Induction of soluble tumour necrosis factor receptors during treatment with interleukin-2

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Summary
Interleukin-2 (IL-2) treatment induces other cytokines such as tumour necrosis factor (TNF). TNF may mediate some of the anti-tumour activity of IL-2, but conversely, may contribute to its dose limiting toxicities. Cleaved extracellular domains of the p55 and the p75 TNF receptors (sTNFR-R1 and R2) bind to and inhibit the biological activity of TNF in vitro, but may also act as carrier molecules. We have assayed TNF and sTNFR-1 and 2 in the plasma of advanced cancer patients, before and during treatment with IL-2. Plasma levels of TNF in 22 patients were not significantly different from 25 normal controls, but levels of sTNFR-1 and sTNFR-2 were higher (P < 0.001). Levels of TNF and both its soluble receptors were significantly increased in 13 patients receiving IL-2 therapy. Maximum induced levels of sTNFR-1 and sTNFR-2 correlated closely with maximum induced levels of TNF (P < 0.001), but peak levels of sTNFR-1 and two were achieved 24–48 h after peak TNF. Levels of TNF and sTNF-Rs did not correlate with toxicity. Treatment with IL-2 leads not only to induction of TNF but also soluble binding proteins at levels which may modulate its biological activity.

Administration of interleukin-2 [IL-2] by bolus or continuous infusion leads to induction of other cytokines including tumour necrosis factor [TNF] (Lotze et al., 1985; Gemlo et al., 1988). TNF has been shown to be involved in the classical CTL response to antigen (Ranges et al., 1987); the generation of MHC-unrestricted LAK activity (Owen-Shaub et al., 1988; Chouaib et al., 1988); and necrosis of animal tumours via effects on the vascular endothelium (Gerlach et al., 1989). Induction of TNF during IL-2 therapy may be important therefore, in modulating its anti-tumour activity, and levels of TNF circulating during therapy with IL-2 have been reported as being predictive of response to treatment in one clinical study (Blay et al., 1990). Conversely, TNF plays a role in the pathogenesis of endotoxic shock (Tracey et al., 1987), and may mediate some of the toxic effects of IL-2 (Herberman, 1989).

Biological responsiveness to TNF requires interaction with specific cell membrane receptors. Activated lymphocytes have been shown to express receptors for TNF (Munker et al., 1987; Scheurich et al., 1987), and the observation that IL-2 activated lymphocytes respond to exogenous TNF with augmented function in vitro (Ostensen et al., 1987; Owen-Schaub et al., 1988) suggested that IL-2 may stimulate TNF receptor expression. IL-2 increased the percentage of TNF binding peripheral blood mononuclear cells and TNF receptor density (Owen-Schaub et al., 1989). Whether or not up-regulation of the TNF receptor is a direct effect of IL-2 or is mediated through other cytokines remains unclear. Interferon-gamma (IFN-γ), also induced during therapy with IL-2, has previously been shown to induce synthesis of the TNF receptor (Ruggiero et al., 1986).

Two immunologically distinct TNF binding proteins (Mwt = 30 kDa) have been isolated, which inhibit the biological activity of TNF by preventing the binding of TNF to its cellular receptor (Seckinger et al., 1988). Evidence suggests that these binding proteins (sTNFR-R1 and sTNF-2) are formed by proteolytic cleavage of the extracellular domain of the transmembrane portions of the p55 and p75 TNF receptors respectively (Porteu et al., 1991). In a phase I study of recombinant TNF and IFN-γ in patients with advanced cancer, infusion of recombinant TNF led to release into the circulation of a TNF binding protein (Lantz et al., 1990). Injection of IFN-γ alone did not result in an increase in TNF binding protein. In this study, we demonstrate that IL-2 therapy not only induces in vivo production of TNF but also induces both forms of its soluble receptor. Peak levels of both types of binding proteins correlate closely with maximum induced levels of TNF.

