Human Corneal Fibrillogenesis
Collagen V Structural Analysis and Fibrillar Assembly by Stromal Fibroblasts in Culture

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Purpose. The stroma of the developing cornea is a highly organized extracellular matrix formed essentially by uniform, small-diameter collagen fibrils with constant interfibrillar spacing. Unlike the fibrillogenesis of chicken cornea, the assembly and maturation of human corneal fibrils have been poorly investigated. In the current study, the authors aimed to ascertain the heterotypic organization (collagens I and V) of the human corneal fibrils at the supramolecular level. To gain more insight into the molecular structure of collagen V, its cellular source, and its role in fibrillogenesis, the authors used cultured human corneal fibroblasts.

Methods. The structure of human corneal stroma after brief homogenization of the tissue was analyzed by immunogold labeling using specific polyclonal antibodies and rotary shadowing. Biochemical, electron microscopic, and immunolabeling approaches were used to investigate the collagen fibril formation and the extracellular matrix synthesis using human corneal fibroblasts grown in culture as a model system.

Results. The authors showed that in human corneal stroma, collagen I is distributed uniformly along the striated fibrils, in contrast to collagen V, which could be identified only at sites at which the fibrils partially were disrupted. Rotary shadowing observations of the homogenate revealed that collagen VI, a major component of the human cornea, was associated closely with the collagen fibril surface. Corneal fibroblasts synthesize and deposit a collagenous matrix with fibrils resembling those of the human cornea in appearance and collagen composition. Biochemical data indicate that a high concentration (20% to 30%) of collagen V is synthesized by stromal fibroblasts and that collagen V molecules are processed similarly to matrix forms in which the extension peptides are retained on the molecules.

Conclusions. The heterotypic nature (collagens I and V) of human corneal fibrils was determined. Results indicate that human corneal fibroblasts synthesize the major collagen types in human cornea (collagens I, V, and VI) and express all the posttranslational equipment for correct collagen molecular assembly and processing in a manner that closely resembles the situation in situ, offering the opportunity for more detailed study of this process, which is essential for optical transparency. Invest Ophthalmol Vis Sci. 1996;37:1749-1760.

The corneal stroma is a highly organized tissue composed of orthogonal layers of uniform, small-diameter collagen fibrils, with relatively constant spacing. In chicken, the primary stroma, laid down by the corneal epithelium, is thought to provide a template for the elaboration of the secondary stroma by the corneal fibroblasts.1-3 Contrary to chicken, no primary stroma is noticeable in humans, and this led to questions about the absolute requirement of a preformed structure for corneal morphogenesis by fibroblasts. The precise packing order and the regularity of fibril diameter of the corneal stroma are supposed to contribute to optical transparency, unlike other connective tissues of high collagen content. Although collagen I is the predominant collagen in stroma, the minor collagens V and VI can be considered essential components of corneal stroma.4-6 Collagen III is detected in mam-
The content of collagen V in chicken cornea is relatively high (20%) with respect to collagen I when compared with other matrices, such as tendon, dermis, or bone (<5%). The amount of collagen V in human cornea has been estimated to be approximately 7% to 10%, and the fibril diameter is approximately 25 nm. One particular feature of chicken corneal fibrils is to incorporate both collagens I and V in heterotypic banded fibrils. Collagen V is essential in modulating fibril diameter, and increasing the molar ratio of collagen V to collagen I in vitro self-assembly assay progressively decreased the fibril diameter. The importance of the relative ratio of these two collagens is emphasized in a study showing the corneal morphogenesis in the collagen I-deficient mouse mutant. Indeed, the mutant cornea is characterized by a lack of orthogonal organization and a thinner diameter of collagen fibrils. In addition to the importance of the relative ratio of the two collagens, the retention of the N-terminal globular domain is responsible for this modulation because it has a steric influence on fibril growth. Consistent with this hypothesis, it was shown recently that a targeted mutation in the region of the col5a2 gene coding for the N-procollagen domain reveals disorganized and larger corneal fibrils than those in normal mouse cornea.

