Blood-Brain Barrier Transport of ^{125}I-Labeled Basic Fibroblast Growth Factor

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Purpose. This study was carried out to examine the blood-brain barrier (BBB) transport of human basic fibroblast growth factor (bFGF) and investigate its mechanism.

Methods. The BBB transport of ^{125}I-bFGF was measured by several *in vivo* methods including intravenous administration, *in situ* internal carotid artery perfusion, and intracerebral microinjection. The *in vitro* binding of ^{125}I-bFGF was characterized using freshly prepared bovine brain capillaries.

Results. The distribution volume of ^{125}I-bFGF in the postvascular supernatant increased with the perfusion time, and exceeded the space occupied by the brain microvasculature and its trichloroacetic acid (TCA) precipitability was more than 90%. ^{125}I-bFGF avidly bound to isolated bovine brain capillaries with a B_{max} of 206 ± 48 pmol/mg protein, and a K_d of 36.5 ± 15.7 nM. This binding was significantly inhibited by unlabeled bFGF and heparin in a concentration-dependent manner. The cationic peptides, protamine and poly-L-lysine (each 300 μM), produced over 85% inhibition of ^{125}I-bFGF binding to brain capillaries. Furthermore, glycosaminoglycans with a sulfate residue, chondroitin sulfate B and C (each 10 μg/mL) also inhibited the binding of ^{125}I-bFGF. The *in vivo* transcytosis of ^{125}I-bFGF from the luminal side to the brain was also inhibited by the presence of heparin (10 μg/mL) and poly-L-lysine (300 μM), whereas neither hyaruronic acid (10 μg/mL) nor insulin (10 μM) had any effect. In addition to these results, the brain efflux index method was used to confirm that the transcytosis of ^{125}I-bFGF from brain to blood across the BBB was negligible.

Conclusions. These results suggest that ^{125}I-bFGF is transported across the BBB, possibly by an adsorptive-mediated transcytosis mechanism that is triggered by binding to negatively charged species on the luminal membrane surface of the brain microvasculature, such as heparan sulfate proteoglycans.

KEY WORDS: adsorptive-mediated transcytosis; basic fibroblast growth factor; blood-brain barrier; internal carotid artery perfusion; isolated capillaries.

INTRODUCTION

Human basic fibroblast growth factor (bFGF) is an 18 kDa polypeptide composed of 154 amino acid residues. bFGF is found in a wide variety of organs including brain, retina, corpus luteum, prostate, cartilage and bone (1). bFGF exerts a mitogenic or differentiating action *in vitro* on a large number of cell types, and also plays important roles in promoting wound healing and tumor growth through angiogenesis (2). In the central nervous system, bFGF can act as a neurotrophic agent promoting the survival of cerebral cortical neurons (3), and protecting neurons against transient brain ischemia (4) and excitatory amino acid toxicity (5,6). These activities of bFGF in the brain are supported by evidence that the expression of bFGF mRNA is induced by transient brain ischemia (7), and that bFGF transgenic mice are able to resist the effects of a complex hypoxic-ischemic cerebral insult (8). Therefore, bFGF is a potentially useful pharmacological agent that could be used to treat neurodegenerative disorders, but only if it is able to gain access to injured neuronal cells via the blood-brain barrier (BBB) from the blood circulation. Autoradiographic examination after intravenous administration of ^{111}In-labeled bFGF has shown that the labeled peptide does not, in fact, cross the BBB (4). However, doubt has been cast on the three-dimensional structure of the radiolabeled form, because uniformly labeled ^{14}C-bFGF is transported through the BBB to some extent (9). Thus, it is not completely clear that intravenously administered bFGF can be delivered into the brain interstitial space beyond the continuous nonfenestrated endothelium which makes up the BBB.

The delivery of blood-borne peptides and proteins, including insulin and transferrin (10), LDL (11), leptin (12), and angiotensin II (13), into the brain is facilitated by receptor-mediated transport mechanisms via specific receptors on the BBB. In contrast to these highly specialized receptors, the lectin wheatgerm agglutinin (WGA) (14), and cationic proteins (15) cross the BBB by an adsorptive-mediated transcytosis mechanism after interaction with anionic sites on the brain endothelial cell membrane. Biochemical studies using cultured endothelial cells from peripheral organs have shown that bFGF is internalized via binding to heparan sulfate proteoglycans (HSPG) on the cell membrane (16,17). A similar endocytosis mechanism may operate at the BBB.

The present study, therefore, was carried out to examine the BBB transport of exogenously administered bFGF and investigate its mechanism. In this study, ^{125}I-labeled bFGF was prepared by the modified lactoperoxidase method. Adsorptive-mediated transcytosis of ^{125}I-bFGF can be demonstrated by *in vivo* studies using an internal carotid artery perfusion/capillary depletion technique, and by *in vitro* binding studies using freshly prepared bovine brain capillary.

