

Interferon- α inhibits *in vitro* osteoclast differentiation and renal cell carcinoma-induced angiogenesis

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Abstract. Bone is a common site of osteolytic and richly vascularized metastases of renal cell carcinoma (RCC) and Interferon (IFN)- α based therapies have been considered for the treatment of patients affected by this disease. The effects of IFN- α on metastatic RCC patients have been related to its immunomodulatory, and cytotoxic activity on tumor cells, but there could be an effect also on tumor induced osteoclast differentiation and bone angiogenesis. When osteoclasts obtained from human peripheral blood mononuclear cells, cultured in the presence of receptor activator of nuclear factor- κ B (RANKL) and macrophage-colony stimulating factor (M-CSF), were treated with IFN- α , the expression of bone tartrate resistant acid phosphatase (TRACP) type 5b was reduced, as well as calcium-phosphate resorption activity and expression of pro-osteoclastic transcription factor c-Fos. IFN- α modulation of angiogenesis was studied by analysis of proliferation, survival, and migration of a bone endothelial cell line (BBE), and by the analysis of pro-angiogenic factor expression in RCC cell lines. IFN- α inhibited bone endothelial cell proliferation and the expression of FGF-2, while the vascular endothelial growth (VEGF) did not show any significant variation. Moreover, IFN- α inhibited the migration induced by the RCC through the impairment of fibroblast growth factor-2 (FGF-2) secretion. These data demonstrate multiple activities of IFN- α on renal cancer-induced bone disease, in addition to its recognized role as a cytotoxic and immunomodulatory agent, because they indicate its ability to reduce bone resorption and to impair tumor-associated angiogenesis, and they also suggest the use of IFN- α to treat skeletal metastases of other carcinomas.

Introduction

Interferon (IFN)- α , the first cytokine to be produced by recombinant DNA technology, demonstrated direct and indirect anti-tumor activity in a variety of solid and hematologic malignancies, including metastatic renal cell carcinoma (1-3). Immunotherapies using interferons and/or cytokines, mainly interleukin-2, are currently the treatment of choice for metastatic renal cell carcinoma (RCC) in which response rates to IFN- α are approximately 15% (4), and recent randomized studies demonstrated a survival advantage for patients receiving systemic IFN-based therapy (5). Metastases of RCC are discovered at the time of diagnosis in almost one-third of the patients, and >50% of these involve the skeleton (6,7). IFN- α effects on tumor include prolongation of the cell cycle time of malignant cells, inhibition of biosynthetic enzymes and apoptosis, interaction with other cytokines, and immunomodulatory effects (1,2). However, other important determinants of bone metastases development, that involve interactions between tumor cells and the bone microenvironment, may be affected. Cancer cells migrated to the bone may have the ability to produce growth factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF-2), which attract endothelial cells in the bone marrow microenvironment, in order to produce neovascularization, as demonstrated in breast cancer (8,9). This allows the cancer cells in the bone marrow to multiply and cause destruction of bone by attracting osteoclast progenitors from circulation at the metastatic site. Although the tumor cell/bone interactions responsible for bone destruction in bone metastases of RCC are not known, bone lesions in metastatic RCC are richly vascularized and osteolytic, and histologic analysis of bone metastases of RCC typically shows a large amount of osteoclasts around the tumor cells, causing extensive bone destruction (10). Therefore, angiogenesis and osteoclastogenesis can be possible effective targets for the treatment of this disease. IFN- γ and IFN- β are already known to inhibit osteoclast differentiation and bone resorption *in vitro* and *in vivo* (11). On the contrary, the ability of IFN- α to modulate bone metabolism is largely unknown. Since IFN- α and IFN- β interact with the same membrane receptor (12), it is reasonable to speculate that also IFN- α can impair osteoclast differentiation. On the other hand, few and controversial results

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are reported on IFN- α effect on RCC-induced angiogenesis (13,14). In this report we sought to analyze if the effectiveness of the IFN- α treatment in the aggressive RCC is also due to impairment of osteoclast differentiation and bone resorption, as well as of tumor-associated neo-angiogenesis.

