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Re-Engineering Erythropoietin as an IgG Fusion Protein That Penetrates the Blood−Brain Barrier in the Mouse

Qing-Hui Zhou,[†] Ruben J. Boado,[†,‡] Jeff Zhiqiang Lu,[‡] Eric Ka-Wai Hui,[‡] and William M. Pardridge*[,†]

Department of Medicine, UCLA, Los Angeles, California 90024, United States, and ArmaGen Technologies, Inc., Santa Monica, California 90401, United States

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Abstract: Erythropoietin (EPO) is a potent neuroprotective agent that could be developed for the treatment of multiple brain disorders. However, EPO does not cross the blood−brain barrier (BBB). A brain-penetrating form of EPO, specific for the mouse, was engineered by fusion of the 166 amino acid EPO to the carboxyl terminus of the heavy chain of a chimeric monoclonal antibody (mAb) against the mouse transferrin receptor (TfR), and this new fusion protein is designated cTfRMAb-EPO. The fusion protein was expressed in stably transfected Chinese hamster ovary cells and purified by protein G chromatography. The fusion protein was homogeneous on SDS−PAGE and Western blotting, and bound the mouse EPO receptor (EPOR) with high affinity, ED50 = 0.33 ± 0.04 nM. The cTfRMAb-EPO fusion protein was radiolabeled by tritiation and injected intravenously (iv) into adult mice for measurements of the plasma pharmacokinetics and brain uptake. The ^{3}H-fusion protein was rapidly cleared from the blood with a clearance rate of 5.9 ± 0.3 mL/min/kg, which is 14-fold faster than the clearance of EPO in the mouse. The cTfRMAb-EPO fusion protein penetrated the BBB in vivo, as shown by the capillary depletion method. The brain uptake of the cTfRMAb-EPO fusion protein was 2.0 ± 0.1% injected dose/g of brain following iv administration. The high level of brain uptake of the fusion protein enables pharmacologic increases in exogenous EPO in the mouse brain following the systemic injection of the cTfRMAb-EPO fusion protein. In conclusion, EPO has been re-engineered as an IgG fusion protein that binds dual receptors: the mouse TfR, to enable penetration of the BBB, and the mouse EPOR, to produce neuroprotection in brain behind the intact BBB.

Keywords: Blood−brain barrier; drug targeting; drug delivery; monoclonal antibody

Introduction

Erythropoietin (EPO) is a potent neuroprotective agent that could be developed as a new therapeutic for both acute brain disorders, such as brain ischemia,[1] traumatic brain injury,[2] or spinal cord injury,[3] and chronic brain disorders, including Parkinson's disease (PD)[4] or amyotrophic lateral sclerosis.[5] However, a recent clinical trial of intravenous EPO administration within the first 6 h of human stroke failed to show any neuroprotective effect.[6] The blood−brain barrier (BBB) is intact in the early hours after stroke,[7,8] when neuroprotection is still possible.[9] Therefore, if EPO is to be neuro-

* Address correspondence to this author. Mailing address: UCLA Warren Hall 13-164, 900 Veteran Ave., Los Angeles, CA 90024. Phone: 310-825-8858. Fax: 310-206-5163. E-mail: wpardridge@mednet.ucla.edu.

† UCLA.

‡ ArmaGen Technologies, Inc.

(1) Sakanaka, M.; Wen, T.-C.; Matsuda, S.; Masuda, S.; Morishita, E.; Nagao, M.; Sasaki, R. In vivo evidence that erythropoietin protects neurons from ischemic damage. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 4635–4640.

(2) Grasso, G.; Sfacteria, A.; Meli, F.; Fodale, V.; Buemi, M.; Iacopino, D. G. Neuroprotection by erythropoietin adminstration after experimental traumatic brain injury. *Brain Res.* **2007**, *1182*, 99–105.

(3) Matis, G. K.; Birbilis, T. A. Erythropoietin in spinal cord injury. *Eur. Spine J.* **2009**, *18*, 314–323.

(4) Xue, Y. Q.; Zhao, L. R.; Guo, W. P.; Duan, W. M. Intrastriatal adminstration of erythropoietin protects dopaminergic neurons and improves neurobehavioral outcome in a rat model of Parkinson's disease. *Neuroscience* **2007**, *146*, 1245–1258.

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protective in stroke, the molecule must cross the BBB. However, direct measurements of BBB transport of EPO in the mouse,[10] rat,[11] or Rhesus monkey[12] all show that EPO does not cross the intact BBB. Systemic EPO does distribute to cerebrospinal fluid (CSF) following intravenous (iv) administration.[13] However, CSF is an ultrafiltrate of plasma, and all proteins in plasma distribute into CSF,[14] inversely related to molecular weight. The finding of protein entry into CSF is expected for any protein in plasma, and is not evidence the protein crosses the BBB and enters brain parenchyma following iv administration. Neuroprotection with EPO requires the development of EPO variants that cross the BBB.

