A Tumor Necrosis Factor (TNF) Receptor-IgG Heavy Chain Chimeric Protein as a Bivalent Antagonist of TNF Activity

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Summary

Using a multistep polymerase chain reaction method, we have produced a construct in which a cDNA sequence encoding the extracellular domain of the human 55-kD tumor necrosis factor (TNF) receptor is attached to a sequence encoding the Fc portion and hinge region of a mouse IgG1 heavy chain through an oligomer encoding a thrombin-sensitive peptide linker. This construct was placed downstream from a cytomegalovirus promoter sequence, and expressed in Chinese hamster ovary cells. A secreted protein, capable of binding TNF and inactivating it, was produced by the transfected cells. Molecular characterization revealed that this soluble version of the TNF receptor was dimeric. Moreover, the protein could be quantitatively cleaved by treatment with thrombin. However, the monovalent extracellular domain prepared in this way has a greatly reduced TNF inhibitory activity compared with that of the bivalent inhibitor. Perhaps because of its high affinity for TNF, the chimeric protein is far more effective as a TNF inhibitor than are neutralizing monoclonal antibodies. This molecule may prove very useful as a reagent for the antagonism and assay of TNF and lymphotoxin from diverse species in health and disease, and as a means of deciphering the exact mechanism through which TNF interacts with the 55-kD receptor.

The recent cloning of the 55-kD (1–3) and 75-kD (3–5) TNF receptors has opened the way for further studies of TNF effects and signal transduction. Moreover, it appears that truncated receptor molecules, lacking the transmembrane or cytoplasmic domains, are capable of interacting with TNF, and therefore have been isolated from urine (6, 7) and serum (2) as TNF inhibitors. We considered that derivatives of such molecules might prove useful as antagonists of TNF action in vivo, as high affinity ligands to be applied as the basis of a more sensitive assay for TNF, and as reagents to be used in defining the molecular interaction between TNF and its receptor.

Unfortunately, truncated forms of the TNF receptor are highly unstable in vivo, and therefore are poor substitutes for antibodies as a means of antagonizing TNF action in living animals. The naturally occurring TNF receptor fragments are univalent and therefore have an avidity that is effectively far lower than that of a bivalent ligand. The production of large quantities of a truncated receptor by recombinant means has, in our hands, been problematic, since the protein is produced in an inactive and insoluble form in bacteria (Peppel, K., and B. Beutler, unpublished observations). This difficulty may arise from the highly cysteine-rich structure of the receptor-binding domain (1, 2). When produced in mam-

malian cells through recombinant techniques, the soluble receptor fragment is active, but produced at low levels, and therefore difficult to purify (Beutler, B., and T. Brown, unpublished observations).

To circumvent these problems, we have engineered a chimeric protein in which the extracellular domain of the TNF receptor, which normally engages the TNF molecule, is covalently linked to domains C_H2 through C_H3 of a mouse IgG1 heavy chain. Interposed between the two polypeptide sequences is a hexapeptide sensitive to cleavage by thrombin. The chimeric protein is expressed and secreted by Chinese hamster ovary (CHO)[1] cells. It is highly active as a TNF inhibitor, is readily purified by affinity chromatography using an anti–mouse IgG column, and is quantitatively cleaved by thrombin to yield the extracellular domain in an active form.

Materials and Methods

Cell Culture. The human skin melanoma cell line SK MEL 109 was grown in FM medium supplemented with 10% FCS. CHO

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[1] *Abbreviations used in this paper:* CHO, Chinese hamster ovary; CHX, cycloheximide; TNFR-ED, TNF receptor extracellular domain.

cells were grown in F12 medium supplemented with 10% FCS. Transfected CHO cells were grown in monolayer or spinner cultures. For production of the TNF inhibitor, CHO cells in spinner culture were maintained in serum-free medium (90% F12:10% complete WRC 935; Amicon, Beverly, MA) for 3 d, after which cells were removed and the conditioned medium containing the inhibitor was processed.

