Antiangiogenic Characteristics of Astrocytes From Optic Nerve Heads With Primary Open-angle Glaucoma

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Objective: To evaluate the angiogenic status of the human optic nerve head (ONH) with primary open-angle glaucoma (POAG).

Methods: Using real-time reverse transcription–polymerase chain reaction and Western blot, we analyzed the expression of proangiogenic and antiangiogenic factors in cultured ONH astrocytes from healthy donors and donors with POAG. Immunohistochemical analysis was used to localize those factors in human ONHs from healthy donors and donors with POAG. Cocultures of normal and POAG astrocytes with human umbilical vein endothelial cells were performed to obtain functional data on angiogenesis.

Results: The ONH astrocytes from donors with POAG decreased expression of proangiogenic factors (vascular endothelial growth factor C and platelet-derived growth factor A) and increased expression of antiangiogenic factors (collagen XVIII and ADAMTSL-3) when compared with normal ONH astrocytes. Vascular endothelial growth factor C and platelet-derived growth factor A were markedly reduced in the lamina cribrosa of the ONHs of donors with POAG. Endostatin immunolabeling increased in the lamina cribrosa of the ONHs of donors with POAG. When cocultured with human umbilical vein endothelial cells, POAG astrocytes induced less tube formation than normal ONH astrocytes.

Conclusion: The ONH astrocytes from donors with POAG display antiangiogenic characteristics when compared with normal ONH astrocytes.

Clinical Relevance: The study supports the clinical observation of decreased angiogenesis in patients with POAG.

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Primary open-angle glaucoma (POAG), the most frequent form of glaucoma, is characterized by progressive optic nerve fiber loss and retinal ganglion cell death. Increased intraocular pressure is considered a main risk factor. In glaucoma, optic nerve fiber degeneration initially occurs at the lamina cribrosa, a fibroelastic structure formed by extracellular matrix (ECM) and quiescent astrocytes that provides mechanical and biological support for optic nerve axons. Chronic elevated intraocular pressure causes ECM remodeling and quiescent astrocytes to become reactive. Reactive astrocytes express new ECM proteins, such as elastin, tenascin C, and proteoglycans; vary their expression of cell adhesion molecules, growth factors, and cytokines; and synthesize excessive nitric oxide. Some of these products are believed to be neurotoxic for the retinal ganglion cells.

Angiogenesis, a process by which proliferating endothelial cells from preexisting vessels form new vessel sprouts and expand the vascular network, occurs if the balance between proangiogenic and antiangiogenic factors favors the former. The vascular endothelial growth factor (VEGF) family consists of positive regulators of angiogenesis in the retina. Different molecules with known antiangiogenic activity include small fragments from cleaved ECM proteins such as endostatin or tumstatin. Endostatin is the product of the cleavage of its full-length precursor collagen XVIII, a process performed by elastase and some of the matrix metalloproteinases. Collagen XVIII is a constituent of the inner limiting membrane of the retina and is also expressed by optic nerve head (ONH) astrocytes and endothelial cells. The absence of collagen XVIII leads to abnormal retinal vascularization and deposits under the retinal pigment epithelium. Other proteins with antiangiogenic activity, such as the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family of related proteins, have thrombospondin motifs that are antiangiogenic.

Under retinal ischemic conditions, such as diabetic retinopathy and retinal branch...
Because astrocytes are the main source of proangiogenic factors during development, we studied the angiogenic status of human ONH astrocytes from patients with glaucoma. Our results indicate a decreased proangiogenic activity of POAG astrocytes when compared with normal astrocytes. We also show that the ECM of the lamina cribrosa acts as a reservoir for endostatin and VEGF-C in human ONHs.

**REAL-TIME QUANTITATIVE REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION**

Cytoplasmic RNA was isolated from cultured ONH astrocytes (passage 3) from 6 different glaucoma donors and 6 different healthy donors as previously described. RNA from cultured ONH astrocytes belonging to 1 eye of each of the donors was used. Real-time quantitative reverse transcription–polymerase chain reaction conditions and primer sequences are given in eTable 2 (available at: http://www.archophthalmol.com). For each cell culture, polymerase chain reactions were performed in triplicate. After 22 hours, 3 fields were randomly selected from each culture and photographs were acquired (Axiovision 4.6; Zeiss, Go¨ ttingen, Germany), and each value was normalized in pixels, using image analysis software (Axiovision 4.6; Zeiss, Go¨ ttingen, Germany). For negative controls, the primary antibody was replaced with non-immune serum. Serial sections of healthy eyes and eyes with glaucoma were stained simultaneously to control for variations in immunostaining.

