
Detection of specific collagen types in normal and keratoconus corneas

David A. Newsome, Jean-Michel Foidart, John R. Hassell, Jay H. Krachmer, Merlyn M. Rodrigues, and Stephen I. Katz

Keratoconus is a corneal disease of unknown cause that involves a progressive thinning and scarring of the corneal connective tissue. We examined normal human and keratoconus corneas, including one healed penetrating keratoplasty specimen. Organ cell cultures of normal and keratoconus corneal specimens were labeled with radioactive proline and analyzed by CM-cellulose chromatography and slab gel electrophoresis to determine collagen biosynthesis. Collagen types I and III were synthesized in similar amounts by normal and keratoconus stromocytes in culture. Specifically purified antibodies were used to determine the distribution of collagen types in tissue sections by immunofluorescence. The distribution of collagen types I, III, and IV in keratoconus was also similar to that in normal corneas, except that scarred regions in keratoconus and at the host-graft juncture were largely type III. Immunofluorescent reaction of the anti—type IV collagen antibodies with Bowman's layer, in particular, and Descemet's membrane in keratoconus specimens indicated extensive destruction. Basement membrane destruction may play an important role in this disease.

Key words: keratoconus, corneal collagens, collagen types, immunofluorescence, normal and abnormal corneal extracellular matrix

Keratoconus is a slowly progressive, serious corneal disorder of uncertain heritability and unknown cause. The clinical hallmark of the disease is the irregular, cone-shaped protrusion of the central cornea which results from weakening and thinning of the stromal elements. Although the disease has been reported to exhibit familial patterns and an au-

tosomal recessive mode of inheritance has been postulated,¹ most cases appear to be sporadic. In other clinical reports, keratoconus has been linked to a heritable disorder of connective tissue, Ehlers-Danlos syndrome.²⁻⁴

Extensive biomicroscopic and pathological studies of keratoconus have so far failed to demonstrate conclusively whether the primary site of the disease is the corneal stroma or the epithelium and its basement membrane.⁵ Ectasia of the stroma likely involves either primary or secondary alterations in its major structural macromolecules or in their interactions with other matrix elements. Collagen, the most abundant stromal element (about 71% of dry weight) has naturally received much attention. Histopathological studies have documented stromal scarring and degradative changes in the stromal lamellae but have been of little help in elucidating pathogenetic mechanisms.

From the Section on Retinal and Ocular Connective Tissue Diseases (D. N., J. H.) and Ocular Pathology (M. R.), Clinical Branch, National Eye Institute, the Laboratory of Developmental Biology and Anomalies, National Institute of Dental Research (J-M. F.), Dermatology Branch, National Cancer Institute (S. K.), National Institutes of Health, Department of Health, Education and Welfare, Bethesda, Md., and the Department of Ophthalmology, University of Iowa (J. K.). J-M. F. is a Fellow of the FNRS in Belgium. Submitted for publication July 5, 1979.

Reprint requests: Dr. David Newsome, Building 10, Room 10 D-17, National Institutes of Health, Bethesda, Md. 20205.



Fig. 1. Fluorescence micrograph of a frozen section of normal cornea reacted with anti-type IV collagen antibodies demonstrates a wide, discrete area of fluorescence in the epithelial basement membrane area. ($\times 250$.)

In the present study we have used specifically purified antibodies to characterize the distribution of collagen types in normal human corneal and anterior scleral tissue as well as in keratoplasty specimens from patients with well-documented keratoconus, including one postmortem specimen from a patient who had successful perforating keratoplasty for keratoconus. We also characterized biochemically the collagens synthesized by normal and keratoconus stromal cells and normal scleral cells. Our data demonstrate the cornea-specific distribution of collagen types as well as some alterations in collagenous components in keratoconus specimens.

Materials and methods

Corneal specimens. Five normal and five keratoconus corneas were studied. Normal tissue was

obtained at autopsy from individuals aged 7, 22, 28, 35, and 56 years. Corneoscleral specimens were embedded in OCT embedding medium (Ames) and then frozen in liquid nitrogen. Frozen sections ($4 \mu\text{m}$ thick) were transferred to albumin-coated glass slides to serve as substrate for a modified direct immunofluorescent staining reaction. Keratoconus specimens from patients in the age range 28 to 49 years, all with typical clinical criteria for the disease as documented by one of us (J. K.), were placed into cold M-K corneal storage medium at the time of surgery or autopsy and embedded, frozen, and sectioned within 20 hr by the methods described above. The keratoconus specimens had variable amounts of obvious scarring; all had irregular thinning and Fleisher pigmented rings.

Corneal stromal cell cultures were established as previously described⁶ from explants of pure stroma prepared under a dissecting stereomicroscope. Cell cultures were similarly started from cleanly dissected explants of sclera and conjunctival

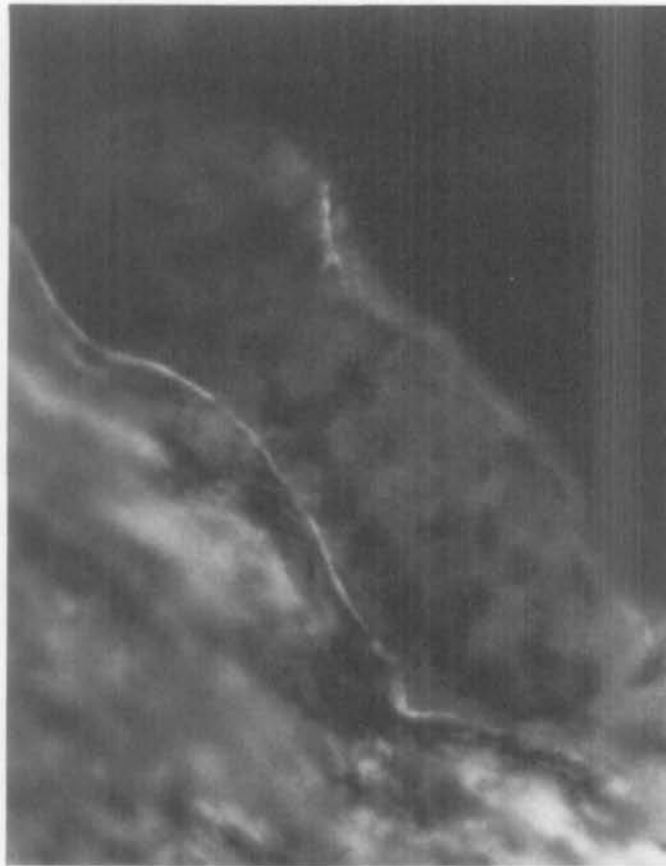


Fig. 2. Fluorescence micrograph of a frozen section of keratoconus cornea reacted with anti-type IV collagen antibodies demonstrates a marked reduction in and discontinuity of the amount of fluorescence in the epithelial basement membrane area as compared with that seen in the normal specimen in Fig. 1. ($\times 500$.)

