Exp. Eye Res. (1994) **59**, 323–333

Transforming Growth Factor Beta 2 is the Predominant Isoform in the Neural Retina, Retinal Pigment Epithelium–Choroid and Vitreous of the Monkey Eye

BRUCE A. PFEFFER*§, KATHLEEN C. FLANDERS†, CHRISTOPHER J. GUÉRIN‡, DAVID DANIELPOUR† AND DON H. ANDERSON‡

** Laboratory of Retinal Cell and Molecular Biology, National Eye Institute, NIH, Bethesda, MD 20892, U.S.A., † Laboratory of Chemoprevention, National Cancer Institute, NIH, Bethesda, MD 20892, U.S.A. and ‡ Neuroscience Institute, University of California, Santa Barbara, CA 93106, U.S.A.*

(*Received Seattle 4 January 1994 and accepted in revised form 10 May 1994*)

Several techniques were utilized to assess the levels, disposition and cellular sources of isoforms 1 and 2 of transforming growth factor beta (TGF-β) in the posterior pole of the monkey eye. Freshly dissected tissues, as well as the saline vehicles in which dissections were performed, were analysed by sandwich enzyme-linked immunosorbent assay. In all tissues TGF-β2 was the predominant isoform, with $\beta 2:\beta 1$ ratios of 6:1 for neural retina (as ng g^{-1}) and 425:1 for vitreous (as pmol l^{-1}). Retinal pigment epithelium (RPE)-Bruch's membrane-choroid complex contained approximately 10 times the amount of both TGF-β isoforms as neural retina. For first passage cultures of monkey RPE, TGF-β2, but not TGF-β1, accumulated over time in conditioned media samples. Immunoreactivity for TGF-β2 was detected both in tissue sections of posterior pole, specifically in rod outer segments and RPE, and also in the first passage cultures of RPE. Antibodies to specific peptide sequences of both isoforms localized TGF-β to the outer segments of rod photoreceptors. The apparent sequestration of TGF-β2 in photoreceptor outer segments, as well as the in vitro evidence for possible synthesis and release by RPE, suggest that TGF-β2 is an important modulator of visual function acting at the retina–RPE interface.

Key words: transforming growth factor beta; retinal pigment epithelium; photoreceptor; monkey; immunocytochemistry; epithelial cell culture.

1. Introduction

Several isoforms of transforming growth factor beta (TGF-β) have been described thus far (Roberts et al., 1990). The virtually ubiquitous occurrence of TGF-β in tissues of the body implies that the site-specific actions of this growth factor are profoundly influenced by mechanisms involving secretion, activation of latent forms, extracellular accumulation and gradient formation, and the binding of ligand to various receptors (Wakefield et al., 1988, Lyons and Moses, 1990; Moses et al., 1990; Massagué, Heino and Laiho, 1991; Chen et al., 1993). Such interactions may underlie the participation of TGF-β in morphogenesis, wound healing, extracellular matrix turnover and disease processes (Ignotz and Massagué, 1987; Rizzino, 1988). To date, the most extensively studied isoforms of TGF-β have been isoforms 1 and 2, which share 72% sequence homology, and are distinguished by their biological activities (Daopin et al., 1992; Qian et al., 1992). It has been demonstrated that in eyes from human subjects affected by proliferative vitreoretinopathy, vitreal levels of TGF-β2 are elevated compared to amounts of this growth factor in intraocular fluid aspirates taken from eyes with retinal detachments, but *without* clinical signs of proliferative vitreoretinopathy (Connor et al., 1989). There is, however, little information available on the cellular source(s) of ocular TGF-β2. Production of TGF-β2 by cultured ciliary body epithelial cells (Helbig et al., 1991) and immunohistochemical detection of TGF-β2 in the ciliary epithelia in situ (Pasquale et al., 1993; Peress and Perillo, 1994) implicate these cells as a source of TGF-β2 in the anterior segment of the eye, and in the aqueous in particular (Cousins et al., 1991). The immunohistochemical localization of TGF-β1 and -β2 to photoreceptors (Lutty et al., 1991, 1993) is the first suggestion of a retinal site of TGF-β and invites speculation as to its function in the outer retina.

We have now extended previous studies by quantifying normal endogenous levels of TGF-β types 1 and 2 in the neural retina, in the retinal pigment epithelium–choroid complex, and in the vitreous of normal monkey eyes. Additionally, we demonstrate that TGF-β2 can be detected immunocytochemically in cultured monkey retinal pigment epithelium (RPE) and that RPE in vitro releases the TGF-β2 isoform into the culture medium where it accumulates over time. Finally, we have elucidated the subcellular compartmentalization of TGF-β2 within the photoreceptors.

§ Corresponding author. Present address: Storz Division of American Cyanamid, Building 96, Room 225A, 401 N. Middletown Rd, Pearl River, NY 10965, U.S.A.

0014-4835/94/090323+11 $08.00/0

These results suggest an unrecognized in vivo role for TGF-β2 in retinal homeostasis.

2. Materials and Methods

Isolation of Ocular Tissues and Conditioned Saline for TGF-β Analysis

Ocular tissues utilized for these experiments were obtained from the eyes of rhesus monkeys (*Macaca mulatta*; see Acknowledgements). All animals were killed by exsanguination under deep sodium pentobarbital anaesthesia, in strict accordance with NIH animal research guidelines. Enucleated eyes were transported and stored in cold, modified Hanks balanced saline (HBSS; in mmol l^{-1}: NaCl, 138·5; KCl, 3·5; $NaH_2PO_4 \cdot H_2O$ 1; $CaCl_2 \cdot 2H_2O$, 0·5; $MgCl_2 \cdot 6H_2O$, 0·27; $MgSO_4 \cdot 7H_2O$, 0·37; Hepes, 15; $NaHCO_3$, 4·2; dextrose, 8·3; pH, 7·2) until further processing, which commenced within 0·5 hr of removal of the eyes.

After the globe of each eye was trimmed free of muscle, fat and other extraneous tissues to expose the sclera, the anterior segments were removed by a circumferential incision just posterior to the ora serrata. All liquid vitreous issuing from the inverted eye was cut away, collected in 1-ml portions, and snap-frozen on dry ice. A small amount of residual vitreous remained adherent to the retinal surface. The poles were cut into quadrants through the optic disc, and each set was placed in a separate well of a six-well cluster (Falcon, Becton-Dickinson, Ventura, CA). The tissues were then inundated with 12 ml of HBSS (as above) and incubated at room temperature in the dark for 1·5 hr. After this interval, much of the neural retina in the quadrants had detached from the underlying RPE and the remainder could be coaxed gently from the RPE with fine jeweller's forceps, yielding a cleanly separated piece of neural retina devoid of pigment. Neural retina tissue was harvested in this way from six eyes, rapidly frozen (three retinas per tube) on dry ice, and stored at −60°C for further analysis. The HBSS in which the retinal separation and harvesting took place was retrieved and frozen, as 'BSS-1'. The remaining portions of the quadrants were then transferred to 10 ml of fresh HBSS, and the RPE-Bruch's membrane–choroid (RPEBC) complexes from three eyecups were teased away from the underlying sclera. Approximately 20 to 30 min elapsed during the completion of these dissections. RPEBC tissue representing three eyes was rapidly frozen on dry ice and stored as for the other samples. The HBSS conditioned during the latter dissection (BSS-2) also was frozen and saved for analysis, to control for diffusion or other release of TGF-β into the buffer during the tissue handling and dissection.

