A Hypoxia-Responsive Glial Cell–Specific Gene Therapy Vector for Targeting Retinal Neovascularization

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Purpose. Müller cells, the major glial cell in the retina, play a significant role in retinal neovascularization in response to tissue hypoxia. We previously designed and tested a vector using a hypoxia-responsive domain and a glial fibrillary acidic protein (GFAP) promoter to drive green fluorescent protein (GFP) expression in Müller cells in the murine model of oxygen-induced retinopathy (OIR). This study compares the efficacy of regulated and unregulated Müller cell delivery of endostatin in preventing neovascularization in the OIR model.

Methods. Endostatin cDNA was cloned into plasmids with hypoxia-regulated GFAP or unregulated GFAP promoters, and packaged into self-complementary adeno-associated virus serotype 2 vectors (scAAV2). Before placement in hyperoxia on postnatal day (P)7, mice were given intravitreal injections of regulated or unregulated scAAV2, capsid, or PBS. Five days after return to room air, on P17, neovascular and avascular areas, as well as expression of the transgene and vascular endothelial growth factor (VEGF), were compared in OIR animals treated with a vector, capsid, or PBS.

Results. The hypoxia-regulated, glial-specific, vector-expressing endostatin reduced neovascularization by 93% and reduced the central vaso-obliteration area by 90%, matching the results with the unregulated GFAP-Endo vector. Retinas treated with the regulated endostatin vector expressed substantial amounts of endostatin protein, and significantly reduced VEGF protein. Endostatin production from the regulated vector was undetectable in retinas with undamaged vasculature.

Conclusions. These findings suggest that the hypoxia-regulated, glial cell–specific vector expressing endostatin may be useful for treatment of neovascularization in proliferative diabetic retinopathy.

Keywords: hypoxia, GFAP, Müller cell, HIF-1 responsive element, OIR model, gene therapy

Neovascularization in age-related macular degeneration and diabetic retinopathy remains a major cause of vision loss and legal blindness in the world today. Reduction, occlusion, or loss of retinal or choroidal vessels are recognized as risk factors for retinal, vitreal, or choroidal neovascularization in diabetic retinopathy, retinopathy of prematurity, and age-related macular degeneration. The consequent retinal hypoxia increases levels of HIF-1, the master regulator of angiogenesis, which regulates expression of many target genes, including at least 31 proangiogenic factors and nine antiangiogenic factors.[1]

The major growth factor associated with both physiological and pathological angiogenesis is VEGF, expressed by several ocular cell types, including Müller cells, RPE cells, pericytes, vascular endothelial cells, and ganglion cells.[2–5] Vascular endothelial growth factor mediates multiple events in an angiogenic program characterized by increased vascular permeability (causing macular edema), recruitment, proliferation, migration, adhesion, and organization of endothelial cells to form tubular new vessels. The critical role of VEGF in many forms of ocular neovascularization made it an attractive target for development of VEGF-targeting therapies.[6–8] The introduction of successful anti-VEGF treatments has dramatically reduced vision loss in diabetic macular edema[9,10] and neovascular forms of age-related macular degeneration.[11,12] However, better therapies are needed: the requirements for multiple injections and frequent office visits place a burden on patients, neovascularization may reappear when the treatment is stopped, and the injections are associated with a low risk for elevated intraocular pressure, uveitis, vascular occlusion, vitreous hemorrhage, or retinal detachment.[13–15] Moreover, there is wide variation in patient responses to treatment; robust gain in vision is observed in approximately 30% of patients, and 10% of patients do not respond to anti-VEGF treatments.[8,16–18] A recent large study ($n = 835$ patients) found no link between SNPs in VEGF receptors, and patient responses,[19] suggesting that continuous suppression of the VEGF signaling may be more efficacious than monthly injections, and/or that other pathways are contributing to the angiogenic responses to ocular hypoxia.

Gene therapy offers the advantage of local and sustained delivery of anti-angiogenic molecules, and has proven to efficiently suppress neovascularization in animal models.[20–24] Restricting expression of anti-VEGF molecules to periods when the disease process is active should reduce the potential for complications associated with prolonged reduction in VEGF, an important survival factor for photoreceptors, endothelial cells, ganglion cells,[25] RPE, and the ciliary process. Inhibition of VEGF has been associated with thinning of the outer nuclear layer in patients[26]; vessel occlusion in animals and patients[27,28]; and in animals with decreased ERGs,[29] reduced thickness of the inner and outer nuclear layers[30] and damage to RPE.[31] (Despite these observations, the overall safety record of intravitreal anti-VEGF drugs has been good; the changes reported may be too subtle for routine clinical observation.)

Since hypoxia is a common feature of pathological neovascularization (NV) leading to vision loss, physiologically regulated gene delivery by low oxygen levels offers great potential for more controlled gene therapy for ischemic ocular diseases. In order to target genes to hypoxic cells, several hypoxia-sensitive gene switches have been developed, all of which are based on hypoxia responsive elements (HRE).[32–38]

The hypoxia responsive elements are targeted by hypoxia inducible factor—a heterodimer of HIF-1α and HIF-1β. In normoxia, HIF-1α is hydroxylated and rapidly degraded by the proteasome. In hypoxic conditions, HIF1α is stabilized and accumulates in the cytoplasm to form HIF1 dimers that bind the HREs in target genes. Incorporating HREs in our gene therapy make it HIF-1 regulated, so the therapy will be activated only in retinal regions experiencing hypoxia, or other pathological conditions activating HIF-1 regulated angiogenesis.

