Journal of Clinical & Experimental Dermatology Research

Blaya et al., J Clin Exp Dermatol Res 2012, 3:5
DOI: 10.4172/2155-9554.1000159

Research Article Open Access

Antitumoral Activity of Multiferon® (HulFN-alpha-Le) in Experimental Murine Melanoma Model

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Abstract

Multiferon® (HulFN-alpha-Le) (MF) is a natural interferon alpha used in several countries for the treatment of selected viral infections and as an adjuvant treatment in different types of cancer, including melanoma.

We have examined the influence of MF *in vitro* and *in vivo* in murine melanoma cells. B16F10 cells were treated with MF at different concentrations ranging from 0 to 1000 IU/mL and this substance had no effect on cell proliferation, migration or clonogenic abilities of the tumoral cells. However, *in vivo* studies with murine models after subcutaneous implantation of B16F10 cells showed that tumor volumes measured during the experiment and at sacrifice were significantly smaller in mice receiving MF (90 IU/mice 3 times a week) than in the control group ($p<0.005$). MF was well tolerated and no body weight loss or other sign of toxicity was observed in all groups.

These results indicate that MF is active *in vivo* in murine experimental melanoma model without showing any direct antimelanoma activity in *in vitro* assays when used at doses mimicking the serum levels found in clinical practice in humans. We believe that the activation of the immune system following the administration of MF may be one of the mechanisms that explain the anti-tumoral effect of this drug.

Keywords: Multiferon; Melanoma; Antitumor activity

Abbreviations: MF: Multiferon; IFN-alpha: Interferon-alpha; DTIC: Dacarbazine; DMEM: Dulbecco's Modified Eagle Medium; PBS: Phosphate-Buffered Saline; FCS: Fetal Calf Serum

Introduction

Melanoma is a type of cancer arising from the melanocyte cells of the skin. The prognosis of the disease is variable, and a better outcome has been observed when early diagnosis and surgical treatment can be done, due to the lack of efficacy of complementary treatments. Interferon alpha (IFN-alpha) has been used worldwide for decades to treat some viral infections and different types of cancer including melanoma. Multiferon (MF) is a natural interferon alpha consisting of 6 IFN-alpha subtypes and produced in human leukocytes following induction with Sendai virus. It has been approved in several countries for the treatment of hepatitis B and C (especially after failure of recombinant IFN-alpha) [1,2] and melanoma as an adjuvant therapy in stages IIb-III in combination with dacarbazine (DTIC) [3]. The dosing conventionally used for the latter indication is 3 million IU three times a week for 6 months after 2 initial cycles of DTIC (850 mg/m^2). The objective of this study was to assess the antitumoral activity of MF in monotherapy in an experimental melanoma model, starting at a cellular level with a cell proliferation assay, then with a clonogenic test and a migration ability test. The *in vitro* tests were followed by *in vivo* experiments where mice were inoculated with murine melanoma cells and treated with MF.

Materials and Methods

Proliferation assay

B16F10 cells (10^4) were seeded in DMEM culture medium and incubated overnight to promote cell adherence to the surface of 96-well microplates. Cells were treated with Multiferon® (HuIFN-alpha-Le) (Swedish Orphan Biovitrum AB, Stockholm, Sweden) solved in PBS for 48 hours at different concentrations ranging from 0 to 1000 IU/mL (0, 15, 31, 62, 125, 250, 500 and 1000 IU/mL). Cells treated with PBS alone (MF 0 UI/mL) were taken as a control group. Cell viability was estimated via metabolic activity using the XTT assay (Roche Molecular Biochemicals, Indianapolis, IN, USA). The concentrations of MF were deduced after considering the usual dosing (3 M IU three times a week) and the volume of distribution of this drug in humans. We took as a reference the summary of product characteristics of IntronA® (IFN-alpha 2b) 3 M IU/0.5 ml solution for injection or infusion (Schering-Plough Corporation, Kenilworth, New Jersey, USA) available at the European Medicines Agency webpage [4]. According to this document, the levels of this drug after intravenous administration in clinical assays in humans peaked by the end of the infusion (135 to 273 IU/mL). Experiments were repeated three times in triplicate. Cells were observed using an optic microscope (Nikon AZ100, Nikon Corporation, Tokyo, Japan) to assess their viability and morphology.

