Changes in IGF Activities in Human Diabetic Vitreous

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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

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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.

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 electroretinogram 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 marker α 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-

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 tractional 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 tractional 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 30–60 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 vitrectomy 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 vitrectomy for diabetes-related ocular complications, 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 stored at −80°C until use in this study. Data concerning 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_2HPO_4, 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 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 (#A-7511; Sigma). Aliquots of cells (50 μl) suspended in contraction medium at 400,000 cells per milliliter 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. 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 FBS, 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 FBS 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 FBS, 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-

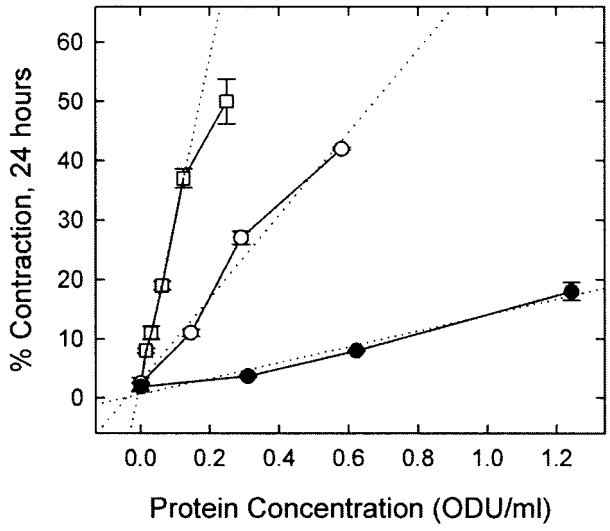

FIG. 1. Müller cell extracellular matrix contraction is stimulated by diabetic vitreous. Müller cells attached to collagen gels were incubated in medium containing varying concentrations of vitreous protein sample 1 (○), sample 2 (●), or FBS as a positive control (□). Data are the mean and SD of results 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.

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. 2*A* 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. 2*C* 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

