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Immunological Study of Acidic Fibroblast Growth Factor (aFGF) Distribution in the Eye

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During the last ten years, several groups, including the present authors, have detected growth factor activities in various ocular tissues, and the presence of a ubiquitous Eye-Derived Growth Factor (EDGF) has been described. More recently, isolation and characterization of this growth factor activity from the retina led to the identification of two molecules. These molecules were shown to be identical to other growth factors isolated from neuronal and non-neuronal tissues and are now designated as acidic and basic fibroblast growth factor (aFGF, bFGF). The biological function and the reason for the ubiquitous distribution of these factors remain unclear. Understanding may be improved by quantification of this distribution in various tissues during development. In the present study, specific polyclonal antibodies were raised against acidic FGF, aFGF was determined in various ocular tissues by enzyme immunoassay, and the localization of immunoreactive aFGF by immunohistological staining with fluorescent antibodies or with enzyme- or gold-labeled antibodies was studied.

In almost all tissues tested aFGF was found; but the retina, cornea, and vitreous body contained the highest levels of aFGF per gram of tissue. In the retina, aFGF was associated primarily with the nerve fiber layer and the inner and outer segments of the photoreceptors, whereas corneal aFGF was detected in the cytoplasma of the basal layer of epithelial cells.

Key words: growth factor, aFGF, immunoassay, eye, vitreous body, cornea, retina, lens

Growth-factor activity has been reported in various ocular tissues by several groups in the last ten years [1–4]. Although the original aim of the present authors was to characterize growth factors from the retina and to study their role in the control of lens growth and differentiation, the presence of growth factor activity in most ocular tissues—including the iris, vitreous body, ciliary processes, retina, and choroid—has been demonstrated [2]. These factors have been designated *eye-derived growth factors* (EDGF). Two (EDGF I and II), [5–7] which have a high affinity for

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heparin, were identical to acidic and basic fibroblast growth factor (FGF) found in other nonocular tissues, and a third factor (EDGF III) was characterized [8]. A retina-derived growth factor has been also shown to be identical to aFGF [9], and both forms have been characterized by other groups.

The biological and physiological significance of these growth factors is unknown, but they are believed to have key roles in tissue growth and homeostasis throughout the whole life span.

High levels of bFGF have been reported in the vitreous bodies of patients with vasoproliferative disorders of the retina [10], suggesting pathological involvement. However, these data are subject to debate [11] (Clément et al., manuscript in preparation).

Accurate knowledge of the distribution of growth factors in various tissues of healthy and pathological eyes may improve understanding of their physiological roles and physiopathological implications.

Specific antibodies were raised against aFGF, an enzyme immunoassay was developed, and immunohistological studies were done to quantify and localize aFGF in normal bovine and human eyes.

MATERIALS AND METHODS

Antigen Purification and Immunization Procedure

As already described, aFGF was extracted from bovine brain [6]. Using this procedure, aFGF was shown to be completely pure (99%) after heparin-Sepharose chromatography. Over a 6-week period, female New Zealand albino rabbits were given weekly intradermal injections of 50 to 100 μg of aFGF emulsified in complete Freund's adjuvant (Sigma). Rabbits subsequently received monthly booster injections of 50 μg of aFGF emulsified in Freund's incomplete adjuvant. Rabbits were bled monthly ten days after each booster injection. Antibody production was monitored by enzyme linked immunosorbent assay (ELISA) [12].

Characterization of Antibodies

Immunoassays and immunohistological studies were carried out with sera from two different rabbits.

The characterization, specificity, and properties of the immune serum used for immunoassay have been reported elsewhere [13]. The criteria presented include ELISA, immunoblot, and inhibition of aFGF-induced biological activity by these anti-aFGF antibodies. No cross-reactivity with bFGF could be detected by any of these methods (fewer than 8/1,000).

Immunohistological studies were performed with another polyclonal antibody, which also showed no cross-reactivity with bFGF, as judged by ELISA.

Protein content was determined using the method of Bradford (14) with bovine serum albumin as the standard.

Immunoassay Procedure

All reagents used in the assays were diluted in 0.1 M phosphate buffer, pH 7.4, containing 0.4 M NaCl, 10^{-3} M EDTA, 0.1% bovine serum albumin (BSA), 0.01% NaN^3 (EIA buffer).

