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Drug Targeting of Erythropoietin Across the Primate Blood-Brain Barrier with an IgG Molecular Trojan Horse[S]

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ABSTRACT

Erythropoietin (EPO) is a neurotrophic factor that could be developed as a new drug for brain disorders. However, EPO does not cross the blood-brain barrier (BBB). In the present study, human EPO was re-engineered by fusion to the carboxyl terminus of the heavy chain of a chimeric monoclonal antibody (MAb) to the human insulin receptor (HIR). The HIRMAb acts as a molecular Trojan horse to ferry the EPO into the brain via receptor-mediated transport on the endogenous BBB insulin receptor. The HIRMAb-EPO fusion protein was immunoreactive with antibodies to both human IgG and EPO. The HIRMAb-EPO fusion protein bound with high affinity to the extracellular domain of both the HIR (ED_{50} = 0.21 ± 0.05 nM) and the EPO receptor (ED_{50} = 0.30 ± 0.01 nM) and activated thymidine incorporation into human TF-1 cells with an ED_{50} of 0.1 nM. Differentially radiolabeled EPO and the HIRMAb-EPO fusion protein were injected intravenously into adult rhesus monkeys. Whereas EPO did not cross the primate BBB, the HIRMAb-EPO fusion protein was rapidly transported into brain, at levels that produce pharmacologic elevations in brain EPO at small systemic doses. The HIRMAb fusion protein selectively targeted the brain relative to peripheral organs. In conclusion, a novel IgG-EPO fusion protein has been engineered, expressed, and shown to be bifunctional with retention of high-affinity binding to both the insulin and EPO receptors. The IgG-EPO fusion protein represents a new class of EPO neurotherapeutics that has been specifically re-engineered to penetrate the human BBB.

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Erythropoietin (EPO) is a neurotrophic factor made in the brain, which activates EPO receptors (EPORs) in the brain (Sakanaka et al., 1998). EPO could potentially be developed as a new drug for the treatment of acute brain conditions, such as stroke or traumatic brain injury, or chronic neurodegeneration, such as Parkinson's disease (PD) (Sakanaka et al., 1998; Grasso et al., 2007; Xue et al., 2007). The brain drug development of EPO is limited by the lack of transport of large-molecule pharmaceuticals across the brain capillary endothelial wall, which forms the blood-brain barrier (BBB) in vivo. Similar to other large-molecule drugs, EPO does not cross the BBB in the absence of BBB disruption (Lieutaud et al., 2008). Neurotrophic factors such as EPO can be re-engineered to cross the BBB as fusion proteins with BBB molecular Trojan horses (Pardridge, 2008). A molecular Trojan horse is an endogenous peptide, or peptidomimetic monoclonal antibody (MAb), that crosses the BBB via receptor-mediated transport on an endogenous BBB receptor. The most potent BBB molecular Trojan horse is a MAb to the human insulin receptor (HIR) (Pardridge et al., 1995). Genetically engineered forms of the HIRMAb have been produced, and both chimeric and humanized HIRMAbs bind the human BBB insulin receptor in vitro and cross the rhesus monkey BBB in vivo (Boado et al., 2007a). HIRMAb-neurotrophin fusion proteins have been engineered for brain-derived neurotrophic factor (Boado et al., 2007b) and glial-derived neurotrophic factor (Boado et al., 2008). The HIRMAb-brain-derived neurotrophic factor fusion protein induces neuroprotection in human neural cells and crosses the BBB of the adult rhesus monkey in vivo (Boado et al., 2007b). The HIRMAb-glial-derived neurotrophic factor fusion protein activates the c-ret kinase in human neural cells, induces neuroprotection in

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ABBREVIATIONS: EPO, erythropoietin; EPOR, EPO receptor; BBB, blood-brain barrier; MAb, monoclonal antibody; PD, Parkinson's disease; HIR, human insulin receptor; ID, injected dose; HC, heavy chain; LC, light chain; TV, tandem vector; SFM, serum-free medium; CHO, Chinese hamster ovary; TCA, trichloroacetic acid; V_D, volume of distribution; PK, pharmacokinetics; PS, permeability-surface area; AUC, area under the plasma concentration curve; AUC_{ss}, steady-state AUC; AA, amino acid; ECD, extracellular domain; ELISA, enzyme-linked immunosorbent assay; DHFR, dihydrofolate reductase; PAGE, polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; CSF, cerebrospinal fluid; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography.

regional brain ischemia (Boado et al., 2008), and exhibits no toxicity, effects on blood glucose, or dose-related immune reactions in primate toxicology studies (Pardridge and Boado, 2009).

The present studies describe the re-engineering of human EPO as an IgG fusion protein to enable receptor-mediated transport of EPO across the human BBB. This work describes the genetic engineering, expression, and characterization of a HIRMAb-EPO fusion protein. Mature human EPO was fused to the carboxyl terminus of the heavy chain (HC) of the chimeric HIRMAb, as depicted in Supplemental Fig. 1. The HIRMAb-EPO fusion protein was expressed in COS cells transiently and stably transfected Chinese hamster ovary (CHO) cells and affinity-purified from serum-free medium (SFM). The bifunctional properties of the HIRMAb-EPO fusion protein were examined with respect to dual binding to both the HIR and the human EPOR. The biological activity of the HIRMAb-EPO fusion protein was confirmed with a bioassay using human TF-1 cells. The differential transport of recombinant EPO and the HIRMAb-EPO fusion protein across the BBB in vivo was examined in the rhesus monkey.

Materials and Methods

Engineering of HIRMAb-EPO Fusion Protein Tandem Expression Vector. The human EPO cDNA encoding for amino acids Ala^{28}–Arg^{193} (GenBank accession number NP_000790), and excluding the 27-amino acid (AA) signal peptide, was synthesized at Retrogen (San Diego, CA). The EPO artificial gene has a StuI site on the 5′ end followed by CA to maintain the open reading frame and introduce a Ser-Ser-Ser linker between the CH3 region of the HIRMAb HC and the amino terminus of the EPO minus the signal peptide. The 3′ end of the EPO cDNA was engineered with a StuI site immediately after the stop codon, TGA. An internal StuI site in the EPO cDNA was removed by use of an alternative codon for Glu^{186} in the design of the synthetic EPO gene. The EPO cDNA was obtained from Retrogen subcloned into the pCR-Blunt vector (Invitrogen, Carlsbad, CA). The 515-nucleotide EPO cDNA sequence was confirmed by bidirectional DNA sequencing.

