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Characterization of a Recombinant Extracellular Domain of the Type 1 Tumor Necrosis Factor Receptor: Evidence for Tumor Necrosis Factor-α Induced Receptor Aggregation

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ABSTRACT: An expression plasmid encoding the extracellular portion of the human tumor necrosis factor (TNF) type 1 receptor (TNF-R1) was constructed and used to generate a stable cell line secreting soluble TNF-R1 (sTNF-R1). The sTNF-R1 was purified, and its biochemical properties and its interactions with human TNF-α were examined. SDS-PAGE resolved the purified sTNF-R1 into three bands of approximate M_r 24 200, 28 200, and 32 800. Sedimentation equilibrium analysis gave a molecular weight of 25 000 for sTNF-R1 whereas the molecular weight obtained by gel filtration chromatography was approximately 55 000–60 000. Scatchard analysis of [^{125}I]TNF-α binding to sTNF-R1 revealed high-affinity binding (K_d = 93 pM), comparable to that observed for the intact receptor on whole cells. Competitive binding experiments showed that sTNF-R1 has a 50–60-fold higher affinity for TNF-α than for TNF-β, in contrast to the equal affinities of TNF-α and TNF-β for the full-length TNF-R1 transiently expressed in mammalian cells. The sTNF-R1 was found to block the cytotoxicity of TNF-α and TNF-β on a murine L-M cell assay. The sizes of the sTNF-R1·TNF-α complex determined by gel filtration chromatography and sedimentation equilibrium were approximately 141 and 115 kDa, respectively. The stoichiometry of the complex was examined by Scatchard analysis, size-exclusion chromatography, HPLC separation, amino acid composition, sequence analysis, and sedimentation equilibrium. The data from these studies suggest that at least two molecules of sTNF-R1 can bind to a single TNF-α trimer. We propose that the initiation of signaling by TNF-R1 involves TNF-α-induced receptor oligomerization.

TNF-α and TNF-β are related cytokines produced by activated macrophages and lymphocytes, respectively (Beutler & Cerami, 1989). Originally described for their ability to induce hemorrhagic necrosis of certain murine tumors and for their cytotoxicity to some tumor cell lines (Carswell et al., 1975), they have now been shown to mediate a wide variety of biological responses both in vivo and in vitro (Goeddel et al., 1986; Beutler & Cerami, 1988a,b). Binding studies with [^{125}I]-labeled TNF-α and TNF-β have revealed the presence of specific TNF receptors on the majority of somatic cell types examined (Rubin et al., 1985; Kull et al., 1985; Baglioni et al., 1985; Aggarwal et al., 1985; Tsujimoto et al., 1985; Yoshie et al., 1986). Although the biochemical events underlying TNF action have not yet been elucidated, it appears that binding of TNF to these receptors initiates the biological responses of TNF. However, the molecular forms of active TNF-α and TNF-β are still somewhat unclear. It has been shown by crystallization studies and sedimentation equilibrium that TNF-α exists as a closely packed trimer (Wingfield et al., 1987; Eck et al., 1988; Lewit-Bently et al., 1988; Jones et al., 1989); however, there are many conflicting reports in the literature as to whether the active form of TNF-α, that

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triggers a response through receptor binding, is a trimer, dimer, or monomer (Smith & Baglioni, 1987; Peterson et al., 1989; Heller et al., 1990).

Two distinct TNF receptors have been recently identified, cloned, and expressed from both human and mouse (Loetscher et al., 1990; Schall et al., 1990; Gray et al., 1990; Smith et al., 1990; Himmler et al., 1990; Nophar et al., 1990; Lewis et al., 1991; Goodwin et al., 1991; Kohno et al., 1990; Dembic et al., 1990). These receptors, designated TNF-R1 (p55) and TNF-R2 (p75), bind both TNF-α and TNF-β with similar affinities. They show only about 30% amino acid identity in a 170 amino acid extracellular region, characterized by 4 cysteine-rich repeat units, that is presumed to be the ligand binding domain. Their intracellular regions, however, show no homology. The 4-fold cysteine-rich repeat region of the type 1 receptor has 34% amino acid sequence identity to the extracellular region of the nerve growth factor (NGF) receptor (Johnson et al., 1986) and is also homologous to an open reading frame of the Shope fibroma virus (Smith et al., 1991), the B lymphocyte activation molecule Bp50 (Stamenkovic et al., 1989), and the MRC OX40 antigen (Mallet et al., 1990). The existence of these homologies establishes that the TNF receptors belong to a diverse superfamily of proteins.

Since TNFs induce such a wide spectrum of activities, it is likely that some of these responses are mediated by TNF-R1, others by TNF-R2. It has recently been shown that receptor clustering may provide a sufficient signal for many TNF effects since TNF-R1 can be triggered to induce a variety of TNF activities, even in the absence of TNF, by cross-linking with anti-TNF receptor 1 specific antibodies (Engelmann et al., 1990b; Espevik et al., 1990; Tartaglia et al., 1991). Similar experiments with antibodies to TNF-R2 demonstrate that this receptor can signal T-cell and thymocyte proliferation (Tartaglia et al., 1991).

