Purposes. Previous studies from this laboratory revealed that vitreous insulin-like growth factor biological activity increases in diabetes and that this change can precede the onset of proliferative diabetic retinopathy. The goal of this study was to characterize this phenomenon in an animal model of alloxan-induced diabetes.

Methods. Swine made diabetic with intravenous alloxan were euthanized at times varying from 0 to 90 days. Vitreous samples from normal and diabetic swine were evaluated for changes in Müller cell contraction-promoting activity, the presence of insulin-like growth factor binding protein (IGFBP), and carbonic anhydrase-I and -II. Ocular tissues from these animals were also evaluated for changes in contraction-promoting growth factors and IGFBP message levels.

Results. Alloxan-induced diabetes is associated with significant increases in vitreous Müller cell contraction-promoting activity that are present in as few as 30 days and are sustained for at least 90 days. Biochemical studies revealed that the increases cannot be attributed to loss of growth factor-attenuating IGFBPs, changes in local expression of contraction-promoting growth factors, or vitreous hemorrhage.

Conclusions. The previously reported increases in Müller cell contraction-promoting activity detected in human diabetic vitreous are present in diabetic swine within 30 days of chemical induction. The increase does not appear to be attributable to loss of growth factor control, increases in local growth factor expression, or vitreous hemorrhage, suggesting that other mechanisms are involved. It is the authors’ speculation that diabetes induces blood-vitreous barrier changes that allow a different subset of plasma proteins to enter vitreous fluids. (Invest Ophthalmol Vis Sci. 2011;52:7485–7491) DOI:10.1167/ iovs.11-7781

Proliferative diabetic retinopathy (PDR) is a late-stage complication of diabetes in which fibrovascular tissues emerging from the retina exert tractional forces that can cause retinal detachment.1,2 There is considerable interest in identifying the causal cells and the stimuli driving their pathogenic responses because the ability to arrest or attenuate any of these processes would represent a significant gain in control of this complication. Immunohistochemical studies of diabetic epiptirenal tissues have identified a number of different cell types, including glia, immune cells, retinal pigment epithelial cells, and fibroblast-like cells of uncertain origin.3–8

Abundant circumstantial evidence indicates that Müller cells are a source of the tractional forces that cause retinal detachment in PDR. Müller cells, the principal retinal glia, are consistently identified in diabetic fibrovascular scar tissue,5,6,8,9 and studies from this laboratory revealed that the fibroblast-like cells are also derived from phenotypically altered Müller cells.9–11 Although phenotypically normal Müller cells lack the capacity to generate tractional forces, this is not the case with the fibroblast-like cells.11,12 Systematic study of Müller cell tractional force generation in vitro also revealed that this activity is not constitutive for the fibroblast phenotype but is stimulated by exogenous growth factors, including members of the insulin-like growth factor (IGF) and platelet-derived growth factor (PDGF) families.11,12

One study from this laboratory with findings particularly relevant to diabetic retinopathy examined changes in Müller cell contraction-promoting activity in normal and diabetic vitreous. It was determined that normal vitreous contains little or no stimulatory activity, but it is increased in diabetes and PDR.13 Studies with growth factor-neutralizing antibodies attributed the biological activity in diabetic vitreous to IGFs rather than to PDGF, and increased IGF activity in vitreous did not correlate with hemorrhage as a diagnostic feature, suggesting that the growth factors originate from local production or enter vitreous fluid through another mechanism. Finally, and perhaps of greatest interest, increased IGF activity was detected in diabetic vitreous fluids in eyes without PDR, suggesting that this change can precede the onset of clinically significant proliferative disease.

That changes in vitreous biological activity temporally precede the onset of proliferative retinal disease is suggestive of a causal relationship and is worthy of additional study. Unfortunately, the limited availability of vitreous samples from patients in the early disease states precludes systematic study of this relationship in humans. For this biochemically oriented vitreous study, we made use of an established large animal model of hyperglycemia,14,15 the goal of which was to evaluate the temporal relationship that exists between diabetes and the increases in Müller cell contraction-promoting activity detected in diabetic vitreous.