The EPO cDNA was released from the pCR vector with StuI, and the approximately 500-base pair EPO cDNA fragment was isolated by agarose gel electrophoresis using the QIAGEN gel extraction kit (Valencia, CA). The EPO cDNA was inserted into the pHIRMAb eukaryotic tandem expression plasmid, which has been described previously (Boado et al., 2007b), at the HpaI site, and this expression plasmid was designated pHIRMAb-EPO. The pHIRMAb tandem vector (TV) encodes the light chain (LC) of the chimeric HIRMAb, the HC of the chimeric HIRMAb, dihydrofolate reductase (DHFR), and the neomycin resistance gene. The latter genes allowed for selection of high-producing host cell lines with methotrexate and selection with G418. The entire open reading frames for the LC, HC, and DHFR expression cassettes of the pHIRMAb-EPO plasmid were confirmed by bidirectional DNA sequencing performed at Eurofins MWG Operon (Huntsville, AL) using custom sequencing oligodeoxynucleotides synthesized at Midland Certified Reagent Co. (Midland, TX).

Transient Expression of HIRMAb-EPO Fusion Protein in COS Cells. COS cells were transfected with the pHIRMAb-EPO TV by using Lipofectamine 2000, with a ratio of 1:2.5 μg DNA/μL Lipofectamine. After transfection, the cells were cultured in VP-SFM (Invitrogen). The conditioned SFM was collected at 3 and 7 days. Transgene expression and fusion protein secretion to the medium was assayed by measurement of human IgG in the conditioned medium. Human IgG enzyme-linked immunosorbent assay (ELISA) was performed in Immulon 2 high binding plates (Dynex Technologies, Chantilly, VA) with COS cell conditioned medium as described previously (Boado et al., 2007b).

Production of Stably Transfected CHO Line. The TV was linearized, and DG44 CHO cells were electroporated, followed by selection in hypoxanthine-thymine-deficient medium and amplification with graded increases in methotrexate up to 80 nM in SFM. The CHO line underwent two successive rounds of one cell/well dilutional cloning, and positive clones were selected by measurement of medium human IgG concentrations by ELISA. The CHO line was stable through multiple generations and produced medium IgG levels of 10 to 30 mg/liter in shake flasks at a cell density of 1 million to 2 million cells/ml. The CHO cells were propagated in 1-liter bottles until 2.4 liters of conditioned SFM was collected. The medium was ultra-filtered with a 0.2-μm Sartopore-2 sterile-filter unit (Sartorius Stedim Biotech, Goettingen, Germany) before protein A chromatography.

Protein A Chromatography. SFM conditioned by either transiently transfected COS cells or stably transfected CHO cells was filtered and applied to a 25-ml protein A-Sepharose 4 Fast Flow (GE Life Sciences, Chicago, IL) column equilibrated in 25 mM Tris, 25 mM NaCl, 5 mM EDTA, pH 7.1. After application of the sample, the column was washed with 25 mM Tris, 1 M NaCl, 5 mM EDTA, pH 7.1, and the fusion protein was eluted with 0.1 M sodium acetate, pH 3.7. The acid eluate was pooled, Tris was added to 0.05 M, NaCl was added to 0.15 M, the pH was increased to 6.0, and the solution was stored sterile-filtered at 4°C.

SDS-PAGE and Western Blotting. The homogeneity of protein A-purified fusion protein was evaluated with a reducing 12% SDS-polyacrylamide gel electrophoresis (PAGE), followed by Coomasie Blue staining. For Western blotting, immunoreactivity was tested with a primary rabbit antibody to human EPO (R&D Systems, Minneapolis, MN) or a primary goat antibody against human IgG HCs and LCs (Vector Laboratories, Burlingame, CA). Human recombinant EPO was purchased from R&D Systems.

Size Exclusion Chromatography. Size exclusion chromatography (SEC) high-performance liquid chromatography (HPLC) of the protein A-purified HIRMAb-EPO fusion protein was performed with two 7.8-mm × 30-cm TSK-GEL $G3000SW_{XL}$ columns (Tosoh Bioscience, Tokyo, Japan) in series, under isocratic conditions at a flow rate of 0.5 ml/min with a PerkinElmer Series 200 pump (PerkinElmer Life and Analytical Sciences, Waltham, MA). The absorbance at 280 nm was detected with a Shimadzu (Kyoto, Japan) SPD-10A UV-visible detector and a Shimadzu CR-8 chart recorder. The elution of molecular weight standards (GE Healthcare, Little Chalfont, Buckinghamshire, UK), blue dextran-2000, aldolase, and ovalbumin was measured under the same elution conditions.

HIR Receptor Assay. The affinity of the fusion protein for the HIR extracellular domain (ECD) was determined with an ELISA using the lectin affinity-purified HIR ECD. CHO cells permanently transfected with the HIR ECD were grown in SFM, and the HIR ECD was purified with a wheat germ agglutinin affinity column as described previously (Coloma et al., 2000). The HIR ECD (0.2 μg/well) was plated on Immulon 2 high binding 96-well plates, and the binding of the chimeric HIRMAb or the HIRMAb-EPO fusion protein was detected with a biotinylated goat anti-human IgG (H+L) antibody (0.3 μg/well) and the ABC Elite detection system (Vector Laboratories). The concentration that caused 50% binding to the HIR ECD, the ED_{50}, was determined by nonlinear regression analysis.

EPO Receptor Assay. Binding of the HIRMAb-EPO fusion protein to recombinant human EPOR was evaluated by using a fusion protein of human IgG Fc and the ECD of recombinant human EPOR, which was obtained from R&D Systems, and plated in 96-well plates overnight at 0.2 μg/well. Wells were blocked with Tris-buffered saline and 1% bovine serum albumin. Various concentrations of either the HIRMAb-EPO fusion protein or human IgG1 were plated for 2 h at room temperature. After aspiration, the wells were washed with Tris-buffered saline/0.05% Tween 20, a conjugate of alkaline phosphatase and a goat anti-human κ LC antibody (Sigma-Aldrich, St. Louis, MO) was plated, and detection at 405 nm was performed with

an ELISA plate reader after color development with *para*-nitrophenylphosphate (Sigma-Aldrich). The concentration that caused 50% binding to the EPOR ECD, the ED_{50}, was determined by nonlinear regression analysis.

