Light- and Electron-Microscopic Localization of Basic Fibroblast Growth Factor in Adult Rat Retina

By

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Summary: An antiserum against basic fibroblast growth factor (bFGF) was characterized by immunoblot and used to localize bFGF-immunoreactivity in the adult rat retina. Crude homogenate of the retina, when examined with immunoblotting, exhibited a main band with the same molecular weight (18kDa) as bFGF. Light microscopic immunohistochemistry demonstrated intense bFGF staining in the photoreceptor outer segments and the outer plexiform layer, and weak immunostaining in some cells of the outer nuclear layer, inner nuclear layer and ganglion cell layer. The most intense bFGF-immunoreactivity was noted in glial (Müller) cells with vertically oriented thin processes. There were also immunoreactive pigment epithelial cells and mesenchymal cells in the choroid. Under electron microscopy, the outer plexiform layer was found to contain immunoreactive products exclusively in the axon terminals of photoreceptor cells which formed synapses with bipolar cell processes. The more widespread and uneven localization of bFGF-immunoreactivity in the mature rat retina than previously demonstrated suggests that endogenous bFGF is involved in complex physiological events other than retinal regeneration and histogenesis.

Basic fibroblast growth factor (bFGF), isolated from the brain and pituitary (Bohlen *et al.*, 1984; Gospodarowicz *et al.*, 1984; Esch *et al.*, 1985; Pettmann *et al.* 1985), has been shown to stimulate the proliferation and differentiation of mesodermally derived cells as well as to facilitate the survival and neurite extension of neurons cultured from different brain regions (Gospodarowicz *et al.*, 1983; Gospodarowicz *et al.*, 1986; Morrison *et al.*, 1986; Walicke *et al.*, 1986; Gensburger *et al.*, 1987; Walicke *et al.*, 1988). Immunohistochemical studies have demonstrated it in neurons and astrocytes of the brain (Pettmann *et al.*, 1986; Janet *et al.*, 1987; Grothe *et al.*, 1991; Gomez-Pinilla *et al.*, 1992; Matsuda *et al.*, 1992; Weise *et al.*, 1992) and in certain peripheral tissues (DiMario *et al.*, 1989; Grothe *et al.*, 1989; Joseph-Silverstein *et al.*, 1989; Kardami *et al.*, 1989; Okada *et al.*, 1992). bFGF also mediates the differentiation and maintenance of photoreceptors (Faktorovich *et al.*, 1990; Lavail *et al.*, 1991) and induces retinal regeneration in the chick embryo (Park *et al.*, 1989). Previous physiological studies have shown that bFGF has retinotrophic functions in all the species examined to date,

but there are discrepancies in the recent immunohistochemical studies on retinal bFGF in cows, lampreys and goldfish (Hanneken *et al.*, 1989; Rubunson *et al.*, 1991; Raymond *et al.*, 1992). In the bovine retina, only developing capillary endothelial cells are immunoreactive for bFGF (Hanneken *et al.*, 1989), whereas bFGF is widely distributed in the retinas of the fish species (Rubunson *et al.*, 1991; Raymond *et al.*, 1992). More recently, Gao and Hollyfield (1992) have shown bFGF in Muller cells and pigment epithelial cells of the adult rat retina, using only light microscopic immunohistochemistry. However, the selective demonstration of bFGF in non-neuronal cells of the adult rat retina is not necessarily reconciled with previous studies which suggest that bFGF is detected in neurons of the mature rat brain (Pettmann *et al.*, 1986; Janet *et al.*, 1987; Grothe *et al.*, 1991; Gomez-Pinilla *et al.*, 1992; Matsuda *et al.*, 1992; Weise *et al.*, 1992). With immunoblot, and light- and electron-microscopic immunohistochemistry, the present study was designed to re-investigate the location of bFGF-like molecules in the mature rat retina.

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Materials and Methods

Wistar male rats weighing approximately 200 g were housed at constant temperature (22°C) with a 12:12 light-dark cycle and given food and water *ad libitum*. The following experiments were conducted in accordance with the Guide for Animal Experimentation at Ehime University School of Medicine.

Immunoblot analysis

Four animals were sacrificed by decapitation; the retinas were frozen in liquid nitrogen and kept (for no longer than two days) at -80°C till use. They were homogenized in a solution consisting of 320 mM sucrose, 2 mM EDTA and 2 mM PMSF. In a study of the reactivities of bFGF antiserum to acidic FGF (aFGF), bFGF and crude retinal extract, both factors (10 ng each) and 10 μg of retinal homogenate were subjected to SDS-polyacrylamide gel electrophoresis and subsequent silver staining or immunoblot analysis as described elsewhere (Towbin *et al.*, 1979; Okumura *et al.*, 1989; Desaki *et al.*, 1992; Okada *et al.*, 1992).