We chose to target Müller cells, the major retinal glial cell, since it traverses the retina, and would therefore experience hypoxic stress following capillary loss in diabetes[39,40] and expresses VEGF in oxygen-induced retinopathy.[3] It is known that Müller cell–derived VEGF is a significant contributor to NV[41] in the retina.[42] Gene therapy targeting Müller cells is an important addition to existing gene therapies that target RPE cells; together they provide a means to selectively deliver antiangiogenic therapy to either the inner retina or outer retina/choroid.

To suppress ocular neovascularization, we selected endostatin for its profound effects on angiogenesis affecting more than VEGF-dependent pathways. A cleavage product of collagen XVIII, it inhibits endothelial cell proliferation, migration and survival,[43] and it increases expression of antiangiogenic factors (e.g., thrombospondin), and inhibits levels of proangiogenic factors (e.g., HIF1a, ephrins, Ids).[44] Endostatin blocks VEGF-induced microvascular permeability by rapidly stabilizing occludin and tight junctions in vessels.[45] Following internalization into endothelial cells, either through endocytosis or through clathrin-coated pits,[46] endostatin localizes to the nucleus[47] and reduces expression of VEGF and increases the expression of pigment epithelium-derived factor (PEDF).[48] The C-terminal peptide of endostatin competes with VEGF for binding to VEGF receptor 3.[49]

We previously reported that a promoter containing a hypoxia-responsive domain together with a GFAP-cell specific promoter maintained cell-specificity and was hypoxia-inducible both in vitro and in vivo.[38] The current study tested the hypothesis that our hypoxia-regulated, glial cell–specific AAV vector expressing endostatin would be as effective in reducing neovascularization in the OIR model as gene therapy with constitutively expressed endostatin.[20,50]

Methods

Construction of Plasmids and Generation of scAAV2

Previously tested promoter cassettes[38] having the GFAP promoter or the REG-GFAP (hypoxia regulated GFAP) promoter were cloned into the self-complementary adeno-associated virus serotype 2 vectors (scAAV) plasmid. The human GFAP promoter (GfaABC1D domain of GFAP promoter, 681-bp size)—a gift from Michael Brenner, PhD, University of Alabama—is a truncated promoter with subfragments that in combination, were previously shown to drive astrocyte-specific gene expression in transgenic mice.[51] To make this promoter hypoxia responsive, we incorporated two domains immediately upstream of the 5′ end of GFAP promoter: first, positioned most 5 prime, we inserted a silencing region (122 bp, HRSE; provided by Keith Webster, University of Miami Miller School of Medicine); and second, between the HRSE and the GFAP promoter we inserted six copies of the previously described hypoxia response element (6XHRE; total size 108 bp) of the phosphoglycerate kinase gene.[35] To make endostatin encoding vectors, the endostatin open reading frame of 612-bp size (NCBI id NM_030582.3) without the stop codon was amplified from a commercially available plasmid (Catalog no. puno1-hendo18; InvivoGen, San Diego, CA, USA) by PCR using the following primers: forward primer; 5′-GACaaccggtATGTA CAGGATGCAACTC-3′ and reverse primer; 5-GTATacgcgtC TACTTGGAGGCAGTCATG-3′, and cloned into self-complementary adeno-associated virus (scAAV) plasmid. To detect exogenous endostatin a DNA sequence encoding a FLAG epitope tag (FLAG: DYKDDDDK) and including appropriate restriction sites was synthesized (IDT DNA Technology, Coralville, IA, USA) and cloned into the 3′ end of endostatin (Fig. 1). The new constructs were named as GFAP-Endo (scAAV-GFAP-Endostatin-FLAG) and REG-Endo (scAAV-HRSE-6XHRE-GFAP-Endostatin-FLAG). We used ScAAV serotype-2 viruses produced at the Gene Therapy Vector Core, University of North Carolina (Chapel Hill, NC, USA) and titers were determined by standard dot blot analysis.

Intravitreal Injection and Oxygen-Induced Retinopathy (OIR) Model

All procedures involving mice were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic Research and approved by the Institutional Animal Care and Use Committee at Florida Atlantic University. The murine OIR model was used to compare the efficacy of the unregulated GFAP-Endo and the hypoxia-regulated REG-Endo. Briefly, 40 pups aged P7 (C57 Bl/6J mouse strain) were anesthetized with ketamine/xylazine and given intravitreal injections of 1 μL (2×10^9 viral particles) of either GFAP-Endo or REG-Endo (Fig. 1) in one eye and phosphate buffered saline or the empty AAV2 capsid vector in the contralateral eye. The pups aged P7, with their mothers, were then either maintained in normal room air, (room air controls 21% oxygen) or exposed to 75% O_2 for 5 days in a chamber (OIR animals). Pups were moved to room air conditions for 5 days at P12. Exposure of neonatal mice to high oxygen (P7–P12) causes central vaso-obliteration by P12; the reduced retinal vessels are insufficient in room air and the consequent physiologic hypoxia induces peripheral neovascularization over the period P12 through P17. All the neonatal animals used for this study developed neovascularization. Following ketamine/xylazine euthanasia at P17, eyes were rapidly collected for preparation of flatmounts or for analysis of endostatin and protein expression.