TF-1 Bioassay. Human TF-1 cells obtained from the American Type Culture Collection (Manassas, VA) were cultured in RPMI medium 1640 with 10% fetal bovine serum and 2 ng/ml human recombinant granulocyte-macrophage colony stimulating factor (PeproTech, Rocky Hill, NJ) (Kitamura et al., 1989). Cells were plated in 96-well plates at 400,000 cells/well and cultured overnight in medium containing no granulocyte-macrophage colony stimulating factor. The next day, the HIRMAb-EPO fusion protein was added followed by incubation for 44 h. The medium was then supplemented with [^{3}H]thymidine (PerkinElmer Life and Analytical Sciences) at a final concentration of 0.5 μCi/well. The wells were incubated at 37°C for 4 h, and intracellular radioactivity was determined after washing the cells in a Cell Harvester (Millipore Corporation, Billerica, MA) over glass fiber/C filters under vacuum. The filter was washed three times with cold 10% trichloroacetic acid (TCA), and the cell lysate was solubilized in 1 N NaOH. Radioactivity was determined with a PerkinElmer Life and Analytical Sciences liquid scintillation spectrometer, and cell protein was determined with the bicinchoninic acid protein assay (Thermo Fisher Scientific, Waltham, MA). The cell radioactivity was divided by the thymidine specific activity (6.7 μCi/nmol), and thymidine incorporation was expressed as fmol/mg of protein.

Radiolabeling of Proteins. ^{125}I-Bolton-Hunter reagent was purchased from American Radiolabeled Chemicals (St. Louis, MO). Human recombinant EPO was purchased from R&D Systems and shown to be homogenous by SDS-PAGE. The EPO (6 μg) was radiolabeled with 2 mCi of fresh Bolton-Hunter reagent to a specific activity of 67 μCi/μg and a TCA precipitability of >99% after purification with a 1.0 × 28-cm column of Sephadex G-25 and elution with 0.01 M NaH_2PO_4/0.15 M NaCl (pH 7.4)/0.05% Tween 20. The TCA precipitation of the labeled EPO remained >99% at 24 h after iodination, and the EPO was administered to the primate within 24 h of radiolabeling. [^{3}H]*N*-succinimidyl propionate was purchased from American Radiolabeled Chemicals. The HIRMAb-EPO fusion protein was radiolabeled with fresh [^{3}H]*N*-succinimidyl propionate to a specific activity of 2.9 μCi/μg and a TCA precipitability of 96% after purification with a 1.0 × 28-cm column of Sephadex G-25 and elution with 0.02 M 4-morpholineethanesulfonic acid, 0.15 M NaCl (pH 6.0), 0.05% Tween 20. The solution was buffer-exchanged with 0.02 M 4-morpholineethanesulfonic acid, 0.15 M NaCl (pH 6.0), 0.05% Tween 20, 0.1% bovine serum albumin and an Ultra-15 microconcentrator (Millipore Corporation), which increased the TCA precipitability to 99%. The ^{3}H-labeled HIRMAb-EPO fusion protein was labeled in advance of the primate study and stored at −70°C.

EPO Radio Receptor Assay. The retention of high-affinity EPOR binding by the [^{125}I]EPO after radiolabeling with the Bolton-Hunter reagent was examined with a radio receptor assay. A mouse anti-human IgG1 Fc antibody (Invitrogen/Zymed Laboratories, South San Francisco) was plated overnight to capture a Fc fusion protein of the human EPOR ECD (R&D Systems). The wells were washed with phosphate-buffered saline (PBS), followed by the addition of 100 μl/well of a comixture of [^{125}I]EPO at a concentration of 0.01 μCi/well (0.15 ng/well) and various concentrations of unlabeled human EPO (R&D Systems), followed by a 3-h incubation at room temperature. The wells were emptied by aspiration and washed with cold PBS, and 250 μl/well of 1 N NaOH was added, followed by heating at 60°C for 30 min. Radioactivity was counted in Ultima Gold (PerkinElmer Life and Analytical Sciences) in a PerkinElmer Tricarb 2100TR liquid scintillation counter, and the fractional binding per well was computed. The half-saturation constant, K_D, of EPO binding to the EPOR was determined by nonlinear regression analysis.

Primate Brain Uptake and Capillary Depletion Analysis. An adult female rhesus monkey (5.6 kg) was obtained from Covance (Alice, TX). The animal was injected intravenously with 2132 μCi of [^{3}H]HIRMAb-EPO fusion protein and 330 μCi of [^{125}I]EPO in 3.0 ml by bolus injection over 30 s in the left femoral vein. The injection dose (ID) of the HIRMAb-EPO fusion protein was 130 μg/kg, and the ID for EPO was 0.9 μg/kg. The animal was initially anesthetized with intramuscular ketamine, and anesthesia was maintained by 1% isoflurane by inhalation. All procedures were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the National Institutes of Health. After intravenous drug administration, femoral venous plasma was obtained at 1, 2.5, 5, 15, 30, 60, and 120 min for determination of ^{3}H and ^{125}I radioactivity. The animal was euthanized at 120 min after drug injection, and samples of major organs (heart, liver, spleen, lung, skeletal muscle, kidney, 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 radioactivity determination.

Samples (approximately 2 g) of frontal cortex were removed for capillary depletion analysis as described previously (Triguero et al., 1990). The brain was homogenized in 8 ml of cold PBS in a tissue grinder. The homogenate was supplemented with 9.4 ml of cold 40% dextran (70 kDa; Sigma-Aldrich), and an aliquot of the homogenate was taken for radioactivity measurement. The homogenate was centrifuged at 3200*g* at 4°C for 10 min in a fixed angle rotor. The brain microvasculature quantitatively sediments as the pellet (Triguero et al., 1990), and the postvascular supernatant is a measure of capillary-depleted brain parenchyma. The vascular pellet and supernatant were counted for ^{3}H and ^{125}I radioactivity in parallel with the homogenate. The volume of distribution (V_D) was determined for each of the three fractions from the ratio of total ^{125}I or ^{3}H radioactivity in the fraction divided by the total ^{125}I or ^{3}H 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 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 by using chloroform as the quench agent to compute the efficiency of counting of ^{125}I in the ^{3}H window as described previously (Boado and Pardridge, 2009). All samples for ^{3}H counting were solubilized in Soluene-350 and counted in the liquid scintillation counter in Opti-Fluor O (PerkinElmer Life and Analytical Sciences).

