Maturation of Tumor Vasculature by Interferon-β Disrupts the Vascular Niche of Glioma Stem Cells

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Abstract. *Background: The vascular niche necessary for cancer stem cell maintenance is a potential target for cancer therapy. Materials and Methods: Human glioma xenografts were treated with IFN-β delivered systemically via a liver-targeted, adeno-associated viral vector. The vascular niche was examined with immunofluorescence for glioma stem cells, endothelial cells, and perivascular cells. Results: Although IFN-β was not directly toxic to glioma stem cells in vitro, IFN-β decreased tumor size and the number of stem cells recovered in both heterotopic and orthotopic models. Treatment with IFN-β increased perivascular cells investing the tumor vasculature (6-fold) distancing stem cells from endothelial cells. Additionally, vascular smooth muscle cells co-cultured between stem cells and endothelial cells decreased stem cell recovery. Conclusion: Continuous delivery of IFN-β decreased the number of stem cells in glioma xenografts by disrupting the vascular niche through an increase in perivascular cells, which created a barrier between the glioma stem cells and the endothelial cells.*

Stem cells in the brain are clustered in the subventricular zone and the dentate gyrus of the hippocampus, where they are closely associated with endothelial cells (1-4). In fact, the nervous and vascular systems are related throughout development, as they both originate from the neural tube, and their growth and differentiation are dependent on cytokines secreted from progenitors of each system (5-7). Regions of the brain where neural stem cells reside have specialized contacts between blood vessels and neural cells to allow for vascular cytokines to influence neural stem cells (8).

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Cancer stem cells (tumor-initiating cells or cancer stem-like cells) have recently been discovered in leukemia and many solid tumors including gliomas (9-11). These cells appear to be resistant to radiation therapy and chemotherapy and, therefore, likely contribute to tumor recurrence and relapse (12-14). Glioma stem cells arise from transformed neural stem cells and have phenotypic and functional characteristics similar to those of neural stem cells (9, 15). Glioma stem cells are highly tumorigenic, forming tumors with as few as 100 to 1000 cells (16). Tumor regrowth after treatment can be attributed to glioma stem cells and, therefore, these cells have become the focus of research for new chemotherapeutic agents (17).

There is increasing evidence to support the concept of a microvascular niche for tumor stem cells, which may be a potential target for attacking glioma stem cells (18-21). Calabrese *et al*. (20) showed that stem cells in brain tumor xenografts reside in close proximity to endothelial cells, and stem cell recovery increased when glioma stem cells were co-cultured with endothelial cells. They, along with others, have also reported a decrease in stem cells when tumors are treated with anti-angiogenic agents, supporting the hypothesis of a specialized vascular niche for stem cells (18-20).

Interferon-beta (IFN-β) is a regulatory cytokine produced by host cells in response to foreign antigens. It has multiple cellular effects including induction of apoptosis, modulation of angiogenesis, and immunomodulation (22-26). IFN-β is active against many types of cancer, though its clinical utility is limited by its short half-life and systemic toxicity. We have previously reported that treatment of human tumor xenografts, including glioma, with IFN-β results in significant inhibition of tumor growth. We also noted that this therapy matured the tumor vasculature with increased investment of the vessels with perivascular cells (27). We hypothesized that this increased coverage of endothelial cells by perivascular cells may disrupt the microvascular niche of glioma stem cells, leading to a decrease in the number of cancer stem cells and a resultant decrease in tumor growth.

Materials and Methods

Cells. Luciferase-expressing U87 cells provided by Dr. Robert Carter (Boston, MA, USA) were maintained in Dulbecco's modified Eagle's medium (Cellgro, Mediatech Inc, Herndon, VA, USA) with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% penicillin, streptomycin, and glutamine supplements (GIBCO BRL, Grand Island, NY, USA). Human umbilical vein endothelial cells (HUVECs; Lonza, Walkersville Inc, Walkersville, MD, USA) were maintained in EGM2 medium (Lonza) supplemented with growth factors (SingleQuots; Lonza). Umbilical artery smooth muscle cells (UASMCs; Lonza) were maintained in SmGM-2 (Lonza) supplemented with growth factors (SingleQuots; Lonza).

Stem cells were recovered from heterotopic and orthotopic U87 gliomas by harvesting the tumors in phosphate-buffered saline. Tumors were minced and incubated for 30 minutes in a mixture of collagenase and hyaluronidase. Once the tissue was dissociated, the tumor suspension was filtered through a 40-μm filter, and the supernatant was centrifuged to recover a cell pellet. Cells were then placed in neurobasal medium (Invitrogen, Carlsbad, CA, USA) supplemented with 20 ng/ml epidermal growth factor (Invitrogen), 20 ng/ml basic fibroblast growth factor (Invitrogen), and 50 μg/ml 7.5% bovine serum albumin to select for glioma stem cells (2). Tumor spheres were collected after 2 weeks of culture.