**TUBE CORD FORMATION ASSAY**

Tube formation assay was performed on a 24-well plate coated with 230 µL of extracellular matrix (Matrigel; Sigma) per well as previously described. Human umbilical vein endothelial cells (HUVECs; Clonetics Cambrex Bio Science, Walkerville, Maryland) were plated alone in 2% serum and used as a basal control for tube length formation. The HUVECs were also incubated with VEGF (7 ng/mL; Sigma) to show that the batch of cells used were responsive to VEGF. Cocultured HUVECs and astrocytes were plated at the same time on extracellular matrix at a density of 60,000 cells and 40,000 cells, respectively. Astrocytes from 4 different donors with glaucoma and 4 healthy donors were tested, and all cocultures of HUVECs and astrocytes were performed in triplicate. After 22 hours, 3 fields were randomly selected from each culture and photographed were acquired with a CCD (charge-coupled device) camera. Total tube length was measured on the digitalized photograph as length in pixels, using image analysis software (Axiovision 4.6; Zeiss, Go¨ ttingen, Germany), and each value was normalized to the mean tube length in the basal control. Quantitative measurements of tube length were performed by 2 independent observers (M.N.R. and L.C.) in a masked fashion. Significant differences between the means were set at P<.05 (analysis of variance followed by Bonferroni test; Prism 3.0; GraphPad).

**WESTERN BLOTS**

Normal (n=6) and POAG (n=6) ONH astrocytes were grown to 95% confluence, and Western blot analysis was performed as previously described. We used mouse monoclonal antibody against endostatin (1:500; Upstate Biotechnology, Lake Placid, New York) and rabbit polyclonal against platelet-derived growth factor A (PDGF-A; 1:1000; Santa Cruz Biotechnology, Santa Cruz, California). Membranes were washed in Tris buffer saline triton and then incubated with the appropriate secondary antibody conjugated to horseradish peroxidase for 1 hour. For detection of secondary antibodies, we used a chemiluminescence plus detection system (Roche Diagnostics GmbH, Boehringer Mannheim, Mannheim, Germany). Both PDGF-A and endostatin protein were normalized to actin and measured by densitometry for significance. Samples were run and analyzed 3 independent times in groups of 3 normal and 3 POAG astrocytes.

**IMMUNOHISTOCHEMICAL ANALYSIS**

Eight eyes from donors with glaucoma (eTable 3; available at: http://www.archophthalmol.com) and 8 eyes from healthy donors were used. Tissues were fixed with 4% paraformaldehyde in 0.1M phosphate-buffered saline (pH 7.4). Two slides that contained at least two 6-µm optic nerve sections each were stained per donor. Rabbit polyclonal antibodies against VEGF-C and PDGF-A (1:100; Santa Cruz Biotechnology) and a monoclonal antibody against endostatin (1:100; Upstate Biotechnology) were used as primary antibodies. Double immunohistochemical analysis used glial acidic fibrillary protein (GFAP; 1:200; Sigma, St Louis, Missouri) as an astrocyte marker. We used secondary antibodies labeled with Alexa 488 and Alexa 568 (1:800; Molecular Probes, Eugene, Oregon). For negative controls, the primary antibody was replaced with nonimmune serum. Serial sections of healthy eyes and eyes with glaucoma were stained simultaneously to control for variations in immunostaining.

**RESULTS**

**DECREASE IN PROANGIOGENIC FACTORS IN ONH ASTROCYTES FROM DONORS WITH POAG**

Messenger RNA (mRNA) expression levels for proangiogenic factors VEGF-C and PDGF-A were significantly downregulated in POAG astrocytes compared with normal ONH astrocytes. All data are given as mean (SEM) unless otherwise indicated. Relative mRNA expression for VEGF-C in normal ONH astrocytes was 1.1 (0.07), whereas POAG ONH astrocyte expression was 0.7 (0.04).
Relative mRNA expression for PDGF-A was 4.2 (0.8) in normal astrocytes and 0.65 (0.1) in POAG astrocytes (P = .04) (Figure 1B). Relative expression for VEGF-A was not statistically significant (P = .25): 14.4 (3.1) in normal astrocytes and 23.3 (6.6) in POAG astrocytes.

The α6 integrin, a positive regulator of PDGF-A signaling in glial cells, was significantly downregulated by POAG astrocytes (P = .04); the relative mRNA expression values were 4.6 (0.6) in normal astrocytes and 1.5 (0.3) in POAG astrocytes (Figure 1C). No statistically significant differences were found for the mRNA expression of the proangiogenic factors insulinlike growth factor–binding protein 5 or parathyroid hormone–like hormone when normal ONH astrocytes were compared with POAG ONH astrocytes (data not shown). Relative mRNA expression values for angiopoietin-1 were significantly different (P = .004): 0.9 (0.1) in normal ONH astrocytes and 1.8 (0.2) in POAG ONH astrocytes (Figure 1D). Significantly decreased mRNA expression was found for endothelial tyrosine kinase receptor, the receptor for angiopoietin-1 in POAG ONH astrocytes, when compared with normal ONH astrocytes (P = .049); expression values were 3.8 (0.7) in normal astrocytes, and 1.4 (0.2) in POAG astrocytes. Western blot analysis revealed a decrease in PDGF-A protein levels by POAG ONH astrocytes (Figure 2A) when compared with normal astrocytes (P = .04) (Figure 2B). Thus, the decrease
in PDGF-A mRNA expression is translated into a decrease in PDGF-A protein in POAG astrocytes.

