Neuroprotection with a Brain-Penetrating Biologic Tumor Necrosis Factor Inhibitor

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Abbreviations: BBB, blood-brain barrier; MAb, monoclonal antibody; TNF, tumor necrosis factor; TNFI, TNF inhibitor; TNFR, TNF receptor; PD, Parkinson’s disease; AD, Alzheimer’s disease; TiR, transferrin receptor; cTiRMAb, chimeric MAb against mouse TiR; RMT, receptor-mediated transport; ECD, extracellular domain; HIR, human insulin receptor; TH, tyrosine hydroxylase; ID, injected dose; CHO, Chinese hamster ovary; RRA, radio-receptor assay

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ABSTRACT

Biologic tumor necrosis factor (TNF)-α inhibitors do not cross the blood-brain barrier (BBB). A BBB-penetrating TNFα-inhibitor was engineered by fusion of the extracellular domain of the type II human TNF receptor (TNFR) to the carboxyl terminus of the heavy chain of a mouse/rat chimeric monoclonal antibody (MAb) against the mouse transferrin receptor (TfR), and this fusion protein is designated cTfRMAb-TNFR. The cTfRMAb-TNFR fusion protein and etanercept bound human TNFα with high affinity and a KD of 374 ± 77 pM and 280 ± 80 pM, respectively. Neuroprotection in brain in vivo following intravenous (IV) administration of the fusion protein was examined in a mouse model of Parkinson’s disease. Mice were also treated with saline or a non-BBB penetrating TNF decoy receptor, etanercept. Following intra-cerebral injection of the nigral-striatal toxin, 6-hydroxydopamine, mice were treated every other day for 3 weeks. Treatment with the cTfRMAb-TNFR fusion protein caused a 83% decrease in apomorphine-induced rotation, a 67% decrease in amphetamine-induced rotation, a 82% increase in vibrissae-elicited forelimb placing, and a 130% increase in striatal tyrosine hydroxylase (TH) enzyme activity. In contrast, chronic treatment with etanercept, which does not cross the BBB, had no effect on neurobehavior or striatal TH enzyme activity. A bridging ELISA specific for the cTfRMAb-TNFR fusion protein showed the immune response generated in the mice was low titer. In conclusion, a biologic TNF-inhibitor is neuroprotective following intravenous administration in a mouse model of neurodegeneration, providing the TNF decoy receptor is re-engineered to cross the BBB.
Tumor necrosis factor (TNF)-α is a pro-inflammatory cytokine in peripheral tissues, and the leading TNFα-inhibitors (TNFI) are biologic drugs, including etanercept, a TNF decoy receptor-Fc fusion protein, infliximab, a chimeric anti-TNFα monoclonal antibody (MAb), and adalimumab, a human anti-TNFα MAb (Tansey and Szymkowski, 2009). TNFα also plays a pathologic role in brain disorders, including Parkinson’s disease (PD) (McCoy et al, 2006), Alzheimer’s disease (AD) (He et al, 2007), and depression (Himmerich et al, 2008). However, the biologic TNFI’s cannot be developed for treatment of the brain, because these large molecule drugs do not cross the blood-brain barrier (BBB).

