Brief Definitive Report

A HUMAN INHIBITOR OF TUMOR NECROSIS FACTOR α

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TNF is a protein identified by its ability to induce cytotoxicity for tumor cells and to inhibit their growth in culture (1). TNF-α (also termed cachectin), mainly produced by monocytes/macrophages, mediates and participates in a wide range of biological activities (2, 3). In addition, TNF-α shares several of its activities with IL-1, among them prostaglandin E_2 (PGE_2) and collagenase production by human dermal fibroblasts and synovial cells (4). Both IL-1 and TNF-α induce fever by stimulating hypothalamic PGE_2 synthesis (5).

To our knowledge, no inhibitory factor directed against TNF activity has been reported yet. We previously isolated a specific IL-1 inhibitor from the urine of febrile patients (6–8). As TNF and IL-1 are both mediators of fever, we wondered whether such urine might also contain a TNF-α inhibitory (TNF-α INH) activity. Indeed, we found an inhibitory factor of 40–60 kD, therefore proving the existence, as in the case of IL-1, of at least one negative feedback regulator.

Materials and Methods

Urine Collection. Urine (15 liters) was freshly obtained before any treatment from a pool of five patients, two of whom were suffering from small-cell carcinoma, one from malignant histiocytosis, one from polymyositis, and one from sepsis. All patients were highly febrile (>38.5°C) and devoid of urinary infections. Urine was concentrated and ammonium sulfate precipitated as described earlier (6–8).

Reagents and Media. PBS, MEM, FCS, penicillin, streptomycin, and glutamine were obtained from Gibco (Paisley, Scotland). Human rTNF-α (hrTNF-α) was produced in *Escherichia coli* at Biogen S. H., Geneva, Switzerland.

Bioassay of TNF-α INH. TNF-α INH activity was measured in an assay of cytotoxicity using a TNF-susceptible cell line L929, as previously described (9).

Quantitation of TNF-α INH Activitiy. The percentage of TNF-α INH was determined by assuming that OD values from cells stimulated by actinomycin D corresponded to 100% of inhibition, whereas OD from cells cultured with actinomycin D and hrTNF-α corresponded to maximal cell mortality of 0% of TNF-α INH. The percentage of TNF-α INH in the assay of cytotoxicity was calculated by the following formula: Percentage of TNF-α INH = $100 \times \{[(\text{OD with actinomycin D} + \text{hrTNF-}\alpha + \text{TNF INH}) - (\text{OD with actinomycin D} + \text{hrTNF-}\alpha)]/[(\text{OD with actinomycin D}) - (\text{OD with actinomycin D} + \text{hrTNF-}\alpha)]\}$

Molecular Sieve Chromatography. All dialysis and chromatographic steps were performed at 4°C. Gel filtration chromatography was carried out on a Sephacryl S-200 column (0.9 × 60 cm) (Pharmacia Fine Chemicals, Uppsala, Sweden), equilibrated in 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl. 20 mg of protein was applied

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(0.8 ml) to the column and eluted with the same buffer at a flow rate of 5.4 ml/h. Fractions (1.35 ml) were collected and tested for TNF-α INH activity.

FPLC Chromatofocusing. Chromatofocusing was performed with a Mono P prepacked column (HR 5/20, 5 × 200 mm; Pharmacia Fine Chemicals) equilibrated in 25 mM Bis-Tris buffer adjusted to pH 7.1 with iminodiacetic acid (Fluka, Buchs, Switzerland). 30 mg of protein was applied to the column and eluted with a polybuffer 74/iminodiacetic acid at pH 4.0. The actual pH of each fraction (1 ml) was determined with a pH meter, and TNF-α INH inactivity was evaluated.

Trypsin Digestion. 50 μl of trypsin (10 mg/ml) (Sigma Chemical Co., St. Louis, MO) in 0.2 M Tris-HCl buffer, pH 8.0, containing 1 mM calcium chloride was added to 500 μl of Sephacryl S-200 semipurified urine dialyzed against the above-mentioned buffer. After 4 h of incubation at 37°C, a second 50-μl sample of trypsin was added to the digestion mixture. The reaction was terminated after 24 h by adding 100 μl soybean trypsin inhibitor (20 mg/ml) (Sigma Chemical Co.). Trypsin digest and controls were tested at a 1:20 final dilution on L929 cells and in the presence of hrTNF-α at 0.2 ng/ml concentration.

