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DISTRIBUTION AND TUMOR NECROSIS FACTOR-ALPHA ISOFORM BINDING SPECIFICITY OF LOCALLY ADMINISTERED ETANERCEPT INTO INJURED AND UNINJURED RAT SCIATIC NERVE

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Abstract—Tumor necrosis factor-alpha (TNF) is a pro-inflammatory cytokine that is implicated in the initiation of neuropathic pain. Locally administered TNF antagonist etanercept offers a promising new treatment approach to target neuropathic pain. Here we evaluate the distribution and binding specificity for TNF isoforms of locally administered etanercept into injured and uninjured rat sciatic nerve. Distribution and co-localization of etanercept and TNF in the injured and uninjured nerve was evaluated at 1, 24, 48 and 96 h after etanercept local application using immunohistochemistry. In addition, binding specificity of etanercept for TNF isoforms was analyzed using immunoblot assay system in nerve lysates. A new observation was that locally administered etanercept reached the endoneurium of the injured but not the uninjured nerve 1 h after its application and mainly co-localized with TNF-positive structures, morphologically similar to Schwann cells and macrophages. We further noticed that immunoblot analyses for etanercept demonstrated its preferential binding to transmembrane and trimer TNF isoforms. Finally, locally administered etanercept inhibited pain-related behaviors in a rat sciatic nerve crush model. We conclude that locally administered etanercept reaches the endoneurial space in the injured nerve and preferentially binds to transmembrane and bioactive trimer TNF isoforms to modulate neuropathic pain. Locally administered etanercept has potential as a targeted immunomodulating agent to treat local pathogenesis in neuropathic pain after peripheral nerve injury.

Key words: peripheral nerve injury, neuropathic pain, tumor necrosis factor, etanercept.

Tumor necrosis factor-alpha (TNF) is a pro-inflammatory cytokine that plays crucial roles in peripheral nerve injury (Wagner and Myers, 1996b; George et al., 1999; Myers et al., 2006). Nerve injury causes local upregulation of TNF in activated Schwann cells, macrophages and other resident cells (Stoll et al., 1993; Sommer and Schroder, 1995; Wagner and Myers, 1996a). The local increase in TNF activity initiates an inflammatory cascade that produces severe persistent neuropathic pain (Redford et al., 1995; Wagner and Myers, 1996b; Schafers et al., 2003).

Several TNF inhibitors, including etanercept (Enbrel®, Amgen, Inc., Thousand Oaks, CA, USA) that is a dimeric fusion protein consisting of the extracellular ligand-binding portion of the 75 kDa tumor necrosis factor-alpha receptor 2 (TNFR2) and the Fc portion of human immunoglobulin G (IgG), have been shown to reduce pain-related behaviors in a neuropathic pain model (Sommer et al., 2001; Schafers et al., 2003; Zanella et al., 2008). However, several adverse effects including the reemergence of latent tuberculosis, severe infections, and new and recurrent malignancies have been reported due to the chronic use of systemically delivered TNF inhibitors in patients (Scheinfeld, 2004; Bongartz et al., 2006; Hochberg et al., 2005). More recently, it has been reported that locally administered etanercept provides analgesic effects in a rat neuropathic pain model (Sommer et al., 2001; Zanella et al., 2008) and patients with sciatica and cervical radiculopathy (Tobinick and Davoodifar, 2004). Zanella et al. (2008) reported that systemic etanercept reduced thermal hyperalgesia but required a 10-fold higher dose than a locally administered compound in a rat chronic constriction injury model. These results are strongly consistent with potential efficacy for the proposed local treatment approach permitting the use of lower drug dosage, which should minimize side effects and complications of TNF inhibitor administration. However, etanercept is a macromolecule with an apparent molecular weight (MW) of approximately 150 kDa which is not expected to cross the blood–nerve barrier (Zhou, 2005; Stephen et al., 2006). After nerve injury, the blood–nerve barriers become more permeable (Weerasuriya et al., 1980), but little is known about the effects of nerve injury on drug access to the nerve. In addition, etanercept is a fusion protein consisting of TNFR2 that preferentially binds to transmembrane TNF isoforms (Grell et al., 1995), but the binding specificity for TNF isoforms of locally administered etanercept into nerve tissues has not been clarified. Binding to transmembrane TNF by TNF receptors, or even TNF antagonists, can induce reverse signaling through this membrane-anchored ligand and can