In vitro studies. Stem cells recovered from intracranial U87 tumors were plated as single cell suspensions in a 96-well plate with 10.000 cells/ml plated in the top row with serial 1:2 dilutions for the remaining rows. Once tumor spheres had formed, stem cells were exposed to 100 units/ml and 10.000 units/ml of recombinant IFN-β (Avonex, Biogen Idec Inc, Cambridge, MA, USA) for 72 hours.

Neurospheres were co-cultured with HUVECs and UASMCs to determine the effect of the presence of these cells on stem cells *in vitro*. Neurospheres were gently dispersed and placed in EGM2 medium supplemented as stated above in 6-well plates. UASMCs and HUVECs were resuspended in complete EGM2 medium and were plated at high density on 6- and 12-well hanging cell culture inserts with 1.0-μm pores, respectively (Millipore, Billerica, MA, USA). Control wells contained neurospheres only. UASMC-only wells contained neurospheres and UASMC cells plated on 6-well hanging inserts. HUVEC-only wells contained neurospheres and HUVECs plated on 12-well hanging inserts placed inside empty 6-well hanging inserts. HUVEC and UASMC co-culture wells contained neurospheres with HUVECs plated on 12 well-hanging inserts placed inside 6 well-hanging inserts containing UASMC cells (Figure 1A). All experiments were performed in triplicate. Hanging inserts containing HUVECs and UASMCs were removed after 5 days of co-culture with neurospheres. Seventy-two hours after removal of HUVECs and UASMCs, glioma cells were harvested and re-plated at a concentration of 1×10^5 cells/well. Tumor spheres were counted 10 days after re-seeding.

Tumor sphere initiation assay. Single-cell suspensions were plated in a 96-well plate with serial dilutions (10,000 to 1 cells/well) for the initiation assay. After 10-14 days of culture, spheres were counted and reported as tumor spheres formed per cells plated.

Adeno-associated virus vector production. Adeno-associated virus vectors (AAV) were used to establish continuous, long-term delivery of human IFN-β *in vivo*. Construction of the pAV2 hIFN-β vector plasmid has been described previously (28). This vector plasmid includes the CMV-IE enhancer, β-actin promoter, a chicken β-actin/rabbit β-globin composite intron and a rabbit β-globin polyadenylation signal mediating the expression of the cDNA for human IFN-β. The hIFN-β cDNA was purchased from InvivoGen (San Diego, CA, USA). Recombinant AAV vectors pseudotyped with serotype 8 capsid were generated by the method described previously (29) using the pAAV8-2 plasmid provided by J. Wilson (Philadelphia, PA, USA). These AAV2/8 vectors were purified using ion exchange chromatography (30).

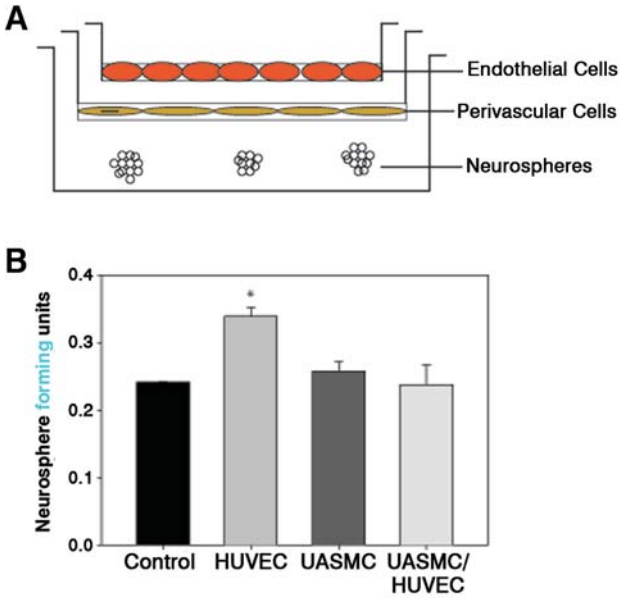

Figure 1. *Endothelial cells increase stem cell recovery in vitro while the addition of perivascular cells decreases stem cell recovery. A: Diagram of in vitro experiment to assess the interaction between stem cells, endothelial cells and perivascular cells. B: When stem cells were co-cultured with HUVECs alone, harvested cells formed more neurospheres than when stem cells were cultured alone, with pericytes or in combination with pericytes and endothelial cells. *p=0.002.*

