Müller cells, the principal glia of the retina, generate tractional forces in response to IGF-I and platelet-derived growth factor and are present in diabetic fibrovascular scar tissues causing traction retinal detachment. While diabetes-associated increases in vitreous IGFs have been reported, paradoxically high concentrations of these same growth factors in normal vitreous suggest the presence of more complex mechanisms regulating growth factor bioavailability. To define diabetes-associated changes in vitreous biological activity, the stimulatory effects of 68 samples were evaluated using Müller cell tractional force generation as a target bioassay. Dose-response profiles were used to calculate vitreous specific activity (per unit protein) and total vitreous activity (per unit volume). Vitreous samples from patients lacking diabetes or other retinal pathology had undetectable or low activities, whereas diabetic retinopathy was associated with 6.9- and 8.7-fold increases in vitreous specific and total activities, respectively. Secondary analyses revealed no activity differences associated with patient sex, age, or the presence of vitreous hemorrhage. However, compared with diabetes alone, the presence of proliferative diabetic retinopathy was associated with additional 2.3-fold increases in vitreous specific and total activities. Vitreous dose-response assays performed with and without growth factor–neutralizing antibodies enable attribution of vitreous activity to IGFs (53.9%) and, to a lesser extent, platelet-derived growth factors (14.5%). Because the observed increases in vitreous growth factor activity grossly exceed the reported increases in growth factor concentration, these data indicate that diabetes-associated changes in vitreous biological activity involve more complex biochemical changes that ultimately yield increased growth factor bioavailability and/or Müller cell responsiveness. Diabetes 53: 2428–2435, 2004

Diabetes affects nearly 16 million people in the U.S. alone, and diabetic retinopathy, the principal ocular complication, remains the leading cause of vision loss between the second and seventh decades of life (1–3). Diabetes-associated changes in retinal physiology precede clinically detectable changes in the retinal vasculature, and experimental evidence from studies of human diabetes and animal models of hyperglycemia suggest that these early changes involve Müller cells, the principal retinal glia. Changes in electoretinogram b-waves (attributed to Müller cells and/or bipolar cells) and functional impairment of Müller cell glutamate transporter systems are evident within 2 weeks of hyperglycemia in streptozotocin-induced diabetic rats (4–6). Sustained diabetes is sufficient to induce dramatic changes in the Müller cell phenotype, including cellular hyperplasia, decreased glutamine synthetase activity, increased inducible nitric oxide synthetase activity, and de novo expression of the glial fibrillary acidic protein, which is thought to reflect physiological stress or damage (5,7–10). In response to ischemia, Müller cells express vascular endothelial cell growth factor, the principal angiogenic growth factor, leading to retinal neovascularization characteristic of advanced diabetic retinopathy (11–13). Finally, Müller cells have been detected in fibrovascular scar tissue associated with proliferative diabetic retinopathy (PDR), a late-stage complication leading to traction retinal detachment and vision loss (14–16).

Studies of Müller cells isolated from normal human and porcine retina revealed an extraordinary capacity for changes in cell phenotype that include de novo expression of the myoid maker smooth muscle actin and, with this, the capacity to generate tractional forces (17–19). Interestingly, Müller cell tractional force generation is not constitutive but is stimulated by the presence of certain exogenous growth factors, including members of the platelet-derived growth factor (PDGF) family and IGF-I (18,19). Although vitreous PDGF reportedly increases in diabetes, the mean reported concentration of 100 pg/ml (20) is below the threshold of Müller cell sensitivity (18,19) and seems unlikely to drive tractional force generation. Vitreous concentrations of IGF-I also increase diabetes to mean concentrations ranging from 1.4 to 6.3 ng/ml (21–27). In contrast to PDGF, these levels are well above the threshold of Müller cell sensitivity (18,19), suggesting that IGF-I is present in diabetic vitreous in quantities sufficient to drive Müller cell fibrocontractive responses. Although the evidence in support of IGF-I as a vitreal Müller cell stimulus is compelling, other observations suggest that this relationship may be more complex. Vitreous IGF-I levels reported for normal populations and control sub-

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FBS, fetal bovine serum; IGFBP, IGF binding protein; PDGF, platelet-derived growth factor; PDR, proliferative diabetic retinopathy; TGF, transforming growth factor.

