Fibroblast Growth Factor-18 Reduced Infarct Volumes and Behavioral Deficits After Transient Occlusion of the Middle Cerebral Artery in Rats

Jeff L. Ellsworth, PhD; Richard Garcia, BS; Jin Yu, MD; Mark S. Kindy, PhD

Background and Purpose—Fibroblast growth factor 18 (FGF18) is expressed in rodent brain and is a trophic factor for neuron-derived cells in culture. The purpose of the present study was to evaluate whether FGF18 was neuroprotective in a rat model of cerebral ischemia and to compare the results with those obtained with FGF2.

Methods—Cerebral ischemia was produced in rats by a transient 2-hour occlusion of the middle cerebral artery (MCAo) with an intraluminal filament followed by 22-hour reperfusion. Starting 15 minutes after MCAo, FGF18 or FGF2 was administered by a 3-hour intravenous infusion. Infarct volumes and behavioral deficits were measured 24 hours after MCAo.

Results—Infusion of FGF18 produced dose-dependent reductions in infarct volumes and improvements in tests of reference and working memory, motor ability, and exploratory behavior. FGF18 was more efficacious than FGF2 on virtually all measures examined. The reductions in infarct volume and behavioral deficit were associated with FGF-mediated increases in regional cerebral blood flow.

Conclusions—These results demonstrate that FGF18 is an effective neuroprotective agent in a rat model of transient MCAo. (Stroke. 2003;34:1507-1512.)

Key Words: animal models ▪ growth factors ▪ neuroprotection ▪ rats

Each year, ~600,000 people in the United States suffer a new or recurrent stroke.1 About one third of stroke patients die within the first year, with the remaining two thirds living on average an additional 6 years.1 Although many patients spontaneously recover from stroke, it is the major cause of long-term disability.1,2 Interruption of the ischemic cascade has been attempted by administration of various neuroprotective factors.3 Several members of the fibroblast growth factor (FGF) family of proteins have been studied as neuroprotectants in animal models of cerebral ischemia.4–6 Of these, FGF2 has been studied most extensively.5

We recently identified a novel FGF, called FGF18,7 that is expressed within brain tissue both during development and in the adult.8,9 FGF18 promoted the survival of neurons in serum-free cell culture (J.L. Ellsworth, PhD, unpublished data) and stimulated neurite outgrowth from PC-12 cells.10 These neurotrophic activities suggested that FGF18 might be neuroprotective after stroke. To this end, we examined the efficacy of FGF18 after middle cerebral artery occlusion (MCAo) in rats and compared the results with those obtained with FGF2.

Materials and Methods

MCA Occlusion

Male Sprague-Dawley rats (250 to 275 g, Harlan, Indianapolis, Ind) were given free access to food and water before the experiment. All procedures were within institutional guidelines for animal use. Rats were anesthetized with halothane (1% in 70%/30% NO2/O2), and their bilateral femoral arteries were cannulated with PE-50 tubing for monitoring of mean arterial blood pressure (MABP) and for blood collection. The right femoral veins were cannulated for infusion of test articles. Body and brain temperatures were monitored with a rectal thermometer and a thermistor probe inserted into the temporalis muscle, respectively, and were maintained at 37°C by use of a water-jacketed heating pad. Temperatures and MABP were monitored continuously from 1 hour before to 6 hours after ischemia. The left common carotid artery of each rat was exposed through a midline incision in the neck. The superior thyroid and occipital arteries were electrocoagulated and divided. A microsurgical clip was placed around the origin of the external carotid artery (ECA). The distal end of the ECA was ligated with 6-0 silk and transected, and 6-0 silk was tied loosely around the ECA stump. The clip was removed, and the blunted tip of a 4-0 nylon suture was inserted into the ECA stump.11 The loop of the 6-0 silk was tightened around the stump, and the nylon suture was advanced through the internal carotid artery until it rested in the anterior cerebral artery. After the nylon suture had been in place for 2 hours, it was pulled back into the ECA, and the incision was closed. Fifteen minutes after the onset of ischemia, vehicle, or FGF18 (16, 66, or 133 μg·kg−1·h−1) or FGF2 (24, 72, or 132 μg·kg−1·h−1) was administered intravenously by infusion at a rate of 0.5 mL/h over a 3-hour period. The FGF dose range chosen was based on literature values.6 Investigators performing both the infusions and the analyses were blinded to the contents of the infusion vials.