Methods

Diabetic Swine

The methods used in this study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Before alloxan induction, 12 male Yorkshire swine (~32 kg) were tested for insulin responsiveness in intravenous glucose tolerance tests after an overnight fast.14 The animals received glucose in a 50% solution (0.5 g/kg), with glucose levels measured at 10-minute intervals up to 60 minutes. The animals were fluid-loaded with 1000 mL normal saline to avoid renal complications and then induced with intravenous alloxan (150

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mg/kg; Sigma, St. Louis, MO) at 100 mg/mL in 0.15 M NaCl/0.1 M NaOH. After injection, food was provided ad libitum, and fasting blood glucose levels were monitored daily until hyperglycemia (>200 mg/dL) was confirmed on 3 consecutive days, after which blood glucose levels were tested weekly. Subcutaneous insulin injections (NPH 70/30, ultralente) were given to animals with preprandial AM blood glucose levels above 400 mg/dL. Dilated ocular examinations by the collaborating ophthalmologist (JOM) were performed while the animal was maintained under inhaled anesthesia (1%-2% isoflurane) delivered with a nose cone. Eyes were diluted with 1% tropicamide ophthalmic solution and 2.5% phenylephrine hydrochloride solution, and examinations were performed with a 15-diopter lens and an indirect ophthalmoscope.

Ocular Tissues
Immediately after euthanization by pentobarbital overdose, eyes were removed and immersed in ice-cold saline. While they were maintained on ice, the globes were cleaned of extraneous tissue, rinsed with ice-cold saline, and opened with a hemispheric cut using a razor blade and iris scissors. The vitreous body was removed and stored frozen until use in experiments.

Immunohistochemistry
Tissues processed for immunohistochemistry were fixed for 24 hours at 4°C with 2% paraformaldehyde in phosphate buffer (0.1 M Na₂HPO₄, pH 7.0), rinsed with additional phosphate buffer, infiltrated for 1 hour on ice with one part tissue-embedding media (Fisher Diagnostics, Fair Lawn, NJ) and two parts 30% sucrose in phosphate buffer (0.1 M Na₂HPO₄, pH 7.0), and quick-frozen with cryospray. Blocks were cryosectioned at 5-μm thickness, four sections per slide, dried at 50°C for 60 minutes, and stored at ~80°C. After postfixation with acetone for 3 minutes and the addition of a Papanicolaou ring, cryosections were blocked with 20% nonimmune serum in PBS (0.15 M NaCl, 0.01 M Na₂HPO₄, pH 7.0) for 60 minutes at room temperature. Primary and secondary antibody treatments with previously characterized immunochemicals were conducted for 60 minutes at room temperature using 2% goat serum in PBS with three 5-minute washes

Collagen Gel Contraction Assays
Müller cells were isolated from porcine retina and maintained in culture using previously published procedures from this laboratory. Assays in which Müller cells contracted collagen gels were also performed using our published procedures and compared vitreous stimulatory activity to that of fetal bovine serum quantified as optical density units (ODU) measured at 280 nm using a 1-cm light path. Phase-contrast photomicrographs of Müller cells on collagen gels were taken using an inverted microscope (TMS; Nikon) equipped with epifluorescence illumination (Optiphot-2; Nikon, Tokyo, Japan) and with a digital camera (RETIGA EXi; QImaging, Burnaby, BC, Canada).

Western Blot and Western Ligand Blot Analyses
Experiments to evaluate vitreous IGFBPs by Western blot and Western ligand blot analyses were performed using our previously reported reagents and protocols. Briefly, vitreous samples were electrophoresed under nonreducing conditions, transferred to nitrocellulose, and probed with antibodies against individual IGFBPs or biotinylated IGF-II. Detection was with horseradish peroxidase-conjugated avidin, respectively, with chemiluminescence development followed by exposure to x-ray film.

RT-PCR Analysis
Total RNA was isolated from porcine ocular tissue and liver with an extraction reagent according to the manufacturer's instructions (TRizol; Invitrogen-Gibco, Grand Island, NY). RT-PCR reactions were performed with 1 μg total RNA, 20 pm each primer, and commercial RT-PCR reagents (Ready-To-Go; Amersham Pharmacia Biotech, Piscataway, NJ). Primers used in this study included IGF-I f-catcacatcctcttcgcatc r-aggcttgaggggtgcga, IGF-II f-cctcgctgctgctctttctt r-tgacgcttgctgctcttc, IGFBP-2 f-cgacaggtgtgcacagacagtgcttgatcagctgccaggtgc, IGFBP-3 f-fccagaaacgcagctgtacgcttc r-tccatgcgtgactgactgccagc, PDGF-B f-clocktagacgcacccagccagtctgcctgcatcagagaacggcagtgagtcc r-tccatgcgtgactgactgccagc, G3PDH f-accacagtccatgccatcac r-tccatgcgtgactgactgccagc. Reaction programs were run on a thermocycler (MiniCycler model PTC-150; MJ Research, Watertown, MA) and included a reverse transcription program of 20 minutes at 42°C and 5 minutes at 95°C; 35 cycles of 1 minute at 95°C, 45 seconds at the appropriate annealing temperature, and 45 seconds at 72°C; and 5 minutes at 72°C. For negative control reactions, reverse transcriptase was inactivated at 95°C for 10 minutes before the addition of the primer and template. PCR products were separated on 2% agarose gels, visualized with ethidium bromide, and photodocumented (ImageQuant 400; GE Healthcare, Piscataway, NJ).

