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Evaluation of the antitumoral effect mediated by IL-12 and HSV-tk genes when delivered by a novel lipid-based system

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Abstract

In the present work, we used a novel albumin-associated lipoplex formulation, containing the cationic lipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-ethylphosphocholine (EPOPC) and cholesterol (Chol), to evaluate the antitumoral efficacy of two gene therapy strategies: immuno-gene therapy, mediated by IL-12 gene expression, and "suicide" gene therapy, mediated by HSV-tk gene expression followed by ganciclovir (GCV) treatment. Our data show that, in an animal model bearing a subcutaneous TSA (mouse mammary adenocarcinoma) tumor, intratumoral administration of the albumin-associated complexes containing the plasmid encoding IL-12 results in a strong antitumoral effect, as demonstrated by the smaller tumor size, the higher T-lymphocyte tumor infiltration and the more extensive tumor necrotic and hemorrhagic areas, as compared to that observed in animals treated with control complexes. On the other hand, the application of the "suicide" gene therapy strategy results in a significant antitumoral activity, which is similar to that achieved with the immuno-gene therapy strategy, although involving different antineoplastic mechanisms. For the tested model, albumin-associated complexes were shown to efficiently mediate intratumoral delivery of therapeutic genes, thus leading to a significant antitumoral effect. This finding is particularly relevant since TSA tumors are characterized for being poorly immunogenic, aggressive and exhibiting high proliferation capacity.

Keywords: Cancer gene therapy; "Suicide" gene therapy; Immunotherapy; Gene delivery; Cationic liposomes; Transfection

1. Introduction

Over the last years, gene therapy has emerged as a promising strategy for cancer treatment [1,2]. However, some limitations are associated to the clinical application of gene therapy, the reduced ability to deliver functional therapeutic genes into target cells being the major one. Therefore, research in gene therapy has been focused on the development of suitable carriers that, while exhibiting adequate features for in vivo use, would also mediate efficient intracellular delivery of genetic material [3–6].

Cationic liposome/DNA complexes ("lipoplexes") have been extensively studied aiming at developing appropriate non-viral gene delivery systems [7,8]. Much effort has been devoted to the synthesis of new cationic lipids, selection of different helper lipids and association of proteins or fusogenic peptides aiming at enhancing lipoplex biological activity [4,5]. Our previous observations indicated that association of albumin to lipoplexes strongly increases their transfection activity, namely in the presence of serum [9]. Therefore, in this study we tested the efficacy of an albumin-associated lipoplex formulation, containing the new cationic lipid EPOPC and Chol, prepared at the 4/1 lipid/DNA (+/−) charge ratio, in antitumoral gene therapy strategies.

Among cancer gene therapy approaches, immuno-gene therapy mediated by IL-12 gene expression and "suicide" gene therapy mediated by HSV-tk gene expression, followed by ganciclovir (GCV) treatment, have emerged as promising strategies for cancer treatment [10–13]. IL-12 plays a key antitumoral effect by enhancing the proliferation and the cytotoxic activity of both T and NK cells and inducing the production of IFN-γ and other cytokines, resulting, consequently, in

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tumoral cell death and in the inhibition of both angiogenesis and metastasis formation [10,14,15]. The application of the HSV-tk "suicide" gene therapy results in the conversion of the prodrug GCV into a toxic active metabolite that causes cell death. The cells expressing the HSV-tk metabolize the prodrug to ganciclovir monophosphate, which is further converted into ganciclovir triphosphate by cellular kinases. The resulting guanosin analog either inhibits the DNA polymerase directly and/or is incorporated into cellular DNA, resulting in chain termination and tumoral cell death [16–18]. A key feature of this strategy is the "bystander effect", by which a high percentage of tumoral cell death can occur even when only a low percentage of cells have been transfected [17,19–21].

In the present work, we evaluated the antitumoral activity resulting from the application of the above mentioned therapeutic strategies, using a novel albumin-associated lipoplex formulation. These studies were performed both in vitro, by measuring cell viability, and in an animal model, by determining the tumor size and animal survival, and assessing both tumoral histology and infiltration of T-lymphocytes.

2. Material and methods

2.1. Tumor cell line and mice

TSA is an aggressive and poorly immunogenic cell line established from the first in vivo transplant of a moderately differentiated mammary adenocarcinoma that was generated spontaneously in a BALB/c female mouse (a gift of Dr. M. Colombo, Instituto per lo Studio e la Cura dei Tumor, Milan, Itali) [22]. TSA cells were maintained at 37 °C, under 5% CO_2, in Dulbecco's modified Eagle's medium-high glucose (DMEM-HG) (Irvine Scientific, Santa Ana, CA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO), penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (4 mM). TSA cells grow in monolayer and were detached by treatment with a trypsin solution (0.25%) (Sigma Chemical). For in vitro transfection studies, 0.4×10^5 TSA cells were seeded in 1 ml of medium in 48-well culture plates, 24 h before transfection, and used at 50–70% confluence. For in vivo studies, after being detached by trypsin treated cells were resuspended in saline buffer (PBS), to obtain a cell density of 500×10^3 cells/ml. 200 μl of the cell suspension were immediately injected subcutaneously in the left flank of 8-week-old female BALB/c mice (Charles River Laboratories, Barcelona, Spain), which were handled in accordance with the European Community guidelines.

2.2. Preparation of cationic liposomes and their complexes with DNA

Small unilamellar cationic liposomes (SUV) were prepared from a 1:1 (mol ratio) mixture of EPOPC and Chol, by extrusion of multilamellar liposomes (MLV). Briefly, lipids (Avanti Polar Lipids, Alabaster, AL) dissolved in $CHCl_3$ were mixed at the desired molar ratio and dried under vacuum in a rotatory evaporator. The dried lipid films were hydrated with deionized water to a final lipid concentration of 6 mM (in vitro studies) or 60 mM (in vivo studies) 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 deionized water (in vitro studies) and filter-sterilized utilizing 0.22 μm pore-diameter filters (Schleicher & Schuell). For in vitro studies, lipoplexes were prepared by sequentially mixing 100 μl of a HEPES-buffered saline solution (HBS) (100 mM NaCl, 20 mM HEPES, pH 7.4), with liposomes (volume was dependent on the desired (+/−) lipid/DNA charge ratio) and with 100 μl of HBS solution containing 1 μg of pCMVluc encoding luciferase (a gift of Dr. P. Felgner (Vical, San Diego, CA)). The mixture was further incubated for 15 min at room temperature. For complexes containing albumin (HSA), liposomes were pre-incubated with this protein (32 μg HSA/μg of DNA) for 15 min, followed by a further 15 min incubation with plasmid DNA solution at room temperature. Different plasmids were used: pCMVlacZ encoding β-galactosidase (Gibco BRL, Gaithersburg, USA); pCMVluc (a gift of Dr. P. Felgner, Vical, San Diego, CA); pCMVIL-12 encoding IL-12 (a gift of Prof. D. Mahvi, University of Wisconsin-Madison, Madison, USA); pCMVtk encoding HSV-tk (a gift of Prof. N. Düzgünes, University of Pacific, San Francisco, USA).