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From ZymoGenetics, Inc, Seattle, Wash (J.L.E., R.G.); Department of Physiology and Neuroscience, Stroke Program and Center on Aging, Medical University of South Carolina, and Ralph H. Johnson, Veterans Affairs Medical Center, Charleston (J.Y., M.S.K.); and Molecular Therapeutics, Inc, Mt Pleasant, SC (M.S.K.).

Correspondence to Jeff L. Ellsworth, PhD, ZymoGenetics, Inc, 1201 Eastlake Ave E, Seattle, WA 98102. E-mail jefe@zgi.com

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1507
average scores were relative measures: scores of 0, 1, 2, 3 and 4 equaled

FGF18 and FGF2 reduced infarct volumes in MCAo rats. Differences were significant vs vehicle (*P<0.0001).

Measurement of Infarct Volume
Each rat was anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) at 24 hours after ischemia. The brains were transected pericardially perfused with 10% phosphate-buffered saline, removed, chilled for 15 minutes at −20°C, and placed in a Rodent Brain Matrix (Electron Microscopy Sciences). Coronal sections were prepared and subjected to 2% triphenyltetrazolium chloride (TTC) staining. The infarct area in each section was determined with National Institutes of Health Image Analysis Software, version 1.55. Total infarct volume for each brain was calculated by summation of the infarct areas of all brain slices (area times slice thickness [1 mm]) for each hemisphere and corrected for edema as follows: infarct volume \( (\text{mm}^3) = \text{infarct area} - \text{TIV} - \text{TCV} \), where TIV is total ipsilateral volume and TCV is total contralateral volume.

Behavioral Assessment
Animals were preconditioned to all tasks and were tested before and after ischemia. The Morris water maze was used to measure reference and working memory. The pool consisted of a 60×60×100 cm (diameter×height) circular metal tank that had a removable 14.5-cm-wide, 34-cm-high metal platform. The water level was 1 cm above the surface of the platform, with the water temperature maintained at 19°C to 21°C. Nontoxic black powdered paint was added to the water to obscure the visual appearance of the platform. Video records of the performance of each animal were analyzed with a Videomax V (Columbus Instruments). In tests of reference memory, video records were analyzed over three 90-second trials for target zone latency (TTL), time taken to reach the former location of the platform; search time, percent of time during each of three 30-second intervals of the probe trial that the subject spent in the platform; relative target visits (RTVs), percent of visits to the target zone of the total number of visits to all 4 zones. In the working memory test, video records of two 60-second trials were analyzed for TTL. The difference in finding the platform in the 2 trials by rats before and after ischemia were called D1 and D2, respectively. A working memory deficit was calculated as the difference between D1 and D2.

General locomotion and activity within a novel, open-field environment were assessed in exploratory behavior and general activity tests. The test apparatus consisted of a 60×60×100 cm (length×width×height) plastic box painted black. A video motion analyzer recorded the activity of each rat during each 15-minute session. The average scores were relative measures: scores of 0, 1, 2, 3 and 4 equaled 100%, 75%, 50%, 25%, and 0% of normal, respectively.

Motor performance was evaluated with the beam walk test. The time required to traverse the beam was converted to an ordinal scale: latencies for all 3 trials <10 seconds=1.0; latencies for all 3 trials >10 and <25 seconds=2.0; latencies for all 3 trials >25 and <60 seconds=3.0; failure to complete all 3 trials but completion of 1 or 2 with latencies <25 seconds=4.0; failure to complete all 3 trials but completion of 1 or 2 with latencies >25 seconds=5.0; and failure to complete all trials=6.0.

For all behavioral tests, the dashed lines in the figures represent the average scores of normal pre-MCAo rats.

Measurement of Cerebral Blood Flow
Regional cerebral blood flow (rCBF) was monitored by laser Doppler flowmetry every 30 minutes over the period 1 hour before to 6 hours after MCAo. Animals were anesthetized with halothane (1% in 70%/30% O2/N2), and a 2.0-mm hole was drilled in the skull, with the probe positioned 0.1 mm above the dura over the cortical surface. In the hemisphere ipsilateral to the occlusion, coordinates were as follows: point A, 1 mm posterior to the bregma and 5.4 mm lateral to the midline; point B, 1 mm posterior to the bregma and 2.1 mm lateral to the midline; and point C, 1 mm anterior to the bregma and 3.4 mm lateral to the midline. The mean values of rCBF measured before MCAo were taken as baseline, and the data thereafter were expressed as percentages of this value.

