Secretion of collagen types I and II by epithelial and endothelial cells in the developing chick cornea demonstrated by *in situ* hybridization and immunohistochemistry

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Summary

Cells involved in the synthesis of collagen types I and II in the cornea of developing chick embryos have been studied by using *in situ* hybridization and immunohistochemistry. Corneas processed for *in situ* hybridization with the type I and II collagen probes demonstrated specific mRNAs in the epithelium of embryos at stage 18 with an increase at stages between 26 and 31, and then gradual decrease to the background level in the next several days. In the endothelium, a small amount of specific mRNA was recognized through these stages. In the stroma, only sections hybridized with the type I probe demonstrated mRNA in fibroblasts. Immunostaining demonstrated specific collagen types in the stroma at sites which were closely associated with cells containing specific mRNAs. Both collagens type I and II were present beneath the epithelium as narrow bands at stage 18; as the thicker primary stroma at stages 20 and 26; and as subepithelial, subendothelial and stromal staining at stage 31. Thereafter, type I collagen was increased in the stroma but it was also noted in the subepithelial and, to a lesser degree, subendothelial regions, whereas type II collagen was gradually confined to the subendothelial matrix. Electron microscopic examination of sections from 5-day-old (stage-27) embryo corneas using antibodies against the carboxyl propeptides of type I and II procollagens revealed the presence of these procollagens within the cisternae of the endoplasmic reticulum and Golgi vesicles in both epithelial and endothelial cells. In the epithelial cells both the periderm and basal cells contained these procollagens within the cytoplasmic organelles. These results indicate that not only the epithelial cells, but also the endothelial cells secrete collagen types I and II during the formation of the primary corneal stroma and for several days after invasion of fibroblasts.

Key words: collagen, cornea, *in situ* hybridization, immunohistochemistry, epithelial cell, endothelial cell, chick embryo.

Introduction

The cornea of the embryonic chick has been a model tissue to investigate the roles of collagen during embryonic morphogenesis (Hay, 1973). Previous biochemical (Trelstad et al. 1974; Linsenmayer et al. 1977, 1982), morphological (Hay & Revel, 1969; Trelstad & Coulombre, 1971; Hay, 1980) and immunofluorescence (von der Mark et al. 1977; Hendrix et al. 1982) studies provide ample evidence that the corneal epithelium of the chick embryos secretes collagen types I and II, together with proteoglycans, basally to produce the primary corneal stroma. On the other hand, the corneal endothelium formed on the posterior surface of the primary stroma secretes hyaluronate (Toole & Trelstad, 1971) and at later stages the elements of Descemet’s membrane (Hay et al. 1979). Hyaluronate induces hydration and swelling of the corneal stroma before the invasion by mesenchymal cells. Although the endothelium has
never been shown to be a source of type I and II collagens in the primary corneal stroma (Hay et al., 1979), von der Mark et al. (1977) noted that the corneal endothelium was stained with antibody to type II collagen prior to deposition of Descemet's membrane.

In the present study, we have reexamined this well-studied tissue with particular focus on the role of epithelial and endothelial cells in the formation of early corneal stroma. *In situ* hybridization (Hayashi et al. 1986) was used to detect specific collagen mRNAs in corneal cells involved in the synthesis of collagens and immunostaining to localize collagens secreted from these cells into the stroma. Furthermore, at a stage of peak production of collagen in the epithelium, immunostaining for the carboxyl propptides of procollagens was examined by electron microscopy to demonstrate specific procollagens within the secretory organelles of collagen-synthesizing cells. The results indicate that, in the embryonic chick cornea, the epithelium and, to a lesser degree, the endothelium secretes collagen types I and II during the formation of primary corneal stroma and for several days after invasion of the stroma by mesenchymal cells. In the epithelium, both the basal cells and periderm secrete the two collagen types.

