

Increased Serum Levels of Soluble Receptors for Tumor Necrosis Factor in Cancer Patients¹

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ABSTRACT

Soluble forms of the two molecular species of the cell surface receptors for tumor necrosis factor (TNF) have been detected in normal urine. Using enzyme-linked immunosorbent assays for these soluble receptors, we determined their levels in the sera of 40 healthy subjects and 59 patients with solid tumors. The mean \pm SD concentrations of both the soluble type I (p55) and type II (p75) receptors were significantly higher in the cancer patients than in the healthy controls: 1.96 ± 1.19 versus 0.79 ± 0.19 ng/ml ($P < 0.001$) and 6.43 ± 4.8 versus 3.2 ± 0.6 ng/ml ($P < 0.001$), respectively. The incidence and the extent of the increase correlated with the staging of disease. Sera of the cancer patients had a marked inhibitory effect on the *in vitro* cytotoxic activity of TNF. This inhibition was proportional to the content of soluble TNF receptors and could be fully abolished by the addition to the sera of specific antibodies against the receptors. Among the cancer patients, the incidence of increase in the concentrations of soluble TNF receptors (about 70%) greatly exceeded that of the serum carcinoembryonic antigen (about 26%), a commonly used cancer marker. The origin of the serum soluble TNF receptors in cancer patients and the physiological implications of their effect on TNF function remain to be elucidated.

INTRODUCTION

Tumor necrosis factor, a polypeptide cytokine produced primarily by mononuclear phagocytes, initiates its multiple effects on cell function (1, 2) by binding to specific, high-affinity cell surface receptors. There are two known molecular species of the TNF³ receptors (3, 4). Each species exists not only as a cell surface form but, as recently found, also as soluble molecules (3, 5-8). The cell surface forms of the TNF receptors (TNF-Rs) provide the intracellular signaling for cell response to TNF (8). The accessibility of TNF to them is, therefore, a precondition for the initiation of the cellular response to the cytokine. On the other hand, the soluble forms of these receptors, because of their ability to compete with the cell surface forms for TNF, may function as inhibitors of TNF bioactivity (3, 5-7). Evidence indicating that the soluble forms are derived from the cell surface receptors by proteolytic cleavage suggests that the mere process of their formation constitutes a mode of down-regulation of the receptors, resulting in decreased responsiveness to TNF (9-11).

Comparative studies of the response of various cultured cell lines to the cytotoxic effect of TNF, as well as studies of the control of the *in vivo* response to this cytokine, indicate the existence of mechanisms which can suppress its function (12-

14). In exploring the nature of these mechanisms we found that in cell cultures of several lines derived from solid tumors, soluble forms of the TNF-Rs are produced continuously.⁴ In view of this finding, we undertook the present study in order to determine whether, and to what extent, the serum of cancer patients contains soluble TNF-Rs.

PATIENTS AND METHODS

Subjects

The study population consisted of 59 consecutive cancer patients hospitalized between November 1989 and May 1990. Their mean age was 64.9 ± 12.6 (SD) years (range, 33-80).

Exclusion criteria included recent surgery (within 1 month), a recent or active infective process, fever, and concurrent therapy with corticosteroids or immunosuppressive or chemotherapeutic agents.

The control group consisted of 40 consecutive individuals (age 51.1 ± 8.5 years, range, 34-70) seen in our outpatient clinic for a routine annual checkup.

Both the patients and the healthy controls had a detailed medical history taken and underwent a thorough physical examination by two of us (G. K. and A. D.). Laboratory tests included complete blood count, liver and kidney functions, blood lipid profile, thyroid function, erythrocyte sedimentation rate, and stool examination for occult blood. All control subjects had a chest X-ray, and the women underwent a gynecological examination, a Papanicolaou smear, and mammography if indicated. Serum levels of the CEA were determined in 54 of the cancer patients.

We also included, as a separate group, five patients with benign tumors and three postmalignancy patients who were considered cured at the time of the study.

Methods

Serum Samples. Blood obtained by venipuncture was allowed to clot at room temperature for 1-3 h. The serum was stored at -20°C .

Cancer Staging. Staging of colorectal cancer was according to the American Joint Committee TNM Classification (15). Classification of breast cancer followed the standard system devised by the American Joint Committee on Cancer Staging (16).