Enzyme Immunoassay (EIA) was performed as previously described according to the second antibody solid-phase EIA protocol [15].

Briefly, mouse antirabbit immunoglobulin G (IgG) monoclonal antibodies were adsorbed on 96-well microtiter plates and 1% BSA was added to saturate protein-binding sites. Plates were washed with 10^{-2} M phosphate buffer, pH 7.4, containing 0.05% Tween 20, each well was filled with 300 μl of EIA buffer, and the plates were stored at 4°C until use. Assays were performed in a total volume of 150 μl, with each component added in a 50-μl volume: standard aFGF solution, aFGF enzyme conjugate, and diluted anti-aFGF antiserum. Enzymatic tracer (aFGF-AChE) was obtained by coupling aFGF with the tetrameric form of acetylcholineesterase from the electric organs of the electric eel *Electrophorus electricus* [16].

After overnight incubation at 22°C, the plates were washed as described above, and 200 μl of Ellman's reagent [17] were added to each well. After 30 min to 2 h, the absorbance at 414 nm was measured using a Dynatech spectrophotometer plate reader.

Ocular Tissue Extracts

Ocular tissues were dissected from freshly slaughtered bovines and kept at 4°C. Homogeneous batches of specific tissues were crushed in a Moulinex blender and then homogenized in a tight-fitting potter homogenizer after the addition of 100 mM phosphate buffer pH 6.7, 2 M NaCl. After 1 h of continuous vigorous shaking at 4°C, samples were centrifuged (30 min, 20,000 g) and the ionic strength of the supernatant was adjusted to 0.65 M before application to a heparin-Sepharose chromatography column (50 mg of heparin-Sepharose were routinely used for extracts).

Preparation of Tissue Section

Tissues were fixed, within 30 min of death for bovines and within 4 h of death for humans in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 4 h at 4°C. The tissues were dehydrated in a graded series of ethanol, cleared in toluene, and embedded in paraffin. Sections were cut to a thickness of 5 μm, mounted on slides, dried for 24 h at 37°C, and then stored at 4°C. Fixed frozen sections were compared with sections of paraffin.

Immunohistological Technique

The distribution of aFGF protein was analyzed by indirect immunofluorescence and immunoperoxidase or immunogold methods, enhanced by silver staining. Sections were preincubated in normal goat serum (10% in phosphate-buffered saline (PBS) 0.4 M NaCl, 0.1% BSA, 0.3% Triton x 100) for 30 min at 22°C. Preincubation medium then was discarded and, without washing, the sections were incubated in specific antiserum diluted 1/50 and 1/100 in a moist chamber at 22°C for 2 h. After incubation, sections were rinsed in PBS and incubated with goat antirabbit IgG antibodies (1/100 dilution) for 1 h at 22°C. This second antibody was goat antirabbit IgG antibody, which was conjugated with fluorescein isothiocyanate (FITC) (Tago) or with peroxidase (Pasteur Production) revealed by diaminobenzidine (Sigma) or labeled with colloidal gold [18] (Jansen) and treated by silver enhancement [19].

Several controls were performed without primary antibodies to control nonspecific immunoreactivity of reagents, with aFGF-absorbed primary antibody and preimmune serum.

RESULTS

Quantification of aFGF in Ocular Tissues

The sensitivity and specificity of EIA are presented in Figure 1. The minimal detection level was 252 pg/ml, with 50% sensitivity at 1.48 ng/ml of aFGF. At this level of sensitivity, the aFGF content of one eye was adequate for quantification.

The quantification of affinity-purified immunoreactive aFGF in ocular tissues is presented in Table I (sclera, choroid, and retinal pigment epithelium were extracted whole).