The molecular organization of striated fibrils is complex, and the precise role of the different components of the corneal matrix has not been investigated fully. It has been proposed, however, that fibril constituents stabilize and organize the corneal stroma by interacting with proteoglycans or other collagenous components of the matrix. In this context, collagen VI, a minor collagen, is thought to contribute to the regular spacing between corneal fibrils with regard to its ubiquitous distribution as a 100 nm periodic filament interwoven banded heterotypic fibril. Collagen VI also has been shown to bind collagen I in an in vitro binding assay and to interact with a large variety of cells, suggesting a possible role in matrix–matrix interactions as well as in cell–matrix interactions. Whether these collagens are mainly responsible for the elaboration of a highly organized corneal stroma remains questionable. The purpose of our study was to provide more insight into the molecular composition and organization of embryonic human corneal fibrils, which have been investigated poorly. One immunolocalization study had been performed on human corneal stroma in aged corneas. To investigate the early steps of fibrillogenesis, fibroblasts isolated from embryonic human cornea were cultured. We show that human corneal fibroblasts grown in culture retain their capacity to synthesize, process, and deposit small-diameter fibrils resulting from the coassembly of collagens I and V. Results indicate that these cells do express all the posttranslational equipment for correct collagen I and collagen V molecular assembly and processing in a manner that closely resembles the situation in vivo. We also show that collagen VI interacts with heteropolymeric fibrils in a perpendicular fashion, suggesting a role for collagen VI in matrix–matrix interactions.

**MATERIALS AND METHODS**

Human adult corneas deemed unsuitable for grafting were obtained from the Department of Ophthalmology at the Hôpital Édouard Herriot (Lyon, France). Eighteen- to 22-week-old embryos were obtained after spontaneous expulsion and were provided by the Department of Anatomy and Pathology at the Hôpital Édouard Herriot. Embryonic corneas were removed at the hospital, and all procedures were performed in full accordance with the tenets of the Declaration of Helsinki.

Rabbit polyclonal antibodies against human collagens I, IV, and V were provided by the Institut Pasteur and have been characterized. Rabbit polyclonal antibodies against human collagen VI were purchased from Heyl (Berlin, Germany). Monoclonal antibody against the aminopropetide of the procollagen I (SP1D8) was obtained from the Developmental Studies Hybridoma Bank (Baltimore, MD) and has been characterized. Pepsinized collagens I and V and intact collagen V were extracted and purified as previously described.

**Cell Culture**

Explants of 0.5 mm³ were dissected from the entire cornea of the 18- to 22-week-old embryos after careful scraping of the epithelial and endothelial contaminating cells, placed onto plastic dishes, cultured in Dulbecco's modified Eagle's medium plus 10% fetal calf serum, and supplemented with glutamine and antibiotics (all reagents from Eurobio, les Ulis, France) in 5% CO₂ in air at 37°C. The corneal fibroblasts had grown to confluency in 10 to 15 days; at this point, cells were passaged and subcultured in the presence of sodium ascorbate (50 µg/ml) until used. In some cases, subconfluent cells were rinsed in phosphate-buffered saline (PBS) and cultured for 48 hours in serum-free medium, supplemented with 50 µg/ml L-aminopropionitrile and sodium ascorbate (50 µg/ml).

**Immunofluorescence Staining**

Cryostat sections (5 to 7 µm) of human adult and embryonic corneas were fixed in 2.5% paraformaldehyde for 30 minutes at room temperature, washed in PBS, and blocked in 1% bovine serum albumin in
PBS. Before blocking sections, some samples were treated with diluted acetic acid for 30 minutes at 4°C to detect possible buried collagen V. Sections were overlaid with the primary antibodies for 2 hours at room temperature, rinsed in PBS, and incubated with fluorescein-conjugated goat anti-rabbit serum (Dakopatts, Copenhagen, Denmark). For double immunofluorescence staining, samples were incubated with monoclonal collagen I antibody (SP1D8) followed by fluorescein-conjugated goat anti-mouse serum and with polyclonal antibodies against collagen V followed by rhodamin-conjugated swine anti-rabbit serum. Samples were mounted in PBS–glycerol (1:1) and examined with an universal Zeiss (Lyon, France) microscope equipped with epifluorescence optics.

Cells cultured for 24 hours were fixed in 25% paraformaldehyde for 20 minutes and permeabilized in 0.1% Triton ×100 for 20 minutes. Subsequent steps were as described above.

