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Interferon-α-Induced Inhibition of B16 Melanoma Cell Proliferation: Interference with the bFGF Autocrine Growth Circuit

Maria Torcia,*,†,[1] Maria Lucibello,* Giovanna De Chiara,* Danilo Labardi,* Lucia Nencioni,* Paolo Bonini,* Enrico Garaci,* and Federico Cozzolino*,‡

**Department of Experimental Medicine and Biochemical Sciences, University of Rome "Tor Vergata," Rome, Italy;*
†Department of Clinical Physiopathology, University of Firenze, Firenze, Italy; and
‡Institute of Experimental Medicine, National Research Council, Rome, Italy

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The molecular mechanisms underlying the growth inhibition induced by interferon-α (IFN-α) in B16 murine melanoma cells were investigated. IFN-α did not induce cell apoptosis, but strongly interfered with the synthesis of basic fibroblast growth factor (bFGF), which acts as an autocrine growth factor in this system. Inhibition of bFGF synthesis was observed at the same concentrations (50–500 pM, 10–100 U/ml) of IFN-α able to induce growth arrest of B16 melanoma cells. Although the synthesis of acidic (a)FGF was only slightly affected by IFN-α, the cytokine induced release of an aFGF-related low-molecular-weight peptide, which was able to interfere with bFGF binding to surface receptors. Thus, the molecular mechanisms of IFN-α activity on melanoma cells include a specific modulation of the bFGF autocrine circuit. © 1999 Academic Press

The activation of the bFGF gene has been reported as an essential event in the progression from melanocytic precursor lesions to malignant melanoma (1, 2). Experiments in vitro with human malignant melanoma cell lines documented that proliferation of these neoplastic cells is inhibited by antisense oligodeoxynucleotides targeted against different regions of human bFGF mRNA (3), thus confirming that the autocrine production of bFGF represents a prerequisite to sustain the proliferation of human malignant melanomas. More recently, it has been reported that antisense oligodeoxynucleotides against the translation start site of human FGF-receptor 1 (FGFR1) not only inhibit the proliferation of human normal and malignant melanocytes, but also cause dendrite formation and severe disruption of cell-cell contact, indicating that the interference with FGF-R expression and function could attain more dramatic effects on melanoma cell viability than those obtained with the sole deprivation of growth factor (4). Given the importance of bFGF and FGF-R in the autocrine growth of melanoma cells, a lot of efforts were devoted to design synthetic drugs or gene therapy strategies able to block FGF synthesis or FGF-R function (5–7).

The interferon (IFN) family consists of three mayor glycoproteins, IFN-α, IFN-β, IFN-γ. Although IFNs were first described as antiviral agents, it is now clear that they also compromise growth and differentiation of a large variety of cellular types, e.g., by limiting oncogene expression and modulating various aspects of host immunity (8). It has been reported that IFN-α and IFN-β can modulate the synthesis of FGF-like molecules in normal endothelial cells (9), human carcinomas, and bladder tumor cell lines (10, 11). Since IFN-α and IFN-β showed antitumor activity in metastatic malignant melanoma (12, 13), both as single-agent therapy or in combination with chemotherapeutic drugs, we wanted to study whether the cytostatic effect could be related to an interference with the autocrine growth circuit sustained by bFGF in melanoma cells. The paper reports that IFN-α down regulates the production of bFGF molecules by B16 murine melanoma cells and induces the release of aFGF peptides able to interfere with bFGF binding to surface receptor.

MATERIALS AND METHODS

Reagents. Human recombinant (hr) bFGF and mouse monoclonal anti-human bFGF were a generous gift of Dr. Federico Bertolero (Pharmacia, Nerviano, Italy). The proliferative activity of hr bFGF and the neutralizing activity of the monoclonal antibodies were tested on mouse endothelial cells obtained from American Type Culture Collection (ATCC, Rockeville, MD). hr aFGF and rabbit

[1] To whom correspondence should be addressed at Dipartimento di Fisiopatologia Clinica, Università degli Studi di Firenze, Viale Pieraccini 6, Firenze, I-50139. Fax: +39 055 427 1413. E-mail: g.torcia@dfc.unifi.it.

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anti-human aFGF were a generous gift of Dr. Ken Thomas (Merck, Sharp & Dohme, West Point, PA). The anti-aFGF antibodies recognize epitopes in all the three exons of aFGF molecule, as assessed by western blot analysis of synthetic peptides derived from aFGF molecule (data not shown). Mouse r IFN-α and mouse purified IFN-α/β were obtained by Lee Biomolecular (specific activity: 10^7 UI/mg).

Cell cultures. B16 murine melanoma cell line was obtained from ATCC and grown in RPMI 1640 medium supplemented with 2% fetal bovine serum (FBS, Hyclone, Milano, Italy) and 1 mM L-glutamine.

