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Distribution and Expression of Transforming Growth Factor-β and Platelet-derived Growth Factor in the Normal and Glaucomatous Monkey Optic Nerve Heads

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Purpose: Remodeling of the extracellular matrix occurs in the lamina cribrosa in progressed glaucomatous optic nerve damage including disc cupping. We examined immunohistochemical changes in the transforming growth factor (TGF)-β and platelet-derived growth factor (PDGF) in the optic nerve head in an experimentally induced glaucoma model.

Methods: We used 3 cynomolgus and 2 Japanese monkey eyes. Glaucoma was induced by repeated argon laser photocoagulation of the chamber angle. Eyes were enucleated after disc cupping had formed 3 to 5 months after photocoagulation. The optic nerve head was examined for expression of TGF-β1, -β2 and -β3 and PDGF-A and -B in frozen sections and by the biotin ExtraAvidin-alkali phosphatase method.

Results: Normal monkey eyes showed TGF-β1, -β2 and -β3, and PDGF-A and -B in the optic nerve head including the nerve fibers, glial cells, and vascular cells. Glaucomatous eyes showed stronger expression of TGF-β1 and -β2 in the glial cells around the lamina cribrosa. The staining intensities for TGF-β3, PDGF-A and -B were the same as in normal eyes.

Conclusions: Eyes with experimental glaucoma showed higher expression of TGF-β1 and -β2 around the lamina cribrosa. These findings may show upregulation of extracellular matrix production as related to remodeling of the lamina cribrosa in glaucoma. **Jpn J Ophthalmol 2001;45:592–599** © 2001 Japanese Ophthalmological Society

Key Words: Glaucoma, growth factor, optic nerve head, platelet-derived growth factor, transforming growth factor-β.

Introduction

Glaucomatous optic nerve damage has been postulated to be induced in the optic nerve head, particularly around the lamina cribrosa.[1,2] The lamina cribrosa is a connective tissue that supports optic nerve fibers both mechanically and functionally.[3,4] Previous investigations over approximately 15 years clarified many phenomena which occur in the glaucomatous lamina cribrosa.[5–21] This tissue is mainly composed of extracellular matrix (ECM), including collagen fibers, elastic fibers, and proteoglycans. The phenomena seen in the glaucomatous lamina cribrosa have been recognized as remodeling of the ECM.[5,10,13–21] This suggests that continuous destruction, followed by regeneration, should cause backward bowing of the tissue. Electron microscopic examinations showed destructive collagenous lamellae[3,14] without effective association of elastic fibers[14–16] or sulfated proteoglycans[10] in the glaucomatous eyes. In contrast, while the human optic nerve head expressed higher mRNA of collagens and elastin,[19,20] metalloproteinases (MMP) were activated in primate models.[21] The metabolism of ECM should be regulated by many mechanisms, such as a balance of MMP and tissue inhibitors of MMP (TIMP) or a complex cascade of cytokines and

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growth factors.[22,23] Transforming growth factor (TGF)-β and platelet-derived growth factor (PDGF) are the typical growth factors related to ECM metabolism.[22,24,25] In this study, we examined the distribution and expression of TGF-β and PDGF in the optic nerve head using the experimental monkey model of glaucoma to evaluate whether growth factors could be related to the progression of the optic disc cupping and nerve damage in glaucoma.

Materials and Methods

Five eyes from 5 normal adult monkeys (3 cynomolgus and 2 Japanese monkeys) were treated with an argon laser to induce experimental glaucoma as described previously.[8,10,14] The 3 cynomolgus monkeys were approximately 3 years old, but the age of the Japanese monkeys was unknown. The fellow eyes were used as normal controls. All animals were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Resolution on the Use of Animals in Research. The laser treatments were repeated weekly for 3 to 5 weeks. The intraocular pressure (IOP) of laser-treated eyes increased from 25 to 45 mm Hg after repeated laser treatments, and that of normal eyes remained at 15 to 21 mm Hg.