Materials and methods

Cell cultures. As models of RCC, we used both the CRBM-1990 cell line, previously isolated from a bone metastasis of RCC (15), and the ACHN cell line, originally derived from primary RCC (ATCC, Manassas, VA). The endothelial cell line BBE was cloned from fetal bovine sternum (16). Cell lines were maintained in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen, Carlsbad, CA) or Ham's F12 medium modified by Coon (Sigma, St. Louis, MO), supplemented with 10% fetal bovine serum (FBS) (Mascia Brunelli, Milan, Italy), L-glutamin (2 mM) (Sigma), penicillin (100 U/ml) (Invitrogen), and streptomycin (100 μ g/ml) (Invitrogen), depending on the assay.

Osteoclast cell formation assay. Osteoclasts were obtained from peripheral blood mononuclear cells (PBMC) (17). Fresh buffy coats (AVIS, Bologna, Italy) were diluted 1:1 with PBS, layered over 4 ml Hystopaque (Sigma), and centrifuged at 900 g for 30 min. A layer of mononuclear cells was extracted from interphase of the PBS and Hystopaque and centrifuged at 400 g for 5 min. Cells were rinsed in PBS and seeded on tissue-culture glass or plastic ware in DMEM (Euroclone, Milan, Italy) supplemented with 10% FBS (Hyclone, Logan, UT) and incubated at 37°C in a humidified 5% CO₂ atmosphere. After 1 h the medium was discarded and replaced with differentiating medium (receptor activator of nuclear factor- κ B (RANKL) 30 ng/ml, Peprotech, Rocky Hill, NJ; macrophage-colony stimulating factor (M-CSF) 25 ng/ml, Peprotech). In order to verify the differentiation of mononuclear cells to osteoclasts, after 7 days of continuous culture, cells were analysed for tartrate resistant acid phosphatase (TRACP) activity by cytochemistry (Acid Phosphatase, Leukocyte assay, Sigma). TRACP-positive cells containing 3 or more nuclei were considered to be differentiated osteoclasts.

Effects of IFN- α on osteoclast differentiation. To determine the effect of IFN- α treatment on osteoclast differentiation and formation, the BoneTRACP Assay (SBA Sciences, Turku, Finland) was used for the quantification of the active isoform 5b of TRACP (TRACP5b). Compared to TRACP, which may be expressed by different cells of the mononuclear phagocyte system (18,19), this is a specific marker that is enclosed in vesicular compartment of osteoclasts (20), and quantification of TRACP5b is representative of the differentiation of osteoclasts. PBMC seeded in differentiating medium in the presence of different concentrations (0, 250, 500, 1000 U/ml, respectively) of IFN- α (extracted from leukocyte, kindly provided by Alfa Wassermann, Bologna, Italy), were lysated at 7 days of culture, with Triton X-100 1% in PBS for 10 min, centrifuged at 1500 rpm, and evaluated for TRACP5b activity. Each treatment was carried out in quadruplicate and the experiment was replicated twice. The medium was changed after 3-4 days of

culture. IC₅₀ (drug concentration resulting 50% inhibition of TRACP5b activity) values were determined by seeding PBMCs with the same protocol described above. One hour after seeding, increasing doses of the drug were added.

Effects of IFN- α on osteoclast activity. To study the influence of IFN- α on osteoclast-mediated resorption, PBMC were cultured under differentiating conditions on a submicron calcium phosphate substrate (Osteologic Multitest Slide, BD Biosciences, Erembodegem, Belgium) (21), and incubated with different concentrations of IFN- α (0, 250, 500, 1000 U/ml, respectively) for 10 days. Medium was changed every 3-4 days, and each treatment was carried out in duplicate, and experiments were repeated three times. At the end of incubation, cells were removed by exposure to 0.01% NaOCl. Slides were rinsed twice in distilled water and observed by light microscopy with a 20x lens. The area resorbed by osteoclasts was quantified by image analysis by Lucia Software (Nikon, Tokyo, Japan) and expressed as percentage of resorbed area with respect to total area.