Conditioned Media from Cultured Monkey RPE

Confluent first passage cultures of monkey RPE were utilized to generate conditioned media samples. Detailed methodology for establishing the RPE cultures is presented elsewhere (Pfeffer, 1990). Briefly stated, cells were maintained in 12-well clusters (Costar, Cambridge, MA) for 2 weeks in medium with the following formulation: (1) the basal medium was a modification of a 1:1 mixture of Medium 199 and Dulbecco's modified Eagle's medium containing 1·5 g l^{-1} $NaHCO_3$, 4 g l^{-1} Hepes, 1·5 g l^{-1} glucose, 225 mg l^{-1} fructose, and $[Ca^{2+}]$ at 0·5 mmol l^{-1}; (2) bovine calf serum (Hyclone, Logan UT) was reduced to 1% (v/v) and the medium supplemented with the following additions: 0·5% bovine retinal extract (v/v; approximately 1·5 mg protein ml^{-1} in 200× stock), Albumax at 200 mg l^{-1} (a source of fatty acids; Gibco BRL, Gaithersburg, MD), and a variety of hormones, transport factors and trace nutrients, including 5 mg l^{-1} insulin (Collaborative Biomedical, Bedford, MA) and 15 mg l^{-1} transferrin (Boehringer-Mannheim, Indianapolis, IN). Under these conditions the cultured RPE cells can be maintained at confluence as a stable monolayer, exhibiting typical epithelial morphology, that will express a variety of biochemical and physiological markers characteristic of the differentiated tissue in vivo (Pfeffer, 1990). Confluent wells contained 6×10^5 cells each, as determined by hemacytometer counting of parallel cultures.

In order to determine whether TGF-β species were released by RPE cells into conditioned medium, the monolayers were first rinsed three times (1×1 ml $well^{-1}$, $2 \times 0{\cdot}5$ ml $well^{-1}$) with a more defined medium formulation, namely the same medium as described above, but lacking calf serum and retina extract. In this way any traces of pre-existing or exogenously supplied (i.e. medium-derived) growth factors were eliminated, as shown for similarly performed rinses in a previous study of de novo production of insulin-like growth factor and its binding proteins by cultured monkey and human RPE (Waldbillig et al., 1991, 1992). Cultured RPE cells in six wells, with 1 ml of medium per well, were then incubated for 16 hr with defined medium, after which the conditioned medium was pooled and immediately frozen for further analysis. This same group of cultures then underwent a fresh change of defined medium, and the conditioning period was repeated for another 9 hr, followed, again, by harvesting and freezing of the conditioned medium. A final 24-hr incubation was also performed with a separate group of RPE cells (of the same cell strain used for the other time points) in six wells. Freshly made defined medium not exposed to cells served as a zero time point control.

Immunochemical Detection and Measurement of TGF-β

Ocular tissues, including vitreous and saline samples, were acid–ethanol extracted by a modification of the method of Roberts et al. (1980), as described by Danielpour (1993). Briefly, 4·0 ml of cold acid–ethanol solution [93% (v/v) ethanol, 2% con-

centrated HCl, 85 μg ml^{-1} phenylmethyl sulfonyl fluoride (PMSF), and 5 μg ml^{-1} pepstatin A] were added for each 1 g of tissue utilized. This mixture was immediately homogenized for 1–2 min at 4°C. After overnight extraction at 4°C with gentle agitation, extracts were clarified by centrifugation at 10000 *g* for 10 min, and the resulting supernatants were dialysed extensively (3 × 100 volumes) against 4 mmol l^{-1} HCl at 4°C, using a 3500 MW cutoff Spectropore dialysis tubing (Spectrum Medical Industries, Los Angeles, CA). Samples were reclarified by centrifugation before analysis. Conditioned media were processed by the method of Danielpour et al. (1989). Sandwich ELISA (SELISA) determinations of TGF-β1 and -β2 were carried out using anti-TGF-β1 or -β2 polyclonal antibodies raised against native, mature porcine TGF-β dimers, produced in rabbit (for the coating antibody) and in turkey (for the second antibody) and affinity-purified on TGF-β-linked Sepharose 4B (Pharmacia LKB, Piscataway, NJ) as previously described (Danielpour et al., 1989). The complete specificity of the antibodies utilized for SELISA for one or the other TGF-β isoform has been reported in detail elsewhere (Danielpour et al., 1989). In general, these antibodies are most appropriately used for detection of native TGF-β molecules found in cell extracts and conditioned media. The assays of RPE-conditioned media were performed using seven serial dilutions of the initial extract for each time point.

For Western analysis, the retina–RPE–choroid complex was dissected rapidly, in one piece, from the eyes of six rhesus monkeys (in contrast to procedures described above), and immediately frozen until subsequent thawing and homogenization; the latter was carried out in 10 mmol l^{-1} Tris, pH 7·5, containing 1 mmol l^{-1} ethylenediaminetetraacetic acid, 10 μmol l^{-1} benzamidine HCl (United States Biochemical, Cleveland, OH), 1 mmol l^{-1} PMSF (Calbiochem, LaJolla, CA), 1 μg ml^{-1} O-phenanthroline (Sigma Chemical Co., St. Louis, MO), 10 μg ml^{-1} aprotinin (U.S. Biochemical), 10 μg ml^{-1} leupeptin (U.S. Biochemical) and 10 μg ml^{-1} pepstatin A (U.S. Biochemical). Homogenates were spun at 10000 *g* for 30 min at 4°C to remove cellular debris. Tissue homogenate or acid-ethanol extract was loaded (150 μg protein) on to each lane of a 17% polyacrylamide gel and SDS polyacrylamide gel electrophoresis was carried out using standard methodology. Separated proteins were blotted overnight on nitrocellulose. The nitrocellulose membrane was blocked in 5% powdered milk in phosphate buffered saline and incubated overnight at 4°C in either of two primary anti-peptide antibodies to TGF-β2 (see below) at an IgG concentration of 400 ng ml^{-1}. After rinsing, the blots were incubated overnight once again in a secondary antibody conjugate (goat anti-rabbit-horseradish peroxidase; BioRad Laboratories, Richmond, CA) used at a 1 : 1000 dilution from stock. The antigen was detected using either diaminobenzidine or the Amersham ECL chemiluminescence system (Amersham International, Amersham, U.K.).

Immunohistochemistry

The morphological and immunohistochemical methods we employed in this study have been described in detail elsewhere (Erickson et al., 1993; Matsumoto and Hale, 1993). All procedures complied with the NIH Guide for the Care and Use of Laboratory Animals. For conventional light and electron microscopic immunolocalizations, monkey ocular tissues were obtained from anesthetized animals that had undergone an intracardiac perfusion of a mixed aldehyde fixative (1% formaldehyde from paraformaldehyde and 1% glutaraldehyde in 0·1 mol l^{-1} sodium phosphate buffer). Following perfusion, the globes were rapidly enucleated and the posterior poles were fixed for an additional hour in the same fixative. After fixation the eyecup was trimmed into quadrants, which were rinsed twice in 0·5 mol l^{-1} sodium maleate buffer, pH 5·2, followed by post-fixation in 2% uranyl acetate in 0·2 mol l^{-1} maleate buffer, pH 4·75. The tissues were dehydrated in a graded methanol series and infiltrated and embedded in LR White resin (Polysciences, Warrington, PA).

Tissue sections were blocked by incubation in a 1:100 dilution of normal goat serum (Vector, Burlingame, CA). The blocking step was followed by overnight exposure at room temperature to one of the following antibodies, diluted in 0·1 mol l^{-1} sodium phosphate buffer, pH 7, containing 0·5% bovine serum albumin (Sigma, Fraction V) and 0·1% sodium azide: (1) an affinity-purified IgG fraction of polyclonal antiserum raised against a peptide fragment of TGF-β1 [266–278] (15 μg ml^{-1}; Flanders et al., 1989); (2) a similar fraction raised against a peptide fragment of TGF-β2 [50–75] (1 μg ml^{-1}; Flanders et al., 1990a); or (3) a commercially prepared anti-peptide IgG fraction from a polyclonal antiserum raised against the same TGF-β2 [50–75] sequence (1 μg ml^{-1}; Santa Cruz Biotechnology, Santa Cruz, CA). These above mentioned peptides were derived from the primary sequences for human TGF-β1 and -β2, respectively. The TGF-β1 peptide sequence represents part of the inactive precursor molecule that is eventually cleaved from the mature TGF-β species (Flanders et al., 1989), while the TGF-β2 fragment constitutes that part of the mature molecule which may confer specificity of biological activity (Daopin et al., 1992; Qian et al., 1992). Both anti-peptide antibodies cross-react with specific TGF-β counterparts from a wide variety of vertebrates (Flanders et al., 1990a), attesting to the high degree of conservation of the sequences of these epitopes across species lines. These amino acid sequences are preferentially revealed in denatured molecules that would be the expected result of tissue fixation and processing for immunocytochemistry. At the IgG concentrations utilized for immuno-

histochemistry (1–15 $\mu g\ ml^{-1}$), neither antibody has any cross reactivity with other TGF-β isoforms, as Western immunoblotting has confirmed the specificity of these antibodies for the appropriate isoform (Flanders et al., 1989, 1990a). Control sections were incubated with pre-immune or non-immune rabbit serum at dilutions reflecting the IgG concentration of the primary antibody. Specific antibody binding to the retinal tissue was detected by means of secondary antibodies complexed with 1-nm gold particles (Janssen Amersham, Arlington Heights, IL), followed by silver enhancement (IntenSE II, Amersham). At the end of the procedure, plastic sections were stained with a mixture of toluidine blue, azure II, and methylene blue in sodium borate, pH 9. One-micrometer-thick sections were examined and photographed using a Zeiss Photomicroscope II (Thornwood, NY) with bright field optics.

