c-fos Protein Expression and Ischemic Changes in Neurons Vulnerable to Ischemia/Hypoxia, Correlated with Basic Fibroblast Growth Factor Immunoreactivity

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Abstract. Injuries to the brain induce rapid expression of c-fos and c-jun proto-oncogenes in neurons. The protein products (Fos and Jun) of these cellular immediate early genes are thought to regulate target genes that participate in fundamental biological responses. In recent studies of rat brain infarct we demonstrated that gliosis and angiogenesis, two of the fundamental biological responses, are related to neuronal expression of basic fibroblast growth factor (bFGF). In the present study, we explore the linkage between c-fos and bFGF genes by comparing the temporal and spatial domains of Fos and bFGF immunoreactivities (IR) in brain infarct and in transient global ischemia. We demonstrate colocalization of Fos-IR and ischemic changes in neurons at infarct periphery and in regions of "selective vulnerability" beginning 3 hours post-infarction and lasting up to 1-2 weeks. These are: cortical neurons in layers II-III and V, interneurons in hippocampal formation, cerebellar Purkinje cells, and many subcortical nuclei and brainstem nuclei. bFGF-IR appears 12-24 hours later than Fos-IR in the same region but in non-ischemic neurons and the expression persists beyond 2 weeks. Persistent and not transient c-fos expression appears to be associated with ischemic neuronal death, although some of these neurons may survive beyond 2 weeks post-infarction.

Key Words: Angiogenesis; Brain; Fibroblast growth factor; Gliosis; Infarct; Proto-oncogenes.

INTRODUCTION

Brain infarction induces adaptive responses characterized by proliferation of glial cells (gliosis) (1), blood vessels (angiogenesis) and macrophages (2). The regulation of the adaptive response involves transcriptional induction of cellular genes. Among the first set of genes to be activated by ischemia are proto-oncogenes c-fos and c-jun, members of a class of inducible genes termed cellular immediate early genes (IEGs) (3-5). Their protein products Fos and Jun form a heterodimeric complex that binds to specific regulatory DNA sequences known as AP-1 binding sites. Activation of the AP-1 transcription factors induces or inhibits transcription of target genes that are postulated to be involved in cell growth and other long-term responses. c-fos is rapidly induced in cultured neurons and other non-dividing cells by a variety of factors including serum (6), polypeptide growth factors (7) and neurotransmitters (8). In vivo c-fos expression in hippocampus and neocortex can be induced by ischemia/hypoxia (9-12), seizures (13), sensory stimulation (14), and trauma (15).

Information on the nature of the target genes is just beginning to emerge. The proenkephalin gene (16), dynorphin gene (17), nerve growth factor (NGF) gene and brain-derived neurotrophic factor (BDNF) gene (18) have been described as target genes regulated by the IEGs. The relationship between IEGs and other growth factor genes is not known.

In recent studies of ischemic brain infarct in rats, we have demonstrated neuronal activation with expression of basic fibroblast growth factor (bFGF) at infarct periphery and in distant neocortex, hippocampus and subcortical nuclei. This is followed within a day by gliosis and angiogenesis (2). The distribution of the neuronal bFGF immunoreactivities (IR) in our study is similar to that of the c-fos following experimental brain ischemia (9-12) and suggests a possible linkage between c-fos and bFGF induction. In the present study, we compare the temporal progression and topographic distribution of Fos-IR and bFGF-IR in rat brain subsequent to focal infarct and global ischemia and correlate these findings with histopathological parameters.

MATERIALS AND METHODS

Animal Model of Focal Brain Infarct

Ischemic infarct was created in a group of 24 male Sprague-Dawley rats weighing 300-400 g according to the procedure described by Chen (19). Under general anesthesia induced by intraperitoneal injection of pentobarbital (40 mg/kg body weight), the parietal branch of the left middle cerebral artery was ligated with a 10-0 nylon suture. The left common carotid artery (CCA) was ligated and the right CCA was occluded with a metal clip for 1 hour (h). The rectal temperature was maintained at 37°C with a heat lamp.

