Enhanced Neuroprotective Effects of Basic Fibroblast Growth Factor in Regional Brain Ischemia after Conjugation to a Blood-Brain Barrier Delivery Vector

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ABSTRACT

Basic fibroblast growth factor (bFGF) has minimal pharmacological effects in the central nervous system in the absence of blood-brain barrier (BBB) disruption. BBB transport of bFGF occurs via an absorptive-mediated transcytosis mechanism, which is relatively inefficient. To enhance the BBB transport of bFGF, this neurotrophin was reformulated to enable receptor-mediated transport across the BBB via the transferrin receptor. bFGF was monobiotinylated and coupled to a BBB drug-delivery vector comprised of streptavidin (SA) and the OX26 monoclonal antibody to the rat transferrin receptor. The entire conjugate of biotinylated bFGF bound to the OX26-SA is designated bio-bFGF/OX26-SA. The bFGF retains receptor-binding affinity and has increased brain uptake following conjugation to OX26-SA. The bio-bFGF/OX26-SA conjugate protects cortical cell cultures against hypoxia/reoxygenation insult in a dose-dependent manner in vitro. A single intravenous injection of bio-bFGF/OX26-SA, equivalent to a dose of 25 μg/kg bFGF, produces an 80% reduction in infarct volume in the brain of rats subjected to permanent occlusion of the middle cerebral artery in parallel with a significant improvement of neurologic deficit. The neuroprotection is time-dependent, and there is a 67% reduction in stroke volume if the conjugate is administered at 60 min after arterial occlusion, whereas no significant reduction in stroke volume is observed if treatment is delayed 2 h. In conclusion, neuroprotection in regional brain ischemia is possible following the delayed intravenous injection of low doses of bFGF providing the neurotrophin is conjugated to a BBB drug-targeting system.

Human basic fibroblast growth factor (bFGF) is an endogenous neurotrophin that is neuroprotective in cerebral ischemia following i.c.v. injection (Lyons et al., 1991). The bFGF was administered by i.c.v. injection because prior work had shown that bFGF does not cross the blood-brain barrier (BBB) in pharmacologically significant amounts (Whalen et al., 1989). In the absence of BBB disruption, the intravenous administration of bFGF does not cause neuroprotection in focal brain ischemia using the middle cerebral artery occlusion (MCAO) model (Roberts et al., 1995; Harukuni et al., 1998). If BBB disruption is present in experimental brain ischemia, then bFGF is neuroprotective following the intravenous administration of high doses (135 μg/kg) in rats subjected to the MCAO model (Fisher et al., 1995; Ay et al., 1999). However, clinical trials of bFGF in human subjects show dose-associated side effects (Clark et al., 2000; Laham et al., 2000). The administration of high doses of the neurotrophin is required due to the modest rate of bFGF penetration into the brain from blood across the BBB. The BBB transport of 125I-bFGF is relatively slow and occurs via absorptive-mediated transcytosis of this cationic peptide (Deguchi et al., 2000).

The BBB transport of bFGF could be increased by reformulation of the neurotrophin by conjugation of bFGF to a BBB transport vector that undergoes receptor-mediated transport through the BBB in vivo. The mouse OX26 monoclonal antibody to the transferrin receptor enters the brain from blood via the BBB transferrin receptor (Pardridge et al., 1991), and conjugation of drugs to the BBB transport vectors is facilitated with avidin-biotin technology (Pardridge, 2001). In this approach, a conjugate of the OX26 monoclonal antibody and streptavidin (SA), designated OX26-SA, is prepared in parallel with monobiotinylation of the neurotherapeutic. In the present study, bFGF is monobiotinylated and conjugated to OX26-SA, and this overall conjugate is designated bio-bFGF/OX26-SA. The bio-bFGF/OX26-SA conjugate retains high affinity for the BBB recep-

ABBREVIATIONS: bFGF, basic fibroblast growth factor; bio-bFGF, biotinylated bFGF; BBB, blood-brain barrier; SA, streptavidin; MCAO, middle cerebral artery occlusion; BDNF, brain-derived neurotrophic factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; ANOVA, analysis of variance.
tor and has enhanced brain uptake following a single i.v. injection in the rat, in parallel with reduced peripheral organ uptake (Wu et al., 2002). The purpose of the present study was to test the neuroprotective effects of bFGF after refor-
mulation and conjugation to a BBB delivery vector using the mixed rat cortical cell culture model in vitro and the perme-
antly middle cerebral artery occlusion model in vivo. These studies show neuroprotection in regional brain ischemia fol-
lowing the delayed intravenous administration of low doses (25 μg/kg) of bFGF, providing the neurotrophin is conjugated to a BBB drug-targeting system.

