SYNTHESIS OF TYPE III COLLAGEN BY FIBROBLASTS FROM THE EMBRYONIC CHICK CORNEA

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ABSTRACT

Synthesis of collagen types I, II, III, and IV in cells from the embryonic chick cornea was studied using specific antibodies and immunofluorescence. Synthesis of radioactively labeled collagen types I and III was followed by fluorographic detection of cyanogen bromide peptides on polyacrylamide slab gels and by carboxymethylcellulose chromatography followed by disc gel electrophoresis.

Type III collagen had been detected previously by indirect immunofluorescence in the corneal epithelial cells at Hamburger-Hamilton stages 20–30 but not in the stroma at any age. Intact corneas from embryos older than stage 30 contain and synthesize type I collagen but no detectable type III collagen. However, whole stromata subjected to collagenase treatment and scraping (to remove epithelium and endothelium) and stromal fibroblasts from such corneas inoculated in vitro begin synthesis of type III collagen within a few hours while continuing to synthesize type I collagen. As demonstrated by double-antibody staining, most corneal fibroblasts contain collagen types I and III simultaneously. Collagen type III was identified biochemically in cell layers and media after chromatography on carboxymethylcellulose by detection of disulfide-linked $\alpha 1(III)_3$ by SDS gel electrophoresis.

The conditions under which the corneal fibroblasts gain the ability to synthesize type III collagen are the same as those under which they lose the ability to synthesize the specific proteoglycan of the cornea: the presence of corneal-type keratan sulfate.

KEY WORDS collagen · cornea · fibroblasts · immunofluorescence · antibodies

The factors that normally control the synthesis of type III collagen remain to be elucidated. Fibrous connective tissues generally contain both collagen type I and collagen type III, but the ratio between them varies with the tissue: the medial layer of the aorta contains, and aortic smooth muscle cells synthesize, ~70% type III and ~30% type I (5, 30); dermis contains, and dermal fibroblasts synthesize, ~10–20% type III and ~80–90% type I (5, 13); tendon contains mostly type I (40) with some type III, which is located in the endotendineum (2).

Under constant conditions in vitro, the ratio between collagen types I and III synthesized remains constant for many cell generations (21). However, changes in serum concentration or cell density can alter the ratio, with the highest proportions of type III collagen being synthesized in

lower serum concentrations (31) and at higher cell densities (1) than those optimal for type I collagen.

Corneal stromata of many animal species contain mostly type I collagen (33), with perhaps some type III collagen (36; von der Mark, unpublished) and some as yet incompletely characterized collagen types (15, 25; von der Mark, unpublished). Human corneal fibroblasts in vitro synthesize almost exclusively type I collagen (37), with some A- and B-type collagen (48). The chick cornea, on the other hand, appears to be unique, for, after Hamburger-Hamilton development stage 30, it contains no detectable type III collagen (38, 39, 44; see data below). Between stages 20 and 30, antibodies to type III collagen bind to the corneal epithelial cells (44) but not to the primary stroma. From stage 30 onward, the primary and secondary stroma of the chick cornea contains type I collagen but no type III. Some type II collagen is present in the anterior part of the stroma, probably synthesized by the corneal epithelium, although this activity of the corneal epithelium has so far been shown only for stage 26 (28). The region of the corneal stroma after stage 30 that is devoid of type III collagen is identical with the region that stains metachromatically for sulfated glycosaminoglycans (e.g., keratan sulfate) (4, 44). In this study, we present biochemical and immunological evidence that exposure of the corneal stroma to proteolytic enzymes and release of the fibroblasts from the extracellular matrix affect the regulation of collagen synthesis in that they initiate type III collagen synthesis in vitro in the corneal stroma fibroblasts.

MATERIALS AND METHODS

Tissue Isolations and Labeling

Whole corneas were removed from White Leghorn chicken embryos at 14–18 d of incubation, transferred to Simm's balanced salt solution (for study of whole corneas) or to Ca^{+2}- and Mg^{+2}-free saline G with 10% (vol/vol) fetal calf serum (for separation of corneal cell types), and trimmed free of noncorneal tissues. Whole, trimmed corneas were then incubated in nutrient medium that contained radioactive amino acids (see below). Alternatively, for separation of corneal cell types (see reference 8 for details), whole, trimmed corneas were treated for 10 min with 0.25% collagenase and scraped to separate the stromal matrix, which contains only the corneal fibroblasts, from the epithelium and endothelium. The scraped stromas then either were rinsed with nutrient medium and incubated directly with radioactive amino acids or were dissociated completely by a second, longer collagenase treatment (30 min). Liberated stromal fibroblast cell suspensions were centrifuged, resuspended in nutrient medium, plated into plastic tissue culture Petri dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.), and incubated at 37°C. Only primary cultures were studied.

The nutrient medium used was either Dulbecco's modification of Eagle's medium (lacks proline) or Ham's F-12 (contains proline). Dulbecco's medium was used for labeling whole, trimmed corneas; F-12 medium was used for growing and labeling separated corneal tissues and primary cell cultures. Both media contained 10% (vol/vol) fetal calf serum, sodium ascorbate (200 μg/ml), penicillin (100 U/ml), and streptomycin sulfate (100 μg/ml). When used for isotopic labeling, the media also contained β-aminopropionitrile fumarate (50 μg/ml).

Cell populations either were labeled immediately (during their first 12 h in vitro; $t = 0$ labeling) or were grown for 7–8 d in vitro and then labeled for a subsequent 24-h period. For labeling at $t = 0$, the medium contained 100 μCi/ml of L-[2,3-^{3}H(*N*)]proline (20–25 Ci/mM; NEN Chemicals GmbH, Dreieichenhain, W. Germany) and 100 μCi/ml of [2-^{3}H]glycine (3–23 Ci/mM; The Radiochemical Centre Ltd., Amersham, Buckinghamshire, England). For labeling at other times, the medium contained 20 μCi/ml of both labeled amino acids. At the end of the labeling periods, cells and labeling media were separated to allow individual analysis and then mixed with an equal volume of 2 M NaCl containing 0.1 M Tris-HCl, pH 7.5, 0.05 M EDTA, 0.002 M phenylmethylsulfonyl fluoride, and 0.002 M N-ethylmaleimide and stored frozen at −70°C until analyzed.