MATERIALS AND METHODS

Materials

Recombinant human basic fibroblast growth factor (154 amino acid residues, theoretically 17,122.67 Da, pI = 10.1 (28)) was kindly provided by Kaken Pharmaceutical Co., Ltd., (Tokyo, Japan). [U-^{14}C] sucrose (17.6 GBq/mmol) and ^{125}I-Na (3.8 GBq/mL) were supplied by NEN Life Science Products Inc. (Boston, MA) and Amersham Pharmacia Biotech. Ltd. (Bucks, UK), respectively. Clear-sol I (liquid scintillation cocktail) was obtained from Nacalai Tesque, Inc., (Kyoto, Japan). Heparin sodium, salmon roe protamine sulfate, chondroitin sulfate A (CS-A) sodium salt, chondroitin sulfate C (CS-C) sodium

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salt, and rooster comb hyaluronic acid sodium salt were purchased from Wako Pure Chemical Industries, Ltd., (Osaka, Japan). Dextran (industrial grade, average M.W., 80,000), bovine serum albumin (BSA, Fraction V), poly-L-lysine hydrobromide (average M.W., 4,000), poly-L-glutamic acid sodium salt (average M.W., 14,000), chondroitin sulfate B (CS-B) and fucus vesiculosus fucoidan were purchased from Sigma Chemical Co., (St. Louis, MO). All other chemicals were of analytical grade and were used without further purification.

Animals

Adult male Wistar rats weighing 250 to 300 g were purchased from Japan SLC (Shizuoka, Japan). They were housed in a room with controlled temperature (24 ± 4°C) and humidity (55 ± 5%), and had free access to food and water.

Preparation of ^{125}I-bFGF

Iodinated bFGF (^{125}I-bFGF: 20.76 MBq/mL) was prepared by the modified lactoperoxidase method described by Yuge *et al.*(18). The radiochemical purity of ^{125}I-bFGF was confirmed to be 98% or more by HPLC using a TSKgel G2000 SW_{XL} gel filtration column (Tosoh, Tokyo, Japan).

Isolation of Brain Capillaries

Bovine brains were obtained from the Meat Inspection Center of Shizuoka City (Shizuoka, Japan). Capillaries were freshly prepared by a mechanical homogenization technique (19). These capillaries were then resuspended in buffer B (103 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 15 mM HEPES, pH 7.4) and the protein content was determined by BCA protein assay (Pierce, Chemical Co., Rockford, IL). Purification of capillaries was monitored by assaying the activity of a marker enzyme, γ-glutamyl transpeptidase, using the method of Dallaire *et al.* (20).

In Vitro Binding Studies

Binding of ^{125}I-bFGF to brain capillaries was examined by the method of Kumagai *et al.* (19) and Terasaki *et al.* (21). Brain capillaries (approximately 200 mg of protein) were preincubated in 180 μL incubation buffer (141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM HEPES, 10 mM D-glucose, and 0.1% BSA) at 4°C for 20 min. Then, the capillary suspension was mixed with ^{125}I-bFGF (0.925 kBq, 31.5 nM) and ^{14}C-sucrose (3.7 kBq), an extracellular marker, in the presence and absence of various compounds, including unlabeled bFGF (0.1–8 μM), polycations (protamine and poly-L-lysine, each 300 μM), an anion (poly-L-glutamic acid, 300 μM), and peptides (transferrin and insulin, each 10 μM) and WGA (50 μg/mL). At designated times (10–60 min) after the start of incubation, the suspension was centrifuged at 10,000 × g for 45 sec in a microcentrifuge (MRX-150, TOMY, Tokyo, Japan). In a separate experiment, either heparin (0.1 ~ 1,000 μg/mL) or a variety of glycosaminoglycans (chondroitin sulfate A, chondroitin sulfate B, chondroitin sulfate C, hyaluronic acid, fucoidan and dextran, each 10 μg/mL) was added to the suspension after ^{125}I-bFGF was pre-incubated with the capillaries at 4°C for 60 min. Then, the mixture was incubated for another 60 min, and then centrifuged as described above. The capillary pellet was solubilized with 500 μL 1 N NaOH in a vial for 30 min at 56°C. One hundred microliters supernatant was removed to measure the ^{125}I and ^{14}C radioactivity, and the remainder was used for TCA assay. Binding was expressed as the percentage of medium counts bound per milligram of capillary protein as follows:

$$\%\ ^{125}\text{I-bFGF bound/mg protein} = \left\{ \frac{^{125}\text{I-bFGF (dpm/mg capillary protein)}}{^{125}\text{I-bFGF (dpm/mL medium)} \times \text{fraction of TCA precipitable}} - \frac{^{14}\text{C-sucrose (dpm/mg protein)}}{^{14}\text{C-sucrose (dpm/mL medium)}} \right\} \times 100 \quad (1)$$

