Localization of Collagen Types I and IV mRNAs in Human Optic Nerve Head by In Situ Hybridization

M. Rosario Hernandez, Nan Wang, Nancy M. Hanley, and Arthur H. Neufeld

Using in situ hybridization, individual cells expressing mRNAs for collagen types I and IV were localized in fixed-tissue sections of adult and fetal human optic nerve heads. Astroglial cells lining the cribiform plates and cells inside the cribiform plates of the lamina cribrosa had mRNA for collagen type IV. Cells in the glial columns, pial septa, and vascular wall also contained mRNA collagen type IV. Collagen type I mRNA was expressed by cells of the cribiform plates of the lamina cribrosa of adults. Few cells in the glial columns, pial septa, and blood vessels had mRNA for collagen type I. Scleral fibroblasts contained mRNA for collagen type I. These results indicated that the expression of mRNA for both collagen types I and IV paralleled the localization of these extracellular matrix proteins in the optic nerve head and suggested that both collagen types were synthesized in this tissue throughout life.

Cupping of the optic nerve head associated with primary open-angle glaucoma apparently results from compression, stretching, and rearrangement of the connective tissue of the lamina cribrosa in response to elevated intraocular pressure. Changes in the composition and distribution of the extracellular matrix of the lamina cribrosa were found in humans and primates with glaucoma. In normal human eyes, age-related increases in the connective tissue area and in extracellular matrix components occurred in the lamina cribrosa. We postulated that changes in the extracellular matrix of the lamina cribrosa may influence the degeneration of nerve fibers that occurs in glaucoma.

The lamina cribrosa is composed of stacks of connective tissue plates, the cribiform plates, aligned in register, to form channels through which the axons pass. Previous studies using immunocytochemistry found that the cribiform plates contain substantial amounts of collagen type IV, a ubiquitous basement membrane component. Collagen type IV forms lamellar structures, apparently part of the basement membranes of the astrocytes lining the cribiform plates, and also extends linearly into the core of the plates, forming a network of fibrillar material. Collagen type I, an interstitial form of fibrillar collagen, is present in the core of the cribiform plates and increases in amount as the plates expand with age.

Our current knowledge of the extracellular matrix of the optic nerve head comes from previous ultrastructural, biochemical, or immunocytochemical studies. However, these techniques cannot distinguish newly synthesized extracellular matrix from older material, nor can they identify cells responsible for the biosynthesis of accumulated material. Moreover, biochemical determinations of structural and temporal changes in the extracellular matrix of individual optic nerve heads are difficult, if not impossible, to do because of the small amount of tissue available.

In situ hybridization is a technique that allows the localization of specific mRNAs in cells or tissues by using a labeled complementary DNA (cDNA) or RNA probe. The availability of highly specific cDNA probes for the mRNAs of several extracellular matrix macromolecules made possible the study of gene expression and its regulation in various tissues and cells. We used in situ hybridization to localize mRNAs for collagen types I and IV in individual cells in human fetal and adult optic nerve heads. Our ob-
servations indicate that mRNAs for both macromolecules are present in the human optic nerve head throughout life.

Materials and Methods

Preparation of the Tissue Sections

Five pairs of human eyes (patient ages, 15, 42, 55, 56, and 87 yr) without a history of eye disease and two pairs of human fetal eyes (age, 20 and 21 weeks of gestation) were enucleated shortly after death and fixed immediately in freshly prepared, buffered 4% paraformaldehyde for 2–4 hr.\(^{13,21}\) The optic nerve heads were then dissected and rinsed several times in phosphate-buffered saline (PBS) containing 0.1 M glycine and dehydrated in a graded series of ethanol (50%, 70%, 95%, and 100% ethanol) for 30 min each followed by three changes of xylene for 30 min each. The tissues were embedded in paraffin according to standard procedures. In each pair of eyes, one optic nerve head was oriented for sagittal sections, and the other for cross sections. Six-micron sections were cut and placed on subbed slides to prevent detachment of the sections during hybridization. The slides were subbed by incubating them in single-concentrated Denhardt’s solution, 10 mM dithiothreitol (DTT) yeast tRNA (100 ng/ml), and 10% dextran sulfate.\(^{24}\) The hybridization mixture (20 \(\mu\)l/slide) containing 2–3 \(\times 10^6\) cpm of labeled RNA probe was applied to the tissue sections, which were covered with siliconized cover slips and incubated in a closed moist chamber at 42°C for 18 hr to allow hybridization. After hybridization, the cover slips were removed gently in twice-concentrated SSC at 37°C, and then the slides were washed in 50% deionized formamide, twice-concentrated SSC, and 10 mM EDTA at 37°C for 15 min. The slides then were treated with RNase A (50 \(\mu\)g/ml; Sigma) in fourfold-concentrated SSC at 37°C for 30 min. After RNase A digestion, they were washed in fourfold-concentrated SSC containing 10 mM Tris and 1 mM EDTA at 37°C for 15 min. The slides were then washed in 50% formamide, twice-concentrated SSC, and 10 mM DTT at 37°C for 30 min, followed by washing in fourfold-concentrated SSC containing 10 mM Tris and 1 mM EDTA at 37°C for 15 min. The slides were then washed in five changes of xylene for 30 min each. They then were washed three times in xylene for 10 min each and twice in 100% ethanol for 10 min each and then allowed to air dry. They were fixed in 4% paraformaldehyde for 20 min, washed three times in PBS–glycine for 5 min each, dehydrated in a graded series of ethanol, and air dried.