Biologic TNFIs can be re-engineered for BBB penetration by engineering fusion proteins of the TNFI and a BBB molecular Trojan horse (MTH) (Pardridge, 2010). The latter is a peptide or peptidomimetic MAb that traverses the BBB via transport on an endogenous receptor-mediated transport (RMT) system, such as the insulin receptor or the transferrin receptor (TfR). The most potent BBB MTH is a genetically engineered MAb against the human insulin receptor (HIR) (Boado et al, 2007). A fusion protein of the HIRMAb and the extracellular domain (ECD) of the type II TNF receptor (TNFR) has been engineered, and is designated the HIRMAb-TNFR fusion protein (Hui et al, 2009). The brain uptake of the HIRMAb-TNFR fusion protein in the Rhesus monkey is high, >3% of injected dose (ID)/brain, whereas etanercept does not cross the BBB in vivo (Boado et al, 2010). However, the HIRMAb only cross reacts with the insulin receptor of the Old World primate, such as the Rhesus monkey (Pardridge et al, 1995), and does not cross react with the insulin receptor of New World primates or lower animals. There is no known MAb against the mouse insulin receptor that can be used as a BBB MTH in the mouse. Therefore, a surrogate MTH for the mouse has been engineered, which is a chimeric MAb against the mouse TfR, designated the cTfRMAb (Boado et al, 2009). A fusion protein of the
cTfRMAb and the ECD of the TNFR-II has been engineered (Zhou et al, 2011a), and the structure of the cTfRMAb-TNFR fusion protein is shown in Figure 1A. The decoy receptor is fused to the carboxyl terminus of the heavy chain of the IgG part of the fusion protein. In contrast, for the engineering of etanercept (Peppel et al, 1997), the TNFR decoy receptor is fused to the amino terminus of the heavy chain of the Fc fragment (Figure 1B).

The present investigation tests the neuroprotective actions of both the cTfRMAb-TNFR fusion protein and etanercept in the 6-hydroxydopamine mouse model of PD. The fusion proteins are administered every other day over a 3-week period by intravenous injection in the tail vein. Neuroprotection is assessed with 3 assays of neurobehavior, with striatal tyrosine hydroxylase (TH) enzyme activity and TH immunocytochemistry. The level of immune response in the mice caused by chronic administration of the cTfRMAb-TNFR fusion protein is assessed with a bridging ELISA.
Methods

**Mouse Parkinson’s disease model and treatment.** All procedures were approved by the UCLA Animal Research Committee. Adult male C57BL/6J mice (Jackson Labs, Bar Harbor, ME) weighing 25-32 g were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) intraperitoneally. Animals received a unilateral intra-cerebral injection of a total of 12 ug of 6-hydroxydopamine·HBr (Sigma Chemical Co.) dissolved in 0.02% ascorbic acid in 0.9% saline. The 6-hydroxydopamine (6 ug in 2 uL) was injected into the right striatum at 2 locations as described previously (Fu et al, 2010). The toxin was injected into the striatum at sites with the following stereotaxic coordinates: +1.0 mm relative to bregma, 2.1 mm relative to midline, 2.9 mm below the skull surface (site 1); +0.3 mm relative to bregma, 2.3 mm relative to midline, 2.9 mm below the skull surface (site 2). Mice were treated intravenously with either 1 of 3 treatment drugs: saline (n=10 mice); etanercept, 1.0 mg/kg (n=10 mice); or the cTfRMAb-TNFR fusion protein, 1.0 mg/kg (n=10 mice), every 2 days over the following 3 weeks, with the first dose given 1 hour after toxin injection into the brain. Treatment drugs were injected IV via the tail vein in a volume of 50 uL/mouse. The mice were euthanized at 3 weeks following toxin administration for measurement of striatal TH enzyme activity.

Etanercept (Enbrel®) was obtained from the UCLA Pharmacy. The cTfRMAb-TNFR fusion protein was purified by protein G affinity chromatography of serum free medium conditioned by stably transfected Chinese hamster ovary (CHO) cells as described previously (Zhou et al, 2011a). The 235 amino acid extracellular domain of the type II human TNFR, minus the signal peptide, was fused to the carboxyl terminus of the heavy chain of the cTfRMAb (Figure 1A) as described previously (Zhou et al, 2011a). The fusion protein was formulated in 0.01 M sodium acetated buffered saline, pH=6.5, and was stored either sterile filtered at 4ºC or at
-70°C. The molecular weight (MW) of the cTfRMAb-TNFR fusion protein is 195,200 Da (Zhou et al., 2011a), whereas the MW of etanercept is 51,200 Da. Therefore, at a systemic dose of each fusion protein of 1 mg/kg, a nearly 4-fold molar excess of etanercept was administered.