Expression of c-Fos and FGF-2 protein by Western-blotting. To evaluate c-Fos protein expression in IFN- α treated osteoclast precursor cells, PBMC were seeded on Petri dishes in the presence of differentiating medium with different concentrations of IFN- α (0, 250, 500, 1000 U/ml). After 2 days, protein lysates were obtained in boiling Laemmli buffer (22) and sonicated. Protein content was quantified by BCA system (Pierce, Rockford, IL), and equal amounts of protein (80 μ g) were loaded into each lane. Proteins were separated by 6% polyacrylamide gel electrophoresis and transferred to a nitrocellulose sheet. Membranes were stained with Ponceau red to confirm equal amount protein loading. Blots were probed with the anti-c-Fos (Ab-2) human polyclonal antibody (Calbiochem, La Jolla, CA) diluted 1:100 for 2 h and then with a horseradish peroxidase-conjugated anti-rabbit polyclonal antibody (Amersham Biosciences, Buckinghamshire, UK) 1:500 for 30 min. The reaction was revealed by chemiluminescence. FGF-2 protein expression in RCC IFN- α -treated cells was determined by the same protocol. Protein lysates were obtained after 3 and 5 days for ACHN cells, and after 4 and 6 days for CRBM-1990 cells cultured in the presence of different concentrations of IFN- α (0, 1000, 2500, 5000 U/ml). Equal amounts of protein (50 μ g) were loaded into a 15% polyacrylamide gel. The membrane was incubated with the rabbit polyclonal anti-FGF2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500 for 2 h. The reaction was revealed by chemiluminescence.

Semiquantitative RT-PCR. CRBM-1990 and ACHN cells were seeded in IMDM plus 10% FBS. After 24 h, medium was changed with different concentrations of IFN- α (0, 1000, 2500, 5000 U/ml). After 3 days for ACHN cells and 4 days for CRBM-1990 cells, RNA was isolated using RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). Total RNA was reverse transcribed into cDNA using the Advantage RT-for-PCR Kit (Clontech Laboratories, Palo Alto, CA). Semiquantitative RT-PCR amplification for human FGF-2 was determined using forward and reverse primers (GenBank™ accession no. NM_002006). The RT-PCR consisted in one denaturation

at 94°C for 5 min, and then 30 cycles of amplification of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec and extension at 72°C for 45 sec, and final extension at 72°C for 7 min. Specific cDNA for VEGF-A was determined using forward and reverse primers (GenBank accession no. NM_003376). The RT-PCR consisted in one denaturation at 94°C for 10 min and 30 cycles of amplification of denaturation at 94°C for 60 sec, annealing at 55°C for 30 sec and extension at 72°C for 45 sec before final extension at 72°C for 7 min. Parallel reactions were performed for every assay using primers designed to amplify human β -actin (GenBank accession no. NM_001101). Specific cDNA for β -actin levels was assayed by denaturation at 94°C for 10 min, and then 30 cycles of amplification of denaturation at 94°C for 30 sec, annealing at 65°C for 45 sec and extension at 72°C for 30 sec, and final extension at 72°C for 10 min. Primer sequences were: FGF-2: 5'-CTCTTTCAGCATTACAC-3' and 5'-TCCCCTAACACAACTTAC-3'; VEGF-A: 5'-CTTCCAGGAGTACCCTGA-3' and 5'-GCCTCGGCTTGTTCCACA-3'; β -actin: 5'-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3' and 5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3'. The products were separated by electrophoresis using 2% agarose gel stained with ethidium bromide (0.5 μ g/ml). The picture of the gel were transferred to the computer by camcorder and quantified by dedicated software for densitometric evaluation of the bands (Quantity One, Biorad Laboratories Headquarters, Hercules, CA, USA). The specific bands were normalized to β -actin signals determined in parallel for each sample. RT-PCR was replicated four times.

Chemotaxis assay. The inhibitory effect of IFN- α on the endothelial cell migration induced by CRBM-1990 and ACHN was tested. The chemotaxis assay was performed with the Boyden chamber technique using a 24-well plate. In the lower compartment of the wells RCC cell lines were seeded and maintained in Ham's F12 medium modified by Coon with 10% FBS for 24 h. Medium was then changed with serum-free medium with IFN- α (2500 U/ml). After 72 h BBE were resuspended in serum-free medium, at a final density of 5×10^4 cells/ml. After placing polycarbonate filters (Transwells, Corning, Corning, NY) with a pore size of 8 μ m over the wells, 200 μ l of the BBE suspension were seeded in the upper compartment. FGF-2 (10 ng/ml, Sigma) was added to some wells where ACHN or CRBM-1990 were seeded. BBE were allowed to migrate for 5 h at 37°C in a humidified atmosphere with 5% CO₂. The filter was then removed, and migrated cells on the lower side were fixed in 11% glutaraldehyde, stained with crystal-violet solution, and counted from nine random 20x optical fields in each well. This assay was performed in triplicate and replicated twice.

