Correlation Between Angiogenesis and Basic Fibroblast Growth Factor Expression in Experimental Brain Infarct

Hsing Hong Chen, MD; Chung-Ho Chien, MD; H. Mei Liu, MD

Background and Purpose Cerebral endothelial cells are quiescent under normal conditions; they are stimulated to proliferate around an infarct, although the mechanism is unclear. In the present study we explored the relation between angiogenesis and the expression of basic fibroblast growth factor (bFGF) by participating cells in brain infarct.

Methods Brain infarct was created in rats by ligation of a branch of the left middle cerebral artery followed by permanent occlusion of the left common carotid artery and temporary occlusion of the right common carotid artery. The brains were removed after 1 to 14 days and studied with histological and immunohistochemical methods. Bromodeoxyuridine (BrdU) was used as an S-phase marker for the proliferative cells.

Results Enhanced bFGF immunoreactivity was observed in neurons adjacent to the infarct after 1 day, and the change subsequently spread to distant neurons in the ipsilateral hemisphere. After 2 days blood vessels and glial cells around the infarct began to incorporate BrdU. During the first week new capillaries accompanied by macrophages extended into the infarct. The macrophages, endothelial cells, and reactive astrocytes expressed mild to moderate bFGF immunoreactivity.

Conclusions The spatial and temporal correlation between bFGF expression and angiogenesis in conjunction with the well-known biological properties of bFGF suggest that bFGF produced by neurons, macrophages, and glial cells may participate in angiogenesis in brain infarct. (Stroke. 1994;25:1651-1657.)

Key Words • growth factors • cerebral infarction • endothelial • angiogenesis

Operative Procedure

The surgical procedure used to create a brain infarct was adapted from the method described by Chen. A total of 14 adult Sprague-Dawley rats weighing 500 to 600 g were used. After intraperitoneal injection of pentobarbital (40 mg/kg body wt), the rat was laid down on its right side. A 1.5-cm longitudinal incision centered at a point between the left eye and left ear was made. The scalp flaps were reflected, and the temporalis muscle was dissected away. Under an operating microscope and by use of a dental drill, a 2-mm burr hole was made at the junction of the zygoma and temporal squamosa. The dura and arachnoid membrane were carefully opened, revealing the parietal branch of the middle cerebral artery, which was ligated with a 10-0 nylon suture. A small piece of absorbable gelatin sponge was placed in the burr hole, the temporalis muscle was repaired, and the scalp incision was closed with continuous silk sutures. The rat was then placed in a supine position. A ventral midline incision was made over the neck. Both common carotid arteries (CCAs) were exposed after retracting the anterior margin of the sternoclidomastoid muscles. The left CCA was permanently occluded with a silver clip (Edward Weak Research Inc), and the right CCA was occluded with a metal clip for 1
FIG 1. Photograph shows 7-day-old infarct created by ligation of parietal branch of left middle cerebral artery followed by permanent left common carotid artery occlusion and temporary right common carotid artery occlusion. Evans blue dye was injected intraperitoneally 24 hours before removal of the brain. The discoloration indicates breakdown of the blood-brain barrier (original magnification x7).

hour. After ensuring that the circulation was reestablished in the right CCA, the neck wound was closed with silk sutures.

Sample Collection

The brains were removed for examination at intervals of 1, 2, 3, 5, and 10 days and 1 and 2 weeks after the operation. One day before the removal of the brain, the rats were given an intraperitoneal injection of a tracer (1 mL of 1% Evans blue dye in 5% bovine serum albumin). Two hours before death, each animal received BRdU 5 µg/g IP. The rats were deeply anesthetized and perfused intracardially with 100 mL of physiological saline followed by 250 mL of 3% paraformaldehyde and 0.5% glutaraldehyde. The brains were removed and fixed in the same fixative for an additional 24 hours. Coronal sectioned brain slices through the infarct were embedded for paraffin sections.

Histological Study

Paraffin sections cut at 4 µm were stained with hematoxylin-eosin, Wilder’s reticulum stain, and Sevier-Munger silver stain for neuraxons.

Immunohistochemical Study

Paraffin sections were stained according to the standard streptavidin-biotin peroxidase technique using a Dako LSAB kit. The bFGF antibody, purchased from Oncogene Science, is a rabbit affinity purified polyclonal antibody against peptide

FIG 2. Photomicrographs. a, One-day infarct immunostained for basic fibroblast growth factor (bFGF) to show enhanced bFGF immunoreactivity, particularly in neurons adjacent to the infarct (arrows). The large pyramidal neurons are more prominently stained than small neurons. Choroid plexus (double arrows) is strongly stained (original magnification x32). b, Close view of cortical neurons near the infarct shows heavy bFGF staining of cytoplasmic granules (original magnification x512). c, Cortical neurons in a normal brain immunostained for bFGF to show weak reactivity (original magnification x512).
FIG 3. Photomicrograph of bromodeoxyuridine labeling of endothelial cells and pericytes in proliferative branching capillaries adjacent to a 3-day-old infarct. Labeled cells have dark nuclei; unlabeled cells have lightly stained nuclei (original magnification ×210).