At the end of some labeling periods, cells in duplicate, nonlabeled dishes were suspended with 0.5% trypsin–0.1% EDTA (10 min, 37°C). Total cell numbers were then determined directly with a haemocytometer counting chamber.

Isolation of Collagen Fractions

Except where noted otherwise, all steps were performed at 0–4°C. Carrier collagen was only added before carboxymethylcellulose chromatography.

16-D CORNEAS AND AORTAS: After labeling, trimmed corneas and aortas were separated from the labeling medium and digested with pepsin (0.3 mg/ml in 0.5 M acetic acid adjusted to pH 2 with HCl; Serva GmbH, Heidelberg, W. Germany) at 4°C for 18 h with stirring. Samples were then titrated to pH 8 with NaOH and centrifuged (500 *g*, 15 min, 4°C). Pellets were subjected to cyanogen bromide (CNBr) cleavage (see below). Supernates were dialyzed against three changes of 0.02 M Na_2HPO_4, pH 8.5, containing 17.5% (wt/vol) KCl to precipitate collagens and then centrifuged (12,000 *g*, 30 min, 4°C). Supernates were discarded. Precipitates were redissolved in 0.5 M acetic acid and dialyzed against the same solution.

CELL CULTURES AND TISSUES FROM 14-D AND 18-D CORNEAS AND 9-D AORTAS: Labeling media (with cell or tissue rinses) were dialyzed against 0.15 M NaCl, 0.025 M EDTA, 0.05 M Tris-HCl, pH 7.5 (radioimmunoassay buffer) and stored frozen until analyzed. Cells alone were first dialyzed against radioimmunoassay buffer and then centrifuged (12,000 *g*, 30 min, 4°C). Supernates and the samples described above that were derived from labeling media were designated neutral salt-soluble fractions and were stored frozen. Pellets remaining after extraction of cell samples were digested with pepsin in the cold as described above, titrated to pH 8 with NaOH, and centrifuged (12,000 *g*, 1 h, 4°C). These supernates were designated pepsin-soluble fractions and were stored frozen. The small pellets remaining after neutral salt extraction and pepsin digestion were solubilized completely by CNBr cleavage and were designated CNBr-soluble fractions; they contained minor amounts of radioactivity and were not further analyzed.

Radioactivity was determined by mixing aqueous samples, made up to 1 ml with 10 ml of Scintigel (Roth Chemie GmbH, Karlsruhe, W. Germany) or with 10 ml of Biofluor (NEN Chemicals GmbH, Dreieichenhain, W. Germany).

Total Collagen

To approximate the amount of radioactivity incorporated into total collagen, aliquots (containing $1-2 \times 10^5$ cpm) of the neutral salt-, and pepsin-soluble fractions were hydrolyzed in 6 N HCl at 108°C for 24 h under a nitrogen atmosphere. The hydrolysate was chromatographed on a Beckman M 72 ion exchange resin (0.9 × 60 cm) (Beckman Instruments, Inc., Fullerton, Calif.) using a regular amino acid analysis program without ninhydrin. The percent of collagen synthesis, normalized for comparison with noncollagenous proteins (11), is calculated:

$$\frac{\text{Hypro cpm}}{2 \times \text{total Pro cpm} - \text{Hypro cpm}} \times 100$$

CNBr Cleavage and Electrophoretic Separation of Peptides

The ^{3}H-labeled samples and appropriate unlabeled standards of collagen types I, II, and III were cleaved with CNBr as previously described (14, 43). CNBr-derived peptides were separated and identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on slab gels containing 0.1% SDS and a gradient of 10–18% acrylamide (150 V for 1–2 h, then 250 V for 3–4 h). Gels were stained with Coomassie Brilliant Blue R-250, destained, photographed, and then processed for fluorography (3).

Carboxymethylcellulose Chromatography

Samples that had been treated with pepsin and inactivated at pH 8 as described above were dialyzed against 0.08 M sodium acetate, pH 4.8, and stored frozen. Carboxymethylcellulose (CM-cellulose) chromatography was carried out as previously described (34, 43). Fractions containing the β1, 2 and α2 chains as well as the α1(III) and α1(III)$_3$ chains (24) were pooled, dialyzed against 0.02 M acetic acid, and lyophilized.

SDS-PAGE of α Chains

Whole α chains obtained by CM-cellulose chromatography were identified by SDS-PAGE in 0.1% SDS on 5% acrylamide tube gels (0.5 × 10 cm) containing 0.1 M sodium phosphate, pH 7.0, and 0.5 M urea (20) in the presence or absence of β-mercaptoethanol (1–2%). After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250, destained, photographed, frozen, cut into 1.0-mm slices with a Mickle Gel Slicer (Mickle Engineering Co., Gomshall, Surrey, England), and placed in liquid-scintillation vials containing 10 ml of 3% Protosol and 0.4% Omnifluor in toluene (NEN Chemicals GmbH). After digestion overnight at 37°C, the vials were cooled and counted directly (26). This method gives ^{3}H counting efficiencies of 45–48% regardless of the presence or absence of a Coomasie Blue-stained band in the gel slice and releases as much radioactivity from the gel slices as conventional H_2O_2 methods.

Collagen Antibodies

Antibody against type I collagen was made in guinea pigs (41) and antibody against type III collagen was made in goats.[1] Antibodies specific for single collagen types were obtained by cross-absorbing antisera on columns of heterologous collagen types followed by affinity chromatography on homologous collagen types (41).