Effects of IFN- α on cell growth of BBE. To study the effect of IFN- α on growth of BBE cells, 60,000 cells/well were seeded in 12-well plates in Coon's medium plus 10% FBS. After 24 h, medium was changed with medium with different concentrations of IFN- α (0, 1000, 2500, 5000 U/ml). Cell growth was evaluated on harvested cells at different time-points (24, 48, 72 h) by trypan blue method. The growth curve was repeated three times, and for each condition two different wells were seeded.

Soft-agar assay. Anchorage-independent growth of RCC cells was analyzed after exposure to IFN- α . Briefly, cells were exposed to IFN- α (1000, 2500 or 5000 U/ml, respectively) for 4 or 5 days, corresponding to their doubling time, and seeded in 0.33% agarose (SeaPlaque; FMC BioProducts, Rockland, ME) with a 0.5% agarose underlay. Cells (3300) were plated in a semisolid medium (IMDM plus 10% FBS plus agar 0.33%) and incubated at 37°C in a humidified 5% CO₂ atmosphere. Colonies were counted after 10 days. The experiment was performed in triplicate and repeated twice.

Statistical analysis. Statistical analysis was performed with the StatView™ 5.0.1 software (SAS Institute Inc., Cary, NC). Due to the low number of the experiments data were considered distributed not normally and the differences between groups were evaluated by the non-parametric Mann Whitney U test. In figures, although results of assays are reported as mean \pm standard error of the means (SEM), median values are also indicated. The mRNA expression for angiogenic growth factors was analyzed by Wilcoxon test. The level for significance was set at $p < 0.05$.

Results

Inhibition of osteoclast formation and bone resorption. Osteoclasts derived from PBMC, as previously described, were characterized by the presence of multinuclearity (Fig. 1A) and by TRACP expression. To quantify the inhibition of osteoclast formation and activity by IFN- α , TRACP5b activity and *in vitro* ability to degrade a layer of calcium-phosphate were also evaluated. TRACP5b was significantly reduced after 7 days of treatment with the lower concentration of IFN- α (250 U/ml, $p=0.0495$) (Fig. 1B). IC₅₀ value obtained for this assay is 403.8 U/ml. IFN- α treatment also significantly impaired the degradation of the calcium-phosphate layer even at its lowest concentration (250 U/ml, $p=0.0455$) (Fig. 1C). To analyze if IFN- α effect on osteoclast precursors involves RANK signal transduction pathway via Fos, c-Fos protein level was evaluated by Western blotting after IFN- α treatment. As shown in Fig. 2, c-Fos expression was markedly inhibited after 48-h treatment with 250 U/ml IFN- α , confirming the observed inhibition of osteoclast differentiation under the same exposure conditions.

Inhibition of *in vitro* tumorigenesis of RCC cells. To evaluate IFN- α inhibition of tumorigenesis, colony formation of RCC cells in soft-agar, a recognized *in vitro* assay of transformation (23), was used. Both ACHN and CRBM-1990 cells treated with IFN- α showed a marked and a significant inhibition of colony formation in soft-agar compared to untreated cells (Table I).

Inhibition of angiogenesis. IFN- α treatment decreased significantly the proliferation of endothelial cells at all the end-points ($p=0.0209$, Fig. 3). In chemotaxis assay untreated ACHN cells increased the number of migrated BBE (mean \pm SEM, 69.8 ± 9.2 vs. 25.8 ± 8.9 spontaneously migrated BBE cells), as well as CRBM-1990 (53.3 ± 3.1). IFN- α did not directly inhibit the migration of BBE towards the neoplastic cells, as demonstrated by the non-significant differences