Animal models. All experiments were conducted with male CB17 SCID mice (Charles River Laboratories, Boston, MA, USA) at 4-6 weeks of age housed in the Animal Resource Center at St Jude Children's Research Hospital in compliance with an animal protocol. Heterotopic tumors were established by injection of 3×10^6 U87 cells in the subcutaneous space of the flank under isoflurane anesthesia. Tumor size was determined by direct measurement with handheld calipers in two dimensions, and volumes were calculated as follows: (length×width^2)/2. Orthotopic tumors were established by implanting 5×10^5 luciferase-expressing U87 cells intracranially *via* a right-sided, parietal burr hole using an M844 surgical microscope system (Leica Microsystems Inc, Allendale, NJ, USA). Tumor burden was assessed with bioluminescence imaging by giving each mouse a 3-mg intraperitoneal injection of luciferin (Xenogen Corporation, Alameda, CA, USA) 10 minutes before imaging with an IVIS Imaging System 100 Series (Xenogen). Bioluminescence has been shown to correlate with tumor burden (31, 32). Systemic levels of human IFN-β in mouse plasma were determined using a commercially available enzyme-linked immunosorbent assay (Biosource International,

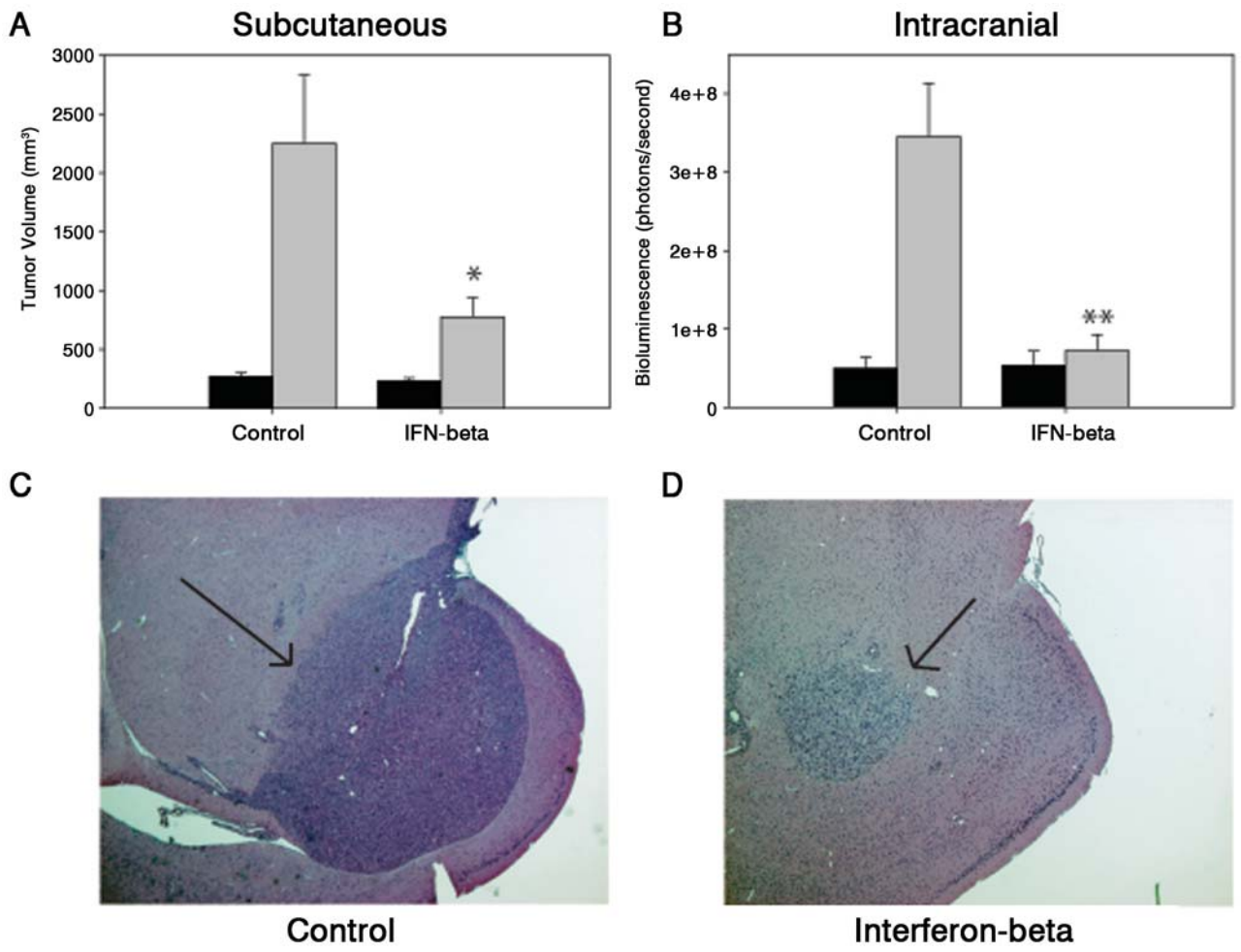

Figure 2. *Continuous IFN-β decreases glioma growth in heterotopic and orthotopic murine xenografts. Bar graphs represent average tumor size of (A) subcutaneous and (B) intracranial xenografts after 0 (black bar) and 14 (open bar) days of treatment with IFN-β. Representative hematoxylin and eosin-stained images of control (C) and treated (D) intracranial tumors. Arrows indicate tumor. *p=0.05, **p=0.03.*

Camarillo, CA, USA). Mice were euthanized with carbon dioxide after two weeks of treatment or if neurologic symptoms occurred prior to finishing the treatment course. All studies were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee of St. Jude Children's Research Hospital.

