TNF Inhibitor with a Low Molecular Weight Found in the Human Sera

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SHIMODA, A., HANAUMI, K, and KUMAGAI, K. TNF Inhibitor with a Low Molecular Weight Found in the Human Sera. Tohoku J. Exp. Med., 1995, 177 (4), 327-335 — We found a TNF inhibitory factor with a molecular weight of 5 to 10 kDa in the human sera. The activity was detected by inhibiting the activity of serum to TNF-induced cytotoxicity against target cells. It was found in sera of all the healthy donors tested without any febrile diseases. Moreover, our results demonstrated that TNF inhibitory factor decreases in the serum of patients on regular hemodialysis treatment and in the serum of diabetes mellitus patients. The activity found in human sera was eluted from DEAE-cellulose column (Mono Q) at 0.25 and 0.45 M NaCl, and was labile to incubation for 60 min at 56°C and susceptible to treatment with trypsin, which destroyed 60% of its biological activity. TNF inhibitory factor may act as a regulator of the biological activity of TNF and could have beneficial effects in certain inflammatory conditions, and therefore, could be useful in clinical application. —— TNF inhibitor; TNF inhibitory factor; cytotoxicity; human serum

Tumor necrosis factor (TNF-α), the cytokine mainly produced from monocytes, has a cytolytic activity on various tumor cell lines (Old 1985). TNF-α has pleiotropic activities, including cytotoxic effects against tumors and virus-infected cells, stimulation of interleukin-1 secretion, stimulation of prostaglandin E2 and collagen production, and stimulation of various immune effector cells (Dayer et al. 1985). Its related cytokine, lymphotoxin (TNF-β), is synthesized by activated lymphocytes and shares many of the same biological activities as TNF-α (Aggarwal et al. 1985). Clinical interest has focused on TNF because it appears to be a common mediator of inflammation, endotoxin-induced shock, and the wasting syndrome commonly observed in chronic infections and neoplastic diseases. Attempts to control the adverse effects of TNF/cachectin would be of clinical importance. Early studies (Dinarello et al. 1986) have revealed that injection of endotoxin in a sublethal dose results in development of tolerance to toxic effects of TNF. Moreover TNF itself induces an increased and prolonged

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resistance to the cytotoxic effect of TNF. Heterokaryons derived by fusion of TNF-resistant and sensitive cells survived the cytotoxic effect of TNF. All these data indicate the existence of mechanisms for the protection against the cytotoxic effect of TNF. In view of the potent bioactivity of TNF, a TNF inhibitor could have an important physiological role. Inhibitory activity toward TNF was originally detected in the urine of febrile patients and later in the urine from normal donors (Olsson et al. 1991). The inhibitor was purified from the urine collected from febrile patients by ion exchange chromatography, gel filtration, TNF affinity chromatography and reverse-phase chromatography (Seckinger et al. 1988; Nophar et al. 1990). A protein with a molecular weight of 33 kDa was obtained.

Engelmann et al. (1989) purified a TNF-binding protein from the urine by ion exchange chromatography and reverse-phase chromatography. A protein with an apparent molecular weight of 27 kDa was obtained, which blocked the binding of TNF to its receptors (Engelmann et al. 1989). The TNF binding protein found by Seckinger et al. (1988) seems to be identical with the TNF binding protein described above (Loetscher et al. 1991). We recently demonstrated that the serum obtained from normal donors contained factor(s) that could inhibit the cytolytic activity of both recombinant human TNF and lymphotoxin on LM cells, a clone of murine L929 fibroblast cell line, in vitro (Shimoda et al. 1990).

