Stabilization of the Bioactivity of Tumor Necrosis Factor by Its Soluble Receptors

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Summary

The receptors for tumor necrosis factor (TNF) exist in cell-associated as well as soluble forms, both binding specifically to TNF. Since the soluble forms of TNF receptors (sTNF-Rs) can compete with the cell-associated TNF receptors for TNF, it was suggested that they function as inhibitors of TNF activity; at high concentrations, the sTNF-Rs indeed inhibit TNF effects. However, we report here that in the presence of low concentrations of the sTNF-Rs, effects of TNF whose induction depend on prolonged treatment with this cytokine are augmented, reflecting an attenuation by the sTNF-Rs of spontaneous TNF activity decay. Evidence that this stabilization of TNF activity by the sTNF-Rs follows from stabilization of TNF structure within the complexes that TNF forms with the sTNF-Rs is presented here, suggesting that the sTNF-Rs can affect TNF activity not only by interfering with its binding to cells but also by stabilizing its structure and preserving its activity, thus augmenting some of its effects.

TNF, a polypeptide cytokine, produced primarily by mononuclear phagocytes, plays a key role in the initiation of the inflammatory response. It has a variety of effects, of different, even contrasting nature (1, 2). Evidence that some of these effects can be detrimental to the host have attracted attention to the mechanisms that regulate TNF function. The intracellular signals for the response to TNF are provided by cell surface receptors, of two distinct molecular species, to which TNF binds at high affinity (3, 4). Both receptors for TNF exist also in soluble forms (5–8), apparently derived by proteolytic cleavage from the cell surface forms (9). These soluble TNF receptors (sTNF-Rs)[1] can compete for TNF with the cell surface receptors and thus block its activity (5–8). It was therefore suggested that they function as physiological attenuators of the activity of TNF, safeguarding against its potentially harmful effects. We report here that the sTNF-Rs affect TNF function also by stabilizing its activity, most likely by preventing dissociation of the homotrimeric TNF molecules (10–13) to inactive monomers.

Materials and Methods

TNF and Its Soluble Receptors. Recombinant human TNF-α (TNF; 6 × 10^7 U/mg of protein), produced by Genentech Co.

(San Francisco, CA), was kindly provided by Dr. G. Adolf, of the Boehringer Institute (Vienna, Austria). Radiolabeling of the TNF with ^{125}I was performed by the chloramine-T method, as previously described (14). Native TNF was produced by stimulation of human peripheral mononuclear phagocytes with bacterial lipopolysaccharide for 6 h, as described elsewhere (15), and used without further purification. The soluble forms of the type I (p55) and type II (p75) TNF receptors (sTNF-RI and sTNF-RII, previously called TBPI and TBPII [8]) were isolated from normal urine by ligand (TNF) affinity purification followed by reversed-phase HPLC, as described before (8).

Cells. Mononuclear leukocytes of B-chronic lymphocytic leukemia (B-CLL) patients were isolated from the peripheral blood by centrifugation on a Ficoll-Hypaque cushion (Pharmacia, Uppsala, Sweden). To enrich the leukocyte fraction for leukemic cells, the mononuclear leukocytes were depleted of T cells by rosetting with sheep erythrocytes (16), and then depleted of the mononuclear phagocytes by adherence to plastic (17). The leukocytes were cultured in RPMI 1640, supplemented with 10% FCS. The same culture medium was used for the growth of human foreskin fibroblasts, strain FS11 (18), and murine A9 cells (19).

Cytocidal and Growth Stimulatory Activities of TNF. B-CLL cells and human foreskin fibroblasts were cultured in 96-well microtiter plates at densities of 2.5 × 10^5 cells/0.2 ml/well and 5 × 10^3 cells/0.2 ml/well, respectively. The rate of cell growth after the indicated culture time was assessed by measuring the incorporation of [^{3}H]thymidine into the DNA of the cells. Labeled thymidine (25 Ci/mmol; Amersham Corp., Amersham, UK) was applied to the cultures (1 μCi/well) for the last 8 h of incubation and the amount of radioactivity incorporated into DNA was then

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[1] *Abbreviations used in this paper:* B-CLL, B-chronic lymphocytic leukemia; sTNF-R, soluble tumor necrosis factor receptor.

determined after harvesting the cells with the aid of a-PHD-cell harvester (Cambridge Technology Inc., Watertown, MA). The cells were lysed by washing with distilled water, and the label bound to the filter was measured by liquid scintillation counting. Detachment of the fibroblasts from the culture plates, before harvesting, was accomplished using trypsin. The increase in fibroblast number during incubation was assessed by determining cell neutral red dye uptake (20).

