Collagen IV mRNA Expression During Development of the Mouse Retina: An In Situ Hybridization Study

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**Purpose.** During development of the nervous system, neuronal migration and axonal growth depend on specific interactions with molecules in the extracellular matrix. In a recent study of laminin expression, it was shown that laminin B1 mRNA was expressed by both nonneural cells and the retinal ganglion cells during development of the mouse retina. Because collagen IV is associated with laminin in basement membranes, this report examined whether collagen IV mRNA also is expressed by neurons and nonneural cells during retinal development.

**Methods.** Collagen IV was localized by immunocytochemistry, whereas the sites of collagen IV mRNA synthesis were identified by in situ hybridization.

**Results.** Collagen IV immunostaining was detected at embryonic day 12 (E-12), the earliest stage examined. At E-12 and E-15, collagen was found in the lens, the embryonic (hyaloid) blood vessels, and the internal limiting membrane (ILM) of the retina. At E-17, immunostaining was reduced in the ILM, whereas the lens and hyaloid were strongly stained. Collagen was barely detected in the ILM in postnatal retinas. In the in situ hybridization experiments, at E-12, collagen IV mRNA was found in the lens and the hyaloid vessels. Only sparsely labeled cells were present in the retina. After E-17, the density of labeling in these structures decreased dramatically. Collagen IV mRNA was not found in the retina at any stage in development or in the adult. Northern blot analysis showed that a 6 Kb collagen IV transcript was present in the eye.

**Conclusions.** These findings establish that high levels of collagen IV are present at the ILM only during early development (E-12 to E-17), when most axonal growth occurs. Retinal collagen IV is possibly derived from nonretinal sources, such as the lens, or more likely from the hyaloid vessels. Invest Ophthalmol Vis Sci. 1993;34:145–152.

In the developing nervous system, neuronal migration and axonal outgrowth are mediated by interactions with molecules present in the extracellular matrix. Using mono-specific antibodies as molecular probes, several extracellular (ECM) molecules, such as laminin, fibronectin, collagen, and heparan sulfate proteoglycan, have been identified in the nervous system. Although laminin is the most prevalent and best studied of the ECM molecules examined so far, others, such as collagens, also may play key roles in nervous system development. Collagen I and Collagen IV have been reported to promote neurite outgrowth from peripheral neurons and also may interact with some central nervous system neurons. In addition, collagen IV has been found in both synaptic and extrasynaptic basal lamina and might participate in synaptic differentiation.
In the vertebrate retina, an early study found an absence of hydroxyproline, and it was suggested that the retina did not contain collagen. Immunostaining experiments, however, showed that collagen is present in the internal limiting membrane (ILM) of the retina. The ILM is a basement membrane located at the interface of the retina and the vitreous; it contains a network of proteoglycans and collagen fibrils.

Embryonic retina also has been shown to synthesize collagens. Recent experiments with dissociated chick retinal neurons report that embryonic cells of all ages attach effectively to collagen IV. Neurite outgrowth, however, was relatively modest compared to that on laminin. It has been suggested that the vitreal collagen is derived from Müller (glia) cells in the retina. Collagen I and Collagen IV mRNAs have been reported recently in fetal and adult human optic nerves.

We are interested in identifying cells that produce ECM molecules in the developing nervous system and in characterizing molecular interactions involved in axonal outgrowth. As an initial step, we recently attempted to identify cells that produce laminin in the retina. Because laminin-specific antibodies have not been particularly successful in identifying sites of laminin synthesis, we decided to localize laminin B1 mRNA by in situ hybridization.

The experimental results showed there are at least two principal sites of laminin B1 mRNA synthesis in the developing mouse retina: (1) the hyaloid blood vessels during the major period of axonal outgrowth; and (2) the retinal ganglion cells at later developmental stages. These data suggest that in addition to non-neuronal cells, certain retinal neurons also may synthesize and secrete extracellular matrix molecules such as laminin. Because retinal ILM contains collagen and retina has been reported to synthesize collagens, we are interested in identifying the sites of collagen IV synthesis in the retina. For the present study, we used in situ hybridization to localize the sites of collagen IV mRNA expression during development of the mouse retina. Our results show that retinal collagen IV is not derived from any of the retinal cell types, but instead is likely to originate from outside the retina from the lens, or more likely from vascular cells of the vitreous, the hyaloid vessels.

MATERIALS AND METHODS

Animals were used in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Developmental studies of collagen IV gene expression were carried out with fetal and postnatal animals. Mice from embryonic day 12, when retinal development starts, up to postnatal day 21, when the retina is mature, were studied.