Other tumors were classified as follows: Stage I, a localized mass of less than 2 cm in diameter (no metastases); Stage II, a localized tumor mass greater than 2 cm in diameter, with no identifiable macroscopic or microscopic metastases; Stage III, metastases limited to lymph nodes, irrespective of tumor size; Stage IV, distant metastases present.

For each tumor we also recorded the degree of differentiation, the presence of necrosis or ulceration, and the type of cellular infiltrate in and around the lesion.

ELISA for Soluble TNF Receptors. ELISA plates (Maxisorp Nunc, Denmark) were coated with monoclonal antibodies to the soluble forms of either type I or type II TNF-R (8)⁴ by incubation of the plates for 2 h at 37°C with a solution of 25 $\mu\text{g/ml}$ of the antibodies in PBS containing 0.02% NaN_3 . After an additional 2 h of incubation at 37°C with PBS containing 1% bovine serum albumin, 0.02% NaN_3 , and 0.05% Tween 20 (blocking solution) to block nonspecific binding of protein, the plates were rinsed with PBS containing 0.05% Tween 20 (washing solution). Samples for testing were serially diluted in a solu-

⁴ Unpublished data.

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³ The abbreviations used are: TNF, tumor necrosis factor; TNF-R, tumor necrosis factor receptor; sTNF-R, soluble tumor necrosis factor receptor; CEA, carcinoembryonic antigen; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.

Table 1 Concentrations of soluble TNF-RI in the serum at different stages of cancer (ng/ml)

Tumor	No. of patients	Stage I	Stage II	Stage III	Stage IV
Colon	29	1.22 ± 0.35 ^a (3) ^b	1.42 ± 0.54 ^c (11)	1.76 ± 1.18 ^a (8)	2.41 ± 0.60 ^c (7)
Breast	12	0.88 ± 0.39 ^d (2)	1.41 ± 0.72 ^d (5)	1.43 ± 0.53 ^a (4)	0.87 (1)
Pancreas	6		1.25 ± 0.77 ^d (2)		3.61 ± 2.70 ^a (4)
Stomach	3			1.00 (1)	1.73 ± 0.12 ^c (2)
Lung	2				2.57 ± 1.30 ^d (2)
Bile ducts	2				3.20 ± 0.28 ^c (2)
Adrenal	2				2.58 ± 1.07 ^a (2)
Esophagus	1				2.29 (1)
Kidney	1	0.46 (1)			
Ovary	1				4.90 (1)
Total	59	0.98 ± 0.41 ^d (6)	1.4 ± 0.57 ^c (18)	1.61 ± 0.96 ^c (13)	2.68 ± 1.3 ^c (23)

^a $P < 0.05$, compared to healthy controls.

^b Numbers in parentheses, number of patients.

^c $P < 0.001$, compared to healthy controls.

^d Not statistically significant.

^e $P < 0.01$, compared to healthy controls.

tion containing 0.65 M NaCl, 10 mM sodium phosphate buffer, pH 7.0, 0.05% Tween 20, 0.1% Nonidet P-40, and 0.02% NaN₃ before being added to the plates in aliquots of 80 μ l/well. The plates were incubated for 2 h at room temperature and rinsed 3 times with washing solution. Rabbit polyclonal antiserum against the relevant soluble receptor (3), diluted 1:500 in blocking solution, was then added to the wells. After being incubated for a further 12 h at room temperature, the plates were rinsed again with washing solution and incubated for 2 h at 37°C with horseradish-peroxidase-conjugated affinity purified goat anti-rabbit IgG (Biomakor, Israel). The assay was developed for 30 min by using 2,2'-azino-bis(3-ethylbenzthiazoline-6 sulfonic acid) as a substrate (Sigma). The enzymatic product was determined colorimetrically at 405 nm. Purified urine-derived soluble forms of the two receptors (3) served as standards. The detection limit of the assay was 30 pg/ml. Interassay variation was 8.7% for the serum levels of sTNF-RI and 5.5% for sTNF-RII. Validity of the assay was confirmed by the following tests. (a) Normal sera and sera of cancer patients containing increasing sTNF-Rs were spiked with the urine-derived purified TNF receptors, in amounts similar to those present in the sera, and assayed as above. In all cases full recovery was observed. (b) In either assay, replacement of the coating monoclonal antibody with two monoclonal antibodies recognizing other antigenic determinants of sTNF-RI or II (8)^a resulted in identical measurements. (c) Addition of the coating monoclonal antibody at a concentration of 1 mg/ml to the tested sera, or omission of the step of coating of the plate with the antibody, resulted in null readings. (d) No cross-reactivity was found for the two species of receptors in the two assays. The readings obtained were not affected by varying of the clotting time of the blood over a range of 1 to 48 h, nor by the use of plasma (collected with heparin) instead of serum. This rules out the possibility that release of sTNF-Rs by the leukocytes in response to clotting contributes to any extent to the measured serum levels of the receptors. The readings were also unaffected by repeated freezing and thawing of the sera. Adding TNF, at a concentration of 25 ng/ml, to tested samples of sTNF-Rs resulted in identical measurements. Addition of TNF at a concentration of 100 ng/ml to the sTNF-Rs resulted in about 25% decrease of the signal obtained for 4 ng/ml sTNF-RI and had no effect on the measurement of sTNF-RII.