Several groups have isolated two immunologically distinct species of TNF-specific binding proteins from urine and serum which have been hypothesized to function as physiological inhibitors of TNF activity (Kohno et al., 1990; Peetre et al., 1988; Seckinger et al., 1989; Novick et al., 1989; Engelmann et al., 1990a; Gatanaga et al., 1990; Lantz et al., 1990). Comparison of the N-terminal sequences of these proteins with those deduced from cDNA cloning of TNF receptors demonstrates that these binding proteins are shed forms of the two cell-surface TNF receptors. Virtually nothing is known about the role of these soluble receptor forms in vivo. The availability of TNF receptor cDNAs has now made possible the production of large quantities of these TNF binding proteins. This will permit an analysis of the interaction between TNF-α and the extracellular domain of TNF-R1 and should help clarify the mechanism by which TNF-α signals through this receptor. In this paper, we describe binding studies with recombinant soluble TNF receptor 1 and TNF-α or TNF-β and characterization of the sTNF-R1·TNF-α complex in solution.

Experimental Procedures

Reagents. Recombinant human TNF-α (4.75×10^7 units/mg) and TNF-β (2.1×10^8 units/mg) were provided by the Genentech, Inc., manufacturing group. [^{125}I]TNF-α (specific activity, 24.6 μCi/μg) was prepared using the lactoperoxidase method (Miyata et al., 1985).

Expression of sTNF-R1 in Mammalian Cells. The mammalian expression plasmid pRK5-sTNF-R1 consisted of a 659 bp fragment inserted between the cytomegalovirus immediate early promoter and the SV40 termination and polyadenylation signals of the vector pRK5 (Schall et al., 1990). The fragment contains 651 bp of the cDNA for human TNF-R1 (nucleotides 158–809) (Schall et al., 1990) coding for a 29 amino acid signal peptide and 180 amino acids of the extracellular domain followed by a stop codon that was inserted using synthetic DNA oligomers.

The plasmid pRSV neo, containing a neomycin resistance gene, was cotransfected with pRK5-sTNF-R1 into the human embryonic kidney cell line 293 S (Graham et al., 1977) by the calcium phosphate precipitation method (Gorman, 1985). G418-resistant clones were isolated and tested for sTNF-R1 secretion by dot blot analysis (Heller et al., 1990) of the media with [^{125}I]TNF-α. A single clone, sTNF-R1-11, was found to secrete sTNF-R1 and was selected for further experiments.

Purification of sTNF-R1. A 0.5–1.0 L sample of medium (F12 supplemented with insulin, transferrin, and trace elements) conditioned for 48 h from confluent cultures of the sTNF-R1-11 cell line was concentrated approximately 75-fold by ultrafiltration through a 10000 molecular weight cutoff YM-10 membrane. The concentrate was applied to a 1 × 5 mm TNF-α affinity column (human TNF-α coupled to CH-Sepharose 4B; Pharmacia). Bound protein was eluted from the affinity column with 0.2 M glycine (pH 3.0). Fractions were analyzed for sTNF-R1 by SDS–PAGE analysis and then further purified by reverse-phase HPLC on a 4.6 × 100 mm C_{18} column (J. T. Baker). The C_{18} column was eluted at a flow rate of 0.5 mL/min with a gradient of mobile phase A [0.1% trifluoroacetic acid (TFA) in H_2O] and mobile phase B (0.07% TFA in acetonitrile). Protein was monitored at 214 nm. The peak fractions (0.5 mL) were collected in tubes containing 0.3 mL of phosphate-buffered saline, pH 7.4 (PBS), lyophilized, and resuspended in water. Protein concentrations were deteremined by amino acid composition of aliquots.

Binding Assays. In competitive binding experiments, 4.2×10^{-11} M sTNF-R1 was incubated with 1.15×10^{-10} M [^{125}I]TNF-α trimer in binding buffer (PBS/0.5% BSA) in a final volume of 0.15 mL for 2 h at room temperature, alone or in the presence of increasing concentrations of unlabeled human TNF-α or TNF-β. At the end of the 2-h incubation, 440 ng (1.9×10^{-8} M final concentration) of 993 monoclonal antibody against sTNF-R1 (Genentech, Inc.) was added to each reaction and incubated for an additional 2 h at room temperature. Monoclonal antibody 993 was produced by hyperimmunizing BALB/c mice in the hind footpads with sTNF-R1 in RIBI adjuvant (RIBI ImmunoChem Research, Hamilton, MT) and fusing the draining inguinal and popliteal lymph node cells with the mouse myeloma cell line X63-Ag8.653 (Kearney et al., 1979). Purified 993 was produced from ascites fluid using protein A–Sepharose (Repligen Corp., Cambridge, MA) and established affinity chromatography methods (Goding, 1978). Monoclonal antibody 993 (IgG2a, κ isotypes) will bind to both sTNF-R1·TNF-α complex or free sTNF-R1 in solution and does not block formation of the complex (data not shown). Ten microliters of a 10% suspension of Pansorbin (Calbiochem) was then added to each reaction and incubated for 45 min at 4 °C. The reactions were stopped by spinning at 15800g for 1 min at 4 °C. The pellets were washed once with 200 μL of cold binding buffer, and the amount of [^{125}I]TNF-α bound to sTNF-R1 was determined by counting the cell pellets in an Isodata γ counter. Nonspecific binding was determined by omitting either the sTNF-R1 or the monoclonal antibody from the reaction.

Saturation isotherm experiments were performed by incubating increasing concentrations of [^{125}I]TNF-α with a fixed concentration (4.2×10^{-11} M) of sTNF-R1 in a volume of 0.15 mL. The samples were processed as described above for the competitive binding experiments. Nonspecific binding was

determined by adding a 100-fold molar excess of unlabeled TNF-α to an identical set of reactions. Both displacement and saturation reactions were performed in duplicate. The data were plotted with the Scatchard equation using nonlinear least-squared regression.