Cloning of a cDNA Encoding the TNF Receptor Extracellular Domain. The TNF receptor extracellular domain (TNFR-ED) was cloned from total cytoplasmic RNA prepared from HL60 cells using a reverse transcriptase/PCR protocol (8). Briefly, an oligonucleotide primer with the sequence cttaagcttagtactcaTGTGGTGCCT-GAGTCCTCAG, corresponding to the 3' end of the extracellular domain, was used to direct first-strand cDNA synthesis in a total volume of 20 μl. The reaction was then diluted to 100 μl in PCR buffer (8) and a second primer with the sequence gcgcatcgaTCTGG-CATGGGCCTCTCCACC, corresponding to the 5' end of the human TNF receptor, was added. The reaction was subjected to 40 cycles of denaturation and synthesis in an automated temperature cycler (Perkin Elmer Cetus Instrs., Norwalk, CT). The band corresponding to the TNFR-ED was purified by gel electrophoresis and ligated into the vector pGEM-3Z.

Preparation of the Chimeric Construct. A plasmid encoding a murine IgG1 heavy chain cDNA was obtained from Dr. C. Haseman of the Howard Hughes Medical Institute. The TNFR-ED and the IgG heavy chain cDNAs were separately amplified by PCR using primers gcgcatcgaTCTGGCATGGGCCTCTCCACC (corresponding to the 5' end of the TNFR-ED moiety), ggatccacgcggaaccag-TGTGGTGCCTGAGTCCTC (corresponding to the 3' TNFR-ED moiety and the thrombin cleavage site), ctggttccgcgtggatccGTGCC-CAGGGATTGTGGT (corresponding to the thrombin cleavage site and the 5' end of the IgG moiety, and attaagcattctagaTCATTTACC-AGGAGAGTG (corresponding to the 3' end of the IgG moiety).

The PCR products obtained after the first synthetic PCR reaction thus carry the thrombin cleavage site on their 3' (TNFR-ED) and 5' (IgG) ends (Fig. 1). The PCR products were isolated on a low melting point gel and slices containing the correct DNAs were combined and used for a second round of PCR amplification, using only primers corresponding to the 5' end of the TNFR-ED and 3' end of the IgG. This PCR reaction effectively joins the TNFR-ED and the IgG through the thrombin cleavage site.

The construct was then digested with ClaI and XbaI (encoded in the 5' TNFR-ED and 3' IgG primers) and ligated into the vector pCMV4 (9). The entire sequence was verified by dideoxynucleotide sequencing (10) on both strands.

Assays of TNF and TNF Inhibitor Activity. TNF inhibitor activity was determined through inhibition of TNF cytotoxicity toward human SK MEL 109 cells in the presence of the protein synthesis inhibitor cycloheximide (CHX). Typically, a constant amount of inhibitor was incubated with a series of standard concentrations of TNF in FM medium containing 10% FCS and CHX at a final concentration of 50 μg/ml. The final system volume was 150 μl. TNF was allowed to preincubate with the inhibitor for 1 h (Figs. 3, 6–8) at 37°C in a 96-well microtiter plate. 50 μl of FM containing 10% FCS and 5 $\times$ 10^4 SK MEL 109 cells was then added to each well. A further incubation was then carried out overnight at 37°C in a 5% CO$_2$ incubator. At the end of the incubation the plate was washed and stained with crystal violet. The cell-bound dye was dissolved in 40% acetic acid and staining was quantitated in an automatic plate reader (Intermed, Thousand Oaks, CA). All assays were performed in duplicate and the SE in all experiments was <10%. mAbs against TNF were used for comparative purposes in some assays. One such antibody (designated mAb 1) was

provided by Suntory, Inc. (Osaka, Japan). A second antibody (SW 18.1; designated mAb 2) was provided by Dr. S. Wolpe (Genetics Institute, Cambridge, MA). A third antibody (designated mAb 3) was provided by Dr. R. Schreiber (Washington University, St. Louis, MO). mAbs 1 and 2 neutralize human TNF, whereas mAb 3 neutralizes mouse TNF.

CHO Cell Transfection. CHO cells were cotransfected with the inhibitor cDNA in pCMV4 (pCMV4-I), and with the vector pcDNAneo, in a 10:1 ratio, or with pcDNAneo alone, using the calcium phosphate precipitation method of Chen and Okayama (11). Cells resistant to 1 mg/ml G418 were selected over a 3-wk period and pooled. Each transfection yielded ~200–300 independent transfectants.