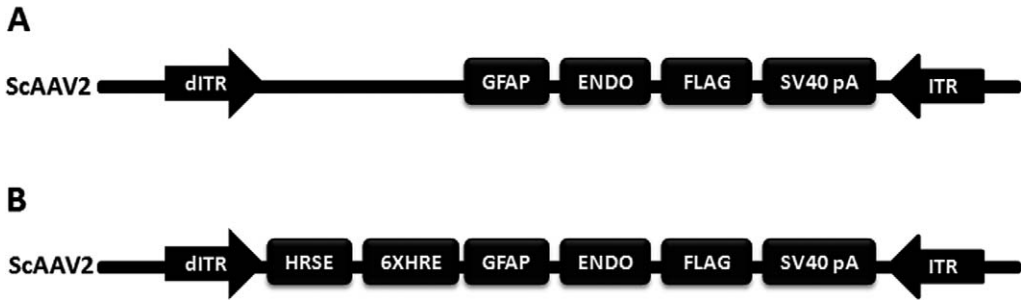

FIGURE 1. Schematic diagram of vectors used in this study. The promoter elements and endostatin cDNA are incorporated into scAAV2. (**A**) GFAP-Endo vector: this vector has human GFAP promoter (681 bp) and human endostatin (Endo) cDNA open reading frame (612 bp) fused with FLAG epitope. (**B**) REG-Endo vector: this vector includes hypoxia-regulated sequence (HRSE-6XHRE, 230 bp) along with GFAP promoter and endostatin cDNA fused with FLAG epitope; SV40 poly A (SV40pA) sequence and inverted tandem repeat sequences (ITR) are present in scAAV2 vectors (dITR denoted the mutated ITR in 5′ end). Construction of above vectors is described in methods section.

Flat Mounts

Following removal of the cornea and lens, the eye cups were fixed with 4% paraformaldehyde for 1 hour and washed in PBS. The retina was isolated from the eye cup and stained with Isolectin B4-594 (AlexaFluor 594, I21413; Life Technologies, Grand Island, NY, USA) at room temperature overnight. The retina was divided into quadrants, which were flattened on a slide with ganglion cells down, cover-slipped with slide mounting medium (Fluoromount-G; Qiagen, Valencia, CA, USA) and imaged using an inverted fluorescence microscope (Nikon Corp., Tokyo, Japan). Flat mount techniques were successfully practiced in room air control animals.

Analysis of Vaso-Obliteration and Neovascularization

The percentage of vaso-obliteration was calculated by comparing the central avascular area to total retinal area using a photo editing program (Photoshop; Adobe Corp., Mountain View, CA, USA).[52,53] To quantify peripheral neovascularization, the outer two-thirds of each retinal quadrant was selected. Using lasso tool the peripheral vascular area was selected and neovascular tufts were quantified using magic tool as described by Connor et al.[52] The ratio of peripheral neovascular area to the total vascular area was measured and expressed as percentage. For each retina, neovascular areas in each quadrant were averaged to represent that retina.

Western Blotting and ELISA

The retina was collected in radioimmunoprecipitation assay buffer with protease inhibitors, homogenized on ice, and centrifuged. For Western blots, supernatant proteins (20 μg/well) were electrophoresed on a 12% Tris glycine gel, transferred to nitrocellulose, blocked for 1 hour with 5% dried milk in Tris-buffered saline, and incubated overnight with rabbit anti-FLAG primary antibody (Sigma-Aldrich Corp., St. Louis, MO, USA). We used B-actin antibody as loading control for each sample. Endostatin bands were detected by enhanced chemiluminescence using horse-radish peroxidase conjugated secondary antibodies, and following the manufacturer's instructions (Thermo Fisher Scientific, Inc., Rockford, IL, USA).

Endostatin and VEGF concentrations in retinal lysates were measured using human endostatin and the mouse VEGF ELISA kits (Ray Biotech, Inc., Norcross, GA, USA) according to the recommended protocol. The concentration of endostatin expressed by either GFAP-Endo or REG-Endo vector was normalized to that in the PBS-treated eyes. Vascular endothelial growth factor was normalized to the amount of protein in each sample. Each experiment was performed in duplicate ($n = 2$), and each data point was performed in triplicate.

Statistical Analysis

Data are expressed as mean ± SEM. Significance was determined using analysis of variance with Newman-Keuls multiple comparison test). Differences between conditions were regarded as significant if $P < 0.05$.

RESULTS

Reduction in Peripheral Neovascular and Central Avascular Areas

Robust neovascularization was observed in both capsid and PBS-treated eyes (Fig. 2) from oxygen-exposed mice. In comparison, the angiostatic effects of both endostatin-expressing vectors were clearly visible in flatmounted retinas (compare A and B in Figs. 2 and 3, respectively). Eyes treated with either GFAP-Endo or REG-Endo had neovascular areas that were 90% lower ($P < 0.001$ for both) than those in PBS- or capsid-injected eyes (Fig. 4). Neovascular areas in PBS and capsid-injected eyes were not significantly different.

Comparison of the vector-treated eyes (Figs. 2A, 3C) with their contralateral control eyes treated with capsid or PBS (Figs. 2B, 3D) reveals the dramatic reduction in central avascular area. The mean avascular areas in the PBS- and capsid-treated eyes were almost 10-fold larger than in eyes treated with GFAP-Endo or REG-Endo ($P < 0.001$; Fig. 5).

Vector-Driven Endostatin Expression

Western blots clearly illustrate that exogenous endostatin levels achieved in the OIR retinas were greater in the GFAP-Endo treated eyes than in the REG-Endo eyes. As expected, the flagged endostatin was barely detectable in the REG-Endo–treated eyes of animals maintained in room air (Fig. 6). Enzyme-linked immunosorbent assay of the flagged human endostatin revealed 13-fold greater expression of endostatin by REG-Endo in OIR retinas than in room air retinas (2.7 ± 0.8 ng/mg versus 0.2 ± 0.3 ng/mg, respectively; $P < 0.01$; Fig. 7). In room-air mice, endostatin levels were 10-fold higher in GFAP-Endo treated retinas than in REG-Endo treated retinas (2.3 ± 0.4 ng/mg versus 0.2 ± 0.3 ng/mg, respectively; $P < 0.01$; Fig. 7). The 13-fold difference in endostatin expression by REG-Endo in room air and in OIR retinas is comparable with the 12-fold difference observed in our in vitro experiments (Fig. 4 in our previous manuscript[38]). Endostatin expression in GFAP-Endo injected eyes was not significantly different in OIR animals by comparison to room air animals.