stroma. Primary outgrowths of cells as well as cells in the third serial passage forming confluent layers were used. Cells were maintained in polystyrene culture dishes (Falcon) in Eagle's minimal essential medium with 5% fetal calf serum, 100 u/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin, and exposed to 37°, 100% humidity, and a 95% air-5% CO_2 atmosphere. Confluent cultures were radiolabeled by 24 hr incubation in serum-free Dulbecco-Vogt medium (NIH) with 100 $\mu\text{Ci}/\text{ml}$ ^3H -proline (NET-323; New England Nuclear, Boston), 100 $\mu\text{g}/\text{ml}$ vitamin C, and 50 $\mu\text{g}/\text{ml}$ β -aminopropionitrile fumarate as a lathyrogen. Medium and cells together with carrier collagen from skins of lathyritic rats were extracted in 0.5M acetic acid containing 1.0 mg/ml pepsin. The collagen was purified by NaCl precipitations (25%; two precipitations) in the presence of carrier collagen from skins of lathyritic rats and eluted from a carboxymethyl (CM)-cellulose chromatography col-

umn (1.6 by 5 cm) as described elsewhere, which permitted an average ($n = 4$) recovery of 74% of applied counts.⁷⁻⁹ Peak fractions in the effluent were pooled, lyophilized, and electrophoresed in the presence of 0.05M urea on sodium dodecyl sulfate (SDS)-polyacrylamide slab gels.¹⁰ Purified types I and III collagen standards were included with each electrophoresis. Reduced samples were obtained by dividing the original 250 μl samples into two and adding 5 μl of β -mercaptoethanol to one set of the samples. After electrophoresis the gels were stained with Coomassie blue and were impregnated with 2,5-diphenyloxazole (PPO) in dimethylsulfoxide. Autofluororadiograms on Kodak X-Omat film were prepared by exposing the film at -74° for periods up to 7 days.

Preparation of antigens. Types I and III collagens were isolated from fetal calf skin,⁸ type II from a rat chondrosarcoma,¹¹ and type IV from a murine sarcoma.¹² Type I procollagen was ex-

tracted from the skin of dermatosporactic calves and was a gift from Dr. C. M. Lapiere. Type III procollagen was purified from fetal calf skin.⁸ The identity and purity of the collagens was determined by amino acid analysis after hydrolysis in 6N HCl at 110° for 18 hr, by CM-cellulose and diethylaminoethyl-cellulose elution profiles, and by SDS-polyacrylamide slab gel electrophoresis with and without β -mercaptoethanol reduction and with and without cyanogen bromide digestion. Human fibronectin was purified from fresh human plasma by affinity chromatography on a gelatin column. The material eluted with 1M potassium bromide was electrophoresed after reduction of the disulfide bonds with 20 mM dithio-treitol. The 220,000 dalton chain of fibronectin was eluted from slices of the acrylamide gels and used to immunize rabbits.

Preparation of antibodies to collagens. Rabbits or guinea pigs were immunized by repeated subcutaneous or foot pad injections of 0.5 to 5.0 mg of the antigen in Freund's complete adjuvant. Antibodies were isolated and purified by cross-immunoabsorption and checked for cross-reactivity to collagen and proteoglycans by indirect immunofluorescence, micropassive hemagglutination assay,¹³ and radioimmunoassay. Antibodies to collagens were purified from the antisera by affinity chromatography and cross-immunoabsorption.¹⁴ Serum from immunized animals was passed sequentially over columns of types II and III collagen and types IV and V collagens which had been covalently bound to Sepharose 4B. The unbound material was then passed over a type I collagen column which was extensively rinsed with phosphate-buffered saline (PBS). Antibodies were eluted from the column with 0.5N acetic acid-0.5M NaCl. This eluate was neutralized with 3M Tris, dialyzed against PBS, and concentrated by filtration on a UM20 Amicon filter. Antibodies to the other antigens were purified according to a similar protocol.

Testing of antibodies to collagens. The specificity of the antibodies was determined by radioimmunoassay (RIA), by immunofluorescence labeling of target tissues, and by enzyme-linked immunoabsorbent assay (ELISA) as reported previously.¹⁵ For RIA, types I and III collagens were labeled with iodine-125 by the chloramine-T method¹⁶ (sp. act. 1 to 2 μ Ci/ μ g). The RIA was performed according to the method of Rohde et al.¹⁷ In quantitative inhibition tests preincubation of the purified antibody with increasing amounts of the autologous collagen inhibited the binding of the antibody with the labeled antigen, whereas no

Table I. Distribution of ocular connective tissue proteins in normal and keratoconus specimens as detected by immunofluorescence

Site	Proteins detected
Corneal epithelium	No reaction for collagens/fibronectin
Corneal epithelial basement membrane	Type IV collagen
Corneal stroma*	Type I collagen; fibronectin
Descemet's membrane	Type IV collagen; fibronectin
Corneal scars	Type III collagen; some type I
Conjunctival blood vessels	Type IV collagen
Sclera	Type III collagen; some type I

*Type III collagen has been extracted from corneal stroma and characterized biochemically but was not reliably detected by immunofluorescence in our studies.

significant inhibition was observed when the antibody was preincubated with the heterologous collagen. Indirect immunofluorescence studies were performed on fresh-frozen sections of human skin, liver, tendon, and spleen. They established that the purified antibodies reacted only with those structures known to contain these molecules. Finally, indirect immunofluorescence blocking and competition studies confirmed the specificity of the anti-type I and anticollagen antibodies, since a preincubation with the specific antigen blocked the binding of antibodies to known high-affinity substrates. For example, a preincubation with type I collagen blocked the binding of anti-type I collagen antibodies to human skin but did not interfere with the binding of anti-type III collagen antibody.

Preparation of antibodies to fibronectin. Specific antibody to fibronectin was purified by affinity chromatography.¹⁴ Serum from immunized rabbits was passed over a column of fibronectin which had been covalently bound to Sepharose 4B. The unbound material was then washed away with PBS. Antibody to fibronectin was then eluted from the column with 0.5M acetic acid, pH 2.9, neutralized with 3M Tris, dialyzed against PBS, and concentrated on a UM20 Amicon filter.