Pharmacokinetics and Organ PS Product. The ^{3}H or ^{125}I radioactivity in plasma, disintegrations per minute per milliliter, was converted to %ID/ml, and the %ID/ml was fit to a monoexponential or biexponential equation. The intercepts (A1, A2) and the slopes (k1, k2) were used to compute the median residence time, the central volume of distribution (*V*c), the steady-state volume of distribution (V_{ss}), the area under the plasma concentration curve (AUC) at 120 min, the steady-state AUC (AUC_{ss}), and the systemic clearance as described previously (Pardridge et al., 1995). Nonlinear regression analysis used the AR subroutine of BMDP Statistical Software (Statistical Solutions Ltd, Cork, Ireland). Data were weighted by $1/(\%ID/ml)^2$.

The organ clearance (μl/min/g), also called the permeability-surface area (PS) product, was computed from the terminal organ uptake (%ID/g) and the 120-min plasma AUC (%IDmin/ml) as follows: organ PS product = [(%ID/g)/AUC] × 1000.

Gel Filtration Chromatography of Primate Plasma. Primate plasma removed at 120 min after intravenous injection of the [^{3}H]HIRMAb-EPO fusion protein and [^{125}I]EPO was analyzed with gel filtration chromatography using a 1 × 30 cm Superose 6HR column (GE Life Sciences) and a PerkinElmer Series 200 pump. The absorbance at 280 nm was detected with a Shimadzu SPD-10A UV-VIS detector and a Shimadzu CR-8 chart recorder. The elution volume of blue dextran-2000 was used as a measure of the column

void volume. The injection sample was comprised of 50 μl of plasma diluted to 200 μl with PBS containing 0.05% Tween 20. The column was eluted at 0.25 ml/min in PBS containing 0.05% Tween 20. Fractions were counted for ^{3}H and ^{125}I as described above.

Results

DNA sequencing of the pHIRMAb-EPO plasmid encompassed 9036 nucleotides, which spanned the expression cassettes for the HC fusion gene, the LC gene, and the DHFR gene. The fusion HC expression cassette included a 1986-nucleotide open reading frame, which encoded for a 631-AA protein, comprised of a 19-AA IgG signal peptide, the 443-AA HIRMAb HC, a 3-AA linker (Ser-Ser-Ser), and the 166-AA human EPO minus the signal peptide, which was 100% identical to the AA sequence from Ala^{28} to Arg^{193} of human EPO (GenBank accession number NP_000790). The predicted molecular mass of the HC fusion protein, minus glycosylation, is 67,226 Da, with a predicted isoelectric point of 8.75. The LC was comprised of 234 AA, which included a 20-AA signal peptide, a 108-AA variable region of the LC of the HIRMAb LC, and a 106-AA human κ LC constant (C)-region. The predicted molecular mass of the LC is 23,398 Da with a predicted isoelectric point of 5.45.

Lipofection of COS cells with the pHIRMAb-EPO TV resulted in high medium human IgG levels (Table 1), as determined with a human Fc-specific ELISA. The HIRMAb-EPO fusion protein was purified by protein A affinity chromatography. After SDS-PAGE and Coomasie Blue staining, the size of the LC was the same for both the HIRMAb and the HIRMAb-EPO fusion protein (Supplemental Fig. 2). The size of the HC of the fusion protein was approximately 35 kDa larger than the HC of the HIRMAb (Supplemental Fig. 2). During Western blotting, the LC of either the HIRMAb or the HIRMAb-EPO fusion protein reacted equally with a primary antibody directed against the human IgG (H+L), as shown in Supplemental Fig. 3A. The size of the HC of the fusion protein was approximately 35 kDa larger than the size of the HC of the HIRMAb on Western blots using either the anti-human IgG primary antibody (Supplemental Fig. 3A) or the anti-human EPO primary antibody (Supplemental Fig. 3B). The anti-EPO primary antibody reacts with the HC of the fusion protein and recombinant EPO, but it does not react with the HIRMAb (Supplemental Fig. 3B). The HIRMAb-EPO fusion protein eluted as a single peak, with <1% aggregation on SEC HPLC (Supplemental Fig. 4).

The affinity of the fusion protein for the HIR ECD was determined with a ligand binding assay using lectin affinity-purified HIR ECD (see *Materials and Methods*). There is comparable binding of either the chimeric HIRMAb or the HIRMAb-EPO fusion protein to the HIR ECD with ED_{50} of 0.20 ± 0.03 and 0.21 ± 0.05 nM, respectively (Fig. 1).

TABLE 1
Secretion of immunoreactive human HIRMAb-EPO fusion protein to medium of transfected COS cells

Day	Medium IgG	
	Lipofectamine 2000 Only	pHIRMAb-EPO TV
	ng/ml	
3	<3	1825 ± 80
7	<3	6424 ± 596

Mean ± S.E. (n = 3 dishes).

The affinity of the HIRMAb-EPO fusion protein for the ECD of the human EPOR was measured with an ELISA (see *Materials and Methods*). There was no binding of human IgG1κ to the EPOR, whereas saturable binding of the HIRMAb-EPO fusion protein was observed (Fig. 2). The affinity of the HIRMAb- EPO fusion protein for the EPOR was high, with an ED_{50} of 0.30 ± 0.01 nM (Fig. 2). The biologic activity of the HIRMAb-EPO fusion protein was evaluated with a bioassay in human TF-1 cells. Thymidine incorporation into TF-1 cells was increased via a saturable mechanism by the HIRMAb-EPO fusion protein, with an ED_{50} of 0.1 nM (Fig. 3).

CHO cells stably transfected with the TV encoding the HIRMAb-EPO fusion protein were subjected to limited dilutional cloning, a high-producing CHO line was isolated, and the fusion protein was purified by protein A affinity chromatography. The results of the SDS-PAGE, Western blotting, SEC HPLC, and HIR and EPOR binding assays were identical to the results obtained for the COS-derived fusion pro-

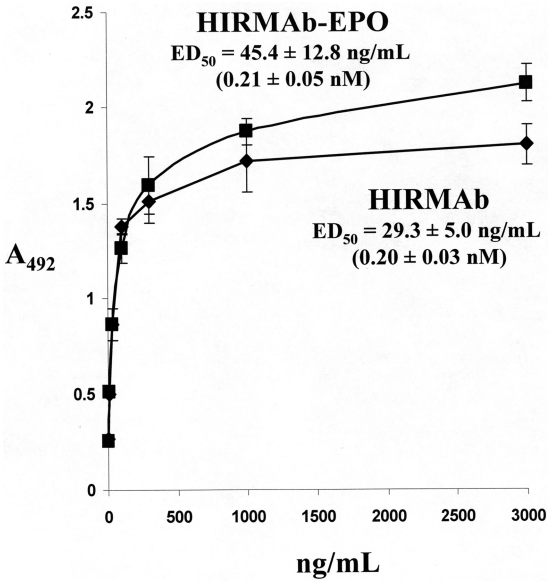

Fig. 1. Binding of either the chimeric HIRMAb or the HIRMAb-EPO fusion protein to the HIR ECD is saturable. The ED_{50} of HIRMAb-EPO binding to the HIR ECD is comparable with the ED_{50} of the binding of the chimeric HIRMAb. Data are mean ± S.E. (n = 3 dishes/point).