For confocal microscopy, monkey eyes were obtained from animals following a lethal intravenous injection of sodium pentobarbitol. The globes were enucleated immediately, and fixed by immersion overnight in 4% paraformaldehyde in 0·1 M sodium cacodylate buffer. After approximately 1 hr, the anterior segment was cut away and the remaining posterior segment was stored in fixative at 4°C. For sectioning purposes, small wedges of retina–RPE–choroid near the posterior pole were cut away from the posterior segment, rinsed several times in 0·1 mol l^{-1} sodium phosphate buffer, and then embedded in low melting point agarose (Type VI; Sigma Chemical Co., St. Louis, MO). One-hundred-micrometer-thick sections were cut using a vibratome and incubated with primary antibody as described above. For visualization of TGF-β immunoreactivity in the laser scanning confocal microscope, specific antibody binding to the retinal tissue was detected by means of appropriate secondary antibodies (goat anti-rabbit IgG) conjugated to Cy3 (indorecarbocyanine; Jackson Immunoresearch Laboratories, West Grove, PA). Secondary antibody conjugates were used at a dilution of 1:200 from stock. Vibratome sections were examined using a BioRad 500 Confocal Laser Scanning Microscope.

For immunocytochemical demonstrations of TGF-β and cellular retinaldehyde-binding protein (CRALBP) in cultured cells, first-passage monkey RPE was seeded on the uncoated glass substrates of four-well ChamberSlides (Nunc, Naperville, IL) and cultivated using methods described above. After the cells had reached confluence and acquired the expected epithelial phenotype, the ChamberSlides containing the cells were briefly rinsed in modified HBSS, and then the cells were fixed for 1 hr in 4% formaldehyde (from paraformaldehyde) in 0·1 mol l^{-1} sodium cacodylate buffer, pH 7·4. Following rinses in several changes of buffer, the slides were stored in buffer. The immunofluorescence protocol was identical to the immunohistochemical procedures used in the processing of the plastic embedded sections, and included similar controls (see above). In addition, a polyclonal antiserum to CRALBP was included in the protocol to serve as a positive control.

For electron microscopic visualization, 60–80-nm ultrathin sections of normal monkey retina, fixed and embedded in equivalent manner to that used for light microscopy, were placed on nickel grids and processed using the same protocol outlined above, except that goat anti-rabbit IgG secondary antibodies conjugated with either 5-nm or 15-nm gold particles were used in place of the 1-nm reagent, and the silver enhancement step was eliminated. Control grids were exposed to one of four different reagents in place of the primary anti-TGF-β2 antibody: (1) normal rabbit serum, diluted 1:40 in 0·1 mol l^{-1} Tris-buffered saline (TBS; pH 7·5); (2) normal rabbit IgG in TBS, at a concentration of 1 $\mu m\ ml^{-1}$; (3) TBS plus 0·5% (w/v) bovine serum albumin; or (4) TGF-β2 antibody (1 $\mu g\ ml^{-1}$) pre-incubated with a 10-fold excess of the specific TGF-β2 peptide antigen (see above) in TBS. Following processing, the grids were stained with aqueous solutions of uranyl acetate and lead citrate, and then examined in a Philips CM10 electron microscope (Mahwah, NJ).

3. Results

Immunohistochemistry

All of the anti-TGF-β antibodies employed reacted specifically with photoreceptor outer segments of monkey retina. At the light microscope level, the intensity of the outer segment labeling generated by the two antibodies to peptide fragment [50–75] of TGF-β [Fig. 1(A)] was much greater than that produced by the TGF-β1 [266–278] peptide antibody (results not shown). Rod outer segments showed the most immunoreactivity, while cone outer segment labeling was only occasionally apparent, and at a much lower level; this held true for both peripheral retina and the macular region. Control incubations yielded no immunohistochemical reaction product in outer segments [Fig. 1(B)]. No detectable immunoreactivity was noted over the rod or cone inner segments or cell bodies.

Electron micrographs confirmed that the anti-TGF-β2 immunolabeling associated with the outer segments was strictly intracellular, and was not due to labeling of the interphotoreceptor matrix (IPM), the extracellular compartment between the photoreceptor outer segments and the RPE [Fig. 2(A)]. The specificity of this outer segment labeling was further documented by the absence or nearly complete diminution of labeling in control sections exposed to either normal (non-immune) rabbit serum (Fig. 2(B)], control (non-immune) IgG, TBS-BSA, or anti-TGF-β2 [50–75] plus an excess of the appropriate peptide [Fig. 2(C)]. The gold particles appeared to be associated primarily with the disc membranes that comprise the outer

