Restriction of neuroblastoma angiogenesis and growth by interferon-α/β

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Purpose. *We tested the hypothesis that the antiangiogenic activity of the type I interferons (IFNs), could affect tumor engraftment and growth in murine xenograft models of neuroblastoma.*
Methods. *Subcutaneous and retroperitoneal human neuroblastoma xenografts were established in SCID mice. Five days after tumor cell inoculation, daily subcutaneous injections of human IFN-α at several different doses were initiated and continued for 30 days. The effectiveness of continuous delivery of low-dose interferon was then tested using a gene therapy approach in which an adeno-associated virus vector encoding IFN-β (rAAV-IFN-β) was used to mediate expression prior to retroperitoneal tumor implantation.*
Results. *Subcutaneous and retroperitoneal tumors were significantly smaller in IFN-α–treated mice, as compared with control mice. Intratumoral basic fibroblast growth factor and vascular endothelial growth factor expression were also decreased, as was mean intratumoral endothelial cell density. Interestingly, the lower doses of IFN-α were more effective than the higher dose. No tumors developed in any of the mice given rAAV-IFN–β, whereas each of the mice that received control vector developed large tumors.*
Conclusions. *Treatment with IFN had a significant impact on neuroblastoma engraftment and growth in mice, particularly when delivered continuously using a gene therapy approach. This activity appears to be mediated in part by inhibition of tumor-induced angiogenesis through the downregulation of tumor-elaborated factors, including basic fibroblast growth factor and vascular endothelial growth factor.*
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INHIBITION OF TUMOR-INDUCED ANGIOGENESIS may restrict tumor growth, inhibit metastatic spread, and potentially cause tumor regression.[1] Endothelial cells that support tumor neovascularization are attractive targets for anticancer therapy because these genetically stable cells are unlikely to develop drug resistance. However, effective antiangiogenic therapy is often tumorostatic; therefore, long-term protein delivery may be required for successful cancer treatment.[1] Gene therapy-mediated expression of an angiogenesis inhibitor is a practical alternative to chronic daily protein administration. Furthermore, frequent low-level "metronomic" dosing of anticancer agents, or continuous delivery as is established with a gene therapy-mediated approach, may achieve the greatest efficacy while avoiding side effects seen in current chemotherapy protocols that use high-dose pulse administration of a drug.[2]

The interferons (IFNs) are a family of endogenously expressed glycoproteins initially discovered in the 1950s because of their antiviral properties. Today the IFNs are some of the most commonly studied agents used in the biological therapy of cancer. IFNs are multifunctional regulatory cytokines that control cell function and regulation. The antitumor efficacy of IFN is based on its pleiotropic effects. The direct antiproliferative effects of IFN-α/β, which include inhibition of cell growth by promoting arrest in the G1 phase of the cell cycle,[3] are often assumed to be the primary mechanism involved in their use as anticancer agents.

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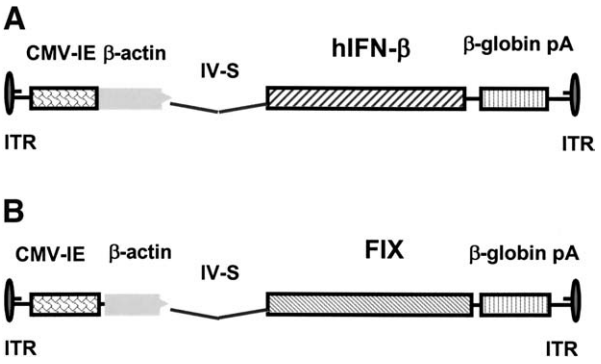

Fig 1. Adeno-associated viral vectors. Recombinant AAV vectors were constructed using a CAGG β-actin promoter and a rabbit β globin polyadenylation signal mediating the expression of the cDNA for either **(A)** human interferon-beta (hIFN-β) or **(B)** human clotting factor IX (control).

