Inhibition of Angiogenesis and Vascular Tumor Growth by Interferon-Producing Cells

A Gene Therapy Approach

Adriana Albini,* Chiara Marchisone,[†]
Federica Del Grosso,[†] Roberto Benelli,*
Luciana Masiello,* Carlo Tacchetti,[‡] Maria Bono,[‡]
Maria Ferrantini,[§] Carmela Rozera,[§]
Mauro Truini,[¶] Filippo Belardelli,[§]
Leonardo Santi,^{*†} and Douglas M. Noonan*

From the Molecular Biology Laboratory and the Tumor Progression Section,* Istituto Nazionale per la Ricerca sul Cancro, the Centro di Biotecnologie Avanzate,[†] Genova; the Department of Experimental Medicine, Anatomy Section,[‡] Università di Genova, Genova; the Istituto Superiore di Sanità,[§] Rome; and the Servizio di Anatomia, Istologia e Citologia Patologica,[¶] Ospedale San Martino, Genova, Italy

We developed an *in vivo* gene therapy approach to characterize and optimize the anti-angiogenic activity of class I interferons (IFNs), using packaging cell lines producing an amphotropic LXS-based retrovirus expressing either IFN- α 1 (α 1Am12), IFN- β (β Am12) murine cDNAs, or the vector alone (neoAm12). Pretreatment of endothelial-like EAhy926 cells *in vitro* with conditioned media (CM) from α 1Am12 or β Am12 cells for 48 hours significantly inhibited their migration and invasion as compared to neoAm12-CM-treated cells. β Am12-CM also inhibited the formation of capillary-like structures on Matrigel by EAhy926 cells. *In vivo*, inclusion of the β Am12 cells strongly inhibited, and α 1Am12 partially inhibited, the angiogenic response in the Matrigel sponge model in both immune-competent and athymic nude mice. Electron microscopy showed a reduction of host cell infiltration in α 1Am12- and β Am12-containing sponges and reduction of invading tubular clefts of host cells as compared to controls. Finally, inoculation of either α 1Am12 or β Am12 cells (10%) along with a highly angiogenic Kaposi's sarcoma cell line (90%) resulted in a powerful reduction of tumor growth in nude mice *in vivo*, as did infection with the interferon- α -producing retroviruses. These data suggest that a gene therapy approach using class I interferons can effectively inhibit tumor angiogenesis and growth of vascular tumors. (*Am J Pathol* 2000, 156:1381-1393)

Anti-angiogenic therapy is one of the most promising strategies to inhibit tumor growth and metastatic dissemination. Most solid tumors cannot expand without generation of new blood vessels ensuring an adequate supply of oxygen and nutrients.¹ Furthermore, the formation of metastasis by solid tumors also appears to be dependent on neovascularization of the primary tumor. The complex process of angiogenesis includes the recruitment of nearby endothelial cells, their activation, degradation of the vascular basement membrane, proliferation and migration toward the angiogenic stimulus, and linkage to the preexisting vascular network to form a new capillary. Angiogenesis is mediated by multiple positive and negative regulatory molecules released by both tumor cells and the surrounding normal cells: the balance of these mediators determines the outcome of this process.^{2,3} Anti-angiogenic therapy is based on the use of negative regulators of neovascularization aimed at suppressing the pro-angiogenic signal or increasing the inhibitory signals. Class I interferons (IFNs), including the IFN- α leukocyte family and fibroblast IFN- β , are well known antiviral immunomodulatory molecules with an antiproliferative activity *in vitro* and *in vivo*.^{4,5} They were one of the first cytokines to be used therapeutically in clinical trials of human cancer, particularly in association with traditional chemotherapeutic agents. IFNs have been found to have anti-angiogenic properties as well. IFN- β is able to decrease 72-kd gelatinase (MMP-2) production by suppressing gene expression,⁶ and IFN- β appears to down-regulate bFGF mRNA expression and protein synthesis.⁷

We have previously demonstrated that these molecules are effective inhibitors of metastatic cell migration and invasion,⁸ as well as endothelial cell migration and invasion during the angiogenesis process.^{9,10} Further-

Supported by grants from the Compagnia di San Paolo, Torino, Italy (to A. A.); the Ministero dell'Università e Ricerca Scientifica e Tecnologica (MURST, to L. S. and C. T.); the Associazione Italiana per la Ricerca sul Cancro (AIRC, to A. A. and D. N.); the Ministero della Sanità, Il Programma Nazionale di Ricerca sull'AIDS (to A. A.); and CNR Target Project Biotechnology (to C. T.). A. A. is a participant in the EC Biomed II concerted action "HIV and Kaposi's Sarcoma."

Accepted for publication December 22, 1999.

Address reprint requests to Dr. Douglas Noonan, Modulo di Progressione Neoplastica, Istituto Nazionale per la Ricerca sul Cancro, Centro di Biotecnologie Avanzate, 16132, Genova, Italy. E-mail: noonan@ermes.cba.unige.it.

more, both IFN- α and IFN- β are able to suppress angiogenesis *in vivo*, with the effects of IFN- β being synergistically enhanced by 13-*cis* retinoic acid.^{9,10}

A major challenge in developing a successful anti-angiogenic approach is optimization of the route of administration. An anti-angiogenic strategy requires chronic, local delivery of the therapeutic molecule to the tumor in quantities able to inhibit neovascularization but without interfering with the normal physiology of the host vessels. The gene therapy approach may be ideal for these requirements, particularly for protein inhibitors.¹¹ Recombinant IFNs administered intravenously have a short half-life, rapid clearance, and systemic toxicity that limit delivery of the molecule to the tumor due to side effects. Local IFN- α or IFN- β gene transfer could overcome these difficulties providing chronic, high regional concentrations of the protein, potentially with minimal systemic side effects. However, there are several practical restrictions of the current vector systems, including the relative difficulty of obtaining high titers of LXS-based retroviruses producing high levels of an antiviral cytostatic agent such as class I IFNs. We have used a preclinical anti-angiogenic cell and gene therapy approach, with cDNAs encoding either IFN- α or IFN- β inserted into the recombinant retroviral vector LXS and transfected into a packaging cell line. The released infectious retroviruses contain the transfected gene and express it, but they are replication-incompetent. In addition, these lines also produce high levels of IFN protein. The strategy permits continuous and direct delivery of both IFN protein and retroviral IFN vectors to the tumor. The potential application to the clinic of direct introduction of packaging cells has already been tested in pilot clinical trials.^{12,13}

We tested the anti-angiogenic properties exerted by GP+*env* Am12 packaging cells transfected with an LXS genome expressing either murine IFN- α 1 (α Am12) or IFN- β (β Am12) cDNAs *in vitro* and *in vivo*. We show that conditioned media (CM) from both α Am12 and β Am12 cells have inhibitory activity on endothelial activation in chemotaxis and invasion assays. However, only β Am12-CM blocked the differentiation into capillary-like structures of endothelial cells on Matrigel, indicating that an influence on endothelial cell differentiation is exerted by IFN- β . This corresponded to the observation that β Am12 cells significantly inhibited angiogenesis *in vivo* in both immune-competent and in athymic nude mice.

