Production of Platelet-Derived Growth Factor by Interleukin-1β and Transforming Growth Factor-β-Stimulated Retinal Pigment Epithelial Cells Leads to Contraction of Collagen Gels

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Purpose. In an in vitro model of the later contractile stages of proliferative vitreoretinopathy, interleukin-1β (IL-1β) and transforming growth factor-β (TGF-β) stimulate the contraction of collagen gels by retinal pigment epithelial (RPE) cells. This contraction occurs after a lag period and appears not to be a direct effect of the cytokines but is mediated by another factor produced in the presence of the two cytokines. The nature of this factor has been investigated.

Methods. Human RPE cells were seeded onto collagen gels in the presence of IL-1β and TGF-β. After 24 hours, the conditioned medium was removed and added to new collagen gels seeded with RPE cells, and the diameter of the collagen gels was measured after various intervals. The ability of the conditioned medium to effect contraction was determined after various treatments, including size fractionation, heating, trypsin digestion, and binding to heparin-Sepharose. The involvement of platelet-derived growth factor (PDGF) as a stimulator of contraction was tested with neutralizing antibodies and by polymerase chain reaction analyses of specific mRNAs.

Results. IL-1β and TGF-β cause RPE cells to contract after a delay of up to 24 hours, whereas conditioned medium from cytokine-treated cells results in immediate contraction in a manner similar to that of serum. The factor in the conditioned medium causing immediate contraction was found to be heat-stable, trypsin-sensitive, and resistant to extremes of pH. It has a size of between 30 and 50 kDa and binds heparin. The factor in conditioned medium from cytokine-treated cells does not act in the presence of C-kinase inhibitors or cycloheximide, suggesting that signaling is mediated by way of protein kinase C and new protein synthesis. Stimulation of contraction by conditioned medium is inhibited by anti-PDGF antibodies, and contraction is stimulated by human PDGF.

Conclusions. Contraction in the presence of cytokines is mediated by the production of PDGF or a PDGF-like molecule. This factor could have implications in the pathogenesis of proliferative vitreoretinopathy. Invest Ophthalmol Vis Sci. 1997;38:824–833.

Retinal pigment epithelial (RPE) cells are located between the choroid and the photoreceptors and form one component of the blood–retinal barrier.1,2 Under normal conditions, these cells do not divide but, as a result of trauma, RPE and other cells may proliferate extensively and migrate from their normal location in the outer retina through the neural retina into the vitreous humor. Here the proliferating cells form epiretinal membranes consisting of cells embedded within a disorganized extracellular matrix, extending into the vitreous humor and attached to the vitreous surface of the neural retina. Epiretinal membrane contraction exerts tractional forces on the attached underlying retina, causing detachment and leading to blindness.3 This sequence of events is known as proliferative vitreoretinopathy (PVR). There is an inflammatory component to PVR, probably resulting from rupture of the blood–retinal barrier and entry of serum components and leukocytes into the
reinita. These inflammatory cells may synthesize cytokines locally, and high levels of interleukin-1 (IL-1) and transforming growth factor-β (TGF-β) have been found in the vitreous humor of patients with PVR.5-7

Retinal pigment epithelial cells produce numerous cytokines in culture6-13 and exhibit a wound-healing response in which metalloproteinases and extracellular matrix components are synthesized,14-16 moreover, they contract gels comprising ECM components,16-20 mimicking the final stages of PVR. This contraction, which is dependent on new protein synthesis and signaling by way of protein kinase C, is greater in the presence of serum or the cytokines IL-1β plus TGF-β.17 Serum-induced and cytokine-induced contractions of collagen gels differ in their kinetics.18 In the presence of serum, contraction occurs almost immediately, whereas contraction stimulated by the cytokines shows a lag of up to 24 hours, although eventually, serum- and cytokine-stimulated contraction often reach a similar extent.19 This lag and the necessity for protein synthesis suggest that contraction may not be stimulated directly by the cytokines and that another factor may be involved. In this article, this factor is shown to be platelet-derived growth factor (PDGF) or a similar protein.