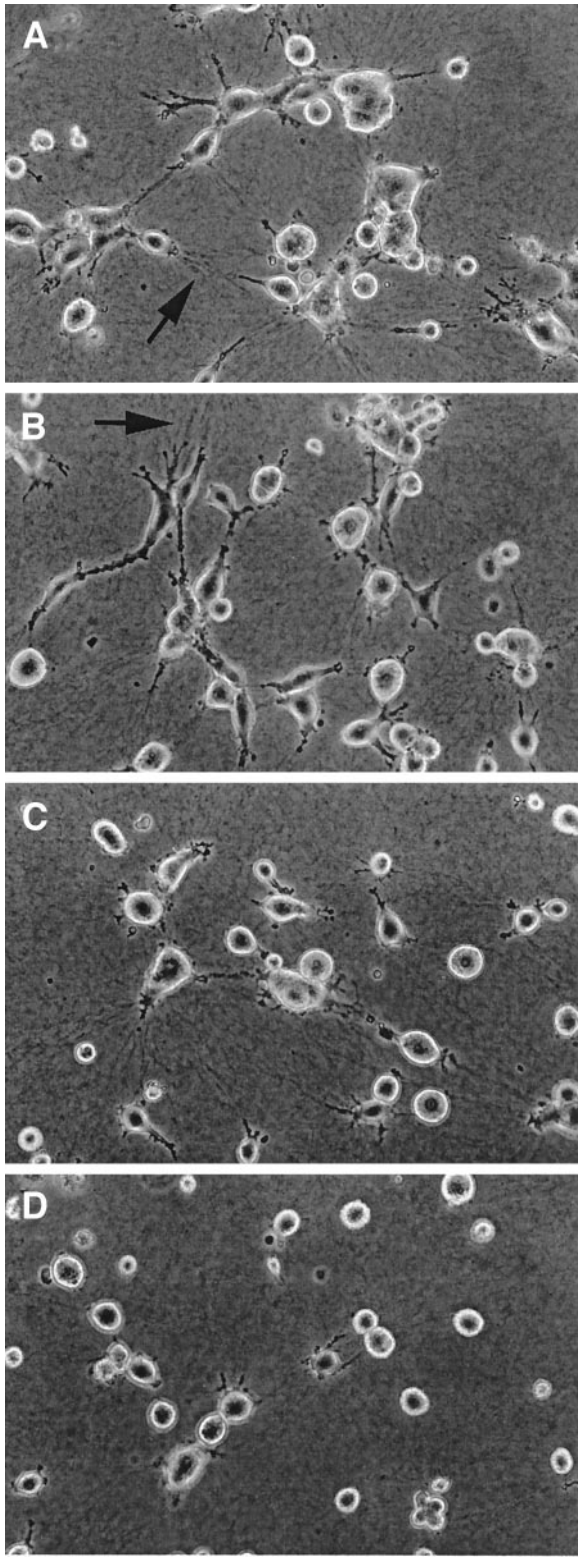

FIG. 2. Müller cell morphologies during extracellular matrix contraction in response to vitreous and serum proteins as described in Fig. 1. Müller cells attached to collagen gels were incubated in medium containing 0.25 ODU/ml FBS as a positive control (*A*), 0.58 ODU/ml sample 1 (*B*), 1.24 ODU/ml sample 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×.

using the same procedures. The mean specific activities of vitreous fluids removed from patients with diabetic retinopathy were approximately sevenfold higher than control

TABLE 1
Summary of vitreous activities and characteristics

Category	Specific activity*	P†	Vitreous protein‡	P†	Vitreous activity§
Normal‖ vs.	0.06 ± 0.04 (4)		6.40 ± 1.64 (4)		0.39 ± 0.28
Diabetes	0.42 ± 0.30 (64)	**0.01**	8.16 ± 3.30 (48)	0.08	3.41 ± 2.84
Female vs.	0.40 ± 0.27 (31)		7.97 ± 3.32 (24)		3.16 ± 2.51
Male	0.45 ± 0.34 (33)	0.26	8.06 ± 3.02 (24)	0.37	3.59 ± 3.02
22–39 years	0.57 ± 0.32 (10)		5.73 ± 2.19 (5)		3.25 ± 2.19
40–49 years	0.49 ± 0.31 (8)		5.96 ± 3.13 (9)		2.93 ± 2.41
50–59 years	0.31 ± 0.23 (19)		9.19 ± 3.54 (14)		2.82 ± 2.39
60–69 years	0.44 ± 0.36 (16)		8.81 ± 2.12 (11)		3.87 ± 3.31
70–80 years	0.41 ± 0.22 (10)	0.25	9.32 ± 3.00 (9)	0.05	3.83 ± 2.42
Hemorrhage (−) vs.	0.45 ± 0.31 (20)		8.47 ± 2.82 (8)		3.82 ± 2.89
Hemorrhage (+)	0.40 ± 0.30 (44)	0.29	8.10 ± 3.43 (40)	0.39	3.27 ± 2.81
PDR (−) vs.	0.19 ± 0.07 (5)		8.56 ± 2.53 (4)		1.59 ± 0.76
PDR (+)	0.44 ± 0.31 (59)	**0.04**	8.12 ± 3.36 (44)	0.40	3.55 ± 2.90
PDR (+) hemorrhage (−) vs.	0.51 ± 0.30 (17)		8.08 ± 3.04 (6)		4.09 ± 2.86
PDR (+) hemorrhage (+)	0.41 ± 0.31 (42)	0.07	8.13 ± 3.41 (38)	0.49	3.33 ± 2.87

Data are means ± SD or means ± SD (n) unless otherwise indicated. *Specific activity represents 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. P values in bold were considered significant. ‡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. ‖Normal 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

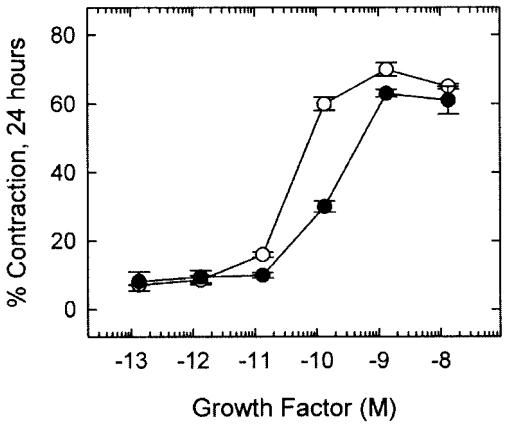

FIG. 3. Dose response of extracellular matrix contraction by Müller cells stimulated with IGF-I and IGF-II. Müller cells attached to collagen gels were incubated in serum-free medium containing varying concentrations of IGF-I (○) or IGF-II (●). Data are the mean and SD of results from triplicate cultures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