Materials and Methods

Materials

Human recombinant IFN- α -2a (hr-IFN- α ; Roferon-A, Roche, Basel, Switzerland) and IFN- β (hr-IFN- β ; Frone 3.000.000, Serono, Geneva, Switzerland) were dissolved in phosphate-buffered saline (PBS), aliquoted, and stored at -80°C . Heparin (Clarisco, Schwarz Pharma S.p.A, Milan, Italy) was used *in vivo* at a concentration between 32 and 36 U/ml. Human synthetic HIV-1 Tat protein¹⁴ (Tecnogen, Cesna, Italy) was dissolved in PBS containing 0.1% bovine serum albumin (BSA), aliquoted, and stored at -80°C . Its

purity was verified by Western blot analysis and silver-staining. Human recombinant tumor necrosis factor- α (TNF- α , Sigma, St. Louis, MO) was diluted in PBS-0.1% BSA to a concentration range of 0.1 to 10 ng/ml and stored at -20°C . Matrigel was purified from the EHS tumor as previously described,¹⁵ and is commercialized by Collaborative Biomedical Products (Bedford, MA).

Cells

The human endothelial-like EAhy926 cell line, derived from the fusion of human umbilical vein endothelial cells with the A549 carcinoma cell line,¹⁶ was maintained in Dulbecco's modified essential medium (DMEM, Celbio, Milano, Italy) with 10% heat-inactivated fetal calf serum (FCS, Seromed, Berlin, Germany) and supplemented with glutamine (300 $\mu\text{g/ml}$). This line has the characteristics of endothelial cells,¹⁶ and immunofluorescence staining with an anti-human Factor VIII antibody demonstrated that these cells continue to synthesize Factor VIII similar to human umbilical vein endothelial (HUVE) cells. HUVE cells were obtained from the American Type Culture Collection and cultured in M199 containing 10% FCS, 10 ng/ml aFGF and 10 ng/ml bFGF (Peprotech, Rocky Hill, NJ), and 50 ng/ml of heparin (ICN, Irvine, CA) in gelatin-coated flasks. CM from Kaposi's sarcoma (KS) and NIH-3T3 cells were obtained by incubating a subconfluent T75 cell-culture flask of cells with 8 ml of serum-free DMEM (SFM) for 24 hours. Supernatants were collected, centrifuged, and stored at -20°C . KS-IMM cells, an immortalized KS cell line¹⁷ which forms highly angiogenic tumors *in vivo*¹⁸ was cultured in DMEM with 10% heat-inactivated FCS and supplemented with glutamine (300 $\mu\text{g/ml}$).

The amphotropic packaging cell line GP+*env* Am12,¹⁹ which produces a recombinant amphotropic retrovirus when transfected with the LXS genome²⁰ was maintained in DMEM and 10% FCS supplemented with 0.4 mg/ml of G418 (Calbiochem, La Jolla, CA). These cells were transfected with the plasmid form of the LXS retroviral vector,²⁰ the LXS plasmid containing a full cDNA insert encoding either murine IFN- α 1 (LMuIFN α 1SN) or IFN- β (LMuIFN β SN) in the LXS genome.²¹ The LMuIFN α 1SN-transduced cells (α Am12) produce between 4000 to 8000 U/10⁶ cells/day of IFN- α ; the LMuIFN β SN-transduced cells (β Am12) produce approximately 4000 U/10⁶ cells/day of IFN- β . The titers of biologically active IFN were measured by testing the antiviral activity of the supernatants on mouse L929 cells as previously described.²²

CM from a semiconfluent flask of α Am12 or neoAm12 cells were collected, filtered with a 0.2- μm filter (Millipore, Bedford, MA), and used to infect a semiconfluent flask of KS-IMM cells. The infection was repeated three times at 24-hour intervals. The infected cells were selected for resistance to neomycin using 0.8 mg/ml G418 in the media. The surviving cells were grown, pooled, and the supernatant of a confluent flask was collected and titrated for IFN- α production. Supernatants of the pooled IFN- α transduced KS-IMM cells contained 256 U/ml of IFN- α , whereas in supernatants of the pooled KS-IMM-

Figure 1. Chemotactic response of EAhy926 endothelial cells pretreated with human recombinant IFN- α and - β to Tat (a) and KS-CM (b). Recombinant class I IFNs were strong inhibitors of Tat- and KS-CM-induced migration.

neo-transduced cells (empty virus) no detectable IFN was found.

Methods

Chemotaxis Assays

This test was carried out in Boyden chambers as described²³ using different chemoattractants which induce an angiogenic response, including Tat with heparin, KS-CM, or NIH-3T3 cell-conditioned medium (NIH-3T3-CM), as indicated. To assess the effect of IFNs on EAhy926 cell migration, EAhy926 cells were pretreated with 10,000 U/ml of hr-IFN- α or hr-IFN- β in complete medium for 48 hours. SFM containing 0.1% BSA was used as a negative control.

