Vitreous IGFBP-3 Effects on Müller Cell Proliferation and Tractional Force Generation

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PURPOSE. Previous studies from this laboratory revealed that vitreous insulin-like growth factor binding protein-3 (IGFBP-3) is a biologically active fragment of the intact protein. The goal of this study was to characterize its effects on Müller cell proliferation and tractional force generation, activities relevant to proliferative diabetic retinopathy (PDR).

METHODS. Müller cells were isolated from normal porcine retina. The vitreous-type IGFBP-3 fragment was isolated from normal human plasma and compared with intact recombinant protein for the ability to modulate Müller cell proliferation and tractional force generation in tissue culture models.

RESULTS. Müller cells were stimulated to proliferate by serum and platelet-derived growth factor (PDGF), but not insulin-like growth factor (IGF)-I or IGF-II. The cells were similarly unresponsive to IGFBP-3 or the IGFBP-3 fragment alone or in combination with IGF-I or IGF-II. In contrast, Müller cells demonstrated robust extracellular matrix contraction in response to IGF-I, IGF-II, and PDGF. Intact IGFBP-3 attenuated extracellular matrix contraction in response to IGF-I and IGF-II while the IGFBP-3 fragment modulated cell responses to IGF-II only. Neither binding protein altered cell responses to PDGF.

CONCLUSIONS. Intact IGFBP-3 modulates Müller cell tractional force generation stimulated by IGF-I and IGF-II while the effects of the vitreous-type fragment are limited to IGF-II. Porcine Müller cells proliferate in response to PDGF, but not IGF-I or IGF-II. Both forms of IGFBP-3 are also without mitogenic effects alone or in combination with IGFs. It appears that Müller cell tractional force generation in PDR is driven by vitreous IGF activity and proliferation is stimulated by growth factors outside of the IGF system. (Invest Ophthalmol Vis Sci. 2012;53:93–99) DOI:10.1167/iovs.11-8683

Proliferative diabetic retinopathy (PDR) is a late-stage complication of diabetes in which fibrovascular tissues emerging from the retina exert tractional forces that cause retinal detachment.1,2 PDR is ultimately a cellular disorder with proliferation and tractional force generation as major pathogenic activities.2 There has been considerable interest in identifying the causal cells and the stimuli driving these pathogenic activities as the ability to arrest them would represent a significant gain toward controlling this complication.

Immunohistochemical studies of diabetic epiretinal tissues identified different cell types including glia, immune cells, retinal pigmented epithelial cells, and fibroblast-like cells of uncertain origins.3–9 Müller cells, the principal retinal glia, are consistently identified in diabetic fibrovascular scar6,8,9 and there is abundant evidence to indicate that they are a source of the fibroblast-like cells in PDR.9–11 Systematic studies of Müller cell tractional force generation in vitro revealed that this activity develops in concert with acquisition of the fibroblast-like phenotype and is not constitutive, but is stimulated by certain exogenous promoters including members of the insulin-like growth factor (IGF) and platelet-derived growth factor (PDGF) families.10,11 In addition, IGFs and PDGF are reported to be Müller cell mitogens and may play important roles in driving cell proliferation.12–14

Recent studies from this laboratory examined vitreous from normal and diabetic eyes for the ability to stimulate Müller cell tractional force generation in vitro.15 It was determined that normal vitreous induces little or no response while samples from patients with diabetes or PDR stimulate significant responses, the magnitude of which correlate with disease severity. More recently, we detected similar vitreous changes in swine with chemically induced diabetes suggesting that the vitreous changes may precede the development of retinal disease.16 In addition, studies with growth factor-neutralizing antibodies were able to attribute the stimulatory activity in human diabetes to IGFs rather than PDGF. As a consequence of these findings, there is now considerable interest in gaining an improved understanding of vitreous changes in diabetes and, in particular, those that result in increased IGF biological activity.