2.3. In vitro transfection activity

TSA cells were covered with 0.3 ml of DMEM-HG before lipid/DNA complexes were added. The complexes (containing 1 μg of DNA) were added gently to cells in a volume of 0.2 ml per well. After 4 h incubation (in 5% CO_2 at 37 °C) the medium was replaced with DMEM-HG and the cells were further incubated for 48 h. The cells were then washed twice with phosphate-buffered saline solution (PBS) and 100 μl of lysis buffer (1 mM DTT; 1 mM EDTA; 25 mM Tris-phosphate (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). 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 RLU of luciferase per mg of total cell protein.

2.4. In vitro antitumoral activity

The in vitro antitumoral activity resulting from transfection of the cells mediated by HSA-EPOPC:Chol/DNA (+/−) (4/1) or EPOPC:Chol/DNA (+/−) (4/1) complexes containing the pCMVtk plasmid ("suicide" gene approach), was evaluated in TSA cells. Following 4 h incubation with the complexes or HBS (no transfected cells), the medium was replaced with DMEM-HG containing or not (control cells and transfected cells non-treated with GCV) different concentrations of GCV (1, 25, 50 or 100 μM). Cells were further incubated for 6 days in cultured conditions (in 5% CO_2 at 37 °C). The medium with or without GCV was replaced daily and the cell viability was accessed every other day by a modified Alamar Blue assay [23]. Briefly, 1 ml of 10% (v/v) Alamar Blue dye in complete DMEM-HG medium was added to each well. After 3 h of incubation at 37 °C, 200 μl of the supernatant were collected from each well and transferred to 96-well plates. The absorbance at 570 nm and 600 nm was measured in a Mediators PhL luminometer (Mediators Diagnostika, Vienna, Austria). Cell viability (as a percentage of control cells) was calculated according to the formula $(A_{570}-A_{600})$ of treated cells $\times 100/(A_{570}-A_{600})$ of control cells.

2.5. Ex vivo antitumoral activity

TSA cells were transfected in vitro using the HSA-EPOPC:Chol/DNA (+/−) (4/1) complexes containing the different plasmids (pCMVluc; pCMVIL-12; pCMVtk) or simply incubated with HBS. After 4 h incubation (in 5% CO_2 at 37 °C) with the different complexes or HBS, the medium was replaced with DMEM-HG containing 10% FBS and the cells were further incubated for 24 h. After being detached with trypsin and washed two times with PBS, the cells were resuspended in PBS saline buffer, to obtain a cell density of 500×10^3 cells/ml. 200 μl of this cell suspension (100×10^3 cells) were immediately injected subcutaneously in the left flank of female 8-week-old BALB/c mice (six animals per group). The animals injected with cells previously transfected with the HSV-tk gene (pCMVtk plasmid) were submitted to seven intraperitonial administrations of GCV (75 mg/kg), performed from the day of cell injection during 7 consecutive days. Tumor growth was monitored every 5 days by measuring two perpendicular tumor diameters with a calliper. Mice were sacrificed when the tumor volume reached approximately 1 cm^3.

2.6. In vivo antitumoral activity: tumor implantation and treatment

After being detached with trypsin and washed two times with PBS, TSA cells were resuspended in PBS saline buffer, to obtain a final cell density of 500×10^3 cells/ml. 200 μl of this cell suspension (100×10^3 cells) were immediately injected subcutaneously in the left flank of female 8-week-old BALB/c mice. When the

tumor volume reached approximately 0.02 cm^3, usually 9 days after cell injection, the animals were submitted to different treatments (six animals per group). These consisted of four intratumoral administrations of 50 μl of the various HSA-EPOPC:Chol/DNA (+/−) (4/1) complexes (prepared with different plasmids and using 10, 20 or 40 μg of DNA/administration) at days 0, 3, 5 and 7. The animals that were treated with the complexes containing the pCMVtk plasmid were submitted to five (100 mg/kg) or seven (75 mg/kg) intraperitonial administrations of GCV, performed from the third day after the first treatment during 5 or 7 consecutive days, respectively. Tumor growth was monitored every 5 days by measuring two perpendicular tumor diameters with a calliper. Mice were sacrificed when the tumor volume reached approximately 1 cm^3.

2.7. In vivo antitumoral activity: tumoral infiltration of T cells and histological analysis

The subcutaneous tumors induced with TSA cells in female 8-week-old BALB/c mice were treated as mentioned in the previous section. Twelve days after the beginning of treatment, animals were sacrificed and tumors were extracted, embedded in OCT compound (Miles Laboratories, Inc., Elkhart, IN), snap-frozen in liquid nitrogen and stored at − 80 °C. For immunohistochemistry, 6 μm cryostat sections were fixed in acetone, blocked using 10% FBS and incubated for 1 h with a FITC-labeled rat antimouse mAb against CD3 protein (Pharmingen, BD Biosciences, San José, USA). Unbound antibody was removed by washing with PBS and tumor sections were counterstained with hematoxylin. Sections were then observed by fluorescence microscopy using a Zeizz Axiovert 200 microscope (Zeizz, Göttingen, Germany) and the number of immunostained cells was counted under a microscopic field at 400× magnification. For histological analysis, 6 μm cryostat tumor sections were fixed in acetone and stained with hematoxylin and eosin (H&E) and then observed under a Nikon Eclipse E600 microscope (Nikon, Japan).

