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Continuous Local Delivery of Interferon- β Stabilizes Tumor Vasculature in an Orthotopic Glioblastoma Xenograft Resection Model

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Abstract

Background—High-grade glioblastomas have immature, leaky tumor blood vessels that impede the efficacy of adjuvant therapy. We assessed the ability of human interferon-beta (hIFN- β) delivered locally via gene transfer to effect vascular stabilization in an orthotopic glioblastoma xenograft resection model.

Methods—Xenografts were established by injecting three grade IV glioblastoma cell lines (GBM6-luc, MT330-luc, and SJG2-luc) into the cerebral cortex of nude rats. Tumors underwent subtotal resection, and then had gel foam containing an adeno-associated virus vector encoding either hIFN- β or green fluorescence protein (GFP, control) placed in the resection cavity. The primary end point was stabilization of tumor vasculature, as evidenced by CD34, α SMA, and CA IX staining. Overall survival was a secondary endpoint.

Results—hIFN- β treatment altered the tumor vasculature of GBM6-luc and SJG2-luc xenografts, decreasing the density of endothelial cells, stabilizing vessels with pericytes, and decreasing tumor hypoxia. The mean survival for rats with these tumors was not significantly improved, however. In rats with MT330-luc xenografts, hIFN- β resulted in tumor regression, with a 6-month survival of 55% (INF- β group) and 9% (control group).

Conclusion—The use of AAV hIFN- β in our orthotopic glioblastoma resection model stabilized tumor vasculature, and dramatically improved survival in rats with MT330 xenografts.

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Background

Glioblastoma multiforme (GBM) is a highly aggressive tumor with a diffusely infiltrative growth pattern, making complete surgical resection impossible. Historically, median survival for patients with GBM has been less than one year, and even with the addition of modern radiotherapy and chemotherapy, the median survival is only 14.6 months.^{1,2} Glioblastomas demonstrate vigorous angiogenesis which aids in their rapid growth and invasion.³ Studies have demonstrated that these tumors express increased levels of angiogenic factors, such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and angiopoietins.^{4–7} The overproduction of these angiogenic factors leads to the development of a network of immature leaky tumor blood vessels providing heterogeneous perfusion to the tumor.^{3,8} Thus, glioblastomas contain areas of hypoxia and acidosis that are relatively resistant to radiotherapy and traditional chemotherapy.^{9–12} We have hypothesized that stabilization of the abnormal tumor vasculature leads to improved tumor perfusion as well as limiting further tumor growth, because of the inability of the vessels to grow and meet the expanding needs of the growing tumor.^{13,14}

Previously, we demonstrated that the tumor vasculature of subcutaneous glioblastoma xenografts underwent maturation and stabilization upon exposure to continuous human interferon- β (hIFN- β) delivered through a gene therapy approach.¹³ Intratumoral vessels had decreased permeability, increased structural support by pericytes, and improved overall tumor perfusion. Additionally, we have shown that the vasculature stabilization leads to improved delivery of chemotherapeutic agents and increased sensitivity to radiotherapy.^{14,15} We sought to improve upon our murine glioblastoma models. An orthotopic infiltrating glioblastoma model that includes a subtotal surgical resection would more closely mirror the clinical treatment of glioblastoma in humans. Additionally, an orthotopic surgical resection model would allow us to evaluate the effects of hIFN- β delivered locally via gene transfer. We have been successful in developing an orthotopic infiltrative glioblastoma resection model in nude rats. Therefore, in this study we sought to assess the ability of hIFN- β delivered locally via gene transfer to effect vasculature stabilization (primary endpoint) and impact survival (secondary endpoint).

Methods

Cell lines

Three grade IV glioblastoma cell lines with infiltrative patterns of growth were engineered to express the firefly luciferase gene: GBM6-luc (provided by Dr. D. James, San Francisco, CA), MT330-luc (provided by Dr. C. Duntch, Memphis, TN), and SJG2-luc (provided by C. Morton, Memphis, TN). These three cell lines were used for all in vitro and in vivo experiments. Hs294T (American Type Culture Collection, Manassas, VA) a melanoma cell was used as a positive control for in vitro studies.