Immunofluorescence and immunohistochemical analysis. Immunofluorescence staining was conducted on formalin-fixed, paraffin-embedded tumor samples with a rabbit anti-human nestin antibody (Millipore AB5922), a rat anti-mouse CD34 antibody (14-0341-85, eBioscience, San Diego, CA, USA), and a mouse anti-human smooth muscle actin (SMA, M0851, Clone 1A4; DAKO Carpinteria, CA, USA). Secondary antibodies were goat anti-rabbit Alexa Fluor 488, goat anti-rat Alexa Fluor 647, and goat anti-mouse Alexa Fluor 555 (Invitrogen).

Immunohistochemical staining for SMA also was conducted on formalin-fixed, paraffin-embedded 4 μm-thick tumor sections with mouse anti-human smooth muscle actin (DAKO) antibody as previously described (33).

The distance from nestin-positive glioma stem cells to CD34-positive endothelial cells was measured on dual-stained brain tumor samples with the line-measuring function on ImageJ (NIH analysis software). Stem cells present in each sample were determined by counting the number of nestin-positive cells present in an image taken at ×400. Images were captured in well-vascularized areas without necrosis. Three fields were evaluated per tumor sample. SMA staining was quantified by capturing images with a camera at ×200 magnification. Background hematoxylin staining was removed, and images were transformed to grayscale and then monochrome with Adobe Photoshop (Adobe Systems, Inc, San Jose, CA, USA). The amount of positive staining was quantified with ImageJ and reported as the number of pixelsviahigh-power field (HPF).

Statistical analysis. Continuous variables are reported as mean±standard error and were compared using an unpaired Student's *t*-test with Sigmaplot software (Version 9, SPSS, Inc, Chicago, IL). A *p* value of less than 0.05 was considered significant.

