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Influence of Age on the Response to Fibroblast Growth Factor-2 Treatment in a Rat Model of Stroke

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Abstract

Basic fibroblast growth factor (FGF-2) has been reported to protect against ischemic injury in the brains of young adult rodents. However, little is known about whether FGF-2 retains this capability in the aged ischemic brain. Since stroke in human is much more common in older people than among younger adults, to address this question is clinically important. In this study, aged (24-month-old) rats were treated with intracerebroventricular infusion of FGF-2 or vehicle for 3 days, beginning 48 hr before (pre-ischemia), 24 hr after (early post-ischemia), or 96 hr after (late post-ischemia) 60 min of middle cerebral artery occlusion, and were killed 10 days after ischemia. Aged rats given FGF-2 pre-ischemia showed better symmetry of movement and forepaw outstretching, and reduced infarct volumes, compared to rats treated with vehicle, but no significant improvement was found in aged rats given FGF-2 after focal ischemia. In contrast, young adult (3-month-old) rats treated with FGF-2 for 3 days beginning 24 hr post-ischemia showed significant neurobehavioral improvement and better histological outcome. In addition, we also found that newborn neurons in the rostral subventricular zone (SVZ) were increased in aged rats treated with FGF-2 prior to ischemia. However, unlike in young adult ischemic rats, only a few of newly generated cells migrated into the damaged region in aged brain after focal ischemia. These findings point to differences in the response of aged versus young adult rats to FGF-2 in cerebral ischemia, and suggest that such differences need to be considered in the development of neuroprotective agents for stroke.

Keywords

aging; growth factor; ischemia; brain; neurogenesis; neuroprotection

1. Introduction

Despite a large number of animal studies with promising neuroprotective agents, no clinically successful strategy for neuroprotection has emerged (Pitkanen, 2003). Whether this discrepancy is due to species differences or differences between the pathophysiology of human stroke and the animal models used is uncertain. Although stroke in humans usually afflicts the elderly (Arnold, 1981; Ramirez-Lassepas, 1998), most experimental studies on stroke have used young adult animals due to their greater availability, lower cost and fewer health problems. This is the case notwithstanding that abnormalities in glycolytic flux, lactate production, oxidation and energy production are more pronounced with advancing age, suggesting a

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reduced ability of the brain to adapt to stress (Hoyer, 1987). For example, ischemic lesion in the hippocampal CA1 region after global ischemia and in cerebral infarct size after focal ischemia increase in severity with increasing age (Arnold, 1981; Hoyer, 1987; Yao et al., 1991). After ischemia, mild memory impairment is observed in aged rats, while changes in some exploratory behaviors are observed in young adult rats (Andersen et al., 1999). These findings suggest that the outcome after cerebral ischemia might be affected significantly by advancing age.

Basic fibroblast growth factor (bFGF or FGF-2) is a polypeptide with potent trophic and protective effects on the brain. FGF-2 has been reported to exert neuroprotection against a wide variety of insults, including ischemic neuronal injury (Wada et al., 2003). A number of studies showed that administration of FGF-2 significantly reduced infarct volume and improved limb placing tests and rotarod fall latency in a model of focal cerebral ischemia in the rat compared to animals treated with vehicle alone (Ay et al., 1999; Bethel et al., 1997; Li and Stephenson, 2002). The mechanisms of enhanced functional recovery may include stimulation of neural sprouting and neural stem/progenitor cells in brain (Berry et al., 2005; Wada et al., 2003). However, little is known about the efficacy of FGF-2 in aged rodents subjected to focal ischemia (Ooboshi et al., 2000).

In this study, we investigated the effect FGF-2, given at different times in relation to the onset of focal cerebral ischemia, in aged compared to young adult rats. We found differences between these two groups in the effective time window for FGF-2 administration and in the neurogenesis response to FGF-2. Because of these differences, models of focal cerebral ischemia that employ aged animals may be more relevant to human stroke, and could have greater predictive value in the search for new modes of treatment.

2. Results

Physiological data including arterial pressure, arterial blood gas and blood glucose were measured in rats treated with or without FGF-2 before and after focal ischemia, and no significant difference was found between groups before MCA occlusion and early reperfusion.

