ORIGINAL ARTICLES—Laboratory science

Transforming growth factor β isoforms in human optic nerve heads

Janethe D O Pena, Andrew W Taylor, Cynthia S Ricard, Ivonne Vidal, M Rosario Hernandez

Abstract

***Aim*—To determine if the isoforms of transforming growth factor β (TGF-β) are present in fetal, normal adult, and glaucomatous optic nerve heads.**

***Methods*—To localise cells synthesising TGF-β, optic nerve heads were stained using antibodies to TGF-β1, TGF-β2, and TGF-β3. To demonstrate synthesis, human optic nerve heads from fetal, glaucomatous, and normal age matched subjects were explanted, cultured overnight, and the culture supernatant was assayed for the presence of TGF-β1 and TGF-β2 by bioassay. In addition, semiquantitative RT-PCR was performed to determine the gene expression pattern of TGF-β2.**

***Results*—Immunohistochemistry of glaucomatous samples revealed the presence of intense staining for TGF-β2 primarily in astrocytes, whereas TGF-β1 was localised to blood vessels. No TGF-β3 immunoreactivity was observed. There was little or no expression of TGF-β in normal optic nerve heads. Optic nerve heads from glaucomatous eyes released 70–100-fold more TGF-β2 than normal age matched optic nerve heads. Fetal optic nerve heads released 90–100-fold more TGF-β2 than normal adult optic nerve heads. TGF-β1 was undetectable by bioassay in all samples tested. There was no apparent increase in TGF-β2 gene expression in glaucomatous and fetal eyes, suggesting post-transcriptional regulatory mechanisms.**

***Conclusions*—These results demonstrate that TGF-β2 is produced in high levels in the fetal and glaucomatous optic nerve heads, perhaps by a mechanism of post-transcriptional regulation. TGF-β may be important during development of the optic nerve head and, in glaucoma, TGF-β2 may be a mediator of astrocyte reactivation and extracellular matrix remodelling in the lamina cribrosa.**

(*Br J Ophthalmol* 1999;**83**:209–218)

Department of Ophthalmology and Visual Sciences, Washington University School of Medicine, St Louis, USA
J D O Pena
C S Ricard
I Vidal*
M R Hernandez

Department of Anatomy and Neurobiology, Washington University School of Medicine, St Louis, USA
M R Hernandez

Schepens Eye Research Institute and the Department of Ophthalmology, Harvard Medical School, Boston, USA
A W Taylor

*Present address:
Department of Neuroscience Graduate Program, University of Pennsylvania, Philadelphia, PA, USA.

Correspondence to:
M Rosario Hernandez, DDS, Department of Ophthalmology and Visual Sciences, Washington University School of Medicine, 660 S Euclid Avenue, Box 8096, St Louis, MO 63110, USA.

Accepted for publication 30 July 1998

Remodelling of the extracellular matrix (ECM) in the optic nerve head, including changes in fibrillar collagens, basement membrane components, and elastin is a morphological feature of glaucomatous optic neuropathy.[1] The changes in the ECM of the optic nerve head are associated with the collapse of the cribriform plates and the cupping of the optic disc. The remodelling of the ECM may contribute, secondarily, to the loss of axons and visual impairment in glaucoma by disrupting the nutritional and mechanical support to the axons from the retinal ganglion cells as they pass through the lamina cribrosa of optic nerve head.

Transforming growth factor β (TGF-β) plays a fundamental role in the biology of extracellular matrix, regulating not only the synthesis and degradation of specific components, but also the ability of cells to interact with ECM macromolecules. Control of these cell-matrix interactions may represent one of the principal mechanisms by which TGF-β affects cell migration, differentiation, and growth in various tissues[2] during normal morphogenesis and wound healing.

