Comparison of Blood-Brain Barrier Transport of Glial-Derived Neurotrophic Factor (GDNF) and an IgG-GDNF Fusion Protein in the Rhesus Monkey

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ABSTRACT:

The brain drug development of glial-derived neurotrophic factor (GDNF) is prevented by the lack of transport of this protein across the blood-brain barrier (BBB). GDNF transport across the BBB can be made possible by re-engineering the neurotrophin as a fusion protein with a genetically engineered monoclonal antibody (MAb) against the human insulin receptor (HIR), which crosses the BBB on the endogenous insulin receptor. The present work was designed to compare the BBB transport in vivo of GDNF and the HIR MAb-GDNF fusion protein. Owing to species specificity of HIR MAb binding to the insulin receptor, the present studies were performed in the adult rhesus monkey. The brain uptake of human IgG1 was determined to assess the uptake of a brain plasma volume marker.

The brain clearance of GDNF was no different from the clearance of the IgG1, which indicated GDNF does not cross the primate BBB in vivo. In contrast, BBB transport of the HIR MAb-GDNF fusion protein was shown with film and emulsion autoradiography, as well as the capillary depletion method. In parallel with the increased brain uptake, fusion of the GDNF to the HIR MAb resulted in a decrease in the uptake of GDNF by liver, spleen, and kidney. Administration of the HIR MAb-GDNF fusion protein had no effect on glycemic control. The brain uptake parameters show that a systemic dose of the HIR MAb-GDNF fusion protein of 0.2 mg/kg may generate a 10-fold increase in the cerebral concentration of GDNF in the human brain.

Glial-derived neurotrophic factor (GDNF) is a neurotrophin that could be developed as a neurotherapeutic for multiple brain diseases, including stroke, Parkinson's disease (PD), drug addiction, and alcohol addiction (Lapchak et al., 1997; Ron and Janak, 2005; Boado et al., 2008; Carnicella et al., 2008). However, GDNF does not cross the blood-brain barrier (BBB). Owing to the BBB problem, the delivery of GDNF to brain in subjects with PD was attempted with a transcranial drug delivery method: convection-enhanced diffusion (CED) (Lang et al., 2006). However, the PD trial was halted because of lack of efficacy, and primate studies showed limited distribution of GDNF in brain following intracerebral infusion (Salvatore et al., 2006).

GDNF can be delivered through the BBB following peripheral administration, providing the biopharmaceutical is re-engineered as a fusion protein with a BBB molecular Trojan horse (Pardridge, 2008). The latter is an endogenous peptide, or a peptidomimetic monoclonal antibody (MAb), that undergoes receptor-mediated transport across the BBB via an endogenous peptide receptor/transporter. The most

potent BBB Trojan horse known is a MAb against the human insulin receptor (HIR) (Pardridge et al., 1995). Genetically engineered forms of the HIR MAb have been produced, and the chimeric or humanized HIR MAb rapidly crosses the BBB in the rhesus monkey (Boado et al., 2007b). The HIR MAb cross-reacts with the insulin receptor of Old World primates, such as the rhesus monkey, but does not react with the insulin receptor in New World primates, such as the squirrel monkey (Pardridge et al., 1995), and does not react with the rodent insulin receptor. There is no known MAb against the rodent insulin receptor that can be used as a BBB molecular Trojan horse in rats or mice. Therefore, biological testing of the HIR MAb in vivo must be performed in the rhesus monkey.

A HIR MAb-GDNF fusion protein has been genetically engineered, expressed, and shown to be a bifunctional protein, which binds with high affinity both to the HIR and to the GDNF receptor $\alpha 1$ (Boado et al., 2008). The "head" of the fusion protein binds the BBB HIR to mediate transport across the BBB, and the "tail" of the fusion protein binds the GDNF receptor $\alpha 1$ to mediate GDNF pharmacologic action in brain. The purpose of the present study was to compare the BBB transport of human recombinant GDNF and the HIR MAb-GDNF fusion protein in the adult rhesus monkey. The results of these pharmacokinetic (PK) and in vivo brain uptake studies can be used to design future preclinical investigations on the efficacy and toxicity of the HIR MAb-GDNF fusion protein.