Testing of antibody to fibronectin. The specificity of the antibody was determined by RIA, by ELISA,¹⁵ by radial immunodiffusion according to Ouchterlony, and by immunoelectrophoresis. For RIA, labeled fibronectin was purified from the culture medium of human fibroblasts grown with

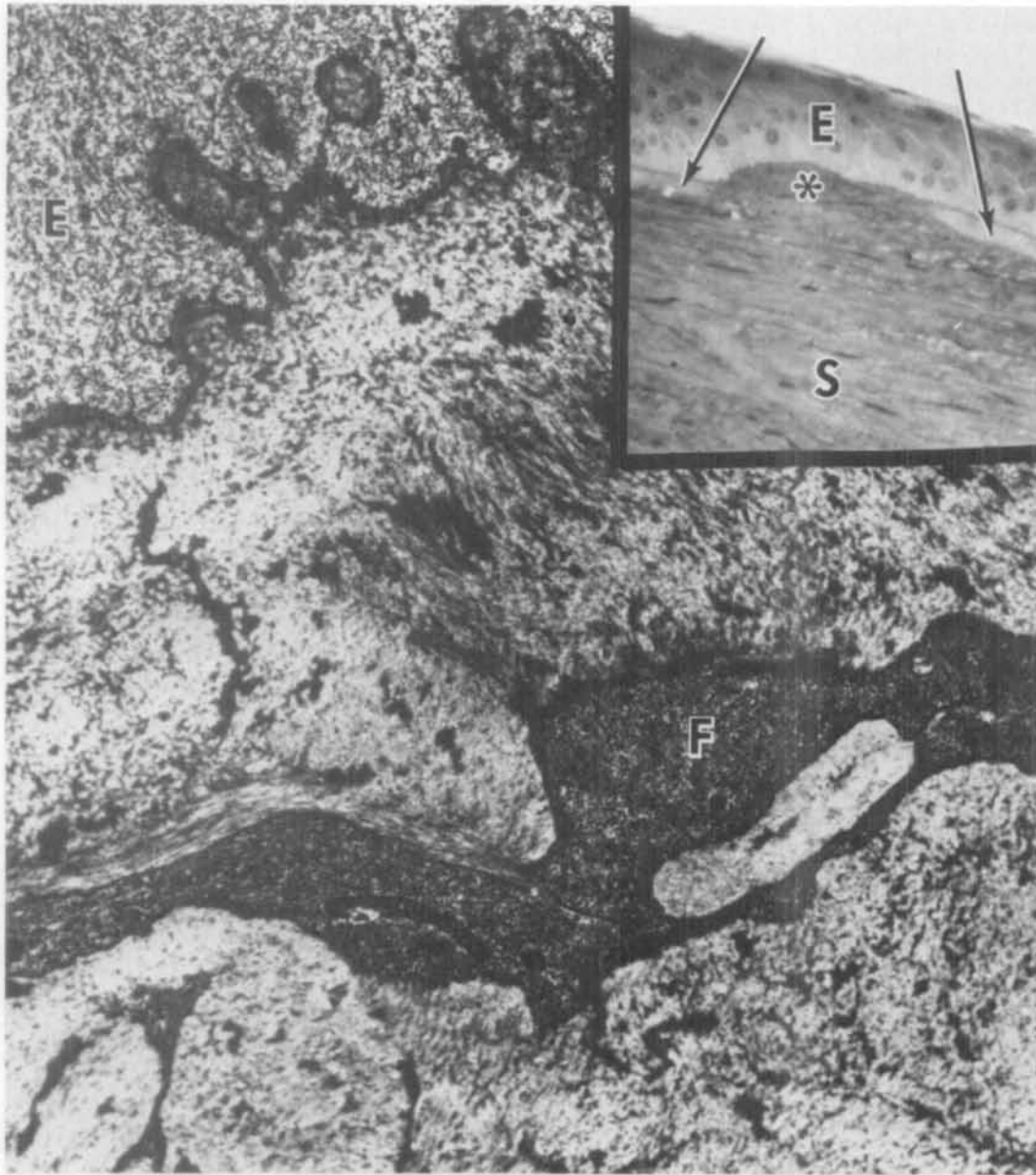


Fig. 3. Inset of the superficial area of a scarred keratoconus cornea shows disruption (asterisk) of Bowman's layer (arrows) and superficial stromal fibrosis. *E*, epithelium; *S*, stroma. (Toluidine blue; $\times 350$.) The transmission electron micrograph demonstrates irregular subepithelial connective tissue excrescences in the basal epithelium (*E*). Bowman's layer is replaced by irregular connective tissue. *F*, fibroblast. ($\times 27,000$.)

[U- 3 H]leucine.¹⁸ Fibronectin was also labeled with 125 I by the chloramine-T method.¹⁶ Preincubation of the antibody with other antigens, including types I and III collagens, laminin, and type IV collagen, did not inhibit the binding of the antibody to labeled fibronectin. By radial immu-

nodiffusion and immunoelectrophoresis a single line of precipitation was obtained with an antifibronectin antibody and purified fibronectin or normal human serum.

Immunofluorescence reaction. Modified direct immunofluorescent staining was performed on

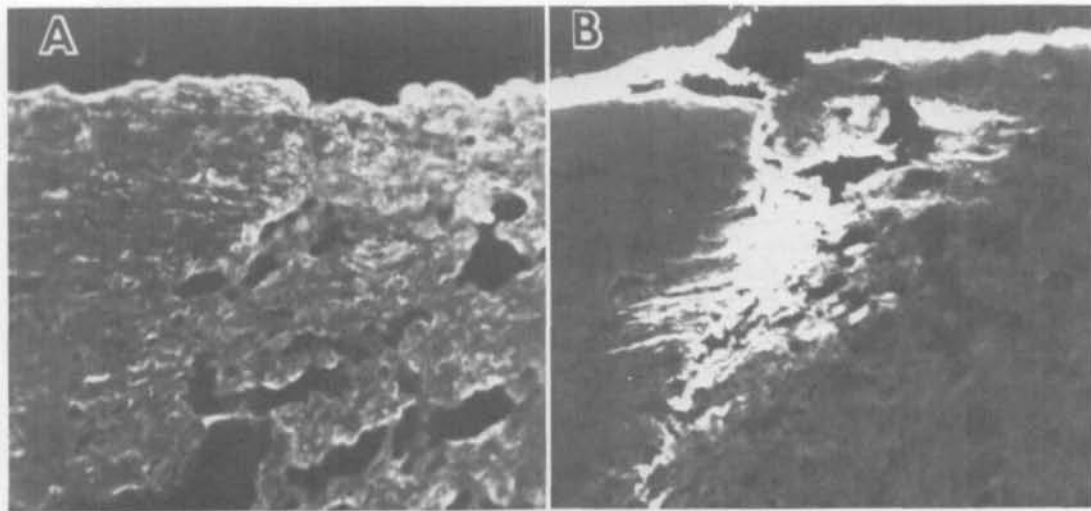


Fig. 4. These sections of a healed perforating keratoplasty specimen from a patient with keratoconus were reacted with anti-type I (A) and anti-type III (B) antibodies. Note the strong fluorescence of the scar with anti-type III and the similarity of staining of the graft and host stromas with both antibodies. ($\times 300$.)