Immunocytochemistry

The indirect immunofluorescence technique was used to localize collagen in cryostat sections of the mouse retina according to protocols described earlier. Adult mice were anesthetized with ether and killed by cervical dislocation; pups were anesthetized and decapitated. After enucleation, eyes were removed and fixed in freshly prepared 4% paraformaldehyde. The fixed tissue was left in 15% sucrose overnight in the refrigerator. Eyes were embedded in OCT compound (VWR Scientific Division, San Francisco, CA) and sectioned at 12 μm on a cryostat maintained at −20°C. Tissue sections were collected on gelatin-coated slides. After the blocking, sections were treated with collagen IV antisera. The antigen was visualized after sections were stained with fluorescein isothiocyanate-goat anti-rabbit IgG. When pre-adsorbed (Collagen IV; Chemicon, El Segundo, CA) and pre-immune sera were used as controls, no staining of the sections was observed. Collagen IV antibodies were purchased from Chemicon.

In Situ Hybridization

RNA localization was carried out according to published protocols. 35S-labeled DNA probes were prepared by nick translation of a cDNA probe that contained a 850 Kb PstI/Avai fragment, which is part of the mouse α1 (IV) collagen gene coding sequence. Eyes from adult and postnatal mice or whole heads from embryonic mice were fixed in 4% paraformaldehyde, frozen sectioned at 12 μm, and collected on gelatin-coated slides. After incubation with proteinase K (1 mg/ml), sections were washed and treated with 0.25% acetic anhydride in 0.1 mol/l tris-ethanolamine, pH 8.0. Hybridizations were carried out overnight at 45°C in 0.15 mol/l NaCl, 50% formamide, and 1X Denhardt’s solution at a probe concentration of 2 μg/ml. After a series of washes, with a final rinse in 0.1 X sodium chloride/sodium citrate buffer (SSC) for 30 min at 60°C or in 0.5 × SSC for 30 min at 50°C, the slides were dried and processed for autoradiography. Tissue sections were counterstained with cresyl violet and examined under bright and dark field optics. Autoradiograms were developed after 3–10 days of exposure. To ascertain the specificity of hybridization, some retinal sections were pretreated with RNase A and RNase T1 before use. With embryonic tissue, a 3–5 day exposure was sufficient; for postnatal retinas, longer exposures (6–9 days) were needed to demonstrate distinct cellular labeling. This often led to an increase in tissue background. When comparing label distribution in retinas from different ages, similar regions of retina were examined.

RNA Analysis

For Northern blot analysis, total RNA was extracted from 6–12 eyes, and 10 μg RNA was electrophoresed.
in a 1.5% agarose gel containing formaldehyde. RNA was transferred to nitrocellulose, and the blot was hybridized to 32P-labeled collagen IV probe overnight at 42°C and washed several times, ending in a final wash in 0.2 X SSC and 0.1% sodium dodecyl sulfate at 42°C. The blot was developed after 1 or 2 days of exposure. The locations of ribosomal RNA markers were determined by staining the blots with methylene blue.

RESULTS

Immunocytochemical Localization

Collagen IV was localized in the developing mouse eye by the indirect immunofluorescence method. Figure 1 shows the pattern of immunostaining observed in embryonic eyes. At embryonic day E-12, several ocular structures showed intense staining (Figs. 1A, B). In the lens, staining was strongest at the cortex and decreased toward the nucleus. In the retina, a band of intense reactivity was observed at the inner limiting membrane. In addition, weak staining of the neural retina was noted. Immunostaining also was seen on the apical side of the retina, probably arising from the pigment epithelium and Bruch's membrane. In addition, a mass of cells located between the retina and the lens were strongly labeled. These cells likely belonged to the hyaloid (blood) vessels that are present in the embryonic eye. A similar pattern of labeling also was noted in E-15 eyes (Figs. 1C, D). The intensity of labeling in the ILM was, however, somewhat reduced at this stage. In addition, collagen-immunostaining extended from the central retina to the periphery (Figs. 1C, D). By E-20, only weak immunoreactivity was found in the inner limiting membrane, and the stained structures were associated with the blood vessels (Fig. 1E). In contrast, the pigment epithelium and Bruch's membrane were strongly labeled (Fig. 1E). When pre-absorbed antisera were used, there was little immunostaining in the tissue (Fig. 1F).

