Identification of Tumor Necrosis Factor (TNF) Amino Acids Crucial for Binding to the Murine p75 TNF Receptor and Construction of Receptor-selective Mutants

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The bioactivity of tumor necrosis factor (TNF) is mediated by two TNF receptors (TNF-Rs), more particularly TNF-RI and TNF-RII. Although human TNF (hTNF) and murine TNF (mTNF) are very homologous, mTNF binds only to mTNF-RI. By measuring the binding of a panel of mTNF/hTNF chimeras to both mTNF-R, we pinpointed the TNF region that mediates the interaction with mTNF-RII. Using site-specific mutagenesis, we identified amino acids 71–73 and 89 as the main interacting residues. Mutein hTNF-S71D/T72Y/H73E interacts with both types of mTNF-R and is active in CT6 cell proliferation assays mediated by mTNF-RII. Mutein mTNF-D71S/Y72T/A73H/E89T binds to mTNF-RII only and is no longer active on CT6 cells. However, the L929s cytotoxicity of this mutein (an effect mediated by mTNF-RII triggering) was also 100-fold lower than that of wild-type mTNF due to enhanced dissociation during incubation at subnanomolar concentrations. The additional mutation of amino acid 102, resulting in the mutein mTNF-D71S/Y72T/A73H/E89T/P102Q, restored the trimolecular stability, which led to an enhanced specific activity on L929s cells. Hence the specific activity of a TNF species is governed not only by its receptor binding characteristics but also by its trimer stability after incubation at subnanomolar concentrations. In conclusion, the mutation of TNF amino acids 71–73, 89, and 102 is sufficient to obtain a mTNF mutein selective for mTNF-RI and a hTNF mutein that, unlike wild-type hTNF, acts on mTNF-RII.

Tumor necrosis factor (TNF) is a pleiotropic cytokine with a wide range of biological activities including cytotoxicity, immune cell proliferation, and mediation of inflammatory responses (1–3). It exerts both direct and indirect antitumor effects on a number of tumors in vivo. However, its therapeutic application in the treatment of tumors is severely hampered by pathogenic side effects such as hypotension and liver toxicity (4). The multiple biological effects of TNF are mediated by two cell surface TNF receptors (TNF-Rs), namely TNF-RI and TNF-RII (5, 6). These receptors bind in the grove regions between TNF subunits; hence one trimeric TNF molecule binds three receptor molecules (7). Clustering of surface-bound receptors initiates a signaling cascade in the cell. Moreover, receptor-specific muteins of hTNF have been obtained by mutating amino acids located at the intersubunit grooves (8–10).

Although murine TNF (mTNF) and human TNF (hTNF) are 79% identical at the amino acid level, hTNF interacts with mTNF-R but not with mTNF-RII. Due to the high homology, chimeric TNF genes can be obtained by exchanging homologous regions between the hTNF and mTNF genes. Bacterial expression of such in-frame chimeric genes results in chimeric TNF subunits that are able to trimerize into bioactive molecules. These chimeric proteins are subsequently used to localize the epitopes for species-specific TNF monoclonal antibodies (11).

We created and characterized different mTNF/hTNF chimeras to identify the region(s) responsible for interaction with mTNF-RII. Subsequently we obtained receptor-specific muteins by replacing murine amino acids in this region by their human counterparts and vice versa. This resulted in a mTNF-RI-specific mutein of mTNF and a hTNF mutein also binding efficiently to mTNF-RII.

MATERIALS AND METHODS

Cytokines and Antisera—Recombinant mTNF and hTNF were produced in our laboratory and had a specific biological activity of 2.1 × 10^8 and 6.7 × 10^7 units/mg, respectively. TNF muteins were purified to near homogeneity as described previously (11). The extracellular domains of mTNF-RI and mTNF-RII were expressed in the baculovirus/Sf9 system and purified as follows. The cell supernatant was subjected to ammonium sulfate precipitation (20–80% cut). The pellet was dissolved in 50 mM Tris-HCl (pH 8.5) and passed over a Q-Sepharose column (Amersham Pharmacia Biotech). The flow-through with soluble receptors was dialyzed against 50 mM sodium acetate (pH 5.3), applied on an SP-Sepharose column (Amersham Pharmacia Biotech), and eluted with a linear NaCl gradient. Peak fractions had a purity of >90% as measured by SDS-polyacrylamide gel electrophoresis. Expression and purification of bivalent chimeric proteins formed with the Fc region of human IgG1 and the ligand-binding domains of hTNF-RI (hTNF-RI/Fc) or hTNF-RII (hTNF-RII/Fc) were described previously (12, 13).