However, IFNs are also immunomodulatory,[4] regulate cellular responses to inducers of apoptosis,[5] and affect angiogenesis.[6] Their antiangiogenic properties appear to be mediated in part by their ability to downregulate expression of tumor-induced proangiogenic factors including basic fibroblast growth factor (bFGF),[7] vascular endothelial growth factor (VEGF),[8] and matrix metalloproteinase-9 (MMP-9).[7,9]

Clinical experience has proven the antiangiogenic potential of IFN-α in the treatment of a variety of pediatric vascular neoplasms, including hemangiomas, malignant hemangiopericytoma, and pulmonary hemangiomatosis.[10] In addition, IFN-α/β is currently the most commonly used cytokine in human patients[11] and is used in the adjuvant therapy of metastatic melanoma[12] and renal cell cancer.[13] Clinical experience using type I IFN therapy for high-risk neuroblastoma (NB) has been limited. A single phase I clinical trial has evaluated all-trans-retinoic acid administered in combination with interferon-α-2a in pediatric patients with refractory cancer, including 6 patients with NB. This combination was well tolerated and an objective response was seen in 1 patient with bone metastasis, whereas stable disease was seen in a second patient.[14] Although IFN-α/β has great promise for the treatment of solid neoplasms, clinical trials often have failed to show efficacy or have been limited by systemic side effects from the high-dose administration thought to be required for effective therapy.[15] Pharmacokinetic studies have demonstrated that daily intravenous and subcutaneous dosing is associated with a systemic half-life of less than 5 hours,[16] perhaps limiting efficacy by decreasing exposure to sites of primary tumor and metastatic implants. Furthermore, previous research has shown that optimization of the dose and frequency of IFN delivery is necessary for maximal antitumor efficacy.[7] Potentially, frequent lower dose "metronomic" delivery or continuous gene therapy-mediated delivery may address the limitations of current IFN clinical protocols while avoiding some of the systemic side effects. The objective of this study was to test the hypothesis that the antiangiogenic activity of interferon could be utilized to affect NB xenograft engraftment and growth in a murine model using either "metronomic" recombinant-IFN scheduling or through AAV-mediated gene therapy delivery of IFN.

MATERIAL AND METHODS

Cell lines. The human NB cell lines IMR-32, purchased from American Type Culture Collection (Manassas, Va) and NB-1691, provided by Dr P. Houghton (Memphis, Tenn) were maintained in RPMI-1640 (Bio-Whittaker, Walkersville, Md) supplemented with 10% heat-inactivated fetal bovine serum (Mediatech Cellgro, Herndon, Va), 100 units per ml penicillin and 100 μg per ml streptomycin (GIBCO BRL, Grand Island, NY), and 2 mmol L-glutamine (GIBCO). 293T Cells (human embryonic kidney cells expressing SV40 large T antigen [American Type Culture Collection]) were maintained in Dulbecco modified Eagle medium (Mediatech) supplemented with penicillin-streptomycin and L-glutamine, as above.

rAAV vector construction, production, and purification. Construction of the pAV5 CAGG FIX vector plasmid has been described previously.[17] The rAAV vector plasmid, pAV5 CAGG hIFN-β, was constructed by simply excising the FIX cDNA from the pAV5 CAGG FIX vector plasmid and inserting the hIFN-β cDNA (InvivoGen, San Diego, Calif). These plasmids include the CMV-IE enhancer, β-actin promoter, a chicken β-actin/rabbit β globin composite intron and a rabbit β globin polyadenylation signal (CAGG) mediating the expression of the cDNA for human IFN-β. This CAGG hIFN-β expression cassette was flanked by the AAV-5 internal terminal repeats (Fig 1). rAAV-5 vectors were made by the transient transfection method described previously.[17] The necessary AAV5 vector plasmid containing the type 5 internal terminal repeats (pAAV5-7D05) and packaging plasmid (pAAV5-2) were provided by Dr Robert Kotin (NIH).[18] rAAV5 virions were purified using mucin affinity column chromatography.[19]

Murine tumor models. Subcutaneous (heterotopic) human NB xenografts were established in

C.B-17 SCID (Jackson Laboratory, Bar Harbor, Me) mice by right flank injection of 2.0×10^6 IMR-32 cells in 200 μl PBS (n = 20). Retroperitoneal (orthotopic) xenografts were established in C.B-17 SCID mice by injection of 1.5×10^6 IMR-32 cells in 150 μl PBS (n = 20) behind the left adrenal gland via a left subcostal incision during administration of 2% isoflurane. Five days after tumor cell inoculation, daily subcutaneous injections of human IFN-α (PBL Biomedical) were initiated. Groups of mice in both the heterotopic (n = 20) and orthotopic cohort (n = 20) received daily subcutaneous injections of IFN-α, (5,000, 10,000, or 20,000 units per day, n = 5/group) or PBS (control, n = 5/group) in a total volume of 200 μl for 30 days. Measurements of the subcutaneous tumors were performed in 2 dimensions with calipers and volumes calculated as width^2 × length × 0.5. Mice with retroperitoneal tumors were killed 30 days after completion of therapy, at which time their tumors were excised. Portions of the tumors were snap-frozen in liquid nitrogen or fixed in formalin.