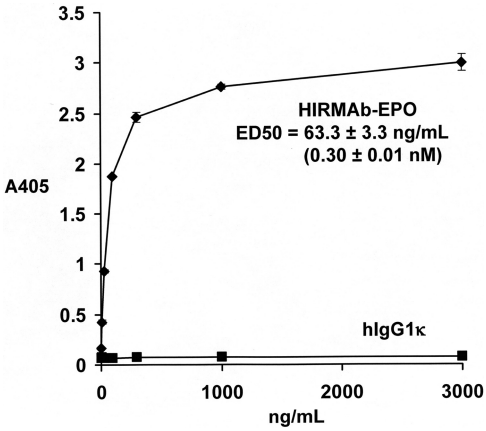

Fig. 2. Binding of either human IgG1κ or the HIRMAb-EPO fusion protein to the EPOR ECD is detected by ELISA. There is no binding to the EPOR by the human IgG1κ. Data are mean ± S.E. (n = 3 dishes/point).

tein. The CHO-derived HIRMAb-EPO fusion protein was used for the primate brain uptake study.

The HIRMAb-EPO fusion protein was radiolabeled with ^{3}H, and the recombinant human EPO was radiolabeled with ^{125}I. The retention of biological activity of the EPO after radiolabeling with the ^{125}I-Bolton-Hunter reagent was confirmed with a radio receptor assay. The design of the radio receptor assay is shown in Fig. 4A. Binding of the [^{125}I]EPO to the EPOR is displaced by unlabeled EPO with a K_D of 0.17 ± 0.09 nM (Fig. 4B). This assay shows the high-affinity binding of EPO to the EPOR is retained after radiolabeling with the Bolton-Hunter reagent.

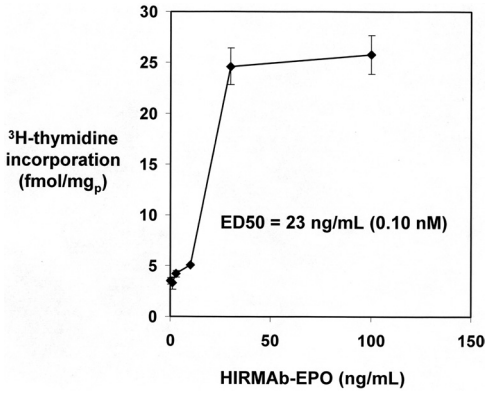

Fig. 3. EPO bioassay in human TF-1 cells shows a saturable increase in thymidine incorporation into the cells in response to graded increases in medium concentration of HIRMAb-EPO fusion protein. Data are mean ± S.E. (n = 4 dishes/point).

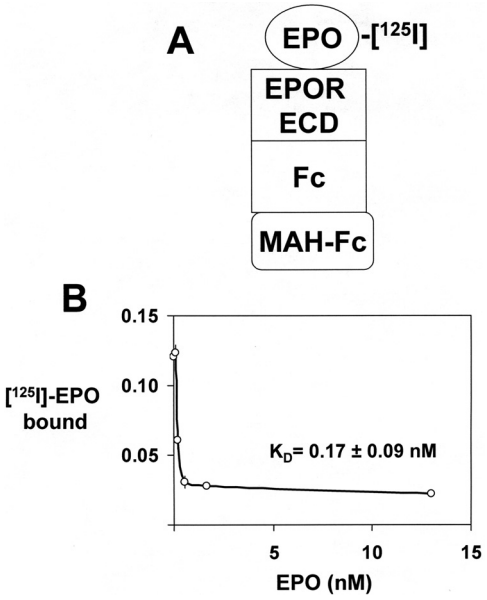

Fig. 4. A, outline of radio receptor assay for measurement of the binding of Bolton-Hunter reagent-labeled [^{125}I]EPO to the EPOR. A mouse anti-human (MAH) IgG1 Fc was plated, which bound the Fc region of a Fc fusion of the EPOR ECD. The EPOR binds to the [^{125}I]EPO, which is displaced by the addition of unlabeled EPO. B, the saturable binding was analyzed by a nonlinear regression analysis to yield the concentration, K_D, which produced 50% inhibition of [^{125}I]EPO binding to the EPOR.

The [^{3}H]HIRMAb-EPO fusion protein and the [^{125}I]EPO were coinjected intravenously into an adult rhesus monkey. The clearance of the plasma radioactivity is shown in Fig. 5A for the [^{125}I]EPO and the [^{3}H]HIRMAb-EPO fusion protein, and these data show that the HIRMAb-EPO fusion protein was removed from plasma much faster than was EPO. The plasma radioactivity that was precipitable with TCA was >99% for [^{125}I]EPO at all time points (Fig. 5B). The plasma radioactivity that was TCA-precipitable for the [^{3}H]HIRMAb-EPO fusion protein was >99% through 30 min, 97% at 60 min, and 93% at 120 min after administration (Fig. 5B). The plasma clearance profiles (Fig. 5A) were fit to a biexponential function for the HIRMAb-EPO fusion protein and a monoexponential function for EPO for estimation of the pharmacokinetics (PK) parameters, which are shown in Table 2 for each protein. The estimated plasma AUC_{ss} for EPO is 13-fold higher than the plasma AUC_{ss} for the HIRMAb-EPO fusion protein (Table 2). The uptake of the proteins by brain and peripheral organs was measured as a %ID/100 g tissue, and these values are given in Table 3. The brain V_D of the proteins was measured with the capillary depletion method, and the V_D values for the homogenate, vascular pellet, and postvascular supernatant are shown in Table 4. The radioactivity in the postvascular supernatant represents intact fusion protein as the TCA precipitability of the postvascular supernatant was 91 ± 1% (Table 4).

