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Combined Use of Carboxyl-Directed Protein Pegylation and Vector-Mediated Blood-Brain Barrier Drug Delivery System Optimizes Brain Uptake of Brain-Derived Neurotrophic Factor Following Intravenous Administration

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Purpose. Peptide drug delivery to the brain requires optimization of (a) plasma pharmacokinetics and (b) blood-brain barrier (BBB) permeability. In the present studies, plasma pharmacokinetics are improved with protein pegylation and BBB transport is facilitated with the use of vector-mediated drug delivery using the OX26 monoclonal antibody (MAb) to the rat transferrin receptor, which undergoes receptor-mediated transcytosis through the BBB in vivo.

Methods. A conjugate of OX26 and streptavidin (SA), designated OX26/SA, was prepared in parallel with the carboxyl-directed pegylation of brain-derived neurotrophic factor (BDNF). A novel bifunctional polyethyleneglycol (PEG) was used in which a hydrazide (Hz) was attached at one end and a biotin moiety was attached to the other end. This allowed for conjugation of BDNF-PEG-biotin to OX26/SA.

Results. The brain uptake of BDNF-PEG-biotin was increased following conjugation to OX26/SA to a level of 0.144 ± 0.004% injected dose per g brain and a BBB permeability-surface area product of 2.0±0.2 μL/min/g.

Conclusions. These studies demonstrate that peptide drug delivery to the brain can be achieved with advanced formulation of protein-based therapeutics. The formulation is intended to (a) minimize rapid systemic clearance of the peptide, and (b) allow for vector-mediated drug delivery through the BBB in vivo. Following this dual formulation, the brain uptake of a neurotrophin such as BDNF achieves a value that is approximately 2-fold greater than that of morphine, a neuroactive small molecule.

KEY WORDS: pegylation; blood-brain barrier; pharmacokinetics.

INTRODUCTION

Brain-derived neurotrophic factor (BDNF) is a potential neuropharmaceutical that could be used for the treatment of a variety of neurodegenerative disorders (1,2). However, BDNF, like other neurotrophins or protein-based therapeutics, does not undergo significant transport through the brain capillary endothelial wall (3,4), which makes up the blood-brain barrier (BBB) in vivo. A second problem limiting brain uptake of BDNF, or the other neurotrophins that are cationic proteins, is the rapid removal from blood, principally by liver, of the protein following systemic administration (3,4). Since brain uptake of a drug is a dual function of (a) the blood-brain barrier permeability -surface area (PS) product, a measure of BBB permeability, and (b) the plasma area under the concentration curve (AUC), a measure of the plasma pharmacokinetics of the drug, both factors (PS product, plasma AUC) must be optimized in order to increase brain delivery of the neurotrophin to a level that exerts CNS pharmacologic effects in vivo (5).

Previous studies demonstrated that the BBB permeability of BDNF could be increased by conjugating this neurotrophin to a BBB drug delivery system, such as the OX26 murine monoclonal antibody to the rat transferrin receptor (3). Owing to the abundance of the transferrin receptor on the brain capillary endothelium (6,7), the OX26 monoclonal antibody (MAb) is able to undergo receptor-mediated transcytosis through the BBB (8–10). BDNF was attached to a conjugate of the OX26 MAb and neutral avidin following mono-biotinylation of the neurotrophin (3). In these previous studies, the neurotrophin was biotinylated at the ε-amino group of surface lysine residues (3), a reaction that is predicted to inactivate the biologic activity of the neurotrophin, since similar modifications inactivate nerve growth factor (NGF) (11). Therefore, the present studies describe mono-biotinylation of BDNF at carboxyl moieties on surface glutamate or aspartate residues. However, owing to the unfavorable plasma pharmacokinetics of BDNF (3,4), it was expected that brain uptake of the neurotrophin would not be optimized by conjugation to a BBB drug delivery system without parallel re-formulation of the neurotrophin that also optimizes plasma pharmacokinetics.

The plasma AUC of BDNF may be increased by protein pegylation of the neurotrophin (4). In previous studies, BDNF was pegylated with polyethyleneglycol (PEG) moieties of 2,000 Daltons (PEG^{2000}) at surface carboxyl residues using hydrazide linkers, and these studies demonstrated complete retention of the biologic activity of BDNF following carboxyl-directed pegylation (4). Both BBB PS product and plasma AUC could be optimized by the use of a novel bifunctional PEG linker in which a hydrazide moiety is attached to one end of the PEG polymer and a biotin moiety is attached to the other end. The availability of such a bifunctional PEG would allow for attachment of a pegylated BDNF molecule to a conjugate of the OX26 MAb and an avidin analogue. In the present studies, streptavidin (SA) is used, as the use of a neutral avidin analog optimizes pharmacokinetics as compared to the use of the cationic avidin (12).