Hybridization Probes

The cDNAs encoding for human \(\alpha_2(1)\) collagen\(^ {22}\) and human \(\alpha_1(IV)\) collagen\(^ {23}\) (gifts of Dr. Francesco Ramirez, Mount Sinai Medical School, New York, NY) were used in this study. A 1000 base-pair \(Eco\ RI–Pst\ I\) restriction fragment encoding from amino acid position 450 to the middle of the carboxy-terminal propeptide of human \(\alpha_2(1)\) collagen and a 1200 base-pair \(Eco\ RI–Hind\ III\) restriction fragment encoding the terminal fragment of the 3’-untranslated region of the human \(\alpha_2(IV)\) collagen chain were subcloned into a Gemini transcription vector (Promega, Madison, WI) and used as a template to obtain antisense and sense riboprobes. Antisense \(^{35}\)S-labeled RNA probes were transcribed by SP6 RNA polymerase on \(Eco\ RI\) linearized DNA templates. Sense RNA probes were generated by T7 RNA polymerase on DNA templates linearized with Hind III (collagen type IV clone)\(^ {23}\) or \(Pst\ I\) (collagen type I clone).\(^ {22}\) The RNA transcripts were labeled with \(^{35}\)S-adenosine triphosphate (specific activity, 1000–1500 Ci/mMol; New England Nuclear, Boston, MA). The transcription reactions were done under conditions suggested by the manufacturer. Both antisense and sense RNA probes were labeled to a specific activity of 3–5 \(\times 10^7\) cpm/\(\mu\)g RNA.

In Situ Hybridization

Before hybridization, the sections were incubated in 1 \(\mu\)g/ml Proteinase K (Sigma, St. Louis, MO), 0.1 M Tris (pH 8), and 50 mM ethylenediaminetetraacetic acid (EDTA) at 37°C for 30 min to improve the accessibility of the radiolabeled hybridization probe.\(^ {21}\) The slides were immersed in 4% paraformaldehyde for 5 min to arrest proteolysis and then washed three times in PBS–glycine for 5 min each. They then were immersed in freshly prepared 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8) for 10 min to reduce the background of the autoradiographs\(^ {21}\) and then were washed in twice-concentrated SSC, dehydrated in a graded series of ethanol, and air dried.

Hybridization was done in a mixture containing 50% deionized formamide, single-concentrated Denhardt’s solution, 10 mM dithiothreitol (DTT) yeast tRNA (100 ng/ml), and 10% dextran sulfate.\(^ {24}\) The hybridization mixture (20 \(\mu\)l/slide) containing 2–3 \(\times 10^6\) cpm of labeled RNA probe was applied to the tissue sections, which were covered with siliconized cover slips and incubated in a closed moist chamber at 42°C for 15–18 hr to allow hybridization. After hybridization, the cover slips were removed gently in twice-concentrated SSC at 37°C, and then the slides were washed in 50% deionized formamide, twice-concentrated SSC, and 10 mM DTT at 37°C for 30 min, followed by washing in fourfold-concentrated SSC containing 10 mM Tris and 1 mM EDTA at 37°C for 15 min. The slides then were treated with RNase A (50 \(\mu\)g/ml; Sigma) in fourfold-concentrated SSC at 37°C for 30 min. After RNase A digestion, they were washed in fourfold-concentrated SSC at 37°C for 30 min; then in 50% formamide, twice-concentrated SSC, and 10 mM DTT at 65°C for 45 min; and finally in twice-concentrated SSC to bring the mixture to room temperature. The slides were dehydrated in serial graded ethanol and allowed to dry overnight.
Later the slides were dipped in NTB-2 autoradiographic emulsion (Kodak, Rochester, NY), diluted 1:1, dried, and exposed in the dark at 4°C for 7-21 days. They were developed in D-19 (Kodak), diluted 1:1, at 19°C for 6 min, and then fixed and stained with hematoxylin and eosin. The autoradiographs were viewed under bright- and dark-field illumination and photographed using a Nikon Optiphot photographic microscope (Garden City, NY).