The specificity of the guinea pig antibodies for type I collagen and of goat antibodies for type III collagen was demonstrated by passive hemagglutination (41) and by radioimmunoassay.[1] In addition, sections of 17-d embryonic chick corneas were stained with the same preparation of goat antibodies to type III collagen that was used for the immunofluorescence staining of corneal fibroblasts in vitro. Failure of the corneal stroma to stain indicated that this antibody did not cross-react with type I collagen (Fig. 1).

Immunofluorescence of Cultured Cells

Cell cultures grown on plastic tissue culture dishes were rinsed three times with phosphate-buffered saline, fixed with 70% ethanol for 5 min, extracted with ethanol-ether (1:1, vol/vol) for 5 min, and air-dried (all steps performed at room temperature). Staining for immunofluorescence with antibodies to collagen types I and III was then performed as described previously (12). Double staining for collagen types I and III was achieved by using rabbit antibodies to type III procollagen (32) (kindly provided by Dr. R. Timpl) counterstained with rhodamine-labeled goat anti-rabbit γ-globulin (Nordic Pharmaceuticals, Tilburg, The Netherlands) (1:40 dilution) and guinea pig antibodies to type I collagen counterstained with fluorescein-labeled rabbit anti-guinea pig γ-globulin (Behringwerke AG, Marburg/Lahn, W. Germany) (1:20 dilution), as previously described (43).

RESULTS

Absence of Type III Collagen in the Intact Chick Cornea after Day 7 of Development

Previous work has demonstrated that at embryonic ages older than stage 30 (~7 d), type III collagen can no longer be detected in sections of the chick cornea by indirect immunofluorescence with antibodies to type III procollagen (44). We confirmed this on a section of a 17-d embryonic chick cornea by using goat antibodies to chick type III collagen (Fig. 1). The antibody reacted with mesenchyme from limbus, fibrous sclera, and nictitating membrane but not with the corneal stroma. It was important to confirm biochemically the absence of type III collagen.

Whole, trimmed corneas and aortas from 16-d embryos were labeled for 24 h with [^{3}H]proline and [^{3}H]glycine. Aorta tissue is known to synthesize large amounts of type III collagen, and it was used here as a standard. Tissues were digested with pepsin, and solubilized collagen was precipitated with 17% KCl and redissolved in 0.5 M acetic acid

[1] Herrmann, H., W. Dessau, L. Fessler, and K. von der Mark. In preparation.

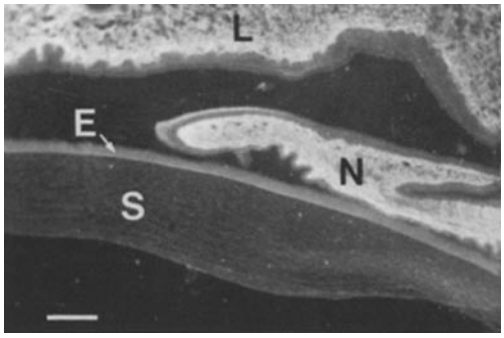

FIGURE 1 Immunofluorescence staining of a 17-d embryonic chick cornea with goat antibodies against chick type III collagen; counterstaining with fluorescein-conjugated rabbit anti-goat γ-globulin. Type III collagen is present in the mesenchyme of the eyelid (*L*), nictitating membrane (*N*), and fibrous sclera (not shown) but absent from the corneal stroma (*S*) and the epithelium (*E*). The stroma contains type I collagen (44). Bar, 50 μm. × 140.

as described in Materials and Methods. Subsequent analysis of these pepsin-soluble fractions by CNBr cleavage, SDS-PAGE on slab gels, and fluorography revealed type III collagen synthesis by the aorta (presence of α1[III]CB5), but no type III collagen was detected in the cornea by Coomassie Blue staining or fluorography (Fig. 2).

Initiation of Type III Collagen Biosynthesis When Corneal Fibroblasts Alone are Incubated In Vitro

IMMUNOFLUORESCENT STAINING: Corneal fibroblasts were isolated from 14- and 18-d embryos, and special care was taken to exclude all fibrous sclera, a tissue previously shown to contain type III collagen-synthesizing cells (44). Corneal fibroblasts were inoculated in vitro and then processed for indirect immunofluorescent staining with collagen antibodies at various times.

After 12–18 h in vitro, only about half of the cells stained intracellularly with antibodies to type I collagen (Fig. 3), and about half the cells stained with antibodies to type III collagen (Fig. 3).

After 3–15 d in vitro, almost all the fibroblasts stained with both type I and III collagen antibodies. These results were obtained when cells in separate cultures were stained with antibodies to type I collagen, type III collagen, or type III procollagen (Fig. 4), and they were confirmed when antibodies to type I collagen and to type III procollagen were applied sequentially to the same cell preparations in the double-antibody localization procedure described in Materials and Methods (Fig. 5). At all times in vitro, the corneal fibroblasts were unable to bind antibodies to type II collagen and were negative to ± in their ability to bind antibodies to type IV collagen (data not shown).

BIOCHEMICAL IDENTIFICATION OF TYPE III COLLAGEN SYNTHESIS: To confirm the synthesis of type III collagen by methods independent of antibodies, 7-d cultures of corneal fibroblasts were incubated with [^{3}H]proline for 24 h. The medium was dialyzed, digested with pepsin to convert procollagen into collagen and then lyophilized. Cell layers were extracted with 1 M NaCl and digested with pepsin, as described in Materials and Methods.