First, we conducted neurobehavioral testing, including tests for symmetry of movement and forepaw outstretching, and found normal results (score=3) in all young adult and aged rats before MCAO, but profound behavioral deficits (score=0) in all after 60 minutes of MCAO. Thus, none of the animals tested was excluded because of an inadequate degree of cerebral ischemia. Symmetry of limb movement and of forepaw outstretching was monitored daily for 10 days. Aged rats treated with FGF-2 48 hr before ischemia (Plan A) showed significant improvement in symmetry of movement and forepaw outstretching, compared to vehicle-treated aged rats ($p<0.05$; two-way ANOVA with the Bonferroni correction). Significant improvement of symmetry of movement ($p<0.05$), but not forepaw outstretching ($p>0.05$), was observed when aged rats were given FGF-2 beginning 24 hr (Plan B) after ischemia. No significant improvement was observed when aged rats were given FGF-2 beginning 96 hr (Plan C) after ischemia ($p>0.05$; two-way ANOVA with the Bonferroni correction). In contrast, young adult rats did show improvement when treated according to Plan B ($p<0.05$).

Next, we evaluated the effects of FGF-2 treatment on infarct volume and histological changes after focal ischemia. In accord with their improved neurobehavioral status, aged rats treated with FGF-2 prior to ischemia showed a reduction in infarct volume, measured by cresyl violet and TTC staining, compared to vehicle-treated animals ($P<0.05$, Fig. 2a and b). Similar findings were observed in young adult rats given FGF-2 beginning 24 hr after ischemia, but not in aged rats treated with FGF-2 post-ischemia ($P>0.05$, Fig. 2a and b).

In vehicle-treated aged rats examined 10 d after MCAO, caspase-3 activation and DNA damage were detected in the cortical ischemic penumbra. However, after treatment with FGF-2 beginning 48 hr prior to ischemia, the number of cells showing caspase-3 activation or DNA damage in the corresponding cortical penumbra region was significantly reduced, but post-ischemic treatment did not (Fig. 2b).

In young adult rats, FGF-2 is not only neuroprotective, but also stimulates the proliferation of neuronal stem cells in the SVZ of the lateral ventricle (Wada et al., 2003). A subpopulation of these cells then migrates into ischemic brain areas, where they could be involved in repair and functional recovery. Therefore, we asked whether FGF-2 affects neurogenesis in the SVZ of aged rats. As shown in Figure 3a–b, BrdU (Bromodeoxyuridine)-positive cells were increased in the SVZ of aged rats treated with FGF-2 48 hr before induction of focal ischemia. Double-immunolabeling (Fig. 3d–e) showed that after MCAO, most of these cells expressed the immature neuronal marker DCX (Francis et al., 1999), consistent with neuronal lineage. As expected, a number of nestin-positive neural precursor cells migrated into the damaged regions in young adult rats, however, most of nestin-positive cells remained in the SVZ in aged rats after focal ischemia with pre- or post-treatment of FGF-2 (Fig. 4). This observation suggested that FGF-2-mediated outcome improvement in aged rats after focal ischemia may be through neuroprotection, but not through neurogenesis.

3. Discussion

We found that in aged rats with stroke from MCAO, pretreatment with FGF-2 improves outcome measures including neurobehavioral status, infarct volume, and cell death. Symmetry of movement, but not forepaw outstretching, was also improved when aged rats were given FGF-2 beginning 24 hr after ischemia. No significant improvement of behavior and infarct volume was observed when aged rats were given FGF-2 beginning 96 hr after ischemia, however, young adult rats benefited from treatment started 24 hr after MCAO. Both aged and young adult rats with MCAO showed increased FGF-2-induced neurogenesis, demonstrated by increased BrdU and DCX staining, but only a few of these newly generated cells migrated in the cortical penumbra in aged ischemic rats treated with FGF-2 before or after focal cerebral ischemia, suggesting that a discrepancy exists between aged and young adult rats in response to FGF-2 treatment in ischemic models.