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. 4*A*). 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-I–neutralizing antibody. The results obtained after 24 h revealed dose-dependent inhibition of IGF-I and IGF-II (Fig. 4*B*). In both cases, maximal inhibition was achieved at a concentration ≥10 μg/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

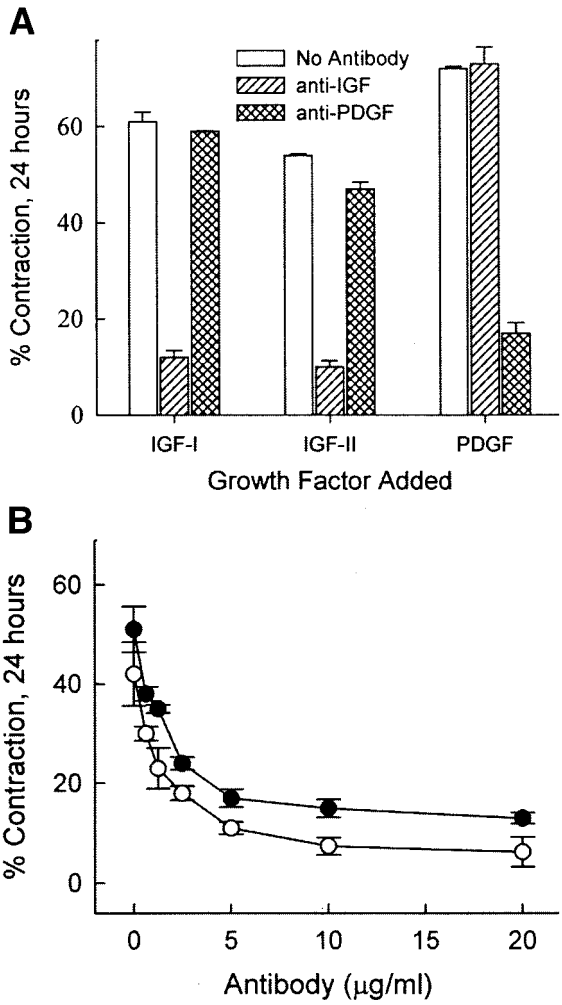

FIG. 4. Effects of growth factor–neutralizing antibodies on IGF-I, IGF-II, and PDGF. Müller cells attached to collagen gels were incubated in serum-free medium containing 1 nmol/l IGF-I, 1 nmol/l IGF-II, or 5 nmol/l PDGF combined with 10 μg/ml anti-IGF, 20 μg/ml anti-PDGF, or antibody vehicle alone. 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 are indicated in *B*. Data are the mean and range of results obtained from duplicate cultures under each condition after 24 h of incubation.

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 tractional 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-

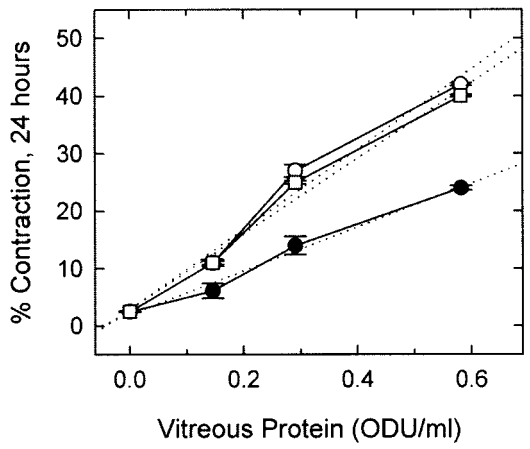

FIG. 5. Inhibition of diabetic vitreous-stimulated extracellular matrix contraction with neutralizing antibodies against IGF and PDGF. Müller cells attached to collagen gels were incubated in medium containing identical dilutions of a single vitreous sample, combined with antibody vehicle alone (○), 10 μg/ml anti-IGF (●), or 20 μg/ml anti-PDGF (□). The results presented are the mean and range of results obtained from duplicate cultures under each condition after 24 h of incubation.