Soluble cytokine binding proteins in biological fluids have been shown in some cases to represent "shed" forms of cell surface cytokine receptors (Seckinger et al. 1988). To ascertain whether this was the case for the TNF inhibitor, we purified a TNF inhibitory protein from human serum. Primary characterization indicated that the inhibitory factor is a protein, as it is sensitive to trypsin and has an apparent mol wt of 0.5-1.0 × 10^4. Furthermore, this factor inhibited the sensitivity of LM cell to TNF dependent cytotoxicity without binding or modifying TNF molecules. Although inhibitory mechanisms remain obscure, TNF inhibitory factor may be a regulator of the biological activity of TNF and counteract potentially harmful effects of TNF/cachectin (Olsson et al. 1991). In the present report we describe the purification of TNF inhibitory factor from serum of healthy donors as well as some of its physicochemical and biological properties.

**Materials and Methods**

**TNF assay.** LM cells, a clone of murine L929 fibroblast cell line, were cultured with 100 U/ml of TNF and serially diluted test samples in a 96-well, flat-bottomed microcytotoxicity plate (3040; Falcon Plastics, Oxnard, CA, USA) at 37°C for 24 hr in a humidified CO₂ incubator in the presence of actinomycin D (1 μg/ml). After incubation, the plates were washed, and cell lysis was determined by staining the plate with crystal violet (0.2%) methanol (2%) in water. Dye uptake was calculated by using automated micro ELISA autoreader (Multi-
scan MC, titerhek, Flow Laboratories Inc., Tokyo). One unit of TNF activity is defined as the amount required to lyse 50% of the LM cells (Tartaglia et al. 1991). TNF inhibitory activity is defined as the percentage to inhibit the lysis of LM cells by TNF (100 U/ml).

**TNF-affinity column.** A TNF-affinity column was prepared by coupling 9.918 mg of human natural TNF at 1.0 g of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) in a coupling buffer (0.1 mol/liter NaHCO₃, 1 mol/liter NaCl, pH 9.0). TNF-Sepharose was poured into a 5 ml column and was washed three times in 100 ml of 1 mol/liter NaCl, 0.1 mol/liter sodium acetate, pH 8.0, and 1 mol/ml NaCl, 0.1 mol/liter boric acid, pH 4.0, alternatively before use (Olsson et al. 1989).

Five hundred ml of a dialyzed sample was applied to the TNF-affinity column at a flow rate of 10 ml/hr. The column was then washed with 0.2 mol/liter NaCl, 10 mmol/liter Tris-HCl, pH 8.0 until the absorbance at 280 nm of the output solution was negative. The column was then eluted consecutively with 5 ml aliquots of 0.2 M glycine-HCl, pH 2.5, and total eluate was dialyzed against phosphate-buffered saline (PBS, pH 7.4) at 4°C for 24 hr. Protein concentration was determined by absorbance at 260/280 nm in a Beckman spectrophotometer (DU-65; Beckman, Fullerton, CA, USA, Gatanaga et al. 1990).

**Cell line and growth condition.** LM cells, a clone of murine L929 fibroblast cell line, were maintained in RPMI 1640 supplemented with 5% fetal calf serum (FCS) and 0.2% glucose.

**Molecular sieve chromatography.** Gel filtration chromatography was carried out on a Sephacryl S-200 column (2.0×90 cm) (Pharmacia Fine Chemicals), equilibrated in PBS. A total of 200 mg protein was applied (5.0 ml) to the column and eluted with the same buffer at a flow rate of 35 ml/hr. Fraction (7.0 ml) was collected and tested for TNF inhibitory activity. The molecular weight (Mr) of the activity in this gel filtration was calibrated by using the standard markers, bovine serum albumin (Mr 67,000), ovalbumin (Mr 43,000), chymotrypsinogen A (Mr 25,000) and ribonuclease A (Mr 13,700) (Lantz et al. 1990).

**MonoQ column.** Samples of inhibitory factor were applied on a FPLC-MonoQ prepacked column (HR 10/10, 15×100 mm: Pharmacia Fine Chemicals) equilibrated in 0.02 mol/liter Na₂HPO₄ • NaH₂PO₄, pH 7.2 (Seckinger et al. 1988; Lantz et al. 1990). The column was washed with four column volumes of the starting buffer prior to initiation of a linear 0 to 500 mmol/liter sodium chloride gradient. The flow rate was approximately 2 ml/min. Fractions (1.0 ml) were collected, and the samples were prepared for the assay.

