Exp. Eye Res. (1996), **62**, 309–324

Fate of Biotinylated Basic Fibroblast Growth Factor in the Retina Following Intravitreal Injection

GEOFFREY P. LEWIS[a*], STEVEN K. FISHER[a,b], AND DON H. ANDERSON[a]

[a] *Neuroscience Research Institute and* [b] *Department of Biological Sciences University of California, Santa Barbara, CA 93016*

(*Received Cleveland 15 August 1995 and accepted in revised form 22 November 1995*)

Exogenous basic fibroblast growth factor (bFGF) stimulates proliferation of non-neuronal retinal cells in vivo. To help understand how this proliferative effect is mediated, we followed the fate of biotinylated bFGF after injection into the vitreous of normal rabbit eyes. The retinal distributions, binding, and processing of biotinylated bFGF (bFGF-biotin) was examined from 2 hr to 7 days after intravitreal injection using laser scanning confocal microscopy, electron microscopy and Western blot analysis. At 2 hr, bFGF-biotin was detected throughout the extracellular space and on retinal basement membranes. At 6 hr, discrete punctate material first appeared within the cytoplasm of Müller cells, astrocytes, endothelial cells, retinal pigment epithelial (RPE) cells, and ganglion cells. Labeling was also present in the invaginations of the photoreceptor synaptic terminals at this time. This general pattern persisted up to 4 days after injection but was greatly attenuated by post-injection day 7. Labeling in the inner retina decreased progressively over the seven days; whereas labeling in the outer retina, primarily within the RPE, increased at 4 days post-injection and then gradually decreased to nearly undetectable levels by 7 days. Western analysis of retinal protein homogenates following injection showed that an 18 kDa component representing intact bFGF, can be identified up to 1 week following injection. This component, as well as a 15 and 9 kDa biotinylated fragment, showed a progressive reduction during the one week post-injection period. Cross-linking experiments demonstrated that bFGF-biotin binds to three putative receptors with approximate molecular weights of 54, 62, and 110 kDa. These data are consistent with binding of exogenous bFGF to: (a) low affinity bFGF receptors associated with retinal basement membranes; (b) invaginations at the base of photoreceptor synapses; and (c) putative high affinity bFGF receptors on the plasma membranes of glial cells, endothelial cells, RPE cells and ganglion cells. bFGF-biotin apparently binds to, and is then internalized by, the same non-neuronal cell types that are stimulated to proliferate following retinal injuries such as detachment.

Key words: bFGF; retina; Müller cell; ganglion cell; astrocyte; endothelial cell; biotinylation; receptor.

1. Introduction

Basic fibroblast growth factor (bFGF) is a multifunctional growth factor that has been implicated in a number of proliferative, regenerative, and survival-related cellular events in the central nervous system (see Baird and Bohlen, 1990; Hicks et al., 1990, for reviews). Levels of endogenous bFGF have been shown to increase at sites of injury suggesting that its release and/or activation from intracellular and extracellular sources may play an important regulatory role in this process (Finklestein et al., 1988; Logan 1990, 1992; Frautschy et al., 1991; Eckenstein et al., 1991; Gomez-Pinilla et al., 1992; Kostyk et al., 1994). Exogenous administration of bFGF has been shown not only to mimic injury-related events but also to aid in neuronal survival after injury. In the retina, exogenous bFGF induces proliferation in non-neuronal retinal cells (de Juan et al., 1990; Eclancher et al., 1990; Lewis et al., 1992), and increases the expression of intermediate filament proteins in retinal glia (Morrison et al., 1985; Perraud et al., 1988; Lewis et al., 1992). It also prevents or delays photoreceptor cell degeneration and loss in retinal dystrophic rats (Faktorovich et al., 1990) and in light-damaged retinas (Faktorovich et al., 1992; La Vail et al., 1992), promotes the regeneration of ganglion cell axons after optic nerve transection (Sievers et al., 1987), and stimulates regeneration of whole retina in retinectomized chick embryos (Park et al., 1989). Understanding the steps by which bFGF is processed by retinal cells in vivo is likely to be important in revealing a mechanism by which these effects occur.

Basic FGF is known to bind to both high affinity cell surface tyrosine kinase receptors, and to low affinity receptors in the extracellular matrix. Binding of bFGF to the latter heparan sulfate-containing molecules appears to be necessary for functional interaction with the high-affinity receptors (Yayon et al., 1991; Rapraeger et al., 1991). As with most growth factors, the receptor-ligand complex is then internalized. Unlike other growth factors, however, which undergo rapid degradation by lysosomes, internalized bFGF appears to be somewhat more stable, and is processed into a subset of smaller peptides that remain intact for

* For correspondence at: Neuroscience Research Institute, University of California, Santa Barbara, CA 93106, U.S.A.

0014–4835/96/030309+16 $18.00/0

some time (Moscatelli et al., 1988; Moenner et al., 1989; Walicke and Baird, 1991; Hawker and Granger, 1992; Gonzalez et al., 1991; Ferguson et al., 1990).

The retina contains high concentrations of bFGF (Glaser et al., 1980; Baird et al., 1985); immunolocalization and in situ hybridization studies confirm that it is present at various cellular and extracellular sites (Hanneken et al., 1989; Hageman et al., 1991; Gao and Hollyfield, 1992; Hanneken and Baird, 1992; Morimoto et al., 1993; Noji et al., 1990). When ^{125}I bFGF is applied to sections, binding is detected on retinal basement membranes, and in association with the inner and outer plexiform layers (Jeanny et al., 1987; Fayein et al., 1990; Cirillo et al., 1990; Faucheux et al., 1991). Putative high affinity bFGF receptors have been identified on a number of retinal cell types including photoreceptors (Mascarelli et al., 1989; Fayein et al., 1990; Faucheux et al., 1991; Raymond et al., 1992), Müller cells (Mascarelli et al., 1991), retinal pigment epithelial cells (Sternfeld et al., 1989; Fayein et al., 1990; Rakoczy et al., 1993) and ganglion cells (Ferguson et al., 1990; Toriglia et al., 1994).

As in the rest of the CNS (Giulian et al., 1989; Bradshaw and Cavanaugh, 1990; Puro et al., 1990; Sporn and Roberts, 1990), the retina's response to injury is characterized by gliosis, microglial activation, cell proliferation and macrophage invasion (Anderson et al., 1983; Erickson et al., 1983; Hjelmeland and Harvey, 1988; Fisher et al., 1991). Previously, we showed that the proliferative and gliotic responses can be mimicked by injecting bFGF into an otherwise normal eye (Lewis et al., 1992). In order to more clearly establish a direct link between injected bFGF and these cellular events, we traced the fate of bFGF-biotin following injection of the factor into the vitreous. The results show that the injected factor diffuses into the neural retina where it can be detected for up to 7 days. It apparently binds to, and is then internalized by, the same non-neuronal cell types that are later induced to proliferate in what appears to be a receptor-mediated fashion.

2. Materials and Methods

Biotinylation of bFGF

We used a slight modification of the procedure described by Lee et al., (1989) to biotinylate the four cysteine residues contained in human recombinant bFGF. Briefly, one hundred micrograms of human recombinant bFGF (PeproTech Inc., Rocky Hill, NJ, U.S.A. or Scios Nova, Mt. View, CA, U.S.A.) was dissolved in 200 μl of 100 mM sodium phosphate, pH 7·0. To this solution was added 10 μl of a 1 mg ml^{-1} solution of biotin (long arm) maleimide (Vector Labs; Burlingame, CA, U.S.A.) in dimethylformamide. Biotinylation was allowed to proceed for 2·5 hr at room temperature and for a succeeding 20 hr at 4°C. The reaction was stopped by adding 10 μl of a 1 mg ml^{-1} solution of cysteine in distilled water. Using this procedure, biotinylated bFGF has been shown to be indistinguishable from non-biotinylated bFGF in its ability to bind the high affinity receptor, to inhibit high affinity receptor binding, and to stimulate phosphorylation of the high affinity receptor (Lee et al., 1989).

Intraocular Injection of bFGF-Biotin

Adult New Zealand White rabbits were anesthetized with 20 mg kg^{-1} Ketaset (ketamine HCL; Parke-Davis, Morris Plains, NJ, U.S.A.) and 5 mg kg^{-1} Rompun (xylazine; Miles Labs, Shawne, KS, U.S.A.) intramuscularly. The right eyes received a single injection of 5 μg of bFGF-biotin in 50 μl of balanced salt solution (BSS; Alcon, Forth Worth, TX, U.S.A.) into the vitreous cavity using a 30 gauge needle. As controls, some eyes received a single injection of 5 μg of biotinylated dextran (dextran-biotin; 10 kDa; Molecular Probes, Eugene, OR, U.S.A.), free biotin (Vector Labs), or no injection at all. Animals were killed 2 hr, 6 hr, 18 hr, 48 hr, 4 days and 7 days after the intraocular injection by an overdose of sodium pentobarbital. Two animals were done at each time-point for the bFGF-biotin, dextran-biotin and free biotin. No signs of intraocular inflammation were observed using indirect ophthalmoscopy at any time following injection.

Confocal Fluorescence Microscopy

Eyes that had been injected with bFGF-biotin were immersion fixed in 4% paraformaldehyde and 0·1% glutaraldehyde in sodium cacodylate buffer (0·1 N; pH 7·4). Initially samples were prepared after a 2 hr fixation. Subsequent samples were prepared after varying lengths of fixation. It was found that storing the tissue in the fixative did not affect the ability of the avidin to bind to the bFGF-biotin receptor complex. After fixation, retinal tissue samples approximately 2 mm square were excised and embedded in 5% agarose (Sigma, St. Louis, MO, U.S.A.) in PBS as described by Matsumoto and Hale (1993). Sections 100 μm thick were cut on a Vibratome (Technical Products International, Polysciences, Warington, PA, U.S.A.) and incubated in avidin conjugated to the fluorochrome Cy3 (1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, U.S.A.) in PBS containing 0·1% Triton X-100 overnight at 4°C on a rotator. Uninjected control retinas were also labeled with avidin-Cy3 to test for endogenous biotin binding sites. The sections were then rinsed in PBS, mounted in 5% n-propyl gallate in glycerol and viewed using a laser scanning confocal microscope (Bio-Rad 500, Hercules, CA, U.S.A.).

A competition assay was used to show that

unlabeled bFGF can compete for the same binding sites on sections as those occupied by bFGF-biotin. Retinal sections from normal uninjected rabbit, cat and monkey eyes were fixed, embedded, and cut on a Vibratome as described above. Twenty ng ml^{-1} of bFGF-biotin was then applied directly to the sections and incubated overnight at 4°C. Some sections were also incubated with a 50-fold excess of non-biotinylated bFGF (1000 ng) along with the biotinylated-bFGF. The next day the sections were rinsed in PBS and incubated in avidin-Cy3 (1:200) overnight at 4°C. After rinsing, the sections were mounted and viewed as described above.