The cytocidal activity of TNF was determined in A9 cells, as described before (20). The cells were plated in 96-well plates, 18 h before the assay, at a density of 3×10^4 cells/well. TNF was applied to the cells, in serial dilutions, together with cycloheximide (25 µg/ml) and, after further incubation for 10 h, cell viability was assessed by measuring neutral red dye uptake. 1 U of cytocidal activity is defined as the activity that results in 50% cell death.

Quantification of the sTNF-Rs by ELISA. The concentrations of the sTNF-Rs were determined by a two-site capture ELISA, using mouse mAbs and rabbit antisera against the sTNF-Rs, as described elsewhere (21).

Results

Enhancement of TNF-induced Growth of Cells by the sTNF-Rs. In contrast to previously reported inhibitory effects of the sTNF-Rs on TNF function, we observed an increase in the enhancement of cell growth by TNF in the presence of these proteins. The TNF-induced growth of B-CLL cells (22, 23) was affected by the sTNF-Rs in a bimodal fashion. Up to certain concentrations, the sTNF-Rs enhanced cell growth; at higher concentrations they were inhibitory (Fig. 1). A similar degree of enhancement was observed with the soluble forms of both the type I (p55) and type II (p75) TNF receptors (sTNF-RI and sTNF-RII, respectively). However, the optimal concentration of the sTNF-RII for enhancement of cell

growth was higher than that of the sTNF-RI. In the absence of TNF, the sTNF-Rs had no effect on the B-CLL cells (Fig. 1, *dashed lines*).

The TNF-induced growth of normal fibroblasts (24) was also enhanced by the sTNF-Rs. Both the increase in fibroblast number in response to TNF throughout the incubation period (Fig. 2 *A*), and the extent of stimulation of cell growth on the last day of incubation (Fig. 2 *B*), were augmented in the presence of the soluble receptors up to certain concentrations of the sTNF-Rs; at higher concentrations they were inhibitory. No effect of the sTNF-Rs could be observed in the absence of TNF (Fig. 2, *A* and *B*, *dashed lines*).

Decay and Stabilization TNF Bioactivity in Diluted Solutions. The stimulatory effects of TNF on B-CLL and fibroblast cell growth are assessed after treatment of the cells with TNF for several days (22–24). A clue to the mechanism whereby the sTNF-Rs enhance these effects was found on examining the residual activity of TNF in the fibroblast media at the end of this long incubation period. This was determined by applying samples of the fibroblast growth media to A9 cells, which are sensitive to the rapidly induced cytocidal effect of TNF (Fig. 2 *C*). Several features could be noted: (*a*) TNF activity in the growth media on day 9 (1–10 U/ml) was significantly lower than that of the TNF originally applied to the cells (200 U/ml). (*b*) The decrease in TNF activity in cultures also containing the sTNF-Rs was lower than when TNF was applied alone. (*c*) The degree of preservation of TNF activity in the fibroblast growth media by the sTNF-Rs was dependent on their concentration. Optimal concentrations were identical to those found for the effect of the sTNF-Rs on fibroblast growth (compare Fig. 2 *C*, to *A* and *B*).

