Rabbit Corneal Endothelial Cells Modulated by Polymorphonuclear Leukocytes Are Fibroblasts

Comparison With Keratocytes

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The authors previously reported that polymorphonuclear leukocytes modulate rabbit corneal endothelial cells into fibroblasts, which acquire the characteristics of fibroblasts. The progeny of the fibroblastic corneal endothelial cells (FCEC) were further studied to compare the characteristics of the fibroblast with those of keratocytes as a function of culture age. During 11 days in culture, FCEC showed 32 population doublings, whereas keratocytes underwent 10 population doublings. When collagen phenotypes of both cultures were analyzed as a function of culture age, labeled collagens in both cultures were fractionated into types I, III, and V. The proportion of each collagen was relatively unchanged in keratocytes regardless of culture age: type I accounted for 92–96%, type III for 2–6% and type V for 2–5%. In contrast, the profiles were significantly changed in FCEC: at day 2, type I accounted for 57%, type III for 37.5%, and type V 5.5%. Over the following 2 days, type I increased to approximately 75%, whereas type III collagen decreased to approximately 20%. As FCEC multilayered, type I collagen synthesis reached a stationary level of 80%, with 12% of type III. When the stoichiometry of type I collagen was compared, the α1/α2 ratio was 6.2 in FCEC and the ratio was 3.5 in keratocytes at day 2. The ratio reached a normal value at day 7 in FCEC and at day 3 in keratocytes. The synthesis of type I trimer and transient alteration of type I/III and the rapid growth rate at early stages of growth, indicate that FCEC behave like cells seen in wound healing or other rapidly growing tissues, in contrast to the stabilized keratocytes. Invest Ophthalmol Vis Sci 27:891–897, 1986

Polymorphonuclear leukocytes (PMNs) have been reported to modulate polygonal corneal endothelial cells into fibroblastic cells that synthesize predominantly type I procollagen rather than type IV collagen seen in Descemet's membrane/corneal endothelium complex.1–3 When injury is inflicted on the corneal endothelium, PMNs migrating into the anterior chamber directly influence the endothelium, whereas PMNs that infiltrate into the stroma may exert an influence through the release of inflammatory mediators or lysosomal enzymes.4 Although PMNs maintain the integrity of host defense against microbial infections and develop an inflammatory response, some enzymes released by PMNs escape and attack the host tissues.5,6 Under such conditions, atypical repair mechanism may occur at the Descemet's membrane/endothelium complex. Retrocorneal fibrous membrane (RCFM), seen in vivo and in experimental animals, is the ultimate outcome of such atypical wound repair.7–9 RCFM present posterior to the pre-existing Descemet's membrane is composed of collagen fibrils, amorphous substances, and fibroblastic cells interspersed between extracellular matrices. The cells composing RCFMs synthesize predominantly type I procollagen.9

To understand the atypical repair mechanism in Descemet's membrane/corneal endothelium complex, we studied the effect of polymorphonuclear leukocytes on the primary cultures of rabbit corneal endothelial cells. In previous studies1,2 we reported that a subpopulation of corneal endothelial cells responded either when exposed directly to PMNs or to PMN-conditioned medium. The responding endothelial cells changed their shape from polygonal to that of fibroblast and irreversibly changed their collagen phenotypes from type IV to type I. In the present study, we further characterized the fibroblastic endothelial cells by comparing them with keratocytes. The fibroblastic corneal endothelial cells had collagen phenotypes similar to those of keratocytes. In addition, they have further characteristics of cells seen in wound healing: rapid growth rate, altered ratios of type I/III collagen and the presence of type I trimer at early stages of growth:

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in contrast, keratocytes maintain the controlled ratio of type I/III collagen and stoichiometry of type I collagen.

Materials and Methods

Cell Cultures

Modulated rabbit corneal endothelial cells were established by using PMN-conditioned medium as described previously. Primary rabbit keratocytes were donated by Dr. Michael Delmage (Estelle Doheny Eye Foundation, Los Angeles, CA). Isolation and establishment of keratocytes in culture were previously described. Polymorphonuclear leukocytes (PMNs) were obtained from the peritoneal cavity of New Zealand rabbits after glycogen stimulation as described previously. The isolated and purified PMNs were resuspended in DMEM supplemented with 10% fetal calf serum and 50 μg/ml gentamicin. PMN-conditioned medium was obtained from 1-day-old culture, which was incubated at 37°C in a humidified atmosphere of 7.5% CO₂ in air. Medium was then collected and sterilized. The rabbits used in this study were handled in accordance with the ARVO Resolution on the Use of Animals in Research.