At 3 to 5 months after the initial IOP elevation, the monkeys were sacrificed with an overdose of intravenous pentobarbital and intramuscular ketamine hydrochloride. Soon after *enucleation*, the bilateral optic nerve heads were carefully dissected and rinsed in 0.01

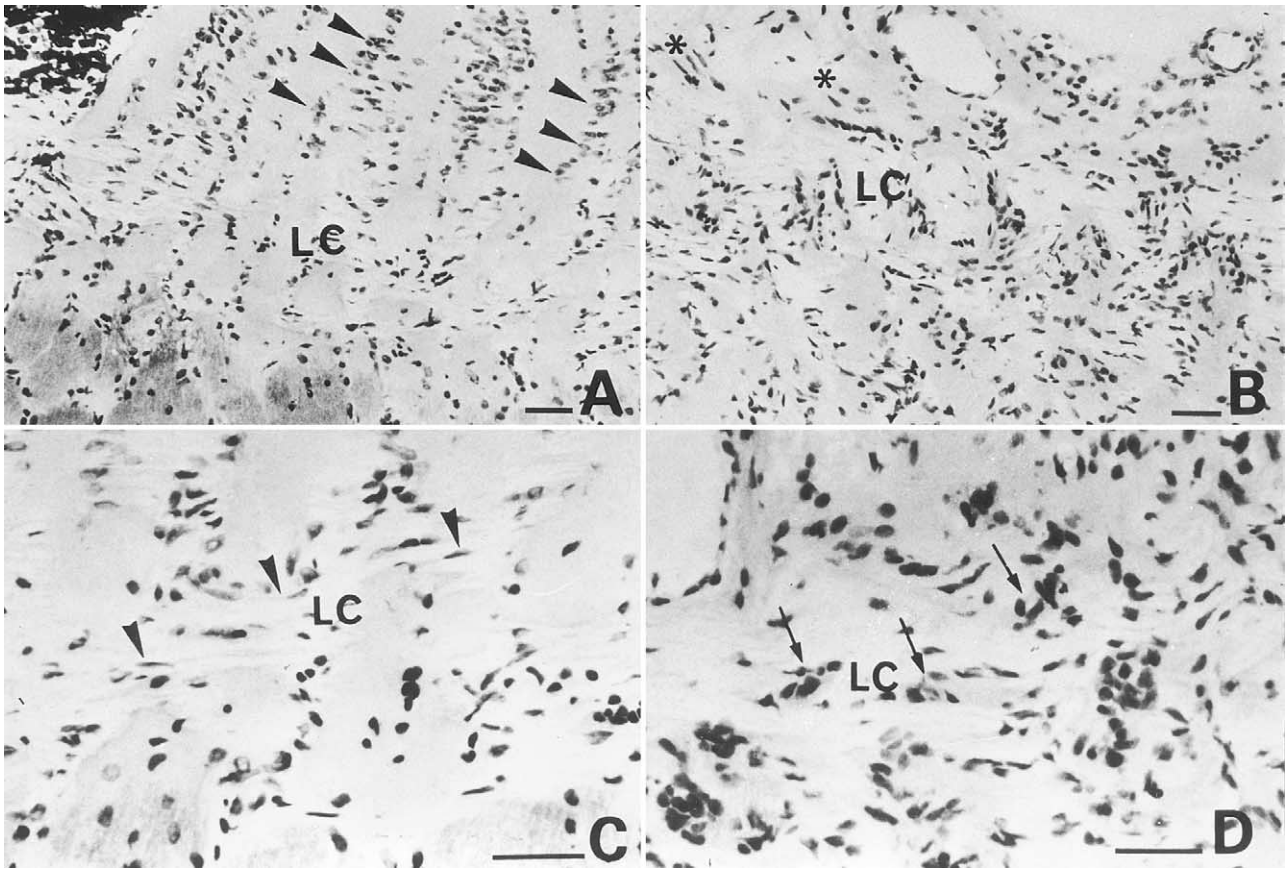

Figure 1. Histological sections of optic nerve heads from normal (**A**, **C**) and experimental glaucoma (**B**, **D**) eyes of monkeys. Nuclear staining by Meyer's hematoxylin. (**A**) In prelaminar portion, glial cells, probably astrocytes, are regularly arranged making glial columns (arrowheads) in normal optic nerve heads. Lamina cribrosa and sclera are connected to each other. Nonmyelinated optic nerve fibers extend to myelinated nerve fibers in this area. (**B**) Disarrangement of glial cells is marked in prelaminar tissues from experimental glaucoma eyes. Number of glial cells increased in lamina cribrosa but decreased in prelaminar area (*). (**C**) High power view shows that the laminar beams are arranged crossing scleral canal in normal lamina cribrosa. On the surface of laminar beams, flat cell nuclei, probably of astrocytes, are shown (arrowheads). (D) Glaucomatous lamina cribrosa became thicker and more circuitous. Glial cells around lamina cribrosa increased in number and are irregularly arranged (arrows). LC: lamina cribrosa. Bar = 100 μm.