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ABBREVIATIONS: GDNF, glial-derived neurotrophic factor; PD, Parkinson's disease; BBB, blood-brain barrier; CED, convection-enhanced diffusion; MAb, monoclonal antibody; HIR, human insulin receptor; PK, pharmacokinetics; hIgG1, human IgG1; TCA, trichloroacetic acid; VD, volume of distribution; ID, injected dose; AUC, area under the plasma concentration curve; ELISA, enzyme-linked immunosorbent assay; ECD, extracellular domain; TBS, Tris-buffered saline; TBST, Tris-buffered saline/Tween 20.

Fig. 1. A, structure of sandwich ELISA used to measure immunoreactive HIR MAb-GDNF fusion protein. The capture reagent is a complex of a murine MAb against the HIR, which binds the lectin affinity-purified HIR ECD. The HIR MAb part of the HIR MAb-GDNF fusion protein binds the HIR ECD, and the detection reagent is a goat anti-GDNF antibody complexed by a rabbit anti-goat IgG conjugate of alkaline phosphatase. B, plasma concentration of immunoreactive HIR MAb-GDNF fusion protein in the rhesus monkey following the intravenous injection of 0.2 mg/kg fusion protein.

Materials and Methods

Radiolabeling of Proteins. The HIR MAb-GDNF fusion protein was purified from serum-free conditioned medium of permanently transfected Chinese hamster ovary cells with protein A affinity chromatography, cation exchange chromatography, and anion exchange chromatography. Human (h) IgG1 κ , the isotype control of the HIR MAb part of the fusion protein, was isolated from human myeloma plasma and purchased from Sigma-Aldrich (St. Louis, MO). Human recombinant GDNF, produced in bacteria, was purchased from PeproTech (Rocky Hill, NJ). 125 I-Iodine and 3 H-N-succinimidyl propionate were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). 125 I-Bolton-Hunter reagent was purchased from American Radiolabeled Chemicals (St. Louis, MO). The HIR MAb-GDNF fusion protein was labeled with 125 I-iodine and chloramine T, as described previously (Boado et al., 2007b), to a specific activity of 11 μ Ci/ μ g and a trichloroacetic acid (TCA) precipitability of >99%. The TCA precipitation of the labeled HIR MAb-GDNF fusion protein remained >99% at 24 h after radiolabeling. The HIR MAb-GDNF fusion protein was radiolabeled on the same day as administration to the primate. The hIgG1 was tritiated with 3 H-N-succinimidyl propionate, as described previously (Boado et al., 2007a), to a specific activity of 2.8 μ Ci/ μ g and a TCA precipitability of 96.4%, was labeled before primate injection, and was stored at -70°C . The TCA precipitation of the 3 H-hIgG1 remained >96% before injection. The GDNF was labeled with 125 I-iodine and chloramine T to a specific activity of 11 μ Ci/ μ g and a TCA precipitation of 97.1%. However, the chloramine T-labeled GDNF was not stable, as the TCA precipitation decreased to 82% by 24 h after labeling. Therefore, the GDNF was labeled with the 125 I-Bolton-Hunter reagent to a specific activity of 32 μ Ci/ μ g and a TCA precipitation >99%. The TCA precipitation of the Bolton-Hunter-labeled GDNF remained >99% at 24 h after radiolabeling. The GDNF was labeled 1 day before injection in the primate.

Primate Drug Administration. Four adult female rhesus monkeys (4.3–7.2 kg) were investigated. Primates were obtained from Covance (Alice, TX). Animals received 650 to 1080 μ Ci intravenous injections of 125 I-HIR MAb-GDNF fusion protein, which was coinjected with unlabeled HIR MAb-GDNF fusion protein, at a dose of 0.2 mg/kg. Animals receiving 125 I-GDNF were administered 790 to 800 μ Ci of the labeled GDNF, which was coinjected with unlabeled GDNF, at a dose of 0.05 mg/kg, and with 810 μ Ci of 3 H-hIgG1. Animals were initially anesthetized with intramuscular ketamine, and anesthesia was maintained by 1% isoflurane by inhalation. All the procedures were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) as adopted and promulgated by the U.S. National Institutes of Health. After intravenous drug administration, venous plasma was obtained at 1, 2.5, 5, 15, 30, 60, and 120 min for determination of radioactivity or glucose concentration. Animals were euthanized, and samples of major organs (heart, liver, spleen, lung, kidney, skeletal muscle, and omental fat) were removed, weighed, and processed for determination of radioactivity. The cranium was opened, and the brain was removed. Samples of frontal cortical gray matter, frontal cortical white matter, cerebellar gray matter, and cerebellar white matter were removed for radioac-