The purpose of the present studies was to perform carboxyl-directed mono-biotinylation of BDNF with a PEG^{2000} linker and attach this complex to the OX26/SA BBB drug delivery system. The biologic activity of the neurotrophin fol-

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ABBREVIATIONS: BDNF, brain-derived neurotrophic factor; PEG, polyethyleneglycol; Hz, hydrazide; PEG^{2000}, PEG of 2,000 Dalton molecular weight; MAb, monoclonal antibody; NGF, nerve growth factor; PS, permeability-surface area; AUC, plasma area under the concentration curve; SA, streptavidin; OX26, murine MAb to the rat transferrin receptor; TCA, trichloroacetic acid; HABA, 2-hydroxyazobenzene-4′-carboxylic acid; BSA, bovine serum albumin; EDAC, N-methyl-N′-3-(dimethylaminopropyl)carbodiimide hydrochloride; BBB, blood-brain barrier; V_D, brain volume of distribution; Cl, systemic clearance; V_C, systemic volume of distribution; MRT, plasma mean residence time; ID, injected dose; bio, biotinylated; PK, pharmacokinetics; OX26/SA or SA-OX26, conjugate of SA and OX26 MAb.

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lowing attachment to the drug delivery system was examined as was the in vivo plasma pharmacokinetics and brain uptake of the neurotrophin.

MATERIALS AND METHODS

Materials

Human recombinant BDNF was produced in E. coli (13) and was obtained from Dr. James Miller (Amgen, Thousand Oaks, CA). [^{125}I]iodine was obtained from Amersham Corp. (Chicago, IL). Methoxy-PEG-hydrazide (Hz) (MW = 2,000 Da), designated PEG^{2000}-Hz, and biotin-PEG-Hz (MW = 2,000 Da), designated biotin-PEG^{2000}-Hz, were synthesized by Shearwater Polymers, Inc. (Huntsville, AL). N-methyl-N'-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDAC), biotin hydrazide, and all other reagents were obtained from Sigma Chemical Corp. (St. Louis, MO). High-Trap columns (5 ml) were obtained from Pharmacia (Piscataway, NJ). 3T3 cells transfected with the TrkB gene, designated TrkB-Fl#14/NIH3T3, were provided by Dr. Andrew Welcher, Amgen Corp (14). The CellTiter 96™ Aq_{ueous} non-radioactive cell proliferation assay was obtained from Promega Corp. (Madison, WI). The anti-phosphotyrosine antibody was purchased from Upstate Biotechnology (Lake Placid, NY), and the avidin-biotin peroxidase kit was obtained from Vector Labs (Burlingame, CA). The anti-TrkB antiserum was provided by Dr. Andrew Welcher (Amgen Inc.). Adult male Sprague-Dawley rats (250 g) were purchased from Harlan-Sprague-Dawley, Inc. (Indianapolis, IN).

Biotinyl-Pegylation of BDNF

Both pegylation and mono-biotinylation of BDNF residues was achieved with the combined use of methoxy-PEG^{2000}-Hz and biotinyl-PEG^{2000}-Hz, using reactions analogous to the carboxyl-directed biotinylation of NGF with biotin hydrazide (11). In this reaction, 1.0 mg of BDNF was dissolved in 0.2 ml of 0.1 M pyridine (pH = 4.8), and to this solution was added the following: 39.6 mg of PEG^{2000}-Hz, 3.0 mg biotin-PEG^{2000}-Hz, and 60 μl of 70 mg/ml EDAC. Following mixing, the reaction tube sat overnight at room temperature, and the next day, buffer A was added to 1.0 ml final volume, and the solution was applied to a 5 ml HiTrap copper affinity column, where buffer A = 0.05 M Na_2HPO_4/0.5 M NaCl/pH = 7.0. The HiTrap copper affinity column bound pegylated BDNF but not PEG polymers that were un-incorporated into BDNF protein (4). Prior to sample application, the HiTrap column was washed with 15 ml of water, 2.5 ml of 0.1 M $CuSO_4$, and 15 ml of water. Following application of the 1.0 ml of BDNF-PEG^{2000}-biotin, the column was washed with 20 ml of buffer A, which eluted the unreacted PEG polymers, and the pegylated BDNF was eluted with 15 ml of 50 mM EDTA in buffer A, with collection of 1.0 ml fractions. The copper/EDTA was removed by pooling the eluate and adding buffer A to a volume of 20 ml. A Centriprep-10 microconcentrator (Amicon Corp., Beverly, MA) was used to reduce the volume to 2 ml, and this cycle was repeated for 2 more times with a final reduction to 1.0 ml, measurement of protein content with the bicinchoninic acid (BCA) assay (Pierce Chemical Co., Rockford, IL.), and storage at −20°C until use. In this synthesis, the molar ratio of PEG-Hz/EDAC/BDNF carboxy residues was 22/22/1, and 7.0% of the total PEG-Hz contained biotin at the terminus of the PEG strand opposite of the Hz moiety. A 7.0% biotin content was determined with the HABA assay to yield, on average, of 1.0 biotin residues incorporated per BDNF homodimer.

The change in molecular weight of BDNF following pegylation, and the incorporation of biotin at the tip of the PEG strand, was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. BDNF-PEG^{2000}-biotin that eluted from the copper affinity column was applied to a 12% SDS-PAGE minigel (BioRad, Richmond, CA), and electrophoresis was performed at constant current (30 mAmp) for 2 hours at 4°C. Three identical samples of [^{125}I]-BDNF-PEG^{2000}-biotin were applied to the gel, and following SDS-PAGE, the gel was divided into 3 pieces. One gel slab was stained for protein content with Coomassie Blue; another gel slab was dried and applied to Kodak XO-Mat X-ray film for autoradiography; a third gel piece was blotted to nylon without methanol (100 Volts, 60 minutes, 4°C) and the nylon filter was subsequently stained for biotin content with the ABC kit (Vector Labs) using avidin and biotinylated peroxidase and diaminobenzidine as chromagen.