For proliferation studies, cells were cultured in 96-well plates at the concentration of 3×10^3 cells/well in RPMI/FBS, in the presence or absence of the indicated stimuli at 37°C. Cells were pulsed with 0.5 μCi of ^{3}H-thymidine (Amersham, Milano) in the last 8 h of culture, harvested on a glass fiber filter and the radioactivity recorded by a β-counter.

For apoptosis studies, cells were cultured in T75 flasks in the presence or absence of IFN-α (500 pM), bFGF (20 ng/ml), IFN-α + bFGF, or with puromycin (20 μg/ml, Sigma). After 12 h, cultures were diluted 1:1.5 with 0.5% Triton-X-100 in 5 mM Tris, 20 mM EDTA and lysed. Intact chromatin was separated by fragmented DNA by centrifugation for 20 min at 27,000 $\times$ *g* and stained by diphenylamine reagent as reported (14).

For immunoprecipitation studies, cells were cultured in 6-well plates in RPMI 1640 medium without methionine and cysteine (GIBCO, Milano) for 3 h, washed and resuspendend in the same medium, supplemented with 1% (v/v) dialyzed FBS, L-glutamine, 0.4 μCi/ml ^{35}S-methionine and 0.4 μCi/ml ^{35}S-cysteine (Amersham, specific activity 800 Ci/mmol) for 3 h at 37°C in 5% CO_2. At the end of the incubation time, supernatants were removed and cells washed with cold PBS and lysed in 0.25% NonIdet P-40 (NP-40) plus 1 mM Phenylmethylsulfonyl fluoride (PMSF). Supernatants and cell lysates were immunoprecipitated with 1 μg/ml of mouse monoclonal anti-bFGF antibodies or rabbit polyclonal anti-aFGF antibodies, in the presence or absence of 10 μg/ml cold bFGF or aFGF. Immunocomplexes were bound to protein G-sepharose, washed three times with PBS, eluted by boiling in SDS Laemmli buffer, and run on 15% SDS-polyacrilamide gel electrophoresis slabs, which were treated with Amplify (Amersham), dried, and exposed to Hyperfilm MP (Amersham).

For western blot analysis, NP-40 (0.25% in PBS) lysates from 2×10^7 cells and TCA-concentrated supernatants were diluted 1:1 in 2-mercaptoethanol Laemmli buffer, run in 15% SDS-PAGE as above, blotted against nitrocellulose filters, and immunostained with the appropriate antibodies (rabbit anti-aFGF, 1 μg/ml; mouse anti-bFGF, 1 μg/ml) or with pre-immune immunoglobulins. The antigen-antibody complexes were visualized using appropriate secondary antibodies and the ECL detection system, as recommended by the manufacturer (Amersham).

For radioligand binding studies, B16 cells were cultured in 24-well plates. Just before reaching the confluence, cells were washed and incubated in binding medium (RPMI 1640, HEPES 20 mM, 0.15% gelatin) with different concentrations of ^{125}I-bFGF (Amersham, specific activity 50 μCi/μg), in the presence or absence of 100-fold unlabeled hr bFGF for 2 h at 4°C under continous shaking. In parallel experiments, cells were pre-treated with RPMI 1640 medium at pH 3.0 for 40 s at 4°C, before incubation with the radioligand. Cells were then washed and the FGF bound to low affinity receptors eluted with 2.0 M NaCl. Cells were then lysed with 0.1% Triton-X 100 in phosphate buffer, pH 8.0, and bound radioactivity recorded in a γ-counter (Beckman). Specific binding was calculated for each experimental point, and data were analyzed by a scientific program (Fig. P, Biosoft).

For FGF receptor competition assays, B16 cells were cultured in T75 flasks, in the presence of 500 pM IFN-α in RPMI 1640 medium supplemented with 1% nutridoma (v/v) (Sigma). Supernatants were vacuum-concentrated, dialyzed against PBS and ultrafiltered on Amicon filters with different molecular weight (MW) cut-offs. Low and high MW fractions were both tested for the capacity to displace the FGF binding to B16 cells cultured in 24-well plates, in the presence of 0.5 nM ^{125}I-bFGF, in the same experimental conditions described above.

For northern blot analysis, mRNA was extracted from 5×10^7 cells (pre-confluent cultures), treated or not with 50 or 500 pM IFN-α for 12 h, by a purification kit (Quiagen), fractionated on a 1% agarose gel containing 2.2 M formaldehyde in MOPS buffer, and transferred to nitrocellulose filters in 10$\times$ SSC. The filters were probed with 100 ng of a randomly primed ^{32}P-labeled synthetic probe obtained from mouse a-FGF gene sequence and washed once in 1$\times$ SSC with 0.1% SDS, three times for 20 min each at 68°C in 0.2$\times$ SSC with 0.1% SDS, dried, and exposed for 20 h, at $-$70°C, to Kodak XAR film with an intensifying screen.