late Müller cell tractional force generation, adding impetus to current and future investigations into avenues of intervention. Secondary analyses of these results included examination of other variables including sex and age, both of which were negative. Although there was an interesting trend toward higher specific activities and lower protein concentrations in younger patients, these results lacked overall statistical significance. The influence of vitreous hemorrhage on specific and total vitreous activities was also insignificant, which was surprising when considering that both relevant growth factor families are present in whole blood and capable of stimulating Müller cell responses (18). In contrast, significant differences were detected when biological activities were examined in relation to the presence of proliferative complications. Vitreous specific and total activities are two- to threefold higher when PDR is a diagnostic feature compared with 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 were generally consistent with several other studies of diabetic vitreous, reporting IGF-I concentrations of 1.4 (22), 3.8 (24), 5.0 (25), 6.3 (23), and 6.8 ng/ml (21) and IGF-II concentrations of 23 (23) and 45 (25) ng/ml. 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 popu-

TABLE 2
Summary of anti–growth factor neutralization data

			Anti-IGF			Anti-PDGF		
Sample	Specific activity*	r_{corr}†	Specific activity	r_{corr}	% Inhibition‡	Specific activity	r_{corr}	% Inhibition
1	36.5	0.91	11.3	0.95	69.0	32.0	0.97	12.3
2	72.0	0.97	21.4	0.81	70.2	62.1	0.94	13.7
3	25.3	0.96	9.9	0.93	61.0	18.7	0.97	26.2
4	39.6	0.99	18.1	0.99	54.2	36.3	0.99	8.3
5	67.2	0.97	22.9	0.94	65.9	56.3	0.96	16.7
6	12.6	0.98	7.2	0.98	43.3	10.9	0.98	13.9
7	137.4	0.97	87.9	0.96	40.4	96.7	0.86	29.6
8	62.5	0.94	37.5	0.91	40.0	62.3	0.96	0.2
9	34.7	0.93	18.1	0.82	47.8	32.2	0.95	7.2
10	77.3	0.97	39.9	0.96	48.4	61.1	0.97	20.9
					% Inhibition + anti-IGF			% Inhibition + anti-PDGF
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.

lations reported values of 0.3 (22), 0.7 (26,27), 1.4 (25), and 2.7 ng/ml IGF-I (23) and 18.1 (23), 21.3 (26,27), and 25 ng/ml IGF-II (25). In these examples, vitreous growth factor molarities range from 0.03 to 0.35 nmol/l IGF-I and 2.4 to 3.3 nmol/l IGF-II. When these data are considered in light of demonstrated Müller cell sensitivities to IGF-I and IGF-II, it is reasonable to conclude that normal vitreous contains ample quantities of IGF to drive Müller cell responses in our assay system. The virtual absence of detectable activity is compelling evidence of a vitreous control mechanism that most likely involves growth factor attenuation. In support of this premise, a recent study by Simo et al. (33) measured unbound IGF-I in control vitreous samples, reporting a mean concentration of 0.1 ng/ml, which is well below our observed threshold of Müller cell sensitivity for this ligand. Importantly, this concentration of free IGF-I is also 3- to 25-fold lower than the total IGF-I concentrations reported by other investigators, suggesting that the majority of this growth factor is sequestered. While free IGF-II was not examined in the study by Simo et al., based on the low levels of IGF-related activity in control samples, it seems reasonable to speculate that free IGF-II would also be below the limit of Müller cell sensitivity.

In addition to the ligands IGF-I and IGF-II, the IGF system contains at least six high-affinity IGF binding proteins (IGFBPs) capable of binding to and modulating growth factor activities (34–36). IGFBP-2 and -3 are reportedly present in normal vitreous, and it seems likely that one or both of these IGFBPs function as a growth factor "sink," sequestering and thus controlling IGF-I activity (22,25–27,37–39). This interpretation, however, is complicated by the fact that IGFBP's effects on growth factor activities can vary from inhibition to potentiation, depending on the IGFBP species, growth factor, and experimental system examined and that IGFBPs can have direct effects on cells that are independent of growth factor affinity (13,40,41). Unfortunately, the effects of individual IGFBPs on Müller cells and/or Müller cell growth factor responsiveness are unknown and, to reconcile the increases in growth factor activity against changes in IGF system components, it is necessary that we develop a more complete understanding of the effects of individual IGFBPs in this unique ocular system.