Cytotoxicity Assay. Two-fold serial dilutions of sTNF-R1 were mixed with a 0.12 ng/mL aliquot of either TNF-α (5.7 units/mL; 2.3 pM) or TNF-β (25.6 units/mL; 2.4 pM) in the presence of 10 μg/mL cycloheximide. Samples were assayed for cytolytic activity on mouse L-M fibroblasts as described previously (Kramer & Carver, 1986).

Gel Filtration. Gel filtration was performed at room temperature by FPLC using a Superose 6 column (10 × 300 mm, Pharmacia) equilibrated and eluted with PBS. Recombinant TNF-α and sTNF-R1, either alone or mixed at varying ratios in a final volume of 0.1 mL, were incubated for 2 h at room temperature before being applied to the column. The elution profiles of sTNF-R1, TNF-α, or the complex were monitored by the absorbance at 214 nm. The elution position (relative molecular weight) of the complex, free TNF-α and free sTNF-R1, was determined with respect to the following proteins of known molecular weight: thyroglobulin (bovine), 670 000; γ-globulin (bovine), 158 000; ovalbumin (chicken), 44 000; myoglobin (horse), 17 000; and vitamin B-12, 1350 (Bio-Rad).

N-Terminal Sequence Analysis. Gel slices from SDS–PAGE were ground up in a 1.5-mL microcentrifuge tube, and protein was eluted by incubating for 18 h at 37 °C in 500 μL of 0.1% SDS/0.2 M Tris-HCl (pH 8.0). The eluted protein was then applied to a reverse-phase sequencing cartridge (Hewlett Packard prototype sequencer, patent number EPO 25773S) and sequenced. Fractions from the Superose 6 column for sequencing were directly applied to the sequencing cartridge. The sequencing yields determined for TNF-α or sTNF-R1 were corrected on the basis of the recovery from sequencing known amounts of TNF-α or sTNF-R1 (concentration determined by amino acid analysis). The recoveries were approximately 31% for TNF-α and 36% for sTNF-R1.

Amino Acid Analysis. Protein samples collected from HPLC were lyophilized and then transferred to 6 × 50 mm glass tubes with two washes of 70% acetonitrile, 29% H_2O, and 1% trifluoroacetic acid; 1.25 nmol of norleucine was added as an internal standard; then the samples dried and vapor-phase-hydrolyzed at 110 °C for 24 h in a Waters Pico-tag workstation.

Samples collected from Superose 6 runs were dried in 12 × 75 mm glass tubes and acidified by adding 250 μL of 6 N HCl to each tube before evacuating it in the workstation. All samples were analyzed with a ninhydrin chemistry amino acid analyzer (Beckman 6300). The amount of protein analyzed was usually between 2 and 10 μg. The norleucine internal standard was used to correct for transfer losses.

Analytical Ultracentrifugation. One hundred microliter samples of TNF-α, sTNF-R1, and sTNF-R1·TNF-α complex in PBS were loaded into charcoal-filled Epon 6 channel equilibrium cells equipped with quartz windows. The sTNF-R1·TNF-α complex was prepared by incubating 2.5 μM sTNF-R1 with 0.83 μM TNF-α overnight at 4 °C followed by 2 h at room temperature prior to loading of the sample. The cells were loaded into an An-60 Ti rotor and centrifuged at either 15 000 or 18 000 rpm at 20 °C in a Beckman Optima XLA analytical ultracentrifuge for 18–24 h. The concentration gradient in the cell was determined by UV absorption at either 230, 240, or 280 nm depending on the total protein concentration. Absorbance values determined by the centrifuge were checked versus KNO_3 and tryptophan absorbance standards, and wavelength accuracy was verified with a holmium oxide filter. The partial specific volumes for TNF-α, 0.74 mL/g, and nonglycosylated sTNF-R1, 0.70 mL/g, were computed from the amino acid compositions using the additivity rule and values for the individual amino acid residues (Cohn & Edsall, 1965). The density at 20 °C, 1.005 g/mL, for PBS was determined with a Paar DMA 35 digital density meter. The buoyant molecular weights, $M(1 - \bar{v}\rho)$, were determined by analyzing the absorbance gradient as a single ideal species for TNF-α and sTNF-R1 (three samples), and as two ideal species for the mixture of TNF-α and sTNF-R1 (two samples). The data were fit to the models either using the Simplex algorithm implemented in Turbo Pascal v5.5 (Shire et al., 1991) or with the general curve-fitting routines in the commercially available graphics software package KaleidaGraph.