Plasmids and Proteins—mTNF and hTNF chimeric plasmids were obtained by exchanging restriction fragments between previously constructed pPLHTNFI, pATTrpMTNF, pPLMHTNF-11, and pPLMHTNF-22 (11). The TNF coding information of these plasmids comprises a unique 5′NcoI and 3′HindIII restriction site as well as a unique HindIII site at amino acids 89–90 (see Fig. 1). pATTrpMHTNF-23 was created by ligation of the fragments NcoI/pPLMHTNF-22/HindIII and HindIII/pATTrpMTNF/HindIII into pATTrpMTNF. pATTrpMHTNF-24 was con-
structed by ligation of the fragments NeoI/pATTrpMTNF/HindIII and HindIII/pPLLcMTNF/HindIII into pATTrpMTNF. pPLLcMTNF-15 was obtained by ligation of the fragments NeoI/pPLLcMTNF/HindIII and HindIII/pPLLcMTNF/HindIII into pATTrpMTNF. All original plasmids were opened with NeoI and HindIII. The encoded proteins were called MHTNF-23, MHTNF-24, MHTNF-15, and MHTNF-16, respectively.

Site-specific Mutagenesis—The bacterial expression plasmid pMatHTNF (14) was mutated by the gapped heteroduplex method using a Transformer Site-Directed Mutagenesis kit (CLONTECH, Palo Alto, CA). The nucleotides used were 5'-CTAGTTGCTAGC-3' (GAGGGTTGTAAC-5') (5635, +KpnI), 5'-TTGTACACTTAATCTTACCAACGCCTCGGAC (5'-GAACTTGCTGGGTACAACTC-3') (V83I, +AccI), 5'-GGCCCTCCACCACTGCTCTCCACCA-3' (D71S/Y72T/A73H, +PmlI), 5'-GACTAGTCTGCTTAAAGCCACCCATCTAC (V86I, +HpaI), 5'-AGCCGGATCCTCCCGTAT (F83I, +NruI), 5'-CGATTGCTGGGTACAACTC-3' (85V, +BsaI), 5'-TCATACACGAAAGTTAACACTC-3' (E89T, +HpaI), and 5'-AGACGGCCCTGCGCAGGACACCC-3' (P102Q, -StyI) (lowercase, altered nucleotides; italics, new codons). To mutate the hTNF gene, we used the bacterial expression plasmid pMatHTNF (15) and the oligonucleotides 5'-GGCTGGCCGCCTACTGCTCTGCCACAGTAAGTTGTTACCCACCCATCTAC-3' (85V, +HpaI), and 5'-AAGACGGCCCTGCGCAGGACACCC-3' (P102Q, -StyI) (lowercase, altered nucleotides; italics, new codons). Mutant clones were screened for the presence of a newly created (or eliminated) restriction site and were confirmed by sequence analysis.

L929s Cytotoxicity Assays—L929s murine fibroblastoma cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin sulfate (100 coils/ml), and L-glutamine, 1.1 mM sodium pyruvate, and 50% (smTNF) I, smTNF-RII, hTNF-RI/Fc, or hTNF-RII/Fc were passed through different mAb coating sensor-chips flow cells (association phase) followed by HEPES-buffered saline (dissociation phase). The changes in response between TNF and mAb coated sensor-chip flow cells (association phase) followed by HEPES-buffered saline (dissociation phase). The changes in response between TNF and mAb coated sensor-chip flow cells (association phase) followed by HEPES-buffered saline (dissociation phase).