Electron Microscopy

Vibratome sections, 100 μm thick, cut from the same eyes that were injected with bFGF-biotin and used for the confocal microscopy, were incubated in 0·3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidases. Sections were then rinsed in PBS after which avidin-HRP (1:50, Vector Labs) was added overnight at 4°C. The next day sections were rinsed in Tris buffer (0·1 M, pH 7·6) and incubated in diaminobenzidene (1 mg ml^{-1}) in 0·1 M Tris buffer containing 0·02% hydrogen peroxide for 7–15 min. Sections were again rinsed in buffer and fixed in 1% paraformaldehyde and 1% glutaraldehyde overnight at 4°C. After rinsing, the sections were post-fixed in 1% osmium tetroxide for 1 hr, rinsed in distilled water, dehydrated in graded ethanol and water, and placed in propylene oxide. The sections were then embedded in Spurs resin (Polysciences) according to the manufacturer's instructions. Thin (90 nm) sections were cut with a diamond knife, placed on grids, and viewed on a Philips CM-10 electron microscope.

Biochemistry

Two animals were killed 2 hr, 18 hr, 48 hr, 4 days and 7 days after an injection of 5 μg of bFGF-biotin into the vitreous cavity. Retinal protein homogenates were prepared for SDS–PAGE and Western blot analysis. They were homogenized at 4°C in 0·02 M PBS containing 0·1 M phenyl-methylsulfonylfluoride, 10 mM EDTA, 0·5% Triton X-100 and 2·55% SDS. Protein concentrations were determined by the BCA Protein Assay (Pierce Chemical Co., Rockford, IL, U.S.A.). Fifty migrograms of protein was loaded in each lane of a 16·5% polyacrylamide gel. The separated retinal proteins were transferred to nitrocellulose paper and blocked in PBS/BSA (2·5%) for 1 hr. Blots were washed in PBS containing 0·1% Tween (TPBS) and incubated with streptavidin–alkaline phosphatase (avidin-AP; 1:3000; Bio-Rad) for 1 hr. After washing in TPBS and PBS, the blots were placed in developing reagent (NBT-BCIP, Pierce, Rockford, IL, U.S.A.) for 15 min, rinsed in distilled water and air dried.

bFGF-Biotin Receptor Cross-linking

Two animals were killed 2 hr and 18 hr after injection of 5 μg of bFGF-biotin into the vitreous. Following enucleation, retinas were immediately peeled away from the retinal pigment epithelium, rinsed three times in ice-cold Hepes buffer, and placed in 400 μM disuccinimidyl suberate (DSS) i DMSO for 30 min at 4°C. The reaction was stopped by adding 20 mM Tris NaCl for approximately 10 min. The retinas were then homogenized, and protein concentrations were determined. Fifty micrograms of protein was loaded in each lane of a 16·5% SDS–PAGE gel as described above. Separated proteins were then transferred to nitrocellulose and probed with avidin-AP to visualize the biotinylated components as described above.

In Vivo Cell Proliferation Assay

The retinal cell proliferation assay described in Lewis et al. (1992) was employed. In brief, 200 μCi of ^{3}H-thymidine (2·59 TBq mmol^{-1}; Amersham, Arlington Heights, IL, U.S.A.) in 100 μl of BSS was injected into the vitreous cavity of two rabbits 3 days following a similar intravitreal injection of 5 μg bFGF-biotin. Animals were then killed by intravenous injection of sodium pentobarbital 24 hr later. Following enucleation and dissection of the anterior segments, the posterior segments were immersion-fixed for 1 hr in 1% paraformaldehyde and 1% glutaraldehyde in 0·086 M sodium phosphate buffer (pH 7·2). The tissue was then post-fixed in 2% uranyl acetate in maleate buffer for 2 hr, dehydrated in increasing concentrations of methanol and embedded in LR White resin (Polysciences). One-micrometer-thick sections were placed on glass slides and dipped under sodium vapor illumination into a 1:1 mixture of Kodak NTB-2 emulsion (Rochester, NY, U.S.A.) and distilled water at 43°C. The autoradiograms were exposed for 7 days at 4°C, developed for 2 min in full-strength D-19 (Kodak) at 20 °C, washed, fixed, and stained with methylene blue-azure II.

Neurite Extension Assay

PC12 cells (1×10^4 per cm^2) were plated on polylysine coated eight-well tissue culture plates. The cells remained in Dulbeco's modified Eagle's media, which was supplemented with 5% calf serum and 5% horse serum, for 24 hr at which time 20 ng ml^{-1} of native or biotinylated bFGF was added to the wells every other day. Biotinylated bFGF was added to four of the wells, two wells were treated with non-biotinylated bFGF, and two wells contained no bFGF.

After 7 days in culture, each of the wells was photographed, and representative measurements of neurite outgrowth were collected.

Cell Proliferation Assay

Induction of cell proliferation by human recombinant bFGF and bFGF-biotin was evaluated in a 3T3 cell proliferation assay as described by Klagsburn (1978).

3. Results

Biological Activity of bFGF-Biotin

We evaluated the biological activity of bFGF-biotin under both in vitro and in vivo conditions. In an in vivo retinal cell proliferation assay (Lewis et al., 1992), tissue autoradiograms showed that cells representing all non-neuronal cell types within the retina were induced to proliferate following intravitreal injection of bFGF-biotin. These included capillary endothelial cells, pericytes, astrocytes, Müller cells, microglia and RPE cells [Fig. 1(A)]. Control eyes ($n = 2$) that received an intravitreal injection of balanced salt solution instead of bFGF-biotin showed virtually no ^{3}H-thymidine labeled cells [Fig. 1(B)]. These results conform with previously published results using non-biotinylated recombinant bFGF (Lewis et al., 1992), and demonstrate that bFGF-biotin retains biological activity sufficient to induce proliferation following intravitreal injection.

The biological activity of bFGF-biotin was also demonstrable in two in vitro assays. In a neurite outgrowth assay (Togari et al., 1985), the induction of neurite outgrowth by bFGF-biotin in PC12 cells was indistinguishable from the non-biotinylated factor. After 7 days in culture, cells treated with biotinylated bFGF had extensive neuritic processes, as did the cultures containing native bFGF. However, neurites were absent in cultures that were not exposed to bFGF (data not shown). Furthermore, induction of cell proliferation by bFGF-biotin was not significantly different from that induced by native bFGF in a 3T3 cell proliferation assay (Klagsburn, 1978). Both biotinylated and non-biotinylated factor had activity levels in the range of 35–41 units ng^{-1} bFGF where 1 unit of 3T3 activity is defined as the amount of bFGF needed to elicit half-maximal ^{3}H-thymidine incorporation (maximal ^{3}H-thymidine incorporation is obtained by adding 20% serum).

Diffusion, Binding and Internalization of bFGF-Biotin

Two hours after injection of bFGF-biotin, labeling appears to be primarily extracellular, extending throughout the extracellular space and terminating at the outer border of the inner plexiform layer (IPL) [Fig. 2(A)]. The labeling distinctly outlines processes in the vitreal one-third of the retina, defining profiles of ganglion cells and their axons, astrocytes, and the endfeet of the Müller cells, i.e. the radial glia that extend the entire width of the retina. At this timepoint, bFGF-biotin binding is associated with the basement membranes surrounding the blood vessels overlying the optic nerve axon bundle in the vitreous, as well as the basement membrane that lines the vitreal border of the retina (i.e. the inner limiting membrane; ILM). The endothelial cell surfaces within these vessels are also labeled. Labeling of the inner plexiform layer (IPL) is concentrated in three distinct strata, with labeling most intense in the vitreal tier.

At 6 hr after injection, the labeling now appears at the outer limiting membrane and within the RPE cytoplasm. Widespread intracellular punctate labeling

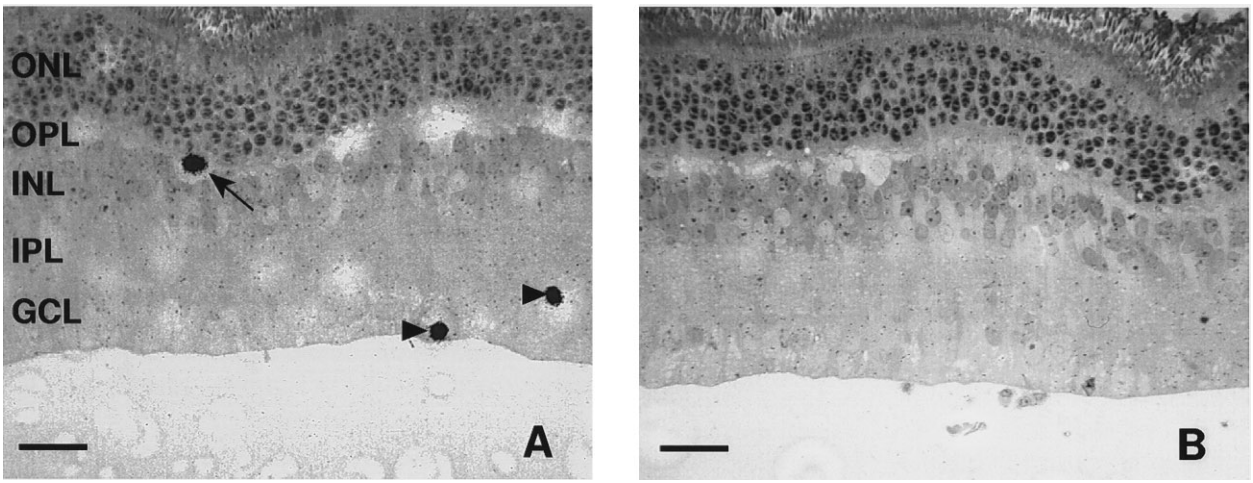

FIG. 1. ^{3}H-thymidine autoradiography of retinal sections after intravitreal injection of bFGF-biotin. (A) 4 days after injection of bFGF–biotin all non-neuronal cell types were found to incorporate 3H-thymidine demonstrating that biotinylation of bFGF does not affect its biological activity. In this section a microglial cell (arrow) and two astrocytes (arrowheads) are shown labeled. (B) Control retina 4 days after injection of saline, instead of bFGF–biotin, no cells are labeled. (GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer.) Bar, 40 μm.