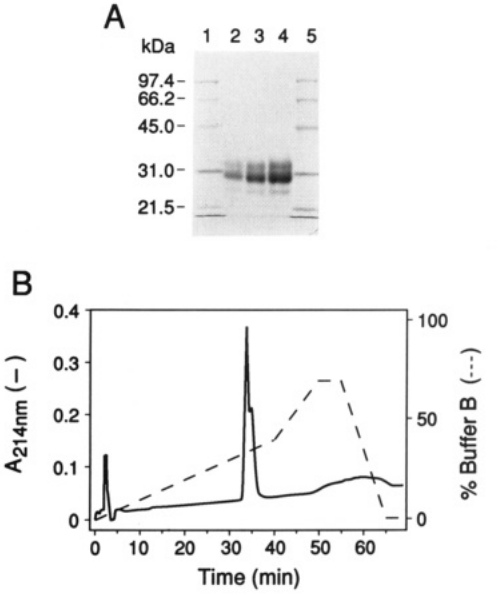

FIGURE 1: SDS–PAGE analysis and reverse-phase HPLC purification of sTNF-R1. (A) SDS–PAGE analysis of fractions eluted from a TNF-α affinity column. Medium from 293 S cells secreting sTNF-R1 was enriched for sTNF-R1 by TNF-α affinity chromatography as described under Experimental Procedures. Electrophoresis was performed on 12% acrylamide gels (Laemmli, 1970) using a Novex mini gel system. Lanes 1 and 5 indicate the positions of marker proteins (low molecular mass standards, Bio-Rad). Lanes 2–4 are aliquots of fractions 4–6 from the TNF-α affinity column showing the peak of protein eluted. (B) Reverse-phase HPLC of sTNF-R1. Fractions 4–6 from the TNF-α affinity column step were pooled and fractionated on a C_{18} reverse-phase column with a linear acetonitrile gradient (dashed line) in a solvent system containing 0.1% TFA. The absorption at 214 nm was monitored, and 0.5-mL fractions were collected in 0.3 mL of PBS. The peak fractions, retention time = 32–37 min (profile is shown), were pooled, lyophilized, and then used for all subsequent studies.

Results

Expression and Purification of sTNF-R1. A cDNA coding for the extracellular domain of human TNF-R1 was inserted into a mammalian expression vector and transfected into human embryonic kidney 293 S cells. The stable cell line sTNF-R1-11 that secretes sTNF-R1 into the media was isolated. Conditioned serum-free medium from this cell line was collected from confluent cultures, concentrated, and purified by TNF-α affinity chromatography. The eluted fractions were analyzed by [^{125}I]TNF-α dot blot analysis (Heller et al., 1990) and SDS–PAGE (Figure 1A). The sTNF-R1 preparation migrates on SDS–PAGE as two major bands with apparent

molecular weights of approximately 32 800 and 28 200 and a faint minor band at $M_r \approx 24\,200$. Since small amounts of TNF-α were found to leach from the affinity column (data not shown), we included a reverse-phase HPLC step to remove the TNF-α and any other minor contaminating proteins. After HPLC purification, no TNF-α could be detected in the sTNF-R1 fractions as judged by the absence of a 17-kDa band on a silver-stained SDS–polyacrylamide gel. The two closely resolving peaks (retention time ≈32–37 min) observed after HPLC fractionation (Figure 1B) correspond to the two major molecular weight forms of sTNF-R1. The yield of purified sTNF-R1 was approximately 0.5–1.0 μg/mL of culture media.

Characterization of sTNF-R1. Two N-terminal forms of the naturally occurring shed TNF type 1 receptor (TNF binding protein) have been isolated from human urine (Himmler et al., 1990). These investigators found that approximately 80% of the shed TNF-R1 had the amino-terminal sequence Asp-Ser-Val- while the remainder was longer by 11 amino acids beginning with Leu-Val-Pro-. The carboxy terminus of their preparation was determined to be -Ile-Glu-Asn, making the Leu and Asp N-terminal forms of the TNF binding protein 172 and 161 amino acids long, respectively. Our sTNF-R1 was engineered to terminate with -Asp-Ser-Gly, immediately preceding the transmembrane region, which is longer by eight residues than that determined for the urinary TNF binding protein.

In order to further characterize our recombinant sTNF-R1 from the 293 S cells, the HPLC-purified preparation was analyzed by SDS–PAGE, and the two major protein bands ($M_r \approx 32\,800$ and 28 200) were individually subjected to N-terminal sequence analysis. Both bands were found to contain the same degree of N-terminal heterogeneity: about 74% had aspartic acid as the N-terminal residue, and 26% contained the N-terminal leucine, which agrees well with the corresponding proteins isolated from urine. Since both bands were found to contain two different N-terminal sequences, there are at least four different species of sTNF-R1 distinguishable not only by their N-terminus but also by differences in carbohydrate composition or differential processing of the C-terminus. sTNF-R1 has three potential N-linked glycosylation sites at positions 14, 105, and 111 (Schall et al., 1990).

Binding of TNF-α to sTNF-R1. The functional integrity of the TNF-α binding domain of the sTNF-R1 was examined by [^{125}I]TNF-α binding analysis (Figure 2). A saturation isotherm for the binding of [^{125}I]-labeled TNF-α to sTNF-R1 demonstrated that this binding was specific and saturable (Figure 2A). Scatchard analysis of the data (Figure 2A inset) indicates a single class of binding sites with a K_d = 93 pM. This value is somewhat lower than previously reported for the high-affinity site of the full-length membrane-associated TNF-R1 transiently expressed in mammalian cells (0.5–0.66 nM) (Loetscher et al., 1990; Schall et al., 1990). However, using the present preparation of [^{125}I]TNF-α, we have determined a K_d of approximately 0.3 nM for the full-length cellular TNF-R1 (data not shown). The Scatchard analysis of the binding data (Figure 2A) indicates that 2.3 molecules of sTNF-R1 bind to a single TNF-α trimer. Similar experiments performed using two additional monoclonal antibodies to precipitate the sTNF-R1·TNF-α complex gave stoichiometries of 2.3 and 2.0, respectively (data not shown).

