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ScienceDirect
Biochimica et Biophysica Acta 1768 (2007) 1093–1102
Biochimica et Biophysica Acta BBA
www.elsevier.com/locate/bbamem

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

H. Faneca [a], A.S. Cabrita [b], S. Simões [a,c], M.C. Pedroso de Lima [a,d,*]

[a] Center for Neuroscience and Cell Biology, University of Coimbra, 3000 Coimbra, Portugal
[b] Institute of Experimental Pathology, Faculty of Medicine, University of Coimbra, 3000 Coimbra, Portugal
[c] Laboratory of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra, 3000 Coimbra, Portugal
[d] Department of Biochemistry, Faculty of Science and Technology, University of Coimbra, 3000 Coimbra, Portugal

Received 3 August 2006; received in revised form 30 November 2006; accepted 5 December 2006
Available online 8 January 2007

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

* Corresponding author. Tel.: +351 239 820 190; fax: +351 239 853 607.
E-mail address: mdelima@ci.uc.pt (M.C. Pedroso de Lima).

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doi:10.1016/j.bbamem.2006.12.017

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