Collagen Gel Contraction by Cells Associated With Proliferative Vitreoretinopathy

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The capacities of porcine choroidal fibroblasts, retinal glial cells, and retinal pigment epithelial cells to contract collagen gels in vitro were compared. Experiments with varied cell numbers indicated that glial cells are the most effective, followed by choroidal fibroblasts and retinal pigment epithelial cells. Analysis of the secretory products from cultures of these cell types revealed that retinal pigment epithelial cells synthesize and secrete peptides that promote fibroblast contraction of collagen gels in vitro. The mechanism of action of the retinal pigment epithelial cell-secreted contraction promoter was compared with that found in serum (type A) and secreted by cultured endothelial cells (type B). Like the serum factor, the retinal pigment epithelial cell-secreted factor was not dependent on active protein synthesis by the target cell and must be present continuously to promote contraction. Invest Ophthalmol Vis Sci 33:2429–2435, 1992

Proliferative vitreoretinopathy is a common and severe complication of retinal detachment surgery that can lead to visual impairment and blindness. The pathogenesis of this disorder is defined currently by the activities of extravitreal cells that gain access to the vitreous cavity through retinal defects.†–3 These cells proliferate and migrate over (or under) the vitreal surface of the retina to form a contractile connective tissue membrane.4 Contractile forces originating in the resulting membrane ultimately can detach the retina.

Several studies have examined the cellular content of epiretinal membranes to determine which cells contribute to this process. Immunohistologic and light and electron microscopic analyses have identified retinal pigment epithelial cells (RPEC),5–6 fibroblasts,7 astrocytes,7–10 Muller’s glia,11,12 and macrophages13,14 in these membranes, suggesting that many different cells may be involved. These conclusions were supported further by studies in which purified populations of each cell type were injected into the vitreous cavity of animals. Each was observed to induce formation of contractile epiretinal membranes.15–22

Our interest in the mechanisms of cellular contraction led us to question which of these cell types contributes to the tractional forces that ultimately cause retinal detachment. Previous studies concluded that RPEC,2,23–25 fibroblasts,26 and glial cells27 each can cause contraction of collagen matrices in vitro. However, the contraction potentials of each in relation to the other cells that are potentially involved are not known. In this study, we directly compared the contractile activities of RPEC, choroidal fibroblasts, and retinal glial cells.

Another aspect of this study was based on our earlier report that certain cells can stimulate matrix contraction through secreted promoters that influence other cells.28–30 Analysis of the contraction-promoting factors secreted by endothelial cells and those present in serum suggest the presence of at least two functionally different classes of promoters.29,30 The two classes of contraction promoters, designated types A and B, differ in: (1) the length of exposure necessary to stimulate contractile activity and (2) the requirement for de novo protein synthesis by the contracting cells. Skin fibroblasts contracting collagen gels in the presence of serum (type A promoter) required continuous exposure to the serum for contraction to proceed, and the contraction process was not sensitive to cycloheximide. Skin fibroblasts contracting collagen gels in the presence of endothelial cell conditioned media (type B promoter) would continue to contract after only a brief exposure to the media, but contraction was inhibited by cycloheximide. In this study, we examined the ability of RPEC to stimulate contraction in other cell types through secreted factors.

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Materials and Methods

Cells

Cultures of human skin fibroblasts were established from explanted foreskin obtained at circumcisions. These were maintained in Dulbecco's modified Eagle's medium (GibCo, Grand Island, NY) with fetal bovine serum 10% (FBS; Hyclone, Logan, UT).28 Porcine eyes (obtained from a local slaughterhouse; Polar Meats, Bessemer, AL) were used to isolate other cell types. Choroidal fibroblasts were harvested from explants of choroid. Strips of choroid were dissected into 1-2-mm cubes and incubated in minimal amounts of minimal essential medium (MEM; Gibco) containing FBS 15% to promote tissue adherence. The fibroblasts migrating from these tissues were harvested after approximately 3 wk.