**Materials and methods**

White Leghorn chick embryos at stages 18, 20, 26, 31, 37, 39 and 43 were selected according to Hamburger & Hamilton (1951). At each stage, at least four to five embryos were studied by the following techniques. Eyes to be examined by *in situ* hybridization were fixed in 4 % formaldehyde in 0-01 M-triethanolamine buffer, pH 8.0 for 10 min. The tissues were dehydrated in ethanol and embedded in paraffin. Eyes from stage-27 embryos were examined by electron microscopic immunohistochemistry using the protocol described below.

**In situ hybridization**

Hybridization procedures used in this study were essentially the same as those described previously (Hayashi et al. 1986), except for a minor change in post hybridization washing to prevent eye sections detaching from the slides.

Briefly, deparaffinized sections mounted on subbed microscope slides were treated with pronase (0-25 mg ml⁻¹ in 50 mm-Tris–HCl, pH 7.6 and 5 mm-EDTA) for 10 min and acetylated with a freshly diluted acetic anhydride (0-25 % in 0-1 M-triethanolamine buffer, pH 8.0) for 10 min. The treated sections were processed for *in situ* hybridization at 45°C for 18–20 h in a mixture containing the tritiated cDNA probe (2 µg ml⁻¹), yeast t-RNA (500 µg ml⁻¹), salmon sperm DNA (80 µg ml⁻¹), 50 % formamide, 10 mm-Tris–HCl, pH 7.0, 0.15 M-NaCl, 1 mm-EDTA, pH 7.0, 1 × Denhardt's mixture (1966) and 10 % dextran sulphate. Collagen cDNA probes employed were: (a) a 530-base-pair BamHI–PvuII fragment from pYN535 (Ninomiya & Olsen, 1984), a probe α2(I)-specific recombinant plasmid and (b) a 603-base pair BamHI–PvuII fragment from pYN2142 (Ninomiya et al. 1984), a probe α1(II)-specific clone. Probes were labelled with ³H-dUTP by nick translation to a specific activity of 1–2 × 10⁶ cts min⁻¹ µg⁻¹ DNA.

After hybridization and removal of the cover glass by immersing the slides in 2 × standard saline–citrate buffer (SSC) (1 × SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate, pH 7.0) for 1 h at room temperature, sections were washed three times in 2 × SSC for 10 min each at room temperature, three times in 1 × SSC containing 10 % formamide for 10 min each at 45°C, and three times in 0.1 × SSC for 10 min each at 45°C. The last wash in 0.1 × SSC was cooled at room temperature for an additional 10 min. Afterwards, the slides were dehydrated in ethanol, dried in air, immersed in Kodak NTB-2 nuclear track emulsion and exposed for 10 days at 4°C. The exposed slides were developed in Kodak D-19 developer for 2-5 min at 18°C. The sections were stained with haematoxylin.

**Immunohistochemistry**

**Light microscopic study**

After deparaffinization by heat and in xylene, and a rinse in 100 % ethanol (Hayashi et al. 1987), sections were treated with 1 % hydrogen peroxide in 50 % methanol for 30 min to minimize endogenous peroxidatic activity in the tissue and washed in PBS. Sections were treated with 100 units ml⁻¹ testicular hyaluronidase (Type V, Sigma Chemical Co., St Louis, MO) in PBS for 20 min at 37°C and rinsed in PBS. For comparison, some sections were not treated with the enzyme. The slides were then placed in a moist chamber and sections were covered with 5 % bovine serum albumin in PBS for 15 min. The excess albumin was removed by blotting and sections were covered with the primary antibody solution and incubated overnight at 4°C. The primary antibodies were monoclonal antibodies directed against (a) type I collagen from chick skin (Linsenmayer et al. 1979) and (b) type II collagen from sternal cartilage (Linsenmayer & Hendrix, 1980). The antibody to type I collagen was used at a concentration of 5 µg ml⁻¹ and the antibody to type II collagen at 25 µg ml⁻¹ in PBS containing 1 % bovine serum albumin and 0.05 % NaN₃.