Determination of Carcinoembryonic Antigen. CEA levels were measured by a double antibody ¹²⁵I-radioimmunoassay (Diagnostic Products Corporation, Whitney, United Kingdom). A serum level of 5 ng CEA/ml was considered as the upper limit of normal.

Determination of Inhibitory Effect of Sera from Cancer Patients on TNF Cytotoxicity. The cytotoxic activity of TNF was determined by using murine A9 cells (17) as targets. The cells were seeded in 96-well microplates at a density of 20,000 cells/well. After 24 h the supernatants were decanted. The cells were placed on ice and human recombinant human TNF (5 units/ml, 6 × 10⁷ units/mg protein; Genentech Co., San Francisco, CA) was applied alone or together with serum samples

with or without added antibodies to the sTNF-Rs (8)⁵ or with samples of the purified sTNF-Rs isolated from the urine (3). After additional incubation on ice for 90 min the samples were decanted and the plates were rinsed twice with cold medium. This was followed by addition of Dulbecco's modified Eagle's minimal essential medium containing 10% fetal calf serum and 25 μ g/ml cycloheximide. Cell viability was determined 12 h later by the neutral red uptake assay (18).

Statistics. The data were analyzed by the Student's *t* test. Correlation coefficients were calculated and regression equations were derived when appropriate.

RESULTS

Serum concentrations of the soluble forms of type I (p55) and type II (p75) TNF-Rs were determined by ELISA in 29 patients with colon carcinoma, in 30 patients with various other solid tumors (see Tables 1 and 2), and in 40 healthy controls.

In the healthy subjects, the average concentrations (mean ± SD) of sTNF-RI and sTNF-RII were 0.79 ± 0.19 and 3.2 ± 0.6 ng/ml, respectively. These values were independent of age and sex. The corresponding values in cancer patients were significantly higher, 1.96 ± 1.19 ng/ml ($P < 0.001$) and 6.43 ± 4.82 ng/ml ($P < 0.001$), respectively. In contrast, sTNF-RI and sTNF-RII concentrations were within the normal range in the group of 5 patients with benign tumors (adenoma of the breast and colon and a benign insulinoma, 0.68 ± 0.19 and 2.96 ± 0.69 ng/ml, respectively) and 3 patients considered cured of malignancy (2 breast and 1 stomach cancer) (0.9 ± 0.09 and 3.32 ± 0.28 ng/ml, respectively).

Of the 29 patients with colon carcinoma, 21 (72.4%) exhibited a significant increase (more than 2 SD above the normal mean) in serum levels of type I and type II sTNF-Rs. The incidence of such increase and its extent clearly correlated with the staging of the disease. Thus, whereas among the Stage I patients only 1 in 3 showed an increase in receptor concentrations, significant increases were shown by 7 of 11 patients at Stage II (63%), 6 of 8 at Stage III (75%), and all patients (7 of 7) at Stage IV (Tables 1 and 2; Fig. 1, open circles). A similar pattern was also observed for patients with other malignancies (Tables 1 and 2; Fig. 1, solid circles). The extent of increase in the serum concentrations of sTNF-Rs also correlated with the staging of the disease, being higher with more advanced disease (Tables 1 and 2; Fig. 1).