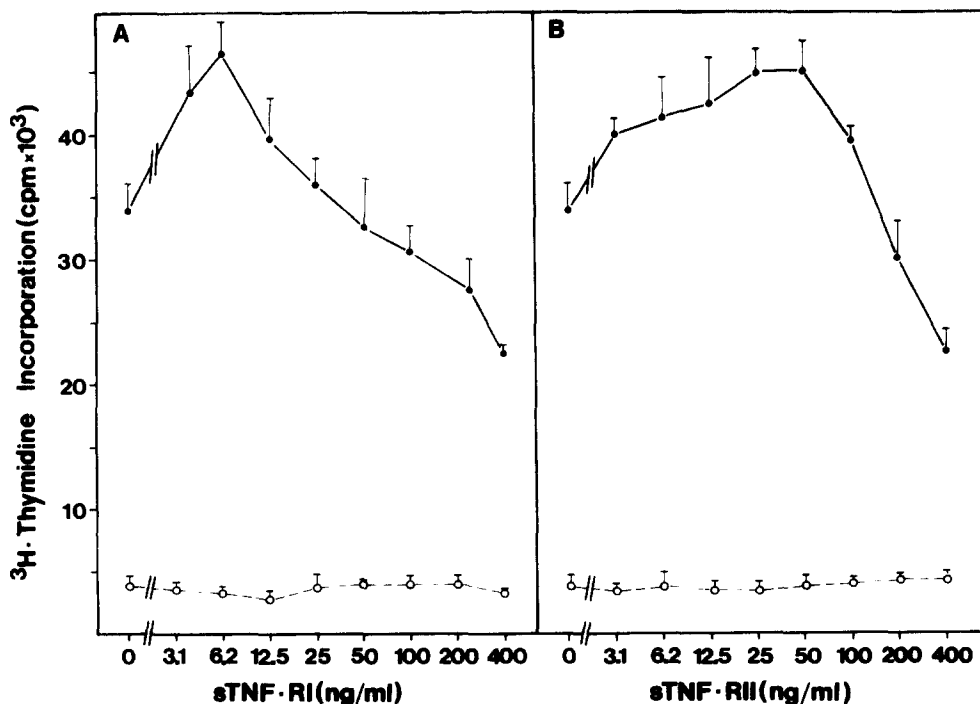Spontaneous decay of TNF activity has been reported (11,

Figure 1. The sTNF-Rs enhance TNF-induced growth of B-CLL cells. Growth of leukemic B lymphocytes isolated from the peripheral blood of a B-CLL patient in the presence or absence of TNF (10 ng/ml; *solid* and *dashed lines*, respectively) and either sTNF-RI (*A*) or sTNF-RII (*B*), at the indicated concentrations, was quantified on day 7 of incubation by determining the incorporation of [³H]thymidine into the cellular DNA. Data were confirmed by tests on lymphocytes of three other patients.

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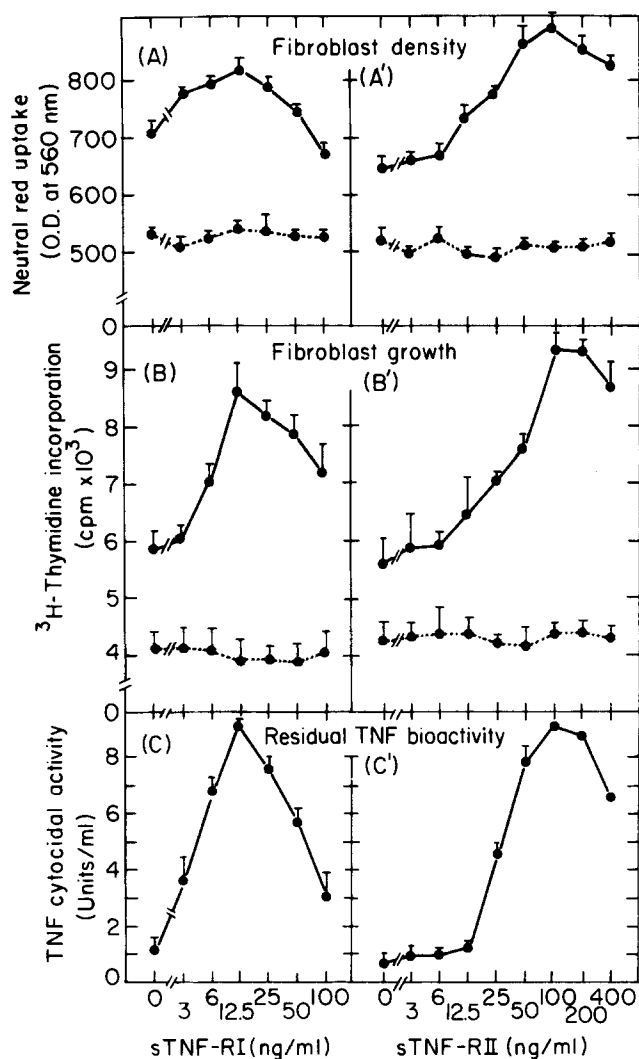
25). This decay, assessed by determining the cytotoxic activity of TNF after various periods of preincubation, was found to depend upon TNF concentration; negligible at high TNF concentrations, it was significant at those concentrations of TNF usually used in bioassays (Fig. 3).