INCREASE IN ANTIANGIOGENIC FACTORS IN ONH ASTROCYTES FROM DONORS WITH POAG

Among antiangiogenic genes, a significant up-regulation for collagen XVIII and ADAMTSL-3 was detected in POAG astrocytes. Collagen XVIII relative mRNA expression values were 0.8 (0.2) in normal ONH astrocytes and 7.0 (1.7) in POAG astrocytes (P = .02) (Figure 1E). ADAMTSL-3 relative mRNA expression values were 0.4 (0.1) in normal ONH astrocytes and 3.4 (0.9) in POAG astrocytes (P = .03) (Figure 1F). Thrombospondin 2, a molecule with important antiangiogenic activity in the eye, did not significantly change its expression in POAG ONH astrocytes (data not shown). As a marker of collagen XVIII presence, POAG astrocytes increased endostatin protein expression 1.8-fold when compared with normal astrocytes (P = .04) (Figure 2B).

TUBE CORD FORMATION ASSAY

The HUVECs plated on extracellular matrix form a tubular network within 22 hours when grown in high serum concentration (10%) 34-35. When grown in 2% serum, HUVEC tube formation is low (Figure 3A). Tube length formation by HUVEC in 2% serum (HUVEC basal control) was considered baseline and used as control for normalization (Figure 3E). When VEGF (7 ng/mL) was added to the culture, HUVECs significantly increased the tube length by 1.5-fold (P < .001) (Figure 3B and E). A more robust increase in tube length was observed when normal astrocytes were added to the culture (Figure 3C). Addition of normal astrocytes increased tube length 2.2-fold when compared with baseline (no astrocytes) (P < .001) (Figure 3C and E). When HUVECs were co-cultured with ONH astrocytes from donors with POAG,
tube length formation was increased only 1.5-fold when compared with control (HUVEC basal control) (Figure 3D and E). Comparing tube length formation by POAG astrocytes to normal ONH astrocytes, a significant 35% reduction was found ($P = .001$) (Figure 3E). These results indicate decreased angiogenic stimuli to the tube, forming endothelial cells by POAG ONH astrocytes.

**IMMUNOHISTOCHEMICAL ANALYSIS**

Double immunolabeling for VEGF-C and GFAP showed that expression of VEGF-C was readily observable in the cribriform plates and in the vessels of the ONH (Figure 4A and B). Immunolabeling of VEGF-C localizes between the astrocytic processes and follows a linear pattern (arrowheads in Figure 4B). In the ONH from donors with POAG, markedly less VEGF-C immunolabeling at the cribriform plates was observed (Figure 4C). The reduction in VEGF-C immunolabeling occurs specifically in the spaces between the astrocytic processes because VEGF-C immunolabeling is low in the lamina cribrosa but high in the vessels (V) (arrow in the lamina cribrosa in optic nerves with POAG). The arrowhead indicates VEGF-C immunolabeling in the cribriform plate. E, The PDGF-A (red) immunolabeling in the lamina cribrosa of normal optic nerves (arrowheads). F, Double immunolabeling for PDGF-A (red) and GFAP (green) shows that PDGF-A colocalizes with the astrocytic processes (arrowheads) and is abundant between the astrocytic processes of the CP and around V, G and H. Marked decrease of PDGF-A immunolabeling in the cribriform plates of optic nerves with POAG. I, Representative micrographs of collagen XVIII (red) immunolabeling on the lamina cribrosa of normal optic nerves. J, Double immunolabeling for collagen XVIII (red) and GFAP (green) shows that collagen XVIII immunolabeling is low in the lamina cribrosa but high in the vessels (V, K and L). Increased collagen XVIII immunolabeling at the cribriform plates (CP) of optic nerves with POAG. Collagen XVIII localizes to the space of astrocytic processes (arrowheads) and is abundant at the core of the CP. NB indicates nerve bundles. Magnification bar, 35 µm.

Clinical evidence of decreased angiogenesis during glaucoma was first reported in 1966, when a differential evo...
lution of diabetic retinopathy was first described for patients with POAG. \textsuperscript{36,37} More recently, histologic analysis of the post–laminar bulbar region of the optic nerve revealed a reduction in the number of capillaries in eyes with POAG.\textsuperscript{38} This result indicates not only that capillaries are lost but also that newer capillaries are not being produced.