**Kinetic studies.** Fifty µl of partially purified TNF inhibitor or control samples were added to LM cells in the microplate assays at 24 hr before or at 0, 1, 6 and 23 hr after the addition of TNF. The effects on cytolysis were measured as described above.
RESULTS

Identification of a serum-derived TNF inhibitory factor. Samples (50 μl) of healthy human serum and the ultrafiltrate were tested for their ability to inhibit 100 units/ml of TNF activity in vitro. When LM cells were incubated with human sera for 24 hr, TNF dependent cytotoxicity on LM cells was markedly inhibited (Fig. 1). There was a concentration dependence of serum on the killing of TNF. While the data were not shown, similar results were observed when serum was held constant and TNF was varied. Human serum was separated by using three molecular size ultrafiltrate membrane tubes 5 kDa, 20 kDa, 50 kDa. The inside and outside solutions of these ultrafiltrate membrane were tested for inhibitory activity (Engelmann et al. 1990). Inhibitory activity was recovered by an apparent molecular mass of 5-20 kDa (Fig. 1).

Partial purification and physicochemical characterization of TNF inhibitor. Salt-precipitated human serum was subjected to Sephacryl S-200 gel filtration. The inhibitory activity was eluted from the gel in a single peak, and the maximal inhibitory activity showed an apparent molecular mass of 5-10 kDa (Fig. 2). The results of ion exchange chromatography are shown in Fig. 3. TNF inhibitor was eluted by approximately 0.25 mol/liter and 0.45 mol/liter NaCl from DEAE-column (MonoQ). Accordingly, in the present experiments, we used the mixture of 0.25 mol and 0.45 mol fractions. The partially purified TNF inhibitor was used for physicochemical studies. TNF inhibitor was unstable to incubation at 56°C for 60 min, and susceptible to treatment with trypsin, which destroyed 60%
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Fig 2. Elution profile of human TNF-inhibitor on Sephacryl S-200. Serum of healthy volunteers was passed through molecular sieve chromatography as described in Materials and Methods. Column fractions were tested at 1:16 dilution for the effect in rhTNF (100 U/ml) cytotoxicity assay in the presence of actinomycin D (1 μg/ml) (●).

Fig 3. Elution profile of human serum TNF-inhibitor on FPLC-MonoQ column chromatography. Serum of healthy volunteers was chromatofocused as in Materials and Methods. Column fractions were tested at 1:16 dilution for the effect in rhTNF (100 U/ml) cytotoxicity assay in the presence of actinomycin D (1 μg/ml) (●).

of its biological activity (data not shown).

**TNF affinity chromatography.** Affinity chromatography on immobilized TNF was then utilized in the next step of the purification procedure. The degree of purification was not determined at this stage, because we found that most TNF inhibitory activity eluted together with the buffer solution (data not shown).

**Inhibition kinetics of serum factor on TNF-induced LM cell lysis.** TNF-induced cell destruction in vitro can be divided into multiple stages. The first stage occurring within the first 1–2 hr, however, is an interaction of TNF with cell membrane receptors. The other stages occur subsequent to TNF binding. In the following experiments serum inhibitor was added at various intervals before or after exposure of target LM cells to TNF. Serum factors inhibited only the very
early stages (1 hr) of the cytolytic reaction for TNF, but addition of serum factors after 6 or 23 hr had no inhibitory activity against TNF (data not shown).

**Fig. 4.** TNF inhibitory activity in serum of healthy volunteers, diabetes mellitus patients and patients on regular hemodialysis. Samples of serum were tested for their ability to inhibit TNF activity in vitro. Means±s.d. were follows: (a) healthy volunteers: 49.4±19.0% (n=32); (b) diabetes mellitus patients: 88.9±14.1% (n=16); (c) patients with chronic renal failure on regular hemodialysis treatment: 86.8±15.1% (n=20).