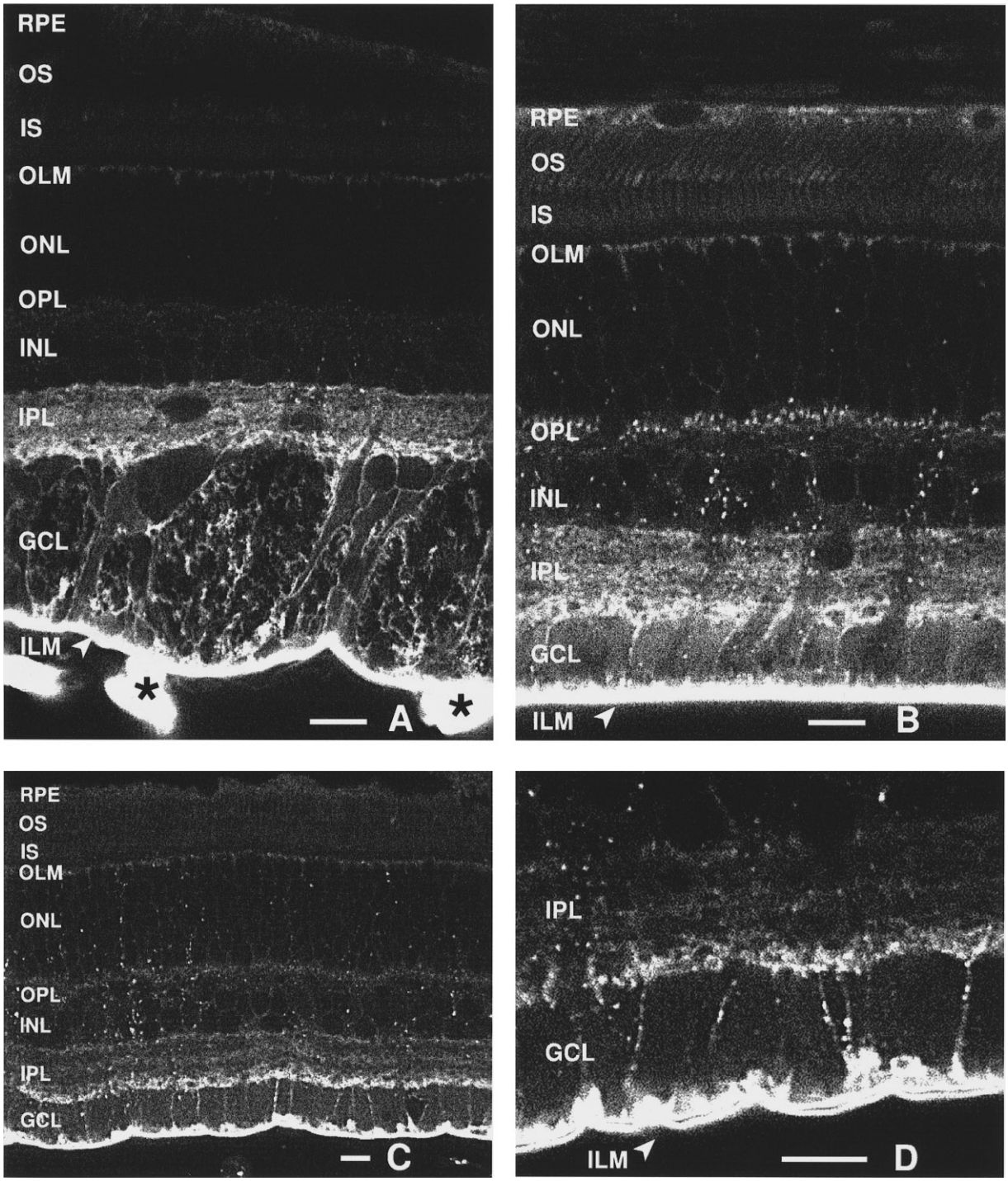

FIG. 2. bFGF–biotin localization on Vibratome sections of rabbit retina after intravitreal injection and viewed with the laser scanning confocal microscope. (A) 2 hr after injection, labeling occurs on the basement membranes of the vessels (*) in the vitreous and the inner limiting membrane (ILM; arrowhead), throughout the extracellular space and terminating at the outer border of the inner plexiform layer. The labeling distinctly outlines processes in the vitreal one-third of the retina, defining profiles of ganglion cells and their axons, the endfeet of the Müller cells, and astrocytes. The inner plexiform layer (IPL) displays a stratified labeling pattern. (B) 6 hr after injection, labeled punctate material appears primarily in the inner retina. The punctate labeling in the outer plexiform layer (OPL) corresponds closely with the distribution of the synaptic invaginations at the photoreceptor axon terminals. Diffuse labeling appears along the lateral borders of the Müller cell endfoot processes, within the Müller cell and RPE cytoplasm, and at the level of the IPL and OLM. (C) At 18 hr after injection, labeled punctate material appears in vertical columns that extend from the vitreal surface through the outer nuclear layer (ONL) corresponding to the distribution of the Müller cells. (D) Higher magnification reveals labeling within the endfeet cytoplasm of the Müller cells that is separate from the ILM. In addition, the cell surface labeling on the lateral borders of the Müller cells in the ganglion cell layer (GCL) consists of a string of beaded material. Tissue was taken from various retinal locations which accounts for the different appearance and thickness of the sections as well as the presence or absence of vessels. (INL, inner nuclear layer; OLM, outer limiting membrane; IS, inner segments; OS, outer segments; RPE, retinal pigment epithelium.) Bar, 20 μm.

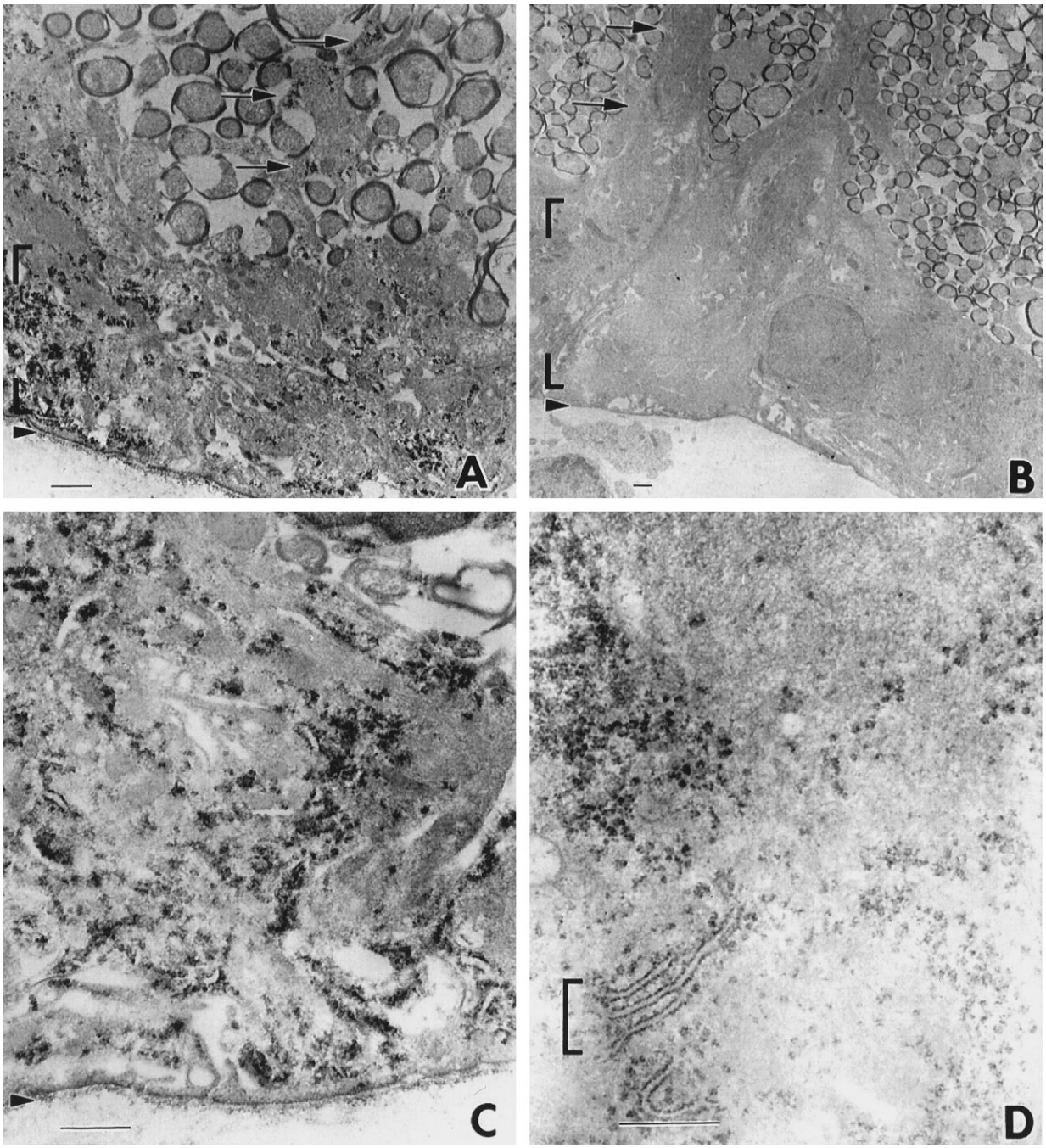

FIG. 3. Electron microscopic localization of injected bFGF-biotin on thin sections of rabbit retina. (A) Labeled material (appears black) is present in the endfoot region of the Müller cells (brackets) and in Müller cell processes (arrows) extending through the nerve fiber bundle (48 hr). The internal limiting membrane (ILM; arrowhead) is also labeled. (B) Control section of a rabbit retina that did not receive an injection of bFGF–biotin (48 hr). No labeled material is present within the cytoplasm of cells nor is the ILM labeled. (C) Higher magnification of labeled material, approximately 50–100 nm in diameter, present within a Müller cell endfoot and along the ILM (arrowhead; 18 hr). (D) High magnification of vesicular labeled material within the Müller cell cytoplasm. The rough endoplasmic reticulum (bracket) is not labeled but appears dark due to osmication of the tissue. Bar, 1 μm.

appears for the first time, primarily in the inner retina [Fig. 2(B)]. Diffuse labeling persists along the lateral borders of the Müller cell endfoot processes and within their cytoplasm. Punctate labeling is also present within the outer plexiform layer (OPL), and at higher magnification it appears that this labeling represents bFGF-biotin binding to invaginations at the rod and cone synaptic terminals (Figs 2(B) and 8(A) and (B)].

At 18 hr post-injection, the labeling pattern is essentially the same as that observed at the 6 hr timepoint: labeled punctate material appears in vertical 'columns' that extend from the vitreal surface through the ONL [Fig. 2(C)]. These columns correspond to the distribution of the Müller cell processes. At higher magnification, the labeling along the inner surface of the retina can be separated into two distinct

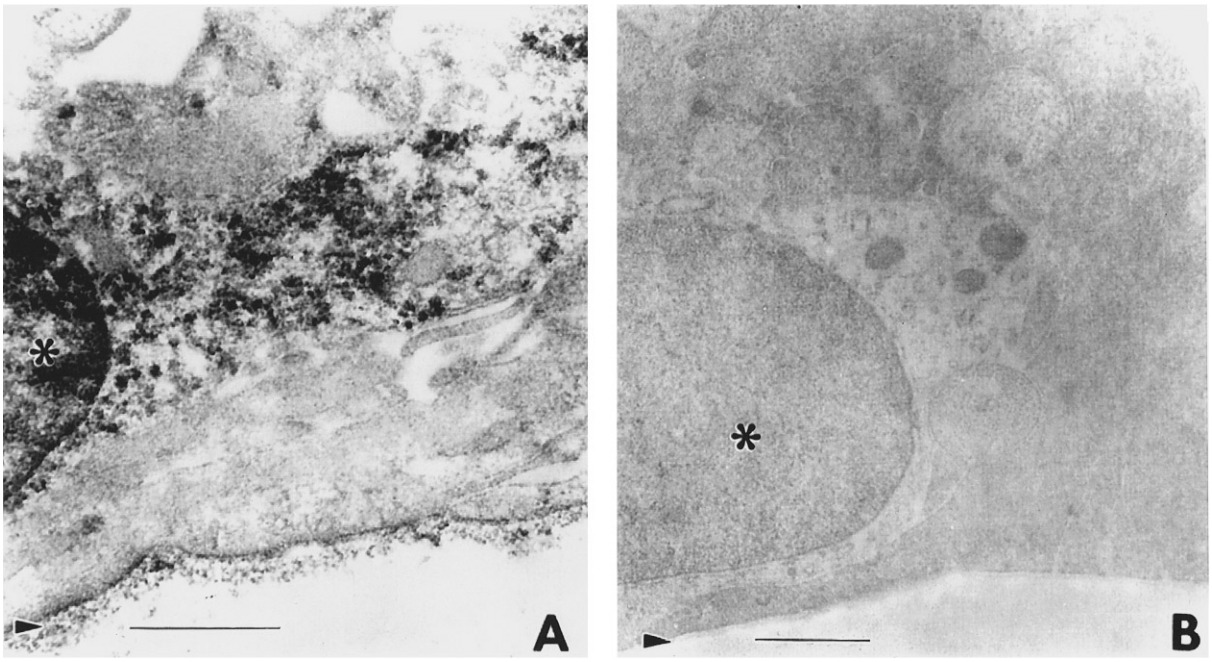

FIG. 4. Electron microscopic localization of injected bFGF–biotin on thin sections of rabbit retina. (A) An astrocyte nucleus (*) and its process are labeled (48 hr). (B) Control eye that did not receive an intravitreal injection of bFGF–biotin. No labeling is present in the astrocyte nucleus (*) or its process. Arrowhead, inner limiting membrane; bar, 1 μm.