Interestingly, when one considers the IGF concentrations reported for normal vitreous,17–22 there should be higher levels of biological activity than we observe15,23 suggesting that the growth factors are in some way controlled or attenuated. This led us to speculate that diabetes-associated increases in vitreous IGF activity can arise from increases in growth factor levels or from loss of normal control. We have since determined that the insulin-like growth factor binding proteins (IGFBP) present in normal vitreous, IGFBP-2 and IGFBP-3, are able to neutralize IGF-I and IGF-II effects on Müller cell tractional force generation.24 Our studies have provided a better understanding of IGFBP-2 effects on Müller cells, but IGFBP-3 is complicated by the fact that it exists in vitreous as a proteolytic fragment of the intact protein.25 This fragment appears to originate in plasma, cross the blood-vitreous barrier, possesses growth factor affinity and thus has the potential to influence IGF biological activity. However, our understanding of its effects on Müller cells is otherwise limited by the absence of experimental evidence. The goal of this study was to evaluate the direct and indirect effects of the vitreous IGFBP-3 fragment on Müller cell proliferation and tractional force generation to improve our understanding of its role in vitreous biochemistry in normal and disease states.
METHODS

Isolation and Culture of Porcine Müller Cells

The methods used to secure animal tissues complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Review Board at the University of Alabama at Birmingham. Müller cells were isolated from normal porcine retina and maintained in culture using previously published methods. Briefly, eyes removed from anesthetized animals were maintained in ice-cold saline until dissection. Retinas were digested sequentially with papain and DNase, and the cells were released by trituration. Supernatants enriched with morphologically recognizable Müller cells were plated in growth medium composed of Dulbecco’s minimum essential medium (DMEM) supplemented with 20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid and 10% fetal bovine serum (FBS). The cells were permitted to adhere for 30 to 60 minutes at 37°C, after which the nonadherent population was removed and the medium was replaced with fresh growth medium. Müller cells isolated by this method are all positive for glial fibrillary acidic protein (GFAP) and glutamine synthetase and negative for alpha smooth muscle actin. Cultures were maintained in a humidified incubator at 37°C with 5% CO₂.

IGFRII Fragment Purification

Using previously published protocols, human plasma (100 mL) was acidified by the addition of 5.75 mL of glacial acetic acid, stirred for 30 minutes on ice and then centrifuged to remove insoluble material. Ethanol was added successively to the supernatants to achieve 40% (vol/vol) and 80% (vol/vol) after which the mixtures were stirred on ice for 60 minutes and centrifuged to collect the insoluble material. The 80% ethanol pellet was resuspended and dialyzed overnight against PBS after which the nonadherent population was released with 0.05% trypsin and 0.53 mM EDTA and replated in fresh growth medium.

Cell Proliferation Assays

Cells detached with trypsin/EDTA as for routine subculture were plated in 8-well chamber slides (Nunc, Rochester, NY) at 40,000 cells per well in 0.2 mL growth medium. On the second day the growth medium was removed, the wells washed with serum-free medium and the incubation continued with 0.2 mL serum-free DMEM containing 1 mg/mL crystalline BSA (DMEM-BSA). On the third day the DMEM-BSA was removed and replaced with 0.2 mL DMEM-BSA containing the experimental additives including human IGF-I (Gropep, Inc., Adelaide, Australia) human IGF-II (Gropep, Inc.) and human PDGF-AB (Upstate Biotechnology, Inc., Lake Placid, NY). After 24 hours the medium was removed and the slides were fixed with 2% paraformaldehyde in phosphate buffer (0.1 M Na2HPO4, pH 7.0) for 1 hour at room temperature, washed with PBS (0.01 M NaCl, pH 7.4), permeabilized by a 10-minute treatment with PBS containing 0.1% Triton X-100 and blocked with 20% nonimmune goat serum (The Binding Site, Ltd., Birmingham, UK) in PBS for 60 minutes at room temperature. Primary and secondary antibody treatments with anti-Ki67 (Dako North America Inc., Carpenteria, CA) and Cy2-conjugated goat anti-mouse antibody (Jackson Immunoresearch Inc., West Grove, PA) were for 60 minutes at room temperature using 2% goat serum in PBS with three 5-minute washes in between. The slides were then rinsed briefly with PBS, reacted with 1 μg/mL 4’,6-diamidino-2-phenylindole (DAPI) in PBS for 5 minutes and were rinsed again with PBS. Photomicrographs were taken with a microscope equipped with epi-fluorescence illumination and phase-contrast optics (Optiphoto; Nikon, Tokyo, Japan) and with a digital camera (RETIGA EXi; QImaging Corp., Burnaby, Canada). Successive paired photomicrographs of Ki67-associate and DAPI fluorescence were taken during a north to south transit across the center of each well were quantified by an observer naïve to the experimental conditions counting a minimum of three successive fields and a minimum of 300 DAPI-positive cells. Images were assembled into composite photographs with image management software (Adobe Photoshop C2; Adobe Systems, Inc., San Jose, CA).

