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A COMPARISON OF HYPOXIA-INDUCIBLE FACTOR- α IN SURGICALLY EXCISED NEOVASCULAR MEMBRANES OF PATIENTS WITH DIABETES COMPARED WITH IDIOPATHIC EPIRETINAL MEMBRANES IN NONDIABETIC PATIENTS

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Abstract

Purpose—The purpose of this study was to first determine whether hypoxia-inducible factor-1 α (HIF-1 α) was detectable in diabetic preretinal membranes and to compare the presence of HIF-1 α in fibrovascular proliferative diabetic retinopathy membranes with nondiabetic, idiopathic, epiretinal membranes.

Methods—Twelve patients with proliferative diabetic retinopathy membranes requiring pars plana vitrectomy and nine nondiabetic patients with idiopathic epiretinal membranes requiring pars plana vitrectomy underwent excision of these membranes. Immunohistochemical staining for the presence of HIF-1 α was performed on the excised membranes. The degree of staining for HIF-1 α (1+, 2+, and 3+ scale) and the cellular location of staining were determined for each specimen. Institutional Review Board approval and informed consent were obtained for all patients.

Results—Eleven of 12 (92%) diabetic preretinal membranes were positive for HIF-1 α , and most had intense (2+ to 3+) cytoplasmic staining with occasional focal nuclear positivity. Five of 9 (55%) nondiabetic epiretinal membranes were positive for HIF-1 α with significantly weaker cytoplasmic staining (1+ to 2+) with occasional focal punctuate nuclear staining.

Conclusion—Hypoxia-inducible factor-1 α is found more often and more intensely in diabetic preretinal membranes compared with nondiabetic idiopathic epiretinal membranes.

Keywords

epiretinal membranes; hypoxic-inducible factor; ischemia; proliferative diabetic retinopathy

Vascular endothelial growth factor (VEGF) has been implicated in the cause of proliferative diabetic retinopathy (PDR).¹ Indeed, regression of PDR has been shown in response to anti-VEGF treatment of macular edema.² It is known that hypoxia results in the release of angiogenic factors such as VEGF,³ connective tissue growth factor,⁴ and hypoxia-inducible factor-1 (HIF-1).^{5–8} VEGF production is known to be highly upregulated in eyes with ischemic retinal disorders.¹ Hypoxia-inducible factor-1 is a recently identified stimulator of angiogenesis that is upregulated in the presence of tissue hypoxia.^{5–8} Not much information exists regarding HIF-1 and diabetic retinopathy.⁹

Determination of the presence and degree of HIF-1 α expression in patients with retinal vascular disease will help determine the degree of HIF-1 overexpression in ischemic eyes. If HIF-1 is indeed overexpressed in diabetic eyes, future attempts to therapeutically target HIF-1 activity may help to prevent the pathologic fibrovascular retinal response to hypoxia and the subsequent anatomical complications. Because vitrectomy is often used to treat these complications, we planned to analyze the excised fibrovascular tissue specimens for the presence of HIF-1 α .

Such excised membranes from patients with diabetes have been used to determine the presence of growth factors and receptors.^{4,10}

The purpose of this study was to determine whether HIF-1 α was detectable in diabetic membranes, to determine whether the extent of HIF-1 α expression correlated with vascular activity, and to compare the presence of HIF-1 α in excised PDR membranes with its presence in excised, nondiabetic, idiopathic, epiretinal membranes (ERMs).

Materials and Methods

Vitreous and Preretinal Membrane Collection

Patients undergoing pars plana vitrectomy for complications related to PDR and those undergoing pars plana vitrectomy for idiopathic ERMs were eligible for inclusion. Patients with diabetes were included if there were fibrovascular ERMs causing tractional retinal elevation or vitreous hemorrhages with associated fibrovascular proliferation that required pars plana vitrectomy. Fibrovascular membranes were defined as showing either blood vessels containing erythrocytes within the proliferative extraretinal tissue (active fibrovascular membrane) or ghost vessels in fibrotic extraretinal membranes (fibrotic fibrovascular membrane). Patients without diabetic retinopathy and with idiopathic ERMs causing visual distortion or blurred vision and requiring pars plana vitrectomy were included as nondiabetic ERMs. Informed consent was obtained from each patient before entry into the study.