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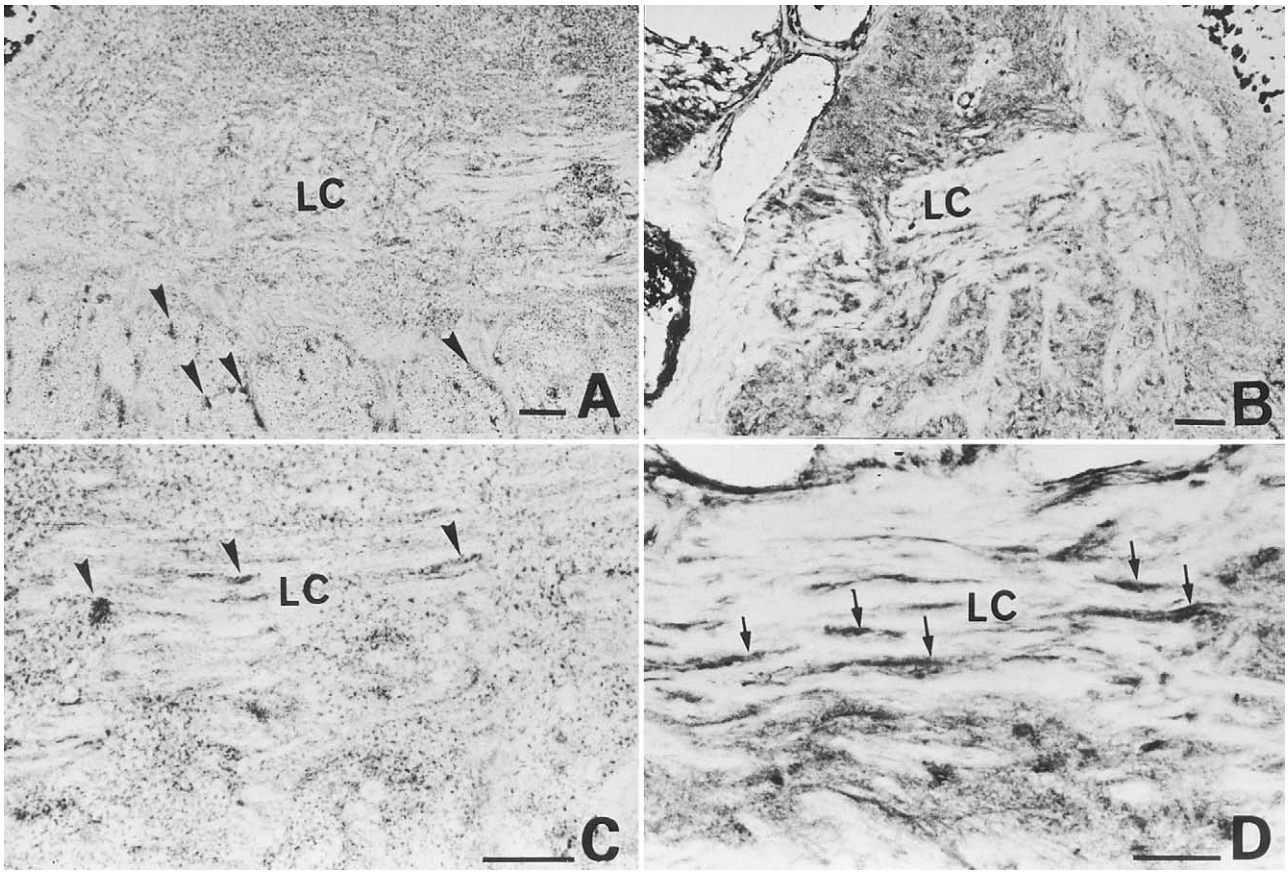

Figure 2. Immunostaining of transforming growth factor (TGF)-β1 in optic nerve heads from normal (**A**, **C**) and experimental glaucoma eyes (**B**, **D**) of monkeys. (**A**) TGF-β1 expressed diffusely in optic nerve fibers and glial cells in normal optic nerve heads. Some glial cells are strongly stained in postlaminar area (arrowheads). (**B**) Entire optic nerve head shows higher expression of TGF-β 1 in experimental glaucoma eyes, particularly in lamina cribrosa. (**C**) Normal lamina cribrosa contains some glial cells expressing TGF-β 1 (arrowheads) as well as nerve fibers diffusely stained. (**D**) Cells expressing TGF-β 1 are increased in number and in staining intensity around lamina cribrosa (arrows) in experimental glaucoma eyes. LC: lamina cribrosa. Bar = 100 μm.

