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Original article

Interferon-β gene transfer induces a strong cytotoxic bystander effect on melanoma cells

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ABSTRACT

A local gene therapy scheme for the delivery of type I interferons could be an alternative for the treatment of melanoma. We evaluated the cytotoxic effects of interferon-β (IFNβ) gene lipofection on tumor cell lines derived from three human cutaneous and four canine mucosal melanomas. The cytotoxicity of human IFNβ gene lipofection resulted higher or equivalent to that of the corresponding addition of the recombinant protein (rhIFNβ) to human cells. IFNβ gene lipofection was not cytotoxic for only one canine melanoma cell line. When cultured as monolayers, three human and three canine IFNβ-lipofected melanoma cell lines displayed a remarkable bystander effect. As spheroids, the same six cell lines were sensitive to IFNβ gene transfer, two displaying a significant multicell resistance phenotype.

The effects of conditioned IFNβ-lipofected canine melanoma cell culture media suggested the release of at least one soluble thermolabile cytotoxic factor that could not be detected in human melanoma cells. By using a secretion signal-free truncated human IFNβ, we showed that its intracellular expression was enough to induce cytotoxicity in two human melanoma cell lines. The lower cytoplasmatic levels of reactive oxygen species detected after intracellular IFNβ expression could be related to the resistance displayed by one human melanoma cell line. As IFNβ gene transfer was effective against most of the assayed melanomas in a way not limited by relatively low lipofection efficiencies, the clinical potential of this approach is strongly supported.

1. Introduction

Malignant melanoma is an aggressive cancer whose incidence continues to increase worldwide [1,2]. Melanomas most often derive from epidermal melanocytes of the skin, although they can also derive from non-cutaneous melanocytes such as those lining the choroidal layer of the eye, gastrointestinal and urogenital mucosal surfaces, or the meninges [3]. Canine malignant melanoma appears clinically similar to human melanoma. Being chemo and radioresistant, both diseases do not respond well to treatment with conventional biological response modifiers and share similar metastatic phenotypes and site selectivity [4–6]. While some different veterinary immunogene therapy trials were attempted [7], the *in vitro* screening of new approaches can provide new options of effective treatments for the canine disease. Once safety and efficacy are assessed in veterinary clinical trials, the successful treatments could be readily translated for testing on human melanoma patients [8].

Systemic treatment with IFNα-2b is a FDA approved adjuvant therapy for patients with stage IIb or III resected melanoma. While improving disease free survival (3.8 *vs.* 2.8 years) [9], the overall survival advantage associated with this treatment is relatively small [10]. IFNβ has antitumor effects against melanoma, and in general is more potent than IFNα [11–13]. However in a phase II clinical trial for metastatic melanoma, high doses of IFNβ displayed a low response rate while increased serum levels of pro-apoptotic cytokines (TRAIL and IL-1) and immunomodulatory and anti-angiogenic chemokines (CXCL10 and CCL8); and diminished the levels of pro-angiogenic peptides VEGF and CXCL5 [12]. Since the half life of circulating interferons is 3–5 h, the lack of sustained levels could be responsible of their inefficacy for inhibiting or eradicating solid tumors [14,15]. A local nonviral gene therapy mediated approach for the delivery of this cytokine could be an alternative strategy for IFN-based therapy for melanoma, enabling a sustained exposure to IFN protein produced

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by both tumor and non-tumor cells. Interferons can display direct antitumor activity and significant bystander effect, the latter characterized by the activation of host effector cells, the enhancement of apoptosis, and the inhibition of angiogenesis [16].

Multicell spheroids constitute an *in vitro* cell culture model with *in vivo* tumor microenvironmental features that are not present in monolayer cultures. Spheroids display tridimensional intercellular interactions, higher levels of anti-apoptotic proteins and inner regions with a low cell growth rate due to lower availability of oxygen and nutrients. Spheroids rather than monolayer based assays are better predictors of the responses to the treatments for solid tumors [17–20].

As it was reported earlier, IFNβ gene lipofection was cytotoxic for various tumor cell types including *M8* melanoma cell line, and there is evidence supporting a strong bystander effect on *EW7* Ewing sarcoma cell line [21]. To get a deeper insight on the subject, we explored the direct cytotoxic effects of IFNβ gene lipofection on monolayers and spheroids of three cell lines derived from cutaneous human melanoma and four cell lines derived from mucosal canine melanoma.

2. Materials and methods

2.1. Cell cultures

Cultured cells derived from four surgically excised oral (*Bk*, *Ch*, *Ol*) and ocular (*Ak*) canine melanomas were obtained by enzymatic digestion of tumor fragments with 0.01% Pronase (Sigma, St. Louis, MO) and 0.035% DNase (Sigma) or by mechanical disruption in serum free culture medium [19]. Both canine melanoma and human cutaneous melanoma *M8* [22], *A375* [23] and *SB2* [23] were cultured as monolayers and spheroids at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 with DMEM/F12 medium (Invitrogen, Carlsbad, CA) containing 10% FBS (PAA, Germany), 10 mM HEPES (pH 7.4) and antibiotics. Serial passages were done by trypsinization (0.25% trypsin and 0.02% EDTA in PBS) of sub-confluent monolayers.