The BBB PS products for the HIRMAb-EPO fusion protein and recombinant EPO were computed from the 2-h plasma AUC (Table 2) and the brain uptake (Table 3), and the PS products are given in Fig. 6C. Figure 6 also displays the 2-h plasma AUC, the %ID/100g, and the BBB PS product for EPO and the HIRMAb-EPO fusion protein compared with the same parameters for a vascular space marker, human IgG1. The brain uptake (%ID/100g) and the BBB PS product for EPO were not significantly different from the same values for the IgG1 brain plasma volume marker. The PS products for peripheral organs were similarly computed for the HIRMAb-EPO fusion protein and recombinant EPO, and these data are given in Table 5. The ratio of the PS product for the HIRMAb-EPO fusion protein, relative to the PS product for the recombinant EPO, in each organ is plotted in Fig. 7.

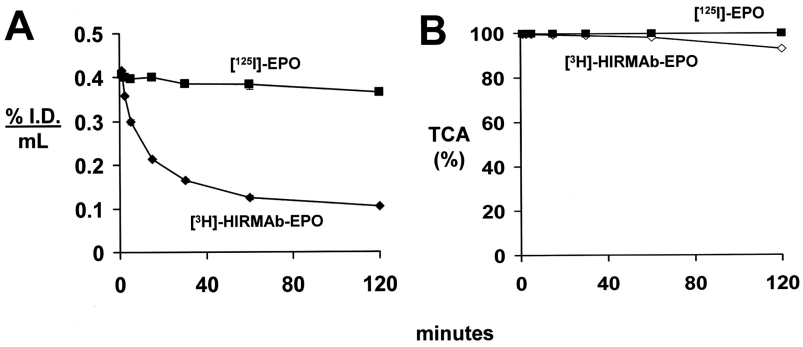

Fig. 5. A, the plasma concentration of [^{125}I]EPO and [^{3}H]HIRMAb-EPO fusion protein is plotted versus the time after a single intravenous injection of the proteins in the adult rhesus monkey. Data are expressed as percentage of ID/ml. B, the percentage of plasma radioactivity that is precipitable by 10% TCA is plotted versus the time after injection for both proteins. Data are mean ± S.E. of three replicates from a single primate.

TABLE 2
Pharmacokinetic parameters estimated from the plasma clearance data in Fig. 5A

Parameter	Unit	[^{125}I]EPO	[^{3}H]HIRMAb-EPO
A1	%ID/ml	0.400 ± 0.002	0.251 ± 0.021
A2	%ID/ml	–	0.174 ± 0.015
k1	min^{-1}	0.00083 ± 0.00010	0.110 ± 0.025
k2	min^{-1}	–	0.0049 ± 0.0010
Median residence time	min	1208 ± 151	191 ± 37
V_c	ml/kg	44 ± 1	42 ± 2
V_{ss}	ml/kg	–	91 ± 7
AUC at 120 min	%IDmin/ml	46.0 ± 0.2	18.0 ± 0.4
AUC_{ss}	%IDmin/ml	486 ± 59	37.5 ± 4.6
Clearance	ml/min/kg	0.037 ± 0.004	0.44 ± 0.05

TABLE 3
Organ uptake of [^{125}I]EPO and [^{3}H]HIRMAb-EPO in the rhesus monkey
Data are %ID/100 g; mean ± S.E. of three replicates from a single primate.

Organ	[^{125}I]EPO	[^{3}H]HIRMAb-EPO
Frontal gray matter	0.34 ± 0.02	2.1 ± 0.1
Frontal white matter	0.17 ± 0.02	1.6 ± 0.1
Cerebellar gray matter	0.34 ± 0.01	1.6 ± 0.1
Cerebellar white matter	0.21 ± 0.02	1.2 ± 0.1
Heart	2.2 ± 0.19	1.4 ± 0.2
Liver	6.9 ± 0.3	17.8 ± 6.3
Spleen	6.0 ± 0.1	15.9 ± 4.6
Lung	9.5 ± 0.7	4.5 ± 0.6
Skeletal muscle	0.39 ± 0.01	0.17 ± 0.03
Fat	0.41 ± 0.05	0.26 ± 0.01
Kidney	11.1 ± 0.2	5.2 ± 0.4

TABLE 4
Capillary depletion analysis of HIRMAb-EPO and EPO distribution in brain
Mean ± S.E. of three replicates from a single primate. n.m., not measured.

Parameter	EPO	HIRMAb-EPO
Homogenate V_D	7.7 ± 0.7	260 ± 11
Postvascular supernatant V_D	6.3 ± 0.2	156 ± 8
Brain capillary pellet V_D	0.22 ± 0.03	32 ± 9
TCA precipitation (%)	n.m.	91 ± 1

The metabolic stability of the [^{3}H]HIRMAb-EPO fusion protein and [^{125}I]EPO in the primate plasma at 120 min after intravenous injection was verified by gel filtration chromatography. The elution of the [^{3}H]HIRMAb-EPO fusion protein and [^{125}I]EPO is shown in Fig. 8, A and B, respectively. The elution volumes of blue dextran-2000 (void volume) and IgG are indicated by the arrows in Fig. 8A. The peak elution of the [^{3}H]HIRMAb-EPO fusion protein and the [^{125}I]EPO was fraction 25 and 29, respectively (Fig. 8), which correlated with the elution peaks of the [^{3}H]HIRMAb-EPO fusion protein and [^{125}I]EPO standards.

Discussion

An IgG-EPO fusion protein could be engineered by fusion of the EPO to either the amino terminus or the carboxyl terminus of either the HC or LC of the IgG, such as the HIRMAb. However, only one of these conformations constrains the EPO in a dimeric configuration, and that is the fusion of the EPO to the carboxyl terminus of the HC, as shown in Supplemental Fig. 1. EPO dimers may be more active than EPO monomers (Sytkowski et al., 1999). EPO

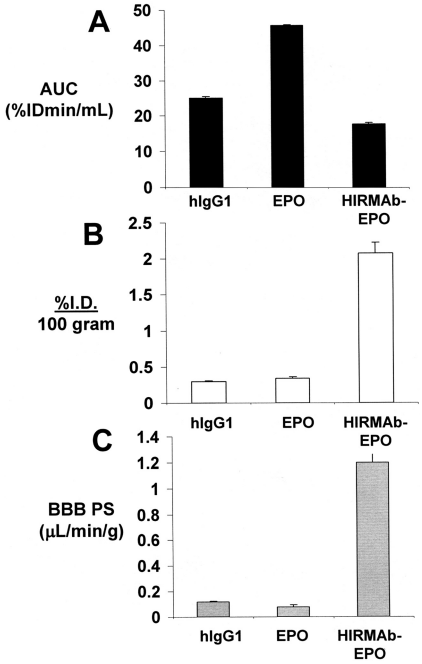

Fig. 6. The AUC (A), the brain uptake or percentage of ID per 100 g brain (B), and the BBB PS product (C) are plotted for EPO, the HIRMAb-EPO fusion protein, and a brain plasma volume marker, human IgG1 (hIgG1). The IgG1 data are from Boado and Pardridge (2009). All measurements were made at 2 h after intravenous administration of the protein in the rhesus monkey. Data are mean ± S.E. of three replicates from a single primate.