domains: a narrow, clearly defined band that represents the inner limiting membrane and a broader, more diffuse, and less regular zone that appears to originate from labeling within the Müller cells' endfoot cytoplasm [Fig. 2(D)]. At the electron microscopic level the labeling within the cytoplasm of the astrocytes, the glia that surrounds the ganglion cell axons, and within the Müller cells, is resolved as discrete particles approximately 50–100 nm in diameter [Figs 3(A), (C) and (D) and 4(A)]. In addition, reaction product also appears within astrocyte nuclei [Fig. 4(A)]. Labeled 50–100 nm particles were absent in control eyes that were processed similarly, but had not received intravitreal injections of bFGF-biotin. [Figs 3(B) and 4(B)]. At the ultrastructural level, we were able to visualize only traces of reaction product at retinal locations distal to the IPL. Punctate material in the proximal portion of inner nuclear layer (INL), the site where Müller cell bodies reside, was resolved in the light microscopic level; however, it could not be detected at the electron microscopic level.

At 48 hr after injection of bFGF-biotin, the overall labeling pattern appears similar to that observed at 6 and 18 hr, although intracellular punctate labeling is more robust. Labeling occurs specifically in cell bodies within the INL [Fig. 5(A)], within ganglion cell bodies [Fig. 5(A)], in the cell bodies and the processes of astrocytes in the nerve fiber layer [Fig. 5(B)], throughout the Müller cell cytoplasm [Fig. 5(C)], and in the cell bodies and nuclei of endothelial cells [Fig. 5(B) and (C)]. The intense basement membrane labeling of the vasculature, however, often obscures the endothelial cell labeling. The labeling within these cells, therefore, is more easily identified on 0·5 μm thick resin embedded sections. [Fig. 6(A) and (B)]. No intracellular endothelial cell labeling is observed in control retinas that did not receive an injection [Fig. 6(C)]. The punctate labeled material frequently occurs in groups or clusters within the astrocytes' and Müller cells' cytoplasm [Fig. 5(B) and (C)]. Such clusters are observed at the 48 hr timepoint and at the 4 day timepoint in these glial cells [Fig. 7(A)], but they are notably absent by day 7 [Fig. 7(C)].

There is an apparent overall decrease in labeling of the inner retina from the nerve fiber bundle on the ONL at post-injection day 4 [Fig. 7(A)], but intense labeling is now detected in the RPE cytoplasm [Fig. 7(B)]. Labeling of the outer limiting membrane is also more intense at the 4 day timepoint [Fig. 7(B)]. However, there is no detectable labeling associated with interphotoreceptor matrix, with the basal lamina of the RPE, or the immediately adjacent choriocapillaris. No significant labeling is observed in the retina or the RPE at 7 days following injection [Fig. 7(C)].

To demonstrate the specificity of bFGF-biotin receptor binding, 20 ng ml^{-1} of bFGF-biotin or the same concentration plus a 100-fold excess of non-biotinylated bFGF was applied to retinal sections taken from normal rabbit, cat or monkey eyes. The ex vivo labeling pattern is similar to the pattern obtained following bFGF-biotin injection in vivo. In addition, the pattern of labeling is similar in all three species tested. Labeling is associated with the basements membranes of the ILM, vasculature, and Bruch's membrane as well as the IPL and OPL [Fig. 8(A) and (B); cat retina]. This labeling is blocked almost completely by the addition of excess unlabeled bFGF to the sections [Fig. 8(C)].

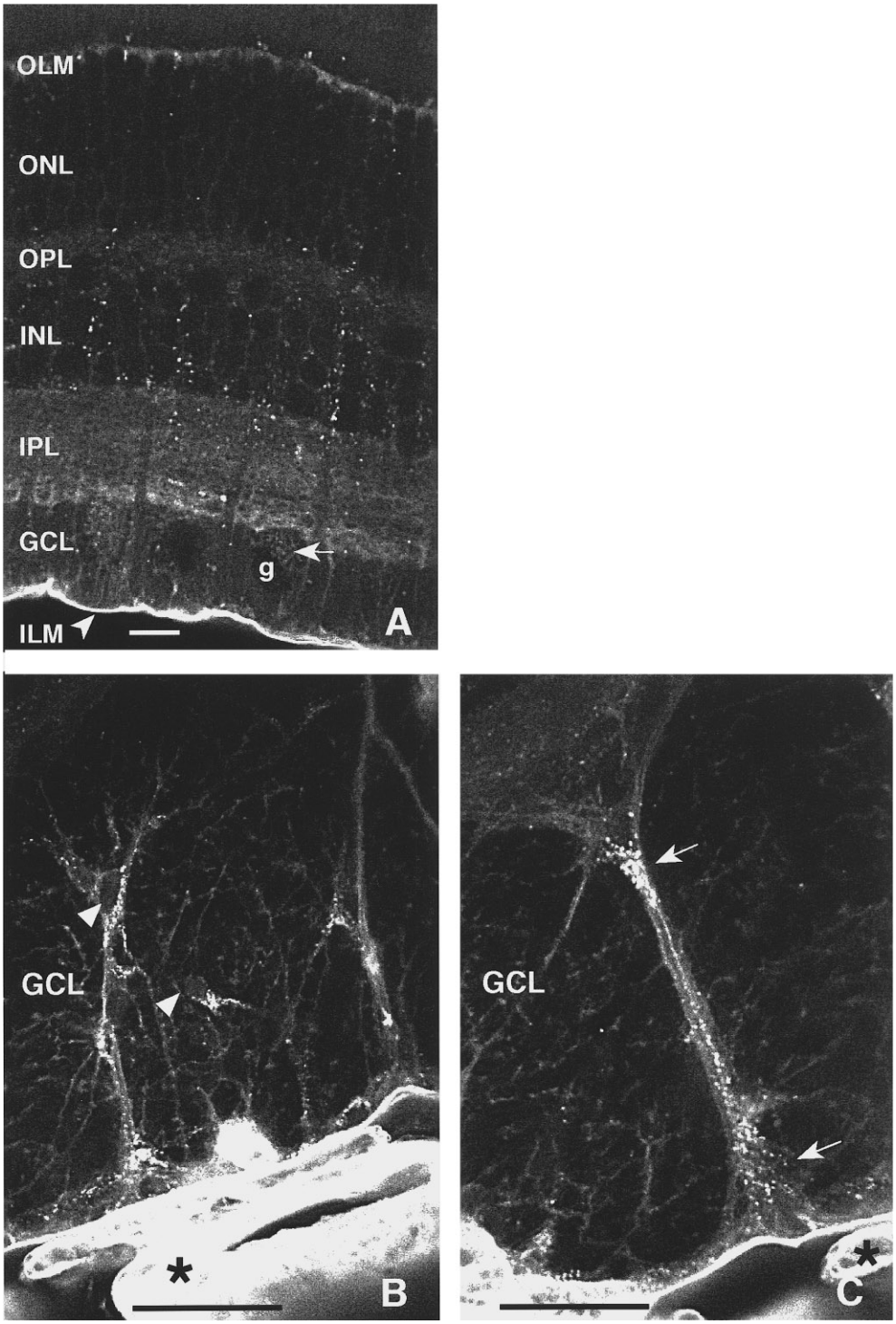

FIG. 5. bFGF-biotin localization on Vibratome sections of rabbit retina and viewed with the laser scanning confocal microscope. (A) 48 hr after intravitreal injection, the pattern of labelling is similar to that observed at 18 hr. Labeling also occurs within the ganglion cell cytoplasm (arrow); the nucleus which contains the 'g', is unlabeled. (B) High magnification of the punctate material in the astrocyte cell bodies (arrowheads) and processes (48 hr). (C) Groups of punctate material also appear in the Müller cells at this time (48 hr; arrows). The bright fluorescence at the bottom of (B) and (C) represents labeling of the basement membrane surrounding the blood vessels (*) as well as the vascular endothelial cells, located in the vitreous cavity. Tissue was taken from various retinal locations which accounts for the different appearance and thickness of the sections. (ILM, inner limiting membrane; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; OLM, outer limiting membrane; g, ganglion cell.) Bar, 20 μm.

Diffusion of Biotinylated Dextrans

Intravitreal injections of biotinylated dextrans (dextran–biotin) were used to determine if a biologically inactive molecule of similar molecular mass is processed similarly by the retina. The results show that dextran–biotin generates a distinctly different pattern of retinal labeling when compared to bFGF-biotin. At