Results

Identification of a Urine-derived TNF-α INH Activity. Amicon-concentrated and ammonium sulfate–precipitated urine (40–80%) showed TNF-α INH activity when tested in a cytotoxicity assay with the TNF-susceptible cell line L929 in the presence of actinomycin D. Thus, at a 1:20 final urine dilution, total inhibition of the cytotoxic effect induced by hrTNF-α was observed so that OD_{570} value was identical to that measured in the presence of actinomycin D alone (OD_{570} = 1.5). Moreover, this material showed inhibitory activity in urine dilutions of up to 1:160 on cells (OD_{570} = 0.83) whereas control value of hrTNF-α at a final concentration of 0.2 ng/ml measured in the presence of actinomycin D was lower (OD_{570} = 0.73), so that 50% of inhibition was observed at a dilution of ~1:100 (OD_{570} ~ 1.10). The TNF-α INH had no effect on cell viability when tested without actinomycin D (data not shown).

Characterization of the Urinary TNF-α INH. Salt-precipitated urine was subjected to Sephacryl S-200 gel filtration. The inhibitory activity was eluted from the gel in a single peak and maximal inhibitory activity showed an apparent molecular mass of 40–60 kD (Fig. 1). In another experiment, the salt-precipitated urine was chromatofocused on a Mono-P HR 5/20 column as described in Materials and Methods. Fig. 2 shows that eluted fractions between pH 5.5 and 6.1 contained the bulk of TNF-α INH activity.

Experiments were performed to establish whether the TNF-α INH activity was due to a protein. Heating of Sephacryl S-200 semi-purified TNF-α INH at 56, 75, and 95°C reduced its activity in a time- and temperature-dependent fashion (Table I). In another experiment, trypsin digestion for 24 h at 37°C reduced the inhibitory activity by ~60% (Table I). The possibility still remained that a molecule of low molecular weight binding to a protein was the active moiety of the inhibitory activity. Consequently, 1 ml of Sephacryl S-200 fraction was adjusted to 2 M urea and extensively dialyzed at 4°C against PBS containing 2 M urea. This material was dialyzed against PBS again before the bioassay; the inhibitory activity was found to be unaffected (data not shown).

We next investigated whether an excess of TNF-α was capable of overcoming the effect of the inhibitor. A consistent amount (25 μl) of Sephacryl S-200

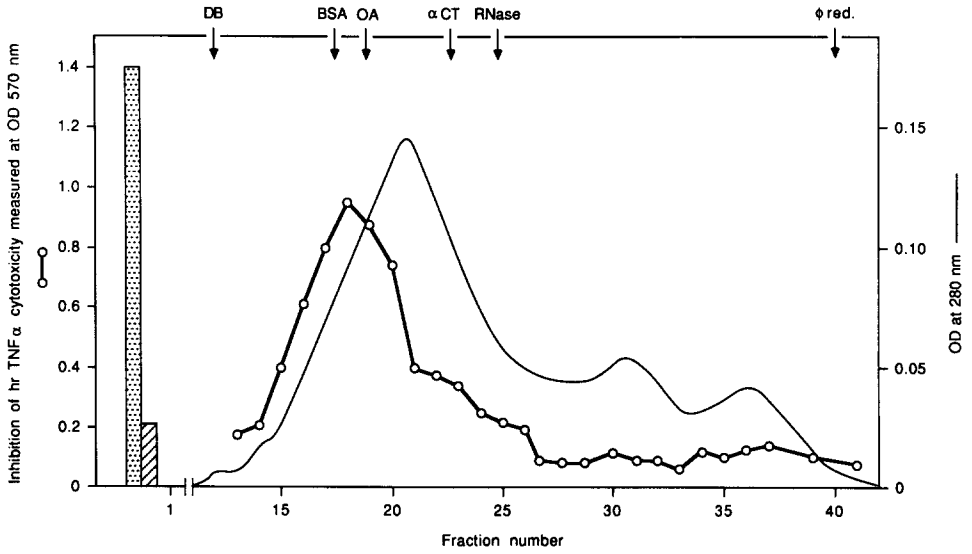

FIGURE 1. Urinary TNF-α INH activity profile of Sephacryl S-200 gel filtration. Urine of febrile patients was concentrated, ammonium sulfate–precipitated, and passed through molecular sieve chromatography as described in Materials and Methods. Column fractions were tested at 1:10 dilution for effect in the hrTNF-α (1.0 ng/ml) cytotoxicity assay in the presence of actinomycin D (1 μg/ml) (○). Bars represent cell lysis measured by dye uptake at 570 nm in response to actinomycin D (▨) and to actinomycin D plus hrTNF-α (▨) without urine. Molecular weight markers are dextran blue (*DB*); BSA; OVA; α-chymotrypsinogen-A (αCT); RNase, and phenol-red (Φ-*red*).