Three isoforms, TGF-β1, TGF-β2, and TGF-β3, have been identified in human tissues, in many instances in a tissue specific pattern of distribution. In neural tissues, TGF-β1 and TGF-β2 are expressed preferentially in the central nervous system (CNS); whereas TGF-β1 is the predominant isotype in peripheric nerves.[3] In the eye, TGF-β1 and

Table 1 Clinical data of glaucoma cases

Sample	*Age/ sex*	*Cup/ disc ratio*	*Visual field**	*Experiment†*
1	58/M	0.9	4	IHC
2	74/F	0.9	3	IHC
3	78/M	0.9	3	IHC
4	71/M	0.6	2	IHC
5	51/F	0.9	4	IHC
6	51/F	0.9	4	IHC
7	91/F	0.7	2	IHC
8	67/F	0.5	2	IHC
9	67/F	0.4	2	IHC
10	69/M	NA	2	IHC
11	92/M	NA	2	IHC
12	77/M	0.9	4	BA
13	77/M	0.9	4	BA
14	77/M	NA	2	BA
15	53/M	NA	2	RT-PCR
16	67/F	NA	3	RT-PCR
17	92/M	NA	3	RT-PCR

*Visual field: 1 = no defect; 2 = increasing scotoma; 3 = significant defect; 4 = loss of central field.
†IHC = immunohistochemistry; BA = bioassay; RT-PCR = reverse transcriptase polymerase chain reaction.
NA = information not available.