Finally, while we now have compelling evidence of IGF system ligand contributions to the vitreous biological activity of interest, several important limitations merit discussion. The inability of the IGF-neutralizing antibody to distinguish between IGF-I and IGF-II precluded the determination of which of the two IGF species contributed to the vitreous activity. However, given that IGFBP affinities for IGF-II are consistently higher than those for IGF-I (34,42), increases in vitreal concentrations of either ligand would likely yield net increases in free IGF-I. With this in mind, IGF-I, rather than IGF-II, is most likely the ligand responsible for the IGF-related activity detected. Along this same line, 20–39% of vitreous biological activity was not accounted for in the growth factor–neutralization experiments. Studies performed with Müller and other cell types resulted in the identification of a number of growth factors, cytokines, and lipid mediators capable of promoting tractional force generation in the absence of other stimuli. At the least, these include the two growth factor 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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REFERENCES

1. Davis M: Diabetic retinopathy: a clinical overview. *Diabetes Care* 15:1844–1874, 1992
2. Aiello LP, Gardner TW, King GL, Blankenship G, Cavallerano JD, Ferris FL 3rd, Klein R: Diabetic retinopathy. *Diabetes Care* 21:143–156, 1998
3. Neely K, Quillen D, Schahat A, Gardner T, Blankenship G: Diabetic retinopathy. *Med Clin North Am* 82:847–876, 1998
4. Tzekov R, Arder GB: The electroretinogram in diabetic retinopathy. *Surv Opthalmol* 44:53–60, 1999
5. Li Q, Zemel E, Miller B, Perlman I: Early retinal damage in experimental diabetes: electroretinographical and morphological observations. *Exp Eye Res* 74:615–625, 2002
6. Li Q, Puro DG: Diabetes-induced dysfunction of the glutamate transporter in retinal Muller cells. *Invest Ophthalmol Vis Sci* 43:3109–3116, 2002
7. Mizutani M, Gerhardinger C, Lorenzi M: Müller cell changes in human diabetic retinopathy. *Diabetes* 47:445–449, 1998
8. Rungger-Brandle E, Dosso AA, Leuenberger PM: Glial reactivity, an early feature of diabetic retinopathy. *Invest Ophthalmol Vis Sci* 41:1971–1980, 2000
9. Leith W, Barber AJ, Xu B, Dice C, Ratz MJ, Tanase D, Strother JM: Glial reactivity and impaired glutamate metabolism in short-term experimental diabetic retinopathy. *Diabetes* 47:815–820, 1998
10. Abu El-Asrar AM, Desmet S, Meersschaert A, Dralands L, Missotten L, Geboes K: Expression of the inducible isoform of nitric oxide synthase in the retinas of human subjects with diabetes mellitus. *Am J Ophthalmol* 132:551–556, 2001
11. Vinores SA, Youssri AI, Luna JD, Chen YS, Bhargave S, Vinores MA, Schoenfeld CL, Peng B, Chan CC, LaRochelle W, Green WR, Campochiaro PA: Upregulation of vascular endothelial growth factor in ischemic and non-ischemic human and experimental retinal disease. *Histol Histopathol* 12:99–109, 1997
12. Murata T, Nakagawa K, Khalil A, Ishibashi T, Inomata H, Sueishi K: The relation between expression of vascular endothelial growth factor and breakdown of the blood-retinal barrier in diabetic rat retinas. *Lab Invest* 74:819–825, 1996
13. Amin RH, Frank RN, Kennedy A, Eliott D, Puklin JE, Abrams GW: Vascular endothelial growth factor is present in glial cells of the retina and optic nerve of human subjects with nonproliferative diabetic retinopathy. *Invest Ophthalmol Vis Sci* 38:36–47, 1997
14. Nork TM, Wallow IHL, Sramek SJ, Anderson G: Muller's cell involvement in proliferative diabetic retinopathy. *Arch Ophthalmol* 105:1424–1429, 1987
15. Sramek SJ, Wallow IH, Stevens TS, Nork TM: Immunostaining of preretinal membranes for actin, fibronectin, and glial fibrillary acidic protein. *Ophthalmology* 96:835–841, 1989