sections of cornea and corneoscleral tissue with antibodies and control sera in two dilutions: 10 and 20 $\mu\text{g/ml}$ PBS. After a 30 min incubation the sections were washed in PBS and then exposed to fluorescein-conjugated goat-anti-rabbit immunoglobulin for 30 min, washed again in PBS, and mounted with glycerine. Specimens were examined with a Leitz ultraviolet microscope equipped with a camera attachment and a BG 12 filter. Exposures were made with an automatic exposure meter on Kodak Ektachrome 200 film.

Results

With the use of the techniques described herein it was not generally possible to assess immunofluorescent reactivity quantitatively. We were able to localize the heterogeneous population of human corneal collagens to specific sites. This distribution is summarized in Table I.

Corneal epithelium. The epithelial layer itself did not react with any antibodies. This finding was similar in normal and keratoconus corneas. The zone just deep to the epithelium showed a strong reaction with antibodies to type IV collagen (Fig. 1). In the keratoconus specimens with more advanced stromal thinning, the reaction with anti-type IV in the epithelial basement membrane

area was strikingly decreased and irregular (Fig. 2).

Bowman's layer. Bowman's layer reacted similarly with antibodies to types I and III collagen as did the anterior stroma. However, the positivity for anti-type I was noticeably reduced as compared with more posterior layers of the stroma. Bowman's layer reacted more strongly with antibody to fibronectin than did the stroma. Except for the presence of type III-containing scars that included part of the Bowman's area in some keratoconus specimens, there was no difference between the types of detectable collagens in the normal and keratoconus material. The histological appearance of a typical scar can be seen in Fig. 3.

Corneal stroma. In both normal and keratoconus tissue the stroma was strongly positive for type I. Procollagen type I reaction was consistently weaker in all specimens of cornea. In some of the heavily scarred keratoconus specimens there was a patchy increase in detectable procollagen type I in the subepithelial anterior stroma.

Type III collagen was not clearly detected in the normal corneal stroma by fluorescence methods. Consistent reactivity in normal tis-

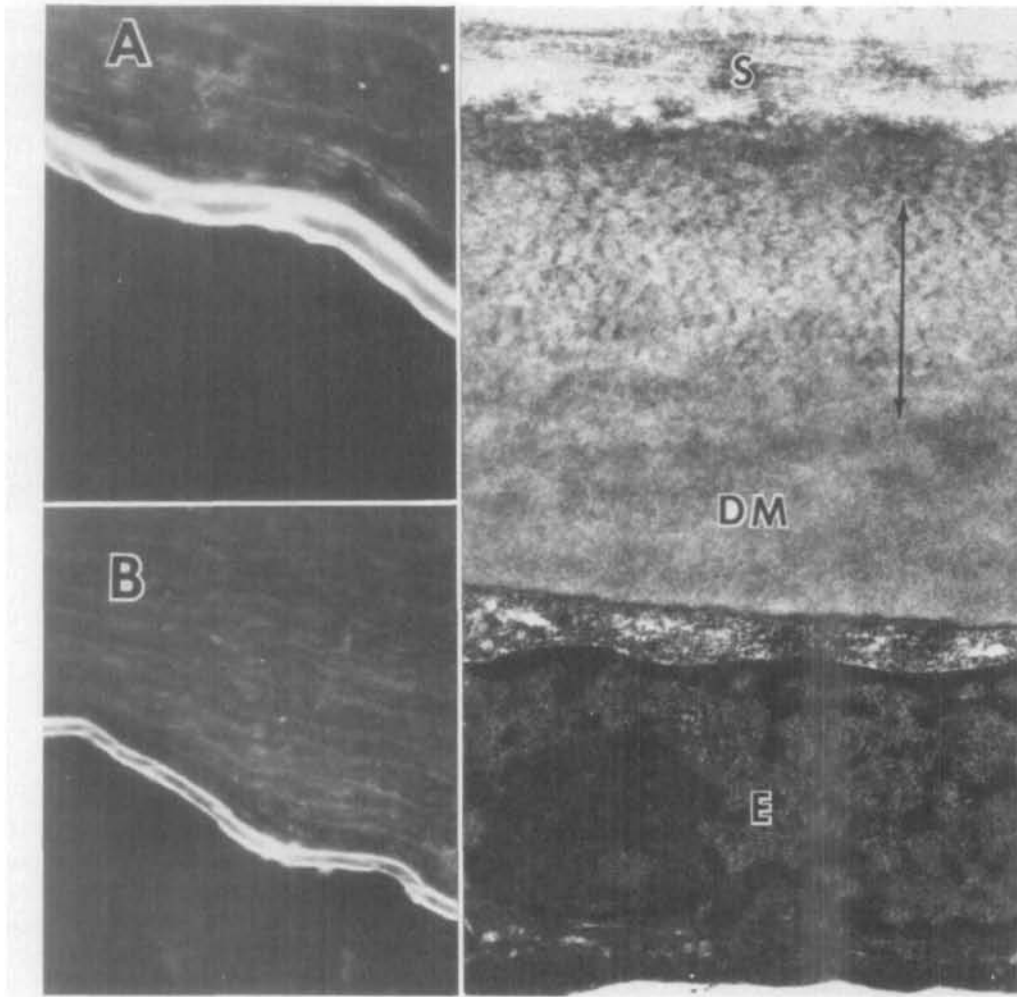


Fig. 5. Fluorescence micrographs (A, $\times 450$; B, $\times 300$) of a section of normal cornea reacted with antifibronectin antibody exhibits fluorescent anterior and posterior portions of Descemet's membrane identical to that pattern seen with anti-type IV antibodies. **Right panel,** Transmission electron micrograph of normal tissue. The fluorescence pattern was seen in both normal and undisrupted keratoconus Descemet's membrane and may correspond to the normal 100 nm wide-spaced collagen arrow zone (immunofluorescent dark interleaf) and anterior and posterior granular basement membrane-like zones of material. S, stroma; DM, posterior Descemet's membrane; E, endothelial cell. ($\times 30,000$.) Fibronectin is also detectable in stroma itself in the fluorescence micrographs.

sues was detected only on the edges of stromal lamellae. No differences were visible between the reaction for type III and procollagen type III in normal and keratoconus unscarred stroma.