To test the influence of neoAm12, α 1Am12, or β Am12 cell CM on endothelial cell migration, different flasks of EAhy926 cells were treated for 48 hours with CM from the packaging cells as indicated. CM were prepared by incubating $\sim 3 \times 10^6$ Am12 cells with 8 ml of medium for 24 hours. G-418 selection was omitted 48 hours before the incubation. SFM containing 0.1% BSA was used as negative control. Briefly, EAhy926 cells were harvested with trypsin/ethylenediaminetetraacetic acid solution (0.05/0.02% in PBS, Seromed), collected by centrifugation, and resuspended in SFM with 0.1% BSA. The lower compartment of Boyden chambers (200 μ l) was filled with che-

Figure 2. Effects of the pretreatment with neo-, α -, or β Am12 packaging cell-CM on migration and invasion of EAhy926 endothelial cells. **a:** Chemotaxis. The pretreatment with Am12-CMs did not significantly alter the background migration of EAhy926 cells (SFM), whereas α - or β Am12-CM pretreatment showed a clear inhibitory effect on NIH-3T3-CM induced migration. **b:** Invasion. α - or β Am12-CM pretreatment showed a clear inhibition of NIH-3T3-CM-induced invasion of EAhy926 cells.

moattractant. EAhy926 cells ($1.2 \times 10^5/400 \mu$ l/chamber) were placed in the upper compartment. The two compartments were separated by a polycarbonate filter (12- μ m pore size, Costar, Acton, MA) coated with gelatin (5 mg/L)

Figure 3. Effects of β Am12-CM on the migration HUVE cells and reversal of inhibition with an anti-IFN- β antibody. Treatment of HUVE cells for 48 hours with β Am12-CM caused a significant (*, $P = 0.005$) reduction in HUVE cell migration toward the KS-CM chemoattractant. Addition of 5 μ l of an anti-IFN- β antibody strongly reverted, and of 12 μ l completely reverted the inhibition of migration by β Am12-CM, demonstrating the direct involvement of IFN- β . SFM, background migration in the absence of chemoattractant.

Figure 4. Effects of neoAm12-CM, α 1Am12-CM, and β Am12-CM on growth and organization of EAhy926 endothelial cells in the Matrigel morphogenesis assay. EAhy926 cells spontaneously form capillary-like structures after 24 hours of incubation at 37°C. EAhy926 cells treated with neoAm12-CM and α 1Am12-CM were not affected in their ability to form these cellular networks. In contrast, β Am12-CM completely inhibited this process as well as the growth of endothelial cells on Matrigel.

to allow for cell adhesion. The chambers were incubated for 6 hours at 37°C in a humidified atmosphere containing 5% CO₂. After incubation, cells on the upper side of the filter were removed. The cells, which had migrated to the lower side of the filter, were fixed in 100% ethanol, washed in H₂O,

and stained with toluidine blue. Five to eight units/field per filter were counted at $\times 160$ magnification with a microscope (Zeiss, Göttingen, Germany).

The inhibitory effects of β Am12-CM were also tested on HUVE cell migration as above. To confirm a direct

Figure 5. MTT assay on EAhy926 cells treated with human recombinant IFN- α or IFN- β . Cells were grown in the presence of 10,000 Units/ml of either IFN- α or IFN- β in culture medium. Untreated control cells showed an eight-fold increase of the metabolic activity after 96 hours as compared to time 0, whereas IFN-treated cells showed only a twofold increase of the metabolic activity at 96 hours as compared to time 0. No loss of metabolic activity was observed in any of the samples.

involvement of IFN- β in the inhibition of migration, β Am12-CM were preincubated for 1 hour at 37°C before incubation with HUVE cells with either 5 μ l or 15 μ l of neutralizing monoclonal anti-IFN- β antibody (clone 7F-D3²⁴) at a titer of 4×10^6 inhibitory units/U of IFN- β . Preincubated β Am12-CM without antibody and DMEM with 5 μ l of antibody served as controls. The antibody alone did not significantly affect migration.

3-[4,5-Dimethylthiazol-2-yl]2,5-Diphenyltetrazolium Bromide Metabolic Assay

Cytostatic and potential toxic effects of IFN were tested using the MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide) metabolic growth assay. The EAhy926 cell line was plated in a 96-multiwell plate at 800 cells/well and samples treated in sextuplicate with 10^5 U/ml of either IFN- β or IFN- α or DMEM as a control. The cell growth number was assessed at 24-hour periods over 4 consecutive days. After the indicated hours, 50 μ l of 5 mg/ml MTT was added to each well and incubated at 37°C for 4 hours. MTT is reduced by live cells into an insoluble blue formazan product that was solubilized by addition of dimethyl sulfoxide and clarified by centrifugation. The absorbance was read with a multiwell scanning spectrophotometer at 540 nm.

Chemoinvasion Assay

The chemoinvasion assay^{23,25} was performed similarly to the chemotaxis assay, but with polycarbonate filters coated with 40 μ g/50 μ l/filter of Matrigel, a reconstituted basement membrane. This assay assesses the invasive capability of endothelial cells, mimicking the process of extravasation through the vascular basement membrane.

Matrigel Morphogenesis Assay

A 24-microwell plate, prechilled at -20°C, was carefully filled with 300 μ l/well of Matrigel (10 mg/ml) with a cold pipette, avoiding bubbles. The Matrigel was polymerized for 1 hour at 37°C. EAhy926 cells (70,000 cells/well) were suspended in the CM from either neoAm12, α 1Am12, or β Am12 cells and then carefully layered on top of the polymerized gel and incubated at 37°C. The effects of the CM on the growth and morphogenesis of endothelial cells was already evident after as little as 6 hours, and after 24 hours the wells were photographed with CCD optics and a digital analysis system (Image Pro Plus, Media Cybernetics, Silver Spring, MD).

In Vivo Angiogenesis

In vivo angiogenesis was determined with the Matrigel sponge model of angiogenesis²⁶ as modified.²⁷ Briefly, either neoAm12, α 1Am12, or β Am12 cells (1×10^6 cells in 100 μ l) were added to unpolymerized Matrigel solution containing either a potent cocktail of angiogenic factors (Tat-TNF α -heparin), or heparin and the packaging cells at 4°C to a final volume of 700 μ l. Tat was used at 100 ng/ml, TNF- α at 2 ng/ml, and heparin at 32 to 36 U/pellet. The Matrigel suspension was slowly injected subcutaneously into the flanks of C57/bl6 or nu/nu mice (Charles River, Milan, Italy) using a cold syringe. *In vivo* Matrigel quickly polymerizes and forms a solid gel. Between 8 and 12 gels were used for each treatment; experiments were performed three times.

After 4 days, the gels were collected and weighed; the samples were then minced and diluted in water to evaluate the hemoglobin content by a Drabkin reagent kit (Sigma), using a standard curve of mouse blood hemoglobin and normalizing to 100 mg of recovered gel. Some samples were fixed in formalin, embedded in paraffin, and sections stained with hematoxylin and eosin for histological analysis; others were prepared for electron microscopy.