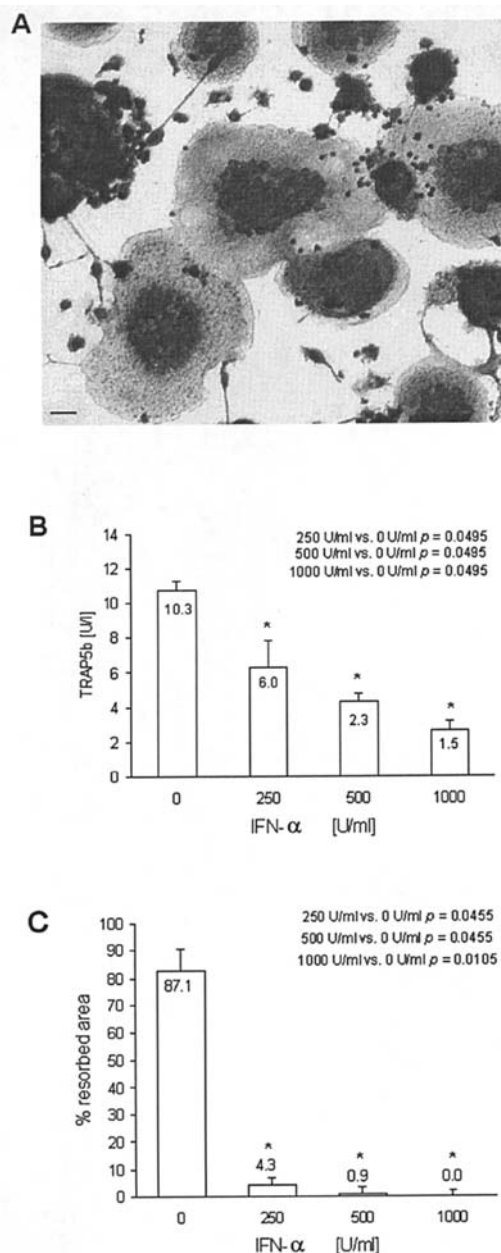


Figure 1. IFN- α effect on osteoclasts. Human mononuclear cells from peripheral blood were seeded in DMEM plus 10% FCS, RANKL, and MCS-F. (A) Multinucleated giant osteoclasts induced to differentiate by RANKL and M-CSF from PBMC at 7 days of culture (representative pictures, Giemsa staining) (bars, 20 μ m); (B) TRAP5b activity; (C) *In vitro* resorption activity was evaluated as fraction of absent calcium-phosphate layer with respect to the total area. IFN- α strongly inhibited osteoclast activity and differentiation. Results represent means \pm SEM. Median values are also reported (labels). In TRAP5b assay, each treatment was carried out in quadruplicate and the experiment was replicated twice, while in resorption assay each treatment was carried out in duplicate, and the experiments were repeated three times. *Significantly difference between medians TRAP5b (B) or medians percentage of resorbed area (C) by Mann Whitney U test ($p < 0.05$).

between the BBE migrated versus non-IFN- α medium (25.8 ± 8.9) and the BBE migrated versus the IFN- α added medium (19.7 ± 0.5). The treatment of CRBM-1990 with IFN- α induced a significant reduction in BBE migration ($p = 0.0495$) (Fig. 4). The percentage of inhibition was 24.1%. FGF-2 significantly reverted the inhibitory activity of IFN- α on BBE migration towards CRBM-1990 ($p = 0.0495$). The

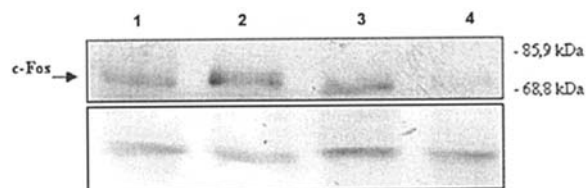


Figure 2. Western blot analysis of c-Fos expression level in PBMC cells stimulated with RANKL and MCS-F and treated with IFN- α (representative figure of a single experiment). IFN- α was added to the culture 1 h after seeding, while M-CSF and RANK-L were also added to the medium. Protein extracts were collected after 2 days. In the lower panel equal amounts of protein loaded were checked by Ponceau red staining. Lanes 1-4, 0-50-100-250 U/ml IFN- α . IFN- α induced a down-regulation of pro-osteoclast differentiation transcription factor c-Fos. The analysis of c-Fos expression was replicated twice.

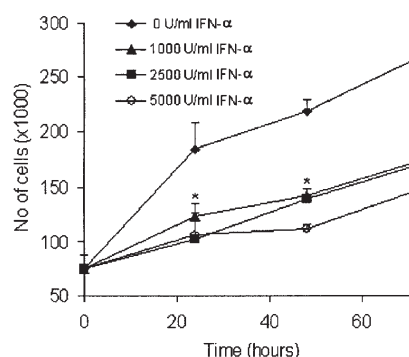


Figure 3. Survival of BBE cells at different concentration of IFN- α . Cells were seeded on 12-well plates. The number of viable cells per well was determined by Trypan blue cell counting at different times. The growth curve was repeated three times, and for each condition two different wells were seeded. Results represent means \pm SEM. Statistical differences between median values (medians are not shown) were analysed by Mann Whitney U test. All treatments at different concentrations of IFN- α , in respect to the control, at each time-point, were responsible of a significant inhibition (p -value is the same at all time-points and at all conditions, $p = 0.0209$).