Examining the kinetics of this decay in the presence of the sTNF-Rs revealed a rather complex modulation of TNF activity by these proteins. Initially, the sTNF-Rs were found, as reported before, to inhibit the cytocidal activity of TNF (Fig. 4, B and E, and at time 0 in Fig. 4, A and D). Yet, after TNF incubation, an additional effect of these proteins was observed. While in the absence of the sTNF-Rs the activity of TNF gradually decreased, in the presence of these proteins TNF activity remained quite stable (Fig. 4, A and D). The over-all pattern of the effect of the sTNF-Rs on the cytocidal activity of TNF, after 7 d of incubation, displayed the same "bell-shaped" concentration dependence curve as found for the effects of TNF on the growth of B-CLL cells and fibroblasts (compare Fig. 4, C and F to Figs. 1 and 2).

These findings suggest that decay in TNF activity can be a limiting factor for its long-term effects. Attenuation of this decay by the soluble receptors accounts for their ability to augment these effects.

Spontaneous decay of TNF activity occurred irrespective

Figure 2. The sTNF-Rs enhance the TNF-induced growth of normal fibroblasts. Effects of sTNF-RI (left) and sTNF-RII (right) on the growth of human foreskin fibroblasts (strain FS11) in the presence or absence of TNF (10 ng/ml; solid and dashed lines, respectively), were assessed after 9 d of incubation, in the following two ways: (A and A') by measuring cell density, based on determining the uptake of neutral red dye by the cells; and (B and B') by measuring the rate of cell growth as shown by incorporation of [³H]thymidine into DNA. The residual bio-activity of TNF in the growth media of the cells, on day 9 of incubation (C and C') was determined by measuring the cytocidal effect of samples of the growth media on A9 cells. See Materials and Methods for additional details.
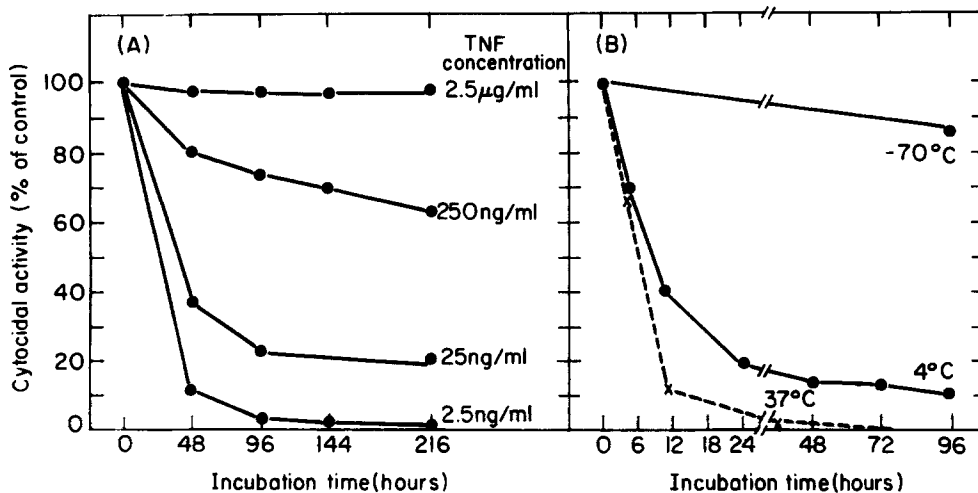

