and incubation time, a higher toxicity was observed in SCC-7 cells. Our results illustrate that cell death induced by GCV is a relatively slow process, since after 3 days of cell treatment with GCV only 30–40% reduction in cell viability was achieved for the highest concentration tested. Independently of the incubation time, no further toxicity was observed for GCV concentrations above 100 μM (data not shown). These results

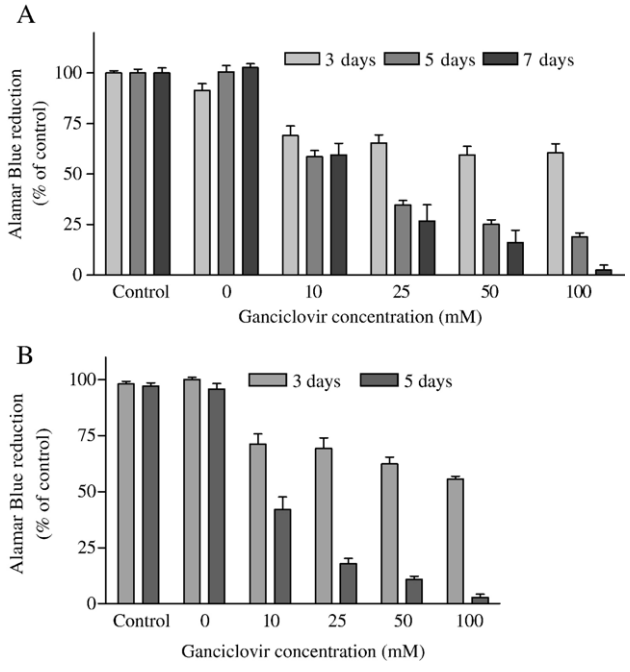

Fig. 3. Effect of GCV concentration and incubation time on the viability of HSC-3 cells (A) and SCC-7 cells (B) transfected with HSV-tk. Cells were transfected with Tf-lipoplexes composed of DOTAP:Chol/ pCMV-tk /transferrin, (3/2 (+/−) charge ratio) followed by GCV treatment at the indicated doses and incubation times. Cell viability was evaluated by the Alamar Blue assay. Control represents cells (non-transfected) treated with 100 μM GCV. Data represent the mean ± S.D. of at least three independent experiments performed in triplicate.

suggest that for GCV concentrations higher than 100 μM, the limiting factor affecting cytotoxicity is the level of thymidine kinase expression.

3.3. Cellular mechanisms involved in the bystander effect

Evaluation of transfection efficiency mediated by Tf-lipoplexes, as assessed by scoring the percentage of cells that were positive for β-galactosidase staining, indicates that only about 5–10% of cells were successfully transfected (data not shown). Based on this finding and taking into account that: (i) the results illustrated in Fig. 2 clearly indicate that transgene expression under the control of CMV promoter (used for both luciferase and thymidine kinase plasmids) is transient when transfection is mediated by Tf-lipoplexes; (ii) TK and luciferase exhibit similar short half-lives (0.5 and 3 h, respectively) [27,28]; (iii) phosphorylated GCV exhibits a short half-life (3–12 h) [29] and (iv) a large extent of cytotoxicity after 7 days is still observed when cell treatment with GCV is restricted to the first 3 days following transfection (data not shown), it can be suggested that the observed delayed cytotoxic response should be attributed to a rapid diffusion of phosphorylated GCV to non-transfected cells, thus triggering further mechanisms of cell death, which can require long times to occur. However, the hypothesis of signalling pathways triggered by the DNA damages observed in transfected cells having a slow effect in the neighbouring cells cannot be ruled out.

On the other hand, the possibility of phosphorylated ganciclovir to be present in the cell culture medium and thus available to reach non-transfected cells is also excluded. This was demonstrated by the absence of cell death when the supernatant of GCV-treated, transfected cells, collected after 2, 5 or 7 days incubation with this drug, was added to non-treated, transfected cells (Fig. 4). These results suggest that the observed cytotoxicity is due to diffusion of the toxic agent into neighbouring cells via intercellular communication pathways, such as gap junctions.

