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Infliximab Induces Potent Anti-inflammatory Responses by Outside-to-Inside Signals Through Transmembrane TNF-α

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Background & Aims: **Both infliximab (chimeric anti-tumor necrosis factor [TNF]-α antibody) and etanercept (p75 TNF-α receptor/immunoglobulin G fusion protein) are effective against rheumatoid arthritis, but only infliximab induces clinical remission in Crohn's disease. To clarify this difference in clinical efficacy, we investigated reverse signaling through transmembrane TNF-α (mTNF) by these 2 anti-TNF agents.** ***Methods:*** **We stably transfected wild-type and cytoplasmic serine-replaced mutant forms of mTNF in human Jurkat T cells. Cells were stimulated with infliximab and etanercept and then analyzed for E-selectin expression, reactive oxygen species accumulation, apoptosis, and cell cycle distribution by flow cytometry. Interleukin-10 and interferon-γ were measured by enzyme-linked immunosorbent assay. Phospho-c-Jun NH2-terminal kinase, Bax, Bak, $p21^{WAF1/CIP1}$, caspase-8, and caspase-3 were examined by immunoblotting.** ***Results:*** **Both anti-TNF agents induced E-selectin expression, but only infliximab induced interleukin-10 production, apoptosis, and G0/G1 cell cycle arrest. Apoptosis and cell cycle arrest were abolished by substitution of all 3 cytoplasmic serine residues of mTNF by alanine residues. Infliximab induced accumulation of reactive oxygen species and up-regulation of Bax, Bak, and $p21^{WAF1/CIP1}$ proteins, suggesting the involvement of p53 activation. Moreover, phosphorylation of c-Jun NH2-terminal kinase was necessary for infliximab-induced apoptosis and cell cycle arrest.** ***Conclusions:*** **We revealed the mTNF motifs and the downstream intracellular molecular events essential for reverse signaling through mTNF. The biologic effects of mTNF elicited by infliximab should be important action mechanisms of this potent anti-inflammatory agent in addition to the neutralization of soluble TNF-α. These observations will provide insight into the novel role of mTNF in inflammation.**

Tumor necrosis factor (TNF)-α is a potent proinflammatory cytokine that orchestrates various inflammatory responses. The precursor form of TNF-α, called *transmembrane TNF-α* (mTNF), is expressed as a 26-kilodalton cell surface type II polypeptide on activated macrophages and lymphocytes, as well as on other cell types. mTNF consists of N-terminal 30 amino acid (aa) residues of the cytoplasmic domain, 26 aa of the transmembrane domain, and 177 aa of the extracellular domain. The C-terminal 157 aa are processed by TNF-α-converting enzyme (TACE) between residues Ala^{76} and Val^{77}.[1,2] The secreted soluble form of 17-kilodalton polypeptide binds to type 1 and type 2 TNF receptors (TNF-RI, TNF-RII) as a homotrimer and mediates pleiotropic effects, such as cytokine production, cell adhesion molecule expression, and proliferation as well as apoptosis.[3–5]

mTNF is constitutively expressed on resting natural killer (NK) cells[6] and is also induced upon activation on various types of cells such as monocytes and T lymphocytes.[7,8] mTNF has also been shown to mediate biologic functions in a cell-to-cell contact fashion and to be involved in cytotoxic activity by monocytes[7,9] and NK cells,[6] polyclonal B-cell activation induced by human immunodeficiency virus (HIV)-infected $CD4^+$ T cells[10] and by human T-cell leukemia virus-infected $CD4^+$ T cells,[11] IL-10 production from monocytes,[8] and expression of adhesion molecules such as intercellular adhesion molecule (ICAM)-1 and tissue factor from endothelial cells.[12,13] Costimulatory signals for interleukin (IL)-4-dependent immunoglobulin synthesis are also provided by mTNF.[14]

mTNF not only acts as a ligand but also mediates reverse signaling into cells expressing this molecule. Our group and others have shown that stimulation of mTNF with anti-human TNF-α polyclonal Ab or TNF-RII

Abbreviations used in this paper: aa, amino acid; CHX, cycloheximide; Eta, etanercept; Ifx, infliximab; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; mTNF, transmembrane TNF-α; ROS, reactive oxygen species; Ser, serine; TACE, TNF-α converting enzyme; TNF, tumor necrosis factor; TNF-R, TNF receptor; WT, wild-type.

