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Gene Transfer of IFN-γ into Established Brain Tumors Represses Growth by Antiangiogenesis

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The experiments in this paper were designed to examine the therapeutic effects of adenoviral-mediated gene transfer of IFN-γ into a mouse model of an established metastatic brain tumor. Temperature-sensitive replication-defective adenovirus was generated for gene transfer of IFN-γ (AdIFN) and β-galactosidase (AdBGAL) cDNAs in vivo. In this model, treatment with AdIFN elicits prolonged survival times and brain tumor rejection. Evidence against an immune-mediated response accounting for this result include: 1) absence of a memory immune response upon challenge, 2) lack of antitumor effects at sites distal to inoculation of AdIFN, and 3) preservation of the therapeutic effects of AdIFN in *scid* and beige mice and in inducible NO synthase (iNOS) knockouts. High concentrations of IFN-γ do not inhibit tumor growth in vitro making it unlikely that the antitumor effect of this treatment acts directly on the growth of the tumor cells. However, gene transfer of IFN-γ inhibits neovascularization of the tumor in a 3LL-Matrigel assay in vivo, and AdIFN induces apoptosis of endothelial cells in vivo, supporting the idea that AdIFN represses tumor growth by inhibiting angiogenesis. The substantial non-immune-mediated therapeutic benefits of AdIFN in animals paves the way for devising novel strategies for treating human brain tumors. *The Journal of Immunology,* **2000, 164: 217–222.**

Despite many aggressive multimodality conventional therapeutic protocols over the past two decades, the prognosis of patients diagnosed with primary and metastatic malignant brain tumors remains poor (1). Furthermore, data from cancer registries from around the world show a persistent increase in the incidence of primary brain tumors particularly in the elderly. This is of concern because recent evidence suggests that the persistent rising trend may not be artifactual (2). In many cases, systemic malignancies also metastasize to the nervous system with a prevalence that is about twice as common as primary neoplasms in adults. These facts highlight the need for novel molecular strategies for malignant brain tumors that have shown success in pre-phase I animal models.

Tumor growth in a live organism is dependent not only on its ability to evade immune surveillance but also on its capacity to maintain a continuous and stable supply of nutrients and oxygen for proliferation. Tumor angiogenesis, or the capacity to form new capillaries from preexisting microcapillaries, is a common characteristic and a limiting step in the growth of most solid tumors. The ability to induce neovascularization either 1) is produced by molecules secreted by the tumor itself or from a host cell recruited by the tumor, 2) may arise from molecules derived from the extracellular matrix, or 3) may be caused by loss of physiological inhibition of endothelial cell proliferation (3–5).

Data from this laboratory showed that priming intracerebrally with a rat malignant glioma cell line (RT2), genetically modified to secrete "high amounts" of IFN-γ, elicits prolongation of survival times and tumor rejection (6). The animals acquired a memory response characterized by systemic and CNS antitumor immunity, suggesting that the antitumor effects in this rat model are at least in part immune mediated. Unlike RT2, most human tumors are not immunogenic (7). The following preclinical gene therapy experiments were designed to examine the potential benefits of adenoviral-mediated gene transfer of IFN-γ into a model of established metastatic brain tumors in mice. A mouse Lewis lung carcinoma (3LL) was selected because it is malignant and poorly immunogenic. Unexpectedly, we were led to the findings that gene transfer of IFN-γ in vivo represses growth of established 3LL brain tumors not because of an immune response, but rather by inhibiting angiogenesis.

Materials and Methods

Cell lines and animals

C57BL/6 wild-type (wt),[3] *scid*, and beige mice were purchased from The Jackson Laboratory (Bar Harbor, ME). 3LL (Lewis lung carcinoma, C57BL/6) was a generous gift from Dr. I. Fidler (M. D. Anderson Cancer Center, Houston, TX).

Generation of shuttle plasmids

Subcloning was conducted by standard recombinant DNA techniques. The expression cassettes that drive the expression of IFN-γ and *lacZ* were digested out of the plasmids pGIFN and pGBGAL (6), and cloned into pXCJL.2 to generate pXIFN and pXBGAL, respectively (Fig. 1).

