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Time window of infarct reduction by intravenous basic fibroblast growth factor in focal cerebral ischemia

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Abstract

Basic fibroblast growth factor (bFGF) is a heparin-binding polypeptide with potent trophic and protective effects on brain neurons, glia and endothelia. In previous studies, we showed that intravenously administered bFGF reduced the volume of cerebral infarcts following permanent occlusion of the middle cerebral artery in rats. In the current study, we examined the time dependence of bFGF infusion on infarct reduction, and the effect of co-infusion of bFGF with heparin. We found a significant reduction in infarct volume when the bFGF infusion (50 μg/kg per h for 3 h) was begun up to 3 h, but not 4 h after the onset of ischemia. The infarct reducing effects of bFGF were not altered by co-infusion of heparin. These results are potentially important in light of the ongoing clinical trials of intravenous bFGF in acute stroke.

Keywords: bFGF (basic fibroblast growth factor); Cerebral ischemia, focal

1. Introduction

Basic fibroblast growth factor (bFGF) is a 18-kDa polypeptide with potent survival-promoting and protective effects on central nervous system cells, including neurons, glia and endothelial cells (Baird, 1994; Chen and Finklestein, in press; Walicke, 1988). This peptide is also a potent vasodilator in rat brain (Regli et al., 1994; Rosenblatt et al., 1994). Basic FGF binds avidly to heparin, and co-administration of heparin markedly prolongs the circulating half-life of intravenously administered bFGF (Klagsbrun, 1985; Whalen et al., 1989).

In recent studies, we found that intraventricularly or intravenously administered bFGF reduces the volume of cerebral infarction in models of focal cerebral ischemia in both rodents and cats (Bethel et al., in press; Fisher et al., 1995; Jiang et al., 1996; Koketsu et al., 1994). In particular, using a model of permanent middle cerebral artery occlusion by intraluminal suture in mature rats, we found that the intravenous administration of bFGF (50 μg/kg per h) for 3 h, starting at 0.5 h after the onset of ischemia, reduced infarct volume and corresponding neurological disability by 40–50% when measured one day after infarction (Fisher et al., 1995). Intravenous bFGF crosses the damaged blood-brain barrier to penetrate ischemic brain tissue (Fisher et al., 1995). The mechanism of infarct reduction may include direct protection of vulnerable brain cells as well as effects on cerebral blood flow (Fisher et al., 1995). Preclinical data in various animal models have recently led to ongoing human clinical trials of intravenous bFGF in acute stroke.

In the current study, we examined the 'therapeutic window' during which intravenous bFGF can reduce infarct volume following permanent focal ischemia in rats. Moreover, because of the known interaction between heparin and bFGF, we explored the effects of co-administration of heparin and bFGF on reduction in infarct volume.

2. Materials and methods

2.1. Ischemia surgery

Focal cerebral infarcts were made by permanent intra-arterial suture occlusion of the proximal right middle cerebral artery under institutional guidelines, as described previously (Fisher et al., 1995). Briefly, mature male Wistar rats (250–300 g, Charles River Laboratories, Wilmington, MA, USA) were allowed free access to food and water before surgery. Animals were anesthetized with

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chloral hydrate (400 mg/kg/i.p.), and rectal temperature was maintained at 37 ± 0.5°C using a heating blanket connected to a temperature controller (Model 73A, Yellow Springs Instrument, Yellow Springs, OH, USA) (we showed previously that rectal temperature closely parallels brain temperature in this model, and that intravenous bFGF infusion does not alter body or brain temperature (Fisher et al., 1995). A silicone catheter (Technical Products, Decatur, GA, USA) was introduced into the femoral artery for monitoring of mean arterial blood pressure (MacLab Data Acquisition System, AD Instruments, Castle Hill, Australia). Samples for arterial blood gases (pH, pCO_2 and pO_2; Model 248 Blood Gas Analyzer, Ciba Corning Diagnostics, Norwood, MA, USA) were also obtained from this catheter or from puncture of the tail artery. Another catheter was introduced into the femoral vein for drug infusions. Catheters were drawn through a subcutaneous tunnel and exited through the dorsal neck.