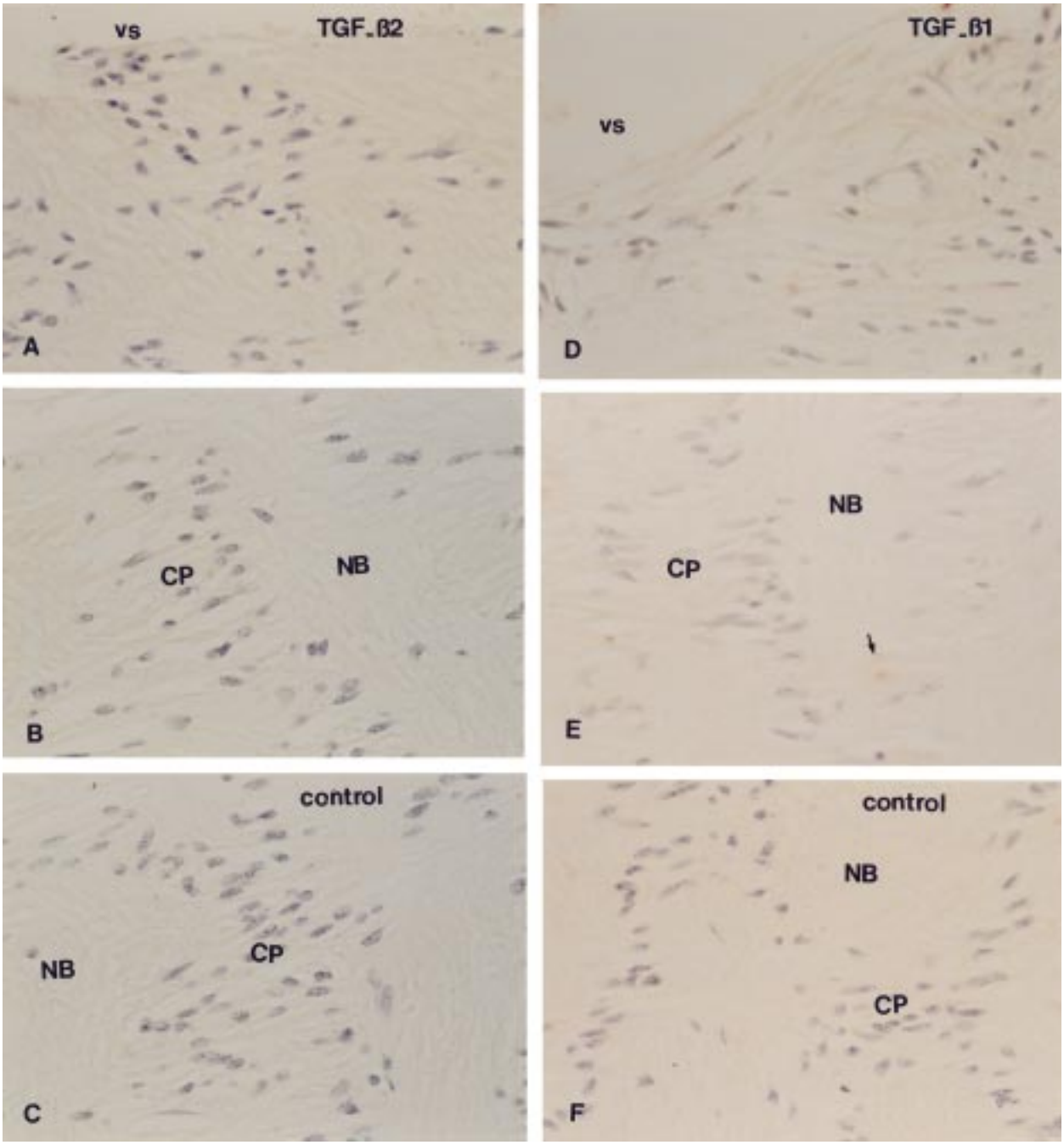

Figure 1 TGF-β1 and TGF-β2 immunoperoxidase staining in normal optic nerve head, donor age 68 years old. Little or no staining for TGF-β2 is observed. Few blood vessels were stained with TGF-β1 (arrow, E). (A) and (D) Prelaminar region; (B) and (E) lamina cribrosa; (C) and (F) negative controls. vs = vitreal surface; CP = cribriform plates, NB = nerve bundles. ×375.

TGF-β2 are present in the anterior segment of human eyes[4] and TGF-β1, TGF-β2, and TGF-β3 have been identified in the retina of humans, monkeys, and cats.[5] In a preliminary report, TGF-β1 has been identified in the postlaminar (myelinated) optic nerve by immunocytochemistry.[6]

Considering the effects of TGF-β on extracellular matrix synthesis and degradation, and the reported presence of TGF-β family members in other compartments of the human eye, we localised the cellular source of TGF-β1, TGF-β2, and TGF-β3 by immunohistochemistry in fetal eyes and glaucomatous and normal age matched adult human tissues. We also investigated TGF-β2 gene expression by relative reverse transcriptase polymerase chain reaction (RT-PCR) and the activity of TGF-β1 and TGF-β2 in explanted fetal, normal adult, and glaucomatous human optic nerve heads.

Materials and methods

IMMUNOHISTOCHEMISTRY FOR TGF-β PROTEIN

Four pairs of human eyes (donor ages 65–81 years old) with no history of eye disease, seven eyes from seven different donors with history of advanced primary open angle glaucoma (donor ages 51–91 years old, samples 1–7 in Table 1), and four eyes from mild primary open angle glaucoma (donor ages 67–92 years