Competitive binding experiments were performed by adding increasing concentrations of either TNF-α or TNF-β to fixed amounts of [^{125}I]TNF-α and sTNF-R1. Both TNF-α and TNF-β caused a dose-dependent decrease in specific [^{125}I]-TNF-α binding to sTNF-R1 (Figure 2B). The data indicate

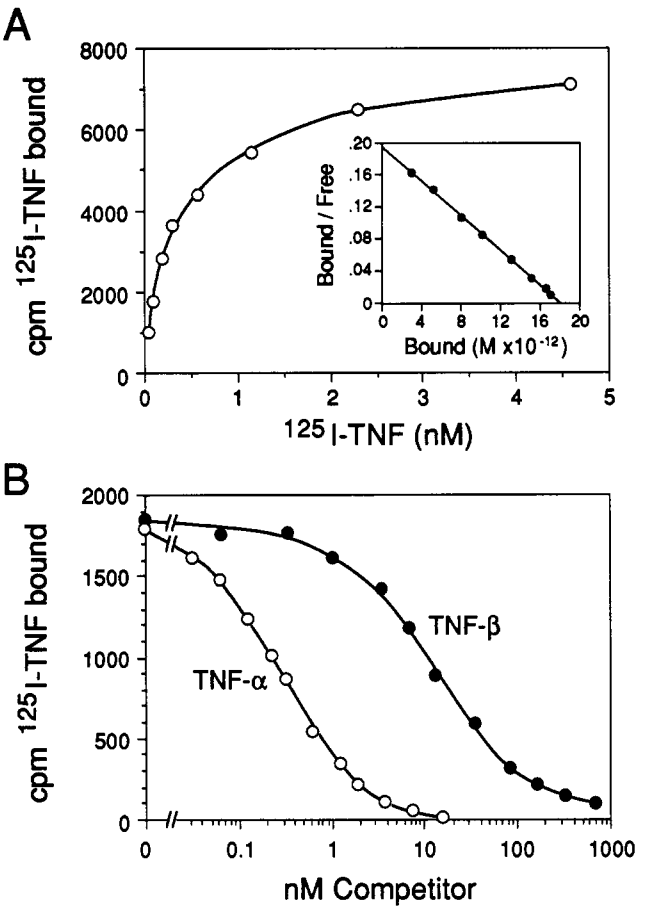

FIGURE 2: Binding of [^{125}I]TNF-α to sTNF-R1. (A) Saturation isotherm of specific binding of [^{125}I]TNF-α to sTNF-R1. Duplicate samples of sTNF-R1 (4.2×10^{-11} M) were incubated with increasing concentrations of [^{125}I]TNF-α alone or in the presence of a 100-fold molar excess of unlabeled TNF-α, and the specific binding at each concentration was determined. The inset presents the data transformed by Scatchard analysis. (B) Displacement curves showing the inhibition of specific binding of [^{125}I]TNF-α by unlabeled TNF-α (○) or TNF-β (●). Increasing concentrations of either TNF-α (32 pM–15 nM) or TNF-β (68 pM–676 nM) were added to a fixed amount (1.15×10^{-10} M) of [^{125}I]TNF-α and sTNF-R1 (4.2×10^{-11} M) and processed as described under Experimental Procedures. All binding experiments were performed 2–3 times with similar results. The results from a single representative experiment are shown.

sTNF-R1 has about a 50-fold lower affinity for TNF-β than for TNF-α. K_i values of 0.22 and 11.7 nM were obtained for TNF-α and TNF-β, respectively. This is in contrast to what has been observed for the transiently expressed full-length receptor in mammalian cells where both TNF-α and -β have equal affinities for TNF-R1 (Schall et al., 1990).

Neutralization of TNF-α and -β Bioactivity by sTNF-R1. Soluble TNF-R1 was tested for its ability to inhibit the cytolytic activity of TNF-α and TNF-β on mouse L-M fibroblasts. Constant amounts of either TNF-α or TNF-β were incubated with increasing amounts of sTNF-R1 and the mixtures assayed for cytotoxicity on L-M cells. Relatively high concentrations of sTNF-R1 were needed to inhibit the cytotoxic activity of TNF-α or TNF-β (Figure 3). However, consistent with the binding analysis, TNF-α was more efficiently neutralized than TNF-β. EC_{50} values of approximately 2 and 80 nM were obtained for TNF-α and TNF-β, respectively.

Gel Filtration of the sTNF-R1·TNF-α Complex. The sTNF-R1·TNF-α complex was also examined by size-exclusion chromatography. TNF-α and sTNF-R1 were first chromatographed separately on Superose 6 under nondenaturing

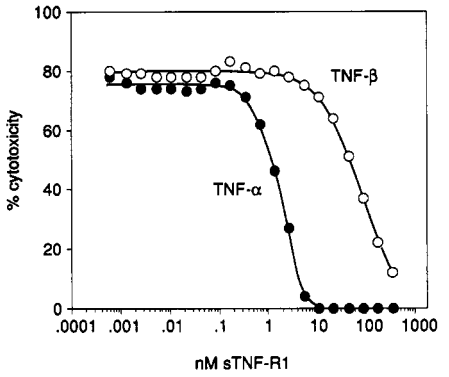

FIGURE 3: Inhibition of TNF-α and TNF-β activity by sTNF-R1. TNF-α or TNF-β were added at a final concentration of 0.12 ng/mL to the wells of a 96-well microtiter plate seeded 24 h earlier with 4×10^4 mouse L-M cells/well. Two-fold serial dilutions of sTNF-R1 [6.8 μg/mL (3.54×10^{-7} M) initial concentration] were made. All wells contained cycloheximide at a final concentration of 10 μg/mL. Triplicate plates were then assayed for viability as previously described (Kramer & Carver, 1986). This experiment was repeated twice. The mean residual cytotoxic activity of TNF-α (●) and TNF-β (○) is plotted as a function of a concentration of sTNF-R1 from a single representative experiment.