MATERIALS AND METHODS

Human Retinal Pigment Epithelial Cell Culture

RPE cells were cultured as described previously21 from human donor eyes obtained from the South Carolina Lions Eye Bank. The cells were grown on laminin-coated 24-well tissue culture plates (Costar, Boston, MA). The cells were stained for keratins using antikeratin monoclonal 8.13 antibody (ICN, Los Angeles, CA) to confirm their epithelial identity. In most experiments, cells were used between the third and fifth passage in culture, at which time, they were 95% to 100% keratin-positive.

Collagen Gel Contraction

Twenty-four-well tissue culture plates were first coated with 2% bovine serum albumin (RIA grade; Sigma, St. Louis, MO) in phosphate-buffered saline-bovine serum albumin (PBS—BSA) overnight to block nonspecific binding. The PBS—BSA was removed, and the wells were washed twice with PBS. Neutralized collagen solution was prepared by mixing one part 10X minimal essential medium (MEM; Life Technologies, Gaithersburg, MD) and one part 0.2 M Hepes, pH 9.0 (Sigma) with eight parts vitrogen (Celtrix Laboratories, Palo Alto, CA), of which 0.5 ml of this mixture was added per well. The plate was incubated at 37°C for 1 hour to allow the collagen to solidify. RPE cells were removed from their culture flask using trypsin, rinsed in serum-containing Dulbecco’s modified Eagle medium (DMEM, Life Technologies) followed by serum-free medium. Approximately 250,000 cells were added to each well with or without additional factors, including 2.5 U (0.5 ng) recombinant human IL-1β (Collaborative Research, Bedford, MA) plus 5 U (1 ng) human TGF-β1 (Sigma) per milliliter or 10% fetal bovine serum (Life Technologies). The plate was incubated at 37°C for 1 hour to allow cells to attach. After 1 hour, 1 ml DMEM was squirted gently into each well so that the gel detached from the wall of the well and floated in the medium. The cytokines or additional factors again were added to maintain the original concentration, and the plate was incubated at 37°C. The diameter of the gel was measured at various times thereafter using an Image-1 analysis system (Universal Imaging, West Chester, PA).

Preparation of Conditioned Medium

Cells were grown on collagen gels in serum-free medium in the presence of IL-1β and TGF-β for 24 hours. The medium was removed and passed through a 0.45-μm filter.

Heparin–Sepharose Chromatography

Two milliliter conditioned medium was applied to a 2 ml heparin–Sepharose column (Sigma) preequilibrated with 0.01 M Tris–HCl (pH 7.5). The column was washed with equilibration buffer and then eluted with 0.2 M NaCl, 0.5 M NaCl, 1.0 M NaCl, 1.5 M NaCl, and 2 M NaCl in 0.01 M Tris–HCl (pH 7.5). Fractions were collected, desalted, restored to the original volume of 2 ml, and assayed for stimulation of contraction.

Molecular Weight Range Determination

Conditioned medium was centrifuged through Centricon-100, Centricon-50, Centricon-30, Centricon-10, and Centricon-3 microconcentrators (Amicon, Beverly, MA), which retain proteins with an approximate molecular mass of greater than 100 kDa, 50 kDa, 30 kDa, 10 kDa, and 3 kDa, respectively. These fractions then were adjusted to their original volumes and assayed for their ability to stimulate contraction.

Biochemical Characterization

Conditioned medium was subjected to the following treatments: heating at 100°C in a boiling water bath for 10 minutes; incubation at 37°C for 4 hours with 200 μg bovine pancreatic trypsin (Sigma) per milliliter, after which 400 μg soya bean trypsin inhibitor (Sigma) per milliliter was added followed by a further incubation at 37°C for 4 hours; incubation with 5 mM dithiothreitol (DTT) (Sigma); adjusting to pH 3 by the addition of 0.1 N HCl or pH 10 by addition of 0.1 HCl.