Clonogenic assay

B16F10 cells were seeded at limiting dilutions in DMEM culture medium and incubated overnight to promote their adherence to the 24-well microplates. Cells were exposed to MF at different concentrations (0, 500 and 1000 IU/mL) during seven days, after which the cultures were washed with PBS and the cells were fixed in 70% ethanol and stained with crystal violet. Colonies > 5 cells were counted under microscope (Nikon AZ100, Nikon Corporation, Tokyo, Japan). Experiments were repeated three times in triplicate.

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Received August 11, 2012; **Accepted** September 07, 2012; **Published** September 23, 2012

Citation: Blaya B, Ortega-Martínez I, Tejerina EA, Granda TG, Boyano MD (2012) Antitumoral Activity of Multiferon® (HulFN-alpha-Le) in Experimental Murine Melanoma Model. J Clin Exp Dermatol Res 3:159. doi:10.4172/2155-9554.1000159

Migration assay

5×10^3 B16F10 cells were seeded in the upper compartment of transwell cell culture chambers (Falcon Cell Culture Inserts, Becton-Dickinson Laboratories, Orangeburg, NY, USA) in 24-well microplates and cultured in 1% FCS medium with 1000 IU/mL of MF (Figure 2). After 48 hours, melanoma cells migrated to the filter (8 µm diameter pores) and adhered to the lower surface of the 24-well microplate. Cells were fixed in 70% ethanol and stained with crystal violet. Areas from stained cells were measured using a Nikon microscope AZ 100 with digital camera Sight DS-5Mg. Positive area for crystal violet were analysed using Image J software. Values are shown as the cell-stained areas in arbitrary units ± standard deviations of at least three independent experiments performed in quadruplicate.

In vivo study

All the animal experiments were approved by the local authorities and were performed according to the European animal testing regulations.

A group of 20 syngenic C57BL/6 mice were inoculated subcutaneously in the right flank with 5×10^5 B16F10 cells. All of them developed a clinically palpable tumor 72 hours later. Five days after the implantation (on day 6), the animals were divided into two groups: (Group I, n=10) treated with 50 µL of MF in PBS at a concentration of 1800 IU/mL and (Control, n=10) treated with 50 µL of PBS. All animals were injected intraperitoneally three times a week, on days 6, 8, 10 and 12. Tumors were measured three times a week before the treatments using a caliper. Tumor size (mm^3) was determined by the equation: tumor volume = (length × $width^2$/2). The animals were sacrificed on day 14 and the tumors where measured and weighted after the necropsy.

Statistical analysis

Statistical analysis of the data was performed using SPSS version 18 for Windows (SPSS, Inc., 2009, Chicago, IL, USA). The difference of means between samples was determined using the Student t-test. The level of statistical significance was set at $p<0.05$.

Results

We observed that MF concentrations ranging from 0 to 1000 IU/mL did not modulate the proliferation of B16F10 mouse melanoma cells (Figure 1). Moreover, no direct cytotoxic effect or any noticeable cell morphology alteration was observed at any dose of MF. We could not demonstrate any effect of MF on the clonogenic and migration ability of melanoma cells either (Table 1). In the *in vivo* study, the administration of MF reduced the growth of melanoma tumors in mice when compared to the control group ($p<0.05$). Tumor weight after sacrifice was lower in the group treated with MF (median 1.09 ± 0.5 grams) than in the control group (median 1.81 ± 0.5 grams) (Figure 3) ($p=0.02$). Moreover, MF did not appear to induce toxicity, as the body weight (data not shown) and the clinical signs of mice recorded during the length of the experiment were not different from controls.

Discussion

In the study presented herein we have observed that MF shows antitumoral activity against melanoma *in vivo* when administered to mice models. However, we failed to demonstrate any direct antitumoral effect of this substance on melanoma B16F10 cells *in vitro* when used at doses ranging from 0 to 1000 IU/mL. In fact, neither the proliferation rate of these cells nor their ability to form colonies or their migration capacity was affected by MF when compared with control cells.