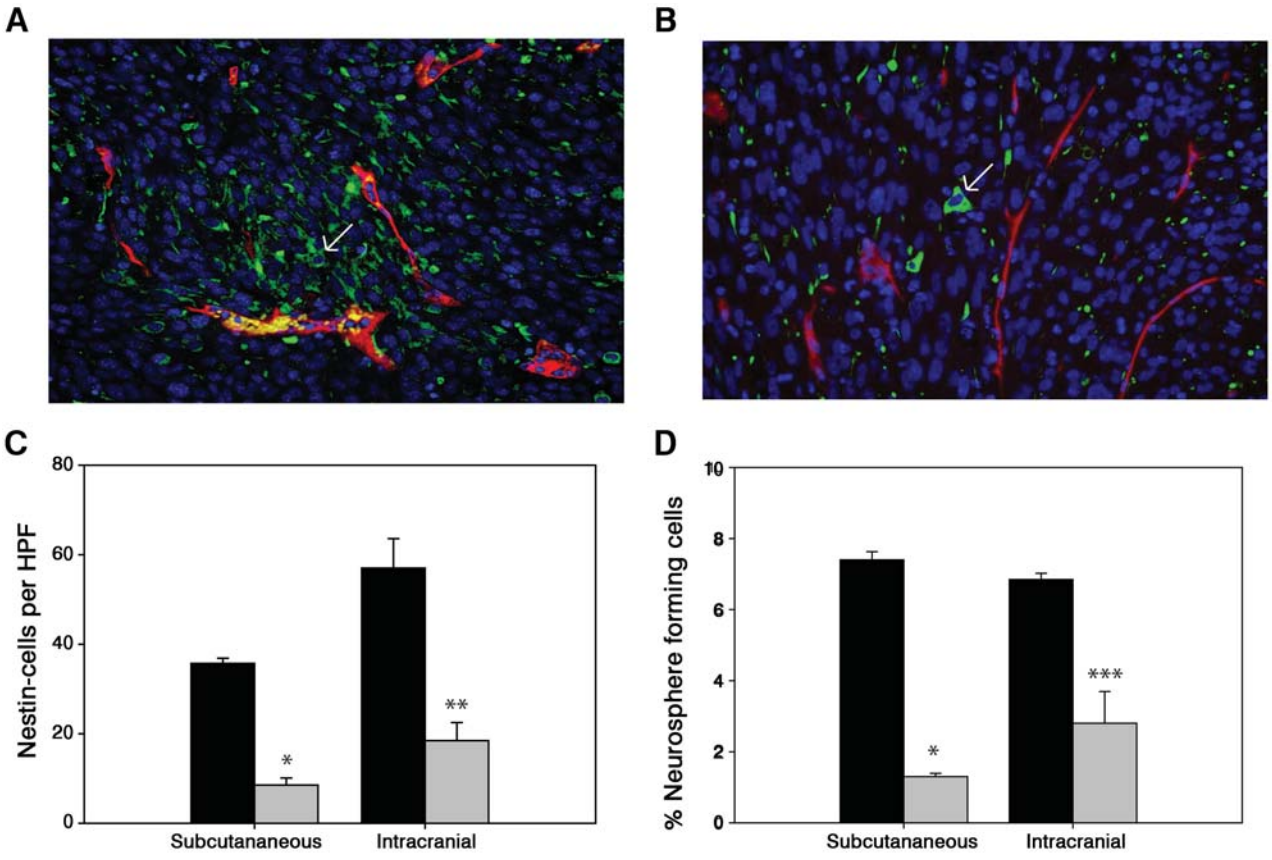

Figure 3. *IFN-β decreases stem cells in gliomas. A: Representative images (×400) of control (A) and IFN-β (B) treated U87 gliomas with immunofluorescent staining for nestin (green), CD34 (red), and DAPI (blue). Arrows represent nestin-positive glioma stem cells. Fewer stem cells were present in IFN-treated gliomas based on (C) immunofluorescent staining and (D) neurosphere recovery. * p<0.0001, ** p=0.0003, *** p=0.006.*

Results

IFN-β inhibits glioma xenograft growth. Subcutaneous gliomas were treated with continuous IFN-β (4.66×10^9 AAV-hIFN-β vector particles per mouse *via* tail vein) when they reached an average size of 249.8±20.9 mm^3. After 2 weeks of treatment, treated tumors were significantly smaller than control tumors (Figure 2A, 777±163 *vs.* 2254±579 mm^3, p=0.05). Orthotopic, intracranial tumors were size-matched based on bioluminescent signal, with an average signal of $5.1\text{x}10^7\pm1.1\times10^7$ photons/second 2 weeks after tumor cell inoculation. Treatment with AAV-hIFN-β (4.66×10^9 vector particles per mouse) restricted tumor growth compared with controls after 2 weeks of treatment (Figure 2B, $7.28\times10^7\pm2.0\times10^7$ *vs.* $3.5\times10^8\pm6.8\times10^7$ photons/second, p=0.03). Because normalizing the tumor vasculature, with resultant improvement in tumor oxygenation, may overestimate the bioluminescent signal in IFN-β-treated tumors, differences in tumor burden as assessed with bioluminescence were confirmed at the time of harvest with hematoxylin and eosin stained tissue through the largest portion of the tumor. Treated tumors were clearly smaller than controls based on microscopic evaluation as well (Figure 2C/D). Plasma hIFN-β levels at the time of euthanasia averaged 3.46±5.2 ng/ml in mice that received AAV-hIFN-β. Thus, continuous systemic delivery of human IFN-β resulted in a significant restriction in the growth of heterotopic and orthoptic glioma xenografts.