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Additional Factors Tested on Collagen Gels

Staurosporine (Sigma) was dissolved in methanol at a concentration of 200 μM and added to the collagen gels at a final concentration of 5 nM. Calphostin (Sigma) was dissolved in methanol at a concentration of 200 μM and added to the collagen gels at a final concentration of 7.5 nM. Cycloheximide was dissolved in methanol at a concentration of 1 mg/ml and added at a final concentration of 25 μg/ml of medium. Human PDGF (R & D Systems, Minneapolis, MN) was diluted in DMEM containing 1 mg BSA/ml and used at a concentration of 10 ng/ml medium. Polyclonal goat anti-human PDGF (Collaborative Research) was used at a concentration of 50 half-maximal units per milliliter of medium (50 μg/ml). Control assays used the vehicles alone. In these contraction inhibition assays, the viability of the cells was checked at the end of the experiment by trypan blue staining.

Reverse Transcriptase–Polymerase Chain Reaction

RNA was extracted from cells using RNAStat (Tel-Test, B, Friendswood, TX). After removal of the medium, 1 ml RNAStat was added to three collagen gels, each containing approximately 300,000 RPE cells grown under the same conditions. The gel/RNAStat mixture then was passed through a hypodermic needle (21-gauge) to break up the gels. The phenolic and aqueous components were separated into two phases by centrifugation after addition of 0.2 ml chloroform, and RNA was precipitated from the aqueous phase using 0.5 ml isopropyl alcohol at −18°C. The RNA was redissolved in water and the purity and concentration determined from the absorbance at 280 and 260 nm.

The mRNAs were copied into cDNA using a 25-μl reaction mix containing total cellular RNA (1 μg); oligo (dT)15 (0.5 μg); RNase H minus Moloney murine virus reverse transcriptase (100 U; Life Technologies); RNAsin (40 U; Promega, Madison, WI); 800 μM dATP, dCTP, dGTP, and dTTP; 2 mM DTT; 4 mM magnesium chloride; 50 mM potassium chloride; and RNA was precipitated from the upper phase (75 μl) contained 10 mM Tris-HCl (pH 8.3), Taq polymerase (2.5 U), and 5 μl of the diluted reverse transcription mix. This was subjected to 40 cycles of PCR (95°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute), a 10-minute incubation at 72°C, and then an indefinite incubation at 4°C. The products were separated on Metaphor (FMC Bioproducts, Rockland, ME) gels. Markers consisted of a 100-base pair (bp) DNA ladder (Life Technologies). The PCR fragments were subjected to restriction endonuclease digestion to confirm their identity. The digestion products were separated on Metaphor gels.

Oligonucleotides were designed using sequences obtained from Genbank and the Oligo program (National Biosciences, Plymouth, MN). The amplification primers for actin were 5’CGTGGGATCCGGGAAAGC and 5’CTCGCCACATTGTTGAACCT, which give rise to a 484-bp product when mRNA is subjected to reverse transcription–polymerase chain reaction (RT–PCR). The amplification primers for PDGF-A were 5’AGGGGCGCAAGGGGGAATAC and 5’GGGCGACCCTCTCATCCTAC, for PDGF-B were 5’CGAGGACCAGGGCGAACAAAC and 5’CAAAGAGGCGACCACCATCTG, and for connective tissue growth factor (CTGF) were 5’TCCCTFCAGTACCGTGGTA and 5’TCCCTCATTATGTTCTCT. These give rise to PCR products of 467, 456, and 458 bp, respectively. Each pair of primers spans at least one exon/exon boundary.

Statistical Analysis

With the exception of a series of experiments to test whether the contraction-factor in conditioned medium had the properties of PDGF, all experiments were performed at least three times. The data from these experiments, which used low-passage cultures of RPE cells from different donors, were combined and analyzed by analysis of variance. The mean, standard deviation, and significance level are given in the legends to the figures. In all cases, n refers to the number of experiments carried out using cells from different donors.