Electron Microscopy

Tissues were fixed with 2.5% glutaraldehyde (Polyscience, Warrington, PA) in 0.1 mol/L of cacodylate buffer, pH 7.3, and postfixed with 1% OsO₄ (Polyscience) in the same buffer. After *en bloc* staining with 1% uranyl acetate and dehydration with ethanol and propylene oxide, samples were embedded in LX112 (Polyscience).²⁷ One- μ m thick sections were stained with toluidine blue and observed with a Leica DM/DR microscope equipped with Nomarsky optics. Gray-silver sections were stained with uranyl acetate and lead citrate and observed with Zeiss EM 10C or EM902 electron microscopes.

Tumor Growth in Vivo

The IFN- α -transduced and parental KS-IMM cells were suspended (5×10^6 cells) in Matrigel (10 mg/ml) and

Figure 6. Effects of neoAm12-CM, α 1Am12-CM, and β Am12 cells on angiogenesis *in vivo*. Histology of the Matrigel sponges collected from C57/black mice after 4 days *in vivo* and stained with hematoxylin and eosin. Vascular structures containing erythrocytes and lined by endothelial cells are readily visible in implants containing the angiogenic stimulus TTh (Tat, TNF- α , and heparin) alone or with neoAm12 cells. The sponges containing TTh and α Am12 cells do not show vessels but small lacunae lacking endothelial lining. The presence of β Am12 cells almost completely inhibited TTh-induced angiogenesis. Magnifications: **left**, $\times 100$; **right**, $\times 400$.

Figure 7. Effects of neoAm12-CM, α Am12-CM, and β Am12 cells on angiogenesis *in vivo* in C57/black immunocompetent (a) or athymic nu/nu (b) mice. The angiogenic response in Matrigel sponges was measured by the hemoglobin content. β Am12 cells significantly inhibited angiogenesis as demonstrated by the reduced hemoglobin content of the implants in both immunocompetent mice with TTh (a; * $P = 0.037$) or nude mice with the packaging cells alone (b; * $P = 0.033$). Inclusion of α Am12 cells did not significantly reduce TTh-induced angiogenesis (a).

injected subcutaneously in the flanks of nude (nu/nu) mice.

To test the potential of IFN- α or IFN- β to inhibit the tumor growth *in vivo*, 5×10^6 KS-IMM cells was mixed with 6.25×10^5 (the number of cells releasing approximately 2500 U of IFN/day) of either α Am12 or β Am12, suspended in Matrigel (10 mg/ml), and injected subcutaneously in nude mice. For the positive controls, animals were injected with 5×10^6 of parental KS-IMM cells or 5×10^6 KS-IMM mixed with neoAm12 cells. Each sample was performed in sestuplicate. On the 15th day after inoculation, sera from each animal were collected, diluted in DMEM with 2% FCS, and tested for IFN as above.

Tumor size was measured regularly, the animals were sacrificed and tumors were collected at the 22nd day, according to current ethical practice. Each sample was fixed in formalin, paraffin embedded, sectioned, and stained with hematoxylin and eosin. The samples were also stained immunohistochemically for Factor VIII with a polyclonal anti-murine Factor VIII antibody (DAKO, Carpinteria, CA) and an alkaline phosphatase-conjugated secondary antibody (DAKO).

Statistical analyses were performed using Student's *t*-test with the Prism (Graphpad, San Diego, CA) software package.

Results

Chemotaxis and Chemoinvasion

Endothelial cell responses to angiogenic factors can be measured *in vitro* by assessment of induction of endothelial cell chemotaxis and chemoinvasion.²³ Supernatants from KS cells,²⁷ which contain bFGF and VEGF, and Tat, which binds the VEGF receptor KDR,²⁸ strongly induce

angiogenesis. We first tested the effect of recombinant IFN- α or IFN- β on EAhy926 cell migration and invasion to these stimuli. Pretreatment of endothelial cells with 10,000 U/ml of recombinant human IFN inhibited Tat-induced migration by 96% for IFN- α and 85% for IFN- β (Figure 1a). EAhy926 cell migration to KS-CM was inhibited by 41% for IFN- α and 53% for IFN- β (Figure 1b). This observation confirms the ability of class I IFNs to inhibit the endothelial cell migration and invasion induced by angiogenic stimuli.

Because recombinant IFNs showed potent inhibition of endothelial cell migration, we first tested the effects of treatment with α Am12 or β Am12 cell supernatants on the endothelial cell response to the angiogenic factors produced by NIH-3T3 cells (NIH-3T3-CM) before use *in vivo*. Treatment of EAhy926 cells for 48 hours with media from neoAm12 cells had little effect on endothelial cell invasion and enhanced endothelial cell migration. In contrast, treatment with media from either α Am12 or β Am12 cells inhibited endothelial cell migration (Figure 2a) and repressed endothelial cell invasion down to background levels (Figure 2b). There was no significant difference between the level of inhibition produced by IFN- α or IFN- β . Pretreatment with the neoAm12-CM actually appeared to further activate EAhy926 cell chemotaxis to NIH-3T3-CM. Treatment of HUVE cells with β Am12 cell supernatants gave a similar inhibition of migration (Figure 3). This inhibition was completely reverted by addition of an anti-IFN- β antibody, confirming the direct involvement of IFN in inhibition of endothelial cell chemotaxis. These data suggest that the treatment of endothelial cells with α Am12 or β Am12 cell supernatants is able to suppress the chemotactic and invasive behavior of endothelial cells, and that the level of inhibition of endothelial cell migration and invasion was similar

Figure 8. Effects of neoAm12-CM, α 1Am12-CM, and β Am12 cells in a Matrigel implant containing heparin alone, *in vivo*. Histology of the Matrigel sponges collected from C57/black mice after 4 days *in vivo* and stained with hematoxylin and eosin. Gels containing neoAm12 cells showed the presence of blood-filled vascular lacunae, which were conspicuously absent in gels containing α 1Am12 or β Am12 cells. Magnification, $\times 200$.

independent of whether murine or human IFNs were being delivered.