Transfection of 293 cells and production of viral particles

Shuttle plasmids and pBHGts were cotransfected via liposomes into 293 cells grown at 32°C. Successful recombination produces infectious virions that induce total lysis of 293 cells within 5–8 wk. Viral DNA isolated from 100–200 μl of supernatant from the wells with complete lysis is examined by PCR to determine whether virions contain the appropriate cDNA and to

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[3] Abbreviations used in this paper: wt, wild type; iNOS, inducible NO synthase; L-NAME, N^G-nitro-L-arginine methyl ester; H&E, hematoxylin and eosin; o.m., original magnification; AdIFN, adenovirus IFN; AdBGAL, adenovirus β-galactosidase..

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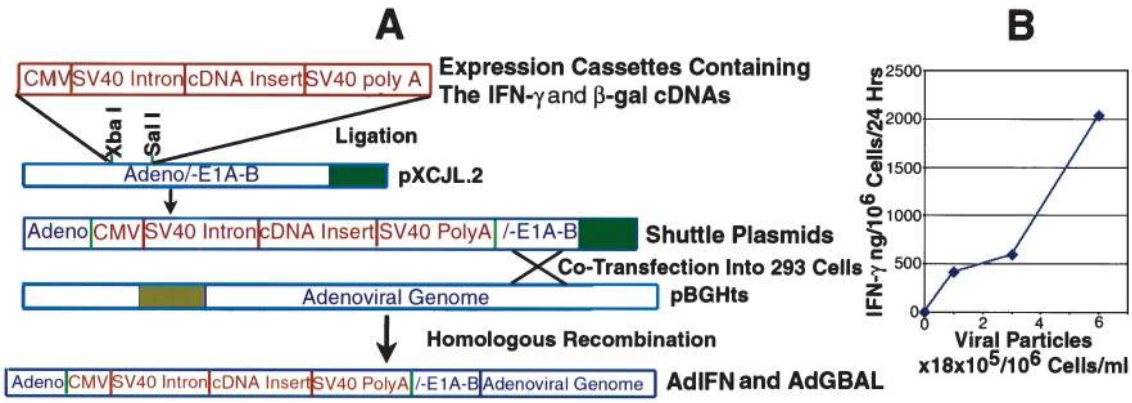

FIGURE 1. Generation of the live recombinant replication-defective temperature-sensitive AdIFN and AdBGAL. *A,* Cartoon depicting the strategy for generating the recombinant adenoviruses. Two days after culturing 10^6 3LL cells with titrated amounts of AdIFN in triplicates, amounts of IFN-γ present in the supernatant fluid were quantified by ELISA (*B*).

detect wt virus contamination using E1 region primers. The recombinant virus is plaque purified and grown on 293 cells at 32°C. Tissue culture supernatants containing adenovirus are concentrated by centrifugation over cesium chloride using the method of Graham and Prevec. Viral titer is determined by counting plaques using an agarose overlay method as described by Graham and Prevec (8). The titers of AdIFN and AdBGAL of viral particles/PFU ratios are 43 and 81, respectively.

Intracerebral tumor/viral implantation

C57BL/6 mice were anesthetized by i.p. xylazine (10 mg/kg) and ketamine (90 mg/kg) and then placed in a stereotactic frame. The bregma was identified, a burr hole was drilled and the dura was exposed. Using a Hamilton syringe, the dura was punctured, and a needle was inserted 3.5 mm (1 mm anterior and 2 mm to the right of the bregma) into the brain. After 3 min, 3 μl containing 1500 tumor cells mixed 50:50 with 30% methylcellulose, or 10 μl of viral particles suspended in 10 μl of PBS, were injected over 3 min. After another 3 min, the needle was withdrawn slowly. Where indicated, 2 days later, the mice were injected at the same coordinate with viral particles (AdIFN or AdBGAL) in a total volume of 10 μl.

ELISA

Supernatants of transduced cells or sera of animals treated by AdIFN were assayed for murine IFN-γ (R&D, Minneapolis, MN) by ELISA following the manufacturer's specifications.