3. Results

3.1. In vitro biological activity of the cationic liposome/DNA complexes

The results presented in Fig. 1 show the influence of complex composition and charge ratio (+/−) on their biological activity in TSA cells, as assessed by luciferase gene expression. As evident from this figure, the biological activity mediated by the complexes EPOPC:Chol/DNA prepared at 2/1 (+/−) charge ratio is higher than that observed when these are prepared at 1/1 and 4/1 (+/−) charge ratios. Moreover, it is possible to verify that association of albumin to the EPOPC:Chol/DNA complexes strongly potentiates their biological activity for any charge ratio. However, the highest biological activity increase, induced by albumin association, was observed for complexes prepared at 4/1 (+/−) charge ratio, this being the most efficient HSA-lipoplex formulation (Fig. 1). Since HSA-EPOPC:Chol/DNA (+/−) (4/1) complexes are those mediating the highest levels of transfection and, as demonstrated in our previous work [9], exhibiting favourable properties for gene delivery (high degree of DNA protection, surface charge close to neutrality and reduced interaction with serum components), this formulation was selected for further studies involving the delivery of therapeutic genes both *in vitro* and *in vivo* cancer models.

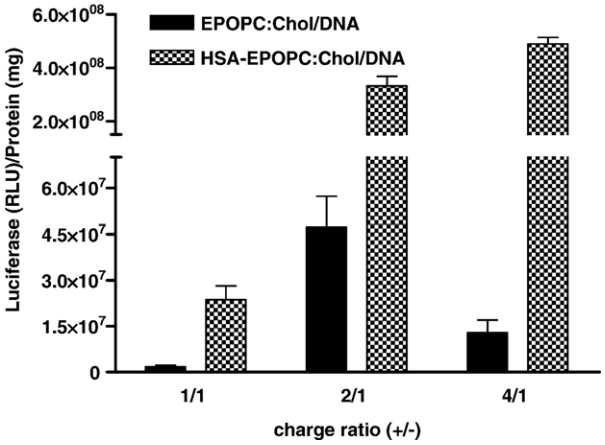

Fig. 1. Effect of complex composition and charge ratio (+/−) on luciferase gene expression in TSA cells. Cells were covered with 0.3 ml of DMEM-HG prior to the addition of cationic liposome/DNA complexes. EPOPC:Chol liposomes pre-incubated or not with HSA (32 μg/μg of DNA), were complexed with 1 μg of pCMVluc at the indicated lipid/DNA charge ratios. After 4 h incubation, the medium was replaced with DMEM-HG and the cells were further incubated for 48 h. The level of luciferase gene expression was evaluated as described in Materials and methods. The data are expressed as RLU of luciferase per mg of total cell protein (mean ± standard deviation obtained from triplicates), and are representative of at least three independent experiments.

3.2. In vitro antitumoral activity of the HSV-tk/GCV strategy

Before testing the antitumoral effect of HSV-tk/GCV approach in an animal model, we studied the in vitro sensitivity of TSA cells to this strategy, in order to predict its in vivo therapeutic potential.

Fig. 2 shows the cytotoxic effect of ganciclovir in TSA cells previously transfected with EPOPC:Chol/DNA (+/−) (4/1) or HSA-EPOPC:Chol/DNA (+/−) (4/1) complexes, containing the HSV-tk gene. The results are expressed as the percentage of cell viability with respect to control cells, cells that were not treated with GCV. The results presented show that GCV treatment promotes a significant death of TSA transfected cells, which increases with both incubation time and GCV concentration. However, the extent of cytotoxicity observed for TSA cells transfected with EPOPC:Chol/DNA (+/−) (4/1) complexes, followed by 6 days of GCV treatment (100 μM), was much lower than that observed for TSA cells transfected with HSA-EPOPC:Chol/DNA (+/−) (4/1) complexes under the same experimental conditions. Two days after transfection with HSA-EPOPC:Chol/DNA (+/−) (4/1) complexes followed by GCV treatment, only a slight decrease in the cell viability was observed. However, 6 days after transfection following GCV treatment a significant extent of cytotoxicity is achieved for concentrations of GCV higher than 25 μM, approximately 95% of cell death being observed upon cell treatment with 100 μM GCV. This high cytotoxicity is not due to the drug itself or transfection protocol, since 6 days after incubation of non-transfected TSA cells with 100 μM GCV or transfection in the absence of GCV no significant cell death was observed (Fig. 2).

3.3. Ex vivo antitumoral activity of the gene therapy strategies

In order to evaluate the potential of the different therapeutic strategies for ex vivo application, cells were transfected with the

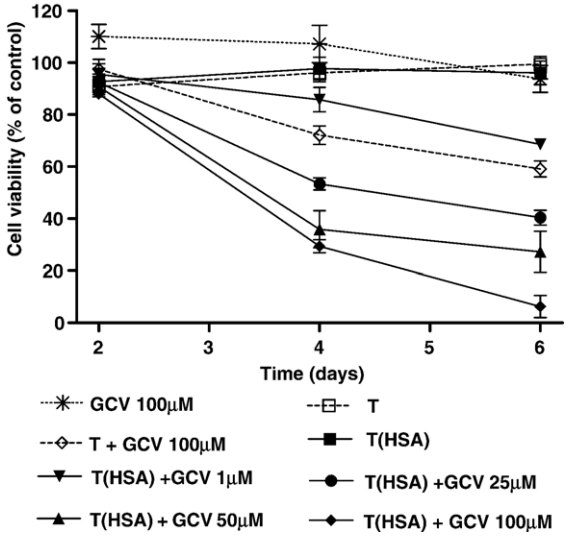

Fig. 2. Effect of ganciclovir concentration on the viability of TSA cells. Cells were covered with 0.3 ml of DMEM-HG before the addition of 200 μl of HSA-EPOPC:Chol/DNA (+/−) (4/1) complexes (transfected cells-T(HSA): solid line) or 200 μl of EPOPC:Chol/DNA (+/−) (4/1) complexes (transfected cells-T: broken line), containing the pCMVtk plasmid, or 200 μl of HBS (non-transfected cells: dot line). After 4 h incubation, the medium was replaced with DMEM-HG (control cells and transfected cells non-treated with GCV) or with DMEM-HG containing different concentrations of ganciclovir (1, 25, 50 or 100 μM) and the cells were further incubated for 6 days. The medium with or without GCV was replaced daily and cell viability was assessed by the Alamar Blue assay, as described in Materials and methods. Cell viability (as a percentage of control cells) was calculated according to the formula $(A_{570}-A_{600})$ of treated cells $\times 100/(A_{570}-A_{600})$ of control cells.

complexes containing the therapeutic genes before being inoculated to induce the tumor. The percentage of transfected cells was approximately 15%.