SDS-PAGE on slab gels of the lyophilized samples revealed that most of the collagen secreted

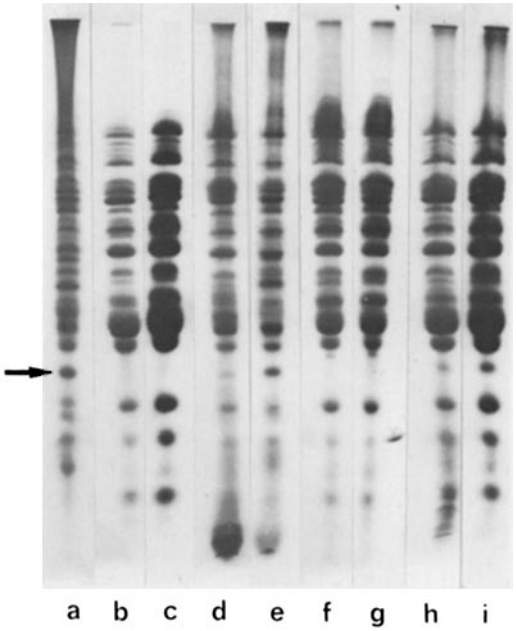

FIGURE 2 SDS-PAGE on gradient slab gels (10–18% acrylamide) of CNBr-derived peptides from ^{3}H-labeled 16-d embryonic chick cornea (*b* and *c*; *f* and *g*) and aorta (*d* and *e*; *h* and *i*). Coomassie Blue staining of samples is seen in *a*, *b*, *d*, *f*, and *h*; fluorographic detection of radioactive bands in the same samples is seen in *c*, *e*, *g*, and *i*. (*a*). CNBr peptide pattern of a mixture of type I and type III collagen. Arrow indicates marker peptide for type III collagen, α1(III)CB5, Commassie Blue. (*b* and *c*) 16-d cornea, pepsin-soluble collagen. (*d* and *e*) 16-d aorta, pepsin-soluble collagen. (*f* and *g*) 16-d cornea, pepsin-insoluble residue. (*h* and *i*) 16-d aorta, pepsin-insoluble residue.

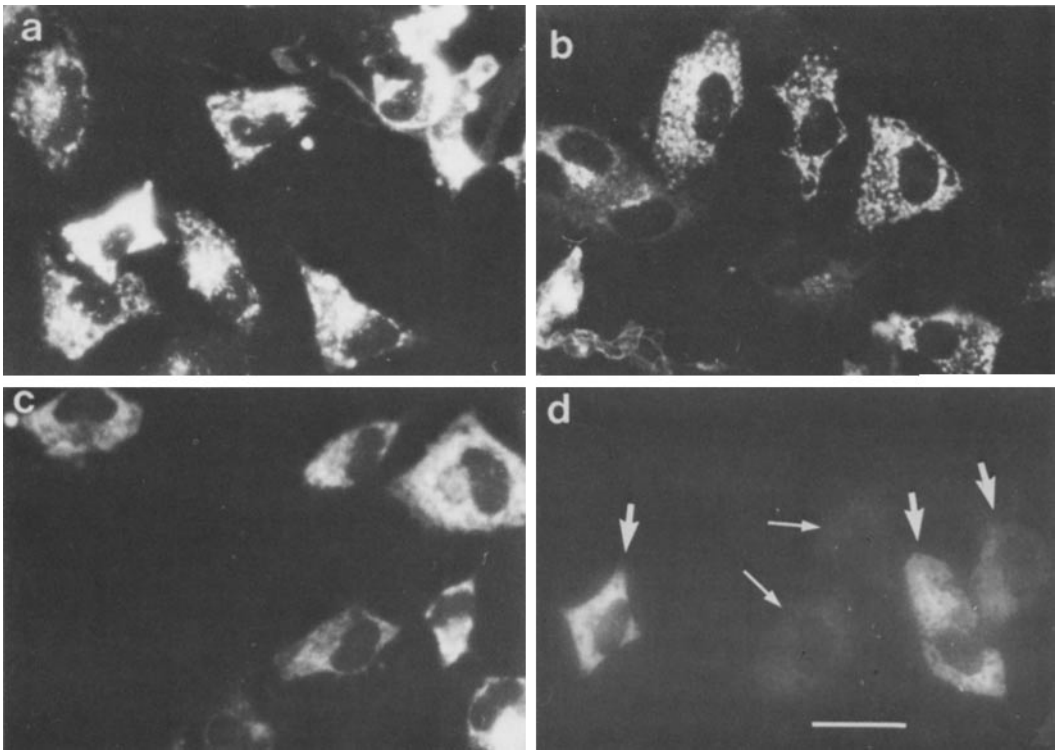

FIGURE 3 Immunofluorescence localization of intracellular type I collagen (*a* and *b*) and type III collagen (*c* and *d*) in corneal fibroblasts from 14-d chick embryos 12 h after inoculation in vitro ($t = 0$ cells). (*a*) Guinea pig antibodies to chick type I collagen. (*b*) Rabbit antibodies to type I collagen. (*c* and *d*) Goat antibodies to chick type III collagen (same antibodies as used for staining shown in Fig. 1). Cells labeled with large arrows contain type III collagen; those labeled with small arrows are negative. Bar, 20 μm. × 600.

into the medium is type I collagen (Fig. 6). Besides α1(I) and α2 chains, considerable amounts of B chain of type V collagen are visible in the fluorogram; A chains may be hidden under α1(I) (42). The presence of A and B chains in the cornea has been suggested in a study by Freeman (15).

The production of type III collagen is indicated by the appearance of a disulfide-linked molecule in the γ region that migrates in the position of α1(III) (slightly faster than α1[I]) after reduction with β-mercaptoethanol (Fig. 6). Similar collagen patterns were obtained from cell layer samples (not shown).