**Experimental Procedures**

**Materials.** Male Sprague-Dawley rats weighing 280 to 320 g and pregnant rats of 16-day gestation age were purchased from Harlan (Indianapolis, IN). Recombinant human bFGF was provided by Scio.

Sciences Inc. (Sunnyvale, CA). DMEM (with high glucose), fetal bovine serum (FBS), and antibiotics were purchased from Invitrogen (Carlsbad, CA). Biotin-XX-NHS was obtained from Calbiochem, (San Diego, CA), where NHS is N-hydroxysuccinimide, and XX is bis-aminohex-

anoyl. 2-Iminothiolane (Traut’s reagent), m-maleimidobenzoyl-N′-hydroxysuccinimide ester, and BCA protein assay reagents were purchased from Pierce (Rockford, IL). Recombinant streptavidin, 3-(4,5-
dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,3,5-
triphenyltetrazolium chloride, and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

**Mixed Rat Cortical Cell Cultures.** Mixed rat cortical cells were cultured according to Cazevieille et al. (1993). Briefly, fetal brain tissue was obtained from two pregnant rats of 16-day gestation age. Bilateral forebrain cortices were removed into 2 ml of ice-cold Heps-buffered saline solution containing 0.05% trypsin. The tissue masses were dissected using microscissors. At the end of incubation in a water bath with gentle shaking at 37°C for 30 min, trypsin inhibitor was added to a final concentration of 0.1%. After standing at room tem-

terature for 20 min, the supernatant was aspirated, and the pellets were suspended in DMEM supplemented with 10% FBS and antibi-

otics. After standing at room temperature for 15 min, the cell sus-
pension was transferred to a sterile tube, and the tissue pellets were discarded. The cells were plated into 24-well cluster dishes (Costar Corp., Cambridge, MA), which were precoated with 0.1 mg/ml poly-
t-l-lysine, at a density of 106 cells/ml in 1 ml of DMEM supple-
mented with 10% FBS and antibiotics. The cultures were maintained at 37°C with 5% CO2/95% air and saturating humidity. The medium was changed twice a week.

**Biotinylation of bFGF.** Forty-three nanomoles of recombinant human bFGF (Scios product code P8504, mol. wt. 16,400) were added to 300 μl of 0.05 M NaHCO3 (pH 8.3) and mixed with 430 nmol of biotin-XX-NHS in 12.3 μl of dimethyl sulfoxide. The reaction pro-
ceeded at room temperature for 1 h with shaking and was stopped by the addition of 10 μmol of glycine. The products were transferred into a dialysis bag (Spectrum Laboratories, Inc., Fort Lauderdale, FL; molecular weight cutoff 6,000 to 8,000), and were dialyzed three times against 1 liter of 10 mM phosphate buffer, pH 7.4, at 4°C for 12 h. The final yield of bio-bFGF, as determined by BCA protein assay, was approximately 85% of the original bFGF used. The molar ratio of biotin incorporated into bFGF protein was 1.1, based on the 4′-hydroxazobenzene-2-carboxylic acid assay (Kang and Pardridge, 1994).