Immunohistological Studies

Immunolocalization was achieved using a second antibody labeled with a fluorochrome (FITC), an enzyme (peroxidase), or colloidal gold (silver enhancement as

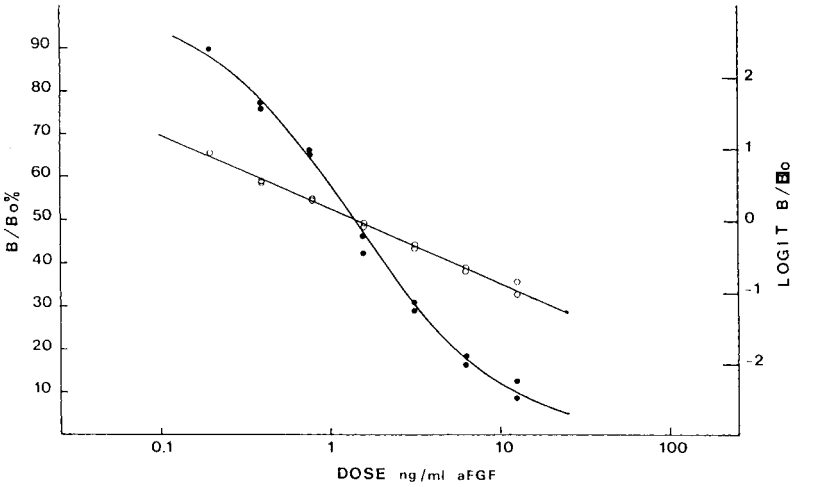

Fig. 1. Standard curve of AchE-aFGF (EIA). Displacement curve of AchE-aFGF in EIA (●) generated by standard aFGF [13] and corresponding linear log-log scale representation (○). The maximal sensitivity (defined as 50% B/BO) was 1.48 ng/ml and the minimal detection level (defined as 80% B/BO) was 252 pg/ml.

TABLE I. aFGF Distribution in Ocular Tissues*

Ocular tissue	Total content in one eye (ng)	ng of aFGF per g of tissue
Cornea	928	412
Aqueous humor	80	35
Lens	249	115
Vitreous body	4,790	295
Retina	243	479
Pigment epithelium choroid and sclera	1,376	15
Optic nerve		818

*Enzyme Immunoassays were performed with polyclonal anti-aFGF. Sclera, retinal pigment epithelium, and choroid were dissected whole. Weight of aqueous humor was calculated from volume measurements assuming a density of 1. The mean values from three independent experiments are given. Decreasing amounts of aFGF were noted from the optic nerve to the aqueous humor through retina, vitreous body, lens. aFGF was present in comparable amounts in the cornea and retina.

described in methods). Positive immunoreactions were noted at the same sites with these different techniques, whatever the treatment of sections.

Vitreous body and retina. Immunoperoxidase staining of a whole section of human retina is shown in Figure 2a–c. Generalized labeling of most cells can be seen at two magnifications (Fig. 2a, b), whereas no labeling was noted in the control with aFGF-absorbed primary antibodies (Fig. 2c).

A more detailed study of the different layers of the retina is presented in Figure 3a–c. Figure 3a shows marked immunoreaction of axon fibers; Figure 3b shows diffuse labeling of ganglion cells and intense labeling of some isolated cells in the plexiform layers. The intense labeling seen in Figure 3c is located in the inner and outer segments of the photoreceptors. Immunoperoxidase staining of the inner part of the retina (Fig. 4) confirmed the staining of the internal limiting membrane, nerve fibers, ganglion cells, stout columns, and footplates of the Müller cells (glial). Gold staining of the same region (Fig. 5) was noted in the nerve fiber layer and detached vitreous body.

aFGF in cornea and lens. Immunofluorescence of a healthy human cornea is presented in Figure 6a, b. Intense labeling was clearly located in the cytoplasm of the basal epithelial cell layer. Diffuse labeling was also seen throughout the epithelium. No significant aFGF immunoreaction was observed in the stroma, basal membranes (Bowman and Descemet), and endothelium (not shown).

Immunoperoxidase localization of aFGF in the lens (not shown) indicated that only the lens epithelium (i.e., the anterior cell layer beneath the anterior capsula, including the equatorial zone) contained significant amounts of immunoreactive material.

DISCUSSION

The availability of specific aFGF antibodies enabled us to perform rapid quantitative and qualitative studies in normal and pathological tissues.

The results presented here illustrate the complementarity of two techniques: the almost ubiquitous distribution of aFGF in ocular tissues illustrated by EIA and by histological staining.

The distribution of FGF in various ocular tissues has been reported and quantified by biological assay in previous studies. However, at that time, EDGF/FGF was partially purified and the existence of two biologically and chemically distinct forms of FGF was not suspected.