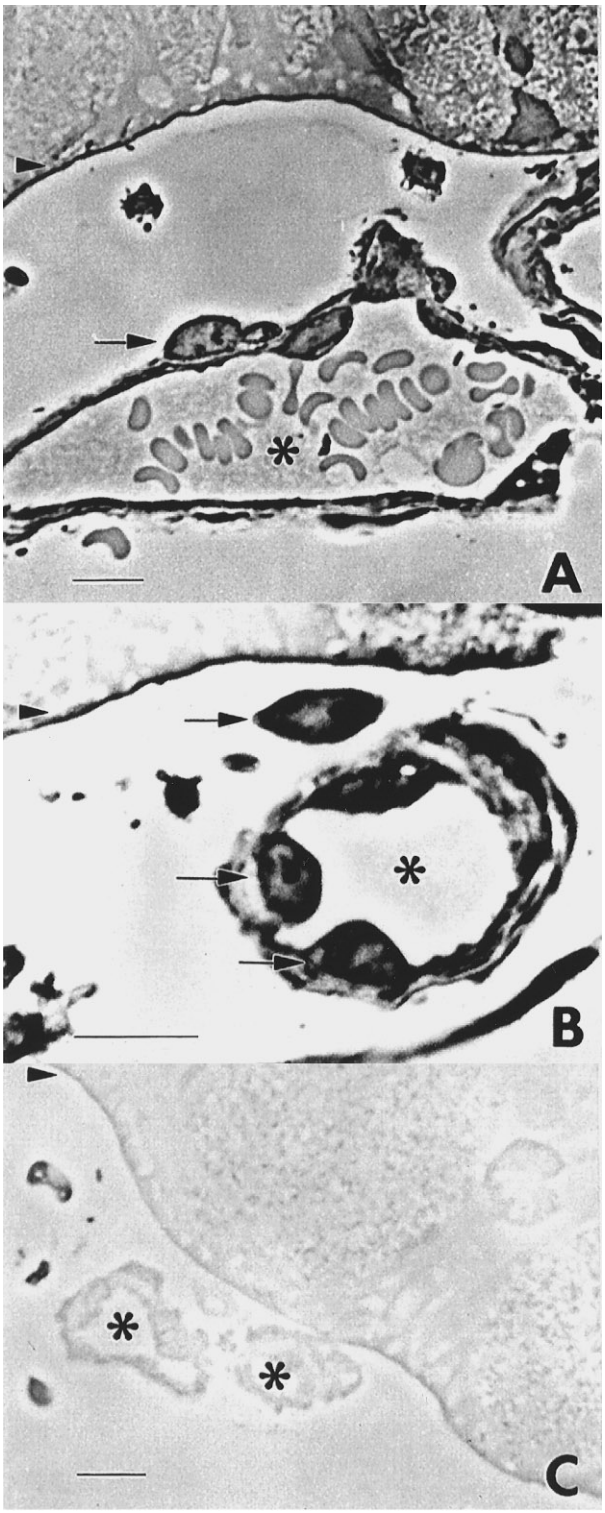

FIG. 6. Light microscopic localization of injected bFGF–biotin on 0·5 μm thick resin embedded sections of rabbit retina. (A) Labeling occurs in basement membranes surrounding vessels (*) present in the vitreous cavity, along the internal limiting membrane (ILM; arrowhead), and in vascular endothelial cells (arrow; 48 hr). (B) Higher magnification photograph demonstrating labeling of vascular endothelial cells and their nuclei (arrows). The ILM (arrowhead) and the basement membranes surrounding the vessels (*) are also labeled. (C) Control sections that did not receive an injection of bFGF–biotin. No labeling is present along the ILM (arrowhead), the basement membrane surrounding the vessels (*) or vascular endothelial cells. Bar, 10 μm.

2 hr following the injection, the dextrans are distributed diffusely throughout the extracellular space of the retina, extending from the ILM to the distal tips of the outer segments [Fig. 9(A)]. The weak labeling of the OLM seen with injected dextran-biotin is, however, present on sections from eyes that had been injected with free biotin [Fig. 9(C)] and on sections that had been treated with avidin-Cy3 alone (not shown) indicating that the labeling of the OLM is, at least in part, non-specific. At 48 hr there is some intracellular particulate labeling within the cytoplasm of Müller cells and astrocytes [Fig. 9(B)].

BFGF-Biotin is Processed into Lower Molecular Weight Fragments Following Intravitreal Injection

The retinas from bFGF-biotin injected eyes were dissected as 2, 18 and 48 hr, and at 4 and 7 days following the injection. Western blots of separated retinal proteins were then probed with avidin-AP in order to detect the biotinylated components. Blots of the recombinant bFGF-biotin used for injection revealed two biotinylated components: an 18 kDa component which corresponds to the predicted molecular mass of bFGF-biotin, and a 36 kDa component that presumably represents a bFGF-biotin dimer (Fig. 10, lane A). At 2 hr following injection, however, four major biotinylated components (at 9, 15, 18 and 36 kDa), and several minor components, are detected (Fig. 10, lane C). Of those four, the 18 kDa and 36 kDa components show the most intense reactivity. The levels of these two unprocessed components decline progressively with time following injection, but remain detectable up to 7 days post-injection (Fig. 10, lanes D–G). The levels of the 9 and 15 kDa components diminish somewhat more rapidly such that, by 4 days after injection, they are undetectable. It is unclear why the 9 and 15 kDa components are present at the 2 and 48 hr post-injection intervals (Fig. 10, lanes C and E), but are not detected at the intervening 18 hr timepoint (lane D) although this may represent animal to animal variation.

BFGF-Biotin Binds to Three Putative Receptors in the Retina

Retinas were obtained from two eyes that had been injected with bFGF-biotin 2 and 18 hr earlier. Following exposure to the DSS cross-linking agent, separation of retinal proteins by SDS–PAGE, blotting and subsequently probing of the blots with avidin-AP, biotinylated bands are apparent at approximately 72, 80 and 125 kDa (Fig. 11, lanes C and D). These bands represent the sum of the molecular masses of bFGF-biotin (at 18 kDa) and the mass(es) of the putative receptor(s). The 125 kDa band most likely represents

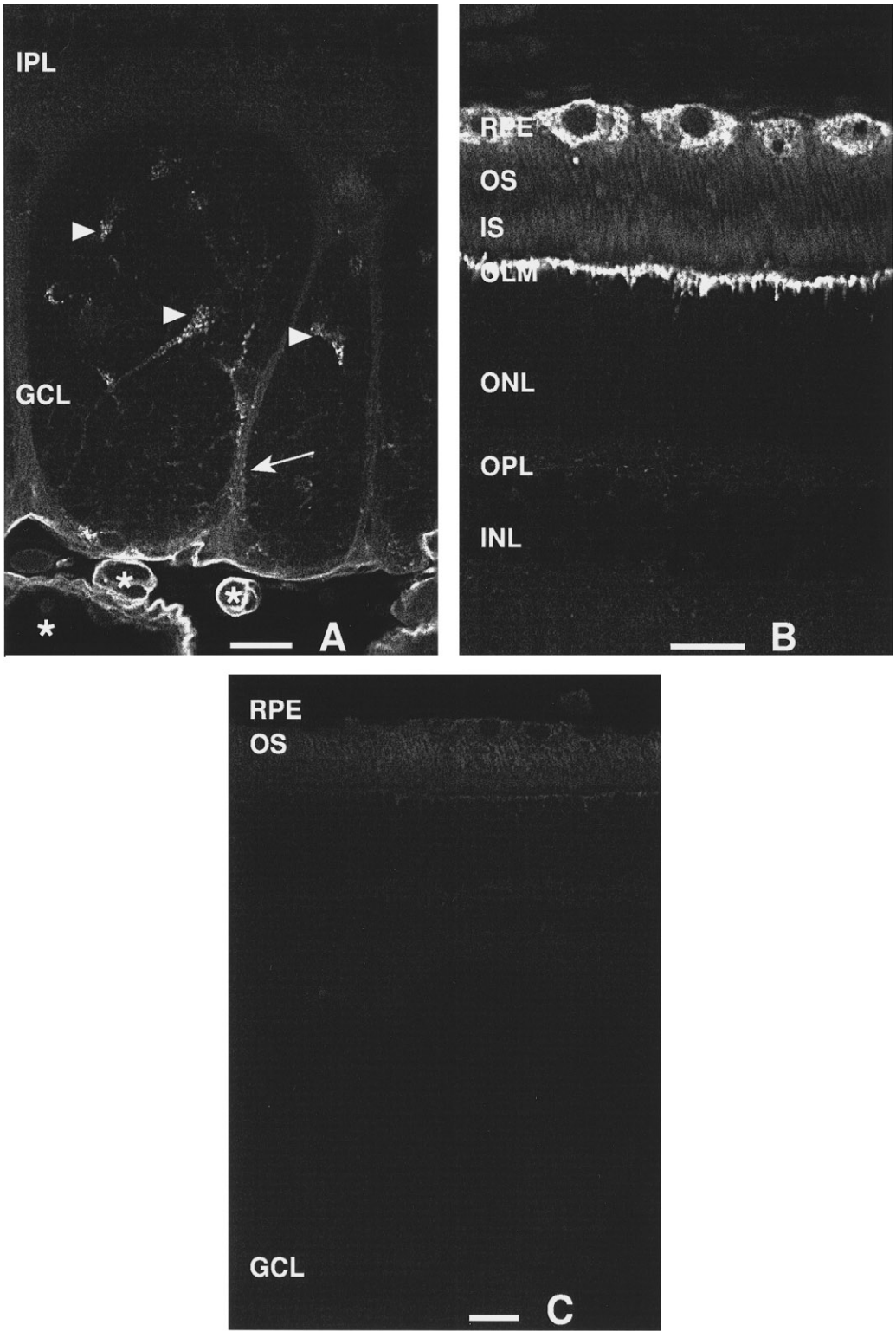

FIG. 7. bFGF–biotin localization on Vibratome sections of rabbit retina and viewed with the laser scanning confocal microscope. (A) 4 days after intravitreal injection, the labeling intensity on the basement membranes of the blood vessels (*) in the vitreous is less compared to earlier timepoints although punctate material is still present within the Müller cells (arrows) and astrocytes (arrowheads). (B) An increase in the intensity of labeling of the outer limiting membrane (OLM) and retinal pigment epithelium (RPE) are seen at this time (4 days). No labeling is present in the choroid located above the RPE. (C) 7 days after injection, labeling of all these structures is barely detectable. (GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; IS, inner segments; OS, outer segments.) Bar, 20 μm.

the sum of the 18 kDa bFGF monomer plus a receptor with an estimated mass of approximately 110 kDa. Previous estimates of the molecular mass of the bFGF high affinity receptor using SDS–PAGE range from approximately 110–150 kDa (see Baird and Bohlen, 1990 for review). Likewise, the 72 and 80 kDa

