Basic Fibroblast Growth Factor Stimulates $^3$H-Thymidine Uptake in Retinal Venular and Capillary Endothelial Cells in Vivo

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Recent studies have found basic fibroblast growth factor (bFGF), an angiogenic peptide, in retina and have suggested that bFGF is responsible for retinal vascular proliferation. To test the hypothesis that bFGF stimulates $^3$H-thymidine uptake in retinal vascular cells in vivo, we injected bFGF (100 ng) into the vitreous cavity of six cats at 0 hr and again at 24 hr. Eight control eyes received boiled bFGF or no injection. After 46 hr, $^3$H-thymidine was injected into the vitreous cavity of all eyes and 2 hr later the eyes were enucleated. Intense $^3$H-thymidine uptake was seen in eyes with bFGF (56 ± 20 SD positive cells per section) but not in control eyes (7-10 positive cells per section $P < 0.001$). Trypsin digest preparations showed that the thymidine uptake was predominantly in the venular (89%) and capillary (10%) endothelium and not in arterioles (1%) $P < 0.001$. The data suggest that retinal venular endothelial cells respond preferentially to exogenous bFGF, and in part may explain their prominent role in the neovascular process. In a second group of experiments to test the hypothesis that retinal ischemia releases a diffusable factor similar to bFGF that can cause $^3$H-thymidine uptake in retinal vascular cells, we created branch retinal vein occlusion in six cat eyes. The fellow eyes received no injections. In the eyes with branch vein occlusion there was an intense $^3$H-thymidine uptake within the distribution of the occluded vein (84 ± 77 SD positive cells per section), but none in the areas outside the occluded vein $P < 0.001$. The data suggested that branch vein occlusion does result in a significant expression of endothelial cell mitogenic activity within the distribution of the occluded vein, but this activity did not appear to be mediated by a diffusible factor that increased mitogenic activity in the retina outside the occluded vein's distribution. Invest Ophthalmol Vis Sci 31:1238-1244, 1990

Retinal neovascularization in proliferative diabetic retinopathy, retinal vein occlusion, sickle cell anemia, and retinopathy of prematurity is hypothesized to be mediated by a release of angiogenic factors from ischemic retina.1-3 This hypothesis is attractive because it appears to explain new vessel growth outside the distribution of a branch vein occlusion or the increased risk of iris neovascularization after removal of the lens and vitreous in patients with diabetic retinopathy.4-6 The finding that normal retina contains substantial amounts of an angiogenic peptide, basic fibroblast growth factor (bFGF), supports this hypothesis.7,8

bFGF is an 146-amino-acid polypeptide that is present in retina and is widely distributed in the body.9 In several bioassays it is reported to be angiogenic even at low (1-10 ng/ml) concentrations.9-11 It causes endothelial cell mitosis, chemotaxis, and invasion into collagen gels in vitro.12-15 According to one report, elevated amounts of bFGF are present in vitreous of diabetic patients.16

If bFGF serves such a function in retinovascular disease, it should be able to elicit a proliferative response from the retinal vasculature. To test this hypothesis we injected bFGF in the vitreous cavity of cats and measured $^3$H-thymidine uptake by the retinal vasculature. In a related experiment, we tested the hypothesis that retinal ischemia releases an angiogenic growth factor similar to bFGF, by creating branch retinal vein occlusions in the cat and studying the proliferative response both within the distribution of the occlusion and outside the area of the vein occlusion.

Materials and Methods

Cats (2-4 kg) of both sexes were used in the experiments and were treated according to ARVO Resolution on Animal Experimentation. Under intramus-
cular ketamine (20–40 mg/kg) and xylazine (2 mg/kg) anesthesia, 100 ng recombinant bovine bFGF (Amgen, Mountain View, CA) in 0.1 ml phosphate buffered saline (PBS) was injected through the pars plana (4 mm behind the limbus) temporally using a tuberculin syringe with a 27-gauge needle into the right eye of six cats. The left eye was used as an untreated control. Three eyes were injected with 100 ng bFGF that had been boiled for 3 min and dissolved in 0.1 PBS as a control. After 24 hr the procedure was repeated.

In a second group of six cats, branch retinal vein occlusions were produced in the right eye by placing a 20-gauge diathermy probe (OMS, Danvers, MA) through a pars plana sclerotomy and by using an indirect ophthalmoscope to place the probe over the retinal veins (superior and nasal only). Then by applying current (intensity level 2) in the diathermy unit (OMS), the vein was occluded usually with one application. The arteries were avoided.