More recently, the purification of both forms of factors from the retina have enabled us to quantify the FGF content in the retina by biochemical and biological assays [8]. Results obtained with these assays indicated a content of 0.25 μg of pure, biologically active aFGF per 1,000 retinas [3, 8]. Several other groups reported similar values of FGF activity in the retina and vitreous body [9, 11, 20]. The value obtained for retina with EIA was almost the same (Table I). It is worth noting that this high correlation between biochemical and immunological quantification was obtained only after heparin-Sepharose chromatography of the homogenized tissue extracts. EIA of crude extracts routinely yielded higher and less reproducible values (not shown), although no cross-reactivity (below 8/1,000) with bFGF could be detected.

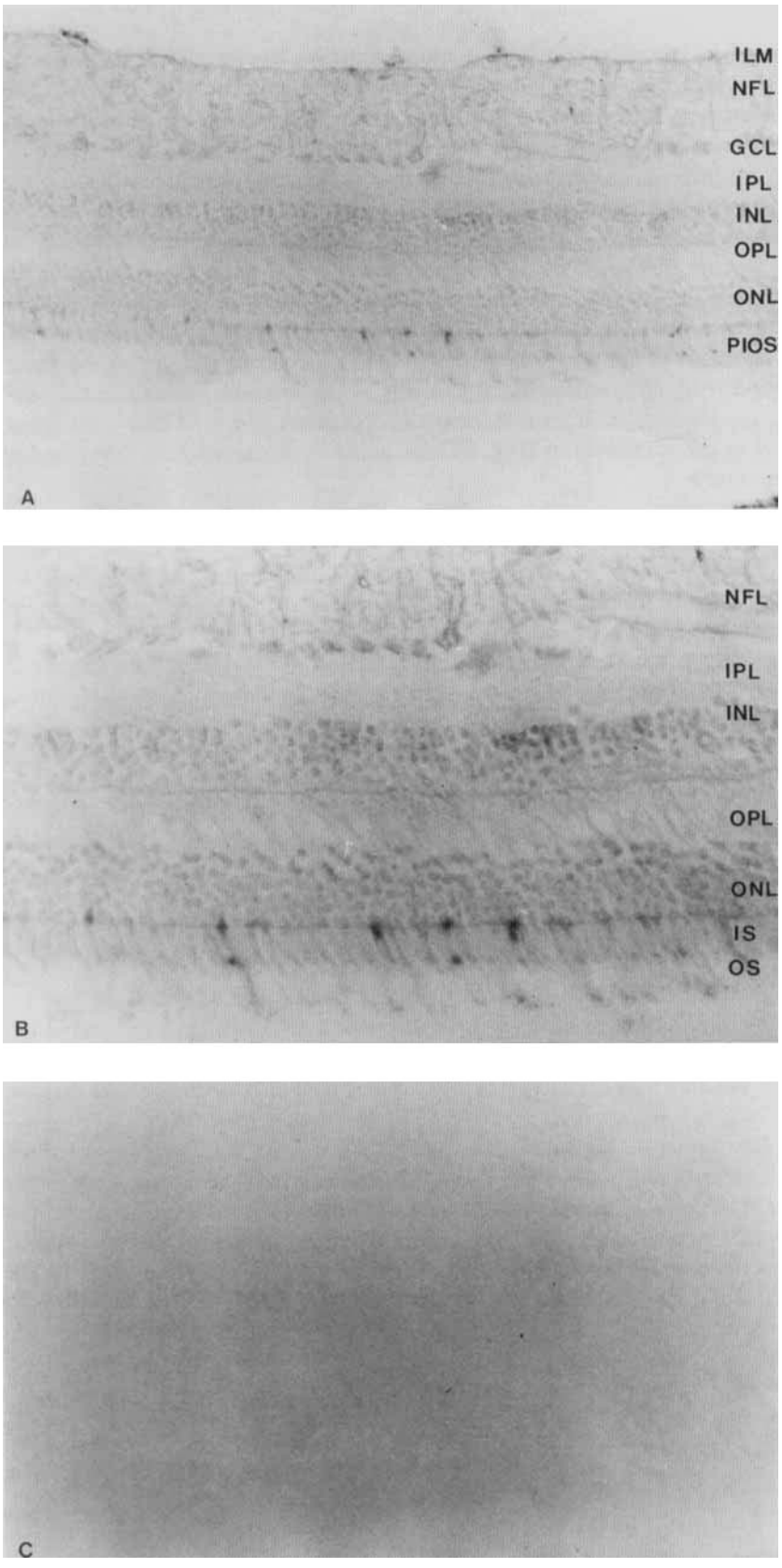

Figure 2.