In a second set of experiments, rAAV CAGG IFN-β (1×10^{11}, 2.5×10^{11}, or 5×10^{11} vector genomes/mouse, n = 4/group) or rAAV CAGG FIX, the control vector generating expression of human clotting factor IX (1×10^{11} vector genomes, n = 6), was injected in a maximum volume of 500 μl, into the tail vein of C.B-17 SCID male mice. After 3 weeks, systemic IFN- β expression in the serum was measured by enzyme-linked immunosorbent assay (ELISA). Retroperitoneal xenografts were then established by injection of 1.5×10^6 NB1691 cells in 150 μl PBS (n = 20). After 30 days, the mice were killed. All murine experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of St. Jude Children's Research Hospital.

Human IFN-β immunoassay. Quantitation of AAV-mediated human IFN-β expression in mouse sera was performed by a commercially available sandwich immunoassay (ELISA, PBL Biomedical). The sensitivity range for this assay is 250 to 10,000 pg/ml.

Protein extraction and measurement of angiogenic protein expression. Protein lysates were made by homogenizing snap-frozen tumor specimens with a Dounce (Kontes) homogenizer in 1 ml of lysis buffer. The homogenates were incubated on ice for 30 minutes and centrifuged at 10,000 × *g* for 10 minutes at 4°C. The supernatants were then recentrifuged, collected, and frozen at −70°C for later use. Total protein was quantified for each sample using the Bradford assay. The levels of intratumoral bFGF, VEGF, and MMP-9 protein were analyzed by Quantikine ELISA kits for the respective proangiogenic factor (R&D Systems, Minneapolis, Minn). The minimal detectable levels of protein with these assays were: bFGF, 3 pg/ml; VEGF, 5 pg/ml; and MMP-9, 150 pg/ml.

Tumor immunohistochemistry and Tdt-mediated dUTP nick end labeling (TUNEL) assay. Formalin-fixed, paraffin-embedded 5 μl tumor specimens were analyzed by immunohistochemistry with an anti-CD34 antibody to determine endothelial cell density, as previously described.[20] Endothelial cell density in hot spots was determined with light microscopy at 400× by the method described previously by Weidner et al.[21] Apoptosis in subcutaneous and retroperitoneal tumors was determined by the TUNEL method using a commercially available in situ apoptosis detection kit (Serologicals, Norcross, Ga). Densities of apoptotic cells were determined by 400x light microscopy in the field with the highest density of apoptotic cells in a region that had no evidence of necrosis.[22]

Statistical analyses. Results are reported as means +/− SE. The Sigmaplot program (SPSS Inc, Chicago, Ill) was used to analyze and graphically present the data. An unpaired Student *t* test was used to analyze statistical differences between final xenograft weights and volumes, tumor angiogenic protein expression, intratumoral microvessel density, and apoptosis. A *P* value less than .05 was considered to be statistically significant.

RESULTS

Restriction of NB xenograft engraftment and growth with daily IFN-α treatment. The efficacy of daily recombinant human IFN-α administration was tested in both heterotopic (SQ) and orthotopic (RP) human NB tumor models. Five days after tumor cell implantation, treatment with a fixed dose of IFN or PBS was begun. Subcutaneous tumors were significantly smaller in IFN-treated mice after 6 weeks, as compared with controls (742 +/− 198 mm^3), with the greatest efficacy—tumors that were 96% smaller than in control mice—seen after low-dose administration (5,000 units/day, 29 +/− 14 mm^3, $P < .005$, Fig 2, *A*). Similarly, retroperitoneal tumor growth was restricted in all IFN-treated mice with the greatest effect—tumors that were 86% smaller—seen with lower doses of IFN-α (10,000 units/day, 475 +/− 338 mm^3, $P < .05$, Fig 2, *B*) as compared with tumors in control (3313 +/− 1146 mm^3) mice.