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jects without diabetic retinopathy range from 0.25 to 2.85 ng/ml (21–23,25–27) and are also above the threshold of Müller cell sensitivity. However, direct measurements of Müller cell responses to normal vitreous revealed little or no stimulatory activity, suggesting that IGF-I activity is in some way controlled or attenuated in normal vitreous (28). With this paradoxical relationship in mind, several important questions about the potential role of vitreous IGF-I in diabetic retinopathy remain unanswered. Is vitreous biological activity to which Müller cells respond actually increased in PDR? If so, can this activity be attributed to IGF-I or is the activity of this growth factor attenuated, as appears to be the case in normal vitreous? Adding to the potential complexity of this relationship are reports of vitreous IGF-II concentrations 10- to 30-fold higher than those of IGF-I (25–27). While the effects of IGF-II on Müller cell contractile force generation are unknown, even modest activity by this ligand would further increase the levels of unaccounted for biological activity in normal vitreous. Studies performed to address these questions directly examined the relationship between diabetes and its ocular complications on vitreous biological activity using Müller cell contractile force generation as a biologically relevant target assay. Additionally, the effects of IGF-II and growth factor–neutralizing antibodies on vitreous biological activity were examined to assess the contributions of IGF- and PDGF-related species.

RESEARCH DESIGN AND METHODS

Isolation and culture of porcine Müller cells. Müller cells were isolated from papain and DNase-dissociated retinas and maintained in culture as previously described, with minor modifications (18). 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 repeated trituration. Supernatants enriched with morphologically recognizable Müller cells were combined and plated in growth medium composed of Dulbecco’s minimum essential medium supplemented with 20 mmol/l N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid and 10% fetal bovine serum (FBS). The cells were permitted to adhere for 30 to 60 min at 37°C, after which the nonadherent population was removed and the medium replaced with fresh growth medium. By indirect immunofluorescence, these cells were more than 95% positive for carbonic anhydrase II, cellular retinaldehyde binding protein, glial fibrillary acidic protein, and vimentin (17,18). The cells were maintained at 37°C with growth medium changes every 3–4 days until confluence, when they were released with 0.05% trypsin and 0.53 mmol/l ethylenediaminetetraacetic acid and replated in fresh growth medium. For the experiments described here, cells were used between passages 3 and 9.

Vitreous samples and patient information. Vitreous samples were collected from patients undergoing vitreectomy at the Eye Foundation Hospital (Birmingham, AL) in the normal course of treatment for their particular disorder. While the majority of the vitreous samples were obtained from patients undergoing vitreectomy for diabetes-related ocular complications, some samples were also obtained from patients with and without diabetes, requiring vitreous removal for reasons unrelated to retinal pathology, including vitreous opacities, intraocular lens placement, and removal of an intraocular lens–derived foreign body. The collection protocol was reviewed by the Institutional Board for Human Use at the University of Alabama at Birmingham, and informed consent was obtained after explanation of the nature and possible consequences of the procedure. Samples were removed during infusion with balanced salt solution alone or during infusion with balanced salt solution modified by the addition of sodium fluorescein to enable quantification of vitreous dilution as described previously (28,29). All samples were maintained on ice until transported to the laboratory, centrifuged to remove debris, and assayed for protein content by optical density at 280 nm (optical density units [ODU]), and, when applicable, samples and the infusion solution were assessed for fluorescein content to determine the extent of vitreous dilution. All samples were maintained at –80°C until use in this study. Data for this clinical presentation were provided by the operating surgeon on the day of the procedure without knowledge of sample physical characteristics or biological activity. In addition to general information, the surgeons were asked to provide binary assessments of the presence or absence of diabetic retinopathy, PDR, and vitreous hemorrhage.

Contraction assays. Collagen gels were prepared from native type I collagen isolated from rat-tail tendons by limited pepsin digestion and sequential salt precipitation (30). Acid-soluble collagen dissolved in 12 mmol/l HCl was adjusted to physiological pH and ionic strength using 10× PBS (0.1 mol/l Na(HPO)_, 1.5 mol/l NaCl), and 0.1 mol/l NaOH while maintained on ice. Alqouts (0.2 ml) of the collagen solution were added to circular scores on the bottom of 24-well tissue culture plates and incubated at 37°C for 90 min 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 (PA-7511, Sigma). Alqouts of cells (50 µl) suspended in contraction medium at 400,000 cells per milliliter or 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. Repeated measurements of this type indicate that these values are reproducible to 1.25% of the original gel thickness (31,32).