RESULTS

Conditioned Medium From Interleukin-1β- and Transforming Growth Factor-β-Treated Cells Abolishes the Lag Period Before the Onset of Contraction

Previous studies using collagen gels have shown that whereas cytokine-stimulated contraction manifests a lag of up to 24 hours, contraction produced by serum begins almost immediately (Fig. 1). This suggests that IL-1β and TGF-β may induce the production of a “contraction factor” whereas serum already may contain it. To test this hypothesis, cells were grown on collagen gels for 24 hours in serum-free medium supplemented with the two cytokines. Conditioned
FIGURE 1. Effect of conditioned medium on the contraction of collagen gels. Retinal pigment epithelial cells seeded on collagen gels were incubated with serum-free medium (●), serum-free medium containing interleukin-1β (IL-1β) (○), serum-containing medium (Δ), or filtered medium from cells grown for 24 hours on collagen gels in the presence of IL-1β and TGF-β (i.e., conditioned medium [■]). In similar experiments with cells from different donors, the average gel diameters after a 48-hour incubation (as a percentage of that at zero time) were: serum-free medium; 89 ± 3.6 (n = 3, P < 0.02 when compared to contraction in the presence of IL-1β and TGF-β); serum-containing medium; 67 ± 11.5 (n = 3, P > 0.2); conditioned medium; 56 ± 7.9 (n = 3, P < 0.005); IL-1β–TGF-β-containing medium; 71 ± 14.

Contraction-Stimulating Effect of Conditioned Medium Appears to be Dependent on Protein Synthesis and Protein Kinase C

It has been shown that the contraction stimulated by cells in the presence of IL-1β and TGF-β is dependent on new protein synthesis and is signaled by way of protein kinase C. To determine whether new protein synthesis also is required after formation of the contraction factor, cycloheximide was added to the cells in the presence of conditioned medium and, again, contraction of collagen gels was inhibited (Fig. 2A). This suggests that synthesis of new proteins occurs during this phase of the process of contraction as well. Moreover, a protein kinase C-mediated event appears to be involved in this process because contraction also was inhibited when the cells were incubated with staurosporine (Fig. 2A) or calphostin (Fig. 2B), inhibitors of protein kinase C.

FIGURE 2. Effect of G-kinase inhibitors and cycloheximide on gel contraction. (A) Retinal pigment epithelial (RPE) cells, seeded on collagen gels, were treated with conditioned medium (■), serum-free medium containing interleukin-1β (IL-1β) (○), serum-containing medium (Δ), or conditioned medium plus 5 nM staurosporine (○), or conditioned medium plus 25 μg cycloheximide/ml (△). In similar experiments with cells from different donors, the average gel diameter (as a percentage of that at zero time) after a 72-hour incubation were: conditioned medium plus staurosporine: 91 ± 1.5 (n = 5, P < 0.0001); conditioned medium plus cycloheximide: 95 ± 1.5 (n = 5, P < 0.0001); IL-1β–TGF-β-containing medium: 81.0 ± 1.7 (n = 3, P < 0.0007); conditioned medium: 73 ± 4 (n = 3). (B) RPE cells were grown in serum-containing medium (Δ), conditioned medium (■), serum-free medium (○), or conditioned medium plus calphostin (△).
Time (hours)

FIGURE 3. The heparin-binding affinity of the contraction factor. Conditioned medium was passed through a heparin-Sepharose column and eluted with various concentrations of sodium chloride. The fractions were collected, desalted, and assayed for contraction activity. (▲) Material that did not bind to the column. (○) Material that eluted with 0.2 M NaCl. (▼) Material that eluted with 0.5 M NaCl. (●) Material that eluted with 1 M NaCl. (●) Material that eluted with 1.5 M NaCl. (△) Material that eluted with 2 M NaCl. (■) Unfractionated conditioned medium.

lease various growth factors from heparin and, after removal of salt, these were tested for their ability to stimulate contraction. The salt concentrations used were 0.2 M NaCl, which has been reported to elute TNF-α; 0.5 M NaCl, which elutes PDGF; 1.0 M NaCl, which elutes an epidermal growth factor-like heparin-binding growth factor secreted by several cell types including macrophages; 1.5 M NaCl, which elutes fibroblast growth factor-1; and 2.0 M NaCl, which elutes fibroblast growth factor-2. TGF-β, TGF-α, and IL-1 do not bind to heparin. Contraction-stimulating activity was found to be associated with fractions eluted with 0.2 M and 0.5 M NaCl, the salt concentrations that elute TNF-α and PDGF, respectively (Fig. 3).