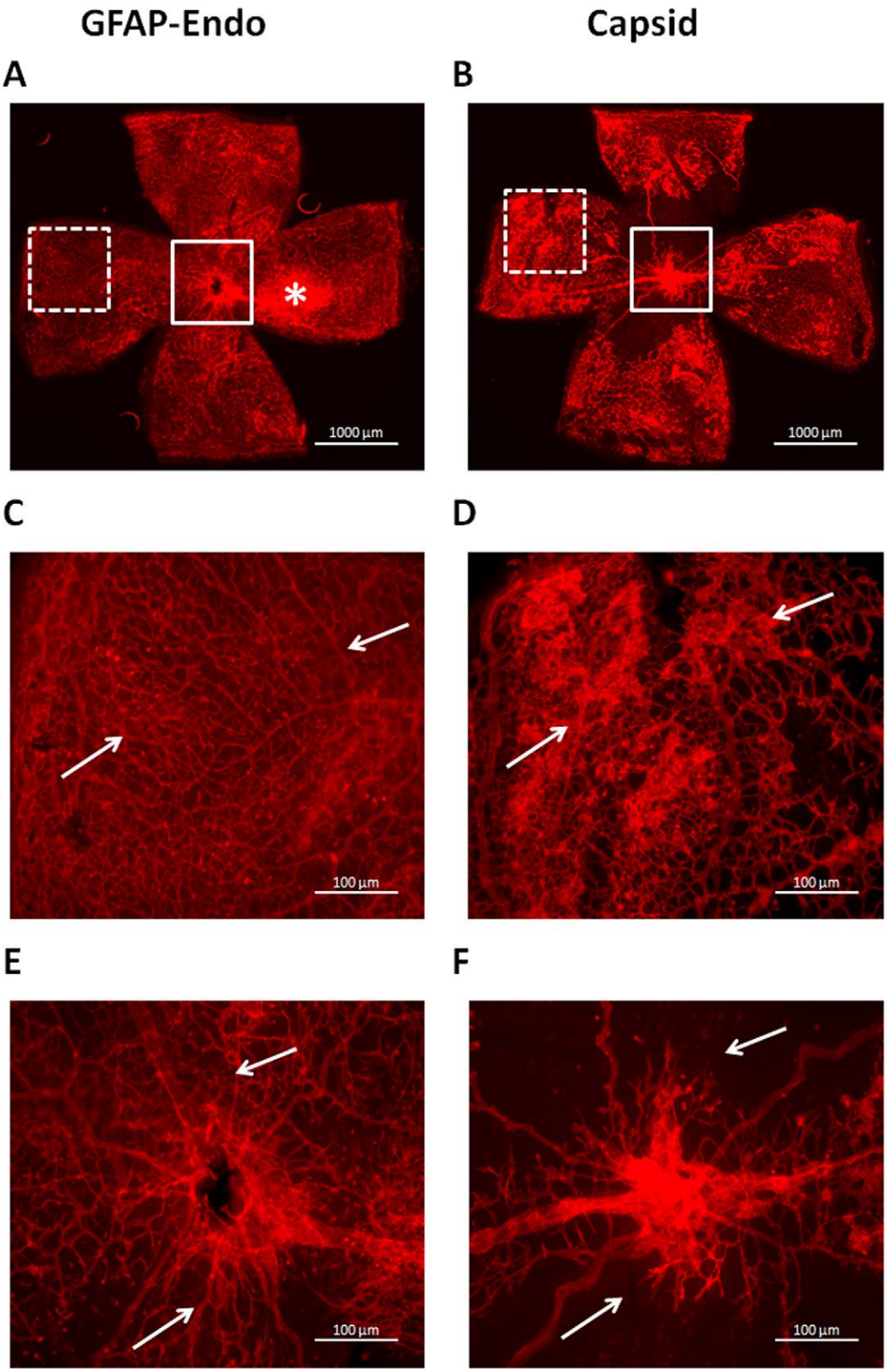

Figure 2. Representative micrographs of flatmounted P17 retinas treated with GFAP-Endo. Light micrographs of retinas from mice exposed to high oxygen were isolated, retinal wholemounts made, and blood vessels identified with lectin staining. In response to high oxygen followed by room air, the central retina loses much of the normal vasculature, whereas neovascularization occurs in the peripheral retina indicated by the formation of neovascular tufts. Retina from GFAP-Endo–treated eye (**A**) shows reduction of both the central avascular area (*solid white square*) and peripheral neovascular area (*square with dashed white lines*) compared with contralateral capsid injected eye (**B**). Magnified view of the peripheral retina shows reduced neovascular tufts in GFAP-Endo–treated eye (*arrows*, [**C**]) compared with capsid-injected eye (*arrows*, [**D**]). Magnified view of central avascular area shows reduction of vaso-obliteration and increased vascularity in GFAP-Endo–treated eye (*arrows*, [**E**]) compared with capsid-injected eyes (*arrows*, [**F**]). Note, the "hot spot" in the retina (*asterisk*, [**A**]) is an artifact due to accidental puncture during the injection. *Scale bars*: 1000 µm; (**A**, **B**); 100 µm (**C**–**F**).