In a previous study [5], a different type of recombinant human IFN-alpha (Wellferon), showed an antiproliferative effect on melanoma DX3-azac cells *in vitro* in concentrations ranging from 100 to 1000 IU/mL, but the authors disclosed that this effect disappeared when cells were grown at densities of 5×10^4 cells/dish. In our study, we seeded the cells at a concentration of 10×10^4 cells/dish, and we did not see any direct antiproliferative effect of MF.

Our results appear to be in contradiction with those published by Martínez et al. [6] where IFN-alpha showed decreased cell survival in a time and dose-dependent manner when used in B16F10 cells, but

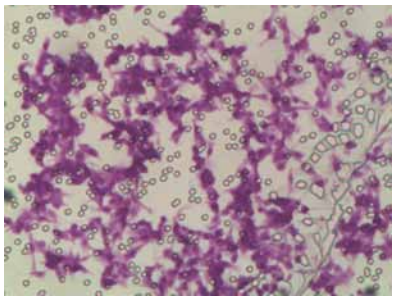

Figure 1: (Crystal violet, x400) Microphotography of the cell migration assay. Cells ($5x10^3$) were seeded in the upper compartment of transwell culture chambers (Falcon Cell Culture Inserts, Becton-Dickinson Laboratories, Orangeburg, NY, USA) in 24-well microplates and cultured in 1% FCS medium with 1000 IU/mL of MF. After 48 hours, melanoma cells migrated to the filter (8 µm diameter pores) and adhered to the lower surface of the 24-well microplate.

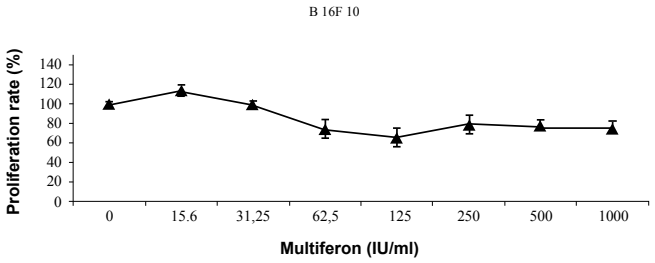

Figure 2: Effect of Multiferon on cell viability of melanoma cells. Cells (10^4) were seeded in normal culture medium and incubated overnight for the adherence to the surface of 96-wells microplates. Then, cells were treated with the indicated concentrations of Multiferon (OrphanBiovitrum, Stockholm, Sweden) for 48h and viability was estimated by means of metabolic activity using XTT assay (Roche Molecular Biochemicals, Indianapolis, IN). Multiferon concentration ranging 0-1000 IU/mL did not modulate the proliferation of B16F10 mouse melanoma cell line. Values are shown as percentage of untreated control cells ± SD of at least three independent experiments performed in quadruplicate.

Multiferon (U/ml)	Clonogenic ability Number of colonies	Migratory ability (area, arbitrary units)
0	29,5 ± 3,5	37,2 ± 3,5
500	31,0 ± 0,5	nd
1000	30,4 ± 6,7	35,7 ± 0,2

Table 1: Effect of Multiferon on clonogenic and migratory ability of B16F10 melanoma cells. The number of colonies was counted under microscope and values were expressed as the mean ± SD. In the migratory assay, positive area for crystal violet stained cells were analysed using Image J software. Values are expressed as the cell stained areas in arbitrary units ± SD of at least three independent experiments performed in quadruplicate.