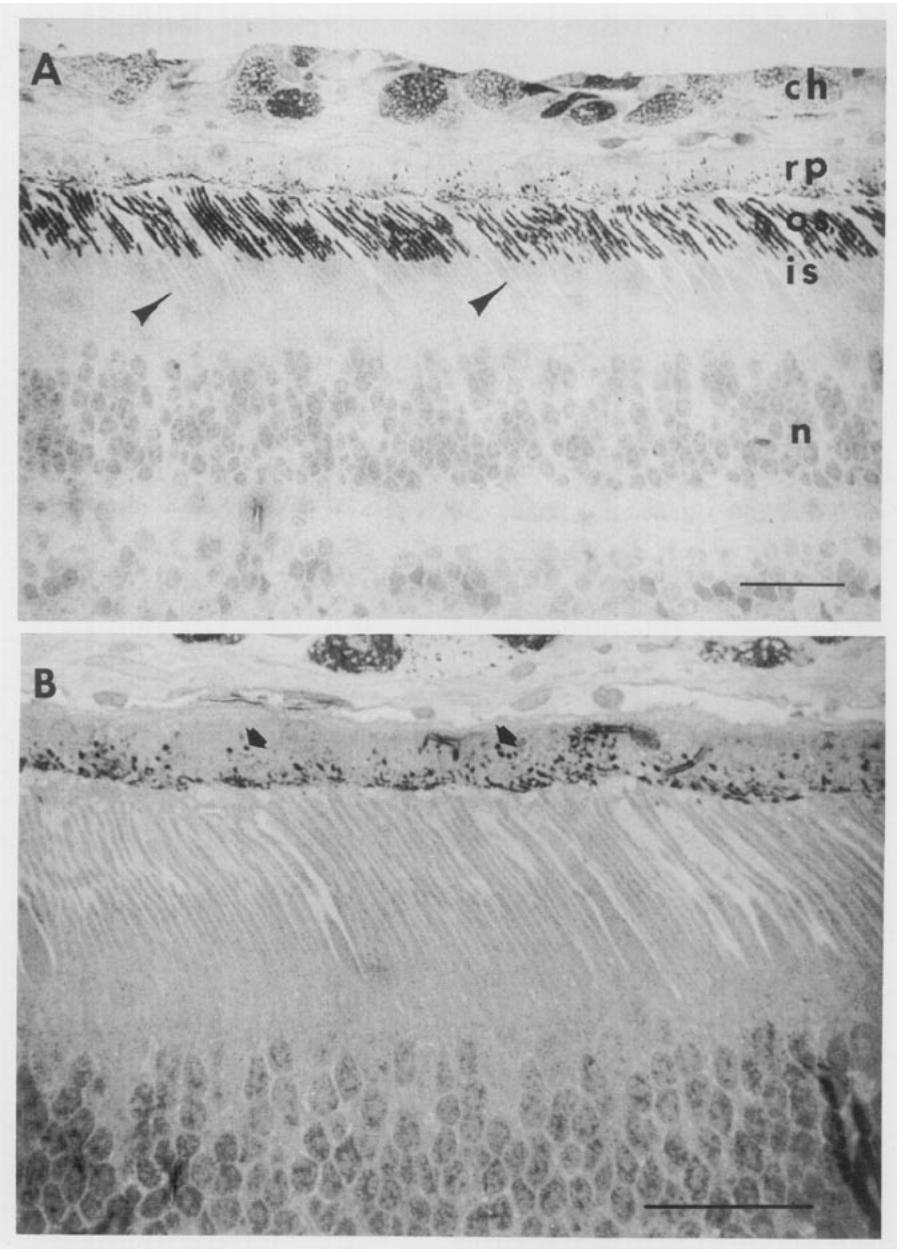

FIG. 1. Light microscopic immunolocalization of TGF-β2 in the normal rhesus monkey retina. (A) The distal portion of the photoreceptor cell layer, corresponding to the location of the photoreceptor outer segments, is labeled uniformly when antibodies to TGF-β2 [50–75] are applied to one micron plastic sections, followed by processing using an immunogold, silver-enhanced procedure to allow visualization of the antigen. ch, choroid; rp, retinal pigment epithelium; os, outer segment layer; is, inner segment layer; n, photoreceptor nuclear layer; arrowheads point to cone inner segments. Bar = 100 μm. (B) Control sections, exposed to preimmune rabbit serum in place of the primary antibody and then processed similarly, show only background labeling. Arrows point to melanin granules in RPE. Bar = 100 μm.

segments, with relatively few particles present at or near the enclosing plasma membranes. The particle distribution throughout the outer segment appeared to be uniform with no differential concentration at either the base or apex of the outer segments. There was no labeling above background in any other photoreceptor cell compartments or organelles.

Cultured RPE cells showed positive immunoreactivity both for TGF-β2 [Fig. 3(A)] and for CRALBP, the latter a marker for RPE (Eisenfeld et al., 1985) [Fig. 3(B)]. However, the intracellular fluorescent labeling pattern was distinctly different for these two proteins when viewed by confocal microscopy. Whereas the labeling for CRALBP was diffusely distributed in the cytoplasm and of virtually uniform intensity, the anti-TGF-β2 labeling consisted of large numbers of discrete globules scattered throughout the cells' cytoplasm. The number of fluorescent inclusions and their relative fluorescent intensity varied somewhat from cell to cell. Control preparations displayed no fluorescent reaction for TGF-β2 (not shown). TGF-β1 was not demonstrable in cultured cells by immunohistological means.

In comparison to rod outer segments, positive labeling for TGF-β2 was undetectable in the RPE in situ with conventional light microscope immunohistochemistry on 1-μm plastic sections [Fig. 1(A)].

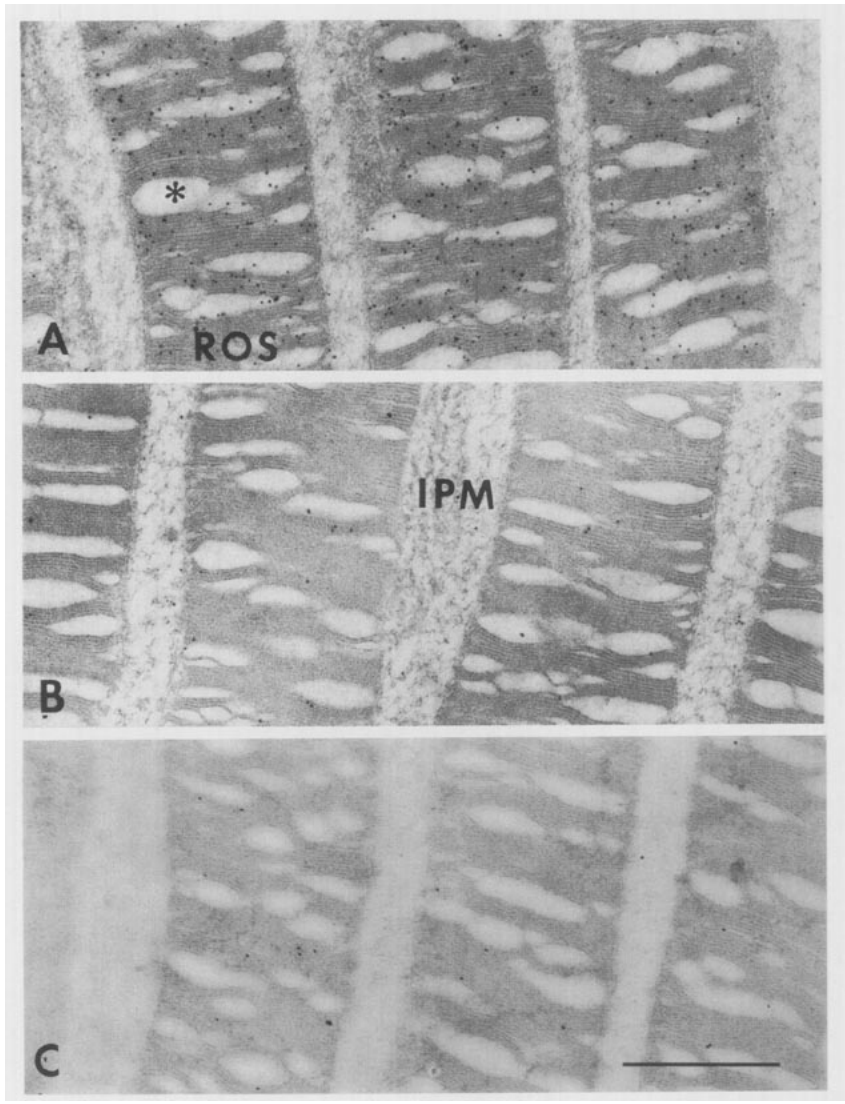

FIG. 2. Electron microscopic immunolocalization of TGF-β2 in rod photoreceptor outer segments (ROS) of the normal monkey retina. Tissue was sectioned along the longitudinal axis of the rod outer segments. The original magnifications for A, B, and C are all 36000; bar = 1 μm. (A) Immunogold labeling of rod outer segment cytoplasm. The 15-nm gold particles, which denote sites of anti-TGF-β2 binding, are confined to the intracellular domain of ROS. Virtually no particles are present in the IPM, the extracellular matrix adjacent to the ROS. ROS, rod outer segment; asterisk indicates artefactually expanded intradiscal space. (B) Control, incubated with normal (non-immune) rabbit serum in place of the primary TGF-β2 antibody. Only low level background labeling is apparent. IPM, interphotoreceptor matrix. (C) Control, prepared by incubation of the primary TGF-β2 [50–75] antibody with an excess of the corresponding peptide antigen. ROS labeling is blocked, providing added evidence of the specificity of the antibody labeling in (A).

However, when intact posterior pole tissue was processed for confocal microscopy and probed with either the commercially prepared (Santa Cruz Biotechnology) anti-TGF-β2 antibody [Fig. 1(C)] or our other anti-TGF-β2 immunoreagent (also against the [50–75] peptide), a positive result was obtained. The immunofluorescent granules were similar to those observed in cultured cells, except that the inclusions were more densely accumulated at the apicolateral borders of the cells [Fig. 3(C)].