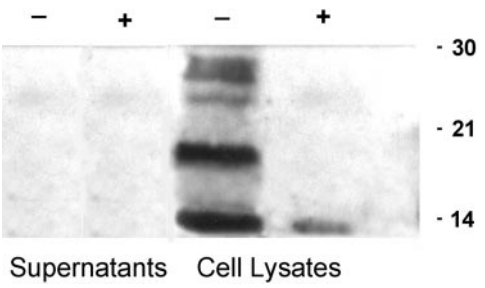

FIG. 1. bFGF production by B16 melanoma cells. Confluent B16 cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine for 4 h at 37°C. Supernatants and cell lysates were immunoprecipitated with anti-bFGF antibodies and protein G-sepharose, in the absence ($-$) or in the presence (+) of 1 μg/ml cold bFGF. B16 cells produce bFGF molecules with MW of 18 and 25 KDa.

RESULTS

bFGF is an autocrine growth factor for B16 melanoma cells. Murine B16 melanoma cells have been described to produce bFGF-like proteins with MW ranging between 26 and 46 KDa, but only highly metastatic B16 cell clones are able to release such proteins in the supernatants (15). In order to ascertain whether or not bFGF acts as an autocrine growth factor in B16 cells, we first studied bFGF production and release. Supernatants and cell lysates of metabolically labelled confluent B16 cells were immunoprecipitated with anti-bFGF monoclonal antibodies and analyzed in SDS-PAGE. Two forms of bFGF protein with MW of $\approx$18 and 25 KDa were detected, whose immunoprecipitation was specifically blocked by cold bFGF. These proteins were found only in cell-associated fractions (Fig. 1a).

A second set of experiments was performed to study the expression of bFGF receptors by B16 cells. Binding studies with ^{125}I-bFGF, in the presence or absence of excess cold bFGF, revealed the presence of both high ($K_d \approx$ 67 pM, 24,000 binding sites/cell) and low affinity ($K_d \approx$ 1 nM, $\approx 10^6$ binding sites/cell) receptors for bFGF on B16 cells.

Finally, proliferation studies, performed with hr bFGF, as well as neutralizing anti-bFGF antibodies, showed that the cytokine induced a dose-dependent increase in ^{3}H-thymidine incorporation by B16 cells in

TABLE 1

Effect of hr bFGF and of Neutralizing Anti-bFGF Antibodies on the Spontaneous Proliferation of B16 Melanoma Cells

	[^{3}H]thymidine incorporation (cpm)				
	0	5	10	15	20
bFGF (ng/ml)	2,835	6,574	12,321	13,538	13,465
Heat-inactivated bFGF (ng/ml)	—	2,285	2,365	2,987	2,934
Anti-bFGF antibodies (μg/ml)	—	1,847	1,168	995	965
Control mouse IgG (μg/ml)	—	2,974	2,843	2,867	2,543

Note. B16 melanoma cells were cultured at 2×10^3 cells/well in the presence of hr bFGF, the heat-inactivated cytokine, neutralizing monoclonal anti-bFGF antibodies, or control mouse IgG for 36 h. Cells were labeled with 0.5 μCi of [^{3}H]thymidine in the last 8 h of culture. Data, representative of one experiment of three performed, are expressed as mean [^{3}H]thymidine incorporation of triplicate cultures. SD was always <15%.

vitro, while neutralization of the autocrine bFGF by anti-bFGF monoclonal antibodies induced inhibition of the spontaneous B16 cell growth in vitro (Table 1). On the whole, these data show that bFGF is an autocrine growth factor for B16 cells.

Effect of IFN-α on B16 cell proliferation. It is known that IFN-α is able to inhibit the growth of human melanoma cells, both in vitro and in vivo (12, 13). When tested on B16 cells a dose-dependent inhibition of cell proliferation was observed, which reached a plateau at 500 pM (100 U/ml), with an half-maximal activity at ≈50 pM (Fig. 2a). A time-course analysis showed that the growth inhibition induced by IFN-α was maximal after 12 h of incubation (Fig. 2b). Thus, the cytokine showed a remarkable inhibitory activity at concentrations definitely lower than those reported so far (10, 11).