Preparation of Fibril Suspension

Cornea and cell layers were washed in PBS containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM N-ethylmaleimide, 2.5 mM ethylenediaminetetraacetic acid), frozen in liquid nitrogen, and homogenized in a tissue homogenizer (Spex Industries, Metuchen, NJ). The homogenate was then washed twice in PBS containing protease inhibitors and treated with 1 mg/ml hyaluronidase (type III; Sigma, St. Louis, MO) for 30 minutes at 37°C. The resultant suspension was centrifuged at 2000g for 30 minutes, and the supernatant was replaced by fresh PBS. This procedure was repeated twice to obtain a homogeneous suspension of the extracellular matrix components produced in culture. For fibril diameter determination, suspensions of extracellular matrix components were dropped onto Formvar–copper grids, washed with PBS, and stained negatively with 2% phosphotungstic acid at pH 7.4 for 20 seconds or stained positively with 0.4% phosphotungstic acid at pH 3.5 for 15 minutes, followed by 1% uranyl acetate in water for 10 minutes. Fibril diameters were measured with a micrometer, and values were analyzed using the Abacus Concepts Stat View II program (Abacus Concepts, Berkeley, CA).

Rotary Shadowing

Homogenates were then set to dialyze against 0.1 M ammonium acetate before rotary shadowing and were sprayed onto a freshly cleaved mica sheet. Rotary shadowing was performed as described elsewhere.

Immunoelectron Microscopy

Cell Culture. After 5 days in culture, confluent corneal fibroblasts were rinsed with PBS and subsequently fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature. To facilitate the penetration of antibodies, cell layers were digested with 0.5 mg/ml hyaluronidase (type III; Sigma). Some samples were treated with diluted acetic acid at 4°C for 30 minutes to determine whether collagen V is buried into collagen fibrils. Samples were quenched in sodium borohydride (500 μg/ml) for 60 minutes at 4°C to reduce any free aldehydes, washed several times, and incubated with 1% bovine serum albumin in PBS. Cells were incubated with specific antibodies for either 2 hours at room temperature or overnight at 4°C, followed by 2 hours incubation with 1:50 5 nm colloidal gold particles coated with goat anti-rabbit IgG. After extensive washes, cells were fixed in 1% glutaraldehyde in PBS for 30 minutes, postfixed in 1% OsO4, and PBS for 30 minutes, dehydrated in a graded ethanol series, and embedded in epoxy resin. Ultrathin sections were contrasted with uranyl acetate and lead citrate.

Fibril Homogenate. The suspension of cell layer homogenate was absorbed onto carbon–collodion-coated nickel grids, washed with PBS, treated for 30 minutes with 1% bovine serum albumin in PBS, and incubated for 2 hours with collagen type-specific polyclonal antibodies at various dilutions in blocking solution. After washing with PBS, the grids were floated for 1 hour on drops of 5 nm colloidal gold particles coated with goat anti-rabbit IgG in blocking solution. Grids were fixed slightly with 1% glutaraldehyde, and, after washing in PBS, the grids were stained negatively or positively as described above. Control experiments were performed with rabbit preimmune serum. Observations were achieved with a 1200 EX Jeol electron microscope (CMEABG, Villeurbanne, France).

Collagen Extraction. Cell layers from confluent cultures were dialyzed against 0.5 M acetic acid and digested with pepsin (50 mg/ml; Sigma) overnight at 4°C. The soluble material was lyophilized and then dissolved in sample buffer containing β-mercaptoethanol before electrophoresis and Western blot analysis. Acetic acid extraction was performed on cell layers by stirring for 48 hours the homogenate at 4°C in 0.5 M acetic acid containing 0.2 M NaCl. After removing insoluble material by centrifugation, the supernatant was dialyzed against acetic acid and then lyophilized. The media collected from cultured cells treated with βAPN was dialyzed extensively against acetic acid, centrifuged to eliminate the precipitate, and lyophilized. The cell layer also was solubilized directly by adding, in the culture petri dish, a small volume of sample buffer containing β mercaptoethanol.