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Abbreviations: ANOVA, analyses of variance; BSA, bovine serum albumin; DAB, 3′-3-diamino-benzidine; DAPI, 4,6-diamidino-2-phenylindole; DRG, dorsal root ganglia; ECL, enhanced chemiluminescence; Fc, fragment crystallizable; IgG, immunoglobulin G; LTα, lymphotoxin-alpha; MW, molecular weight; SNC, sciatic nerve crush; sTNF, soluble tumor necrosis factor-alpha; tmTNF, transmembrane tumor necrosis factor-alpha; TNF, tumor necrosis factor-alpha; TNFR2, tumor necrosis factor alpha receptor 2.

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trigger cell activation, cytokine suppression or apoptosis of the transmembrane TNF-bearing cell (Tracey et al., 2008). Thus, evaluation of the distribution and binding specificity for TNF isoforms of locally administered etanercept may be important for understanding the utility of etanercept for locally treating the neuropathological changes in neuropathic pain.

In the present study, we established an immunohistochemical method of etanercept detection using an antibody for human IgG, and evaluated the dynamics of etanercept uptake and distribution to injured and uninjured sciatic nerve in rats. In addition, binding specificity of etanercept for TNF isoforms was analyzed using immunoblot assay system in nerve lysates. Finally, we confirmed the pain-relieving effect of locally administered etanercept in a rat sciatic nerve crush (SNC) model using behavioral tests of mechanical sensitivity.

EXPERIMENTAL PROCEDURES

Animals and anesthesia

A total of 52 Adult female Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN, USA) weighing 200–250 g were used. Rats were housed in pairs with a 12-h light/dark cycle with free access to food and water. The experimental protocols were approved by the VA Healthcare System Committee on Animal Research, and conform to the NIH Guidelines for Animal Use. All efforts were made to minimize animal suffering and to reduce the number of animals used. The animals were anesthetized with 4% isoflurane (IsoSol; Vedco, St. Joseph, MO, USA).

Surgical procedure

All surgical procedures were performed in an aseptic manner using microsurgical techniques. Rats were placed in a lateral position and a skin incision was made from the greater trochanter to the mid-thigh. The left sciatic nerve was exposed through a gluteal muscle splitting incision. Rats were divided into four groups: (1) the control group was injected intraneurially with 125 μg etanercept (Enbrel®, Amgen, Inc.) in 5 μl of sterile bacteriostatic water into the endoneurial space of the uninjured rat sciatic nerves using a Hamilton syringe (Hamilton Company, Reno, NV), a 30-gauge-needle (n=2). (2) The sham group was subjected to an operation in which the left sciatic nerve was exposed and 100 μg etanercept in 50 μl of sterile bacteriostatic water (Sterile Water for Injections, Hospira, Inc., Lake Forest, IL, USA) was applied into the epineurial space immediately adjacent to the nerve (n=16). Careful insertion of the needle into the epineurial space of the crushed nerve site and slow injections to avoid an overflow were ensured. (3) The SNC group was subjected to the crush operation using smooth-surface forceps once for 5 s (n=24). The muscle layer was closed using silk suture and the skin was closed with metal clips. (4) The vehicle-treated SNC group was subjected to the crush operation as described above, and then 50 μl of sterile bacteriostatic water was applied into the epineurial space immediately adjacent to the nerve 24 h after the first surgery (n=10). The sterile bacteriostatic water that we used contains 0.9% benzyl alcohol. Although benzyl alcohol in this low concentration has been widely used as a preservative in parenteral preparations, it is known to have potential neurotoxic effects at higher concentrations (Deland, 1973).