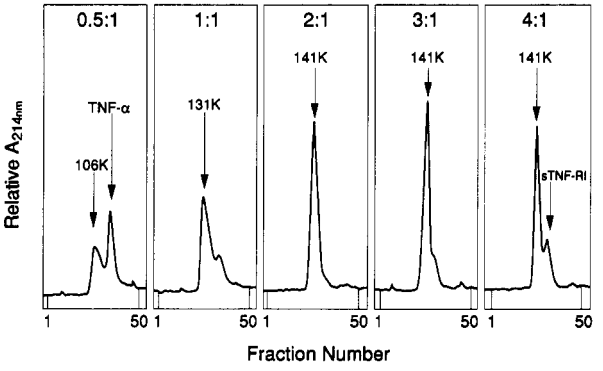

FIGURE 4: Gel filtration chromatography of various ratios of TNF-α and sTNF-R1. TNF-α and sTNF-R1 were mixed at the indicated molar ratios, assuming that TNF-α is a trimer and sTNF-R1 a monomer: 0.5:1, 0.55 μM sTNF-R1 and 1.09 μM TNF-α; 1:1, 0.95 μM of each; 2:1, 1.5 μM sTNF-R1 and 0.75 μM TNF-α; 3:1, 1.85 μM sTNF-R1 and 0.62 μM TNF-α; 4:1, 2.10 μM sTNF-R1 and 0.53 μM TNF-α. The concentrations of TNF-α and sTNF-R1 were determined by amino acid composition analysis. The total micrograms in each mixture was identical. TNF-α and sTNF-R1 were incubated for $\geq$2 h at room temperature in a volume of 0.1 mL of PBS. The mixtures were chromatographed on an FPLC system using a Superose 6 column eluted with PBS at 0.35 mL/min. Peaks were monitored for the absorbance at 215 nm.

conditions in order to determine their elution positions in the uncomplexed form. By this analysis, sTNF-R1 elutes at a position corresponding to a molecular mass of 55–60 kDa, suggesting that it exists as a dimer in solution or it is an elongated protein. The molecular mass of TNF-α determined by Superose 6 chromatography is 30 kDa instead of the predicted trimer size of 52 kDa. Other investigators have observed sizes for TNF-α by gel filtration ranging from 33 to 46 kDa (Aggarwal et al., 1985; Arakawa & Yphantis, 1987; Schoenfeld et al., 1991).

Soluble TNF-R1 (monomer, nonglycosylated molecular weight of 19218) and TNF-α (trimer, M_r = 52050) were incubated in molar ratios of 0.5:1, 1:1, 2:1, 3:1, and 4:1 and analyzed by gel filtration on Superose 6 (Figure 4). At a ratio of 0.5:1 (sTNF-R1 to TNF-α), two peaks of apparent size 106 and 30 kDa are seen corresponding to complex and free TNF-α, respectively. At a 1:1 ratio, the complex peak shifts to a slightly higher apparent molecular mass of 131 kDa. At a 2:1, 3:1, and 4:1 ratio, the complex migrates at approximately

Table I: Molecular Weight Analysis by Sedimentation Equilibrium

sample	$M(1-\bar{v}\rho)$	$\bar{v}$	M_r[a]	stoichiometry[b]
TNF-α	12550 ± 22	0.739	48.8 ● 0.1	
sTNF-R1[c]	7970 ± 870	0.680 ● 0.010	25.2 ± 3.6	
complex[d]	33150 ± 1600	0.710[e]	115.7 ± 5.6	2.5 ± 0.5
		0.704[f]	113.3 ± 5.5	2.4 ± 0.6

[a] Molecular weight ± one standard deviation. [b] Calculated stoichiometry of the complex expressed as moles of sTNF-R1 per mole of TNF-α trimer. [c] Partial specific volume calculated as described in the text assuming a range of 10–30% for the carbohydrate content of sTNF-R1. [d] Data analyzed as a two-component ideal model with 20% glycosylation assumed for sTNF-R1. [e] Partial specific volume calculated assuming the sTNF-R1·TNF-α complex exists at a stoichiometry of 2:1. [f] Partial specific volume calculated assuming the sTNF-R1·TNF-α complex exists at a stoichiometry of 3:1.

141 kDa. However, at the 4:1 ratio, a distinct peak of free soluble receptor begins to accumulate, whereas at the 2:1 and 3:1 ratios, most of the sTNF-R1 and TNF-α are bound in a complex. The presence of both TNF-α and sTNF-R1 in the complex was determined by SDS–PAGE analysis of aliquots of the column fractions. These results suggest that two or three molecules of sTNF-R1 bind to a single TNF-α trimer.

Stoichiometry of the sTNF-R1·TNF-α Complex. In order to accurately determine the stoichiometry of the complex, sTNF-R1 and TNF-α were mixed at a molar ratio of 3:1, and the mixture was resolved by gel filtration on a Superose 6 column. The peak of the complex as monitored by both absorbance and SDS–PAGE was found in fractions 24 and 25 (data not shown) and corresponds to the 141-kDa complex obtained at the 3:1 ratio in Figure 4. Three independent measurements to determine the stoichiometry of the complex were then performed on these two fractions. First, the ratio of sTNF-R1 to TNF-α was determined by quantitating the yield from the first cycle of sequencing of the complex, which contains TNF-α (Val) and the two N-terminal forms of sTNF-R1. Fractions 24 and 25 were found to contain molar ratios of sTNF-R1 to TNF-α (trimer) of 2.31 and 2.66, respectively.[1] This suggests that the complex peak could contain a mixture of two and three sTNF-R1 molecules per TNF-α trimer.