tivity determination. Samples (~1 g) of frontal cortex were removed for capillary depletion analysis, as described previously (Pardridge et al., 1995). For the capillary depletion analysis, the brain sample was divided into three fractions: total brain homogenate, postvascular supernatant, and capillary pellet; the volume of distribution (VD) was determined for each of the three fractions from the ratio of total 125 I radioactivity in the fraction divided by the total 125 I radioactivity in the 120-min terminal plasma. Plasma and tissue samples were analyzed for 125 I radioactivity with a gamma counter (Wizard 1470; PerkinElmer Life and Analytical Sciences) and were analyzed for 3 H radioactivity with a liquid scintillation counter (Tricarb 2100TR; PerkinElmer Life and Analytical Sciences). The 125 I isotope emits radiation that is detected in the 3 H channel (0–12 keV) of the liquid scintillation counter. Therefore, quench curves were produced, using chloroform as the quench agent, to compute the efficiency of counting of 125 I in the 3 H window. Based on this standard curve, as well as the 125 I radioactivity in the sample determined with gamma counting, the net 3 H radioactivity in the sample was determined.

Brain Autoradiography. The brain was cut into coronal slabs, and blocks were frozen in optimal cutting temperature embedding medium. Frozen sections (20 μ m) were cut with a cryostat at -15°C ; the sections were air-dried and exposed to Kodak (Rochester, NY) Biomax film for 2 weeks followed by X-ray film development. The films were scanned, and the image was saved in Photoshop (Adobe Systems, Mountain View, CA) and colorized with National Institutes of Health (Bethesda, MD) Image software. Frozen sections of brain were also subbed to slides coated with HyperCoat LM-1 emulsion (GE Healthcare, Little Chalfont, Buckinghamshire, UK); the slides were developed in the dark for 6 weeks and processed with D-19 developer and fixer (Eastman Kodak, Rochester, NY). Some of the slides were counterstained with Mayer's hematoxylin.

Pharmacokinetics. The TCA-precipitable radioactivity in plasma (disintegrations per minute per milliliter) was converted to percentage injected dose (ID) per milliliter, and the percentage ID per milliliter was fit to either a single or biexponential equation: $\%ID/ml = A_1e^{-k_1t} + A_2e^{-k_2t}$.

The intercepts (A_1 or A_2) and the slopes (k_1 , k_2) were used to compute the median residence time, the central volume of distribution, the steady-state volume of distribution, the area under the plasma concentration curve (AUC), and the systemic clearance as described previously (Pardridge et al., 1995). Nonlinear regression analysis used the AR subroutine of the BMDP Statistical Software (Statistical Solutions Ltd., Cork, Ireland). Data were weighted by $1/(\%ID/ml)^2$. The organ clearance (microliter per minute per gram), also called the permeability-surface area product, is computed from the terminal organ uptake (percentage ID per gram) and the 120-min plasma AUC (percentage ID per minute per milliliter) as follows: organ clearance = $[(\%ID/g)/AUC] \times 1000$.

HIR MAb-GDNF Enzyme-Linked Immunosorbent Assay. The concentration of the HIR MAb-GDNF fusion protein in plasma was measured with a two-site sandwich enzyme-linked immunosorbent assay (ELISA), which is outlined in Fig. 1A. The assay uses the HIR extracellular domain (ECD) as the

TABLE 1

Concentration of HIR MAb-GDNF fusion protein as determined by measurements of either radioactivity or sandwich ELISA

For ELISA data, expressed as microgram per milliliter in plasma (see Fig. 1B). Data are mean $\pm$ S.E.