Under the operating microscope, the bifurcation of the right common carotid artery was exposed through a midline incision in the neck. A 4-0 monofilament nylon suture with its tip rounded near a flame was introduced into the right external carotid artery and advanced into the internal carotid artery for a length of 17 mm from the bifurcation. These methods place the tip of the suture at the origin of the anterior cerebral artery, thereby occluding the middle cerebral artery (Fisher et al., 1995). The suture was left in place until death.

Following middle cerebral artery occlusion, animals were allowed to awaken from anesthesia and were placed in a rodent swivel and tethering harness device (Stoelting, Wood Dale, IL, USA), allowing free movement during drug infusions. After infusion, catheters were tied and cut, and animals were released from the harness and returned to their cages. Surgical mortality was $< 10\%$ and occurred in all cases during the anesthetized period, before animals could receive infusions.

2.2. Drug formulation and infusion

Recombinant human bFGF was obtained as a concentrated stock solution (2 mg/ml, Scios, Mountain View, CA, USA) and stored at -80°C before use. The stock solution was diluted into 0.9% NaCl containing 100 μg/ml bovine serum albumin (Boehringer-Mannheim, Indianapolis, IN, USA, Cat. No. 711454), pH 7.4, to give a final bFGF concentration of 30 μg/ml. The bFGF solution was infused intravenously at 0.5 ml/h via a microprocessor controlled syringe pump (Stoelting) to deliver a dose of 50 μg/kg per h for 3 h. This dose was chosen as the dose used in previous studies in which significant reduction in infarct volume was found (Fisher et al., 1995). Heparin was obtained as a stock solution of 1000 units/ml (Elkins-Sinn, Cherry Hill, NJ, USA) and was diluted into 0.9% NaCl and infused at 0.5 ml/h to deliver a dose of 15 units/kg per h for 3 h. This dose was chosen as a standard intravenous dose of heparin used as an anticoagulant (equivalent to 1000 units/h for a 70 kg man). Vehicle solutions infused into control animals contained no bFGF or heparin but all other constituents at the same concentrations.

2.3. Experimental design

In Experiment 1, we examined the time dependence of infarct reduction by intravenous bFGF. bFGF (50 μg/kg per h) or vehicle was infused for 3 h, beginning at 0.5, 2, 3 or 4 h after ischemia. At each time point, vehicle- as well as bFGF-treated animals were prepared contemporaneously. In Experiment 2, we tested the effects of co-infusion of heparin with bFGF. Animals received vehicle, heparin alone (15 units/kg per h), bFGF alone (50 μg/kg per h), or heparin plus bFGF (15 units/kg per h plus 50 μg/kg per h, respectively) for 3 h, beginning at 0.5 h after ischemia. For both experiments, blood pressure was recorded just before and just after stroke surgery, and before and at the end of infusions. Blood gases were recorded before stroke surgery and at the end of infusions. Animals were assigned treatments in a random fashion. The experimenter performing the surgery, infusions and infarct volume determinations was blinded to treatment assignment until all data had been collected.

2.4. Infarct volume determination and data analysis

At 24 h after ischemia, animals were killed by an overdose of chloral hydrate, and brains were removed and cut into seven 2 mm coronal slices using a rat brain matrix (RBM 4000C, ASI Instruments, Warren, MI, USA). The slices were then stained with 2,3,5-triphenyltetrazolium hydrochloride (TTC) at room temperature for 30 min and fixed in 10% buffered formalin. After 48 h, the infarct area on each slice was determined using a computerized image analyzer (Bioquant, R&M Biometrics, Nashville, TN, USA) using the 'indirect method' (area of the intact contralateral (left) hemisphere − area of intact regions of the ipsilateral (right) hemisphere) to correct for brain edema (Jiang et al., 1996). Infarct volume was expressed as a percentage of intact contralateral hemispheric volume. All data were expressed as mean ± S.E.M. and analyzed by one- or two-way analysis of variance, followed by appropriate pairwise two-tailed *t*-tests with Bonferroni correction for multiple comparisons.