In vitro hIFN- β sensitivity studies

GBM6-luc and MT330-luc cells were plated at a density of 5×10^4 cells per well in a 96-well plate. SJG2-luc and Hs294T cells were plated at a density of 3×10^4 cells per well in a 96-well plate. After 24 hours, each row of cells was treated with an escalating dose of recombinant human IFN- β (rhIFN- β) (Biogen, Cambridge, MA) 0, 10, 100, 1,000, or 10,000 U/ml. After 96 hours, cell viability was assessed by adding AlamarBlue Cell Viability Reagent (Invitrogen, Carlsbad, CA) to all wells and measuring the absorbance on a Bio Tek Synergy 2 plate reader (MTX Lab Systems Inc, Vienna, VA).

AAV vector production and functional studies

Vector plasmids containing hIFN- β , green fluorescence protein (GFP), and firefly luciferase (luc) genes were constructed as previously described.^{16,17} The AAV-2/2, AAV-2/5, and AAV-2/8 vectors were produced and purified using transient transfection and ion exchange chromatography methods.¹⁸ Standard quantitative polymerase chain reaction analysis was used to determine the vector particle titer. To assess in vivo functionality and tissue specificity of each AAV vector serotype, the vectors possessing the firefly luciferase gene were injected in the murine cerebral cortex at a concentration of 1×10^7 vector particles per 10 μ L of PBS and followed with bioluminescence imaging, as described below.

hIFN- β detection

Levels of hIFN- β in plasma and protein extracted from brain parenchyma were determined after administration of vector using a commercially available enzyme-linked immunosorbent assay kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

Animal models

All animal experiments were performed on male CB-17 SCID mice and 6–10 week-old male Nude rats (Charles' River Laboratories, Wilmington, MA). For all surgical procedures, anesthesia was administered with a mixture of ketamine and xylazine. Animals were prepped and placed on a warming pad in a stereotactic frame. After exposing the skull, a burr hole (2 $\times$ 3 mm for mice and 4 $\times$ 6 mm for rats) was made posterior to the bregma over the right cerebral hemisphere and the underlying dura mater was exposed (Figure 1). Injection of the desired number of vector particles or 1×10^6 glioblastoma cells was performed at a depth of 2–3mm. Lastly, the skin and subcutaneous tissue was closed over the skull defect using skin glue or 3-0 chromic suture.

Vector expression in mice and tumor burden in rats were monitored by bioluminescence imaging using the IVIS Imaging System 100 Series (Xenogen Corporation, Alameda, CA) postoperatively on a weekly basis. Previous studies have shown that the bioluminescence signal correlates with tumor burden.^{19,20} When the bioluminescence signal was at least 1×10^5 photons/sec, approximately 2 weeks after tumor implantation, tumor resection was performed.

We used a technique similar to that published previously²¹ for resection of our orthotopic glioblastoma xenografts. Briefly, after administering anesthesia, prepping, and exposing the burr hole, the dura mater overlying the tumor was removed. Next, a neuro tip suction device was used to perform a subtotal tumor resection. A post-resection bioluminescence image was obtained to confirm subtotal resection and quantify the postoperative tumor burden (Figure 1). A small piece of gel foam soaked with PBS containing 1×10^{11} vector particles of AAV-2/5 GFP (control) or hIFN- β was then placed in the resection cavity. In order to decrease cerebral edema in the postoperative period, animals received intraperitoneal injections of mannitol (1 g/kg), dexamethasone (3 mg/kg), depo-medrol (4 mg/kg), and Lactated Ringer's Solution (5 ml) every 12 hours for 3 days. Tumor burden was monitored in all animals after resection with weekly bioluminescence imaging. All animals were sacrificed when they developed neurologic symptoms such as right or left sided weakness, ataxia, paralysis, or seizures. At that time, blood and tissue were collected for further analysis, and the number of days post tumor resection was recorded and used to define overall survival.