**TNFα radio-receptor assay.** The saturable binding of human TNFα to either etanercept, or the cTfRMAb-TNFR fusion protein, was determined with a radio-receptor assay (RRA), as described previously (Hui et al., 2009). For TNFα binding to either the cTfRMAb-TNFR fusion protein, or to a mouse IgG1 negative control, a goat anti-mouse IgG1 Fc antibody (Bethyl Labs) was plated in 96-well plates (0.2 ug/well). For TNFα binding to either etanercept, or to a human IgG1 negative control, a mouse anti-human IgG1 Fc antibody (Invitrogen) was plated in 96-well plates (0.2 ug/well). The fusion protein, or negative control antibody, was plated (100 ng/well), followed by a 1 hour incubation at room temperature. The wells were then washed with PBS, followed by the addition of 100 uL/well of a co-mixture of [125I]-human TNFα (Perkin Elmer, Boston, MA; specific activity=91 uCi/ug) at a concentration of 0.01 uCi/well (0.1 uCi/mL; 1.1 ng/mL; 60 pM) and various concentrations of unlabeled human TNFα, followed by a 3 hour incubation at room temperature. The wells were washed and bound radioactivity determined as described previously (Hui et al., 2009). The half-saturation constant, KD, of TNFα binding to the cTfRMAb-TNFR or etanercept fusion protein was determined by non-linear regression analysis using the BMDP2007e software (Statistical Solutions, Cork, Ireland), following fitting of binding data to the following equation:

\[
\text{Bound} = \frac{(B_{\text{max}})(C)}{(KD+C)},
\]

where Bmax is the maximal binding and C= the concentration of TNFα.

**Behavioral testing.** Beginning 1 week after the toxin administration, mice were tested weekly for apomorphine- and amphetamine-induced rotation, which was performed on separate
days, as described previously (Fu et al, 2010). A vibrissae-elicited forelimb placing trial in the mice was performed at the end of the 3 weeks of treatment (Fu et al, 2010).

**Tyrosine hydroxylase enzyme activity** Homogenates of mouse brain striatum (left and right side) and frontal cortex were prepared with a Polytron homogenizer in 5 mM KPO4/0.1% Triton X-100, pH=6.3 followed by centrifugation. After an aliquot was removed for measurement of protein with the bicinchoninic acid (BCA) assay, dithiothreitol was added to the supernatant to 1 mM, and the supernatant was stored at -70C until assay. The TH enzyme activity in the supernatant was measured with [3,5-3H]-L-tyrosine (Perkin Elmer, Boston, MA) as substrate. The purity of the [3,5-3H]-L-tyrosine was assessed by thin layer chromatography. TH enzyme activity converts [3,5-3H]-L-tyrosine to L-DOPA and [3H]-water. The [3H]-water product was separated from the [3H]-tyrosine substrate with a charcoal separation technique, as described previously (Fu et al, 2010). Any residual [3H]-water present in the [3,5-3H]-L-tyrosine was accounted for with determinations of assay blanks in each assay. The assay was validated with [3H]-water (Perkin Elmer), which showed the [3H]-water was 100% recovered in the supernatant after removal of amino acid by the charcoal. TH enzyme activity was measured at 37ºC for 30 min, and expressed as pmol/hour/mg protein.

**Tyrosine hydroxylase immunocytochemistry.** The brain was removed and coronal blocks were frozen in powdered dry ice, followed by embedding in Tissue Tek OCT medium and re-frozen and blocks were stored at -70C. Frozen sections (20 micron thickness) were prepared at -20C on a Micron Instruments (San Marcos, CA) cryostat. Sections were fixed in cold acetone/methanol (1:1) at -20C for 20 minutes. Immune staining was performed with an affinity purified rabbit antibody against rat TH, which cross reacts with all forms of mammalian TH (Pel-Freez, Rogers, AR), which was diluted 1:1000 in phosphate buffered saline (PBS) with 0.3%
Triton X-100 and 3% horse serum. The secondary antibody was 2 μg/mL biotinylated horse anti-rabbit IgG (Vector Labs, Burlingame, CA). Immune detection was performed with the ImmPACT DAB kit (Vector Labs) using dianaminobenzidine. The sections were not counterstained, and were scanned with a UMAX PowerLook III scanner with transparency adapter. The striatal immunostaining on the lesioned and non-lesioned side was quantitated by determination of optical density using the NIH Image software (version 1.62). The entire striatum on both the lesioned and non-lesioned side was outlined for measurement of density of staining. In addition, the cortex was outlined as a measure of the background, and the cortical density was subtracted from the striatal density to yield the background-corrected density of striatal immunostaining.

