Type I collagen and fibronectin synthesis by retrocorneal fibrous membrane

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The primary cultures obtained from the experimentally induced retrocorneal fibrous membrane synthesized and secreted into the medium mainly type I procollagen. This collagen was characterized after limited pepsin treatment and identified as type I collagen by the following criteria: (1) it contained two α1 chains and one α2 chain, (2) its sedimentation behavior was identical to that of type I collagen from skin, and (3) its peptide map after limited proteolysis with Staphylococcus aureus V8 protease was identical to that of type I collagen. The medium contained procollagen I, which was converted into α size chains by limited pepsin treatment, whereas the cellular fraction contained type I collagen already processed to its end product. Type III collagen and basement membrane collagen were present as minor components in this system. Fibronectin, one of the major glycoproteins in extracellular matrices, was also synthesized and secreted into the medium. In contrast, normal corneal endothelial cells produce mainly basement membrane collagen. (INVEST OPHTHALMOL VIS SCI 22:200-212, 1982.)

Key words: retrocorneal fibrous membrane, endothelium, tissue culture, organ culture, procollagen, collagen, basement membrane collagen, fibronectin, ultracentrifugation

The retrocorneal fibrous membrane (RCFM), which was first described by Fuchs in 1901, has been observed in various clinical conditions associated with disease and damage of the corneal endothelium. The presence of RCFM posterior to the pre-existing Descemet's membrane is thought to represent an end-stage disease process of the corneal endothelium, which results in functional alterations of the corneal endothelium, leading to corneal opacity and blindness.

Investigators have studied the morphologic changes during RCFM formation in an attempt to determine the origin of the "fibroblast-like" cell present within this membrane. Currently there are two basic theories regarding their origin; (1) participation by the stromal fibroblast or (2) metaplasia of the corneal endothelial cell to a fibroblast-like cell. To date there are no data regarding the biochemical alterations within this membrane. In this paper, we present our results concerning collagen and fibronectin synthesis by cells isolated from freeze-induced RCFMs. The major collagen type synthesized in this system was type I collagen, which was secreted into the medium in its precursor forms. On the other hand, normal corneal endothelial cells in cul...
ture produced basement membrane collagen.

Methods and materials

Production of RCFMs. Eight-week-old New Zealand white rabbits were used in all the experiments. Animals were sedated with ketamine, and anesthesia was induced with sodium pentobarbital administered intravenously through an ear vein. Proparacaine solution (Alcaine; Alcon Laboratories, Inc., Ft. Worth, Texas) was applied topically to the cornea, and a lid speculum was inserted. Transcorneal freezing was performed with an Alcon surgical cryostat unit (DNE-300 U) with nitrous oxide and a probe (diameter 2.5 mm) temperature of -80°C. The probe was applied to the midcornea for 30 sec, removed for 30 sec, then reapplied for 30 sec. In a similar fashion, the superior, inferior, temporal, and nasal corneal quadrants were frozen with slight overlap of the central application spot. After the procedure, atropine and gentamicin sulfate (Garamycin; Schering Corp., Kenilworth, N.J.) were instilled in each eye. Freezing was performed at evenly spaced bi-weekly intervals for four sessions. The animals were followed by external eye examination and slit-lamp microscopy and were sacrificed 1 week after final cryoapplication. At least one eye per cryoapplication group was examined histologically to determine whether there was contamination by corneal keratocytes.

Cell culture. Cell cultures of RCFMs were prepared as follows. The corneal epithelium was removed by scraping with a No. 15 surgical blade. The central cornea containing the RCFM was then excised with curved corneal scissors. The membrane was scraped from the overlying corneal stroma under a dissecting microscope. The RCFM was rinsed in sterile HEPES-Spinner solution (Millipore Corp., Bedford, Mass.) for 3 min at room temperature. The RCFM was then incubated in a solution containing 0.2% collagenase (Millipore) and 0.05% hyaluronidase (Millipore) at 37°C for 90 min and centrifuged at 1000 rpm for 5 min; the pellet was resuspended in 0.2% trypsin containing 1 mM dithiothreitol at room temperature. The trypsin reaction was stopped after 3 min by the addition of an equal volume of 20% fetal calf serum in Dulbecco’s modified Eagle medium (DMEM). This suspension was centrifuged at 1000 rpm for 5 min, and the supernatant was discarded. The pellet was resuspended in DMEM supplemented with 20% fetal calf serum and plated on 100 mm culture dishes (Falcon). Primary cultures were maintained in DMEM supplemented with 10% fetal calf serum and 50 μg/ml gentamicin. All cultures were incubated at 37°C under a humidified atmosphere of 7.5% CO₂ in air.