Tissue staining

Abs used are against mouse IFN-γ (American Type Culture Collection, Manassas, VA), mouse CD31 (anti-platelet endothelial cell adhesion molecule (PECAM)-1, PharMingen, San Diego, CA), human factor VIII (Dako Corporation, Carpinteria, CA). For immunohistochemistry, anti-IFN-γ and anti-CD31 were reacted with biotin following the manufacturer's specifications (Vector, Burlingame, CA). Fresh frozen sections of 6–10 μm thickness were reacted with biotinylated Abs, developed, and then counterstained in 1% methyl green as previously described (6). Reactions with anti-IFN-γ were performed in the presence of saponin. For immunofluorescence, tissue sections were sequentially reacted with rabbit anti-factor VIII then Cyan3-coupled anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). For the TUNEL reaction, sections were processed using the Fluorescein-FragEL DNA fragmentation detection kit following the manufacturer's specifications (Oncogene Research Product, Cambridge, MA). Processed sections were viewed by epifluorescence microscopy (Axioplan 2; Carl Zeiss, Thornwood, NY).

Matrigel assay for angiogenesis

Angiogenesis assays were performed as described elsewhere (9); briefly C57BL/6 mice were injected with 0.5 ml of Matrigel (Collaborative Biomedical Products, Bedford, MA) mixed with 1×10^6 3LL cells and AdIFN or AdBGAL (22×10^9 viral particles) in the abdominal midline (day 0 in all experiments). To quantify angiogenesis, Matrigel pellets were harvested on day 6, all surrounding tissue was dissected away, 0.3 ml Matrisperse was added (Collaborative Biomedical Products), and the gel was liquefied by incubation at 4°C overnight. Hemoglobin content of the liquefied pellets was determined by the Drabkin method (Sigma Diagnostic, St. Louis, MO) following the manufacturer's specifications.

Statistical analysis

Statistical calculations were performed by the JMP software (SAS Institute, Cary, NC).

Results

Generation of recombinant adenovirus and transfer of IFN-γ gene into normal brain and tumor cells

Shuttle plasmids pXIFN and pXCLON were constructed to contain the mouse IFN-γ and *Lac*Z cDNAs cloned 3′ to CMV promoters and SV40 splicer donor sites and 5′ to the adenovirus recognition sequence, respectively (Fig. 1*A*). The recombinant temperature-sensitive replication-defective adenoviral vectors AdIFN and AdBGAL were generated by cotransfecting pXIFN or pXCLON with pBHGts into 293 cells respectively. Cultured 3LL cells transduced by AdBGAL or AdIFN express *LacZ* or secrete IFN-γ in vitro, respectively (Fig. 1*B*). AdIFN and AdBGAL transduce normal brain to express IFN-γ and β-galactosidase in vivo, respectively (Fig. 2). Furthermore, IFN-γ secreted in the brain is detected in serum; 2 days after receiving 11.5×10^9 viral particles of AdIFN intracerebrally, mice sera ($n = 2$) contain 1.44 ng/ml of IFN-γ as measured by ELISA. *LacZ* expression in the brain is persistent for longer than 6 wk. AdIFN-treated mice show no overt signs of neurological dysfunction.

AdIFN generates an anti-brain tumor response

To test for therapeutic effects, animals implanted intracranially with 3LL were injected 2 days later with AdIFN or AdBGAL (Fig.

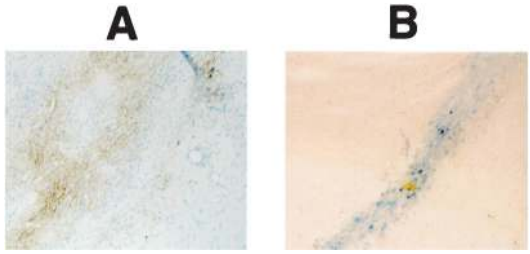

FIGURE 2. AdIFN induces IFN-γ expression and anti-tumor effects in vivo. Two weeks after intracerebral injection of AdIFN (5 μl, 2.4×10^9 viral particles/μl); and AdBGAL (5 μl, 3.5×10^9 viral particles/μl), mice brains were reacted with biotinylated anti-IFN-γ Ab (*A*) and stained for *LacZ* (*B*), original magnification (o.m.) ×100.