In the specimen of a successful graft of normal donor into a keratoconus corneal bed, both areas showed the same reaction with antibodies to types I and III. In the grafted

specimen the healed junction between donor keratoconus recipient reacted strongly with antibodies to type III and procollagen type III. The zone of scarring extended into both the host and donor tissues (Fig. 4). Type III collagen could be clearly detected also in the more thinned and scarred keratoconus specimens as patches corresponding to stromal scars.

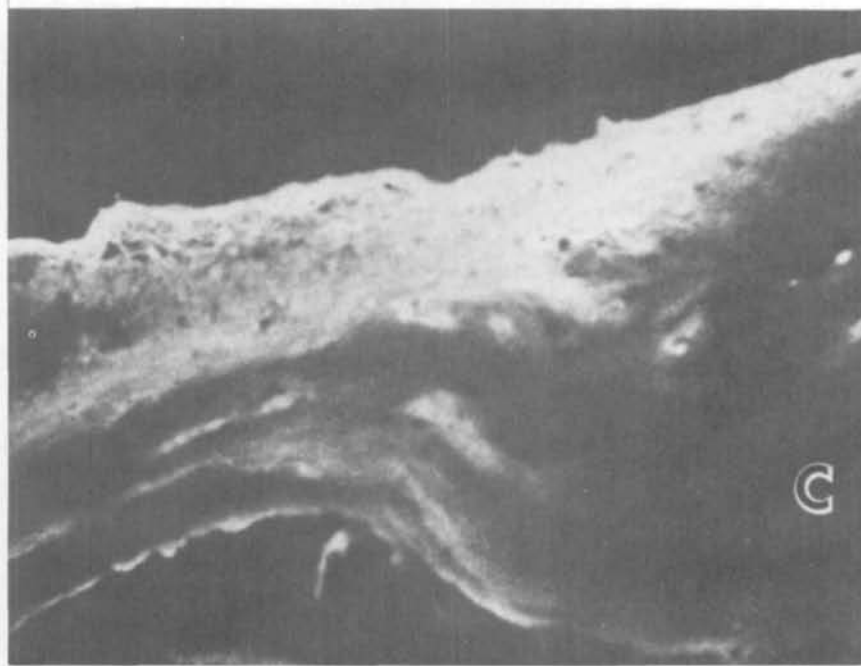


Fig. 6. Fluorescence micrograph of a normal sclerocorneal section reacted with anti-type III antibodies reveals strong fluorescence in Tenon's and the sclera at the limbal transition zone. Note the relative lack of reaction in the clear cornea (C). This strong reaction was evident in all areas of sclera studied in normals and keratoconus. ($\times 450$.)

Both normal and keratoconus corneal stromas exhibited high levels of detectable fibronectin. There was a particularly concentrated positive reaction at the level of the stroma adjacent to Descemet's membrane and in the epithelial and Descemet's basement membrane areas themselves (Fig. 5).

Descemet's membrane. Descemet's membrane reacted strongly with antibodies to type IV collagen, exhibiting a striking laminar pattern with bright fluorescence on the stromal and endothelial faces and a dark, apparently unstained interleaf (Fig. 5). When measured on a fluorescence micrograph, the bright leaves each were about $1 \mu\text{m}$ wide at $4\times$, and the dark interleaf $2 \mu\text{m}$. These relative widths were comparable to the three anatomical zones discernible ultrastructurally: the anterior zone of granular basement membrane-like material at the stromal interfaces, the middle zone of wide-spaced collagen, and the posterior zone of basement membrane-like material (Fig. 5). In keratoconus specimens, the staining pattern of

Descemet's membrane was often irregular, and the laminar pattern less defined. This finding appeared to be associated with the frequent folds in Descemet's membrane near the thinned stroma.

Normal Descemet's membrane had definitely detectable fibronectin by the immunofluorescent stain. The distinctive laminar pattern was present, with more intense staining on the stromal as compared with the endothelial surface. The interruption of the pattern of fibronectin reaction in most of the keratoconus specimens was in contrast to the picture in the normal tissues (Fig. 5).

Sclera. Normal human sclera had a preponderance of detectable type III collagen over type I collagen. This pattern, opposite to that seen in the corneal stroma, was most apparent at the scleral spur region, where the two tissues are juxtaposed (Fig. 6). Procollagen type I reaction was consistently weaker in all scleral specimens than that of type I itself.

Conjunctiva. Normal human conjunctival stroma reacted strongly with antibodies to