Methods

Patient samples
Plasma samples from 22 patients with metastatic carcinoma were taken prior to treatment with IL-2 (breast n = 12, melanoma n = 6 and renal cell carcinoma n = 4). Thirteen patients were studied during treatment with IL-2. The tumour types were: metastatic breast cancer (n = 8), renal cell carcinoma (n = 3) and metastatic melanoma (n = 2). The patients with breast cancer were treated on one of two schedules:
1. 9 x 10⁶ IU/m²/day for 4 days per week escalating by increments of 3 x 10⁶ IU/m² per infusion to reach a final dose of 18 x 10⁶ IU/m²/day at week four (n = 4, EuroCetus protocol ECL20410). In this group, patients had received at least one chemotherapy regimen for metastatic disease.
2. A 5 day infusion of IL-2 (18 x 10⁶ IU/m²/day) prior to combination chemotherapy for metastatic disease. Renal cell carcinoma patients were treated as part of the EuroCetus phase II study (EC MP003), with two 5 day infusions of IL-2 (18 x 10⁶ IU/m²/day) with an intervening 2 day rest period. Patients with metastatic melanoma were treated with DTIC 250 mg/m² on five consecutive days, followed 2 weeks later by two 5 day infusions of IL-2 as above (EuroCetus protocol EC MP001). Plasma samples were collected at frequent time points during the IL-2 treatment period. Samples were collected into EDTA, centrifuged at 4°C and stored at −20°C prior to assay.

TNF-α immunoradiometric assay
Plasma TNF levels were determined by IRMA (Medgenix Ltd.) according to the kit procedure. Briefly, standards or
samples were added to anti-TNF tubes in the presence of 
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-labeled antibody directed against a different TNF epi-
Tope. After 18 h incubation at room temperature, tubes
were washed with Tween 20 and the remaining radioactivity,
reflecting the TNF concentration, was measured on a gamma
counter. This assay measures both free TNF and TNF bound
to its receptor (Radoux & De Groote, 1992).

**ELISA for soluble TNF receptors**

ELISA plates (Maxisorp Nunc, Denmark) were coated with
monoclonal antibodies to the soluble forms of either sTNF-R1
or sTNF-2 and the assay carried out as described previously
(Engelmann et al., 1990; Aderka et al., 1991). Purified urine
derived soluble forms of the two receptors served as stan-
dards. The detection limit of the assay was 30 pg ml\(^{-1}\)
and no cross-reactivity was found for the two species of receptors
in the two assays. Addition of 25 ng ml\(^{-1}\) recombinant
TNF to tested samples of sTNF-Rs did not affect the estimates
of binding protein.

**Results**

Plasma TNF and TNF binding proteins (sTNF-R1 and R2)
were measured in 22 patients with advanced cancer prior to
administration of IL-2, and during IL-2 treatment in 13 of
these. IL-2 was given using three different treatment
regimens. The mean pre-treatment levels of TNF in this
group of patients with advanced cancer prior to therapy with
IL-2 was not significantly different from levels found in 25
normal controls (8.3 ± 1.5 pg ml\(^{-1}\) vs 7.4 ± 0.6 pg ml\(^{-1}\)).
The mean pretreatment sTNF-R1 level in patients was however
significantly higher compared with 53 normal controls
(1.6 ± 0.1 ng ml\(^{-1}\) vs 0.7 ± 0.2 ng ml\(^{-1}\) P < 0.001, Table I). Simi-
larly, the mean pretreatment sTNF-R2 level in patients was
3.1 ± 1.4 ng ml\(^{-1}\) compared with 2.1 ± 0.6 ng ml\(^{-1}\) in normal
controls (P < 0.001).

In nine patients, TNF and sTNF-R were measured 6 h
after the start of the IL-2 infusion. In all nine patients, TNF
levels were raised at 6 h and the mean level was significantly
higher compared with pretreatment levels (25.1 pg ml\(^{-1}\)
vs 6.8 pg ml\(^{-1}\), P = 0.005) as shown in Table II. In five of
the nine patients, levels of sTNF-R1 and R2 were raised at 6 h
compared with pretreatment levels. However, the mean levels
for the group as a whole were not significantly higher at this
time point.