IFN-β decreases the number of glioma stem cells in vivo but not in vitro. To determine the effect of continuous delivery of IFN-β on the glioma stem cells in treated tumors, tumors were harvested, and stem cell content was determined by immunofluorescent staining and a functional recovery assay. Immunofluorescence revealed that samples from U87 tumors treated with continuous IFN-β for 2 weeks contained fewer stem cells, as identified by nestin staining (Figure 3A/B) than control (SQ: 35.7±1.2 cells/HPF *vs.* 8.5±1.6 cells/HPF, p<0.0001, IC: 54.5+6.9 cells/HPF *vs.* 18.4+4.0 cells/HPF, p=0.0003, Figure 3C). To further determine the effect of IFN-β on stem cells, tumor spheres were cultured from heterotopic and orthotopic tumors, because they have been

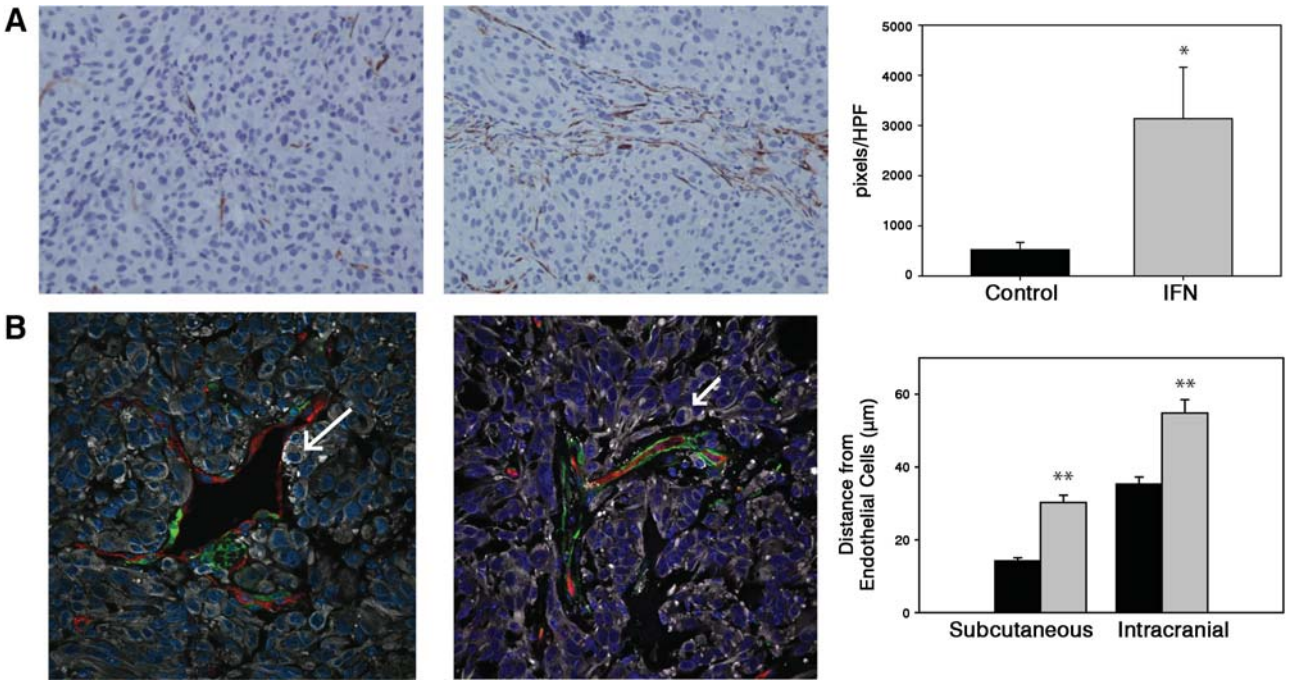

Figure 4. *IFN-β alters the microvascular niche of the glioma stem cells. A: U87 gliomas treated with IFN-β had an increase in the number of perivascular cells compared with control tumors (×200). B: Immunofluorescence staining of nestin (stem cells, white), CD34 (endothelial cells, red) and SMA (perivascular cells, green) showing distance of stem cells from endothelial cells in control and IFN-β–treated tumors (×400). Arrows indicate nestin-positive glioma cells. Bar graph with quantitative assessment of distance. * p=0.035, ** p<0.0001.*