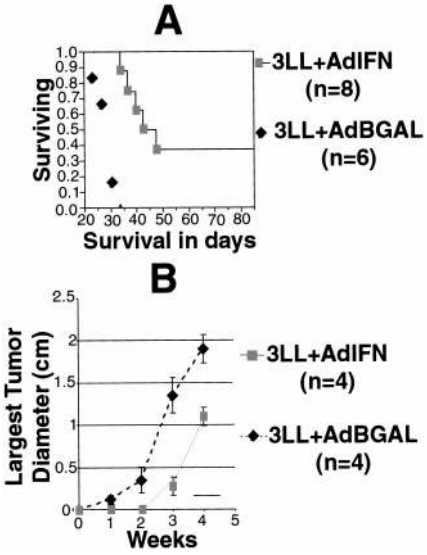

FIGURE 3. AdIFN generates antitumor effects. Mice were implanted intracerebrally with 3 μl containing 1500 3LL cells. Two days later, they were treated by either AdIFN (10 μl, 2.4×10^9 viral particles/μl; $n = 8$) or AdBGAL (10 μl, 3.5×10^9 viral particles/μl; $n = 6$) at the same coordinates (*A*). Survival times were examined by Kaplan-Meier analysis. Mean survival times are 29.7 days for AdBGAL and longer than 57.1 days for AdIFN (Log-Rank, $p = 0.0003$). AdIFN retards growth of s.c.-implanted tumors significantly; mice were injected s.c. in the flank with 1×10^6 3LL cells mixed with AdIFN (24×10^9 viral particles, $n = 4$) or AdBGAL (35×10^9 viral particles, $n = 4$). Largest tumor diameter was measured by calipers (*B*).

3*A*). Unlike those treated by AdBGAL, mice treated by AdIFN show statistically significant prolongation of survival times (Log-Rank test, $p = 0.0003$). Furthermore, 3/8 (38%) of the mice survived longer than 85 days after injection and rejected the tumor because histological analysis revealed cavity formation and no tumor mass. To examine whether AdIFN generates similar systemic antitumor effects, groups of mice were implanted s.c. with 1×10^6 3LL cells mixed with either AdIFN or AdBGAL. Unlike with AdBGAL, 3LL cells mixed with AdIFN fail to produce palpable tumors 2 wk after injection, although tumor growth occurred after 3 wk (Fig. 3*B*).

Evidence against an immune-mediated response

To study whether AdIFN generates a memory antitumor response, animals that had rejected intracerebrally implanted 3LL were rechallenged by wt 3LL s.c. Unexpectedly, growth in naive mice was similar to growth in mice that had previously rejected intracerebral 3LL cells (Fig. 4*A*). To further examine for antitumor effects at a distance, mice implanted by 3LL cells in both hemispheres were treated by AdIFN or AdBGAL only on the right side. None of these animals rejected intracerebral 3LL, and the mean survival times associated with AdIFN and AdBGAL were 26 and 21 days, respectively (Fig. 4*B*). At autopsy, visual inspection revealed that, whereas brains of all mice injected with AdBGAL carried large bilateral tumor masses (8/8), 6/9 animals implanted with AdIFN showed tumors only on the left side. Histological examination of the latter showed large tumors on the left and cavity formation on the right (Fig. 4, *C* and *D*). Thus the data support the hypothesis that AdIFN elicits "local" antitumor effects.

To examine whether the effects of AdIFN are mediated by B, T, or NK cells, *scid* and beige C57BL/6 mice injected intracerebrally with 3LL cells were treated by AdIFN or AdBGAL. Kaplan Meier analysis reveals that the therapeutic effects of AdIFN on prolonging survival times are preserved both in *scid* (Log-Rank $p = 0.0001$; Fig. 5*A*) and beige mice (Log-Rank, $p = 0.0125$; Fig. 5*B*). In addition, histological analysis shows that 4/9 (44%) *scid* and 3/10 (33%) beige AdIFN-treated mice reject 3LL. IFN-γ may enhance nonspecific phagocytic activity in microglia by inducing expression of inducible NO synthase (iNOS). However, the antitumor effects generated by AdIFN are independent of iNOS expression because they are preserved in C57BL/6 iNOS knockouts