The biotin-PEG^{2000}-Hz was custom-synthesized by Shearwater Polymers, Inc., and was 100% incorporated with a single hydrazide residue and 100% incorporated with a single biotin residue as determined by NMR. The molecular weight of the bifunctional PEG was 2,493 Daltons, as determined by MALDI mass spectroscopy.

Iodination of BDNF-PEG^{2000}-Biotin

BDNF-PEG^{2000}-biotin was iodinated with ^{125}I and lactoperoxidase, as described previously (4), to a specific activity of 3.1 μCi/μg and a trichloroacetic acid (TCA) precipitability of 97%.

HABA Assay

The incorporation of biotin into BDNF at the end of the PEG^{2000} that was incorporated at surface carboxyl moieties, was determined with the avidin-HABA assay (15). A 0.3 ml mixture of HABA (72 μg/ml) and avidin (200 μg/ml) in 0.05 M Na_2HPO_4/0.15 M NaCl/pH = 6.0 was added to a spectrophotometer microcuvette. The absorbance at 500 nm was measured with a Beckmann spectrophotometer, and the cuvette was successively enriched with 10 μl-20μl of either 50 μM biotin standard, buffer A control, methoxy-PEG^{2000}-Hz, or BDNF-PEG^{2000}-biotin. The measurement of absorbance at 500 nm was repeated, and the decrease in absorbance at 500 nm caused by biotin displacement of HABA from avidin was recorded with each successive addition of 5–7 aliquots of either biotin standard or experimental sample.

Survival and Auto-Phosphorylation Assays

3T3 cells transfected with the TrkB gene were plated in 96-well culture dishes and grown in Dulbecco's modified Eagle medium with 10% fetal calf serum (FCS) and 400 μg/ml G418 (12,000 cells/well) (16). On day 2, the cells were rinsed with assay medium (RPMI 1640 medium containing 0.3 mg/ml L-glutamine and no calf serum), and then 100 μl of assay medium was added to each well followed by the addition of 50 μl BDNF

solutions. The latter was either BDNF, OX26/SA alone, bio-PEG^{2000}-BDNF without OX26/SA, or bio-PEG^{2000}-BDNF conjugated to OX26/SA. The CellTiter 96™ Aq_{ueous} non-radioactive cell proliferation assay (Promega) was performed exactly as described previously (4).

BDNF-induced auto-phosphorylation (14) of the 3T3-TrkB cells was assayed by incubating serum-starved cells (0.5% FCS overnight) with 0, 1, 10, or 100 ng/ml of BDNF solution. The latter contained either unconjugated BDNF, BDNF-PEG^{2000}-biotin, BDNF-PEG^{2000}-biotin conjugated to OX26/SA, or comparable concentrations of OX26/SA without BDNF. Following exposure of the cells to BDNF solutions for 15 minutes at 37°C, media was aspirated, and cells were lysed, immunoprecipitated with the anti-TrkB antiserum and protein G sepharose, and subjected to 7.5% SDS-PAGE exactly as described previously (4). Following SDS-PAGE, the gel was blotted to Immobilon-P with 10% methanol at 100 Volts for 60 minutes at 4°C, and the filter was stained with 2 μg/ml anti-phosphotyrosine antibody, as described previously (4). The filter was scanned with a Hewlett-Packard scanner, and the signal was quantitated by NIH Image and a Adobe Photoshop software on a Power Macintosh computer. The molecular size of the TrkB immunoprecipitate was estimated with biotinylated molecular weight standards and was 144 kDa.

Sephacryl S300 Gel Filtration Chromatography

Owing to the multivalency of streptavidin binding of biotin, the incorporation of more than one biotin residue per BDNF homodimer would create the formation of high molecular weight aggregates that would be selectively cleared by the liver and reticulo-endothelial system following intravenous administration in vivo. The aggregates of BDNF-PEG^{2000}-biotin with high degrees of biotinylation and OX26/SA were removed from either mono-biotinylated BDNF-PEG-biotin conjugated to OX26/SA or BDNF-PEG that contained no biotin by Sephacryl S300 gel filtration chromatography. [^{125}I]-BDNF-PEG^{2000}-biotin (117 μCi) was added to 200 μg of OX26/SA and applied to 2.6 × 96 cm column of Sephacryl S300 (Pharmacia). Elution was performed with 0.01 M Na_2HPO_4/0.5 M NaCl/0.05% Tween-20/pH = 7.4 with a flow rate of 15 ml/hour, and 3 ml fractions were collected. Three peaks were identified: the aggregate peak containing BDNF with high degrees of biotinylation, a middle peak containing mono-biotinylated BDNF-PEG^{2000}-biotin conjugated to OX26/SA, and a third peak that contained BDNF-PEG^{2000} that contained no incorporated biotin. The fractions for the three peaks were pooled and concentrated with a Centriprep-10 microconcentrator prior to intravenous injection in rats for plasma pharmacokinetics and brain uptake measurements.