Intravenous Administration

Rats were anesthetized with *i.m.* ketamine (235 mg/kg), and the left femoral artery was cannulated using polyethylene tubing (SP-31, Natsume Seisakusho Ltd., Tokyo, Japan). Following an intravenous bolus injection of ^{125}I-bFGF (1.10 MBq/116 μg/kg) via a femoral vein, approximately 0.25 mL blood was withdrawn at 1, 5, 15, 30, and 60 min. At 60 min, rats were killed by exsanguination from the descending aorta and immediately cleared of any remaining circulating blood by perfusion with ice-cold saline. Brain, kidney and liver were rapidly removed, rinsed with ice-cold saline, blotted, and weighed. Plasma was obtained by centrifugation at 10,000×g for 5 min, and brain tissue was subjected to the capillary depletion technique (15). An aliquot of plasma was used to measure TCA precipitability.

Intracarotid Artery Perfusion

In vivo BBB transport of ^{125}I-bFGF was measured in ketamine-anesthetized rats using the intracarotid artery perfusion/capillary depletion technique (15). The perfusate consisted of Krebs-Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, and 10 mM D-glucose, and 0.1% BSA, pH 7.4) containing 7 ~ 14 kBq/mL ^{125}I-bFGF with sufficient ^{14}C-sucrose (an intravascular marker) to give a ^{14}C/^{125}I ratio of 4:1. In separate studies, various compounds, including heparin (10 μg/mL), poly-L-lysine (300 μM), hyaluronic acid (10 μg/mL) or insulin (10 μM), were added to the perfusate with the isotopes. Perfusion was carried out at a flow rate of 1.2 mL/min. At designated times (1, 2.5, 5 and 10 min), rat brains were quickly removed and separated into postvascular supernatant and pellet by the capillary depletion technique (15).

An investigation was also carried out to see if there was any artifactual dissociation of nonspecifically bound radioisotope from the microvasculature during the capillary depletion step. Rat brain was perfused with 53 kBq/mL ^{125}I-bFGF for 5 min and the capillary pellet was obtained by the capillary depletion method as described above. The pellet was mixed with 30 kBq ^{14}C-sucrose and the supernatant obtained. The suspension was centrifused at 5,700×g for 10 min at 4°C and the capillary pellet and postvascular supernatant were separated again. The radioactivity in both fractions was counted. The percentage of ^{125}I-bFGF and ^{14}C-sucrose released from the capillary pellet was calculated as follows:

$$\text{Release (\%)} = \frac{^{125}\text{I or } ^{14}\text{C supernatant} \times 100}{^{125}\text{I or } ^{14}\text{C in supernatant} + ^{125}\text{I or } ^{14}\text{C in pellet}} \quad (2)$$

Brain Efflux Method

The brain efflux method (22) was used to examine the efflux of ^{125}I-bFGF through the BBB. Briefly, rats were anesthetized with *i.m.* ketamine (235 mg/kg) and placed in a stereotaxic frame (SR-6, Narishige Scientific Instrument Lab., Tokyo, Japan). A hole approximately 1-mm was made 0.2 mm anterior and 5.5 mm lateral to the bregma using a dental drill (MINITOR, Narishige Scientific Instrument Lab.). The needle of the microsyringe (#1801, GASTIGHT, HAMILTON) was inserted to a depth of 4.6 mm below the bone surface (Par2 region of cerebrum). One microliter ECF buffer (122 mM NaCl, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 0.4 mM K_2HPO_4, 10 mM D-glucose, 10 mM HEPES, pH 7.4, 0.1% BSA) (20) containing ^{125}I-bFGF (41.8 ~ 148 kBq/mL, 3.04 ~ 9.12 μM) and ^{14}C-inulin (100 ~ 300 kBq/mL) was injected into the brain. At 10, 60, and 120 min after injection, CSF was sampled, and then rats were decapitated, and the left and right cerebrum were removed. Brain samples were solubilized with Soluene 350, and ^{125}I and ^{14}C radioactivity measured.