Tissue processing

For immunohistochemical experiments, SNC group rats were anesthetized with 4% isoflurane 24 h after the first surgery and the sciatic nerve was exposed and 100 μg etanercept in 50 μl of sterile bacteriostatic water was applied into the epineurial space immediately adjacent to the nerve as described above, and then the incision was closed. The dose of etanercept was based on previous studies (Sommer et al., 2001; Zanella et al., 2008). Immunohistologic examinations were performed in the control (n=2 at 1 h after the application of etanercept), the sham and SNC (n=8 each at 1, 24, 48 and 96 h after the application of etanercept) and the vehicle-treated SNC groups (n=2 at 96 h after the application of sterile bacteriostatic water). Rats were anesthetized using an i.p. injection of a cocktail containing sodium pentobarbital (Nembutal, 50 mg/ml; Abbott Labs, North Chicago, IL, USA) diazepam (5 mg/ml, Steris Laboratories, Phoenix, AZ, USA) and saline (0.9%, Steris Laboratories) in a volume proportion of 1:1:2, respectively and perfused with fresh 4% paraformaldehyde in 0.1 mol/l phosphate buffer. Bilateral sciatic nerves were removed and post-fixed briefly in 4% paraformaldehyde overnight. Tissue was processed in an Autotechnicon Cycler TP 1010 (Leica Microsystems, Inc., Bannockburn, IL, USA), and embedded in paraffin.

For immunoblotting analysis, rats (n=8) were sacrificed at 1 and 5 days after SNC by overdose of i.p. injection of the cocktail as described above followed by intracardiac Euthasol (Virbac, Fort Worth, TX, USA). Rats were decapitated rapidly under anesthesia, and bilateral sciatic nerves were removed and frozen in liquid nitrogen.

Immunohistochemistry

Sections (10 μm) of the nerves were cut from each sample and placed on slides. Sections were deparaffinized with xylenes and rehydrated in graded ethanol, and endogenous peroxidase was blocked with 3% H_2O_2. Non-specific binding sites were blocked with 10% normal horse or rabbit serum. Anti-human IgG (AbD Serotec, Oxford, UK; 1:500) or rat-TNF (R&D Systems, Minneapolis, MN, USA; 1:100) antibodies were incubated overnight at 4 °C in 1% horse or rabbit serum. Slides were rinsed in phosphate-buffered saline and subsequently incubated for 30 min with biotinylated conjugated horseradish peroxidase antimouse or goat antibodies (Vector Laboratories, Burlingame, CA, USA). Sections were incubated for 30 min with avidin–biotin–peroxidase complex (ABC Elite, Vector Laboratories), developed with 3′-3-diaminobenzidine (DAB) (Vector) and counterstained with Methyl Green. For dual immunofluorescence, sections were immersed in 0.5% sodium borohydride followed by antigen retrieval and nonspecific binding block as described above, then primary antibody incubation overnight at 4 °C, rabbit antimouse Alexa 488 (green) fluorescent antibody (Molecular Probes, 1:400) or rabbit anti-goat Alexa 594 (red) fluorescent antibody (Molecular Probes, 1:400) for 1 h, and nuclear 4,6-diamidino-2-phenylindole (DAPI) stain (Molecular Probes, 1:20,000, blue) for 5 min. Sections were mounted using Slowfade gold antifade reagent (Molecular Probes). Some sections were run without primary antibodies as controls or were preabsorbed, showing no human IgG or TNF immunoreactivity. Imaging was done on a Leica microscope using Open Laboratory Software (Improvision, Coventry, UK).

Immunoblotting assay

Samples were extracted using lysis buffer (50 mM Tris–HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 10% glycerol, 0.1% SDS, 5 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin and leupeptin) and reduced with 10% β-mercaptoethanol (Fisher). Equal amounts of protein (60 μg, by Pierce BCA Protein Assay) per lane were run on 12.5% Tris–glycine SDS–polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA) for 1 h at 50–80 mA, and then transferred to nitrocellulose membranes using iBlot dry blotting system (Invitrogen) at 20 V for 7 min. The membranes were blocked with 5% non-fat milk (Bio-Rad), incubated with etanercept (25 mg/ml Amgen, Inc.; 1:1000) for 2 h at room temperature and