All animal experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of St. Jude Children's Research Hospital.

AAV vector distribution

Upon sacrifice, tissues from some rats were collected in order to confirm and quantify the presence of vector in various tissues. Tissue from the tumor, peritumoral ipsilateral hemisphere, uninvolved ipsilateral hemisphere, contralateral hemisphere, liver, and testes were snap frozen in liquid nitrogen. Genomic DNA was extracted from the tissues and quantitative polymerase chain reaction (qPCR) was performed as described previously.²²

Immunohistochemistry

Intratumoral vasculature and hypoxia were evaluated with immunohistochemical staining. Goat anti-rat CD34 antibody (R&D Systems, Minneapolis, MN) was used to highlight endothelial cells and mouse anti-human α -smooth muscle actin (α SMA) (clone 1A4, Dako, Carpinteria, CA) was used to identify the perivascular cells stabilizing the vessels, as previously described.²³ Intratumoral hypoxia was assessed by staining for carbonic anhydrase IX (CA IX), with size-matched tumors sections being used for comparison.²⁴ Images from the appropriately stained slides were obtained and analyzed as previously described.^{13,25}

Statistical analysis

Continuous variables are reported as mean $\pm$ SEM, and were compared using an unpaired Student's t test. Survival data are presented using Kaplan-Meier curves, and were compared using a log rank test. A p value of less than 0.05 was considered significant. The data were analyzed and presented graphically using SigmaPlot (Version 10; SPSS Inc, Chicago, IL) and GraphPad Prism (Version 5; GraphPad Software Inc, San Diego, CA).

Results

Glioblastomas are minimally sensitive to rhIFN- β in vitro

We sought to assess the direct effect of rhIFN- β on glioblastoma viability in vitro. GBM6-luc, MT330-luc, and SJG2-luc cells were plated at an appropriate density in 96-well plates and allowed to adhere overnight. The next day, the cells were exposed to increasing doses of rhIFN- β from 0 to 10,000 U/ml. After 96 hours, the AlamarBlue assay was used to quantify the number of viable cells. All three cell lines were found to be only slightly sensitive to hIFN- β ; GBM6, MT330, and SJG2 were respectively 78%, 81%, and 67% of control at a rhIFN- β concentration of 10,000 U/ml (Figure 2). These in vitro results suggest that rhIFN- β alone has minimal anti-proliferative or cytotoxic effects on glioblastoma cells.

AAV-2/5 best localizes to the brain and produces hIFN- β

In order to determine which vector serotype was able to produce the best local expression of our transgene in the brain parenchyma, we performed intracranial injections of 1×10^7 vector particles of AAV-2/2, AAV-2/5, and AAV-2/8 containing the firefly luciferase gene in CB-17 SCID mice. Two weeks after injection of AAV containing the three serotypes, the animals were imaged with bioluminescence (Figure 3). Serotype 5 preferentially infected the brain parenchyma leading to high levels of local expression of firefly luciferase, while serotypes 2 and 8 showed a higher degree of transduction of other remote sites. Thus, AAV-2/5 was chosen for use in the orthotopic resection model as it provides the best local expression of the hIFN- β gene.

Local and systemic levels of hIFN- β expression were determined after intracranial injections of 1×10^{11} vector particles of AAV-2/5 GFP and hIFN- β in CB-17 SCID mice. Two weeks after injection of the vector, the animals were sacrificed, a portion of brain surrounding the injection site was snap frozen, and plasma was collected. An ELISA was used to measure

hIFN- β in the brain parenchyma and plasma. In the mice that received the AAV-2/5 GFP vector, no hIFN- β was detected in the brain parenchyma or the plasma. Animals that received the AAV-2/5 hIFN- β vector had a mean hIFN- β concentration of 4.77 ± 0.95 pg/200 μ g protein in the brain and 123.6 ± 44.3 pg/ml in the plasma. These results demonstrate that AAV-2/5 hIFN- β produces excellent local expression of hIFN- β with minimal spill into the systemic circulation, which should minimize systemic toxicity.