Protein Synthesis

The cells were labeled for 20 hr with DMEM* containing 200 μCi[5-3H]proline (27 Ci/mM) (Amersham Corp.; Arlington Heights, IL), 2% fetal calf serum, 50 μg/ml 2-aminopropionitrile, and 50 μg/ml ascorbate. Medium was collected and the cell debris was removed by centrifugation. An aliquot of medium factor was dialyzed against 0.5 M acetic acid followed by pepsin treatment (100 Aig/ml) at 4°C for 24 hr. The enzyme was stopped by raising pH to 8.0. The reaction mixture was then dialyzed against 0.5 M acetic acid, lyophilized, and redissolved in 0.15 M NaCl, 0.05 M Tris–HCl, pH 7.4, 0.1% Triton-X-100.

Determination of DNA

DNA was assayed by fluorometric analysis. Cell layers were digested with pronase for 24 hr at 55°C, and the released DNA was precipitated by cetyltrimethylammoniumbromide. The precipitate was collected and reacted with diaminobenzoic acid for fluorometric analysis.

RESULTS

Although fibroblastic corneal endothelial cells (FCEC) have been reported to assume the characteristics of fibroblasts, comparison has not been made with any known fibroblast cell line. Johnson et al and Noden presented evidence, using quail–chicken transplantation experiments, that neural crest gives rise to the endothelium as well as to the fibroblasts of the avian cornea. Keratocytes in culture were therefore used to compare with FCEC the aspects of growth behavior, collagen synthetic capacity, and collagen phenotypes. Due to the fact that the 24th passage of FCEC had shown to express identical collagen phenotypes observed in the earlier passage cultures (8–10th), the 20th passage culture was compared with the third passage keratocyte cultures which showed identical collagen phenotypes with the primary and the first passage cultures (results not shown). It should be noted that the terminology of fibroblast has been descriptively used for representing any cell that demonstrates spindle-shaped morphology and subsequently produces an extracellular matrix consisting predominantly of type I collagen. All data were based on two or more analyses of triplicate cultures.

Comparison of Growth and Morphology

FCEC or keratocytes were inoculated at 4 × 10⁵ cells per 60-mm culture dish and allowed to attach to the substratum. Cells were then recovered from trypsinization at days 1, 2, 3, 4, 7, 9, and 11 and counted. The growth behaviors of the two cell lines were compared as a function of days in culture. Figure 1A shows the characteristic growth curves of FCEC and keratocytes. Both cultures required 1 day to attach and appeared to lose half of the initially inoculated cell density. The number of FCEC present on day 2 (1.15 × 10⁶ cells/dish) was approximately six times higher than the

* Abbreviations used in text: DMEM, Dulbecco-Vogt Modified eagle’s medium; EDTA, ethylenedinitrilotetraacetic acid; PAGE, polyacrylamide gel electrophoresis.

SDS-Slab Gel Electrophoresis

Polypeptides were electrophoresed under the conditions described by Laemmli, and fluorograms developed by Kodak RPX-OMAT processor (Eastman Kodak; Rochester, NY). For the quantitation of individual collagen types, the medium fractions treated with pepsin were separated by disc gel electrophoresis. The radioactive gels were cut into 1-mm slices, hydrolyzed in 0.2 ml of 0.4 M NaOH at 55°C overnight, neutralized, and counted.

Enzyme Digestion

Bacterial collagenase digestion was performed as described by Peterkofsky and Diegelmann.

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plated cell density (0.20 X 10^6 cells/dish), whereas that of keratocytes was approximately three times higher than the plated cell density (0.20 X 10^6 cells on day 1 and 0.65 X 10^6 cells on day 2). Although cells continued to replicate during the entire 11-day period in culture, such exponential growth was not observed in either FCEC or keratocytes on the subsequent days. At day 11, cell density of FCEC (6.45 X 10^6 cells) was 32-fold greater than the plated cell density, whereas that of keratocyte (2.10 X 10^6 cells) was ten-fold greater. FCEC, having a rapid growth rate, result in multilayers of cells, whereas keratocytes, having a lower growth rate, maintain monolayer. When the amounts of DNA from the recovered cells were measured at each day, the slopes were approximately parallel to those of the cell numbers (Fig. 1B), suggesting that the amounts of DNA in both cultures are consistent with the numbers of cells present.