Time	%ID/ml (Based on Radioactivity)	%ID/ml (Based on ELISA)
<i>min</i>		
1	0.21 $\pm$ 0.01	0.128 $\pm$ 0.01
2.5	0.16 $\pm$ 0.01	0.113 $\pm$ 0.01
5	0.14 $\pm$ 0.01	0.108 $\pm$ 0.01
15	0.083 $\pm$ 0.006	0.092 $\pm$ 0.006
30	0.062 $\pm$ 0.001	0.086 $\pm$ 0.001
60	0.060 $\pm$ 0.001	0.074 $\pm$ 0.001
120	0.066 $\pm$ 0.003	0.058 $\pm$ 0.003

capture reagent and an anti-GDNF antibody as the detector reagent. The assay was designed so that immunoreactivity would be measured only on the intact HIR MAb-GDNF fusion protein, and reactivity requires the presence of both parts of the fusion protein: the HIR MAb moiety and the GDNF moiety. A murine MAb against the HIR (Abcam, Cambridge, MA) was plated in 96-well plates overnight at 4°C in 0.1 M NaHCO₃/8.3 (100 ng/well); this antibody binds an epitope on the HIR that is spatially removed from the HIR MAb binding site. The solution was removed by aspiration; the wells were washed with 0.01 M Tris/0.15 M NaCl/7.4 (TBS); and 100 μ l (200 ng)/well of lectin affinity-purified HIR ECD was added followed by 90-min incubation at room temperature. The wells were washed with TBS/0.05% Tween 20 (TBST), and either the plasma or the HIR MAb-GDNF reference standard was added in 100 μ l/well followed by 60-min incubation at room temperature. A goat anti-human GDNF antibody (R&D Systems, Minneapolis, MN) was applied in a volume of 100 μ l (400 ng)/well followed by 30-min incubation at room temperature. After washing with TBST, a conjugate of rabbit anti-goat IgG and alkaline phosphatase (Vector Laboratories, Burlingame, CA) was applied in a volume of 100 μ l (500 ng)/well followed by 45-min incubation at room temperature. The wells were washed with TBST, and 100 μ l/well of *p*-nitrophenylphosphate (Sigma-Aldrich) was incubated in the dark at room temperature for 15 to 30 min. The color development was terminated by the addition of 100 μ l/well of 1.2 M NaOH, and color development was measured with an ELISA plate reader at 405 nm. The limit of detection was 1 ng/well HIR MAb-GDNF fusion protein. The standard curve was determined with 0- to 300-ng/ml solutions of HIR MAb-GDNF fusion protein and was curvilinear, which was fit with a nonlinear regression analysis using the AR subroutine of the BMDP software. The curve fitting yielded a correlation coefficient (r^2) of >0.97 .

Results

The concentration in plasma of the HIR MAb-GDNF fusion protein was determined by both radioactivity measurements and by ELISA, and these data are comparable (Table 1). The concentration of the HIR MAb-GDNF fusion protein in plasma, as determined with the ELISA, decreases rapidly (Fig. 1B), despite the large size, 190 kDa, of the fusion protein, owing to uptake by tissues expressing the insulin receptor. There is no effect of administration of 0.2 mg/kg HIR MAb-GDNF fusion protein on glycemic control in the primate (Table 2). The plasma concentration data in Table 1 were used to generate the PK parameters for the HIR MAb-GDNF fusion protein, which are given in Table 3. The organ uptake of the HIR MAb-GDNF fusion protein at 120 min after injection is given in Table 4. The brain VD at 120 min after injection for the HIR MAb-GDNF fusion protein, as determined with the capillary depletion method, is given in Table 5. This method separates brain homogenate radioactivity into the capillary and postvascular supernatant compartments. The high VD in the postvascular supernatant, relative to the capillary pellet, is indicative of transport of the HIR MAb-GDNF fusion protein across the BBB and into brain parenchyma.

TABLE 2

Plasma glucose after injection of the HIR MAb-GDNF fusion protein

Data are mean $\pm$ S.E.