Receptor concentrations did not correlate with the ESR or

⁵ Unpublished study.

Table 2 Concentrations of soluble TNF-RII in the serum at different stages of cancer (ng/ml)

Tumor	No. of patients	Stage I	Stage II	Stage III	Stage IV
Colon	29	3.93 ± 1.15 ^a (2) ^b	4.9 ± 1.79 ^c (11)	6.49 ± 2.62 ^c (8)	7.57 ± 2.70 ^d (7)
Breast	12	2.64 ± 1.05 ^a (2)	3.41 ± 1.07 ^a (5)	5.46 ± 1.29 ^c (4)	3.50 (1)
Pancreas	6		5.49 ± 1.40 ^a (2)		16.10 ± 11.27 ^a (4)
Stomach	3			4.04 (1)	6.65 ± 0.47 ^c (2)
Lung	2				6.59 ± 3.31 ^a (2)
Bile ducts	2				12.50 ± 8.90 ^a (2)
Adrenal	2				5.74 ± 2.62 ^a (2)
Esophagus	1				6.15 (1)
Kidney	1	5.00			
Ovary	1				18.10
Total	59	3.68 ± 1.25 ^a (6)	4.55 ± 1.68 ^c (18)	6.01 ± 2.21 ^d (13)	8.88 ± 5.42 ^d (23)

^a Not statistically significant.
^b Numbers in parentheses, number of patients.
^c P < 0.01, compared to healthy controls.
^d P < 0.001, compared to healthy controls.
^e P < 0.05, compared to healthy controls.

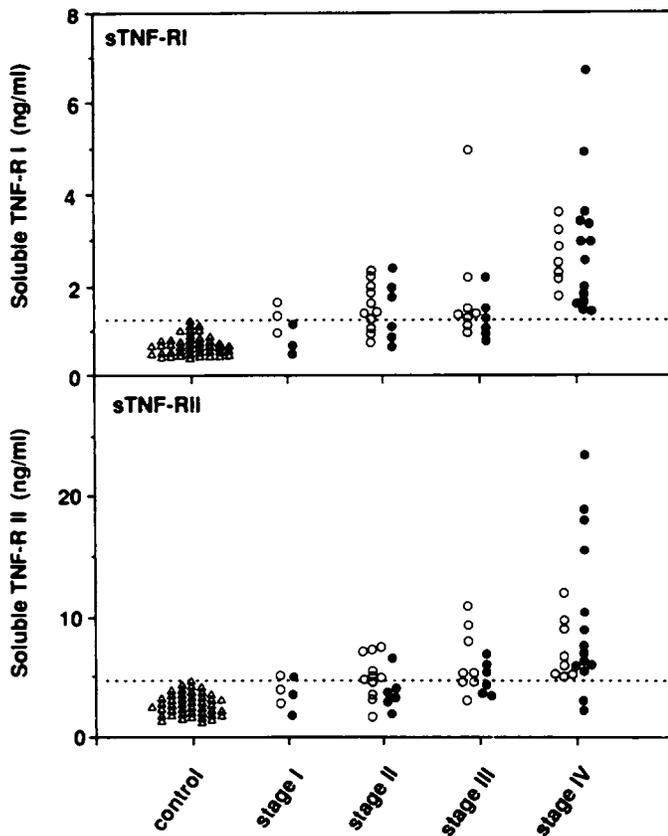


Fig. 1. Serum levels of sTNF-Rs in normal controls and cancer patients. (Δ) Controls. (○) Patients with colon carcinoma. (●) Patients with other solid tumors. ---, value of the mean ± 2 SD of the normal control.

with serum levels of cholesterol or calcium (Table 3). Nor did they correlate with necrosis, ulceration, type of cellular infiltrate, or degree of differentiation of the tumors.

An apparent correlation was found between the increase in levels of serum sTNF-Rs and the extent of weight loss observed in the patients ($r = 0.69$; $P < 0.01$). Consistently with its association with disease stage, the increase in sTNF-R levels correlated negatively with hemoglobin and albumin concentrations (both of which are expected to decrease in advanced cancer) and positively with serum levels of alkaline phosphatase (Table 3). The increase in sTNF-Rs also correlated positively with the number of circulating WBC (Table 3), even though no

correlation was observed between the numbers of leukocytes and the cancer staging ($r = 0.07$; P not significant).

