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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.

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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

Fig. 1. Cytotoxic effects of IFNβ lipofection on monolayers (ML) and spheroids (SP). Transiently β-gal or IFNβ-lipofected melanoma cells were cultured as ML or SP and cell growth was quantified by APH, as described in Section 2. $^{***}p \leq 0.001$ with respect to βgal.

flow cytometry using a 485-nm excitation beam and a 538-nm band pass filter. The mean fluorescence intensity was quantified using WinMDI software. Data were normalized as a percentage of the value of the non-lipofected control cells.

2.9. Statistical analysis

Data were represented as the means ± standard error of the mean (SEM) from three or more independent experiments. Results were analyzed using unpaired Student's *t*-test (if two groups), one-way ANOVA followed by Tukey's test (if more than two groups) or two-way ANOVA followed by Tukey's test (if two nominal variables). $P < 0.05$ values were considered statistically significant.

3. Results and discussion

3.1. Lipofection with IFNβ gene displayed a significant cytotoxic effect on melanoma cells

In order to test the effects of a nonviral gene transfer achieved by lipoplexes carrying the IFNβ gene, we lipofected three cell lines derived from cutaneous human melanoma (*A375*, *M8*, *SB2*) and four derived from mucosal canine melanoma (*Ak*, *Bk*, *Ch*, *Ol*) (Fig. 1). The lipofection with βgal had no effect on cells survival and was used as control. Lipofection rates were <10% in five cell lines (*Bk*: 5.0 ± 0.9, *Ol*: 8.5 ± 0.8, *A375*: 7.5 ± 1.0, *SB2*: 1.7 ± 0.3, *M8*: 2.9 ± 0.8%) and <30% in the remaining two (*Ak*: 28.0 ± 1.9, *Ch*: 18.0 ± 1.07%). *Ak*, *Bk*, *Ch*, *A375*, *M8* and *SB2* monolayers were highly sensitive to IFNβ gene lipofection, while *Ol* was resistant. Taking into account the fair lipofection efficiency observed for *Ol*, this resistance would be due to an inherent lack of sensitivity.

When cultured as spheroids, six out of seven cell lines were sensitive to IFNβ gene transfer: four displaying high (*Ch* and *Ak*) to medium (*Bk* and *SB2*) and two displaying low (*A375* and *M8*) responses (Fig. 1). *A375* and *M8* spheroids were significantly less sensitive to IFNβ gene than their respective monolayers evidencing a strong multicell resistance phenotype [19]. In this case, the cytotoxic activity of IFNβ lipofection was diminished due to their low spheroids growth rate. This behavior is a common feature of many solid tumors that is known as limiting the initial effectiveness of many conventional cell cycle-dependent chemotherapeutic agents [18]. Spheroids assembled from IFNβ-lipofected cells were much less compact than those assembled from βgal-lipofected