Cultures of porcine retinal glial cells were established from fragments of peripheral retina, essentially according to an earlier method.31 These cells, however, were established without antibiotics and maintained in MEM with FBS 15%. Cells prepared for immunofluorescent staining on cover slips stained positively (> 80%) with a polyclonal antibody against glial fibrillary acidic protein (G-9269; Sigma, St. Louis, MO).

Porcine RPEC were isolated from freshly enucleated eyes using a modification of a previous method.32 The cells released from posterior eyecups by treatment with trypsin 0.05% and ethylenediaminetetraacetic acid 0.02% (EDTA; GibCo) frequently were found to contain nonpigmented cells. To remove these, the cells were centrifuged at room temperature for 5 min at 500 × g through a cushion composed of Percol 40% (Pharmacia, Piscataway, NJ) with 0.01 mol/l Na2PO4 and 0.15 mol/l NaCl, pH 7.4. In this procedure, the pigmented cells were recovered in the pellet, and the nonpigmented cells remained near the top of the cushion. The RPEC were grown in MEM containing FBS 15%. All cells were harvested for subculture or experimentation using trypsin 0.05% and EDTA 0.02%.

Assay of Collagen Gel Contraction

Native collagen gels were prepared as described previously.28,29 Vitrogen 100 (Collagen Corp., Palo Alto, CA) was adjusted to physiologic ionic strength, pH, and a concentration of 1.5 mg/ml with 10% of 10× phosphate-buffered saline (1.5 mol/l NaCl and 0.1 mol/l Na2HPO4) and 0.1 mol/l NaOH while it was maintained at 4°C. An aliquot of this solution (0.2 ml) was added to the center of a 12-mm circular score on the bottom of a 24-well tissue culture plate (Falcon, Oxnard, CA) and polymerized at 37°C for 90 min. This procedure produced gels approximately 2-mm thick attached only to the bottom surface of the well (Fig. 1A).

Cells released with trypsin and EDTA treatment were washed once with growth medium (containing serum) and again with serum-free medium before they were placed onto the top of the gels in 0.05-ml serum-free medium. These were incubated for an additional 30 min at 37°C to allow cell adhesion; after this, the gels were covered with 1 ml of medium (Fig. 1A).

Gel contraction was observed as a function of reduced gel thickness (Fig. 1B). The gels were measured on an inverted phase-contrast microscope (Nikon TMS; Garden City, NY) by adjusting the plane of focus from the bottom to the top of the gel and recording the distance of stage movement. This method was determined to be reproducible to 25 μm (1.25% of the initial gel thickness).28

Preparation of Conditioned Media

Serum-free conditioned media were prepared according to established procedures used in our laboratory.29,30 Briefly, the cells were grown to near confluence in media containing serum. After this, the cul-

Fig. 1. Contraction assay model. (A) A polymerizing solution of type I collagen (0.2 ml) is placed within circular scores on the bottom of wells. Cells suspended in media are placed on top of the polymerized gel and allowed to attach before the well is flooded with medium. (B) Gel contraction is measured as reduced gel thickness in the center of the gel, at a mark applied during scoring.
tures were rinsed and replaced with serum-free media and incubated at 37°C. Media conditioned by the cells in this manner were collected daily for 5 d, and then the cultures were discarded. The collected media were centrifuged to remove cell debris and stored at −20°C until use.

As a control for contamination by serum proteins, an enzyme-linked immunosorbent assay-type test was used to determine the level of serum albumin in the conditioned media was less than 0.15 μg/ml, corresponding to a FBS concentration 0.001%.

Other

Protein concentrations were determined according to an earlier method. Cycloheximide and other chemicals were obtained from Sigma.