Decreased tumor-elaborated angiogenic factor expression. We next sought to determine whether

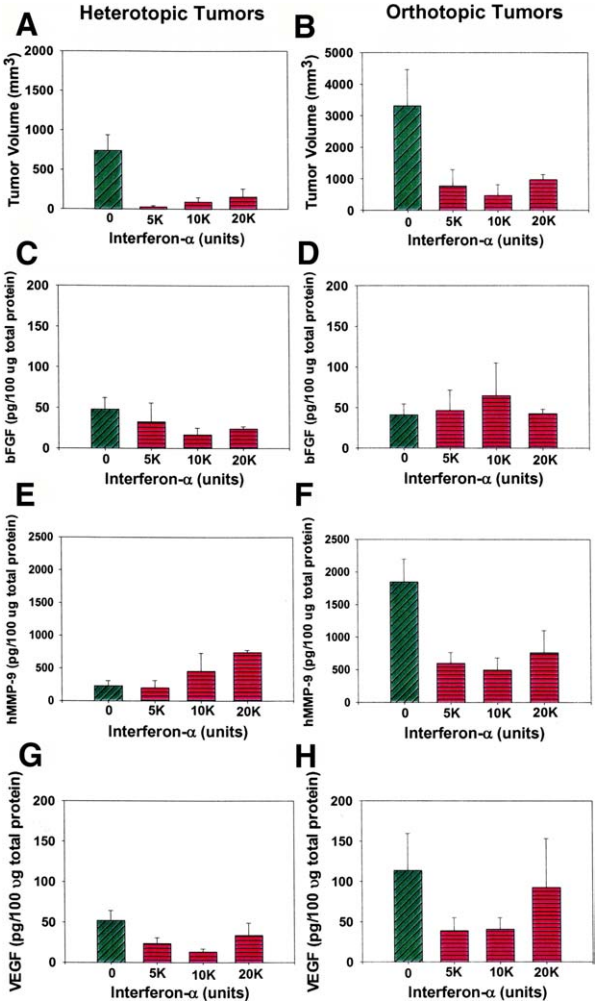

Fig 2. Inhibition of tumor proliferation and angiogenic factor expression. Tumor volume was significantly smaller in **(A)** subcutaneous and **(B)** retroperitoneal NB xenografts with the greatest restriction in growth seen after daily treatment with lower doses of recombinant IFN-α. Levels of intratumoral bFGF expression were decreased in subcutaneous tumors **(C),** whereas no difference was seen in retroperitoneal tumors **(D)** after IFN-α treatment. A similar site-dependent effect on human MMP-9 expression was seen where retroperitoneal tumors responded to IFN treatment **(F)**, whereas baseline MMP-9 levels were low in control subcutaneous tumors **(E)**. Intratumoral VEGF expression was lower than controls in both subcutaneous **(G)** and retroperitoneal tumors **(H)**, with the greatest effect again seen after treatment with lower doses of IFN-α.

the restriction in xenograft growth seen in both heterotopic and orthotopic tumor models occurred as a result of IFN-mediated inhibition of tumor-induced angiogenesis. After protein extraction from all frozen tumor specimens, ELISA was performed to determine the levels of expression of several proangiogenic factors. Differences in proangiogenic factor expression based on tumor site and IFN-α treatment group were found. Decreased levels of intratumoral bFGF protein expression were seen in all heterotopic tumors treated with IFN. In these subcutaneous tumors, the greatest reduction in bFGF levels was seen after low-dose IFN-α administration (17 +/− 5 pg/100 μg total protein), as compared with tumors in control mice (48 +/− 11 pg /100 μg total protein, $P < .031$, Fig 2, *C*). Interestingly, bFGF levels were unchanged in retroperitoneal tumors after IFN treatment (Fig 2, *D*). In contrast, intratumoral MMP-9 expression was decreased in all orthotopic tumors, with the greatest reduction in MMP-9 levels again seen in the low-dose IFN-α–treated mice (0.50 +/− 0.18 ng/100 μg total protein) as compared with controls (1.85 +/− 0.35 ng/100 μg total protein, $P < .017$, Fig 2, *F*). No decrease in MMP-9 levels was seen after IFN-α treatment in the heterotopic tumors, as baseline levels were already low (Fig 2, *E*). Decreased intratumoral VEGF expression was seen in both orthotopic and heterotopic NB models. As demonstrated with bFGF and MMP-9 expression, the greatest decrease in VEGF expression was seen after treatment of subcutaneous tumors with lower doses of IFN-α (52 +/ 12 pg /100 μg total protein) as compared with controls (13 +/− 4 pg / 100 μg total protein, $P < .05$, Fig 2, *G*). A similar decrease in VEGF expression was seen in the orthotopic tumors (Fig 2, *H*).