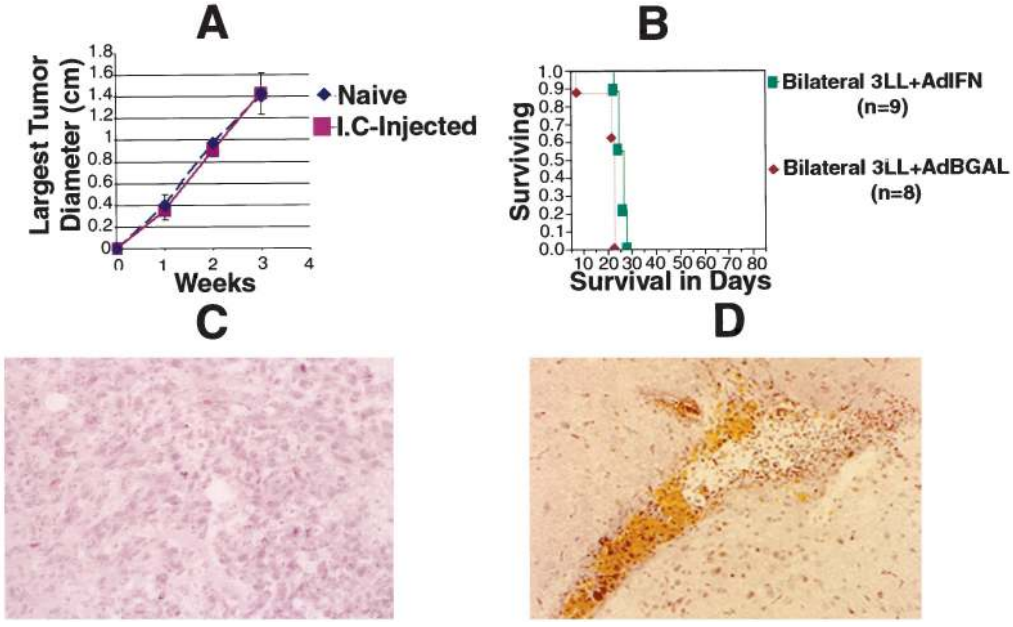

FIGURE 4. AdIFN does not elicit a memory response or antitumor effects at distance. AdIFN-treated mice that rejected 3LL (intracerebrally (I.C.) injected, $n = 3$) and naive animals ($n = 4$) were reimplanted with 1×10^6 wt 3LL cells sc (*A*). Largest tumor diameter was measured using calipers. Mice were implanted with 3LL in both hemispheres and then treated by AdIFN or AdBGAL on the right side only and followed for survival. Survival times were examined by Kaplan-Meier analysis (*B*). Mean survival times were 26 days for AdIFN and 21 days for AdBGAL. H&E staining of AdIFN-injected animals showed tumors on the left side (*C*) and cavity formation on the right (*D*) in the same brain (o.m. $\times$200).

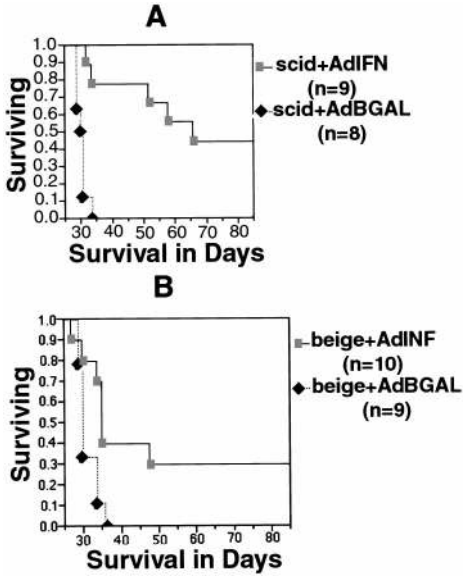

FIGURE 5. T, B, and NK cells are not necessary for the effects of AdIFN. The antitumor effects of AdIFN are preserved in *scid* (*A*) and beige mice (*B*); groups of these mice, inoculated by 3LL, were treated by either AdIFN (10 μl, 2.5×10^9 viral particles/μl; *scid*, $n = 9$; beige, $n = 10$) or AdBGAL (10 μl, 2.5×10^9 viral particles/μl; *scid*, $n = 8$; beige, $n = 9$). Survival times were examined by Kaplan-Meier analysis. Log-rank p values were 0.0001 and 0.0125 for *scid* and beige mice, respectively.

and in wt mice treated by N^G-nitro-L-arginine methyl ester (L-NAME) (data not shown). These results strongly suggest that AdIFN generates its antitumor effects by a mechanism other than an adaptive cellular immune response.