Northern Blot Analysis. Total cytoplasmic RNA was prepared from transfected CHO cells as previously described (12). For Northern blot analysis the RNA was glyoxylated, resolved in a 1.2% agarose gel, and electroblotted onto a nylon membrane. It was then hybridized to ^{32}P-labeled antisense riboprobes corresponding to the TNFR-ED or to human glyceraldehyde-3-phosphate dehydrogenase. After hybridization the blot was washed twice in 2$\times$ SSC at 70°C and finally in 0.1$\times$ SSC at 70°C. Blots were allowed to expose x-ray film overnight at -80°C.

Production and Purification of the Chimeric TNF Inhibitor. For production of inhibitor, cells were incubated in serum-free medium (90% F12:10% complete WRC 935) for 72 h. Cells were then removed by centrifugation followed by filtration. The conditioned medium was then passed over a column of goat anti–mouse heavy chain IgG coupled to Sepharose (Sigma Chemical Co., St. Louis, MO). The affinity resin was then washed with a solution containing 500 mM NaCl, 10 mM NaH$_2$PO$_4$ (pH 7.2), and 1 mM EDTA. Bound protein was eluted with a solution containing 50 mM acetic acid (pH 2.4) and 150 mM NaCl. 500-μl fractions were collected and neutralized by addition of 75 μl of 1 M Tris (pH 8.0). Fractions were dot blotted onto nitrocellulose and incubated with alkaline phosphatase–conjugated goat anti–mouse IgG to permit detection and quantitation of the inhibitor.

Thrombin Cleavage. 100 μl of column eluate containing ~300 ng pure inhibitor was mixed with 100 μl of digestion buffer (at 10 mM CaCl$_2$, 10 mM MgCl$_2$, and 100 mM tris, pH 7.0). 5 μl of a preparation containing 0.5 U of thrombin (Boehringer Mannheim Biochemicals, Indianapolis, IN) was then added and the reaction was incubated at 25°C for various periods of time. Aliquots were removed at intervals and the reaction was terminated by the addition of SDS sample buffer.

Iodination of TNF. Human recombinant TNF was iodinated using the iodogen method (13). Radiolabeled TNF was separated from unincorporated ^{125}I by chromatography on Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, NJ). The specific activity of the final preparation was 10^4 cpm/ng TNF.

Cross-linking of TNF to Inhibitor. 25 ng of ^{125}I TNF was incubated for 1 h at 37°C with 100 ng of purified inhibitor, or inhibitor that had been cleaved with thrombin, in 50 μl of buffer (25 mM Hepes, pH 7.2, 50 mM NaCl, 0.1% BSA). Where indicated, 2 μg of unlabeled competitor TNF was included in the reaction; 2.5 μl of disuccinimidylsuberate (DSS; Pierce Chemical Co., Rockford, IL; 9.3 mg/ml in DMSO) was added and the reaction was incubated for 30 min at 37°C. The reaction was then terminated by the addition of SDS sample buffer.

SDS-PAGE. SDS-PAGE of the inhibitor and of mouse IgG, before and after thrombin cleavage, under reducing and nonreducing conditions, was carried out as previously described (14). Western blot analysis was performed after electrotransfer of the protein to nitrocellulose membranes. An alkaline phosphatase–conjugated goat

anti–mouse IgG was used to detect the chimeric receptor IgG proteins.

In Vitro Translation. A 2.7-kb SacI fragment containing the entire inhibitor cDNA was subcloned into pGEM4. After linearization with XhoI, the plasmid was transcribed in vitro with SP6 polymerase in the presence of m7GpppG to yield 5'-capped RNA transcripts. 20% of the transcription reaction was used to program rabbit reticulocyte lysates (Promega Corp., Madison, WI) in a standard translation reaction prepared according to instructions provided by the manufacturer in the presence of ³⁵S-translabel (ICN Biochemicals, Costa Mesa, CA). After synthesis, the proteins were resolved by SDS-PAGE and analyzed by autoradiography.

Results

Construction of TNF Inhibitor and Expression in CHO Cells. The construct used to produce a chimeric protein, consisting of the TNF receptor-binding domain attached to the IgG heavy chain domains C_H2 through C_H3, is illustrated in Fig. 1 *a*, and a schematic depiction of the protein itself in (Fig. 1 *b*).