All specimens were hybridized with both probes, and at least two slides containing three sections each of each specimen were examined for each exposure time (7, 10, 12, and 21 days). Longer exposures showed better cellular labeling but increased tissue background. When examining the distribution of labeled cells in optic nerve heads from different specimens, similar regions of the nerve were analyzed. The specificity of the hybridization was tested using 35S-labeled probes in the sense orientation on slides processed in parallel to the antisense probes under the same hybridization and washing conditions.

**Results**

Figure 1 shows matching dark- and bright-field micrographs of sagittal sections of the optic nerve head at the level of the lamina cribrosa hybridized to antisense RNA probe for collagen type IV (Figs. 1A–B) and to a corresponding sense RNA probe used as a control (Figs. 1C–D). The intensity of the hybridization signal, as evidenced by the high density of silver grains localized to cells and blood vessels of the connective tissue of the lamina cribrosa, was apparent using the collagen type IV antisense RNA probe. In contrast, little or no hybridization, as evidenced by a low density of silver grains representing the background level and nonspecific binding, was observed using the corresponding sense riboprobe.
At a higher magnification, cells labeled with collagen type IV antisense RNA probe were localized in the core of the cribriform plates of the lamina cribrosa and lined the interface between the connective tissue and nerve bundles (Fig. 2). Vascular endothelia also were labeled throughout the lamina cribrosa and could be identified distinctly from interstitial cells. In fetal eyes, few cells expressing mRNA for collagen type IV were present in the cribriform plates; most labeled cells were associated with blood vessels in the plates (Fig. 3).

In the prelaminar region of adult eyes, clusters of labeled cells were present in glial columns (Fig. 4). Glial cells lining the pial septa in the myelinated nerve were labeled specifically; whereas, fibroblasts inside the septa did not hybridize the probe (Fig. 5A). Cells lining the arachnoid membrane also were labeled (Fig. 5B). As an internal positive control for collagen type IV mRNA expression, silver grains were localized to blood vessels throughout the optic nerve head in fetal and adult eyes. As an internal negative control, silver grains were not localized to fibroblasts of the sclera (Fig. 5C).

Fibroblasts labeled with collagen type I antisense RNA probe were observed in the sclera adjacent to the optic nerve head in adult and fetal tissue (Figs. 6A, 6C). Labeled fibroblasts were more numerous in fetal than adult scleral tissue. No labeling, shown by lack of silver grain localization over fibroblasts, was seen in sections hybridized with the corresponding sense RNA probe (Figs. 6B, 6D).

In the adult lamina cribrosa, cells inside the core and also those lining the cribriform plates hybridized the antisense collagen type I RNA probe (Fig. 7A). Few labeled cells were seen in the fetal lamina cribrosa (Fig. 7B). In the prelaminar region of the adult optic nerve head, hybridization of collagen type I antisense RNA probe was seen occasionally in cells of the glial columns (Fig. 8A). Similarly, only a few cells in the pial septa showed labeling with this probe (Fig. 8B). No labeling of specific cell types was seen when sections were hybridized with control sense RNA probe (Figs. 8C–D). Specific hybridization of collagen type I antisense RNA probe was observed sporadically in the vascular wall throughout the fetal and adult optic nerve head. Because collagen type I mRNA is expressed minimally by vascular endothelial cells, the label probably was associated with pericytes or smooth muscle cells.
Fig. 4. Dark- and bright-field micrographs of a cross-sectional view of the prelaminar region hybridized to $^{35}$S-antisense RNA probe for collagen type IV in a 42-year-old eye. Arrows show cluster of labeled cells in the glial columns (GC) at 12 days of exposure ($\times 450$). N = nerve bundles.