M phosphate-buffered saline (PBS; pH 7.2). Small pieces of the optic nerve heads were embedded in OCT compound and a plastic embedding capsule and were then flash-frozen in dry-ice isopentane. Eight-micrometer-thick frozen sections were cut at −20°C with a microtome-cryostat. They were kept in the freezer at −70°C until the experiment was ready to begin.

Immunohistochemical staining was performed using a biotin-ExtraAvidin-alkali phosphatase method (Sigma, St. Louis, MO, USA). Frozen sections were fixed in 4% paraformaldehyde in 0.01 M PBS at 4°C for 15 minutes, followed by washing in 0.01 M PBS (pH 7.2) three times. After incubation for 30 minutes at room temperature in 10% normal goat serum to reduce nonspecific background, slides were allowed to react for 16 hours at 4°C with one of the following antibodies: rabbit anti-human TGF-β1 (1:100), rabbit anti-human TGF-β2 (1:100), rabbit anti-human TGF-β3 (1:100), rabbit anti-human PDGF-A (1:100), rabbit anti-human PDGF-B (1:100). All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The specificity of each primary antibody was confirmed by Western blotting. As negative control, some sections were incubated with nonimmune rabbit immunoglobulin G (IgG; Santa Cruz Biotech) with the same dilution as each primary antibody. All were diluted with 1% bovine serum albumin (BSA) containing 0.01 M PBS (1% BSA/PBS). Sections were washed with PBS and overlaid with biotinylated anti-rabbit IgG (Sigma) for 90 minutes diluted 1:50 in 1% BSA/PBS. Then slides were exposed to biotin-ExtraAvidin-alkali phosphatase (Sigma) mixed 1:50 in 1% BSA/PBS for 90 minutes. After washing with PBS twice and 0.01 M Tris-HCl buffer (pH 7.4) once, sections were developed for 5 to 20 minutes in Fast-Red TR/Naphthol AS-MX (Sigma) in 10 mM

Tris-HCl buffer (pH 7.2), and washed with Tris-HCl buffer and distilled water. To reduce nonspecific reaction against endogenous alkali phosphatase, we added Revamisol (Vector, Burlingame, CA, USA) into Tris buffer for developing solution. Sections were mounted with mounting media, "Crystal Mount" (Biomeda, Foster, CA, USA) and examined under a light microscope. Another section from each specimen was stained with Meyer's hematoxylin to observe the histological changes in the optic nerve head.

Results

The optic nerve head tissues both from normal and experimental glaucoma monkey eyes showed intense immunostaining for TGF-β1, -β2, -β3 PDGF-A and -B. Capillaries in the postlaminar and retrobulbar optic nerve were stained a little in the sections treated with rabbit nonimmune IgG as negative controls.

In the normal monkey optic nerve heads, positive staining for TGF-β1, -β2, -β3 were detectable along the nonmyelinated nerve fibers in the prelaminar portion, glial columns, astrocytes around the lamina cribrosa, glial cells in the postlaminar portion and vascular systems. The thick and disarranged lamina cribrosa as well as the destroyed glial columns and the loss of prelaminar tissues were noted in the experimental glaucoma eyes (Figure 1). Optic nerve fibers and glial cells were stained against the antibody for TGF-β1 in the normal optic nerve heads. Some glial cells showed marked staining in the postlaminar portion (Figures 2A, C). By high magnification view,