Fig. 3 shows the results of tumor growth after a subcutaneous injection, in the left flank of female 8-week-old BALB/c mice, of 100×10^3 TSA cells previously submitted to different transfection conditions. As can be observed, inoculation of HBS-treated cells induces tumors that exhibit a higher size and growth kinetics than cells previously transfected with HSA-EPOPC:Chol/DNA (+/−) (4/1) complexes, containing the luciferase gene (control complexes). On the other hand, tumors resulting from subcutaneous injection of cells previously transfected with HSA-EPOPC:Chol/DNA (+/−) (4/1) complexes containing the IL-12 gene or the HSV-tk gene (in this case mice were submitted to seven intraperitonial administrations of GCV (75 mg/kg), performed from the day of cell injection during 7 consecutive days) are not only much smaller, but begin to develop later, as compared to those induced upon inoculation of cells transfected with the control complexes. Moreover, under both conditions, 33 days after inoculation of the cells, the tumor volume is approximately three times smaller than that of animals injected with cells previously transfected with the control complexes (Fig. 3). It should be emphasized that differences between tumor sizes cannot be due to differences in the viability of injected cells, since for all experimental conditions each animal was injected with 100×10^3 viable TSA cells.

3.4. In vivo antitumoral activity of the gene therapy strategies

Three distinct therapeutic strategies were evaluated in vivo using the HSA-EPOPC:Chol/DNA (+/−) (4/1) complexes: the "suicide" gene therapy, previously tested in vitro, involving intratumoral administration of complexes containing the HSV-tk gene followed by GCV treatment; the immuno-gene-therapy, involving intratumoral administration of complexes containing the IL-12 gene; the combination of both strategies, involving the intratumoral administration of complexes containing both the IL-12 and the HSV-tk genes, and subsequent treatment with GCV. These strategies were tested in female BALB/c mice bearing a subcutaneous tumor with a volume of approximately 0.02 cm^3, resulting from the inoculation of 100×10^3 TSA cells in the left flank 9 days before the first gene treatment. These subcutaneous tumors are characterized for being aggressive, poorly immunogenic and exhibiting high proliferation capacity [14,24].

Fig. 4 shows the results obtained for the inhibition of tumor growth after application of the different gene therapy strategies. These consisted of four intratumoral administrations of 50 μl of HSA-EPOPC:Chol/DNA (+/−) (4/1) complexes or HBS (Fig. 4B), performed on days 0, 3, 5 and 7 after tumor implantation. The animals treated with complexes containing the HSV-tk gene were submitted to seven intraperitonial administrations of GCV (75 mg/kg), performed from day 3 during 7 consecutive days. As shown in Fig. 4A, four intratumoral administrations of the complexes containing 40 μg of plasmid encoding either IL-12 or HSV-tk or four intratumoral administrations of complexes

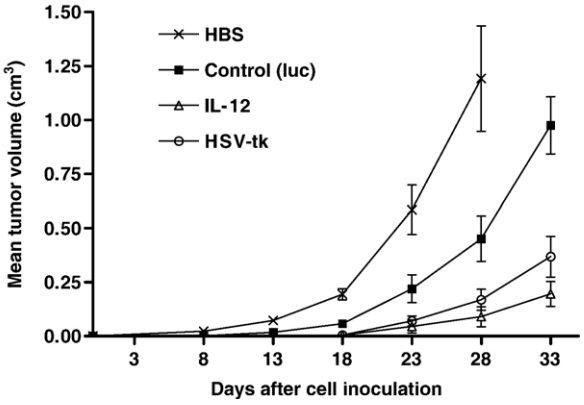

Fig. 3. Effect of the in vitro pre-treatment of TSA cells on the subcutaneous tumor growth in BALB/c mice. TSA cells were transfected in vitro with the HSA-EPOPC:Chol/DNA (+/−) (4/1) complexes, containing the different plasmids (pCMVIL-12, pCMVtk or pCMVluc), or incubated with HBS. After 4 h incubation, the medium was replaced with DMEM-HG containing 10% FBS and the cells were further incubated for 24 h. Cells were then resuspended in PBS saline buffer, in order to obtain a final cell density of 500×10^3 cells/ml, and 200 μl of the cell suspension were immediately injected subcutaneously in the left flank of female 8-week-old BALB/c mice. Animals injected with cells transfected with the complexes containing the pCMVtk plasmid were submitted to seven intraperitonial administrations of GCV (75 mg/kg), performed from the day of cell injection during 7 consecutive days. Tumor growth was monitored every 5 days by measuring two perpendicular tumor diameters with a calliper. The data are expressed as mean tumor volume (cm^3) and represent the mean ± standard deviation obtained from six animals. Mice were sacrificed when the tumor volume reached approximately 1 cm^3.