**Immunity ELISA.** The presence of anti-cTfRMAb-TNFR fusion protein antibodies in mouse plasma was measured with a 2-site sandwich ELISA described previously (Zhou et al, 2011b). The cTfRMAb-TNFR fusion protein is used as the capture reagent and biotinylated cTfRMAb-TNFR fusion protein as the detector reagent. The mouse plasma was diluted 1:50 in PBS. The capture reagent was plated overnight at 4C in 96-wells in 100 μL (250 ng)/well in 0.05 M NaHCO3/8.3. The wells were blocked with PBS containing 1% bovine serum albumin (PBSB), followed by the addition of 100 μL/well of the 1:50 dilution of mouse plasma. After a 60 min incubation at 37C, the wells were washed with PBSB, and the wells were incubated with biotinylated cTfRMAb-TNFR fusion protein (25 ng/well) for 60 min. The wells were washed with PBSB, followed by incubation with 100 μL (500 ng/well) of a streptavidin-peroxidase conjugate (#SA-5004, Vector Labs) for 30 min at room temperature (RT). The wells were washed with PBSB, and 100 μL/well of o-phenylenediamine/H2O2 developing solution (#P5412, Sigma Chemical Co, St. Louis, MO) was added for a 15 min incubation in the dark at RT. The reaction was stopped by the addition of 100 μL/well of 1 M HCl, followed by the measurement.
of absorbance at 492 nm and 650 nm. The A650 was subtracted from the A492. The (A492-A650) for the PBSB blank was then subtracted from the (A492-A650) for the sample.

The cTfRMAb-TNFR fusion protein was biotinylated with sulfo-biotin-LC-LC-N-hydroxysuccinimide, where LC=long chain (#21338, Pierce Chemical Co.). The biotinylation of the cTfRMAb-TNFR fusion protein was confirmed by SDS-PAGE and Western blotting, where the blot was probed with avidin and biotinylated peroxidase. The non-biotinylated cTfRMAb-TNFR fusion protein was tested as a negative control.

**Statistical analysis.** Statistical differences between saline, etanercept, and fusion protein treated mice were determined with analysis of variance (ANOVA) with Bonferroni correction. Statistical differences between striatal immuno-staining on the lesioned and non-lesioned side was determined with Student’s t-test.
Results

TNFα bound to both the cTfRMAb-TNFR fusion protein and to etanercept with high affinity with a KD of 374 ± 77 pM and 280 ± 80 pM, respectively (Figure 2).