In postnatal retinas, the ILM showed relatively weak immunostaining, whereas the blood vessels again were strongly immunoreactive (Figs. 2A–D). Furthermore, the ciliary body was strongly labeled at P-3 and P-5 stages (Figs. 2E, F). In summary, the immunostaining experiments show that at E-12 to E-17, collagen IV

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**FIGURE 1.** Immunocytochemical localization of collagen IV in embryonic eye. Cryostat sections of the eye were processed for immunocytochemistry, as described in “Materials and Methods.” (A, B) E-12. (C, D) E-15. (E) E-20. (F) E-12, control. r, retina. le, lens. hv, hyaloid vessels. Note immunostaining of lens, blood vessels (arrows), inner limiting membrane (open arrow), and pigment epithelium and choroid (arrow head).
is present at the ILM. At these developmental stages, the lens, hyaloid vessels, pigment epithelium, and Bruch's membrane also were labeled. Immunostaining was first observed in the ciliary body at E-17. In postnatal eyes, the blood vessels, lens, ciliary body, and Bruch's membrane were stained, whereas the ILM was labeled faintly. Furthermore, immunostaining seen in the optic nerve always was associated with blood vessels (Fig. 2A).

Collagen IV mRNA in Embryonic Eyes

To identify the cellular sites of collagen synthesis, we localized collagen IV mRNA by in situ hybridization. Figure 3 presents collagen mRNA localization in embryonic day 12 eye. At this stage, strong labeling was found over the lens, the hyaloid vessels, and the pigment epithelium/Bruch's membrane region (Figs. 3 A–D). In contrast, comparatively little label was associated with retinal cells (Figs. 3E, F). At E-15 and subsequent developmental stages, labeling again was intense and closely associated with extra-retinal structures, the hyaloid vessels, and the lens (Figs. 3A–C).

For example, in E-20 retina, the blood vessels at the ILM were the only structures labeled (Figs. 4A–C). In the postnatal stages, no labeling was seen in the retina; however, the blood vessels and the pigment epithelium were clearly labeled (Fig. 5).

Collagen IV mRNA Characterization

To characterize the collagen IV transcript found in the developing eye, we carried out a Northern blot analysis of total RNA isolated from postnatal stages. As shown in Figure 6, we found a major transcript of ~6–7 kb size in RNA extracted from P-3 and adult eyes. Moreover, collagen IV mRNA level was higher in the P-3 eye. In addition to the major transcript, a minor band of ~5 Kb was noticed in these blots. We have not carried out Northern blot analysis on isolated retinas from embryonic eyes, because we have found it difficult to obtain good RNA preparations. Furthermore, no col IV mRNA was detected in RNA from P-3 retinas (data not shown). This contrasts with laminin, because laminin B1 transcript was abundant in the isolated P-3 retina.22
**DISCUSSION**

**Sites of Collagen IV mRNA Synthesis**

Our immunocytochemical data show that high levels of collagen IV are present in the internal limiting membrane of the retina early during development (E-12 to E-17) and are barely detected later. In mRNA localization experiments, we found that collagen IV mRNA was present at high levels in the lens, the ciliary body, the pigment epithelium/Bruch’s membrane complex, and the hyaloid vessels. In addition, collagen IV mRNA was found in blood vessels in postnatal eyes.

**FIGURE 3.** Localization of collagen IV mRNA by in situ hybridization in embryonic eye. RNA localization was carried out as described in detail in “Materials and Methods.” (A, B) E-12. (C, D) E-12. (F) E-20. A and C are bright, and B and D are dark-field micrographs. Note strong labeling of lens (le), blood vessels (arrow), and pigment epithelium (arrow head). No labeled cells were seen in the retina (r).

**FIGURE 4.** Sites of collagen IV mRNA synthesis in embryonic eye. (A) E-20. (B) E-15. (C) E-20. Note strong labeling of hyaloid vessels (hv; arrow) and pigment epithelium (arrow head). No labeled cells were seen in the ganglion cell layer (gcl) or in the neuroepithelial layer (nel).
with a recent report in which no collagen α1 (IV) mRNA was detected in newborn mouse retina. In using the collagen IV probe, we noted that the background labeling was higher than that observed with the laminin B1 probe. Whether this is the result of differences in mRNA levels or is the result of cross hybridization is not known. Unfortunately, the higher background in the tissue made it difficult to distinguish between no labeling and very low levels of collagen IV mRNA expression in the embryonic retina.