Plasma Pharmacokinetics

Plasma pharmacokinetics and organ uptake were measured exactly as described previously (3), except the data were fit to a mono-exponential function rather than a bi-exponential equation. Ketamine-xylazine anesthetized male Sprague-Dawley rats were administered either (a) [^{125}I]-BDNF (5 μCi/rat), (b) [^{125}I]-BDNF-PEG^{2000}-biotin (5 μCi/rat), or (c) [^{125}I]-BDNF-PEG^{2000}-biotin (5 μCi/rat) conjugated to OX26/SA (10 μg/rat). These doses were equivalent to 0.06 and 0.05 nmol of BDNF-PEG^{2000}-biotin and OX26/SA, respectively. Since there are 3–4 biotin binding sites per OX26/SA conjugate (12), 30% of the biotin binding sites were occupied by the [^{125}I]-BDNF-PEG^{2000}-biotin. These methods adhere to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985). Brain uptake parameters were calculated from measurements of total brain radioactivity. Previous studies using the capillary depletion method have shown that in the case of the OX26 MAb, measurements of total brain radioactivity reflect antibody delivered through the BBB into brain (8).

RESULTS

The formulation of BDNF attached to OX26/SA is depicted in Figure 1 and involves the use of pegylated BDNF that contains a PEG^{2000} linker between the BDNF carboxyl residue and the biotin moiety. This formulation allows for the use of carboxyl-directed pegylation of BDNF, to optimize plasma pharmacokinetics, and for mono-biotinylation of the BDNF-PEG^{2000} to allow for attachment to the OX26/SA BBB drug delivery system. Following attachment of PEG^{2000} to BDNF carboxyl residues, the molecular weight of the BDNF monomer was increased from 14,000 Daltons to approximately 40,000 Daltons (Figure 2, Coomassie Blue stain). Film autoradiography demonstrated the radiolabeled form of BDNF had a molecular weight identical to the BDNF detected by Coomassie Blue staining (Figure 2). Western blotting allowed for detection of the biotin residue on the BDNF-PEG^{2000}-biotin, and the molecular weight of the BDNF determined by Western blotting was identical to that detected with either Coomassie Blue staining or film autoradiography (Figure 2).

The biologic activity of the BDNF-PEG^{2000}-biotin either free or attached to the OX26/SA conjugate was retained based

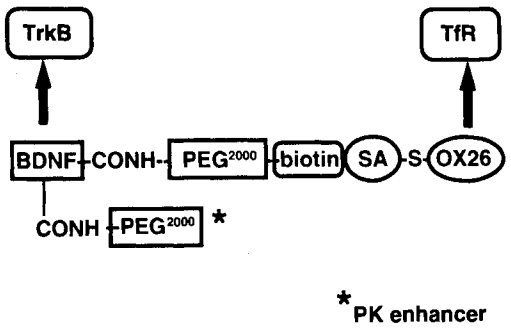

Fig. 1. Structure of conjugate of BDNF attached to the OX26 MAb using both pegylation and avidin-biotin technology. The stable thioether (-S-) linkage between OX26 and streptavidin (SA) is prepared in parallel with mono-biotinylation of BDNF. BDNF is mono-biotinylated using a bifunctional PEG^{2000} derivative that contains a hydrazide moiety at one end for attachment to BDNF carboxyl moieties and a biotin group at the other end of the PEG strand. The BDNF is pegylated with PEG^{2000}-hydrazide and 7% of the PEG^{2000} contains the biotin-PEG^{2000}-hydrazide. The BDNF-PEG^{2000}-biotin is then conjugated to OX26/SA. PEG:polyethyleneglycol. The tri-functionality of the BDNF formulation is illustrated: (a) binding to TrkB for pharmacologic effect, (b) binding to the transferrin receptor (TfR) for BBB transport, and (c) pegylation as a pharmacokinetic (PK) enhancer; in this context, a PK enhancer is a moiety that delays the clearance of drug from plasma to enhance the plasma AUC.

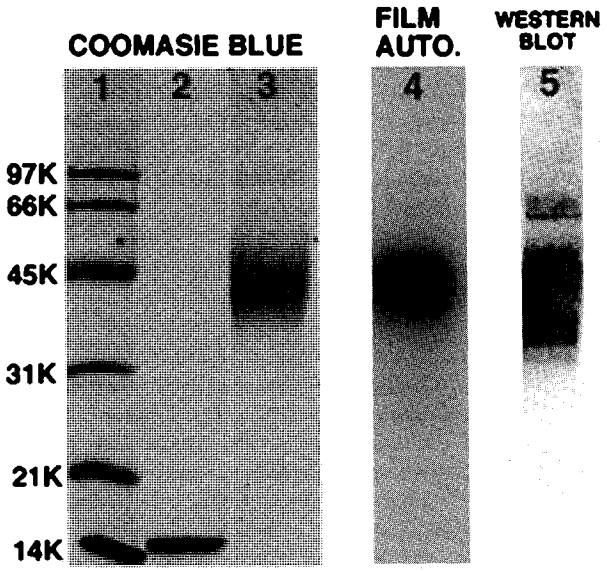

Fig. 2. Either native BDNF (lane 2) or [^{125}I]BDNF-PEG^{2000}-biotin (lanes 3, 4, 5) was applied to a 12% SDS-PAGE gel. One-third of the gel was subjected to Coomassie Blue staining (lanes 1–3). The Coomassie Blue stain of molecular weight standards (lane 1) or unconjugated BDNF (lane 2) is shown. Another third of the gel was dried and exposed for film autoradiography to show the migration of the radiolabeled form of the BDNF-PEG^{2000}-biotin (lane 4). Another third of the gel was blotted to a filter, which was then stained with avidin and biotinylated peroxidase to visualize the attachment of biotin to the BDNF-PEG^{2000} (lane 5).

