Abstract. Background: Interferon-α (IFN-α) subtypes bind to the same receptor and are expected to have the same biological functions. Whether or not leukocyte IFN, containing six major IFN-α proteins had the same anti-tumor effect as one subtype, recombinant IFN-α2b, was investigated. Materials and Methods: Three melanoma lines were treated with both types of IFN, and the effect on proliferation and survival was estimated both after short-term and prolonged treatment. Results: All the melanoma cell lines were sensitive to the antiproliferative effects of both IFN species during short-term treatment. However, upon prolonged culture, the frequency of resistant colony formation was significantly higher in cultures treated with IFN-α2b compared to those treated with leukocyte IFN. There was a qualitative difference between the resistant colonies selected by the two IFN species with respect to morphology, growth rate and sensitivity to apoptosis. Conclusion: The development of resistant clones occurred at a lower rate during long-term treatment with leukocyte IFN containing six major subtypes of IFN-α as compared to IFN-α2b.

Malignant melanoma represents an aggressive tumor with rapid metastatic spread. Surgery of the primary tumor followed by chemotherapy based on staging is the established therapy (1), but the results are not encouraging due to extensive genome instability and development of resistance (2-5).

Type I interferons (IFNs) have been used as adjuvant therapy after surgery. Depending on the dose, effects have been seen on relapse-free survival, but the effects on overall survival are less clear (6-7). In a critical review (8), some encouraging results have been reported on IFN-α therapy in combination with the cytostatic drug dacarbazine (DTIC) and other drugs. A recent study demonstrated that adjuvant treatment of cutaneous melanoma with DTIC and low doses of a leukocyte-derived IFN (leuIFN), Multiferon®, increased both relapse-free survival and overall survival (9).

Type I IFNs include both one IFN-β gene product and 13 IFN-α gene products, usually called subtypes. Currently, treatment with single subtype recombinant IFN (rIFN) dominates the therapeutic arsenal. Conversely, natural leuIFN used in this study comprises a mixture of six different IFN-α gene products.

Since the discovery of the IFN-α gene family, speculation has been raised as to the biological significance of its genetic complexity, especially as all type I IFNs bind to the same receptor. It is known that there are differences in potency, and qualitative differences have been claimed between the type I IFNs.

The objective of this study was to assess whether the single subtype IFN-α2 had the same effect on the growth and survival of melanoma cell lines in vitro as leuIFN which includes this subtype.

**Materials and Methods**

**Reagents.** LeuIFN (Multiferon®) was obtained from ViraNative AB, Umeå Sweden, each ampoule containing 6 MIU/mL. rIFN-α2b (IntronA) was obtained from Schering-Plough (Kenilworth, NJ, USA), each ampoule containing 10 MIU of lyophilized material. Both IFNs were dissolved in phosphate-buffered saline (PBS) with 1 mg/ml human albumin. For all the experiments, the protein concentration of each IFN was adjusted to give equimolar concentrations. The molecular weight of rIFN-α2b is 19.3 kDa. The calculated average MW of the six major IFN-α subtypes constituting Multiferon® was 19.7 kDa. 3H-methyl-thymidine was obtained from GE-healthcare (Uppsala Sweden). WM-266-4 and SKmel-28 cells were purchased from ATCC (LGC Promochem, Middlesex, UK) and Mewo cells from IPD-ESTDAB (http://www.ebi.ac.uk/ipd/estdab/ordercells.html). Daudi cells were obtained from Flow Laboratories (Irvine, UK).

**Cell culture.** The melanoma cells were propagated in Minimal Essential Medium with Earles Salts supplemented with 20 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate, 1x non-
essential amino acids, antibiotics (all from Invitrogen, Stockholm, Sweden) and 10% foetal bovine serum (Integro, Dieren, The Netherlands). The Daudi cells were grown in RPMI supplemented with 10 mM Hepes, 1 mM sodium pyruvate, appropriate antibiotics and 10% foetal bovine serum.