Fig. 5. In situ hybridization of $^{35}$S-antisense collagen type IV RNA probe to sections of the human optic nerve. Postlaminar region (A). Glial cells (arrowheads) lining the pial septa express collagen type IV mRNA; fibroblasts (F) inside the septa (PS) show no hybridization to this probe in 87-year-old eye at 10 days of exposure ($\times 530$). Arachnoid membrane (B). Mesothelial cells lining the arachnoid trabeculae show positive hybridization in a 42-year-old eye at 21 days of exposure ($\times 420$). Serving as a negative internal control, fibroblasts of the sclera adjacent to the optic nerve head show no hybridization to this probe in a 56-year-old eye at 12 days of exposure ($\times 530$) (C).
In general, few, if any, silver grains were present over the nerve bundles as detected by both antisense RNAs probes in this study. Hybridization was not apparent in the axons or glial cells inside nerve bundles. This finding is consistent with the absence of mRNA in axons and the absence of collagen types I and IV inside the nerve bundles in the human optic nerve.8

Discussion

In situ hybridization is a technique that identifies cells expressing mRNA for a specific protein, thus indicating the expression of a particular gene in tissue containing those cells.13,14 Regarding extracellular matrix collagens, translation of mRNA, in most cases, correlates with steady-state mRNA levels and with the protein synthesis rate.26 Thus, this technique of demonstrating gene expression may be useful for localizing specific cells in a tissue that are responsible for synthesizing extracellular matrix collagens.

We used in situ hybridization to identify cells that have mRNAs for collagens type I and IV in human optic nerve heads. Because these studies were done in...
fixed-tissue sections, the anatomic structure of the optic nerve head was preserved. Sectioning the tissue allowed us to determine the presence of specific mRNAs in individual optic nerve heads.

In our study, mRNAs encoding for collagen types I and IV were found throughout the human optic nerve head. The distribution of the labeled cells, presumably synthesizing collagen types I and IV, paralleled the localization of extracellular matrix proteins as previously described in ultrastructural and immunocytochemical studies.

In the prelaminar region, cells clustered in glial columns expressed mRNA for collagen type IV, which is a component of the basement membrane and is associated with blood vessels in the glial columns. Cells labeled in this study by in situ hybridization that presumably synthesize collagen type IV may be endothelial cells, pericytes, and/or astroglial cells surrounding the vessels. Collagen type I mRNA was found in a few cells in the glial columns; this was consistent with the presence of small amounts of collagen type I around blood vessels in the glial columns.

By immunocytochemistry, collagen type IV was present in basement membranes lining the cribiform plates and also formed a network of fibrillar material inside these plates in adult eyes. Our previous work showed that collagen type IV increased with age inside the cribiform plates. In the lamina cribrosa, collagen type IV mRNA was found in astroglial cells lining the cribiform plates and in cells inside the core.
of the plates. These results suggest that age-related accumulation of collagen type IV in cribriform plates is due to its continuous synthesis throughout life.

Collagen type IV mRNA was not abundant in cells of fetal optic nerve heads. Only cells associated with blood vessels were labeled clearly; very few cells in the cribriform plates were labeled. In humans, the precise age when basement membranes appear in the optic nerve is unknown. However, previous microscopic observations indicate that the lamina cribrosa is fully formed at 21 weeks of gestation and that the vascular connective tissue contributes to the development of the connective tissue of the cribriform plates. The expression of mRNA for collagen type IV preferentially in blood vessels throughout the lamina cribrosa in fetal eyes is consistent with this suggestion.

Our previous work and that of others showed that collagen type I is not abundant in the optic nerve head at birth but increases with age as the connective tissue of the lamina cribrosa increases. Our results consistently demonstrated mRNA expression for collagen type I in many cells of the cribriform plates in adult eyes but in only a few cells in fetal eyes. Thus, the accumulation of collagen type I with age is apparently a result of biosynthesis in adult tissue. By contrast, many cells expressed mRNA for collagen type I in the fetal and adult sclera; collagen type I is the major collagen type in this fibrous tissue.

Using in situ hybridization, sufficiently detectable mRNA in human tissues obtained at autopsy and fixed within 5 hr of death, was reported. Our observations suggest that in situ hybridization can be used effectively to investigate age-related and glaucomatous changes in the biosynthesis of extracellular matrix macromolecules in the lamina cribrosa. Future studies will use fresh tissue and archival paraffin blocks of normal and pathologic optic nerve heads to determine the gene expression of extracellular matrix macromolecules or other proteins.

Key words: collagen, mRNA, optic nerve head, in situ hybridization, human, glaucoma

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References