treatment of ACHN with IFN- α had no significant effect although there is a trend of a reduced BBE migration in IFN- α treated cells (percentage of inhibition 17.4%) (Fig. 4). IFN- α treatment of ACHN for 72 h did not inhibit significantly the expression of mRNAs specific for VEGF-A, and FGF-2. However, in CRBM-1990 cells at 96 h a significant trend of a reduced expression of FGF-2 was demonstrated by the semiquantitative PCR (Fig. 5). By Western blotting, FGF-2 protein was also down-regulated (18 kDa FGF-2 fraction) (Fig. 6). On the contrary IFN- α after 72 h had no effect on growth factor gene expression by ACHN, as it was verified by the semiquantitative PCR and by Western blotting (data not shown).

Discussion

IFN- α has undergone extensive clinical evaluation for the treatment of advanced RCC, often in combination with other anti-tumor agents. The mechanism of action of this cytokine remains to be fully elucidated, even though anti-tumor effects have been associated to a direct cytotoxic activity (24,25), and to the killing of tumor cells by activated T cells and NK

Table I. Growth in soft agar of RCC cells treated with IFN- α .

Cells	Conc. IFN- α [U/ml]	No. of colonies ^a Median Range	% Inhibition	p-value
ACHN	0	342.5 322.0-363.0		
ACHN	1000	128.5 102.0-155.0	62	0.0495
ACHN	2500	84.5 77.0-92.0	75	0.0495
ACHN	5000	68.0 64.0-72.0	80	0.0495
CRBM-1990	0	379.5 368.0-391.0		
CRBM-1990	1000	210.0 171.0-210.0	45	0.0495
CRBM-1990	2500	259.5 245.0-274.0	32	0.0495
CRBM-1990	5000	179.0 173.0-185.0	53	0.0495

^aCells were seeded at a concentration of 3300 cells, and the number of colonies was determined after 10 days of growth in 10% FCS medium. Data are expressed as median of 3 plates, the experiment was repeated twice and p-value was calculated by Mann Whitney U test.

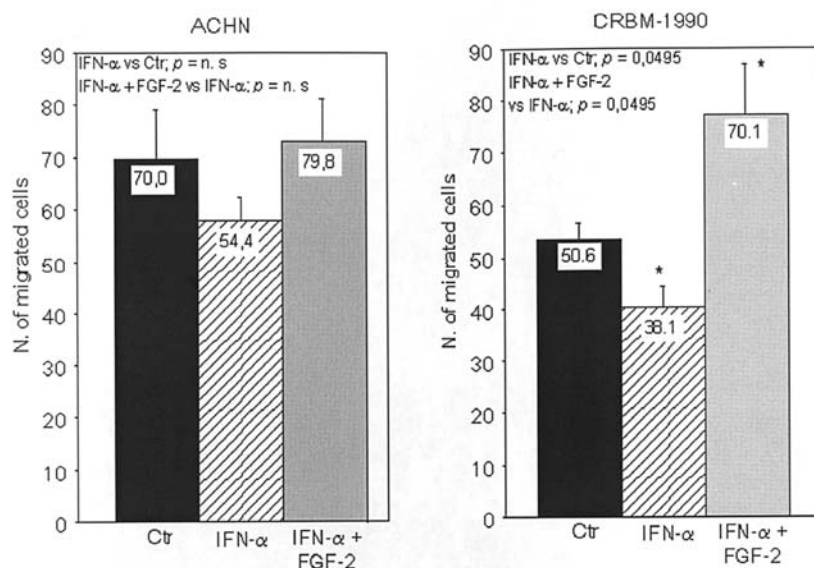


Figure 4. Effect of IFN- α on endothelial cell chemotaxis, induced by FGF-2 secreted by RCC. BBE were plated in the upper compartment of a Boyden chamber in which previously, ACHN (left panel) or CRBM-1990 (right panel) were seeded in the lower compartment, and treated with IFN- α (2500 U/ml) for 3 days. At the end of the incubation period of RCC with IFN- α , FGF-2 was added in a fraction of wells. The number of migrated BBE were determined by cell counting after crystal violet staining. The experiment was performed in triplicate, and repeated twice. * $p < 0.05$ by Mann Whitney U test. Data are reported as mean \pm SEM, and median values are also indicated (labels). In CRBM-1990 cells IFN- α significantly inhibited migration, and this effect was reverted by the addition of FGF-2 in the medium.