Apoptosis analysis by flow cytometry. Apoptosis was analyzed by staining the cells with propidium iodide. Fifteen thousand cells/well were seeded into 12-well plates, the final volume was 2 ml/well. Staining was performed by suspending pelleted cells in 0.5 ml of 20 mM Tris-HCl, pH 7.6, 100 mM sodium chloride, 0.1% NP-40, 20 µg/ml RNaseA and 50 µg/ml propidium iodide. After incubation in the cold, for at least 1 h, stained cells were analyzed on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) using Cellquest Pro software. Apoptotic cells were recorded in the sub G1-DNA compartment.

Determination of cell viability. The melanoma cells were seeded into 96-well plates at densities of 500, 750 and 400 cells/well for the WM-266-4, Mewo and SKmel-28 cells respectively and allowed to attach overnight. Cell viability was analyzed 6 days after addition of IFNs using the WST-1 assay (Roche Bioscience, Mannheim, Germany). This assay is based on the activity of succinate-tetrazolium reductase, which exists in mitochondrial respiratory chain and is active only in viable cells. After 2 h, the absorbances were read at 450 nm with a reference reading at 690 nm. The Daudi cells were seeded at 10⁶ cells/well in 96-well plates and grown in the presence of the IFNs for three days and then pulsed overnight with 1 µCi/well of ³H-thymidine followed by scintillation counting.

Selection of resistant clones. Fifty thousand WM-266-4 cells were seeded into 25 cm² flasks in 15 ml medium containing 167 pM IFN, and 7.5 ml medium with IFN was replenished every 4-5 days. Four weeks later the bulk culture of surviving cells was seeded into 96-well plates in medium without IFN at densities of either 0.1x10⁴ or 0.3x10⁴ cells/well. After 24 days individual clones were further expanded. In total 18 colonies from each treatment were obtained, six single colonies from the 3 plates seeded with 0.1x10⁴ cells/well and 12 colonies from two plates with 0.3x10⁴ cells/well.

Results

Cell growth inhibition was studied by counting cells treated with both IFNs in three concentrations, 50, 167 and 500 pM every second day. Figure 1 shows a typical experiment at 167 pM with a reduction of cell number noted after 3 days, and after 11 days it had reached very low levels, although 3.5 fold higher (p=0.0019) in cells treated with rIFN-α2b. After two weeks small colonies of cells were seen, but at a higher frequency in cells grown in rIFN-α2b. Two weeks later visual inspection revealed the appearance of large colonies and some scattered individual cells (Figure 2). The number of large colonies was dramatically higher in cultures treated with rIFN-α2b compared to leuIFN, i.e. leuIFN treatment gave rise to substantially fewer resistant cells than rIFN-α2b. The leuIFN-treated cultures had only a few large colonies and virtually no scattered cells.
To determine a mechanism for the higher rate of resistant cell development in the rIFN-α2b cultures, the direct effect on cell viability was analyzed using the WST-1 assay and the results are given in Figure 3. The three melanoma cell lines that were tested showed similar responses to both IFN-α preparations over the dose range used. The WM-266-4 cell line was more sensitive than SKmel-28 and Mewo. Thus, a 50% reduction of WST-1 reactivity was seen at 10 pM in WM-266-4 cells while at least a 10-fold greater concentration of IFN was needed in the other lines. The subtle difference noted between the two IFN preparations in all three cell lines was within the sensitivity of the assay.

The suppliers of the IFNs indicate specific activities of 2.6x10^8 IU/mg protein and 1.1x10^8 IU/mg protein for rIFN-α2b and leuIFN respectively. The unit designations are based on antiviral activity, which means that with the molar concentrations used, more antiviral units of rIFN-α2b are given to the cultures. The IFN-sensitive Daudi cell line was used to demonstrate that the IFN preparations used had the expected potency. The rIFN-α2b was slightly more potent using 3H-thymidine uptake, closely reflecting the differences in specific activity (Figure 4).

As the WM-266-4 cells were more sensitive, that cell line was focused on for the continuing studies. During IFN treatment the rate of cell death increased with time to the same degree for both IFNs (Figure 5). DNA laddering and a positive Tunel reaction suggested apoptotic cell death (data not shown).