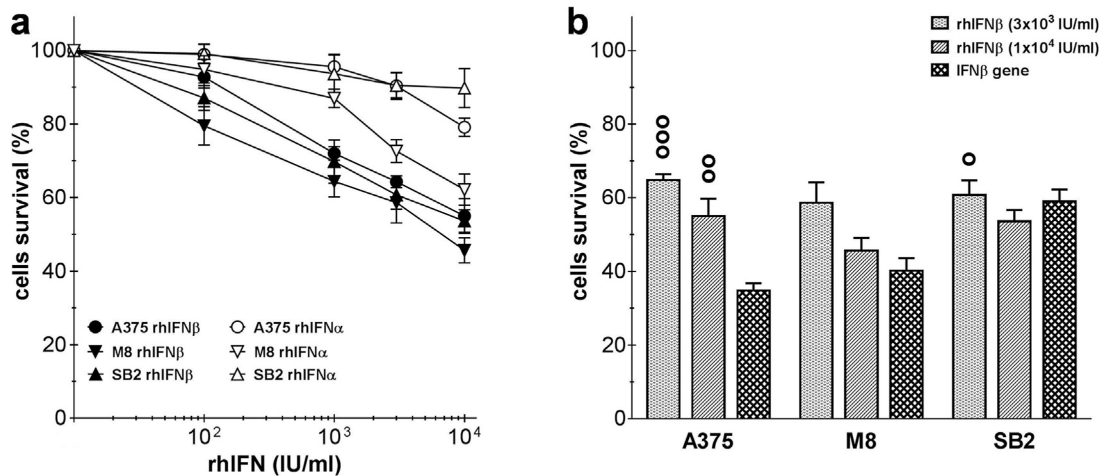

Fig. 2. Cytotoxic effects of exogenously added interferon (IFN) recombinant proteins on monolayers. (a) Cell monolayers were grown in the presence of increasing concentrations of recombinant proteins (rhIFNα or rhIFNβ) as indicated and cell growth was quantified by APH assay as described in Section 2. (b) Cytotoxic effects of rhIFNβ with respect to IFNβ gene. $^{\circ\circ\circ}p \leq 0.001$; $^{\circ\circ}p \leq 0.01$; $^{\circ}p \leq 0.05$ with respect to IFNβ gene.

cells (Fig. 1), probably because of neighbor cells death induced by IFNβ cytotoxicity rather than a diminution of cell adhesion that is currently associated to tumor invasion and poor prognosis [32].

3.2. Lipofection with IFNβ gene appeared to be more effective than the addition of rhIFNβ protein

The three human cell lines were sensitive to recombinant human IFNβ (rhIFNβ) in a dose dependent way (Fig. 2a). *A375* and *SB2* cells started to be significantly sensitive to rhIFNβ from 1×10^3 UI/ml and *M8* did so from 1×10^2 UI/ml. While *SB2* was insensitive to recombinant human IFNα (rhIFNα), *A375* was sensitive at the maximal assayed concentration (10,000 UI/ml) and *M8* at a lower concentration (3000 UI/ml). At the same concentrations, cytotoxicities were always higher for rhIFNβ than for rhIFNα.

IFNβ gene transfer could display a pharmacokinetics with lower systemic toxicity, but the continuous release of low cytokine concentrations would be less effective than a single high dose of rhIFNβ. To discern between these two options, we compared the effects produced by hIFNβ gene transfer and the addition of high dose of rhIFNβ protein (Fig. 2b). In the three assayed human melanoma cell lines, cytokine gene transfer was always equally or more effective than rhIFNβ: in *A375* hIFNβ gene transfer was significantly more effective than 10,000 UI of rhIFNβ.

3.3. IFNβ transgene expression displayed a remarkable bystander effect

The cell death rates were always significantly higher than lipofection rates suggesting a strong bystander effect. In *SB2* whose lipofection efficiency was only 2%, hIFNβ gene expression diminished cells survival about 41% as compared to control βgal expression. Such bystander effect was already found in various tumor cell lines for adenoviral IFNα gene transfer [33], and non-viral IFNβ in a Ewing sarcoma cell line [21]. To evaluate the degree of the bystander effect involvement we assayed the survival of IFNβ-lipofected melanoma cells diluted with the corresponding βgal-lipofected cells.

As shown in Fig. 3a and b, cells survival was always lower than those expected without bystander effect. A 1:2 dilution (IFNβ-/βgal-lipofected cells) did not affect the IFNβ cytotoxic effects on spheroids (all; Fig. 3b) or monolayers (*Ak*, *Ch* and *SB2*; Fig. 3a). In most of the cases, a significant cytotoxicity appeared with only 10% of IFNβ-lipofected cells in the mixture. At this ratio, *Ak* monolayers and *Ch* spheroids did not display dilution effects (Fig. 3a–c). This suggests that IFNβ-lipofected cells spread the cytotoxicity to neighbor βgal-lipofected cells and would allow using this approach *in vivo* where lipofection rates are usually lower than *in vitro*.

The *Ch* cells bystander effect was much stronger in spheroids than in monolayers (Fig. 3a and b), probably due to a lower number of cell-to-cell contacts in 2D compared to 3D cultures. This was explored by studying this effect in monolayers seeded at different cell densities (2×10^3, 4×10^3 and 8×10^3 cells/well; Fig. 3d). Both the maximal cytotoxicity (Fig. 3d) and the bystander effect (Fig. 3d) diminished at lower cell densities (2×10^3 cells per well) as compared to standard cell densities (4×10^3 cells per well). Conversely at higher cell densities (8×10^3 cells per well), the maximal cytotoxic effect was not modified, but the bystander effect increased (Fig. 3d) as evidenced by the dilution of the cytokine gene. Therefore, cell-to-cell contact or close proximity between cells expressing IFNβ and neighbor cells not expressing, increased the bystander effect.