Statistical Analysis
Results are expressed as mean±SD. Differences were analyzed by use of 1-way analysis of variance (ANOVA), and repeated-measures ANOVA was computed on the monitoring data (Instat 2.03). Two-group comparisons were evaluated by Student’s t test with Bonferroni’s correction for multiple comparisons. Comparisons between FGF18 and FGF2 were evaluated by fitting a linear regression model including the treatment (FGF18 or FGF2) and the dose as predictors of the outcome variable using STATA 7 (Stata-Corp 2001). Regression results are presented as the regression coefficient (rc) estimating the difference in outcome (the units varied for different outcomes) between the FGF2 and FGF18 groups averaged across dose groups. Each point in the figures represents the mean±SD for 12 animals per group.

Expression and Purification of FGF18
_Escherichia coli_–derived human FGF18 was purified from culture media as described. _E_ coli–derived human FGF2 was purchased from Peprotech, Inc. Bioactivities were evaluated by use of BaF3 cells stably expressing Fgfr 3-(IIIc). FGFs were dissolved in vehicle (0.05 M NaPO4, 0.094 M NaCl, 50 μg BSA/mL, pH 7.2) prior to use.

Results
As visualized by staining of coronal sections with TTC, MCAo produced a unilateral infarction in the striatum and in large areas of the cerebral cortex. Infusion of FGF18 reduced infarct volumes (Figure 1). Infarct volumes were also reduced by infusion of FGF2, but the response was less than that observed with FGF18 (Figure 1, rc=15.1±1.9 mm³ [mean±SE], P<0.0005).

Infusion of FGF18 reduced the TTL in the third (final) trial of the reference memory test (Figure 2a). TTL decreased from 52.7±3.4 seconds in the vehicle-treated group to 26.8±3.6 seconds (P<0.0001) in rats infused with 133 μg FGF18 · kg⁻¹ · h⁻¹. Infusion of FGF2 also reduced the TTL (Figure 2a) but to a lesser extent (rc=−7.25±1.13 seconds per 90-second trial, P<0.0005). Infusion of FGF18 increased the acquisition and retention of reference memory over the 3 trials (Figure 2b). Little dose response was seen with infusion of FGF2 (Figure 2b), and the effect observed was less than...
that seen with FGF18 (rc = -1.29 ± 0.28 seconds per 90 seconds per trial, P < 0.0005). Infusion of either FGF18 or FGF2 reduced the search time deficit, calculated as the difference between the pre- and post-MCAo values for each group (Figure 2c). For pre-MCAo rats, ∼50% of their quadrant visits are to the target zone in the third trial (Figure 2d). In vehicle-treated MCAo rats, the RTVs were reduced to 25% (Figure 2d; pre- versus post-MCAo, P < 0.0001). Infusion of FGF18 increased RTV (Figure 2d). Relative to vehicle-treated animals, low-, medium-, or high-dose FGF2 decreased, increased, or produced no significant change in RTV, respectively (Figure 2d). Overall mobility of rats in the water maze was increased by infusion of either FGF18 or FGF2 (Figure 2e), but the effect observed with FGF2 was less than that seen with FGF18 (rc = 1.83 ± 0.34 visits per 90-second trial, P < 0.0005).

In the working memory test, the time to locate the platform decreased from 34.8 ± 2.6 seconds in trial 1 to 24.2 ± 2.9 seconds in trial 2 (P < 0.0001, n = 13 rats per group) for pre-MCAo rats. For vehicle-treated MCAo rats, the time to locate the platform in trials 1 and 2 increased to 54.4 ± 3.3 and 52.9 ± 4.9 seconds, respectively (P < 0.0001, pre- versus post-MCAo). Infusion of FGF18 reduced the working memory deficit (Figure 2f). Infusion of FGF-2 also reduced the working memory deficit, but the effects were not as robust as those seen with FGF18 (Figure 2f, rc = -3.14 ± 0.66 seconds per 60-second trial, P < 0.0005).