3. Results

As described previously (Fisher et al., 1995), permanent unilateral occlusion of the proximal middle cerebral artery by intra-arterial suture produced large infarcts in the ipsilateral dorsolateral cerebral cortex and underlying striatum (Fig. 1). In Experiment 1, we examined the 'therapeutic window' of infarct reduction by intravenous bFGF. Ani-

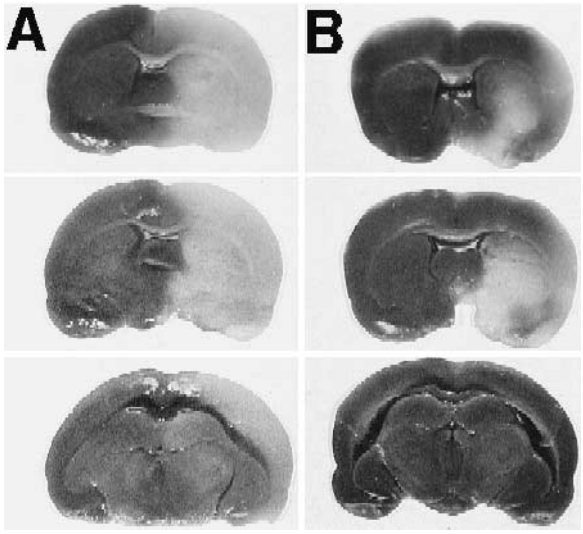

Fig. 1. Reduction in infarct volume by intravenous bFGF. (A) TTC-stained coronal sections from an animal receiving a 3-h infusion of vehicle, beginning at 30 min after middle cerebral artery occlusion. A large infarct is seen (pale region) involving the cerebral cortex and underlying striatum. (B) TTC-stained sections from an animal receiving a 3-h infusion of bFGF (50 μg/kg per h), beginning at 30 min after middle cerebral artery occlusion. A smaller infarct (pale region) is seen.

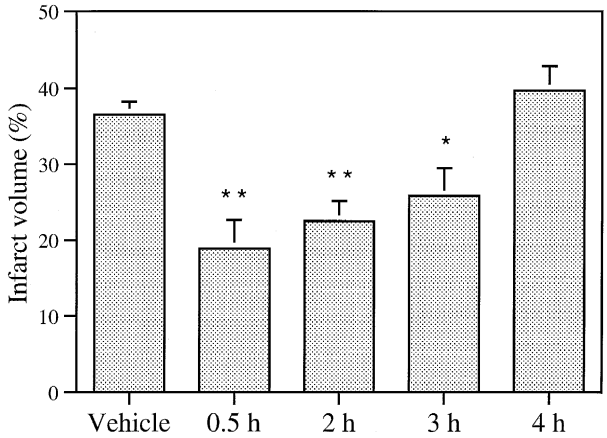

Fig. 2. Time dependence of infarct reduction by intravenous bFGF. Infarct volume in animals receiving bFGF infusion starting at 0.5 h ($n=6$), 2 h ($n=6$), 3 h ($n=5$), or 4 h ($n=5$) after the onset of ischemia were compared to that of pooled vehicle-treated controls ($n=18$). Data are mean ± S.E.M. One-way ANOVA: $F=11.16$, $P=0.0001$. Asterisks indicate values different from control animals by $P<0.01$ (**) or $P<0.05$ (*).