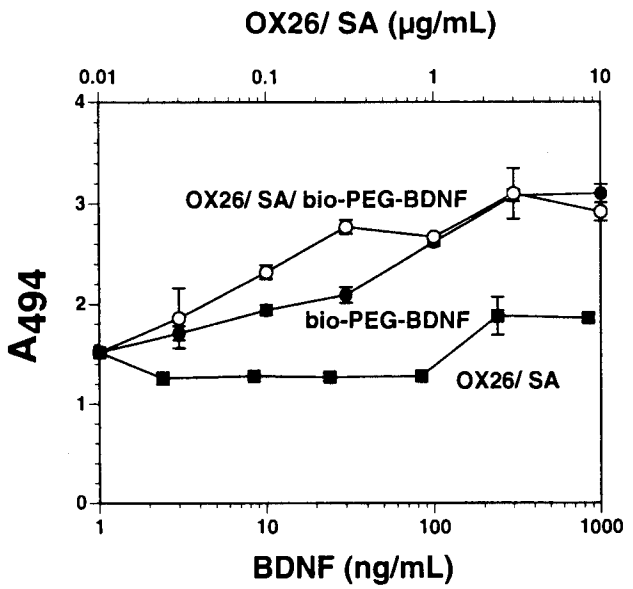

Fig. 3. 3T3-TrkB cell survival in the presence of OX26/SA without BDNF, in the presence of biotinylated (bio)-PEG-BDNF without OX26/SA, and bio-PEG-BDNF attached to the OX26/SA vector, designated by the closed squares, closed circles, and open circles, respectively. The concentration for either the BDNF or the OX26/SA vector is given. Data are mean ± SE (n = 3 replicates per point). SA = streptavidin. PEG = polyethyleneglycol; bio = biotinylated.

on either the cell survival studies (Figure 3) or the TrkB autophosphorylation assay (Figure 4).

BDNF was pegylated with R-PEG^{2000}-Hz, where R = either a methoxy residue or a biotin moiety, and the percent of total PEG that contained the biotin moiety (relative to the methoxy terminus) was varied from 7% to 17% (Figure 5A). The HABA assay demonstrated the use of a 7% biotin formulation achieved the incorporation of one biotin moiety per BDNF dimer (Figure 5B). However, the HABA assay reflects the average incorporation of biotin into BDNF molecules and some neurotrophin molecules may contain no biotin and some neurotrophin molecules may contain 2 or more biotin residues per BDNF dimer; the latter formulations involving multi-biotinylation would lead to the formation of high molecular weight aggregates following conjugation to OX26/SA. These high molecular weight aggregates were removed by Sephacryl S300 gel filtration chromatography. Following the conjugation of BDNF-PEG^{2000}-biotin to OX26/SA using a formulation that, based on the HABA assay, contained one biotin moiety incorporated per BDNF dimer, the elution from the Sephacryl S300 column demonstrated 3 peaks (Figure 6A). Peak 1 is the high molecular weight peak and contains aggregates. Peak 2 contains mono-biotinylated forms of BDNF-PEG^{2000}-biotin conjugated to OX26/SA, and peak 3 contains BDNF-PEG^{2000} that has no incorporated biotin. These 3 formulations of BDNF were injected separately into anesthetized rats, and the plasma concentration curves for the first 30 minutes after injection are shown in Figure 6B. The peak 3 BDNF, which contains no biotin, is cleared very slowly from blood, consistent with the effects of pegyla-

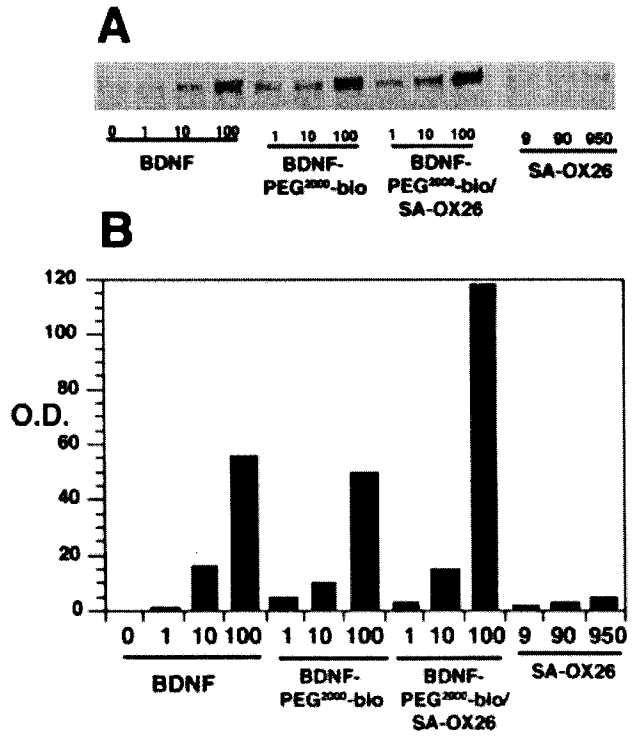

Fig. 4. The induction of TrkB autophosphorylation by BDNF, BDNF-PEG^{2000}-biotin, BDNF-PEG^{2000}-biotin attached to the OX26/SA vector, or OX26/SA without BDNF is shown in serum starved 3T3-TrkB cells. (A) Western blot after staining with an anti-phosphotyrosine antibody. The immunoreactive TrkB migrated at a molecular weight of 144 kDa. (B) Optical density (OD) of the scanned film at each concentration of BDNF or SA-OX26 conjugate given in ng/ml. The SA-OX26 designation is identical to OX26/SA.