The question remained open as to whether type III collagen synthesis in corneal fibroblasts was initiated by the dissociation of the corneal matrix and plating of isolated fibroblasts on culture dishes, or whether it was a consequence of the collagenase treatment required for removal of endothelium and epithelium and for dissociation of the stroma. In order to answer this question and to further elucidate conditions controlling synthesis of type III collagen, attempts were made to follow the onset of type III collagen synthesis quantitatively in intact (but scraped) stromata that had been exposed for 10 min to collagenase but not dissociated and in cell cultures of freshly inoculated corneal fibroblasts. Intact corneal stromata from 14-d chick embryos from which epithelium and endothelium had been removed and freshly inoculated corneal fibroblasts were labeled with [^{3}H]proline and [^{3}H]glycine for 12 h; for comparison, 7-d fibroblast cultures from 14-d and 18-d embryonic chick corneas were labeled with the same amino acids, but for 24 h.

Cell layers and tissues were extracted, as described in Materials and Methods, to yield neutral salt-, pepsin-, and CNBr-soluble fractions. Cell layer extracts and labeling media were dialyzed and aliquots were used for analysis of ^{3}H-labeled

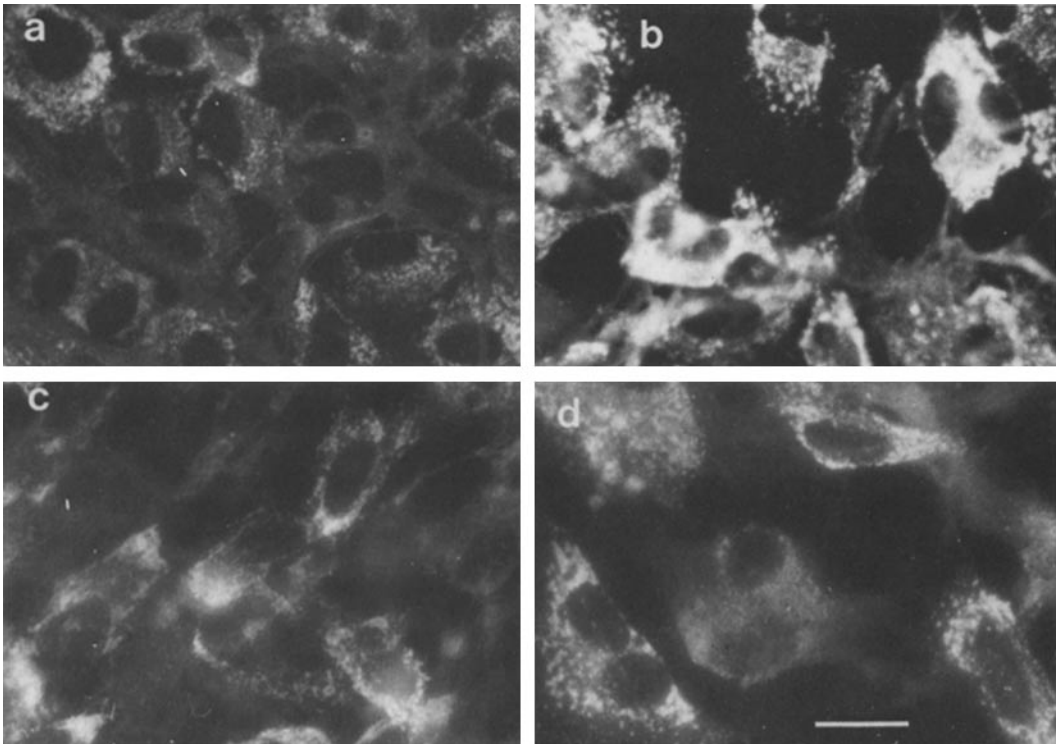

FIGURE 4 Immunofluorescence localization of intracellular type I collagen (*a* and *b*) and type III collagen (*c* and *d*) in corneal fibroblasts from 14-d chick embryos 11–15 d after inoculation in vitro. (*a*) Guinea pig antibodies to type I collagen. (*b*) Rabbit antibodies to type I collagen. (*c*) Goat antibodies to type III collagen (same antibodies as used for staining shown in Fig. 1). (*d*) Rabbit antibodies to procollagen type III (p_N α1[III]) from calf. Bar, 20 μm. × 600.

amino acids and CM-cellulose chromatography. For CM-cellulose chromatography, neutral salt-soluble fractions were also digested with pepsin to convert procollagen to collagen. For detection of type III collagen, the fractions eluting in the α2 and β1, 2 regions were pooled, dialyzed, lyophilized, and subjected to SDS-PAGE on tube gels under reducing and nonreducing conditions. By this method, type III collagen was detected as a γ-compound reducible with β-mercaptoethanol to chains of α size in the tissue extract of 14-d scraped stromata (Fig. 7 *a* and *b*), in the medium of 7-d cultures of corneal fibroblasts (Fig. 7 *c* and *d*), as well as in the cell layer of such cultures (not shown). This material comigrated with standard type III collagen from chick skin (24) (Fig. 7 *f* and *g*) and was present in sufficient quantities to be detected as a Coomassie Blue-staining band (Fig. 7) as well as a radioactive peak after slicing and counting of the gels (Fig. 8). This suggests that considerable synthesis of type III collagen is initiated in the corneal stroma in tissue culture after exposure to collagenase and removal of epithelium and endothelium. No type III collagen synthesis was observed when intact, whole cornea was labeled with [^{3}H]proline for 12 h under identical conditions.[2]

TOTAL COLLAGEN CONTENT OF FRACTIONS: To allow estimation of collagen content, neutral salt-, and pepsin-soluble fractions of the samples were subjected to amino acid analysis and the percent of label incorporated into collagen was estimated from the incorporation into hydroxyproline and proline (Table I). The analysis suggested that the stromal fibroblasts left within the matrix of the intact, scraped stromata synthesized a very high proportion of collagen, but that, when fibroblasts from exactly the same number of stromata were released from the matrix by the final collagenase dissociation step and inoculated in vitro as

[2] Schell, A., and K. von der Mark. In preparation.