**Synthesis of OX26-SA Conjugate.** The OX26-SA conjugate was prepared as described previously (Kang and Pardridge, 1994). Briefly, 20 mg of murine monoclonal antibody OX26 was thiocolinated with a 10:1 M ratio of 2-iminothiolane. In parallel, 7 mg of recombi-
nant SA was activated with a 20:1 M ratio of m-maleimidobenzoyl-
N′-hydroxysuccinimide ester. At the end of the protein thiolation/ activation, the two samples were mixed and allowed to stand at room temperature for 3 h for conjugation. The conjugate was labeled with 2.5 μCi of [3H]biotin and was purified on a 2.6 × 92-cm column of Sephar 

acyl S300HR (Pharmacia, Peapack, NJ) following by elution in 0.01 M NaHPO4/0.15 M NaCl, pH 7.4, 0.15% Tween 20 at 30 ml/h, and 3-ml fractions were collected. The conjugate peak eluted be-
tween fractions 70 to 89 and was well separated from unconjugated SA (fractions 98–107). The number of biotin binding sites per OX26-SA conjugate was approximately three, as determined with a [3H]biotin binding assay, as described previously (Kang and Pardridge, 1994).

**Hypoxic Insult and MTT Assay.** In vitro neuroprotective effect of bFGF analogs was assessed using the MTT assay as reported by Dore et al. (1997). The mixed rat frontal cortical cells were cultured for 10 days. One day prior to the test, the medium was replaced with 0.5 ml of serum-free DMEM per well supplemented with 0.1% bovine serum albumin, glucose, and antibiotics, which stops cell division and arrests the cells in the G1/G0 phase of cell growth (Kiyokawa et al., 1997). Three graded doses (0.1, 1.0, or 10 ng/ml) either of native bFGF, bio-bFGF, or bio-bFGF/OX26-SA were added to the cultures and incubated for 24 h. The doses of bio-bFGF/OX26-SA contained 110 ng/ml or 0.55 nmol of OX26-SA. Designated wells were enriched with medium only or corresponding doses of OX26-SA as controls. On the experimental day, the medium was replaced with 0.3 ml of fresh medium per well, and bFGF and its analogs were added at the same concentrations as above. All the cell plates were placed in a custom-

made hypoxia chamber maintained in a 37°C water bath and aerated with 95% N2/5% CO2 at a rate of 1.2 l/min for 24 h. After 4 h of reoxygenation, 0.5 ml of freshly made MTT solution (0.5 mg/ml, passed through a 0.2-μm filter) was added to each well and followed by 2 h of incubation in the cell culture incubator. At the end of the incubation, the cells and MTT formazan crystals were solubilized by addition of 1.0 ml of anhydrous isopropanol/0.1 N HCl per well. The total reduced MTT was quantitated spectrophotometrically at 570 nm. Background correction was performed with extracts of cells not treated with MTT. The average reduced MTT in designated cell wells without exposure to the hypoxia/reoxygenation insult was consid-

ered 100%. To supplement the MTT assay, medium lactate dehydro-
genase activity was measured spectrophotometrically. However, en-

zyme release to the medium was only detected with the combined exposure of the cells to hypoxia and glucose deprivation. This assay was not used further, since glucose was included in the medium to reflect physiologic conditions.

**Focal Cerebral Ischemia Model.** After fasting overnight, male Sprague-Dawley rats weighing 280 to 320 g were lightly anesthe-
tized with inhalation of halothane and orotracheally intubated by transillumination as previously reported by Cambron et al. (1995). The animals were artificially ventilated with a mixture of 70% N2O/30% O2, and 0.5% halothane at a rate of 90 strokes/min and a volume of 5 ml/stroke. Body temperature was maintained with a Harvard thermal blanket with a rectal probe (Harvard Apparatus, Holliston, MA). Systolic blood pressure was measured by a model 29 rat tail arterial pulse amplifier (ITTC Inc./Life Science Instruments, Wood-

land Hills, CA). The left femoral artery was cannulated with PE50 tubing from which blood was collected for the measurement of blood pH, pO2, and pCO2 using a model 238 pH/blood gas analyzer (Chiron Corp., Emeryville, CA). After all the physiologic parameters were stabilized, a ventral midline neck incision was made, and a perma-

nent MCAO was introduced by an intraluminal suture (3-0) (Fisher et al. 1995). The suture was prepared with a rounded tip by heating near a flame, and the size of the tip was checked with a hemocytom-

eter under a microscope to be approximately 0.3 to 0.4 mm. All the physiologic parameters were rechecked 10 min after MCAO, and the incision was sutured. The animal was allowed to recover under a heating lamp for 4 h, and then individually housed in the vivarium with free access to food and water. The animals were anesthetized 24 h after MCAO with inhalation of halothane and decapitated for removal of the brain. Coronal sections were cut to 2-mm thickness using a rat brain matrix. The brain sections were incubated in 2% 3,5,3′-triphenyltetrazolium chloride solution at 37°C for 30 min. The
stained sections were fixed in 10% formalin/10 mM phosphate buffer, pH 7.4, and stored at 4°C. The experimental protocol was approved by the UCLA Animal Research Committee.