Several previous studies have reported that vitreous collagen is derived from retina as well as the hyalocytes. During development of the chick eye, vitreous collagen was found to be derived mainly from the neural retina at early stages (26-27 and 29-30) and by the hyalocytes in the vitreous at later stages (40). Although the identity of the collagen chain was not established, indirect evidence suggested that the vitreous collagen probably was type II. Biochemical analysis of collagens secreted by embryonic neural retina cells showed that these cells synthesize two distinct collagens, one of which (~90% abundance) is the precursor to collagen type II. Recent in situ hybridization studies show that the ciliary region of the eyecup may be the source of type II collagen synthesized by the retina. In monolayer cultures obtained from explants of rabbit retina, however, ~90% of the collagen secreted is type I, suggesting possible species differ-

**FIGURE 5.** Collagen IV mRNA localization in postnatal (P-3) retina. Notice that the labeled cells are located under the retina close to the vitreous. No labeled cells are present in the ganglion cell layer (gcl) or in the neuroepithelial layer (nel). Pigment epithelium (pe) and blood vessels (arrows) are labeled.

In contrast, comparatively low amounts of collagen IV mRNA were found in the retinal cells at any stage in development or in the adult. These observations agree

**FIGURE 6.** Northern blot of collagen IV mRNA. RNA was extracted from eyes and electrophoresed in a 1.5% agarose gel. After blotting onto nitrocellulose, collagen IV transcripts were detected by hybridization with 32P-labeled probe. Both adult (A) and P-3 eyes show an ~6 Kb transcript (arrow head). Collagen IV mRNA level was higher in the P-3 eye.
ences in retinal collagen synthesis.\textsuperscript{17} Although we found large amounts of collagen IV mRNA in the ciliary body, the retina itself showed relatively low levels of the transcript. Our experimental results and the findings of Stone and Laurie\textsuperscript{32} strongly suggest that collagen \( \alpha \) (IV) transcripts are present at very low levels or are absent in the neonatal retina.

Because of the comparatively low levels of collagen IV mRNA in the retina, we have proposed that collagen IV in the ILM possibly originates from the hyaloid artery or the lens. This suggestion, however, raises the question regarding how collagen IV synthesized in extra-retinal sites could be secreted and deposited at the ILM. An alternative interpretation is that collagen IV in the ILM is derived from the low levels of collagen IV mRNA synthesized by retinal cells throughout embryonic development. The reduced collagen IV immunostaining in postnatal ILM might result from antigen masking, because of the presence of other ILM components.

**Identity of Collagen IV mRNA**

Type IV collagen is a major constituent of structural proteins of basement membranes.\textsuperscript{35} Molecular cloning studies and sequence analysis have shown that it generally consists of \( \alpha 1(IV) \) and \( \alpha 2(IV) \) chains with a heteromer composition of \((\alpha 1(IV))_2(\alpha 2(IV)) \) or \((\alpha 1(IV))_3\), depending on the basement membrane.\textsuperscript{28-33} RNA blotting shows there are two mRNAs (6.7 Kb and 5.4 Kb size) for human \( \alpha 2(1) \) and \( \alpha 1(1) \) and chick \( \alpha 2(1) \) with the difference in the length of 3' untranslated end.\textsuperscript{28-34} In Northern blots probed with full length \( \alpha 1(IV) \) cDNA, we found a major transcript of \( \sim 6-7 \) Kb mRNA in postnatal and adult mouse eyes. Because of the close homology between \( \alpha 1 \) and \( \alpha 2 \) mRNAs, our experiments cannot distinguish between the two transcripts. We presume that retinal collagen also is made up of \( \alpha 1 \) and \( \alpha 2 \) chains. In addition to a major band at \( \sim 6-7 \) Kb, we also noticed a minor band of smaller size (\( \sim 5 \) Kb) in our blots. We do not know whether the minor transcript is a degradation product or a separate collagen IV mRNA species.

In summary, we found that collagen IV is present at the internal limiting membrane of the retina early during development (E-12 to E-17) and is barely detected subsequently. In previous work, we had shown that laminin is present at the ILM in embryonic and postnatal stages of the mouse retina. In situ hybridization experiments also showed that collagen IV mRNA and laminin B\textsubscript{1} mRNA were present in high levels at these stages. Thus, it appears that early in development, the ILM contains high levels of laminin and collagen, whereas at later stages (E-20 and after), the ILM predominantly contains laminin. The functional significance of the changes in matrix composition of the ILM is not known.

Immunocytochemical studies have shown that the retinal internal limiting membrane also contains many of the components integral to basement membranes.\textsuperscript{1,36} In addition to laminin and collagen IV, other matrix components, such as fibronectin, vitronectin, and proteoglycans, also have been found in the internal limiting membrane.\textsuperscript{19,26,37,38} The identity of the cell types that synthesize these proteins remains to be established.

**Key Words**

Collagen, development, extracellular matrix, hyaloid, retina.

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**References**

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