Animal Model of Global Ischemia

Global ischemia was created in a group of 24 rats by bilateral clamping of CCA for 1 h under general anesthesia followed by reperfusion.

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Immunohistochemical Methods

At different time points of 0 h (control), 3 h, 6 h, 12 h, 1 day (d), 2 d, 3 d, 5 d, 7 d, 10 d, 1 week (wk) and 2 wk post-lesion, two rats were deeply anesthetized with 60 mg/kg pentobarbital and perfused intracardially with 100 ml of warm phosphate-buffered saline (PBS) followed by 400 ml of ice cold 3% paraformaldehyde in PBS for 1 h. The brains were removed and fixed in the same fixative overnight. Coronal sections were embedded in paraffin. Sections cut at 4 μm were routinely stained with hematoxylin and eosin (H&E). Immunohistochemistry was performed according to standard streptavidin-biotin peroxidase technique using a Dako LSAB kit (Carpinteria, CA). Antibody against Fos (Oncogene Science, Uniondale, NY) is a rabbit affinity-purified polyclonal antibody raised against the peptide (TPTPTGPNCVPKNTD) corresponding to amino acids 73–87 in the N-terminal of Fos. The specificity of the anti-Fos serum was verified by the fact that preincubation of the antibody with an excess of HPLC-purified Fos peptide (Oncogene) abolishes the IR. The bFGF antibody (Oncogene) is a rabbit affinity-purified polyclonal antibody against peptide (AILFLPM) corresponding to amino acids 147–153 of human bFGF. The specificity of the bFGF antibody was confirmed by the fact that the IR was abolished by prior incubation of the antibody (1 μg/ml) with 2 μg recombinant bFGF (Oncogene). After blocking in 3% H₂O₂ for 15 minutes (min) and in a blocking solution for 20 min to eliminate nonspecific binding, the sections were incubated overnight at 4°C in primary antibodies followed by sequential incubations in biotinylated secondary antibody and streptavidin horseradish peroxidase complex for 30 min each at room temperature. The sections were reacted in 3-amino-9-ethylcarbazole or in 3′3′ diaminobenzidine in the presence of 0.003% H₂O₂ for 10 min and counterstained with hematoxylin. Negative controls were stained with irrelevant antibodies (anti-epithelial membrane antigen and anti-desmin antibodies).

RESULTS

Animals with Focal Infarct

3–12 h Post-Infarction: The post-infarction brains show considerable individual variations in the extent and distribution of neuronal ischemic changes and Fos-IR. In brains 3 h post-lesion, Fos-IR is strongly expressed in neuronal nuclei of hippocampal formation, bilateral neocortex, piriform gyrus and entorhinal cortex (Fig. 1). In pre-infarction rat brains, Fos is expressed at a uniformly low basic level in neuronal nuclei throughout the brain (Fig. 2B); only a few isolated neurons showing enhanced Fos-IR are seen in neocortex, hippocampus and hypothalamus. A low level of bFGF-IR is seen in hippocampal neurons but not in other regions. In neocortex of post-infarction brains, Fos-IR neurons are randomly distributed in all layers, but are densest in layers II–III, in large pyramidal cells in layer V, and focally in layer IV (Fig. 2A). The Fos-IR neurons in the infarct invariably show cytological changes characteristic of ischemia/hypoxia (Figs. 3A, B, D, E, 4A, B, D, E). The nuclei are atrophic, dense and homogeneous with angular or elongated outlines. The perikaryon is deeply stained and has wavy or corkscrew-like apical dendrites. In hippocampus, the first neurons to exhibit Fos-IR and ischemic changes are the polymorphic and horizontal interneurons at the hilar region (CA4) and the adjacent CA3 subfields (Figs. 1, 4A, B). CA1 is affected less frequently and CA2 appears to be least involved. In the dentate gyrus, Fos-IR and ischemic changes are first seen in a band of neurons inside the granule cell layer or wedged among them (Fig. 4D, E). These cells resemble basket cells. The dentate granule cells and hippocampal pyramidal cells appear to be more resistant to ischemia and to Fos induction than the interneurons. However, in certain instances large numbers of granule cells and pyramidal cells in all segments may exhibit Fos-IR and ischemic changes.