Treatment Schedule. The rats with MCAO were randomly assigned to four groups, and all rats received pharmacologic treatment via a single femoral vein injection. The first group received 1.2 ml/kg vehicle (10 mM phosphate-buffered saline containing 1% bovine albumin). The second group received 25 μg/kg bio-bFGF (1.56 nmol/kg). The third group received 150 μg/kg OX26-SA (0.75 nmol/kg), and the fourth group received the conjugate of bio-bFGF/OX26-SA equivalent to 25 μg/kg bio-bFGF and 150 μg/kg OX26-SA. The intravenous injection was administered at 0, 1, 2, and 3 h after MCAO. One group of animals was treated immediately after MCAO with a lower dose of the conjugate, e.g., 5 μg/kg bio-bFGF coupled to 30 μg/kg OX26-SA by a single i.v. injection in 1.2 ml/kg vehicle.

Neurologic Deficit Scores. The neurologic deficit status of the animals was evaluated 2 and 24 h post-MCAO according to Liu et al. (1999) by a 0- to 5-point scale: grade 0, no visible neurologic deficit; grade 1, failure to extend the right forepaw fully; grade 2, intermittent circling; grade 3, sustained circling without moving forward; grade 4, failure to walk spontaneously with a depressed level of consciousness; and grade 5, death.

Calculation of Infarct Volume. The stained and fixed brain sections were photographed on both sides, using an Epson model 650 digital camera (Epson America, Torrance, CA). Infarct areas were measured using the NIH Image Software (version 1.61) and calibrated using a glass circle (10-mm diameter) and a square (12 × 12 mm). The infarct area was corrected to compensate for the effect of brain edema based on the area ratio of the ipsilateral (ischemic) to contralateral (nonischemic) hemispheres. The infarct volume was calculated by summed infarct areas from each section and multiplied by section thickness (2 mm).

Statistical Analysis. Data were presented as the mean ± S.D. of triplicate of each group of animals. The statistical differences between infarct volumes were assessed with Student’s t test for the in vitro data and analysis of variance (ANOVA) using the Bonferroni correction for the 's volumes were assessed with Student’s t test for the in vitro data and analysis of variance (ANOVA) using the Bonferroni correction for the

Results

In Vitro Neuroprotection. The hypoxia/reoxygenation insult produced severe inhibition of MTT reduction in the mixed rat cortical cell cultures without treatment (Fig. 1). Pretreatment of the cultures with bFGF resulted in elevated MTT reduction in a dose-dependent manner, and statistically significant effects were observed at the doses of 1.0 ng/ml or greater. The conjugate of bio-bFGF/OX26-SA also showed dose-dependent neuroprotective effects, whereas the vector OX26-SA alone did not have a significant effect compared with the nontreated cultures (Fig. 1).

In Vivo Neuroprotection. All physiologic parameters were stable before and 10 min after MCAO (Table 1). The infarct volumes in the animals treated immediately after MCAO are shown in Fig. 2. The OX26-SA vector alone (150 μg/kg) did not have any significant effects on the brain infarct volume compared with the vehicle group. Bio-bFGF (25 μg/kg) alone showed a marginal reduction (16%) of infarct volume, but this was not statistically significant compared with the vehicle-treated group. By contrast, a single i.v. injection of the conjugate of bio-bFGF/OX26-SA, at a dose equivalent to 25 μg/kg bFGF, resulted in a marked reduction of infarct volume of 80%. Figure 3 shows the neurologic deficit at 2 and 24 h post-MCAO. Treatment with vehicle, the OX26-SA vector alone, or the bio-bFGF alone caused no changes in neurologic scores (Fig. 3). However, the conjugate of bio-bFGF/OX26-SA significantly improved the neurologic deficit at 2 and 24 h.