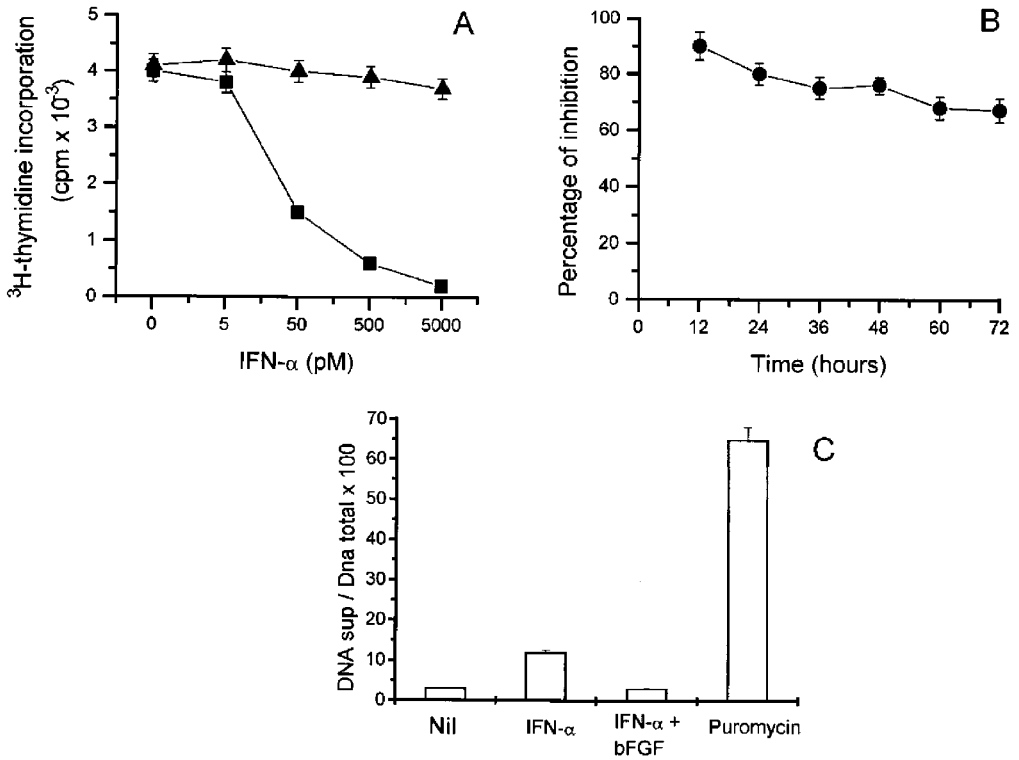

FIG. 2. (**A**) Dose-dependent effect of IFN-α on B16 melanoma cell proliferation. B16 cells were cultured at 3×10^3 cells/well in the presence of the indicated concentrations of IFN-α (■), or of the heat-inactivated molecule (▲) for 24 h. Cells were pulsed with 1 μCi of ^{3}H-thymidine in the last 8 h of cultures. Data represent the mean ^{3}H-thymidine incorporation of triplicate cultures. Data shown are representative of one experiment out of three performed. (**B**) Time-dependent effect of IFN-α on B16 cell proliferation. Cells were cultured at 3×10^3 cells/well in the presence of 500 pM IFN-α for the indicated times. (**C**) Effect of IFN-α on B16 cell apoptosis. Cells were incubated with 500 pM IFN-α, 20 μg/ml puromycin, or IFN-α + 20 ng/ml bFGF for 12 h, then lysed. The ratio of fragmented to intact DNA, as a measure of cells undergoing apoptosis, was determined by the diphenylamine method.