AdIFN inhibits tumor angiogenesis

To examine whether IFN-γ suppresses tumor growth in vitro, 3LL cells were grown in the presence of titrated concentrations of IFN-γ, and cell numbers were counted at variable time intervals. The log growth curves demonstrate that physiological (12.5 ng/ml; 1×) and high concentrations (1.25 μg/ml; 100×) of IFN-γ only slightly retard growth of 3LL in vitro and only after 7 days (Fig. 6*A*). Thus, the direct effects of IFN-γ on tumor growth in vitro do not explain the in vivo data, namely that AdIFN elicits rejection of 3LL brain tumors in 38% and 44% of C57BL/6 and *scid* mice, respectively (Figs. 3*A* and 5*A*).

To test the hypothesis that AdIFN inhibits tumor angiogenesis in vivo, animals were implanted in the avascular s.c. space by 10^6 3LL cells mixed in Matrigel with either AdIFN or AdBGAL. Neovascularization was assayed 6 days later by quantifying hemoglobin content and assaying for endothelial cell penetration of the tumor by immunohistochemical staining for CD31. Matrigels mixed with AdIFN contain significantly less hemoglobin than AdBGAL (Student's t test, $p < 0.0001$; Fig. 6*B*). Furthermore, immunohistochemical analysis revealed that, whereas 3LL cells mixed with AdBGAL grew as a continuous tumor penetrated by blood vessels, those mixed with AdIFN grew in spheroid-like islands devoid of endothelial cells (Fig. 6*C*). Therefore, it appears that AdIFN inhibits tumor neovascularization in vivo.

Regional administration of "high" amounts of IFN-γ and TNF in combination with melphalan, by the limb perfusion technique, results in over an 80% complete response in patients with limb melanoma. In these patients, "high local" concentrations of TNF and IFN-γ cause selective disruption of the tumor vasculature by reducing the activation of $\alpha_v\beta_3$ integrin, leading to apoptosis of angiogenic endothelial cells (10). This information raised the question whether, in this model, "high local" concentrations of IFN-γ (Fig. 2) induce apoptosis of endothelial cells in vivo. Three days after treating 3LL brain tumors with virus, unlike AdBGAL, H&E staining of AdIFN-injected brains reveals intense perivascular infiltrates and fragmented nuclei in tumor blood vessels (Fig. 7*A*). Double immunofluorescence staining for fragmented DNA (TUNEL method) and factor VIII positive endothelial cells reveal apoptosis in blood vessels in the tumor bed (Fig. 7, *B–D*). Furthermore, 10 days after receiving adenovirus, unlike brains injected with AdBGAL that harbor large "healthy" well-vascularized tumors (Fig. 7, *E* and *G*), tumors treated by AdIFN are devoid of blood vessels and show massive apoptosis (Fig. 7, *F* and *H*).

Discussion

We initially expected gene transfer of IFN-γ to produce an immune reaction because IFN-γ up-regulates expression of MHC class I molecules in 3LL (data not shown) and drives microglia, a putative APC of the brain, into an immune-active state (11). Our