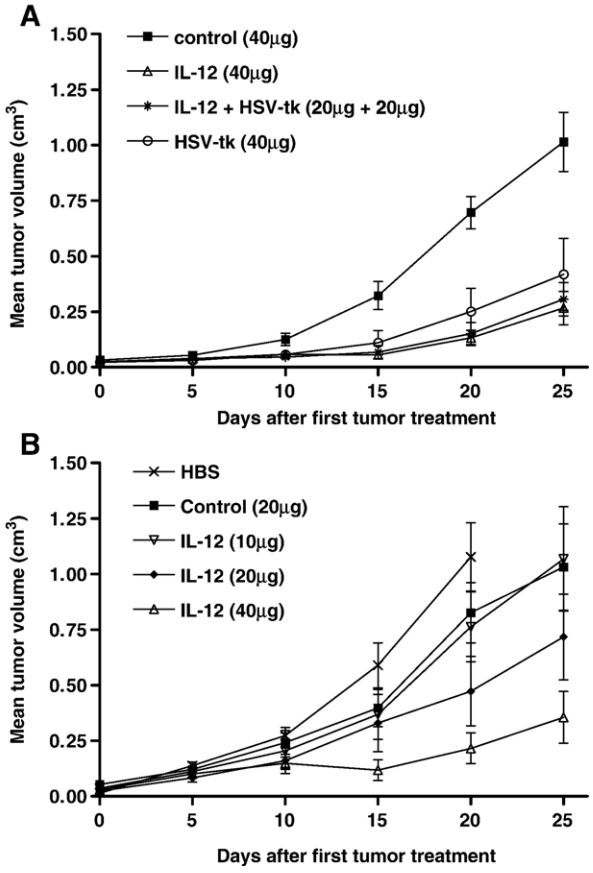

Fig. 4. Antitumoral effect of different gene therapy strategies against subcutaneous tumors induced with TSA cells in BALB/c mice. Animals were treated with HSA-EPOPC:Chol/DNA (+/−) (4/1) complexes containing: (A) 40 μg of plasmid DNA or (B) different amounts of plasmid DNA (10, 20 or 40 μg). 200 μl of TSA cells (100×10^3 cells) were injected subcutaneously into the left flank of female 8-week-old BALB/c mice. When tumor volume reached approximately 0.02 cm^3, usually 9 days after cell injection, the animals were submitted to different treatments, which consisted of four intratumoral administrations of 50 μl of HSA-EPOPC:Chol/DNA (+/−) (4/1) complexes or 50 μl of HBS (non-treated) performed on days 0, 3, 5 and 7. The therapeutic complexes contained different amounts of the pCMVIL-12 plasmid (IL-12) or pCMVtk plasmid (HSV-tk), while control complexes contained pCMVluc plasmid. Animals treated with the complexes containing the pCMVtk plasmid were submitted to seven intraperitonial administrations of GCV (75 mg/kg), performed from day 3 after the first gene treatment during 7 consecutive days. Tumor growth was monitored every 5 days by measuring two perpendicular tumor diameters with a calliper. The data are expressed as mean tumor volume (cm^3) and represent the mean±standard deviation obtained from six animals. Mice were sacrificed when the tumor volume reached approximately 1 cm^3.

containing 20 μg of each plasmid, induced a significant antitumoral effect ($P<0.01$) when compared to that resulting from four administrations of control complexes (complexes containing 40 μg of plasmid encoding luciferase). The antitumoral activity of the combined gene therapy strategy was similar to that observed when only immuno-gene therapy or "suicide" gene therapy were tested (Fig. 4A). It should be noted that, in the group of six animals submitted to the immuno-gene therapy treatment, a complete tumor regression was observed in one of the mice.

Fig. 4B shows the importance of the therapeutic gene dose on the observed antitumoral effect. As illustrated, four intratumoral administrations of complexes containing 10 μg of plasmid encoding IL-12 have the same effect as that for control complexes, while four intratumoral administrations of complexes prepared with 40 μg of plasmid containing the IL-12 gene resulted in a significant reduction in tumor growth ($P<0.01$), when compared to that of control complexes. However, four intratumoral administrations of control complexes promoted, by itself, a slight antitumoral effect ($P<0.05$) when compared to that observed in HBS-treated animals (Fig. 4B).

It should be noted that parallel studies involving two intratumoral administrations of the complexes, resulted in a smaller antitumoral effect than that achieved upon four intratumoral administrations (data not show).

3.5. In vivo antitumoral activity: evaluation of T-lymphocyte tumor infiltration

The antitumoral activity of the immuno-gene therapy and "suicide" gene therapy strategies involves the activation of the immune system against the neoplastic tissue, although most likely through different pathways. Since T-lymphocytes greatly contribute to the antitumoral responses induced by the immune system, we evaluated the tumoral infiltration of T-lymphocytes following application of the above mentioned strategies.

Fig. 5 illustrates the tumoral infiltration of T-lymphocytes, as assessed by immunohistochemistry using a rat antimouse FITC-labeled mAb against CD3 protein, 12 days after the first gene therapy treatment of TSA subcutaneous tumors. Tumors from animals submitted to four intratumoral administrations of control complexes (HSA-EPOPC:Chol/DNA (+/−) (4/1) complexes prepared with 40 μg of plasmid containing the luciferase gene) presented a more extensive T-lymphocyte infiltration than those from non-treated animals. However, for animals treated with four intratumoral administrations of HSA-EPOPC:Chol/DNA (+/−) (4/1) complexes, containing 40 μg of plasmid encoding IL-12, extensive T-lymphocyte infiltration was elicited, this being much more significant than that registered in tumors from animals treated with control complexes. It is interesting to observe that in the case of animals submitted to four intratumoral administrations of the complexes, containing 40 μg of plasmid encoding HSV-tk, T-lymphocyte infiltration was higher than that in tumors from animals treated with control complexes, but less extensive than in tumors from animals submitted to the immuno-gene therapy protocol (Fig. 5).

3.6. In vivo antitumoral activity: analysis of tumor histology

The studies of tumoral infiltration of T-lymphocytes were complemented by tumor histological analysis, performed 12 days after the first gene therapy treatment of TSA subcutaneous tumors, using the hematoxylin and eosin assay. The results shown in Fig. 6 demonstrate the absence of any significant necrotic and hemorrhagic areas or leukocyte infiltration in tumors from non-treated animals. In the case of tumors from animals submitted to four intratumoral administrations of control