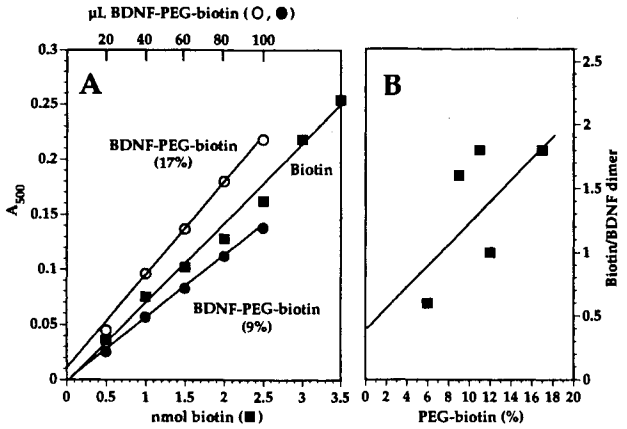

Fig. 5. The binding of 2-hydroxyazobenzene-4'-carboxylic acid (HABA) to avidin is proportional to the absorbance at 500 nm (A_{500}), and the HABA binding to avidin is inhibited by biotin or biotin analogues. (A) The nmol of biotin standard or the μL of BDNF-PEG^{2000}-biotin is plotted versus the decrease in absorbance at 500 nm. Two different formulations of BDNF-PEG-biotin are shown, wherein the fraction of hydrazide-PEG-biotin relative to the total hydrazide-PEG was 9% or 17%, respectively. (B) The incorporation of biotin per BDNF dimer is plotted versus the % of hydrazide-PEG-biotin that contains biotin-PEG-hydrazide in the pegylation reaction.

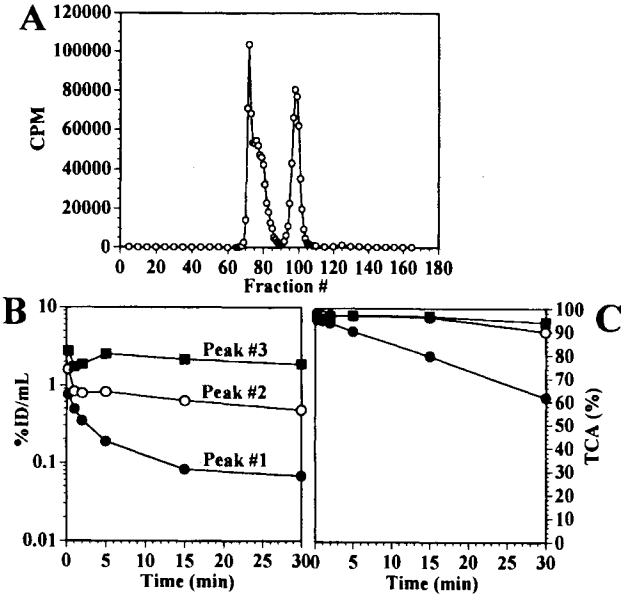

Fig. 6. (A) Sephacryl S300 gel filtration chromatographic separation of [^{125}I]BDNF-PEG^{2000}-biotin attached to the OX26/SA vector. Three peaks are visualized. The peak migrating at 70–72 ml is the high molecular weight aggregate peak formed by OX26/SA and multi-biotinylated forms of BDNF-PEG-biotin; peak 2 migrating at fractions 76–80 contains mono-biotinylated BDNF-PEG-biotin attached to OX26/SA, and peak 3 migrating at tubes 90-100 represents BDNF-PEG that contains no incorporated biotin and is not attached to the OX26/SA vector. (B) The clearance from rat plasma in vivo of [^{125}I]BDNF-PEG^{2000}-biotin following injection of peaks 1, 2, or 3 into separate rats, respectively. (C) The plasma trichloroacetic acid (TCA) precipitability of peak 1 (closed circle), peak 2 (open circle), or peak 3 (closed square), respectively.

tion on plasma clearance of the neurotrophin, and is slowly degraded in vivo based on measurement of TCA soluble radioactivity (Figure 6C). Peak 1 is rapidly removed from plasma following intravenous injection and is rapidly converted to TCA soluble metabolites (Figures 6B–C). Conversely, the BDNF formulation in peak 2, which contains mono-biotinylated BDNF conjugated to OX26/SA, is cleared with intermediate plasma pharmacokinetics and is metabolically stable (Figures 6B–C). The peak 2 formulation was subsequently used for brain uptake and plasma pharmacokinetic measurements.

