

Dimeric fibroblast growth factor-2 enhances functional recovery after focal cerebral ischemia

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Abstract. *Purpose:* The purpose of this study was to examine the effects of dimerized basic fibroblast growth factor (dFGF), a novel engineered growth factor, in a model of functional recovery following focal cerebral infarction (stroke) in rats.

Methods: A focal stroke was made in mature male rats by occlusion of the middle cerebral artery (MCA). dFGF was administered by intracisternal injection at one and three days after stroke. Tests to evaluate sensorimotor recovery of the contralateral limbs were done during the next three weeks after stroke.

Results: dFGF significantly enhanced recovery of sensorimotor function in limb placing and body swing tests compared to vehicle treatment. There were no differences in body weight or infarct volume in dFGF- vs. vehicle-treated animals.

Conclusions: dFGF represents a potential treatment to enhance functional recovery after stroke and offers several advantages over bFGF, including stability and independence from extracellular heparin sulfates.

Keywords: Cerebral ischemia, stroke, recovery, fibroblast growth factor

1. Introduction

Previous preclinical studies have demonstrated the potential usefulness of basic fibroblast growth factor (bFGF) in animal models of acute stroke and stroke recovery. If bFGF is given intracerebroventricularly or intravenously within four hours after the onset of permanent or temporary focal ischemia in rats, infarct size is reduced, likely due, in part, to reduction of apoptotic cell death at the borders of focal infarcts [1–3]. If,

on the other hand, bFGF is given intracisternally starting at one day after permanent focal ischemia in rats, infarct size is not reduced, but recovery of neurological function is enhanced [4–6]. Mechanisms of this recovery-promoting effect likely include: (1) stimulation of new axonal sprouting and new synapse formation in undamaged regions of brain in both the ipsilateral and contralateral hemispheres [5,6], and (2) stimulation of proliferation, migration, and differentiation of progenitor cell populations in brain [7]. Clinical trials of intravenous bFGF initiated for acute stroke failed to show efficacy, but suggest a potential benefit of bFGF treatment in the subacute, recovery phase following

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stroke [8].

In the process of bFGF signaling, two bFGF monomers come together to promote dimerization of high-affinity FGF cell surface receptors, thus initiating the signal transduction cascade [9,10]. Covalent bonding of two bFGF monomers with a tripeptide linker puts the monomers in an optimal conformation for this process [10]. The resulting molecule, dimerized bFGF (dFGF) is stable and more potent on a molar basis than monomeric bFGF in several *in vitro* and *in vivo* assays, including endothelial cell proliferation and angiogenesis [10]. dFGF is also less dependent than bFGF on binding to extracellular heparin sulfate moieties to achieve a maximal biological effect [10]. In the current study, we examined the effects of dFGF in promoting neurological recovery in a model of focal cerebral ischemia in rats.

2. Methods

2.1. Construction of dFGF

dFGF was cloned and purified as previously described [10]. Briefly, a template of wild-type bFGF was used and modified by incorporation of DNA sequences corresponding to a his tag, a T7 tag, a tripeptide linker, and a thrombin cleavage site through PCR. Introduction of the restriction sites *NdeI*, *SacI*, and *SpeI* to the 5' and 3' of two bFGF genes enabled controlled subcloning of two differentially modified bFGF genes into the pET14b variant expression vector (gift of Dr. David Ornitz, Washington University, Saint Louis, MO). The vector was transfected into the *E. Coli* strain BL21, enabling expression of the protein. The protein was then purified by Ni²⁺ chromatography [11, 12], and subsequent T7-affinity chromatography, as described by the manufacturer (Novagen, Madison, WI). The concentration of the expressed and purified protein was determined by enzyme-linked immunosorbent assay. dFGF was stored in PBS containing imidazole (1.2 mM) and Tris HCl (48 M), pH 7.4, at -85 °C until use. After production, and again before use, the activity of dFGF was validated using a BaF3 proliferation assay as described [11,13] with slight modification. Briefly, BaF3 cells previously transfected with FGFR1c were plated and treated with PBS, 50 ng/ml dFGF alone, 50 ng/ml bFGF, or 50 ng/ml bFGF supplemented with 500 ng/ml heparin. Cell proliferation was determined after three days by whole cell count. dFGF was considered active if the proliferative capacity of dFGF was the same as bFGF with heparin, and significantly greater than bFGF alone.