Figure 1 demonstrates the induction of TNF, sTNF-R1
and sTNF-R2 in three patients receiving a 5 day course of
IL-2 at a dose of 18 × 10\(^6\) IU IL-2 m\(^{-2}\) day for 5 days and one
patient in whom treatment was terminated after four days
because of toxicity. In all cases, treatment with IL-2 led
to induction of TNF as well as induction of both types of TNF
soluble receptor. Induced levels of sTNF-R2 were higher than
sTNF-R1. Peak levels of sTNF-R were noted 24–48 h after
the peak of induced TNF and although levels of sTNF-R
decreased after the end of treatment with IL-2, they were still
elevated compared with pre-treatment values. Figure 2 shows
the effects of IL-2 administration for two 5 day infusions
separated by a 2 day rest period. Once again the rise in TNF
generally appears simultaneously with a rise in sTNF-R1 and
R2. Peak sTNF-R1 and R2 sometimes coincide and some-
times follow, peak TNF levels. During the 2 day rest period
between courses of treatment, levels of sTNF-R1 and R2 did
not fall to baseline, although levels of TNF generally did. In
this figure, the data are plotted on a logarithmic scale. This
highlights the relative concentrations of the binding proteins
and the cytokine and the fact that levels of sTNF-R1 and
R2 rise concordantly. We also studied the induction of TNF
and sTNF-R in an escalating dose schedule of IL-2, com-
mencing at an apparent dose of 9 × 10\(^6\) IU IL-2 m\(^{-2}\) day
increasing by 3 × 10\(^6\) IU m\(^{-2}\) per treatment period to a final
dose of 18 × 10\(^6\) IU IL-2 m\(^{-2}\) day (data not shown). Levels of
TNF and sTNF-R1 and R2 rose at the start of each infusion,
but levels of induced TNF and sTNF-R were much lower in
this group of patients than those observed in the other
treatment regimens. Although this effect may have been due
to the treatment schedule, this, the first of our IL-2 studies,
was performed at a time when the requirement for the addi-
tion of albumin during reconstitution of rIL-2 intended for
infusion was not made clear. We have previously demon-
strated that failure to reconstitute IL-2 intended for infusion
with a small amount of albumin may lead to a significant
decrease in bioavailability in vitro (Miles et al., 1990). It is
interesting to note, however, that in this series of samples
peak levels of TNF and sTNF-R were not necessarily seen at
the higher doses of IL-2, indeed in two of the patients, peak
levels of TNF and sTNF-R fell with increasing doses of IL-2.

Considering the group as a whole, there was a strong
correlation between the maximum levels of induced TNF
and peak levels of induced sTNF-R1 and sTNF-R2 (R = 0.835,
P < 0.001 and R = 0.785, P < 0.001 respectively, see Figure 3).

There was no obvious correlation between peak levels of
TNF or sTNF-R1 or 2 and clinical manifestations of IL-2
toxicity in terms of blood pressure, temperature and weight
gain. No responses to treatment were recorded in this group
of patients.

**Discussion**

In this study we have demonstrated that although pre-treat-
ment levels of TNF in patients with advanced cancer are
similar to those found in normal controls, levels of sTNF-R1
and R2 are significantly higher. Aderka et al. (1991) have
previously noted elevated levels of sTNF-R in patients with
advanced cancer. We have also demonstrated that admini-
stration of rIL-2 leads to induction of both forms of the
soluble TNF receptor as well as induction of TNF itself with
induced levels of sTNF-R2 being significantly higher than
peak levels of sTNF-R1. In patients from whom blood was
taken at early time points, levels of TNF were raised in all
patients after 6 h of IL-2, though levels of sTNF-R were
raised in only five of the nine patients at this time point.
After 24 h of IL-2, levels of sTNF-R were raised in all
patients treated. Early induction of sTNF-R may be due to
a combination of upregulation of TNF receptors by induction
of mRNA and protein and shedding of the extracellular
domain of both TNF receptors. Activation of T-cells is
associated with a rapid induction of sTNF-R2 mRNA and
protein (Ware et al., 1991). Similarly, rapid induction of
sTNF-R2 and subsequently sTNF-R1 has been documented in
activated B-lymphocytes (Heilig et al., 1991). Ware et al.,
also demonstrated that further stimulation of activated

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**Table I** Circulating TNF and TNF binding proteins in normals and patients with advanced cancer prior to IL-2

| Group       | TNF (pg ml\(^{-1}\) ± s.e.) | sTNF-R1 (ng ml\(^{-1}\) ± s.e.) | sTNF-R2 (ng ml\(^{-1}\) ± s.e.) |
|-------------|-----------------------------|-------------------------------|-------------------------------|
| Normals     | 7.4 ± 0.6                   | 0.7 ± 0.1                     | 2.1 ± 0.1                     |
|             | (n = 25)                    | (n = 53)                      | (n = 53)                      |
| Pre-treatment | 8.3 ± 1.5                   | 1.6 ± 0.2*                    | 3.1 ± 0.3*                    |

*Statistically significantly different from normal samples (P < 0.0001).