AAV-2/5 is present in the resection bed

Approximately two weeks after tumor implantation, the rats underwent tumor resection. A post-resection bioluminescence image was obtained to confirm that a subtotal tumor resection had been performed. Next, a piece of gel foam soaked with PBS containing 1×10^{11} vector particles of AAV-2/5 GFP or hIFN- β was placed in the resection cavity. When the rats developed neurologic symptoms the animals were sacrificed and tissue was harvested from the intracranial tumor, ipsilateral peritumoral hemisphere, uninvolved ipsilateral hemisphere, contralateral hemisphere, liver, and testes and snap frozen in liquid nitrogen. Genomic DNA was extracted from the tissues and CMV primers were used to check for the presence of the DNA containing the transgene. The concentration of vector particles found in the tumor, peritumoral, and ipsilateral cerebral hemisphere was much higher than in the contralateral brain, liver, and testes (Figure 4). The mean concentration of vector particles per μ g of DNA found in the tissue from the resection bed (tumor and peritumoral cerebral hemisphere) was about 50 times greater than that found in the distant tissues (liver and testes). Thus, our method of administering AAV-2/5 into the resection cavity leads to infection of the cells adjacent to the resection cavity and a very low rate of infection outside the brain.

hIFN- β delivered via gene transfer stabilizes tumor vasculature

The primary purpose of our study was to assess the effects of hIFN- β delivered via gene transfer on tumor vasculature. To evaluate this, cross-sections of the orthotopic glioblastoma xenografts were stained with a CD34 antibody to highlight the endothelial cells, an α SMA antibody to evaluate the presence of stabilizing pericytes, and a CA IX antibody in order to determine if vascular changes affected tumor hypoxia.

Treatment with hIFN- β altered tumor vasculature in orthotopic GBM6-luc and SJG2-luc xenografts. Tumors in the hIFN- β group demonstrated a decreased density of endothelial cells; CD34 pixels per HPF for GBM6 was $1.8 \times 10^5 \pm 2.4 \times 10^4$ (control) and $6.8 \times 10^4 \pm 1.1 \times 10^4$ (hIFN- β), $p=0.0006$, and for SJG2 was $1.1 \times 10^5 \pm 1.4 \times 10^4$ (control) and $4.1 \times 10^4 \pm 8.9 \times 10^3$ (hIFN- β), $p=0.0007$. Also, treatment with hIFN- β stabilized tumor vessels with pericytes; α SMA pixels per HPF for GBM6 was $9.9 \times 10^3 \pm 1.6 \times 10^3$ (control) and $1.8 \times 10^4 \pm 3.8 \times 10^3$ (hIFN- β), $p=0.045$, and for SJG2 was $7.9 \times 10^3 \pm 2.2 \times 10^3$ (control) and $2.6 \times 10^4 \pm 3.7 \times 10^3$ (hIFN- β), $p=0.0008$. The changes in tumor vasculature brought on by treatment with hIFN- β led to a decrease in tumor hypoxia; CA IX pixels per HPF for GBM6 was $3.1 \times 10^5 \pm 5.3 \times 10^4$ (control) and $8.6 \times 10^4 \pm 2.5 \times 10^4$ (hIFN- β), $p=0.0009$, and for SJG2 was $1.8 \times 10^5 \pm 4.9 \times 10^4$ (control) and $2.4 \times 10^4 \pm 4.2 \times 10^3$ (hIFN- β), $p=0.0065$. Thus, glioblastoma xenografts treated with hIFN- β demonstrated evidence of tumor vascular stabilization, a decrease in immature endothelial-lined vessels and an increased density of stabilizing pericytes, and a concomitant decrease in tumor hypoxia (Figure 5).