3.4. Only the conditioned medium from IFNβ-lipofected canine melanoma cells was cytotoxic

To determine the possible involvement of secreted cytotoxic factors by IFNβ-lipofected cells in the bystander effect, we took aliquots of conditioned culture media (CCM) of IFNβ-, βgal-lipofected or control melanoma cells 24 h after lipofection. Media were filtered and frozen for further assays on the corresponding untreated cell monolayer cultures. Untreated control cell or βgal/CCM had a very slight or no effect on cells survival.

While CCM of cIFNβ-lipofected canine melanoma cells (cIFNβ/CCM) was highly cytotoxic for canine melanoma unlipofected cells, it was only slightly but significantly cytotoxic (survival ≥90%) for human melanoma cells (Fig. 4a). Although direct cIFNβ-lipofection was more cytotoxic in *Ak* and *Ch* than cIFNβ/CCMs (survival ratios: *Ak*: 1.7-fold; *Ch*: 2.0-fold), it was almost equivalent to cIFNβ/CCM in *Bk* (survival ratio: 1.1). All these data suggest that at least part of the bystander effect in canine melanoma would be mediated by a soluble cytotoxic factor released by cIFNβ-lipofected cells.

We were not able to find a similar soluble factor released to CCMs by human melanoma cells after hIFNβ gene lipofection even though harvesting them 48 or 72 h post-lipofection or reducing the volume for concentration (data not shown).