Isolation and establishment of corneal endothelial cells in culture were previously described.¹³

Isolation of collagen synthesized by RCFM cells. Cells grown to confluence (7 to 10 days) were labeled for 24 hr with 10 ml of DMEM containing 200 μCi of [3H] proline (27 Ci/mM) (Amersham), 2% fetal calf serum, 100 μg/ml ascorbic acid, and 64 μg/ml 2-aminopropionitrile (β-APN). Medium fraction was harvested while cellular fraction was extracted in 0.05M Tris HCl, pH 7.4, 1.0M NaCl, 0.1% Triton-X-100. To the medium and cellular fractions the following protease inhibitors were added: 1 mM phenylmethyl sulfonyl fluoride, 10 mM N-ethylmaleimide, 4 mM ethylenedinitrilotetraacetic acid (EDTA), and 0.1 mM α,α’-dipyridyl. Ammonium sulfate was added to the medium fraction to 45% of saturation after centrifugation for removal of cell debris. The precipitate was collected by centrifugation at 10,000 × g for 15 min at 4°C and redissolved in 2.0M urea and buffer I (0.05M Tris HCl, pH 7.4, 0.15M NaCl, 0.1% Triton-X-100) followed by dialysis into the same buffer.

Extraction of collagen synthesized by RCFM organ culture. RCFMs prepared as described in the previous section were labeled for 4 hr with DMEM containing 5[3H] proline, 10% fetal calf serum, 100 μg/ml ascorbic acid, and 64 μg/ml β-APN. The membranes were separated from the medium fraction and extracted in 0.05M Tris HCl, pH 7.4, 1.0M NaCl, 0.1% Triton-X-100 followed by dialysis into buffer I.

Velocity ultracentrifugation. The sample (before or after pepsin treatment) was layered on a 5% to 20% sucrose gradient containing 2.0M urea and buffer I, placed over a 50% sucrose cushion, and sedimented in a Beckman SW 40 rotor at 40K rpm for 48 hr at 4°C. The gradient was fractionated. Fractions containing collagenous protein were pooled and dialyzed against 6.0M urea in buffer I at 4°C. The samples were then denatured by heating to 55°C for 30 min and sedimented on a 5% to 20% sucrose gradient containing 6.0M urea and buffer I in a Beckman SW 40 rotor at 40K rpm for 48 hr at 20°C.

Sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis (SDS-PAGE). Polypeptides were electrophoresed under the conditions described by Laemmli,¹⁴ and flurograms were developed by a Kodak RPX-OMAT processor.¹⁵,¹⁶
Fig. 1A. Light micrograph of freeze-injured rabbit cornea taken 1 week after final cryoapplication. An extensive RCFM was present posterior to Descemet's membrane (arrow). This membrane was composed of multiple fibrous layers containing fibroblastic-like cells (Periodic acid-Schiff stain; X150.)

Peptide mapping by *Staphylococcus aureus* V8 protease digestion. Protein bands separated by electrophoresis and localized by fluorograph were swelled with 0.125M Tris, pH 6.8, 0.1% SDS, 1 mM EDTA (buffer II) and then placed onto 3% stacking gel slots. A 10 or 20 μg amount of the protease in buffer II containing 20% glycerol was overlaid, and electrophoresis was carried out as described by Cleveland et al.17

Enzyme digestions. Samples were incubated with 100 μg/ml pepsin for 24 hr at 4° C or 24 hr at 15° C. Pepsin activity was stopped by raising the pH to 8.0 with the addition of solid Tris and boiling for 1 min in the presence of 0.2% SDS. Protease-free bacterial collagenase digestion was performed as described by Peterkofsky and Diegelman.18 The reaction was stopped by addition of SDS to a final concentration of 0.2%, followed by boiling.