hIFN- β delivered via gene transfer impacts survival in MT330

Lastly, we sought to determine whether hIFN- β delivered locally via gene transfer impacted tumor progression or overall survival. After the animals underwent subtotal xenograft resection and received vector with GFP (control) or hIFN- β , tumor burden was followed with weekly bioluminescence imaging. When neurologic symptoms developed the animals

were sacrificed and overall survival (numbers of days from date of resection to date of death) was recorded. MT330-luc tumors were resected in 22 rats. Post-resection, the mean bioluminescence signal was 1.2×10^5 , with the signal on average being lower in the control group ($7.7 \times 10^5 \pm 1.2 \times 10^6$ vs. $1.5 \times 10^6 \pm 2.2 \times 10^6$, $p=0.3$). Two weeks postoperatively, the hIFN- β group had a mean bioluminescence signal about 20% that of the control group ($4.4 \times 10^6 \pm 6.4 \times 10^6$ vs. $2.1 \times 10^7 \pm 3.8 \times 10^7$, $p=0.2$). Additionally, the hIFN- β group had a survival advantage with 6-month survival of 55% versus 9% (GFP), $p=0.01$ (Figure 6). GBM6-luc tumors were resected in 18 rats. Post-resection tumor burden was similar in both groups, tumors progressed comparably, and there was no difference in mean overall survival (12 days GFP v. 10 days hIFN- β). Thirteen SJG2-luc tumors underwent resection. Post-resection tumor burden, tumor progression, and overall survival (12 days GFP v. 14 days hIFN- β) did not differ between groups. Thus, in our orthotopic xenograft resection model, AAV-2/5 hIFN- β slowed the rate of tumor progression and prolonged survival in rats with MT330 luc tumors, with 55% of hIFN- β rats being cured.

Discussion

Glioblastomas are aggressive highly vascular tumors with a dismal prognosis that produce excessive amounts of multiple angiogenic factors, such as bFGF, VEGF, and angiopoietins.⁴⁻⁷ Our lab has previously demonstrated that systemic hIFN- β delivered through a gene therapy approach stabilizes the vasculature of subcutaneous (heterotopic) glioblastoma xenografts by increasing the number of pericytes.¹³ We sought to assess the ability of hIFN- β delivered locally through gene transfer to stabilize the vasculature of orthotopic glioblastoma xenografts in our recently developed resection model, and determine whether local delivery of hIFN- β impacted overall survival.

We successfully developed an orthotopic glioblastoma xenograft model that closely mirrors the clinical scenario seen in humans. Our model starts with establishing orthotopic infiltrating xenografts in nude rats. Next, the tumors undergo a subtotal surgical resection and AAV-2/5 GFP or hIFN- β is placed within the resection bed. The surgical resection is highly reproducible, with a procedural related mortality of less than 5%, and with 100% of untreated tumors demonstrating progressive growth. Over the ensuing few days the cells infected with AAV-2/5 hIFN- β begin to produce continuous local hIFN- β . As we demonstrated with our vector distribution studies, the promoter and transgene are present in the tissue surrounding the resection cavity with few vector particles being present outside the brain. Continuous low-level local expression of the transgene keeps the systemic level of hIFN- β to a minimum, thus limiting systemic toxicity commonly seen with intravenous administration of interferon. Our clinically-relevant orthotopic glioblastoma xenograft model has enabled us to investigate hIFN- β delivered locally via gene transfer, a novel treatment.