^{14}C and ^{125}I Double Isotope Counting

The radioactivity of ^{14}C-sucrose in the presence of ^{125}I-bFGF was counted in a liquid scintillation system using an Aloka LSC-5100 spectrometer (Aloka, Co. Ltd., Tokyo, Japan). To minimize the spillover of ^{125}I energy (due to the multiple non γ emissions of ^{125}I) into the ^{14}C channel, the counting windows for ^{14}C were set in an interval of 60 ~ 156 keV. The measurement error for ^{14}C under these conditions was less than 7%, even for a sample with a 5:1 ratio of ^{125}I and ^{14}C radiation activity. The ^{125}I radioactivity was counted using a γ-counter (Model 530, Packard).

Data Analysis

Pharmacokinetic analysis of the plasma elimination curve of ^{125}I-bFGF after *i.v.* injection was fitted to a two-compartment open model using the nonlinear regression analysis program MULTI (23) in order to estimate the parameters for a two-compartment open model (A, B, α, β), total body clearance (CL_{tot}) and volume of distribution at steady-state (Vd_{ss}).

The data for the binding of ^{125}I-bFGF to the isolated capillaries were analyzed by a model that involved saturable and nonsaturable binding as follows:

$$\text{Bound} = \frac{B_{max} \times C_f}{K_d + C_f} + \alpha \times C_f \quad (3)$$

where B_{max} and K_d are the maximal number of binding sites and dissociation constant, respectively. α represents a nonsaturable binding constant. C_f is the free concentration of intact ^{125}I-bFGF in the incubation medium, corrected by TCA precipitability.

The efflux of ^{125}I-bFGF from the brain across the BBB was evaluated by the brain efflux index (BEI) method described by Kakee *et al.* (22).

All data are presented as mean ± S.E., except where otherwise noted. Statistical analysis of data was performed by a one-way analysis of variance (ANOVA) followed by Dunnet's test for multiple comparison.

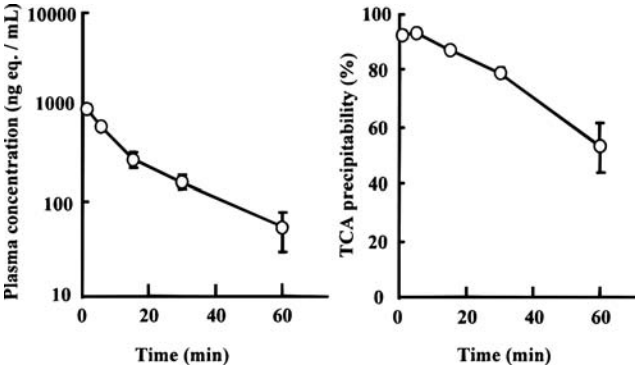

Fig. 1. (Left panel) Profiles of the TCA-precipitable plasma concentration of ^{125}I-bFGF after *i.v.* injection of a dose of 1.10 MBq/116 μg/kg) to rats. (Right panel) Time-course of the percentage of plasma radioactivity that is precipitable with TCA after *i.v.* injection. Each value represents the mean ± S. E. of three experiments.

RESULTS

Purification of ^{125}I-bFGF

^{125}I-bFGF was prepared by the modified lactoperoxidase method, protecting the thiol residues of bFGF using sodium tetrathionate to suppress dimerization of bFGF; then it was purified using an ion exchange HPLC column and a Sephadex G25 column. The ^{125}I-bFGF obtained migrated as a single peak through the TSK gel filtration HPLC column and the elution volume (20 mL) was identical to that of unlabeled bFGF. The ^{125}I-bFGF obtained was confirmed to be identical with unlabelled bFGF by examining the immunoreactivity with mouse monoclonal bFGF antibody (4.2, 48.1, 66.1, Scios Inc. (Mountain View, CA)) and the proliferation activity using baby hamster kidney 21 cells (18).

Intravenous Administration

The time-courses of the plasma concentration and TCA precipitability after *i.v.* injection of ^{125}I-bFGF are shown in Fig. 1. The pharmacokinetic parameters generated by fitting the plasma concentration profile to a two-compartment open model are listed in Table I. ^{125}I-bFGF was removed from plasma with a half-life of 20 min. The distribution volume (V_d) of ^{125}I-bFGF 60 min after *i.v.* bolus injection reached 48.0 mL/g for liver

Table I. Pharmacokinetic Parameters of ^{125}I-bFGF in Rats

Parameters	
A (ng eq./mL)	764 ± 61
B (ng eq./ mL)	420 ± 138
α (min^{-1})	0.257 ± 0.083
β (min^{-1})	0.0342 ± 0.0069
CL_{tot} (mL/min/kg)	7.56 ± 1.16
Vd_{ss} (mL/kg)	173 ± 15