Electrophoresis and Immunoblotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out with 6% acrylamide gels according to Laemmli. After Coomassie
blue staining, the electrophoretic gels of the unreduced pepsinized material were analyzed by light densitometry (junior plus scanning densitometer; Helena-France, St. leu la Forêt). Proteins were electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Molsheim, France) overnight in 10 mM CAPS, 5% methanol, pH 11. After saturation, membranes were incubated with appropriate anti-collagen antibodies followed by secondary antibodies conjugated to alkaline phosphatase (Dakopatts). Immunoblots were revealed with the alkaline phosphatase conjugate substrate kit from BioRad (BioRad, Ivry-sur-Seine, France).

RESULTS

Collagen Composition of Human Embryonic Corneal Stroma

Immunofluorescence Staining. To control and confirm the specificity of antibodies used for the immunoelectron microscopy studies, frozen tissue sections were processed for immunofluorescence microscopy. Representative results are presented in Figure 1. Type I collagen was distributed throughout the entire human cornea (Figs. 1A, 1B) and was not detected in a posterior layer corresponding to the Descemet’s membrane (Fig. 1B). Collagen V was restricted to the Bowman’s membrane with a diffuse staining at the anterior surface of the corneal stroma (Fig. 1C). When sections were treated with diluted acetic acid, collagen V was detected throughout the entire corneal stroma (Fig. 1D). Collagen VI presented a pattern identical to that observed with collagen I (Figs. 1E, 1F). In some areas, collagen VI appeared as long fibrous strands underlying collagen bundles (Fig. 1F). Controls using nonimmune serum showed no reactivity (not shown).

Immunogold Labeling and Rotary Shadowing. The human cornea is a very compact tissue with a high content in proteoglycans, thus, to facilitate the immunolabeling of the corneal stroma, we isolated fibrils from embryonic cornea. The isolated fibrils obtained from embryonic cornea homogenate were strongly labelled with antibodies to collagen I (Fig. 2A), whereas antibodies to collagen IV used as control were not reactive (Fig. 2A, insert). Treatment with diluted acetic acid led to partially disrupted fibrils fragments. Collagen V was detected at the damaged portions of the fibrils but was not present along the intact structure of the fibrils (Fig. 2B). No immunogold particles were observed along fibrils when acetic acid treatment was omitted in negative controls (Fig. 2B, insert). Collagen VI was located on filaments close to striated fibrils after immunolabeling (not shown) or it was visualized directly after rotary shadowing as characteristic beaded filaments or tetramers that appeared to be attached to the fibrils by their globular ends (Figs. 2C to 2E).

Matrix Deposition and Molecular Organization in Cell Culture

Immunofluorescence Staining. After 24 hours in culture, corneal fibroblasts showed similar intracellular fluorescence with antibodies against collagens I (Fig. 3A), V (Fig. 3B), and VI (Fig. 3C). Double immunofluorescence staining for collagens I and V showed that the cells stained for both collagens, indicating that subpopulations of secreting cells specific for collagens I and V do not exist. Nonimmune rabbit sera used as a control showed no immunofluorescence staining (Fig. 3D). No extracellular staining was detected at this time of culture, indicating that no remaining extracellular matrix from parental tissue was present before cells were seeded. A deposition of noticeable extracellular matrix as a fibrous network was observed from 4 to 5 days in culture in the presence of sodium ascorbate (not shown).

Negative Staining and Rotary Shadowing. The extracellular matrix laid down by cultured fibroblasts consisted of small-diameter fibrils interwoven in a thin filamentous network, exhibiting no apparent orthogonal distribution. Diameters of these fibrils were uniform (mean value, 18.5 ± 4.3 nm; Fig. 4A) and presented a cross-bandung pattern (Fig. 4B). Rotary shadowing observations of the collagen fibril homogenate obtained from cell culture layers revealed the presence of molecules emerging perpendicularly from the fibril surface, more or less periodically arranged along the fibrils (Fig. 4B, inset). These structures probably represent collagen VI tetramers that interact with striated fibrils, as observed for native corneal collagen fibrils (Figs. 2C to 2E).