*Preparation of marker collagen.* Fibroblasts from embryonic rabbit skin were cultured by conventional techniques.20 Secondary cultures were then labeled and the collagen purified.21

**Results**

Morphologic characterization of RCFMs and their cell cultures. Multiple freeze injury to rabbit corneas resulted in RCFM formation in more than 90% of the treated eyes. These membranes covered the central area of the cornea posterior to Descemet's membrane and were composed of multiple layers of fibrous tissue containing fibroblast-like
Type I collagen and fibronectin synthesis

Fig. 1B. Light micrograph of isolated RCFM from freeze-injured rabbit cornea. The RCFM appears folded with fibrous tissue bordered on both sides by Descemet's membrane (arrow). The anterior borders of Descemet's membrane appeared to be uncontaminated by corneal fibroblasts. Broken portions of Descemet's membrane in this section appear to be artifically induced (open arrow). (Periodic acid-Schiff stain; X300.)

cells (Fig. 1A). Membranes isolated for tissue culture contained, in addition to the RCFM, normal peripheral endothelial cells and Descemet's membrane. Stromal contamination of the anterior side of Descemet's membrane was not identified by routine histologic techniques (Fig. 1B). Membranes introduced into culture exhibited at least two cell populations: fibroblast-like cells and flattened senescent cells (Fig. 2A). At confluency the principal cell type was the fibroblast-like cell, which had a tendency to multilayer, exhibiting characteristic of transformed cells (Fig. 2B). In contrast, confluent primary cultures from normal corneal endothelium exhibited characteristic polygonal shaped cells and contact inhibition (Fig. 2C).

Isolation and purification of collagenous peptides synthesized by RCFM culture. Collagenous peptides were primarily characterized from the soluble fraction recovered from the medium. Initial isolation of the collagen was performed by ultracentrifugation under native conditions. Fig. 3A shows the typical sedimentation profile of the medium fraction concentrated with ammonium sulfate followed by limited pepsin treatment. The major peak (fraction numbers 19 to 22) co-sedimented with known type I marker collagen, which was sedimented in a companion tube at the same time. This peak was rich in type I collagen present in its helical conformation. Type III collagen was also present as a minor component. A minor fast-sedimenting peak (fraction numbers 27 to 29) contained basement membrane collagen which was designated as CE (still held by interchain disulfide bridges after limited pepsin treatment). This CE collagen was identical to that synthesized by normal corneal endothelial cells in terms of sedimentation behavior, electrophoretic mobility, and insensitivity to
Fig. 2A. Phase-contrast micrograph of primary RCFM cells at 4 days after inoculation. Arrow indicates flattened senescent cells. (×170.)

Fig. 2B. Phase-contrast micrograph of primary RCFM cells at confluence. (×170.)
protease. The fractions separated by sedimentation were further analyzed on SDS-PAGE (Fig. 3B). It was apparent that type III collagen existed in a trimeric form with an intrahelical disulfide bond and that type I collagen yielded monomeric $\alpha_1$ and $\alpha_2$ chains (fraction numbers 19 to 22). The shoulder area of this major peak (fraction numbers 16 to 18) contained a number of smaller pepsin-resistant peptides that could be removed by bacterial collagenase.

For further purification, fractions containing type I and III collagen were pooled, denatured, and then subjected to a second ultracentrifugation under denaturing conditions. Fig. 4 shows one major peak that contained mostly denatured type I collagen yielding a mixture of $\alpha$ chains (fraction numbers 20 to 23) and disulfide-linked type III collagen (fraction numbers 24 and 25). The type I fraction showed an $\alpha_1/\alpha_2$ ratio of 2, in contrast to type III–rich fractions, which contained mostly $\alpha_1$ chains.

**Comparison of organ culture product to cell culture product.** When RCFMs were incubated in organ culture for 4 hr with $[^3]H$ proline, approximately 80% of the total nondialyzable $[^3]H$proline–labeled proteins was present in the tissue fractions. Comparison of the collagen molecules obtained from organ culture to those obtained from the cell culture medium subjected to limited pepsin treatment demonstrated that type I collagen was the major collagenous peptide in both systems (Fig. 5). Appreciable amounts of disulfide-linked type III collagen and CE collagen were also present in the cell culture medium, whereas the presence of these two molecules was markedly reduced in the organ culture products. It is of interest to note that type I collagen obtained from organ culture was already converted into $\alpha$ size chains, suggesting that the tissue contains very specific proteases which cleave off non-collagenous extensions of the procollagen molecule. Such enzymes, amino and carboxyl procollagen peptidases, have been demonstrated in chick embryo tendon cell culture. 21

**Characterization to type I collagen synthesized by RCFM culture.** Since monomeric
Fig. 3A. Velocity sedimentation under native condition. Ammonium sulfate precipitate of the medium protein was pepticized and sedimented in a Beckman SW 40 rotor at 40K rpm for 48 hr at 4°C. Closed arrow, Type I collagen marker; open arrow, position of basement membrane collagen.