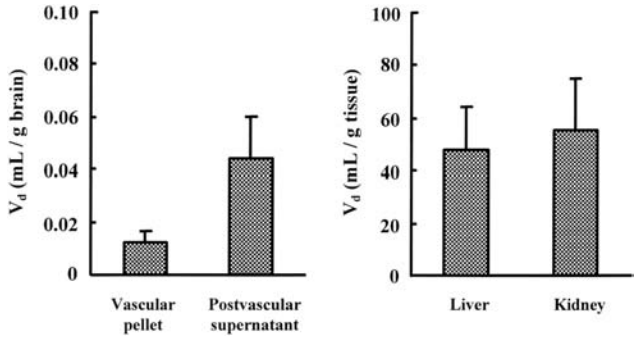

Fig. 2. Distribution to brain (left panel), liver and kidney (right panel) of ^{125}I-bFGF at 60 min after *i.v.* administration. The brain was separated into a postvascular supernatant and a vascular pellet using the capillary depletion technique. Each value represents the mean ± S.E. of three experiments.

and 55.2 mL/g for kidney (right panel of Fig. 2). On the other hand, the V_d values of TCA-precipitable ^{125}I-bFGF in the postvascular supernatant and the vascular pellet of the brain were 0.0447 mL/g brain and 0.0122 mL/g brain, respectively (left panel of Fig. 2).

Internal Carotid Artery Perfusion

The distribution volume (V_d) of ^{125}I-bFGF in the vascular pellet and postvascular supernatant increased with the perfusion time of the labeled peptide (Fig. 3). On the other hand, the V_d of ^{14}C-sucrose, a vascular reference compound, kept nearly constant in both fractions, suggesting that the BBB remains intact during the perfusion. The V_d of ^{125}I-bFGF in the postvascular supernatant reached 21.3 ± 3.3 μL/g brain (n = 10) during the 5-min perfusion, which was 5-fold greater than that of ^{14}C-sucrose (4.11 ± 0.41 μL/g brain (n = 8)). The TCA precipitability of the radioactivity taken up by the postvascular supernatant during perfusion for 1 to 10 min was over 90%.

The percentage release, which represents dissociation of ^{125}I-bFGF from the vascular pellet during the capillary depletion procedure, was 18.0 ± 3.0%. On the other hand, the percentage release of ^{14}C-sucrose, used as an extracellular marker, was 93% of the amount added. These results suggest that only a small amount of ^{125}I-bFGF is released from the vascular pellet during the capillary depletion procedure.

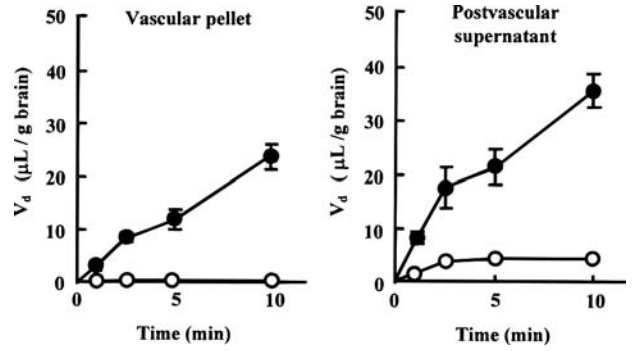

Fig. 3. Plots of the distribution volume (V_d) of ^{125}I-bFGF in the vascular pellet (left panel) and postvascular supernatant (right panel) versus time following internal carotid artery perfusion of labeled peptide. Key, ●: ^{125}I-bFGF; ○: ^{14}C-sucrose. Each value represents the mean ± S.E. of three to ten experiments.

Identification and Characterization of ^{125}I-bFGF Binding in Bovine Brain Capillaries

The binding of ^{125}I-bFGF to isolated brain capillaries at 4°C increased with time and reached equilibrium after 10 min of incubation. The concentration-dependence of ^{125}I-bFGF binding to capillaries under equilibrium conditions (incubation for 60 min) is shown in the left panel of Fig. 4. Fitting the data to eq. (3) gave B_{max} = 206 ± 48 pmol/mg protein, K_d = 36.5 ± 15.7 nM, and α = 0.608 ± 0.050 pmol/mg protein/nM. In addition, the binding of ^{125}I-bFGF to brain capillaries was inhibited by increasing the concentration of unlabeled bFGF (Fig. 4 (right panel)). These results suggest the existence of a binding site for ^{125}I-bFGF on bovine brain capillaries.

As shown in Table II, the binding of ^{125}I-bFGF to capillaries was not affected by bovine insulin (10 μM), bovine holotransferrin (10 μM) and WGA (50 μg/mL). In contrast, protamine, poly-L-lysine, and poly-L-glutamate (each 300 μM), significantly inhibited ^{125}I-bFGF binding to brain capillaries at 4°C by more than 85%.