Sections were then covered with the second antibody, goat anti-mouse IgG (Cooper Biomedical, Inc., Malvern, PA) at a 1:20 dilution for 1 h, rinsed in PBS and covered with mouse peroxidase–antiperoxidase (PAP) (Jackson ImmunoResearch Laboratories, Inc., Avondale, PA) at a 1:50 dilution for 1 h at room temperature and rinsed in PBS. Antigenic sites on sections were demonstrated by reacting the sections with a mixture of 0.05 % 3,3′-diaminobenzidine tetrahydrochloride (Grade II, Sigma Chemical Co.) in 0.05 M-Tris–HCl buffer, pH 7.6 and 0.01 % hydrogen peroxide for 7 min. The sections were then counterstained with haematoxylin, dehydrated in ethanol, cleared...
Electron microscopy

Corneas from 5-day-old (stage-27) embryos were fixed in a mixture of 4 % formaldehyde and 0·025 % glutaraldehyde in 0·1 M-phosphate buffer, pH7·4 for 4 h at 4°C, washed in three changes of 0·1 M-phosphate buffer containing 7 % sucrose overnight at 4°C. The whole corneas were treated with 10 % methanol for 10 min, 20 % methanol for 10 min, 1 % hydrogen peroxide in 20 % methanol for 30 min, 10 % methanol for 10 min, washed in PBS and immersed in 5 % bovine serum albumin in PBS for 10 min. The immunostaining was carried out by incubating the corneas en bloc with the primary antibodies, rabbit antibodies (Pesciotta et al. 1982) against the carboxyl propeptides of type I and type II procollagens at the concentration of 20 μg ml⁻¹ overnight at 4°C.

They were washed in PBS, incubated in goat anti-rabbit IgG (Cooper Biomedical, Inc.) at a 1:20 dilution for 1 h at room temperature, washed and incubated in rabbit peroxidase–antiperoxidase complex (Cooper Biomedical, Inc.) at a 1:50 dilution for 1 h.

After incubation with the antibodies, the corneas were fixed in 2 % glutaraldehyde in PBS for 20 min at 0°C, washed and placed in the diaminobenzidine mixture containing 1 % dimethyl sulphoxide, but without hydrogen peroxide for 30 min and for an additional 3 min in the same mixture in the presence of hydrogen peroxide. They were washed and reacted with 1 % osmium tetroxide in 0·1 M-phosphate buffer, pH7·4 for 1 h, washed, dehydrated in ethanol and embedded in Epon–Araldite. Thin sections were examined in a Philips 420 transmission electron microscope.

Negative controls for immunohistochemical staining included replacing the primary antibody with the nonimmune serum of animals used to raise the antibody, or incubating in the medium lacking the primary antibody.

Results

In situ hybridization of collagen mRNAs

In the eye of an embryonic chick at stage 18, the ectoderm detaches from the underlying lens and becomes the presumptive corneal epithelium. The corneal sections processed for in situ hybridization to the type I collagen cDNA probe (Fig. 1A) demonstrated a few autoradiographic silver grains over the corneal epithelium. Sections labelled with the type II collagen probe (Fig. 1B) exhibited a large number of silver grains over the epithelium and a moderate number over the lens epithelium. The labelling with the type II probe of the corneal epithelium extended beyond the area anterior to the lip of the optic cup (data not shown). At stage 26, the primary corneal stroma is covered completely on its posterior surface by the newly migrated endothelium. Corneal sections labelled with the type I probe (Fig. 1C) showed a moderate number of silver grains over the epithelium and some over the endothelium. Sections labelled with the type II probe (Fig. 1D) showed large numbers of silver grains, exceeding that of the previous date examined, over the epithelium and some over the endothelium. Again, the labelling of the epithelium with the type II probe passed the limit of the corneal surface and covered more peripheral regions of the ectoderm. Labelling of the peripheral ectoderm was not obvious with the type I probe. At stage 31, the corneal stroma is already invaded by a number of fibroblasts. Corneal sections labelled with the type I probe (Fig. 1E) exhibited many silver grains over most of the fibroblasts with the number of silver grains over the epithelium being comparable with the previous stage. Sections labelled with the type II probe (Fig. 1F) contained many grains over the corneal epithelium and the nearby ectoderm, and some over the endothelium.