In order to investigate the possible role of gap junctions in the observed cytotoxic effect, we have evaluated the extent of connexin expression in HSC-3 and SCC-7 cells, as well as the effect on modulation of expression and functionality of gap junctions upon incubation of the cells with an activator (db-cAMP) and an inhibitor (AGA) (Fig. 5). A significant enhancement of connexin expression was observed in both HSC-3 and SCC-7 cells (two fold increases), upon their incubation for 24 h with db-cAMP, as assessed by flow cytometry using an antibody anti-connexin 43. In contrast, incubation of the cells with AGA did not result in a significant effect in connexin 43 expression. In addition, we evaluated the effect of the gap junctions' modulators on phosphorylation of connexins, using a specific antibody against the dephosphorylated form of connexin 43. It is known that connexin phosphorylation or dephosphorylation play a role in the conductivity of gap junctions (by opening or closing) [30]. Our results demonstrate that AGA induces dephosphorylation of connexin 43 in both cell lines. On the other hand, in the presence of db-cAMP an increase in phosphorylated connexin 43 expression was observed.

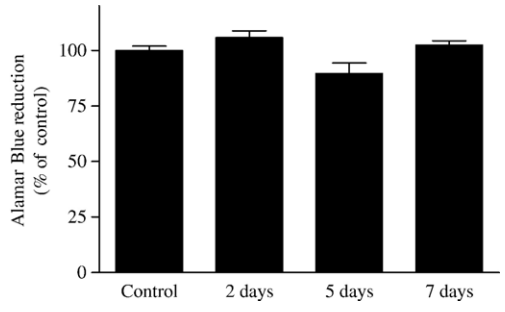

Fig. 4. Viability of non-transfected HCS-3 cells upon incubation with medium collected from cells transfected with HSV-tk and treated with 100 μM GCV. Cell viability was evaluated by Alamar Blue assay over different incubation periods. Data represent the mean±S.D. of at least three independent experiments performed in triplicate.

In order to further confirm the role of gap junctions in the bystander effect, the influence of activating or inhibiting gap junctions on the extent of cytotoxicity was assessed by treating both HSC-3 and SCC- 7 cells with db-cAMP and AGA. In both cases, we observed a concentration-dependent effect on GCV cytotoxicity only after 5 days incubation with ganciclovir in the presence of gap junctions' modulators. Upon optimization of the experimental conditions, a significant inhibition of cytotoxicity was observed when both cell types were pre-treated with 50 μM AGA (60% cell viability was achieved at 5 days incubation with 50 μM GCV, as compared to 25% cell viability in the absence of AGA) (Fig. 6B). On the other hand, for the same incubation time, cytotoxicity mediated by 25 μM ganciclovir increases from 25% to 60% when the cells were pre-treated with 500 μM db-cAMP (Fig. 6A). Incubation of the cells with the gap junctions' modulators at the tested concentrations did not result in any toxicity (data not shown).

3.4. Molecular mechanisms of cell death mediated by HSV-tk/GCV gene therapy

Aiming at gaining insights into the molecular mechanisms involved in the process of cell death induced by the HSV-tk/GCV system, different experimental approaches were used to evaluate the relative extent of apoptosis versus necrosis.

At a first approach, following 48 h of transfection with HSV-tk, HSC-3 cells (treated or not with GCV) were fixed and stained using a May–Grünwald solution and a Giemsa solution, as described in Materials and methods. As can be observed in Fig. 7A, the morphology of transfected cells treated with GCV suggests that an apoptotic process is occurring, as indicated by the presence of apoptotic vesicles and condensed nuclei. In order to confirm these apoptotic events, and to be able to distinguish among viable, apoptotic and necrotic cells, HSC-3 cells were analysed by fluorescence microscopy upon their incubation with SYTO-13 or PI.

As illustrated in Fig. 7B, 10% of cells are apoptotic in transfected cultures not treated with GCV, as assessed by scoring the cells for fragmented or condensed DNA. However, following treatment with 100 μM GCV for 2 days, the