Matrigel Morphogenesis Assay

Although chemotaxis and chemoinvasion assays describe the initial steps of endothelial cell activation, the morphogenic assay on Matrigel indicates the ability of endothelial cells to differentiate into capillary-like structures.²³ Untreated EAhy926 cells showed the typical

anastomosed cellular network 24 hours after plating. The addition of either neoAm12 or α 1Am12 supernatants did not influence endothelial cell morphogenesis (Figure 4). In contrast, treatment with β Am12 cell-conditioned medium completely inhibited the formation of these capillary structures, blocking growth and the morphogenic organization of EAhy926 cells on Matrigel (Figure 4).

Similar results were obtained with recombinant IFN- α and - β (not shown and see Refs. 9 and 10). Analysis of EAhy926 cell growth using the metabolic MTT assay indicated that these doses of IFN were cytostatic rather than being cytotoxic (Figure 5), with no loss of cell viability observed in the treated groups over 4 days.

In Vivo Angiogenesis

Our observations *in vitro* led us to test the IFN-based gene therapy approach to inhibit the angiogenic process *in vivo*. A combination of heparin, Tat, which specifically binds and activates the VEGF receptor KDR,²⁸ and TNF- α which up-regulates and activates KDR,²⁹ is a very potent angiogenic cocktail (TTh).¹⁸ Subcutaneous injection of the TTh cocktail in Matrigel sponges induced a strong angiogenic reaction, with the formation of vascular lacunae derived from host cells (Figure 6) corresponding to an increase in hemoglobin content (Figure 7a). Sponges containing TTh with neoAm12 control cells also showed a strong angiogenic response, with vessels lined by endothelial cells (Figure 6) and an elevated hemoglobin content (Figure 7a). The co-injection of TTh and α 1Am12 cells produced a limited inhibitory effect on angiogenesis, with smaller vessels lined by discontinuous endothelium and scanty host cell infiltration (Figure 6), as well as a lower hemoglobin content (Figure 7a). β Am12 cells markedly inhibited TTh-induced angiogenesis as indicated by the lack of vessels (Figure 6) and a dramatic reduction of hemoglobin content in Matrigel implants (Figure 7a). The Am12 packaging cell system is derived from NIH-3T3 cells, which also produce angiogenic factors. As expected, inclusion of neoAm12 packaging cells with heparin in Matrigel produced a substantial angiogenic response *in vivo* (Figure 8). The angiogenic response to α Am12 cells was reduced as compared to the neoAm12 controls, and there was little angiogenic response to β Am12 cells (Figure 8). To confirm that the inhibition of angiogenesis *in vivo* was due to direct effects of IFN- β on endothelial cells and not to a T-cell mediated response, similar experiments were performed in nude mice. In athymic nude mice a strong angiogenic reaction was observed in the Matrigel gels containing heparin and neoAm12 cells, whereas a significantly less angiogenic reaction ($P < 0.05$) was obtained when β Am12 cells were present (Figure 7b). No significant inhibition of angiogenesis was observed for α Am12-containing gels (not shown).

Microscopic observation of sections of implanted Matrigel samples mixed with either control TTh alone or TTh+neoAm12 cells showed the presence of invading clefts of cells, often surrounding a lumen (Figure 6). Ultrastructural analysis of the sample confirms these ob-

Figure 9. Electron micrographs of selected areas of implanted Matrigel containing TTh and mixed without packaging cells (**a**), with neoAm12 cells (**b**), with α Am12 cells (**c**, **e**), or with β Am12 cells (**d**). **a:** A continuous layer of cells surrounding an empty lumen. A neutrophil is present nearby (**asterisk**). **b:** A cluster of neutrophils (**asterisk**) close to host invading cells. **c:** An invading neutrophil. **d:** A neutrophil leading invading cells. A red blood cell (rbc) is entrapped among them. **e:** Several viral particles (**arrows**) either secreted or budding from an α Am12 cell mixed with the Matrigel. Scale bars: **a**, 2.9 μ m; **b**, 3 μ m; **c**, 1.6 μ m; **d**, 2.7 μ m; **e**, 0.18 μ m.

Figure 10. *In vivo* KS-IMM tumor growth. 5×10^6 KS-IMM were injected in nude mice alone or mixed with 6.25×10^5 of either neoAm12, α 1Am12, or β Am12 cells. In addition, 5×10^6 KS-IMM cells transduced with the supernatants of α 1Am12 cells were also injected (*inset*). The presence of IFNs either in the form of packaging cells or transduced into the tumor cells (*inset*) strongly inhibited tumor cell growth.

servations, demonstrating the presence of empty lumen surrounded by a continuous layer of cells (Figure 9). These vascular cells do not show evidence of budding viral particles, indicating that they originated from the host. Neutrophils are observed in the proximity of the invading cells, sometimes forming clusters (Figure 9, a and b).

Matrigel implants containing either α 1Am12 or β Am12 cells rarely showed cleft formation (Figure 6), however, invading neutrophils were observed (Figure 9, c and d). The virus-producing Am12 cells were mostly organized in round clusters (Figure 6). Injected Am12 cells retained the ability to produce viral particles as shown by the evident budding in Figure 9e (neoAm12 cells shown). These observations suggest that, unlike Matrigel samples containing either TTh or TTh+neoAm12 cells, the presence of α 1Am12 or β Am12 cells impaired the formation of invading tubular clefts with a general reduction of cellular recruitment. Some PMNs were still observed; however, the vascularization of the gel was reduced with α 1Am12 cells and virtually absent with β Am12 cells. The lacunae found in the presence of neoAm12 cells were lined by cells, whereas the lacunae found in the presence of α 1Am12 cells were not. These observations indicate that class I IFNs inhibited endothelial cell recruitment *in vivo*. The significantly lower hemoglobin content in the β Am12(+) cell samples may also be linked to the additional inhibition of endothelial cell differentiation by IFN- β . Electron microscopy of the gels implanted in athymic nude mice showed similar results, with β Am12-containing samples showing a reduced PMN infiltration as compared to controls.

In Vivo Tumor Growth Inhibition

We then investigated whether IFN- α and IFN- β were able to inhibit angiogenic tumor growth *in vivo* of the highly vascular KS-IMM cell line. KS-IMM cells were incubated with α 1Am12 cell supernatants and transduced cell lines

expressing high levels of IFN- α were isolated. When these lines were inoculated in nude mice *in vivo*, they demonstrated a markedly reduced capacity to form tumors *in vivo* as compared to KS-IMM cells which had not been transduced (Figure 10 inset). These data suggested that high local levels of IFN could inhibit tumor cell growth.