FIG 4. Photomicrograph shows angiogenesis in a 5-day-old infarct. Branches of new capillaries are derived from preexisting vessels and extend into the infarct (Wilder’s reticulum, original magnification ×160).

Results

Gross Findings
In brains removed 1 to 7 days postlesion, the infarct appeared as a circumscribed area of softening and bluish discoloration due to extravasation of the circulating Evans blue dye as the result of breakdown of the BBB (Fig 1). After 10 days the infarct was no longer stained but appeared as a depressed glial scar, indicating a return of the BBB function.

Histological and Immunocytochemical Findings
In normal rat brain, strong bFGF staining was seen in the choroid plexus, ependymal lining of the ventricles, and blood vessel walls. Neurons throughout the brain showed weak bFGF immunoreactivity; the large pyramidal neurons of the cerebral cortex and hippocampus, cerebellar Purkinje cells, and cranial and spinal motor neurons contained more cytoplasmic granular reactive products than the smaller neurons. No distinct bFGF immunoreactivity was seen in astrocytes in normal
brain, although reactive astrocytes that proliferated around the infarct showed mild to moderate bFGF reactivity (see below).

Brains removed 1 to 2 days postlesion showed a localized area of ischemic neuronal necrosis and edema of the neuropil in the surrounding area. After 1 day the cortical neurons in adjacent areas and to a lesser extent in the distant ipsilateral hemisphere exhibited enhanced bFGF immunoreactivity (Fig 2a). The reaction products appeared as coarse intracytoplasmic granules and were particularly prominent in large pyramidal cells of the cerebral cortex (Fig 2b and 2c). In 2-day infarct there was a sudden onset of vascular proliferation manifested initially as BrdU labeling of endothelial cells and pericytes in blood vessels adjacent to the infarct (Fig 3). From 3 to 6 days intense capillary proliferation was noted, and the new capillaries accompanied by macrophages extended into the infarct (Fig 4). At the same time, enhanced neuronal bFGF expression and BrdU labeling of glial cells spread from the infarct to the entire ipsilateral hemisphere. The 7-day infarct was a well-delineated lesion largely filled in by new capillaries and debris-laden macrophages and surrounded by reactive astrocytes (Figs 5 and 6). The macrophages exhibited moderate bFGF reactivity in the form of perinuclear collections of fine granules (Fig 7). The endothelial cells of the blood vessels and the reactive glial cells also exhibited mild to moderate bFGF immunoreactivity (Fig 8). After 10 days blood vessels in the vicinity of the infarct no longer exhibited BrdU labeling, although a small number of labeled glial cells were seen around the infarct. With the removal of debris by macrophages, a steady decrease in the size of the infarct and in the number of blood vessels and macrophages was noted. By 2 weeks the infarct appeared as a collapsed cavity surrounded by a dense collection of reactive astrocytes. The chronological order of histological changes is presented in Fig 9.
FIG 7. Photomicrograph shows macrophages in a 5-day-old infarct immunostained for basic fibroblast growth factor to show perinuclear collections of intracytoplasmic granules (arrows) (original magnification ×560).

Discussion

We have demonstrated enhanced bFGF expression in neurons adjacent to the infarct beginning 1 day postlesion. This was followed within a day by capillary proliferation and suggests an association between bFGF and angiogenesis. This suggestion was supported by the well-documented angiogenic property of bFGF.

Molecules capable of triggering proliferation of endothelial cells (angiogenic, and factors) are known to be present in a wide variety of tissues. The major sources are most if not all solid tumors, macrophages, and brain. The brain-derived endothelial cell growth factors are a family of closely related molecules showing affinity to heparin and heparan sulfate proteoglycan. The biological activities of these molecules are potentiated by heparin, hence the term heparin-binding growth factors. The major FGFs are acidic FGF (aFGF), with pI of 5.6, and bFGF, with pI of 9.6; their molecular weights range between 16,000 and 18,000, and they show a 55% sequence homology. FGFs induce angiogenesis in nanogram amounts in addition to being potent mitogens for a wide variety of mesoderm-derived cells as well as glial cells and Schwann cells. bFGF is considered to be a neurotrophic factor because it can induce nerve fiber extension and support survival of central nervous system, sensory, and sympathetic neurons in tissue culture. The facts that FGFs have neurotrophic, angiogenic, and gliogenic capacities and that brain is a ready source of FGFs suggest that these molecules may play vital pathophysiological roles in the nervous system.

The concentration of FGFs present in the brain is relatively low (35 to 50 μg/kg) compared with that in the pituitary gland (350 to 600 μg/kg). An important question is the type of cells that synthesize FGFs in the brain. Our study and those of several previous investigators have shown that FGFs are localized mainly in neurons. In other studies bFGF was predominantly localized in nuclei and cytoplasm of astrocytes throughout the brain and in cultured astrocytes. It is apparent that both neurons and glial cells are capable of producing bFGF. The amount of FGFs present in normal brains is so low that FGFs are barely detectable with immunohistochemistry. In our material the highest concentration of bFGF was found in choroid plexus epithelial cells. Subsequent to injury, the neurons and the proliferative nonneural cells (glial cells, endothelial cells, and macrophages) are induced to express increased amount of FGFs. The nature of the inducing factor(s) is not known; it could be products of the injured cells and/or extravasated plasma components.