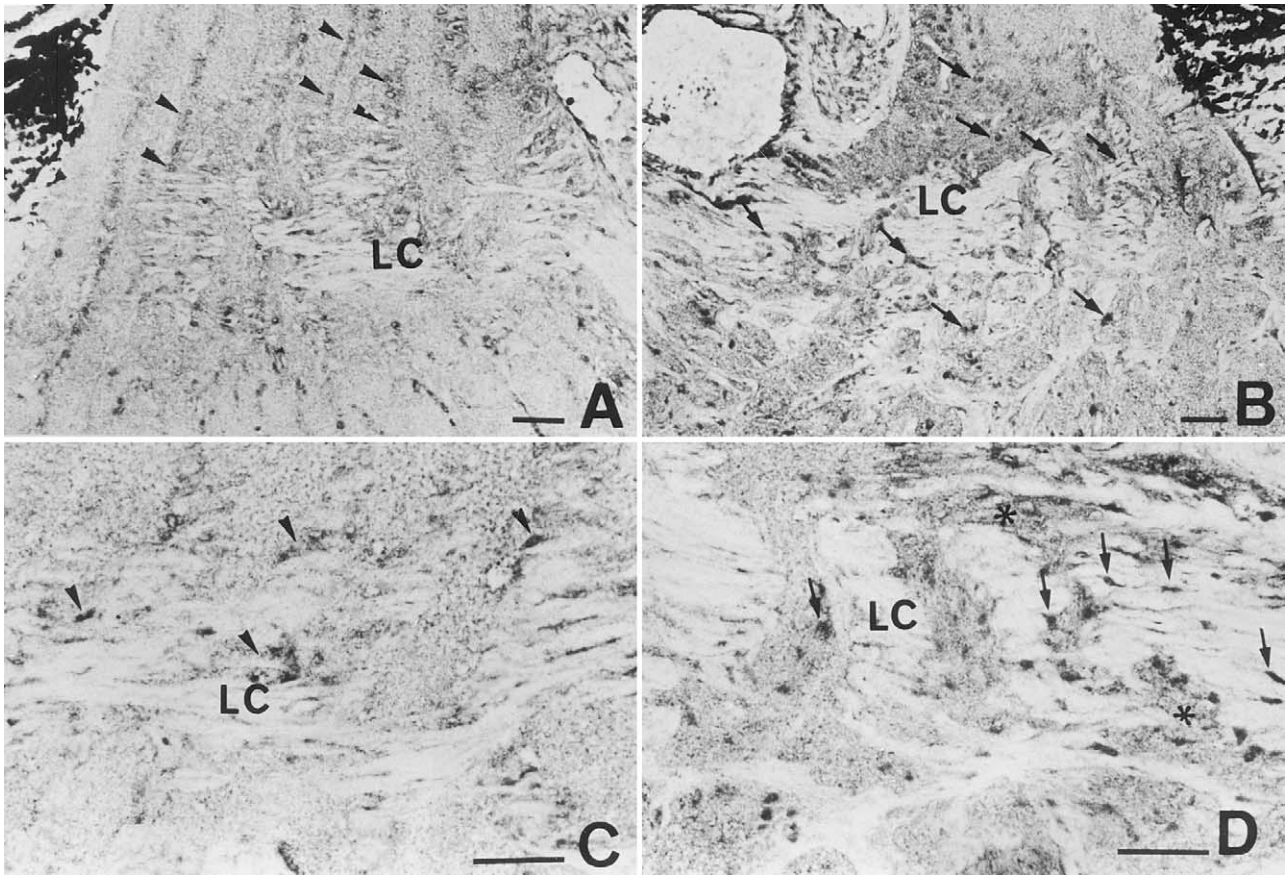

Figure 3. Immunostaining of transforming growth factor (TGF)-β2 in optic nerve heads of normal (**A**, **C**) and experimental glaucoma eyes (**B**, **D**) of monkeys. (**A**) TGF-β2 is expressed diffusely both in optic nerve fibers and glial cells in normal optic nerve heads. Glial cells, particularly astrocytes, in prelaminar and lamina cribrosa areas are stained intensely compared with those expressing in TGF-β1 (arrowheads). (**B**) Experimental glaucoma eye shows obvious morphological changes in lamina cribrosa covered by dense immunostaining for TGF-β2. Many glial cells express TGF-β2 from the prelaminar to postlaminar areas (arrows). (**C**) Normal lamina cribrosa shows some glial cells expressing TGF-β1 within and on surface of laminar beams (arrowheads) as well as diffuse staining in optic nerve fibers. (**D**) In experimental eyes, diffuse staining of optic nerve fibers is stronger than that in normal eyes in lamina cribrosa (*). Cells with expression of TGF-β2 increased in number and in staining intensity around lamina cribrosa (arrows) with experimental glaucoma. LC: lamina cribrosa. Bar = 100 μm.