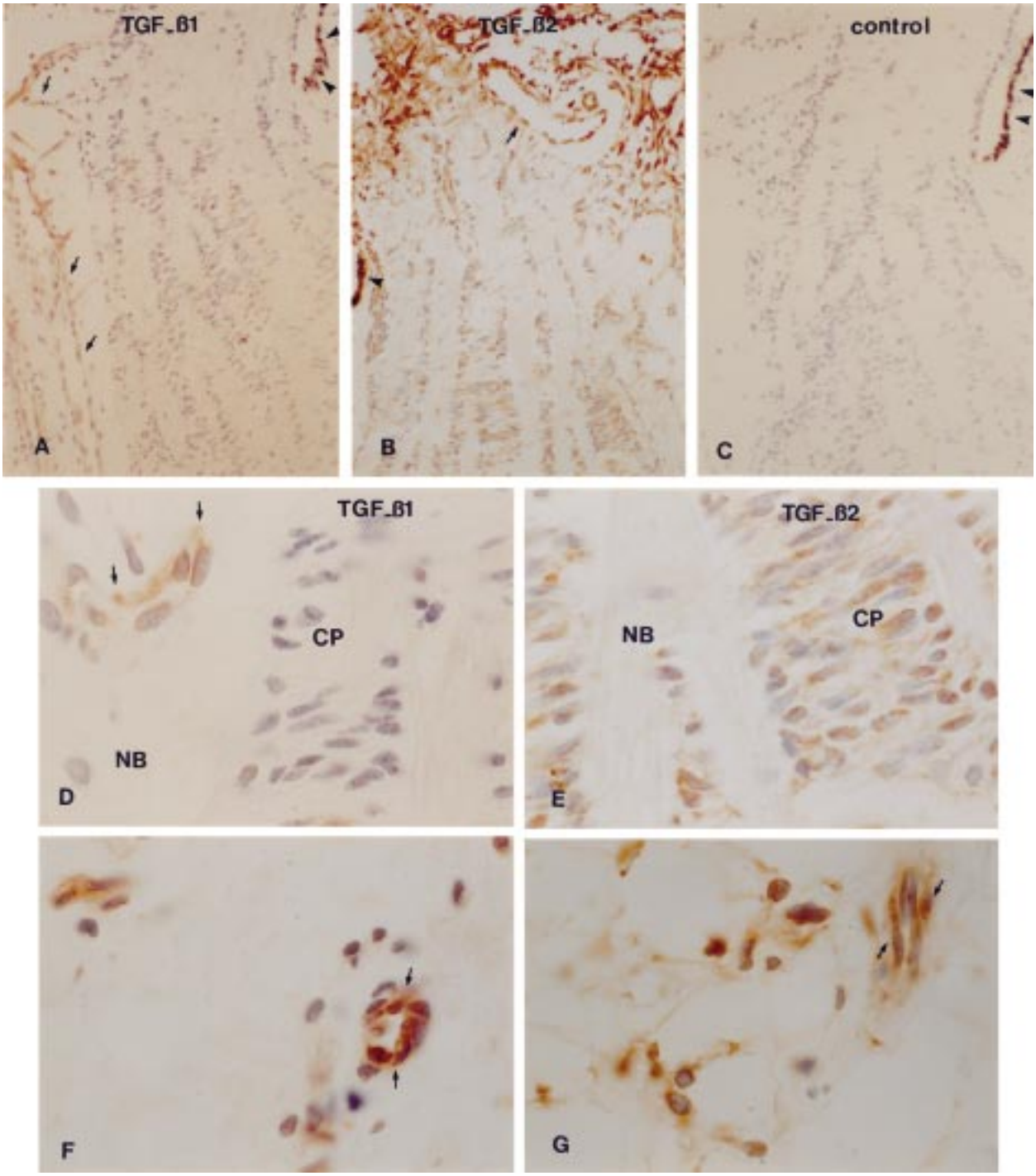

Figure 2 TGF-β1 and TGF-β2 immunoperoxidase staining in fetal optic nerve head (gestational age 24 weeks). TGF-β1 immunoreactivity was restricted to blood vessels throughout the optic nerve head (A, D) and TGF-β2 reactivity was localised to the extracellular matrix and cells in the optic nerve head (B, E). In the sclera, TGF-β1 was detected in association with blood vessels (F) and TGF-β2 was associated with the vessels and fibroblasts (G). (C) Illustrates the negative control. Arrows point to blood vessels and arrowheads point to the retinal pigment epithelium. NB = nerve bundles; CP = cribriform plates. (A–C) ×75; (D–G) ×750.

old, samples 8–11 in Table 1) were obtained from eye banks throughout the United States and from the Glaucoma Research Foundation (San Francisco, CA, USA). Eyes from donors with documented mild to advanced primary open angle glaucoma had cup/disc ratios of 0.4 to 0.9, demonstrated visual field defects and were reportedly treated for glaucoma by ophthalmologists (Table 1). The interval between death and fixation was 2–4 hours for the adult eyes. Three pairs of fetal eyes (gestational ages 18, 20, and 24 weeks) were obtained through the Anatomical Gift Foundation (Laurel, MD, USA). Optic nerve heads from these fetal eyes were fixed immediately after enucleation in 10% buffered formaldehyde for at least 6 hours. The optic nerves were dissected, washed several times in phosphate buffered saline (PBS) containing 0.1% glycine, and processed for paraffin embedding accord-