Twenty-one patients underwent pars plana vitrectomy and membrane peeling. None of the patients received any intraocular injections of steroids or anti-VEGF drugs before surgery. All eyes with PDR received panretinal photocoagulation in the past. None had fresh (within 2 months) laser treatment.

During the pars plana vitrectomy, the posterior hyaloid was first removed from the retinal surface. Care was taken to ensure that the posterior hyaloid was removed entirely from the surface before any diabetic membrane dissection or removal of the nondiabetic ERM. Intraocular forceps, a lighted knife, and vertical scissors were used to dissect fibrovascular and fibrotic proliferative membranes from the retinal surface in diabetic eyes. For nondiabetic membranes, intraocular pic forceps and a Michels pic were used to carefully and atraumatically dissect the idiopathic ERM from the retinal surface. Membranes were removed from the eyes using intraocular pic forceps and placed into sterile containers filled with balanced salt solution. The excised membranes in the balanced salt solution-filled containers were placed on ice for immediate transport to the laboratory.

In the laboratory, preretinal membranes were snap-frozen within 1 hour of removal in optimal cutting temperature compound and kept at -70°C . Eight-micron sections were cut and then stained using immunohistochemical staining.

Immunohistochemistry was used to determine the presence of HIF-1 α in the membranes. Preretinal membrane tissues were cryostat sectioned at 8- μm sections, air-dried, rehydrated

with phosphate-buffered saline (PBS, pH 7.4), and blocked with 5% normal goat serum for 15 minutes.

Confocal Immunofluorescence

Staining for Hypoxic-Inducible Factor-1 α and Cell Types—Thawed 8- μ m frozen sections were air-dried, fixed with reagent-grade acetone for 5 minutes, and washed with PBS (pH 7.4). After endogenous peroxide was blocked by treatment with 0.3% hydrogen peroxide, sections were blocked for 15 minutes with 1% bovine serum albumin (Sigma, St. Louis, MO) in Tris buffer. For single labeling, sections were incubated first with polyclonal anti-HIF-1 α antibody (rabbit anti-HIF-1 α polyclonal antibody, R & D Systems, Minneapolis, MN) for 30 minutes and then with fluorescein isothiocyanate conjugated antirabbit secondary antibody (biotinylated secondary antirabbit antibody; 1:400; Vector Laboratories, Burlingame, CA). The cellular types present in the membranes were determined by immunohistochemical staining. The presence of endothelial cells was assessed by staining with CD-31. The presence of myofibroblasts was assessed by staining with antismooth muscle actin. The presence of neural tissue was assessed by staining for astroglial cells using glial fibrillary acidic protein (GFAP). The presence of retinal pigment epithelium cells or transdifferentiated retinal pigment epithelium cells was assessed by staining with cytokeratin.

Double staining was performed for HIF-1 α and VEGF, HIF-1 α and cytokeratin, HIF-1 α and CD-31, HIF-1 α and smooth muscle actin, HIF-1 α and cytokeratin, and HIF-1 α and GFAP. In this set, the first (primary) antibody was placed on the sample, diluted with 1% bovine serum albumin/PBS, and kept overnight at 4°C. The slide was then washed with PBS 3 times (5 minutes each time). The secondary antibody was conjugated to fluorescein isothiocyanate and incubated for 30 minutes in the dark. The specimen was then blocked with 1% bovine serum albumin/PBS for 1 hour. The second antibody, diluted in bovine serum albumin/PBS solution, was then added and allowed to react for 1 hour. The slide was then washed with PBS 3 times (5 minutes each time). The rhodamine conjugated secondary antibody (red color) was incubated for 30 minutes in the dark. The slide was washed 3 times (5 minutes each time).

Staining for Hypoxic-Inducible Factor-1 α Within the Cell—To localize the HIF-1 α within the cell, the nuclei of the cells were stained using 4',6 diamidino-2-phenylindole (DAPI) fluorescent mounting media (Vector Labs). 4',6 diamidino-2-phenylindole binds to DNA of nuclei and thus can be used to identify cells on specimens. It appears blue on the sections. The HIF-1 α antibody stain was conjugated with fluorescein isothiocyanate and thus appeared green on the sections.