Measurement of contraction-stimulating activity. Vitreous samples were thawed, centrifuged to remove insoluble material, and serially diluted with contraction medium to yield a range of vitreous protein concentrations beginning at 0.25 ODU. Each assay also contained a similar dose-response series using a single lot of PBS, which also served as a positive control. Müller cell contractile responses to these solutions were routinely assessed after 6 h of incubation, and linear regression analyses were performed on the dose-response data to yield a slope representing the percent contraction per unit protein. Calculated vitreous specific activities were normalized to PBS activities to control for day-to-day variations in cell responsiveness and enable direct comparisons of data generated in different experiments.

Growth factor neutralization assays. A single serial dilution of vitreous protein, prepared as above, was subdivided into three sets of tubes to which mouse monoclonal anti–IGF-I (clone SM1.2; Upstate Biotechnology, Lake Placid, NY), rabbit anti–PDGF (R&D Systems, Minneapolis, MN), or PBS was added to yield final antibody concentrations of 10, 20, and 0 µg/ml, respectively. They were preincubated for 30 min at room temperature before use in the assay. Vitreous activities were assessed after 24 h of incubation and calculated as described above. Percent inhibition was calculated by dividing the activity of each antibody-inhibited sample by that of the vehicle alone, subtracting this value from 1, and then multiplying by 100.

Reagents. Tissue culture media and sera were obtained from Life Technologies (Rockville, MD). Recombinant human growth factors obtained from commercial suppliers included IGF-I (GroPeP), IGF-II (GroPeP), and PDGF-AB (Upstate Biotechnology). Other chemicals and reagents were obtained from Sigma.

RESULTS

Diabetic vitreous stimulates extracellular matrix contraction by Müller cells. To determine if vitreous fluids from diabetic patients contain extracellular matrix contraction-stimulating activity, the effects of varying vitreous protein concentrations were tested on Müller cells attached to collagen gels and compared with control cultures containing PBS, a known source of contraction-promoting activity. Negative control cultures were incubated in medium containing BSA. Data presented in Fig. 1 demonstrate the effects of two vitreous samples, one removed from a patient with PDR with vitreous hemorrhage (sample 1) and the second from a patient with diabetic retinopathy without evidence of proliferation or hemorrhage (sample 2). Müller cell responses measured after 24 h of incubation demonstrate protein concentration–dependent increases in extracellular matrix contrac-
tion in both samples as well as FBS. Müller cell responses to the different levels of stimuli were also reflected by differences in cell morphology assessed after 6 h of incubation in the highest concentration of each sample. Cells exposed to 0.25 ODU/ml FBS or 0.58 ODU/ml of sample 1 were similar in that active gel contraction was evident from the lines of tension radiating from cell processes (Fig. 2A and B, respectively). Müller cell morphologies in 1.24 ODU/ml of sample 2 or 1 mg/ml BSA were also similar in that the cells remained rounded with limited evidence of active matrix contraction (Fig. 2C and D, respectively).

**Correlation of vitreous contraction-stimulating activity and protein concentration with clinical presentation.** Vitreous activities measured in these assays were readily quantified by regression analyses, which provided the percent contraction per unit of vitreous protein or specific activity of each sample. For the two vitreous samples presented in Fig. 1 (dotted lines), these analyses yielded 84.2 and 13.4% contraction per ODU protein, respectively. To enable direct comparisons of these data to vitreous samples analyzed in other assays, these data were also normalized to the specific activity of the FBS-positive control (272.7% contraction per ODU) because this would eliminate day-to-day variation in cell responsiveness as a confounding variable. In the examples presented in Fig. 1, FBS-normalized specific activities of samples 1 and 2 were calculated to be 0.31 and 0.05, respectively.

To examine the relationship between diabetes and diabetes-associated complications on vitreous biological activity, this same approach was used to assess the specific activities of 64 vitreous samples removed from patients diagnosed as having diabetic retinopathy and undergoing surgery requiring removal of vitreous fluids. These were compared with four vitreous samples removed from patients with vitreous opacities, but lacking retinal pathology, as an estimate of normal activity in samples collected using the same procedures. The mean specific activities of vitreous fluids removed from patients with diabetic retinopathy were approximately sevenfold higher than control

**FIG. 1.** Müller cell extracellular matrix contraction is stimulated by diabetic vitreous. Müller cells attached to collagen gels were incubated in medium containing varying concentrations of vitreous protein sample 1 ( ), sample 2 ( ), or FBS as a positive control ( ). Data are the mean and SD of results obtained after 24 h of incubation from triplicate cultures under each condition. The function resulting from regression analyses of each dose-response profile is represented by the dotted lines.