Table 1
Mean arterial blood pressure of animals before and after stroke surgery and before and at the end of intravenous infusions

	n	Mean arterial blood pressure (± S.E.M.)			
		Before stroke (mmHg)	After stroke (mmHg)	Before infusion (mmHg)	End of infusion (mmHg)
Experiment 1					
0.5 h					
Vehicle	6	67.0 ± 15.5	74.0 ± 15.0	75.0 ± 8.9	84.5 ± 14.8
bFGF	6	76.5 ± 6.3	79.3 ± 9.1	84.0 ± 10.3	74.0 ± 2.8
Mean	12	71.8 ± 12.8	76.7 ± 12.2	79.5 ± 10.3	79.3 ± 10.6
2 h					
Vehicle	6	83.3 ± 13.4	87.5 ± 8.5	97.0 ± 14.3	105.3 ± 4.6
bFGF	6	75.3 ± 10.6	88.0 ± 9.5	90.3 ± 12.6	89.2 ± 8.6
Mean	12	79.3 ± 12.2	87.8 ± 8.6	93.7 ± 13.3	97.2 ± 10.7
3 h					
Vehicle	3	77.3 ± 8.1	80.3 ± 12.7	113.7 ± 4.0	99.7 ± 8.4
bFGF	5	89.0 ± 13.7	96.4 ± 16.6	90.3 ± 12.6	111.4 ± 33.0
Mean	8	84.6 ± 12.7	90.4 ± 16.5	121.5 ± 10.2	107.0 ± 26.0
4 h					
Vehicle	3	90.6 ± 16.3	95.3 ± 7.2	129.3 ± 14	127.3 ± 8.4
bFGF	5	99.8 ± 4.4	100.2 ± 13.2	121.4 ± 10.7	97.3 ± 18.0
Mean	8	96.4 ± 10.4	98.4 ± 11.0	124.3 ± 11.8	110.1 ± 21.1
Experiment 2					
Vehicle	6	77.8 ± 6.2	79.7 ± 8.8	80.8 ± 11.9	82.7 ± 14.3
Heparine alone	6	83.5 ± 9.5	80.3 ± 7.0	84.2 ± 13.3	88.0 ± 14.0
bFGF	6	72.2 ± 9.2	80.2 ± 6.0	77.3 ± 7.1	81.2 ± 6.2
bFGF + heparin	6	78.7 ± 6.3	78.3 ± 6.0	79.0 ± 2.9	78.2 ± 5.4
Mean	24	78.0 ± 8.5	79.6 ± 6.6	79.8 ± 9.6	82.7 ± 10.7

Data are expressed as mean ± S.E.M. and are complete for all cells, except for values in four vehicle-treated and four bFGF-treated animals in the 0.5 h group and one bFGF-treated animal in the 4 h group at the end of infusions in Experiment 1, due to thrombosed femoral artery catheters. Mean baseline and subsequent blood pressures varied between groups studied at different time points in Experiment 1, reflecting differences in the time of blood pressure measurement in relation to the anesthetized period during stroke surgery. However, no differences were found among vehicle- and bFGF-treated animals studied at any given time point by two-way repeated measures analysis of variance. Similarly, no blood pressure differences were found between groups studied in Experiment 2.

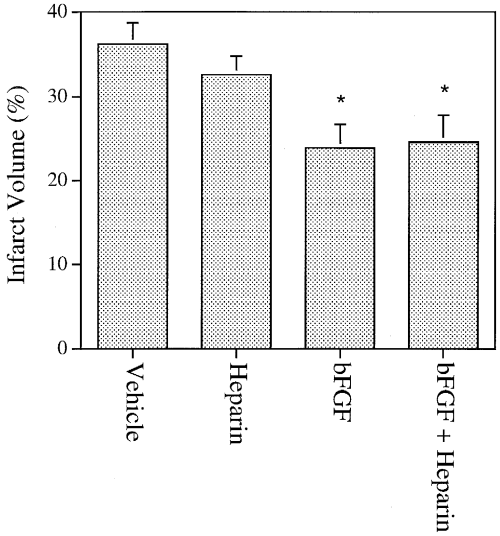

Fig. 3. Effect of heparin on infarct reduction by bFGF. Animals received an intravenous infusion of vehicle ($n = 6$), heparin ($n = 6$), bFGF ($n = 6$), or bFGF + heparin ($n = 6$), starting at 0.5 h after the onset of ischemia. Data are mean ± S.E.M. One-way ANOVA: $F = 5.33$, $P = 0.007$. Asterisks indicate values different from those in vehicle-treated controls by $P < 0.05$.

mals received intravenous infusions of bFGF (50 μg/kg per h) for 3 h, starting at 0.5, 2, 3 or 4 h after the onset of ischemia. Vehicle-treated controls were prepared contemporaneously at each time point. Infarct volumes were not different among control animals ($F = 1.44$, p-.s.), so that these data were pooled in the analysis. Compared to vehicle-treated controls, significant infarct reduction was observed when bFGF was infused beginning at 0.5, 2 and 3, but not 4 h after the onset of ischemia (reductions of 48, 37 and 29%, respectively; Figs. 1 and 2).