After 46 hr, 10 μCi 3H-thymidine (specific activity 20 Ci/mM; New England Nuclear, Boston, MA) was injected into the vitreous cavity of all eyes. Two hours later the cats were sacrificed by an overdose of intracardiac phenobarbital and the eyes removed. After a 24-hr fixation in buffered formalin, the anterior segments were removed at the pars plana and a 10-mm horizontal section containing the optic nerve was prepared for paraplast embedding and autoradiography. The retina from the superior collettes was removed and trypsin digests were prepared. For autoradiology, 5-μm paraplast retinal sections were cut or the trypsin digests were placed on gelatin coated slides. The slides were then immersed in Kodak (Rochester, NY) NTB-2 photographic emulsion and allowed to dry under red-light conditions. The slides were placed in a light-tight black slide box with desiccant and stored in the dark for 7 days and then developed, fixed, and stained.

3H-thymidine uptake of unstained retinal cross sections was counted by two observers who were unaware of the surgical manipulation of the eyes; these counts then were averaged. The results are expressed in terms of counts per section with a standard deviation.

3H-thymidine uptake was quantified in the trypsin digests by counting all available tissue and expressing the counts as positive cells per low-power field. In eyes with a branch vein occlusion, counts were made separately in areas corresponding to the distribution of the occluded vein(s). These counts were made by a single observer who was aware of the surgical manipulations, because the distribution of the occluded vein needed to be known for accurate comparisons.

Similarly, the ratio of positive cells in venules, capillaries, and arterioles was calculated after counts of more than 100 positive cells in each section had been reached. Results are expressed as cell number and a standard deviation.

**Results**

**Effects of bFGF Injected Intravitreally into Normal Cat Eyes**

*Clinical observations: Injection of 100 ng bFGF into the normal cat vitreous appeared to cause no immediate effect. There was no immediate dilatation of the vasculature or evidence of inflammation. After 24 hr there again was no noticeable change in the eye. The injection was repeated. Forty-six hr after the original injection of bFGF there appeared some mild dilation of the retinal vasculature. Six bFGF injected eyes did not appear clinically different than the three eyes injected with boiled bFGF.*

*Histologic and autoradiographic findings: The tissue sections of the posterior segments of the normal cat eyes fixed 48 hr after the injection of bFGF showed no apparent histologic abnormalities. There was no retinal edema or evidence of inflammatory infiltration into the retina in any of the eyes injected. Autoradiographic studies of the retinal tissue sections from the six uninjected eyes showed only minimal uptake of 3H-thymidine in the retina or retina vasculature (7 ± 5 positive cells per 5-μm retinal section, mean ± SD). This was also true of the three boiled-bFGF-injected eyes. In these eyes we averaged 10 ± 1 positive cells per section. However, in the six eyes that received active bFGF there was a 5-fold increase in the number of labeled cells in the tissue sections (average 56 ± 20 positive cells per section). A student t-test comparing the boiled bFGF and active bFGF injected eyes revealed that this result was highly statistically significant (P < 0.001). In the tissue sections, many labeled cells, which were located in the nuclear layers, appeared to be vascular endothelial cells.*

*Trypsin digest preparations of bFGF-injected eyes: In an effort to isolate the surrounding retinal tissue from the retinal vasculature, trypsin digests were prepared in three normal eyes, in the three boiled-bFGF-injected eyes, and in three active-bFGF-injected eyes. Autoradiographic preparations of the trypsin digests allowed a straightforward determination of capillaries, venules, and arterioles. Labeled cells were distinguished easily. Trypsin digests of the eye that had not received bFGF or had received boiled bFGF had very little label in the vascular cells (Fig. 1). However, in the eyes that received active*
Fig. 1. Autoradiographic preparation of trypsin digest from cat retina 48 hr after intravitreous injection of boiled bFGF. No uptake of 3H-thymidine is evident (X100).

bFGF there was an intense labeling of the vasculature, primarily in endothelial cells (Figs. 2A, B). To determine the distribution of the labeled cells, we recorded the location of over 100 cells in each of the preparations. The location was subdivided into arterioles, capillaries, and venules. The results were averaged and tabulated. We found that 89% of the labeled cells were located in the venules and to a much lesser extent in the capillaries. The arterioles took up almost no label (Fig. 3).

Branch Vein Occlusion Studies

Clinical observations: The six eyes in which branch vein occlusions were induced developed immediate venous engorgement and by 48 hr showed intraretinal hemorrhage and retinal edema and occasionally serious retinal detachment.

Histologic and autoradiographic observations: In the eyes with branch retinal vein occlusion, there were varying degrees of inner retinal edema and cell loss consistent with inner retinal ischemia.

Autoradiography of tissue sections of the involved segments of these eyes showed a heavy labeling of the inner retina (Fig. 4). Many of the sections contained labeled vessels that were surrounded by a diffuse inner retinal edema (Figs. 5A, B).

To quantitate the labeling we counted the labeled cells in the areas where there was histologic evidence of branch retinal vein occlusion (i.e., inner retinal edema) and expressed the counts as number of labeled cells per ×100 field. We found that within the...
distribution of the occluded vein there was a very high labeling index (83 ± 77 SD labeled cells per ×100 fluid field; n = 6).