2.2. Middle Cerebral Artery (MCA) Occlusion

Proximal MCA occlusion was employed to induce focal infarcts of the right dorsolateral cerebral cortex and underlying striatum [4–7]. Male Sprague-Dawley rats (Charles River Breeding Laboratories), 300–350 g, were handled daily for one week before surgery. One day prior to surgery, animals were injected intraperitoneally with 40 mg/kg cefazolin sodium. Rats were anesthetized with 2% isoflurane in a nitrous oxide/oxygen mixture (2:1). Core body temperature was thermostatically maintained at 37 °C during the procedure. The proximal right MCA was electrocoagulated from just proximal to the olfactory tract to the inferior cerebral vein, and subsequently transected. The surgery was performed sparing the facial nerve and without removing the zygomatic arch. Following surgery, animals were again injected intraperitoneally with 40 mg/kg cefazolin sodium and were placed into individual cages. All rats were weighed the day prior to surgery and on days of behavioral assessment thereafter.

2.3. dFGF Administration

Recombinant dFGF was diluted to a concentration of 10 µg/ml in PBS supplemented with 100 µg/ml BSA (Boehringer Mannheim, Cat. #711454). The vehicle solution contained the same constituents without dFGF. Prior to intracisternal injections, rats were re-anesthetized with 2% isoflurane in nitrous oxide/oxygen (2:1) and placed into a stereotaxic frame. Rats received either 50 µl of dFGF in vehicle (0.5 µg) or vehicle alone by percutaneous injection into the cisterna magna, as described previously [5–7]. Intracisternal injections of dFGF or vehicle were administered at 24 and 72 hours after MCA occlusion (total dose of dFGF administered = 1 µg).

2.4. Behavioral testing

Rats were examined for behavioral function by employing standardized tests to assess sensorimotor function in the limbs as well as vestibulomotor function. Limb placing tests and body swing tests were performed one day prior to surgery, one day after surgery but prior to injection, day 3, and then every fourth day through day 19 after intracisternal treatment. Limb placing tests were performed as described previously [5,6]. The forelimb placing test determined sensorimotor function in response to whisker tactile,

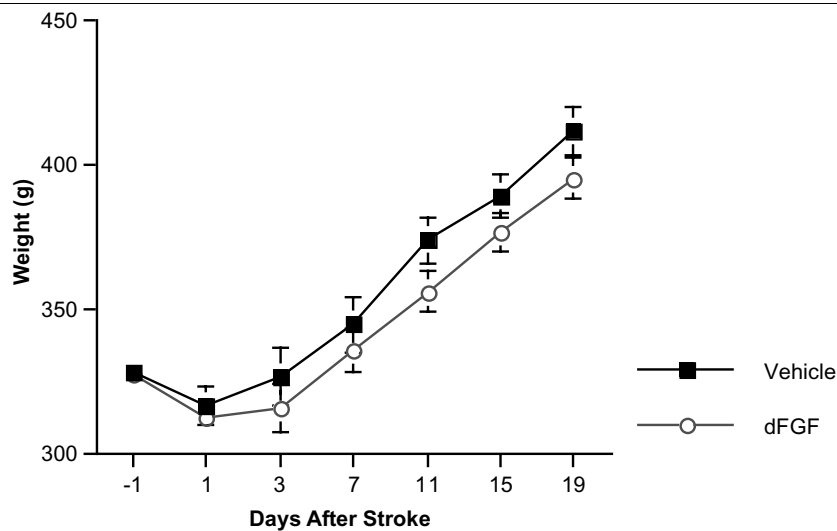


Fig. 1. Body Weight. There was no difference in body weight after surgery between vehicle- and dFGF-treated animals (N = 10 vs. 10, p-n.s.).