Immunohistochemistry

Four animals were anesthetized with pentobarbital (40 mg/kg) and perfused transcardially, first with 50 ml of ice-cold saline, then with 300 ml of 4% paraformaldehyde-0.075% glutaraldehyde-0.2% picric acid in 0.1 M phosphate buffer (PB) (pH 7.4) (Somogyi *et al.*, 1982). Immediately after perfusion, the retina was dissected out and cut into 60 μm sections with a microslicer. The sections were immunostained with bFGF antiserum as described previously (Desaki *et al.*, 1992; Matsuda *et al.*, 1992). Half of the sections were mounted on gelatin-coated slides for light microscopic observation. The others were postfixed with 1% osmium tetroxide, dehydrated in a graded series of ethanol while being stained with 1% uranyl acetate in 70% ethanol, transferred to propylene oxide and embedded in epoxy resin. Ultrathin sections were cut with a Reichert ultramicrotome and observed in an electron microscope. Control sections were incubated with the primary antiserum that had been adsorbed with an excess of bFGF, then processed as described above.

Results

Immunoblot

The bFGF antiserum used in this study recognized purified bFGF with a molecular weight of 18 kDa, but not aFGF (Fig. 1). Crude homogenate of the retina, when examined with immunoblotting,

Fig. 1. aFGF (lanes 1, 3) and bFGF (lanes 2, 4) were subjected to SDS-polyacrylamide gel electrophoresis and then silver staining (lanes 1, 2) or immunoblot analysis (lanes 3, 4) with bFGF antiserum. Note that this antiserum recognizes bFGF but not aFGF (lanes 3, 4). Crude homogenate of the rat retina, when examined with immunoblotting, also exhibits a main band with the same molecular weight (18 kDa) as bFGF (lane 5). Lane 6 shows silver staining of the retinal homogenate.

exhibited a main band of the same molecular weight (18 kDa) as bFGF (Fig. 1).

Immunohistochemistry

Under light microscopy, bFGF-immunoreactivity was noted in the inner part of photoreceptor cell outer segments, whereas photoreceptor cell bodies were only occasionally labeled with bFGF antiserum (Fig. 2). The outer plexiform layer close to the innermost part of the outer nuclear layer was also intensely immunoreactive for bFGF (Fig. 2). A small number of cells in the inner nuclear layer exhibited feeble immunostaining; cells in the innermost cell row appeared to be more frequently immunopositive than those in the outer cell rows (Fig. 2). A few ganglion neurons also contained immunoreaction materials in the cytoplasm. Besides the above retinal cells of neuronal origin, many glial (Müller) cells with vertically oriented thin processes, some mesenchymal cells in the choroid and pigment epithelial cells were endowed with bFGF immunoreactivity (Fig. 2). Pretreatment of bFGF antiserum with an excess of bFGF abolished all immunoreactions (Fig. 2d).

The immunoreactive outer plexiform layer in proximity to the inner nuclear layer, when examined with electron microscopy, was found to contain bFGF-like materials in the axon terminals of photoreceptor cells which formed synapses with bipolar cell dendrites devoid of immunoreaction deposits (Fig. 3a). Part of the photoreceptor (possibly cone) outer

Fig. 2. a: Diagram of photoreceptor, bipolar and ganglion cells in the retina. OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; IPL, inner plexiform layer; GCL, ganglion cell layer; NFL, nerve fiber layer. b: Bright-field photomicrograph showing individual retinal layers in a semi-thin section stained with toluidine blue. c: Bright-field photomicrograph showing bFGF immunoreactivity in the mature rat retina. d: Bright-field photomicrograph showing disappearance of bFGF-immunoreactive structures in a negative control section of rat retina. bar = 50 μ m.

segments with a lamellar structure was immunoreactive for bFGF (Fig. 3b). The above immunoreactions were not seen in control electron micrographs (Fig. 3c, d). Müller cells were most intensely labeled with bFGF antiserum as expected from the light microscopic observation, and they were seen in close apposition to retinal ganglion cells with or without bFGF-immunoreactivity (Fig. 3e). In the choroid, long tapering processes of putative mesenchymal cells were immunopositive, whereas vascular endothelial cells did not exhibit bFGF immunoreactivity (Fig. 3f).