From 6 to 12 h, neurons in the infarct core begin to disintegrate with loss of Fos-IR. Fos-IR appears in many subcortical nuclei: septal nucleus, preoptic nucleus, bed nucleus, accumbens nucleus, islands of Calleja, amygdala, striatum, dorsolateral thalamus, substantia nigra and
hypothalamus. After 12 h, cerebellar Purkinje cells begin to exhibit enhanced Fos-IR as well as ischemic changes characterized by cytoplasmic vacuolization and eosinophilia and nuclear pyknosis (Fig. 3D, E). Twelve h post-infarction, bFGF-IR begins to appear in neurons at the infarct periphery and in bilateral neocortex, hippocampus and subcortical nuclei.

1–3 d Post-Infarction: After 1 d, a focal infarct is established (Fig. 5). The neurons in the infarct core are necrotic and have lost their Fos-IR. Pyknotic Fos-IR neurons are concentrated at the infarct periphery, and more Fos-IR neurons are seen throughout the ipsilateral and contralateral neocortex. Meanwhile, bFGF-IR is strongly manifest in bilateral cortex and hippocampus. Although Fos-IR and bFGF-IR are located in the same topographic regions, they are expressed by different neuronal groups. Unlike Fos-IR which is located in ischemic neurons, bFGF is expressed by non-ischemic neurons that show only a low level of Fos-IR in their nuclei (Fig. 3B, C). In hippocampus, strong Fos-IR is seen in ischemic presumed interneurons, whereas bFGF is strongly expressed by all the non-ischemic pyramidal cells (Fig. 4B, C). In dentate gyrus, strong Fos-IR is primarily seen in presumed basket cells while bFGF is expressed by granule cells (Fig. 4E, F). The great majority of cerebellar Purkinje cells show strong Fos-IR but not bFGF (Fig. 3E, F). Strong Fos-IR is seen in deep cerebellar nuclei and many nuclei in midbrain, pons and medulla. Some of these cells show ischemic changes and Fos-IR, others express bFGF-IR.

4–7 d Post-Infarction: On day 4, the numbers of Fos-IR neurons in neocortex and hippocampus appear to have reached a maximum. Many of the Fos-IR neurons undergo further atrophy and appear to be degenerating. Glial cells in general do not clearly express Fos-IR, although a few astrocytes around the infarct, in white matter of the cerebellum and in periventricular regions exhibit Fos-IR in their nuclei and cytoplasmic processes.

1–2 wk Post-Infarction: A slow decline in the number of Fos-IR neurons is observed after the first week. The apparent decrease could be the result of ischemic neuronal death. Even after 2 wk, severely atrophic ischemic neurons displaying Fos-IR are numerous in the neocortex, hippocampus, Purkinje layer and brainstem nuclei. The bFGF-IR of neurons persists beyond 2 wk.

Global Ischemia

Bilateral temporary clamping of CCA followed by reperfusion induces Fos expression and ischemic changes within 3 h in a small number of neurons in neocortex, hippocampus, piriform gyrus, hypothalamus and cerebellum. No frank tissue necrosis nor gliosis is seen in this
transient global ischemia model. A mild bFGF-IR is observed diffusely in non-ischemic hippocampal pyramidal cells. By the end of 4 d, Fos-IR and bFGF-IR had largely disappeared. This is in striking contrast to the persistent Fos-IR in the post-infarction brains.

A comparison of the time courses of Fos-IR and bFGF-IR resulting from infarct and global ischemia is presented in Figure 6.