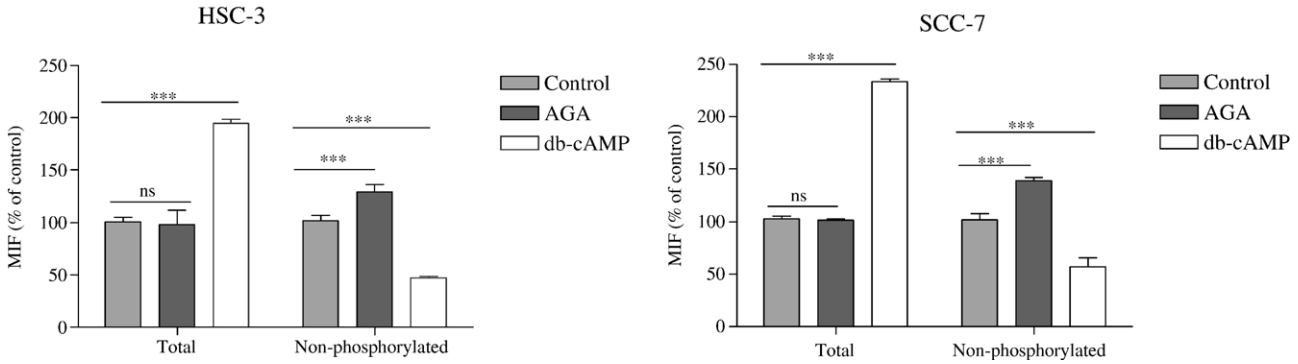

Fig. 5. Modulation of connexin expression and phosphorylation by gap junction modulators. Cells were incubated for 24 h with 50 μM AGA or 500 μM db-cAMP. Connexin 43 expression (total) was determined by FACS analysis using a mouse antibody anti-connexin 43 (Santa Cruz Biotechnology, California, USA) and the extent of connexin 43 phosphorylation was assessed using a mouse antibody anti-dephosphorylated connexin 43 (Invitrogen, California, USA). Data are shown as mean fluorescence intensity (MIF) (percentage of untreated cells (control)). Data represent the mean ± S.D. of at least two independent experiments. Statistical significance between experimental groups was determined by one-way ANOVA analysis (***$p<0.001$; ns $p>0.05$).

percentage of apoptotic cells increases up to 30%, but no necrotic cells are observed (as assessed by scoring the cells incorporating PI).

In order to quantify the extent of apoptosis, we used a flow cytometry assay based on staining the cells with FITC-AV or PI. With this assay it is possible to distinguish non-apoptotic live cells (AV negative/PI negative), early apoptotic cells (AV positive/PI negative) and late apoptotic or necrotic cells (PI positive) [24]. AV binds with high affinity to negatively charged phospholipids, namely phosphatidylserine, which is exposed at