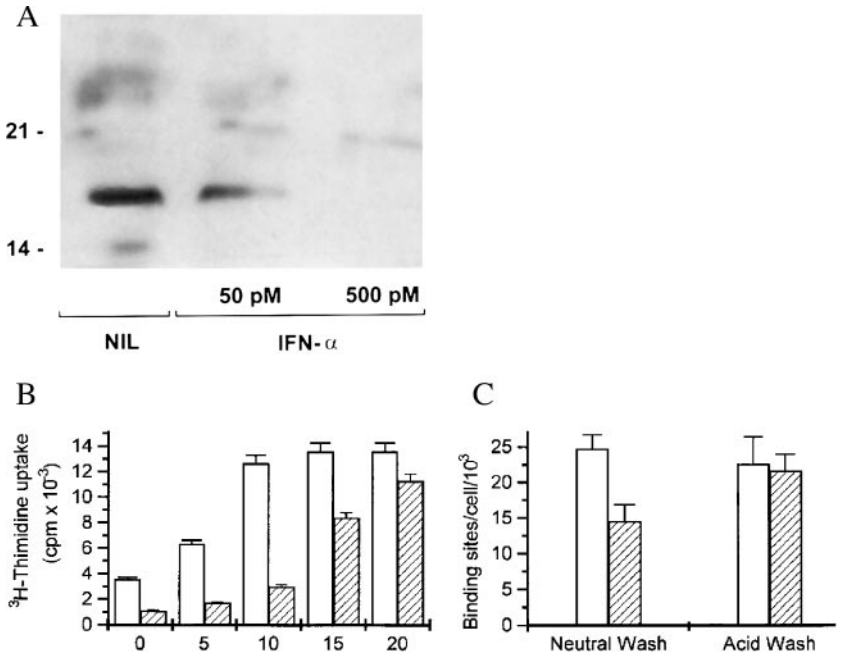

FIG. 3. IFN-α interferes with the FGF-induced autocrine growth circuit. (**A**) B16 cells were cultured in RPMI plus 1% nutridoma in the presence of 50 or 500 pM IFN-α for 18 h. Cells were lysed, blotted on nitrocellulose, and immunostained with anti-bFGF antibodies. (**B**) Effect of IFN-α treatment on bFGF-induced B16 cell proliferation. Cells were pre-treated with 500 pm IFN-α for 12 h at 37°C, washed, and cultured in the presence or absence of the indicated concentrations of hr bFGF at 3×10^3/well for an additional 36 h. Parallel cultures were set up with untreated cells. Cultured cells were pulsed with 0.5 μCi of ^{3}H-thymidine in the last 8 h of culture. Data, representative of one experiment out of three performed, are expressed as mean ^{3}H-thymidine incorporation of triplicate cultures. (**C**) Effect of IFN-α on functional parameters of FGF receptors expressed by B16 cells. Cells were cultured in the presence or absence of 500 pM IFN-α for 18 h at 37°C, washed with binding medium at pH 7.4 (NW) or with the same medium at pH 3.5 (AW), and incubated with ^{125}I-bFGF in the presence or absence of excess unlabeled bFGF. Data of specific binding were analyzed by a BIOsoft program. IFN-α inhibits bFGF synthesis and interferes with bFGF-induced proliferation of B16 cells, through the modulation of FGF-receptor availability.

In order to define the molecular basis of the IFN-α-induced growth inhibition, we first studied whether the cytokine was able to induce apoptosis in B16 cells and whether this phenomenon could be reverted by bFGF. B16 cells were incubated with 500 pM IFN-α, in the presence or absence of bFGF, or with puromycin as a positive control for 12 h, lysed, and the ratio of fragmented to intact DNA, as a measure of cells undergoing apoptosis, was recorded. Only 12% of B16 cells underwent apoptosis within 12 h of treatment and this effect could be completely reverted by bFGF (Fig. 2c), indicating that a cytostatic activity, rather than induction of apoptosis, was involved.

Effect of IFN-α on the bFGF autocrine growth circuit. Based on the above evidence, we studied the effect of IFN-α on the bFGF synthesis by B16 cells. Cells were incubated with 50 and 500 pM IFN-α for 18 h, then their lysates were run in SDS-PAGE. Western blot analysis performed with anti-bFGF antibodies showed that IFN-α strongly inhibited the synthesis of bFGF (Fig. 3a). Interestingly, the cytokine concentrations and the incubation times needed to obtain this effect were similar to those able to induce cytostatic acitivity.

To ascertain whether the inhibition of bFGF synthesis was reversible, IFN-α-treated B16 cells were washed, cultured for an additional 60 h, and then labelled with ^{35}S-methionine and ^{35}S-cysteine. Immunoprecipitation of cell lysates with anti bFGF antibodies revealed de novo synthesis of bFGF molecules (data not shown). Furthermore, pre-treatment of B16 cells with IFN-α for 12 h did not abolish their ability to proliferate in response to exogenous bFGF (Fig. 3b), even though higher doses of the factor were needed to obtain the same proliferative response. This observation strongly suggested that the cytokine could also affect the kynetic parameters of FGF receptors on B16 cells.

B16 cells were therefore treated with with 500 pM IFN-α for 18 h, washed, and incubated with ^{125}I-bFGF in classical binding experiments. Figure 3c shows that a reduction of the binding capacity was evident. In order to rule out a possible interference of endogenous ligands released by the cells, parallel experiments were performed by acid-washing the cells before incubation with ^{125}I-bFGF. Under these conditions, no alteration in the number or in the affinity of FGF receptors was detected. This set of data indicated that IFN-α did not modulate synthesis and/or expression of FGF receptors