Contraction Factor Is Heat Stable, Sensitive to Trypsin, Partially Sensitive to Dithioreitol, and Resistant to Extremes in pH

Treatment of conditioned medium with trypsin at a concentration of 200 μg/ml for 4 hours caused it to lose 85% of its ability to effect contraction, whereas, in the presence of soybean trypsin inhibitor, the effect of trypsin was abolished completely, indicating the proteinaceous nature of the factor. Treatment with 5 mM DTT caused partial loss (46 ± 6.6%, n = 3) of contraction activity, suggesting that the factor probably is disulfide-bonded. When conditioned medium was treated at 100°C for 10 minutes in a boiling water bath, however, there was little loss of contraction activity (8 ± 4.6%, n = 3), indicating that the active factor is heat-stable. Moreover, when it was incubated at various pH values from pH 3.0 to pH 10.0, all activity was maintained. All of these properties are consistent with the factor being PDGF, which is heat stable, sensitive to DTT and trypsin, and resistant to extremes in pH. TNF-α, on the contrary, is a heat-labile protein.

Contraction Is Inhibited by Antiplatelet-Derived Growth Factor Antibody

Because the properties of the conditioned medium factor suggest that it is PDGF, a neutralizing anti-PDGF antibody was added to RPE cells in collagen gel contraction assays. This antibody completely blocked the contraction of cells in the presence of IL-1β and TGF-β; moreover, in the presence of antibody, the normal contraction observed in the absence of cytokines also was abolished (Fig. 5). When the anti-PDGF antibody

Contraction Factor in Conditioned Medium Has an Approximate Size of 30 to 50 kDa

To characterize the factor further, its approximate molecular weight was determined using a series of Centricon microconcentrators (Amicon), which separate the components of conditioned medium according to their molecular weights. The fractions were adjusted to their original volume and filter-sterilized before testing them in a contraction assay. The fraction with a size range of 30 to 50 kDa stimulated greater contraction than the others, which showed a similar level of contraction to medium without serum or the cytokines (Fig. 4). This size is consistent with the contraction-stimulating factor being PDGF.

FIGURE 4. Size fractionation of the contraction activity in conditioned medium. Conditioned medium sequentially was passed through Centricon filters (Amicon, Beverly, MA) that retained proteins greater than a specific size. The retained fractions were assayed for contraction activity. Unfractionated conditioned medium (■). Proteins with an approximate size of: >100 kDa (▼), 50 to 100 kDa (△), 30 to 50 kDa (○), 10 to 30 kDa (○), 3 to 10 kDa (▲).
PDGF and RPE Cell Contraction

FIGURE 5. Effect of anti-platelet-derived growth factor (PDGF) antibody on contraction of collagen gels. Retinal pigment epithelial cells seeded on collagen gels were treated with interleukin-1β (IL-1β) and transforming growth factor-β (TGF-β) (TGFB) or serum in the presence or absence of neutralizing anti-PDGF antibody at a concentration of 50 μg/ml. (●) Cells growing in serum-free medium. (△) Cells growing in serum-containing medium. (○) Cells growing in medium containing IL-1β and TGF-β. (□) Cells growing in medium containing IL-1β and TGF-β together with neutralizing anti-PDGF antibody. (▲) Cells growing in medium containing serum together with neutralizing anti-PDGF antibody. In similar experiments with cells from different donors, the average gel diameters (as a percentage of that at zero time) after a 72-hour incubation were: serum-free medium: 86 ± 5.4 (n = 4, P < 0.029 when compared to serum-free medium containing IL-1β and TGF-β); serum-containing medium: 63 ± 11 (n = 4, P < 0.0001); serum-free medium containing IL-1β and TGF-β plus anti-PDGF antibody: 93 ± 2.3 (n = 4, P < 0.0001); serum-containing medium plus anti-PDGF antibody: 66 ± 11.5 (n = 3, P < 0.0001); serum-free medium containing IL-1β and TGF-β: 81 ± 3.