Results

Analysis of Contraction Potentials of Choroidal Fibroblasts, Retinal Glial Cells, and RPEC

Cultures of choroidal fibroblasts, retinal glial cells, and RPEC were established from porcine eyes as described. Contraction assays were done in which choroidal fibroblasts, glial cells, or RPEC were seeded onto collagen gels at a single density of 25,000 cells per gel and incubated at 37°C in media containing serum. During the course of a 96-hr incubation, the fibroblasts and glial cells reduced gel thicknesses by more than 50% (Fig. 2). During the same period, the RPEC contracted the matrices approximately 7% (Fig. 2). No mitotic figures were observed during the 4-d incubation.

Another series of experiments were done in which the numbers of each cell type were varied 0–25,000 cells per gel. After 96 hr of incubation, the glial cells appeared to be the most active, followed by choroidal fibroblasts (Fig. 3A). As before, at this cell density, the RPEC did not produce significant changes in gel thickness. To explore this further, additional experiments were done in which the numbers of cells were varied from 0–200,000 cells per gel. Increased num-
bers of RPEC caused substantial changes in gel thickness (Fig. 3B).

Linear-regression analysis of the data in Figure 3 yielded functions describing the linear aspects of each activity profile, the slope of which reflects the percent contraction per thousand cells (Fig. 4). We concluded that glial cells were the most active in causing matrix contraction, followed by choroidal fibroblasts and then RPEC.

RPEC Secrete Contraction Promoters

To examine whether or not RPEC secrete contraction-promoting factors in vitro, confluent cultures of RPEC were incubated with serum-free MEM. Cell culture medium conditioned in this manner (RPEC-CM) was tested for contraction-promoting activity using skin fibroblasts seeded onto collagen gels as the target cells according to previously established protocols. Fibroblasts incubated in the conditioned medium reduced gel thickness by 45% during 5 hr of incubation compared with 40% in the presence of FBS and less than 1% in serum-free media (Fig. 5). Thus, soluble factors secreted by RPEC can promote contraction by fibroblasts.

To measure the specific activity of RPEC-CM, varying amounts of RPEC-secreted protein were added to fibroblasts attached to collagen gels as before. After 8 hr of incubation, gel thicknesses were measured, yielding a concentration-dependent profile (Fig. 6). Linear-regression analysis of data points 1-5 yielded the function \( y = 4.28x + 1.99 \), the slope of which (4.28%) represents the percent contraction per microgram of protein. The specific activity of RPEC-CM (4.28%/µg) is considerably higher than earlier values reported for fibroblast and glial conditioned medium (0.32%/µg). This suggests that RPEC are a potent source of contraction-promoting activity.
To confirm that the contraction-promoting activity recovered in RPEC-CM was actually a secretory product of RPEC, media were collected from RPEC cultures in which protein synthesis was inhibited by addition of cycloheximide. As a control, media to which cycloheximide was added after conditioning also were analyzed. All media samples were dialyzed against fresh media before use in a contraction assay. Media conditioned in the presence of cycloheximide did not promote choroidal fibroblast contraction (Table 1). Samples to which cycloheximide was added after conditioning retained approximately 75% of the activity of untreated RPEC-CM. These data demonstrate that the contraction promoter(s) observed in the conditioned media was a secretory product.

RPEC Secrete a Type A Contraction Promoter

To characterize the contraction-promoting activity present in RPEC-CM, we assayed the contraction using human skin fibroblasts, according to the previously established protocols. Skin fibroblasts placed on top of collagen matrices were incubated in RPEC-CM. At various times, the media were removed and replaced with serum-free media, and the incubation was continued (Fig. 7). Removal of the RPEC-CM halted contraction during the rest of the assay.

The dependence of RPEC-CM-promoted contraction on de novo protein synthesis was determined in assays in which fibroblasts contracting in response to the conditioned medium were blocked in protein synthesis with cycloheximide (Fig. 8). Treatment with the inhibitor only marginally reduced the RPEC-CM-promoted fibroblast contraction, resulting in an effect similar to that observed with serum. This response was in marked contrast with the complete inhibition observed when cycloheximide-blocked fibroblasts were stimulated with either endothelial cell conditioned medium or endothelin 1. Together, these two experiments suggested that the RPEC-secreted promoter has characteristics similar to those observed with type A promoters, such as serum, and distinct from those found in endothelial cell conditioned media.