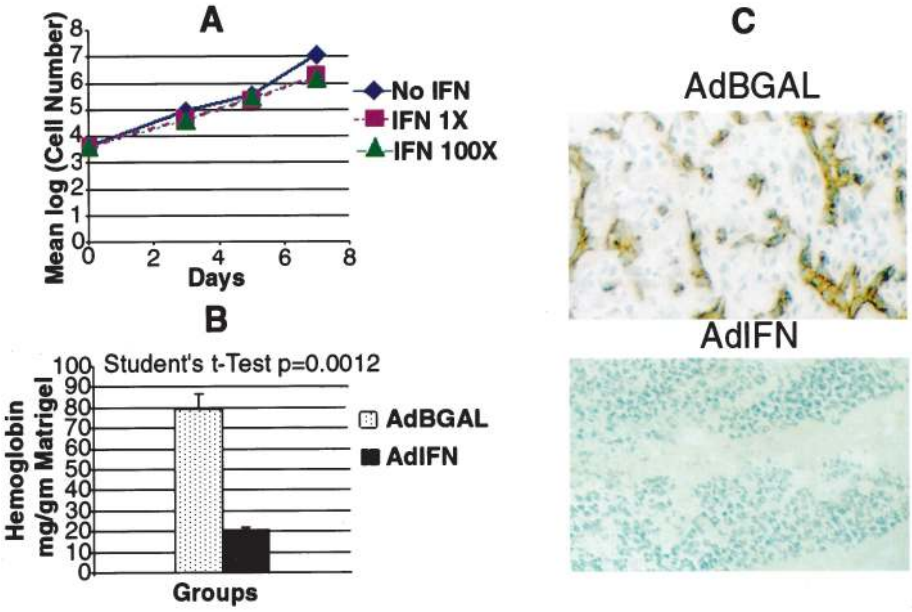

FIGURE 6. AdIFN inhibits tumor angiogenesis in vivo. First, the effects of IFN-γ on 3LL in vitro do not explain the antitumor effects of AdIFN in vivo; 4×10^3 3LL cells were cultured in triplicates in the absence or presence of IFN-γ at 12.5 ng/ml (1×) and 1.25 μg/ml (100×). At variable time intervals, plates were treated by 0.25% trypsin, and viable cells were counted by trypan blue exclusion (*A*). Second, angiogenesis assays in vivo were performed by injecting mice with 0.5 ml of Matrigel (*B* and *C*) mixed with 1×10^6 3LL cells and AdIFN or AdBGAL (22×10^9 viral particles) in the abdominal midline (9). Frozen sections of Matrigels mixed with virus were reacted immunohistochemically with anti-CD31 (*C*; o.m. ×200) and PBS as negative control.