TGB-β Levels Detected in Fresh Ocular Tissues

The results of the SELISA assays showed unequivocally that, on a weight per weight comparison, the RPE-Bruch's membrane–choroid (RPEBC) complex contained much higher levels of both the β1 and β2 isoforms of TGF-β than did the neural retina (Table I). For the β2 isoform, the tissue concentration was nearly 10-fold higher in the RPEBC samples than in those from retina, whereas, for the β1 isoform, that differential was even higher (approximately 22:1). The data also showed clearly that β2 is the predominant species both in the ocular tissues and in the saline washes derived from those same samples (Table I). The ratio of β2 to β1 ranged from an average of 6·0:1 for neural retina samples, expressed as ng TGF-β g^{-1} of tissue, to 425:1 for vitreous, expressed as pmol l^{-1}. Considering that each monkey retina contained approximately 1·4 ng of TGF-β2, the additional

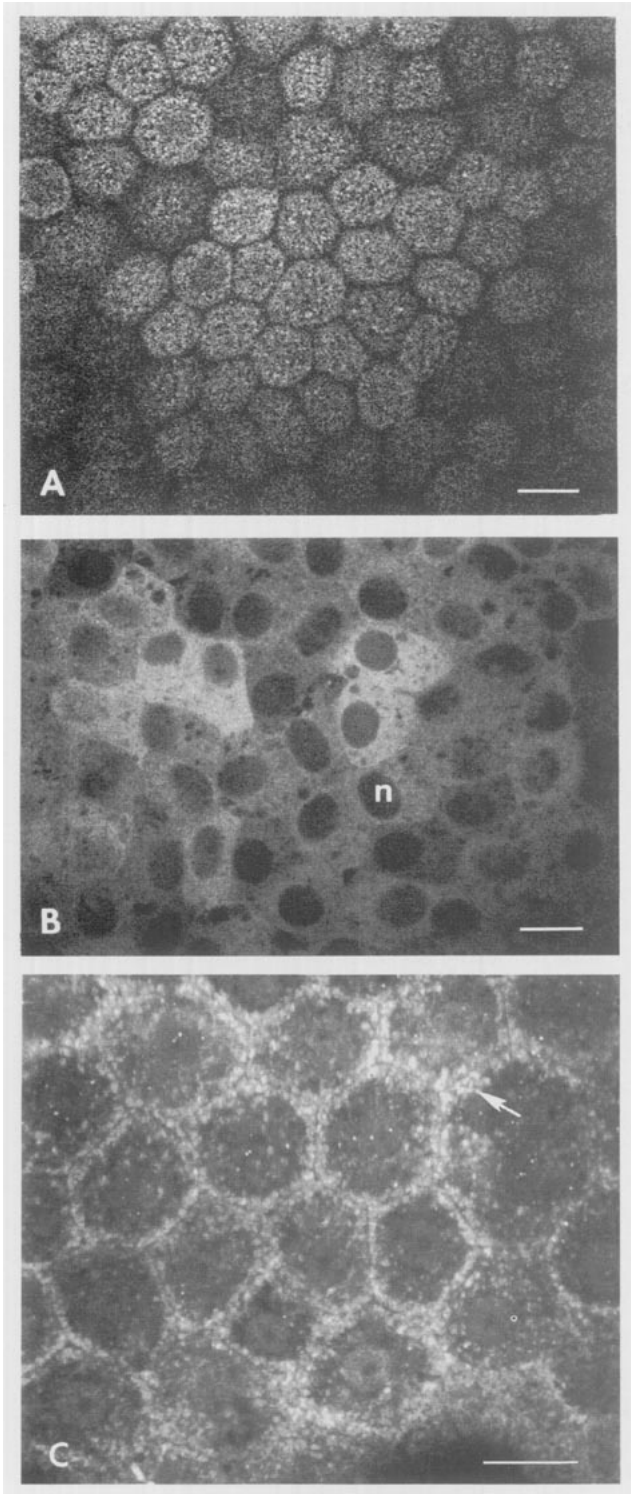

FIG. 3. Confocal fluorescent images of anti-TGF-β2 and anti-CRALBP labeling in cultured monkey RPE and in monkey RPE in situ. (A) Cultured RPE cells exhibit punctate immunofluorescent labeling for TGF-β2 throughout the apical cytoplasm in this 1-μm optical section. Bar = 10 μm. (B) Positive control. A 1-μm optical section showing anti-CRALBP labeling in cultured RPE. In contrast to the punctate fluorescence shown in (A), the anti-CRALBP labeling pattern, as expected, is much more uniformly distributed (see text). The nuclear zone (n) lacking positive immunoreactivity is prominent in most cells. Bar = 10 μm. (C) Anti-TGF-β2 labeling of monkey RPE in situ. Composite digital image of the RPE in tangential section showing the cumulative labeling present in 18 sequential 1-μm optical sections. A punctate pattern of fluorescence is similar to that of the cultured cells in (A). In contrast to the latter, in situ labeling is concentrated primarily in the peripheral cytoplasm at the cells' lateral margins (arrow). Bar = 10 μm.

0·83 ng (normalized per retina) of this isoform that diffused into the HBSS buffer during the initial separation of neural retina from RPE emphasized the importance of taking such diffusion or 'leakage' into account when dealing with such soluble factors. A small amount of cortical vitreous that usually remained adherent to the monkey retinas after the dissection procedure probably contributed a small amount of growth factor to the levels measured in the retina samples. In the vitreous samples, the β1 isoform was present only in trace amount (0·40–0·74 pmol l^{-1}) compared to the much higher concentration range of the β2 isoform (190–295 pmol l^{-1}). This latter value compares very favourably with the mean value of vitreous samples from human patients with uncomplicated retinal detachments (360 pmol l^{-1}), as measured by radio-immunoassay (Connor et al., 1989).

For the SELISA assays, which exploited two different polyclonal antibodies against native dimers of the growth factors, we detected absolute amounts of TGF-β2 per retina in the 0·5 to 1·5 ng range. However, on Western immunoblots of either retinal protein homogenates or acid–ethanol extracts derived from the homogenates, no components were recognized by the either of the anti-TGF-β2 [50–75] antibodies (see Methods) under either reducing or non-reducing conditions, in spite of the fact that both antibodies recognized 25 ng of the appropriate purified native protein on the same blot. In addition, these same anti-peptide antibodies used here for Western analysis had previously evinced positive immunoreactivity for TGF-β2 in tissue sections (above). These findings suggest that the levels of TGF-β isoforms present in our unconcentrated retina homogenates and acid–ethanol extracts precluded analysis by immunoblot, for which the detection threshold is approximately 5 ng.

Release of TGF-β by Cultured Monkey RPE cells

TGF-β2 accumulated in defined media conditioned by monkey RPE in a time-dependent manner (Fig. 4), reaching a concentration of 32·9 pmol l^{-1} after 24 hr. Despite the fact that the cell layers were rinsed several times in serum-free medium before the conditioning intervals began, we subjected the same group of cells to the longer 16-hr incubation first, before the ensuing 9-hr conditioning period, in order to further discern whether TGF-β that might have already accumulated on cell surfaces was merely being dislodged during the incubations. If this had been the case, the subsequent 9-hr exposure to a fresh change of medium would not have been expected to yield as high a value for TGF-β2

TABLE I

Transforming growth factor-β levels in monkey ocular tissues

Tissue or Extract	Total sample weight (g)	TGF-β2		TGF-β1	
		ng g^{-1}	pmol l^{-1}	ng g^{-1}	pmol l^{-1}
Neural retina 1* (3)†	1·8	2·9±0·2		0·6±0·01	
Neural retina 2 (3)	1·9	1·8±0·5		0·25±0·02	
BSS-1 (6)			16·4±0·5		0·8±0·05
RPEBC (6)	0·4	22·0±0·5		9·60±0·43	
BSS-2 (6)			13·3±0·3		2·6±0·9
Vitreous 1 (3)			190·0±17		0·4±0·2
Vitreous 2 (5)			295·0±26		0·74±0·13

* For both the neural retina and vitreous, the two independent samples contain pooled material from separate groups of eyes.
† Number of eyes from which tissues were pooled into individual samples.
Data values are expressed as means±1 S.D.