Platelet-Derived Growth Factor-Stimulated Contraction Can Mimic the Effect of Serum

PDGF is likely to be the contraction-stimulating factor induced by the cytokines because contraction was blocked totally by anti-PDGF antibody. To test whether human PDGF can indeed induce contraction, the purified protein was added to RPE cells growing on collagen gels. PDGF stimulated contraction at a level of 10 ng/ml and mimicked the effect seen with serum (Fig. 6), that is, contraction was rapid and, unlike IL-1β–TGF-β-stimulated contraction, no lag was observed. When PDGF was used in combination with IL-1β and TGF-β, contraction was no greater than that observed with PDGF alone, and again no lag was observed.


Two PDGF polypeptides, the A and B chains, are encoded by separate genes, and all three isoforms (AA, AB, and BB) have been found as secreted dimers. To investigate which PDGF gene expressed by RPE cells is regulated during IL-1β–TGF-β-stimulated contraction, mRNA was extracted from cells growing on collagen gels in serum-free medium, serum-containing medium, and medium containing IL-1β and TGF-β. The RNA was transcribed into cDNA and used in PCR reactions under conditions that give a semiquantitative measure of mRNA levels.

A PCR product of the predicted size (467 nucleotides) for that transcribed from PDGF-A mRNA using PDGF-A-specific primers was observed in reactions from cells growing on collagen gels for 24 hours without serum (Fig. 7A, lane 1). In the presence of IL-1β and TGF-β (lane 2) or of serum (lane 3), the PCR was added to collagen gels seeded with RPE cells in the presence of serum, a reduction of contraction activity was sometimes observed, but this usually was small (Fig. 5).

FIGURE 6. Effect of platelet-derived growth factor (PDGF) on contraction of collagen gels. Retinal pigment epithelial cells on collagen gels were treated with 10 ng/ml of PDGF, and the diameter of the collagen gels was measured at various timepoints. (●) Contraction in serum-free medium, (△) serum-containing medium, (○) serum-free medium containing IL-1β and TGF-β, (□) serum-free medium containing interleukin-1β (IL-1β) and transforming growth factor-β (TGF-β) plus PDGF, (▲) serum-free medium containing PDGF. In similar experiments with cells from different donors, the average gel diameters (as a percentage of that at zero time) after a 72-hour incubation were: serum-free medium: 89 ± 1.1 (n = 3, P < 0.08 when compared to IL-1β–TGF-β-treated cells); serum-containing medium: 67 ± 8 (n = 3, P < 0.0001); serum-free medium with PDGF: 72 ± 8 (n = 3, P < 0.001); serum-free medium containing IL-1β and TGF-β plus PDGF: 75 ± 0 (n = 3, P < 0.01); serum-free medium containing IL-1β and TGF-β: 83 ± 1 (n = 3).
FIGURE 7. Reverse transcriptase-polymerase chain reaction (RT–PCR) analyses of the production of platelet-derived growth factor (PDGF)-A, PDGF-B, and connective tissue growth factor (CTGF) mRNAs by retinal pigment epithelial (RPE) cells. RPE cells were grown on collagen gels in serum-free medium, medium containing IL-1β and TGF-β, or medium with serum. After 24 hours, RNA was extracted from the cells and subjected to RT–PCR. (A) Lanes 1 to 3 and 5, PCR fragments generated using primers specific to human PDGF-A; lanes 6 to 8, PCR fragments generated using specific primers to human PDGF-B. Lanes 1 and 6, RNA from cells grown in serum-free medium; lanes 2 and 7, RNA from cells grown in interleukin-1β (IL-1β)–transforming growth factor-β (TGF-β); lanes 3 and 8, RNA from cells grown in the presence of serum; lane 4, 100-base pair (bp) markers; lane 5, reaction containing water rather than RNA. (B) Lanes 1 to 4, PCR fragments generated using primers specific to human CTGF; lanes 6 to 8, PCR fragments generated using specific primers to human β-actin; lanes 1 and 6, RNA from cells grown in serum-free medium; lanes 2 and 7, RNA from cells grown in IL-1β–TGF-β; lanes 3 and 8, RNA from cells grown in the presence of serum; lane 4, reaction containing water rather than RNA; lane 5, 100-bp markers.