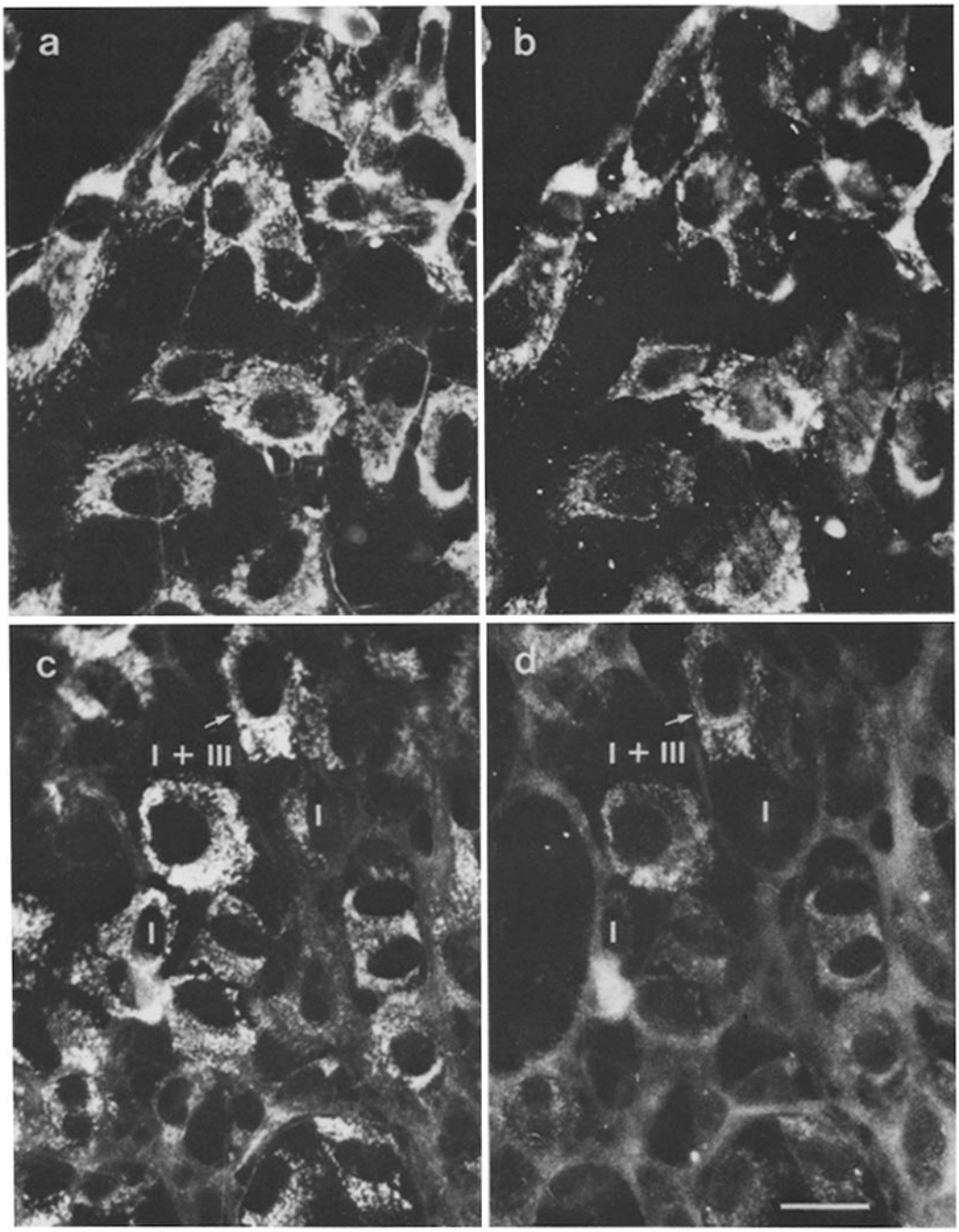

FIGURE 5 Immunofluorescence double-labeling of corneal fibroblasts from 14-d chick embryos 8 d (*a* and *b*) and 15 d (*c* and *d*) after inoculation in vitro. (cf. *a* with *b*, and *c* with *d*). (*a* and *c*) Guinea pig antibodies to type I collagen. (*b* and *d*) Rabbit antibodies to procollagen type III (p_N α1[III]). Most of the cells produced collagen types I and III simultaneously (*I + III*) (*a* and *b*). Approximately 10–20% of the cells stain only for type I collagen (*I*) (*c* and *d*). No cells stained for type III collagen only. Bar, 20 μm. × 600.

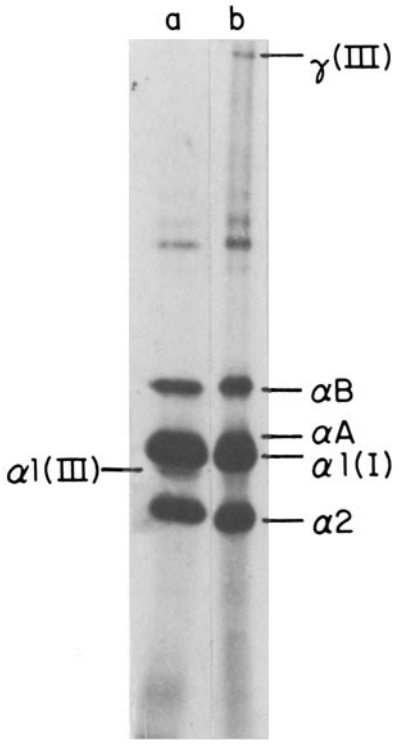

FIGURE 6 SDS-PAGE of ^{3}H-labeled collagen from the medium of 14-d embryonic chick corneal fibroblasts after 7 d in culture (fluorography). Cells were labeled with [^{3}H]proline for 24 h, and the medium was digested with pepsin to convert procollagen into collagen and to remove noncollagenous proteins. Synthesis of type III collagen is evident from a band migrating in the position of collagen γ chains (*b*), which are reducible to α chain size by mercaptoethanol (*a*).

confluent saturated layers, the proportion of collagen synthesized was drastically reduced (Table I). The total incorporation was lowered only slightly (not shown). The results also suggested that, in corneal fibroblast cultures, after 7–8 d, higher proportions of labeled collagen are found in the media than in cell layers. Similar conclusions were drawn from results obtained by immunoprecipitation with specific antibodies of labeled media and cell layers (not shown).