visual, tactile, and proprioceptive stimuli with scoring between 0 and 12, where 12 indicates maximal impairment. Hindlimb placing tests measured sensorimotor function of the hindlimb in response to tactile and proprioceptive stimuli, and scored between 0 and 6, where 6 represents maximal impairment. The body swing test provides a measure of vestibulomotor function. Function was measured as a percentage of the number of body swings towards the side ipsilateral to the MCA occlusion, where 50% is normal, and 0% is maximal impairment. Sensorimotor function of the forelimbs was additionally tested by the cylinder (spontaneous limb use) test [5,14]. In this test, animals are videotaped as they rear up in a narrow glass cylinder. The number of spontaneous movements made by each forelimb to initiate rearing, to land on or to move laterally along the wall of the cylinder, or to land on the floor after rearing are counted, and expressed as an asymmetry score [$((\text{total contralateral forelimb use} - \text{total ipsilateral forelimb use}) / \text{total forelimb use}) \times 100$]. Normally, there is no asymmetry between sides on the test, but, after stroke, the affected (contralateral) side is used less (asymmetry score is > 0). This test was done on the day before and weekly after stroke.

2.5. Infarct volume determination

After the completion of behavioral testing, animals were euthanized by deep anesthesia with chloral hydrate and transcardially perfused with 10% formalin in saline. The brains were subsequently removed, fixed in formalin, dehydrated, and embedded in paraf-

fin. A microtome was used to cut 5 μm coronal sections, which were mounted onto glass slides and stained with hematoxylin/eosin (H&E). Six slices (+4.7, +2.7, +0.7, -1.3, -3.3, -5.3 mm compared with bregma) were used to determine the area of cerebral infarcts via a computer-interfaced imaging system (Bioquant, Nashville, TN), through the "indirect method" (intact contralateral hemispheric area - intact ipsilateral hemisphere area), to correct for brain shrinkage during preparation and processing [15]. Infarct volume was calculated as the sum of infarct areas per brain multiplied by slice thickness and expressed as a percentage of the intact contralateral hemispheric volume. Infarct volumes in the cortex and striatum were also separately determined using the same method. H&E staining also allowed for the examination of gross histological changes.

2.6. Data analysis

Experimenters performing the behavioral assessments and infarct volume determinations were blinded to treatment assignment. Behavioral and weight data were analyzed by repeated measures two-factor ANOVAs (treatment by time), and infarct volume data were analyzed by two-tailed t-tests.

3. Results

Occlusion of the right proximal MCA produced infarction in the dorsolateral cerebral cortex and underly-