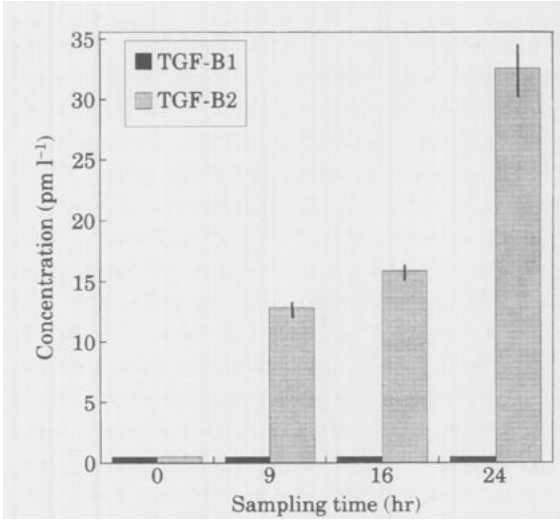

FIG. 4. Graph depicting accumulation of TGF-β2 in defined medium conditioned by monkey RPE cells. Values refer to the average, for two replicate runs, of means of measurements performed at each time point with seven serial dilutions, ±1 S.D., as denoted by error bars.

(Fig. 4). In contrast to TGF-β2, TGF-β1 was below the limits of detection (0·5 pmol l^{-1}) at all time points (Fig. 4).

4. Discussion

This study used three different approaches to ascertain the presence and amounts of TGF-β1 and β2 in tissues of the posterior pole of the monkey eye: (1) SELISA assays of freshly dissected neural retina, RPE-Bruch's membrane-choroid, and vitreous; (2) accumulation of growth factor in conditioned medium of pure RPE cell cultures, also as quantified by SELISA and (3) immunolocalization of TGF-β2 at both the light (by immunogold and immunofluorescence) and ultrastructural levels in native tissue, and by immunofluorescence in cultured RPE. On the basis of our results from all three types of experiments, we conclude that TGF-β2 is clearly the predominant isoform in the posterior pole, with much lower quantities of TGF-β1 present in all of these tissues. It is highly unlikely that the TGF-β2 measured in the three tissues is due to contamination from plasma elements. Blood-derived TGF-β2 can be ruled out as a source of significant contamination because of the exsanguination employed prior to enucleation and dissection of the eyes. Moreover, TGF-β2 is not a major constituent of human serum (Cheifetz et al., 1987; Danielpour et al., 1989, 1990) and, in all likelihood, the same is true for other closely-related primates, including rhesus monkey.

That fact that cultured RPE appears to produce TGF-β2 exclusively, rather than TGF-β1, suggests that this cell type is one source of the β2 isoform detected by SELISA in the RPE–choroid complex. This conclusion is also supported by the positive anti-TGF-β2 immunolabelling demonstrable in the RPE, identified both in vitro and in situ. Since we also detected measurable amounts of TGF-β1 in RPE-choroid samples, it is probable that the choroid, with its combination of endothelial cells, pericytes, melanocytes and fibroblasts, is the predominant in vivo source of the β1 isoform found in this tissue complex. Indeed, there is recent immunohistochemical support for this latter conclusion (Lutty et al., 1993). Southern analysis of polymerase chain reaction products from cultured human RPE showed that TGF-β2 transcripts were predominant, although message for TGF-β1 was also detected in RPE in vitro (Tanihara et al., 1993). Conversely, there is the possibility that the apparent synthesis and export of TGF-β2, to the exclusion of TGF-β1, by the cultured RPE cells simply reflects an adaptation to in vitro conditions. An up-regulation of TGF-β2 expression in cultured cells is suggested by the difference in the number of optical sections contributing to the confocal images in Fig. 3(A) and (C), the latter, representing RPE in situ, being composed of 18 sequential 0·8-μm optical sections. The immunolabeling pattern exhibited by RPE, both in culture and in the intact eye, is consistent with one that might be generated from intracellular secretory granules.

A recent study implicates hyalocytes as one source of the high levels of TGF-β2 we measured in the normal monkey vitreous (Lutty et al., 1993). This study also demonstrated that choroidal tissues are a potential source of TGF-β2, and suggested that transcytosis of the growth factor by RPE in the basal to apical direction is a plausible mechanism for delivery of activated TGF-β2 across the outer blood–retinal barrier. Using the same antibody to TGF-β2 that we employed in the present study for electron microscope immunocytochemistry, reaction product was noted in association with the choroidal stroma and rod outer segments, but RPE was not observed to possess immunoreactivity (Lutty et al., 1993). Our immunohistochemical results utilizing 1-μm plastic sections are certainly consistent with much lower levels of immunoreactivity for TGF-β2 for RPE compared with rod outer segments. With respect to the β1 isoform, in cryosections of aged human retinas, an antibody with apparent specificity for intracellular TGF-β1 (anti-LC [1–30] (Flanders et al., 1989)) labeled the endothelium of the choriocapillaris immediately adjacent to the basal RPE surface; labeling of the basal portion of some RPE cells was noted occasionally (Lutty et al., 1993). Immunoperoxidase labeling was not present in RPE or choroidal tissue using another antibody (anti-CC [1–30]) preferentially recognizing the β1 isoform in extracellular compartments.

It is important to note that we have now identified a number of other subcellular sites of TGF-β1, TGF-β2 and TGF-β immunolabeling in the retina and RPE–choroid of feline, monkey and human eyes (Anderson et al., 1991; Anderson et al., submitted). Labeling at some of these sites is demonstrable only by using different fixation regimens, embedding media and microscopic imaging systems—a result in accordance with previous results in mammalian retinas using antibodies to other growth factors as well as to TGF-β (Hageman et al., 1991; Hanneken and Baird, 1992; Peress and Perillo, 1994). Moderate levels of anti-TGF-β3 labeling can also be detected in the RPE cytoplasm, within the mitochondrial-rich zone of the photoreceptor inner segments (i.e. the ellipsoid), and in the cytoplasm of the various cell types that form the retinal vasculature.

For the neural retina, the quantitative data obtained from the SELISA assays also were consistent with the immunohistochemical results. In a previous study, Lutty et al. (1993) described immunoreactivity associated with the photoreceptor layer in the normal human retina using two polyclonal antibodies, one to a TGF-β1 peptide fragment [1–30], and the other the anti-TGF-β2 peptide fragment [50–75] antibody also employed by us (Flanders et al, 1990a). In this study we have corroborated and extended that observation by demonstrating intracellular immunoreactivity for TGF-β2 at the ultrastructural level that is associated with outer segments, and almost exclusively in rods as opposed to cone photoreceptors. Since the anti-TGF-β2 antibody we utilized for immunocytochemistry reacts with the mature form of the growth factor (Flanders et al., 1990b), the subcellular localization of mature TGF-β2 within photoreceptor (predominantly rod) outer segments may have functional significance (see below).

Without performing a bioassay (Tucker et al., 1984), it cannot be determined whether the TGF-β we have detected via SELISA in ocular tissues or in RPE-conditioned medium represents active or latent TGF-β, particularly since the acid extraction of the tissues would have converted all of the growth factor to active form. Connor et al. (1989) determined that approximately 87% of the vitreal TGF-β was in latent form, and it is generally accepted that the growth factor is at least initially released as an inactive high molecular weight complex (Rizzino, 1988; Miyazuno et al., 1988). Under both in vivo and in vitro conditions, TGF-β has been shown to be externalized together with a proteoglycan receptor designated β-glycan, that may be membrane-anchored or in soluble form (Andres et al., 1989; Segarini, 1990). All of the three receptors currently characterized for TGF-β, including β-glycan, have been detected in primary explants of human neural retina (Massagué et al., 1990). The functional significance of localization of the growth factor within photoreceptor outer segments is still elusive, as noted by Lutty et al. (1993). TGF-β is thought to be a protein whose function ultimately depends upon secretion from cells of synthesis. In contrast, autoradiographic evidence suggests that other protein constituents of the extracellular compartment associated with and synthesized by photoreceptors (for the most part components of the IPM) do not accumulate in outer segments, but are secreted from the inner segment portion of the cell (Feeney, 1973; Hollyfield et al., 1985), where protein synthesis occurs, and where the cellular machinery for processing and release is presumed to reside (Bok, 1985).