Decreased tumor-induced angiogenesis. Having demonstrated site- and dose-dependent inhibition of tumor-elaborated expression of several proangiogenic factors after treatment with IFN-α, we next sought to correlate the restriction of tumor growth in both heterotopic and orthotopic tumors with alterations of tumor-induced neovascularization. All subcutaneous and retroperitoneal tumor specimens were stained with an antibody to CD34, an endothelial cell marker, and the tumor microvessel density was determined. A reduction in microvessel density was seen in all IFN-treated tumors in both models, with the greatest reduction in endothelial cell density occurring after lower dose IFN treatment. Maximal reduction in mean intratumoral microvessel density was seen in subcutaneous tumors after low-dose IFN treatment (6.5 +/− 0.3 vessels/hpf) as compared with tumors in control mice (22.5 +/− 2.2 vessels/hpf, $P < .0004$, Fig 3, *A*). A similar trend toward decreased endothelial cell density was seen after IFN treatment in the retroperitoneal tumors with the greatest reduction in vascularity again seen after treatment with lower doses of IFN (23.6 +/− 3.3 vessels/hpf [control] vs 8.5 +/− 2.6 vessels/hpf

[10,000 unit/day]), $P < .011$, Fig 3, *B*). Representative tumor sections stained with an anti-CD34 antibody are shown in Fig 3. TUNEL assay was performed to determine whether the inhibition of tumor-induced angiogenesis correlated with alterations in levels of intratumoral apoptosis. No significant difference in level of apoptosis was seen after treatment with IFN. However, larger control tumors had high levels of central necrosis, whereas the IFN-treated tumors were well encapsulated with little central necrosis.

Gene therapy with AAV-IFN-β. Having demonstrated that the antiangiogenic potential of recombinant IFN for therapy of human NB xenografts is dependent on the dose and frequency of administration, we next sought to improve the efficacy of IFN by using an alternate method of protein delivery, AAV-mediated gene therapy. We have extensively studied gene therapy with AAV-mediated expression of a variety of angiogenesis inhibitors. We used AAV-FIX for the control vector. Prior work has shown no difference in tumor growth in mice that were untreated and those given the control vector AAV-FIX. Three weeks after tail vein administration of AAV (IFN or FIX), and before tumor implantation, systemic human IFN-β expression was measured by ELISA. Low levels of circulating IFN were demonstrated (mean = 341 +/− 104 pg/ml), as compared with AAV-FIX controls (13 +/− 4 pg/ml). Stable IFN-β expression was demonstrated at 5 (488 +/− 75 pg/ml) and 7 (568 +/− 62 pg/ml) weeks after vector administration, with higher IFN levels seen after higher dose vector administration. Three weeks after vector administration, retroperitoneal human NB cells (NB1691) were implanted in the retroperitoneum of all 18 mice. At the time of sacrifice, 30 days after tumor cell inoculation, no tumors had developed in any of the 12 mice given rAAV-IFN-β. Each of the 6 mice that had received control vector had large tumors at that time (mean weight = 2.92 +/− 0.35g, $P < .0002$).

DISCUSSION

NB, the most common solid malignancy of infancy, provides a particularly difficult therapeutic challenge. Metastatic disease is present in nearly 50% of patients at diagnosis and, despite the development of increasingly aggressive multimodality therapy, overall prognosis remains poor in this high-risk population. New treatment strategies are obviously necessary. NB is usually a highly vascular tumor that grows rapidly and may metastasize early. Therefore, targeting tumor neovascularization is a logical treatment strategy.