ing to standard procedures. Sagittal sections of 6 μm were cut and placed on silane coated slides. Before immunostaining, slides were deparaffinised and incubated for 1 hour in PBS glycine at room temperature to reduce non-specific binding. To visualise cells synthesising TGF-β, rabbit polyclonal antibodies with specificity for TGF-β1, TGF-β2, or TGF-β3 were used (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The anti-TGF-β1 antibody recognises an epitope corresponding to amino acids 328–353 at the carboxy terminus of TGF-β1; the anti-TGF-β2 antibody recognises an epitope corresponding to amino acids 352–377 at the carboxy terminal of TGF-β2, and the anti-TGF-β3 antibody recognises an epitope corresponding to amino acids 350–375 at the carboxy terminal of TGF-β3. Corresponding antigenic peptides (Santa Cruz Biotechnology) or recombinant TGF-β1 and TGF-β2 (R&D Systems, Minneapolis, MN, USA) were obtained and used to preabsorb primary antibody for negative controls. These antibodies were purified by peptide affinity chromatography and tested for isoform specificity by the company, and have also been used in previous publications.[7 8] Antibodies were used at 1:200 dilution and all steps of the staining process were carried out at room temperature. Slides were preincubated with 5% milk for 30 minutes, rinsed, and then incubated with primary antibody for 30 minutes. Biotinylated secondary antibody was placed on the sections and incubated for 30 minutes, washed with PBS, and reacted with the streptavidin-peroxidase conjugate (Vector Laboratories, Burlingame, CA, USA) for 30 minutes. After a PBS wash, the bound antibody-peroxidase complexes on the sections were visualised using a DAB substrate solution consisting of 1.5 mg 3,3-diaminobenzidine tetrahydrochloride (DAB) and 50 μl of 30% hydrogen peroxide, in 10 ml of 0.1 M TRIS, pH 7.6. The sections were incubated in the dark until brown staining appeared, at about 5–7 minutes, washed in PBS, counterstained with haematoxylin or nuclear fast red, dehydrated, and coverslipped with Permount. To confirm that the cells synthesising TGF-β2 were astrocytes, sections from glaucomatous eyes (samples 3 and 11 in Table 1) were double stained using a monoclonal antibody against human glial fibrillary acid protein (GFAP; Sigma Chemical, St Louis, MO, USA) and a polyclonal antibody against TGF-β2 (Santa Cruz Biotechnologies) followed by incubation with Rhodamine-Red and Cy-5 labelled secondary antibody mixture (Molecular Probes, Eugene, OR, USA). Slides were examined in a Nikon Optiphot-2 microscope and images were recorded using Kodak Royal Gold 400 ASA colour print film. Visualisation of the GFAP/TGF-β2 co-localisation was done in a Zeiss LSM 410 laser confocal microscope and images were printed using an Epson Stylus photo printer.

Representative sections of all samples were stained simultaneously to control variation in the reactions. Negative controls were performed by absorbing the primary antibody with TGF-β1, TGF-β2, or TGF-β3 peptides (Santa Cruz Biotechnology) or recombinant TGF-β1 and TGF-β2 (R&D Systems) or by replacing the primary antibody with non-immune rabbit serum.