In our model, hIFN- β delivered via gene transfer effected changes in the intratumoral vasculature. hIFN- β treatment decreased the number of small endothelial-lined blood vessels, improved structural support by recruiting smooth muscle cells to the perivascular space, and lessened tumor hypoxia. These findings are congruous with what we found in subcutaneous (heterotopic) glioblastoma xenografts treated with systemic hIFN- β via gene transfer.¹³ In previous studies involving neuroblastoma xenografts we found that vascular normalization produced by hIFN- β improved tumor perfusion and delivery of chemotherapeutic agents.¹⁵ Additionally, treatment of orthotopic glioblastoma xenografts with hIFN- β via gene transfer improved tumoral oxygenation and sensitivity to ionizing radiation.¹⁴ Since vascular normalization induced by hIFN- β improves tumor perfusion and oxygenation, AAV hIFN- β used as an adjunct to surgical resection should sensitize glioblastomas to adjuvant chemotherapy and ionizing radiation.

Treatment with hIFN- β slowed tumor progression and dramatically improved survival in rats with MT330-luc tumors, but did not impact tumor progression or survival in rats with GBM6-luc or SJG2-luc tumors. Most MT330-luc tumors underwent regression when treated with hIFN- β , while GMB6-luc and SJG2-luc tumor continued to progress. Since, hIFN- β did not demonstrate any direct cytotoxic effects in vitro, presumably the explanation for the drastic sensitivity difference lies within the interplay between the tumor and its microenvironment. In theory, vascular stabilization inhibits the ability of vessels to proliferate and meet the demands of a growing tumor. In our model, the tumor is present in the confined intracranial space and even a small amount of tumor growth may prove to be lethal, thus no survival advantage might be seen in the animals receiving hIFN- β . Nonetheless, since hIFN- β treatment alone did not impact tumor progression or overall survival in GBM6-luc and SJG2-luc tumors, these cell lines are well suited for further studies using AAV hIFN- β as an adjunct to surgical resection, chemotherapy, and ionizing radiation.

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Figure 2. In vitro sensitivity of glioblastomas to rhIFN- β

GBM6-luc, MT330-luc, SJG2-luc, and Hs294T cells were exposed to increasing dose of rhIFN- β for 96 hours, and the number of viable cells was quantified using the AlamarBlue Assay. All three glioblastomas are only slightly sensitive to the direct effects of rhIFN- β in vitro.

Figure 3. AAV serotype tissue specificity

AAV serotypes 2, 5, and 8 containing the firefly luciferase gene were injected into the brain of SCID mice. Serotype 5 showed a high level of local expression, while serotypes 2 and 8 were found to have significant levels of transduction at remote sites.

Figure 4. AAV-2/5 tissue distribution

Genomic DNA was extracted from various tissue sites (tumor, peritumoral ipsilateral hemisphere, uninvolved ipsilateral hemisphere, contralateral hemisphere, testes, and liver) and CMV primers were used to detect the presence of vector by qPCR. High levels of the vector DNA were detected in the tumor and peritumoral tissue, while very low levels were found in the liver and testes. Error bars refer to standard error. $n = 3$ in each group.

Figure 5. hIFN- β stabilizes tumor vasculature and decreases tumor hypoxia

Xenografts were stained for CD34, α SMA, and CAIX. Images of stained slides, magnified 400 times, were quantified as pixels per HPF and treatment groups were compared. A, data for GBM6 xenografts, * $p=0.0006$, ** $p=0.045$, *** $p=0.0009$; B, data for SJG2 xenografts, * $p=0.0007$, ** $p=0.0008$, *** $p=0.0065$; C–D, SJG2 xenografts stained for CD34; E–F, SJG2 xenografts stained for α SMA; G–H, GBM6 xenografts stained for CA IX. Error bars refer to standard error. $n = 5$ in each group.

Figure 6. Effects of AAV hIFN- β on MT330 tumor progression and overall survival
 A, bioluminescence signal preoperative, postoperative, 7 days postoperative, and 14 days postoperative for rats with MT330 tumors. B, Kaplan-Meier survival curve for rats with MT330 tumors, with 6-month survival of 9% for GFP (control) ($n = 11$) and 55% for hIFN- β ($n = 11$), $*p=0.01$. Error bars refer to standard error.