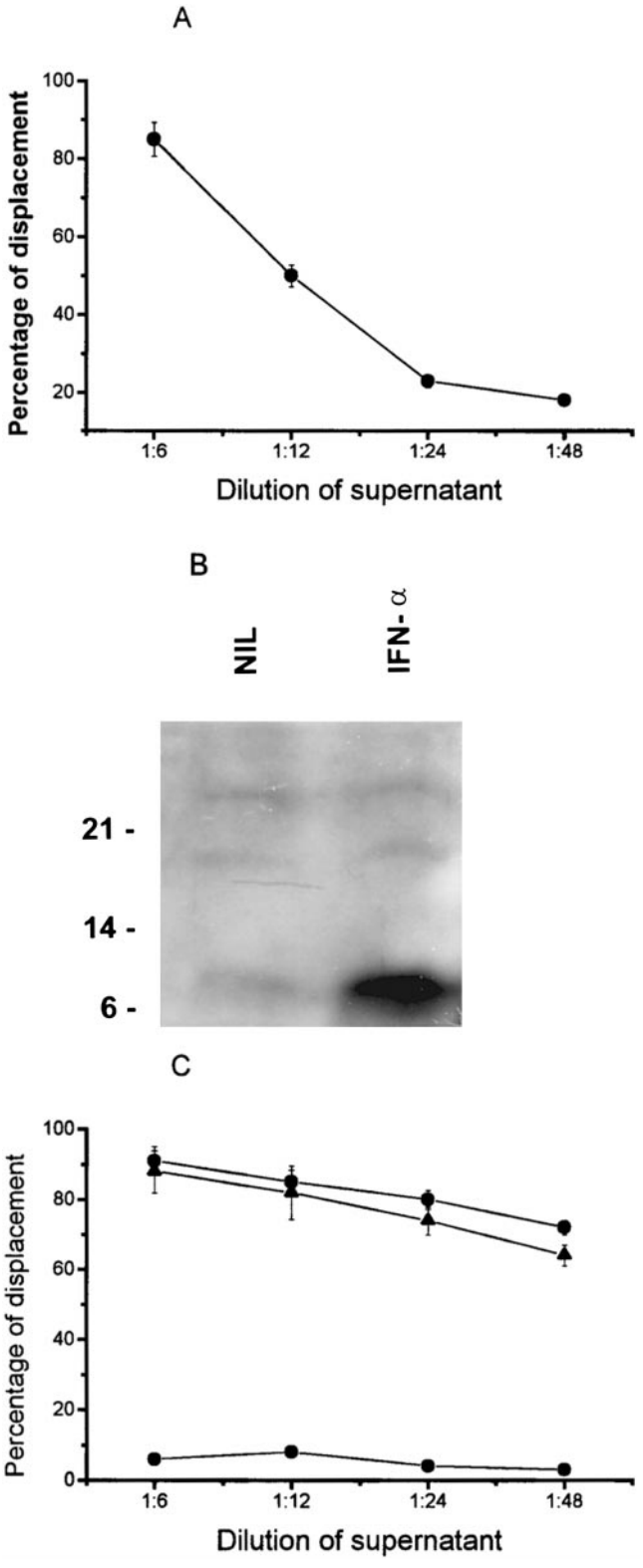

FIG. 4. IFN-α-conditioned medium of B16 melanoma cells is able to displace the binding of ^{125}I-bFGF to surface receptors. (**A**) Supernatants were obtained from IFN-α-treated B16 cells, cultured in T75 flasks in RPMI 1640 supplemented with 1% Nutridoma for 18 h, and vacuum-concentrated. For binding studies, B16 cells were cultured in triplicate in 24-well plates, in the presence of 0.5 nM ^{125}I-bFGF, with or without the indicated dilutions of B16 cell-conditioned medium, or with 100-fold excess cold bFGF. Results are expressed as mean ± SE percentage of the maximal displacement of radioactive ligand obtained with cold bFGF. In the same experimental conditions, the supernatants of unstimulated cultures did not yield any ^{125}I-bFGF displacement. (**B**) Supernatants of unstimulated or IFN-α-stimulated B16 cells, immunoblotted with polyclonal anti-aFGF antibodies, contain a 7.3 KDa MW protein, which is absent in supernatants of unstimulated cultures. aFGF is not evident in either supernatant. (**C**) Supernatants of IFN-α-treated B16 cells, obtained as above, were ultrafiltered on membranes with MW cut-off of 10 KDa, absorbed with rabbit anti-aFGF (●) antibodies, with preimmune IgG (■), or with rabbit anti-bFGF (▲) antibodies, coupled to protein A-sepharose, and tested as above for the ability to displace the binding of ^{125}I-bFGF. The displacement activity is restricted to the low-MW fraction of culture supernatants and is completely abolished by absorption with anti-aFGF antibodies.

on B16 cells, but rather it induced generation of molecule(s) able to occupy FGF receptors.

To test the latter hypothesis, ^{125}I-bFGF binding experiments were set up with supernatants from B16 cells cultured with no stimulus or with 500 pM IFN-α. Figure 4a shows that 5-fold concentrated supernatants from IFN-α-conditioned cells were able to displace the binding of ^{125}I-bFGF to B16 cell surface receptors.

