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.

Materials and Methods

TNF and Its Soluble Receptors. Recombinant human TNF-α (TNF; 6 x 10⁷ 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 ¹²⁵I 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 x 10⁶ cells/0.2 ml/well and 5 x 10⁵ cells/0.2 ml/well, respectively. The rate of cell growth after the indicated culture time was assessed by measuring the incorporation of [³H]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...
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. Detac-
chment 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 cytoidal 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 x 10^4 cells/well. TNF was ap-
plied 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 cytoidal ac-
tivity is defined as the activity that results in 50% cell death.

Quantification of the sTNF-Rs by ELISA. The concentra-
tions 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 opti-
mal concentration of the sTNF-RII for enhancement of cell
growth was higher than that of the sTNF-RI. In the ab-

The TNF-induced growth of normal fibroblasts (24) was
also enhanced by the sTNF-Rs. Both the increase in fibro-
blast 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 aug-
mented 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 Solu-
tions. The stimulatory effects of TNF on B-CLL and fibro-
blast 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 deter-
mired by applying samples of the fibroblast growth media
to A9 cells, which are sensitive to the rapidly induced cyto-
oidal 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 ap-
plied to the cells (200 U/ml). (b) The decrease in TNF ac-
tivity 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 concen-
trations 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,
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 cytotoxic 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 cytotoxic 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 FSll) 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 bioactivity of TNF in the growth media of the cells, on day 9 of incubation (C and C') was determined by measuring the cytotoxic 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 bioactivity 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 bioactivity at various times was determined by measuring the cytotoxic effect of the TNF samples on A9 cells, as described in Materials and Methods.

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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. In Fig. 5.A and B, the incorporation of a 125I-labeled TNF (5 ng, 1.5 × 10⁶ cpn) was incubated 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).
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 (t1/2 ~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.

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).
References