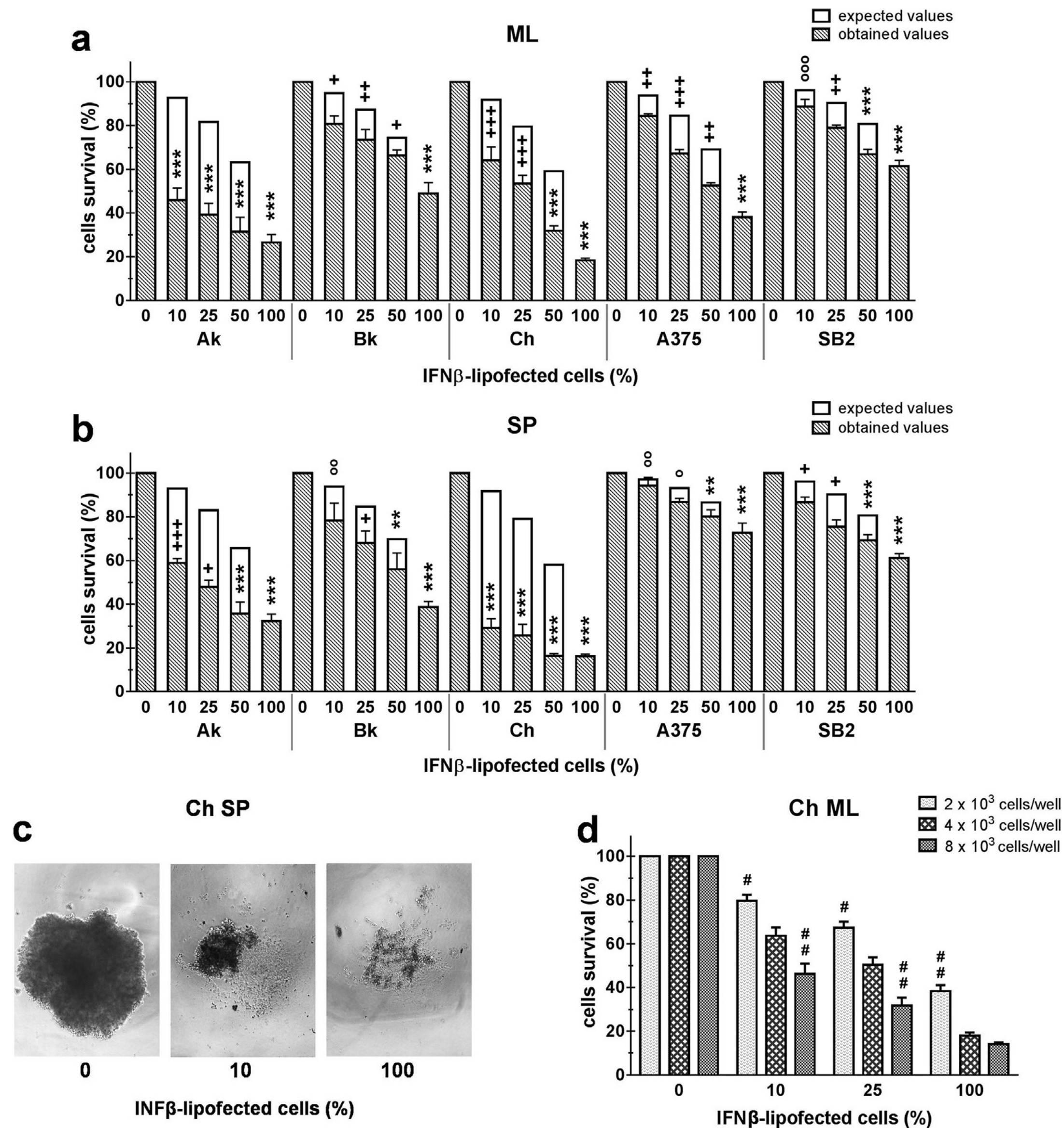

Fig. 3. Bystander effect of IFNβ lipofection on monolayers (a,d) and spheroids (b,c). (a,b) Transiently IFNβ-lipofected cells were mixed with their respective βgal-lipofected controls at proportions of 0, 25, 50, 75 and 100%. The mixtures were seeded as described in Section 2. (c) Representative images of *Ch* spheroids at 100× magnification. (d) The mixtures were seeded at three different cell densities: 8×10^3 (2×), 4×10^3 (1×) or 2×10^3 (0.5×) cells/well. Cell growth was quantified by APH assay as described in Section 2. $^{***}p \leq 0.001$; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$ with respect to βgal. $^{+++}p \leq 0.001$; $^{++}p \leq 0.01$, $^{+}p \leq 0.05$ with respect to βgal and IFNβ; $^{\circ\circ}p \leq 0.01$ with respect to IFNβ; $^{\#\#}p \leq 0.01$ $^{\#}p \leq 0.05$ with respect to the same treatment at 4×10^3 cells/well.

Metalloproteinase-9 cleaves hIFNβ protein and inactivates it [34]. To evaluate the possible involvement of proteases or other factors in the degradation of hIFNβ released by lipofected cells, different dilutions of rhIFNβ protein were made in fresh or conditioned media and were incubated at 37 °C for 24 h but no significant differences in cytotoxicity were found (data not shown). The dissimilar behavior of canine and human cells to the homologous IFNβ gene lipofection, suggests the involvement of more than one cytotoxic mechanism.

3.5. Cytotoxicity of released cIFNβ was thermo-labile and dose-dependent

As expected, dilution with βgal-lipofected CCMs diminished the cytotoxic activity of canine melanoma CCMs after cIFNβ gene lipofection. Being *Ak* and *Ch* CCMs more active than *Bk* CCM, probably because their higher lipofection efficiencies as compared to *Bk* cells (Fig. 4b).

cIFNβ lipofection had no effect on *Ol*, as described above. Nevertheless, *Ol* CCM after cIFNβ lipofection, when added to *Ch* cells, was as active as *Ch* CCM on the autologous cells (Fig. 4a and c). Therefore we confirmed that the lack of effects on *Ol* after cIFNβ gene lipofection was not due to a defective release of the cytotoxic factor but to an intrinsic resistance to it.

A preliminary characterization of the cytotoxic factor present in CCM after cIFNβ gene lipofection, CCMs was performed by incubation at different temperatures (30 min at 23, 53 and 72 °C, and 5 min at 96 °C) followed by dilution (1:1) in fresh culture medium. When added to the monolayers of autologous cell lines, cytotoxicity was abolished at 72 and 96 °C, while was conserved at 23 and 53 °C (Fig. 4d). This pattern of lability is consistent with a protein nature of the factor(s). It is worth to note