2.2. Plasmids

psCMV plasmids carrying the *Escherichia coli* β-galactosidase gene (psCMV-βgal) [24], human IFNβ gen (psCMV-hIFNβ) [21] or canine IFNβ gene (psCMV-cIFNβ) [25] were amplified in *Escherichia coli* DH5α (Invitrogen), grown in LB medium containing 100 mg/ml neomycin and purified by ion-exchange chromatography (Qiagen, Valencia, CA).

With a deletion of the N-terminal secretion signal sequence, the truncated IFNβ gene (IFNβsf) was obtained by PCR-amplification from plasmid psCMV-hIFNβ (oligonucleotides 5′-TACGGATCCATGAGCTACAACTTGC-3′ and 5′-ATATAGCGGCCGCTCAGTTTCGGAGG-3′). The PCR fragments were subcloned in psCMV plasmid yielding psCMV-IFNβsf.

2.3. Liposome preparation and in vitro lipofection

DC-Chol (3β[N-(N′,N′-dimethylaminoethane)-carbamoyl]cholesterol) and DMRIE (1,2-dimyristyl oxypropyl-3-dimethyl-hidroxyethylammonium bromide) were synthesized and kindly provided by BioSidus (Buenos Aires, Argentina). DOPE (1,2-dioleoyl-sn-glycero-3-phosphatidyl ethanolamine) was purchased from Sigma. Liposomes were prepared at lipid/co-lipid molar ratios of 3:2 (DC-Chol:DOPE) or 1:1 (DMRIE:DOPE) by sonication as described [26,27]. Optimal lipid mixtures were determined for every cell line.

In most experiments, cells were seeded into 12-well plates at a density of 3–5 × 10^4 cells/cm^2 and were allowed to adhere overnight. Monolayers were exposed to lipoplexes (0.5 μg plasmid DNA/cm^2 and 1 μl liposome/cm^2) from 2 to 5 h in a serum-free medium. Then the lipofection medium was replaced with fresh complete medium. Unless otherwise indicated, the human and canine cells were lipofected with psCMV-hIFNβ and psCMV-cIFNβ respectively.

2.4. β-Galactosidase staining assay

Transfection frequency was checked by β-galactosidase (βgal) staining to ensure that the lipofection rates were comparable in different experiments. Twenty-four hours after βgal lipofection, cells were trypsinized, fixed in suspension, stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-GAL, Sigma) and counted using an inverted phase contrast microscope [28].

2.5. Generation of conditioned cell media (CCM)

Twenty-four hours after lipofection, the medium was harvested, filtered through a 0.22 μm filter, aliquoted and stored at −20 °C for 24 h [29]. The medium harvested from the non-lipofected control cells was termed Ctrl/CCM, medium from the βgal-lipofected cells was termed βgal/CCM and medium from the IFNβ-lipofected cells was termed IFNβ/CCM. Unless otherwise indicated, CCM was added to untreated monolayer cultures of the same cell line that produced it.

For heat inactivation studies, the CCM was placed in a water bath at 23 °C, 53 °C, 72 °C for 30 min, or at 96 °C for 5 min. After cooling at room temperature, the CCM was diluted 1:2 with complete medium and added to monolayer cultures.

2.6. Cell growth assay

Cells were seeded onto 96-well plates at 1–5 × 10^4 cells/well 24 h after lipofection or 24 h before exposure to CCM or rhIFNβ (BioSidus). To produce a non-adherent condition for the development of spheroids, 96-well plates were pre-coated with 1.5% (w/v) agar (Sigma). Cell growth was quantified with acid phosphatase assay (APH) [30,31]. After 4 days as monolayers (ML) or after 11 days as spheroids (SP), the medium was removed and the culture was washed with phosphate-buffered saline (PBS). Then, 100 μl/well of the assay buffer (0.1 M sodium acetate, 0.1% Triton-X-100, 2 mg/ml p-nitrophenyl phosphate; Sigma) was added and incubated for 60 (ML) or 90 min (SP) at 37 °C. Following incubation, 10 μl of 1 N NaOH was poured to each well, and the absorbance was read at 405 nm in a microplate analyzer. Data were normalized as a percentage of the value of the corresponding untreated cells.

2.7. Bystander effect assay

Twenty-four hours after lipofection, cells were trypsinized and resuspended in complete medium. IFNβ-lipofected cells were mixed with their respective βgal-lipofected controls at proportions of 0, 10, 50 and 100%. The mixtures were seeded into uncoated or agar-coated 96-well plates. After 4 days as monolayers or after 11 days as spheroids, cell growth was quantified by the acid phosphatase assay as described above. Data were normalized as a percentage of the value of the βgal-lipofected cells. The dilution expected values (DEV) of cells survival (without bystander effect) were calculated as follows: $DEV = 100 - (B \times (100 - A))$, being A the actual percent of survival after IFNβ lipofection and B the fraction of IFNβ-lipofected cells (0.1; 0.25; 0.5) when diluted with βgal-lipofected cells [24].

2.8. Measurement of cellular reactive oxygen species (ROS) production

Forty-eight hours after lipofection, cells were trypsinized, washed with PBS and incubated with 5 μM H_2DCF-DA (Sigma) in PBS for 20 min at 37 °C. Then, the cells were washed with PBS, resuspended in complete medium and analyzed by fluorescence