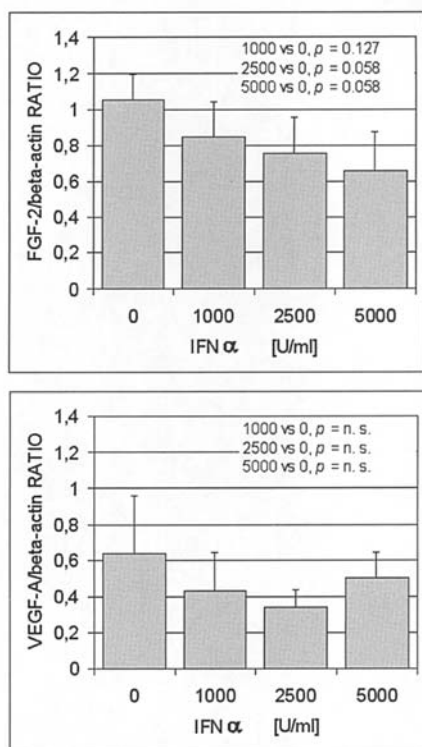


Figure 5. RT-PCR of mRNA of β -actin, FGF-2 (upper panel) and VEGF-A (lower panel) in CRBM-1990. The products were separated by electrophoresis and specific bands were quantified by dedicated software for densitometric evaluation. Each amplified product of the respectively pro-angiogenic gene was normalized to β -actin signals determined in parallel for each sample. RT-PCR was replicated four times.

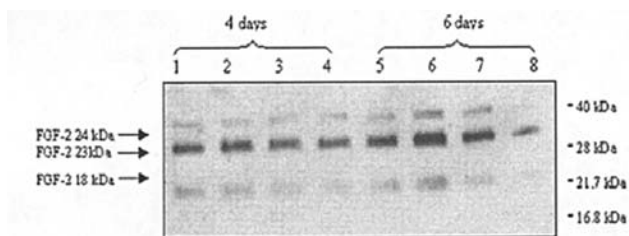


Figure 6. Western blot analysis of FGF-2 expression level in CRBM-1990 cells treated at different concentrations of IFN- α at different time-points (a representative figure). Membranes were stained with Ponceau red to confirm equal amount protein loading (not shown). Lanes 1 and 5, 0 U/ml IFN- α ; lanes 2 and 6, 1000 U/ml IFN- α ; lanes 3 and 7, 2500 U/ml IFN- α ; lanes 4 and 8, 5000 U/ml IFN- α . In CRBM-1990 cells 18 kDa FGF-2 expression decreased progressively with the increase of IFN- α concentration both at 4 and 6 days of treatment.

cells (3,14). We suggest that the benefits of IFN- α treatment in RCC metastatic patients are a result of combined effects on different targets of the tumor-host microenvironment within bone.

By using human PBMC as a source of osteoclast precursors, we found that IFN- α is able to strongly inhibit osteoclast differentiation, as shown by a significant reduction of TRACP5b. In turn, it inhibits *in vitro* resorption ability (calcium-phosphate degradation). The rate of response to IFN- α in TRACP5b assay and in resorption assay is different probably because TRACP5b is an earlier marker of osteo-

clast differentiation than the resorption activity. Moreover, TRACP5b and resorption activity are not directly connected, and it is not clear how much TRACP5b is involved in inorganic matrix and collagen Type I degradation (26). In addition to the known antiproliferative effect on hemopoietic progenitors (27), we found that the inhibition of osteoclast differentiation induced by IFN- α is due to a down-regulation of c-Fos transduction signal, affecting the RANKL cascade to a fully differentiated osteoclast. Therefore IFN- α impairs osteoclast differentiation through the same IFN- β mechanism (11,28). An anti-osteolytic effect has already been demonstrated for other members of the IFN family. In particular, signalling cross talk between RANKL and IFN- β is critical for bone homeostasis, because RANKL attachment to its receptor induces a negative feedback via IFN- β synthesis induction (11). In addition, IFN- γ strongly suppresses osteoclastogenesis by interfering with the RANKL-RANK signaling pathway, via TRAF6 (29).