DISCUSSION

c-fos is Preferentially Expressed in Neurons Vulnerable to Ischemia/Hypoxia

Focal ischemia induces strong and prolonged c-fos expression in neurons at infarct periphery and in bilateral neocortex, entorhinal cortex, cingulate gyrus, piriform gyrus, hippocampal formation, amygdala, hypothalamus, septal nucleus, cerebellar Purkinje cells and many brainstem nuclei. Our study shows minor differences from previous investigations that used different experimental models and animal species. By comparison, clamping of bilateral CCA for 1 h causes minimal ischemic changes and transient Fos-IR in cortical and hippocampal neurons. Therefore, the widespread neuronal changes observed in focal infarct appear to be related to the infarct and not to the 1 h of clamping of the contralateral CCA during the experimental production of the brain infarct.

Within neocortex and hippocampus, c-fos is preferentially induced in neurons known to be vulnerable to ischemia/hypoxia (20–22). These neurons show nuclear pyknosis, cytoplasmic eosinophilia and shrinkage (23, 24). In neocortex, interneurons in layers II–III and pyramidal neurons in layer V are more vulnerable than other layers. In the hippocampal formation, the most vulnerable neurons are interneurons in the hilar region (CA4) and basket cells in the inner zone of the dentate gyrus. Next in vulnerability are pyramidal cells in CA3, and in decreasing order CA1 and CA2 while dentate granule cells are relatively resistant to ischemia and c-fos induction (21, 22).

Mechanisms of Ischemic Neuronal Death and c-fos Induction

It has been recognized that vulnerability to ischemia is based on differences in biochemical and biophysical properties rather than on peculiarity of the vascular supply. According to the excitotoxic hypothesis, ischemic neuronal death is the consequence of a complex reaction induced by the release of the excitatory amino acid glutamate. It activates the N-methyl-D-aspartate (NMDA) receptor-operated calcium channel (ROCC) and voltage-sensitive calcium channel (VSCC) and causes calcium influx from the extracellular to the intracellular space (25–28). Excessive cytosolic Ca++ is considered a major factor in the initiation of biochemical reactions that lead to ischemic neuronal death (29, 30). The Ca++ ions activate many degrading enzymes including protease calpain, endonuclease, and phospholipases; these enzymes cause disintegration of nuclear and cell membranes and generation of prostaglandins and oxygen free radicals (31–33). Glutamate antagonists reduce neuronal injury (26, 34) and the size of brain infarct up to 50% when given prior to permanent middle cerebral artery occlusion in experimental animals (35, 36). It has been shown that cytosolic Ca++ stimulates the induction of the IEGs through either NMDA receptor activation (37) or a Ca++-calmodulin-dependent protein kinase-activated phosphorylation cascade (38). This may explain the co-expression of c-fos and ischemic neuronal change observed in the present study.

Not all Fos-IR neurons are moribund. Subsequent to transient global ischemia, many cortical and hippocampal neurons briefly express Fos; these neurons do not show ischemic changes. Only neurons showing persistent Fos-IR are associated with irreversible ischemic change.

Relationship Between c-fos, Glutamate and Growth Factor Expression

There have been several reports on the increase in neurotrophin synthesis subsequent to cerebral ischemia and kainic acid-induced seizures (18, 39, 40). The topographic distribution of NGF protein increase is similar to that of c-fos and bFGF, namely hippocampus, neocortex and striatum (18, 39). Increases in NGF and BDNF mRNA are induced in hippocampal neurons in vitro and in vivo by depolarization with high K+ and by administration of a glutamate receptor agonist, kainic acid (40, 41). This increase can be blocked by a non-NMDA receptor antagonist (41).

In view of the similarity in the distributions of Fos-IR and bFGF-IR (2) in brain infarct, we have been concerned with the question of whether there is a linkage between induction of c-fos and bFGF genes. In this study, we have demonstrated that neuronal Fos-IR is followed within 12–24 h by bFGF-IR expression in the same general regions, although they are localized in different neuronal populations. The Fos-IR neurons have a characteristic lamellar distribution and are confined to ischemic neurons in layers II–III and V, while bFGF-IR neurons are located in non-ischemic neurons in all cortical layers. In hippocampus, the first neurons to express Fos-IR are presumed interneurons whereas bFGF-IR is localized in non-ischemic pyramidal cells and granule cells. The failure of the ischemic Fos-IR neurons to express bFGF could be due to energy failure and inhibition of protein synthesis.

