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NOCICEPTIVE CHARACTERISTICS OF TUMOR NECROSIS FACTOR-α IN NAIVE AND TUMOR-BEARING MICE

P. W. WACNIK,[a] L. J. EIKMEIER,[b] D. A. SIMONE,[c,d,e] G. L. WILCOX,[a,e] AND A. J. BEITZ[b]*

[a]*Department of Pharmacology, University of Minnesota, School of Medicine, Minneapolis, MN 55455, USA*

[b]*Department of Veterinary and Biomedical Science, University of Minnesota, School of Veterinary Medicine, St. Paul, MN 55108, USA*

[c]*Department of Oral Science, University of Minnesota, School of Dentistry, Minneapolis, MN 55455, USA*

[d]*Department of Psychiatry, University of Minnesota, School of Medicine, Minneapolis, MN 55455, USA*

[e]*Department of Neuroscience, University of Minnesota, School of Medicine, Minneapolis, MN 55455, USA*

Abstract—A nociceptive role for tumor necrosis factor-α (TNF-α) in naive mice and in mice with fibrosarcoma tumor-induced primary hyperalgesia was investigated. The presence of TNF-α mRNA was confirmed in tumor site homogenates by reverse transcription–polymerase chain reaction (RT-PCR), and examination of TNF-α protein levels in tumor-bearing mice indicated a significantly higher concentration of this cytokine in tumor microperfusates and tumor site homogenates compared with that obtained from a similar site on the contralateral limb or in naive mice. Intraplantar injection of TNF-α into naive or fibrosarcoma tumor-bearing mice induced mechanical hypersensitivity, as measured by withdrawal responses evoked by von Frey monofilaments. This hypersensitivity suggests that TNF-α can excite or sensitize primary afferent fibers to mechanical stimulation in both naive and tumor-bearing mice. In addition, the hyperalgesia produced by TNF-α was completely eliminated when the injected TNF-α was pre-incubated with the soluble receptor antagonist TNFR:Fc. Importantly, pre-implantation systemic as well as post-implantation intra-tumor injection of TNFR:Fc partially blocked the mechanical hyperalgesia, indicating that local production of TNF-α may contribute to tumor-induced nociception.

Key words: cancer pain, hyperalgesia, TNF-α antagonist, bone tumor, flow cytometry, etanercept.

Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine produced by several cell types, including mast cells, macrophages, fibroblasts, endothelial cells and Schwann cells (Friedman, 2000) and by certain tumor cells including fibrosarcomas (Fukuda et al., 2001; Selinsky and Howell, 2000). Recently, Li et al. (2004) showed that the mRNAs for both TNF-α receptors (TNFR1 and TNFR2), but not TNF-α, are constitutively expressed and regulated by lipopolysaccharide in all rat dorsal root ganglia neurons, while the mRNAs for TNF-α and both TNF-receptors are expressed and regulated by lipopolysaccharide in non-neuronal cells exclusively. With respect to nociceptive processing, TNF-α has been implicated in the generation of both inflammatory hyperalgesia (Tonussi and Ferreira, 1999) and neuropathic pain (Covey et al., 2002; Friedman, 2000). For example, in the model of complete Freund's adjuvant-induced inflammation, pretreatment with anti-TNF-α antiserum significantly delays the onset of mechanical hyperalgesia (Woolf et al., 1997). In the sciatic nerve chronic constriction injury (CCI) model of neuropathic pain, intra-operative epineural administration of neutralizing antibodies against the TNF receptor, TNFR-1, reduced mechanical allodynia and thermal hyperalgesia (Sommer et al., 1998), whereas administration at 4 days post-CCI reduced thermal, but not mechanical, hyperalgesia (Sommer et al., 2001a).

It has been shown that TNF-α applied to the sciatic nerve causes spontaneous discharge and bursting activity in cutaneous primary afferent fibers (Sorkin et al., 1997). In addition, s.c. injection of TNF-α lowered the mechanical threshold of C-nociceptors but not Aβ-mechanoreceptors recorded from the sural nerve (Junger and Sorkin, 2000). Moreover, acute mechanical allodynia occurs following administration of TNF-α to the sciatic nerve and is dose-dependent (Sorkin and Doom, 2000). Additional behavioral studies have shown that acute intraplantar (IPL) injection of TNF-α produces mechanical and/or thermal hyperalgesia lasting hours after injection (Cunha et al., 1992; Woolf et al., 1997).