Fig. 3B. Electrophoretogram of fractions in Fig. 1A on 4.5% SDS-PAGE under nonreducing condition. 1, Fraction 17; 2, fraction 18; 3, fraction 19; 4, fraction 20; 5, fraction 21; 6, fraction 22; 7 fraction 23; 8, fraction 24; 9, fraction 27; 10, fraction 28; 11, fraction 29; CE, basement membrane collagen.
Type I collagen and fibronectin synthesis

Fig. 4. Velocity sedimentation under denaturing condition. Fractions 19 to 22 of Fig. 3A were pooled, denatured, and sedimented as described in the text. Inset, Electrophoretogram of fractions 19 to 24 on 4.5% SDS-PAGE under reducing condition.

α chains of type I collagen were separated from trimeric type III collagen molecules on SDS-PAGE, each fraction was analyzed for the α1/α2 ratio (Table I). Known type I marker collagen gave a ratio of 2.16 by the technique used, and type I collagen synthesized by RCFM yielded a ratio close to that value. Type I collagen that was extracted from retrocorneal fibrous membranes incubated in organ culture for 4 hr with [3H]proline gave a value of 2.35.

Type I collagen from RCFM was further characterized by limited proteolysis with S. aureus V8 protease in SDS. The α1 and α2 chains from RCFM type I collagen exhibited peptide profiles identical to known type I collagen (Fig. 6).

Processing of procollagen I. Under normal physiological conditions, procollagen I is converted completely to type I collagen. Since RCFM in organ culture demonstrated complete conversion of procollagen to collagen, the capacity of processing in the cell culture was analyzed prior to protease digestion. Fig. 7 shows a comparison of the materials obtained from medium and cellular fractions. The molecules secreted into the medium were predominantly present as both precursor forms (pro α1 and pro α2) and intermediate forms (pN-collagen and pC-collagen). In contrast, the conversion of procollagen to collagen was completed in the cellular fraction. These observations suggest that although there are procollagen peptidase activities in the medium, it is not sufficiently active to convert all the precursors to end products.

Biochemical products by corneal endothelial cell. Corneal endothelial cells in culture synthesized and secreted basement membrane collagen (CE collagen) as a major col-

### Table I. Ratio of α1 to α2 of type I collagen synthesized by RCFM

<table>
<thead>
<tr>
<th>Sample</th>
<th>α1/α2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I collagen*</td>
<td>2.16</td>
</tr>
<tr>
<td>RCFM-1t</td>
<td>2.16</td>
</tr>
<tr>
<td>RCFM-2t</td>
<td>2.29</td>
</tr>
<tr>
<td>RCFM-3t</td>
<td>2.10</td>
</tr>
<tr>
<td>RCFM-4t</td>
<td>2.27</td>
</tr>
<tr>
<td>RCFM organ culture</td>
<td>2.35</td>
</tr>
</tbody>
</table>

Each monomeric band separated by electrophoresis and localized by fluorograph was hydrolyzed with 4N NaOH for 16 hr at 55°C and neutralized with 1M acetic acid. Each sample was counted in a Beckman LS 3133 T counter.

*Type I collagen isolated from rabbit skin fibroblast culture.

†Tissue culture product.  

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Fig. 5. Electrophoretograms of collagenous peptides synthesized by RCFM cell culture or organ culture resolved by 4.5% SDS-PAGE. 1, Collagen marker; 2, to 4, medium fraction that was labeled with $[^3]H$proline for 24 hr and concentrated with ammonium sulfate followed by pepsin treatment; 5, 1.0M salt tissue extract that was labeled in organ culture for 4 hr with $[^3]H$proline.

Lagenous peptide (Fig. 8). The CE collagen was disulfide-linked, sedimented faster than interstitial procollagens, and yielded two bands (CE 1 and CE 2) on SDS-PAGE under reducing condition. CE 1 and CE 2 migrated very close to each other and immediately below $\beta$ components.

Fibronectin synthesis by RCFM cell culture. When the cell culture medium was analyzed by velocity sedimentation prior to subjecting it to protease, a faster sedimenting peak than CE collagen was present. One of the components of the peak is fibronectin (data not shown). The apparent molecular weight of fibronectin (440K) is smaller than that of CE collagen. Fibronectin was also identified with goat anti-fibronectin serum, followed by incubation with Staph A. The precipitate enhanced by this organism was analyzed by SDS-PAGE. Fig. 9 demonstrates that the protein in the immune precipitate was fibronectin and that all other proteins, including procollagen, are unreacive toward antifibronectin serum.