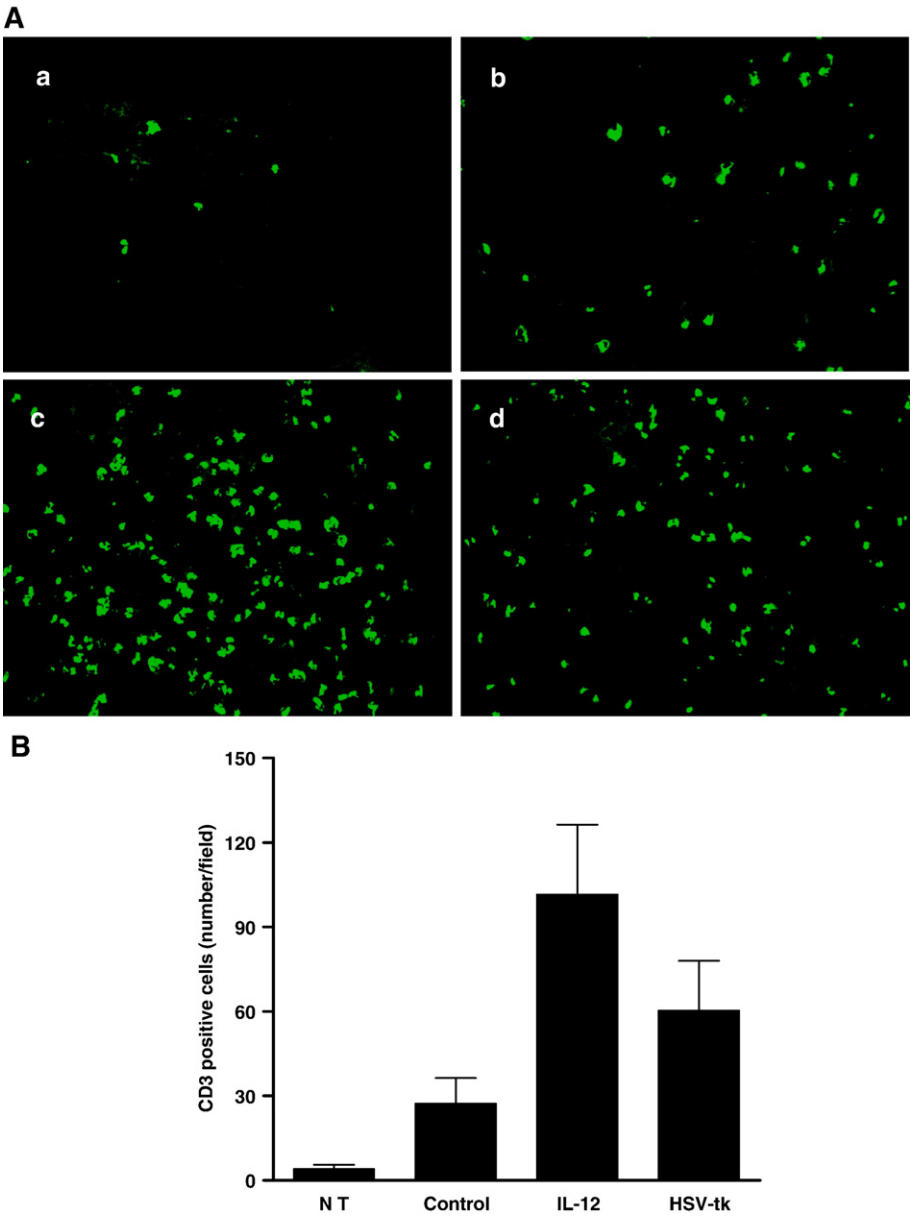

Fig. 5. Effect of different gene therapy strategies on tumor T lymphocyte infiltration. (A) Representative images of T-lymphocyte tumor infiltration obtained by fluorescence microscopy (original magnification: ×400); (B) number of immunostained cells counted under a ×400 microscopic field (mean±standard deviation obtained from 10 fields). (a) and (NT) tumor from non-treated animal; (b) and (Control) tumor from animal treated with complexes containing pCMVluc plasmid; (c) and (IL-12) tumor from animal treated with complexes containing pCMVIL-12 plasmid; (d) and (HSV-tk) tumor from animal treated with complexes prepared with pCMVtk plasmid. Induction of tumor and treatment was performed as described in the legend to Fig. 4. Twelve days after the first of gene therapy treatment, animals were sacrificed, tumors were removed and 6 μm cryostat sections were incubated with a rat antimouse FITC-labeled mAb against CD3 protein.

complexes (HSA-EPOPC:Chol/DNA (+/−) (4/1) complexes containing 40 μg of plasmid encoding luciferase), a few necrotic and hemorrhagic areas as well as low leukocyte infiltration were observed. On the other hand, tumors from animals treated with four intratumoral administrations of complexes containing 40 μg of plasmid encoding either IL-12 or HSV-tk presented extensive areas of necrosis and hemorrhage with leukocyte infiltration in all cases. Although a significant leukocyte infiltration was observed under both therapeutic conditions, this was more evident for the immuno-gene therapy treatment than for the "suicide" gene therapy strategy, not only near these areas of destruction but also in the other areas of the tumor.

4. Discussion

The results obtained in this work show that association of albumin to EPOPC:Chol/DNA lipoplexes, through the establishment of electrostatic interactions between the negative charges of the protein and the positive charge of cationic liposomes, significantly increases transfection activity in TSA cells (Fig. 1). These observations are in agreement with our previous published results which have shown that lipoplexes containing the new cationic lipid EPOPC exhibit a higher transfection activity (more than 2-fold) than the conventional DOTAP:Chol/DNA lipoplexes and that association of albumin