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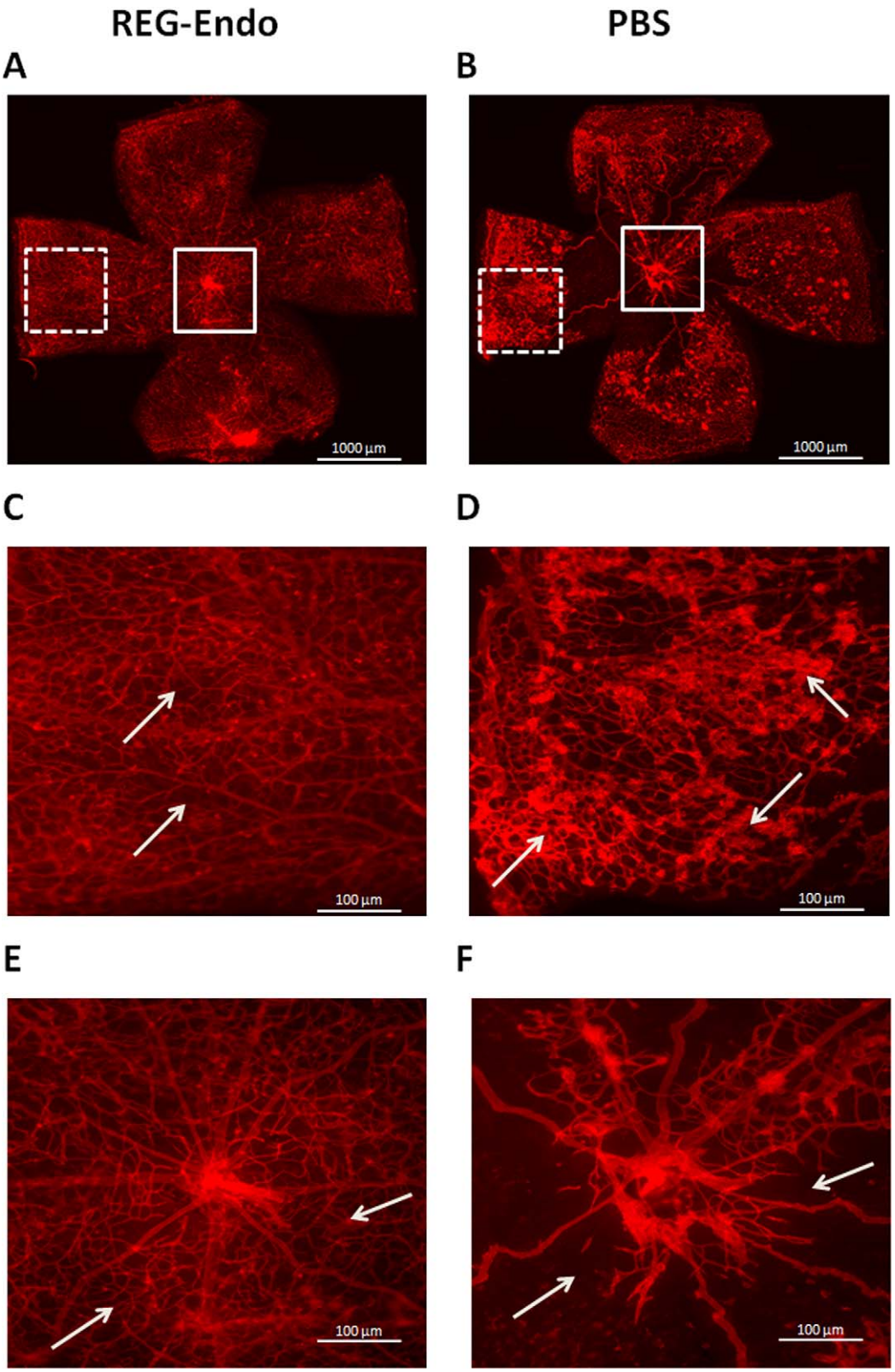

FIGURE 3. Representative micrographs of flatmounted P17 retinas from eyes treated with REG-Endo. Light micrograph of retinal wholemount from REG-Endo–treated eye (**A**) shows reduction of both central avascular area (*white square*) and peripheral neovascular area (*square with dashed white lines*) compared with contralateral PBS-injected eye (**B**). Magnified view of peripheral neovascular retina shows reduced neovascular area in REG-Endo treated eye (*arrows*, [**C**]) compared with PBS-injected eye (*arrows*, [**D**]). Enlarged view of central avascular area shows amelioration of vaso-obliteration and normal vascular patterning in REG-Endo treated eye (*arrows*, [**E**]) compared with contralateral PBS-injected eye (**F**). The bright strands in (**E**) and (**F**) are remnants of hyloid vessels. *Scale bars*: 1000 µm (**A**, **B**); 100 µm (**C**–**F**).

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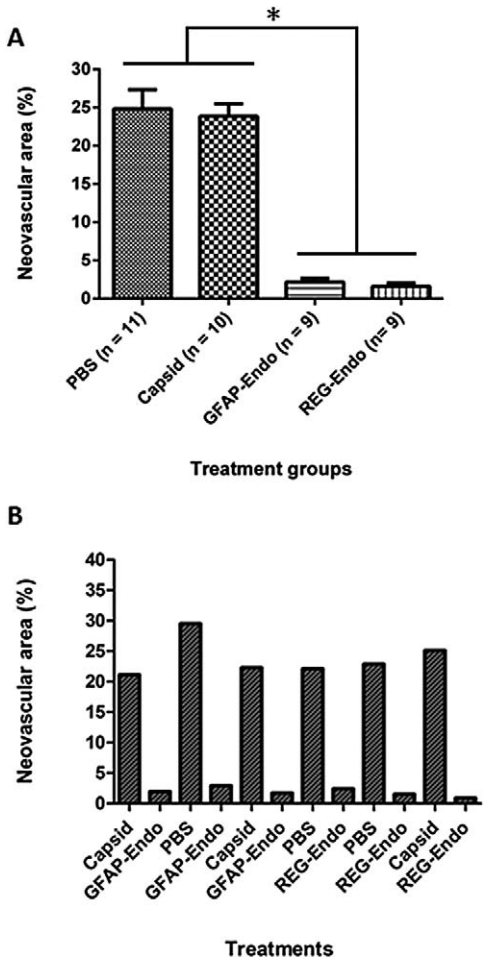