Vitreous body and retina contained high levels of aFGF and immunohistological studies indicated extensive distribution, with 3 regions of higher concentration in the retina. The diffused distribution in the vitreous body could be due to postmortem or artefactual diffusion from the retina or from intravitreous regions containing higher concentrations. This is unlikely, however, as no variation of immunostaining could be detected in the vitreous body and also because aFGF and bFGF for biochemical analysis were purified from vitreous body collected within a few minutes of death (Mereau A., unpublished results).

The ubiquitous presence of aFGF immunoreactive material in the retina and the association of high concentrations of this material with specific regions suggest a possible physiological function for aFGF.

In agreement with previous reports of the presence of aFGF in brain neurons, aFGF was associated with the axon fibers of the neuroretina [21, 22, 23]. This neuronal localization of both acidic and basic FGF, and the in vitro studies [24, 25] indicating that these factors are necessary for growth and survival of neurons, suggest a similar role in vivo. More intriguing is the very intense staining of the inner and outer segments of the photoreceptors.

The presence of bFGF associated with photoreceptors purified by differential centrifugation has already been reported in the bovine retina [26]. However, disruption of the retina could induce the release of bFGF from other regions and nonspecific association with photoreceptors [27]. The presence of bFGF in retinoblastoma has also been reported [28]. Because retinoblastoma is considered to be derived from photoreceptor cells, it has been suggested that photoreceptors could be the source of bFGF in the normal retina. However, these authors [28] clearly ruled out the presence of aFGF in retinoblastoma. Therefore, our observations also indicate either that retinoblastoma cells derive only from a subset of non-aFGF-producing photoreceptor cells or that aFGF gene expression is lost through transformation or during tissue culture.

In the studies presented here, cross-reactivity between aFGF and bFGF has been excluded by ELISA. However, bFGF may have in situ cross-reactive epitopes that are undetected by ELISA. This possibility is unlikely, however, because absorbed primary aFGF antibodies were nonreactive, and retinal pigment epithelium (RPE) could not be immunostained with aFGF antibodies. This absence of the immunodetection of RPE is in good agreement with reports in which nontransformed RPE cells in tissue culture have been shown to synthesize bFGF only [29].

In previous studies, no significant amounts of growth factor activity were found in the lens [2]. The fact that immunoreactive aFGF material is associated with the lens epithelium in the equatorial zone and beneath the anterior capsula, but not with lens fibers, correlates well with what is known of the capacity of these cells to proliferate during lens growth.

Fig. 2. Immunoperoxidase localization of aFGF in human retina. **A,B:** Widespread positive immunoreaction in the different layers of the retina: ILM, inner limiting membrane; NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PIOS, photoreceptor inner and outer segments; IS, inner segment; OS, outer segment. A, ×250; B, ×400. **C:** Negative control: aFGF-absorbed primary antibody. ×250.