16. Hosoda Y, Okada M, Matsumura M, Ogino N, Honda Y, Nagai Y: Epiretinal membrane of proliferative diabetic retinopathy: an immunohistochemical study. *Ophthalmic Res* 25:289–294, 1993
17. Guidry C: Isolation and characterization of porcine Muller cells: myofibroblastic dedifferentiation in culture. *Invest Ophthalmol Vis Sci* 37:740–752, 1996
18. Guidry C: Tractional force generation by porcine Muller cells: development and differential stimulation by growth factors. *Invest Ophthalmol Vis Sci* 38:456–468, 1997
19. Guidry C, Bradley KM, King JL: Tractional force generation by human Muller cells: growth factor responsiveness and integrin receptor involvement. *Invest Ophthalmol Vis Sci* 44:1355–1363, 2003
20. Freyberger H, Brocker M, Yakut H, Hammer J, Effert R, Schifferdecker E, Schatz H, Derwahl M: Increased levels of platelet-derived growth factor in vitreous fluid of patients with proliferative diabetic retinopathy. *Exp Clin Endocrinol Diabetes* 108:106–109, 2000
21. Boulton M, Gregor Z, McLeod D, Charteris D, Jarvis-Evans J, Moriarty P, Khaliq A, Foreman D, Allamby D, Bardsley B: Intravitreal growth factors in proliferative diabetic retinopathy: correlation with neovascular activity and glycaemic management. *Br J Ophthalmol* 81:228–233, 1997
22. Burgos R, Mateo C, Canton A, Hernandez C, Mesa J, Simo R: Vitreous levels of IGF-I, IGF binding protein 1, and IGF binding protein 3 in proliferative diabetic retinopathy: a case-control study. *Diabetes Care* 23:80–83, 2000
23. Grant M, Russel B, Fitzgerald C, Merimee T: Insulin-like growth factors in vitreous: studies in control and diabetic subjects with neovascularization. *Diabetes* 35:416–420, 1986
24. Inokuchi N, Ikeda T, Imamura Y, Sotozono C, Kinoshita S, Uchihori Y, Nakamura K: Vitreous levels of insulin-like growth factor-I in patients with proliferative diabetic retinopathy. *Curr Eye Res* 23:368–371, 2001
25. Meyer-Schwickerath R, Pfeiffer A, Blum WF, Freyberger H, Klein M, Losche C, Rollmann R, Schatz H: Vitreous levels of the insulin-like growth factors I and II, and the insulin-like growth factor binding proteins 2 and 3, increase in neovascular eye disease: studies in nondiabetic and diabetic subjects. *J Clin Invest* 92:2620–2625, 1993
26. Spranger J, Buhnen J, Jansen V, Krieg M, Meyer-Schwickerath R, Blum WF, Schatz H, Pfeiffer AF: Systemic levels contribute significantly to increased intraocular IGF- I, IGF-II and IGF-BP3 [correction of IFG-BP3] in proliferative diabetic retinopathy. *Horm Metab Res* 32:196–200, 2000
27. Spranger J, Mohlig M, Osterhoff M, Buhnen J, Blum WF, Pfeiffer AF: Retinal photocoagulation does not influence intraocular levels of IGF- I, IGF-II and IGF-BP3 in proliferative diabetic retinopathy: evidence for combined treatment of PDR with somatostatin analogues and retinal photocoagulation? *Horm Metab Res* 33:312–316, 2001
28. Hardwick C, Feist R, Morris R, White M, Witherspoon D, Angus R, Guidry C: Tractional force generation by porcine Muller cells: stimulation by growth factors in human vitreous. *Invest Ophthalmol Vis Sci* 38:2053–2063, 1997
29. Hardwick CW Jr, Morris R, Guidry C: Quantitative vitreous collection using a modified infusion fluid during vitrectomy. *Retina* 17:51–56, 1997