Binding of ^{125}I-bFGF to brain capillaries was also inhibited by increasing the concentration of heparin. The concentration of heparin that inhibited ^{125}I-bFGF binding by 50% (IC_{50}) was approximately 0.2 μg/mL (Fig. 5). In addition, CS-B, CS-C and fucoidan (each 10 μg/mL), which contain a sulfate moiety, significantly inhibited the binding of ^{125}I-bFGF to brain capillaries by 42%, 18% and 41%, respectively. On the other hand, neither hyaluronic acid, dextran nor sialic acid (each 10 μg/mL), had any effect (Table II).

Effects of Various Compounds on ^{125}I-bFGF Transcytosis Through the BBB

The effect of various compounds on transcytosis through the BBB was examined using the internal carotid artery perfusion of ^{125}I-bFGF (Fig. 6). The V_d of ^{125}I-bFGF in the capillary pellet and in the postvascular supernatant was significantly

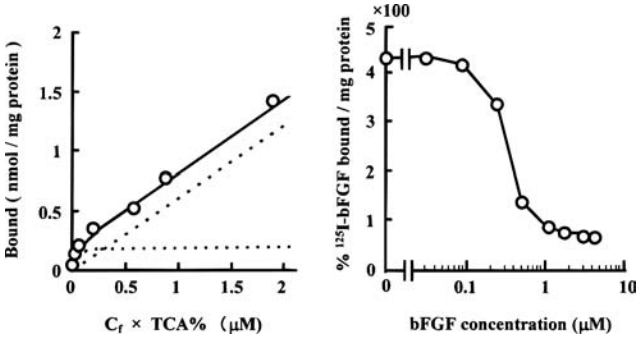

Fig. 4. (Left panel) Concentration-dependence of ^{125}I-bFGF binding to isolated bovine brain capillaries at 4°C. The data were fitted to eq (3) by nonlinear regression analysis. The solid line shows the theoretical value generated by eq. (3) using best fitted parameters. Dotted lines represent saturable and nonsaturable terms of eq. (3). (Right panel) Inhibition of ^{125}I-bFGF binding to isolated brain capillaries by increasing the concentration of unlabeled bFGF. Each value represents the mean of duplicates.

Table II. Effect of Polypeptides and Glycosaminoglycans on ^{125}I-bFGF Binding to Isolated Bovine Brain Capillaries

Inhibitor	Concentration	Relative binding[c] (% of control)
Protamine[a]	300 μM	2.33 ± 0.37***
Poly-L-lysine[a]	300 μM	13.1 ± 1.5***
Poly-L-glutamic acid[a]	300 μM	29.8 ± 1.4***
Insulin[a]	10 μM	108 ± 3
Transferrin[a]	10 μM	109 ± 2
WGA[a]	50 μg/mL	108 ± 2
Heparin[b]	10 μg/mL	13.1 ± 0.4***
CS-A[b]	10 μg/mL	104 ± 5
CS-B[b]	10 μg/mL	58.0 ± 5.1***
CS-C[b]	10 μg/mL	82.0 ± 2.6**
Fucoidan[b]	10 μg/mL	58.9 ± 2.5***
Hyaluronic acid[b]	10 μg/mL	91.7 ± 1.8
Dextran[b]	10 μg/mL	91.8 ± 3.1
Sialic acid[b]	10 μg/mL	100 ± 1

[a] Assays were performed in the presence of various polypeptides by incubating ^{125}I-bFGF (0.925 kBq, 31.5 nM) and ^{14}C-sucrose (3.7 kBq) with the capillary suspension at 4°C for 60 min. The control value for ^{125}I-bFGF binding to isolated brain capillaries was 345 ± 30% bound/mg protein (n = 6).

[b] The capillary suspension was pre-incubated with ^{125}I-bFGF (0.925 kBq, 31.5 nM) for 60 min at 4°C. Then, the suspension was further incubated for 60 min at 4°C by adding various glycosaminoglycans. The control value for ^{125}I-bFGF binding to isolated capillaries under these conditions was 542 ± 19% bound/mg protein (n = 3).

[c] Each value represents the mean ± S.E. of three experiments.

** Significantly different from the control value, $p < 0.01$.

*** $p < 0.001$. WGA: wheatgerm agglutinin; CS: chondroitin sulfate.

inhibited by heparin (10 μg/mL) and poly-L-lysine (300 μM). In contrast, neither hyaluronic acid (10 μg/mL) nor insulin (10 μM) had any effect.