Table 1. Retinal pigment epithelial cell secretion of contraction promoter is sensitive to protein synthesis inhibitors

<table>
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<tr>
<th>Media</th>
<th>Percent contraction</th>
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<tbody>
<tr>
<td>RPE cell conditioned medium</td>
<td>45.5 ± 5.1</td>
</tr>
<tr>
<td>Cycloheximide treatment</td>
<td>13.8 ± 5.7</td>
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<tr>
<td>Minimal essential medium alone</td>
<td>8.7 ± 3.0</td>
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Confluent cultures of retinal pigment epithelial cells were incubated for 24 hr in minimal essential medium with or without added cycloheximide. The media were collected and dialyzed against fresh media to remove the cycloheximide and were tested for contraction-promoting activity with skin fibroblasts. The results shown are the averages and standard deviations of results from quadruplicate cultures after 24 hr of incubation.
Discussion

Our intention was to compare the contraction potentials directly of three different cell types involved in the development of traction retinal detachment. Immunohistologic studies of epiretinal membranes identified RPEC, glial cells, and fibroblasts in isolated tissues. In addition, studies in which viable populations of these cells were introduced into the vitreous indicated that each can induce epiretinal membrane development and cause traction retinal detachment. This also can be induced by introducing activated macrophages (not examined in our study).

To assess the contraction potentials of the cells of interest, we established culture populations of porcine RPEC, retinal glial cells, and fibroblasts from choroid. These populations then were examined for contraction of collagen gels. Our findings showed that there were significant variations in the contraction potentials of the different populations. Glial cells, choroidal fibroblasts, and RPEC, at equal cell densities, were able to cause the contraction of the collagen gels (65%, 58%, and 8%, respectively) during 4 d of incubation. By increasing RPEC densities approximately tenfold, however, we observed substantial contraction. This was consistent with previous studies of RPEC contraction in vitro. Experiments in which cell numbers were varied yielded cell concentration-dependent curves; these were analyzed mathematically to derive specific activities. The percent contraction per 1000 cells was calculated to be 5.3 for retinal glial cells, 2.4 for choroidal fibroblasts, and 0.5 for RPEC under identical conditions. These studies were done with porcine cells and may not reflect the activities of cells derived from human tissues.

Analysis of the secretory products of these cell types indicated that RPEC elaborate potent contraction-promoting factors. Media conditioned by RPEC that were blocked in protein synthesis by cycloheximide were not active in promoting fibroblast contraction, confirming that the contraction promoter was a secretory product rather than serum contamination. Characterization of this factor in the context of previous studies suggested that the RPEC-secreted factor was similar to that found in serum and distinct from that recovered in the conditioned media of endothelial cells. Both FBS and the RPEC-secreted promoter required continuous exposure to the contracting cells. In addition, both the RPEC-secreted factors and FBS were active when the target cells were blocked in protein synthesis by added cycloheximide.

The identity of the RPEC-secreted contraction promoter has not been determined. However, one report found that RPEC secrete detectable quantities of transforming growth factor-beta. This is also active in promoting RPEC contraction in vitro. Transforming growth factor-beta also promotes fibroblast contraction of collagen gels. It is not known whether these factors are active in promoting glial cell contraction. Other investigators report that RPEC secrete factors that are chemotactic and mitogenic for glial cells and fibroblasts. Together, these studies indicate the potential (at least) for RPEC-induced migration, proliferation, and contraction by other cell types through elaborated factors. These findings also suggest that epiretinal membrane formation may be induced through two mechanisms, either directly by attachment of the cell and contraction or indirectly by recruiting other cells. This process in macrophages also probably is stimulated by elaborated chemotactic, mitogenic, and contraction-promoting peptides.

Key words: proliferative vitreoretinopathy, retinal pigment epithelium, fibroblasts, glial cells, contraction

References