Trypsin digest preparations of eyes with branch vein occlusions: The trypsin digest preparations in eyes with branch vein occlusion were not as free of retinal tissue as the bFGF-injected eyes. We speculated that this was due to trypsin inhibitors in serum that had leaked from the vessels. However, the digests were sufficient to determine the distribution of the label in the vasculature. We found in the areas of branch vein occlusion that venules and capillaries contained approximately equal amounts of label. However, as we found with the bFGF-injected eyes, the arterioles contained less than 1% of the label (Fig. 6).

To determine the effect of branch retinal vein occlusion of the uptake of 3H-thymidine in areas outside or distant to the distribution of the occluded vein, we counted labeled cells in the distribution of the occluded vein (n = 6) and outside of these areas in three eyes. We found that within the distribution of the occluded vein there was a high labeling index of the venules and capillaries (48 ± 30 cells per low-power field; n = 6). In marked contrast, we found outside of the distribution of the occluded vein that there was a very low labeling index (1 ± 1 cell per lower power field; n = 3). This difference was highly statistically significant (P < 0.001). When compared to the findings of the bFGF-injected eyes, we found within the distribution of the occluded vein that the labeling index approached that of active bFGF. However, outside the occluded vein the labeling index was normal (Fig. 7).

Discussion

The marked increase in 3H-thymidine uptake in the venular and capillary endothelial cells in the presence of bFGF suggests that bFGF may play a role in retinovascular proliferation. The data shows a very intense labeling in the venular endothelium after bFGF injection. Within 48 hr, large portions of the venules had areas in which the endothelial cells were heavily labeled. In the trypsin digest preparations, the
Fig. 5. (A) Autoradiogram of cross section of retina after branch vein occlusion (no exogenous bFGF). Note 3H-thymidine-labeled cells in distended vein (×100). (B) Higher-power magnification of labeled vessel in inner retina (×400).

Veins contained 89% of all of the label. One possible conclusion from this data is that the veins responded preferentially to bFGF. This correlates well with the clinical and histologic observations that neovascular budding usually begins from the retinal venules.3,19

In the eyes with a branch vein occlusion only, there was a moderate uptake of 3H-thymidine. The occluded vein caused a “blood-and-thunder” appearance and sometimes was associated with an exudative retinal detachment. We have previously demonstrated that the preretinal pO2 is quite low over experimentally induced branch retinal vein occlusions (7 torr, whereas normal = 21 torr).20 Therefore, we reasoned that if ischemic retina does release a soluble angiogenic growth factor into the vitreous cavity, then one might expect that experimentally induced branch vein occlusion in the cat would release such a compound. We further reasoned that if the compound had the same or similar properties as bFGF,

Fig. 6. Relative distribution of vascular cells with 3H-thymidine uptake in trypsin digest in eyes with experimentally induced branch vein occlusion (no bFGF injection). The capillaries and venules contain nearly all label.
then we should see a similar response in the normal retinal vasculature as was caused by exogenous bFGF. However, we found that the branch vein occlusion did not cause any uptake of 3H-thymidine outside the distribution of the vein occlusion. This finding was consistent with a series of experiments we recently completed demonstrating that there is little or no increase in extractable endothelial cell mitogenic activity between branch vein occluded and normal retina.20

Endothelial cell uptake of thymidine was seen predominantly in venules and to a lesser degree in capillaries. Arteriolar endothelial cells did not take up thymidine in response to bFGF injection. This is a striking difference between the arteriolar vascular cells and the venular and capillary endothelial cells. The observation is consistent with those of Michaelson and Williams et al.,19 showing that blood vessel growth in the retina originates from the venous end of the microcirculation (i.e., the postcapillary venules). Our data suggest that the venous side of the microcirculation is particularly sensitive to the exogenous growth factor.

Although this study demonstrates that bFGF can induce thymidine uptake and presumably cell proliferation in vascular and nonvascular cells in the retina, this does not mean that fibroblast growth factor is necessarily the regulatory factor in vascular proliferation. It remains to be demonstrated what role bFGF plays, if any, in normal retina not undergoing mitogenic activity.

Several explanations have been proposed. One is that endogenous bFGF is sequestered in the extracellular matrix, presumably to bound to heparan sulfate in the basement membrane. Moscatelli has demonstrated that there are two major binding sites for bFGF:21 one is a high-affinity receptor that is cell-associated and the other is a low-affinity matrix store. The matrix stores are accessible to the cell as it needs it. Why the large stores of bFGF do not normally cause a mitogenic response remains unexplained. Another possibility is that bFGF in a tissue is normally in an inactive form that in some way becomes active when needed. A third possibility is that the regulation of cell proliferation lies not with the concentration of a mitogenic growth factor, but rather with the cell itself. It is more than conceivable that the cell regulates its receptors and then responds to the surrounding sea of growth factors when needed.

Key words: basic fibroblast growth factor, capillary endothelial cells, retina, venules, 3H-thymidine

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