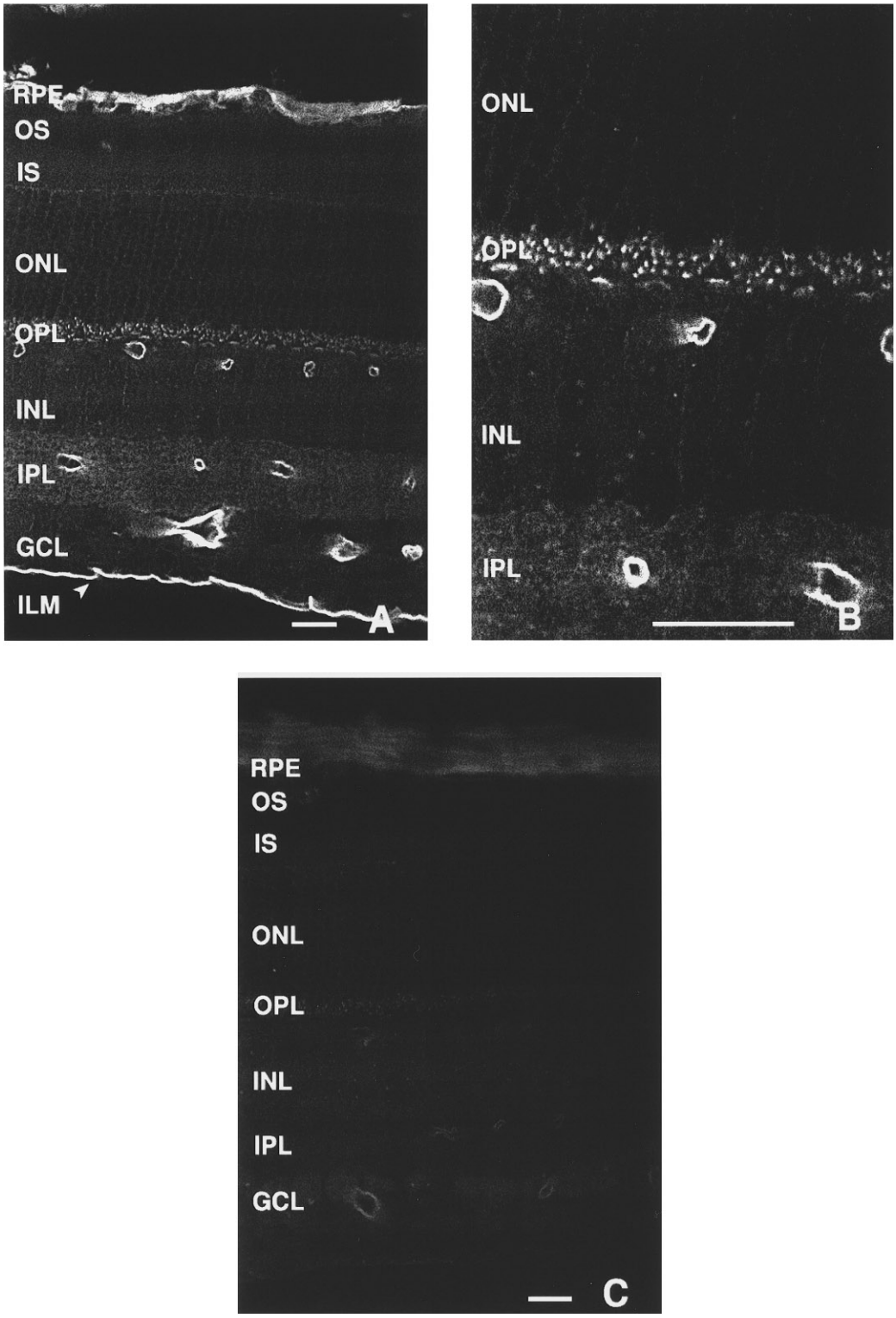

FIG. 8. bFGF–biotin localization on Vibratome sections from un-injected normal cat eyes. (A) bFGF-biotin labeling is present on the basement membranes of the internal limiting membrane (ILM), vasculature, and Bruch's membrane as well as in the inner plexiform layer (IPL) and in the region of the photoreceptor synaptic invaginations in the outer plexiform layer (OPL). (B) Higher magnification showing the labeling in the region of the synaptic invaginations of the rod and cone terminals in the OPL. (C) bFGF–biotin labeling is blocked almost completely when the sections are incubated with 100-fold excess non-biotinylated bFGF. [The region above the RPE in (C) is the tapetum which is autofluorescent.] (GSL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; IS, inner segments; OS, outer segments; RPE, retinal pigment epithelium.) Bar, 20 μm.

components represent the sums of the bFGF monomer plus two putative binding proteins with estimated molecular masses of 54 and 62 kDa respectively. In eyes not injected with bFGF-biotin, avidin-AP recognizes no biotinylated components when it is applied to the blots of retinal proteins. (Fig. 11, lane A).

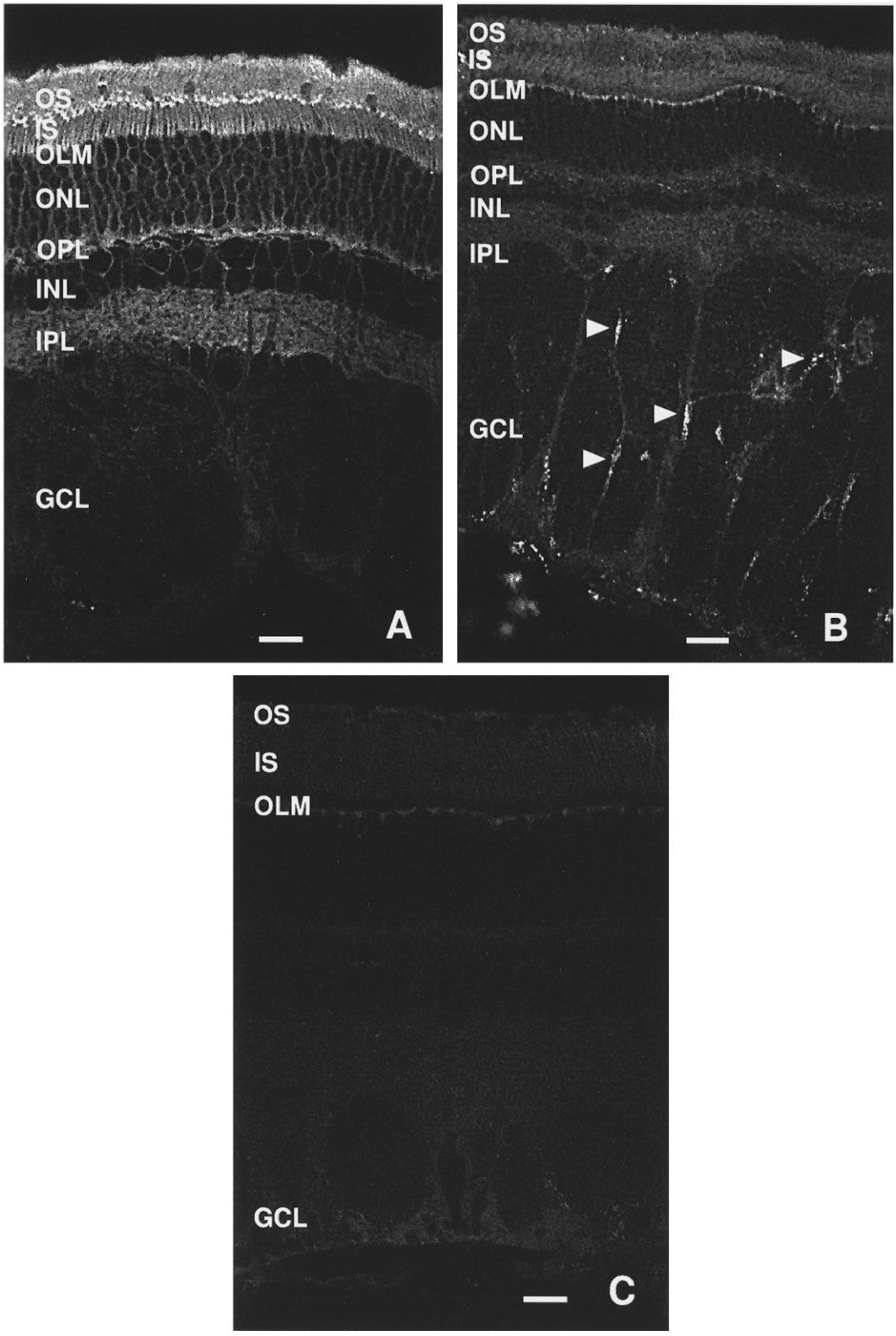

FIG. 9. Control Vibratome sections of rabbit retina viewed with the laser scanning confocal microscope. (A) 2 hr after intravitreal injection of biotinylated dextrans, diffuse labeling is present throughout the extracellular space of the retina. The bright areas between the photoreceptor outer segments (OS) and inner segments (IS) represent a zone of increased extracellular space. (B) 48 hr after injection, the dextrans are internalized in the glial cells (arrowheads). In addition, the outer limiting membrane (OLM) is slightly labeled. (C) 2 hr after injection of free biotin, only the OLM is faintly labeled. (GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer.) Bar, 20 μm.

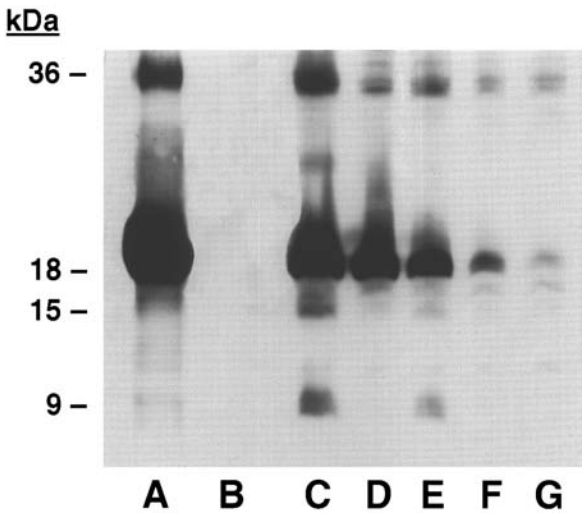

FIG. 10. Western blot of retinal homogenates obtained after injection of bFGF–biotin into the rabbit vitreous. Lane A, Biotinylated human recombinant bFGF cross-reacted with strepavidin–alkaline phosphatase. Bands at 18 and 36 kDa represent native and dimerized bFGF. Lane B, Normal retina receiving no injection of the bFGF–biotin. No biotinylated components are recognized following incubation with streptavidin–alkaline phosphatase. Lanes C, D, E, F, G, 2 hr, 18 hr, 48 hr, 4 days and 7 days respectively after intravitreal injection of bFGF–biotin. Most of the labeling is detected at 18 kDa throughout the 7 day period; however, smaller bands with apparent molecular weights of 9 and 15 kDa are also apparent at earlier time points.

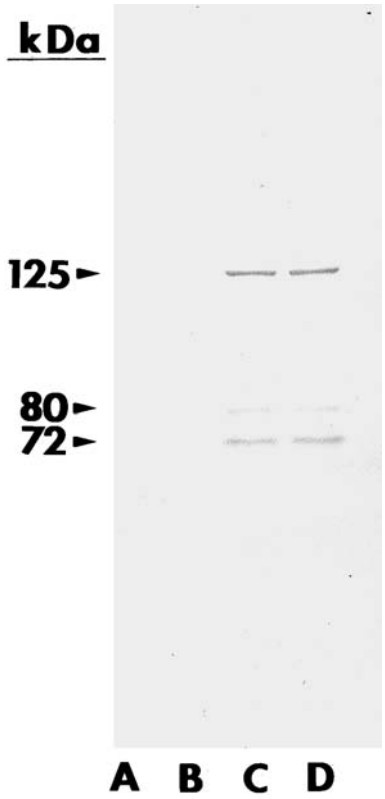

FIG. 11. Western blot of retinal homogenates obtained after cross-linking injected bFGF–biotin to its receptor(s). Lane A, Normal retina receiving no injection of bFGF–biotin. Lane B, An empty lane with no protein loaded. No biotinylated components are recognized in Lanes A and B following incubation with streptavidin–alkaline phosphatase. Lane C and D, 2 hr and 18 hr after intravitreal injection of bFGF–biotin, respectively. Bands are present at 125, 80 and 72 kDa.

4. Discussion

When bFGF is injected into the vitreous cavity of the eye, it induces proliferation in non-neuronal retinal cells, stimulates intermediate filament biosynthesis (DeJuan et al., 1990; Lewis et al., 1992) and, under certain conditions, has a neuroprotective effect on degenerating photoreceptors cells (Faktorovich et al., 1990, 1992; LaVail et al., 1992). The underlying molecular events responsible for producing these effects are not known. The results of this study provide new information with respect to: (a) the time course of bFGF diffusion into, and retention by, the retina following intravitreal injection; (b) binding to specific retinal cell types; and (c) subsequent internalization and processing of the injected factor.

In previous studies, ^{125}I-bFGF has been used on retinal sections to localize putative bFGF receptor binding to retinal basement membranes and to the plexiform layers (Jeanny et al., 1987; Fayein et al., 1990; Cirillo et al., 1990; Faucheux et al., 1991). It has also been used to characterize the cellular processing of the factor into a smaller subset of radiolabeled peptides (Moscatelli et al., 1988; Moenner et al., 1989; Walicke and Baird, 1991; Hawker and Granger, 1992; Gonzalez et al., 1991; Ferguson et al., 1990). In this study, we used a higher resolution, avidin-biotin detection system in an attempt to identify the specific retinal cell types involved in an in vivo context, and to follow the intracellular disposition of the factor once it had been internalized.