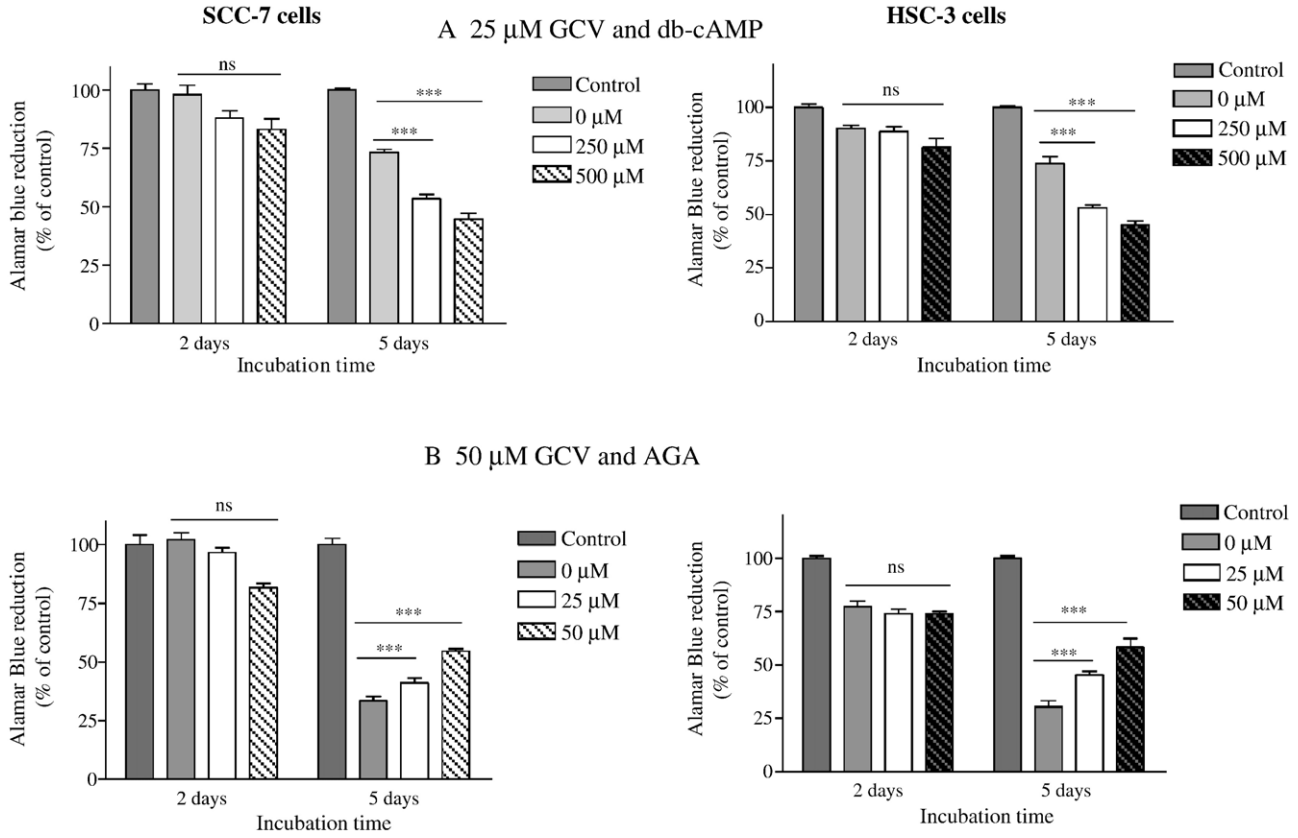

Fig. 6. Effect of modulating gap junctions of oral cancer cells (HSC-3 and SCC-7 cells) on the bystander effect. Cells were transfected with complexes composed of DOTAP:Chol/ pCMV-tk /transferrin (3/2 charge ratio) following treatment with (A) various concentrations of dbAMPc and then incubated with 25 μM GCV or (B) various concentrations of AGA and then incubated with 50 μM GCV. Cell viability was evaluated by the Alamar Blue assay. Data represent the mean ± S.D. of at least three independent experiments performed in triplicate. Statistical significance between experimental groups was determined by one-way ANOVA analysis (***$p<0.001$; ns $p>0.05$).

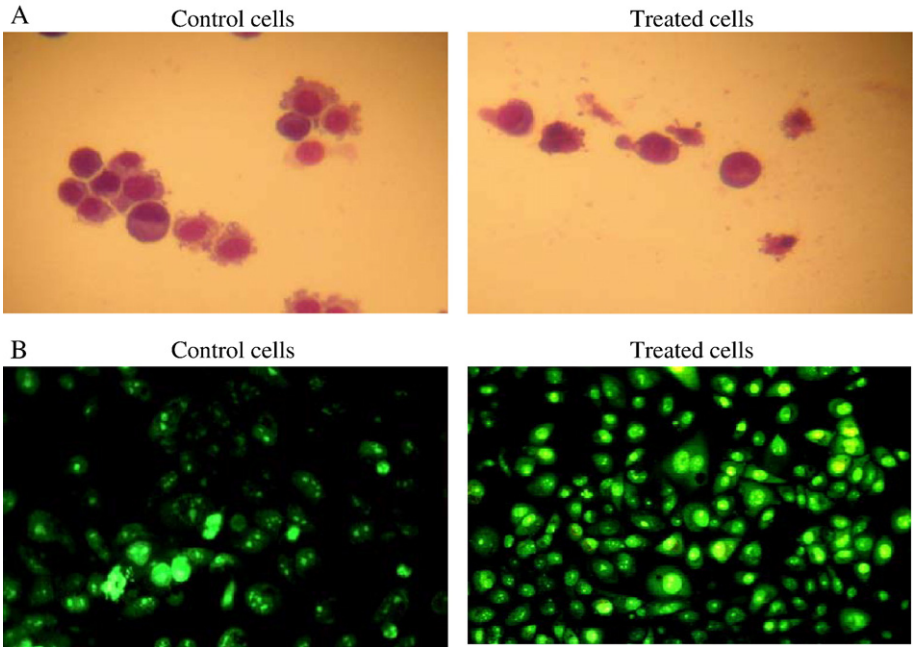

Fig. 7. Morphology of HSC-3 cells as assessed by (A) optical microscopy following cell fixation and staining with May–Grünwald–Giemsa solution and (B) fluorescence microscopy using SYTO-13™ and PI. Cells were analysed 48 h after being transfected with HSV-tk and treated or not with 100 μM GCV.