Brain-penetrating forms of EPO can be re-engineered using BBB molecular Trojan horse (MTH) fusion proteins.[15] A BBB MTH is an endogenous peptide or a peptidomimetic monoclonal antibody (mAb) that undergoes receptor-mediated transport across the BBB via transport on an endogenous BBB receptor, such as the BBB insulin receptor or transferrin receptor (TfR). The most potent MTH is a genetically engineered mAb against the human insulin receptor (HIR).[15] Recently, a fusion protein of EPO and the HIRMAb was engineered and expressed, and shown to undergo rapid transport across the BBB in the Rhesus monkey.[12] Although the HIRMAb cross-reacts with the insulin receptor in Old World primates, such as the Rhesus monkey,[16] the HIRMAb does not cross react with the insulin receptor of lower animals, including the rodent insulin receptor. There is no known mAb against the rodent insulin receptor that could be used as a BBB MTH in rats or mice. Therefore, a surrogate MTH for the mouse has been engineered, which is a chimeric mAb specific for the TfR present at the mouse BBB, and designated the cTfRMAb.[17] The purpose of the present study was to genetically engineer, express, and validate in vitro and in vivo a new IgG fusion protein, wherein human mature EPO is fused to the carboxyl terminus of the heavy chain (HC) of the cTfRMAb. The structure of this fusion protein, which is designated cTfRMAb-EPO, is shown in Figure 1. The present studies show the cTfRMAb-EPO fusion protein is bifunctional, retains high affinity binding for the EPOR, and is rapidly transported across the BBB in the mouse in vivo.

Experimental Section

Production of CHO Line. A tandem vector (TV) containing within a single plasmid DNA the expression cassettes encoding the cTfRMAb heavy chain-EPO fusion protein, the cTfRMAb light chain (LC), and the murine dihydrofolate reductase (DHFR) was engineered similarly to a TV described previously.[18] The cDNA encoding the 166 amino acid (AA) human mature EPO was amplified by PCR as described previously[12] and subcloned at the 3′-end of the cTfRMAb HC to form the pcTfRMAb-EPO tandem vector. The structure of the TV has been described previously.[18] The sequence of the TV was confirmed by bidirectional DNA sequencing performed at Eurofins MWG Operon (Huntsville, AL) using custom sequencing oligodeoxynucleotides synthesized at Midland Certified Reagent Co. (Midland, TX). The TV was linearized and CHO cells were electroporated,

(5) Koh, S. H.; Kim, Y.; Kim, H. Y.; Cho, G. W.; Kim, K. S.; Kim, S. H. Recombinant human erythropoietin suppresses symptom onset and progression of G93A-SOD1 mouse model of ALS by preventing motor neuron death and inflammation. *Eur. J. Neurosci.* **2007**, *25*, 1923–1930.

(6) Ehrenreich, H.; Weissenborn, K.; Prange, H.; Schneider, D.; Weimar, C.; Wartenberg, K.; Schellinger, P. D.; Bohn, M.; Becker, H.; Wegrzyn, M.; Jahnig, P.; Herrmann, M.; Knauth, M.; Bahr, M.; Heide, W.; Wagner, A.; Schwab, S.; Reichmann, H.; Schwendemann, G.; Dengler, R.; Kastrup, A.; Bartels, C. Recombinant human erythropoietin in the treatment of acute ischemic stroke. *Stroke* **2009**, *40*, e647−e656.

(7) Belayev, L.; Busto, R.; Zhao, W.; Ginsberg, M. D. Quanititative evaluation of blood-brain barrier permeability following middle cerebral artery occlusion in rats. *Brain Res.* **1996**, *739*, 88–96.

(8) Latour, L. L.; Kang, D. W.; Ezzeddine, M. A.; Chalela, J. A.; Warach, S. Early blood-brain barrier disruption in human focal brain ischemia. *Ann. Neurol.* **2004**, *56*, 468–477.

(9) Zivin, J. A. Factors determining the therapeutic window for stroke. *Neurology* **1998**, *50*, 599–603.

(10) Banks, W. A.; Jumbe, N. L.; Farrell, C. L.; Niehoff, M. L.; Heatherington, A. C. Passage of erythropoietic agents across the blood-brain barrier: a comparison of human and murine erythropoietin and the analog darbepoetin alfa. *Eur. J. Pharmacol.* **2004**, *505*, 93–101.

(11) Lieutaud, T.; Andrews, P. J. D.; Rhodes, J. K. J.; Williamson, R. Characterization of the pharmacokinetics of human recombinant erythropoietin in blood and brain when administered immedicately after lateral fluid percussion brain injury and its pharmacodynamic effects on IL-1B and MIP-2 in rats. *J Neurotrauma* **2008**, *25*, 1179–1185.

(12) Boado, R. J.; Hui, E. K.; Lu, J. Z.; Pardridge, W. M. Drug targeting of erythropoietin across the primate blood-brain barrier with an IgG molecular Trojan horse. *J. Pharmacol. Exp. Ther.* **2010**, *333*, 961–969.

(13) Juul, S. E.; McPherson, R. J.; Farrell, F. X.; Jolliffe, L.; Ness, D. J.; Gleason, C. A. Erythropoietin concentrations in cerebrospinal fluid of nonhuman primates and fetal sheep following high-dose recombinant erythropoietin. *Biol. Neonate* **2004**, *85*, 138–144.

(14) Reiber, H.; Felgenhauer, K. Protein transfer at the blood cerebrospinal fluid barrier and the quantitation of the humoral immune response within the central nervous system. *Clin. Chim. Acta* **1987**, *163*, 319–328.

(15) Pardridge, W. M. Re-engineering biopharmaceuticals for delivery to brain with molecular Trojan horses. *Bioconjugate Chem.* **2008**, *19*, 1327–1338.

(16) Pardridge, W. M.; Kang, Y. S.; Buciak, J. L.; Yang, J. Human insulin receptor monoclonal antibody undergoes high affinity binding to human brain capillaries in vitro and rapid transcytosis through the blood-brain barrier in vivo in the primate. *Pharm. Res.* **1995**, *12*, 807–816.

(17) Boado, R. J.; Zhang, Y.; Wang, Y.; Pardridge, W. M. Engineering and expression of a chimeric transferrin receptor monoclonal antibody for blood-brain barrier delivery in the mouse. *Biotechnol. Bioeng.* **2009**, *102*, 1251–1258.