RESULTS

Induction and Characterization of Diabetic Swine
Blood glucose levels increased dramatically within a couple of days of successful alloxan administration (Fig. 1A) and remained significantly elevated in all three experimental groups (TRizol; Invitrogen-Gibco, Grand Island, NY). RT-PCR reactions were performed with 1 μg total RNA, 20 pm each primer, and commercial RT-PCR reagents (Ready-To-Go; Amersham Pharmacia Biotech, Piscataway, NJ).
Weekly dilated ocular examinations performed by a collaborating retina specialist before and weekly after alloxan induction detected no retinal changes. However, cataracts developed in all hyperglycemic animals maintained for 60 days or longer and were graded as trace by 60 days and 2+ or 3+ by 90 days. One eye from each animal removed at euthanization was processed for immunohistochemistry to evaluate changes in retinal GFAP content. Although positivity was visible in the astrocyte layers and Müller cells of the normal retina (Figs. 2A, 2B), there was also a progressive increase in GFAP intensity that correlated with the hyperglycemic period (Figs. 2C–H).

Most impressive were GFAP increases in Müller cells, particularly in the region of the outer nuclear layer extending to the outer limiting membrane (compare Figs. 2A and 2G).

**Diabetes-Associated Changes in Vitreous Biological Activity**

Vitreous samples harvested from normal and diabetic animals were evaluated for the ability to stimulate Müller cell tractional force generation using a tissue culture model in which the cells contract three-dimensional collagen gels. In the examples pre-

**FIGURE 2.** Retinal GFAP changes associated with alloxan-induced diabetes. Porcine eyes were cryosectioned and probed for GFAP content by indirect immunofluorescence (A–H). Presented are samples from animals 0 (A, B), 30 (C, D), 60 (E, F), and 90 (G, H) days after alloxan induction. Merged images (B, D, F, H) contain two color channels that represent GFAP (green) and nuclear staining with DAPI (blue). Scale bar, 100 μm.
Müller cells attached to collagen gels were incubated in medium containing equal amounts of protein from fetal bovine serum (FBS), diabetic swine vitreous, or serum albumin. Cells exposed to FBS or vitreous responded with gel contraction detectable within a few hours that progressively increased throughout the incubation period. In contrast, cells incubated in medium containing albumin had little or no response.

Müller cell morphologies under these conditions also reflected the behavioral differences. Cells incubated on collagen gels for 6 hours in medium containing FBS tended to be bipolar, projected processes, and lines of collagen matrix under tension could be observed on the gel surface (Fig. 4A, arrow). Cells incubated in diabetic swine vitreous had similar morphologic features (Fig. 4B). However, cells incubated in medium containing bovine serum albumin (BSA) tended to remain round, projected shorter processes, and generated little or no visible change in the collagen matrix (Fig. 4C).

To better characterize the effects of hyperglycemia on vitreous contraction-stimulating activity, samples were also tested in varying concentrations to enable calculation of specific activities. In the examples provided in Figure 5A, it is apparent that the vitreous samples and FBS stimulate Müller cell responses in a volume-dependent fashion, though the dose-response profile varies considerably. To account for differences in sample protein concentration, Müller cell responses were also plotted against the amount of protein added, and regression analyses of the data obtained from the linear part of each curve permitted calculation of sample specific activities (Fig. 5B).

All normal and diabetic vitreous samples were evaluated for protein concentration and specific contraction-promoting activities using the approach described. Evaluations of vitreous protein concentration detected no statistically significant differences or obvious hyperglycemia-associated trends (Fig. 6A). This was not the case, however, with Müller cell contraction-stimulating activity. The specific activity per unit protein in the 30-day diabetic population was significantly increased over normal, and this increase remained significant through the 60- and 90-day populations (Fig. 6B). We also considered potential changes in total vitreous activity (per unit volume), which was calculated by multiplying individual protein concentrations by specific activities. Although the results varied a bit more, the total vitreous activities remained significantly elevated over those of the normal swine in all three diabetic populations (Fig. 6C). Together, these results indicate that diabetes-associated changes in vitreous include significant increases in Müller cell contraction-stimulating activity.