It is well established that cytokines, such as TNF-α that are either produced by tumors themselves or by surrounding tissue in response to tumor growth, play a role in tumor development and metastasis, and their effects vary depending on the type and location of the tumor. TNF-α has been shown to induce tumor growth, promote angiogenesis, and increase metastasis by down-regulating tumor-suppressor genes and/or increasing adhesion (Dunlop and Campbell, 2000). However, TNF-α is also known to induce apoptosis of tumor cells; thus, limb or organ perfusions of TNF-α have been used as an adjuvant for chemotherapy in cases of non-resectable high-grade sarcomas, melanomas, and liver tumors (reviewed in Ashkenazi, 2002).

*Correspondence to: A. J. Beitz, Department of Veterinary and Biomedical Sciences, University of Minnesota, 295 AnSci./Vet Med. Building, 1988 Fitch Avenue, St. Paul, MN 55108, USA. Tel: +1-612-624-4994; fax: +1-612-625-0204.
E-mail address: alvin@biosci.cbs.umn.edu (A. J. Beitz).

Abbreviations: CBA, cytometric bead array; CCI, chronic constriction injury; DAB, diaminobenzidine tetrahydrochloride; DS-Red2, red-fluorescence-tagged; EDTA, ethylenediaminetetraacetic acid; ET-1, endothelin-1; IHC, immunohistochemistry; IPL, intraplantar; PBS, phosphate-buffered saline; PID, post-injection day; RT-PCR, reverse transcription–polymerase chain reaction; TNF-α, tumor necrosis factor-α.

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Attempts to study the function of TNF-α and its receptor have been difficult because of the lack of specific antagonists. Until recently, the only blockers of TNF-α activity were binding proteins for TNF-α (TNF-α binding protein, TNF-bp), antibodies directed against TNF-α, or non-specific pharmaceuticals like thalidomide. In the current study, we used a fusion protein that binds specifically to free TNF-α and blocks its interaction with cell surface TNF receptors, TNFR:Fc (trade name: Etanercept, a portion of which was a gift from Amgen Thousand Oaks, CA, USA). TNFR:Fc consists of the extracellular ligand-binding portion of the 75 kDa (p75) TNF receptor linked to the Fc portion of human IgG1. Using this antagonist we investigated whether TNF-α contributes to the hyperalgesia observed in a recently developed mouse model of cancer pain (Wacnik et al., 2001). In this model, fibrosarcoma tumor cells are implanted in and around the calcaneus bone. Changes in behavior and electrophysiology following implantation, as well as tumor morphology and neurochemistry have been characterized (Cain et al., 2001; Wacnik et al., 2001). Using this model we have previously demonstrated that the tumor causes spontaneous pain behavior and cutaneous hyperalgesia and that the development of this spontaneous pain and hyperalgesia is associated histologically with bone destruction without significant inflammatory cell infiltrate.

To determine if TNF-α is involved in tumor-induced hyperalgesia we analyzed TNF-α production at the tumor site and investigated the effect of blocking TNF-α activity on tumor-evoked hyperalgesia. Specifically, we tested the efficacy of TNFR:Fc to antagonize the behavioral effects of acute administration of TNF-α in naive mice by co-administering it with exogenous TNF-α. In addition, we administered TNFR:Fc to tumor-bearing mice to ascertain if it would attenuate hyperalgesia produced by the tumor. We hypothesized that the primary hyperalgesia that occurs in this model of cancer pain is partially mediated by TNF-α released by the fibrosarcoma cells.

EXPERIMENTAL PROCEDURES

Animals

A total of 96 C3H/He male mice (National Cancer Institute) aged 8–10 weeks and weighing 24–28 g were used in all experiments. The inbred mouse strain C3H/He is syngeneic to the fibrosarcoma cells used in these experiments. Thus these cells form tumors after implantation without rejection (Clohisy et al., 1996). Mice were housed in boxes of eight in a temperature- and humidity-controlled environment on a 12-h light/dark cycle with free access to mouse chow and water. The Animal Care and Use Committee of the University of Minnesota approved all experimental protocols. All experimental protocols using animals conformed to NIH guidelines (NIH publication No. 86-23, revised 1985) and to the principles set forth in the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, 1996). The minimal number of animals needed to obtain statistical significance was used in the present study to reduce animal numbers and to minimize animal distress.