Density of silver grains over the epithelium gradually decreased and it reached background levels at stage 37 in sections labelled with type I probe; however, a moderate number of grains were still present at stage 39 in sections labelled with the type II probe (Fig. 1H). A small number of silver grains remained present over the endothelium at these stages in preparations labelled with the type II probe. A large number of grains was present over the stromal fibroblasts labelled with the type I probe at stages 39 (Fig. 1G) and 43.

Immunohistochemistry of collagens

Light microscopic localization of secreted collagens

At stage 18, immunostaining for collagen types I (Fig. 2A) and II (Fig. 2B) was evident as a narrow band of fibrous structure beneath the corneal epithelium and at the edges of the optic cup. Type II collagen was also stained along the lens surface and in the vitreous humour. At stage 20, the subepithelial staining for both collagens was increased and seen as a uniformly stained band of about 10 μm thick; with the staining in other structures unchanged. At stage 26, an intense staining for both collagen types occupied about 20 μm thick corneal stroma (Figs 2C,D) and it was more intense in the subepithelial region than the rest of the matrix. Under high magnification, the staining was seen as a fine mesh work of fibrils filling the entire area of the acellular corneal stroma. At stage 31, type I and II collagens were seen in the subepithelial and subendothelial regions as well as the remaining corneal stroma (Figs 2E,F). The subepithelial collagens of both types I and II were distributed in wavy patterns. Staining for type I collagen in the entire stroma was increased by new collagens produced by a number of fibroblasts and it was more
stromal fibroblasts with the type I probe (G) and a large number over the corneal stroma. In addition, type I collagen was seen as an intensely stained narrow bands beneath the epithelium and endothelium. At stage 43, type I collagen in the stroma was further increased. As to type II collagen, at stage 37, it was confined to narrow bands beneath the epithelium and endothelium in addition to the fine fibrils in the anterior stroma. At stage 39, type II collagen in the subepithelial region reduced from the central area of the corneal surface, whereas collagen in the subendothelial region increased. In some of the sections at this stage, both types I and II collagen in the subendothelial region looked wavy, just like the collagens seen in the subepithelial region at stage 31. At stage 43, type II collagen failed to stain beneath the epithelium; stromal staining diminished; and subendothelial staining became less intense.

**Electron microscopic localization of procollagens**

The corneal epithelium from 5-day-old (stage-27) embryos is made up of the flattened periderm and the columnar basal cells. The latter cells contain more abundant well-developed secretory organelles in the cytoplasm than the former. Immunostaining with antibodies to the carboxyl propeptides of procollagen types I (Figs 3A,C) and II (Figs 3B,D) demonstrated procollagens in both the periderm (Figs 3C,D) and basal cells (Figs 3A,B) as electron-dense fine granules or amorphous deposits. Here the stained procollagens filled within the variously distended cisternae of the rough endoplasmic reticulum of these epithelial cells and also accumulated in the intercellular space surrounded by these cells (Fig. 3D). Procollagens were present in the stroma beneath the basal cells but not on the free surface of the periderm. The corneal endothelium contained type I (Fig. 3E) and II (Fig. 3F) procollagens within fewer cisternae of the endoplasmic reticulum than those of the epithelium. Procollagens of both types were also demonstrable in expansions of the cisternae of the Golgi apparatus (Figs 3G,H) and in the large condensing vacuoles in both epithelial and endothelial cells.

**Discussion**

Combined results from *in situ* hybridization to collagen mRNAs and from immunostaining of collagen proteins indicate that, in the developing chick cornea, both the epithelium and endothelium secrete collagen types I and II during the formation of the acellular primary stroma and for the next several days after invasion of fibroblasts to the stroma. Epithelial collagen production reaches its peak between stages 26 and 31, whereas the endothelial collagen is produced at a low level throughout the period of development. Some collagens secreted by these two types of epithelial cells may stay in the matrix immediately beneath these cells and associate with the basement membrane, while the majority distributes through the stroma and forms a fine mesh work with a spiralling orthogonal arrangement (Trelstad & Coulombre, 1971).