FIGURE 1. Morphologies of Müller cells incubated with different mitogens. Phase-contrast photomicrographs were taken of Müller cells 24 hours after incubation in medium containing 10% FBS (A), no additions (B), 10 nM IGF-I (C), or 10 nM PDGF (D). Scale bar, 100 μm.
Collagen Gel Contraction Assays

Collagen gels were prepared from native type I collagen isolated from rat tail tendons by limited pepsin digestion and sequential salt precipitation.\(^\text{10,27}\) Collagen dissolved in 12 mmol/L HCl was adjusted to physiological pH and ionic strength using 10x PBS (0.1 mol/L Na2HPO4, 1.5 mol/L NaCl) and 0.1 mol/L NaOH while maintained on ice. Aliquots (0.2 mL) of the collagen solution were added to circular wells (15 mm in diameter) on the bottom of 24-well tissue culture plates and incubated at 37°C for 90 minutes to allow the gels to polymerize. Cells harvested with trypsin/EDTA were washed with growth medium to inactivate the trypsin and again with contraction medium composed of Dulbecco’s minimum essential medium with reduced sodium bicarbonate (2.7 g/L) and 1 mg/mL crystalline BSA (#A-7511; Sigma Chemical Company, St. Louis, MO). Aliquots of cells (50 µL) suspended in contraction medium at 400,000 cells per µL were applied to the gel surface and then incubated at 37°C to permit cell adhesion. The wells were then flooded with an additional 0.75 mL contraction medium containing test substances. Immediately after flooding, the initial thickness of each gel was measured using an inverted, phase-contrast microscope equipped with a Z-axis digitizer (LaSico, Los Angeles, CA) by adjusting the plane of focus from the surface of the culture vessel to the cell layer. This measurement provided the initial gel thickness against which all subsequent measurements were compared. The subsequent gel thickness was divided by the initial gel thickness, multiplied by 100, and then subtracted from 100 to yield the percentage reduction in gel thickness reported as the percent contraction.

RESULTS

Müller Cell Proliferation in Response to IGF System Ligands

To evaluate the effects of IGF system proteins on Müller cell proliferation, cells attached to collagen-coated coverslips were incubated in serum-free medium for 24 hours followed by an additional 24-hour incubation in medium containing FBS, albumin, 10 nM IGF-I or 10 nM PDGF. Phase-contrast microscopic observation at the end of the incubations revealed that cell morphologies did not differ in response to the different stimuli (Fig. 1) and, more importantly, the cells were attached and well spread at the conclusion of the experimental protocol. The cells were then fixed and probed with DAPI to detect cell nuclei and the Ki67 antigen as indicator of cell proliferation. As one might reasonably predict, cells incubated in medium containing serum were abundant and a substantial fraction were positive for Ki67 (Figs. 2A, 2B). In contrast, cells incubated in serum-free medium appeared to be less abundant and were generally negative for Ki67 (Figs. 2C, 2D). Surprisingly, cell cultures treated with IGF-I more closely resembled the unstimulated cells with respect to culture density and Ki67 expression (Figs. 2E, 2F) while cultures stimulated with PDGF resembled the serum-stimulated cells (Figs. 2G, 2H).