**FIG. 2.** Müller cell morphologies during extracellular matrix contraction in response to vitreous and serum proteins as described in Fig. 1. Müller cells attached to collagen gels were incubated in medium containing 0.25 ODU/ml FBS as a positive control (A), 0.58 ODU/ml sample 1 (B), 1.24 ODU/ml sample 2 (C), or BSA alone (D). Phase-contrast photomicrographs were taken after 6 h of incubation. Arrows indicate regions of collagen matrix under tension. Original magnification = 167×.
TABLE 1
Summary of vitreous activities and characteristics

<table>
<thead>
<tr>
<th>Category</th>
<th>Specific activity*</th>
<th>P†</th>
<th>Vitreous protein‡</th>
<th>P†</th>
<th>Vitreous activity§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal vs.</td>
<td>0.06 ± 0.04 (4)</td>
<td></td>
<td>6.40 ± 1.64 (4)</td>
<td></td>
<td>0.39 ± 0.28</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0.42 ± 0.30 (64)</td>
<td>0.01</td>
<td>8.16 ± 3.30 (48)</td>
<td>0.08</td>
<td>3.41 ± 2.84</td>
</tr>
<tr>
<td>Female vs.</td>
<td>0.40 ± 0.27 (31)</td>
<td></td>
<td>7.97 ± 3.32 (24)</td>
<td></td>
<td>3.16 ± 2.51</td>
</tr>
<tr>
<td>Male</td>
<td>0.45 ± 0.34 (33)</td>
<td>0.26</td>
<td>8.06 ± 3.02 (24)</td>
<td>0.37</td>
<td>3.16 ± 2.51</td>
</tr>
<tr>
<td>22–39 years</td>
<td>0.57 ± 0.32 (10)</td>
<td></td>
<td>5.73 ± 2.19 (5)</td>
<td></td>
<td>3.25 ± 2.19</td>
</tr>
<tr>
<td>40–49 years</td>
<td>0.49 ± 0.31 (8)</td>
<td></td>
<td>5.96 ± 3.13 (9)</td>
<td></td>
<td>2.93 ± 2.41</td>
</tr>
<tr>
<td>50–59 years</td>
<td>0.31 ± 0.23 (19)</td>
<td></td>
<td>9.19 ± 3.54 (14)</td>
<td></td>
<td>2.82 ± 2.39</td>
</tr>
<tr>
<td>60–69 years</td>
<td>0.44 ± 0.36 (16)</td>
<td></td>
<td>8.81 ± 2.12 (11)</td>
<td></td>
<td>3.87 ± 3.31</td>
</tr>
<tr>
<td>70–80 years</td>
<td>0.41 ± 0.22 (10)</td>
<td>0.25</td>
<td>8.32 ± 3.00 (9)</td>
<td>0.05</td>
<td>3.83 ± 2.42</td>
</tr>
<tr>
<td>Hemorrhage (−) vs.</td>
<td>0.45 ± 0.31 (20)</td>
<td></td>
<td>8.47 ± 2.82 (8)</td>
<td></td>
<td>3.82 ± 2.89</td>
</tr>
<tr>
<td>Hemorrhage (+)</td>
<td>0.40 ± 0.30 (44)</td>
<td>0.29</td>
<td>8.10 ± 3.43 (40)</td>
<td>0.39</td>
<td>3.27 ± 2.81</td>
</tr>
<tr>
<td>PDR (−) vs.</td>
<td>0.19 ± 0.07 (5)</td>
<td></td>
<td>8.56 ± 2.53 (4)</td>
<td></td>
<td>1.59 ± 0.76</td>
</tr>
<tr>
<td>PDR (+)</td>
<td>0.44 ± 0.31 (59)</td>
<td>0.04</td>
<td>8.12 ± 3.36 (44)</td>
<td>0.40</td>
<td>3.55 ± 2.90</td>
</tr>
<tr>
<td>PDR (+) hemorrhage (−) vs.</td>
<td>0.51 ± 0.30 (17)</td>
<td>0.07</td>
<td>8.08 ± 3.04 (6)</td>
<td></td>
<td>4.09 ± 2.86</td>
</tr>
<tr>
<td>PDR (+) hemorrhage (+)</td>
<td>0.41 ± 0.31 (42)</td>
<td></td>
<td>8.13 ± 3.41 (38)</td>
<td>0.49</td>
<td>3.33 ± 2.87</td>
</tr>
</tbody>
</table>