A number of studies have shown that administration of FGF-2 to young adult rats reduces infarct volume and improves behavioral outcome from stroke (Ay et al., 1999; Ay et al., 2001; Bethel et al., 1997; Li and Stephenson, 2002). Some studies showed that a significant reduction in infarct volume was found when the FGF-2 infusion (50 µg/kg/hr for 3 hr) was begun up to 3 hr, but not 4 hr after the onset of ischemia (Ren and Finklestein, 1997), however, the enhanced functional recovery could be found if FGF-2 was given 24 hr after focal ischemia (Berry et al., 2005). Since the effect of FGF-2 on the outcome in young adult rats after focal ischemia is well documented, we mainly focus on the role of FGF-2 in the aged brain after MCAO in this study. We found that infarct volume was reduced when FGF-2 was infused at 24 hr after focal ischemia. The discrepancy may be due to the different duration, amount and route of FGF-2 administration and the different stroke model used. In addition, a number of physiologic changes occurring in aging animals could produce confounding results. For example, aged human's temperature regulation may be impaired and there could be differences in collateral blood flow (Kenney and Munce, 2003), which could affect infarct volume independent of FGF-2. We found that FGF-2 was protective in aged rats. This was true only when FGF-2 treatment was begun before the onset of ischemia, whereas young adult animals were protected even when treatment was delayed until 24 hr post-ischemia. Nevertheless, the extent of reduction in infarct size (~25%) was similar in aged rats treated before, and in young adult rats treated 24 hr after, MCAO.

Fibroblast growth factors and their receptors are expressed in the developing brain and in neuroproliferative regions of the adult brain, consistent with a role in neurogenesis (Cameron et al., 1998; Wagner et al., 1999). Moreover, infusion of FGF-2 into the lateral ventricle of adult rats stimulates neurogenesis in the SVZ and increases the number of neurons migrating from SVZ to the olfactory bulb (Kuhn et al., 1997). Although basal and stroke-induced (Jin et al., 2004) neurogenesis decline with age, neuronal precursors in the SVZ retain the ability to respond to FGF-2 with an increase in neuronal proliferation (Jin et al., 2003). It is, therefore, not surprising that in this study; FGF-2 treatment enhanced neurogenesis in the SVZ of both young-adult and aged rats undergoing MCAO. However, we found that majority of newborn cells migrate to the cortical damaged regions in young adult rats but not in aged rats after focal ischemia. The functional significance of this effect is uncertain, due to the fact that maturation of newly generated neuron may take 3–4 weeks, although current evidence suggests that ischemia-induced neurogenesis influences outcome from cerebral ischemia (Raber et al., 2004).

We conclude that FGF-2 improves outcome and stimulates neurogenesis after MCAO in aged rats, as reported previously in young adult rats. This implies that growth-factor treatment for stroke may retain efficacy despite advancing age. However, as we also observed, the time window for efficacy may be narrower in the aged brain. Thus, our results provide additional evidence that it may be important to conduct preclinical testing of prospective stroke therapies in aged animals, whose responses may correspond more closely to those of the aged human population that is most often affected by stroke. We understand that stroke from ischemia/reperfusion is different than stroke from permanent ischemia, therefore, our findings may only pertain to the model we used. In addition, we are aware that the time window of effectiveness of FGF-2 in rat models of stroke is likely to be different for human stroke. In particular, the time course of functional recovery in rats is 2–3 weeks, whereas in humans it is 2–3 months. So, the effective time window for a recovery drug may be 24–96 hr in rats, but perhaps much longer in humans.

4. Experimental Procedures

4.1 Transient focal cerebral ischemia

Aged (24-month-old) and young adult (3-month-old) male Fisher-344 rats were obtained from the National Institute on Aging. All animal experiments were in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals. Rats ($n = 4–6$ each group) were anesthetized with 2.5% isoflurane in 70% N_2O and 30% O_2 . Both common carotid arteries (CCAs) were exposed through a vertical midline neck incision. The animal was placed in a left lateral decubitus position, and the right middle cerebral artery (MCA) was exposed using the modified technique of Tamura (Tamura et al., 1981). Under the surgical microscope, a 2 cm incision was made between the right orbit and tragus. The temporal muscle was retracted laterally and a 3-mm diameter craniotomy was made just rostral to the foramen ovale. The dura was incised with a 26-gauge needle and the MCA was exposed. The MCA was occluded with a microclip and interruption of the blood flow was confirmed visually under the microscope. Immediately thereafter, both CCAs were occluded with microclips and rats underwent simultaneous occlusion of the MCA and both CCAs for 60 min. This model produces an infarct restricted to the cerebral cortex. Recirculation was established by removing the microclips and restoration of MCA blood flow was verified visually. Blood flow was monitored during the MCAO using laser Doppler in selected rats. The temporal muscle was repositioned and the skin was closed. A femoral artery was cannulated for blood pressure monitoring and arterial blood gas sampling before and after focal ischemia.