Additional experiments were performed to evaluate Müller cell dose-dependent responses to these stimuli. Cells incubated under the same conditions as in Figure 1, but in 0%–10% serum were stimulated to express Ki67 in a dose-dependent fashion, achieving a maximal response of approximately 60% and a background approaching 0% (Fig. 3A). Cells exposed to varying amounts of PDGF had a similar dose-dependent response at concentrations above 1.25 nM (Fig. 3B). In contrast, cells exposed to IGF-I or IGF-II in concentrations as high as 40 nM did not have significant increases in Ki67 expression (Fig. 3B).

The absence of IGF-I stimulated Müller cell proliferation was observed in different assay systems (electronic counts and MTT [3(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]), different Müller cell isolates, growth factor preparations, and in experiments in which the culture medium was supplemented with low levels of FBS (not shown). To confirm that the cells and growth factor preparations were functioning normally, Müller cell extracellular matrix contraction assays were performed on successive days using the same cell isolates and growth factor preparations tested in Figure 3. Müller cells treated with IGF-I or IGF-II were stimulated to contract the collagen gels at concentrations above 50 pM (Fig. 4). Cells treated with PDGF were also stimulated to contract the gels at concentrations comparable with those required to stimulate cell growth (Fig. 4). Together, these studies indicated that IGF-I and IGF-II are not significant mitogens for Müller cells under the conditions in which these growth factors were tested.

IGFBP-3 Biological Activities

To examine the potential for Müller cell direct and indirect mitogenic effects, cells were incubated with varying concentrations of intact IGFBP-3 with and without IGF-I, IGF-II, or PDGF. IGFBP-3 was without significant effects at all concentrations tested (Fig. 5A). Similarly, there was no effect when it was combined with any of the three ligands tested. Similar experiments were performed to test the IGFBP-3 fragment mitogenic activity. Whether tested alone or in combination with IGF-I,
IGF-II, or PDGF, we also observed no significant increases in Müller cell proliferation (Fig. 5B).

We next examined the effects of the IGFBP-3 fragment on Müller cell extracellular matrix contraction and performed side by side comparisons with the intact binding protein. In these experiments cells attached to collagen gels were stimulated with 0.5 nM IGF-I or 0.5 nM IGF-II combined with varying amounts of intact IGFBP-3 or the purified fragment. Intact IGFBP-3 significantly decreased Müller cell responses to both IGF-I and IGF-II in a dose-dependent fashion (Fig. 6A). In contrast, the IGFBP-3 fragment had no effect on IGF-I, but was able to attenuate IGF-II effects to a significant extent (Fig. 6B).

Also, as we observed with cell proliferation, there were no direct effects detected when the cells were incubated with the IGFBP-3 fragment alone or with PDGF.

These observations lead us to conclude that neither IGFBP-3 nor the vitreous-associated fragment of this binding protein has detectable effects on Müller cell proliferation when added alone or in combination with the IGF system ligands. In addition, these proteins do not appear to influence Müller cell responses to unrelated growth factors such as PDGF. This is not the case, however, with tractional force generation as the vitreous-associated fragment can modulate Müller cell tractional force generation in response to IGF-II, but not IGF-I.

These conclusions are further supported by calculations of the percent inhibition per mole and extrapolation of the molar excess of each binding protein necessary to completely inhibit growth factor activity (Table 1). IGFBP-3 effectively inhibits IGF-I and IGF-II responses in relatively low molar excesses of 5.5- and 1.5-fold, respectively. In contrast, the IGFBP-3 frag-

**FIGURE 3.** Quantified Müller cell responses to mitogens. Müller cells were incubated in medium containing varying concentrations of mitogens, fixed and probed with DAPI and for Ki67 by indirect immunofluorescence as described in Figure 2. Presented are the results achieved with the indicated concentrations of FBS (A) and growth factors (B) including IGF-I (○), IGF-II (●), and PDGF (□). Each data point represents the means and standard deviations of results obtained from triplicate evaluations of each experimental condition.