Data are means ± SD or means ± SD (n) unless otherwise indicated. *Specific activity is the percent contraction per ODU protein, normalized to the experimental FBS standard as described in Fig. 1. †Unpaired Student’s t tests were used to evaluate differences attributed to sample characteristics except age-related differences, which were evaluated using single-factor ANOVA. ‡Vitreous protein is the vitreous sample protein concentration divided by the fractional percent vitreous as determined by fluorescein dilution. §Vitreous activity is the product of specific activity and vitreous protein. Norm refers to vitreous samples removed from nondiabetic patients with vitreous opacities and without other retinal pathology.

subjects, and this difference was significant (P < 0.02) by an independent t test (Table 1).

To explore the relationship between vitreous biological activity and patient sex, age, vitreous hemorrhage, and proliferative disease, the results from samples grouped according to these features were examined. Although the mean specific activities in each group were significantly elevated above those of the control subjects (P < 0.03), samples grouped according to sex were not significantly different (P = 0.26). Consideration of age as a variable revealed an interesting trend suggestive of elevated specific activities in samples from younger compared with older individuals, but these differences were not significant by single-factor ANOVA (P = 0.25). Because two of the growth factors in serum are potent promoters of Müller cell contractile activity, it was surprising that samples grouped according to the presence or absence of vitreous hemorrhage revealed little variation in mean specific activities and that these samples were not significantly different (P = 0.29). This was not the case, however, with samples grouped according to the presence or absence of PDR. The mean specific activity of the population with PDR was significantly higher than the group without PDR (P = 0.04). Finally, the influence of hemorrhage on vitreous relative activity was reexamined in the higher-activity sample population diagnosed as having active proliferative disease. As before, the mean activity of samples without hemorrhage was slightly higher than that of samples with hemorrhage, but these differences were not considered significant (P = 0.07).

The subset of vitreous samples collected using the fluorescein-dilution method enabled determinations of undiluted vitreous protein concentration. While the mean protein concentrations were higher in diabetic compared with control samples (Table 1), these differences lacked statistical significance (P = 0.08). Similarly, there were no significant differences from normal when the other variables, including sex, age, vitreous hemorrhage, or PDR, were evaluated. Interestingly, there was another age-related trend suggestive of lower protein concentrations in the younger compared with older populations. Nonetheless, statistical analyses of the sample populations by single-factor ANOVA revealed that these variations approached, but did not attain, statistical significance (P = 0.052).

Finally, the products of vitreous specific activity and protein concentration were calculated to provide an estimate of total vitreous activity in the undiluted state. Compared with the control group, the smallest increase was approximately fourfold in the group lacking proliferative complications. Undiluted vitreous activity in the remaining groups was elevated from 7- to 10-fold over control subjects. Together, the assessed changes in vitreous activity, vitreous protein, and total activity estimates indicate that diabetic retinopathy is associated with substantial increases in growth factors to which Müller cells respond.

Analysis of vitreous growth factors. To identify the growth factors responsible for diabetes-associated increases in vitreous contraction-stimulating activity, the contributions of IGF and PDGF were evaluated because members of each family are known to stimulate tractional force generation by Müller cells. Because vitreous IGF-II is reportedly increased in diabetes but its effects on Müller cell contraction are unknown, the potency of this growth factor was first evaluated. Müller cells attached to collagen gels were incubated in medium containing varying concentrations of IGF-II and, for comparison, IGF-I. The dose-response profiles obtained after 24 h of incubation indicate that IGF-II, on a per-molar basis, is approximately half as active as IGF-I (Fig. 3). However, half-maximal response to IGF-II was achieved at ~0.2 nmol/l, indicating that IGF-II is nonetheless a potent Müller cell stimulus.