One group of the experimental rats was treated with a lower dose of the conjugate, 5 μg/kg, which is one-fifth the regular dose used in the study, and the infarct volume was reduced by 34% (Table 2). To assess the time window of the neuroprotective effect, the regular dose (25 μg/kg) of bio-bFGF/OX26-SA was given at 1, 2, and 3 h after MCAO. As shown in Table 2, the treatment with the 1-h delay produced a significant 66% reduction of infarct volume, and there was significant improvement in the neurologic deficit score at both 2 and 24 h as well. However, the delay in treatment for either 2 or 3 h after MCAO showed neither reduction of infarct volume nor improvement of neurologic deficit (Table 2).

Discussion

The results of the present studies are consistent with the following conclusions. First, unconjugated bio-bFGF and the bio-bFGF/OX26-SA conjugate retain neuroprotective effects comparable with the native bFGF in the hypoxia/reoxygenation insult assay in the mixed rat cortical cell cultures (Fig. 1). Second, after a single i.v. injection of bio-bFGF/OX26-SA, equivalent to 25 μg/kg bFGF, there is an 80% reduction in stroke volume with significant improvement of neurologic deficit. In contrast, this dose of unconjugated bio-bFGF does not have a statistically significant effect on either stroke volume or neurologic deficit (Figs. 2 and 3). Third, the neuroprotection of bio-bFGF/OX26-SA is time-dependent with an effective time window of at least 1 h post-MCAO.

MTT reduction is an indicator of the mitochondrial activity in living cells and has been used as an indicator of neuronal injury and death (Dore et al., 1997). As shown in Fig. 1, hypoxia/reoxygenation insult produces markedly decreased MTT reduction in the mixed rat forebrain cortical cell cultures. Preincubation with either the native bFGF, free bio-bFGF, or bio-bFGF/OX26-SA conjugate protects the cortical
Each bar represents the mean group received 25 g/kg OX26-SA conjugate in tissue culture are consistent with previous studies showing that the bFGF still binds to the high affinity bFGF receptor despite conjugation to the OX26 antibody (Wu et al., 2002). These combined results indicate that the biological activity of bFGF is retained following monobiotinylation and conjugation to OX26-SA.

The bFGF/OX26 conjugate is also neuroprotective in vivo in the MCAO model of regional brain ischemia following the delayed intravenous injection of the conjugate (Table 2, Figs. 2 and 3). In contrast, the unconjugated bFGF is not neuroprotective in the MCAO model following the intravenous injection of a dose of the neurotrophin of 25 μg/kg (Figs. 2 and 3). Unconjugated bFGF is neuroprotective in the MCAO model providing high doses (135 μg/kg) are administered in a setting where the BBB is disrupted in the region of the infarction (Fisher et al., 1995; Ay et al., 1999). However, in the absence of hyperglycemia-induced vasculopathy (Kawai et al., 1997), the BBB is intact for 4 to 6 h following regional brain ischemia (Wu and Pardridge, 1999; Menzies et al., 1993; Belayev et al., 1996; Albayrak et al., 1997). Therefore, if bFGF is to be used as an effective neuroprotective agent in stroke following a delayed intravenous administration, then the neurotrophin must be enabled to cross the BBB in pharmacologically significant amounts. BBB transport is possible if the neurotrophin is conjugated to a BBB drug-targeting system, such as the OX26 antibody to the transferrin receptor. This antibody accesses the endogenous transferrin transport system within the BBB and undergoes receptor-mediated transcytosis through the intact BBB in vivo (Bickel et al., 1994). The time window of neuroprotection with the bFGF conjugate is 1 to 2 h following a single intravenous injection of low doses (5–25 μg/kg) of the neurotrophin (Table 2). This period is less than the 3-h time window of neuroprotection following the constant intravenous infusion of high doses (135 μg/kg) of unconjugated bFGF (Ren and Finkelstein, 1997). The therapeutic time window for the bFGF conjugate may be prolonged either by increasing the dose or administering the conjugate by constant intravenous infusion.