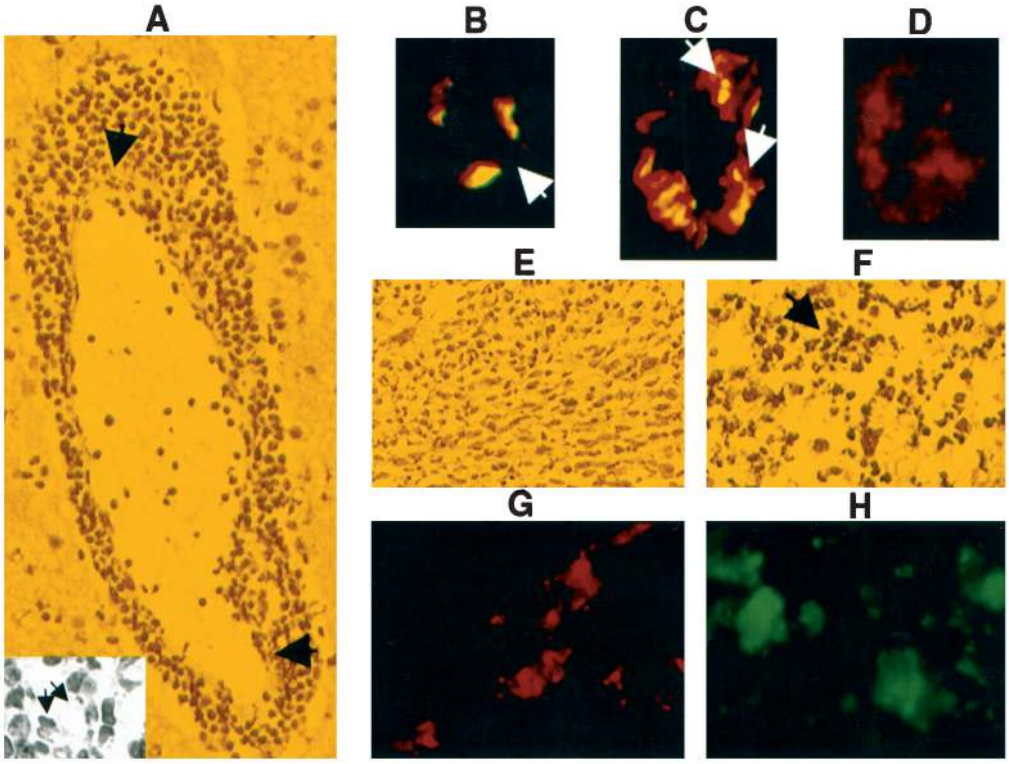

FIGURE 7. AdIFN induces endothelial cell apoptosis. Mice were implanted intracerebrally by 3LL and then treated, 2–4 days later, by either AdIFN or AdBGAL (10 μl, 2.0×10^9 viral particles/μl) at the same coordinates. Frozen sections of brains, injected by AdIFN (*A–C*, *F*, and *H*; $n = 2$ in each group) or AdBGAL (*D*, *E*, and *G*; $n = 2$ in each group), harvested 3 (*A–D*) and 10 days later (*E–H*; $n = 2$ in each group), were examined by H&E (*A*, o.m. of the large and insert images are ×200 and ×1000, respectively. *E* and *F*, o.m. ×400 and double immunofluorescence staining (*B–D*, *G,* and *H*; o.m. ×400) for fragmented DNA (TUNEL method, green) and factor VIII (red). Arrows point to two of multiple areas showing fragmented nuclei in large blood vessels (*A;* H&E) and endothelial cell apoptosis in small blood vessels (yellow, *B* and *C*) in the beds of tumors treated by AdIFN. Ten days after receiving adenovirus, unlike brains injected by AdBGAL, which harbor large "healthy" well-vascularized tumors (*E* and *G*), tumors treated by AdIFN are devoid of blood vessels and show massive apoptosis (*F* and *H*).

findings reveal that intracranial inoculation of AdIFN elicits prolonged survival times and rejection of tumors established in the brain for 2 days while s.c. inoculation directly at the tumor site retards growth of 3LL implanted peripherally. Evidence against an immune-mediated response accounting for this result include 1) absence of a memory immune response upon challenge, 2) lack of antitumor effects at sites distal to inoculation of AdIFN, and 3) preservation of the therapeutic effects of AdIFN in *scid* and beige mice, and in C57BL/6 iNOS knockouts and wt mice treated by L-NAME. High concentrations of IFN-γ do not inhibit tumor growth in vitro, making it unlikely that the antitumor effect of this treatment acts directly on the growth of tumor cells. However, gene transfer of IFN-γ inhibits neovascularization of the tumor implanted with Matrigel in the avascular s.c. space in vivo; furthermore, AdIFN induces apoptosis of endothelial cells in vivo, supporting the idea that AdIFN represses tumor growth by inhibiting angiogenesis. The effects of IFN-γ on angiogenesis have been described in human disease as well as many in vivo and in vitro models. IFN-γ inhibits proliferation and migration of human endothelial cells in vitro (12, 13) and represses lymphocyte-induced tumor angiogenesis in experimental animals (14, 15). IFN-γ also elicits significant inhibition of tube formation in a HUVEC/Matrigel in vivo model (16).

In our model, treatment with AdIFN generates rejection of 3LL brain tumors in 38% and 44% of C57BL/6 and *scid* mice, respectively (Figs. 3*A* and 5*A*). The question of how an antiangiogenic molecule eradicates a tumor is intriguing. A biological precedence was established in experiments where tumor-bearing animals were treated by multiple injections of endostatin. Whereas tumors typically regrow after a single cycle of endostatin, they fail to regrow, showing prolonged dormancy after repeated cycles (17). It is possible that induction of continuous "high" local concentrations of IFN-γ for weeks is equivalent to multiple cycles of antiangiogenic therapy. In this model, the end result is tumor cell apoptosis, probably caused by ischemia (Fig. 7*H*). The effects of IFN-γ on endothelial cells may be mediated either by direct effects or through secondary molecules such as IFN-inducible protein 10 (IP-10) or monokine induced by IFN-γ (MIG). These chemokines, which react with the CXCR3 receptor, inhibit endothelial cell proliferation in vitro and repress tumor vascularization in vivo (9, 18).

The therapeutic implications are important; because this model shows that, regardless of tumor immunogenicity, the strategy of inducing high local concentration of IFN-γ is successful in generating a substantial anti-brain tumor response mediated by antiangiogenesis. The results also suggest that, in the rat glioma model where animals were implanted by RT2 genetically modified in vitro to secrete IFN-γ, the observed antitumor response may be caused primarily by antiangiogenesis and that the acquired immunity reaction against wt RT2 may be secondary (6). The data constitute a proof of principle in animals that sets the stage for devising novel strategies that may lead to clinical trials.

Acknowledgments

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