Also examined were the effects of growth factor–neutralizing antibodies on IGF-I, IGF-II, and PDGF. Of particular interest was whether the anti-IGF–neutralizing antibody raised against IGF-I would also modulate IGF-II
activity. Müller cells attached to collagen gels were incubated in 1 nmol/l IGF-I, 1 nmol/l IGF-II, or 5 nmol/l PDGF-AB alone or combined with 10 μg/ml anti-IGF-I or 20 μg/ml anti-PDGF. The results obtained after 24 h of incubation indicated that anti-IGF-I inhibited IGF-II and IGF-I activities but had no effect on PDGF (Fig. 4A). Similarly, anti-PDGF attenuated PDGF-AB activity but did not substantially alter IGF-I or IGF-II activities. To determine the relative effects of the neutralizing antibody on the two IGF species, Müller cells attached to collagen gels were incubated in 1 nmol/l IGF-I or 1 nmol/l IGF-II with varying concentrations of anti-IGF-1-neutralizing antibody. The results obtained after 24 h revealed dose-dependent inhibition of IGF-I and IGF-II (Fig. 4B). In both cases, maximal inhibition was achieved at a concentration \(= 10 \mu g/ml\).

Finally, to assess the contribution of IGF and PDGF to vitreous biological activity, the effects of these growth factor–neutralizing antibodies on vitreous contraction-promoting activity were examined. Müller cells attached to collagen gels were incubated in identical serial dilutions of vitreous protein to which anti–IGF-I (10 μg/ml), anti-PDGF (20 μg/ml), or an equivalent amount of vehicle alone (PBS) was added. The results obtained from one vitreous sample are presented in Fig. 5 and serve to illustrate antibody effects. Müller cells incubated for 24 h in vitreous protein alone were stimulated in a dose-dependent fashion. Addition of anti-PDGF did not significantly alter the dose-response profile, while addition of anti–IGF-I reduced Müller cell responses. Regression analyses of these data yielded specific activities of 39.6, 36.3, and 18.1% contraction per ODU vitreous protein, yielding 8.3 and 54.2% inhibition for anti-PDGF and anti–IGF-I, respectively. These analyses were performed on a total of 10 samples with moderate to high levels of activity from two diagnostic categories, including PDR with \(n = 6\) and PDR without \(n = 4\) vitreous hemorrhage. Although there was substantial variation in the degree of inhibition observed, on average, IGF-related species accounted for the majority of contraction-stimulating activity (Table 2). In contrast, PDGF-related species accounted for lesser amounts of the biological activity. Consideration of the samples with and without hemorrhage as separate populations indicated that IGF-related species accounted for a larger proportion of the activity in the absence of hemorrhage, although these differences lacked statistical significance.

**DISCUSSION**

The first and most important goal of this study was to determine if vitreous fluids from patients with diabetic retinopathy possess increased capacity to stimulate tractive force generation by Müller cells. With respect to this one issue, the results of our analyses were relatively straightforward in that vitreous from patients with diabetic retinopathy contained approximately sevenfold higher stimulatory activity per unit protein and ninefold higher total vitreous activity than that of the control population without retinal pathology. Thus, in diabetic retinopathy, the appropriate stimuli are present to stimu-

![FIG. 3. Dose response of extracellular matrix contraction by Müller cells stimulated with IGF-I and IGF-II. Müller cells attached to collagen gels were incubated in serum-free medium containing varying concentrations of IGF-I (○) or IGF-II (●). Data are the mean and SD of results from triplicate cultures under each condition after 24 h of incubation.](image)

![FIG. 4. Effects of growth factor–neutralizing antibodies on IGF-I, IGF-II, and PDGF. Müller cells attached to collagen gels were incubated in serum-free medium containing 1 nmol/l IGF-I, 1 nmol/l IGF-II, or 5 nmol/l PDGF combined with 10 μg/ml anti-IGF, 20 μg/ml anti-PDGF, or antibody vehicle alone. The results presented in A are the mean and range of results obtained from duplicate cultures under each condition after 24 h of incubation. In a separate experiment, Müller cells attached to collagen gels were incubated in medium containing 1 nmol/l IGF-I (●) or 1 nmol/l IGF-II (○), and the concentrations of anti-IGF-I are indicated in B. Data are the mean and range of results obtained from duplicate cultures under each condition after 24 h of incubation.](image)
The trend toward higher specific activities of which were negative. Although there was an interesting examination of other variables including sex and age, both of which were negative. The results presented are the mean and range of results obtained from duplicate cultures under each condition after 24 h of incubation.

The results presented are the mean and range of results obtained from duplicate cultures under each condition after 24 h of incubation. The consistent absence of substantial stimulatory activity in control populations of vitreous samples suggests that IGF-I and IGF-II concentrations are below the limits of detection in the population without this complication. Importantly, the number of samples characterized as lacking proliferation was small, and while the differences are statistically significant, this conclusion is drawn with caution.