As a preclinical approach, KS-IMM cells were inoculated along with a fraction, consisting of 10% of the total cells inoculated, of either α 1Am12, β Am12, or neoAm12 cells. Co-inoculation with either α 1Am12 or β Am12 cells resulted in a near complete block of tumor growth (Figure 10). In contrast, co-inoculation of the neoAm12 cells resulted in tumor growth equal to or greater than that of the parental KS-IMM cells alone. Growth inhibition was accompanied by a lack of vascular structures and extensive necrosis (Figure 11), a similar pattern was observed in the tumors formed by *ex vivo*-infected KS-IMM cells (Figure 11). In contrast, the control parental KS-IMM tumors had a readily visible vasculature, as confirmed by immuno-histochemical staining for Factor VIII (Figure 11, inset). Detectable levels of IFN were observed in the sera in three of six animals treated with α 1Am12 cells, in one of six in animals treated with IFN- α transduced KS-IMM cells, and in one of six in animals treated with β Am12 cells. No IFN activity was found in any of the sera of the control animals. These data suggest that local rather than systemic IFN was responsible for the inhibition of tumor growth.

Discussion

Tumor angiogenesis is a rate-limiting step in tumor growth and metastatic spread. Tumor-induced endothelial cell activation leads to the acquisition of a phenotype characterized by chemotactic motility, basement membrane invasion, and proliferation, followed by differentiation into a new vessel. Gene therapy appears to be an ideal method for chronic local application of protein inhibitors of angiogenesis.

Class I IFNs, antiviral and immunomodulatory cytokines, are already known as anti-angiogenic drugs. IFN- α is used in the therapy of hemangiomas³⁰ and acts on this vascular tumor inducing apoptotic death.³¹ In addition, Fidler and colleagues³² have shown that the systemic administration of IFN- α in mice carrying a human bladder carcinoma, selected *in vitro* for insensitivity to IFN- α , is also able to inhibit the expression of bFGF and reduce tumor angiogenesis, leading to a block of tumor growth.

IFN- β can modulate growth factor production and function, blocking CSF-1-induced monocyte replication,³³ inhibiting PDGF-induced signal transduction in fibroblasts,³⁴ down-regulating bFGF mRNA in renal carcinoma cells⁷ and reducing EGF receptor expression in breast cancer cells.³⁵ From these observations IFN- β appears to exert its inhibitory effect on growth factors acting directly on their expression, or on receptor expression and function. IFN- β has also been used as an anti-angiogenic drug active against solid tumors in combination with AGM1470, a derivative of fumagillin,³⁶ or with tamoxifen³⁷ in experimental nude mouse models.

Figure 11. Histology of the KS-IMM tumors collected after 22 days. The tissue sections were stained with H&E. Parental KS-IMM cells proliferated rapidly, forming well-vascularized tumors, with Factor VIII-positive mouse endothelial cells (**inset**) in the parental KS-IMM tumor. KS-IMM mixed with $\alpha 1$ Am12 or with β Am12 cells formed small necrotic tumors showing no vascularization. IFN- α transduced KS-IMM cells formed small tumors with necrotic areas and some disorganized vessels which never contained erythrocytes. Magnification, $\times 400$.

We have shown that human recombinant IFN- α 2a and IFN- β are able to exert a direct anti-angiogenic effect on endothelial cells, inhibiting their migration and organization into capillary-like structures.^{9,10} A combination with 13-*cis* retinoic acid synergistically enhanced the effects of IFN- β , which permitted full activity at lower doses, suggesting it may be possible to by-pass the toxicity often observed in clinical use.¹⁰

IFN- α and IFN- β have been shown to share common receptors and it was thought that these would produce similar activities for the two molecules. However, there is substantial evidence indicating clear differences in their antiproliferative, antiviral, immunomodulatory, anti-invasive, and anti-angiogenic effects.²¹

Several studies have shown that IFN- β exerts a more potent anti-angiogenic activity than IFN- α ,^{7,21,38,39} including a reduction of tumor vascularization. Here we have shown that both IFN- α and IFN- β in an amphotropic viral vector affect endothelial cell migration and invasion, and that IFN- β also strongly affects endothelial cell morphogenesis. The multiple effects on IFN- β *in vitro* correlate with a more potent anti-angiogenic effect of retrovirally introduced IFN- β in angiogenesis assays *in vivo*. The anti-angiogenic effects of IFN- β were also evident in athymic nude mice, suggesting that the primary effects on endothelial cells observed *in vitro*, rather than the immunomodulatory activity *in vivo*, may be responsible for the anti-angiogenic activity of IFN- β .

A previous report indicated that human IFN- β only affected human, and not mouse, tumor-induced angiogenesis whereas murine IFN showed a similar specificity for murine tumors, suggesting species specificity.³⁸ However, these assays were all performed in murine model systems, thus the IFN- β activity observed was specific for the tumor type, and not for the host vascular cells recruited in the angiogenic reaction. We have previously shown that human IFN inhibits angiogenesis in murine models *in vivo*^{9,10} and effects murine tumor cells *in vivo*.⁸ These data indicate that human IFN was effective on murine endothelial cells. Here we show that recombinant human IFNs were effective on human cells *in vitro*. In addition, retrovirally-produced murine IFNs were effective on human cells both *in vitro* and *in vivo*. Our data indicate that class I IFN affects endothelial cells equally well when human or murine IFNs are used on human or murine target cells, suggesting that IFN-endothelial cell interactions are not species restricted.

Previous studies have shown that transduction of poorly immunogenic tumor cells (TS/A) with the same vectors can result in host immune-mediated rejection in immunocompetent animals.^{21,40,41} Histological analysis of IFN- β -transduced TS/A tumor cells showed a restricted vascularization of these tumors as compared to controls or IFN- α -transduced TS/A clones.²¹ In contrast, IFN- α appeared to induce a more vigorous immune response to the tumor cells. To confirm that the reduction of vasculature noted in the IFN- β -transduced cells was due to a direct inhibition of angiogenesis, we have shown here that IFN- β inhibits angiogenesis *in vivo* in a tumor cell-free model system, a direct action on endothelial cells was confirmed by *in vitro* assays.