TABLE 5
Organ PS products for EPO and HIRMAb-EPO fusion protein
Data are mean ± S.E. of three replicates from a single primate.

Organ	PS Product	
	EPO	HIRMAb-EPO
	µL/min/g	
Heart	0.49 ± 0.02	0.78 ± 0.11
Liver	1.5 ± 0.1	9.9 ± 3.5
Spleen	1.3 ± 0.1	8.9 ± 2.5
Lung	2.1 ± 0.1	2.5 ± 0.3
Skeletal muscle	0.086 ± 0.002	0.094 ± 0.019
Fat	0.089 ± 0.010	0.14 ± 0.01
Kidney	2.4 ± 0.1	2.9 ± 0.2

binds as a monomer to a dimer of EPORs (Syed et al., 1998). The findings in this study show that fusion of the EPO to the carboxyl terminus of the IgG HC allows for the retention of EPO biological activity, and the HIRMAb-EPO fusion protein binds the EPOR and activates EPO biological activity in the low nanomolar range (Figs. 2 and 3). The ED_{50} of HIRMAb-EPO fusion protein binding to the human EPOR (0.30 ± 0.01 nM) is comparable with the K_D of EPO binding to the EPOR (Elliott et al., 2008). High-affinity binding of the EPO moiety of the HIRMAb-EPO fusion protein is consistent with observations that the amino-terminal portion of EPO is not involved in binding to the EPOR (Syed et al., 1998). With respect to HIRMAb-EPO fusion protein binding to the HIR, fusion of EPO to the carboxyl terminus of the IgG HC leaves free the amino-terminal portions of the IgG chains, which bind to the HIR (Supplemental Fig. 1). The present studies show the HIRMAb-EPO fusion protein binds to the HIR with an affinity equal to the original HIRMAb (Fig. 1).

The EPOR and EPO both are expressed in brain (Saka-

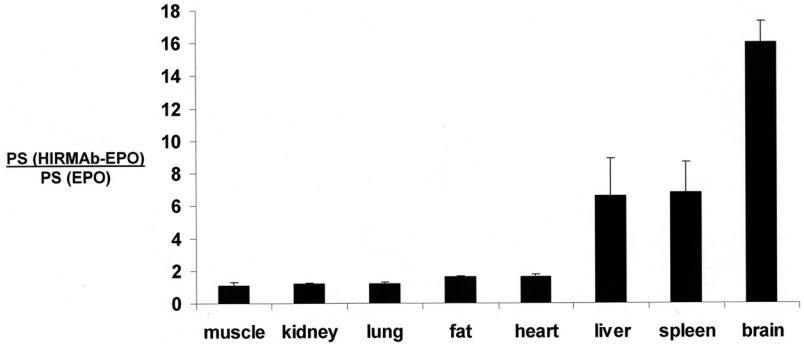

Fig. 7. Ratio of the organ PS product for the HIRMAb-EPO fusion protein, relative to the organ PS product for EPO, is plotted for each organ. Data are mean ± S.E. of three replicates from a single primate.

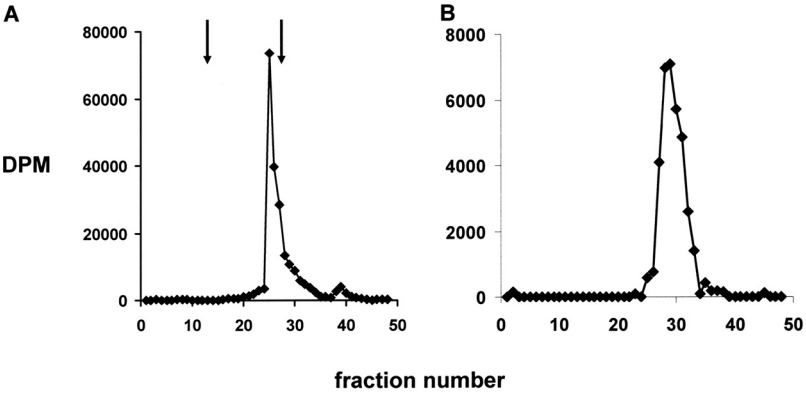

Fig. 8. Elution profile of [^{3}H]HIRMAb-EPO fusion protein (A) and [^{125}I]EPO (B) in primate plasma removed at 120 min after intravenous injection and separated with a Superose 6HR gel filtration column. In A, the left and right arrows represent the elution volume of blue dextran-2000 and IgG, respectively.

naka et al., 1998). EPO has the same characteristics as other neurotrophic factors, because EPO is neuroprotective in neural cells exposed to cytokines, such as tumor necrosis factor-α (Pregi et al., 2009), or toxins such as the Aβ amyloid peptide (Ma et al., 2009). The EPOR that mediates neuroprotection in brain is the same classical EPOR expressed in peripheral tissues (Um et al., 2007). EPO is neuroprotective in transient forebrain ischemia, after the direct intracerebral injection of the peptide (Sakanaka et al., 1998), and peripheral EPO is neuroprotective only when the BBB is disrupted (Catania et al., 2002). The importance of the BBB in EPO action within the brain was also demonstrated in a model of experimental PD. Bypassing the BBB with the intracerebral injection of EPO in rats with experimental PD caused neuroprotection of the nigra-striatal tract in vivo (Xue et al., 2007). However, the peripheral administration of EPO in experimental PD was not neuroprotective (Xue et al., 2007), owing to the lack of EPO transport across the BBB. The lack of EPO transport across the nondisrupted BBB has been confirmed with measurements of immunoreactive EPO in rat brain (Lieutaud et al., 2008). No increase in brain EPO is measureable after the intravenous administration of large doses (5000 U/kg) of EPO when the BBB is not disrupted (Lieutaud et al., 2008). Although the peripheral administration of large doses of EPO does not elevate EPO in brain tissue, there is an increase in the EPO concentration in cerebrospinal fluid (CSF) (Ehrenreich et al., 2002). However, drug penetration into the CSF is an index of blood-CSF barrier permeability, not BBB permeability. CSF is a filtrate of plasma, and all proteins in plasma distribute into CSF, inversely related to the molecular size of the protein (Reiber and Felgenhauer, 1987). Therefore, the detection of a peptide in CSF is not a measure of BBB transport of the peptide.