Among the cancer patients, only partial correlation was observed between the increase in serum concentrations of sTNF-R and CEA. Of 54 patients in whom the serum level of CEA was determined, the concentration of CEA was above the normal (>5 ng/ml) only in 14 (26%). All of the latter also showed an increase in their level of sTNF-Rs; such an increase was however also observed in other patients whose serum CEA level was within the normal range. Altogether, 40 of the cancer patients exhibited an increase in sTNF-R I and 37 in sTNF-R II (74 and 68.5% of the cancer patients, respectively). Similar discrepancies between the incidence of increase in CEA and in sTNF-R levels were noted at all stages of the disease, both in the patients with colorectal cancer and in the group of patients with other kinds of solid tumors (Table 4).

In order to evaluate the bioactivity of the serum TNF-Rs, serum samples were introduced to a TNF cytotoxicity assay. As demonstrated in Fig. 2, the sera of cancer patients and, to a lesser extent, also normal sera protected A9 cells from the cytotoxic effect of TNF. The extent of inhibition correlated with the inhibition observed upon application of the urine-derived purified sTNF-Rs in amounts identical to those present in the sera. Monoclonal antibodies to the sTNF-Rs, which by themselves had no effect on the viability of the A9 cells, blocked the inhibitory effect of the human sera on TNF activity, confirming that this inhibition was solely due to the activity of the sTNF-Rs in these sera.

Table 3 Correlations between the soluble TNF receptors and laboratory parameters

	sTNF-R I		sTNF-R II	
	r	P	r	P
ESR	0.07	NS ^a	0.05	NS
Hemoglobin	-0.41	<0.01	-0.33	<0.55
Total WBC	-0.38	<0.02	0.53	<0.001
Albumin	-0.745	<0.001	-0.595	<0.001
Globulin	0.36	<0.05	0.08	NS
SGOT	0.08	NS	0.10	NS
Alkaline phosphatase	0.58	<0.01	0.52	<0.01
Bilirubin	0.02	NS	0.07	NS
Cholesterol	0.20	NS	0.07	NS
Calcium	-0.04	NS	-0.18	NS
Creatinine	-0.03	NS	0.01	NS

^a NS, not statistically significant.

Table 4 Comparison of the incidence of increase in sTNF-Rs and in CEA in sera of cancer patients^a

	% of patients		
	CEA	sTNF-RI	sTNF-RII
Colorectal cancer			
Stage I (3) ^b	0 (0)	20 (1)	0 (0)
Stage II (10)	20 (2)	70 (7)	70 (7)
Stage III (8)	50 (4)	75 (6)	75 (6)
Stage IV (7)	43 (3)	100 (7)	100 (7)
Total (28)	36 (9)	75 (21)	71 (20)
Other cancers			
Stage I (1)	0	0	0
Stage II (5)	0	60 (3)	20 (1)
Stage III (5)	20 (1)	60 (3)	40 (2)
Stage IV (15)	28 (4)	86 (13)	93 (14)
Total (26)	19 (5)	73 (19)	65 (17)
All cancers			
Stage I (4)	0	25 (1)	0
Stage II (15)	13 (2)	66 (10)	53 (8)
Stage III (13)	38 (5)	69 (9)	61 (8)
Stage IV (22)	33 (7)	91 (20)	95 (21)
Total (54)	25 (14)	74 (40)	68 (37)

^a Percentage of patients in the group with abnormally elevated levels of the examined marker (either CEA or sTNF-Rs, respectively).

^b Numbers in parentheses, number of patients.

DISCUSSION

In the present study, we found significantly increased levels of the two sTNF-Rs in a large proportion of patients with colon carcinoma and other solid malignant tumors. Both the incidence and the extent of increase correlated with the stage of the disease. A significant increase in concentrations of the sTNF-Rs has also been observed in the sera of most patients with chronic lymphocytic leukemia.⁶ The mechanisms involved in this increase, as well as its functional implications, remain to be elucidated. We have previously found that cells of various tumor-derived lines produce the soluble receptors spontaneously in culture.⁴ We therefore suspect that the excessive amounts of receptors in the sera of cancer patients are produced, at least in part, by the tumor cells. Our data do not exclude an alternative possibility, namely, that the receptors are produced by a population of normal cells in reaction to the tumor presence. The two possibilities are not mutually exclusive. The excess of soluble receptors could reflect production both by malignant cells and by nonmalignant cells such as those related to the immune system. In fact, the simultaneous presence of both types of sTNF-Rs in the sera of cancer patients, to a similar extent, points to the involvement of several cell lineages, since the two receptors were found to be expressed differentially in different cells (3, 4).