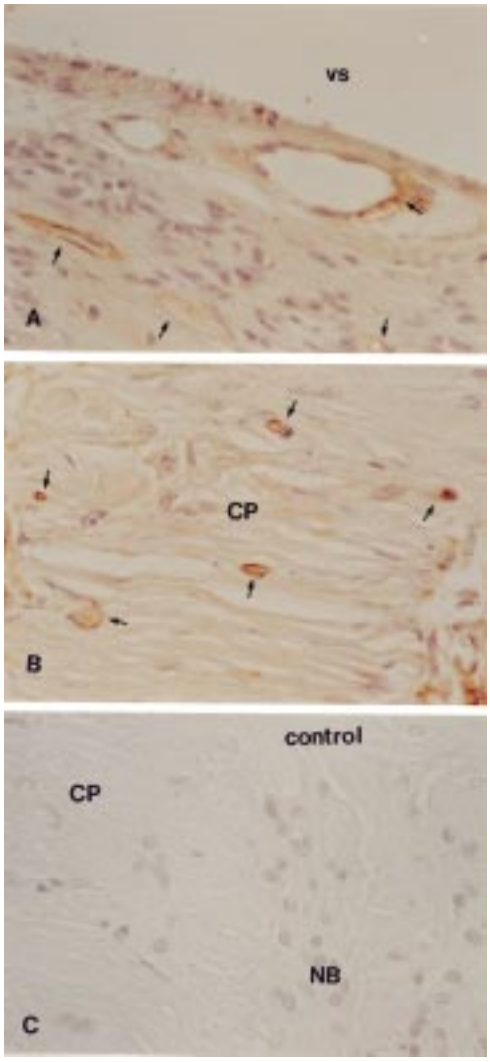

Figure 3 TGF-β1 immunoperoxidase staining in advanced primary open angle glaucoma, donor age 78 years old. Note staining around blood vessels (arrows). (A) Prelaminar region; (B) lamina cribrosa; (C) negative control. vs = vitreal surface; CP = cribriform plates; NB = nerve bundles. ×350.

TGF-β BIOASSAY

Explant preparation

Eight normal human eyes from different individuals (ages 55–85 years old) with no history of eye diseases, and three eyes from two patients with advanced primary open angle glaucoma (age 77 years old, samples 12–14 in Table 1) were used in this study. The eyes were enucleated within 2–6 hours after death, kept at 4°C in a sterile humidified container, and all eyes were processed within 12–18 hours after death. The optic nerves were dissected under a microscope and cleared of surrounding tissues as previously described.[9 10] Each optic nerve head (approximate weight 15 mg) was divided into four pieces and incubated in a 35 mm dish in 0.5 ml of serum free medium, RPMI 1640 media supplemented with a 1/500 dilution of