The present study examined [^{125}I]EPO transport across the BBB in the adult rhesus monkey. The BBB transport, as reflected by the BBB PS product, of [^{125}I]EPO is no different from the same parameters for a brain blood volume marker, human IgG1 (Fig. 6C), which indicates that EPO does not cross the BBB. For peripheral EPO to penetrate the brain across the BBB, it would be necessary for blood-borne EPO to access an EPOR-mediated transport system on the luminal membrane of the brain capillary endothelium, which forms the BBB in vivo. The absence of EPO transport across the BBB in vivo indicates there is no EPOR on the luminal membrane of the BBB. Evidence has been reported for EPOR immunoreactivity at the brain microvasculature, but the receptor is located in a discontinuous pattern on the abluminal side of the endothelium (Brines et al., 2000). These characteristics are typical of receptor expression on the astrocyte foot process that invests the brain capillary.

The re-engineering of EPO as an IgG fusion protein with the HIRMAb molecular Trojan horse produces a brain-penetrating form of EPO. The brain uptake of the HIRMAb-EPO fusion protein, 2.1% of ID/100 g of brain, is high relative to the brain uptake of a molecule confined to the brain plasma volume, such as EPO or human IgG1 (Fig. 6B). Brain uptake is expressed per 100 g of brain, because the brain weight in the adult rhesus monkey is 100 g. The HIRMAb-EPO fusion protein penetrates the BBB and enters brain parenchyma, as demonstrated by the capillary depletion method. The brain V_D of the HIRMAb-EPO fusion protein in the postvascular supernatant is 60% of the total brain homogenate V_D (Table 4), which indicates the majority of the fusion protein bound by the BBB insulin receptor has penetrated brain parenchyma by 2 h after intravenous administration.

Fusion of EPO to the HIRMAb selectively targets EPO to the brain compared with insulin receptor-rich peripheral organs, as demonstrated by the ratio of the organ PS product

for the fusion protein, relative to the organ PS product for EPO (Fig. 7). The PS product reflects transport across the BBB and not sequestration within the brain plasma volume. The PS product for the brain plasma volume marker, human IgG1, should be subtracted from the PS product for both EPO and the HIRMAb-EPO fusion protein. However, because the BBB PS products of the IgG1 and EPO are not significantly different (Fig. 6C), the net PS product for EPO is zero, and a ratio of PS products could not be calculated. Therefore, the PS product ratio for the HIRMAb-EPO fusion protein and EPO for brain shown in Fig. 7 is a minimal estimate of the increased penetration of the BBB by the fusion protein compared with EPO. The PS product ratio for most peripheral organs is near unity for insulin receptor-rich organs such as skeletal muscle, heart, and fat (Fig. 7).

The brain uptake and PK analysis reported here for the primate allows for initial dosing considerations with the HIRMAb-EPO fusion protein. Although EPO is expressed in brain (Sakanaka et al., 1998), the concentration of EPO in the control brain, or CSF, is too low to detect quantitatively by ELISA (Koehne et al., 2002). In those cases where EPO is detectable in human CSF, the EPO concentration is very low, 0.1 pM (Koehne et al., 2002). The concentration of EPO in plasma is also low, 2 to 5 pM (Elliott et al., 2008). In peripheral tissue, the concentration of EPO that causes a 50% increase in pharmacological effect is 12 pM (Elliott et al., 2004), which is equal to 0.4 ng/ml, given an EPO molecular mass of 35,000 Da. Based on the brain uptake of the HIRMAb-EPO fusion protein, 2.1% ID/100 g brain (Fig. 6B), the peripheral injection of a very low dose of the fusion protein (1 μg/kg) in a 5-kg primate would produce a brain concentration of 1 ng/g brain, which is a therapeutic concentration of EPO.

The effect of HIRMAb-EPO administration on hematopoiesis in peripheral tissues is not an issue in the treatment of acute conditions of the brain such as ischemia or trauma with the HIRMAb-EPO fusion protein. However, in chronic treatment of neurodegeneration with the HIRMAb-EPO fusion protein, it is necessary to obtain therapeutic effects in the brain without significant effects on hematopoiesis. This is likely to be the case given the very different PK profiles of EPO and the HIRMAb-EPO fusion protein (Fig. 5A). Fusion of EPO to the HIRMAb reduces the plasma AUC of EPO 13-fold, from 486 ± 59 to 37.5 ± 4.6%ID min/ml (Table 2). The markedly different PK profile for the HIRMAb-EPO fusion protein, compared with EPO, could limit the pharmacologic properties of the fusion protein in peripheral tissues. A peripheral injection of 1 μg/kg of the HIRMAb-EPO fusion protein would be equivalent to a dose of 20 units/kg, because EPO comprises approximately 20% of the amino acid content of the fusion protein (see *Results*). A dose of 20 units/kg approximates a subtherapeutic dose of EPO with respect to hematopoiesis. However, the effect of EPO on the mass of red cells, which persist for approximately 120 days, is primarily a function of the plasma AUC of EPO (Elliott et al., 2008). EPO variants that are cleared from plasma slowly have a greater effect on hematocrit. Conversely, EPO variants that are rapidly cleared from plasma have a diminished effect on hematocrit (Elliott et al., 2004, 2008). Fusion of EPO to the HIRMAb results in a 13-fold reduction in plasma AUC (Table 2). Therefore, doses of the HIRMAb-EPO fusion protein that induce neuroprotection in brain may have minimal effects on hematopoiesis in peripheral tissues.

In conclusion, these studies show it is possible to re-engineer EPO as a HIRMAb fusion protein so that circulating EPO penetrates the BBB via receptor-mediated transport on the BBB insulin receptor. A brain-penetrating form of EPO may cause pharmacologic effects within the brain after peripheral administration, even when the BBB is not disrupted. Further drug development of the HIRMAb-EPO fusion protein will require investigations into the pharmacodynamics and the potential toxicity and immunogenicity of this novel fusion protein.

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