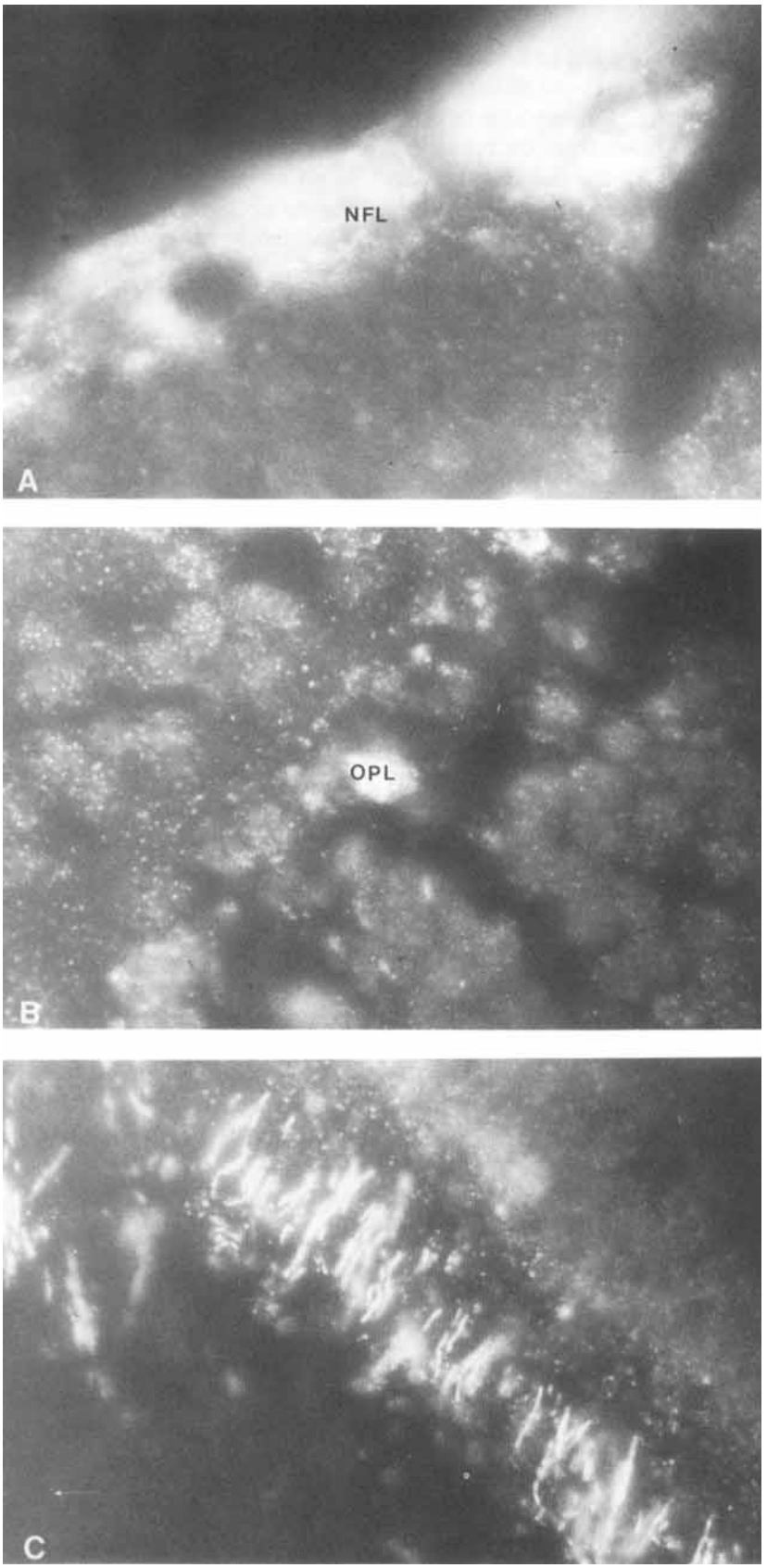

Figure 3.

Interestingly, the addition of labeled aFGF or bFGF to frozen sections of cornea or lens showed that lens capsula and Bowman and Descemet membranes contain receptors to FGF [30]. Under the present experimental conditions, we were unable to visualize significant amounts of FGF associated with these membranes, which suggests either that the antigen (aFGF) is inaccessible to the antibody or that antigen concentration is inadequate. Because it is known that FGF can be removed from Descemet membrane by heparinase treatment [30], it is likely that immunoreactive epitopes hidden in these basement membranes are inaccessible to aFGF antibodies.

Most of the aFGF immunoreactive material found in corneal epithelial lens cells seems to be associated with the basal cell layer, although positive staining was also detected throughout the epithelium and, to a lesser extent, in the corneal stroma.

Corneal epithelial cells are among those with the highest rate of renewal. EDGF (partially purified aFGF and bFGF) has been shown to accelerate reepithelialization of cornea in experimentally induced corneal ulcers [32] as well as in pathological ulcers [33].

The same results also have been obtained with purified aFGF and bFGF [34] and with epidermal growth factor (EGF) [32]. However, in studying EGF cross-reacting material in corneal epithelium, no staining was detected (unpublished results).