Immunogold Labeling. To characterize the composition and organization of collagen fibrils laid down by corneal fibroblasts in culture, 5-day cultures were analyzed at the supramolecular level using immunogold labeling. Antibodies to collagen I labeled cross-striated fibrils, whereas the filamentous network showed weak, if any, label (Fig. 5A). In contrast, antibodies against collagen V were specifically localized to the thin filaments as periodic patches, and the striated fibrils remained undecorated (Fig. 5B). After partial disruption of striated fibril structure provoked by acetic acid treatment, the antibody binding to collagen V was only evident in the damaged portions of striated fibrils (Figs. 5D, 5E). The thin filamentous network was less identified, indicating that this material could have been solubilized by acetic acid treatment. In the same way, little immunolabeling with collagen I antibodies was observed on the disrupted fibrils (Fig. 5F). No immunogold particles were observed on extracellular matrix components of untreated or treated sam-
FIGURE 1. Immunofluorescence labeling of 22-week fetal human cornea (frozen sections) with antibodies to collagen I (A,B), collagen V (C,D), and collagen VI (E,F). The entire anterior portion of the cornea reacted with antibodies against collagen I (A) and VI (E), whereas only the Bowman's membrane was strongly labeled with antibodies to collagen V (C, arrowhead). D represents the same area but has been pretreated with 0.1 N acetic acid to disrupt fibrils before incubation with antibodies to collagen V; a bright fluorescence can be seen throughout the stroma. No labeling is observed on the Descemet membrane with antibodies against collagen I (B) and VI (F). b = Bowman's membrane; S = stroma; d = Descemet membrane; E = epithelium. Magnification, ×150.

Biochemical Study. Pepsinized material from the cell layer appeared as four major bands with the same mobility as pepsinized collagens I and V. When immunoblotted, the two higher bands reacted strongly with antibodies against collagen V, and the two lower bands reacted strongly with antibodies against collagen I. When reduced with β-mercaptoethanol, three bands with apparent molecular masses corresponding to the three constitutive α-chains of pepsinized collagen VI were observed (Fig. 6). Quantification by light densitometry of the pepsinized cell layer material staining after SDS–PAGE (Fig. 6, lane 3) indicates that the...
α1(V):α1(I) chain ratio is 1:3.5. To study whether collagen V was present in the cell layer in its intact form, the cell layer was harvested directly in sample buffer and homogenized after the addition of β-mercaptoethanol. The solubilized material was immunotransferred after SDS-PAGE and probed with antibodies to collagen V. Two bands with a slower mobility than pepsinized collagen V were observed and identified as intact α1(V) and α2(V) chains compared to the migration of the human bone tissue intact form of collagen V (Figs. 7A, 7B). Two higher bands also were observed and probably correspond to intracellular procollagen V (Fig. 7A, lanes 1 and 2). When cell cultures were pretreated with EDTA to remove cells...
FIGURE 3. Intracellular localization of collagens I, V, and VI. Human corneal fibroblasts were grown for 24 hours in the presence of ascorbate, permeabilized, and prepared for indirect immunofluorescence staining using rabbit polyclonal antibodies to collagen V and VI and a monoclonal antibody against collagen I (SP1D8) for double immunostaining. A and B show double immunostaining with collagen I antibody and fluorescein-conjugated goat anti-mouse IgG (A) and collagen V antibodies followed by rhodamin-conjugated swine anti-rabbit IgG (B). Collagen I and V staining patterns are observed in all cells. (C) Antibodies against collagen VI also react strongly within intracellular vesicles. (D) Negative control with preimmune serum. Magnifications: ×120 (A,B); ×70 (C,D).

or were homogenized and centrifuged to harvest the fibrils and not the free molecules, the staining of these two bands decreased and was detected only faintly (Fig. 7A, lanes 3 and 4, respectively). We also observed on samples directly harvested from the petri dishes with sample buffer the presence of two bands comigrating with pepsinized collagen V (Fig. 7A, lanes 1 and 2 compared to Fig. 7B, lane 1).