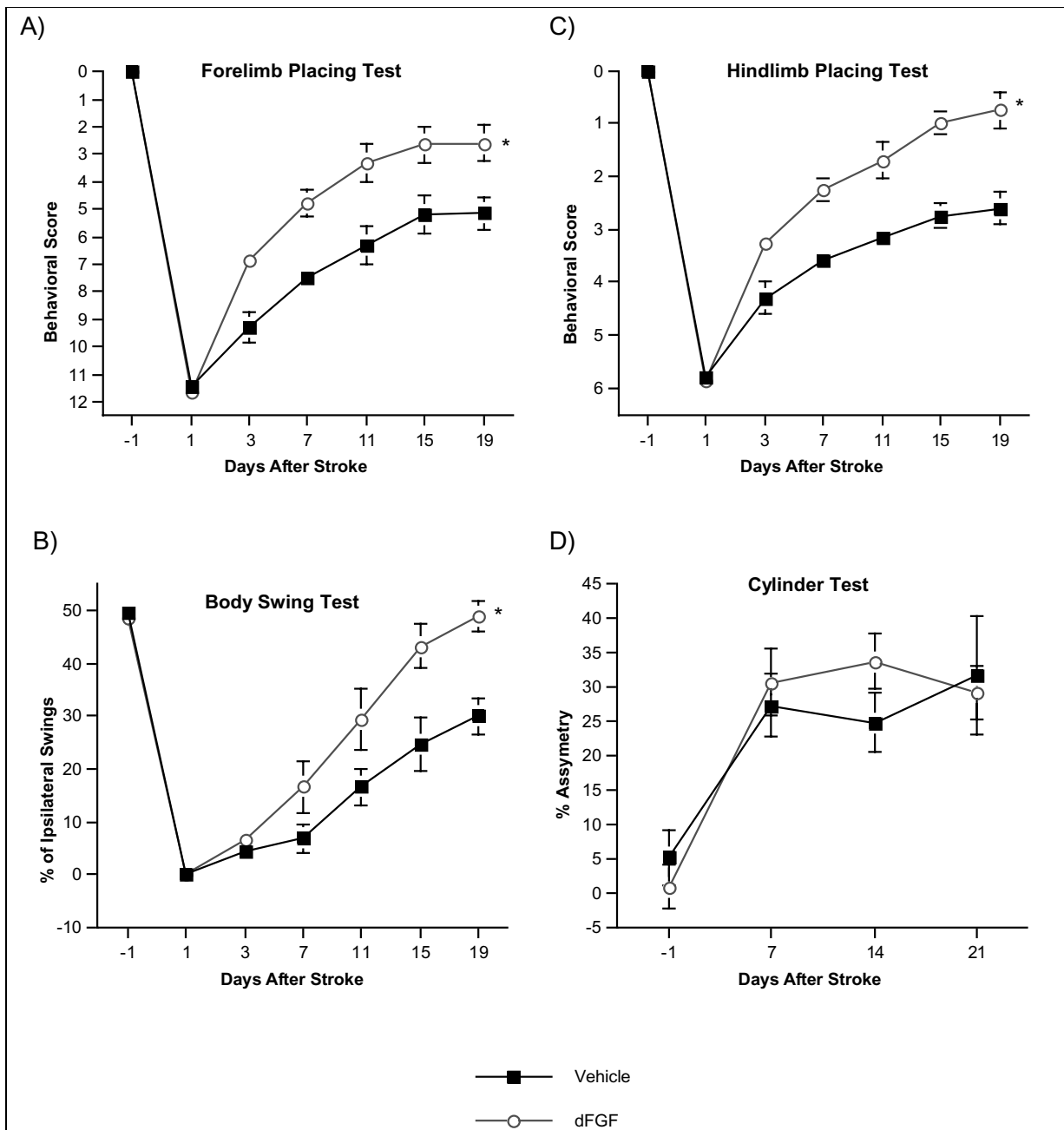


Fig. 2. Behavioral Outcome. Significant enhancement in behavioral recovery was seen in dFGF-treated ($N = 10$) vs. vehicle-treated animals ($N = 10$) in the: (A) forelimb placing test ($p < 0.002$), (B) hindlimb placing test ($p < 0.0001$), and (C) body swing test ($p < 0.005$), but not the (D) spontaneous limb use (cylinder) test (p -n.s.).

ing striatum, as described previously [4–7]. At 30 days after MCA occlusion, total, cortical, or striatal infarct volume were not different between vehicle- or dFGF-treated animals (Table 1).

No hemorrhage, tumor formation, excessive inflammation, or other histological changes, other than in-

fraction, were seen in post-stroke brains. Body weight after surgery was not different between vehicle- and dFGF-treated animals ($F = 1.23$, p -n.s.; Fig. 1).