The results indicate that within 2 hr the injected factor rapidly diffuses throughout the retinal extracellular space. By 6 hr following injection, 50–100 nm diameter biotinylated particles are first identified in astrocytes, endothelial cells, Müller cells, and RPE cells—the same cell types that are later induced to proliferate by exogenous bFGF. The particles most likely represent receptor-bound bFGF-biotin that had been internalized. These observations, along with the cross-linking data (see below), are consistent with in vitro biochemical data which suggest that bFGF binds to high affinity receptors on the plasma membranes of these cell types (Schelling et al., 1988; 1989; Sternfeld et al., 1989; Fayein et al., 1990; Mascarelli et al., 1991; Walicke and Baird, 1991; Hawker and Harris, 1992; Rakoczy et al., 1993).

The results from this study provide strong support for the conclusion that, in the retina under in vivo conditions, internalized bFGF is processed to 9 and 15 kDa molecular weight fragments that are not immediately degraded by lysosomes. In this study, as well as in others (Bouche et al., 1987; Renko et al., 1990; Baldin et al., 1990; Logan, 1990; Walicke and Baird, 1991; Hawker and Granger, 1992; Woodward

et al., 1992), labeled bFGF or a labeled bFGF fragment is localized in the nuclei of endothelial cells and astrocytes. These bFGF fragments may be biologically active since it has been shown that the presence of bFGF in the nucleus increases the synthesis of mRNA coding for specific ribosomal proteins (Amalric et al., 1991).

High affinity receptors for bFGF have been reported to display a range of molecular weights ranging from 110–150 kDa depending on cell type (see Baird and Bohlen, 1990 for review). In the cross-linking experiment, we detected 3 components with estimated molecular masses of 110 kDa, 62 kDa and 54 kDa. The 110 kDa component falls within the accepted range of the high affinity receptor. The 54 and 62 kDa components could represent truncated forms of the high affinity receptor, other variants of the receptor or, perhaps, proteolytic degradation products. Previous studies indicate that alternative splicing by bFGF receptor mRNA generates many different isoforms, with some having molecular weights similar to the two lower values reported here (Johnson et al., 1990; Eisman et al., 1991; Givol and Yayon, 1992; Duan et al., 1992). In addition, the retina and vitreous has recently been reported to contain soluble 70–85 and 55 kDa bFGF binding proteins (Hanneken and Baird, 1994; Hanneken et al., 1995) that are apparently truncated versions of the high affinity receptor. These values are fairly close to the two smaller cross-linked components identified here in retinal protein homogenates.

Although the evidence implicating bFGF high affinity receptor binding in non-neuronal cell proliferation is fairly persuasive, the mechanism(s) responsible for mediating photoreceptor cell survival remain unclear. Our results indicate that injected bFGF is apparently retained in the rabbit retina for at least 7 days following a single intravitreal injection. That could explain why treatment of rodent retinas with bFGF several days prior to injury can arrest photoreceptor cell degeneration (Faktorovich et al., 1992), since it may be continuously available for a number of days following injection.

Putative high affinity bFGF receptors have been identified on the cell surface of photoreceptors, although their precise location is not clear. ^{125}I-bFGF binding has been reported in photoreceptor outer segments (Mascarelli et al., 1989), inner segments, as well as in the OPL (Fayein et al., 1990; Cirillo et al., 1990; Faucheux et al., 1991). Basic FGF high affinity receptor (*flg*) immunoreactivity, on the other hand, has been identified in the goldfish only in association with the axon terminals of the photoreceptors (Raymond et al., 1992). In this study, we find bFGF-biotin binding specifically in the region of the synaptic invaginations at the photoreceptor axon terminals but not to any other photoreceptor sites, thereby corroborating the immunohistochemical results. However, it is still unclear whether this represents binding to the plasma membrane, to the extracellular matrix adjacent to the axon terminals, or even to the dendrites of the post-synaptic neurons.

Immunochemical studies in rat, monkeys, and humans have identified bFGF, or an immunologically related protein, as an insoluble component of interphotoreceptor matrix (IPM) (Hageman et al., 1991; Gao and Hollyfield, 1992). Partial amino acid sequencing of the 18 kDa aqueous insoluble IPM component recognized by anti-bFGF antibodies on Western blots confirm that this component has an amino terminal sequence identical to bFGF (Hageman et al., unpublished data). Paradoxically, however, bFGF-biotin does not bind to the IPM *in vivo*, nor does it bind to the IPM on formaldehyde-fixed retinal sections in any of the species examined to date (Fig. 8). This implies that appropriate binding sites for bFGF–biotin in the IPM are absent, masked, or otherwise unavailable since it does bind to putative high and low affinity receptors at other retinal locations.

Understanding the steps by which bFGF is processed by the retina in vivo is likely to be important in revealing a mechanism by which this factor exerts its proliferative and neuroprotective effects. In this study, we focus on the former effect. Our findings indicate that bFGF-biotin diffuses rapidly into the normal retina following intravitreal injection where it binds to putative low affinity receptors associated with retinal basement membranes. The results also suggest that it binds to putative high affinity receptors on the surfaces of the same retinal cell types that are later induced to proliferate when the retina is experimentally detached or injured, after which it is internalized and processed to lower molecular weight fragments. Further studies, designed to examine the dynamics of bFGF-biotin binding and internalization in degenerating retinas, may yield insights into the mechanism(s) responsible for bFGF's neuroprotective effects on injured photoreceptors.

Acknowledgements

The authors would like to thank Dr Christopher Guerin, Karen Guenther and Scott Geller for their technical assistance; Maura Jess for her assistance with the computer generated layouts of the micrographs; and Dr Patricia D'Amore for her assistance in confirming the biological activity of biotinylated bFGF. Some of the recombinant human bFGF used in this study was obtained as a gift from Scios Nova, Inc., Mountain View, CA, U.S.A. The authors express their appreciation to Scios Nova and, in particular, to Dr Judy Abraham (Scios Nova). The work was supported by US Public Health Service Research Grants EY-02082 (DHA) and EY-00888 (SKF).

References

Anderson, D. H., Stern, W. H., Fisher, S. K., Erickson, P. A. and Borgula, G. A. (1983). Retinal detachment in the cat. The pigment epithelial–photoreceptor interface. *Invest. Ophthalmol. Vis. Sci.* **24**, 906–26.

Amalric, F., Baldin, V., Bosc-Bierne, I., Bugler, B., Couderc, B., Guyader, M., Patry, V., Prats, H., Roman, A. M. and Bouche, G. (1991). Nuclear translocation of basic fibroblast growth factor. *Annals NY Acad. Sci.* **638**, 127–38.

Baird, A., Esch, F., Gospodarowicz, D. and Guillemin, R. (1985). Retina- and eye-derived endothelial growth factors: partial molecular characterization and identity with acidic and basic fibroblast growth factors. *Biochemistry* **24**, 7855–60.

Baird, A. and Bohlen, P. (1990). Fibroblast growth factors. In: *Peptide Growth Factors and their Receptors* (Eds. Sporn, M. B. and Roberts, A. B.). Pp. 369–418. Springer: Berlin.

Baldin, V., Roman, A.-M., Bosc-Bierne, I., Amalric, F. and Bouche, G. (1990). Translocation of bFGF to the nucleus is G1 phase cell cycle specific in bovine aortic endothelial cells. *EMBO J* **9**, 1511–17.

Bouche, G., Gas, N., Prats, H., Balsin, V., Tauber, J. P., Teissie, J. and Amalric, F. (1987). bFGF enters the nucleolus and stimulates the transcription of ribosomal genes in ABAE cells undergoing G0-G1 transition. *Proc. Natl. Acad. Sci., U.S.A.* **84**, 6770–4.

Bradshaw, R. A. and Cavanaugh, K. P. (1990). Isolation and characterization of growth factors. In: *Peptide Growth Factors and their Receptors* (Eds. Sporn, M. B. and Roberts, A. B.). Pp. 17–36. Springer: Berlin.

Cirillo, A., Cristina, A, Courtois, Y. and Jeanny, J. C. (1990). Localization of basic fibroblast growth factor binding sites in the chick embryonic neural retina. *Differentiation* **45**, 161–7.

de Juan, E., Staffansson, E. and Ohira, A. (1990). Basic fibroblast growth stimulates ^{3}H-thymidine uptake in retinal venular and capillary endothelial cells *in vivo*. *Invest. Ophthalmol. Vis. Sci.* **31**, 1238–44.

Duan, D.-S. R., Werner, S. and Williams, L. T. (1992). A naturally occuring secreted form of fibroblast growth factor (FGF) receptor 1 binds basic FGF in preference over acidic FGF. *J. Biol. Chem.* **267**, 16076–80.

Eckenstein, F. P., Shipley, G. D. and Nishi, R. (1991). Acidic and basic fibroblast growth factors in the nervous system: distribution and differential alteration of levels after injury of central versus peripheral nerve. *J. Neurosci.* **11**, 412–19.

Eclancher, F., Perraud, F., Faltin, J., Labourdette, G. and Sensenbrenner, M. (1990). Reactive astrogliosis after basic fibroblast growth factor (bFGF) injection in injured neonatal rat brain. *Glia* **3**, 502–9.

Eismann, A., Ahn, J. A., Graziani, G., Tronick, S. R. and Ron, D. (1991). Alternative splicing generates at least five different isoforms of the human basic-FGF receptor. *Oncogene* **6**: 1195–202.

Erickson, P. A., Fisher, S. K., Anderson, D. H., Stern, W. H. and Borgula, G. A. (1983). Retinal detachment in the cat: the outer nuclear and outer plexiform layers. *Invest. Ophthalmol. Vis. Sci.* **24**, 927–42.

Faktorovich, E. G., Steinberg, R. H., Yasumura, D., Matthes, M. T. and La Vail, M. M. (1990). Photoreceptor degeneration in inherited retinal dystrophy delayed by basic fibroblast growth factor. *Nature* **347**, 83–6.

Faktorovich, E. G., Steinberg, R. H., Yasumura, D. Matthes, M. T. and LaVail, M. M. (1992). Basic fibroblast growth factor and local injury protect photoreceptors from light damage in the rat. *J. Neurosci.* **12**, 3554–67.

Faucheux, B. A., Dupis, C., Delarere, P., Raulais, D., Soubrane, G., Vigney, M., Hauw, J. J., Courtois, Y. and Jeanny, J. C. (1991). Acidic fibroblast growth factor localization and basic fibroblast growth factor binding sites in the eye and optic nerve. In: *Growth Factors and Alzeimer's Disease* (Eds. Hefti, F., Brachet, P. H., Will, B., Christen, Y.). Pp. 165–174. Springer-Verlag: New York, U.S.A.

Fayein, N. A., Courtois, Y. and Jeanny, J. C. (1990). Ontogeny of basic fibroblast growth factor binding sites in mouse ocular tissues. *Exp. Cell Res.* **188**, 75–88.

Ferguson, I. A., Schweitzer, J. B. and Johnson, E. M. Jr. (1990). Basic fibroblast growth factor: receptor mediated internalization, metabolism, and anterograde axonal transport in retinal ganglion cells. *J. Neurosci.* **10**, 176–89.

Finklestein, S. P., Apostilides, P. J., Caday, C. G., Prosser, J., Prosser, J., Philips, M. F. and Klagsbrun, M. (1988). Increased basic fibroblast growth factor (bFGF) immunoreactivity at the site of focal brain wounds. *Brain Res.* **460**, 252–9.