the outer membrane leaflet in apoptotic cells. PI is a non-specific DNA labelling agent that penetrates dead cells due to the loss of membrane integrity, frequently associated with necrosis. As shown in Fig. 8, apoptosis seems to be the main mechanism involved in the death of HSV-tk transfected cells treated with GCV. An increase in the percentage of apoptotic cells with increasing the incubation time with 100 μM GCV is observed (15%, 35% and 50% for 2, 4 and 6 days incubation, respectively). In contrast, the percentage of necrotic cells remains essentially the same (approximately 10%) during the first 4 days of incubation with GCV.

3.5. In vivo studies

To investigate whether intratumoral delivery of the HSV-tk gene mediated by transferrin-associated lipoplexes would result in tumor cell killing and size reduction upon GCV treatment, we used a syngeneic orthotopic murine model for squamous cell carcinoma of the head and neck [25,26]. Twenty-four C3H/HeJ mice with an established tumor in the floor of the mouth (10–25 mm^3) were divided into 3 different experimental groups. The first group of animals was injected intratumorally with PBS (control), while the second and third groups were injected with 40 μg of DNA encoding HSV-tk, either naked or complexed with Tf-lipoplexes, respectively. The second and third groups of animals were subsequently injected twice a day with 25 mg/kg GCV. Animals were observed daily with special attention to tumor size, which was measured in 3 dimensions with callipers. As illustrated in Fig. 9, injection of Tf-lipoplexes is significantly more effective in delaying tumor progression than PBS or naked DNA injections. Eight days after treatment, mice that have been injected with Tf-lipoplexes present a tumor with a size of 500 mm^3, while the tumor size of both control mice and mice that have received naked DNA reach 1000–1500 mm^3.

4. Discussion

Although numerous studies have been reported regarding the anti-tumor effect of the HSV-tk/GCV approach on human oral squamous cell carcinomas, the great majority rely on the use of viral vectors to mediate gene delivery [25,31–33]. The use of non-viral vectors for this specific purpose [34–36] has been scarce, most likely due to their low transfection activity. To our best knowledge, this is the first systematic report on the use of protein-associated lipoplexes aiming at application of the above suicide gene approach for the treatment of human oral

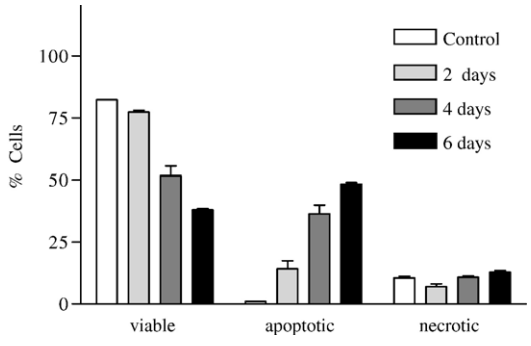

Fig. 8. Extent of apoptosis and necrosis of HSC-3 cells transfected with HSV-tk followed by GCV treatment. Cells were transfected with Tf-lipoplexes followed by treatment with 100 μM GCV. The cells were then incubated with AV and PI and analysed by flow cytometry. Control represents transfected but not GCV treated cells. Results are expressed as the percentage of viable, apoptotic and necrotic cells at different GCV incubation times and represent the mean± standard deviation obtained from duplicate wells.

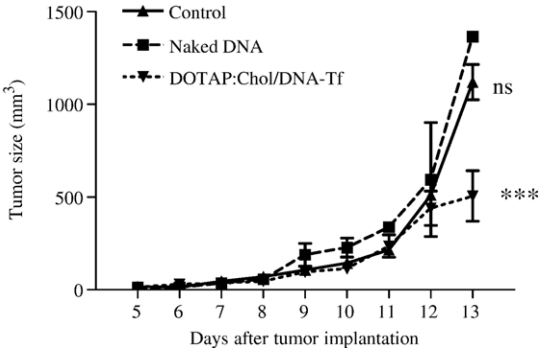

Fig. 9. Antitumor effect of HSV-tk expression followed by treatment with GCV in an orthotopic mouse model of oral carcinoma. Five days after SCC-7 cell implantation, tumors were treated with 40 μg naked DNA or Tf-lipoplexes composed of DOTAP:Chol at 3/2 (+/−) lipid/DNA charge ratio (40 μg DNA). Mice were then injected twice a day with 25 mg/kg GCV for 8 days. Control mice were injected with PBS. The tumor size was measured everyday with callipers and mice were sacrificed 13 days after tumor cell implantation. Results represent the tumor size after treatment (mean±standard deviation) of different experimental groups ($n=8$). Statistical significance between experimental groups was determined by one-way ANOVA analysis (***$p<0.001$; ns $p>0.05$).