Mice tolerated the chronic treatment with either etanercept or the cTfRMAb-TNFR fusion protein, and no mice exhibited signs of immune reaction to the study drugs, and no weight loss was observed in any of the treatment groups. Treatment of PD mice with chronic intravenous cTfRMAb-TNFR fusion protein resulted in a 75-83% reduction in apomorphine-induced rotation as compared to the saline treated mice, whereas etanercept had no significant effect on drug induced rotation (Figure 3). Treatment of PD mice with chronic intravenous cTfRMAb-TNFR fusion protein caused a 45-67% reduction in amphetamine-induced rotation as compared to the saline treated mice, whereas etanercept had no significant effect on drug induced rotation (Figure 4). Treatment of PD mice with chronic intravenous cTfRMAb-TNFR fusion protein caused a 82% increase in vibrissae-elicited forelimb placing score compared to the saline treated mice, whereas etanercept had no significant effect on forelimb placing (Figure 5). Treatment of PD mice with chronic cTfRMAb-TNFR fusion protein caused a 130% increase in striatal tyrosine hydroxylase (TH) enzyme activity on the lesioned (right) side, relative to the saline treated mice, whereas etanercept had no significant effect on striatal TH enzyme activity (Figure 6). Chronic treatment with the cTfRMAb-TNFR fusion protein had no effect on TH enzyme activity in either the frontal cortex or the striatum on the non-lesioned (left) side (Figure 6). The TH enzyme activity results were corroborated with the TH immunocytochemistry which shows the TH immunoreactivity in the striatum on the lesioned and non-lesioned side for 3 mice treated with the cTfRMAb-TNFR fusion protein (Figure 7A-C) and for 3 mice treated with saline (Figure 7D-F). The density of the striatal TH immunostaining on the lesioned side was increased
101% in the mice treated with the cTfRMAb-TNFR fusion protein as compared to the saline treated mice (Table 1).

The design of the immunity ELISA is shown in Figure 8A. The detector reagent is biotinylated cTfRMAb-TNFR fusion protein, and biotinylation of the fusion protein is verified by Western blot analysis (Figure 8B). The pre-injection mouse plasma, and the terminal plasma from the etanercept-treated mice produced no immune reaction with cTfRMAb-TNFR fusion protein (Figure 8C). There was a variable immune reaction observed at the end of the study in the mice treated with the cTfRMAb-TNFR fusion protein at 1:50 dilutions of plasma (Figure 8C).
Discussion

The results of this study are consistent with the following conclusions. First, the cTfRMAb-TNFR fusion protein and the TNFR-Fc fusion protein (etanercept) have comparable affinity of binding of TNFα, with a low a KD <1 nM (Figure 2). Second, chronic intravenous treatment of mice with experimental PD with the cTfRMAb-TNFR fusion protein results in neuroprotection, based on 3 assays of neurobehavior (Figures 3-5), striatal TH enzyme activity (Figure 6), and striatal TH ICC (Figure 7, Table 1). Third, chronic intravenous treatment with a comparable dose of the Fc-TNFR fusion protein (etanercept) has no therapeutic effect in experimental PD (Figures 3-7). Fourth, chronic treatment of mice with the cTfRMAb-TNFR fusion protein causes a low titer immune response against the cTfRMAb part of the fusion protein (Figure 8).

IgG-decoy receptor fusion proteins are formed by fusion of the carboxyl terminus of the decoy receptor to the amino terminus of the IgG heavy chain (Scallon et al, 1995), as shown for etanercept in Figure 1B. The engineering of the cTfRMAb-TNFR fusion protein is a departure from all prior IgG-decoy receptor fusion proteins in that amino terminus of the TNFR-II ECD is fused to the carboxyl terminus of the antibody heavy chain (Figure 1A). Despite this novel structure, the affinity of the cTfRMAb-TNFR fusion protein for TNFα is high, and comparable to the binding affinity of etanercept (Figure 2). The KD of cTfRMAb-TNFR fusion protein binding of human TNFα, 374 ± 77 pM (Figure 2), is comparable to the KD of TNFα binding to the intact membrane receptor, which is 0.3-0.4 nM (Morita et al, 2001). The human TNFR-II receptor binds mouse TNFα to the same extent as human TNFα (Scallon et al, 2002). In addition to TNFα, the cTfRMAb-TNFR fusion protein binds the murine BBB TfR with high affinity, and the brain uptake of the fusion protein in the mouse after IV injection is high, 2.8 ± 0.5 % injected
dose (ID)/gram brain (Zhou et al., 2011a). In contrast, the brain uptake of an IgG that does not cross the BBB is 0.06% ID/gram in the mouse (Lee et al., 2000). Given a brain uptake of 2.8% ID/gram, the brain concentration of the cTfRMAb-TNFR fusion protein is 840 ng/gram brain, or about 6 nM, with the systemic injection dose of 1 mg/kg used in these studies. Since the cerebral concentration of TNFα increases to approximately 0.5 nM in excitotoxic conditions (Shohami et al., 1994), the 1 mg/kg dose of fusion protein is sufficient to sequester the TNFα produced in brain.