Second, the stoichiometry of the peak fractions of the complex was determined by amino acid analysis. Several amino acids that are generally not destroyed or lost during acid hydrolysis [Asx (Asp + Asn), Glx (Glu + Gln), Pro, Ala, Leu, and Lys] were selected for analysis. The experimentally determined mole fractions of this group of amino acids were then compared to the theoretical mole fractions expected for 1:1, 2:1, and 3:1 ratios of sTNF-R1 to TNF-α. The data obtained for Lys, Asx, Leu, and Ala are most consistent with a 2:1 sTNF-R1·TNF-α complex. The data for Glx and Pro suggest a 1:1 ratio (data not shown).[1]

Finally, the stoichiometry of the isolated complex was analyzed by reverse-phase HPLC which separates the TNF-α and the sTNF-R1. The peaks were monitored at 214 nm, and the area under the peaks was determined. The separated sTNF-R1 and TNF-α were also quantitated by amino acid analysis. For these two methods, we found sTNF-R1:TNF-α ratios of 2.50 and 2.78, respectively.[1]

Analytical Ultracentrifugation. TNF-α, sTNF-R1, and the sTNF-R1·TNF-α complex were also analyzed by sedimentation equilibrium. Analysis of TNF-α alone gave a molecular weight of 48800 (Table I). The value of the molecular weight

[1] These data were subjected to the scrutiny of the reviewers and are available upon written request to the authors.

calculated for sTNF-R1 is dependent on the partial specific volume for the glycoprotein. The partial specific volume for sTNF-R1 and resulting molecular weights were calculated assuming that the mass of sTNF-R1 is between 10 and 30% carbohydrate (assumed to consist mainly of mannose with a partial specific volume of 0.61 mL/g). The molecular weight obtained, 25 250 ± 3600, shows that under the conditions of these experiments sTNF-R1 exists predominantly as a monomer in solution.

The sTNF-R1·TNF-α complex was prepared by mixing sTNF-R1 and TNF-α at a 3:1 ratio before analysis by sedimentation equilibrium (Table I). The partial specific volumes for the sTNF-R1·TNF-α complex were estimated for both 3:1 and 2:1 ratios, and for 20% glycosylation of sTNF-R1. The mixture for the centrifuge analysis has two components as determined by FPLC analysis (Figure 4). Accordingly, the centrifuge data for the mixture were analyzed as a two-component ideal species model. The smaller molecular weight species has a similar buoyant molecular weight found for sTNF-R1, and is presumably uncomplexed soluble receptor. The models at 2:1 and 3:1 stoichiometries resulted in ratios of 2.5 ± 0.5 and 2.4 ± 0.6 sTNF-R1 molecules per TNF-α trimer, respectively. Calculations with the same models, but with the assumption of either 10% or 30% glycosylation for sTNF-R1, yield values for the stoichiometric ratios within 10% of the above values. These data are in good agreement with the stoichiometry determined by the various gel permeation chromatography experiments. Taken together, the results from the various analyses are consistent with the sTNF-R1·TNF-α complex containing at least two and possibly three molecules of sTNF-R1 per TNF-α trimer.

Discussion

In the present study, we have expressed, purified, and characterized the extracellular domain (sTNF-R1) of the human TNF-R1 and examined its interaction with TNF-α. The recombinant sTNF-R1 purified from 293 S cells corresponds closely to the naturally shed form of TNF-R1 isolated from human urine (Peetre et al., 1988; Seckinger et al., 1989; Novick et al., 1989; Engelmann et al., 1990a; Gatanaga et al., 1990; Lantz et al., 1990). The urinary TNF binding protein (TNF-BP) has been reported to have a heterogeneous N-terminal sequence: 80% begins with Asp-Ser-Val-, and 20% is longer by 11 amino acids beginning with Leu-Val-Pro- (Himmler et al., 1990). The recombinant sTNF-R1 was found to have the same two N-terminal forms present in roughly these same proportions. The C-terminal sequence of the urinary TNF-BP has been determined as -Ile-Glu-Asn (Smith et al., 1990). Our expression vector was designed to encode a sTNF-R1 containing eight additional amino acids at the carboxy terminus. However, since we have not determined the C-terminal residue of sTNF-R1, we do not know whether sTNF-R1 has undergone the same C-terminal processing as the TNF-BP.

Both TNF receptors (TNF-R1 and TNF-R2) are members of a larger superfamily which includes the p75 NGF receptor (NGF-R). This superfamily is characterized by four cysteine-rich repeat domains in the extracellular region of the protein. A recent study of a recombinant extracellular domain of the NGF-R (Vissavajjhala & Ross, 1990) indicated that its apparent molecular mass determined by analytical gel filtration (118 kDa) was much greater than either its molecular mass predicted from the cDNA sequence (22.3 kDa) or its apparent size on SDS–PAGE (46 kDa). Although it was concluded (Vissavajjhala & Ross, 1990) that both the entire NGF-R and its recombinant extracellular domain probably exist as dimers, there is still a discrepancy between the observed and predicted sizes. These investigators (Vissavajjhala & Ross, 1990) propose that the four cysteine-rich segments of the NGF-R extracellular domain may be folded independently and arranged in a linear pattern, forming an elongated, extended rodlike molecule. Since the extracellular domain of TNF-R1 also contains four homologous cysteine-rich repeats, it is possible that sTNF-R1 may have an extended rodlike conformation. This notion is consistent with the higher molecular mass observed for sTNF-R1 by gel filtration chromatography (55–60 kDa) than by sedimentation equilibrium analysis (25 kDa) under the same nondenaturing conditions. A recent study (Lantz et al., 1990) characterizing urinary TNF-BP also found a higher molecular mass of ~50 kDa for TNF-BP by gel filtration chromatography than by SDS–PAGE analysis (30 kDa). They suggested that TNF-BP either forms a dimer under nondenaturing conditions or has a high excluded volume during gel filtration because of its high carbohydrate content. The sedimentation equilibrium data presented here provide strong evidence that sTNF-R1 is a monomer.