DISCUSSION

Chick corneas normally do not contain type III collagen but contain an abundance of type I collagen. Absence of type III collagen has been demonstrated previously by the inability of type III procollagen antibodies to bind to corneal cells or stroma after day 7 (stage 30) (44) and, as shown here, by the inability of type III collagen antibodies to bind to the corneal cells or stroma and by the absence of α1(III)CB5 during peptide analysis of corneal collagen on SDS-PAGE slab gels. Our data are consistent with earlier findings. Type III collagen has not yet been convincingly demonstrated in any vertebrate cornea. Absence of type III collagen is strongly suggested from CNBr peptide analysis of bovine corneas (33). Schmut's data (36) suggesting the presence of type III collagen in bovine and calf cornea remain inconclusive because the native chains were not shown to migrate as γ chains nor to be disulfide linked, and no peptide analyses were provided.

Thus, the cornea fibroblast seems to be a unique and highly specialized fibroblast type, for it is the

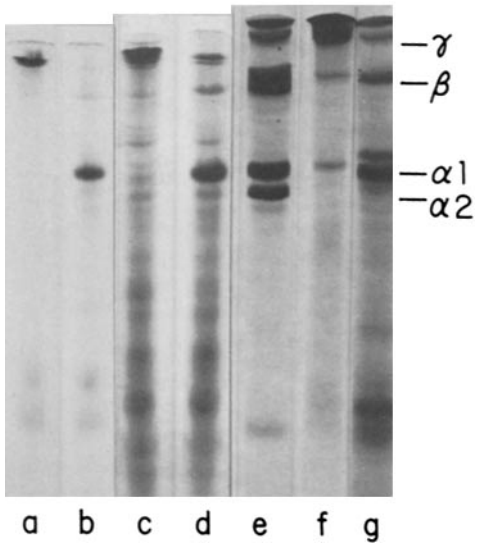

FIGURE 7 SDS-PAGE on tube gels (5% acrylamide) of ^{3}H-labeled type III collagens isolated by CM-cellulose chromatography and of unlabeled standard collagen types I and III. Fractions containing the α2 and β1, 2 chains of the carrier type I collagen were pooled for tube gel electrophoresis. Gels were stained with Coomassie Blue, as shown in this figure, then sliced and counted. The radioactivity patterns in these samples are shown in Fig. 8. Samples *a*, *c*, and *f* were not reduced; samples *b*, *d*, *e*, and *g* were reduced with 2% β-mercaptoethanol. (*a* and *b*) 14-d scraped corneal stroma, $t = 0$, tissues. (*c* and *d*) 18-d corneal fibroblasts, $t = 7$ d in vitro, nutrient medium. (*e*) Standard type I collagen. (*f* and *g*) Standard type III collagen. Reduction caused protein in γ position (α1[III]$_3$) to migrate in α1 position (α1[III]). Experimental samples (*a–d*) from cornea show protein in the γ region, which, after reduction, migrated in the α1 position, the behavior expected of type III collagen.

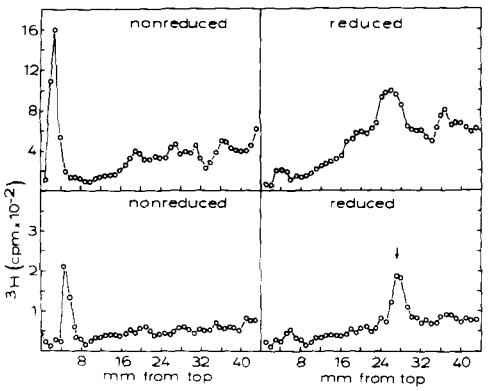

FIGURE 8 SDS-PAGE on tube gels (5% acrylamide) of ^{3}H-labeled type III collagen isolated by CM-cellulose chromatography by pooling material in the $\alpha 2$ region. Top panels: 14-d corneal fibroblasts, t = 8 d in vitro, nutrient medium. Bottom panels: 9-d aorta, t = 0, tissue. Reduced samples were treated with 2% β-mercaptoethanol before electrophoresis. Nonreduced samples were not treated with β-mercaptoethanol. Arrow, position of $\alpha 1$(III).

only one known so far that in vivo produces type I collagen but no type III.

Initiation of type III collagen biosynthesis by chick corneal fibroblasts inoculated in vitro has been demonstrated here by the binding of type III procollagen and collagen antibodies to fixed cells, by SDS-slab gel electrophoresis and fluorography of ^{3}H-labeled corneal fibroblast cultures, and by CM-cellulose chromatography followed by disc gel electrophoresis of the reduced and nonreduced $\alpha 2$ fractions. By immunofluorescence double-staining it was shown that collagen types I and III are produced in the same cells. The rapid appearance (within 12 h) of type III collagen in vitro suggests a change in gene expression of individual cells from synthesis of type I collagen to synthesis of collagen types I and III rather than the emergence and overgrowth of a subpopulation of type III collagen-producing corneal cells.

The ability of individual fibroblast cells to synthesize collagen types I and III simultaneously was first demonstrated by Gay et al. (16) with human skin fibroblasts. Similar, simultaneous synthesis of collagen types I and III was observed in monolayer cultures of embryonic chick tendon fibroblasts.[1] In the latter system, however, the number of cells producing both collagen types I and III increased with the number of passages, although the culture was started with an almost homogeneous population of type I collagen-producing tendon fibroblasts. In corneal fibroblast cultures, however, even 12 h after plating, ~50% of the cells showed positive fluorescence for type III collagen.