TNFα induces apoptosis (Chen and Goeddel, 2002), and is elevated in the brains of patients with PD (Machado et al., 2011). The important role played by TNFα in the pathogenesis of experimental PD was demonstrated in knockout mice. Deletion of the TNFR in mice produced resistance to PD-inducing neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Sriram et al., 2002; Ferger et al., 2004). The intra-cerebral injection of a dominant negative TNFα mutant protein is neuroprotective in experimental PD (McCoy et al., 2006). Intra-cerebral injection of the TNFI was necessary, because large molecule TNFIs do not cross the BBB. The present study shows that a BBB-penetrating TNFI, the cTfRMAb-TNFR fusion protein, is neuroprotective in experimental PD in the mouse following IV administration.

Chronic treatment of 6-hydroxy dopamine-injected mice with intravenous cTfRMAb-TNFR fusion protein causes an improvement in 3 assays of neuro-behavior (Figures 3-5), and a 130% increase in TH enzyme activity in the striatum on the lesioned side (Figure 6), which correlates with an increase in striatal immunoreactive TH as observed with ICC (Figure 7, Table 1).

Chronic treatment of PD mice with intravenous etanercept has no therapeutic effect on neurobehavior (Figures 3-5) or striatal TH enzyme activity (Figure 6). The lack of therapeutic effect of intravenous etanercept is consistent with prior work showing that etanercept does not
cross the BBB (Boado et al, 2010). Owing to the lack of BBB penetration, it was necessary to administer etanercept by direct injection into the spinal cord in rat model of spinal cord injury (Marchand et al, 2009). There is evidence for focal BBB disruption in toxin-induced PD, and mice with knockout of the TNFR have less BBB disruption following MPTP administration (Zhao et al, 2007). Therefore, treatment of PD mice with the cTfRMAb-TNFR fusion protein would be expected to reduce BBB disruption mediated by TNFα. However, any disruption of the BBB that occurs in the 6-hydroxydopamine model in the mouse is insufficient to enable brain penetration of a therapeutic such as etanercept, because chronic dosing of PD mice with etanercept is not therapeutic (Figure 3-6).

Chronic dosing of mice with the cTfRMAb-TNFR fusion protein causes an immune response against the fusion protein, whereas chronic administration of etanercept in mice causes no immune response (Figure 8). This suggests the immune response is directed against the cTfRMAb part of the antibody. However, the immune response against the cTfRMAb-TNFR fusion protein is low titer and produces an average OD reading of 0.2 with a 1:50 dilution of 100 uL of plasma (Figure 8). The low titer immune response in this study equates to 0.1 OD/uL plasma. This titer is 100-fold lower than immune response against biologic drugs that neutralize therapeutic effects of the drug (Dickson et al, 2008). Moreover, recent work has shown that the low titer immune response produced with chronic treatment with cTfRMAb fusion proteins has no neutralizing effect on the TfR in vivo (Zhou et al, 2011b). The rate of brain uptake of fusion protein treated mice and saline treated mice is comparable at the end of 12 weeks of twice/weekly treatment with 2 mg/kg doses of cTfRMAb fusion protein (Zhou et al, 2011b). The low titer, non-neutralizing immune response to the fusion protein may be related to the presence of specific amino acid sequences within the constant region of the heavy chain that
induce T cell immune tolerance (DeGroot et al, 2008). In addition, the fusion protein is produced in CHO cells, which secrete fucosylated glycoproteins, and fucosylated glycoproteins have minimal effects on antibody dependent cytotoxicity (Kanda et al, 2006). Etanercept produced in CHO cells is fucosylated and does not induce antibody dependent cytotoxicity (Shoji-Hosaka, et al, 2006).