Figure 2. Northern blot of TNR inhibitor mRNA produced by transfected CHO cells. 3 μg of total RNA from inhibitor-transfected or control CHO cells of each sample was separated on a 1.2% agarose gel, electroblotted onto nylon, and hybridized either to radiolabeled TNFR-ED antisense RNA (lane *A*) or to radiolabeled glyceraldehyde-3-phosphate dehydrogenase antisense RNA (lane *B*). Arrow indicates the position of the TNF inhibitor mRNA.

CHO cells transfected with the construct express high levels of the chimeric mRNA (Fig. 2). The mRNA detected is not transcribed from the endogenous 55-kD receptor gene, since no such band is discernible in CHO cells transfected only with pcDNA*neo*. Consistent with the fact that high levels of the chimeric mRNA are expressed by CHO cells, high levels of TNF inhibitory activity are secreted by transfected CHO cells (Fig. 3).

Purification of Inhibitor by Affinity Chromatography. The TNF inhibitor could be purified to apparent homogeneity by passing conditioned medium from transfected CHO cell cultures over an anti–mouse IgG–Sepharose column, and eluting the bound protein with 50 mM acetic acid, 150 mM
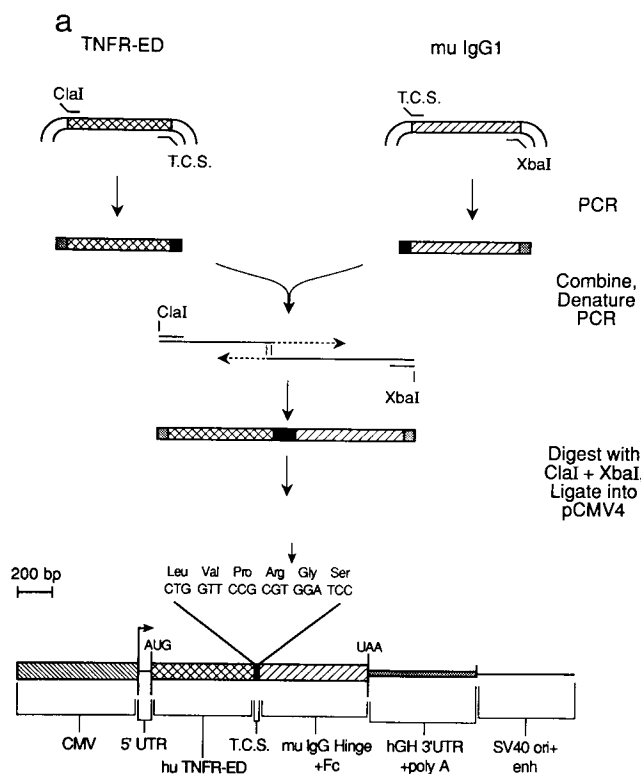