(18) Zhou, Q. H.; Boado, R. J.; Lu, J. Z.; Hui, E. K.; Pardridge, W. M. Monoclonal antibody-glial derived neurotrophic factor fusion protein penetrates the blood-brain barrier in the mouse. *Drug Metab. Dispos.* **2010**, *38*, 566–572.

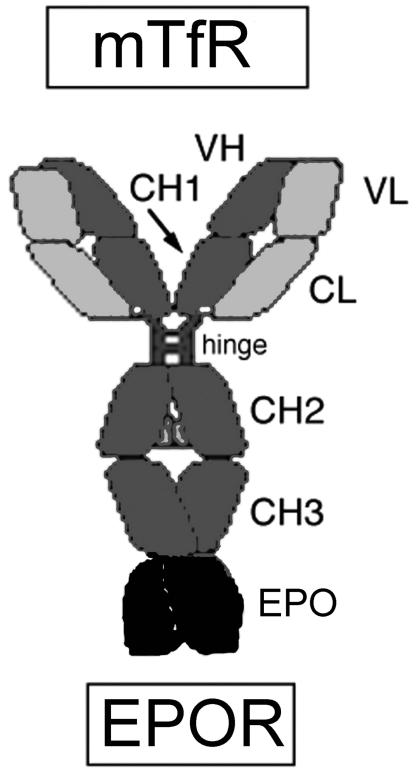

Figure 1. The cTfRMAb-EPO fusion protein is composed of 2 heavy chains and 2 light chains. The heavy chain is formed by fusion of the variable region of the heavy chain (VH) of the rat 8D3 mAb against the mouse transferrin receptor (mTfR) to the amino terminus of mouse IgG1 constant (C)-region, and fusion of human EPO to the carboxyl terminus of the heavy chain C-region. The light chain is formed by fusion of the variable region of the light chain (VL) of the rat 8D3 mAb to the mouse kappa light chain C-region (CL). The heavy chain C-region is composed of 4 domains: CH1, hinge, CH2, and CH3.

followed by selection in hypoxanthine-thymine deficient medium and amplification with graded increases in methotrexate (MTX) up to 80 nM in serum free medium (SFM). The CHO line was dilutionally cloned at 1 cell/well, and high producing clones were selected by measurement of medium mouse IgG concentrations by enzyme-linked immunosorbent assay (ELISA). The CHO line was stable through multiple generations, and produced medium IgG levels of 5−10 mg/L in shake flasks at a cell density of 1−2 million cells/mL.

Protein Purification. The CHO cells were propagated in 1 L bottles, until 2.4 L of conditioned serum free medium was collected. The medium was ultrafiltered with a 0.2 μm Sartopore-2 sterile-filter unit (Sartorius Stedim Biotech, Goettingen, Germany) and applied to a 25 mL protein G Sepharose 4 Fast Flow (GE Life Sciences, Chicago, IL) column equilibrated in 25 mM Tris/25 mM NaCl/5 mM EDTA/pH = 7.1. 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 glycine/pH = 2.8. The acid eluate was pooled and neutralized to pH = 5.5 with 1 M Tris base, concentrated with an Ultra-15 microconcentrator (Millipore, Bedford, MA) and stored sterile-filtered at 4C.

SDS−PAGE and Western Blotting. The homogeneity of the cTfRMAb-EPO fusion protein was evaluated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) under reducing and nonreducing conditions using 12% and 7.5% Tris-HCl gels (Biorad Life Science, Hercules, CA), respectively. Western blot analysis was performed with a goat anti-mouse IgG (H+L) antibody (Bethyl Laboratories, Montgomery, TX) for the mouse IgG Western, and with a rabbit anti-human EPO antibody (R&D Systems, Minneapolis, MN) for the EPO Western. The immunoreactivity of the cTfRMAb-EPO fusion protein was compared with the cTfRMAb described previously.[17]

EPOR Binding Assay. The binding of the cTfRMAb-EPO fusion protein to the mouse or human EPOR was determined by ELISA. A recombinant fusion protein of the human IgG1 Fc fragment and the extracellular domain (ECD) of either the human or mouse EPOR was obtained from R&D Systems. The mouse EPOR ECD encompasses amino acids 25−249 of the mouse EPOR (GenBank P14753), and the human EPOR ECD encompasses amino acids 25−250 of the human EPOR (GenBank NP_000112). The mouse or human EPOR:Fc fusion protein was plated in 96-well plates overnight at 200 ng/well. Following aspiration, washing, and blocking with 1% bovine serum albumin (BSA), either the cTfRMAb-EPO fusion protein, or mouse IgG1κ (Sigma Chemical Co., St. Louis, MO), was plated for 2 h at RT. Following washing, a conjugate of a goat anti-mouse kappa light chain antibody and alkaline phosphatase (Bethyl Laboratories, Montgomery, TX) was plated at 100 ng/well for 45 min at RT, followed by washing, color development with *p*-nitrophenylphosphate (Sigma Chemical Co.), and detection at 405 nm with an ELISA plate reader. The concentration of cTfRMAb-EPO fusion protein that causes a 50% increase in binding is designated the ED50, which was determined by nonlinear regression analysis using the BMDP2007e software (Statistical Solutions, Dublin, Ireland).

Radiolabeling of Fusion Protein. The cTfRMAb-EPO fusion protein, which was injected into mice for a pharmacokinetics analysis, was radiolabeled with [^{3}H]-*N*-succinimidyl propionate (NSP) from American Radiolabeled Chemicals (St. Louis, MO), as described previously.[12] The labeled protein was purified with a 1 × 28 cm Sephadex G-25 gel filtration column, with an elution buffer of 0.01 M sodium acetate/0.14 M NaCl/pH = 5.5/0.2% Tween-20. The cTfRMAb-EPO fusion protein was labeled to a specific activity of 4.0 μCi/μg and a trichloroacetic acid (TCA) precipitability of >97%.