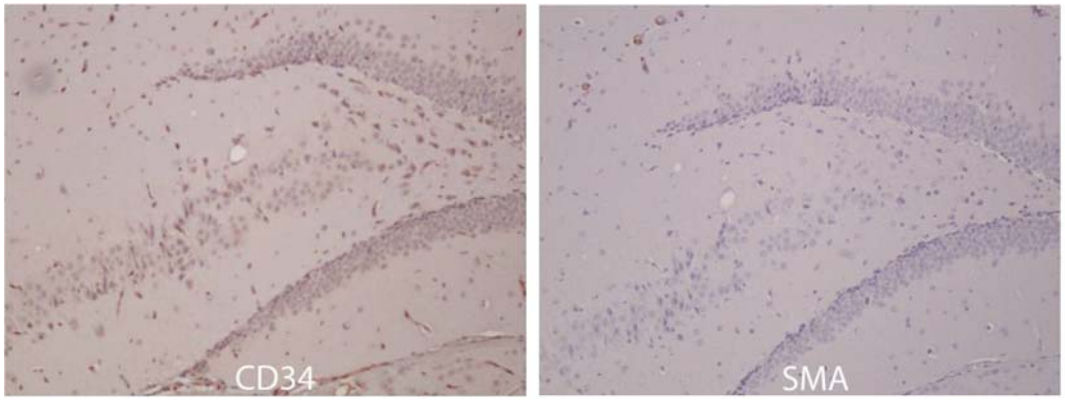

Figure 5. *Vascular niche of neural stem cells. The hippocampus of normal murine brain contains a large number of endothelial cells, detected with anti-CD34 immunohistochemistry (A), although there are few perivascular cells indicated by very few SMA-positive cells (B).*

shown to represent cancer stem cells *in vivo* (34). Fewer stem cells were recovered from IFN-β–treated tumors in both the subcutaneous model (7.4±0.2% *vs*. 1.3±0.1%, $p<0.0001$) and the intracranial model (6.9±0.2% *vs*. 2.8±0.9%, $p=0.006$, Figure 3D). Therefore, continuous delivery of IFN-β decreased the stem cell fraction of both heterotopic and orthotopic gliomas.

Recovered neurospheres were dispersed and injected as a single-cell suspension intracranially into CB17 SCID mice to determine their tumorigenicity. Intracranial tumors were identified consistently 4 weeks after injection of 10,000 stem cells, much less than the 500,000 U87 glioma cells required for tumors of similar size in the same time frame (data not shown).

The direct effect of IFN on stem cells was then evaluated *in vitro* by treating glioma tumor spheres with 100 and 10,000 units/ml recombinant IFN-β. After 72 hours of exposure, no difference was seen in the number of neurospheres present with 100 units/ml (1.76±0.2 *vs.* 1.83±0.09 neurospheres, p=0.78) or 10,000 units/ml (3.3±0.3 *vs.* 3.4±0.4 neurospheres, p=0.85). Because the stem cells form tight spheres of cells, the effect of IFN may not have been evident by counting neurospheres alone. Therefore, cells were dispersed and evaluated for apoptosis with annexin-V staining. No difference in apoptosis was identified with IFN-β treatment (data not shown).

IFN-β affects the vascular niche of the stem cell. We have previously shown that treatment with IFN-β increases the number of intratumoral vascular smooth muscle cells. Therefore, we assessed the effect of IFN-β on the vascular niche of glioma stem cells. IFN-β increased the number of perivascular cells compared with controls after 2 weeks of treatment (Figure 4A, 3136.5±1023.6 *vs.* 523.3±148.0 pixels/HPF, p=0.03). This investment of tumor vessels with perivascular cells had the effect of physically separating the glioma stem cells from the endothelial cells. To confirm the physical separation, the distance of glioma stem cells from endothelial cells was measured in both treated and untreated, subcutaneous and intracranial tumor samples. Nestin-positive cells were farther from endothelial cells in both IFN-β-treated tumor samples (Figure 4B, SQ: 30.2±2.0 μm *vs.* 14.2±0.8 μm, p<0.0001; IC: 54.8+1.9 mm *vs.* 35.3+3.7 mm, p<0.0001).

To better understand the relationship between stem cells, endothelial cells, and perivascular cells, tumor spheres were co-cultured with HUVECs and UASMCs alone and in combination (Figure 1A). The co-culture with HUVECs alone increased the number of stem cells recovered from the culture (0.24±0.001% *vs.* 0.34±0.02%, p=0.002), whereas the addition of UASMCs grown between the HUVECs and neurospheres, acting as a physical barrier, decreased the number of stem cells recovered (0.2±0.03% *vs.* 0.34±0.02%, p=0.036, Figure 1B).

Taken together, these results suggest that glioma stem cells require the close proximity of endothelial cells and their secreted factors for survival. Investing pericytes appear to inhibit this relationship. Interestingly, the vascular niche for normal stem cells within the hippocampus of the brain is also largely devoid of perivascular cells. Although there was a large number of endothelial cells in the hippocampus, there was very little SMA staining, indicating that the blood vessels in the hippocampus had very few perivascular cells (Figure 5).