If the release of TGF-β2 to the culture medium by the RPE in vitro is a valid indication of what might occur in vivo, then this growth factor could conceivably be released from the apical surface of native RPE as a normal constituent of the IPM. Assuming that the results obtained in vitro from the RPE cells' conditioned medium are indicative of the amounts that might accumulate in the IPM, then the 24-hr concentration of around 33 pmol l^{-1} of TGF-β2 attained in vitro would translate into an absolute amount of 16 pg in the approximately 20 μl of soluble IPM present in a single human eye (Adler and Evans, 1985)—an amount well within the total we measured for a single monkey retina (see Table I). TGF-β1, like basic fibroblast growth factor (bFGF), has been shown recently to be a heparin-binding protein (McCaffrey et al., 1992). In theory, then, TGF-β2 might bind to proteoglycans in the neural retina in much the same way that bFGF is sequestered by proteoglycan-rich rod and cone matrix sheaths of the IPM (Hageman et al.,

1991). To date, however, convincing evidence, immunocytochemical or otherwise, indicating that any of the TGF-β isoforms or their receptors are normal constituents of either the soluble or insoluble domain of IPM is lacking.

Given the presence of bFGF in the IPM, it is intriguing that the concentrations of TGF-β attained by RPE-conditioned medium in our culture experiments are of the same order of magnitude as the 80 pmol l^{-1} (2 ng ml^{-1}) TGF-β (isoform unspecified) utilized previously to accomplish partial inhibition of the proliferative effect of bFGF on RPE in vitro (Leschey et al., 1990). In fact, TGF-β is believed to exert many of its effects via synergistic interaction with other growth factors, such as bFGF (Kimelman and Kirschner, 1987; Flaumenhaft et al., 1992). TGF-β has already been shown to influence the proliferation and differentiation of many epithelial cell types (Jetten, Shirley and Stoner, 1986; Silberstein and Daniel, 1987; Coffey et al., 1988; Rizzino, 1988), of which RPE may be included (Leschey et al., 1990). Therefore, the levels of TGF-β that we have shown to be maintained in tissues of the posterior pole of the eye may be significant for normal visual function and also in pathogenesis and tissue repair. In particular, the emerging evidence supporting the possibility that the RPE or the photoreceptors are a source of TGF-β2, a target for TGF-β2, or both, may signify that this growth factor participates in physiologically important interactions, yet to be identified, between these two cell types.

Acknowledgments

Monkey eyes were made available courtesy of John P. Cogan, Chief, In Vivo Vaccine Test Section, Office of Biologics, National Center for Drugs and Biologics, Food and Drug Administration. John Saari, University of Washington, kindly provided anti-CRALBP antibody. Kai Anderson and Christian Kim provided assistance with isolation of vitreous. This work was partially funded by the National Eye Institute (EY 02082 to D. H. Anderson).

References

Adler, A. J. and Evans, C. D. (1985). Proteins of the bovine interphotoreceptor matrix: retinoid binding and other functions. In *The Interphotoreceptor Matrix in Health and Disease* (Eds Bridges, C. D. and Adler, A. J.). Pp. 65–88. Alan R. Liss: New York.

Anderson, D. H., Hageman, G. S., Guérin, C. H. and Flanders, K. C. (1991). Immunolocalization of transforming growth factor TGF-β1, TGF-β2, and TGF-β3 in mammalian retinas. *Invest. Ophthalmol. Vis. Sci. (ARVO Suppl.)* **32**, 754.

Andres, J. L., Stanley, K., Cheifetz, S. and Massagué, J. (1989). Membrane-anchored and soluble forms of betaglycan, a polymorphic proteoglycan that binds transforming growth factor-β. *J. Cell Biol.* **109**, 3137–45.

Bok, D. (1985). Retinal photoreceptor-pigment epithelium interactions. *Invest. Ophthalmol. Vis. Sci.* **26**, 1659–94.

Cheifetz, S., Weatherbee, J. A., Tsang, M. L.-S., Anderson, J. K., Mole, J. E., Lucas, R. and Massagué, J. (1987). The transforming growth factor-β system, a complete pattern of cross-reactive ligands and receptors. *Cell* **48**, 409–15.

Chen, R.-H., Ebner, R. and Derynck, R. (1993). Inactivation of the type I receptor reveals two receptor pathways for the diverse TGF-β activities. *Science* **260**, 1335–8.

Coffey, R. J., Bascom, C. C., Sipes, N. J., Graves-Deal, R., Weissman, B. E. and Moses, H. L. (1988). Selective inhibition of growth-related gene expression in murine keratinocytes by transforming growth factor β. *Mol. Cell. Biol.* **8**, 3088–93.

Connor, T. B., Jr., Roberts, A. B., Sporn, M. B., Danielpour, D., Dart, L. L., Michels, R. G., de Bustros, S., Enger, C., Kato, H., Lansing, M., Hayashi, H. and Glaser, B. (1989). Correlation of fibrosis and transforming growth factor-β type 2 levels in the eye. *J. Clin. Invest.* **83**, 1661–6.

Cousins, S. W., McCabe, M. M., Danielpour, D. and Streilein, J. W. (1991). Identification of transforming growth factor-beta as an immunosuppressive factor in aqueous humor. *Invest. Ophthalmol. Vis. Sci.* **32**, 2201–11.

Danielpour, D., Kim, K. Y., Dart, L. L., Watanabe, S., Roberts, A. B. and Sporn, M. B. (1989). Sandwich enzyme-linked immunosorbent assays (SELISAs) quantitate and distinguish two forms of transforming growth factor-beta (TGF-β1 and TGF-β2) in complex biological fluids. *Growth Factors* **2**, 61–71.

Danielpour, D., Kim, K. Y., Dart, L. L., Watanabe, S., Roberts, A. B. and Sporn, M. B. (1990). Evidence for differential regulation of TGFβ1 and TGFβ2 expression in vivo by sandwich enzyme-linked immunosorbent assays. In *Transforming Growth Factor-βs. Chemistry, Biology, and Therapeutics* (Eds Piez, K. and Sporn, M. B.). *Ann. N. Y. Acad. Sci.* **593**, 300–2.

Danielpour, D. (1993). Improved sandwich enzyme linked immunosorbent assays for transforming growth factor-beta 1. *J. Immunol. Meth.* **158**, 17–25.

Daopin, S., Piez, K. A., Ogawa, Y. and Davies, D. R. (1992). Crystal structure of transforming growth factor-β2: an unusual fold for the superfamily. *Science* **257**, 369–73.

Eisenfeld, A. J., Bunt-Milam, A. H. and Saari, J. C. (1985). Localization of retinoid-binding proteins in developing rat retina. *Expl. Eye Res.* **45**, 187–90.

Erickson, P. E., Lewis, G. and Fisher, S. K. (1993) Post-embedding immunocytochemical techniques for light and electron microscopy. In *Methods in Cell Biology*, Vol. 37. (Ed. Asai, D.). Pp. 283–310. Academic Press: San Diego.

Feeney, L. (1973). Synthesis of interphotoreceptor matrix I. Autoradiography of ^{3}H-fucose incorporation. *Invest. Ophthalmol.* **12**, 739–51.

Flanders, K. C., Thompson, N. L., Cissel, D. S., Van Obberghen-Schilling, E., Baker, C. C., Kass, M. E., Ellingsworth, L. R., Roberts, A. B. and Sporn, M. B. (1989). Transforming growth factor-β1: histochemical localization with antibodies to different epitopes. *J. Cell Biol.* **108**, 653–60.

Flanders, K. C., Cissel, D. S., Mullen, L. T., Danielpour, D., Sporn, M. B. and Roberts, A. B. (1990a). Antibodies to transforming growth factor-beta 2 peptides: specific detection of TGF-β2 in immunoassays. *Growth Factors* **3**, 45–52.

Flanders, K. C., Cissel, D. S., Jakowlew, S. B., Roberts, A. B., Watanabe, S. Danielpour, D. and Sporn, M. B. (1990b). Immunohistochemical localization of TGF-betas 2 and 3 in the nervous system. In *Transforming Growth-Factor-βs, Chemistry, Biology, and Therapeutics* (Eds Piez, K. and Sporn, M. B.), *Ann. N. Y. Acad Sci.* **593**, 338–9.

Flaumenhaft, R., Abe, M., Mignatti, P. and Rifkin, D. B.

(1992). Basic fibroblast growth factor-induced activation of latent transforming growth factor β in endothelial cells: regulation of plasminogen activator activity. *J. Cell Biol.* **118**, 901–9.