Time	Glucose
<i>min</i>	<i>mg/dl</i>
1	81 $\pm$ 1
2.5	79 $\pm$ 3
5	81 $\pm$ 1
15	81 $\pm$ 2
30	82 $\pm$ 2
60	82 $\pm$ 3
120	85 $\pm$ 2

The HIR MAb-GDNF fusion protein was distributed to all parts of brain, as shown by film autoradiography of primate brain (Fig. 2). The uptake of the HIR MAb-GDNF fusion protein in gray matter is higher than in white matter. Emulsion autoradiography of brain sections show a uniform distribution of the fusion protein in the postvascular volume of brain (Fig. 3).

The plasma profile of the GDNF and the hIgG1 is shown in Fig. 4, and the PK parameters for these two proteins are given in Table 3. The systemic clearance of the GDNF is 20-fold faster than the systemic clearance of the hIgG1 and is 8-fold faster than the systemic clearance of the HIR MAb-GDNF fusion protein. The hepatic uptake of the GDNF is 35.5 $\pm$ 0.4% ID/100 g. Because the liver weighs approximately 150 g in a 5-kg rhesus monkey (Davies and Morris, 1993), more than 50% of the injected GDNF is cleared by the liver. The size of the brain in the adult rhesus monkey is approximately 100 g (Davies and Morris, 1993), and the brain uptake of the HIR MAb-GDNF fusion protein, as expressed as %ID/100 g brain, is approximately 17-fold higher than the brain uptake of GDNF (Table 4). However, the brain uptake value for GDNF reflects distribution in the brain plasma volume because the postvascular uptake of GDNF is negligible. The brain VD of GDNF, 17.4 $\pm$ 1.0 μ l/g, is not significantly different from the brain VD for the hIgG1, 20.5 $\pm$ 6.5 μ l/g. The equivalence of the brain VD of GDNF and the hIgG1 vascular space marker indicate the brain penetration of GDNF is restricted to the brain plasma volume.

The relative penetration of hIgG1, GDNF, and the HIR MAb-GDNF fusion protein into brain is quantified by computation of the organ clearance. Organ clearance is derived from the ratio of the organ uptake at 120 min after injection (Table 4), relative to the 120-min plasma AUC (Table 3), as described under *Materials and Methods*. The brain clearance of GDNF is not significantly different from the brain clearance of hIgG1, which represents sequestration within the cerebral plasma volume, whereas the brain clearance of the HIR MAb-GDNF fusion protein is 0.80 $\pm$ 0.02 μ l/min/g (Fig. 5). The organ clearance parameters for hIgG1 and GDNF by peripheral tissues are given in Table 6. The ratio of organ clearance of GDNF relative to hIgG1 clearance by brain is 1 (Fig. 5). However, this ratio ranges from 6 to 14 for heart, lung, skeletal muscle, and fat, and ranges from 20 to 80 for liver, spleen, and kidney (Table 6).

Discussion

The results of these studies are consistent with the following conclusions. First, GDNF does not cross the primate BBB. The brain VD of GDNF is not significantly different from the VD of hIgG1 (see under *Results*), which is equal to the cerebral blood volume (Ito et al., 2005). The brain clearance of GDNF is not significantly different from the clearance of hIgG1 (Fig. 5). Second, the brain clearance of the HIR MAb-GDNF fusion protein is high compared with the brain clearance for either GDNF or hIgG1 (Fig. 5), which is evidence for

TABLE 3
Pharmacokinetic parameters

Parameter	Units	[¹²⁵ I]HIR MAb-GDNF	[³ H]hIgG1	[¹²⁵ I]GDNF
A1	%ID/ml	0.213 ± 0.060	0.214 ± 0.008	0.049 ± 0.027
A2	%ID/ml	0.072 ± 0.016	0.173 ± 0.008	
k1	min ⁻¹	0.254 ± 0.131	0.082 ± 0.007	0.020 ± 0.013
k2	min ⁻¹	0.0062 ± 0.0030	0.0037 ± 0.0004	
MRT	min	151 ± 79	253 ± 41	50.0 ± 32.9
Vc	ml/kg	49 ± 11	56 ± 1	
Vss	ml/kg	168 ± 38	112 ± 5	446 ± 254
AUC ¹²⁰	%ID min/ml	7.0 ± 0.6	25.3 ± 0.3	
AUCss	%ID min/ml	12.5 ± 4.1	48.9 ± 5.4	2.44 ± 0.85
CL	ml/min/kg	1.11 ± 0.34	0.45 ± 0.04	8.9 ± 1.6

A1 and A2, intercepts; k1 and k2, slopes; MRT, median residence time; Vc, central volume of distribution; Vss, steady-state volume of distribution; CL, systemic clearance.