In the present study, we demonstrate a decrease in the immunolabeling of proangiogenic factors and an increase in the immunolabeling of antiangiogenic factors in ONHs of nondiabetic patients with POAG. We also show a differential profile in the expression of genes by ONH astrocytes and functional assay, suggesting that in glaucoma ONH astrocytes are antiangiogenic. Based on our results, we hypothesize that in the ONH of patients with POAG astrocytes prevent angiogenesis by decreasing the expression of proangiogenic factors and by increasing the expression of antiangiogenic factors.

**PROANGIOGENIC AND ANTIANGIOGENIC FACTORS IN THE HUMAN ONH**

The proangiogenic factors VEGF-C and PDGF-A are normally expressed in the core of the cribriform plates of the lamina cribrosa and in the walls of the vessels of human optic nerves. In glaucoma, the marked decrease in VEGF-C and PDGF-A immunolabeling in the lamina cribrosa occurs in the space between the astrocytic processes, whereas immunolabeling for both factors persists in the remnant vessels. Immunolabeling for the antiangiogenic fragment endostatin is weak in the healthy human lamina cribrosa but is markedly increased in the ONH of patients with POAG, where endostatin localizes between the astrocytic processes and the core of the cribriform plates. Our results suggest that the cribriform plates are the reservoir for proangiogenic and antiangiogenic factors.

Active angiogenesis in the eye occurs during retinal development and retinal disease, such as proliferative diabetic retinopathy.\textsuperscript{15,39,60} Astrocytes promote and stimulate this process by providing the support network on which retinal vessels will form and by secreting VEGF, the primary angiogenic factor.\textsuperscript{41,42} Our results from the tube cord formation assay indicate that although ONH astrocytes from healthy adults stimulate angiogenesis in vitro, ONH astrocytes from donors with POAG are less angiogenic than normal astrocytes. We have shown that ONH astrocytes from donors with POAG have a different expression profile of proangiogenic and antiangiogenic factors than astrocytes from healthy donors. Expression of proangiogenic factors by cultured astrocytes from donors with POAG was markedly downregulated. Our data indicate a significant decrease in VEGF-C mRNA production by astrocytes from donors with POAG. This result is consistent with a previous report\textsuperscript{13} of VEGF-C expression downregulation by cultured ONH astrocytes after exposure to increased hydrostatic pressure. Our data indicate that VEGF-A expression levels do not change significantly in ONH astrocytes from nondiabetic donors with POAG when compared with normal astrocytes. In addition, VEGF-C is a regulator of angiogenesis with lymphangiogenic and angiogenic properties. Its angiogenic activities were shown to be mediated through VEGF receptors 2 and 3.\textsuperscript{44}

Expression levels of PDGF-A are significantly downregulated in POAG ONH astrocytes when compared with normal ONH astrocytes. The proangiogenic activity of PDGF-A is mediated by its positive regulation on VEGF-A expression.\textsuperscript{44,45}

Analysis of the expression of antiangiogenic factors revealed a marked upregulation of collagen XVIII and ADAMTSL-3 mRNA by ONH astrocytes from donors with POAG. The antiangiogenic activities of collagen XVIII are based on its 20-kDa fragment endostatin, which inhibits endothelial cell migration.\textsuperscript{40} Recently, collagen XVIII was reported as a negative regulator of astrocyte proliferation around retinal vessels.\textsuperscript{21} Our data show that ONH astrocytes from donors with POAG increase expression of the full-length precursor collagen XVIII mRNA and endostatin protein levels, suggesting that increased collagen XVIII mRNA expression is translated into increased endostatin production.

**DECREASED ANGIOGENESIS AND GLAUCOMA**

We believe that our results explain, in part, the clinically described antiangiogenic status existent during POAG.\textsuperscript{36,37} For active angiogenesis to occur, vascular endothelial tight junctions are lost, allowing endothelial cell migration and proliferation. Our results indicate an increase in angiopoietin-1 and endostatin expression by ONH astrocytes from donors with POAG. Both factors are known to stabilize the blood-retinal barrier.\textsuperscript{47-49} Thus, stabilization of the endothelial tight junction will inhibit the early steps of angiogenesis. If ischemia and hypoxia occur in the ONH during POAG,\textsuperscript{26,27} a decrease in angiogenic stimuli by astrocytes will possibly damage neighboring axons.

During retinal disease and development, astrocytes proliferate and increase the expression of angiogenic factors that precede active angiogenesis. Our results indicate that ONH astrocytes from donors with POAG downregulate important mitogenic and proangiogenic factors and upregulate antiangiogenic factors. Thus, the ONH astrocyte has an active role in decreasing angiogenesis during POAG.

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