Our results with the immunoperoxidase staining were essentially the same as those with immunofluorescence used by previous workers (von der Mark et al. 1977; Hendrix et al. 1982). We have, however, shown more clearly both collagens in the subepithelial and subendothelial regions. This enhanced staining probably was due to the procedures we employed: unmasking of antigenic sites with hyaluronidase and a more sensitive detection by using the peroxidase-antiperoxidase method (Sternberger et al. 1970). We have previously examined (Hayashi et al. 1986) how probe specificity correlates with nucleotide sequence homology and shown that specific hybridizations to collagen types I and II mRNAs, with a minimum of cross-hybridization, are obtained by employing the cdNA probe fragments and hybridization conditions used in this study. The specificity of the monoclonal antibodies to each collagen has been established...
Fig. 2. Chick corneal sections stained with monoclonal antibodies to collagen types I (A,C,E,G) and II (B,D,F,H) by the peroxidase–antiperoxidase technique. At stage 18 (A,B), immunostaining for collagen types I (A) and II (B) is evident as a narrow band beneath the corneal epithelium and at the edges of the optic cup (oc). Type II collagen is also stained along the lens surface and in the vitreous humour (vh). At stage 26 (C,D), an intense staining for type I (C) and type II (D) collagens is seen as mesh work of fibres filling the entire corneal stroma. The staining is more intense in the subepithelial region than the rest of the matrix. At stage 31 (E,F), type I (E) and II (F) collagens are seen in the subepithelial and subendothelial regions as well as the remaining corneal stroma. Subepithelial staining looks very wavy in appearance for both collagen types. Type I collagen (E) in the stroma is markedly increased by collagens produced by newly invaded fibroblasts. Type II collagen (F) is more abundant in the anterior stroma than in the posterior. At stage 39 (G,H), type I collagen (G) fills most of the corneal stroma and is also seen as an intensely stained band beneath the epithelium and a narrow band beneath the endothelium. Type II collagen (H) is confined to the subendothelial region and also is seen as fine fibrils in the anterior stroma. Bar, 20 μm.
biochemically (Linsenmayer et al. 1979, 1980) and histochemically (Hendrix et al. 1982). The rabbit antibodies used in this study bind specifically to each collagen type specific C-propeptide (Pesciotta et al. 1982); and in the sceral cartilage the type II antibody stained only chondrocytes, whereas the type I antibody stained only perichondrial fibroblasts. In each case, no staining was noted in the absence of the primary antibody.

During the development of the cornea, the Golgi apparatus in the basal cells shifts from an apical to basal position (Hay & Revel, 1969; Trelstad, 1970), presumably to facilitate discharge of secretory material basally into the stroma. The present study demonstrates mRNAs in the epithelial cells and collagens in the stroma as early as stage 18 when the majority of the Golgi is located apical to the cell nucleus as well as later stages when the Golgi apparatuses are basally located. The capacity for the production of the primary stroma, judged by the presence of mRNA in the epithelial cytoplasm, thus precedes the structural changes which the cells effect to place their secretory organelles in a basal position. However, an obligatory position of the Golgi apparatus in the basal pole of the cell for excretion of primary stroma does not appear to be necessary. Our electron microscopic observations suggest that at least at day 5, procollagens are secreted from the epithelium into the stroma using two routes: the intercellular space and the basal surface. The periderm may also excrete procollagens into the intercellular space and these procollagens would then have to be transported through intercellular channels to reach the stroma. Herefore, the periderm was not considered a potential contributor to the primary stroma, but the present data provide direct evidence to indicate that it can produce primary stromal materials. The basal cells may thus secrete procollagens into the intercellular space at stages when most of the Golgi apparatuses are located apical or lateral to the cell nucleus and across the basal surface when the Golgi are predominantly in a basal position. The basal portions of the basal cells have been shown (Trelstad, 1971) to contain elongated vacuoles enclosing a dense, slightly fibrillar material and these vacuoles increase in number during the peak production of collagens in the epithelium. In this study, we did not characterize the immunocytochemical properties of the contents of these vacuoles.