In Experiment 2, we examined the effect of co-infusion of heparin (15 units/kg per h) with bFGF (50 μg/kg per h), starting at 0.5 h after the onset of ischemia. Heparin alone had no effect on infarct volume in this intra-arterial suture occlusion model, whereas bFGF reduced infarct volume (Fig. 2) (the magnitude of infarct reduction (34%) was somewhat smaller than that seen in Experiment 1 (48%), possibly reflecting different potencies of the two different lots of bFGF used in these experiments). Co-administration of heparin did not alter the infarct reducing effects of bFGF (Fig. 3).

Baseline (pre-stroke) mean arterial blood pressure varied somewhat among the groups studied at the different time points in Experiment 1, most likely reflecting differences in the degree of anesthesia among groups (Table 1). In addition, mean arterial blood pressures both before and at the end of infusions were higher in groups in which infusions were begun at later times (e.g., 2, 3 and 4 h) compared to the earliest time (0.5 h) after ischemia, re-

Table 2
Arterial blood gases of animals before stroke and at the end of intravenous infusions

	n	Arterial blood gases (± S.E.M.)					
		Before stroke			At the end of infusion		
		pH	pCO_2 (mmHg)	pO_2 (mmHg)	pH	pCO_2 (mmHg)	pO_2 (mmHg)
Experiment 1							
0.5 h							
Vehicle	6	7.38 ± 0.5	44.3 ± 4.5	79.4 ± 3.6	7.40 ± 0.7	33.8 ± 5.5	90.6 ± 13.4
bFGF	6	7.40 ± 0.6	38.7 ± 4.6	88.3 ± 13.0	7.45 ± 0.6	30.7 ± 5.4	94.7 ± 9.7
Mean	12	7.39 ± 0.5	41.5 ± 5.4	84.3 ± 10.6	7.43 ± 0.07	32.1 ± 5.4	92.8 ± 11.1
2 h							
Vehicle	6	7.40 ± 0.0.1	40.2 ± 6.4	85.5 ± 6.3	7.46 ± 0.6	29.2 ± 2.5	93.2 ± 8.3
bFGF	6	7.38 ± 0.4	40.0 ± 3.5	83.0 ± 12.4	7.45 ± 0.6	28.8 ± 3.7	86.3 ± 11.1
Mean	12	7.39 ± 0.03	40.1 ± 4.7	84.2 ± 9.7	7.46 ± 0.05	29.0 ± 3.1	89.5 ± 10.1
3 h							
Vehicle	3	7.35 ± 0.02	41.7 ± 7.2	84.7 ± 19.2	7.43 ± 0.04	30.7 ± 2.8	80.3 ± 4.1
bFGF	5	7.35 ± 0.02	45.8 ± 4.0	97.7 ± 16.6	7.44 ± 0.03	28.8 ± 2.0	92.3 ± 17.8
Mean	8	7.35 ± 0.02	44.3 ± 5.4	92.8 ± 17.5	7.44 ± 0.03	29.5 ± 2.4	87.8 ± 14.9
4 h							
Vehicle	3	7.38 ± 0.03	40.7 ± 2.9	91.9 ± 16	7.45 ± 0.04	29.2 ± 1.4	82.9 ± 6.5
bFGF	5	7.42 ± 0.03	38.0 ± 3.6	86.7 ± 4.6	7.45 ± 0.02	24.3 ± 3.3	93.9 ± 13.3
Mean	8	7.40 ± 0.03	39.0 ± 3.5	88.7 ± 9.5	7.46 ± 0.03	26.4 ± 3.6	89.2 ± 11.7
Experiment 2							
Vehicle	6	7.40 ± 0.1	45.8 ± 8.1	79.2 ± 6.7	7.40 ± 0.1	37.1 ± 6.3	86.4 ± 7.6
Heparine alone	6	7.34 ± 0.4	41.9 ± 6.0	83.6 ± 7.2	7.37 ± 0.4	31.8 ± 1.8	83.0 ± 7.1
bFGF	6	7.30 ± 0.01	43.0 ± 3.2	79.6 ± 11.9	7.40 ± 0.1	32.1 ± 6.1	97.8 ± 18.0
bFGF + heparin	6	7.33 ± 0.3	43.5 ± 3.9	81.0 ± 2.8	7.41 ± 0.4	32.4 ± 3.8	84.8 ± 7.4
Mean	24	7.34 ± 0.04	43.6 ± 5.5	80.8 ± 7.5	7.39 ± 0.05	33.4 ± 5.2	88.2 ± 12.2