reaction product was increased greatly over the control. In contrast, when similar RT–PCR reactions were carried out using PDGF-B-specific primers, no change in the amount of the predicted 456-nucleotide PCR product was seen under the different growth conditions (lanes 6 to 8). No product was seen when water substituted for RNA (lane 5) or when RT was omitted (not shown), confirming that the signals indicate the presence of mRNA; moreover, the primers span at least one exon/exon boundary to distinguish mRNA signals from those generated from contaminating DNA. The identity of the PCR fragments was confirmed by restriction endonuclease digestion (data not shown).

Some antibodies against PDGF have been reported to bind to another growth factor, CTGF, and CTGF is made by RPE cells (see Discussion section). To determine whether CTGF mRNA levels responded to contraction-stimulating factors, similar RT–PCR analyses were carried out using CTGF-specific primers (Fig. 7B, lanes 1 to 3). As with PDGF-A, CTGF-specific PCR products also increased in response to growth in the presence of IL-1β and TGF-β (lane 2) or serum (lane 3). No change in actin mRNA synthesis was detected under the different growth conditions (Fig. 7B, lanes 6 to 8).

DISCUSSION

Retinal pigment epithelial cells contract collagen, a process that involves the reorganization of the collagen matrix and exclusion of water from the gel. Such contractions occur to a greater extent in the presence of fetal bovine serum or a combination of the two cytokines, IL-1β and TGF-β, and are dependent on new protein synthesis; in the case of the cytokines at least, protein kinase C-mediated signal transduction also is required. Despite the similarities between serum- and cytokine-mediated contraction, there is a major difference. Whereas gel contraction in the presence of serum begins almost immediately, contraction stimulated by the two cytokines manifests a lag of up to 24 hours, although the lag period and the rate and extent of contraction vary with the cell donor. This suggests that, in the presence of the cytokines, some intermediary factor may be produced that controls contractile events more directly. Such a possibility is supported by the need for new protein synthesis.

To test for an intermediary factor, medium from RPE cells growing on collagen gels in the presence of IL-1β and TGF-β was used in new contraction assays and found to have an immediate effect with kinetics similar to that of serum-treated RPE cells, that is, no lag period was observed. The contraction-stimulating factor possessed many of the properties of PDGF, including binding to heparin, size, and heat stability. Some activity eluted from a heparin–Sepharose column at a salt concentration expected of TNF-α. This is not surprising because a step elution rather than a continuous gradient elution procedure was used; moreover, the ion concentration at which a protein elutes may be influenced by factors such as the degree of posttranslational modification. Both PDGF-A and PDGF-B are known to be made by human RPE cells as are PDGF-α and PDGF-β receptors, and further support for the direct involvement of PDGF in contraction comes from the observations that authentic human PDGF mimics the effects of conditioned medium; in addition, neutralizing anti-PDGF antibodies almost completely...
inhibit cytokine-stimulated contraction of collagen gels. In contrast, only a partial, usually small, inhibition of serum-induced contraction by anti-PDGF antibodies was observed. Although this suggests that PDGF may play a role in serum-induced contraction, the lack of a complete effect possibly having resulted from insufficient neutralizing antibody, the mode of action of serum may be different. It has been shown, for example, that serum-stimulated contraction by RPE cells is not inhibited by staurosporine, whereas that by IL-1β and TGF-β is inhibited.18