Collagen Synthesis

In order to compare the capacity of protein and collagen synthesis in FCEC with that of keratocytes, the cells were labeled with [3H]proline for 20 hr on the designated days. Radioactive collagens were determined by their susceptibility to purified bacterial collagenase. Synthesis of total medium proteins and collagen was analyzed as a function of culture age (Table 1). Incorporation of [3H]proline into total medium proteins in keratocytes at any stage of growth during the 11 days in culture was approximately twice that of FCEC. On the other hand, collagen synthesis appeared to be similar in both cultures during the early stages of growth. During days 7–9, at which time FCEC multilayered in contrast to the monolayer of keratocytes, collagen synthesis decreased in both cultures, but at different levels: it was almost half rate of the early stages in FCEC in contrast to the marked decrease in keratocyte culture (approximately 20% level of early days). The proportion of collagen in the medium protein of FCEC increased on day 3 (27% of the total medium proteins) and day 4 (24%) and maintained a stationary level of 15% afterwards; keratocyte cultures maintained a 13–15% proportion of collagen synthesis in the early stages of growth, and then the proportion of collagen dramatically reduced when cultures reached the stationary stage of growth.

Fibroblasts in culture have been shown to synthesize both types I and III collagen. However, the ratio of type I to type III collagen synthesized by fibroblasts appears to be controversial: Abe and associates reported that proportionally more type III is produced at high cell density, whereas Hance and Crystal reported that the ratio of type I to III collagen remained constant with time after plating and after subculture. In order to accurately assess the expression of collagen phenotypes of FCEC in comparison with those of keratocytes, synthesis and expression of the individual collagen types were analyzed as a function of culture age. Medium fractions were treated with pepsin prior to analysis on SDS electrophoresis in order to analyze only collagen molecules containing native triple-helical conformation. Labeled collagens obtained from both culture media were fractionated into several components, such as type III as disulfide-bonded molecules,

![Fig. 1. Growth characteristics of fibroblastic corneal endothelial cells (FCEC) and keratocytes. Cells were inoculated at 4 X 10^6 cells per 60-mm culture dish, and then recovered from trypsinization at days indicated prior to counting. The counted cells were assayed for DNA as described in the text. A, cell numbers; B, amounts of DNA. ● FCEC, O, keratocytes.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933129/)

![Table 1. Synthesis of medium collagen by fibroblastic corneal endothelial cells (FCEC) and keratocytes as a function of culture age](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933129/)

<table>
<thead>
<tr>
<th>Cell source</th>
<th>Days in culture</th>
<th>Total incorporation into collagen</th>
<th>Percent in collagen</th>
</tr>
</thead>
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<tr>
<td>FCEC</td>
<td>2</td>
<td>68.7</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>63.2</td>
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<td>7</td>
<td>61.3</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>47.3</td>
<td>6.5</td>
</tr>
<tr>
<td>Keratocyte</td>
<td>2</td>
<td>152.8</td>
<td>20.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>115.0</td>
<td>17.5</td>
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<td>4</td>
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<tr>
<td></td>
<td>9</td>
<td>94.4</td>
<td>3.1</td>
</tr>
</tbody>
</table>

Individual cultures were labeled with [3H]proline for 20 hr. Medium layers were dialyzed against 0.15 M NaCl, 0.05 M Tris HCl, pH 7.4, 0.1% Triton-X-100. A fraction of dialyze was precipitated with cold TCA (to a final 10% concentration) either prior to bacterial collagenase digestion or following the enzyme treatment. The difference of radioactivity in the two TCA precipitates was used for collagens peptides. TCA precipitable counts prior to the enzyme digestion were used for total incorporation.
Fig. 2. Collagen phenotypes synthesized by fibroblastic corneal endothelial cells (FCEC). Cells from the designated days were labeled with [3H]proline for 20 hr. The medium fractions were treated with pepsin prior to electrophoresis on 4.5% SDS-PAGE under nonreduced conditions. 1 and 2, day 1; 3 and 4, day 2; 5 and 6, day 3; 7 and 8, day 4; 9, collagen marker; 10 and 11, day 7; 12 and 13, day 9; 14 and 15, day 11. Marker collagen was prepared from organ culture of rabbit corneal stroma that were labeled with radioactive precursor.

type V collagen as \( \alpha_1(V) \) and \( \alpha_2(V) \), and type I collagen as \( \alpha_1(I) \) and \( \alpha_2(I) \). The identification of the trimeric type III collagen was supported by the characteristic CNBr-peptide profiles on the two-dimensional electrophoresis (result not shown). Type I collagen is the major collagen synthesized by both FCEC (Fig. 2) and keratocytes (Fig. 3), regardless of culture age, whereas types III and V collagen are present as minor components.