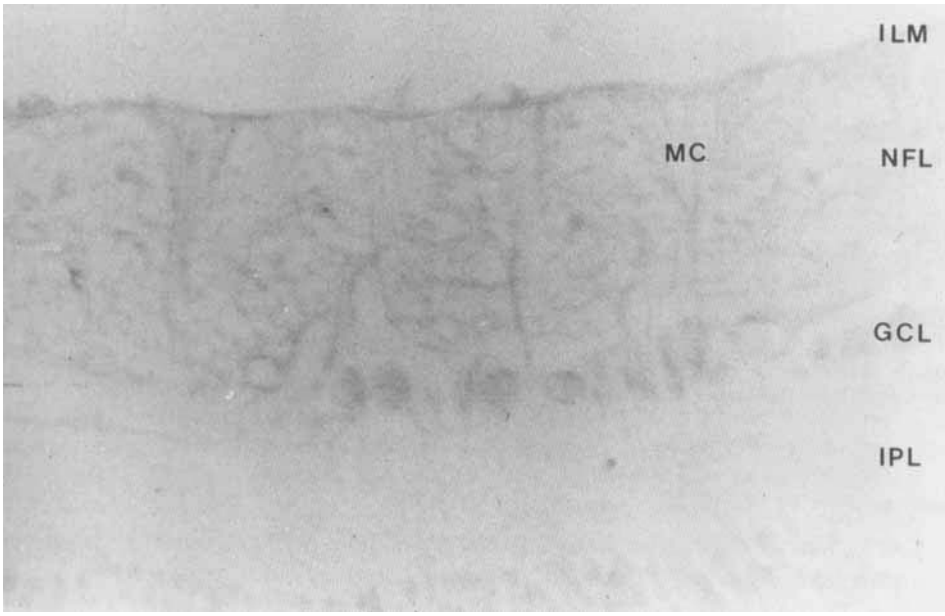

Fig. 4. Immunoperoxidase localization of aFGF in human retina. ×800. Positive immunoreaction in inner limiting membrane (ILM), axons of nerve fiber layer (NFL), ganglion cells (GCL), and the footplates of Müller cells (MC); IPL, inner plexiform layer.

Fig. 3. Immunogold (with silver enhancement) localization of aFGF in bovine retina. **A:** Labeling is apparent over the nerve fiber layer (NFL). **B:** Labeling is apparent in the outer plexiform layer (OPL). **C:** Labeling is apparent in the inner and outer segments of photoreceptors. ×1,000.

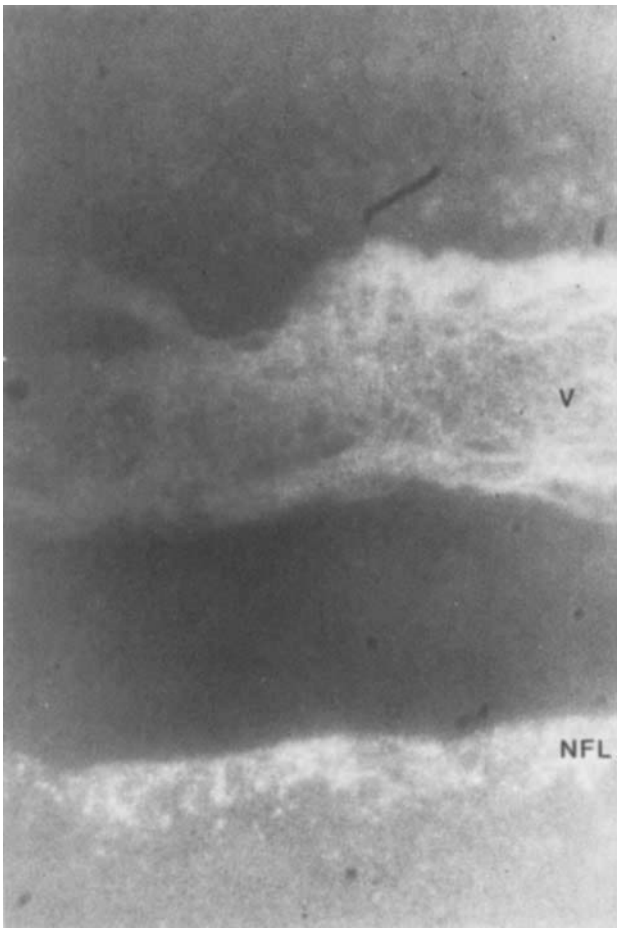

Fig. 5. Immunogold (with silver enhancement) localization of aFGF in bovine retina. Labeling of nerve fiber layer (NFL) and detached vitreous body (V). ×400.