### Origin of Vitreous Biological Activity

We considered the possibility that diabetes induces changes in local ocular tissue expression of growth factors that would, in turn, yield net increases in vitreous Müller cell contraction-promoting activity. Total RNA preparations from normal and diabetic retina and iris-ciliary body complex were evaluated for changes in IGF-I and IGF-II message levels by real-time RT-PCR.
with the results compared to message levels of the ubiquitous glyceraldehyde-3-phosphatedehydrogenase (G-3-PDH). IGF-I message levels in retina and iris/ciliary body either remained unchanged or, when statistical significance was achieved, were slightly decreased (Table 1). IGF-II message levels either remained unchanged in response to hyperglycemia or were modest and mixed and, when significant, were decreased rather than increased. We also considered the possible contribution of increased local expression of PDGF and detected no statistically significant changes in message levels.

We also considered loss of growth factor-attenuating IGFBPs in vitreous as a possible contributing mechanism and evaluated ocular tissues for changes in IGFBP-2 and IGFBP-3 message levels. The results achieved by real time RT-PCR were mixed and, in most cases, failed to achieve statistical significance (Table 1). IGFBP-2 message was undetectable in the normal or diabetic retina but was modestly increased in the diabetic iris/ciliary body. IGFBP-3 followed the same mixed response trend, with modest increases in retina message levels and increases in iris/ciliary body. These results were verified in parallel experiments involving Western ligand blots of normal and diabetic vitreous, which detected no diabetes-associated decreases (Fig. 7A), a finding that was supported by densitometric evaluations of the binding protein intensities (Fig. 7B). Similar results were obtained with Western blot analysis to specifically detect IGFBP-2 and IGFBP-3 (not shown).

FIGURE 5. Evaluation of vitreous biological activity. Müller cells attached to collagen gels were incubated in medium containing the indicated volumes of FBS (□), diabetic (●), or normal (○) animal vitreous (A). Gel thicknesses were measured after 24 hours of incubation. Analyses of Müller cell responses to the same samples presented in A according to protein concentration (B). (dotted lines) Function derived by regression analyses of data from the linear portion of the curve. Results shown are the means ± SD of results obtained from triplicate cultures under each condition.

FIGURE 6. Diabetes-associated changes in vitreous biological activity. Vitreous samples from normal and diabetic swine were evaluated for protein concentration (A), Müller cell contraction-promoting activity per unit protein (B), and total activity (C), which is the product of protein concentration and specific activity. Results are the means ± SD of results obtained from quadruplicate samples.
The positions of the 28.2-kDa and 34.7-kDa molecular weight standards are indicated on the left. Densitometry (B) was performed to quantify reactive band intensity.}

**FIGURE 8.** Carbonic anhydrase isofoms in normal and diabetic swine vitreous. Porcine serum (C) and equal volumes of vitreous from normal (0) and diabetic animals maintained for 30, 60, and 90 days after induction were evaluated in Western blots (A) to detect CA-I and CA-II. The positions of molecular weight standards are indicated on the left. Densitometry (B) was performed to quantify the intensity of anti–CA-I (black bars) and anti–CA-II (gray bars) reactive species.