Efflux of ^{125}I-bFGF from the Brain Across the BBB

The percentages of ^{125}I-bFGF remaining in the cerebrum after microinjection ((100 − BEI)%) were 61.8 ± 5.3% (10 min), 62.8 ± 9.0% (60 min) and 54.7 ± 1.9% (120 min). No appreciable disappearance of ^{125}I-bFGF from the injection site was observed. The low recovery of ^{125}I-bFGF at 10 min after microinjection may be due to adsorption of this peptide to the injection syringe.

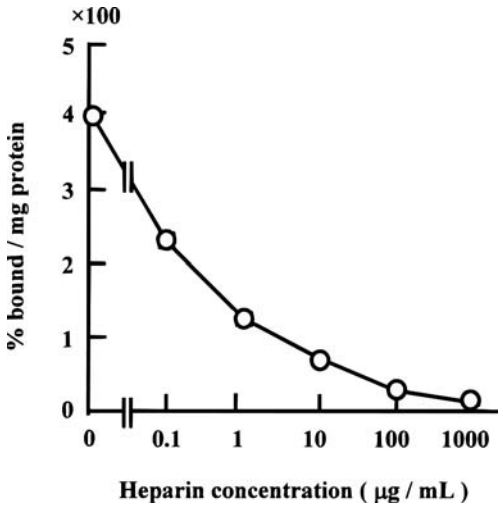

Fig. 5. Inhibition of ^{125}I-bFGF binding to isolated bovine brain capillaries at 4°C by increasing the concentration of heparin. Each value represents the mean ± S.E. of three experiments.

DISCUSSION

This study was designed to quantitatively examine the BBB transport of exogenously administered ^{125}I-bFGF. The results obtained in this study were consistent with the following conclusions. Firstly, ^{125}I-bFGF undergoes significant transport into the brain parenchyma through the BBB, probably via an adsorptive-mediated transcytosis mechanism. Secondly, binding of ^{125}I-bFGF to HSPG present on the brain capillary surface may trigger the transcytosis of this peptide through the BBB.

Pharmacokinetic analysis of the plasma elimination curve of ^{125}I-bFGF after *i.v.* bolus injection showed that ^{125}I-bFGF is removed from the plasma compartment with a CL_{tot} of 7.54 mL/min/kg. This rapid removal may be due to extensive metabolism after avid uptake by liver and kidney (Fig. 2) (18). The Vd_{ss} corresponds well with that of the extracellular space, suggesting that ^{125}I-bFGF is localized in the interstitial fluid of nondisposing organs or trapped by the membrane surface around the microvasculature (18). The brain had less volume of distribution (0.057 mL/g brain). However, the V_d of TCA-precipitable ^{125}I-bFGF in the postvascular supernatant was approximately 5-fold greater than the value reported for ^{14}C-labeled rat albumin (0.013 mL/g brain) (Fig. 2) (24). This result suggests that intact ^{125}I-bFGF may be taken up by the brain through the BBB.

To validate this hypothesis, intracarotid artery perfusion of ^{125}I-bFGF was carried out. A significantly higher and linear increase in V_d for ^{125}I-bFGF (Fig. 3) represents the actual transcytosis of this peptide through the capillary wall. This is supported by the high TCA-precipitability of ^{125}I-bFGF in the capillary-depleted brain (>90%) and a negligible amount of dissociation of capillary-bound ^{125}I-bFGF. The BBB permeability surface area product (PS) of ^{125}I-bFGF was approximately 2.5 μL/min/g brain which corresponded well with that of cationized albumin and cationized IgG reported by Triguero *et al*. (15). These cationic proteins cross the BBB after an electrostatic interaction with negative charges lining the brain capillary membrane surface which triggers adsorptive-mediated transcytosis.

Kinetic analysis of ^{125}I-bFGF binding to isolated bovine brain capillaries showed that there was a saturable binding site with a K_d of 40 nM and a Bmax of 200 pmol/mg protein (left panel of Fig. 4). In the low concentration range of free ^{125}I-bFGF (<5 nM), the specific binding accounted for more than 85% of the total binding. This binding was inhibited by unlabeled bFGF in a concentration-dependent manner (right panel of Fig. 4). In addition, cationic polypeptides protamine and poly-L-lysine, but not insulin and transferrin, which are bound to the specialized receptors, reduced the binding of ^{125}I-bFGF by more than 85% (Table II). These results suggest that the specific binding site for bFGF was blocked by these cationic polypeptides. On the other hand, the binding of ^{125}I-bFGF to the capillary surface was not inhibited by WGA which induces adsorptive endocytosis (14,25). WGA binds to sialic acid and N-acetyl-β-D-glucosaminyl acid negative charges on the cell