There is evidence, primarily from *in vitro* studies, that tumor cells have a greater tendency than nonmalignant cells to produce and shed soluble forms of their cell surface proteins, apparently as a result of enhanced cleavage of the cell surface molecules (reviewed in Refs. 19 and 20). Study of the formation of sTNF-Rs by the tumor, if this is indeed a mechanism bringing to the increased levels of these receptors in cancer patients, may thus provide information on an apparently common feature of tumors and on its relationship to the transformed phenotype. If the soluble receptors are produced by normal cells in response to the tumor, the study of their formation may lead to a better understanding of the mechanisms of tumor recognition and of

⁶ Manuscript in preparation.

inappropriate immune response by the host.

It has been shown that sTNF-Rs can compete with the cell surface receptors for TNF and thus interfere with the cytokine function (3, 5-7). Indeed, we have shown here that the sera of cancer patients effectively inhibit the *in vitro* cytotoxic effect of TNF, proportionally to the content of their sTNF-Rs. These data are consistent with prior evidence that serum infiltrates of cancer patients contain factor(s) that inhibit the cytolytic activity of TNF (21). We found that this inhibition can be abolished by addition of the sera of antibodies to the receptors (Fig. 2). It may therefore be concluded that the soluble receptors in the

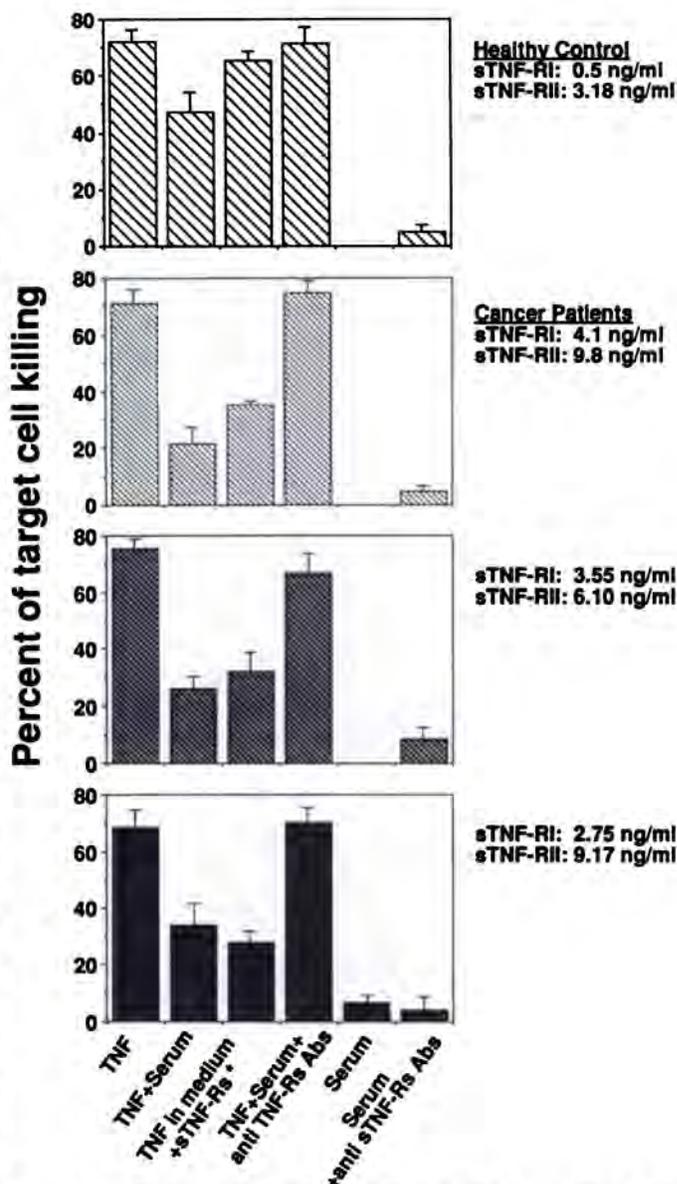


Fig. 2. Inhibition of TNF function by serum sTNF-Rs. The cytotoxic effect of TNF, applied to A9 cells at 5 units/ml, is compared to the extent of cytotoxicity observed upon application of TNF at the same concentration, to a sample of serum from a healthy control, or to samples of sera from 3 cancer patients (applied at concentrations of 90%, with 10% (v/v; Dulbecco's modified Eagle's minimal essential medium). The effects of the donors' sera on TNF activity are compared to the effect of purified urine-derived sTNF-Rs, applied at the same concentrations as in the corresponding sera. Also shown is the extent of cytotoxicity observed when in addition to the sera and TNF, monoclonal antibodies against sTNF-RI and sTNF-RII (8⁴) are applied. The last two columns illustrate the effect of the donors' sera and the monoclonal antibodies (Abs) against the sTNF-Rs when applied on A9 cells in the absence of TNF. *, sTNF-Rs at the concentrations found in the respective serum.

sera of cancer patients are functional and interfere significantly with TNF bioactivity. The implications of this potential interference may not however be fully understood without knowledge of the occurrence and impact of TNF itself in cancer patients. Evidence from *in vitro* studies suggests that TNF is released both by tumor cells (22–25) and by cells of the immune system in response to tumor antigens (26, 27). There is also some evidence, albeit limited and somewhat disputed, for the production of TNF and for its increased inducibility in cancer patients (28–32). In patients with colorectal carcinoma, TNF and its