4.2 Administration of FGF-2 and BrdU

Rats (24-month-old) were anesthetized with 2% isoflurane in 70% N₂O/30% O₂ and an osmotic minipump (Alzet 1003D) was implanted. The cannula was placed in the right lateral ventricle 5.0 mm deep, 1.8 mm posterior to bregma, and 1.3 mm lateral to the midline. Mouse recombinant FGF-2 (R&D Systems; 10 µg/ml) was administered at 1 µl/hr by the intracerebroventricular route for 3 days (total 720 ng FGF-2), beginning 48 hr before (pre-ischemia; Plan A) or 24 hr after (early post-ischemia; Plan B), or 96 hr after (late post-ischemia; Plan C) focal ischemia, in artificial cerebrospinal fluid (aCSF) consisting of 148 mM NaCl, 3 mM KCl, 1.4 mM CaCl₂, 0.8 mM MgCl₂, 0.8 mM Na₂HPO₄, and 0.2 mM NaH₂PO₄. Young adult (3-month-old) rats were treated with FGF-2 for 3 days, beginning 24 hr after MCAO (Plan B). Control rats received aCSF without FGF-2 according to the same schedule. Rats were killed days 10 after MCAO and brains were removed for histological assessment (Fig. 1). BrdU (50 mg/kg in saline) was given twice daily by the intraperitoneal route for the same nine consecutive days.

4.3 Behavioral Tests

Neurobehavioral evaluations were carried out daily starting the day before the surgery and continuing until the end of each experiment according to the protocol developed by Garcia (Garcia et al., 1995), which showed a strong correlation with the number of necrotic neurons in rats with permanent or transient MCA occlusion (Pantoni et al., 1998).

4.3.1 Symmetry in the movement of four limbs—The rat was gently held in the air by the tail to observe symmetry in the movement of the four limbs. Scores indicate the following: 3, all four limbs extended symmetrically; 2, limbs on the left side extended less, or more slowly, than those on the right; 1, limbs on the left side showed minimal movement; and 0, limbs on the left side did not move at all.

4.3.2 Forepaw outstretching—The rat was brought up to the edge of a table and made to walk on its forelimbs while being held by the tail. Symmetry in the outstretching of both forelimbs was observed while the rat reached for the edge of the table and the hindlimbs were kept in the air. Scores indicate the following: 3, both forelimbs were outstretched, and the rat walked symmetrically on its forepaws; 2, left side outstretched less than the right and forepaw walking was impaired; 1, left forelimb moved minimally; and 0, left forelimb did not move.

4.4 Assessment of Injury

Brains were removed, 8-µm coronal brain sections were stained with cresyl violet, and selected brain sections were also stained with triphenyltetrazolium chloride (TTC). Infarct area, left hemisphere area, and total brain area were measured by a blinded observer using the NIH Image program, and areas were multiplied by the distance between sections to obtain the respective volumes. Infarct volume was calculated as a percentage of the volume of the contralateral hemisphere, as described (Swanson et al., 1990). Cell damage was assessed in fresh-frozen 20-µm sections using antibody against activated caspase-3. To determine nonspecific labeling, some sections were incubated without antibody.

4.5 Immunohistochemistry and double immunolabeling

To detect BrdU-labeled cells in brain sections, 8-µm sections were cut and incubated in methanol at -20°C for 10 min and in 2 M HCl at 37°C for 50 min, and rinsed in 0.1 M boric acid (pH 8.5). Sections were incubated in blocking solution (2% goat serum, 0.1% Triton X-100, 1% bovine serum albumin in PBS) for 1 hr at room temperature and then with treated overnight at 4°C with primary antibodies. The primary antibodies used were mouse monoclonal anti-BrdU (Roche; 2 µg/ml) and rabbit anti-activated caspase-3 (Cell signaling; 1:200).

Sections were processed with ABC reagents using a Vector ABC kit (Vector Laboratories). The horseradish peroxidase reaction was detected with diaminobenzidine and H₂O₂.