Infusion of FGF18 decreased the MCAo-induced deficits in exploratory behavior (Figure 3a) and in the beam walk (Figure 3b). Infusion of FGF2 produced no significant improvement in scores in either of these tests (Figure 3a, rc = -0.67 ± 0.11 U, P < 0.0005; Figure 3b, rc = -0.53 ± 0.10 U, P < 0.0005).

rCBF in the ischemic hemisphere was reduced to ∼20% of the pre-MCAo values in all groups (Figure 4a). Removal of the filament after 2 hours increased rCBF, followed by a reactive hyperemia that resolved over the next hour (Figure 4a). Infusion of FGF18 increased rCBF (Figure 4a). The rCBF scale during the 2-hour period of MCAo has been expanded in Figure 4b. The FGF18-mediated increase in rCBF was observed within 1 hour after the start of infusion (Figure 4b) and was maximal with the highest concentration of FGF18 tested at the 120-minute time point. At this time, the rCBF for vehicle- and FGF18-treated rats was 17.9 ± 2.6% and 26.5 ± 2.6% (mean ± SD) (P < 0.0001, n = 12 rats per group), respectively. Although infusion of FGF2 also increased rCBF, the changes were not as dramatic as those seen with FGF18 (Figure 4c). Across all FGF18 and FGF2 dose groups, infarct volumes were negatively correlated with increasing rCBF (r = -0.961).
Infusion of FGF18 nor FGF2 had no significant effects on blood pH or heart rate (Table). Compared with pre-MCAo rats, no significant changes were noted in brain temperatures or MABP for any of the treatment groups (data not shown). Comparing the pre- and post-MCAo values of blood gases revealed that MCAo reduced PCO₂ by 16% and increased PO₂ by a similar amount in vehicle-treated rats (Table). These changes were reversed by infusion of either FGF18 or FGF2 (Table).

**Discussion**

MCAo in rats produced a unilateral striatal infarction that also involved large areas of the cerebral cortex. The MCAo rats exhibited deficits in tests of reference and working memory, general exploratory behavior, and motor activity, observations consistent with the sites of tissue injury. Intravenous infusion of FGF18 beginning 15 minutes after the onset of ischemia reduced the behavioral deficits. The behavioral changes observed were highly correlated with dose-dependent reductions in lesion volumes, which appeared to be due, in large part, to FGF18-mediated increases in rCBF.

At the highest dose of FGF18 infused, rCBF during the period of occlusion was increased from ≈18% to 26% of the preischemia values. Changes in CBF of this magnitude after MCAo in rats produced major differences in infarct volumes observed at the same sites 72 hours later. Similarly, small changes in CBF in tissue plasminogen activator -/- mice relative to wild-type mice accounted for the effect of tissue plasminogen activator deficiency in enlarging the final infarct volumes. FGF2 appears to be a cerebral and peripheral vasodilator acting through a nitric oxide-dependent mechanism. That FGF2 reduced infarct volumes in endothelial nitric oxide synthase -/- mice without changing rCBF, however, suggests multiple mechanisms for FGF2-mediated neuroprotection. Whether the FGF18-mediated increase in rCBF seen in the present study is mediated by one or a combination of these pathways is not yet understood. The disruption of cerebrovascular autoregulation after cerebral ischemia could allow changes in MABP and/or cardiac output to increase cerebral perfusion. Because neither FGF18 nor FGF2 altered MABP or heart rate at the doses used in the

**Figure 3.** FGF18 but not FGF2 reduced MCAo-induced deficits in exploratory behavior (a) and beam walk latency (b). Differences were significant vs vehicle (*P<0.0001).

**Figure 4.** Infusion of FGF18 increased rCBF in MCAo rats. a, rCBF over the entire period of measurement with data taken from reference point A (shaded bar indicates the period of FGF18 infusion); b, rCBF from 30 to 120 minutes after MCAo shown on an expanded scale; panel c, comparative efficacy of FGF18 and FGF2 on rCBF (error bars were omitted from panel a for clarity). In b, differences were significant (*P<0.0001) by repeated-measures ANOVA. c, Vehicle-treated rats (*). Differences were significant for FGF18 (•) vs FGF2 (○): middle, *P<0.001, **P<0.04; right, *P<0.03, **P<0.02.
Physiological Parameters in Rats Before and After MCAo

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Differences significant vs vehicle. *P<0.0001, †P<0.006. Differences significant compared with vehicle-treated rats after MCAo: ‡P<0.0006, §P<0.0012.

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