DISCUSSION
Morphologic data from immunofluorescence and immunoelectron microscopy demonstrate that heterotypic collagen I and V fibrils are the major constituents of the human corneal stroma. The epitopes within the triple helical domain of collagen V are not accessible in intact banded fibrils from human cornea, and disruption of the banded fibrils by acetic acid treatment was necessary to unmask the collagen V epitopes, as described for chicken cornea.9,11,16,28 When differentiated human corneal fibroblasts grew in culture, they retained the capacity to produce uniform, small-diameter fibrils, similar to those observed in parental tissue. We show that the same heterotypic organization probably occurs in the 20 nm diameter banded fibrils produced by corneal fibroblasts in culture. However, in cell culture, we also observed thinner fibrils with no apparent banding striation, which appeared to be formed only with collagen V. We can exclude the possibility that these thin filaments correspond to collagen VI beaded filaments because our polyclonal antibodies do not react with collagen VI in a radioimmunoassay.23 The Bowman's membrane con-

FIGURE 4. Ultrastructural analysis of the small-diameter fibrils produced by human corneal fibroblasts in culture. (A) Fibril diameter distribution was obtained by measuring individual fibrils from negatively stained transmission electron micrographs. Mean diameter is 18.5 ± 4.3 nm. (B) Extracellular matrix formed in culture consists mainly of uniform small-diameter fibrils and presents a typical 67 nm period, revealed by negative staining. Rotary shadowing of the cell layer homogenate reveals also the presence of molecules, resembling the characteristic collagen VI tetramers, which interact with striated fibrils (B, insert arrowheads). Magnification, ×67,000 (B).
FIGURE 5. Organization of collagen I and V into fibrils deposited by human corneal fibroblasts grown in culture for 5 days in the presence of ascorbate. A, B, and C were obtained by labeling cell culture without any pretreatment, whereas D, E, F, and G were pretreated with diluted acetic acid, a condition in which fibril structure partially is disrupted. (A) Uniform small-diameter fibrils are labeled with antibodies to collagen I (arrowheads), but no particles are observed on the filamentous network interwoven the banded fibrils (arrows). (B) Most of the unbanded fibrils are decorated with collagen V antibodies (arrows), whereas the small-diameter banded fibrils are not. (C) No gold particles are observed when the cell layer is incubated with nonimmune serum as a control. (D, E) When pretreated with acetic acid, the fibril structure is obviously disrupted and is strongly labeled with antibodies against collagen V (arrowheads). Note that when fibrils are only partially dissociated, the compact portion of the fibrils is not decorated with gold particles. (F) A scarce distribution of the gold particles is observed when disrupted fibrils are incubated with collagen I antibodies; the acetic acid pretreatment might have solubilized most of the collagen I molecules. (G) Control sample with nonimmune serum. Magnifications: ×56,000 (A, B, C, D, F, G); ×94,000 (E).
contains less organized, unstriated fibrils that are thinner than those observed in the stroma and represent the only structures in the human cornea that reacted with collagen V antibodies without disruption of the fibril integrity (also shown on chicken cornea). Whether these fibrils are only composed of collagen V or are mixed with collagen I is unclear. Because it has been suggested recently that, in chicken, these fibrils might be produced by the corneal epithelium rather than the stromal fibroblasts, we interpret the collagen V thinner fibrils observed in our cultures as an early step of fibrillogenesis or as abortive fibrils caused by culture artifacts. Intracellular localization studies of collagen I, V, and VI in permeabilized cells indicate that most corneal fibroblasts synthesized collagens I, V, and VI. Using double-immunofluorescence labeling for collagens I and V, we show that a subpopulation of cells that only produces one of these two collagen isotypes does not exist. However, it is unknown whether collagens V and I are packaged in the same vesicles before they are deposited. The elucidation of such a proposed event will, however, be a difficult task. Taken together, our data show that biosynthesis of appropriate corneal extracellular components and their subsequent assembly appear to be properties of the human corneal differentiated fibroblasts themselves. Human corneal fibroblasts in culture synthesized collagen V with a relative proportion of 25% to 30% to collagen I. This is noticeable because the amount of collagen V has been estimated by biochemical analysis of human adult cornea to be 10% at the most, whereas more than 80% of collagen I has been found. However, these studies have not been confirmed recently, and they may have failed to extract completely the highly cross-linked collagen V from human adult cornea. The high amount of collagen V that we observed in human corneal fibroblast culture is to be related to the smaller diameter of the fibrils produced in culture (20 nm) compared to that estimated for human corneal stroma (25 to 30 nm). This supports the concept that when both types of collagen molecules are co-assembled, increasing the proportion of collagen V progressively decreases the diameter of the heterotypic fibril formed. There is at least one explanation for such an induction of synthesis of collagen V in culture conditions. Indeed, cells are grown in 10% fetal calf serum that probably contain growth factors; this might stimulate collagen V
205kDa  116kDa  97kDa  66kDa

