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The combination of bleomycin with suicide or *interferon-β* gene transfer is able to efficiently eliminate human melanoma tumor initiating cells

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ABSTRACT

We explored the potential of a chemogene therapy combination to eradicate melanoma tumor initiating cells, key producers of recurrence and metastatic spread. Three new human melanoma cell lines, two obtained from lymph nodes and one from spleen metastasis were established and characterized. They were cultured as monolayers and spheroids and, in both spatial configurations they displayed sensitivity to single treatments with bleomycin (BLM) or human *interferon-β* (*hIFNβ*) gene or herpes simplex virus thymidine kinase/ganciclovir suicide gene (SG) lipofection. However, the combination of bleomycin with *SG* or *hIFNβ* gene transfer displayed greater antitumor efficacy. The three cell lines exhibited a proliferative behavior consistent with melan A and gp100 melanoma antigens expression, and BRAF V600E mutation. BLM and both genetic treatments increased the fraction of more differentiated and treatment-sensitive cells. Simultaneously, they significantly decreased the sub-population of tumor initiating cells. There was a significant correlation between the cytotoxicity of treatments with BLM and gene transfer and the fraction of cells exhibiting (i) high proliferation index, and (ii) high intracellular levels of reactive oxygen species. Conversely, the fraction of cells surviving to our treatments closely paralleled their (i) colony and (ii) melanosphere forming capacity. A very significant finding was that the combination of BLM with *SG* or *hIFNβ* gene almost abrogated the clonogenic capacity of the surviving cells. Altogether, the results presented here suggest that the combined chemo-gene treatments are able to eradicate tumor initiating cells, encouraging further studies aimed to apply this strategy in the clinic.

1. Introduction

Malignant melanoma is an extremely aggressive form of skin cancer whose incidence continues to increase worldwide [1]. While surgical excision can cure localized disease, once distant metastasis has occurred, the overall median survival is about 6–9 months [2]. Melanoma is also among the most common causes of "metastatic cancer of unknown primary", which may reflect a rapid growth of poorly differentiated lesions arising from indolent or unrecognized cutaneous primary lesions [3].

Melanoma control is frequently short lived even when some drugs are proven to be effective. For example, the BRAF inhibitor vemurafenib is approved for the treatment of patients whose melanoma harbors the V600E mutation, which is thought to be a driver mutation [4,5]. However, after treatment with vemurafenib, cancer progression occurs within six months in the vast majority of these patients. On the other hand, the development of ipilimumab immunotherapy displayed an improvement in patients survival [6] while new strategies as oncolytic viruses produced a longer response rates in patients with advanced melanoma [7].

Despite the advances in melanoma research and drug development, 10–20% of clinically disease-free patients relapse 5–10 years following an initial treatment [8]. This phenomenon, which is known as tumor dormancy [8,9], has been related to the existence of therapy-resistant cells with stem-like activity [10,11]. The cancer stem cell theory suggests that rare tumorigenic cells, resistant to conventional therapy, are responsible for relapse, tumor progression and increased tumor aggressiveness. However, in melanoma, tumorigenic capacity is not restricted to a small

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subpopulation of melanoma cells but is widely shared among phenotypically diverse cells [12,13]. Furthermore, the increased plasticity and heterogeneity, is a marker of melanoma malignancy contributing to therapy failure and disease progression [13–15]. Taking into account that the therapeutic choices for melanoma are limited and most treatments fail to improve the quality of life or survival time in a meaningful way [4,5], the discovery and identification of novel therapeutics is urgently needed.

Intratumor non-viral suicide gene therapy with thymidine kinase from the herpes simplex virus (HSV*tk*), in combination with the pro-drug ganciclovir (GCV), was early proposed for treating this malignant disease [16]. The successful eradication of tumors depends on the bystander effect, by which unmodified adjacent tumor cells are also destroyed by HSV*tk*/GCV cytotoxic effect, allowing an effective tumor regression produced by only a minority of genetically modified tumor cells [17].

Interferons (IFNs) are a family of naturally existing glycoproteins known for their antiviral, antiproliferative and immunomodulatory activities. Interferon-β (IFNβ) has antitumor effects against melanoma, and generally is more potent than IFNα [18,19]. Despite the demonstrated clinical effectiveness, the treatment with recombinant hIFNα/β protein is associated with substantial systemic toxicity that worsens the patient's quality of life and often interferes with the therapy completion (about 25% of the treated patients) [20]. The limited performance of hIFNs in cancer therapy trials may have been caused by the lack or insufficiency of sustained delivery of the protein to the tumor site. In previous studies, we demonstrated that the exogenously added recombinant human IFNβ protein (rhIFNβ) can be successfully replaced by the transfer of the corresponding gene *in vitro* [18,19]. Local non-viral delivery of the gene encoding this cytokine provides a localized slow release transgenic system that avoids the adverse events associated to the injection of high doses of recombinant interferon protein while keeping its therapeutic potential [21].