Relationship Between c-fos and Ischemic Neuronal Death

Transient induction of c-fos has been related to a variety of biological activities such as cell proliferation, dif-
Fig. 3. 1 day post-infarction, contralateral rat neocortex (A, B, C) and cerebellar cortex (D, E, F). A: Stained with H&E to show ischemic cortical neurons among non-ischemic neurons. $\times 210$. B: Immunostained with anti-Fos to show intense nuclear and mild apical dendrite IR in ischemic cortical neurons. The non-ischemic neurons show a mild nuclear stain. $\times 210$. C: Immunostained with anti-bFGF to show IR granules in non-ischemic cortical neurons and not in ischemic neurons. $\times 210$. D: Stained with H&E to show ischemic and non-ischemic Purkinje cells. $\times 210$. E: Immunostained with anti-Fos to show strong nuclear IR in ischemic Purkinje cells and not in non-ischemic cells. $\times 210$. F: Immunostained with anti-bFGF to show negative IR in Purkinje cells. $\times 210$. 

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Fig. 4. 2 days post-infarction, rat hippocampus (A, B, C) and dentate gyrus (D, E, F). A: Stained with H&E to show ischemic and non-ischemic neurons in hippocampal CA4. ×175. B: Immunostained with anti-Fos to show strong IR in ischemic hippocampal neurons and not in non-ischemic neurons. ×175. C: Immunostained with anti-bFGF to show IR granules in non-ischemic pyramidal neurons and not in ischemic neurons. ×175. D: Stained with H&E to show ischemic changes in presumed basket cells inside the granule cell layer. ×175. E: Immunostained with anti-Fos to show strong IR in ischemic basket cells and not in the non-ischemic granule cells. ×175. F: Immunostained with anti-bFGF to show mild IR in granule cells and not in the ischemic basket cells. ×175.
differentiation and stimulation (41–43). Persistent c-fos expression, on the other hand, is found to precede physiological cell death (apoptosis) (44, 45) or pathological cell death, such as cerebral ischemia, by hours or days. The prolonged c-fos expression observed in brain infarct could result from excessive Ca++ influx induced by glutamate toxicity. Hence, persistent c-fos expression may be a reflection of an irreversible biochemical derangement associated with impending cell death.

Widespread Induction of c-fos and Growth Factor Genes by Ischemic/Hypoxia and the Question of "Diaschisis"

Severe global ischemia and focal infarct cause activation of many genes including c-fos (9–12), NGF (39, 41), BDNF (18, 40, 41), and bFGF (2). The stereotypic responses and similarity in the topographic distribution of the gene products suggest that these genes may be induced through trans-synaptic activation of the same neuronal circuitry (46, 47). The pathophysiological significance of these responses is not known. The widespread and prolonged gene activation may provide the anatomical substrate for the well-known clinical phenomenon referred to as "diaschisis" in post-infarction patients (3, 46–48).

The present study has demonstrated for the first time coupling of c-fos expression and ischemic changes in neurons known to be vulnerable to ischemia/hypoxia. The induction of c-fos within seconds of ischemia, kindling and other non-lethal stimuli suggests that this early-response gene may be dealing with aspects of acute injury. bFGF has been shown to be a late-response gene and is involved in tissue repair (2, 49, 50). In brain infarct, bFGF-IR appears 12–24 h later in the same topographic regions as Fos-IR but in different sets of neurons. The question of whether bFGF is a target gene of c-fos has to be answered by molecular studies aimed at demonstrating whether the Fos/Jun/AP-1 complex binds to a regulatory sequence in the bFGF gene and whether the DNA binding property reflects a functional interaction.

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