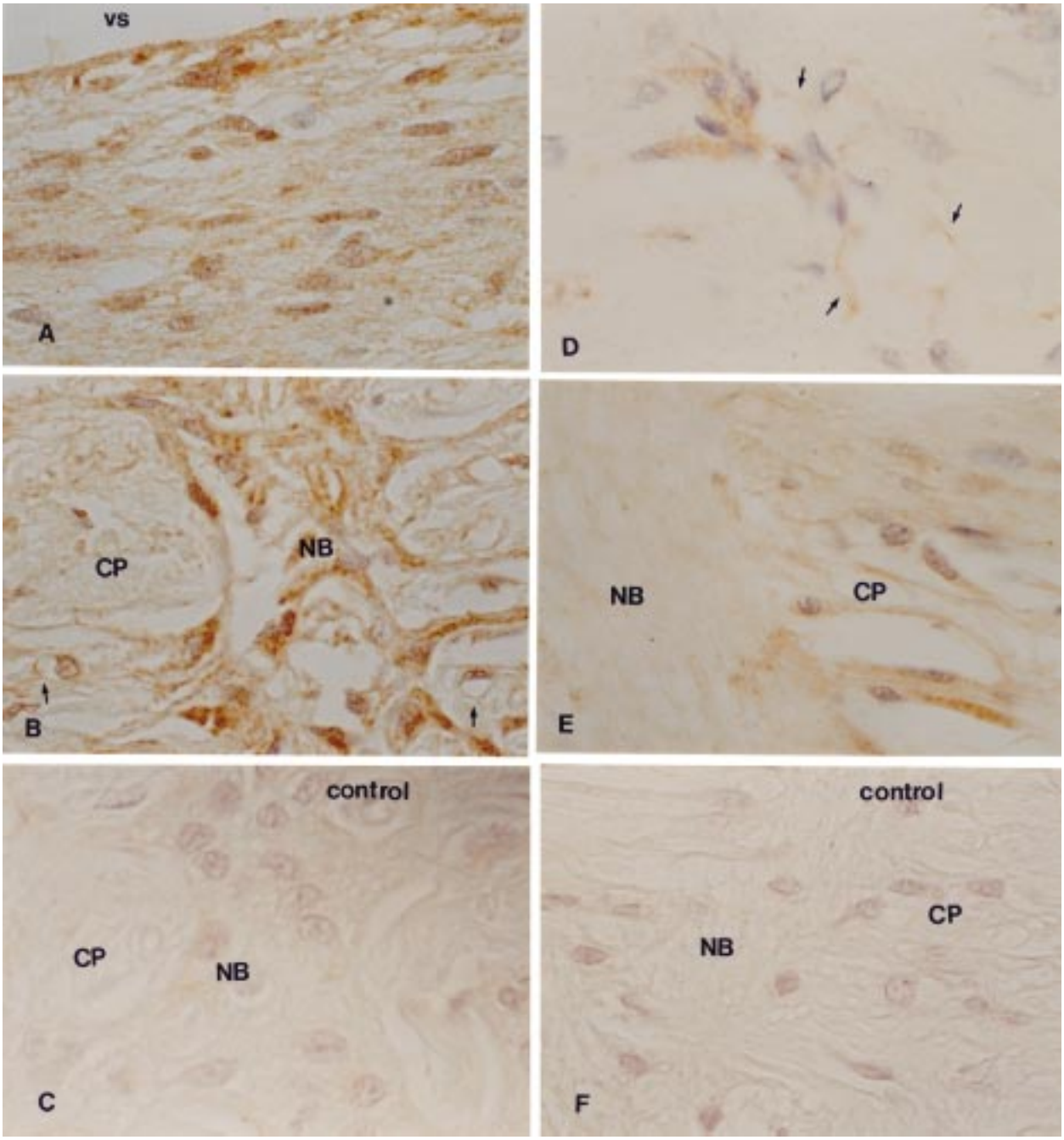

Figure 4 TGF-β2 immunoperoxidase staining in advanced (A, B, and C) and mild (D, E, and F) primary open angle glaucoma, donor ages 74 years old (advanced glaucoma) and 67 years old (mild glaucoma). Note widespread staining of astrocytes and the extracellular matrix throughout the optic nerve head of the sample from with advanced glaucoma (A and B). Reactivity in blood vessels was also noted (arrow). In the mild glaucoma sample, few astrocytes and blood vessels (arrows) were labelled in the prelaminar region (D) and in the lamina cribrosa, with little staining in the extracellular matrix (E). (C) and (F) are negative controls. vs = vitreal surface; NB = nerve bundles; CP = cribriform plates. ×750.

ITS+ (insulin, transferrin, selenium, bovine serum albumin, and linoleic acid) universal culture supplement (Collaborative Biomedical Products, Bedford, MA, USA). In addition to the adult tissues, three pairs of human fetal eyes (ages 18, 20, 22 weeks' gestation) were also used and prepared as above, except that optic nerve heads were left intact and each pair of nerves was combined, so that a similar amount of tissue was used for fetal and adult optic nerves (approximately 15 mg tissue per sample). After 24 hours of incubation at 37°C and 5% carbon dioxide the supernatants were assayed for TGF-β. Cell viability was assessed by culturing the optic nerve explants in EMEM containing 10% fetal bovine serum. Cell outgrowth was observed in all samples used for the bioassays.

Standard Mv1Lu cell assay

To measure total TGF-β, the mink lung epithelial cell (Mv1Lu) bioassay was used. In this study, TGF-β bioassay was used for measuring total TGF-β proteins in the culture supernatants, and because of the acid treatment, it was not possible to distinguish between latent and