The neuroprotective effects of bFGF may be additive with other neurotrophins, such as brain-derived neurotrophic factor (BDNF), which is neuroprotective following direct intracerebral injection in regional brain ischemia (Yamashita et al., 1997). The BDNF must be given directly into the brain because it does not enter the brain following intravenous administration in the absence of BBB disruption (Sakane and Pardridge, 1997). The intravenous administration of unconjugated BDNF provides no neuroprotection in either global or regional brain ischemia (Wu and Pardridge, 1999;...
Zhang and Partridge, 2001a,b). Conversely, the conjugate of BDNF and the OX26 antibody is neuroprotective following the delayed intravenous administration of low doses of the neurotrophin in either global or regional brain ischemia (Wu and Partridge, 1999; Zhang and Partridge, 2001a,b). BDNF is primarily neuroprotective in the cortex of the brain (Yamashita et al., 1997; Zhang and Partridge, 2001b), whereas bFGF is neuroprotective in both cortical and subcortical regions of the brain (Fisher et al., 1995). Therefore, the combined use of bFGF and BDNF conjugates, which are enabled to cross the BBB may have additive effects as neuroprotective agents in brain ischemia. Dual neurotrophin therapy may also increase the therapeutic time window after the stroke during which neuroprotection is still possible.

Clinical trials have shown that bFGF produces dose-dependent hypotension in patients with ischemic heart disease (Laham et al., 2000) and leukocytosis in patients with acute ischemic stroke (Fiblast Safety Study Group, 1998). In the absence of a BBB drug-delivery system, bFGF penetration into the brain is slow and occurs via an absorptive-mediated transcytosis mechanism (Deguchi et al., 2000), and this poor BBB penetration is slow and occurs via an absorptive-mediated transcytosis mechanism (Deguchi et al., 2000), and this poor penetration of the BBB necessitates the administration of high systemic doses of bFGF when the neurotrophin is not reformulated to enable BBB transport (Fisher et al., 1995). The therapeutic effect of bFGF within the brain may be offset by the dose-dependent peripheral side effects caused by the administration of high doses of bFGF. The conjugation of bFGF to the BBB drug-targeting system has dual beneficial effects. First, BBB transport of the bFGF is increased (Wu et al., 2002), which enables neuroprotection with bFGF conjugates at low systemic doses of 25 µg/kg (Fig. 2). Second, conjugation of bFGF to the BBB drug-delivery vector results in decreased peripheral organ distribution (Wu et al., 2002). The size of the OX26-SA conjugate is 200,000 Daltons, and conjugation of bFGF to OX26-SA increases the effective molecular mass of the bFGF from 16,000 to 216,000 Daltons. The larger size of the conjugate restricts transcapillary transport into peripheral tissues, although the conjugate is selectively transported across cerebral capillaries. Therefore, the use of a BBB drug-delivery system optimizes the therapeutic index of bFGF by simultaneously increasing central nervous system uptake and decreasing peptide uptake in peripheral tissues. This phenomenon has been demonstrated previously with a vasoactive intestinal peptide analog, and conjugation of vasoactive intestinal peptide to OX26-SA increased the therapeutic index of the peptide 10-fold (Wu and Partridge, 1996).

In summary, conjugation of bFGF to a BBB drug-delivery vector such as OX26-SA does not diminish the biological activity of the bFGF in a cell culture neuroprotection model (Fig. 1) or in a radio receptor assay (Wu et al., 2002). Neuroprotection is demonstrated in vivo with the permanent MCAO model, and a single intravenous administration of the bFGF/OX26 conjugate results in an 80% reduction in stroke volume at a low systemic dose (25 µg/kg) of bFGF (Fig. 2). This dose of unconjugated bFGF has no significant effect on infarct volume following intravenous administration (Fig. 2). The in vivo neuroprotection of the bFGF/OX26 conjugate is dose-dependent and has an effective time window of at least 1 h post-MCAO. The reformulation of neurotrophins such as bFGF to enable receptor-mediated transport across the BBB may result in improved clinical efficacy in stroke by allowing for neuroprotection in the brain at low systemic doses of the peptide following delayed intravenous administration.

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