The purified sTNF-R1 was found to be functional as determined by its ability to form complexes with TNF-α and TNF-β in solution. TNF-α binds sTNF-R1 with high affinity ($K_d \approx 93$ pM). TNF-β competes rather poorly with TNF-α for binding to sTNF-R1, having a K_i of about 11.7 nM. Purified TNF-BP from human urine was also found to have a higher affinity for TNF-α than TNF-β (Engelmann et al., 1989). It is unclear why sTNF-R1 has a much lower affinity for TNF-β than TNF-α since the intact membrane-bound TNF-R1 has an equal high affinity for both ligands (Schall et al., 1990). Perhaps some specific conformation of TNF-R1 is required for high-affinity binding to TNF-β which is lost when the receptor is not anchored in the cell membrane. It is also possible that other cell-associated molecules are required to form a receptor complex for high-affinity binding of TNF-β.

Consistent with the binding data, sTNF-R1 was found to be more efficient at neutralizing the cytotoxic activity of TNF-α (EC_{50} ~2 nM) than TNF-β (EC_{50} ~80 nM). However, these EC_{50} values are about 5–10-fold higher than the K_i values determined from the binding displacement studies. Possible explanations for this discrepancy are (1) the extreme sensitivity of the L-M cell cytotoxicity assay (detection limit ~1 pg/mL TNF-α) and (2) the ability of TNF-α to simultaneously bind more than one sTNF-R1 molecule (discussed below) which suggests that sTNF-R1·TNF-α complexes with 1:1 stoichiometry may be able to interact with cell-surface TNF receptors.

Several experiments were performed to determine the stoichiometry of binding between sTNF-R1 and TNF-α. In calculating the stoichiometry, we have assumed that TNF-α is a trimer since the majority of literature reports (Wingfield et al., 1987; Eck et al., 1988; Lewit-Bently et al., 1988; Jones et al., 1989), as well as our sedimentation equilibrium data, indicate that TNF-α exists as a compact trimer. Others have suggested that dimer and monomers of TNF-α may also bind TNF receptors (Smith & Baglioni, 1987; Peterson et al., 1989; Heller et al., 1990), which, if true, would complicate this analysis of the stoichiometry. While each of the experimental methods employed has its own inherent potential source of error, data from the experiments are most consistent with a TNF-α trimer being able to form a complex containing two or three molecules of sTNF-R1.

Ligand-induced oligomerization appears to be a general property of receptors for growth factors. For example, aggregation of the tyrosine kinase family of receptors for epi-

dermal growth factor (EGF), platelet-derived growth factor, colony stimulating factor 1, and insulin by the binding of their respective ligands has been reported to be the initiating event of the signal transduction process (Boni-Schnetzler & Pilch, 1987; Yarden & Schlessinger, 1987a,b). One mechanism proposed for the initiation of signal transduction is that ligand binding induces a conformational change in the extracellular domain of the receptor that results in receptor–receptor interactions. Receptor oligomerization then transmits this conformational change from the extracellular domain to the cytoplasmic domain and leads to activation of the kinase function of these receptors (Ullrich & Schlessinger, 1990). A recent study (Lax et al., 1991) demonstrating that EGF induces the oligomerization of a recombinant soluble extracellular domain of the EGF receptor provides support for this proposal. In this case, a single molecule of EGF binds to a single EGF receptor, and receptor aggregation involves receptor–receptor interactions.

In contrast to the EGF·EGF receptor example, our data provide evidence that two or more molecules of sTNF-R1 bind to a single ligand molecule, in this case the TNF-α trimer. Therefore, we propose that TNF-α induces receptor oligomerization on the cell membrane by cross-linking two or more TNF-R1 molecules. Our data suggest that three cell-surface TNF-R1 molecules could potentially interact with one TNF-α trimer. However, due to topological constraints, it is somewhat difficult to imagine this occurring on the cell surface if the three binding sites on TNF-α are equivalent. A definitive answer to this question awaits additional experiments, including cross-linking and crystallization of the complex.

Similar to the data presented here, it has recently been determined that a single human growth hormone molecule can bind two molecules of the extracellular domain of the growth hormone receptor and it has been proposed that receptor dimerization by growth hormone is important for signal transduction (Cunningham et al., 1991). In contrast to TNF-α, however, growth hormone is a monomer and has two distinct receptor binding sites.

Some anti-EGF receptor antibodies that are able to cross-link the EGF receptor can mimic EGF and stimulate DNA synthesis (Schreiber et al., 1981; Chandler et al., 1985; Fernandez-Pol, 1985). A similar phenomenon has been observed for TNF-R1 where it was found that clustering of these receptors by cross-linking with specific antibodies is sufficient to initiate a variety of TNF activities (Engelmann et al., 1990b; Espevik et al., 1990; Tartaglia et al., 1991). Anti-TNF-R2 antibodies have recently been shown to signal thymocyte proliferation (Tartaglia et al., 1991), presumably by the same mechanism. From these observations, along with our present data, we propose that TNF receptor dimerization induced by TNF-α, TNF-β, or receptor-specific antibodies (Engelmann et al., 1990b; Espevik et al., 1990; Tartaglia et al., 1991) is the relevant step that triggers the many biological activities induced by TNF.

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