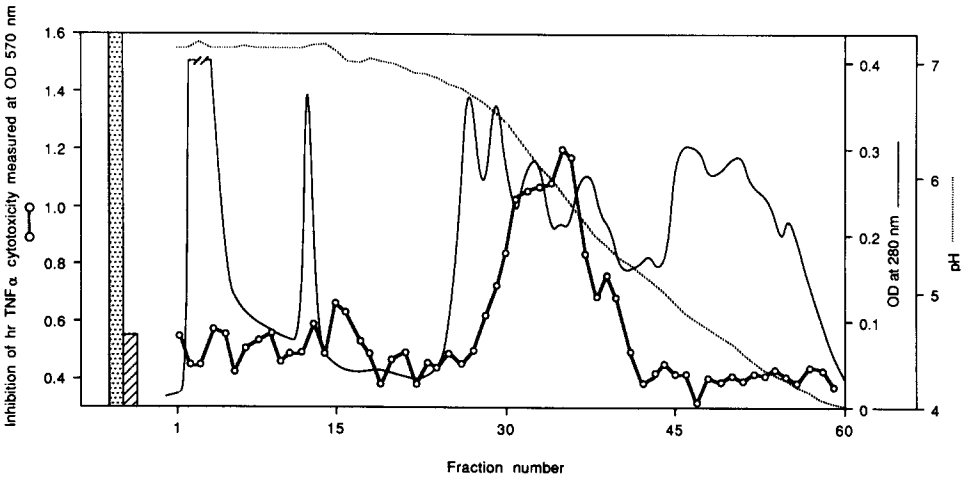

FIGURE 2. Urinary TNF-α INH activity profile of chromatofocusing Mono P column. Urine of febrile patients was concentrated, ammonium sulfate precipitated, and chromatofocussed as described in Materials and Methods. Column fractions (1.0 ml) were tested at 1:10 dilution for the effect in hrTNF-α (0.2 ng/ml) cytotoxicity assay in the presence of actinomycin D (1 μg/ml) (○). Bars represent cell lysis measured by dye uptake at 570 nm in response to actinomycin D (▨) and to actinomycin D plus hrTNF-α (▨) without urine samples.

TABLE I
Biochemical Characterization of TNF-α INH

Inactivation treatment of TNF-α INH activity					
Heat inactivation*			Trypsin inactivation‡		
Temperature	Time	TNF-α INH activity	Conditions	TNF-α INH activity	OD_{570}
	min	%		%	
	10	100	Buffer alone	0	0.71
56°C	20	100			
	60	93	Trypsin + soybean trypsin inhibitor	0	0.70
	10	60	in buffer		
75°C	20	26	Partially purified		
	60	15	Sephadex S-200 urine	61	1.46
	10	27	Partially purified		
95°C	20	10	Sephadex S-200	23	1.03
	60	13	urine digested by trypsin		

* Urine, partially purified by Sephacryl S-200, was heat inactivated at temperatures and times indicated. Remaining TNF-α activity was compared to untreated samples. Percentage of TNF-α INH activity was determined by using the formula reported in Materials and Methods. When exposing cells to actinomycin D alone (1 μg/ml) and to actinomycin D + hrTNF-α (0.2 ng/ml), OD_{570} values were 1.49 and 0.06, respectively.

‡ Urine, partially purified by Sephacryl S-200, was digested with trypsin as described in Materials and Methods. Conditions (1:20 dilution) represent buffer in the absence and presence of trypsin and soybean trypsin inhibitor (SBTI), as well as urine in the absence or presence of trypsin, followed by inactivation with SBTI. Values indicate the percentage of inhibition on hrTNF-α (0.2 ng/ml) - induced cytotoxicity in the presence of actinomycin D (1 μg/ml). When exposing cells to actinomycin D (1 μg/ml) alone and to actinomycin D + hrTNF-α (0.2 ng/ml), OD_{570} were 0.90 and 0.76, respectively.