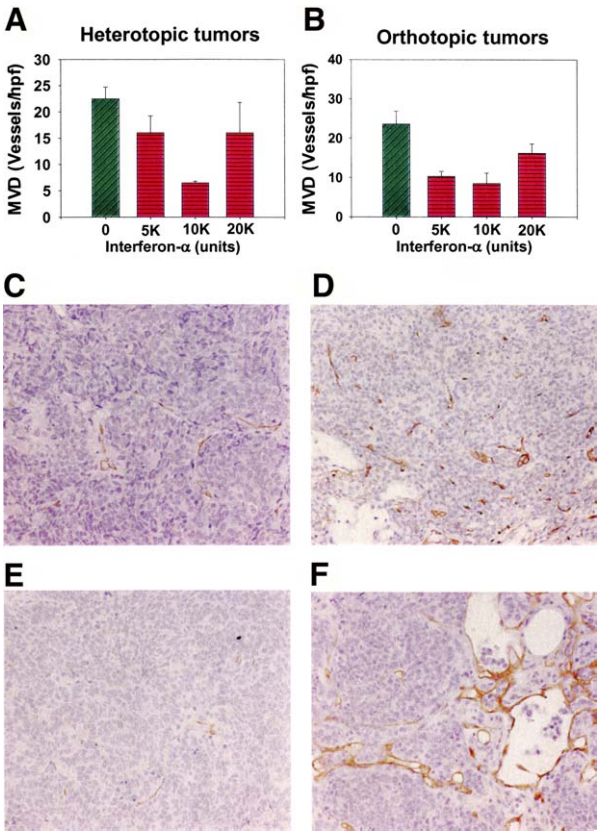

Fig 3. Restriction of tumor-induced angiogenesis. Decreased microvessel density was seen by CD34 immunohistochemistry in **(A)** subcutaneous and **(B)** retroperitoneal NB xenografts in recombinant IFN-α–treated mice. The density of stained endothelial cells was significantly decreased in **(C)** subcutaneous and **(E)** retroperitoneal tumors in IFN-α–treated mice as compared with **(D)** subcutaneous and **(F)** retroperitoneal tumors in PBS-treated mice (magnification ×100).

Many studies have demonstrated the efficacy of IFN-α/β in the biological therapy of cancer. Currently, IFN-α is used as adjuvant therapy for solid malignancies, including metastatic melanoma and renal cell cancer.[23] In addition to its antiangiogenic properties, IFN is a pleiotropic agent that targets multiple antitumor pathways. However, pharmacokinetic studies have demonstrated that daily subcutaneous bolus dosing schedules are associated with a short systemic half-life, potentially intermittently decreasing exposure to sites of tumor growth.[16] Gene therapy-mediated delivery of IFN provides a logical strategy to maintain continuous low level treatment, potentially avoiding periods without tumor exposure to drug allowing recovery and mutagenesis, and avoiding the systemic side-effects seen with the current high dose regimens.

In this study, we have shown that the antiangiogenic potential of the type I interferons can be used as effective prevention of NB engraftment in a murine model. IFN-α and -β share more than 35% sequence homology, activate a similar gene profile, and bind to the same IFN-α/β receptor. Although a potential difference in binding between subtypes of IFN-α and IFN-β exist, in this study we have demonstrated that both of the type I IFNs are able to restrict effective engraftment of human NB. The xenograft studies were performed in immunodeficient mice, thereby limiting the immunomodulatory anticancer effects of IFN, which include stimulation of NK, B, and T cells.[4] Depending on the site of tumor implantation, bFGF (subcutaneous) or MMP-9 (retroperitoneal) expression were downregulated, whereas VEGF expression was downregulated in tumor at both sites. Similar findings of site-dependent expression of angiogenic factors have been previously shown in murine models of melanoma and renal cell carcinoma.[24,25] Interestingly, lower doses of daily recombinant IFN-α administration were more effective than the higher doses, a finding previously demonstrated for IFN in a murine model of bladder carcinoma.[7] Further evidence that the antitumor effect of IFN was antiangiogenic was that the restriction in tumor growth that followed IFN treatment correlated with decreased tumor neovascularization. Again, the greatest decrease in microvessel density, measured by endothelial cell staining, was seen after lower doses of IFN administration.

We were able to improve the efficacy of IFN therapy by using a gene-therapy–mediated approach using an AAV-IFN-β vector. One of the limitations of our model is that pretreatment of the mice with AAV-IFN-β was required before tumor implantation, because AAV-mediated protein expression requires at least 4 to 6 weeks for stable peak production. AAV-IFN-β treatment prevented tumor engraftment in all mice demonstrating the effectiveness of gene therapy-mediated IFN delivery. Importantly, no adverse effects of antiangiogenic therapy were seen. All treated mice appeared healthy during their observation period, with normal wound healing of their abdominal surgical incision. Evaluation of the long-term effects of IFN on normal host-dependent angiogenesis is currently underway. Furthermore, the ability to treat already existing tumors with this strategy remains to be tested.

In summary, in our murine models, the type I IFNs provided effective prevention of NB engraftment and restriction of growth, due at least in part to their antiangiogenic activity, with the greatest impact on xenograft growth seen after gene-therapy–mediated delivery of IFN-β.

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