Immunoblot analysis of these supernatants with anti-bFGF or with anti-aFGF antibodies did not reveal classical FGF molecules. However, a peptide with a MW of 7.3 KDa was specifically detected by anti-aFGF antibodies only in IFN-α conditioned media (Fig. 4b). In order to assess whether this peptide could be responsible for the FGF-R antagonist activity of IFN-α-conditioned media or to define the MW of other inhibitory molecules, we roughly separated the proteic fractions of conditioned media by ultrafiltration through membranes with MW cut-offs of 10, 20, 30, 50 KDa. The two fractions obtained in each ultrafiltration step were 5-fold concentrated and tested for the ability to displace the binding of ^{125}I-bFGF to FGF receptors on B16 cells. The analysis of data obtained clearly showed that the FGF-R antagonist activity resided in a fraction of MW $\leq$ 10 KDa (data not shown). Taking into consideration the presence of aFGF-related peptides, as revealed by western blot analysis, we wanted to ascertain whether the FGF-R antagonist activity could be removed by anti-aFGF antibodies. Figure 4c shows that the displacement of ^{125}I-bFGF, obtained with the $\leq$10 KDa proteic fraction of IFN-α conditioned media, was abolished after absorption with anti-aFGF polyclonal antibodies. Absorption with either monoclonal or polyclonal anti-bFGF antibodies or with control immunoglobulins did not produce any effect in the assay (Fig. 4c). Absorption with anti-EGF or anti-KGF antibodies yielded similar negative results (not shown).

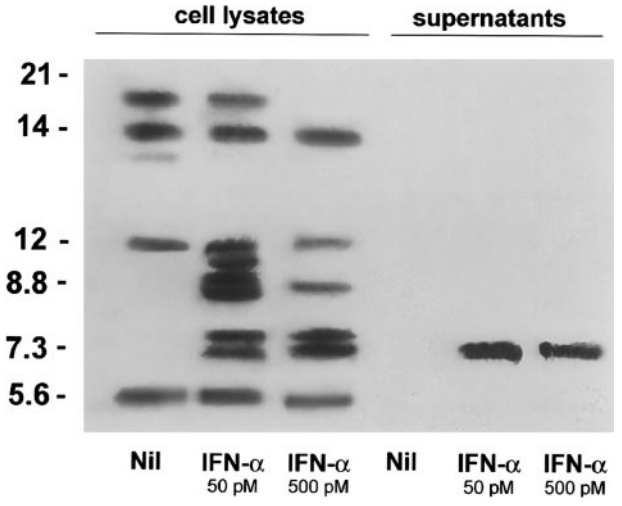

FIG. 5. IFN-α induces generation and release of low MW fragments of aFGF. Confluent B16 cells were metabolically labeled with ^{35}S-methionine and ^{35}S-cysteine for 3 h at 37°C. Supernatants and cell lysates were immunoprecipitated with anti-aFGF antibodies and protein A-sepharose, run in SDS-PAGE and autoradiographed. Low MW aFGF-related peptides were generated in IFN-α-stimulated cultures; a fragment of 7.3 KDa was released in the supernatants.

Assessment of the mechanisms generating aFGF-related peptides. Further experiments were performed to define whether these aFGF-related molecules could be the products of a differential splicing of the aFGF mRNA, resulting in shorter fragments coding for molecules still able to bind the FGF-receptors, as described for the human aFGF mRNA (16). A northern blot analysis of B16 cell mRNA with a murine full-length aFGF probe did not reveal any specific fragment shorter than 470 bp (data not shown). These data suggested that in our system the fragment was probably generated by post-translational modifications of the aFGF molecule. To test the latter hypothesis, we performed metabolic labeling of B16 with ^{35}S-methionine and ^{35}S-cysteine for 3 h. Cell lysates and supernatants were immunoprecipitated with anti-aFGF antibodies, run in SDS-PAGE, and autoradiographed. Figure 5 shows that fragments of MW $<$ 14 KDa were immunoprecipitaded by anti-aFGF antibodies after 3 h of incubation of B16 cells with IFN-α; one of this fragment, with a MW of 7.3 KDa, was also released in culture supernatants. As a whole, these data suggested that the aFGF-related fragments originated from de novo synthesized molecules of aFGF subjected to a high degree of fragmentation.