TABLE 4
Organ uptake of [¹²⁵I]HIR MAb-GDNF and [¹²⁵I]GDNF1 in the rhesus monkey
Data are %ID/100 g tissue (mean ± S.E.).

Organ	[¹²⁵ I]HIR MAb-GDNF	[¹²⁵ I]GDNF
Frontal gray	0.55 ± 0.04	0.033 ± 0.002
Frontal white	0.19 ± 0.01	0.015 ± 0.001
Cerebellar gray	0.52 ± 0.02	0.027 ± 0.004
Cerebellar white	0.22 ± 0.01	0.019 ± 0.001
Heart	15.2 ± 0.3	0.56 ± 0.02
Liver	9.0 ± 0.1	35.5 ± 0.4
Spleen	5.0 ± 0.5	19.5 ± 0.7
Lung	4.4 ± 0.1	1.3 ± 0.1
Kidney	0.62 ± 0.03	104.0 ± 3.6
Skeletal muscle	1.8 ± 0.1	0.18 ± 0.01
Fat	0.55 ± 0.04	0.30 ± 0.02

TABLE 5
Capillary depletion analysis of HIR MAb-GDNF distribution in brain
Data are mean ± S.E.

Fraction	HIR MAb-GDNF VD
	μl/g
Homogenate	76 ± 6
Postvascular supernatant	75 ± 12
Brain capillary pellet	1.4 ± 0.2

brain penetration of the fusion protein. The HIR MAb-GDNF fusion protein distributes to the entire extravascular brain volume as shown by film autoradiography (Fig. 2), emulsion autoradiography (Fig. 3), and the capillary depletion method (Table 5). Third, the HIR MAb-GDNF fusion protein is rapidly removed from plasma, but the plasma species is intact HIR MAb-GDNF fusion protein. This is shown by the equivalence of the blood concentrations of the HIR MAb-GDNF fusion protein, as determined by either radioactivity counting or by ELISA (Table 1). Fourth, there is no change in glycemic control following the intravenous administration of the HIR MAb-GDNF fusion protein (Table 2). Fifth, fusion of GDNF to the HIR MAb redirects distribution of the neurotrophin in peripheral tissues and causes a decrease in the uptake of GDNF by liver, spleen, and kidney (Table 4).

The equivalence of the brain clearance (Fig. 5) of GDNF and the hIgG1 plasma volume marker are evidence that GDNF does not cross the primate BBB. This observation parallels previous work in the mouse, which showed that GDNF does not cross the BBB (Kastin et al., 2003). Owing to lack of GDNF transport across the BBB, recombinant GDNF was administered to PD patients with a transcranial brain delivery method, CED (Lang et al., 2006). However, the penetration of GDNF into the brain parenchyma was minimal with CED and showed the properties of diffusion, not convection (Salvatore et

Fig. 2. Film autoradiography of rhesus monkey brain removed 2 h after an intravenous administration of ¹²⁵I-HIR MAb-GDNF fusion protein. Coronal sections through the cerebrum (top) and hindbrain/cerebellum (bottom) are shown. Intensity increases from red-yellow to blue-violet.

al., 2006). There is an exponential decrease in the concentration of GDNF in brain relative to the short distances from the intracerebral perfusion catheter. The concentration of GDNF at 0 to 2, 2 to 4, 4 to 6, and 6 to 8 mm from the intracerebral catheter is 2200, 600, 20, and <5 ng of GDNF per 100 mg of protein following CED from a reservoir that contains 100,000 ng/ml GDNF (Salvatore et al., 2006). Therefore, to expose parts of brain parenchyma more distal to the catheter to therapeutic concentrations of GDNF, it is necessary to expose more proximal areas of brain to very high concentrations of GDNF, which may be toxic. If successful delivery of therapeutic concentrations of GDNF to the human brain is to be achieved, it is necessary to re-engineer the GDNF to enable transvascular delivery to the brain. This is possible with the re-engineering of GDNF as a fusion protein with a BBB molecular Trojan horse (Pardridge, 2008).