squamous cell carcinomas, encompassing mechanistic studies as well as evaluation of biological activity, both in vitro and in vivo.

In this work, we evaluated the in vitro efficacy of the HSV-tk/GCV system, for treatment of oral squamous cancer cells using Tf-lipoplexes, prepared from cationic liposomes composed of DOTAP:Chol (1:1 mol ratio).

We and others have demonstrated that association of fusogenic peptides or proteins, including albumin or transferrin, to lipoplexes potentiates cellular internalization and cytoplasmic delivery of carried DNA leading to a significant enhancement of transfection both in vitro and in vivo [14,17–20].

The highest levels of transfection activity for both HSC-3 and SCC-7 cells were observed for transferrin-associated lipoplexes prepared at a 3/2 (+/−) charge ratio. A significant enhancement of transfection activity was observed upon association of transferrin, confirming previous observations that this protein promotes both internalization of the complexes and intracellular delivery of DNA.

Our results show that the extent of cell toxicity is GCV dose-dependent, the highest cytotoxicity (essentially 100% cell death) being obtained with 100 μM GCV for both, the human squamous HSC-3 and murine squamous SCC-7 cells. Such concentration of GCV is higher than those reported to result in cytotoxicity in other studies, namely using viral vectors [32], which can be attributed to the lower efficiency of lipid-based carriers to mediate HSV-tk gene transfer. Cell death induced by this approach was revealed to be a slow process, since 100% toxicity was achieved for SCC-7 and HSC-3 cells only after 5 and 7 days incubation with GCV, respectively. However, SCC-7 cells seem to be more sensitive than HSC-3 cells to ganciclovir. The time required for cell death to occur depends on the division rate of the cells. Cells with a higher division rate are more sensitive to phosphorylated GCV than those with a lower division rate, this being attributed to the mechanisms of action of the toxic metabolite. The phosphorylated GCV requires cells under division for effectiveness since it acts during DNA synthesis, being incorporated and causing chain termination and cell death. Some studies with ARA-C, an inhibitor of cell growth, show that cells treated with this drug become less sensitive to phosphorylated GCV that non-treated cells [37].

The transfection studies performed with reporter genes (luciferase) demonstrate that transfection of HSC-3 cells mediated by Tf-lipoplexes is transient, a significant decrease of gene expression being observed 72 h after transfection. In addition, this approach was shown to lead to relatively low values of transfection efficiency (5–10% successfully transfected cells) Accordingly, the high toxicity observed 7 days after transfection of the HSC-3 cells with HSV-tk gene (essentially 100% cell death) cannot be attributed to long term expression of thymidine kinase and continuous phosphorylation of GCV, but most likely to a bystander effect. This phenomenon is clinically important, as genetic modification of all tumor cells is not possible with the available gene transfer approaches.

Different mechanisms have been proposed to explain this effect. As stated above, the diffusion, across the cell membrane, of phosphorylated GCV released from transfected cells to non-transfected cells is excluded. Therefore, other putative mechanisms, namely intercellular transfer of phosphorylated GCV from transfected to neighbouring non-transfected cells through gap junctions were investigated.

The implication of gap junctions in the bystander effect was confirmed by assessing the influence of AGA, a gap junction blocker [38], on the cytotoxicity mediated by GCV. Connexins constitute a family of proteins involved in the establishment of gap junctions, through which intercellular exchange of small hydrophilic molecules occurs [37,39]. AGA exhibits an inhibitory effect on gap junctions via direct interaction with connexins altering connexon particle packing in gap junction plaques and by dephosphorylation of connexin 43. Phosphorylation of this protein may be necessary for the formation of active gap junctions. On the other hand, exposure to AGA may not affect protein synthesis [38,40]. Our results on the effect of gap junction modulators on connexin 43 expression and phosphorylation have confirmed that AGA does not affect connexin 43 expression (as assessed by flow cytometry using an anti-connexin 43 antibody) but promotes its dephosphorylation (as assessed by flow cytometry using an anti-connexin 43 antibody specific to the non-phosphorylated form).