**FIGURE 4.** Müller cell extracellular matrix contraction in response to mitogens. Müller cells attached to collagen gels were incubated for 24 hours in medium containing varying concentrations of IGF-I (○), IGF-II (●), or PDGF (□). Presented are the means and standard deviations of changes in gel thickness by triplicate cultures under each condition.

**FIGURE 5.** Evaluation of IGFBP-3 effects on Müller cell proliferation. Müller cells attached to coverslips were incubated in medium containing the indicated concentrations of intact IGFBP-3 (A) or the vitreous-type IGFBP-3 fragment (B) alone (○), with 10 nM IGF-I (●), 10 nM IGF-II (□), or 10 nM PDGF (■). The cells were probed with DAPI and for Ki67 by indirect immunofluorescence. Presented are the means and standard deviations of results achieved with triplicate evaluations of each experimental condition.
The goal of this study was to characterize the effects of the vitreous type fragment of IGFBP-3 on Müller cell tractional force generation. With respect to Müller cell proliferation the results are relatively straightforward in that intact IGFBP-3 and the vitreous type fragment have no detectable effects on Müller cells. This is the case when the two binding proteins are tested alone or in combination with the ligands IGF-I, IGF-II, or PDGF. In contrast, IGFBP-3 effects on Müller cell tractional force generation are more complex and appear to be form-specific. While neither the intact nor vitreous-type fragment of IGFBP-3 appear to have direct effects on Müller cell tractional force generation, both attenuate IGF stimulation to varying degrees. Intact IGFBP-3 is able to completely inhibit the effects of IGF-I and IGF-II when present in relatively low molar excesses of 5.5- and 1.5-fold, respectively. The IGFBP-3 fragment has no significant inhibitory effects on IGF-I, but is able to completely block IGF-II effects when present at an 8-fold molar excess.

The IGFBP-3 fragment activities we described are generally consistent with previous reports of protein affinities. Ahlsen and colleagues compared intact IGFBP-3 and the plasma fragment’s affinities for IGF-I and IGF-II and reported decreases of 11-fold and 4-fold, respectively, that were largely attributed to an increased off rate. Our studies detected a comparable, 5-fold increase in the molar ratio of the fragment necessary to neutralize IGF-II effects. Similarly, we determined that the IGFBP-3 fragment lost the capacity to attenuate IGF-I activity at physiologically relevant concentrations. Yan and colleagues also demonstrated that the similarly sized pregnancy-associated IGFBP-3 fragment in plasma retains the ability to assemble into the ternary complex with both ligands and the acid labile subunit provided that the C-terminal fragment is also available for assembly. Under these conditions, the fragment retains the ability to inhibit IGF transport across endothelial layers and IGF ligand receptor phosphorylation. While we have no evidence for or against such a complex crossing the blood vitreous barrier, its size should be virtually identical with the ternary complex formed with intact IGFBP-3.

As we have consistently failed to detect intact IGFBP-3 in normal vitreous, these observations led us to speculate that ternary complexes do not cross the blood-vitreous barrier under normal conditions. When viewed as an isolated ligand-binding protein interaction, our observations suggest that the IGFBP-3 fragment can sequester free IGF-II and reduce the pool of bioavailable growth factor in vitreous and so the net effect is to reduce vitreous IGF activity. Importantly, while the specific activity of IGF-II with respect to Müller cell tractional force generation is only one third that of IGF-I, its concentration in normal vitreous is approximately 10-fold higher making it the major source of IGF activity in normal and diabetic vitreous. If, as we speculated, the IGFBP-3 fragment originates in plasma, does it cross the blood-vitreous barrier bound to IGF-II? If so, it may facilitate IGF-II transport across the blood vitreous barrier and have the opposite net effect, increasing levels of IGF biological activity. The vitreous increases may be even further exacerbated by the higher affinity of IGFBP-2 for IGF-II. If bioavailable IGF-II is able to displace a molar equivalent of IGF-I from IGFBP-2, the result would be an additional 3-fold increase in IGF-I activity.