**TABLE 1.** Diabetes-Associated Changes in IGF System Protein Expression

<table>
<thead>
<tr>
<th></th>
<th>ΔCp 0 Days</th>
<th>ΔΔCp 30 Days (P)</th>
<th>ΔΔCp 60 Days (P)</th>
<th>ΔΔCp 90 Days (P)</th>
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<tbody>
<tr>
<td>IGF-I</td>
<td></td>
<td></td>
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<tr>
<td>Retina</td>
<td>2.86 ± 0.51</td>
<td>−0.03 ± 0.40 (0.656)</td>
<td>0.52 ± 1.15 (0.475)</td>
<td>*1.34 ± 0.29 (0.005)</td>
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<tr>
<td>Iris/ciliary body</td>
<td>2.54 ± 0.31</td>
<td>*0.72 ± 0.16 (0.005)</td>
<td>0.73 ± 0.79 (0.141)</td>
<td>*0.86 ± 0.17 (0.005)</td>
</tr>
<tr>
<td>IGF-II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retina</td>
<td>4.34 ± 0.45</td>
<td>*−0.42 ± 0.36 (0.070)</td>
<td>−0.36 ± 0.45 (0.118)</td>
<td>−0.29 ± 0.45 (0.357)</td>
</tr>
<tr>
<td>Iris/ciliary body</td>
<td>1.02 ± 0.33</td>
<td>−0.04 ± 0.33 (0.857)</td>
<td>*0.38 ± 0.44 (0.032)</td>
<td>*0.42 ± 0.37 (0.007)</td>
</tr>
<tr>
<td>IGFBP-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Retina (negative)</td>
<td>−1.82 ± 0.31</td>
<td>*−0.66 ± 0.52 (0.051)</td>
<td>−0.26 ± 0.65 (0.185)</td>
<td>−0.17 ± 0.32 (0.152)</td>
</tr>
<tr>
<td>Iris/ciliary body</td>
<td>2.72 ± 0.86</td>
<td>*0.86 ± 0.26 (0.031)</td>
<td>1.01 ± 0.63 (0.151)</td>
<td>*0.62 ± 0.28 (0.091)</td>
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<tr>
<td>IGFBP-3</td>
<td></td>
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<tr>
<td>Retina</td>
<td>5.04 ± 0.19</td>
<td>*−0.44 ± 0.13 (0.001)</td>
<td>*−0.34 ± 0.10 (0.005)</td>
<td>−0.11 ± 0.31 (0.177)</td>
</tr>
<tr>
<td>Iris/ciliary body</td>
<td>2.72 ± 0.86</td>
<td>*0.86 ± 0.26 (0.031)</td>
<td>1.01 ± 0.63 (0.151)</td>
<td>*0.62 ± 0.28 (0.091)</td>
</tr>
<tr>
<td>PDGF-B</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Retina</td>
<td>5.05 ± 0.82</td>
<td>−0.40 ± 0.67 (0.155)</td>
<td>−0.78 ± 1.35 (0.084)</td>
<td>0.20 ± 0.51 (0.264)</td>
</tr>
<tr>
<td>Iris/ciliary body</td>
<td>2.52 ± 0.61</td>
<td>−0.29 ± 0.25 (0.157)</td>
<td>−0.48 ± 0.47 (0.161)</td>
<td>−0.07 ± 0.26 (0.376)</td>
</tr>
</tbody>
</table>

Asterisks indicate statistically significant differences compared to normal.
those reported in other animal models. Clinically, there were no gross changes in the retina or its vasculature, and the only significant finding was cataract development. Consistent with other models, we observed increases in retinal Müller cell GFAP content but did not detect reduced GFAP in retinal astrocytes. Although the reasons for this difference are uncertain, there are several functional differences that may be contributory, including the duration and level of hyperglycemia, the use of insulin support, and the animal species.

One of the more intriguing questions raised by this study concerns the origin of the vitreous growth factor activity. We considered increased production of known contraction-promoting growth factors by local ocular tissues as a contributing mechanism but found no evidence of changing message levels to support this premise. We also considered the potential loss of growth factor-attenuating insulin-like growth factor-binding proteins and detected no correlative changes in local message levels or vitreous binding proteins, suggesting that the growth factors are from extraocular sources. Plasma is a rich source of IGF-I and, in light of the established relationship between hyperglycemia and vascular dysfunction, hemorrhage seemed to be a likely mechanism. However, unlike the studies by Gao et al., Western blots to detect vitreous hemorrhage based on the presence of erythrocyte carbonic anhydrase-I were negative. These results led us to conclude that hemorrhage was not a contributing factor to the increases in vitreous activity we observed and that the severe vascular dysfunction that permits hemorrhage was not present in our animals within the 90-day experimental period.

Where, then, do vitreous growth factors come from? In the absence of hemorrhage, it is our speculation that plasma growth factors, such as IGF-I, can cross a blood-vitreous barrier in much the same manner as do other plasma proteins, such as albumin. We also recently presented evidence of blood-vitreous barrier protein selectivity in normal eyes that allows transit of an ~30-kDa IGFBP-3 fragment, but not the slightly larger ~44-kDa intact protein, indicating that the normal barrier is also protein species specific. Surprisingly, the normal mechanism through which plasma proteins gain access to vitreous is poorly understood; hence, there is no mechanistic information available with which to speculate about how hyperglycemia induces changes in blood-vitreous barrier function. Although not confirmed, it is our speculation that plasma protein extravasation normally occurs in the ciliary body and that hyperglycemia induces changes in this organ as well as the retinal blood vessels. Regardless of the mechanism, early hyperglycemia-induced blood-vitreous barrier changes may play a causal major role in the progression of diabetic retinopathy. These data should provide the impetus needed to study functional changes in normal and disease states.

References