PDGF has been found to be a primary stimulator of rapid collagen gel contraction by fibroblasts.30-34 With these cells, as with RPE cells, TGF-β-stimulated contraction was slower;15 again suggesting an indirect effect; however, contraction by TGF-β was not altered by anti-PDGF antibodies, implying that in fibroblasts, TGF-β does not act by way of PDGF synthesis. In contrast to RPE cells, IL-1 inhibited fibroblast-stimulated gel contraction and led to dissolution of the gel, probably by stimulating metalloproteinase production.35 Although IL-1 also can stimulate metalloproteinase production by RPE cells,16 this does not seem to result in degradation of type I collagen gels over a period of 72 hours.16 Interrelations between IL-1, TGF-β, and PDGF have been observed in other systems. IL-1 alone,36 TGF-β alone,37,38 or both cytokines together39 have been shown to stimulate PDGF secretion from several cell types. Although in the case of chondrocytes, whereas IL-1 induces PDGF-AA production, TGF-β has an inhibitory effect.40

The observations that the contraction-stimulating factor mediates its effects by way of protein kinase C and requires calcium also are compatible with the factor being PDGF. PDGF activates phospholipase C,24 which catalyzes the degradation of phosphatidyl inositol 4,5-bisphosphate, thereby releasing two second messengers: diacylglycerol, which activates protein kinase C, and inositol-1,4,5-trisphosphate, which releases calcium from endoplasmic reticulum. PDGF also is known to affect adhesion and cytoskeletal organization41-44 and promote cell migration,32,42,45-48 both of which are critical to collagen gel contraction.

Although the data in this article support PDGF, synthesized in the presence of IL-1β and TGF-β, as the factor involved most directly in the cell surface events that lead to gel contraction, RPE cells have been shown to secrete an additional PDGF-like factor whose mitogenic activity can be inhibited by certain anti-PDGF antibodies.49 This factor, known as CTGF, has many other properties in common with PDGF, including induction by TGF-β.27,50 In addition to RPE cells, it also is produced by human umbilical cord vein endothelial cells49 and fibroblasts.56,57 Surprisingly, it shows no primary sequence homology to human PDGF,55 and thus the anti-PDGF antibodies that neutralize it must be recognizing some conformation-dependent epitope. Although authentic PDGF can stimulate rapid gel contraction by RPE cells, the possibility remains that CTGF is the active factor produced in this system because, clearly, neutralization by anti-PDGF antibodies cannot distinguish the two factors. To determine whether RPE cells growing on collagen gels produce CTGF as well as PDGF-A and -B, and whether the levels of any of these factors are modulated by IL-1β and TGF-β, semiquantitative RT-PCR mRNA analysis was carried out. Using RNA extracts from RPE cells growing in the presence and absence of serum or the two cytokines together with primers for PDGF-A, PDGF-B, and CTGF, it was found that the mRNAs for all of these factors are expressed by RPE cells. Because both the mRNAs encoding PDGF-A and CTGF were regulated positively by serum and cytokines, it is not yet possible to distinguish which of the two might be the active factor.

PDGF is a potent growth factor that can initiate many biologic responses associated with inflammation and wound healing. It can stimulate proliferation, chemotaxis, matrix production,24,51 as well as stimulate an increase in release of matrix metalloproteinases.52 Many of these properties may be involved in gel contraction in vitro and epiretinal membrane contraction in vivo and, indeed, there is evidence for the participation of PDGF in epiretinal membrane formation. In a rabbit model system, Yeo et al53 showed that intravitreous injection of fibronectin and PDGF produce intraocular membranes and tractional retinal detachments. Thus, PDGF or similar factors may play a rather significant role in several stages of PVR.

Key Words

collagen gel contraction, cytokines, platelet-derived growth factor, retinal pigment epithelium

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References