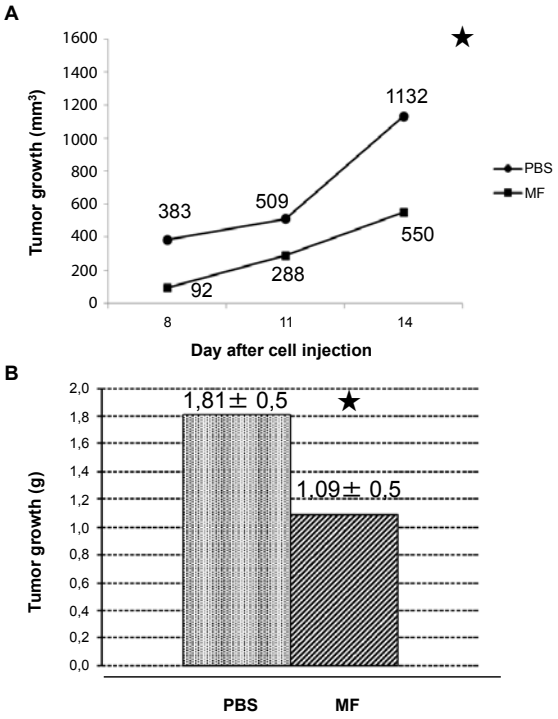

Figure 3: Effect of Multiferon in mice bearing B16F10 melanoma. Mice were inoculated subcutaneously with B16F10 melanoma cells ($5x10^5$), and after 5 days animals (n=10 in each group) were treated with multiferon (1800 U/mL) or the solvent PBS (control group) over 14 days. This experiment was repeated twice and similar results were obtained. The figure represents the data of one of them. (A) Tumor growth curves were determined by measuring tumors with a caliper. Median values of the calculated tumor volume (mm^3) are shown. (B) Mice were killed and tumors were weighted. Histogram represents mean values of the tumors weighted at sacrifice in control (1,81 ± 0,5 g) and treated (1.09 ± 0.5 g) groups. All the mice developed tumors and a higher growth was observed in untreated control group throughout the study. Differences between the two groups were statistically significant throughout the study (p<0,05)

the doses of MF used by this authors (250000-1000000 IU/mL) were much higher than the doses we used in our work (0-1000 IU/mL), and this difference of concentration could explain the lack of antitumoral activity of MF in our *in vitro* study. According to the pharmacokinetic data available on recombinant IFN-alpha [4], we believe that the concentrations of MF that we have used in our experiments (0-1000 IU/mL) are comparable to the real concentration of MF in the intercellular space in patients receiving treatment with this drug, whereas the concentrations previously used in other *in vitro* experimental studies (20000-1000000 IU/mL) [6,7] seem too high.

Habib et al. [8] treated the Cloudman S91murine melanoma cell line with IFN-alpha2b at doses ranging from 78 to 40000 IU/mL and found no evidence of direct antiproliferative effect *in vitro* using an MTT assay, despite the fact that the drug proved to be effective *in vivo* in mice. Other authors have failed to demonstrate a direct proapoptotic effect of IFN-alpha when used at doses below 1000 IU/mL [9].

Our results indicate that MF has neither antiproliferative nor apoptotic or direct cytotoxic effects on B16F10 mouse melanoma cells in the concentrations used in clinical practice in humans.

We believe that the antitumoral activity of MF observed *in vivo* is partially explained by the activation of the immune system and the antiangiogenic effect of this drug [10]. In fact it has been described that IFN-alpha stimulates NK, macrophage and T cell functions [11] that could lead to recognition of tumoral cells by the effectors of the immune system and its subsequent induced apoptosis or lysis. A correlation has been established between lymphocyte infiltration in the tumor and the antitumoral effect of IFN-alpha [12]. More recently it has been published that MF shows activity against Merkel cell carcinoma via modulation of the Merkel cell polyomavirus T-antigens. MF would in fact upregulate the expression of promyelocytic leukemia protein (PML) that would in its turn sequester viral and host proteins that are indispensable for transcription of viral proteins [13].

In our study, we did not compare MF to other commercially available interferons. We think that in the future it would be interesting to compare the antimelanoma activity of the different types of IFN in order to establish a rationale in the management of these drugs.

Conclusion

In vitro, MF did not show antiproliferative nor apoptotic or direct cytotoxic effects on B16F10 mouse melanoma cells at concentrations used in clinical practice in humans. Nevertheless, this drug demonstrated antitumoral activity when used *in vivo* in an experimental tumor model. We believe that this activity could be explained, at least partially, by the activation of the immune system of the host.

This study highlights the importance of using immunocompetent animals and syngenic systems in the study of antineoplastic drugs due to the fact that cellular interactions cannot be evaluated in *in vitro* studies.

Acknowledgements

We thank Swedish Orphan Biovitrum AB for providing Multiferon® (HuIFN-alpha-Le). This study was supported by grants from the Heath Department of the Government of the Basque Country (Exp: 2006111036) and the University of the Basque Country (GIC-10/29).

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