Figure 1. (*a*) Method used to construct the TNF inhibitor. cDNAs encoding the human TNFR-ED and the murine IgG1 heavy chain hinge and Fc region were separately amplified by PCR using primers that add unique restriction enzyme sites (ClaI on the 5' end of the TNFR-ED and XbaI on the 3' end of the IgG1) and a thrombin sensitive peptide linker (on the 3' end of the TNFR-ED and on the 5' end of the IgG). After separation of the PCR products by low melting point agarose electrophoresis, slices containing the fragments of interest were combined and subjected to a second round of amplification using only the 5' end primer for RNFR-ED and the 3' end primer for IgG. The PCR products were again separated by agarose electrophoresis and the fragment corresponding to the full-length inhibitor was isolated, digested with ClaI and XbaI, and subcloned into pCMV4. CMV, cytomegalovirus promoter; 5' UTR, 5' untranslated region; T.C.S., thrombin cleavage site; hGH 3'UTR, human growth hormone 3' untranslated region; SV40 ori + enh, simian virus 40 origin of replication and enhancer. (*b*) Schematic depiction of the protein. Nerve growth factor receptor-like cysteine-rich domains of the TNFR-ED are represented by loops. The bars represent the cysteine residues (oxidation state is unknown). IgG moiety is presented according to the same scheme.
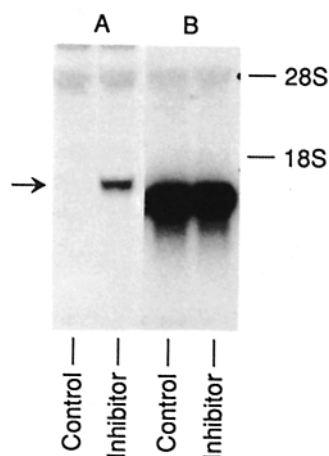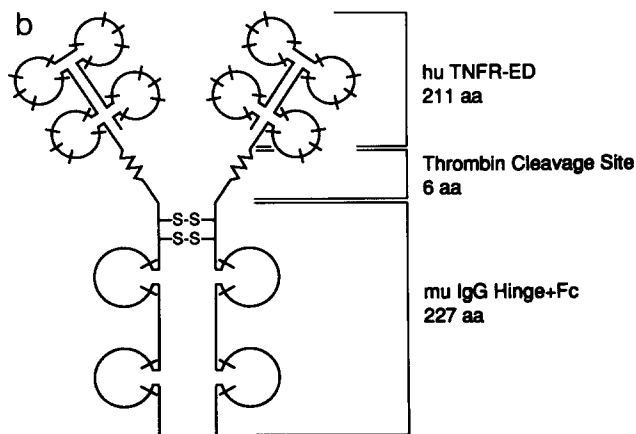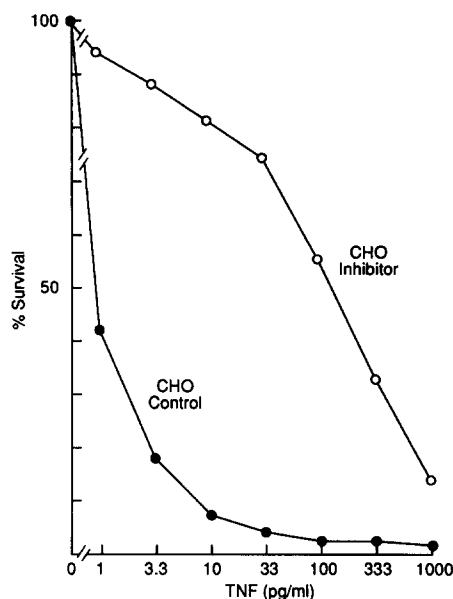
Figure 3. Inhibition of TNF cytotoxicity by secreted inhibitor. 50 μl of CHO-conditioned culture medium (either from cells permanently transfected with the TNF inhibitor cDNA or from control cells transfected with pcDNA*neo* alone) was incubated with varying amounts of human TNF (concentration indicated) in the presence of cycloheximide. After a 1-h incubation at 37°C, 5 × 10⁴ SK MEL 109 cells were added to the system, and the incubation was continued overnight. Cell survival was quantitated by staining with crystal violet.

NaCl. Material so eluted was highly active as a TNF inhibitor. Purified preparations of the inhibitor migrate at a rate consistent with a dimeric structure when subjected to PAGE under nonreducing conditions. When reduced, a monomeric structure is apparent (Fig. 4). The monomer is approximately
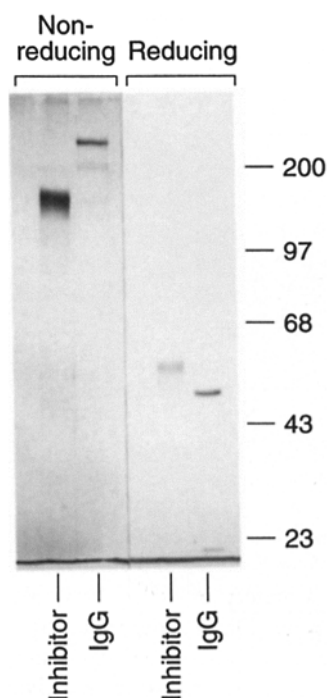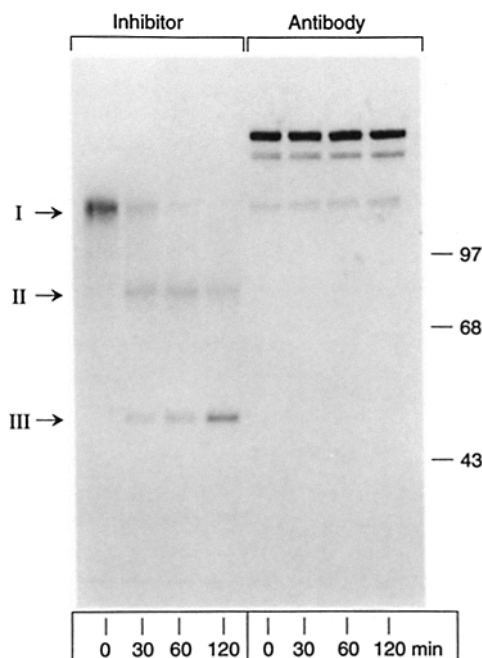