Pharmacokinetics and Brain Uptake in the Mouse. Adult male C57BL/6J mice, weighing 28 g, were obtained from Jackson Laboratories (Bar Harbor, ME). All procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Mice in groups of 4 were anesthetized with intraperitoneal (ip) ketamine (100 mg/kg) and xylazine (10 mg/kg), and injected either intra-

venously (iv) in the tail vein or ip with 0.1 mL (12.3 μCi) of [^{3}H]-cTfRMAb-EPO fusion protein. The injection dose (ID) in each mouse of the cTfRMAb-EPO fusion protein was 110 μg/kg. Since the fusion protein is 20% EPO by amino acid content (Results), the ID of EPO is 22 μg/kg. An aliquot (50 μL) of venous blood was collected at 0.25, 2, 5, 15, 30, and 60 min after injection by sampling the orbital vein. The blood was centrifuged for collection of plasma, which was analyzed for radioactivity. At 60 min after injection, the mice were euthanized and the cerebral hemispheres were removed and weighed for each mouse. One hemisphere was used for total radioactivity after solubilization in Soluene-350 (Perkin-Elmer, Downers Grove, IL), and one hemisphere was homogenized for capillary depletion analysis. Plasma and tissue samples were analyzed for ^{3}H radioactivity with Optifluor-O (Perkin-Elmer) and a liquid scintillation counter (Tricarb 2100TR, Perkin-Elmer). Brain uptake data was expressed as a volume of distribution (VD), which is the ratio of the 60 min organ radioactivity (DPM/g) divided by the 60 min plasma radioactivity (DPM/μL), or as % of injected dose (ID)/gram of tissue. The plasma radioactivity that was precipitable with cold 10% TCA was determined at each time point.

The plasma radioactivity, DPM/mL, was converted to % injected dose (ID)/mL, and the % ID/mL was fit to a biexponential equation,

$$\%\ \mathrm{ID/mL} = A_1\, e^{-k_1 t} + A_2\, e^{-k_2 t}$$

The intercepts (A_1, A_2) and the slopes (k_1, k_2) were used to compute the pharmacokinetics (PK) parameters, including the median residence time (MRT), the central volume of distribution (V_c), the steady state volume of distribution (V_{ss}), the area under the plasma concentration curve (AUC), and the systemic clearance (CL). Nonlinear regression analysis used the AR subroutine of the BMDP Statistical Software. Data were weighted by 1/(% ID/mL).[2]

The organ clearance (μL/min/g), also called the BBB permeability−surface area (PS) product, is computed from the terminal brain uptake (% ID/g) and the 60 min plasma AUC (% ID min/mL) as follows:

$$\mathrm{PS\ product} = [(\%\ \mathrm{ID/g})/\mathrm{AUC}] \times 1000$$

Capillary Depletion Method. One cerebral hemisphere from each mouse was processed for the capillary depletion method as described previously,[18] which separates the brain homogenate into the capillary pellet and the postvascular supernatant. The volume of distribution (VD) was determined for each of the 3 fractions from the ratio of total ^{3}H radioactivity in the fraction, DPM/gram of brain, divided by the ^{3}H radioactivity in the 60 min terminal plasma, DPM/μL. A high VD in the postvascular supernatant, compared to the VD in the capillary pellet, is evidence for transport of the protein through the BBB into brain parenchyma.[19] The measurement of the specific activity of vascular-specific enzymes, such as γ-glutamyl transpeptidase and alkaline phosphatase, in the capillary pellet and postvascular supernatant demonstrates the supernatant is 95%-depleted of the brain vasculature.[19]

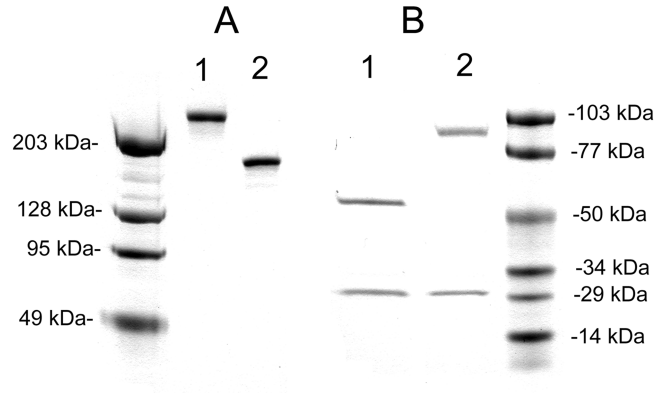

Figure 2. (A) Nonreducing SDS−PAGE of the cTfRMAb-EPO fusion protein (lane 1) and the cTfRMAb (lane 2). (B) Reducing SDS−PAGE of the cTfRMAb (lane 1) and the cTfRMAb-EPO fusion protein (lane 2).