It is therefore tempting to suggest that aFGF (and perhaps bFGF) is the growth factor implicated in vivo in the control of epithelial corneal cell proliferation.

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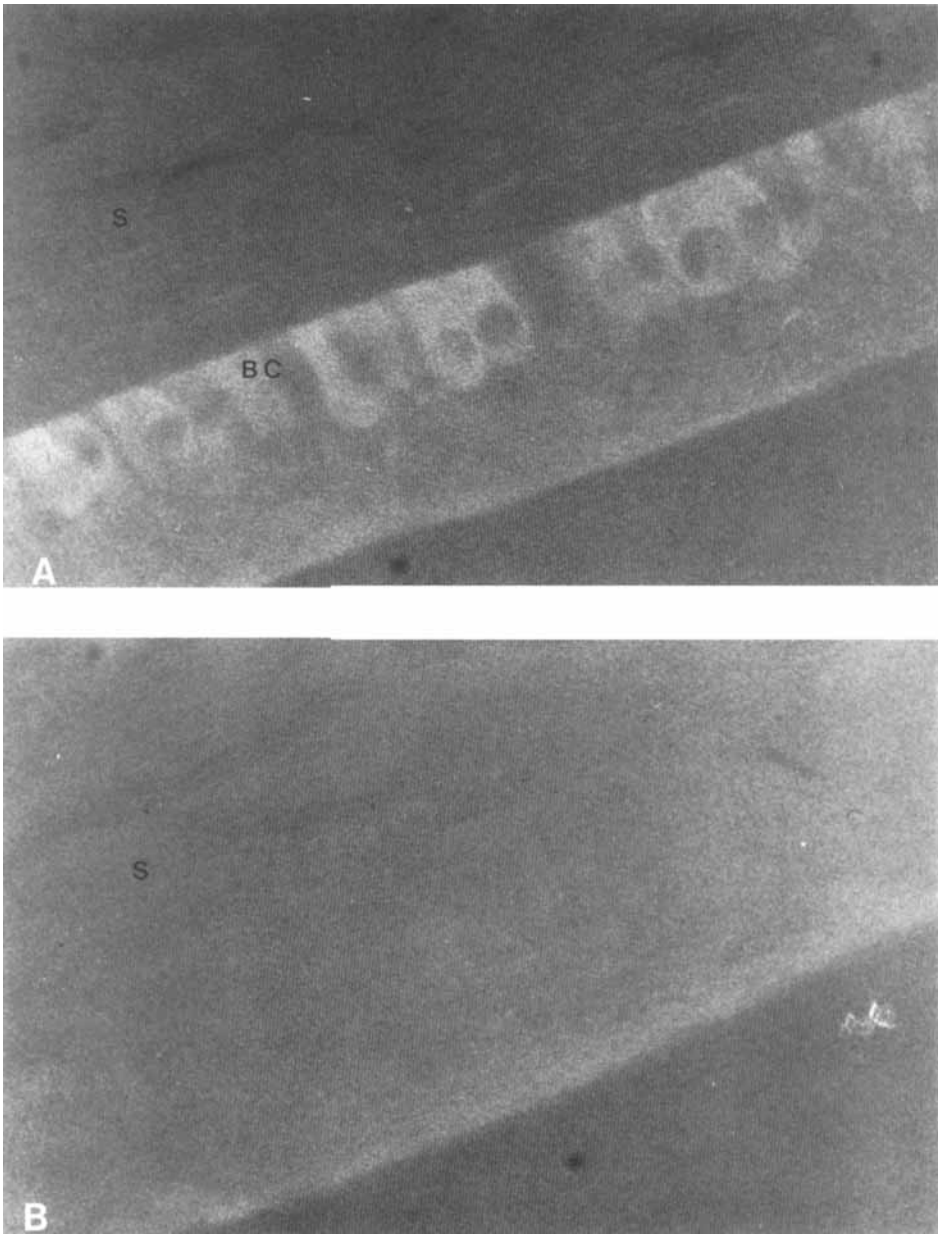

Fig. 6. Immunofluorescence localization of aFGF in human cornea. Tissue fixed in paraformaldehyde and processed for immunofluorescence as described in Materials and Methods. **A:** Intense labeling in the cytoplasma of epithelium basal cells (BC), stroma (S). **B:** negative control; aFGF absorbed primary antibody. ×400.

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