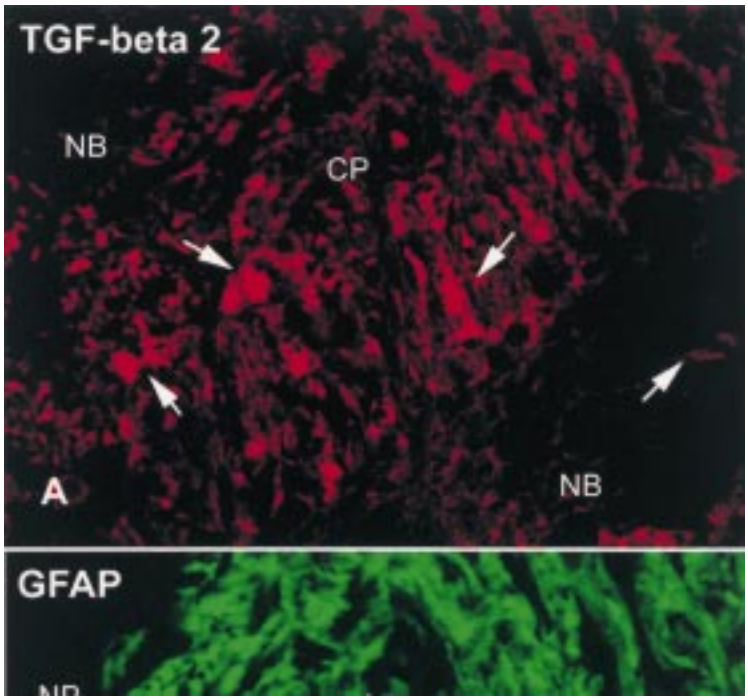

Figure 5 Co-localisation of TGF-β2 and GFAP by immunofluorescence in the lamina cribrosa of a glaucomatous donor (age 92 years old). In (A) immunoreactivity for TGF-β2 is localised to cells inside the cribriform plates (CP) and in the nerve bundles (NB). These cells are also GFAP positive, confirming their identity as astrocytes (B). (C) Illustrates the co-localisation of TGF-β2 and GFAP. Arrows point to some of the cells that show double staining. ×400.

active forms of TGF-β. Specificity was determined by adding neutralising antibodies against specific TGF-β isoforms to the culture supernatants. To perform the assay, supernatants from the optic nerve explants were transiently acidified (pH 2.0) by adding 5 µl of 1 M HCl per 100 µl of supernatant and incubated for 1 hour at 4°C. The acid was neutralised (pH 7.4) by adding 10 µl of a 1:1 (v/v) mixture of 1 M NaOH and 1 M HEPES to the acidic supernatant. To 100 µl of diluted supernatant in the wells of a 96 well flat bottom Falcon plate (Lincoln Park, NJ, USA), 100 µl of 1×10^4 Mv1Lu cells (No CCL64, ATCC) were added in EMEM with 0.5% fetal bovine serum (Hyclone). The cultures were incubated at 37°C for 20 hours and 20 µl of 1 µCi ^{3}H thymidine were added. The explants were then incubated for an additional 4 hours. Incorporated ^{3}H thymidine was measured by scintillation counting as counts per minute (cpm). The amount of TGF-β in the supernatants was determined by comparing cpm of explant supernatant treated cells with a standard curve made from CPM of explants treated with known amounts of TGF-β (R&D Systems). The limits of detection in our assay were between 1 pg/ml and 10 ng/ml. To determine the specific isotype of TGF-β, antisera with neutralising specificity against either human TGF-β1 or TGF-β2 (R&D Systems)[11 12] were used in the TGF-β bioassay. The antibodies were used at 10 times their 50% neutralising dose. The neutralising anti-TGF-β1 was a chicken IgY against recombinant human TGF-β1, used at 10 µg/ml. The neutralising TGF-β2 was a goat anti-porcine TGF-β2 that cross reacts only with TGF-β2 isotypes from mammals, used at 20 µg/ml. The transiently acidified supernatants were incubated with anti-TGF-β1 or anti-TGF-β2 neutralising antibodies for 1 hour at 37°C before being assayed.

RELATIVE RT-PCR FOR TGF-β2

To measure relative levels of TGF-β2 mRNA, total RNA was extracted from minced normal and glaucomatous optic nerve heads (samples 15–17 in Table 1) using TriZol according to manufacturer's recommendations (Gibco/BRL, Gaithesburg, MD, USA) for minimisation of DNA carryover. As positive controls, total RNA was extracted from cultured brain and optic nerve head astrocytes (type 1B astrocytes). For each sample, 1.0 µg total RNA was DNase treated to remove any residual contaminating genomic DNA and reverse transcribed to cDNA with 500 ng of oligo dT and 50 ng random hexamers using Superscript II and kit components (Gibco/BRL) in a 50 µl reaction volume. For PCR, 1 µl of the cDNA was used as template for 25 cycles of amplification in 50 µl reaction volume with either β actin or TGF-β2 amplimers (Clontech). Under these conditions TGF-β2 amplification did not produce PCR products visible by ethidium bromide staining, so a second set of primers was designed for nested PCR. These 20-mer primers were based on sequences immediately adjacent to the 3' ends of the Clontech amplimers in GenBank accession