The association between bFGF, cerebral ischemia/infarct, and angiogenesis has been shown in several recent reports. A twofold to threefold increase in mitogenic activity was demonstrated in tissues surrounding experimental infarct. Increase of bFGF immunoreactivity and its mRNA was demonstrated in rat hippocampus after transient forebrain ischemia. Rats made chronically ischemic by bilateral CCA ligation showed a significant increase in capillary density after intraventricular infusion of bFGF. A single topical application

Fig 8. Photomicrographs. a, Capillaries and glial cells from the contralateral hemisphere showing negative staining (original magnification ×250). b, New capillaries (double arrows) and reactive astrocytes (arrows) at the margin of a 5-day-old infarct immunostained for basic fibroblast growth factor to show moderate cytoplasmic reactivity (original magnification ×250).
of bFGF in the rat cerebral cortex induced capillary overgrowth in the cortex.²⁶

Findings presented in this study in conjunction with available information on bFGF suggest that angiogenesis in the infarct may be initiated by bFGF produced by neurons and sustained by bFGF produced by macrophages, proliferative glial cells, and endothelial cells on a paracrine and autocrine basis. The present study does not rule out the possibility that other growth factors and cytokines may contribute to angiogenesis in the infarct.⁴²⁷ aFGF, also present in the brain,¹⁷ and transforming growth factor-β, produced by virally transformed cells and embryonic tissues, are both potent angiogenic factors.²⁸ Interleukin-1 and interleukin-8, produced by macrophages, were shown to cause chemotaxis and proliferation of endothelial cells and astroglial cells when injected into rat brain²⁹ and hamster cheek pouch.³⁰ However, we were unable to detect any changes in aFGF, transforming growth factor-β, interleukin-1, or interleukin-8 in brain infarct using immunohistochemical methods (H.H.C., C.-H.C., H.M.L., unpublished data, 1993).

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In the present study Chen et al use a polyclonal antibody to basic fibroblast growth factor (bFGF) and immunohistochemical methods to show increased bFGF immunoreactivity in tissue surrounding focal ischemic infarcts in the mature rat brain. Increased bFGF immunoreactivity was found in neurons at 1 day and also became localized in reactive astroglia, macrophages, and endothelial cells at later times after ischemia. This report reemphasizes the current controversy surrounding the immunolocalization of bFGF in the intact mature rodent brain. Using monoclonal antisera, several investigators have shown a widespread localization of bFGF immunoreactivity in astroglial nuclei in the intact rat brain, whereas other investigators (including Chen and colleagues), using both monoclonal and polyclonal sera, have reported that bFGF immunoreactivity is predominantly localized in the cytoplasm of neurons and other cells. The reasons for these different patterns of bFGF immunolocalization remain unclear but may, in fact, depend on different conformations, modifications, or binding sites for the bFGF molecule, as well as on other potential cross-reacting protein species. Regardless of these differences in "baseline" bFGF immunolocalization, however, most studies (including the present one) agree that bFGF immunoreactivity is increased after brain injury or ischemia and that, after the first few days, increased bFGF immunoreactivity is localized prominently in reactive astroglia surrounding brain wounds or infarcts. Increased bFGF immunoreactivity in macrophages and endothelia has also been reported.

The present study by Chen et al suggests two distinct phases of bFGF immunoreactivity after focal ischemia, the first involving neurons alone and the second extending to involve invading macrophages, reactive astrocytes, and endothelial cells. These data are reminiscent of other recent studies showing a biphasic pattern of bFGF gene expression in models of global cerebral ischemia, with one peak of bFGF mRNA expression occurring at early times before cell death and a later peak occurring after cell death has occurred. It is possible that these two phases of bFGF expression play distinct roles. For example, the early expression of bFGF may relate to its trophic properties in increasing cell survival, whereas the later expression of bFGF may relate to its mitogenic properties in promoting cellular proliferation and tissue repair. One persistent puzzle is how and under what circumstances bFGF is released from cells in which it is made, since its gene lacks a classic signal sequence for secretion. Currently, our understanding of the possible functions of bFGF after ischemia remain purely correlative. For example, in the accompanying article, Chen et al note that because of their close temporal association, increased bFGF expression may indeed play an important role in glial and/or vascular proliferation after ischemia. On the other hand, ischemia undoubtedly induces the expression of other trophic and mitogenic molecules, so that it is currently impossible to directly link bFGF with any specific cellular process. Further understanding of the role of bFGF after ischemia must await experiments in which the selective blockade of this factor (either through the use of blocking antisera, antisense technology, or genetically engineered "knockout" animals) can be directly correlated with the extent of cell death, tissue repair, and neurological recovery.

**Seth P. Finklestein, MD, Guest Editor**

**CNS Growth Factor Research Laboratory**

**Massachusetts General Hospital—East**

**Charlestown, Mass**

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H H Chen, C H Chien and H M Liu

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