Fusion of the GDNF to the HIR MAb enables rapid brain penetration of the neurotrophin, as shown by measurements of brain clearance (Fig. 5), film autoradiography (Fig. 2), emulsion autoradiography (Fig. 3), and the capillary depletion method (Table 5). The BBB expresses an insulin receptor (Pardridge et al., 1985), which enables the receptor-mediated transport of circulating insulin across the BBB

FIG. 3. Emulsion autoradiography of 20- μ m sections of rhesus monkey brain removed 2 h after an intravenous administration of 125 I-HIR MAb-GDNF fusion protein. A, the underlying brain tissue is not visible because the section is not counterstained. The underexposed part at the bottom of the image is the area of the slide without brain tissue; the exposed part at the top of the image overlays brain tissue. Magnification bar = 160 μ m. B, oil immersion view of brain after 6 weeks of exposure. Tissue is counterstained with Mayer's hematoxylin. The HIR MAb-GDNF fusion protein is broadly distributed in brain parenchyma. Magnification bar = 16 μ m.

FIG. 4. Plasma concentration of $[^3\text{H}]\text{hIgG1}$ and ^{125}I -GDNF after intravenous injection in the rhesus monkey. Data are expressed as percentage of ID per milliliter.

(Duffy and Pardridge, 1987). Likewise, binding of the murine HIR MAb to the endogenous BBB insulin receptor enables the transport of this antibody into the rhesus monkey brain (Pardridge et al., 1995). The uptake of the HIR MAb-GDNF fusion protein is higher in gray matter, compared with white matter, and this parallels previous observations on the brain uptake of the HIR MAb (Boado et al., 2007b).

FIG. 5. Brain clearance of HIR MAb-GDNF fusion protein, GDNF, and hIgG1. Data are mean $\pm$ S.D. Values are computed from the terminal brain uptake and the 120-min plasma AUC, as described under *Materials and Methods*.

TABLE 6

Organ clearance parameters for hIgG1 and GDNF

Clearance estimates are derived from the organ uptake (Table 4) and the 120-min plasma AUC (Table 3), as described under *Materials and Methods*.

Organ	Clearance		GDNF/hIgG1 Clearance Ratio
	hIgG1 $\mu\text{L}/\text{min}/\text{g}$	GDNF $\mu\text{L}/\text{min}/\text{g}$	
Heart	0.38	2.3	6
Liver	7.4	145	20
Spleen	1.8	80	44
Lung	0.83	5.3	6
Kidney	5.3	426	80
Skeletal muscle	0.087	0.74	8
Fat	0.083	1.2	14

The vascular density in gray matter is approximately 3-fold higher than the vascular density in white matter (Lierse and Horstmann, 1965). The emulsion autoradiography shows the fusion protein is not sequestered to the brain vasculature but is transcytosed through the BBB to enter brain parenchyma. Similar findings were previously observed for the BBB transport of insulin (Duffy and Pardridge, 1987). The autoradiography results are corroborated by the capillary depletion method, which shows transvascular transport of the HIR MAb-GDNF fusion protein across the BBB, as represented by the high VD in the postvascular supernatant relative to the capillary pellet (Table 5).

The HIR MAb-GDNF fusion protein is rapidly removed from plasma (Fig. 1B). However, the species in plasma is the intact fusion protein of the HIR MAb and the GDNF. This is shown by the equivalence of the concentration of the radioactive fusion protein in plasma and the concentration of the immunoreactive fusion protein in plasma (Table 1). Owing to the design of the ELISA (Fig. 1A), both parts of the fusion protein, the HIR MAb and the GDNF, must be intact to be detected in the sandwich ELISA. The plasma concentration of the HIR MAb-GDNF fusion protein is 1 to 2 $\mu\text{g}/\text{mL}$ (Fig. 1B), which is equivalent to a fusion protein concentration of 5 to 10 nM. However, there is no change in glycemic control. The plasma glucose is unchanged in the primate during the 2 h after intravenous injection of 0.2 mg/kg fusion protein (Table 2). Recent studies show that plasma glucose concentrations are not changed from preinjection

values following the intravenous administration of 10-mg/kg doses of the HIR MAb-GDNF fusion protein (Pardridge and Boado, 2009).