In summary, neurodegenerative disease of brain has an inflammatory component triggered in part by TNFα. CNS disease should be treatable with biologic TNF-inhibitors similar to the use of these agents for inflammation in peripheral conditions (Tansey and Szymkowski, 2009). However, the biologic TNF-inhibitors, such as the TNFR decoy receptor, do not cross the BBB (Boado et al, 2010). Systemic etanercept administration has no therapeutic effect in the mouse model of PD, and this is attributed to the lack of etanercept transport across the BBB. However, the same TNFα decoy receptor that forms etanercept is therapeutic after intravenous injection in a mouse model of PD, providing the TNFα decoy receptor is re-engineered to cross the BBB. Fusion of the TNFα decoy receptor to the cTfRMAb, which acts as a BBB molecular Trojan horse, creates a new IgG-TNFR fusion protein. The Trojan horse-TNFR fusion protein both binds the BBB TfR, to enable brain penetration from blood, and binds TNFα, to inhibit the action of this inflammatory cytokine in brain behind the BBB.
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Authorship Contributions

Participated in research design: Zhou, Sumbria, Hui, Lu, Boado, Pardridge

Conducted experiments: Zhou, Sumbria, Hui, Lu, Boado

Contributed new reagents or analytic tools: Boado, Pardridge

Performed data analysis: Zhou, Sumbria, Hui, Lu, Boado, Pardridge

Wrote or contributed to the writing of the manuscript: Zhou, Sumbria, Hui, Lu, Boado, Pardridge
References


dopaminergic neurodegeneration: possible implication in Parkinson's disease incidence.

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Footnotes

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Legends to Figures

Figure 1. (A) The cTfRMAb-TNFR fusion protein is formed by fusion of the extracellular domain (ECD) of the type II human TNFR to the carboxyl terminus of each heavy chain of the cTfRMAb. (B) The etanercept fusion protein is formed by fusion of the same ECD of the type II human TNFR to the amino terminus of the Fc fragment of human IgG1 heavy chain.

Figure 2. Radio-receptor assay shows saturation of binding of human TNFα to either the cTfRMAb-TNFR fusion protein or to etanercept. The binding dissociation constant, KD, was determined by non-linear regression analysis. The horizontal line at 1.5% binding represents the non-specific binding observed when either human IgG1 or mouse IgG1 was plated in lieu of the fusion protein.

Figure 3. Contralateral rotation (over 20 minutes) following the administration of apomorphine to PD mice treated with saline, etanercept, or the cTfRMAb-TNFR fusion protein at 1, 2, and 3 weeks after toxin injection. Data are mean ± S.E. (n=10 mice/group). Statistical differences from the saline treated animals are p<0.01 (*) at weeks 1, 2, and 3, as determined by ANOVA with Bonferroni correction.

Figure 4. Ipsilateral rotation (over 20 minutes) following the administration of amphetamine to PD mice treated with saline, etanercept, or the cTfRMAb-TNFR fusion protein at 1, 2, and 3 weeks after toxin injection. Data are mean ± S.E. (n=10 mice/group). Statistical differences from the saline treated animals are p<0.01 (*) or p<0.05 (‡) at weeks 1, 2, and 3, as determined by ANOVA with Bonferroni correction.
Figure 5. Vibrissae-elicited forelimb placing test scores for the right side, which is ipsilateral to the toxin lesion, and for the left side, which is contralateral to the toxin lesion, for the saline, etanercept, and the cTfRMAb-TNFR fusion protein treated mice. All scores were measured at 3 weeks following toxin injection. Data are mean ± S.E. (n=10 mice/group). Statistical differences from the saline treated animals are p<0.01 (*), as determined by ANOVA with Bonferroni correction.