Hageman, G. S., Kirchoff-Rempe, M. A., Lewis, G. P., Fisher, S. K. and Anderson, D. H. (1991). Sequestration of basic fibroblast growth factor in the primate retinal interphotoreceptor matrix. *Proc. Natl. Acad. Sci., USA* **88**, 6706–10.

Hanneken, A. and Baird, A. (1992). Immunolocalization of basic fibroblast growth factor: dependence on antibody type and tissue fixation. *Exp. Eye Res.* **54**, 1011–4.

Helbig, H., Kittredge, K. L., Coca-Prados, M., Davis, J., Palestine, A. G. and Nussenblatt, R. B. (1991). Mammalian ciliary-body epithelial cells in culture produce transforming growth factor-beta. *Graefes Arch. Clin. Exp. Ophthalmol.* **229**, 84–7.

Hollyfield, J. G., Fliesler, S. J., Rayborn, M. E., Fong, S.-L., Landers, R. A. and Bridges, C. D. (1985). Synthesis and secretion of interstitial retinol-binding protein by the human retina. *Invest. Opthalmol. Vis. Sci.* **26**, 58–67.

Ignotz, R. A. and Massagué, J. (1987). Cell adhesion receptors as targets for transforming growth factor-β action. *Cell* **51**, 189–97.

Jetten, A. M., Shirley, J. E. and Stoner, G. (1986). Regulation of proliferation and differentiation of respiratory tract epithelial cells by TGF-β. *Exp. Cell Res.* **167**, 539–49.

Kimelman, D. and Kirschner, M. (1987). Synergistic induction of mesoderm by FGF and TGF-β and the identification of an mRNA coding for FGF in the early Xenopus embryo. *Cell* **51**, 869–77.

Leschey, K. H., Hackett, S. F., Singer, J. H. and Campochiaro, P. A. (1990). Growth factor responsiveness of human retinal pigment epithelial cells. *Invest. Ophthalmol. Vis. Sci.* **31**, 839–846.

Lutty, G., Ikeda, K., Chandler, C. and McLeod, D. S. (1991). Immunohistochemical localization of transforming growth factor-β in human photoreceptors. *Curr. Eye Res.* **10**, 61–74.

Lutty, G. A., Merges, C., Threlkeld, A. B., Crone, S. and McLeod, D. S. (1993). Heterogeneity in localization of isoforms of TGF-β in human retina, vitreous, and choroid. *Invest. Ophthalmol. Vis. Sci.* **34**, 477–87.

Lyons, R. M. and Moses, H. L. (1990). Transforming growth factors and the regulation of cell proliferation. *Eur. J. Biochem.* **187**, 467–73.

Massagué, J., Cheifetz, S., Boyd, F. T. and Andres, J. L. (1990). TGF-β receptors and TGF-β binding proteoglycans: recent progress in identifying their functional properties. In *Transforming Growth-Factor-βs. Chemistry, Biology, and Therapeutics* (Eds Piez, K. and Sporn, M. B.). *Ann. N. Y. Acad. Sci.* **593**, 59–72.

Massagué, J., Heino, J. and Laiho, M. (1991). Mechanisms in TGF-β action. In *Clinical Applications of TGF-β* (Eds Bock, G. R. and Marsh, J.). *CIBA Foundation Symposium* **157**, 51–65. Wiley: Chichester, UK.

Matsumoto, B. and Hale, I. L. (1993). Preparation of retinas for studying photoreceptors with confocal microscopy. In *Methods in Neurosciences*, Vol. 15 (Ed. Hargrave, P.). Pp. 54–74. Academic Press: San Diego.

McCaffrey, T. A., Falcone, D. J. and Du, B. (1992). Transforming growth factor-β1 is a heparin-binding protein: identification of putative heparin-binding regions and isolation of heparins with varying affinity for TGF-β1. *J. Cell. Physiol.* **152**, 430–40.

Miyazuno, K., Hellman, U., Wernstedt, C. and Heldin, C.-H. (1988). Latent high molecular weight complex of transforming growth factor β1. *J. Biol. Chem.* **263**, 6407–15.

Moses, H. L., Yang, E. Y. and Pietenpol, J. A. (1990). TGF-β stimulation and inhibition of cell proliferation: new mechanistic insights. *Cell* **63**, 245–7.

Pasquale, L. R., Dorman-Pease, M. E., Lutty, G. A., Quigley, H. A. and Jampel, H. D. (1993). Immunolocalization of TGF-β1, TGF-β2, and TGF-β3 in the anterior segment of the human eye. *Invest. Ophthalmol. Vis. Sci.* **34**, 23–30.

Peress, N. S. and Perillo, E. (1994). TGF-β2 and TGF-β3 immunoreactivity within the ciliary epithelium. *Invest. Ophthalmol. Vis. Sci.* **35**, 453–457.

Pfeffer, B. A. (1990). Improved methodology for cell culture of human and monkey retinal pigment epithelium. In *Prog. Ret. Res.*, Vol. 10 (Eds Osborne, N. N. and Chader, G. J.). Pp. 251–91. Pergamon Press: Oxford.

Qian, S. W., Burmester, J. K., Merwin, J. R., Madri, J. A., Sporn, M. B. and Roberts, A. B. (1992). Identification of a structural domain that distinguishes the actions of the type 1 and 2 isoforms of transforming growth factor β on endothelial cells. *Proc. Natl. Acad. Sci., USA* **89**, 6290–4.

Rizzino, A. (1988). Transforming growth factor-β: multiple effects on cell differentiation and extracellular matrices. *Dev. Biol.* **130**, 411–22.

Roberts, A. B., Lamb, L. C., Newton, D. L., Sporn, M. B., DeLarco, J. E. and Todaro, G. J. (1980). Transforming growth factors: isolation of polypeptides from virally and chemically transformed cells by acid ethanol extraction. *Proc. Natl. Acad. Sci., USA* **77**, 3494–8.

Roberts, A. B., Kim, S.-J., Kondaiah, P., Jakowlew, S. B., Denhez, F., Glick, A. B., Geiser, A. G., Watanabe, S., Noma, T., Lechleider, R. and Sporn, M. B. (1990). Transcriptional control of expression of the TGF-βs. In *Transforming Growth Factor-βs. Chemistry, Biology, and Therapeutics* (Eds Piez, K. and Sporn, M. B.). *Ann. N. Y. Acad Sci.* **593**, 43–50.

Segarini, P. (1990). Cell type specificity of TGF-β binding. In *Transforming Growth Factor-βs. Chemistry, Biology, and Therapeutics* (Eds Piez, K. and Sporn, M. B.). *Ann. N. Y. Acad. Sci.* **593**, 73–90.

Silberstein, G. B. and Daniel, C. W. (1987). Reversible inhibition of mammary gland growth by transforming growth factor-β. *Science* **237**, 291–3.

Tanihara, H., Yoshida, M., Matsumoto, M. and Yoshimura, N. (1993). Identification of transforming growth factor-β expressed in cultured human retinal pigment epithelial cells. *Invest. Ophthalmol. Vis. Sci.* **34**, 413–9.

Tucker, R. F., Shipley, G. D., Moses, H. L. and Holley, R. W. (1984). Growth inhibitor from BSC-1 cells is closely related to the platelet type β transforming growth factor. *Science* **226**, 705–7.

Wakefield, L. M., Smith D. M., Flanders, K. C. and Sporn, M. B. (1988). Latent transforming growth factor-β from human platelets. *J. Biol. Chem.* **263**, 7646–54.

Waldbillig, R. J., Pfeffer, B. A., Schoen, T. J., Adler, A. J., Shen-Orr, Z., Scavo, L., LeRoith, D. and Chader, G. J. (1991). Evidence for an insulin-like growth factor autocrine–paracrine system in the retinal photoreceptor–pigment epithelial cell complex. *J. Neurochem* **57**, 1522–33.

Waldbillig, R. J., Schoen, T. J., Chader, G. J. and Pfeffer, B. A. (1992). Monkey retinal pigment epithelial cells in vitro synthesize, secrete, and degrade insulin-like growth factor binding proteins. *J. Cell Physiol.* **150**, 76–83.