Figure 4. Reduction of peripheral neovascularization. (**A**) Mean neovascular areas (mean ± SE) in eyes treated with GFAP or REG-endostatin vector, PBS, or capsid. Eyes treated with either GFAP-Endo or REG-Endo had neovascular areas (2.2% ± 0.5% and 1.6% ± 0.6%, respectively) that were 90% lower than eyes injected with either PBS or capsid-injected eyes (*$P < 0.001$). Neovascular areas in PBS ($n = 11$) and capsid-injected eyes ($n = 10$) were not significantly different (24.8% ± 3.2%, and 22.8% ± 4.1%, respectively; $P < 0.056$). (**B**) Paired comparison of neovascular area in eyes from mice injected with endostatin vector in one eye and capsid or PBS in the contralateral eye. Treatments and mouse number are indicated under each pair of bars.

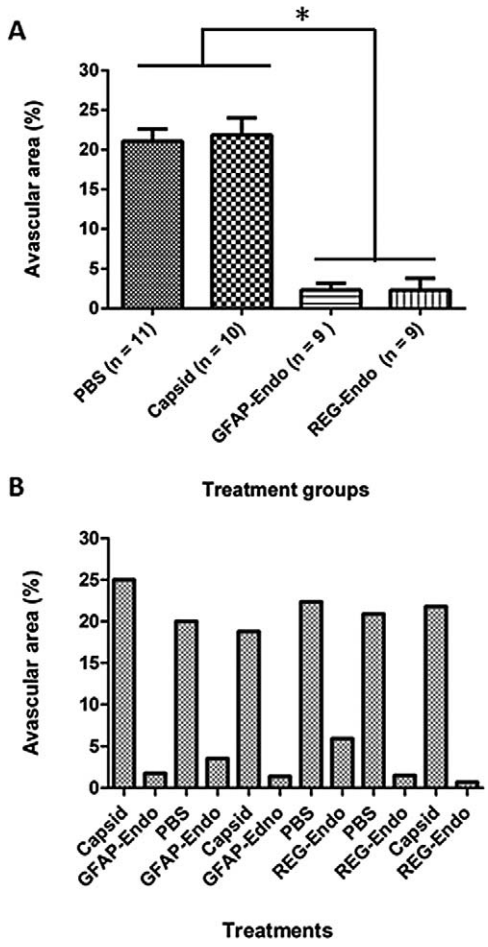

Figure 5. Reduction of central avascular area. (**A**) Central avascular area (mean ± SE) in retinas treated with GFAP or REG-endostatin vector, PBS, or capsid. Significant reduction of the central avascular area was observed in GFAP-Endo (2.34% ± 1.4%, $n = 9$) and REG-Endo (2.31% ± 1.6%, $n = 9$) injected eyes compared with either PBS (21.1% ± 1.9%, $n = 11$) or capsid (21.87% ± 2.5%, $n = 10$) injected eyes. *$P < 0.001$. There was no significant difference in the central avascular area in eyes injected with either PBS or capsid. *$P < 0.02$. (**B**) Paired comparison of avascular areas in eyes injected with endostatin vector in one eye and either capsid or PBS in the contralateral eye (n = number of individual mice).

Reduction of VEGF Levels in OIR Retina

Protein levels of VEGF in P17 control retinas (PBS-injected eyes) exposed to high oxygen were significantly higher than those in room-air animals (365 ± 20 pg/mg vs. 85 ± 23 pg/mg; $P < 0.001$; Fig. 8). Intravitreal injection of either GFAP-Endo (144 ± 28 pg/mg) or REG-Endo (153 ± 38 pg/mg) significantly reduced VEGF levels ($P < 0.001$; Fig. 8) compared with PBS-injected eyes. No significant difference was observed between VEGF levels in eyes treated with GFAP-Endo or REG-Endo vectors.

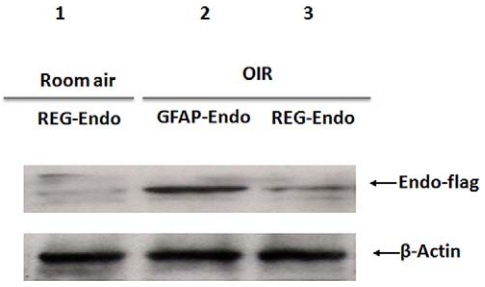

Figure 6. Endostatin FLAG-tagged in mouse retina at P17. Lane 1 shows that eyes treated with REG-Endo vector resulted in barely detectable expression of endostatin in room air. Lane 2 and Lane 3 demonstrate expression of endostatin (22 kDa) by REG-Endo vectors was greater in OIR retinas but still less than the expression from the GFAP-Endo vector.