Figure 5. Cleavage of the TNF inhibitor by thrombin. Affinity-purified TNF inhibitor or anti-TNF mAb 1 was digested with thrombin for varying lengths of time (indicated) and separated by nonreducing SDS-PAGE. After blotting onto a nitrocellulose membrane, the IgG-containing fragments were visualized using an affinity-purified goat anti–mouse IgG antibody conjugated to alkaline phosphatase (Biorad Laboratories, Richmond, CA). Roman numerals I, II, and III refer, respectively, to the undigested form of the TNF inhibitor, and to forms from which one or both TNFR-ED have been removed by the action of thrombin. IgG remains undigested in the presence of thrombin.
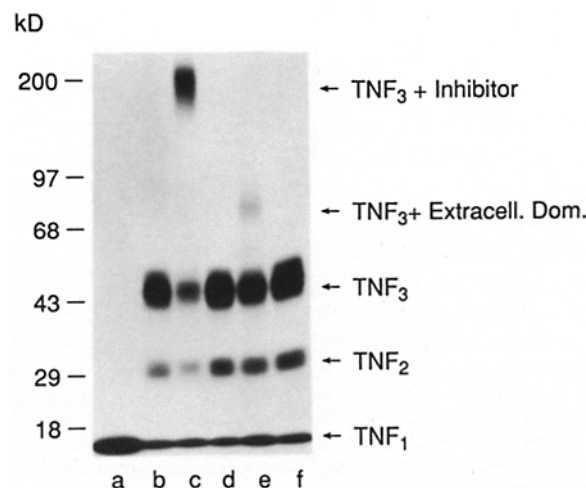

Figure 4. Purification of the TNF inhibitor by affinity chromatography. Conditioned medium from CHO cells transfected with the TNF inhibitor cDNA was passed over a goat anti–mouse IgG sepharose column. The bound protein was eluted with a solution containing 50 mM acetic acid and 150 mM NaCl. The eluate was quickly neutralized by adding 0.1 vol of 1 M tris, pH 8.0. Inhibitor so purified was subjected to reducing (+ β-mercaptoethanol) and nonreducing (– β-mercaptoethanol) SDS-PAGE. 200 ng of an anti-TNF mAb 1 was added to the gel as an additional standard. The gel was silver stained.

Figure 6. Cross-linking of TNF and inhibitor. Lane *a*, ¹²⁵I TNF not cross-linked; lane *b*, ¹²⁵I TNF cross-linked with disuccinimidylsuberate; lane *c*, ¹²⁵I TNF cross-linked to inhibitor; lane *d*, same as lane *c* but unlabeled TNF was included as competitor; lane *e*, ¹²⁵I TNF cross-linked to thrombin digested inhibitor; lane *f*, same as lane *e* but unlabeled TNF was included as competitor.
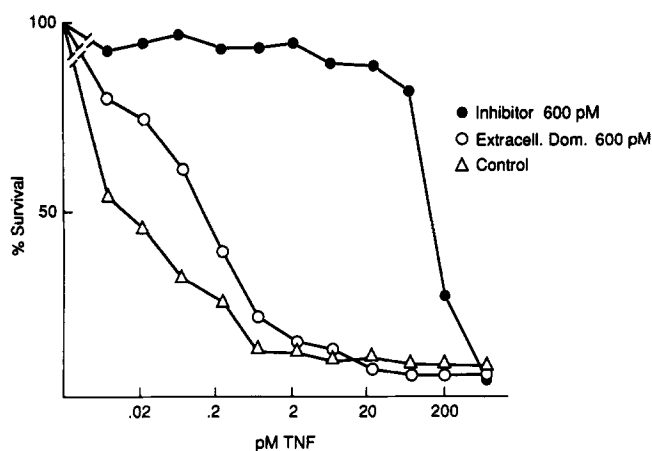
Figure 7. Inhibition of TNF cytotoxicity by inhibitor or extracellular domain. 600 pM of inhibitor or 600 pM of inhibitor cleaved with thrombin was incubated with different amounts of TNF (indicated) in the presence of CHX for 1 h at 37°C. 5×10^4 SK MEL 109 cells were added to each well and incubated overnight. Thrombin alone does not effect survival of SK MEL 109 cells in this assay (data not shown). Cell survival was quantitated by staining with crystal violet.