mRNA could be detected in relatively high amounts in macrophages at the site of the tumor (32). TNF has destructive effects on tumors in some experimental models of cancer (33). There is also some evidence that TNF, formed endogenously in certain experimental animal models of cancer, may play a role in maintenance of the tumor cells in a dormant state (34). It remains to be determined whether the elevated levels of sTNF-Rs in cancer patients represent a tumor “escape” mechanism from the suppressive effects of TNF. It also seems possible that the increased levels of sTNF-Rs, and presumably also of shed soluble forms of receptors to other immune cytokines in the sera of cancer patients, may contribute to the general suppression of certain immune functions observed in late stages of cancer (reviewed in Ref. 35). On the other hand, one should take note of the evidence suggesting that TNF also has facilitatory properties with regard to tumor growth and may, in fact, function as an autocrine growth factor both for certain tumor cells and for normal fibroblasts (23, 36). The latter property, in addition to the angiogenic activity of TNF (37, 38) and its ability to enhance the synthesis of collagenase (39) and other proteases, might support local tumor growth as well as spread of distant metastases. TNF might also affect cancer patients by mediating bone decalcification (22) or cachexia (2). Accordingly, it is possible that the increased formation of sTNF-Rs in these patients might have some beneficial consequences by neutralizing the TNF bioactivity.

An increase in the level of sTNF-Rs in the serum is certainly not distinctive of cancer, since it occurs also in a variety of inflammatory and autoimmune diseases.⁶ Nevertheless, sTNF-Rs might have a diagnostic value, since after exclusion of inflammatory and autoimmune diseases, elevated levels of the soluble TNF receptors can be suggestive of a malignant process.

The incidence of increase in the level of sTNF-Rs in the sera of cancer patients was highest at advanced stages of the disease. However, even at early stages, the quantitation of the receptors may be of diagnostic value. The incidence of their increase at an early stage seems higher than that reported for CEA, a commonly used marker for solid tumors. In colon carcinoma, for example, only about one-fourth of all patients at stages I–III were reported to exhibit an increase in this antigen (Ref. 40; see also Table 4) while in the present study an increase in serum sTNF-Rs was found to occur in more than one-half of the patients at those stages (Table 4).

We conclude that the serum concentrations of sTNF-Rs in cancer patients correlate with the staging of the disease. The soluble TNF receptors are functional and block effectively TNF bioactivity. Their formation may, thus, have bearings in cancer on effects of endogenously formed TNF on the development of the tumor. Furthermore, determination of serum levels of the sTNF-Rs may serve as a diagnostic aid in the early detection, follow-up, and prognosis of cancer.

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