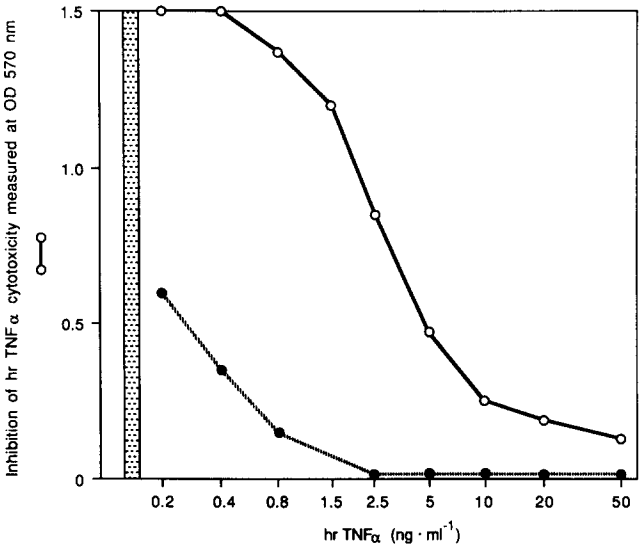

FIGURE 3. Reversibility of TNF-α INH activity. Partially purified Sephacryl S-200 urine was tested at a 1:10 dilution against increasing amounts of hrTNF-α on L929 cells. The bar represents cell lysis measured by dye uptake at 570 nm in response to actinomycin D (1 μg/ml) alone. (○) OD measured in the presence of actinomycin D, an appropriate amount of TNF-α INH and increasing amounts of hrTNF-α. (●) OD in the presence of actinomycin D and increasing amounts of hrTNF-α, without urine.

semipurified inhibitor (equivalent to 1:10 final dilution on cells) was added to increasing amounts of hrTNF-α. As shown in Fig. 3, there was an inverse correlation between the amount of hrTNF-α present in the assay and the degree of inhibition observed. Thus, the inhibitory activity was competitively overcome by increasing concentrations of hrTNF-α.

Discussion

We have found that when tested in a cytotoxicity assay, urine from febrile patients contained a TNF-α inhibitory activity whose nature remains to be determined by purification to homogeneity, many bands being still identified in SDS-PAGE of the Sephacryl S-200 inhibitory fractions. Purification will enable us to establish a relationship with one or more other putative inhibitors. Thus, uromodulin, which binds TNF as well as IL-1, had no inhibitory activity when tested in our cytotoxicity assay, even at final concentrations as high as 2 μg/ml. Primary characterization indicates that the inhibitory activity is due to a protein, as it is sensitive to trypsin and pronase, with a pI ranging from 6.1 to 5.5 and an apparent mol wt of 40–60 $\times$ 10^3. This argues against the hypothesis that denatured TNF is responsible for the TNF-α INH activity by displacing the active native TNF-α from its receptor. Using a Western blotting technique, after a run of the semipurified Sephadex S-200 inhibitory fraction on SDS-PAGE under reducing conditions, no reactivity for any mol wt $<26 \times 10^3$ was seen with polyclonal goat antisera raised against hrTNF-α (a gift from R. V. Ulevitch, Scripps Clinic). Thus, neither a TNF-α precursor nor denatured TNF seems to be responsible for TNF-α INH activity. The TNF-α INH activity shows heat sensitivity, whereas it is fully maintained after urea treatment, eliminating the possibility that a small molecule is responsible for the TNF-α inhibition.

The results indicate that the inhibitor is specific for TNF-α and not due to an actinomycin D binding protein, as TNF-α INH activity was entirely overcome by increasing TNF-α concentrations without changing that of actinomycin D. Our data suggest a competitive mechanism of action perhaps situated at the receptor level itself, as is the case with the IL-1 INH previously observed in urine of similar patients (7, 8, 10). It should be emphasized that urine from some febrile patients seems to be an important source of either IL-1 or TNF-α inhibitory activities and that, in analogy to the IL-1 INH, the relationship between TNF-α INH or IL-1 INH activities and disease still remains to be investigated. The question as to whether the present TNF-α INH is also effective against other bioactivities induced by TNF-α has already been approached, and it has been shown that the PGE_2 production induced by TNF-α on human fibroblasts may well be inhibited by the present TNF-α INH (manuscript in preparation), which means that it is also effective on non–actinomycin D–treated cells. The present TNF-α INH differs unequivocally from the IL-1 inhibitor, since the latter did not inhibit TNF-α-induced cytotoxicity to murine L929 cells. Moreover, the TNF-α INH shows a higher molecular weight and does not inhibit the binding of ^{125}I-IL-1α to the murine thymoma subline EL4-6.1. Like the IL-1 INH, the TNF-α INH is a nonspecies-specific inhibitor as it acts on a murine cell line.

Summary

Urine of some febrile patients exibits a TNF-α inhibitory activity (TNF-α INH), sensitive to heat and trypsin, with an apparent mol wt of 40–60 $\times$ 10^3 and a pI range of 5.5–6.1. As for the Il-1 INH, the TNF INH activity involves a competitive mechanism of action suggesting the existence of a family of negative feedback-regulating molecules interfering with cytokines actions.

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