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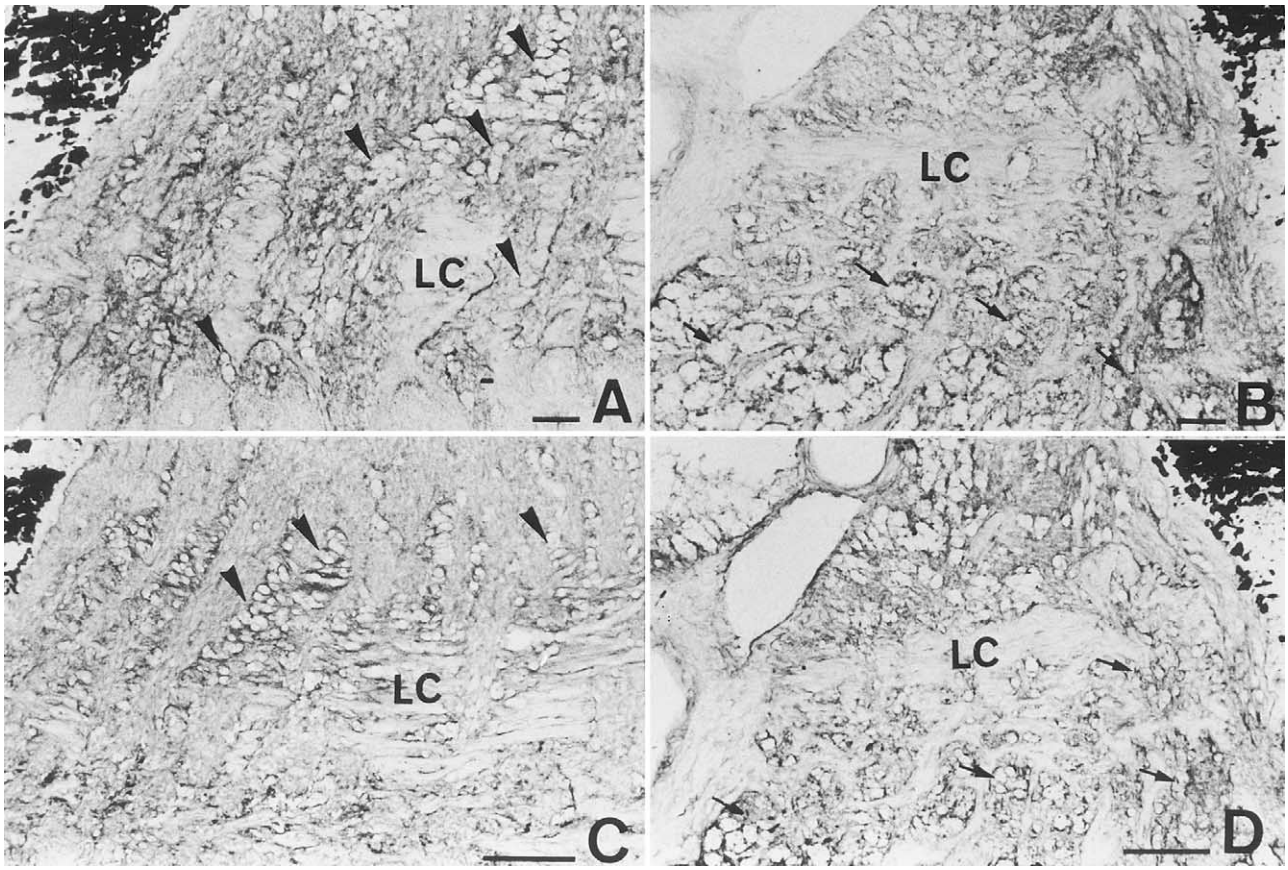

Figure 4. Immunostaining of platelet-derived growth factor (PDGF)-A (**A**, **B**) and PDGF-B (**C**, **D**) in optic nerve heads of normal (**A**, **C**) and experimental glaucoma eyes (**B**, **D**) of monkeys (**A**) PDGF-A expressed diffusely both in optic nerve fibers and glial cells in normal optic nerve heads. Staining for glial cells shows ring-like pattern which suggests PDGF distributed around cell membranes (arrowheads). (**B**) In experimental glaucoma eyes, PDGF is also distributed diffusely in optic nerve heads. Marked change in lamina cribrosa as well as glial cell proliferation in the postlaminar portion are observed with glaucoma (arrows). While expression of PDGF-A in normal and glaucomatous eyes are similar to each other, generally, staining in prelaminar portion was slightly weaker in glaucomatous eyes than in normal eyes. Normal (**C**) and experimental (**D**) eyes show PDGF-B in optic nerve heads. Distribution and staining intensity are similar to PDGF-A. In addition, staining for PDGF-B was also similar in normal and glaucomatous eyes. LC: lamina cribrosa. Bar = 100 μm.