RESULTS

Design and Receptor Specificity of TNF Muteins—Since hTNF and mTNF are very homologous, mTNF/hTNF in-frame chimeras may form bioactive homotrimeric molecules. Because hTNF binds to mTNF-RI only, such chimeras were used to identify the part of the mTNF protein required for binding to mTNF-RI. The interaction of wild-type (wt) and chimeric TNF proteins with both types of mTNF-RI was characterized in a direct in vitro binding assay. Recombinant smTNF-Rs were covalently bound to the surface of a CM5 chip, after which a TNF solution was passed over the receptor-coated surface. The resulting sensorgrams clearly showed that mTFN interacts with both types of mTNF-R and that it dissociates much faster from mTNF-RII than from mTNF-RI. Chimeric proteins with a human sequence between amino acids 50 and 90, viz. MHTNF-22, MHTNF-23, and MHTNF-16, interact with mTNF-RII only, albeit with different efficiency. On the other hand, chimeras with a corresponding murine sequence, viz. MHTNF-11, MHTNF-24, and MHTNF-15, bind to both types of mTNF-R (Fig. 2, A, B, E, and F). We also measured the interaction of these mTFN/hTNF chimeric proteins with both types of hTNF-R/Fc proteins. As mTFN, just like hTNF, interacted with both types of hTNF-R, the chimeric TNF proteins tested also interacted with both hTNF-R/Fc proteins and could not be used to delineate TNF regions important for interaction with a specific hTNF-R (Fig. 2, G and H).

In a next step, a series of mTFN muteins was constructed in which one or more of the 10 amino acids, differing between mTFN and hTFN in the region 50–90 (Fig. 1), had been replaced. The receptor interaction of the resulting muteins was analyzed in vitro. mTFN-D71S/Y72T/A73H/D73N, mTFN-E89T still interacted with both types of mTNF-R. Mutein mTFN-D71S/Y72T/A73H/D89T, which combines both substitutions, did, however, no longer bind to mTNF-RII. The latter mutein, whose amino acids 71–73 and 89 had been replaced, still efficiently interacted with mTNF-RI (Fig. 2, C, and D). Mutein hTNF-S71D/T72Y/H73A/T89E, on the other hand, was able to...
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Biological Activity of TNF Muteins—To test the biological effect of the mutations, the activity of the muteins in L929s cytotoxicity and CT6 proliferation assays was measured. On the basis of activity of agonistic receptor-specific antibodies, cytotoxicity to L929s cells is known to depend on mTNF-RI triggering, and proliferation of CT6 cells is known to depend on mTNF-RII triggering (23). Like hTNF, mTNF-D71S/Y72T/Δ73H/E89T did not induce CT6 proliferation. Mutein hTNF-S71D/T72Y/H73ΔT89E, on the other hand, was biologically active on CT6 cells (Table I). In L929s assays, the bioactivity of hTNF-S71D/T72Y/H73ΔT89E was more pronounced than hTNF; surprisingly, mTNF-D71S/Y72T/Δ73H/E89T was 100-fold less active than mTNF. As such a difference was not in agreement with receptor binding results in vitro, the trimer stability of the different TNF molecules was investigated.

Trimer Stability of Muteins as Measured by Gel Filtration—As bioactive trimeric TNF molecules have been shown to dissociate into inactive monomers during incubation at low concentrations (24), the stability of mTNF and mutein mTNF-D71S/Y72T/Δ73H/E89T was compared. 125I-Labeled proteins (0.4 nM) were incubated at 26 °C for 16 h in Dulbecco’s modified Eagle’s medium (+10% fetal calf serum) as such or in the presence of 4 nM unlabeled mutein, after which they were subjected to size-exclusion chromatography. After incubation, the fraction in the monomeric peak was much greater for mTNF-D71S/Y72T/Δ73H/E89T than for mTNF (Fig. 3, A and B). Mutein mTNF-D71S/Y72T/Δ73H behaved in the same way as mTNF-D71S/Y72T/Δ73H/E89T after preincubation and subsequent gel filtration (data not shown). Based on these results, it may be concluded that the faster dissociation of mTNF-D71S/Y72T/Δ73H/E89T negatively affects its bioactivity in L929s assays where subnanomolar amounts of TNF are used.