Controls—Retinal tissues from normal control postmortem eyes also were stained for HIF-1 α . 4',6 diamidino-2-phenylindole staining of the control retina and colocalization of HIF-1 α and DAPI were also performed. Control rabbit eyes were stained for HIF-1 α . Colocalization of DAPI and HIF-1 α was also performed in the rabbit eyes.

Stained sections were observed using a high-resolution laser scanning confocal microscope (LSM510, Carl Zeiss, Jena, Germany). All confocal images were obtained using standard settings based on positive and negative controls. The amount of fluorescent staining was graded on a scale of 1 to 3 for the presence of HIF-1 α . A score of 1 represented the presence of mild fluorescence, 2 represented moderate fluorescence, and 3 represented intense fluorescence within the entire membrane. The graders (J.I.L. and C.S.) were masked to the source of the membrane (diabetic or nondiabetic) during the grading. Each specimen was step-sectioned, and three to five stained sections were evaluated.

Results

Twelve patients underwent excision of PDR membranes, and nine underwent excision of nondiabetic ERMs. Patients ranged in age from 30 to 77 years. The median ages differed between the groups. The median age was 32 years (range, 30–65 years) for the patients with diabetes and 69 years (range, 48–77 years) for the patients with idiopathic ERM. There were three control postmortem eyes and one rabbit control eye that also underwent staining.

Overall, HIF-1 α staining was substantially present more often in the patients with diabetes than the patients with ERMs. The staining for HIF-1 α was also more intense in the diabetic membranes compared with the idiopathic ERMs. The results are given in Table 1. Eleven of 12 patients (92%) with diabetic membranes were positive for HIF-1 α versus 5 of 9 (56%) with idiopathic membranes ($P = 0.11$, 2-tailed χ^2). Five of the diabetic membranes showed mild positive staining for HIF-1 α , and 6 showed moderate to intense positive staining for HIF-1 α (Figures 1 and 2). 4',6 diamidino-2-phenylindole staining showed that the majority of HIF-1 α staining was cytoplasmic, although there were rare areas of nuclear overlap in the diabetic specimens. Of the nondiabetic idiopathic ERMs, 4 (44%) showed no positive staining for HIF-1 α and 5 showed mild to moderate positive staining for HIF-1 α (Figure 3). The nondiabetic ERMs showed intense staining for GFAP, mild staining for VEGF, mild staining for CD-31, and almost no staining for VEGF receptor-2.

In the diabetic membranes, double staining was markedly positive for HIF-1 α and VEGF and less intense for HIF-1 α and cytokeratin. The degree of positive staining was less intense for predominantly fibrotic (clinically regressed fibrovascular tissue) diabetic membranes compared with predominantly vascular (defined clinically as the appearance of red neovascular vessels on the retinal surface with no to minimal fibrous tissue) diabetic membranes. Fibrotic PDR membranes showed more positive staining for HIF-1 α and GFAP than for active diabetic membranes. Double staining of ERM specimens was minimal for HIF-1 α and VEGF. These ERMs showed little colocalization for HIF-1 α and GFAP (Figure 3) but did show colocalization for HIF-1 α and cytokeratin. These results are summarized in Table 2. Control postmortem eye sections and rabbit controls were negative for HIF-1 α .

Discussion

Hypoxia-inducible factor-1 is a transcription factor that plays an essential role in the systemic homeostasis response to hypoxia. Hypoxia-inducible factor-1 controls the expression of most genes involved in adapting to hypoxic conditions. It is a heterodimer composed of α and β subunits.¹¹ Cellular hypoxia stabilizes HIF-1 α , which then forms an active complex with the β subunit. This results in formation of the heterodimeric transcription factor, HIF-1. Hypoxic-inducible factor-1 triggers the activation of several genes that result in the production of VEGF and other angiogenic factors.^{5–8,12,13} When HIF-1 levels are increased in tissues that are normally poorly vascularized such as the core of solid tumors, angiogenesis occurs.¹⁴

Several researchers have shown that diabetic factors result in HIF-1 production and angiogenesis. Treins et al⁹ have shown that insulin growth factor 1 affects the regulation of HIF-1 in human retinal pigment epithelial cells. Insulin growth factor 1 stimulates HIF-1 accumulation, leading to VEGF mRNA expression. High glucose concentration has been shown to alter hypoxia-induced control of vascular smooth muscle growth through the HIF-1 α -dependent pathway.^{15,16} Vascular endothelial growth factor expression seems to be regulated through dual interdependent mechanisms. One involves HIF-1 directly and the other indirectly through NF-kappa B-mediated cyclo-oxygenase-2 expression and prostaglandin E2 production.¹⁷ Acute intensive insulin therapy exacerbates diabetic blood-

retinal barrier breakdown through HIF-1 and VEGF.¹⁸ This could explain why intensive control can result in transient worsening of diabetic retinopathy.