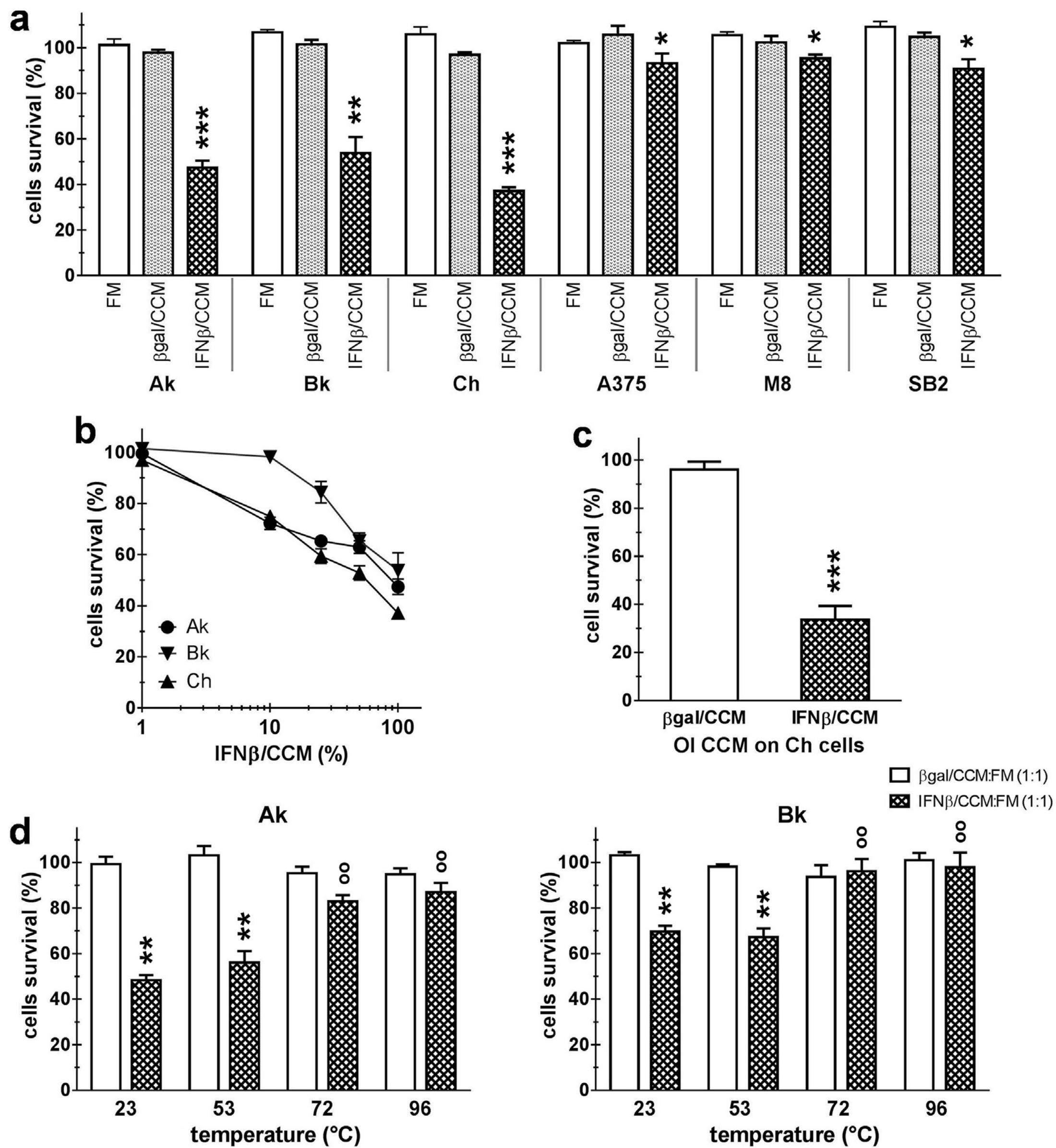

Fig. 4. Effects of conditioned IFNβ or βgal gene lipofected-cell media (IFNβ/CCM or βgal CCM) on homologous cell monolayers (a). Dose response curves of dilutions IFNβ MC on homologous cells (b). Cross-effect of *Ol* IFNβ/CCM on *Ch* cell monolayers (c). Effect of heating on IFNβ/CCM (d). CCMs were prepared by 1:1 mixing with fresh medium (FM) and assayed as described in Section 2. $^{***}p \leq 0.001$, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$ with respect to βgal/CCM; $^{\circ\circ}p \leq 0.01$ with respect to IFNβ/CCM at 23 °C.

that human recombinant IFNβ behaved similarly (data not shown).