the optic nerve fibers showed diffuse staining and there were some strongly stained cells around the lamina cribrosa (Figure 2C). Experimental eyes showed stronger staining for TGF-β1 in almost all the optic nerve head area, especially around the lamina cribrosa. Positive cells for TGF-β1 increased in number and became stronger in staining intensity in the lamina cribrosa in the experimental eyes (Figures 2B, D). TGF-β2 was also positive in the optic nerve fibers and glial cells in the normal optic nerve heads (Figures 3A, C). The staining for TGF-β2 in glial cells particularly in the prelaminar and laminar portions was more significant than that seen for TGF-β1 (Figure 3A). TGF-β2 was expressed diffusely in the optic nerve fibers and glial cells both within and around the lamina cribrosa under examination at high magnification (Figure 3C). The lamina cribrosa in experimental glaucoma eyes showed remarkable changes in morphology associated with the stronger expression of TGF-β2. Many glial cells between the prelaminar and postlaminar portions appeared stronger in TGF-β2 staining (Figure 3B). In addition, while diffuse staining for the nerve fibers become stronger, glial cells around the lamina cribrosa increased both in number and in staining intensity (Figure 3D). Distribution of TGF-β3 in the normal eyes was similar to that of TGF-β2, astrocytes were stained with greater intensity. Experimental eyes did not show any significant differences from normal eyes in staining intensity for TGF-β3.

Normal monkey optic nerve heads showed positive staining for PDGF-A and -B in the nonmyelinated nerve fiber in the retinal nerve fiber layer, the

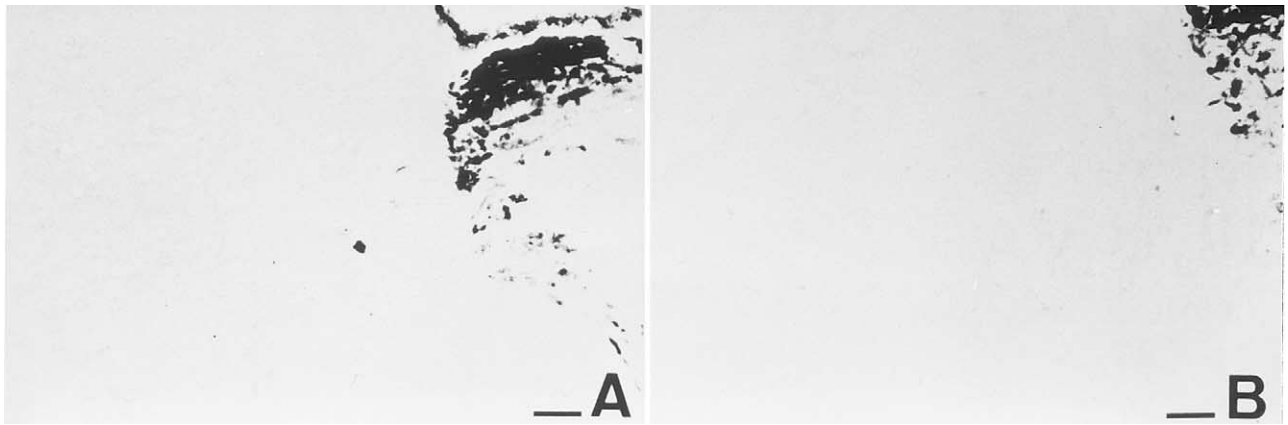

Figure 5. Results from negative controls. Both normal (**A**) and glaucomatous (**B**) optic nerve head tissues showed faint background staining but no significant staining with nonimmune rabbit immunoglobulin G. Bar = 100 μm.

glial columns and the astrocytes around the lamina cribrosa. Myelinated nerve fibers posterior from the postlaminar portion were positive but light in staining. The surface of the lamina cribrosa and glial columns were covered with astrocytes showing remarkable staining. Because PGDF is distributed around the cell membrane, positive staining for glial cells showed a ring-like pattern. Staining for both PDGF-A (Figures 4A, B) and -B (Figures 4C, D) appeared similar between normal and glaucomatous optic nerve heads. In addition, although glaucomatous changes in the optic nerve heads were associated with many proliferative and expanded glial cells posterior from the lamina cribrosa, few if any differences were detectable between normal and glaucomatous optic nerve heads in staining intensity for both PDGF-A and -B (Figures 5A, B). Instead, the tissues in the glaucomatous prelaminar portion tended to be less stained than those in normal eyes.