Discussion

Therapeutic Efficacy

These data represent the first demonstration of the efficacy of hypoxia-regulated, cell-specific, antiangiogenic gene therapy for the retina. In the OIR model, our new REG-Endo vector

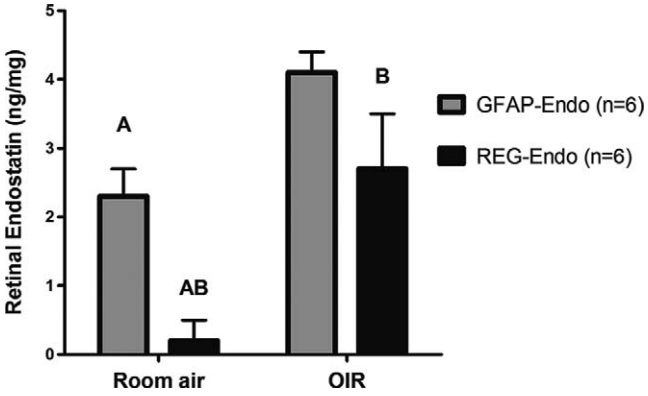

FIGURE 7. Expression of endostatin by regulated and unregulated vectors in room air or OIR retinas. Analysis with ELISA of retinal endostatin expression in mice that were injected with GFAP-Endo or REG-Endo, then placed in oxygen for the OIR model or raised in room air. In the room air groups, retinal endostatin concentration in the REG-Endo mice was only 10% of that in the GFAP-Endo mice. Endostatin concentration in the retinas of the REG-Endo mice in the OIR model was 12-fold greater than in REG-Endo mice in room air. Retinal endostatin expression in the OIR model was statistically equivalent in the REG-Endo and GFAP-Endo mice. Groups with different letters differ significantly ($P < 0.01$ for all comparisons; $n = 6$ in all groups).

reduced neovascular area by 93% and the central avascular area by 90%. These results are comparable with those achieved with the constitutively expressed GFAP-Endo, and with reports that the number of preretinal endothelial cell nuclei was reduced by 86% by AAV-mediated endostatin[50] and by 75% by an adenoviral endostatin vector.[54]

The Müller cell specificity of our promoter has important implications because this major glial cell traverses the width of the retina and may experience a distinct pattern of hypoxia. While our glial cell–specific and hypoxia-regulated vectors will express endostatin selectively in Müller cells, questions remain regarding the effects of the vectors with respect to the location of hypoxia within the retina that may trigger gene expression. For example, a specific defined band of hypoxia in a region of the retina may result in transgene expression from Müller cells that traverse this region; but one constraint may be the magnitude of cell surface for individual Müller cells that experience oxygen deprivation. For a particular Müller cell, once the transgene is induced, it is likely that the endostatin protein product would be secreted from all locations on the Müller cell membrane and therefore at a wide range of depths within the retina.

We have demonstrated regulated expression from our REG-Endo vector both in terms of increased protein product (as shown in ELISA at P17 by comparison to expression in room air) and in terms of functional outcome (inhibition of neovascularization). From the literature and our own data, it is clear that extensive inhibition of neovascularization is obtained with AAV-CMV-endostatin[50] as well as from our own GFAP-Endo and REG-Endo vectors. Importantly, the mechanisms of endostatin action are poorly understood and include inhibition of VEGF-induced permeability, actions in the nucleus, decreased VEGF signaling, and/or production and increased PEDF expression. Hence the production of endostatin ubiquitously under the CMV promoter may result in differences in endostatin action that occur in a very different and progressively changing multicellular microenvironment from that obtained with a Müller-restricted vector or from REG-Endo. A novel feature of our vectors is secretion of endostatin specifically from Müller cells or from hypoxic Müller cells eliciting successful inhibition of neovascularization. Such inhibition of neovascularization by endostatin has not been elicited previously from either Müller cell-specific or combined Müller cell–specific/hypoxia-regulated vectors. While the exact details of endostatin action are only partially understood, it is likely that eliciting the more selective patterns of endostatin distribution and release will result in distinct patterns of vascular inhibition compared with the more diffuse ubiquitous patterns of multicellular secretion obtained with AAV-CMV-endostatin. Furthermore, the REG-Endo vector will result in activated therapy that is disease specific and corresponds to emergence of a hypoxic microenvironment instead of continuous and unvarying rates of production initiating soon after vector delivery. (Note: The activation of our promoter is likely to differ in astrocytes that reside in the inner layers of the retina and therefore may experience different levels of hypoxia compared with cells. As a result, the responses in these two cell types could be different both in timing and in levels of activation.)

It has been suggested that sustained delivery of a transgene may lead to tissue toxicity over time[23]; as discussed earlier, reduced VEGF has been linked to retinal damage in animals. If the transgene is regulated in response to the pathologic state of surrounding tissue, it can be effective during the period of the disease and turned off once the disease progression is stabilized. If the patient has a recurrence of the pathology, the expression will turn on again. Although our vector

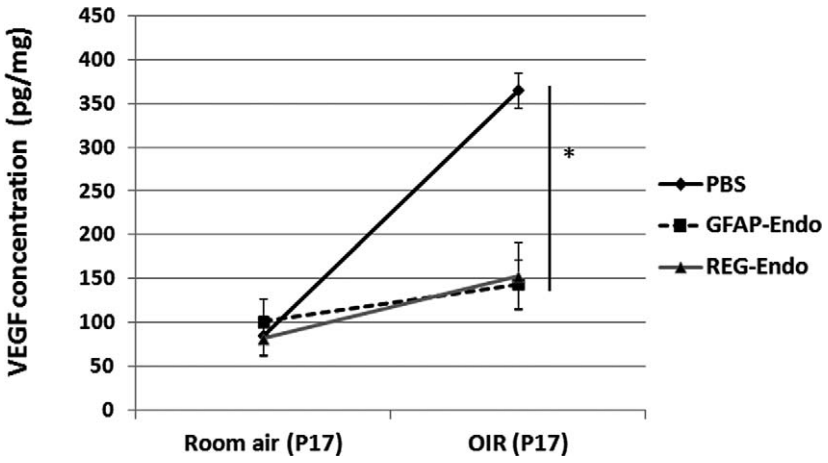

FIGURE 8. Levels of VEGF levels measured by ELISA at P17. Levels of VEGF were higher in retinas from PBS-treated OIR mice than in room air controls. Treatment with either GFAP-Endo or REG-Endo reduced VEGF levels in the OIR retinas. *$P < 0.001$.

successfully inhibited neovascularization within a short period of time, the effectiveness in ensuring more long-term transgene expression when necessary requires further investigation.