Data in all cells are complete, except for values in one bFGF-treated animal in the 4 h group at the end of infusion in Experiment 1. For Experiment 1, no differences were found among mean values of groups studied at different time points, or among values of vehicle- vs. bFGF-treated animals studied at each time point using two-way repeated measures analysis of variance. Similarly, no differences were found among groups studied in Experiment 2.

flecting the fact that animals had awakened from anesthesia at these later but not earliest time points (Table 1). However, there were no differences in blood pressure between contemporaneously prepared vehicle- and bFGF-treated animals studied at any time point (Table 1). There were no differences in blood pressure among any groups in Experiment 2 (Table 1). Likewise, there were no differences in arterial pH, pCO_2, or pO_2 either before stroke surgery or after infusions among any groups studied in either Experiment 1 or 2 (Table 2).

4. Discussion

In summary, using a model of permanent focal ischemia in rats, we found that the intravenous infusion of bFGF reduced infarct volume when begun up to 3 h, but not 4 h after the onset of ischemia. The degree of infarct reduction was not altered by co-infusion of heparin. The infarct-reducing effects of bFGF could not be accounted for by changes in mean arterial blood pressure, arterial blood gases, or core temperature. These data are consistent with several previous reports showing that the intraventricular or systemic administration of bFGF limits infarct size when given within the first few hours after focal ischemia (Bethel et al., in press; Fisher et al., 1995; Jiang et al., 1996; Koketsu et al., 1994; Tanaka et al., 1995). The current report is the first to systematically examine the time window of efficacy of intravenous bFGF, and to examine the interaction of bFGF with heparin.

The mechanism of infarct reduction by intravenous bFGF remains uncertain and may include direct cytoprotection as well as effects on regional cerebral blood flow. In vitro, bFGF protects cultured neurons against a number of toxins and insults thought to be important in the pathogenesis of cell death after ischemia, including anoxia, hypoglycemia, excitatory amino acids, oxygen free radicals, nitric oxide and excess intracellular calcium (Finklestein et al., 1993; Mattson et al., 1989, 1991, 1995; Chen and Finklestein, in press). These effects appear to be mediated through signal transduction cascades initiated by binding of bFGF to its high-affinity cell-associated receptors, resulting in new cellular gene expression and protein synthesis (Mattson et al., 1989). In particular, in cultured neurons, bFGF increases the expression of the free radical scavenging enzyme Cu/Zn superoxide dismutase (SOD), as well as the calcium binding protein calbindin, two proteins that are likely to be neuroprotective (Mattson et al., 1991, 1995). High-affinity bFGF receptors are widely distributed on neurons, glia and some endothelial cells in the rodent brain (Wanaka et al., 1990). Following focal ischemia in rats, intravenous bFGF crosses the damaged blood-brain barrier to enter ischemic, but not non-ischemic brain tissue (Fisher et al., 1995). At the bFGF dose used in the current study (150 μg/kg total), an estimated concentration of 5 ng/ml is reached in ischemic brain tissue (Fisher et al., 1995). In vitro, bFGF exerts direct cytoprotective effects at 1–10 ng/ml (Finklestein et al., 1993). Thus, the infarct reducing effects of bFGF may be due to direct protection of vulnerable cells at the borders of focal infarcts (Fisher et al., 1995; Huang et al., in press; Jiang et al., 1996).