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expressed on HeLa cells induces several biologic effects, including calcium mobilization,[11,15] cytokine production,[11] and E-selectin expression in T cells.[16] The cytoplasmic domain of mTNF should play a critical role in reverse signaling, considering its extreme aa conservation (nearly 90%) among different animal species.[17] Phosphorylation of human mTNF by anti-TNF-α polyclonal Ab in some monocytic cells and in 26-kilodalton precursor TNF-α-transfected HeLa cells has been reported and shown to be restricted to serine (Ser) residues.[18]

The beneficial effect of anti-TNF therapy has been recognized by large-scale, long-term studies of rheumatoid arthritis[19,20] and Crohn's disease.[21] In smaller study-scale studies, the efficacy of anti-TNF therapy has also been demonstrated in patients with psoriasis,[22] ankylosing spondylitis,[23,24] and Behçet's disease.[25] These successful clinical trials have clarified the importance of TNF-α in the pathogenesis of chronic inflammatory disorders. There are 2 types of anti-TNF-α agent: one is an antibody against human TNF-α (infliximab; chimeric anti-TNF-α monoclonal antibody [mAb] with murine variable regions and human IgG1 constant regions),[26] and the other is made of a soluble form of human TNF-RII fusion protein (etanercept; recombinant TNF-RII/IgG1 Fc domain fusion protein).[27] Both of these agents have been shown to bind to soluble TNF and mTNF[26–28] and to be equally effective in rheumatoid arthritis. However, in Crohn's disease, remission is induced only by infliximab and not by etanercept.[29] This difference between infliximab and etanercept in a clinical setting might indicate the existence of additional biologic functions of anti-TNF agents other than mere neutralization of TNF-α.

Here, we demonstrate that outside-to-inside (reverse) signals through mTNF elicited by infliximab resulted in increased apoptosis and cell cycle arrest in a human T-cell line. Moreover, we identified the mTNF intracellular motifs, as well as the downstream intracellular molecules essential for reverse signaling.

Materials and Methods

Cell Line and Reagents

Jurkat cells, a human lymphoblastoid T-cell line, were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 mg/mL streptomycin at 37°C in a 5% CO_2-humidified atmosphere. Infliximab (Remicade) was provided by Tanabe Seiyaku Co. Ltd. (Osaka, Japan), and etanercept (Enbrel) was provided by Wyeth (Tokyo, Japan). Rituximab (chimeric anti-human CD20 mAb), which was used as a control chimeric Ab, was purchased from Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan). Cycloheximide (CHX) was obtained from Sigma (St. Louis, MO); 2′,7′-dichlorofluorescein diacetate was supplied by Wako Chemical (Osaka, Japan); F(ab′)2 fragment of rabbit anti-human IgG was supplied by Dako Cytomation (Kyoto, Japan); [^{3}H] thymidine was obtained from Amersham Biosciences (Arlington Heights, IL); SB 203580 and PD98059 came from Promega (Madison, WI); and SP600125 and z-VAD-fmk were obtained from Calbiochem (La Jolla, CA).

Mutagenesis of mTNF

Mutant mTNF resistant to TACE-mediated cleavage was generated by the oligodeoxyribonucleotide-directed amber method (site-directed mutagenesis) as described previously.[16] In this uncleavable form of mTNF designated as wild-type (WT), Arg at aa residue 77 and Ser at aa residue 78 of the native mTNF were both replaced by Thr (R77T/S78T), which has already been shown to result in the effective reduction of the cleavage of mTNF.[18] The cytoplasmic Ser at aa residues 2, 5, and 27 was sequentially replaced by Ala by site-directed mutagenesis in accordance with the manufacturer's instructions (Mutan-Super Express Km kit; Takara Shuzo, Kyoto, Japan). Briefly, mutations were introduced into the uncleavable form of mTNF (R77T/S78T) subcloned into pKF19 by the following oligonucleotides: TCGGCTTATGGCCACTGAAAGCATGA for S2A, ATGAGCACTGAAGCCATGATCCGG for S5A, and GCCCCAGGGCGCCAGGCGGTG for S27A. The nucleotide sequences for the mutant forms of mTNF were confirmed by direct sequencing using an Amplicycle sequencing kit (Perkin-Elmer, Norwalk, CT).

Stable Expression of mTNF on Jurkat Cells

WT and cytoplasmic Ser-replaced mutant mTNFs were cloned into pCXN2 mammalian expression vector (kindly provided by Dr. Jun-ichi Miyazaki, Osaka University) and transfected into Jurkat cells by electroporation using Gene Pulser apparatus (Bio-Rad Laboratories, Hercules, CA) at 240 V, 960 μF. The cells were immediately plated on prewarmed medium and cultured at 37°C. Two days after transfection, the cells were selected in the presence of G418 (Sigma) 1.6 mg/mL. Clones of each mutant form of mTNF were obtained by limiting dilution.

Surface Staining

Cells were washed twice with the staining medium PBS containing 2% FBS. Cells (5×10^5 per sample) were stained on ice for 40 minutes with FITC-conjugated mAbs in staining medium. Expression of mTNF, TNF-RI, TNF-RII, and Fas was studied using anti-human TNF-α mAb (R&D Systems, Minneapolis, MN), anti-human TNF-RI mAb (Genzyme, Cambridge, MA), anti-human TNF-RII mAb (Genzyme), and anti-human Fas mAb (Medical and Biological Laboratories [MBL], Nagoya, Japan). Induction of E-selectin was studied by using anti-human E-selectin (CD62E) mAb (Ancell, Bayport, MN). FITC-conjugated mouse IgG1 (Dako) was used as a negative control. Stained cells were washed twice with staining medium, and then the expression of cell surface