### Table 1. Summary of IGFBP-3 Inhibition of IGF-I and IGF-II

<table>
<thead>
<tr>
<th></th>
<th>0.5 nM IGF-I, % Inhibition per Mole</th>
<th>$r_{corr}$</th>
<th>100% Inhibition (Molar Ratio)</th>
<th>0.5 nM IGF-II, % Inhibition per Mole</th>
<th>$r_{corr}$</th>
<th>100% Inhibition (Molar Ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFBP-3</td>
<td>$3.75 \times 10^{10}$</td>
<td>0.99</td>
<td>5.5</td>
<td>$1.34 \times 10^{11}$</td>
<td>0.99</td>
<td>1.5</td>
</tr>
<tr>
<td>fragment</td>
<td>$6.03 \times 10^{9}$</td>
<td>0.54</td>
<td>337.1</td>
<td>$2.51 \times 10^{10}$</td>
<td>0.99</td>
<td>7.9</td>
</tr>
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Percent inhibition per mole IGFBP was calculated from regression analysis of the data presented in Figure 6, using a minimum of three binding protein concentrations, each in triplicate. Correlation coefficients ($r_{corr}$) above 0.9 are considered significant. One hundred percent inhibition refers to the calculated molar ratio of IGFBP to growth factor necessary to achieve complete inhibition normalized to growth factor concentration tested by division.
The role of the IGF system with respect to Müller cell proliferation also merits discussion. The results obtained in this study were unequivocal in that we detected no evidence of cell proliferation in response to IGF-I or IGF-II, with or without IGFBP-3 or the IGFBP-3 fragment. We did, however, observe a robust gel contraction response using the same cells and growth factor preparations indicating that both the cells and growth factor preparations were functioning normally. In addition, PDGF proved to be a potent Müller cell mitogen in the same assay system leading us to conclude that the IGFs do not stimulate Müller cell proliferation under these circumstances. While the conclusions about PDGF are consistent with previous reports, this is not the case with IGF-I. Several studies from the mid 90s reported that IGF-I stimulates human Müller cell proliferation in culture. We have compared human Müller cell extracellular matrix contraction with that of porcine cells and determined that their growth factor responses profiles are similar, including comparable sensitivities to IGF-I and PDGF.

Careful comparisons of the techniques used in the present study and those published previously identified a number of differences including the human retina source, cadaveric tissue which is most often from older individuals, the expediting techniques used to isolate the cells, and/or the cell proliferation assays which monitored increases in cell number. On the surface none of these differences appear to account for the disparate results suggesting that it may be something more subtle. One notable difference between the two cell populations was the reported persistence of glutamine synthetase in their cultures. Our primary Müller cell cultures cease expression of glutamine synthetase within a few days of isolation, as they begin to proliferate and adopt the myofibroblast-like phenotype. This suggests that at the least there may be differences in the culture populations used in the two studies.

Finally, if IGFs are not Müller cell mitogens then what vitreous growth factor(s) drive Müller cell growth in fibroproliferative diseases such as PDR? While the results of this and other studies suggest that the PDGF family is involved, not all the data available support this premise. Several studies of PDGF isoforms in diabetic vitreous report concentrations of approximately 100 pg/ml that are well below our observed threshold of Müller cell sensitivity. While we cannot exclude involvement of more exotic mechanisms such as growth factor compartmentalization, it seems more likely that other soluble promoters are involved. Müller cell proliferation is an important pathogenic component in the progression of PDR and systematic studies to positively identify the growth factors involved would likely be of great value.

References