Fig. 3. Collagen phenotypes synthesized by keratocytes. Cells were labeled and prepared as described in Figure 2. 1 and 2, day 1; 3 and 4, day 2; 5 and 6, day 3; 7 and 8, day 4; 9 and 10, day 7; 11 and 12, day 9; 13 and 14, day 11.

Fig. 4. Quantitation of individual collagen types synthesized by fibroblastic corneal endothelial cells (FCEC) and keratocytes as a function of culture age. The cells were labeled with [3H]proline at the designated days. Quantitation procedures were described in the methods.

The proportion of the individual collagen types during the 11 days in culture appeared to be significantly changed as FCEC cultures grew older, but keratocytes appeared to express rather stationary levels of these molecules. Therefore, systematic studies were performed to evaluate the relationship between the expression of each collagen type and culture age. Individual chains were separated by disc gel electrophoresis and then treated mathematically according to the known stoichiometry of chains of each collagen type (Fig. 4). On day 2, type I collagen accounted for 57%, type III for 37.5%, and type V for 5.5% in FCEC. On days 3 and 4, the proportion of type I collagen was increased to approximately 75%, while type III was decreased to approximately 20%. As FCEC multilayered, type I collagen synthesis reached a stationary level of 80% with approximately 12% type III collagen. This finding is different from that previously reported by Hance and Crystal. Unlike their findings, the expression of types I and III collagen appears to be in a dynamic state in FCEC, with a marked transient decrease in type I synthesis and a significant increase in type III synthesis prior to these two molecules reaching stationary levels. Furthermore, this finding also differs from that reported by Abe and colleagues, ie, that the ratio of type I to III becomes lower at high cell density. We found that at high cell density, type III synthesis reached a stationary level, but at low cell density the proportion of this molecule was transiently increased. Synthesis of type V collagen appeared to gradually increase as the cultures aged. On the other
hand, synthesis of these three collagen types by keratocytes showed a very different profile: type I accounted for 92–96% of the total collagen, whereas types III and V collagen respectively ranged from 2–6%, regardless of culture age. The amount of type V collagen synthesized by keratocytes is consistent with the findings of others.19 Although the presence of type III collagen in corneal stroma is controversial,19–21 synthesis of type III collagen in this culture was consistently observed in the range of 2–6% regardless of culture age.

Since FCEC exhibited a rather active state of collagen metabolism the stoichiometry of type I collagen was studied (Table 2). At day 2, $\alpha_1/\alpha_2$ of type I collagen in FCEC was 6.2, which deviates significantly from the expected 2. When mathematically calculated from the actual values, type I collagen at day 2 consisted of equal amounts of $(\alpha_1)_2\alpha_2$ and $(\alpha_1)_3$. As cultures grew older, the $\alpha_1/\alpha_2$ ratio was significantly decreased until it reached the stationary value of 2.4 on day 7. On the other hand, keratocytes showed a slight increase in the $\alpha_1/\alpha_2$ ratio at day 2, indicating that only 16% of type I collagen is in a homopolymeric form. The stoichiometry of the molecule reached normal value from day 2 in this culture. Since type I trimer has been demonstrated in rapidly growing tissues,22 in normal cell cultures,23 in cell lines derived from tumors,24 in tumors,25 and in diseased human gingiva,26 the presence of type I trimer in FCEC on day 2 may suggest that FCEC at this stage retain some characteristics of rapidly growing fibroblasts.

### Discussion

The present results demonstrate that the progeny of fibroblastic rabbit corneal endothelial (FCEC) cells that have been modulated by polymorphonuclear leukocytes (PMN) have some of the characteristics of rabbit keratocytes. FCEC and keratocytes in culture both synthesize types I, III, and V collagen, with type I collagen as a predominant species. Nevertheless, there are significant differences to distinguish these two fibroblastic cell lines: (1) The growth kinetics significantly differ: FCEC divide three times faster than keratocytes; therefore, FCEC multilayer resulted in deposition of extracellular matrices between cell layers, whereas keratocytes maintain confluency but monolayer during 11 days in culture. (2) During the early stages of growth, the proportion of individual collagen types synthesized by FCEC is dramatically changed depending on the culture age, in contrast to the stabilized expression of these collagens in keratocytes. Type I collagen accounted for 57% of the total collagen, type III accounted for 37.5%, and type V for 5.5% in FCEC on day 2.