Figure 6. TH enzyme activity on the lesioned side (right) and the non-lesioned side (left) in the striatum and in the frontal cortex in mice treated with saline, etanercept, or the cTfRMAb-TNFR fusion protein. Brain TH activity was measured at 3 weeks after toxin administration. Data are mean ± S.E. (n=10 mice/group). Statistical differences from the saline treated animals in the right striatum are p<0.01(*), as determined by ANOVA with Bonferroni correction.

Figure 7. TH immunocytochemistry for 3 mice treated with the cTfRMAb-TNFR fusion protein (A-C) and 3 mice treated with saline (D-F). The lesioned side of the brain corresponds to the right side of the figure.

Figure 8. (A) Structure of the 2-site ELISA for detection of antibodies against the cTfRMAb-TNFR fusion protein. The cTfRMAb-TNFR fusion protein is used as the capture reagent, and the biotinylated cTfRMAb-TNFR fusion protein is used as the detector reagent, along with a complex of streptavidin (SA) and horseradish peroxidase (HRP); the biotin moiety is designated, ‘B’. (B) Western blot of non-biotinylated cTfRMAb-TNFR fusion protein (lane 1) and
biotinylated cTfRMAb-TNFR fusion protein (lane 2) with a conjugate of avidin and biotinylated peroxidase. (C) Absorbance at 1:50 dilutions of mouse plasma taken pre-injection, or post-injection after 3 weeks of either IV injections with either etanercept or the cTfRMAb-TNFR fusion protein. Data are shown for all 10 mice in each group.
Table 1. Scanning densitometry of striatal TH immunoreactivity

<table>
<thead>
<tr>
<th>treatment</th>
<th>Density of striatal staining</th>
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<tr>
<td></td>
<td>lesioned</td>
</tr>
<tr>
<td>cTfRMAb-TNFR</td>
<td>0.167 ± 0.028*</td>
</tr>
<tr>
<td>saline</td>
<td>0.083 ± 0.009</td>
</tr>
</tbody>
</table>

*p<0.05 difference from saline treatment. Striatal density corrected for background density over cortex.
Figure 1

A

binds BBB TfR

VH

VL

CH1

CL

CH2

CH3

TNFR-II

binds TNFα

B

binds TNFα

TNFR-II

CH2

CH3
Figure 2

- cTfRMAb-TNFR
  $K_D = 374 \pm 77 \ pM$

- etanercept
  $K_D = 280 \pm 80 \ pM$
Figure 3: The graph shows the total contralateral rotations over 3 weeks for three different groups: saline, etanercept, and cTfRMAb-TNFR. Each bar represents the mean ± standard error of the mean. Significant differences are indicated by asterisks (*).
Figure 4

The graph shows the total ipsilateral rotations over weeks 1, 2, and 3 for three groups: saline, etanercept, and cTfRMAb-TNFR. The y-axis represents the total ipsilateral rotations, ranging from 0 to 160. The x-axis represents weeks 1, 2, and 3.

- **Saline** (black bars): Show a slight decrease in total rotations from week 1 to week 3.
- **Etanercept** (white bars): Show a significant decrease in total rotations compared to saline, especially from week 1 to week 2.
- **cTfRMAb-TNFR** (grey bars): Show the least change in total rotations, with a slight increase from week 1 to week 3.

Statistical significances are indicated by symbols:
- †: Significant difference between etanercept and saline.
- *: Significant difference between cTfRMAb-TNFR and saline.
- ‡: Significant difference between cTfRMAb-TNFR and etanercept.
Figure 5

Bar graph showing placing score for right and left sides for three different groups: saline (black), etanercept (white), and cTfRMAb-TNFR (gray). The graph indicates a significant difference (*) for the left side compared to the right side for the cTfRMAb-TNFR group.
Figure 8

A

SA  HRP

B

cTfRMAb-TNFR

antibody

cTfRMAb-TNFR

B

1  2

170 kDa  130 kDa  95 kDa  72 kDa  55 kDa  43 kDa  34 kDa  26 kDa  17 kDa

C

A490-A655

pre-injection  post-injection- etanercept  post-injection-cTfRMAb-TNFR