Results

A tandem vector was engineered, which contained the expression cassettes for the heavy chain fusion gene, the light gene, and the DHFR gene on a single plasmid DNA. DNA sequence analysis showed the 3 expression cassettes spanned 9,140 nucleotides (nt). The light chain was composed of 234 AA, which included a 20 AA signal peptide, a 108 AA variable region of the light chain (VL) of the cTfRMAb, and a 106 AA mouse kappa light chain constant region, which is 100% identical to AA 113-218 of BAA06141. The predicted molecular weight of the light chain is 23,554 Da with a predicted isoelectric point (p*I*) of 5.73.The fusion protein of the cTfRMAb heavy chain and the EPO was composed of 630 AA, which included a 19 AA signal peptide. The predicted molecular weight of the heavy chain, without glycosylation, is 67,426 Da with a predicted p*I* of 6.73. The domains of the fusion heavy chain include a 118 AA variable region of the heavy chain (VH) of the cTfRMAb, a 324 AA mouse IgG1 constant region, which is 100% identical to AA 140−463 of BAC44885, a 3 AA linker (Ser-Ser-Ser), and the 166 AA human EPO, which is 100% identical to AA 28−193 of NP_000790. The HC contains 4 N-linked glycosylation sites: 1 site in the constant region of the IgG heavy chain and 3 sites in the EPO domain. The predicted molecular weight, without glycosylation, of the heterotetrameric cTfRMAb-EPO fusion protein is 181,906 Da with a p*I* of 6.48.

The CHO-derived cTfRMAb-EPO fusion protein was homogeneous on both nonreducing (Figure 2A) and reducing

(19) Triguero, D.; Buciak, J. B.; Pardridge, W. M. Capillary depletion method for quantifying blood-brain barrier transcytosis of circulating peptides and plasma proteins. *J. Neurochem.* **1990**, *54*, 1882–1888.

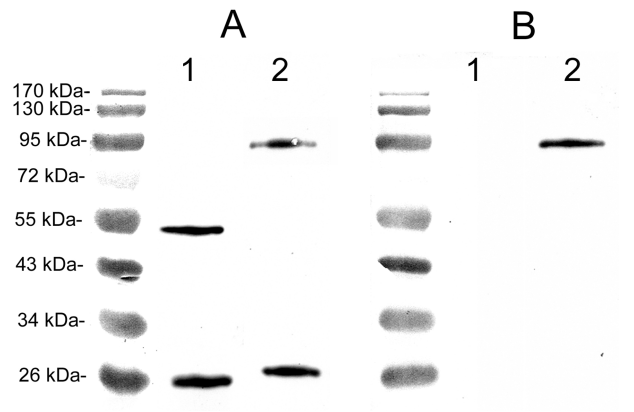

Figure 3. Western blotting with a primary antibody against mouse IgG (A) or against human EPO (B). The anti-mouse antibody reacts with the HC and LC of both the cTfRMAb (lane 1, panel A) and the cTfRMAb-EPO fusion protein (lane 2, panel A). The anti-EPO antibody reacts only with the HC of the cTfRMAb-EPO fusion protein (lane 2, panel B), but does not react with cTfRMAb (lane 1, panel B).

(Figure 2B) SDS−PAGE. The size of the HC of the cTfRMAb-EPO fusion protein on the reducing gel, 90 kDa, was greater than the size of the HC of the cTfRMAb, 55 kDa, owing to fusion of the 35 kDa EPO (Figure 2B). The cTfRMAb-EPO fusion protein migrated at approximately 230 kDa on the nonreducing gel (lane 1, Figure 2A). Western blot analysis with a primary antibody against mouse IgG detected both the HC and the LC of the cTfRMAb (lane 1, Figure 3A) and the cTfRMAb-EPO fusion protein (lane 2, Figure 3A). Western blot analysis with a primary antibody against human EPO reacted with the HC of the cTfRMAb-EPO fusion protein (lane 2, Figure 3B), but did not react with HC of the cTfRMAb (lane 1, Figure 3B).

The cTfRMAb-EPO fusion protein retained high affinity binding to the recombinant mouse EPOR with an ED50 of 0.33 ± 0.04 nM; conversely, there was no binding of mouse IgG1 to the mouse EPOR (Figure 4). When the human EPOR was substituted in this assay for the mouse EPOR (Experimental Section), a similar binding curve was observed, and the ED50 of cTfRMAb-EPO fusion protein binding to the human EPOR was 0.42 ± 0.03 nM.

The cTfRMAb-EPO fusion protein was radiolabeled with the ^{3}H-NSP reagent (Experimental Section) and injected into adult male C57Bl/6J mice via either iv or ip administration. The clearance of the [^{3}H]-cTfRMAb-EPO fusion protein from plasma after iv administration and the appearance of plasma radioactivity after ip administration of the fusion protein are plotted in Figure 5. For the iv injection studies, the plasma radioactivity decay curve was fit to a biexponential equation to yield the pharmacokinetics (PK) parameters shown in Table 1. The [^{3}H]-cTfRMAb-EPO fusion protein was metabolically stable after iv administration, as the plasma radioactivity that was precipitable by TCA was 97.2 ± 0.4, 95.5 ± 0.3, 94.4 ± 0.5, 93.0 ± 0.7, 89.7 ± 1.2, and 74.8 ± 4.8, respectively at 0.25, 2, 5, 15, 30, and 60 min after iv injection. Following ip injection, the concentration of the cTfRMAb-EPO fusion protein in blood increases with time (Figure 5). The plasma AUC of the cTfRMAb-EPO fusion protein at 60 min after ip injection is 245% ID min/mL, which is 46% of the plasma AUC following iv injection (Table 1).

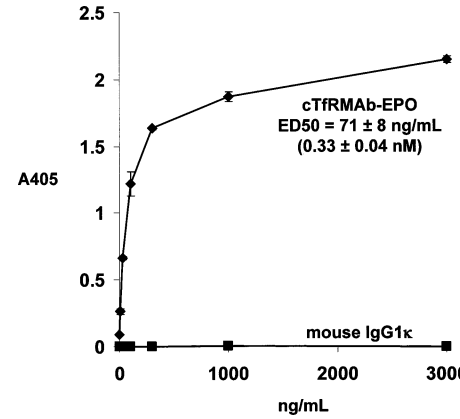

Figure 4. Binding of the cTfRMAb-EPO fusion protein to the mouse EPOR extracellular domain is saturable. The ED50 of cTfRMAb-EPO fusion protein binding was determined by nonlinear regression analysis. There is no binding to the EPOR by mouse IgG1/kappa (mIgG1κ).