Figure 3. Decay of TNF activity in dilute solution. (A) Decay of the activity of pure recombinant TNF, at the indicated concentrations, upon incubation at 37°C. (B) Decay of the activity of crude, monocyte-produced TNF, with an initial bio-activity corresponding to a concentration of 14 ng/ml, at the indicated temperatures. Both TNF preparations were kept for the indicated times in RPMI 1640, supplemented with 10% FCS. The residual bio-activity at various times was determined by measuring the cytocidal effect of the TNF samples on A9 cells, as described in Materials and Methods.
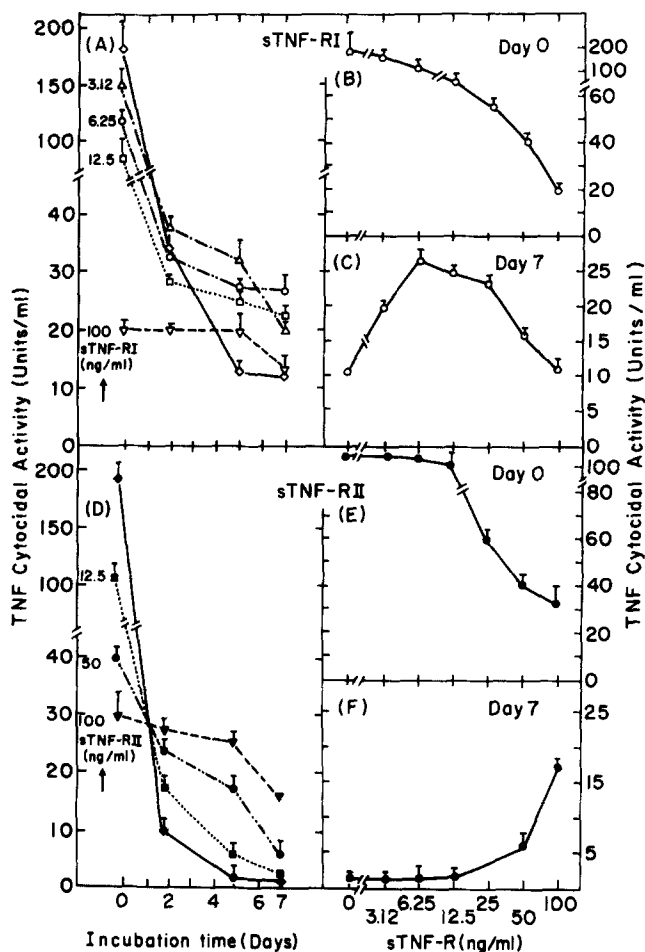
Figure 4. Effect of the sTNF-Rs on decay of TNF activity. TNF (5 ng/ml) was incubated at 37°C either alone or in the presence of the indicated concentrations of sTNF-RI (*A-C*) or of sTNF-RII (*D-F*). At various times, the residual cytocidal activity was determined, as described in Fig. 3. The activity of TNF in the presence of different concentrations of sTNF-RI and sTNF-RII is presented as a function of the duration of incubation (*A* and *D*) and as a function of the concentrations of the sTNF-Rs on day 0 (*B* and *E*) and day 7 (*C* and *F*).

Figure 5. Size exclusion chromatography of TNF and of its complex with sTNF-RI. ^{125}I-labeled TNF (5 ng, 1.5 × 10^6 cpm) was applied, either alone (*A* and *B*) or together with sTNF-RI (50 ng; *C*, *D*, and *E*), to a Superose 12 column (Pharmacia), in 0.15 M NaCl/10 mM sodium phosphate buffer (PBS), either without preincubation (*filled circles*) or after a 3-d preincubation period at 37°C in the same buffer (*open circles*). Fractions of 0.24 ml were collected, at a flow rate of 0.8 ml/min. All fractions were assayed for cytocidal activity (*A* and *C*) and their radioactivity determined in a gamma counter (*B* and *D*). The amounts of sTNF-RI in each fraction (*E*) were determined by ELISA (21). The elution patterns are shown in comparison to the elution volume of molecular mass markers (BSA, 66 kD, OVA, 45 kD, and lysozyme 14.3 kD). The elution pattern of sTNF-RI, after its preincubation with TNF (*E*), was identical to that observed when these two proteins were subjected to chromatographic analysis without preincubation (not shown). The input and recovery of cytocidal activity, in the TNF only samples, were 125 and 95 U, respectively, for samples without preincubation, and 14 and 10 U, respectively, for samples subjected to chromatography after a 3-d preincubation period (*A*). Corresponding values for TNF + sTNF-RI samples (*C*) were 15 and 38 U, respectively, without preincubation, and 14 and 34 U, respectively, for the sample tested after 3 d of preincubation.[2] Similar patterns of elution and similar recoveries of activities were observed when repeating the study presented in this figure with nonlabeled TNF or with sTNF-RII (not shown).