The presence of IGF-related activity in diabetic vitreous suggests that IGF-I and/or IGF-II are present in concentrations above the threshold of sensitivity reflected in the dose-response curves presented in Fig. 4 (−0.04 nmol/l IGF-I and −0.1 nmol/l IGF-II). While this study did not measure vitreous growth factor concentrations, a number of published studies rigorously examined this issue in diabetes. Two recent studies by Spranger et al. (26,27) examined diabetes-associated changes in vitreous IGF-I and IGF-II in conjunction with assessments of changing plasma levels and the effects of retinal photocoagulation. The first study included radioactive immunoassays of 39 vitreous samples from patients with PDR and reported mean vitreous concentrations of 2.3 and 37.9 ng/ml for IGF-I and IGF-II, respectively (26). A second study included eight patients with PDR and reported similar values of 1.1 ng/ml IGF-I and 29.3 ng/ml IGF-II (27). These concentrations translate into vitreous growth factor molarities ranging from 0.15 to 0.89 nmol/l IGF-I and 3.1 to 6.0 nmol/l IGF-II, either of which is sufficient to induce Müller cell responses in our assay system (18,19). Thus, our observations of vitreous biological activity attributable to IGF-related species are consistent with reports of growth factor concentrations in diabetic vitreous.

The consistent absence of substantial stimulatory activity in control populations of vitreous samples suggests that IGF-I and IGF-II concentrations are below the limits of Müller cell sensitivity. As mentioned earlier, studies comparing growth factor concentrations in nondiabetic popula-

### TABLE 2
Summary of anti–growth factor neutralization data

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific activity</th>
<th>r corr</th>
<th>Specific activity</th>
<th>r corr</th>
<th>Specific activity</th>
<th>r corr</th>
<th>% Inhibition</th>
<th>Specific activity</th>
<th>r corr</th>
<th>% Inhibition</th>
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<tr>
<td>1</td>
<td>36.5</td>
<td>0.91</td>
<td>11.3</td>
<td>0.95</td>
<td>69.0</td>
<td></td>
<td></td>
<td>32.0</td>
<td>0.97</td>
<td>12.3</td>
</tr>
<tr>
<td>2</td>
<td>72.0</td>
<td>0.97</td>
<td>21.4</td>
<td>0.81</td>
<td>70.2</td>
<td></td>
<td></td>
<td>62.1</td>
<td>0.94</td>
<td>13.7</td>
</tr>
<tr>
<td>3</td>
<td>25.3</td>
<td>0.96</td>
<td>9.9</td>
<td>0.93</td>
<td>61.0</td>
<td></td>
<td></td>
<td>18.7</td>
<td>0.97</td>
<td>26.2</td>
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<td>4</td>
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<td>0.99</td>
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<td>0.99</td>
<td>8.3</td>
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<td>5</td>
<td>67.2</td>
<td>0.97</td>
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**PDR (n = 10)**

54.04 ± 11.16 14.86 ± 8.42

**PDR without hemorrhage (n = 4)**

63.62 ± 6.50 15.13 ± 6.66

**PDR with hemorrhage (n = 6)**

47.66 ± 8.80 14.68 ± 9.41

Data are means ± SD unless otherwise indicated. *Specific activity refers to calculated percent contraction per ODU protein. †r corr, correlation coefficient. ‡% Inhibition is calculated as 100 − specific activity plus antibody/specific activity without antibody.
DIABETIC VITREOUS STIMULATES MÜLLER CELLS

systems examined in this study (IGF and PDGF) (18,19), transforming growth factor (TGF-β) species (TGF-β1 and TGF-β2) (43,44), a subset of the endothelins (E1, E2, VIC) (32), interleukins-4 and -13 (45), lysophosphatidic acid, and sphingosine 1-phosphate (46). Although we have determined that Müller cells are unresponsive to physiologically relevant concentrations of the TGF-β species and endothelins (18,19), no information is currently available about the effects or potential involvement of the interleukins or lipid mediators. It is also possible that other yet unidentified promoters contribute to vitreous biological activity. Also, given the complexity of tractional force generation as a cellular process, the effects of these promoters may involve modulation of any of the individual processes involved, such as cell adhesion or process extension (47).

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