Fisher, S. K., Erickson, P. A., Lewis, G. P. and Anderson, D. H. (1991). Intraretinal proliferation induced by retinal detachment. *Invest. Ophthalmol. Vis. Sci.* **32**, 1739–48.

Frautschy, S. A., Walicke, P. A. and Baird, A. (1991). Localization of basic FGF and its mRNA after CNS injury. *Brain Res.* **553**, 291–9.

Gao, H. and Hollyfield, J. G. (1992). Basic fibroblast growth factor (bFGF) immunolocalization in the rodent outer retina demonstrated with an anti-rodent bFGF antibody. *Brain Res.* **585**, 355–60.

Giulian, D., Chen, J., Ingeman, J. E., George, J. K. and Naponen, M. (1989). The role of mononuclear phagocytes in wound healing after traumatic injury in adult mammalian brain. *J. Neurosci.* **9**, 4416, 29.

Givol, D. and Yayon, A. (1992). Complexity of FGF receptors: genetic basis for structural diversity and functional specificity. *FASEB* **6**, 3362–9.

Glaser, B. M., D'Amore, P. A., Michels, R. S., Patz, A. and Fenselau, A. (1980). Demonstration of vasoproliferative activity from mammalian retina. *J. Cell Biol.* **84**, 298–304.

Gomez-Pinilla, F., Lee, J. W. and Cotman, C. W. (1992). Basic fibroblast growth factor in adult brain: cellular distribution and response to entorhinal lesion and fimbria-fornix transection. *J. Neurosci.* **12**, 345–55.

Gonzalez, A. M., Buscaglia, M. L., Fuller, J., Carman, L. S. and Baird, A. (1991). Local fate and distribution of locally infused basic FGF. *Annals NY Acad. Sci.* **638**, 417–419.

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., U.S.A.* **88**, 6706–10.

Hanneken, A., Lutty, G. A., McLeod, D. S., Robey, F., Harvey, A. K. and Hjelmeland, L. M. (1989). Localization of basic fibroblast growth factor to the developing capillaries of the bovine retina. *J. Cell Physiol.* **138**, 115–20.

Hanneken, A. and Baird, A. (1992). Immunolocalization of bFGF: dependence on antibody type and tissue fixation. *Exp. Eye Res.* **54**, 1011–14.

Hanneken, A. and Baird, A. (1994). Fibroblast growth factor binding proteins in vitreous fluid. *Invest. Ophthalmol. Vis. Sci.* **35**, (suppl.), 1497.

Hanneken, A., Maher, P. A. and Baird, A. (1995). High affinity immunoreactive FGF receptors in the extracellular matrix of vascular endothelial cells—implications for the modulation of FGF-2. *J. Cell Biol.* **128**, 1221–8.

Hawker, J. R. and Harris, J. G. (1992). Internalized basic fibroblast growth factor translocates to nuclei of venular endothelial cells. *Am. J. Physiol.* **262**, H1525–37.

Hicks, D., Bugra, K., Faucheux, B., Jenny, J. C., Laurent, M., Malecaze, F., Mascarelli, F., Raulais, D., Cohen, Y. and

Courtois, Y. (1990). Fibroblast growth factors in the retina. In *Progress in Retinal Research*, Vol. 11 (Eds. Osborn, N. and Chader, G.). Pp. 333–374, Pergamon Press: New York, U.S.A.

Hjelmeland, L. M. and Harvey, A. K. (1988). Gliosis of the mammalian retina: migration and proliferation of retinal glia. In *Progress in Retinal Research*. Vol. 7 (Eds. Osborn, N., Chader, J.). Pp. 259–281. Pergamon: Oxford.

Jeanny, J. C., Fayein, N., Moenner, M., Chevallier, B., Barritault, D. and Courtois, Y. (1987). Specific fixation of bovine brain and retinal acidic and basic fibroblast growth factors to mouse embryonic eye basement membranes. *Exp. Cell Res.* **171**, 63–75.

Johnson, D. E., Lee, P. L., Lu, J. and Williams, L. T. (1990). Diverse forms of a receptor for acidic and basic fibroblast growth factors. *Molec. Cell Biol.* **10**, 4728–36.

Klagsburn, M. (1978). Human milk stimulates DNA synthesis and cellular proliferation in cultured fibroblasts. *Proc. Natl. Acad. Sci., U.S.A.* **75**, 5057–61.

Kostyk, S. K., D'Amore, P. A., Herman, I. M. and Wagner, J. A. (1994). Optic nerve injury alters basic fibroblast growth factor localization in the retina and optic tract. *J. Neurosci.* **14**, 1441–9.

LaVail, M. M., Unoki, K., Yasumura, D., Matthes, M. T., Yancopoulos, G. D. and Steinberg, R. H. (1992). Multiple growth factors, cytokines, and neurotrophins rescue photoreceptors from the damaging effects of constant light. *Proc. Natl. Acad. Sci., U.S.A.* **89**, 11249–53.

Lee, P. L., Johnson, D. E., Cousens, L. S., Fried, V. A. and Williams, L. T. (1989). Purification and complementary DNA cloning of a receptor for basic fibroblast growth factor. *Science* **245**, 57–60.

Lewis, G. P., Erickson, P. A., Guerin, C. J., Anderson, D. H. and Fisher, S. K. (1992). Basic fibroblast growth factor: a potential regulator of proliferation and intermediate filament expression in the retina. *J. Neurosci.* **12**, 3968–78.

Logan, A. (1990). Acidic and basic fibroblast growth factor genes show individual time-related responses following central nervous system lesion. *J. Endocrinol.* **124**, 47.

Logan, A. (1992). A time course for the focal elevation of synthesis of basic fibroblast growth factor and one of its high-affinity receptors (flg) following a localized cortical brain injury. *J. Neurosci.* **12**, 3828–37.

Mascarelli, F., Raulais, D. and Courtois, Y. (1989). FGF phosphorylation and receptors in rod outer segments. *EMBO J* **8**, 2265–73.

Mascarelli, F., Tassin, J. and Courtois, Y. (1991). Effect of FGFs on adult bovine Müller cells: proliferation, binding and internalization. *Growth Factors* **4**, 81–95.

Matsumoto, B. and Hale, I. L. (1993). Preparation of retinas for studying photoreceptors with confocal microscopy. *Methods Neurosci.* **15**, 54–71.

Moenner, M., Gannoun-Zaki, L., Badet, J. and Barritault, D. (1989). Internalization and limited processing of basic fibroblast growth factor on chinese hamster lung fibroblasts. *Growth Factors* **1**, 115–24.

Morimoto, A., Matsuda, S., Uryu, K., Fujita, H. Okumura, N. and Sakanaka, M. (1993). Light- and electron-microscopic localization of basic fibroblast growth factor in adult rat retina. *Okajimas Folia Anat. Jpn.* **70**, 7–12.

Morrison, R. S., DeVilles, J., Lee, Y. L., Bradshaw, R. and Eng, L. F. (1985). Hormones and growth factors induce the synthesis of glial fibrillary acidic protein in rat brain astrocytes. *J. Neurosci. Res.* **14**, 167–76.

Moscatelli, D. (1988). Metabolism of receptor-bound and matrix-bound basic fibroblast growth factor by bovine capillary endothelial cells. *J. Cell Biol.* **107**, 753–9.

Noji, S., Matsuo, T., Koyama, E., Yamaai, T., Nohno, T., Matsuo, N. and Taniguchi, S. (1990). Expression pattern of acidic and basic fibroblast growth factor genes in adult rat eyes. *Biochem. Biophys. Res. Com.* **168**, 343–9.

Park, C. M. and Hollenberg, M. J. (1989). Basic FGF induces retinal regeneration *in vivo*. *Dev. Biol.* **134**, 201–5.

Puro, D. G., Mano, T., Chan, C. C., Fukuda, M. and Shimada, H. (1990). Thrombin stimulates the proliferation of human retinal glial cells. *Albrecht Von Graefes Arch. Klin. Exp. Ophthalmol.* **228**, 169–73.

Perraud, F., Labourdette, G., Miehe, M., Loret, C. and Sensenbrenner, M. (1988). Comparison of the morphological effects of acidic and basic fibroblast growth factors on rat astroblasts in culture. *J. Neurosci. Res.* **20**, 1–11.

Rakoczy, P. E., Humphrey, M. F., Cavaney, D. M., Chu, Y. and Constable, I. J. (1993). Expression of basic fibroblast growth factor and its receptor in the retina of Royal College of Surgeons Rats. *Invest. Ophthalmol. Vis. Sci.* **34**, 1845–52.

Rapraeger, A. C., Krufka, A. and Olwin, B. B. (1991). Requirement of heparin sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science* **252**, 1705–8.

Raymond, P. A. (1992). Immunolocalization of basic fibroblast growth factor and its receptor in adult goldfish retina. *Exp. Neurol.* **115**, 73–8.

Renko, M., Qurto, N., Morimoto, T. and Rifkin, D. B. (1990). Nuclear and cytoplasmic localization of different basic fibroblast growth factor species. *J. Cell. Physiol.* **44**, 108–14.

Schelling, M. E., Meininger C. J., Hawker, J. R., Granger H. J. (1988). Venule endothelial cells from bovine heart. *Am. J. Physiol.* **254**, H1211–16.

Schelling, M. E., Hawker, J. R. and Granger, H. J. (1989). High and low affinity binding of acidic and basic fibroblast growth factors to their receptor on coronary venule endothelial cells. *FASEB J.* **3**, A1317 (Abstract).

Sievers, J., Hausmann, B., Unsicker, K. and Berry, M. (1987). Fibroblast growth factors promote the survival of adult rat retinal ganglion cells after transection of the optic nerve. *Neurosci Lett.* **76**, 157–62.

Sporn, M. B. and Roberts, A. B. (1990). The multifunctional nature of peptide growth factors. In: Peptide growth factors and their receptors (Eds. Sporn, M. B. and Roberts, A. B.). Pp. 3–15. Springer: Berlin.

Sternfeld, M. D., Robertson, J. E., Shipley, G. D., Tsai, J. and Rosenbaum, J. T. (1989). Cultured human retinal pigment epithelial cells express basic fibroblast growth factor and its receptor. *Curr. Eye Res.* **8**, 1029–37.

Togari, A., Geneva, D., Kuzuya, H. and Guroff, G. (1985). The effect of fibroblast growth factor on PC12 cells. *J. neurosci.* **5**, 307–16.

Torriglia, A., Jeanny, J. C. and Blanquet, P. R. (1994). Immunohistochemical analysis of fibroblast growth factor receptor in bovine retina. *Neurosci. Lett.* **172**, 125–8.

Walicke, P. A., Baird, A. (1991). Internalization and processing of basic fibroblast growth factor by neurons and astrocytes. *J. Neurosci.* **11**, 2249–58.

Woodward, W. R., Nishi, R., Meshul, C. K., Williams, T. E., Coulombe, M. and Eckenstein, F. P. (1992). Nuclear and cytoplasmic localization of basic fibroblast growth factor in astrocytes and CA2 hippocampal neurons. *J. Neurosci.* **12**, 142–52.

Yayon, A., Klagsburn, M., Esko, J. D., Leder, P. and Ornitz, D. M. (1991). Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* **64**, 841–8.