Recently, the presence of HIF-1 α in the diabetic membranes has been shown.¹⁹ Active membranes had more HIF-1 α activity than inactive diabetic membranes. In our study, varying degrees of HIF-1 α staining were seen, possibly related to varying levels of ischemia in our patients. For example, patients with active diabetic membranes (despite previous panretinal photocoagulation (PRP) laser) had intense HIF-1 α staining. In contrast, patients who had fibrotic diabetic membranes had mild to moderate HIF-1 α staining.

Our finding of HIF-1 α in the nondiabetic ERMs is not surprising. It indicates a possible role for hypoxia in the inflammatory response resulting in ERM formation. Armstrong et al¹⁰ have previously reported on the presence of VEGF and tumor necrosis factor- α not only in proliferative diabetic membranes, but also in proliferative vitreoretinopathy membranes and idiopathic macular ERMs. The VEGF levels were lowest in ERMs (216 pg/mg protein) versus proliferative diabetic membranes (5,994 pg/mg protein).

There are potential implications for control of PDR by HIF-1 α blockade. In fact, some researchers have begun to research the use of mammalian target of rapamycin inhibitors, which target HIF-1 α and thus its induced manifestations.²⁰ This study is limited by the few samples. Further work on HIF-1 α and diabetic retinopathy is needed to determine whether the trend observed in our study is real and whether HIF-1 α blockade can be useful clinically in patients with diabetes.

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Fig. 1. Confocal imaging for HIF-1 α and DAPI of an active PDR membrane. The HIF-1 α staining reaction is strongly positive (fluorescein isothiocyanate green; top left). The DAPI stain (blue) shows the nuclei of the cells in the membrane (top right). Double immunofluorescence staining shows the majority of HIF-1 α positivity is cytoplasmic (bottom). Bar = 20 μ m.

Fig. 2. Confocal imaging for HIF-1 α and DAPI of a fibrovascular PDR membrane. The HIF-1 α staining reaction (fluorescein isothiocyanate green) is strongly positive (top left). The DAPI stain (blue) shows many nuclei (top right). Colocalization shows the majority of positive staining is cytoplasmic (bottom). Bar = 20 μ m.

Fig. 3. Epiretinal membrane staining for HIF-1 α , GFAP, VEGF, and VEGF receptor-2 and colocalization of HIF-1 α with GFAP and cytokeratin. ERM staining for HIF-1 α (green) with Dapi counterstain (blue) shows very weak positivity (top left) and moderate positivity (top right). Staining for GFAP (green) was intense (middle left). Although VEGF staining was moderately positive (middle right), VEGF receptor-2 staining was only minimally positive (bottom left). Double staining for HIF-1 α (green) and GFAP (red) did not show colocalization (bottom center). Double staining for HIF-1 α (green) and cytokeratin (red) in contrast was positive (yellow, bottom right).

Table 1Grading of Positive HIF-1 α Staining in Diabetic and Nondiabetic ERMs

Specimen Type	No Staining	1 + Staining	2+ Staining	3+ Staining
Proliferative diabetic (12)	1 (8%)	5 (42%)	3 (25%)	3 (25%)
Idiopathic ERM (9)	4 (44%)	1 (11%)	4 (44%)	0
Postmortem eyes (3)	3 (100%)	0	0	0

Table 2

Grading Results of Confocal Imaging of Diabetic and Nondiabetic ERMs

Double Staining	Proliferative Diabetic (12)	Idiopathic Epiretinal (9)
HIF-1 α and VEGF	2+ to 3+	0
HIF-1 α and cytokeratin	1 +	1 +
HIF-1 α and GFAP	1 +	0