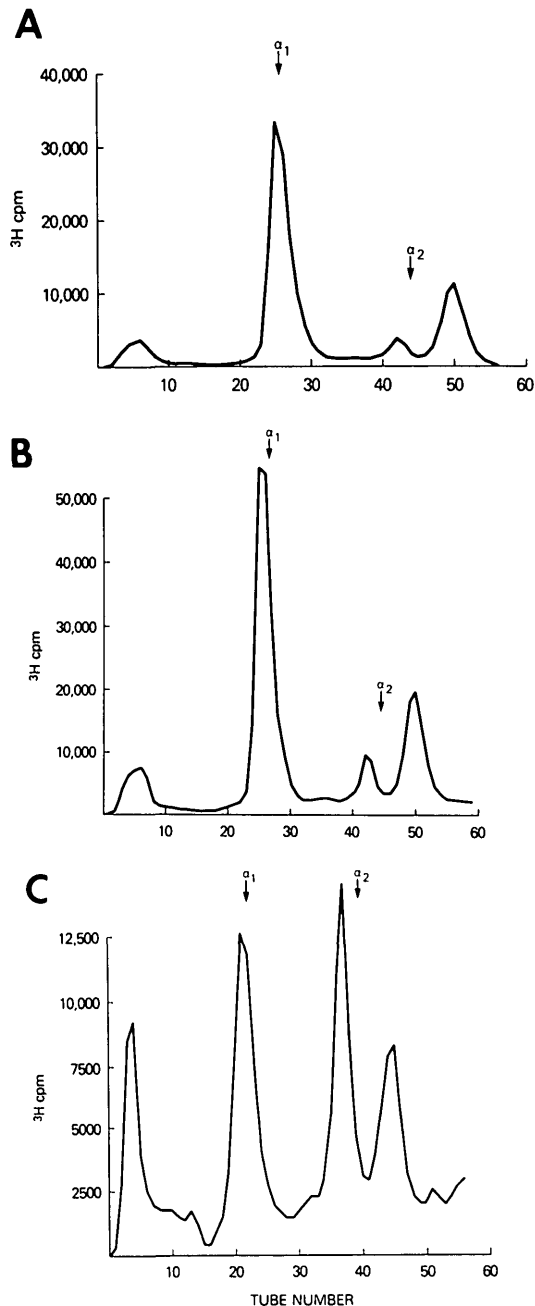


Fig. 7. CM-cellulose chromatograms demonstrate the elution profiles of the radioactively labeled collagens synthesized by cultured corneal stromocytes from normal (A) and keratoconus (B) corneas and sclera (C). Arrows indicate the elution positions of the carrier rat skin type I collagen peaks. Note the large peak containing the type III collagen in C and the similarities of the profiles in A and B.

type I collagen. Walls of blood vessels in the conjunctiva had clearly detectable type IV collagen and fibronectin. The conjunctival epithelium itself did not react with any antibodies.

Identification of collagen synthesized in vitro. Normal and keratoconus corneal stromal cells synthesized both types I and III collagen in vitro. The presence of these two collagens was confirmed by the elution position from CM-cellulose columns (Fig. 7) and SDS slab gel electrophoresis patterns. SDS gel profiles of the migration patterns of pooled fractions from the three collagenous peaks seen on CM-cellulose chromatography showed co-migration of peak 1 collagen chains with purified alpha 1 (I) collagen and peak 3 with authentic alpha 2 collagen chains (Fig. 8). Peak 2 materials migrated largely with the standard type III and exhibited disappearance of the trimeric form when electrophoresed under reducing conditions (Fig. 8).

The ratio of type I to type III collagen as determined from the CM-cellulose profiles was 10:1 for normal corneal tissue and 9:1 for keratoconus tissues (average of five specimens). The preponderance of type III collagen in the sclera was demonstrated by a ratio of 2.1:1, type I to type III (average of five specimens). An example of this large amount of type III collagen synthesized by scleral fibroblasts in culture can be seen in Fig. 7. The material from peak 2 of the chromatogram of the scleral collagens again showed reduction with beta-mercaptoethanol when subjected to SDS gel electrophoresis.

Discussion

Corneal transparency depends upon the orderly deposition of collagen and glycoconjugates in the stroma.^{19, 20} Maintenance of transparency must depend, at least in part, on the controlled turnover of this extracellular matrix. Collagens, the most plentiful group of structural macromolecules in the corneal stroma, are present largely as fibrillar proteins that interact with the stroma-specific proteoglycans to produce a normal optically useful stroma. Thus modifications of the

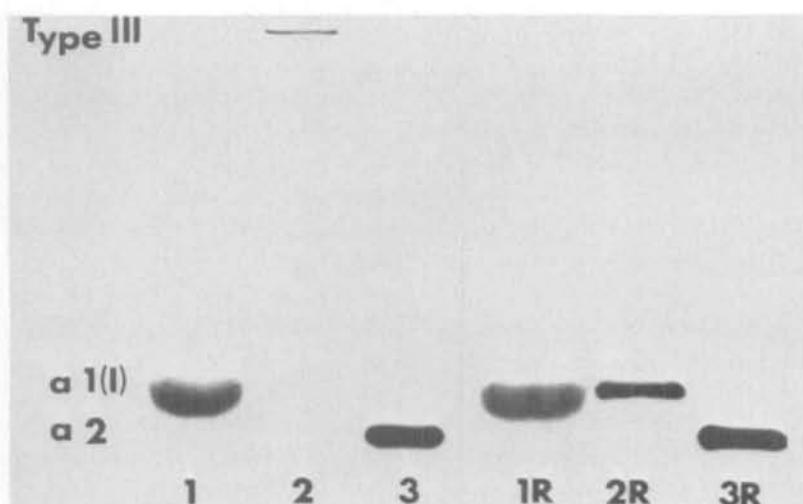


Fig. 8. Fluororadioautograph of an SDS-polyacrylamide slab gel of the collagen chains eluted in the three peaks of the CM-cellulose chromatogram demonstrates the presence of the types I and III collagen synthesized by cultured keratoconus stromacytes. 1, Pooled fractions from the α -1 peak region; 2, pooled from the interpeak; 3, pooled from the α -2 peak. Note the disappearance of the type III trimer after reduction with *b*-mercaptoethanol (2R). The position of the radiolabeled type III collagen was identical to that of coelectrophoresed authentic type III, as was that of the labeled type I. These patterns were similar between normal and keratoconus cell synthetic products; the scleral samples had more prominent type III bands on the gels.

types of collagens synthesized or defects in posttranslational processing of the molecule could lead to corneal disease.

Recent advances in collagen characterization have revealed that there are at least four genetically distinct types which are now referred to as types I, II, III, and IV.²¹ The tissue-specific distribution of collagen types appears to play an important role in morphogenesis, particularly in the cornea,^{22, 23, 27} and in association with the sensory retina.^{24, 25}

Corneal stromal matrix stability may depend in part upon the proportion of the types of collagen present. We have shown that there are two major collagen types synthesized *in vitro* by normal and keratoconus cornea cells, with a similar proportion of types I and III in both. This mixture of types I and III is similar to the collagens that accumulate *in vivo* and have been extracted and identified.²⁶ It is possible that an alteration in the proportion of these major collagen components could destabilize the connective tissue

matrix, as in Ehlers-Danlos type IV, where the synthesis of type III collagen is low to absent.²⁷ Maumenee²⁸ reported that two keratoconus corneal specimens contained increased type III collagen as detected by immunofluorescence. In our specimens type III collagen was not reliably detected in uninvolved corneal stroma. We did demonstrate collections of type III collagen in keratoconus corneal scars and at the site of host-graft wound healing in a perforating keratoplasty specimen. Type III collagen has previously been reported in scars in nonocular tissues, even those such as tendon which do not normally have type III.²⁹

The presence of type III collagen in normal cornea, including human, has been controversial,^{30, 31} and type V collagen preliminarily identified.³² Type III had been identified in bovine material,³³ and type V in lapine.³² Newsome et al.²⁶ have recently submitted evidence from collagenase sensitivity, cyanogen bromide peptides, trypsin sensitivity,

and slab gel electrophoresis with and without reduction identifying types III and V in normal human cornea. These data support the observations of others noted above. Differences with other reports³⁰ may be due at least partially to technique, since type III is heavily crosslinked and difficult to extract from native tissues. It is more difficult to explain the conflict of the present data from cultures,³¹ since extraction is not a problem *in vitro*. We used different media with a short labeling period and early passage cells, in contrast to Stoesser *et al.*³⁴ In the present study preliminary results with anti-type V antibodies revealed a positive reaction pattern identical to that for type IV in both normal and keratoconus corneas. We have not emphasized these results, since our antibodies were not, at the time of this work, rigorously proved to be completely specific. Subsequent purification has, however, confirmed the type V specificity (J-M. F., unpublished data).