**Table II** Circulating TNF and TNF binding proteins pretreatment and 6 h post start of IL-2 infusion (n = 9)

| rIL-2 | TNF sTNF-R1 | sTNF-R2 |
|-------|-------------|---------|
| [T = 0] | 6.8 ± 1.1   | 1.7 ± 0.5 | 3.1 ± 0.8 |
| [T = 6] | 25.1 ± 5.6* | 1.9 ± 0.4 | 3.7 ± 0.6 |

*Statistically significant difference from pretreatment values (P = 0.005).
Figure 1  Levels of TNF and soluble TNF receptors in patients receiving a 5 day courses of IL-2. --- ▲ ---, TNF in plasma (pg ml⁻¹). --- ● ● ●, sTNF-R1 in plasma (ng ml⁻¹). --- ○ ○ ○, sTNF-R2 in plasma (ng ml⁻¹). Patients received 18 x 10⁶ IU IL-2 m⁻² day as an intravenous infusion for 5 days.

Figure 2  --- ▲ ---, TNF in plasma (pg ml⁻¹). --- ● ● ●, sTNF-R1 in plasma (pg ml⁻¹). --- ○ ○ ○, sTNF-R2 in plasma (pg ml⁻¹). Patients received 18 x 10⁶ IU IL-2 m⁻² day as two 5 days infusion separated by a 2 day rest period.
T-cells however results in receptor down regulation possibly due to shedding of receptor. Preferential shedding of sTNF-R2 from neutrophils may occur within minutes of exposure to chemotactic factors by the action of neutrophil elastase (Porte et al., 1991).

Levels of sTNF-R peaked 24-48 h after the maximum level of induced TNF in nine of 13 patients studied. The peak levels of induced sTNF-R correlated strongly with induced levels of TNF. Four patients were treated on an escalating dose schedule in the context of a phase I/II trial of rIL-2 in the treatment of advanced breast cancer. Levels of induced TNF and sTNF-R were much lower than in the other groups, possibly due to decreased bioavailability of the drug. Nevertheless, in the patients treated on this regimen there was not a direct correlation between the dose of IL-2 administered and the levels of sTNF-R induced, indeed the highest levels of sTNF-R2 were seen at the intermediate doses.

Stoichiometric studies of the binding of a recombinant sTNF-R1 have suggested that three molecules of sTNF-R1 bind to one TNFα trimer (Loetscher et al., 1991). The same group also determined that a 10 to 100-fold excess of recombinant sTNF-R1 was required to neutralise TNFα activity. Similarly, Olsson et al. have previously shown that a 10-fold molar excess of TNF binding protein was required to reduce the cytotoxic effects of TNF in a WEHI assay by 50%. In our study the maximum mean induced level of sTNF-R1 for the patient group was 5.93 ng ml\(^{-1}\) compared with a maximum induced TNF of 110 pg ml\(^{-1}\). This represents a 30-fold molar excess of sTNF-R1. Similarly the maximum mean induced level of sTNF-R2 of 17.57 ng ml\(^{-1}\) represents a 90-fold molar excess of this binding protein. Thus, although immunoreactive TNF is induced during treatment with IL-2, binding proteins are also induced at levels which could theoretically neutralise its bioactivity, in the peripheral circulation at least. At the concentrations observed in this study, such binding proteins may also act as carriers for TNF and prolong its half life in the circulation (Aderka et al., 1992). Thus although the bioactivity of TNF in the peripheral circulation may be reduced as a consequence of the presence of soluble receptors, end organ toxicity may be increased as a result of the prolongation of the half-life. The immunoassay used in this study measured both free and bound TNF (Radoux & DeGroote, 1992). Previous studies in vitro have suggested that 75 kDa TNF-R2 receptor and its soluble form is induced directly by IL-2 in T cells (Ware et al., 1991). As TNF-R2 is the major TNF-R expressed on T cells, it is likely that this is a major cellular source of the soluble sTNF-R2 found in this group of patients. The in vitro study of Ware et al. also suggest an explanation for the higher levels of sTNF-R2 compared with sTNF-R1, and for the correspondence between TNF levels and those of its soluble receptors.

A clinical study of rTNF in patients with advanced cancer did however demonstrate that TNF itself could induce TNF binding proteins (Lantz et al., 1990). Our data demonstrate that the induction of sTNF-R follows induction of TNF closely, and that the levels of soluble receptor induced, correlate closely with levels of induced TNF. In this clinical study we are unable to further investigate the mechanisms by which induction of TNF is followed closely by elevation of binding protein levels, but we are able to demonstrate the remarkable concordance between these two parameters in IL-2 treated patients. The relevance of our findings to control of the cytokine network remains to be determined.

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