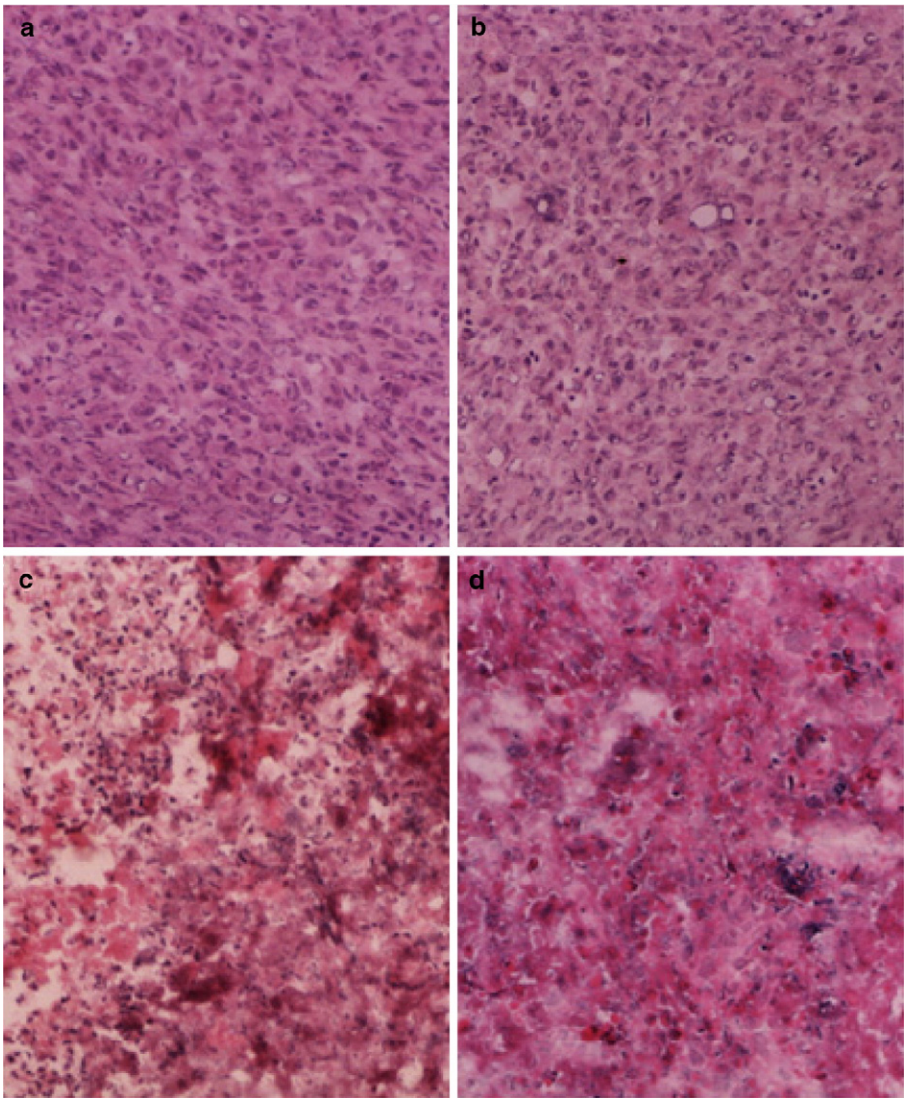

Fig. 6. Effect of different gene therapy strategies on the tumor histology. Representative images: (a) tumor from non-treated animal; (b) tumor from animal treated with complexes prepared with pCMVluc plasmid; (c) tumor from animal treated with complexes containing pCMVIL-12 plasmid; (d) tumor from animal treated with complexes prepared with pCMVtk plasmid. Induction of tumor and treatment was performed as described in the legend to Fig. 4. Twelve days after the first gene therapy treatment, animals were sacrificed, tumors were removed and 6 μm cryostat sections were stained with hematoxylin and eosin (H&E).

strongly increases transfection activity in COS-7 cells [9]. Similarly to what was observed for COS-7 cells, an enhancing effect on transfection of TSA cells was observed upon association of albumin to EPOPC:Chol/DNA lipoplexes, this effect being particularly pronounced at the 4/1 lipid/DNA (+/−) charge ratio (Fig. 1).

Although the exact mechanism of the enhancing effect induced by albumin on the biological activity of the lipoplexes is not known, previous experiments showed that the HSA-lipoplexes could bind non-specifically to cell surface receptors, analogous to scavenger receptors, which in turn mediate their endocytosis, resulting in a transfection enhancement [25,26]. Moreover, albumin can facilitate the escape of DNA from the endocytotic pathway, since this protein has been described as being able to undergo a low pH-induced conformational change, thereby acquiring fusogenic properties [27,28]. Thus, the partial protonation of HSA at endosomal pH and its subsequent interaction with the endosome membrane may be involved in the destabilization of the latter and, consequently, in the cytoplasmic delivery of DNA.

Our previous results also showed that, like in vitro, the HSA-EPOPC:Chol/DNA (+/−) (4/1) complexes are significantly more efficient in mediating in vivo transfection than EPOPC:Chol/DNA (+/−) (4/1) complexes, since upon intravenous administration the biological activity observed with the former was much higher (more than 5-fold) than with the latter [9]. However, the highest levels of transfection for both formulations (complexes with and without albumin) have been observed in the lung. This could be most likely attributed to the large size of the complexes that promotes their rapid elimination from the blood circulation, mainly due to their retention in the lung. This fact limits the intravenous administration of lipoplexes for therapeutic applications that do not involve the lung or blood tissues. However, their in situ

administration could be feasible for some therapeutic approaches, such as those involving solid tumors.

As observed from our in vitro and in vivo studies using a reporter gene, the experiments performed in vitro, using the HSV-tk/GCV "suicide" gene therapy strategy, show that HSA-EPOPC:Chol/DNA (+/−) (4/1) complexes are significantly more efficient in mediating transfection than EPOPC:Chol/DNA (+/−) (4/1) complexes. In fact, the extent of cell death observed for TSA cells transfected with the albumin-associated complexes was much higher than that observed for complexes without albumin (Fig. 2). The application of this gene therapy strategy mediated by HSA-EPOPC:Chol/DNA (+/−) (4/1) lipoplex formulation, resulted in approximately 100% TSA cell death, showing the therapeutic potential of this strategy. This strong cytotoxicity shows the occurrence of the bystander effect, since the percentage of transfected cells (approximately 15%) was much lower than the percentage of cell death. The bystander effect is due to transfer of toxic GCV metabolites from HSV-tk transfected cells, through gap junctions and/or by phagocytosis of "apoptotic" vesicles (containing toxic GCV metabolites), to non-transfected cells [17,19,29–31].

Therefore, based on the above mentioned findings, we tested the efficacy of this new albumin-associated lipoplex formulation (HSA-EPOPC:Chol/DNA (+/−) 4/1 complexes) in antitumoral gene therapy strategies.

The antitumoral activity studies performed in the animal model (female BALB/c mice bearing a flank TSA tumor) show that application of any of the three tested therapeutic strategies, "suicide" gene therapy, immuno-gene therapy and the combination of both strategies, resulted in a significant antitumoral effect. In fact, four intratumoral administrations of the HSA-EPOPC:Chol/DNA (+/−) 4/1 complexes containing 40 μg of plasmid encoding either IL-12 or HSV-tk, or 20 μg of each plasmid, resulted in a significant reduction in tumor growth when compared to the effect of four intratumoral administrations of control complexes (the same formulation containing 40 μg of plasmid encoding luciferase) (Fig. 4A). However, control complexes induced, by itself, a slight antitumoral effect, since tumors from animals treated with these complexes were slightly smaller than those from non-treated (data not shown) or treated animals with HBS (Fig. 4B).