Double-label immunohistochemistry of brain sections was performed as previously described (Jin et al., 2002). The primary antibodies used, in addition to those listed above, were 1) goat polyclonal anti-doublecortin (DCX; Santa Cruz Biotechnology; 1:100); 2) mouse monoclonal anti-human specific nestin (Chemicon; 1:200); 3) goat anti-MCM2 (Santa Cruz, 1:100); 4) rabbit anti-calbindin (Upstate; 1:500); and 5) mouse monoclonal anti-GFAP (Sigma; 1:500). The secondary antibodies were Alexa Fluor 488-, or 594- conjugated donkey anti-mouse, or anti-goat IgG (Molecular Probes; 1:200–500). Images were viewed using a confocal laser-scanning microscope and 3D images were constructed using Imars software

4.6 Quantification of BrdU-labeled cells

BrdU- and DCX-labeled cells in SVZ were counted blindly in five to seven 50- μ m coronal sections per animal, spaced 140 μ m apart; this protocol avoids counting single cells twice due to the thickness of sections and provides accurate estimates of cell numbers. Cells were counted under high-power (200x) on a Nikon E300 microscope with Magnifier digital camera, and the image was displayed on a computer monitor. Results were expressed as the average number of BrdU-positive or DCX-positive cells per section.

4.7 Data analysis

Quantitative data were expressed as mean $\pm$ SEM from 3 – 4 experiments. Two-way ANOVA and Student's *t* test with the Bonferroni correction for multiple pair-wise comparisons were used for statistical analysis. *p* values <0.05 were considered significant.

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Abbreviation

FGF-2	basic fibroblast growth factor
CCAs	common carotid arteries
MCAO	middle cerebral artery occlusion
aCSF	artificial cerebrospinal fluid
BrdU	bromodeoxyuridine
TTC	triphenyltetrazolium chloride

DCX

doublecortin

SVZ

subventricular zone

Fig. 1.

Scheme for administration of FGF-2 before and after MCAO in rats. MCAO was induced in aged rats on day 0. FGF-2 or aCSF was administered by the intraventricular route for 3 days, beginning 2 days before (Plan A), or 1 day (Plan B) or 4 days after (Plan C) MCAO (a). Young adult rats were treated beginning 1 day after MCAO (Plan B) (b). Rats were killed 10 days after MCAO and brains analyzed by cresyl violet staining, BrdU immunohistochemistry, and cell counting.

Fig. 2.

Effects of FGF-2 on infarct volume and cell damage induced by MCAO. FGF-2 or aCSF was administered for 3 days, beginning 2 days before (Plan A), or 1 day (Plan B) or 4 days after (Plan C) MCAO in aged rats, or 1 day (Plan B) after MCAO in young adult rats. Rats were killed 10 days after MCAO. Infarct size was measured on cresyl violet (CV)-stained brain sections (a). In the cerebral cortical region salvaged by FGF-2 (boxes on TTC-stained brain slices), higher magnification views show that FGF-2 decreased ischemic neuronal damage (CV-stained sections), and the number of cells stained with anti-cleaved caspase-3 (Casp-3) (b).

Fig. 3.

Effect of intraventricular FGF2 on BrdU labeling of SVZ cells in aged rats after MCAO. FGF-2 or aCSF was given to aged rats for 3 days, beginning 2 days before MCAO. BrdU was given for the same nine days. Rats were killed 10 days after MCAO and sections were stained with an antibody against BrdU (a). FGF-2 increased the number of BrdU-positive cells in the anterior SVZ in aged rats (b). Double immunolabeling with anti-BrdU (red) and anti-DCX (green) shows apparent co-localization of BrdU and DCX within the same cells (c). Co-localization of BrdU (red) and with DCX (green) was confirmed by 3-D reconstitution of confocal images (d). FGF-2 increased the number of BrdU/DCX-immunopositive cells in SVZ of both young-

adult and aged rats after MCAO (e). *, $p < 0.05$ compared to aCSF (ANOVA and Student-Newman-Keuls tests).

Fig. 4. Migration pattern of nestin-positive cells in aged and young-adult rats after MCAO with FGF-2 treatment. Coronal sections were taken at 10 days of reperfusion in aged rats or young adult rats with FGF-2 treatment for 3 days, beginning days 2 before (pretreatment) or day 1 after (post-treatment) MCAO. Brains were removed at 10 days of reperfusion and immunostaining was performed using anti-nestin antibody. Top panel: low magnification; Bottom panel: high magnification (a). Most of nestin-positive cells (red) in the cortical penumbra in aged rats (pretreatment with FGF-2) expressed GFP (green) (b). A few of nestin-positive cells (red) in these regions expressed mature neuronal marker calbindin (green) (c).