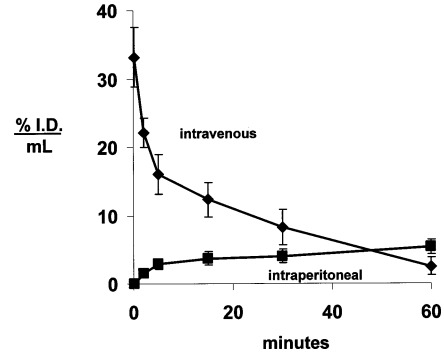

Figure 5. Plasma concentration, expressed as % of injected dose (ID)/mL, of the [^{3}H]-cTfRMAb-EPO fusion protein after intravenous or intraperitoneal injection in the mouse. Data are mean ± SE ($n = 4$ mice/point).

Table 1. Pharmacokinetics Parameters of cTfRMAb-EPO in the Mouse[a]

parameter	units	cTfRMAb-EPO
A_1	% ID/mL	15.2 ± 5.2
A_2	% ID/mL	20.4 ± 2.0
k_1	min^{-1}	0.88 ± 0.63
k_2	min^{-1}	0.034 ± 0.002
MRT	min	28 ± 2
V_c	mL/kg	100 ± 15
V_{ss}	mL/kg	166 ± 12
AUC(60 min)	% ID min/mL	533 ± 25
AUC_{ss}	% ID min/mL	608 ± 29
Cl	mL/min/kg	5.9 ± 0.3

[a] MRT = mean residence time; V_c = plasma volume; V_{ss} = steady state volume of distribution; AUC(60 min) = area under the curve first 60 min; AUC_{ss} = steady state AUC; Cl = clearance from plasma. The injection dose was 110 μg/kg, and the body weight of the mice was 0.028 kg. The parameters were determined from the plasma profile shown in Figure 5 for iv administration.

Table 2. Capillary Depletion Analysis for Brain Uptake of cTfRMAb-EPO Fusion Protein[a]

fraction	VD (μL/g)
brain homogenate	563 ± 55
postvascular supernatant	386 ± 50
vascular pellet	184 ± 10

[a] Mean ± SE (n = 4 mice). The fusion protein was administered by iv injection, and brain measurements were made 60 min following injection.

Table 3. cTfRMAb-EPO Brain Uptake Parameters[a]

parameter	value	units
brain uptake	2.0 ± 0.1	% ID/g
BBB PS product	3.7 ± 0.2	μL/min/g
injection dose	110 ± 5	μg/kg
cTfRMAb-EPO brain concn	62 ± 3	ng/g
EPO brain concn	12 ± 1	ng/g

[a] Mean ± SE (n = 4 mice). The fusion protein was administered by iv injection, and brain measurements made 60 min following injection.

The capillary depletion analysis showed the VD of the cTfRMAb-EPO fusion protein in the brain homogenate was high, 563 ± 55 μL/g (Table 2). The VD of the fusion protein in the postvascular supernatant, 386 ± 50 μL/g (Table 2), is 69% of the homogenate VD, indicating the majority of the cTfRMAb-EPO fusion protein had completed transcytosis through the BBB by 60 min after iv injection.

The uptake of the [^{3}H]-cTfRMAb-EPO fusion protein by brain was determined at 60 min after iv injection, and was 2.0 ± 0.1% ID/g of brain (Table 3). The BBB permeability−surface area (PS) product of the cTfRMAb-EPO fusion protein, which is equal to the ratio of the 60 min % ID/g (Table 3) and the 60 min plasma AUC (Table 1), is 3.7 ± 0.2 μL/min/g. The concentration of the cTfRMAb-EPO fusion protein in brain at 60 min after iv injection is 62 ± 3 ng/g (Table 3). Since the EPO content of the fusion protein is 20% by amino acid content, the brain concentration of EPO at 60 min after iv injection of the fusion protein is 12 ± 1 ng/g (Table 3).

Discussion

The results of this study are consistent with the following conclusions. First, a novel IgG-EPO fusion protein, with dual specificity for the mouse TfR and mouse EPOR (Figure 1), has been genetically engineered and expressed in stably transfected CHO cells in serum free medium (Experimental Section). Second, the cTfRMAb-EPO fusion protein is purified by protein G affinity chromatography to homogeneity on SDS−PAGE (Figure 2), and the purified fusion protein exhibits dual immunoreactivity on Western blotting with antibodies to both mouse IgG and human EPO (Figure 3). Third, the cTfRMAb-EPO fusion protein retains high affinity binding to the mouse EPOR (Figure 4). Fourth, the cTfRMAb-EPO fusion protein exhibits a markedly different PK profile as compared to EPO in the mouse, as shown by the very high rate of systemic clearance from blood of the fusion protein (Table 1). Fifth, the cTfRMAb-EPO fusion protein is BBB penetrating, based on studies with the capillary depletion method (Table 2), and the high brain uptake of the fusion protein, 2.0 ± 0.1% ID/g, enables increases in the brain EPO concentration following iv administration of the fusion protein (Table 3).