Bleomycin (BLM) is a glycopeptide antibiotic with antineoplastic activity due to its endonuclease activity [22]. The cytotoxicity of BLM, a hydrophilic agent with low capability of diffusing through the plasmatic membrane, might be related to the efficiency of drug uptake. Different strategies have been developed to bypass the cytoplasmic membrane [23,24]. In a previous report, we demonstrated that lipoplexes can efficiently facilitate the delivery of BLM into melanoma tumor cells *via* endocytosis [25].

An increasing number of studies have recently shown that immunogene therapy is not only compatible with, but may be synergistic with certain chemotherapies [26–28]. Thus, more studies to explore the combined use of these two modalities are compelling.

Most of the cancer gene therapy studies carried out on animal models use tumor cell lines that were kept in culture for many generations, making them very different from the original tumors. Here, we established and characterized three human melanoma cell lines derived from surgically excised melanoma tumors, to evaluate potential *in vivo* responses of individual spontaneous human melanomas to gene therapy. Our results suggest that bleomycin in combination with suicide or interferon gene treatment is able to effectively eradicate tumor initiating cells.

2. Materials and methods

2.1. Establishment of cell cultures from human melanoma tumors

This research work followed the tenets of the Declaration of Helsinki and all samples were obtained after informed consent from the patients. The clinical samples were approved and in accordance with the institutional review board of the *Instituto de Oncología* "*Ángel H. Roffo*", *Universidad de Buenos Aires*, Argentina. Primary cell lines derived from surgically excised lymph nodes (hM1 and hM2) and spleen metastasis (hM4) of human melanomas were obtained by mechanical disruption of tumor fragments in serum free culture medium [29]. They were cultured as monolayers and multicellular 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 (Invitrogen), 10 mM HEPES (pH 7.4) and antibiotics [29]. Serial passages were done by trypsinization (0.25% trypsin and 0.02% EDTA in PBS) of subconfluent monolayers [29].

For doubling time estimation using GraphPad Prism 6 software (GraphPad Software Inc., USA), cells were trypsinized and 5×10^4 cells were plated in duplicate in 6-well plates and cultured in normal conditions. After trypan blue staining, cells were daily counted in a Neubauer chamber.

2.2. Immunocytochemistry and BRAF mutation detection

Cells attached onto a glass slide were cultured for 48 h in the above described conditions. Cells were then washed, fixed with ethanol, re-hydrated and incubated separately with the following specific monoclonal antibodies as described by the manufacturers: antihuman melan A (BioGenex, San Ramon, CA; clone A103), antihuman S-100 (BioGenex; clone 15E2E2), antihuman GP100 (BioGenex; clone HMB45); antihuman cytokeratin (Dako; clones AE1/AE3). After washing, cells were incubated with Multi-Link immunoglobulins (BioGenex) followed by streptavidin/peroxidase conjugate and developed with 3,3′-diaminobenzidine.

DNA was extracted using the High Pure FFPET DNA Isolation Kit (Roche, Indianapolis, IN). BRAF mutational status was tested using a commercial allele-specific real-time polymerase chain reaction-based assay that can detect five point mutations in codon 600 (V600E, V600K, V600R, V600D, and V600M) when present in as little as 1% of the tissue (B-Raf Mutation Analysis Kit II for detection of B-Raf V600E/K/D/R/M Mutations—Entrogen, Woodland Hills, CA).

2.3. Plasmids and transfection efficiency

Plasmids psCMVβ*gal* (6.8 Kb) [29], psCMV*tk* (4.5 Kb) [29] and psCMV*hIFNβ* (3.9 Kb) [18] carry respectively *Escherichia coli β-galactosidase* gene (3.5 Kb), herpes simplex *thymidine kinase* (1.2 Kb) and human *IFNβ* (0.6 Kb) in the polylinker site of psCMV (3.3 Kb), downstream of the CMV promoter and upstream of poly A sequences. The plasmids (bearing the kanamycin resistance gene for selection *in Escherichia coli*) were amplified, chromatographically purified and quality assessed as described [19]. Plasmid DNA for injection was resuspended to a final concentration of 2.0 mg/ml in sterile PBS.

DC-Chol (3β[*N*-(*N*′,*N*'-dimethylaminoethane)-carbamoyl cholesterol) and DMRIE (1,2-dimyristyl oxypropyl-3-dimethyl-hydroxyethylammonium bromide) were kindly provided by BioSidus (Buenos Aires, Argentina). DOPE (1,2-dioleoyl-sn-glycero-3-phosphatidyl ethanolamine) was purchased from Sigma (Saint Louis, MO). Liposomes were prepared at lipid/co-lipid molar ratios of 3:2 (DCChol: DOPE) or 1:1 (DMRIE:DOPE) by sonication as described [18,29]. Lipids in chloroform solution were evaporated to dryness, and liposomes were prepared by reconstitution in sterile sodium phosphate buffer 0.1 M (pH 7.3) to a final concentration of 1.0 mg/ml, followed by 10 cycles of 15 s sonication at 4 °C. Before lipofection, liposomes and plasmid DNA (1:2, v:v) were mixed and allowed to complex at room temperature for 10 min. Optimal lipid mixtures were determined for every cell line.

In most experiments, cells were seeded into 12-well plates at a density of $3–5 \times 10^4$ cells/cm^2 and were allowed to adhere