Discussion

Our study demonstrated that treatment with IFN-β decreased the proportion of cancer stem cells in human glioblastoma xenografts, in part, by increasing the number of perivascular cells investing the tumor vessels, thereby disrupting the vascular microenvironment upon which the stem cells depend. These findings hold great potential for treatment of recurring and relapsed tumors.

Neural stem cells occupy a specialized vascular niche, which helps maintain their ability to self-renew and differentiate into multiple cell types (35). Though multiple studies have helped to delineate the relationship between the neural stem cells and vascular cells, the precise niche has yet to be fully characterized (2, 3, 5). It is clear that endothelial precursors are closely related to dividing neural precursors, and these cells have a symbiotic relationship (4, 7). It has also been shown that soluble factors released from endothelial cells contribute to neural stem cell maintenance (2, 4, 8, 36). One of these factors is vascular endothelial growth factor (VEGF), which, when infused into stem cell-rich portions of the brain, including the hippocampus and subventricular zone, increases stem cell proliferation (37). We have shown that these stem cell-rich regions of the brain contain numerous endothelial cells, though there are few perivascular cells, which may allow easier diffusion of soluble factors from the endothelial cells necessary for stem cell maintenance.

The dependence of neural stem cell on the vasculature appears to be important to the maintenance and proliferation of glioma stem cells as well. The importance of this vascular niche has been highlighted by recent studies reporting a decrease in glioma stem cells after treatment with angiogenesis inhibitors (13, 18-21, 34). Because these cells require a specialized vascular niche for growth and maintenance, we examined the effect of a vascular normalizing agent, IFN-β, on glioma xenografts. IFN-β decreased tumor growth in both a subcutaneous and intracranial animal model while decreasing the number of stem cells present in the treated tumor samples. Because IFN-β was not directly toxic to stem cells *in vitro*, the decrease in stem cells can be attributed to an indirect effect, an increase in perivascular cells, which acts as a barrier between the stem cells and the endothelial cells. Soluble factors released from endothelial cells are necessary for glioma stem cell growth and allow glioma stem cells to retain their 'stem-like' characteristics, including self-renewal and the de-differentiated state (5, 34, 38). VEGF has been implicated in glioma stem cell tumorigenesis and angiogenesis, and tumor cells expressing increased levels of VEGF are highly malignant and vascular, with decreased survival compared with glioma cells that do not express VEGF (13, 39). The addition of a barrier to these factors would decrease tumor growth and increase stem cell differentiation, decreasing the number of stem cells present in glioma tumor samples.

To confirm that perivascular cells can act as a barrier to soluble factors released from endothelial cells, stem cells were co-cultured with endothelial cells and perivascular smooth muscle cells alone and in combination. The addition of endothelial cells increased stem cell recovery, confirming that

endothelial cells released a factor that supported the stem cells. However, co-culture with perivascular cells had no effect on stem cell recovery, and most of the stem cells placed in culture differentiated into glioma cells. When perivascular smooth muscle cells were cultured between the stem cells and endothelial cells (similar to the effect of IFN-β on tumor samples), fewer stem cells were recovered. This simple *in vitro* model supports the hypothesis that an increase in the number of pericytes between endothelial cells and stem cells would decrease the number of glioma stem cells.

Because gliomas are particularly vascular tumors, antiangiogenic agents have been used in clinical trials with modest results (40-42). Combining these agents, however, with traditional chemotherapy leads to longer survival and decreased tumor growth (34, 43). The effect on the vascular niche has been related to a decrease in endothelial cells, though some angiogenic agents act by 'normalizing' the tumor vasculature without an overall decrease in endothelial cells (27, 44-46). IFN-β normalizes the glioma vasculature by increasing blood vessel investment with perivascular cells, which may have disrupted the vascular niche of the stem cells. Because cancer stem cells have been linked to resistance to traditional therapies and tumor recurrence, it is important to identify novel agents that can target these increasingly important cancer cells. The cells that support tumor vasculature may be important targets for new chemotherapeutic agents to help eliminate the cancer stem cell population.

Conclusion

Treatment with continuous IFN-β decreases the proportion of cancer stem cells in human glioblastoma xenografts by increasing the number of perivascular cells investing the tumor vessels, thereby disrupting the vascular microenvironment upon which the stem cells depend. Further studies focusing on increasing numbers of perivascular cells to decrease cancer stem cell growth is warranted in an attempt to eradicate this self-renewing, multi-potent subpopulation of tumor cells.

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