The brain volume of distribution (V_D) of the [^{125}I]-BDNF, [^{125}I]BDNF-PEG^{2000}-biotin, and [^{125}I]BDNF-PEG^{2000}-biotin conjugated to OX26/SA is shown in Figure 7. The V_D for unconjugated and non-pegylated BDNF is high owing to the brain uptake of low molecular weight metabolites generated from the rapid metabolism of BDNF. Conversely, the brain V_D of BDNF-PEG^{2000}-biotin is reduced owing to the inhibition of BDNF metabolism caused by pegylation. The brain V_D, the BBB PS product, and the brain uptake (%ID/g) of the BDNF-PEG^{2000}-biotin is increased following conjugation to OX26/SA (Figure 7), consistent with drug delivery of the neurotrophin through the BBB mediated by the OX26 MAb delivery system (Figure 1). The pharmacokinetic parameters for the 3 forms of BDNF shown in Figure 7 are given in Table 1.

DISCUSSION

The results of these studies are consistent with the following conclusions. First, BDNF may be formulated with both pegylation (to allow for optimized plasma pharmacokinetics), and mono-biotinylation (to allow for attachment to the OX26/SA BBB drug delivery system), using a novel bifunctional PEG that contains a hydrazide at one end, for attachment to BDNF carboxyl residues, and a biotin moiety at the other end (Figures 1–2). Second, the BDNF-PEG^{2000}-biotin is biologically active despite conjugation to the OX26/SA delivery system based on either 3T3-TrkB cell survival assays (Figure 3) or TrkB autophosphorylation assays (Figure 4). Third, the plasma phar-

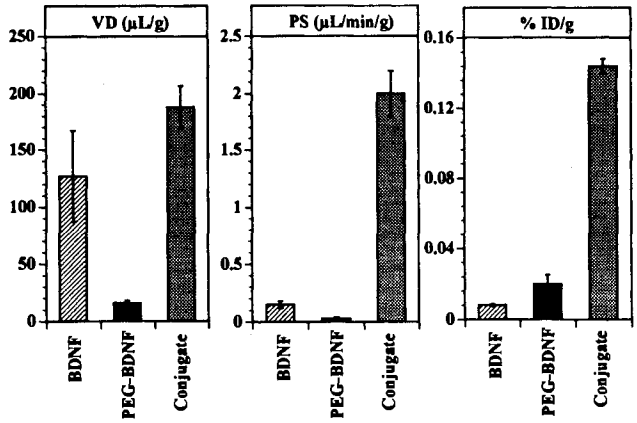

Fig. 7. The brain volume of distribution (V_D), the blood-brain barrier permeability-surface area (PS) product, and the brain uptake, expressed as percent of injected dose (ID) per gram (g) brain is shown for (a) [^{125}I]BDNF, (b) [^{125}I]BDNF-PEG^{2000}-biotin, (designated PEG-BDNF in the Figure), and (c) [^{125}I]BDNF-PEG^{2000}-biotin attached to the OX26/SA conjugate (designated conjugate in the Figure). Data are mean ± SE (n = 3 rats). All measurements made at 60 minutes after intravenous injection.

Table 1. Pharmacokinetic Parameters

Parameter	Units	BDNF[a]	BDNF-PEG^{2000}-biotin[b]	BDNF-PEG^{2000}-bio/SA-OX26[a]
A	%ID/mL	3.9 ± 0.9	7.4 ± 0.5	1.6 ± 0.1
k	min^{-1}	0.068 ± 0.006	0.0062 ± 0.0013	0.014 ± 0.001
$t_{1/2}$	min	10 ± 1	120 ± 22	50 ± 3
$AUC\|_0^t$	%ID·min/mL	58 ± 14	635 ± 79	74 ± 7
$AUC\|_0^\infty$	%ID·min/mL	59 ± 15	1307 ± 292	133 ± 17
V_C	mL/kg	80 ± 16	41 ± 2	135 ± 9
Cl	mL/min/kg	5.5 ± 1.4	0.26 ± 0.06	1.9 ± 0.2
MRT	min	15 ± 1	173 ± 32	73 ± 5

Note: All 3 BDNF formulations were radiolabeled with [^{125}I] and injected intravenously in anesthetized rats. Plasma TCA-precipitable radioactivity over 60–120 minutes was measured, and analyzed by linear regression analysis and a mono-exponential function to generate the intercept (A) and slope (k). The plasma $t_{1/2}$, the area under the plasma concentration curve (AUC), systemic volume of distribution (V_C), systemic clearance (Cl), and plasma mean residence time (MRT) were calculated from A and k as described previously (4).
[a] t = 60 min.
[b] t = 120 min.

macokinetics of BDNF-PEG^{2000}-biotin are unfavorable if multi-biotinylated forms of the neurotrophin are used (Figures 6B, C) with secondary formation of high molecular weight aggregates with OX26/SA, but these aggregates can be removed by gel filtration chromatography (Figure 6A). The brain uptake of mono-biotinylated BDNF-PEG^{2000} that is conjugated to the OX26/SA is high, 0.15%ID/g (Figure 7), and reaches a value that is double that of the brain uptake of morphine.