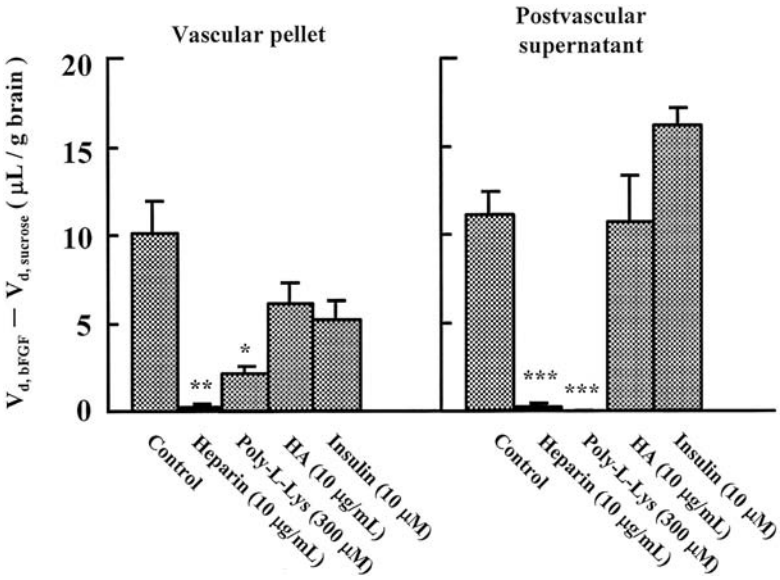

Fig. 6. Effect of heparin, poly-L-lysine (poly-L-Lys), hyaluronic acid (HA) and insulin on the transcytosis of ^{125}I-bFGF through the BBB. Transcytosis of ^{125}I-bFGF through the BBB was measured by intracarotid artery perfusion for 5 min. The brain homogenate was separated into a vascular pellet and a postvascular supernatant by the capillary depletion technique. Each value represents the mean ± S.E. of three to seven experiments. Significantly different from control, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

membrane. Therefore, ^{125}I-bFGF may be bound to regions distinct from these anionic sites. Unexpectedly, the binding of ^{125}I-bFGF to capillaries was significantly inhibited by a polyanionic peptide, poly-L-glutamate, which was used as a negative control. No reason for this could be found in this study. Poly-L-glutamate may occupy the cationic binding site of ^{125}I-bFGF by electrostatic interaction.

The K_d value estimated here (40 nM) corresponded to the affinity for bFGF binding to HSPG on Chinese hamster ovary cells (16). Heparan sulfate consists of a disaccharide repeat unit composed of L-iduronic acid and D-glucosamine which are sulfated and acetylated. The N and O-sulfate groups attached to D-glucosamine give HSPG negative charges which interact with the positive charges of bFGF (26). If such an interaction occurs between ^{125}I-bFGF and the brain capillary surface, the addition of other glycosaminoglycans with sulfate groups to the incubation medium could draw ^{125}I-bFGF from the ^{125}I-bFGF-HSPG complex on the capillary surface. As shown in Fig. 5, the percentage binding was reduced by increasing the concentration of heparin, with an IC_{50} of approximately 0.2 μg/mL. Furthermore, CS-B, CS-C and fucoidan, which are extensively sulfated, reduced the binding of ^{125}I-bFGF to isolated brain capillaries, whereas hyaruronic acid, dextran or sialic acid, all without sulfate groups, had no effect (Table II). These results suggest that HSPG may be a binding site for ^{125}I-bFGF on the brain capillary surface.

The binding of ^{125}I-bFGF to isolated capillaries *in vitro* includes the binding to the abluminal membrane which is richer in HSPG than the luminal side (27). Therefore, two types of *in vivo* studies were performed to examine whether binding to HSPG on the capillary endothelium triggers the BBB transcytosis of ^{125}I-bFGF, directed from the blood side to the brain. As shown in Fig. 6, the binding and uptake of ^{125}I-bFGF by the vascular pellet and postvascular supernatant were significantly inhibited by 10 μg/mL heparin and 300 μM poly-L-lysine. On the other hand, neither 10 μg/mL hyaruronic acid nor 10 μM insulin had any inhibitory effect (Fig. 6). In contrast, no significant efflux of ^{125}I-bFGF from the brain interstitial fluid to the blood circulation across the BBB was observed, suggesting negligible transcytosis of ^{125}I-bFGF from the abluminal side of the capillary membrane. These results suggest that the negative charge on the luminal membrane, possibly HSPG, plays an important role in the initial binding step of the BBB transcytosis of ^{125}I-bFGF.

The present studies demonstrate that blood-borne bFGF can be delivered into the brain across the BBB. However, its efficiency was less than 1% of the infused dose. Therefore, the use of a BBB delivery system may be required in order to obtain maximum efficacy when treating neurodegenerative disorders.

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