Staining for both TGF-β and PDGF was positive in the vascular system, including small capillaries in the optic nerve heads, but there was no significant difference between glaucomatous and normal eyes. Finally, all 5 experimental eyes showed similar findings in immunohistochemical staining.

Discussion

In this study, we presented the immunohistochemical changes in TGF-β and PDGF as cytokines or growth factors, which could relate to ECM changes in the glaucomatous optic nerve head. The findings suggested upregulation of TGF-β, at least TGF-β1 and -β2, in the experimental glaucoma eyes. TGF-β3 showed little or no change. In contrast, the staining for PDGF-A and -B decreased, particularly in the prelaminar area.

TGF-β[22–26] is a multi-potential growth factor having a dimmer formation with a molecular weight of 25 kD. The metabolism for ECM is representative of the functions of TGF-β. Generally, TGF-β can promote ECM production and accumulation by upregulating the transcription of collagen, fibronectin, and proteoglycan, and their receptors and integrins, as well as by suppression of MMP release. Three isoforms, TGF-β1, -β2 and -β3, have been identified for TGF-β in mammalian tissues. On the other hand, PDGF[22,23,27,28] also has many biological functions, such as cell proliferation and motility. PDGF increases ECM production by cell proliferation, especially of fibroblasts. Because PDGF forms a dimmer of PDGF-A and -B, PDGF-AA, PDGF-AB, and PDGF-BB are three isoforms of PDGF. In neural tissues, PDGF would regulate cell differentiation of glial cells. Previously, a few investigations presented the changes of growth factors seen in glaucomatous optic nerve heads. By immunohistochemical study Tripathi et al[29] reported upregulation of TGF-β and γ-interferon in human glaucomatous eyes. Taylor et al[30] showed that culture cells from human optic nerve heads could produce TGF-β2 and PDGF-AA biochemically. In addition, Lambert et al[31] detected the gene expression of TGF-β2 and -β3 as well as TGF-β1 and their receptors, TGF-β RI and RII by cultured human lamina cribrosa cells using reverse transcription-polymerase chain reaction (RT-PCR).

The present study has revealed the following. Three isoforms of TGF-β, -β1, -β2, and -β3 were distributed in the monkey optic nerve heads with almost the same patterns. Both TGF-β1 and -β2 were increased in experimental eyes. Although the precise changes in PDGF could not be detected, the primate optic nerve head also included PDGF-A and -B.

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In the progression of optic disc cupping, the lamina cribrosa should move posterior not by simple compression but by repeated destruction followed by regeneration in the ECM. This was shown by the marked residua of basement membrane-like structures[10,14] or immunohistochemical accumulation of type IV collagen[8,11,12] in the prelaminar area with glaucomatous optic disc cupping. Thus, the phenomenon in the glaucomatous eyes should be recognized as "remodeling." [23] Generally, remodeling is a reaction during the late phase in cases of tissue repair. Tissue injury induces an inflammatory reaction immediately upon injury. In contrast, the optic nerve head tissues never show inflammatory cell infiltration during any phase with glaucoma. Moreover, gliosis by astrocytes is very weak in the surface area of the glaucomatous optic disc. These observations are com-pletely different from general tissue injury.[1,5,32] Unfortunately, the reason for these differences has not been clarified, but may be related to the mechanism that forms the characteristic glaucomatous optic disc cupping as well as the mechanism of onset and progression of glaucomatous optic nerve damage. Growth factors or cytokines have various biological activities that influence each other and regulate many tissue reactions. Because growth factors should work primarily in tissue injury or inflammatory reactions, these play important roles in many disorders. For the findings in the present study, increased expression of TGF-β and the absence of alteration of PDGF or a decrease in PDGF would coincide with ECM production and accumulation in the lamina cribrosa and depressed gliosis in the prelaminar area in glaucomatous eyes. Many growth factors have bifunctionality, and influence and act on each other to cause a fine regulation of the tissue reaction. In this study, we could show that the alteration of various growth factors may relate to the formation of the characteristic optic disc cupping in glaucoma. Glial cells, particularly astrocytes in the prelaminar portion and the lamina cribrosa, should be important as cells that cause these alterations in the optic nerve heads. The alteration of astrocytes may play a key role in the progression of glaucomatous optic nerve damage.[33]

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