To the understanding of mechanisms underlying osteoclastic bone resorption by cancer cells and interactions between tumor cells and bone marrow/bone micro-environment, new evidence has established the significant role of angiogenesis, mediated by endothelial cells of the host, in the development and amplification of bone metastases (9). Therefore, due to the peculiar abundant vascular component of bone metastases from RCC, response to IFN- α treatment in aggressive RCC could be a consequence of impaired neo-angiogenesis, in addition to osteoclastogenesis. Several authors agree that proangiogenic factors, such as VEGF and FGF-2, play an important role in the angiogenesis of RCC. The VEGF pathway is well established as one of the key regulators of angiogenesis in bone metastases. Activation of the VEGF-receptor pathway triggers a network of signalling processes that promote endothelial cell growth, migration, and survival from pre-existing vasculature (30). IFN- α appears to be particularly interesting since it is known to affect angiogenesis in different cancers (31-33).

Besides the direct inhibitory effect of IFN- α on the *in vitro* tumorigenesis of metastatic RCC cells, we found that IFN- α was able to impair the *in vitro* growth of bone endothelial cells. Moreover, IFN- α treatment down-regulated FGF-2 expression in CRBM-1990 as already demonstrated in other human carcinomas, but not in ACHN cells (34). FGF-2 is known to be responsible for the chemotactic signal for endothelial cells (35), and in our experiments IFN- α treatment caused a reduction of endothelial cell migration induced by CRBM-1990 cells in chemotaxis assay, and this inhibitory effect was reverted by FGF-2 addition to the medium. In general ACHN cells were less sensitive to IFN- α treatment in all the evaluated assays. The different rate of response of RCC to IFN- α depending on the cell line tested might be the result a different expression pattern of angiogenic growth factors. In previous studies, ACHN displayed a different angiogenic activity and different expression of both FGF-2 and VEGF, compared to CRBM-1990 (15). This dissimilar behavior might be explained by the genetic evolution of metastatic cells developed during several years after primary tumor removal. Although little is known about the genetic changes in RCC metastases, it has been demonstrated in

previous studies that RCC metastases display different and specific gene expression patterns depending on the tissue in which they migrate. The recent improvement in understanding molecular mechanisms of tumor development underlines how metastases can assume different characteristics in respect to the primary tumor (36,37). This phenomenon is probably due to the clonal origin of the primary tumor, and to the stimuli received in the site of migration, associated with that particular tissue type (38).

Among the different pharmaceutical preparations of IFN- α , IFN- α from leukocytes is constituted by a mixture of subtypes, including at least 15 subtypes, whereas IFN- α recombinant molecules contain single subtypes, such as IFN- α 2a or IFN- α 2b (39). Although the recombinant molecules have the clear advantage of being biologically safe, they also show unequal biological activities, and a mixture of natural IFN- α subtypes produces a more complete biological action than a single subtype, recombinant product (12). For this reason, IFN- α from leukocytes displays a wide range in the efficacy dose according on the specific biologic effect analysed. For example, the peak of concentration for immunostimulating action is at a much lower level than those useful in exerting the antiproliferative activity (39). In our work, a range of doses of 100-1000 U/ml was efficient to inhibit osteoclast differentiation and activity, while a range of 1000-5000 U/ml was needed for anti-angiogenic or anti-tumor effects. In a previous work, IFN- α tested on murine osteoclasts, was efficient to inhibit osteoclast differentiation at a very low dose (1 U/ml) (27). IFN- α and IFN- β interacts with the same receptor, the so-called type I, and they have a similar structure, especially for the subtype IFN- α 2, with 33% of homology (40). Therefore, in this work we used the results obtained with IFN- β to individuate the range of efficient dosage in order for IFN- α to inhibit osteoclast differentiation.

For anti-angiogenic and anti-tumor activity, we chose the range of 1000-5000 U/ml, based on previous work in which IFN- α was tested *in vitro* for the treatment of different cancers (41-43), or for the treatment of endothelial cells (43).

In conclusion, in this work we demonstrated that IFN- α efficacy for the treatment of osteolytic disease in metastatic renal cancer patients can be a consequence of multiple effects on distinct aspects of the pathogenesis of bone metastases. IFN- α effects on osteoclasts, in *in vitro* tumorigenesis and on FGF-2 expression and activity, indicate its use in combination with other drugs with different molecular mechanisms. These findings also evidence the potential benefit of using IFN- α for the treatment of osteolytic bone disease induced also in other metastatic cancers.

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