Our results emphasize some similarities and indicate certain differences between the developing chick cornea and human tissue. Type I collagen was the major detectable collagen in our study of the human corneal stroma, a finding corresponding to what has been reported for chick.³⁵ Type III collagen is a small component of the human cornea but is reportedly absent in the chick. The synthesis of both types I and III by a uniform cell population has not been previously reported for corneal stromocytes but is known to occur with smooth muscle cells³⁶ and fibroblasts.²⁹ We could not reliably detect type III collagen in all normal stromas, perhaps due to the limits of the immunofluorescent reaction. Factors such as masking of type III by other matrix components may also have influenced its detectability.

Type II collagen is in the chick a prominent component of Bowman's layer, the subepithelial stroma, and Descemet's membrane.³⁵ The presence of type II-bearing primary corneal stroma, thought to be critical to the morphogenesis of the adult stroma in the chick, has not been confirmed in human tissue (for review, see ref. 37). We could not demonstrate type II in the Bowman's or sub-

epithelial areas of either the normal or keratoconus specimens.

The laminar staining pattern of Descemet's membrane may indicate a regional distribution of type IV collagen and of the noncollagenous fibronectin within the membrane. Such a specific distribution could explain, at least in part, the distinct ultrastructure of this membrane. Jakus³⁸ and later Hay and Revel³⁹ described the approximately 100 nm periodicity of the anterior third of Descemet's membrane, presumably reflecting the packing of membrane subunits in this area. Such orderly packing could be in response to the presence here of type IV fibrils or aggregates. The interface between the stroma and Descemet's membrane is also distinguished ultrastructurally. Patchy, basement membrane-like material has been reported here by transmission electron microscopy. The strong reaction in this region with antifibronectin suggests that this material consists, at least in part, of fibronectin. The precise localization of fibronectin within the basement membrane-like material at the stromal-Descemet's interface must await ultrastructural studies.

Type IV collagen was also prominently detected at the interface of the endothelium with Descemet's membrane. This observation offers further evidence that this cell layer, so important to the maintenance of proper corneal hydration, synthesizes basement membrane.^{40, 41} The presence of fibronectin here in posterior Descemet's membrane is consistent with its detection in a wide variety of human basement membranes.⁴² This protein is also commonly associated with and synthesized by fibroblasts (for review, see ref. 43). It is possible that the stromocytes synthesize the fibronectin that accumulates in Descemet's membrane. However, the corneal endothelium is embryologically derived from the neural crest, as are the stromocytes proper,⁴⁴ and thus may share common attachment proteins. This observation contrasts with the appearance of the epithelial basement membrane area which had no detectable fibronectin. And, of course, the epithelium is not a neural crest derivative.

The striking amount of type III collagen in

the sclera may in part explain its anatomical differences from the type I-rich corneal stroma. The proportion of collagen types synthesized may affect fibril aggregation and uniformity. In the sclera the matrix fibrils are randomly arrayed in a feltwork with no signs of the orderly lamellae so characteristic of the corneal stroma. Scleral fibril diameters are much more heterogenous, and the fibrils much larger than those of the cornea. Lapiere et al.⁴⁵ have shown that type III collagen influences the size and type of bundles formed by type I collagen in vitro. Such an interaction may condition the collagen bundle organization in various connective tissues.

It is significant that the collagen profiles obtained from cultured corneal, scleral, and conjunctival stromocytes differed among themselves, and that these differences corresponded to differences in immunofluorescent reactivity. For example, the sclera reacted strongly with anti-type III and synthesized the largest proportion of type III collagen in vitro. The persistence of these differences in patterns of synthesis of collagens, each type of which is a distinct gene product, is in contrast to the rapid decay of keratan sulfate proteoglycan synthesis by in vitro corneal stroma, even in organ culture.⁴⁶

The present work has extended our knowledge of the site-specific distribution of the genetically distinct collagen types in normal human and keratoconus corneas and normal sclera. It appears that a normal complement of corneal stromal collagens is present in normal proportions in keratoconus. Thus, if keratoconus is a true collagen disease (i.e., has a defect in collagen on the molecular level), the defect probably occurs as a result of an error in assembly or in the control of turnover, for example, by the overproduction of collagenolytic enzymes. Further investigations of these possibilities are in progress.