The cTfRMAb-EPO fusion protein is a new IgG-EPO fusion protein where the amino terminus of the mature human EPO is fused to the carboxyl terminus of the HC of the chimeric mAb against the mouse TfR. With this design, the EPO is placed in a dimeric configuration (Figure 1). Although EPO is secreted as a monomeric protein, EPO binds a dimer of EPO receptors,[20] and dimeric forms of EPO are biologically more potent forms of EPO in vivo, as compared to the monomeric EPO.[21] The affinity of the cTfRMAb-EPO fusion protein for the mouse EPOR is in the low nM range (Figure 4), and the ED50 of fusion protein binding to the EPOR is comparable to the dissociation constant (K_D) of human EPO binding to the EPOR.[22] The affinity of the cTfRMAb-EPO fusion protein for the mouse EPOR is equal to the affinity of the fusion protein for the human EPOR (Results), which is consistent with the high amino acid identity, 78%, between human EPO (NP_000790) and mouse EPO (NP_031968), and the high amino acid identity, 83%, between the ECD of human EPOR (NP_000112) and mouse EPOR (P14753).

The affinity of the cTfRMAb-EPO fusion protein for the mouse TfR is shown in these studies to be high, as judged by the BBB PS product, which is 3.7 ± 0.2 μL/min/g (Table 3). The BBB PS product for cTfRMAb-EPO fusion protein is a function of the affinity of the cTfRMAb part of the fusion protein for the mouse TfR, which is characterized by a binding K_D of 2.6 ± 0.3 nM.[17] Fusion of neurotrophins such as glial derived neurotrophic factor (GDNF) to the cTfRMAb has no effect on the K_D of fusion protein binding to the mouse TfR,[18] and the BBB PS product of the cTfRMAb-GDNF fusion protein, 3.0 ± 0.3 μL/min/g,[18] is comparable to the BBB PS product for the cTfRMAb-EPO fusion protein (Table 3).

The pharmacokinetics (PK) profile of the cTfRMAb-EPO fusion protein differs from the PK profile of EPO in the mouse. The systemic clearance (Cl) of the cTfRMAb-EPO fusion protein is 5.9 ± 0.3 mL/min/kg, which is 14-fold higher than the systemic clearance of EPO in the mouse,

(20) Syed, R. S.; Reid, S. W.; Li, C.; Cheetham, J. C.; Aoki, K. H.; Liu, B.; Zhan, H.; Osslund, T. D.; Chirino, A. J.; Zhang, J.; Finer-Moore, J.; Elliott, S.; Sitney, K.; Katz, B. A.; Matthews, D. J.; Wendoloski, J. J.; Egrie, J.; Stroud, R. M. Efficiency of signaling through cytokine receptors depends critically on receptor orientation. *Nature* **1998**, *395*, 511–516.

(21) Sytkowski, A. J.; Lunn, E. D.; Davis, K. L.; Feldman, L.; Siekman, S. Human erythropoietin dimmers with markedly enhanced *in vivo* activity. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 1184–1188.

(22) Elliott, S.; Egrie, J.; Browne, J.; Lorenzini, T.; Busse, L.; Rogers, N.; Ponting, I. Control of rHuEPO biological activity: the role of carbohydrate. *Exp. Hematol.* **2004**, *32*, 1146–1155.

0.41 ± 0.03 mL/min/kg.[23] Similarly, the systemic clearance of the HIRMAb-EPO fusion protein in the Rhesus monkey is 12-fold higher than the systemic clearance of EPO.[12] The marked difference in PK profiles of EPO and receptor-specific IgG-EPO fusion proteins is likely to have a significant impact on the potency of the IgG-EPO fusion protein on erythropoiesis. Variants of EPO that are hyper-glycosylated are more slowly cleared from the bloodstream, and have more potent effects on hematopoiesis, despite a reduction in affinity of the EPO variant for the EPOR.[22] The effect of EPO on circulating human red cells, which have a life span of about 120 days, is a function of the plasma AUC,[22] which is inversely related to systemic clearance. Conversely, an EPO variant, such as the cTfRMAb-EPO fusion protein, that undergoes more rapid systemic clearance in vivo is expected to have a diminished effect on erythropoiesis relative to EPO.[22] Thus, fusion of EPO to the cTfRMAb, or the HIRMAb,[12] has the opposite effect on the plasma AUC of EPO as is caused by hyper-glycosylation of EPO. These pharmacokinetics properties of the IgG-EPO fusion proteins are preferred for a brain-penetrating form of EPO that is intended to treat the brain with minimal effects on hematopoiesis. The faster clearance of the cTfRMAb-EPO fusion protein, as compared to EPO, is due to the increased uptake of the fusion protein by TfR-rich organs such as liver or spleen.[12] However, fusion of EPO to an IgG Trojan horse increases the effective molecular weight of EPO from about 20 kDa to about 200 kDa.[12] The larger size of the IgG-EPO fusion protein, relative to EPO, suppresses uptake by peripheral tissues, such as heart, lung, skeletal muscle, fat, and kidney.[12]