Design of a Stable mTNF-RI-specific Mutein—Additional substitution of amino acids in the region 50–90 in mTNF-D71S/Y72T/Δ73H/E89T did not enhance the cytotoxic activity to L929s cells (data not shown). The three-dimensional structure of mTNF (20) shows that amino acids 102–104, which differ in mTNF and hTNF, are located close to amino acids 71–73 (Fig. 4). Hence mutein mTNF-D71S/Y72T/Δ73H/E89T/P102Q was created and tested. The receptor specificity of the latter mutein and mTNF-D71S/Y72T/Δ73H/E89T was similar in direct in vitro receptor binding assays (Fig. 2). As expected, both muteins were inactive in mTNF-RII-mediated CT6 assays. In mTNF-RI-mediated L929s cytotoxicity assays, however, mTNF-D71S/Y72T/Δ73H/E89T/P102Q was 25-fold more active than mTNF-D71S/Y72T/Δ73H/E89T and had a specific activity almost comparable with that of hTNF (Table I). To determine whether this increase in cytotoxic activity was due to increased trimer stability, the TNF mutein was labeled with 125I and subsequently subjected to size-exclusion chromatography. Mutein mTNF-D71S/Y72T/Δ73H/E89T/P102Q appeared to be much more stable than mTNF-D71S/Y72T/Δ73H/E89T and to behave as wt mTNF (Fig. 3C).

**DISCUSSION**

At present, the mechanism of toxicity observed after in vivo administration of TNF-RI-specific muteins remains unclear. In the murine model, where hTNF is a specific agonist for mTNF-RI, the LD50 dose of hTNF is about 50 times higher than that of mTNF, but it still has an antitumor activity that is comparable with that of mTNF (25). In monkeys, administration of hTNF or a TNF-RI-specific mutein of hTNF induced similar changes in important physiological parameters (blood pressure and liver or kidney functions) when measured over a relatively short time interval (26). The pharmacokinetics, however, are very different; the mutein remains in circulation for a much longer time. Hence pathophysiological studies of TNF in rela-
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Table I

Bioactivity of TNF muteins in different murine bioassays

|                | L929s assay | CT6 assay |
|----------------|-------------|-----------|
| mTNF           | 2.1 \times 10^6 | 5 \times 10^5 |
| hTNF           | 6.7 \pm 3.1 \times 10^7 | <500 |
| mTNF-D71S/Y72T/Δ73H/E89T | 1.8 \pm 1.3 \times 10^6 | <500 |
| mTNF-D71S/Y72T/Δ73H/E89T/P102Q | 4.7 \pm 1.3 \times 10^7 | <500 |
| hTNF-S71D/T72Y/H73Δ/T89E | 1.5 \pm 0.6 \times 10^8 | 2.9 \pm 0.9 \times 10^5 |

Fig. 3. Comparison of the stability of wt mTNF and two muteins at subnanomolar concentrations. 125I-Labeled mTNF (A), mTNF-D71S/Y72T/Δ73H/E89T (B), or mTNF-D71S/Y72T/Δ73H/E89T/P102Q (C) were preincubated (16 h, 26 °C) at 200 ng/ml in Dulbecco's modified Eagle's medium/fetal calf serum as such (○) or in the presence of the same unlabeled TNF or mutein at 200 ng/ml (○). Trimeric and monomeric TNF molecules were separated by size-exclusion chromatography, after which the radioactivity in each fraction was determined.