<table>
<thead>
<tr>
<th>Days in culture</th>
<th>$\alpha_1/\alpha_2$ of FCEC*</th>
<th>$\alpha_1/\alpha_2$ of keratocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>6.2</td>
<td>3.5</td>
</tr>
<tr>
<td>3</td>
<td>3.8</td>
<td>2.5</td>
</tr>
<tr>
<td>4</td>
<td>3.2</td>
<td>2.4</td>
</tr>
<tr>
<td>7</td>
<td>2.4</td>
<td>2.2</td>
</tr>
<tr>
<td>9</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>11</td>
<td>2.3</td>
<td>2.3</td>
</tr>
</tbody>
</table>

The $\alpha_1/\alpha_2$ ratio demonstrated in rapidly growing tissues,22 in normal cell cultures,23 in cell lines derived from tumors,24 in tumors,25 and in diseased human gingiva,26 the presence of type I trimer in FCEC on day 2 may suggest that FCEC at this stage retain some characteristics of rapidly growing fibroblasts.

Type I collagen at this stage is composed of equal amounts of $(\alpha_1)_2\alpha_2$ and $(\alpha_1)_3$, indicating that type I trimer constitutes almost 30% of the total collagen on day 2. The proportion of individual collagens reaches stationary levels from day 7 and on, at which time cells multilayer and deposition of extracellular matrix is apparent. At the same time, ratio of $\alpha_1/\alpha_2$ of type I collagen becomes normal. On the other hand, keratocytes do not exhibit any remarkable changes in either the ratio of type I/III or the stoichiometry of type I collagen during 11 days in culture, indicating that keratocytes appear to be programmed to preserve their capacity to synthesize the interstitial collagens in a controlled fashion, whether cells are in a rapidly growing stage or are slowly replicating. However, FCEC that originated from endothelial cells and that had acquired metaplastic characteristics may represent a specific clone of fibroblasts, distinguished from normal fibroblasts. The significant difference in collagen metabolism between FCEC and keratocytes suggests that substances released by polymorphonuclear leukocytes initiate modulation of responding corneal endothelial cells and create selective growth pressures on such cells. The resulting fibroblastic corneal endothelial cells may, therefore, differ from the fibroblasts in normal extracellular milieu. It has been reported that fibroblast populations are heterogeneous with respect to growth potential and various biochemical and physiological parameters.27

Collagens, the major constituents of the extracellular matrix, are known to have fundamental roles in the structure and function of connective tissues. Among the fibril-forming collagens (also called interstitial collagens), type I collagen is the major species in most connective tissues, including skin, tendon, bone, dentin, cornea, fascia, and etc. Type III collagen is also found in appreciable amounts in many connective tissues, but not in dentin or bone. Most pliable tissues
(skin, blood vessels, lung, etc) have appreciable amounts of type III collagen; however, the rigid tissues, such as bone and dentin, contain only type I collagen. The ratio of type I to III collagen also changes under conditions of inflammation and wound healing: early granular tissue has a low I/III ratio, but mature scar has a high I/III ratio. These findings suggest that type I/III ratios are closely associated with the structure and function of the tissues. The amounts of types I and III collagen, therefore, have to be critically regulated in the normal interstitial tissues. On the other hand, when tissues are disrupted by injury or inflammation, cells participating in the wound healing are subjected to the changed extracellular milieu and specific clones of fibroblasts may be transiently selected for the ultimate task of repair. Under such conditions, the relative proportions of types I and III collagen are transiently altered, and synthesis of type I trimer becomes significant. In these aspects, FCEC at day 2 is similar, and may represent such specific clones of fibroblast.

An abnormal collagenous layer appearing between Descemet’s membrane and corneal endothelium has been designated as posterior collagenous layer (PCL), although diverse terms are currently used. Based upon ultrastructural studies of PCL in more than 30 corneal disorders, it can be classified into three types: banded, fibrillar, and fibrocellular. Nevertheless, the specific cell type responsible for the production of PCL, regardless of type of PCL, has not been determined. It has been speculated that endothelium is the most likely source, as demonstrated in numerous experimental model. However, there is no direct evidence that particular corneal endothelial cells are modulated into fibroblasts, which are subsequently responsible for the production of PCL in vivo. Our previous studies in vitro, using polymorphonuclear leukocytes to modulate corneal endothelial cells into fibroblasts, provide evidence that one differentiated cell type (endothelial cells) can be modulated to another cell type (fibroblast).

The data presented here further suggest that fibroblastic corneal endothelial cells showing a dynamic state of cell metabolism, may represent specific fibroblasts such as those that participate in the early stages of wound healing. The counterpart, in vivo, could be selected by a similar mechanism, ie, the continuous influence of inflammation. Therefore, modulation by polymorphonuclear leukocytes and selection of a specific fibroblast clone may represent a mechanism in the production of posterior collagenous layer.

Key words: corneal endothelial cells, keratocytes, fibroblasts, polymorphonuclear leukocytes, collagen

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References