of whether TNF was incubated alone (see legend to Fig. 5), or in solutions supplemented with calf serum proteins (Figs. 3 and 4). It was observed in preparations of pure, *Escherichia coli*-produced, recombinant TNF as well as in crude preparations of TNF obtained from human PBMC (Fig. 3, *A* and *B*). SDS-PAGE analysis of these preparations showed that the loss of the TNF activity was unaccompanied by any change in its subunit molecular size (not shown). These findings as well as other data (11, 25) appear to rule out an involvement of proteolytic degradation of TNF in this process.

Gel Filtration Chromatography Studies of the Molecular Size of TNF, before and after Decay of Its Activity, and of the Complexes It Forms with the sTNF-Rs. Gel filtration chromatography of preparations of TNF having decayed activity suggested, as also indicated in prior studies (11, 25), that this decay is a reflection of a change in the quaternary structure
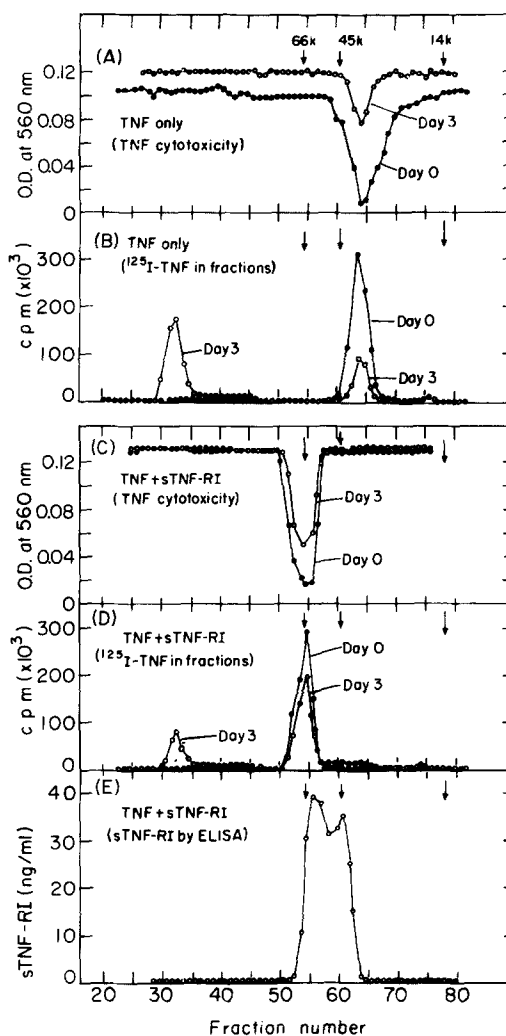
of TNF. The elution pattern normally displayed by TNF is consistent with a molecular mass of ~35 kD (Fig. 5, A and B, thick lines). Yet, after a decay time of 3 d, most of the protein has a retention time corresponding to a much larger mass. A small portion of the protein displayed a smaller mass, ~17 kD (Fig. 5 B, thin line; see also references 11 and 25). It thus appears that the decay of TNF activity is a consequence of a two-step process: the TNF molecules, which normally exist as oligomers, with a subunit molecular mass of 17 kD, dissociate to inactive monomers, which may subsequently form high molecular mass aggregates (25).