3.6. Distinctive cytotoxic responses were obtained by intracellular hIFNβ expression

In IFNα expressing cells some of their related intracellular biological functions can be accomplished without the need of secretion [35]. Since hIFNβ-lipofected human melanoma cells did not generate cytotoxic concentrations of the protein in the CCMs, it would be plausible a direct intracellular action. A truncated version of hIFNβ gene lacking the N-terminal secretion signal (signal-free interferon, hIFNβsf) was cloned in the same plasmid backbone to evaluate this possibility in human melanoma cell lines.

Even with a lower effect than the unmodified hIFNβ gene, hIFNβsf gene lipofection was cytotoxic for *SB2* and *M8* monolayers and spheroids. Conversely, *A375* monolayers and spheroids were not sensitive to hIFNβsf gene lipofection (Fig. 5a and b). In *SB2* and *M8* monolayers hIFNβ gene lipofection was more cytotoxic than hIFNβsf gene. The differences in the extent of cytotoxic effects could be attributed to the secreted IFNβ protein.

As it is the case for hIFNβ and taking into account the low lipofection efficiencies, a bystander mechanism would also be involved in the effects of hIFNβsf gene lipofection.

Previous results showed that hIFNβ lipofection increases intracellular ROS concentration [21]. The effects of hIFNβsf lipofection were evaluated by using a DCF-DA probe. hIFNβsf lipofection increased ROS levels in *A375* and *SB2*. The effect over the βgal-lipofected control levels was similar to hIFNβ lipofection in *SB2* and about a half in *A375* (Fig. 5c). These results together with those of Fig. 5a and b suggest that hIFNβ cytotoxic effects on *A375* would rely on hIFNβ extracellular release but not on its intracellular presence. In *A375*, the mild ROS increase produced by intracellular hIFNβ was not enough for cytotoxicity induction.

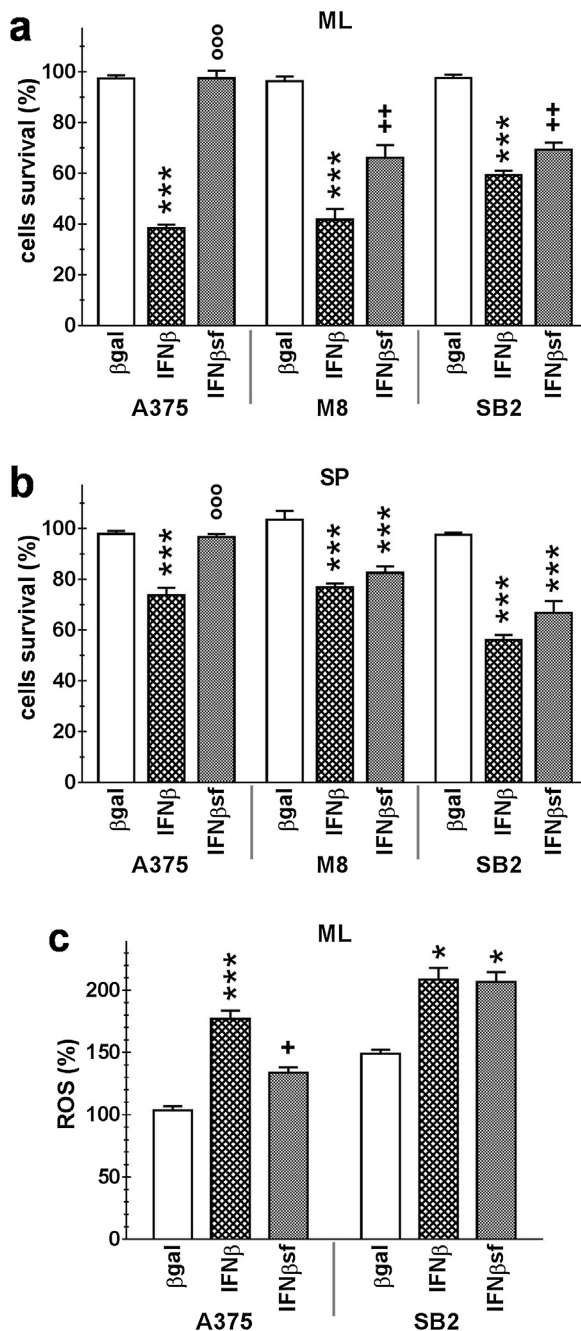

Fig. 5. Effects of IFNβsf lipofection on cell survival (a, b) and intracellular ROS levels (c). Transiently β-gal, IFNβ, or IFNβsf-lipofected melanoma cells were cultured as monolayers (a, c) or spheroids (b). Cell growth and relative intracellular ROS levels were quantified as described in Section 2. $^{***}p \leq 0.001$ with respect to βgal; $^{++}p \leq 0.01$; $^{+}p \leq 0.05$ with respect to βgal and IFNβ; $^{\circ\circ\circ}p \leq 0.001$ with respect to IFNβ.