Comparison of the relative amount of either mRNAs or proteins between the two types of collagens is affected by several variables. Biochemical studies suggested initially (Trelstad et al. 1974) that the corneal epithelium at day 5 produced a mixture of collagen molecules rich in α1 chains, but it was later shown (Linsenmayer et al. 1977, 1982), using peptide-mapping technique, that this mixture contained approximately equal amounts of collagen types I and II. Our results with immunostaining showed a comparable staining for the two collagens at stage 26 (approx. 4.5-5 days) and are consistent with the biochemical data. However, in situ labelling of mRNAs over the epithelial cells with the type I collagen probe was always less intense than that with the type II probe. This difference was largely because the type I probe-specific for pro α2(I) (the BamHI–PvuII fragments of pYN555 (Ninomiya & Olsen, 1984) did not cross-hybridize, under the conditions employed, to mRNAs encoding pro α1(I), which shows only 54% sequence homology with pro α2(I) (Hayashi et al. 1986). Since type I collagen helix consists of two α1 chains and one α2 chain, our data may have demonstrated one third of the total type I collagen mRNA present in these cells. Our results also showed an apparent large accumulation of both collagens in the stroma between stages 18 and 26 (Figs 2A,B, and C,D) but only a minor increase in the amounts of the corresponding mRNAs in the epithelium (Figs 1A,B, and C,D). The relatively small amount of the collagens at stage 18 may be a reflection of a lag time from the accumulation of mRNAs in the epithelium to the deposition of processed and transported collagens in the stroma; or it may be a reflection of some translational regulation of collagen biosynthesis. The relatively large amount of the collagens at stage 26 may be an indication of a slower turnover of secreted collagens (Gross, 1981) in the stroma as compared to mRNAs in the epithelium.

The implications of these observations for the morphogenesis of the primary corneal stroma are several. First, the production of the orthogonal template, used later by the fibroblasts, is the effort of both the epithelium and the endothelium. This might be helpful in explaining the geometry of both the primary and secondary stromas. The primary stroma deposited between stages 20 and 28 shows no cholesteric liquid-crystal-like organization, whereas that produced after stage 28 by the epithelium does (Trelstad & Coulombre, 1971). Since the posterior third of the secondary stroma is an orthogonal set with no angular displacement in the adult, we speculate that the endothelium produces an orthogonal template in which all layers are in alignment. A second implication is that the primary stroma may assemble from molecules discharged into the intercellular space and possibly even from molecules produced by the periderm.

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Fig. 3. Electron micrographs of the corneal epithelium and endothelium from 5-day-old (stage-27) chick embryos stained with antibodies to the carboxyl propeptides of the procollagen types I (A,C,E,G) and II (B,D,F,H) by the peroxidase–antiperoxidase technique. The epithelium is made up of the flattened periderm (p) (C,D) and the columnar basal cells (A,B). Basal cells contain abundant well-developed secretory organelles as compared to the periderm. Immunostaining for procollagen types I (A,C) and II (B,D) demonstrates procollagens as electron-dense fine granules or amorphous deposits (arrows) within the cisternae of the rough endoplasmic reticulum (er) of both the periderm and basal cells and within the intercellular spaces (is). bm indicates basement membrane. The endothelium (E,F) contains types I (E) and II (F) procollagens within fewer cisternae of the endoplasmic reticulum than those of the epithelial basal cells. Some fibrils in the stroma (s) are stained with the two antibodies. In the Golgi apparatus (G) of the endothelium both type I (G) and type II (H) procollagens are demonstrable in expansions of the cisternae (c) and in the large condensing vacuoles (v). Bar, 1 μm.
References


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