30. Miller E, Rhodes R: Preparation and characterization of the different types of collagen. *Meth Enzymol* 82:33–64, 1982
31. Guidry C, Grinnell F: Studies on the mechanism of hydrated collagen gel reorganization by human skin fibroblasts. *J Cell Sci* 79:67–81, 1985
32. Guidry C, Hook M: Endothelins produced by endothelial cells promote collagen gel contraction by fibroblasts. *J Cell Biol* 115:873–880, 1991
33. Simo R, Lecube A, Segura RM, Garcia Arumi J, Hernandez C: Free insulin growth factor-I and vascular endothelial growth factor in the vitreous fluid of patients with proliferative diabetic retinopathy. *Am J Ophthalmol* 134:376–382, 2002
34. Conover CA: Regulation and physiological role of insulin-like growth factor binding proteins. *Endocrine J* 43:S43–S48, 1996
35. Collett-Soberg PR, Cohen P: The role of the insulin-like growth factor binding proteins and the IGFBP proteases in modulating IGF action. *Endocrinol Metab Clin North Am* 25:591–614, 1996
36. LeRoith D: Insulin-like growth factors. *N Engl J Med* 336:633–640, 1997
37. Arnold DR, Moshayedi P, Schoen TJ, Jones BE, Chader GJ, Waldbillig RJ: Distribution of IGF-I and -II, IGF binding proteins (IGFBPs) and IGFBP mRNA in ocular fluids and tissues: potential sites of synthesis of IGFBPs in aqueous and vitreous. *Exp Eye Res* 56:555–565, 1993
38. Pfeiffer A, Spranger J, Meyer-Schwickerath R, Schatz H: Growth factor alterations in advanced diabetic retinopathy: a possible role of blood retina barrier breakdown. *Diabetes* 46 (Suppl. 2):S26–S30, 1997
39. Waldbillig RJ, Jones BE, Schoen TJ, Moshayedi P, Heidersbach S, Bitar MS, van Kuijk FJ, de Juan E, Kador P, Chader GJ: Vitreal insulin-like growth factor binding proteins (IGFBPs) are increased in human and animal diabetics. *Curr Eye Res* 13:539–546, 1994
40. Ferry RJ, Katz LEL, Grimberg A, Cohen P, Weinzimer SA: Cellular actions of insulin-like growth factor binding proteins. *Horm Met Res* 31:192–202, 1998
41. Ferry RJ, Cerri RW, Cohen P: Insulin-like growth factor binding proteins: new proteins, new functions. *Horm Res* 51:53–67, 1999
42. Baxter RC: *Molecular Aspects of Insulin-Like Growth Factor Binding Proteins: Advances in Molecular and Cellular Endocrinology*. Vol. 1. Stamford, CT, JAI Press, 1997, p. 123–159
43. Montesano R, Orci L: Transforming growth factor-beta stimulates collagen-matrix contraction by fibroblasts: implication for wound healing. *Proc Natl Acad Sci U S A* 85:4894–4897, 1988
44. Pena R, Jerdan J, Glaser B: Effects of TGF-β and TGF-β neutralizing antibodies on fibroblast-induced collagen gel contraction: implications for proliferative vitreoretinopathy. *Invest Ophthalmol Vis Sci* 35:2804–2808, 1994
45. Liu X, Kohyama T, Wang H, Zhu YK, Wen FQ, Kim HJ, Romberger DJ, Rennard SI: Th2 cytokine regulation of type I collagen gel contraction mediated by human lung mesenchymal cells. *Am J Physiol Lung Cell Mol Physiol* 282:L1049–L1056, 2002
46. Panetti TS, Magnusson MK, Peyruchaud O, Zhang Q, Cooke ME, Sakai T, Mosher DF: Modulation of cell interactions with extracellular matrix by lysophosphatidic acid and sphingosine 1-phosphate. *Prostaglandins* 64:93–106, 2001
47. Grinnell F: Fibroblast-collagen-matrix contraction: growth-factor signaling and mechanical loading. *Trends Cell Biol* 10:362–365, 2000