Discussion

Using an antiserum against rat bFGF (amino acid 1–23), Gao and Hollyfield (1992) suggested that only Müller cells and pigment epithelial cells contain bFGF-like materials in the adult rat retina. They further showed that antisera against bovine/human bFGF recognize bFGF-like molecules exclusively in the ganglion cell layer, inner nuclear layer and pigment epithelium of the mature rat retina. Since serine residue is present at position 18 in bovine bFGF and absent from the position in rat bFGF, the first 25 amino acid residues of bovine and rat bFGFs, when used as antigens for antibody production, may have yielded antisera that are directed to different epitopes with or without the serine residue (Gao and

Fig. 3. a: Electron micrograph showing bFGF immunoreactivity in the axon terminals of two photoreceptor cells, which form synapses with non-immunoreactive bipolar cell dendrites. bar = 1 μ m.
b: Electron micrograph showing bFGF immunoreactivity in the outer segment of a photoreceptor cell. bar = 1 μ m.
c,d: Electron micrograph showing disappearance of bFGF immunoreactivity within photoreceptor nerve endings (c) and outer segments (d) in negative control sections, c, bar = 1 μ m; d, bar = 1 μ m.
e: Electron micrograph showing bFGF immunoreaction in a Müller cell(M) apposed closely to a ganglion cell(G). bar = 1 μ m.
f: Electron micrograph showing bFGF immunoreaction in mesenchymal cells of the choroid. bar = 1 μ m.

Hollyfield, 1992). To be more specific, Gao and Hollyfield speculated that the differential bFGF staining patterns were caused by the sequence difference between bovine and rat bFGFs. However, they did not take antibody sensitivity into consideration or provide any information on the sensitivity of their antibodies by immunoblot analysis, despite the fact that photoreceptor cells and inner nuclear layer cells are immunoreactive in the developing but not mature retina (Gao and Hollyfield, 1992). The antiserum against bovine bFGF used in the present study reacts well with bFGF-like molecules in 10 µg of retinal crude homogenate, and thus appears to exhibit a higher sensitivity than the other antisera so far employed (Raymond *et al.*, 1992). With the highly sensitive antiserum, the present study showed bFGF immunoreactivity not only in Müller cells and pigment epithelium but also in photoreceptor cells and cells of the inner nuclear layer within the adult rat retina, suggesting that bFGF in the photoreceptor and inner nuclear layers, even though decreasing in content with age, does exist within the mature rat retina. It is likely that our bFGF antiserum recognizes amino acid sequences common to bovine and rat bFGFs. In support of the speculation that our bovine bFGF antiserum crossreacts with rat bFGF, hair matrix cells of the rat vibrissae which are immunostained intensely with the bFGF antiserum have been shown to express rat bFGF mRNA (Okada *et al.*, 1992).

Microslicer-sectioning immediately after perfusion-fixation may also have contributed to satisfactory demonstration of retinal bFGF-immunoreactivity in the present study. In our experience, postfixation of the retina subjected to perfusion-fixation did not yield good immunostaining, possibly due to tissue overfixation which is assumed to preclude preservation of the antigenicity of retinal bFGF (Gao and Hollyfield, 1992). Because the retina is not endowed with blood vessels, transcardial perfusion appeared not to overfix the tissue and immediate sectioning with a microslicer may have made the exposure time of tissue to the fixative as short as possible in this study. Taken together, microslicer-sectioning takes less time for tissue processing than cryostat-sectioning which spends usually 24 hours to clear out the fixative completely from the tissue due to immersion in a cryoprotective reagent, and thus microslicer-sectioning may be adequate for bFGF immunohistochemistry.

The present study demonstrated intense bFGF staining in the axon terminals of photoreceptor cells in synaptic contact with bipolar cell dendrites within the outer plexiform layer. This is in contrast to a previous study which showed more intense immunostaining in the cell bodies and proximal processes of

goldfish photoreceptor cells than in the nerve terminals (Raymond *et al.*, 1992). The discrepancy in retinal bFGF staining between the rat and the goldfish may be attributed to the species difference. The localization of bFGF immunoreactivity in the axon endings of rat photoreceptor cells resembles that of bFGF receptor in goldfish and bovine retinas (Schweigerer *et al.*, 1987; Hanneken *et al.*, 1989). If rat bFGF receptor is present in the same retinal area, bFGF in the photoreceptor nerve endings may act as an autocrine regulator *in situ*, as suggested elsewhere (Schweigerer *et al.*, 1987; Okumura *et al.*, 1989; Logan *et al.*, 1991).

Another feature of immunostaining in the rat retina is the presence of bFGF-like materials in the outer segments of photoreceptor cells, which underwent continuous outward elongation as the pigment epithelium phagocytized and destroyed the tip of photoreceptor outer segments. It is tempting to speculate that bFGF not only facilitates neurite extension of a variety of central neurons (Morrison *et al.*, 1986; Walicke *et al.*, 1986) but also regulates the outward extension of photoreceptor outer segments. In accord with the present immunohistochemical results, a previous biochemical study isolated bFGF from bovine photoreceptor outer segments (Mascarelli *et al.*, 1987).

In lower vertebrates exogenous bFGF has been shown to participate in the transdifferentiation of pigment epithelial cells into photoreceptor cells (Pittack *et al.*, 1991) and in retinal regeneration in response to certain injuries (Park *et al.*, 1989), and endogenous bFGF might act in the same way. The mature rat retina, even though it has less potency of transdifferentiation and/or regeneration, may be under the control of endogenous bFGF in normal and pathologic conditions.

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