As both IFN species seemed to affect the cells in the same way during the first week of treatment, another possibility for the differential appearance of resistant clones was that the IFNs selected preexisting variants. There were distinct differences in morphology of the surviving colonies. In order to further study properties of resistant cells, clones were selected according to “Materials and Methods” by growth at low density for four weeks in presence of IFNs. Recloning was done at very low density without selection pressure during three weeks. Eighteen clones from cultures treated with each IFN species were isolated. It was possible to expand seventeen out of eighteen clones from the cultures treated with rIFN-α2b and eleven out of eighteen from the cultures treated with leuIFN. Interestingly, one
from the rIFN-α2b treated and seven from cultures treated with leuIFN grew slowly with a distinct morphology (Figure 6), while the others had the morphology of the wild-type cells. The resistant cells were enlarged with long slender protrusions. A few individual cells with that morphology were found in the wild-type cultures suggesting that they might be the precursors of the selected resistant cells.

The resistant WM-266-4 cells selected in IFN for four weeks were tested for subsequent IFN sensitivity. The cells were treated with graded concentrations of IFNs for five days. The percentage of apoptotic cells defined as sub-G1 cells was analyzed by flow cytometry at the indicated time points. The number of cells in the sub-G1 compartment was counted as apoptotic cells. Each point represents the mean ± SE of 3 independent experiments. Filled symbols correspond to leuIFN and open symbols to rIFN-α2b.

Discussion

All vertebrates have several IFN-α genes (10). It is known that they differ in potency depending on assay and target cell type. In humans IFN-α1 has the lowest specific activity both in antiviral and antiproliferative assays (11, 12), yet IFN-α1 makes up more than 30% of the IFN protein content expressed by mononuclear cells in peripheral blood after viral challenge. In array studies qualitative differences between type I IFNs have been found (13, 14).

In this study, highly purified IFN-α expressed by leukocytes after viral challenge was used. IFN-α2 made up at least 20% of the protein content. Careful characterization has revealed minute amounts of IL-6, TNF-α and MIP-1α, which in the doses used in the experiments reached levels at least 10,000-fold lower than the highest levels found in plasma during infections. We therefore concluded that the differences noted between leuIFN and rIFN-α2b were not due to contaminating proteins.

Our finding that there was a difference between rIFN-α2b and leuIFN in the ability to select for resistant clones was unexpected and clearly it did not seem to be due to immediate effects on the ability to directly block proliferation and induce apoptosis. A differential effect on cell viability was seen only after six days. Even more interesting was the fact that the IFN-selected clones were qualitatively different with respect to morphology, growth and sensitivity to the apoptotic effect of subsequent IFN-α treatment.
Melanoma cells are notorious for their ability to develop resistance to different kinds of treatments including IFN (2-5, 15). The unexpected finding that the cells could respond to IFNs by apoptosis after four weeks of selection suggested that resistance was reversible and thus regulated and not exclusively a result of selection of mutants. Inhibitors of cytokine actions like SOCS and PIAS are known to negatively regulate cytokine signaling (16, 17). There are very few studies on the effect of IFN signalling during long-term treatment, although such studies are potentially important for the design of clinical studies.

It has been reported that a positively charged C-terminal tail of IFN-α subtypes is important for the antiproliferative response (18). It is notable that IFN-α2 has no net positive charges in the C-terminal tail, while IFN-α14, 8 and 10 in leuIFN have between 3 and 4 net charges. IFN-α2 has been chosen for the development of a type I IFN into a therapeutic remedy and has been registered as such by several companies. The choice was reasonable as IFN-α2 is a major component in IFN preparations obtained from several cell types. However, only few large scale clinical trials have been conducted to evaluate the potential beneficial effect of other IFN-α proteins. Our finding that leuIFN is more potent than rIFN-α2b in suppressing the development of resistant cells and that resistance might be reversible justifies continued clinical studies comparing multi-component IFN-α, such as leuIFN, with single subtype IFN-α.

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References


Figure 7. Apoptosis of cells selected in the presence of IFN. DNA content of the cells treated with IFNs for 5 days was analyzed by flow cytometry after staining with propidium iodide. The bars represent the average number of cells found in the sub-G1 compartment from seven clones ± SD. Closed bars represent treatment with leuIFN and open bars rIFN-α2b. A: clones initially selected in rIFN-α2b. B: clones initially selected in leuIFN. The clones were expanded in IFN-free medium for 6 weeks before they were re-challenged with IFN. The difference between cells selected in rIFN-α2b (A) and leuIFN (B) was significant at all concentrations. (p<0.05).


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