Figure 8. Comparison of anti-TNF activity of two mAbs against TNF with the activity of the TNF inhibitor. Varying amounts of antibody or TNF inhibitor were incubated with human TNF (300 pg/ml) in the presence of CHX for 1 h at 37°C. 5×10^4 SK MEL 109 cells were then added to the system, and the incubation was continued overnight. Cytotoxicity was quantitated by staining with crystal violet.

8–10 kD larger than a protein translated from in vitro transcribed RNA (data not shown). This likely reflects glycosylation at the three N-linked glycosylation sites of the extracellular domain of the TNF receptor as identified by several groups (1, 2). Loetscher et al. (15) reported that TNF receptor isolated from HL60 cells contains 5–10 kD of carbohydrate.

Cleavage of Inhibitor by Thrombin Reduces its Anti-TNF Activity. The inhibitor is quantitatively cleaved by thrombin, as indicated by the fact that the heavy chain fragment visible on SDS-PAGE migrates faster (consistent with proteolysis at the thrombin-sensitive site) after incubation with this enzyme (Fig. 5).

To see whether the cleaved inhibitor was still able to bind TNF, we cross-linked radioiodinated TNF to inhibitor, or to inhibitor that had been cleaved with thrombin and adsorbed to anti-IgG sepharose (to remove the IgG portion of the inhibitor and any undigested, bivalent inhibitor). Both the digested (Fig. 6, lane *e*) and the undigested (Fig. 6, lane *c*) preparation of the inhibitor are capable of binding TNF. The binding was completely abrogated by the addition of 80-fold excess unlabeled TNF (Fig. 6, lanes *d* and *f*). The purified inhibitor is very effective in neutralizing TNF. A threefold molar excess of inhibitor over TNF trimer is capable of more than doubling survival of SK MEL 109 cells in a standardized cytotoxic assay (Fig. 7). A 10-fold excess of inhibitor over TNF trimer affords virtually complete protection. However, cleavage with thrombin greatly reduces the TNF inhibitory activity of the molecule (Fig. 7). See Discussion for possible explanations.

Comparison of Anti-TNF Activity of Inhibitor and mAbs. To compare the anti-TNF activity of the chimeric bivalent inhibitor with the anti-TNF activity of three mAbs known to neutralize human (mAbs 1 and 2) or murine (mAb 3) TNF, a series of dilutions of each reagent was used to block the
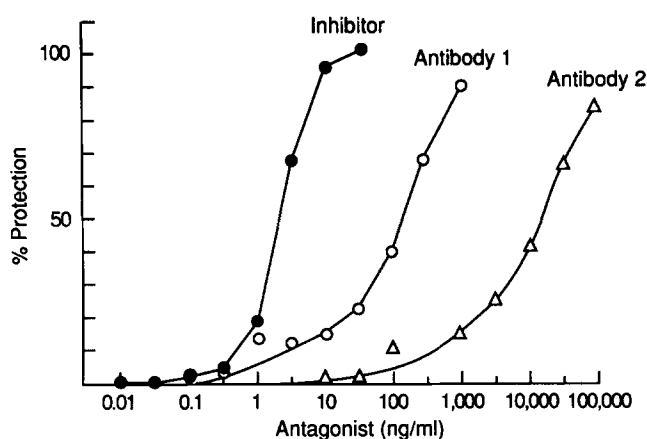
activity of a fixed concentration of human or murine TNF sufficient to cause lysis of >75% of SK MEL cells. The TNF inhibitor is ∼100- and 10,000-fold more effective than the anti-TNF mAbs 1 and 2, respectively, as an antagonist of human TNF activity (Fig. 8). The inhibitor is also ∼100-fold more active against murine TNF than mAb 3 (not shown).