Treatment with intracisternal dFGF produced enhancements of functional recovery in three of four behavioral tests performed, namely the forelimb placing

Table 1
Infarct volume

Treatment	N	Total (%)	Cortex (%)	Striatum (%)
Vehicle	10	30.07 ± 2.53	39.43 ± 3.43	55.01 ± 5.50
dFGF	10	31.15 ± 3.39	41.81 ± 4.66	47.31 ± 5.73

There were no differences in infarct volume between vehicle- and dFGF-treated animals. Data are expressed as percent of intact hemispheric volume (all p-n.s.).

test ($F = 13.58$, $p < 0.002$), hindlimb placing test ($F = 31.86$, $p < 0.0001$), and the body swing test ($F = 10.77$, $p < 0.005$; Fig. 2). No enhancement of function was seen on the spontaneous limb use (cylinder) test ($F = 0.01$, p-n.s.; Fig. 2).

4. Discussion

In summary, we found that intracisternal dFGF enhanced recovery of sensorimotor and vestibulomotor function following unilateral cerebral infarction in rats. These results were most pronounced on forelimb and hindlimb placing tests and the body swing test and not evident on the spontaneous limb use test. These results parallel those that we have obtained using other growth factors, in which we have found that limb placing and body swing tests appear to be more sensitive to treatment effects than the spontaneous limb use test (unpublished data).

In particular, current results obtained with intracisternal dFGF are similar to those obtained in previous studies using equivalent doses and dosing schedules of bFGF [4–6]. Possible mechanisms by which bFGF may enhance neurological recovery after stroke include: (1) enhancement of neural sprouting and new synapse formation in uninjured parts of brain, and (2) stimulation of progenitor cell proliferation, migration, and differentiation in the post-stroke brain. Indeed, in previous studies [5,6], we showed that intracisternal bFGF upregulates the expression of a molecular marker of new axonal sprouting, GAP-43, in cerebral cortex surrounding focal infarcts and in the contralateral homologous cortex. Moreover, inhibition of GAP-43 upregulation by intracisternal co-administration of GAP-43 antisense oligonucleotide with bFGF blocks enhancement of sensorimotor recovery [6]. Intracisternal bFGF also promotes progenitor cell proliferation in the subventricular zone (SVZ) and dentate gyrus of the hippocampus (DG) following focal infarction [7]. Some of these proliferating cells in the SVZ subsequently acquire an immature neuronal phenotype and appear to migrate out of the SVZ, whereas those in the DG remain *in situ*,

acquiring a mature neuronal phenotype [7]. Changes in progenitor cell proliferation, migration, and differentiation following bFGF treatment after stroke may contribute to enhancement of functional recovery [7]. Because dFGF represents a dimeric form of bFGF that is optimized for binding to high-affinity bFGF receptors, the mechanisms of action of dFGF in promoting recovery after stroke are likely to be the same as bFGF.

Both intracisternal bFGF and dFGF may prove useful as treatments to enhance neurological recovery after stroke. Indeed, dFGF may prove superior to bFGF in several respects. First, dFGF is a stable molecule in its active form, as opposed to bFGF, which must interact with a second monomer in an appropriate manner to exert its biological effects. Second, dFGF is not dependent on extracellular concentrations of heparin/heparan-like glycosaminoglycans for its effects [10]. Third, dFGF may prove to be a more potent molecule than bFGF. Indeed, dFGF is more potent than bFGF in *in vitro* assays of smooth muscle cell proliferation and endothelial cell survival, as well as in *in vivo* assays of angiogenesis [10]. Although the effect of intracisternal dFGF in promoting neurological recovery in a rat model of stroke was similar to that previously reported for equivalent doses of bFGF, this does not exclude the possibility that dFGF is more potent at other doses. Further studies are required to resolve this issue.

5. Conclusion

The current results demonstrate that dFGF, a dimeric form of bFGF, shows promise as a potential treatment to enhance neurological recovery following stroke. Further study of dFGF is warranted to explore its full therapeutic potential.

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