The brain uptake (%ID/100 g brain) of the HIR MAb-GDNF fusion protein is 17-fold higher than the uptake of GDNF in cortical gray matter (Table 4). Likewise, the brain clearance of the HIR MAb-GDNF fusion protein is much higher than the brain clearance for GDNF (Fig. 5). However, these brain uptake parameters for GDNF reflect sequestration in the brain plasma volume, as shown by the equivalence of the brain clearance for GDNF and the hIgG1 plasma volume marker (Fig. 5). The brain clearance value for hIgG1 is an apparent value that reflects radioactivity in brain entrapped in the cerebral plasma volume. The trans-BBB clearance of the hIgG1 is zero, and the clearance of hIgG1 should be subtracted from the clearance values for both GDNF and the HIR MAb-GDNF fusion protein to reflect transport across the BBB.

GDNF is rapidly removed from plasma, and the principal organs mediating GDNF clearance from blood are liver, spleen, and kidney (Table 6). Fusion of the GDNF to the HIR MAb causes a modest increase in the uptake of the neurotrophin by certain peripheral tissues, including heart, lung, muscle, and fat (Table 4). However, there is a reduction in GDNF uptake by liver, spleen, and kidney. The GDNF uptake by liver and spleen may be mediated by the cationic charge of the neurotrophin, similar to another cationic neurotrophin, brain-derived neurotrophic factor (Pardridge et al., 1994). The size of the GDNF monomer, 15 kDa, enables glomerular clearance of the neurotrophin in the kidney. In contrast, the size of the HIR MAb-GDNF fusion protein, 190 kDa, restricts renal clearance at the glomerulus. Fusion of GDNF to the HIR MAb causes a redirection of GDNF distribution away from kidney and liver and toward brain.

The present studies showing trans-BBB transport of the HIR MAb-GDNF fusion protein, in conjunction with previous work showing this fusion protein is biologically active in brain in vivo (Boado et al., 2008), suggest that GDNF pharmacologic effects in brain may be achieved following the peripheral administration of the HIR MAb-GDNF fusion protein. The endogenous GDNF concentration in human brain is 0.38 ng/g in the frontal cortex and 0.68 ng/g in the putamen (Michel et al., 2008). Therapeutic effects in brain in PD are possible with relatively modest increases in brain GDNF concentrations (Eslamboli et al., 2005). If the goal of therapeutic dosing of GDNF is a 10-fold increase in brain GDNF, then this would be equal to a putamen GDNF level of approximately 7 ng/g. Because GDNF is approximately 20% of the weight of the HIR MAb-GDNF fusion protein (Boado et al., 2008), the target brain concentration of the fusion protein is 35 ng/g brain. The brain uptake of the HIR MAb-GDNF fusion protein, at a dose of 0.2 mg/kg, is 0.55% ID/100 g brain (Table 4). Given a primate body weight of 5 kg and a fusion protein injection dose of 0.2 mg/kg, the brain concentration of the HIR MAb-GDNF fusion protein increases to 55 ng/g, which is equivalent to 11 ng/g GDNF. These dosing considerations for the primate are representative of the human. The size of the human brain is 10-fold greater than the size of the rhesus monkey brain, which would reduce by approximately 10-fold the brain uptake, when expressed as percentage ID per gram of brain. However, the body size, as well as the total fusion protein dose, would be 10-fold greater in the human compared with the rhesus monkey.

In summary, the present studies show that re-engineering GDNF as an IgG fusion protein can enable brain penetration of the neurotrophin following intravenous administration in parallel with a decrease in GDNF uptake by liver, spleen, and kidney. The brain uptake of the HIR MAb-GDNF fusion protein is sufficient to produce a pharmacologically significant increase in cerebral GDNF.

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