DISCUSSION

It is known that mammalian melanoma cell lines are highly sensitive to the cytostatic effect of IFN-α; consistently, the cytokine is commonly used, both as a single-agent therapy and in combination with chemotherapeutic drugs, in the treatment of human metastatic melanoma (12, 13). Several studies have demonstrated that bFGF is an autocrine growth factor for melanoma cells in vitro and, possibly, also in vivo (2, 3). In the present paper, we confirm the latter notion, describing that bFGF supports proliferation of B16 murine melanoma cells and provide evidence linking the FGF autocrine circuit with IFN-α activity. Infact, IFN-α disrupts the circuit by strongly reducing bFGF synthesis and by inducing the production of a low MW protein, immunoreactive with anti-aFGF antibodies, which competes with the endogenous native bFGF molecule for binding to the specific surface receptors.

Evidence for a negative control exerted by IFN-α on bFGF production was reported in different tumor systems, including renal and urinary bladder carcinomas (10, 11), at dose levels remarkably lower than those yielding a cytostatic effect in vitro, but sufficient to inhibit tumor neoangiogenesis in vivo (11). Thus, in these systems, that do not depend on bFGF, the antitumor activity of IFN-α basically relied on indirect effects. In the bFGF-dependent B16 cells, we observed that low (50–500 pM) concentrations of cytokine were sufficient to inhibit bFGF synthesis and, hence, to induce profound effects on melanoma cell proliferation. Taken together, such considerations might represent a rationale for a lower dosage treatment schedule for human melanomas. It must be stressed, however, that the biologic effect of IFN-α was reversible. A limited cell death was induced by the cytokine and, more important, the cells could regain their capacity to proliferate in response to exogenous bFGF, even though higher doses of cytokine were needed to obtain optimal proliferative responses. This observation underscores the difference between growth and survival factors. Probably, in B16 cells other proteins, such as IGF-1 or integrins (17, 18), are strictly related to cell survival, while bFGF signal might be linked to the cell proliferation machinery only, in keeping with the original studies showing that the sole insertion of a leader sequence in the bFGF coding region was sufficient to induce a transformed phenotype in normal fibroblasts (19). Validation of this hypothesis must await a fine dissection of the specific metabolic pathways triggered by each receptor and a better definition of the complex cross-talks taking place amongst such pathways.

In our experimental conditions, bFGF protein species of 18 and 25 KDa were detectable in cell-associated fractions only, in accordance with Blanckaert and coll. (15). Nevertheless, spontaneous proliferation of B16 cells was inhibited by anti-bFGF neutralizing antibodies, suggesting that low amounts of endogenous bFGF were exported out of the cells and bound to the extracellular matrix or interacted with high affinity surface receptors, delivering their proliferative signals. Similar occurences of "calibrated" factor secretion have been reported, since alternative mechanisms were pro-

posed to explain externalization of bFGF and of other proteins lacking a signal sequence (20–22).

A peptide of ≈7.3 KDa could be specifically detected by anti-aFGF antibodies in culture supernatants of IFN-α-treated B16 cells. A 7 KDa peptide, derived from an alternative splicing of aFGF mRNA, named aFGF′, was reportedly able to displace the binding of bFGF from its high affinity receptors (16), but we could rule out mRNA processing as the source of the fragment in our system. Instead, biosynthetic labelling of B16 cells clearly showed that peptides of MW ranging between 5.6 and 8.8, including a prominent species of 7.3 KDa, were immunoprecipitated by anti-aFGF antibodies as early as 3 h after IFN-α stimulation. These fragments likely originated from the proteolytic cleveage, induced by IFN-α, of de novo synthesized aFGF molecules. Whether IFN-α activated specific endopeptidase or this effect was mediated by accelerated matrix degradation, followed by reduced stability of aFGF molecules, still remains to be established.

Notably, the 7.3 KDa peptide was in a cell-associated fraction, but also present in the culture supernatants, a condition which might provide hints to outline the mechanisms of its generation. In fact, if an intracellular proteolysis of aFGF is involved, the inherent unfolding of the processed peptide may be instrumental for its secretion, which, like that of aFGF itself, is poorly understood, due to the absence of a leader peptide sequence. Likewise, secretion of IL-1, another "leaderless" secretory protein, takes place only after processing of the precursor molecule by Interleukin-1 converting enzyme (23).

The 7.3 KDa peptide was likely responsible for the displacement of ^{125}I-bFGF from surface receptors, since the latter activity was restricted to the low-molecular weight fraction of the conditioned media and could be removed by absorption with anti-aFGF antibodies, but not with unrelated antibodies. Although the aminoacids critical for FGF-R binding have been identified and mapped in position Y24, E96, N101, Y103, L140 (24), several reports indicate that also peptides derived from the primary sequence of aFGF are able to interact with surface receptors, occasionally mediating some biologic activity (16, 25). In this connection, the release of the 7.3 KDa peptide in the extracellular milieau represents an effective mechanism that potentiates the IFN-α-mediated inhibition of the FGF autocrine growth circuit.

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