Transduction of a human KS cell line with the IFN- α expressing vector significantly blocked the growth of this vascular tumor in nude mice *in vivo*. In addition, inoculation with either α 1Am12 or β Am12 cells also produced a potent inhibition of tumor growth. These experiments were performed in nude mice, thus the contribution of a specific tumor response is probably minimal, suggesting that the anti-angiogenic activity plays a key role in the inhibition of tumor growth. Interestingly, both IFN- β and IFN- α were equally potent at inhibition of tumor growth, suggesting that both are able to inhibit tumor angiogenesis *in vivo*. The effect of IFN on tumor growth appears to be quite strong, in that even with a 10:1 ratio of tumor to packaging cell inoculum tumor growth was strongly inhibited.

Monocyte/macrophages and PMNs may play an important role in angiogenesis, particularly in inflammatory conditions. These cells have been shown to produce angiogenic factors, such as interleukin-8 and VEGF.⁴² We have observed that PMNs often appear to be the first cells entering the Matrigel in response to an angiogenic stimulus, followed by macrophages, and later by endothelial cells (see Ref. 27 and submitted for publication). In the IFN-transduced TS/A model, IFN- α -transduced tumor cells showed an intense recruitment of granulocytes and macrophages into the tumors, whereas the recruitment of granulocytes and macrophages by the IFN- β -transduced TS/A tumors was substantially lower. Here we observed that PMN infiltration in response to cell-free antigenic stimuli was substantially reduced in the presence of IFN- β . A limitation of PMN and macrophage infiltration, as well as the release of angiogenic factors by these cells, by IFN- β could also contribute to the inhibition of angiogenesis *in vivo*. In contrast, the presence of these cells in IFN- α -treated samples could contribute to a nonspecific tumor inhibition.

One of the major difficulties in the anti-angiogenic therapy is the necessity for a continuous administration of the drug. This obstacle may be overcome by a gene therapy approach able to produce local high levels of a therapeutic protein. Here we have shown that administration of class I IFNs in a packaging cell system producing an amphotropic retroviral vector reduces endothelial cell invasion and migration *in vitro* and inhibits angiogenesis and tumor growth *in vivo*. Taken together these observations demonstrate that an IFN-based gene therapy approach is not only useful for enhancement of the immune response against tumor cells, but, by inhibiting the angiogenic process, can also directly limit tumor growth.

Acknowledgment

We thank Prof C. Grossi for critical reading of the manuscript.

References

1. Folkman J: Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nature Med* 1995, 1:27-31
2. Liotta L, Steeg P, Stetler-Stevenson W: Cancer metastasis and