homologues (D71S/Y72T/Δ73H and E89T) was sufficient to eliminate the interaction of this mutein with mTNF-RII as measured in vitro. As expected, mutein mTNF-D71S/Y72T/Δ73H/E89T was not active in mTNF-RII-mediated CT6 proliferation assays. In addition, mutein hTNF-S71D/T72Y/H73Δ/T89E, which contains corresponding murine substitutions, interacted with mTNF-RII in vitro and clearly induced proliferation of murine CT6 cells. The results indicate that these amino acids are directly involved in the binding between TNF and mTNF-RII. However, mTNF-RII-mediated cytotoxic activity to L929s cells of mutein mTNF-D71S/Y72T/Δ73H/E89T was 100-fold lower than that of wt mTNF, although this mutein still efficiently interacted with mTNF-RI in vitro. As biologically active trimeric TNF molecules have been shown to dissociate into inactive monomers after incubation at subnanomolar concentrations (27), we investigated the trimer stability of wt and mutant TNF. Size-exclusion chromatography of labeled protein indicated that mutein mTNF-D71S/Y72T/Δ73H/E89T is more prone to dissociation than mTNF during incubation at low concentrations. This trimer instability probably influences the bioactivity in L929s cytotoxicity assays, which are very sensitive and involve an 18-h incubation. The instability, however, does not affect the assessment of in vitro receptor binding using BIAcore as this takes only a few minutes at TNF concentrations of 200 ng/ml. By substituting other amino acids, differing between hTNF and mTNF, and by testing the bioactivity of resulting muteins, mutein mTNF-D71S/Y72T/Δ73H/E89T/P102Q was obtained. This mutein has in vitro receptor binding characteristics similar to those of mTNF-D71S/Y72T/Δ73H/E89T but is as stable as wt mTNF after incubation at low concentrations. Furthermore, mutein mTNF-D71S/Y72T/Δ73H/E89T/P102Q, which is inactive in CT6 proliferation assays, is 25-fold more active than mTNF-D71S/Y72T/Δ73H/E89T in L929s cytotoxicity assays.

The structure of a TNF subunit folds into a β-sandwich, comprising almost entirely antiparallel β-strands, and forms a bell-shaped, rigid, trimeric molecule containing two other subunits. Although the structure of hTNF and mTNF is very similar (20, 28), the amino acids 71–73, 89, and 102 are all located in flexible, surface-exposed loops that differ strongly (loops 64–76, 84–91, and 99–112). Moreover, the amino acids 71–73, 89, and 102 are present near the intersubunit grooves of the TNF trimer (Fig. 4). These intersubunit grooves are the main site for interaction between TNF and TNF-R as clear from mutagenesis studies (8) and crystallography of human lymphotoxin bound to hTNF-RI (7). Furthermore, amino acids 32 and 86 of hTNF, which are crucial for binding to hTNF-RI, are also located near these intersubunit grooves (8–10).

To rationalize the effect of amino acid substitutions at positions 71–73 and 102 on the trimmer stability, theoretical three-dimensional structures were constructed for two mTNF muteins using the crystal structure of mTNF as a template (Fig.
4. Substituting Asp71–Tyr78 with Ser-Thr-His did not affect the internal hydrogen bonds in the predicted model but resulted in the creation of a hydrophilic patch protruding into the aqueous environment. As both Ser and Thr may participate in the formation of hydrogen bonds, we assume increased possibilities to interact with surrounding water molecules, possibly resulting in destabilization of the loop. According to our predicted model, substitution of Pro102 with Gln leads to the formation of a hydrogen bond between Gln102 NE2 and Glu110 OE1, which may enhance the stability of the loop (Fig. 4). Since a Cys69–Cys101 bridge anchors loop 99–112 with loop 64–76, this substitution may affect the top of the molecule and hence influence the overall trimer stability.

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FIG. 4. Structure of mTNF and muteins. A, three-dimensional structure of wt mTNF subunit C as obtained by x-ray crystallography. B, structure of mutein mTNF-D71S/Y72T/A73H/E89T subunit C as predicted by homology modeling (red, residues 71, 72, and 89; yellow, disulfide bond between Cys69 and Cys101, dashed green lines, hydrogen bonds). C and D, detail of the modeled structure of muteins mTNF-D71S/Y72T/A73H/E89T and mTNF-D71S/Y72T/A73H/E89TP102Q, respectively (dashed blue lines, hydrogen bonds).
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