In gel-filtration chromatography of preparations also containing the sTNF-Rs, TNF and the sTNF-Rs eluted as a complex, of an apparent molecular mass larger than that of TNF (Compare Fig. 5, C, D, and E to A and B). The same mass of complex was observed, irrespective of whether or not the TNF and the sTNF-Rs were preincubated together even for extended periods. Furthermore, the proportion of TNF molecules converted to large molecular mass aggregates in this preincubation period was significantly smaller in the presence of the sTNF-Rs (32%) than in their absence (65%) (compare thick and thin lines in Fig. 5 D to those in Fig. 5 B).

Notably, those column fractions corresponding to the complex between TNF and the sTNF-Rs were cytotoxic (Fig. 5 C), implying that upon dissociation this complex yields bio-active TNF.[2]

The effect of the sTNF-RII on the pattern of elution of TNF from the column was almost identical to that of sTNF-RI, except for a slightly lower retention time of the complex formed (not shown).

These findings suggest that prolongation of TNF activity by the sTNF-Rs reflects their ability to associate reversibly with TNF in a complex in which the structure of TNF is stabilized.

Discussion

In their cell surface forms, cytokine receptors function as transducing elements, providing molecular signals for the cytokine effects within the cell. The soluble forms of these receptors, although deficient in intracellular domains, are still capable of binding their agonists, and thus affect their function. The occurrence of such soluble forms extends the spectrum of effects of the receptors also to activities mediated far away from the cells that express them. The present and several prior studies provide examples of two different ways in which the soluble receptors for TNF may affect TNF activity. These proteins, previously demonstrated to compete for TNF with the cell surface receptors (5–8), are now shown also to have the ability to stabilize TNF.

The sTNF-Rs are present constitutively in serum at concentrations that increase significantly in both inflammatory and noninflammatory disease states (21, and unpublished data). The effect of these proteins may differ, however, depending on their concentration at the site of TNF action, the relation of their concentration to the local concentration of TNF, and the rates at which the sTNF-Rs and TNF are cleared from the site of TNF action in relation to the rate of decay of TNF activity. Thus, the sTNF-Rs may in some situations inhibit the effects of TNF, in others, serve as carriers for TNF, and in some cases they may even augment the effects of TNF by prolonging its function. In the circulation, for example, from which the clearance of TNF is rapid ($t_{1/2}$ ~6′) (26), far exceeding the rate of decay of TNF activity, the ability of the sTNF-Rs to stabilize TNF is unlikely to be of any significant consequence, unless it turns out that, once bound to the sTNF-Rs, TNF is cleared at a much slower rate. However, in compartments from which the clearance of TNF is slow, for example, in the synovial spaces (27, 28), the middle ear cavity (29), cerebrospinal fluid (30, 31), the alveolar (32) and peritoneal (33) spaces, or in inflamed tissues where there is lymphatic blockage, the sTNF-Rs may function exactly like in the "closed compartment" formed in a tissue culture well. They would be expected then to function as buffering agents more than as inhibitors. They may mitigate the impact of over-production of TNF, yet, once the formation of TNF ceases, they may also slow down the decrease in its activity, serving as a "slow release reservoir" of bio-active TNF.

The spontaneous decay of TNF activity can be a limiting factor for the function of TNF (31). It seems to reflect denaturation of this protein, resulting in its dissociation to monomers that in turn form multimeric aggregates. Binding of TNF to its soluble receptors slows down this dissociation by stabilizing its trimeric structure.

The mechanism for the spontaneous denaturation of TNF is not known, nor is the way it is inhibited by the sTNF-Rs understood. The stabilization of TNF by its soluble receptors is reminiscent of the stabilization of enzymes by their substrates. In both interactions, stabilization of the protein may be due to an induced conformational change. There is ample evidence for the occurrence of conformational changes in receptors upon binding to their ligand. Such changes occur also in the extracellular, ligand-binding, domains of the receptors (e.g., compare with references 34 and 35) and may, in turn, cause alteration also in the conformation of the bound ligand and thus stabilize it.

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[2] Actually, because of some dissociation of the sTNF-Rs from the TNF during chromatography (compare the patterns of elution of TNF and sTNF-RI in Fig. 5, C and D, respectively), the TNF activity recovered from the column was higher than that applied (see legend to Fig. 5).

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