β APN  -  +  +  +  +

FIGURE 7. (A) Immunoblot analysis of the matrix forms of collagen V in corneal fibroblast layer. The human corneal fibroblasts were grown in the presence (+) or absence (−) of βAPN. (lanes 1, 2) Cell layers were directly extracted with sample buffer, loaded on 6% SDS–PAGE gels under reduced conditions and electrotransferred onto a polyvinylidene difluoride membrane. The membranes were probed with antibodies against collagen V. (lanes 3, 4) The cell layer was treated with ethylenediaminetetraacetic acid as described in Materials and Methods to remove cells (lane 3) or were homogenized with a potter and centrifuged to remove nonfibrillar material (lane 4) before they were extracted and processed as described. (lane 5) Immunoblot of purified intact collagen V from fetal human bones as standard. Note that the higher molecular mass bands, observed in lanes 1 and 2, are observed only faintly in lanes 3 and 4 and probably correspond to the intracellular procollagen V molecules. (B) Purified pepsinized collagen V (pep) and intact collagen V from fetal human bone were run on a 6% SDS–PAGE gel and stained with Coomassie blue. The positions of the pepsinized α1(V) chain, the α2(V) chain (noted pep), and the intact corresponding chains are indicated based on this stained gel.

synthesis because it has been shown recently with TGFβ1, which specifically increased collagen V synthesis in bovine vascular smooth muscle cells.21 There is growing evidence that at least two factors are determinant in the control of heterotypic fibril diameter: the relative proportion of collagens I and V22,23 and the persistence of the N-terminal domain of collagen V molecules, which prevent accretion of the fibril by steric hindrance.24 In the same way, the lack of collagen I in mov13 mutant resulted in the formation of disorganized thinner fibrils in mouse corneal stroma.15 N-processing does not occur completely on collagen V molecules,28–30 and we show for the first time that this process is maintained in culture. Fibroblasts do express the post-translational equipment necessary for correct processing of collagen V because two major bands are detected with migration patterns similar to that of intact collagen V extracted from tissues. It has been postulated that the conformation of the N-propeptides is determinant for the resulting correct cleavage of the N-terminal extensions.35 The corneal fibroblast culture could help to elucidate the nature of the factors responsible for the correct folding and further processing of collagen V molecules. A small proportion of fully processed collagen V, however, is observed in our preparations and could represent proteolytic breakdown during the processing of our samples.

The other characteristic of corneal stroma architecture is the constant interfibrillar spacing. Collagen VI was localized as a filamentous network interweaving banded fibrils in corneal stroma and was thought to act as a spacer between fibrils.34 Immunochemical studies also revealed a tendency for collagen VI filaments to course perpendicular to striated fibrils.35,36 Using the rotary shadowing method, we showed that collagen VI tetramers bound to native collagen fibrils surface by their globular ends in a perpendicular fashion. We observed the same interaction between colla-
gen VI and banded fibrils in cell culture. Such a binding could have been predicted on the basis of previous available data, indicating with a solid phase binding assay that collagen I binds to collagen VI. However, based on the possible presence of the collagen V N-terminal domain emerging from the fibril surface, the existence of a specific binding between intact collagen V and collagen VI is under investigation in our laboratory. These observations strengthen the importance in corneal development of the three major corneal collagens in acting together to influence matrix assembly. Lack of regularity in fibrillar architecture and spacing after an injury is thought to cause opacification of the cornea. Elucidating the molecular mechanisms controlling corneal stroma fibrillogenesis might be essential for a better understanding of morphogenesis and wound repair as well.

**Key Words**
collagen V, fibrillogenesis, fibroblast culture, human cornea, immunolabeling

**Acknowledgments**
The authors thank Agnès Fichard for her interest in this work and for helpful discussions and Dr. Patrick Carroll for assistance with English. They thank Dr. F. Bouvier for human embryonic cornea, and Dr. Hartmann for the antibodies against human collagens I, IV, and V. They also thank Réjane Willems for her skilled technical assistance and Alain Bosch for the excellent artwork.

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