**DISCUSSION**

TNF, a member of the inflammatory cytokines, functions as a significant mediator of cytokine networks and activates various genes by signal transmission through its receptors. Usually in healthy individuals the expression of TNF genes is controlled by its translation level, and it is thought that the function of TNF is also controlled by TNF binding proteins. TNF can produce both beneficial and deleterious manifestations. For example, the proper expression of TNF functions protectively against infectious diseases, whereas the excess of TNF causes septic shock. Moreover, TNF is associated with various autoimmune diseases.

There is much more information concerning the implication of TNF in the
murine model. In MRL-lpr/lpr mice, a striking constant high level of TNF mRNA expression has been reported in the abnormal subpopulation of CD4$^-$ and CD8$^-$ lymphocytes that accumulate in the lymphoid organs (Engelmann et al. 1989). With very low doses of TNF injected for 2 to 4 months, Brennan et al. (1989) observed a marked increase in the mortality from renal diseases. It is assumed that if the mechanism controlling the development of TNF function is elucidated it would be beneficial for the therapy of various diseases.

We demonstrated that serum obtained from healthy donors contained factor(s) that could inhibit the cytolytic activity of both recombinant human TNF and lymphotoxin on LM cells in vitro. These factors are present at high concentrations in the serum from healthy individuals. The TNF inhibitory factor suppressed not only TNF-induced cytotoxicity but also the growth inhibition on murine bone marrow cells by TNF (data not shown). The factor was partially purified by a combination of DEAE-ion exchange chromatography, blue sepharose chromatography and gel chromatography. This factor was a protein with a molecular mass of 5–10 kDa.

Numerous types of cytokine inhibitors are now known. These include (1) receptor antagonist (Hannun et al. 1990); (2) cytokine binding proteins (Engelmann et al. 1989); and (3) autoantibodies to cytokines (Jeffes et al. 1989). When LM cells were washed after preincubation with our serum factor, inhibition to TNF dependent cytotoxicity was still observed, indicating that the serum factor inhibited the sensitivity of LM cell to TNF dependent cytotoxicity rather than to the binding or modification of TNF molecules. Postreceptor mechanisms seem to play a key role in susceptibility to cell killing. Since TNF inhibitory factor is

| Table 1. Comparison of physicochemical and biological properties of TNF-inhibitors |
|---------------------------------------------|-------------------|
| **Source**                                | Urine of febrile patients | Serum of healthy volunteers |
| **Molecular weight**                       | 40–60 kDa          | 5–10 kDa                     |
| **Elution from chromatography**            | at pH 5.5–6.1 from chromato focusing Mono P | at 0.25 M (peak 1) and at 0.45 M (peak 2) from DEAE Mono Q |
| **Inhibitory for**                         | human rTNF in L929 assay | human rTNF human rLT in L929–LM assay |
| **Heat sensitivity**                       | % inactivation     | % inactivation               |
| 56°C 60'                                  | 7%                 | 70–80%                       |
| 75°C 60'                                  | 85%                | ND                            |
| 95°C 60'                                  | 87%                | ND                            |
| **Trypsin sensitivity**                    | % inactivation     | % inactivation               |
| 1 mg/ml, 37°C, 4 hrs                      | 38%                | 60%                           |

ND, not done.
present in the serum of healthy individuals without exception and does not bind TNF itself, we consider that the factor is a blocking factor acting against the action mode of intracellular signal transmission of TNF. Comparison of various properties of other TNF inhibitors with ours, is shown in Table 1.

The presence of the TNF inhibitory factor may serve as a feedback mechanism, reducing the immediate availability of TNF and prolonging its effects. Moreover, these inhibitors may lead to new approaches for the therapy of septic shock and chronic inflammatory diseases.

References