3.7. Conclusion

The sustained IFNα/β slow release obtained by gene transfer might decrease systemic cytotoxicity and increase antitumor activity as compared to the treatment with recombinant IFNα/β protein. IFNβ lipofection was cytotoxic for both human cutaneous and canine mucosal melanoma cells grown as monolayers or spheroids (Fig. 1). Being rhIFNβ more active than rhIFNα and hIFNβ lipofection as active as 10,000 IU/ml rhIFNβ protein, the proposal of an IFNβ gene therapy for melanoma is strongly justified.

The high efficacy of IFNβ gene transfer for inhibiting tumor cell growth when only a low ratio of cells were transfected suggests the presence of a bystander effect. This effect could be mediated by the secreted IFNβ or other cytotoxic factor(s) or alternatively by the cell-to-cell passage of cytotoxic factors through gap junctions. The bystander effect was stronger in *Ak*, *Bk* and *Ch*, and weaker in *A375* and *SB2* (Figs. 3 and 4). This property of the therapeutic approach is essential for its further translation to the *in vivo* situation where gene transfer efficiencies are usually low.

The dilution of IFNβ-lipofected with βgal-lipofected cells had an effect on cytotoxicity that was lower than expected (Fig. 3a and b) evidencing that IFNβ-lipofected induced cytotoxicity on βgal-lipofected cells. On the other hand, physical contact induced by cell density increased the bystander effect (Fig. 3d) is consistent with the passage of a cytotoxic factor through gap junctions or any other cell-to-cell gate. Nevertheless, lindane a gap-junction inhibitor did not affect IFNβ gene cytotoxicity or bystander effect (data not shown).

Cell conditioned media after IFNβ gene lipofection were cytotoxic on autologous canine mucosal melanoma cells, but were weakly effective on human cutaneous melanoma cells (Fig. 4a). Two mechanisms would be involved. In canine melanoma cells the bystander effect was due to a thermolabile cytotoxic factor (Fig. 4d) that is still active when diluted (Fig. 4c). Similar results were reported after adenoviral transduction of IFNα gene to human bladder carcinoma cells [29]. On the other hand hIFNβ-lipofected canine melanoma cells were able to produce significant amounts of hIFNβ in CCMs that resulted cytotoxic to human melanoma cells (data not shown). This set of results is consistent with cIFNβ protein being the main cytotoxic factor in cIFNβ-lipofected canine melanoma cells.

Even though it was not strong, a bystander effect was found for hIFNβ-lipofection of human melanoma cells. This was evidenced by the difference between cytotoxic and lipofection efficiencies. The fact that hIFNβ/CCMs were not active suggests that secretion of hIFNβ protein would not be essential for the cytotoxic effect. Intracellular hIFNβ coded by IFNβsf was effective on *SB2* and *M8* cells, but not on *A375* that was resistant (Fig. 5a and b). The hIFNβsf gene cytotoxic efficiency in *SB2* and *M8* was about 6-fold higher than lipofection efficiency. An intracellular mechanism independent from secretion could be involved as reported for human renal carcinoma lipofected with hIFNβ gene [36]. In that case, cytotoxicity mediated by the intracellular activation of IFN type I receptor was found. On the other hand in *A375* where intracellular mechanisms appeared not to be involved, the secretion of some amount of hIFNβ to contiguous cells would be necessary.

Altogether, the results presented in this work strongly support the hIFNβ gene transfer as a very promising strategy for both canine and human melanoma. Additional treatments will be necessary to overcome the multicellular resistance of tumors like *A375* and *M8* to hIFNβ lipofection, detected when grown as spheroids.

Taking into account the immune and antiangiogenic effects inherent to IFNs that could help in the control of tumor growth [37], it will be interesting to test *in vivo* the validity of this proposal.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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