The retention of biologic activity following carboxyl-directed pegylation/biotinylation of BDNF is analogous to the same findings made for carboxyl-directed biotinylation of NGF (11). Conversely, modification of external lysine residues results in a loss of biologic activity of NGF (11). Surface charge models demonstrate that the NGF-like neurotrophins have segregated areas of cationic and anionic charge on the surface of the protein (17), and that the cationic grooves are involved in high affinity binding to the Trk receptor (18). This segregation of anionic (acidic amino acids) and cationic (basic amino acids) charges on the neurotrophin molecule may explain why the neurotrophin biologic activity is retained despite extensive pegylation (4), as the PEG moieties are selectively attached to carboxyl moieties on surface glutamate or aspartate residues. Placement of PEG polymers at these sites inhibits liver uptake, which optimizes the plasma pharmacokinetics of the neurotrophin, but causes no interference in TrkB binding and biologic activity of BDNF(4). The molecular weight of the BDNF is increased from 14,000 to approximately 40,000 Daltons on SDS-PAGE (Figure 2). Assuming the attachment of each PEG^{2000} moiety increases the molecular weight on SDS-PAGE proportionately, then the studies suggest all 12 glutamate and aspartate residues on the BDNF monomer were pegylated.

The bifunctional PEG derivative that contains a hydrazide moiety at one end, allows for attachment to surface carboxyl residues, and a biotin moiety at the other end, allows for mono-biotinylation of the pegylated neurotrophin, wherein the biotin residue is placed at the tip of the PEG strand. The placement of a biotin moiety, per se, does not influence BBB permeability because the brain uptake of BDNF-PEG^{2000}, as reported previously (4), and the brain uptake of BDNF-PEG^{2000}-biotin (Figure 7), are both low and indicative of negligible BBB transport. The formulation that places the biotin moiety at the tip of the PEG strand was deemed essential because if the biotin was placed on the surface of the neurotrophin molecule, the binding of the OX26 MAb to the BBB transferrin receptor would be impaired by steric interference from the PEG strands attached to the neurotrophin. However, using the molecular formulation depicted in Figure 1, there is no steric hindrance because the biotin is placed at the tip of the PEG strand. Similar strategies have been employed for attachment of the OX26 MAb to the tip of the PEG strand on the surface of liposomes (19). This placement of the OX26/SA drug delivery system, which has a molecular weight approximately 5 times greater than that of the pegylated BDNF, also eliminates any steric hindrance that might be exerted by attachment of the OX26/SA vector to the neurotrophin with respect to neurotrophin binding to the TrkB receptor. Retention of binding to TrkB is demonstrated by both the cell survival assays (Figure 3) and the TrkB autophosphorylation assays (Figure 4).

In order to optimize the plasma pharmacokinetics and the brain delivery of the BDNF-PEG^{2000}-biotin following attachment to the OX26/SA delivery system, it is imperative that any aggregates formed because of multi-biotinylation of the BDNF be removed prior to measurement of plasma pharmacokinetics (Figure 6). Owing to the multivalency of streptavidin binding of biotin, the addition of OX26/SA to multi-biotinylated BDNF-PEG-biotin would result in the formation of high molecular weight aggregates that are rapidly removed following systemic administration (Figure 6). If these aggregates are initially removed by gel filtration, the plasma pharmacokinetics are optimized and the brain uptake is maximized following the intravenous injection of mono-biotinylated BDNF-PEG-biotin attached to the OX26/SA delivery system (Figure 7). The brain uptake at 60 minutes after intravenous injection, 0.15%ID/g, is 2-fold greater than the maximal brain uptake of morphine (20), a neuroactive small molecule. The data in Figure 7 demonstrate a marked decrease of the brain V_D of BDNF following pegylation. The high brain V_D of non-pegylated BDNF shown in Figure 7 is an artifact; as demonstrated previously (4), the brain V_D of [^{125}I]-BDNF decreases with increasing degrees of pegylation. The pegylation decreases systemic degradation of [^{125}I]-BDNF and decreases the release of TCA soluble metabolites, i.e., [^{125}I]-tyrosine, which cross the BBB by amino acid transport systems and yield a spuriously high V_D value for [^{125}I]-BDNF (4). A more accurate measure of the brain uptake of the neurotrophin is demonstrated with BDNF-PEG^{2000}-biotin (Figure 7). The increase in the BBB PS product and the brain

uptake of the BDNF-PEG^{2000}-biotin following biotinylation and attachment of the OX26/SA delivery system is due to the enhancement of BBB transport caused by receptor-mediated transcytosis of the conjugate through the BBB on the brain endothelial transferrin receptor. The affinity of the OX26 MAb for the BBB transferrin receptor is not affected by attachment of streptavidin and the BDNF-PEG^{2000}-biotin, because the BBB PS product of [^{125}I]-BDNF^{2000}-biotin/SA-OX26, 2.0 ± 0.2 uL/min/g (Figure 7), is not significantly different from the BBB PS product for [^{3}H]-OX26, 1.6 ± 0.2 uL/min/g (21).

In summary, these studies demonstrate that pegylation alone will not facilitate brain uptake despite the optimization of the plasma pharmacokinetics. Rather, the neurotrophin must be a special molecular formulation (Figure 1) that incorporates both pegylation technology, to optimize plasma pharmacokinetics, and BBB drug delivery technology, to enable receptor-mediated transcytosis through the BBB, in order to achieve optimal brain uptake of the neurotrophin. The level of brain uptake of BDNF achieved with these studies, 0.15%ID/g (Figure 7), would generate a neurotrophin concentration in brain of approximately 20 ng/g following the intravenous injection of a relatively low systemic dose, 12.5 μg BDNF, in an 0.25 kg rat. This concentration of neurotrophin causes significant autophosphorylation of the TrkB receptor (Figure 4), and is expected to cause CNS pharmacologic effects in vivo.

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