The fact that some toxicity could still be observed was due most likely to an incomplete inhibition of gap junctions by 50 μM AGA. A complete blockage of gap junctional intercellular communication (GJIC) would have required a higher AGA concentration, which could not be achieved due to its cytotoxic effect (data not shown). Guo et al. made the important observation that functional effectiveness of AGA was reduced by serum in a dose-dependent manner most likely due to binding of the drug to serum proteins. In our protocol, we used 10% FBS in the medium, so the incomplete blockage of gap junctions can be explained by these findings [38]. On the other hand, it should be considered that other mechanisms may

also be involved in the bystander effect in HSC-3 and SCC-7 cells.

Since most tumor cells exhibit a reduced level of GJIC [41], an elegant way to increase the extent of the bystander effect induced by the HSV-tk/GCV system would be to promote the GJIC levels [42,43]. This hypothesis was strongly supported by the fact that the bystander effect is absent in cells that lack functional gap junctions, but can be restored or enhanced by connexin gene transfection [44]. cAMP analogs have been reported to enhance GJIC in a variety of cell lines by promoting connexin expression [37,39,45]. Recent studies have indicated that cAMP induces a large increase in connexin mRNA levels in different tumor cell lines, suggesting that this increase accounts for the permeability changes induced by this agent [46,47]. In addition, a different form of control of junctional permeability by cAMP in which the cellular distribution of connexin 43 is modified to increase the gap junctional permeance was reported [48]. Moreover, cAMP can also cause an increase in gap junctional intercellular communications by phosphorylation of connexins via protein kinase A [49]. Our results on an increase of connexin 43 and on a decrease of the non-phosphorylated form of connexin 43, when the cells were incubated with db-cAMP for 24 h, as compared to non-treated cells, were consistent with the above reports.

In this regard, our results show that treatment of both HSC-3 and SCC-7 cells with db-cAMP leads to an increase of the bystander effect, as illustrated by the higher levels of toxicity when transfected cells were treated with GCV in the presence of db-cAMP, as compared to transfected cells treated with GCV in the absence of this drug.

The mechanisms by which cell death induced by the HSV-tk/GCV system occurs are not fully understood. Previous studies have shown that the most likely mechanism of cell death is apoptosis [50–53]. Release of cytochrome C and activation of caspases are known to be involved, while the role of p53 protein in the process of cell death is still a subject of some controversy [54]. Aiming at gaining insights into the molecular mechanisms involved in the process of cell death induced by the HSV-tk/GCV system, different experimental approaches were used to evaluate the relative extent of apoptosis *versus* necrosis in HSC-3 cells.

Our results clearly indicate that apoptosis is the main mechanism involved in the death of HSC-3 cells upon their treatment with the HSV-tk/GCV system, this being particularly notorious upon increasing the incubation time with GCV (an increase up to 50% of apoptotic cells after 6 days incubation *versus* a residual value of 10% of necrotic cells over time).

These findings may be relevant for the clinical application of this particular suicide gene therapy approach, since therapeutic strategies involving tumor cell death through apoptosis are advantageous as compared to those inducing necrosis, in which case the release of death factors to medium can cause toxicity in healthy tissue surrounding the tumor.

Our preliminary results in vivo clearly show an advantage of using Tf-lipoplexes for intra-tumoral administration of HSV-tk genes aiming at applying a suicide gene therapy approach. A significant reduction of tumor growth was achieved using these complexes, as compared to that observed with naked plasmid DNA, which may be attributed not only to the ability of the complexes to carry large copies of plasmid DNA, but also to the recognized role of transferrin to promote cell internalization of the associated complexes. Studies are in progress in our laboratory, aiming at optimizing transfection conditions in order to further improve the observed therapeutic effects, namely in terms of tumor size reduction and ultimately of achieving its complete eradication.

Overall, the present study shows that Tf-lipoplexes constitute a promising system for the successful application of suicide gene therapy approaches aiming at treating human oral carcinomas.

Acknowledgements

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