- angiogenesis: an imbalance of positive and negative regulation. *Cell* 1991, 64:327–336
3. Stetler-Stevenson W: Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention. *J Clin Invest* 1999, 103: 1237–1241
4. Gutterman JU: Cytokine therapeutics: lessons from interferon alpha. *Proc Natl Acad Sci USA* 1994, 91:1198–1205
5. Belardelli F, Gresser I: The neglected role of type I interferon in the T-cell response: implications for its clinical use. *Immunol Today* 1996, 17:369–372
6. Gohji K, Fidler IJ, Tsan R, Radinsky R, Von Eschenbach AC, Tsuruo T, Nakajima M: Human recombinant interferons-beta and -gamma decrease gelatinase production and invasion by human KG-2 renal carcinoma cells. *Int J Cancer* 1994, 58:380–384
7. Singh RK, Gutman M, Bucana CD, Sanchez R, Llansa N, Fidler IJ: Interferons alpha and beta down-regulate the expression of basic fibroblast growth factor in human carcinomas. *Proc Natl Acad Sci USA* 1995, 92:4562–4566
8. Melchiori A, Allavena G, Bohm J, Remy W, Schmidt J, Parodi S, Santi L, Albini A: Interferons inhibit chemotaxis of transformed cells and their invasion of a reconstituted basement membrane. *Anticancer Res* 1987, 7:475–480
9. Benelli R, Masiello L, Pagliarunga G, Noonan DM, Repetto L, Carlone S, Orenco G, Fontanini G, Albini A: IFN-alpha inhibits Tat-induced angiogenesis. HIV and Cytokines. Edited by M Guenounou. Paris, Les Editions INSERM, 1997, pp 447–458
10. Iurlaro M, Benelli R, Masiello L, Rosso M, Albini A: Beta interferon inhibits HIV-1 Tat induced angiogenesis; synergism with 13-cis retinoic acid. *Eur J Cancer* 1998, 34:570–576
11. Kong HL, Crystal RG: Gene therapy strategies for tumor antiangiogenesis. *J Natl Cancer Inst* 1998, 90:273–286
12. Ram Z, Culver KW, Oshiro EM, Viola JJ, DeVroom HL, Otto E, Long Z, Chiang Y, McGarrity GJ, Muul LM, Katz D, Blaese RM, Oldfield EH: Therapy of malignant brain tumors by intratumoral implantation of retroviral vector-producing cells. *Nature Med* 1997, 3:1354–1361
13. Palù G, Cavaggoni A, Calvi P, Franchin E, Pizzato M, Boschetto R, Parolin C, Chilosi M, Ferrini S, Zanuso A, Colombo F: Gene therapy of glioblastoma multiforme via combined expression of suicide and cytokine genes: a pilot study in humans. *Gene Ther* 1999, 6:330–337
14. Ruvo M, Scarallo A, Vecchio G, Palombo G, Fassina G: Synthesis and biological characterization of HIV-1 Tat protein [1–86] BH10 isolate. *Peptides*. Edited by R Ramage, R Epton. Kingswiltford, UK, Mayflower Scientific Ltd, 1996, 1998, pp 771–772
15. Kleinman HK, McGarvey ML, Hassell JR, Star VL, Cannon F, Laurie GW, Martin GR: Basement membranes complexes with biological activity. *Biochemistry* 1986, 25:312–318
16. Edgell CJ, McDonald CC, Graham JB: Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc Natl Acad Sci USA* 1983, 80:3734–3737
17. Albini A, Paglieri I, Orenco G, Carlone S, Aluigi MG, DeMarchi R, Matteucci C, Mantovani A, Carozzi F, Donini S, Benelli R: The b-core fragment of human chorionic gonadotropin (hCG) inhibits growth of Kaposi's sarcoma-derived cells and a new immortalized Kaposi's sarcoma cell line. *AIDS* 1997, 11:713–721
18. Albini A, Florio T, Giunciuglio D, Masiello L, Carlone S, Corsaro A, Thellung S, Cai T, Noonan DM, Schettini G: Somatostatin controls Kaposi's sarcoma tumor growth through inhibition of angiogenesis. *FASEB J* 1999, 13:647–655
19. Markowitz D, Goff S, Bank A: Construction and use of a safe and efficient amphotropic packaging cell line. *Virology* 1988, 167:400–406
20. Miller AD, Rosman GJ: Improved retroviral vectors for gene transfer and expression. *Biotechniques* 1989, 7:980–990
21. Rozera C, Carlei D, Lollini PL, De GC, Musiani P, Di CE, Belardelli F, Ferrantini M: Interferon (IFN)- β gene transfer into TS/A adenocarcinoma cells and comparison with IFN- α : differential effects on tumorigenicity and host response. *Am J Pathol* 1999, 154:1211–1222
22. Belardelli F, Gessani S, Proietti E, Locardi C, Borghi P, Watanabe Y, Kawade Y, Gresser I: Studies on the expression of spontaneous and induced interferons in mouse peritoneal macrophages by means of monoclonal antibodies to mouse interferons. *J Gen Virol* 1987, 68: 2203–2212
23. Albini A: Tumor and endothelial cell invasion of basement membranes. *Pathol Oncol Res* 1998, 4:1–12
24. Kawade Y, Watanabe Y: Characterization of rat monoclonal antibodies to mouse interferon α and β . *The Biology of the Interferon System*. Edited by K Cantell, H Schellekens. Dordrecht, Martinus Nijhoff, 1987, pp 197–201
25. Albini A, Iwamoto Y, Kleinman HK, Martin GR, Aaronson SA, Kozlowski JM, McEwan RN: A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. *Cancer Res* 1987, 47:3239–3245
26. Passaniti A, Taylor RM, Pili R, Guo Y, Long PV, Haney JA: A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. *Lab Invest* 1992, 67:519–528
27. Albini A, Fontanini G, Masiello L, Tacchetti C, Bigini D, Luzzi P, Noonan DM, Stetler-Stevenson WG: Angiogenic potential *in vivo* by Kaposi sarcoma cell-free supernatants and HIV1-tat product: inhibition of KS-like lesions by TIMP-2. *AIDS* 1994, 8:1237–1244
28. Albini A, Soldi R, Giunciuglio D, Giraudo E, Benelli R, Primo L, Noonan D, Salio M, Camussi G, Rockl W, Bussolino F: HIV-1 Tat induced angiogenesis is mediated by activation of the flk-1/KDR tyrosine kinase receptor on vascular endothelial cells. *Nature Med* 1996, 2:1371–1375
29. Giraudo E, Primo L, Audero E, Gerber HP, Koolwijk P, Soker S, Klagsbrun M, Ferrara N, Bussolino F: Tumor necrosis factor- α regulates expression of vascular endothelial growth factor receptor-2 and of its co-receptor neuropilin-1 in human vascular endothelial cells. *J Biol Chem* 1998, 273:22128–22135
30. Folkman J, Ingber D: Inhibition of angiogenesis. *Semin Cancer Biol* 1992, 3:89–96
31. Sgonc R, Fuerhapter C, Boeck G, Swerlick R, Fritsch P, Sepp N: Induction of apoptosis in human dermal microvascular endothelial cells and infantile hemangiomas by interferon-alpha. *Int Arch Allergy Immunol* 1998, 117:209–214
32. Dinney CP, Bielenberg DR, Perrotte P, Reich R, Eve BY, Bucana CD, Fidler IJ: Inhibition of basic fibroblast growth factor expression, angiogenesis, and growth of human bladder carcinoma in mice by systemic interferon-alpha administration. *Cancer Res* 1998, 58:808–814
33. Hamilton JA, Whitty GA, Kola I, Hertzog PJ: Endogenous IFN-alpha beta suppresses colony-stimulating factor (CSF)-1-stimulated macrophage DNA synthesis and mediates inhibitory effects of lipopolysaccharide and TNF-alpha. *J Immunol* 1996, 156:2553–2557
34. Tamm I, Kikuchi T, Kreutter D, Pledger WJ, Pfeffer LM: Selective interferon-alpha/beta effects on platelet-derived growth factor-stimulated processes in quiescent BALB/c-3T3 fibroblasts. *J Interferon Res* 1994, 14:265–273
35. Iacopino F, Ferrandina G, Scambia G, Benedetti PP, Mancuso S, Sica G: Interferons inhibit EGF-stimulated cell growth and reduce EGF binding in human breast cancer cells. *Anticancer Res* 1996, 16: 1919–1924
36. Brem H, Gresser I, Grosfeld J, Folkman J: The combination of anti-angiogenic agents to inhibit primary tumor growth and metastasis. *J Pediatr Surg* 1993, 28:1253–1257
37. Lindner DJ, Borden EC: Effects of tamoxifen and interferon-beta or the combination on tumor-induced angiogenesis. *Int J Cancer* 1997, 71:456–461
38. Sidky YA, Borden EC: Inhibition of angiogenesis by interferons: effects on tumor- and lymphocyte-induced vascular responses. *Cancer Res* 1987, 47:5155–5161
39. Kato N, Nawa A, Tamakoshi K, Kikkawa F, Suganuma N, Okamoto T, Goto S, Tomoda Y, Hamaguchi M, Nakajima M: Suppression of gelatinase production with decreased invasiveness of choriocarcinoma cells by human recombinant interferon beta. *Am J Obstet Gynecol* 1995, 172:601–606
40. Ferrantini M, Giovarelli M, Modesti A, Musiani P, Modica A, Venditti M, Peretti E, Lollini PL, Nanni P, Forni G, Belardelli F: IFN-alpha 1 gene expression into a metastatic murine adenocarcinoma (TS/A) results in CD8+ T cell-mediated tumor rejection and development of antitumor immunity. Comparative studies with IFN-gamma-producing TS/A cells. *J Immunol* 1994, 153:4604–4615
41. Belardelli F, Ferrantini M, Santini SM, Baccarini S, Proietti E, Colombo MP, Sprent J, Tough DF: The induction of *in vivo* proliferation of long-lived CD44hi CD8+ T cells after the injection of tumor cells expressing IFN-alpha1 into syngeneic mice. *Cancer Res* 1998, 58: 5795–5802
42. Cassatella MA: The production of cytokines by polymorphonuclear neutrophils. *Immunol Today* 1995, 16:21