Several studies have demonstrated that lipoplex administration into the lung lumen promotes the production of pro-inflammatory cytokines including TNF-α, IFN-γ and IL-12, which are accompanied by a pulmonary influx of polymorphonuclear cells [32,33]. Moreover, it has been shown that intravenous administration of lipoplexes into mice promotes a similar effect, inducing an immune response that limits subsequent administrations of lipoplexes within a short time period [34]. On the other hand, it has been shown that this effect is not elicited by the lipid component of the cationic liposome/DNA complexes, by itself, and cannot also be attributed to the presence of endotoxins in the plasmid preparation [32–35]. It has been reported that the cytosine–phosphate–guanine (CpG) motifs, present in the DNA plasmid vectors, are responsible for most of the immunostimulatory effects of cationic liposome/DNA complexes, since the methylation or elimination of these motifs resulted in a strong reduction in cytokine production [36–38]. In this context, it is important to emphasize that reduction of tumor growth has been observed following administration of lipoplexes prepared with plasmids lacking therapeutic genes but containing CpG motifs, which is not achieved upon methylation of these sequences [39–41].

Taking into account these findings, the slight antitumoral effect observed after four intratumoral administrations of control HSA-EPOPC:Chol/DNA (+/−) 4/1 complexes was probably due to immunostimulation provoked by CpG motifs, present in the plasmid DNA of the complexes [41]. However, in agreement with other authors, this immunomodulatory effect was enhanced by the complexes, particularly, by the presence of human albumin [42]. Thus, HSA association to lipoplexes may contribute to the antitumoral effect of the tested therapeutic strategies not only by strongly increasing the expression of the therapeutic gene but also leading to an enhancement of the immune response against the tumor [9,42]. The results on the evaluation of T-lymphocyte tumoral infiltration, showing that tumors from animals treated with control complexes present a higher T-lymphocyte infiltration than those from non-treated animals, reflect the immunostimulatory effect of these complexes (Fig. 5). Moreover, results from the histological studies (Fig. 6), showing the presence of some necrotic and hemorrhagic areas and some leukocyte infiltration in mice treated with control complexes, further confirm the involvement of an immune response, in agreement with results reported by other authors [39–41].

On the other hand, the intratumoral administration of the HSA-EPOPC:Chol/DNA (+/−) 4/1 complexes containing the IL-12 gene (prepared with 40 μg of plasmid) resulted in a strong antitumoral effect, as shown by the significant tumor regression (Fig. 4), high T-lymphocyte infiltration (Fig. 5) and the extensive necrotic and hemorrhagic areas (Fig. 6). Although this antitumoral effect was essentially due to the IL-12 expression, a small contribution to this antineoplastic action resulted from the composition of complexes, as discussed above [39–42]. The IL-12 antitumoral effect is due to its capacity to promote proliferation and cytotoxic activity of T-lymphocytes and NK cells, which induce tumor cell killing and damaging of tumor vessels. Moreover, these IL-12 activated cells produce high amounts of INF-γ and others cytokines, such as TNF-α, which strongly further contribute to tumoral cell death and vascular destruction, as well as to angiogenesis inhibition [14,15,43–47]. Among the released cytokines, INF-γ is the main effector of IL-12 antitumor activities, by promoting processes like direct destruction of tumoral cells and angiogenesis inhibition [14,15]; induction of classes I and II MHC glycoprotein expression in tumor cells, thereby increasing their recognition by the immune system [14,17]; production of chemokines, such as IP-10, MIG and other factors by tumoral and endothelial cells and by cells from the immune system, which inhibit tumor angiogenesis and cause vascular damage [46,47].

The application of the "suicide" gene therapy strategy resulted in a significant antitumoral activity that was similar to that observed for the immuno-gene therapy strategy, although these two approaches involve different antineoplastic mechanisms. As previously referred, the "suicide" gene therapy strategy benefits from the bystander effect, which is due to the transfer of toxic GCV metabolites from HSV-tk-transfected to non-transfected tumor cells [17,19,29–31]. However, in vivo, this cell death enhancing effect is also due to HSV-tk transfection and necrosis of tumor endothelial cells which cause vessel rupture and ischemia, thereby inducing a hemorrhagic tumor necrosis. Moreover, in vivo bystander effect also include local inflammatory responses of the cellular immune system probably induced by the release of soluble factors, like TNF-α and IL-1, from HSV-tk-transfected tumor cells and/or the release of cellular debris resulting from the necrosis process [19–21]. It is possible that these mechanisms have been responsible for the significant antineoplastic effect observed after four intratumoral administrations of HSA-EPOPC:Chol/DNA (+/−) (4/1) complexes, containing 40 μg of plasmid encoding HSV-tk, and subsequent GCV administration (Fig. 4A). Our results from the evaluation of T-lymphocyte tumoral infiltration (Fig. 5) and histological studies (Fig. 6) demonstrated the existence of a strong antitumoral action, involving T-lymphocyte infiltration and the presence of necrotic and hemorrhagic areas at a much higher extent than that observed in tumors from animals treated with control complexes. These findings, which are in agreement with those reported by others authors, indicate that the observed host immune responses were involved in the bystander effect of the HSV-tk/GCV "suicide" gene therapy strategy [19–21].

The combination of both immuno-gene therapy and "suicide" gene therapy strategies, through four intratumoral administrations of the HSA-EPOPC:Chol/DNA (+/−) 4/1 complexes containing 20 μg of plasmid encoding IL-12 and 20 μg of plasmid containing the HSV-tk gene, followed by GCV administration, also resulted in a significant antitumor activity when compared to the effect of four intratumoral administrations of control complexes (Fig. 4A). In spite of containing only 20 μg of plasmid encoding IL-12 the observed antitumoral effect mediated by the HSA-associated complexes was similar to that achieved for the best immuno-gene therapy condition (40 μg of plasmid encoding IL-12). This was probably due to the antitumoral action induced by the HSV-tk/GCV strategy, involving the mechanisms referred to above, which compensated the lower immunostimulation elicited by the smaller amount of IL-12 produced in the tumor.

Overall, the present study demonstrates that HSA-EPOPC:Chol/DNA (+/−) 4/1 complexes constitute a promising system for the successful application of any of the tested antitumoral strategies, particularly considering that TSA tumors are characterized for being poorly immunogenic, aggressive and exhibiting high proliferation capacity. However, studies on the optimization of these strategies need to be addressed, namely in terms of extent and duration of transgene expression, aiming at achieving a complete tumor regression.

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