The cTfRMAb-EPO fusion protein is brain penetrating, as demonstrated by the capillary depletion method (Table 2). The capillary depletion method has also been applied to the brain uptake of EPO, and shows that EPO does not cross the BBB in vivo.[12] The distribution of the fusion protein into the postvascular supernatant of brain does not represent metabolites, since gel filtration of plasma shows intact IgG-EPO fusion protein.[12] The brain homogenate volume of distribution (VD) of the fusion protein, 563 ± 55 μL/g (Table 2), is high compared to the brain VD of a brain blood volume marker, such as the OX26 mAb. The latter is a mouse mAb against the rat TfR, and the OX26 mAb does not recognize the mouse TfR.[24] Consequently, the OX26 mAb does not cross the BBB in the mouse, as represented by the very low brain VD for this antibody, which is 10 ± 1 μL/g.[24] The brain VD for the OX26 antibody is equal to the brain blood volume and is 53-fold lower than the brain VD for the cTfRMAb-EPO fusion protein. The fusion protein is not simply sequestered by the brain microvasculature, but is transported across the BBB. The VD in the postvascular supernatant is 69% of the homogenate VD (Table 2), which is evidence for penetration of the brain parenchyma. The brain uptake, measured as % ID/g, is high for the cTfRMAb-EPO fusion protein, 2.0 ± 0.1% ID/g (Table 3), which is 33-fold greater than the brain uptake of the OX26 mAb in the mouse, 0.06 ± 0.01% ID/g.[24] Similarly, the uptake of EPO by mouse brain is 0.05% ID/g.[10] This very low level of brain uptake of either the OX26 mAb or EPO in the mouse reflects sequestration within the brain blood volume of substances that do not cross the BBB.

The brain-penetrating properties of the cTfRMAb-EPO fusion protein enable pharmacologic increases in the brain concentration of EPO following iv administration. At a systemic dose of 110 μg/kg of the fusion protein, the brain EPO concentration is 12 ± 1 ng/g of brain (Table 3). The magnitude of this increase in brain EPO concentration is illustrated by comparison with the endogenous EPO concentration in brain. Under normal conditions, immunoreactive EPO is not measurable in brain.[25] Based on the sensitivity of the assay, the endogenous EPO concentration in brain is <10 pg/g. The concentration of EPO in CSF is very low, <0.1 pg/mL.[26] An endogenous brain EPO concentration of 10 pg/g is >1000-fold lower than the concentration of exogenous EPO in brain produced by an iv administration of 110 μg/kg of the fusion protein (Table 3). A brain concentration of EPO of 12 ng/g is equivalent to a concentration of about 1,000 pM EPO. In contrast, the ED50 of EPO activity in a bioassay of peripheral tissues is 12 pM.[22] With respect to brain, neuronal apoptosis is prevented by concentrations of EPO as low as 25 pM.[27] A 25 pM concentration is equivalent to a brain EPO concentration of 0.2 ng/g of brain, which is 60-fold less than the EPO delivered to brain with an injection dose of 110 μg/kg (Table 3).

The delivery of EPO from blood to brain may cause neuroprotection in the brain, owing to the widespread expression of the EPOR in brain.[28] The EPOR is expressed on both neurons[27,29] and astrocytes[29] in brain. The enhanced delivery to brain of a neuroprotective neurotro-

(23) Kato, M.; Miura, K.; Kamiyama, H.; Okazaki, A.; Kumaki, K.; Kato, Y.; Sugiyama, Y. Pharmacokinetics of erythropoietin in genetically anemic mice. *Drug Metab. Dispos.* **1998**, *26*, 126–131.

(24) Lee, H. J.; Engelhardt, B.; Lesley, J.; Bickel, U.; Pardridge, W. M. Targeting rat anti-mouse transferring receptor monoclonal antibodies through blood-brain barrier in mouse. *J. Pharmacol. Exp. Ther.* **2000**, *292*, 1048–1052.

(25) Statler, P. A.; McPherson, R. J.; Bauer, L. A.; Kellert, B. A.; Juul, S. E. Pharmacokinetics of high-dose recombinant erythropoietin in plasma and brain of neonatal rats. *Pediat. Res.* **2007**, *61*, 671–675.

(26) Koehne, P.; Hochhaus, F.; Felderhoff-Mueser, U.; Ring-Mrozik, E.; Obladen, M.; Buhrer, C. Vascular endothelial growth factor and erythropoietin concentrations in cerebrospinal fluid of children with hydrocephalus. *Child's Nerv. Syst.* **2002**, *18*, 137–141.

(27) Um, M.; Gross, A. W.; Lodish, H. L. A "classical" homodimeric erythropoietin receptor is essential for the antiapoptotic effects of erythropoietin on differentiated neuroblastoma SH-SY5Y and pheochromocytoma PC-12 cells. *Cell. Signalling* **2006**, *19*, 634–645.

(28) Digicaylioglu, M.; Bichet, S.; Marti, H. H.; Wenger, R. H.; Rivas, L. A.; Bauer, C.; Gassmann, M. Localization of specific erythropoetin binding sites in defined areas of the mouse brain. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 3717–3720.

phin such as EPO that is fused to the cTfRMAb Trojan horse is expected to induce neuroprotection in the brain following iv administration, and this has recently been demonstrated in a mouse model of PD. The iv administration of a cTfRMAb-neurotrophin fusion protein to mice with experimental PD resulted in a 272% increase in striatal tyrosine hydroxylase, which correlated with an improvement in neurobehavior.[30]

In summary, EPO has been re-engineered as an IgG fusion protein with dual receptor specificity for both the mouse EPOR and mouse TfR (Figure 1). This new fusion protein can be now evaluated in therapeutic mouse models of brain disorders where EPO may have a neuroprotective effect, including brain ischemia, brain or spinal cord injury, or chronic neurodegenerative disorders.

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(29) Juul, S. E.; Yachnis, A. T.; Rojiani, A. M.; Christensen, R. D. Immunohistochemical localization of erythropoietin and its receptor in the developing human brain. *Pediatr. Dev. Pathol.* **1999**, *2*, 148–158.

(30) Fu, A.; Zhou, Q.-H.; Hui, E. K.-W.; Lu, J. Z.; Boado, R. J.; Pardridge, W. M. Intravenous treatment of experimental Parkinson's disease in the mouse with an IgG-GDNF fusion protein that penetrates the blood-brain barrier. *Brain Res.* **2010**, *1352*, 208–213.