Discussion

The TNF inhibitor that we have produced and characterized finds precedent in the work of Capon et al. (16) and Traunecker et al. (17), who designed secreted chimeric molecules in which the binding moiety of a CD4 molecule was adjoined to an IgG heavy chain. This molecule circulated for a prolonged period of time in vivo. It would thus appear that adding an IgG heavy chain as a partner in chimeric molecules may be a useful strategy for the expression of molecules that would otherwise prove unstable in circulation. The TNF inhibitor may prove useful under many circumstances. Since the molecule is produced by CHO cells in considerable abundance, and since it can easily be purified, it will be possible to produce large quantities of the protein for investigational use. Whereas a mouse IgG molecule was used in these studies, a homologous construct utilizing the human IgG heavy chain could readily be prepared.

The striking efficacy of the inhibitor may result from several of its properties. Engelmann et al. (18) demonstrated that polyclonal antiserum raised against soluble TNF receptors can mimic the cytotoxic activity of TNF in the presence of CHX and can also reproduce a variety of other responses ascribed to TNF. The authors were able to show that cross-linking of TNF receptors on the cell surface by antibodies was both necessary and sufficient to induce lysis in sensitive

target cells. This led the authors to propose that crosslinking of TNF receptors by TNF is the primary mode of signal transduction. Smith and Baglioni (19) have shown the trimer to be the active form of TNF. Mutagenesis studies have shown the subunit interface to be involved in receptor binding (20). Therefore, only a trimer, but not a dimer or a monomer, would allow the binding of the two or three receptors necessary for signal transduction. The inhibitor produced in this study utilizes two TNFR-EDs, and possibly blocks two of the three potential receptor binding sites of TNF. In addition, simultaneous attachment of the two binding domains of the inhibitor to the same trimer increases the effective affinity of this molecule for its ligand. Interestingly, we find that cleavage of the inhibitor with thrombin greatly reduces the TNF inhibitory activity. The monovalent extracellular domain is only able to block one of the three receptor binding domains of TNF; therefore, it is unable to prevent receptor crosslinking by the remaining free binding sites.

The affinity of the interaction between TNF and the combining site of soluble TNF receptors has been estimated by several laboratories, and assigned a K_a value between 10^9 and 10^{10} (6, 7, 21, 22). Since antibodies typically exhibit combining group affinities in the range of 10^5-10^6 K_a, it might be expected that the chimeric protein produced in this study would form far more stable complexes. The sensitivity of a RIA is generally limited by ligand affinity; therefore, the inhibitor might permit the assay of TNF at lower concentrations than could be achieved using a competitive RIA using antibodies. Moreover, the chimeric protein that we have produced should be a particularly versatile reagent, insofar as it should detect TNF from a wide range of mammalian species, whereas most antibody preparations crossreact weakly with TNF from diverse species.

Given the fact that both lymphotoxin (TNF-β) and TNF are bound by the 55-kD receptor with comparable affinity (1-5), the reagent used in these studies should effectively neutralize both of these molecules. This property might lend certain advantages to the use of this inhibitor as a tool for the investigation of TNF function in vivo, and as a means of neutralizing TNF activity for clinical effect. Insofar as the reagent should only recognize functional TNF (i.e., a TNF trimer capable of engaging its receptor), it may be imagined that the protein might permit an analysis of plasma concentrations of active TNF (in contrast with TNF that has been bound by inhibitors or denatured) in various disease processes.

Preliminary studies indicate that the inhibitor has a long half-life in vivo (data not shown). Therefore, it might be useful as a means of assessing the function of TNF under a variety of physiologic and pathologic conditions. Whether intermittently injected, chronically administered via a system similar to that described by Oliff et al. (23), who used CHO cells as a vehicle for the production of recombinant TNF in nude mice, or produced in transgenic animals, the inhibitor might yield valuable insight into the actions of TNF in vivo. Of particular interest might be the effect of the inhibitor upon the course of septic shock, of tumor growth in vivo, and development of the immune system. It would seem likely that the reagent described here will prove useful as a means of defining the interactions that occur between TNF and its receptors. The TNFR-ED can easily be purified after thrombin cleavage. Efforts to crystallize the purified molecule and the thrombin cleavage product, alone and as a complex with the TNF molecule, are in progress.

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