REFERENCES

1. Hammerstein W: Zur Genetik des Keratokonus. Albrecht Von Graefes Arch Klin Exp Ophthalmol 190:293, 1974.
2. McKusick VA: Heritable Disorders of Connective Tissue, ed. 4. St. Louis, 1972, The C. V. Mosby Co.
3. Robertson I: Keratoconus and the Ehlers-Danlos syndrome. A new aspect of keratoconus. Med J Aust 1:571, 1975.
4. Kuming BS and Joffe L: Ehlers-Danlos syndrome associated with keratoconus. A case report. S Afr Med J 52:403, 1977.
5. Pollack FM: Contributions of electronmicroscopy to the study of corneal pathology. Surv Ophthalmol 20:375, 1976.
6. Newsome DA, Takasugi M, Kenyon KR, Stark WF, and Opelz G: Human corneal cells in vitro: morphology and histocompatibility (HL-A) antigens of pure cell populations. INVEST OPHTHALMOL 13:23, 1974.
7. Chung E and Miller EJ: Collagen polymorphism: characterization of molecules with the composition $[\alpha 1(III)]_3$ human tissues. Science 183:1200, 1974.
8. Epstein EH Jr: $[\alpha 1(III)]_3$ human skin collagen: release by pepsin digestion and preponderance in fetal life. J Biol Chem 249:3225, 1974.
9. Trelstad RL: Human aorta collagens: evidence for three distinct species. Biochem Biophys Res Commun 57:717, 1974.
10. Lammi UK: Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature 227:680, 1970.
11. Smith BD, Martin EJ, Miller EJ, Dorfman A, and Swarm R: Nature of the collagen synthesized by a transplanted chondrosarcoma. Arch Biochem Biophys 166:181, 1975.
12. Orkin RW, Gehron P, McGoodwin EB, Martin GR, Valentine T, and Swarm R: A murine tumor producing a matrix of basement membrane. J Exp Med 145:204, 1977.
13. Andreopoulos NA, Mestecky J, Wright GP, and Miller EJ: Characterization of antibodies to the native human collagens and to their component α chains in the sera and joint fluids of patients with rheumatoid arthritis. Immunochemistry 13:709, 1976.
14. Nowack H, Gay S, Wick G, Becker U, and Timpl R: Preparation and use in immunohistology of antibodies specific for types I and III collagen and procollagen. J Immunol Methods 12:117, 1977.
15. Rennard SI, Berg R, Martin GR, Foidart J-M, and Gehron Robey P: Enzyme-linked immunoassay (ELISA) for connective tissue components. Anal Biochem (in press).
16. McConahey PJ and Dixon FJ: A method of trace iodination of proteins for immunological studies. Int Arch Allergy 29:185, 1966.
17. Rohde H, Nowack H, Becker U, and Timpl R: Radioimmunoassay for the aminoterminopeptide of procollagen p $\alpha 1(I)$ chain. J Immunol Methods 11:135, 1976.
18. Engvall E and Rouslahti E: Binding of a soluble form of fibroblast surface protein fibronectin to collagen. Int J Cancer 20:1, 1977.
19. Trelstad RL and Coulombre AJ: Morphogenesis of the collagenous stroma in the chick cornea. J Cell Biol 50:840, 1971.
20. Maurice DM: The structure and transparency of the cornea. J Physiol 136:263, 1957.

21. Martin GR, Beyers PH, and Piez KA: Procollagen. *Adv Enzymol* 42:167, 1975.
22. Dodson JW and Hay ED: Secretion of collagenous stroma by isolated epithelium grown in vitro. *Exp Cell Res* 65:215, 1971.
23. Hay ED: Origin and role of collagen in the embryo. *Am Zoologist* 13:1085, 1973.
24. Newsome DA, Linsenmeyer RF, and Trelstad RL: Vitreous body collagen. Evidence for dual origin from the neural retina and hyalocytes. *J Cell Biol* 71:59, 1976.
25. Smith GN, Linsenmeyer TF, and Newsome DA: Synthesis of type II collagen in vitro by embryonic chick neural retina tissue. *Proc Natl Acad Sci USA* 73:4420, 1976.
26. Newsome DA, Gross J, and Hassell JR: Human corneal stroma contains three distinct collagens. (Manuscript submitted.)
27. Pope FM, Martin GR, Lichtenstein JR, Penttinen R, Gerson C, Rowe DR, and McKusick BA: Patients with Ehlers-Danlos syndrome type IV lack type III collagen. *Proc Natl Acad Sci USA* 72:1314, 1975.
28. Maumenee IH: The cornea in connective tissue disease. *Ophthalmology* 85:1014, 1978.
29. Epstein EH Jr and Munderloh NH: Isolation and characterization of CNBr peptides of human $[\alpha 1(\text{III})]_3$ collagen and tissue distribution of $[\alpha 1(\text{I})]_2\alpha 2$ and $[\alpha 1(\text{III})]_3$ collagens. *J Biol Chem* 250:9304, 1975.
30. Freeman IL: Collagen polymorphism in mature rabbit cornea. *INVEST OPHTHALMOL VIS SCI* 17:171, 1978.
31. Church RL: Procollagen and collagen produced by normal bovine corneal stroma fibroblasts in cell culture. *INVEST OPHTHALMOL VIS SCI* 19:192, 1980.
32. Yue B, Baum JL, and Smith BD: Collagen synthesis by cultures of stromal cells from normal human and keratoconus corneas. *Biochem Biophys Res Commun* 86:465, 1979.
33. Schmut O: The identification of type III collagen in calf and bovine cornea and sclera. *Exp Eye Res* 25:505, 1977.
34. Stoesser TR, Church RL, and Brown SI: Partial characterization of human collagen and procollagen secreted by human corneal stromal fibroblasts in cell culture. *INVEST OPHTHALMOL VIS SCI* 17:264, 1978.
35. von der Mark K, von der Mark H, Timpl R, and Trelstad RL: Immunofluorescent localization of collagen types I, II, and III in the embryonic chick eye. *Dev Biol* 59:75, 1977.
36. Layman DL, Epstein EH Jr, Dodson RF, and Titus JL: Biosynthesis of type I and III collagens by cultured smooth muscle cells human aorta. *Proc Natl Acad Sci USA* 74:671, 1977.
37. Hay ED: Development of the vertebrate cornea. *Int Rev Cytol* (in press).
38. Jakus MA: Studies on the cornea. II. The fine structure of Descemet's membrane. *J Biophys Biochem Cytol* 2(Suppl):243, 1956.
39. Hay ED and Revel JP: Fine Structure of the Developing Avian Cornea. New York, 1969, S. Karger.
40. Perlman M, Baum JL, and Kay GI: Fine structure and collagen synthesis activity of monolayer cultures of rabbit corneal endothelium. *J Cell Biol* 63:306, 1974.
41. Kefalides NW, Cameron JD, Tomiciek EA, and Yanoff M: Biosynthesis of basement membrane collagen by rabbit corneal endothelium in vitro. *J Biol Chem* 251:730, 1976.
42. Stenman S and Vahen A: Distribution of a major connective tissue protein, fibronectin, in normal human tissues. *J Exp Med* 147:1054, 1977.
43. Yamada KM and Oldern K: Fibronectin-adhesive glycoproteins of cell surface and blood. *Nature* 275:179, 1978.
44. Johnston MC, Nodin DM, Hazleton RD, Coulombre JL, and Coulombre AJ: Origins of avian ocular and periocular tissues. *Exp Eye Res* (in press).
45. Lapier CM, Nusgens B, and Pierard GE: Interaction between collagen type I and type III in conditioning bundles organization. *Connect Tissue Res* 5:21, 1977.
46. Klintworth GK and Smith CF: A comparative study of extracellular glycosaminoglycans synthesized by rabbit corneal fibroblasts in organ and confluent cultures. *Lab Invest* 35:258, 1976.