Cell culture and implantation

NCTC clone 2472 fibrosarcoma cells, originally derived from a connective tissue tumor in a C3H mouse, were obtained from the American Type Cell Culture Collection, Rockville, MD, USA. All cells were maintained as described previously (Wacnik et al., 2001). Just prior to implantation, cells were counted, pelleted, resuspended in phosphate-buffered saline (PBS), pelleted a second time, and then resuspended in PBS for implantation. Mice were placed in an enclosed chamber and anesthetized with 2% halothane and the calcaneus bone was implanted with 2×10^5 fibrosarcoma cells in a volume of 10 μl of PBS as previously described (Wacnik et al., 2001). Any mice showing signs of motor dysfunction following implantation were immediately killed.

Drug preparation and administration

TNF-α (Cedarlane, Hornby, Ontario) was reconstituted by first adding 1 ml of sterile water to 5 μg, with all subsequent dilutions made in sterile saline. In all experiments, TNF-α was reconstituted immediately prior to injection from lyophilized powder or from frozen aliquots (<1-month old). TNFR:Fc was reconstituted by first adding 1 ml of sterile water to 10 mg, with all subsequent dilutions made in sterile saline. IPL injections were made in a volume of 20 μl and systemic (i.p.) injections were made in a volume of 100 μl. TNF-α was administered IPL or intratumor at doses ranging from 0.001 pg to 1.0 ng/20 μl. TNFR:Fc was administered i.p. at doses ranging from 100 to 500 μg/mouse and intratumor at a dose of 10 μg/20 μl/mouse.

Immunohistochemistry (IHC) for TNF-α

At post-injection day (PID) 10, three mice were transcardially perfused with 15 ml PBS followed by 30 ml of 4% paraformaldehyde in PBS. Tumors were harvested, cryoprotected in 20% sucrose, and sectioned at 40 μm on a sliding microtome. Sections were processed for IHC using the ABC method biotinylated secondary antibody followed by avidin–horseradish peroxidase complex (Vector Laboratory, Burlingame, CA, USA). The sections were blocked with 2% goat serum in 0.3% Triton X-100 for 1 h and incubated overnight with anti-TNF-α antibody (1:500; Pharmingen, San Diego, CA, USA) or with 2% goat serum in 0.3% Triton X-100 for control sections. Sections were then incubated for 2 h with anti-rat secondary antibody (1:250) and 1 h with ABC complex (1:50). Finally, 0.05% diaminobenzidine tetrahydrochloride (DAB) was used to visualize the enzyme reaction product. Sections were visualized using brightfield microscopy for IHC staining, and fluorescence microscopy to visualize DS-Red-labeled tumor cells. Two control experiments suggest that the immunostaining observed in these experiments is specific for TNF-α. First, TNF-α was used for preabsorption in an initial experiment preabsorption resulted in a loss of all immunostaining for TNF-α from the tumor sections. In subsequent experiments the primary antibody for TNF-α was omitted and the sections subsequently processed with the anti-rat secondary antibody and then with ABC complex followed by a DAB reaction. This also resulted in an absence of immunostaining in the tumor sections.

RNA isolation and reverse transcription–polymerase chain reaction (RT-PCR)

For RT-PCR experiments, 2472 fibrosarcoma cells were analyzed along with a non-osteolytic tumor cell line (G3.26 melanoma cells). The melanoma tumor grows to a similar size as the fibrosarcoma tumor over the same time course, but unlike the fibrosarcoma does not induce mechanical hyperalgesia (Wacnik et al., 1998). RNA was isolated from *in vitro* fibrosarcoma and melanoma cell cultures using RNeasy kit (Qiagen, Valencia, CA, USA); RNA isolation from whole tumors ($n=6$) or contralateral tissue at PID 10 was carried out similarly, with the exception that the protocol for "difficult tissues" was used, as described by the manufacturer (Qiagen). Five micrograms of RNA was used for reverse transcription using Superscript First-Strand Synthesis System for RT-PCR;