The intensity of fluorescence found intracellularly or extracellularly does not give any information on the amount of collagen the cell is producing. Most of the collagen produced in vitro is secreted as a soluble precursor (procollagen) into the medium (Table I). Goldberg (19) detected type III collagen synthesis by mouse and human fibroblasts in vitro and noted the preferential presence of this collagen type in nutrient medium. Although Mayne et al. (29), working with monkey aortic smooth muscle cell cultures, detected type III collagen only in the medium, Wu et al. (45), working with human uterine smooth muscle cell cultures, found type III collagen in the cell layer and in the medium. The rate of secretion of collagen into the culture medium, however, also depends on factors such as cell density, time in culture,[1] and presence of ascorbate.

Type III collagen synthesis in corneal fibroblast cultures may be initiated because proteolytic enzymes are used to dissociate the tissue, because the fibroblasts encounter stimulatory molecules in vitro (e.g., in the fetal calf serum), or because the fibroblasts are removed from contact with an in vivo repressor.

Treatment of cells with proteolytic enzymes, followed by inoculation in vitro, may cause a change in the pattern of proteins synthesized by several cell types. Chondrocytes switch from type II to type I collagen synthesis after release from their cartilage matrix and growth in vitro (35) and begin to produce fibronectin, which they do not make *in situ* (12). Chick tendon fibroblasts, known to secrete only type I collagen immediately after release from tendons (40), begin synthesis of type III collagen when inoculated in vitro.[1] In the present study, evidence is given that exposure of corneal tissue to collagenase alone, without dissociating the stromal matrix, is sufficient to initiate synthesis of type III collagen in corneal fibroblasts. The possibility exists that the change in the cellular phenotype may be caused by alterations of the cell surface as a consequence of protease activity.

Recent experiments, however, also suggest that factors present in fetal calf serum may be involved in regulating type III collagen synthesis. Of eight serum batches tested, seven allowed (or caused) initiation of type III collagen synthesis in corneal fibroblast cultures, although at different rates. In one serum batch, however, type III collagen synthesis was not observed, although cells grew nor-

TABLE I

Collagen Synthesized by Corneal Fibroblasts after Various Periods of Time In Vitro

Sample	Total number of tissues or cells	Number of d in vitro before labeling‡	Cells or NM§	Collagen synthesized*	
				Neutral salt-soluble	Pepsin-soluble
				%	
Fibroblasts‖	50 scraped stromas (stromas intact)	0	Cells	29.0	19.0
			NM	13.0	—
Fibroblasts‖	50 scraped, dissociated stromas (cells plated)	0	Cells	1.8	2.7
			NM	3.5	—
Fibroblasts¶	32.5×10^6 cells**	7	Cells	1.3	0.92
			NM	14.0	—
Fibroblasts	38.0×10^6 cells**	7	Cells	0.37	1.1
			NM	4.3	—
Fibroblasts	33.8×10^6 cells**	8	Cells	0.67	1.0
			NM	22.0	—

* Data derived from incorporation into hydroxyproline and proline as described in Materials and Methods.

‡ Fibroblasts incubated for 0 d: immediately after isolation from embryos, cells were labeled for 12 h with 100 μCi/ml [^{3}H]proline and 100 μCi/ml [^{3}H]glycine. Fibroblasts incubated for 7–8 d: cells were subsequently labeled for 24 h with 20 μCi/ml of each of the two amino acids.

§ NM, nutrient medium (labeling medium).

‖ Scraped, intact stromata were incubated in 6 ml of labeling medium. Fibroblasts from dissociated stromata were inoculated in two 60-mm diameter culture plates (each containing 3 ml of labeling medium). These fibroblasts formed saturated, confluent layers within 2 h.

¶ From 18-d embryos; all other samples from 14-d embryos.

** Total number of cells pooled from five 60-mm diameter culture plates labeled at saturation and counted at the end of the labeling period.

mally and produced large amounts of type I collagen.[2]

The collagen pattern of cornea fibroblasts in vivo may be stabilized by interactions with extracellular factors. At the moment, indirect evidence suggests a repressor, specifically, corneal keratan sulfate proteoglycan. This sulfated glycoprotein normally comprises over 60% of the polysaccharide content of the corneal stromal matrix and is made by the stromal fibroblasts in vivo from the earliest stages of their migration into the stroma (22, 23). Indeed, although maximal levels of keratan sulfate biosynthesis are reached by day 9 (stage 35) of chick development, half-maximal levels already are present in the cornea by day 7 (stage 30) (22), i.e., at the same stage at which the cornea stops making type III collagen (44). In vivo, the fibroblasts make almost all of the keratan sulfate of the cornea (6, 23), but they lose this ability within 24–48 h after their inoculation in vitro (6, 7), a loss also seen in cultured corneal fibroblasts from other animals including humans (9, 10, 17, 18, 27, 46, 47). Corneal fibroblasts in vitro may, therefore, be free to begin synthesis of type III collagen, for they would be losing or would have lost their ability to make keratan sulfate. This freedom from repression might even be experienced by fibroblasts left in the "intact," scraped stroma, for during tissue dissociation the corneas are treated with crude collagenases (containing nonspecific proteases), which probably permeate and begin degrading the stromal matrix even in scraped stromata. Thus, a variety of extracellular molecules, any one of which could be the normal in vivo repressor, are likely to be degraded and extracted during incubation of intact, scraped stromata and could account for their striking ability to synthesize type III collagen. The factors controlling the synthesis of collagen type III by corneal fibroblasts will be interesting to elucidate.

Production of A and B collagen chains does not seem to be affected by the transfer of corneal fibroblasts to tissue culture conditions. A and B collagen chains have been found in the intact cornea (15, 25, 48, and footnote 2), and they are produced in vitro at a higher rate than type III collagen. The function of this collagen in the cornea is still unclear.

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