Discussion
Understanding the functional significance of the molecular diversity of collagen and the control of its biosynthesis by different cells is particularly important in tissues that serve specific functions, such as the light-transmitting cornea. Collagen, the most common protein in the animal kingdom, is a major component of almost every ocular structure. Corneal stroma, which supports an epithelium and an endothelium, is mainly composed of type I collagen, whereas the major constituent of Descemet's membrane is basement membrane collagen. When a
cornea is exposed to a variety of insults, whether causing perforation of Descemet's membrane or not, an RCFM may form. This consists of fibrous connective tissue and elongated cells distinguishable from normal endothelial cells and stroma. Since the RCFM is of a fibrillar nature, we have studied the collagen synthesis by the cells isolated from such a pathological structure.

The fibroblast-like cell population isolated from RCFM (Fig. 2B) was found to synthesize and secrete predominantly procollagen I into the medium in its triple-stranded structure with disulfide bond at noncollagenous extension. Collagen molecules extracted from the cellular fraction demonstrate a-size chains, the final product of processing. These findings suggest that enzymatic activities which cleave off the noncollagenous extensions at NH2 and COOH termini are primarily associated with cell surfaces and/or the extracellular matrix associated with the cell surface.

The major collagenous peptide from RCFM culture medium after pepsin digestion was analyzed by electrophoresis, velocity sedimentation, and limited proteolysis with S. aureus V8 protease. Type I collagen from
Fig. 9. Electrophoretogram of proteins separated by immunoprecipitation. Procedures for the precipitation of fibronectin is described in the text. 1, Supernatant fraction after antigen-antibody-Staph A complex was removed; 2, fibronectin precipitated with antifibronectin serum. FN, Fibronectin.

RCFM shared its electrophoretic identity with type I collagen extracted from rabbit skin fibroblast culture. Analysis by velocity sedimentation also showed that the collagen sedimented as a triple-stranded peptide held noncovalently (β-APN in the medium). The patterns of peptide fragments generated by S. aureus V8 protease also demonstrated that each monomeric chain of the collagen molecule from RCFM was either closely related or identical to those of α1(I) and α2(I). These results indicate that this collagenous peptide was identical to known type I collagen.

Recently, several laboratories have described a collagen with three identical α chains which appear to be structurally related to the α chain of type I collagen (type I trimer).25–29 Amino acid composition and CNBr peptide mapping of type I trimer closely resemble those of α1(I) chain. Freeman26 reported that type I trimer accounts for almost 30% of the collagen present in the 1-week scar of cornea, whereas normal cornea contains 1% of type I trimer. The amount of type I trimer in scarred cornea is progressively reduced by 3 weeks. Since RCFM may represent primarily a scar posterior to Descemet's membrane, the presence of type I trimer was also evaluated. Our findings show that the newly synthesized type I collagen has two α1 chains and one α2 chain at a ratio of 2.35 (Table I). This rules out the presence of significant amounts of type I trimer. This discrepancy of the findings may have resulted from multiple injury vs. single injury, different healing mechanisms in stroma and in endothelium, and the difference in sampling time after introduction of injury to tissue.

It has been reported that cultured endothelial cells derived from vascular tissues synthesize fibronectin.31–33 Fibronectin mediates different adhesive events via multiple binding sites for cells and extracellular components such as collagen, glycosaminoglycans, and hyaluronic acid. Immunofluorescence microscopic studies have demonstrated fibronectin in Descemet's membrane, and fibronectin synthesis was observed in corneal endothelial cell culture (Kay, unpublished data). Our results indicated that fibronectin was synthesized by the cells derived from RCFM. Since a variety of different cells produce fibronectin in the tissue culture system, demonstration of fibronectin in RCFM is required to ensure that fibronectin is indeed one of the constituents of RCFM.

As stated earlier, type I collagen is the predominant collagen species present in transparent corneal stroma. In addition, data presented in this paper clearly demonstrate that type I collagen is also the major collagenous peptide synthesized by the cells derived from opaque RCFM. However, our preliminary findings show that the collagen fibrils in RCFM are not oriented in the manner of un-
injured stroma. This observation opens up more questions. Is there any difference in type I collagen of RCFM from stromal type I collagen at the molecular level? What are the roles of other extracellular macromolecules when collagen self-assembles into fibrils? Even though type I collagen is distributed in a number of tissues (skin, bone, tendon, etc.), it is not understood what dictates its molecular organization in the formation of connective tissues or its mode of association with other extracellular components. It is important to understand how fibrillogenesis takes place and what regulatory mechanisms are responsible for the formation of the dis-oriented fibrils in RCFM. This study, as well as further characterization of type I collagen of RCFM, is being pursued.

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