Two major advantages of this strategy are that the antiangiogenic therapy is delivered as vessels start to form, when they are most amenable to treatment, and that there is a high therapeutic index because therapy is delivered only when and where needed; the remaining healthy retina would be exposed to a much lower dose and only for the duration needed, as expression is suppressed in normoxic tissue.

Retinal Endostatin Levels

Our regulated vector produced approximately 2 to 3 ng of endostatin/mg of retinal homogenate (Fig. 7), which corresponds closely to the 1 to 2 ng of endostatin reported previously with the constitutive VMD2-RPE specific promoter.[21] While these levels are less than 10 to 14 ng of endostatin delivered by a CMV promoter, it is likely that high absolute levels of antiangiogenic protein are not necessary to achieve a substantial therapeutic effect. The key difference between REG-Endo and CMV-endostatin is that for REG-Endo, levels of endostatin produced will be more than 10-fold lower in normoxic retina than in OIR animals.

GFAP Activity and Endostatin Delivery

Endostatin levels were higher in GFAP-Endo treated eyes from OIR mice than in those from similarly treated room air mice (Fig. 7). Müller cells become reactive and induce GFAP in response to most types of retinal injury. Reactive gliosis is a hallmark of the retinal response in the OIR model.[55] As a result, the basal level of the unregulated GFAP-Endo will increase as GFAP expression increases, and will remain active during retinal inflammation and damage. In contrast, REG-Endo will be constitutively silent, activated in hypoxia and shutoff by declining HIF-1 levels, at least by P26, when the OIR retina has "resorbed" the newly formed vessels.[2] Once activated by hypoxia, the increase in GFAP activity that is associated with the reactive phenotype in Müller cells will further boost the production of endostatin.

Change in VEGF Levels

Like others, we found that OIR retinas had significantly higher VEGF than room air retinas. The amount of VEGF produced in the OIR retinas was comparable with that in other studies.[56] Levels of VEGF at P17 were approximately 60% lower in the endostatin eyes. The significant reduction in central avascular area would certainly contribute to a decline in VEGF. It is interesting that the reduction in VEGF in these experiments is comparable with that observed in eyes injected with 500 times higher (1 μg) dose of endostatin on P12 in the OIR model.[57] This might indicate that the REG-Endo produces endostatin at close to saturating levels.

Hypoxia-Regulated Gene Therapy

Hypoxia-regulated gene therapy was introduced by Prentice et al.[36] as a means to deliver cardioprotective genes to myocardium experiencing transient or intermittent ischemia. The field has grown slowly since that time with reports on hypoxia-regulated gene therapy targeting hypoxic regions of tumors, followed by reports on cardiac ischemia and/or ischemia reperfusion injury to heart or neural tissue. Bainbridge et al.[32] first reported hypoxia-regulated expression of a reporter gene in the retina; it was neither cell-specific nor suppressed in normoxic retina. Our group previously reported an RPE-specific AAV vector that was robustly responsive to hypoxia and silenced in normoxia[35] as a potential platform for treatment of choroidal neovascularization. To develop a platform targeting intraretinal neovascularization, a new hypoxia-responsive, glial-specific AAV vector was constructed to target Müller cells, and its specificity and responsiveness were verified in vitro, and in the OIR model of retinal neovascularization.[38]

The highly sensitive response of the REG-Endo vector to hypoxia results from its design. We utilized hypoxia response elements which are consensus DNA-binding sites for the HIF-1 transcription factor, which is kept at low levels in normoxia by the oxygen-dependent degradation of the HIF-1 alpha subunit. Hypoxia-regulated vectors have been of value in different model systems for expressing a range of factors including pro-survival components, angiogenic factors and growth factors.[58–61] By incorporating multimers of the HRE, we increased the response of the promoter to hypoxic conditions; the neuronal silencing element further ensures very low basal expression in normoxia prior to hypoxic induction; REG-Endo was made cell specific by virtue of the 681 bp hybrid regulatory domain from the GFAP gene (see "Methods" section).

HRE-Regulated Switch in Normoxia

A key question regarding timing of transgene expression will be whether endostatin expression ceases once the pathology has subsided. Cell specific and hypoxia-regulated vectors have been shown to switch off in other systems including heart[36,62] and cancer.[63,64] Given the tight correspondence between tissue hypoxia and the induction, maintenance, and termination of HIF signaling, it is anticipated that the activity of REG-Endo will closely correlate with retinal hypoxia.

Implications for the Future

This new strategy for antiangiogenic gene therapy opens an avenue of prophylactic gene therapy for patients at high risk for neovascularization. Theoretically, high-risk patients could be treated with a gene therapy that would only be activated when pathological conditions begin to stimulate neovascularization. Therapy would initiate before the patient experienced any symptoms or loss of vision.

Summary

The major findings of the present study were the use of a hypoxia-regulated, retinal glial cell–specific promoter driving the expression of the human endostatin gene to dramatically reduce peripheral neovascularization and central vaso-obliteration in the OIR mouse model of neovascularization. Our results demonstrated the effect of exogenous endostatin on VEGF expression levels in retina, and confirmed that the regulated vector produced sufficient endostatin to inhibit hypoxia-induced neovascularization in the murine OIR model. These results suggest that the localized production of endostatin in ischemic retina reduces pathological angiogenesis and promotes physiological angiogenesis.

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