In addition to its direct cytoprotective effects, bFGF is also a potent vasodilator and increases regional cerebral blood flow in rat and rabbit brain, an effect that is mediated through nitric oxide production by endothelial nitric oxide synthase (Regli et al., 1994; Rosenblatt et al., 1994). Thus, infarct reduction by bFGF may also be mediated, in part, through increased regional cerebral blood flow. However, recent evidence suggests that the direct cytoprotective effects of bFGF may be more important to infarct reduction than effects on regional cerebral blood flow. For example, following middle cerebral artery occlusion, 'knockout' mice lacking the endothelial nitric oxide synthase gene show no increases in regional cerebral blood flow following intravenous bFGF, but do show a reduction in infarct volume equivalent to wild-type animals (Huang et al., in press).

In the current study, we found that the intravenous infusion of bFGF was effective in reducing infarct volume when begun up to 3 h following the onset of permanent focal ischemia. This time window of efficacy compares favorably with those of several other agents that have been shown to reduce infarct volume in models of focal ischemia (Fisher and Takano, 1995). For example, glutamate receptor antagonists appear to have a therapeutic window of 1 h or less in similar models (Hatfield et al., 1992). On the other hand, some agents (e.g., inducible nitric oxide synthase inhibitors) appear to have much longer (up to 24 h) time windows of efficacy in focal ischemia models (Iadecola et al., 1995).

Basic FGF binds tightly to the glycosaminoglycan heparin, reflecting its binding to heparan sulfate proteoglycan in the extracellular space (Klagsbrun and Baird, 1991). Heparan sulfate proteoglycan can thus be considered the 'low-affinity' bFGF receptor, as distinguished from its family of 'high-affinity' cell-associated tyrosine kinase receptors (Klagsbrun and Baird, 1991). In the rodent, intravenously administered bFGF has a short circulating half-life (0.5–3.0 min) and is sequestered rapidly in several organs, including kidney, liver and spleen (Hondermarck et al., 1990; Whalen et al., 1989). Co-infusion of heparin markedly increases the circulating half-life of bFGF (to 4.5 min), most likely due to displacement from low-affinity binding sites in the extracellular matrix (Whalen et al., 1989). In the current study, we found that co-infusion of heparin did not alter the infarct reducing effects of bFGF. These data may indicate that maximal infarct reduction was achieved at the bFGF dose used (150 μg/kg total), and that no further reduction could be attained even with increased circulating levels of bFGF. On the other hand, we found in previous studies that the infarct-reduc-

ing effects of bFGF are dose-dependent, and that heparin also did not augment the effects of a lower bFGF dose (15 μg/kg total; Ren, Chen and Finklestein, unpublished data). Taken together, these results suggest that the infarct-reducing effects of bFGF are mediated largely through its high-affinity receptors, and that the circulating half-life or degree of binding to low-affinity receptors may be less important than initial access to high-affinity sites.

The current data are potentially important given the recently initiated human clinical trials of intravenous bFGF in acute stroke (Grossbard and Finklestein, unpublished data). In particular, the current data concerning the time window of efficacy are relevant to the consideration of patient entry criteria for such trials. Moreover, since heparin is commonly used as an anticoagulant in stroke patients, it is important to understand possible interactions between bFGF and heparin. However, the current data in rats can only be considered a guide to the possible effects of bFGF in stroke patients, given several notable differences in the biology of rodent vs. human stroke (Wiebers et al., 1990). In particular, cerebral infarction may evolve over longer periods of time in humans compared to rats (Marchal et al., 1996), suggesting that the window of opportunity for treatment with potentially cytoprotective agents such as bFGF may be considerably longer for some patients than suggested by studies in rodents.

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