The EMBO Journal vol.13 no.4 pp.843-850, 1994

Dissociation of TNF-α cytotoxic and proinflammatory activities by p55 receptor- and p75 receptor-selective TNF-α mutants

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Communicated by W.Fiers

Human tumour necrosis factor alpha (TNF-α) is a pleiotropic cytokine capable of killing mammalian tumour cells *in vitro* and *in vivo*, and of enhancing the proinflammatory activity of leucocytes and endothelium, the latter effects limiting its usage as an antitumour agent in humans. Using TNF-α mutants with a selective capacity to bind to the TNF p55 receptor (TNFR55) or to the p75 receptor (TNFR75) we show here that these two major activities of TNF-α can be dissociated. The TNFR55-selective mutants (R32W, E146K and R32W-S86T) which bind poorly to TNFR75 displayed similar potency to wild-type TNF in causing cytotoxicity of a human laryngeal carcinoma-derived cell line (HEp-2) and cytostasis in a human leukaemic cell line (U937). However, these TNFR55-selective mutants exhibited lower proinflammatory activity than wild-type TNF. Specifically, TNF-α's priming of human neutrophils for superoxide production and antibody-dependent cell-mediated cytotoxicity, platelet-activating factor synthesis and adhesion to endothelium were reduced by up to 170-fold. Activation of human endothelial cell functions represented by human umbilical venular endothelial cell (HUVEC) adhesiveness for neutrophils, E-selectin expression, neutrophil transmigration and IL-8 secretion were also reduced by up to 280-fold. On the other hand, D143F, a TNFR75-selective mutant tested either alone or in combination with TNFR55-selective mutants, did not stimulate these activities despite being able to cause cytokine production in TNFR75-transfected PC60 cells. These results demonstrate that (i) TNFR55, in addition to mediating antitumour activity, also mediates neutrophil and endothelial cell activation, (ii) TNFR75 potentiates the role of TNFR55 in neutrophil and endothelial cell activation, and (iii) novel TNF molecules may be constructed which retain full antitumour activity whilst exhibiting reduced proinflammatory activities.

Key words: cytokines/endothelium/inflammation/neutrophils/tumour

Introduction

Tumour necrosis factor-alpha (TNF-α), a 17 kDa protein which exists in the biologically active, physiological form as a homotrimer, is produced primarily by activated macrophages and was originally characterized as a protein that induced necrosis of Meth A sarcomas *in vivo* (Carswell *et al.*, 1975). It exerts direct cytolytic and cytostatic effects on a wide range of human and murine tumour cell lines and displays synergy with other cytotoxic agents and especially the interferons (Sugarman *et al.*, 1985; Fransen *et al.*, 1986). In addition, TNF-α inhibits the growth of normal and leukaemic haematopoietic progenitor cells (Murase *et al.*, 1987). The property of TNF-α in combination with interferon to kill selectively or inhibit many tumour cell lines remains unmatched by any other biological agent. The antitumour action of TNF-α is complex and may be direct or mediated through its effects on endothelium and immune effector cells, depending on the system (Fiers, 1993). The haemorrhagic necrosis of tumours by TNF-α results in part from the activation of endothelium and consequent procoagulation effects (Bevilacqua *et al.*, 1986). The activation of neutrophils, monocytes and lymphocytes (NK cells) can also contribute to the antitumour effect of TNF-α. However, stimulation of these immunomodulatory cells and endothelium also leads to marked proinflammatory effects which have been manifested in human clinical trials by fever, dose-limiting hypotension, hepatotoxicity, pulmonary oedema, thrombocytopenia, intravascular thrombosis and haemorrhage (Hauser *et al.*, 1990; Kilbourn *et al.*, 1990; Van Der Poll *et al.*, 1992; Van Ostade *et al.*, 1993). It is these proinflammatory side-effects which have severely limited the application of systemically administered TNF-α in patients with malignancy (Jones and Selby, 1989; Taguchi and Sohmura, 1991).

The multiple activities of TNF-α are mediated through two high affinity receptors which have recently been cloned (Dembic *et al.*, 1990; Loetscher *et al.*, 1990; Schall *et al.*, 1990; Smith *et al.*, 1990): the TNFR55 (type B, R1) which is ubiquitous and the TNFR75 (type A, R2) which is often more abundant on cells of haematopoietic lineage (Hohmann *et al.*, 1989; Brockhaus *et al.*, 1990; Porteu *et al.*, 1991) and is also expressed on endothelium (Hohmann *et al.*, 1990; Shalaby *et al.*, 1990; Mackay *et al.*, 1993). The expression of two different TNF receptors with unrelated intracellular domains raises the question as to whether they function independently to mediate the different biological activities of TNF-α. To answer this, several groups have used specific receptor antibodies and transfected cell lines expressing the cloned receptors (Engelmann *et al.*, 1990; Espevik *et al.*, 1990; Thoma *et al.*, 1990; Tartaglia *et al.*, 1991; Tartaglia and Goeddel, 1992a; Vandenabeele *et al.*, 1992). However, far from providing definite answers, the results—for example regarding cytotoxicity—have been highly controversial (Heller *et al.*, 1992, 1993; Tartaglia *et al.*, 1993). In

addition, the role of these two receptors in neutrophil priming, mediator release and adhesion to endothelium remains unknown. Considering that in normal mice hTNF is far less lethal than mTNF (Brouckaert *et al.*, 1992) and knowing that in mice hTNF does not interact with TNFR75, it was concluded that TNFR75 contributes specifically to the severe toxicity (Brouckaert *et al.*, 1993; Fiers, 1993). In order to evaluate this different functioning of the two receptors in the human system, hTNF mutants were constructed which interact specifically with one type of receptor (Van Ostade *et al.*, 1993). Such mutants have now been used to characterize responses of human cells important in 'inflammatory responses'.

R32W (arginine at position 32 replaced by tryptophan) (Van Ostade *et al.*, 1993), E146K (glutamate at position 146 replaced by lysine) (Van Ostade *et al.*, 1994) and R32W-S86T (R32W and serine at position 86 replaced by threonine) (Loetscher *et al.*, 1993) are TNFR55-selective mutants which have significantly decreased affinity for TNFR75, with 500-, 3300- and 5000-fold decrease respectively compared with wild-type TNF-α. These mutants have little, if any, reduction in binding to TNFR55, with 1.4-, 2- and 2.2-fold decrease for R32W, E146K and R32W-S86T respectively. It has been shown that R32W maintains full cytotoxic potential *in vitro* and *in vivo* (Van Ostade *et al.*, 1993). D143F (aspartic acid at position 143 replaced by phenylalanine) (Van Ostade *et al.*, 1994) is a TNFR75-selective mutant which has no demonstrable binding to TNFR55 but also exhibits a 30-fold decrease in binding to TNFR75. These mutants represent unique tools which we have used to define the role of the two TNF receptors in the mediation of TNF-α cytotoxicity and proinflammation.

We found that the TNFR55-selective mutants (E146K, R32W-S86T and R32W) have markedly impaired ability to activate neutrophils and HUVEC *in vitro*. Importantly, these TNFR55-selective mutants retain their tumoricidal and tumoristatic potency. However, the TNFR75-selective mutant D143F was unable to stimulate neutrophils or endothelium. These results suggest that TNFR55 alone is sufficient to mediate full TNF cytostasis and cytotoxicity while TNFR75 is necessary for the optimal stimulation of neutrophil and endothelial cell function by TNFR55. These findings have important implications for the clinical use of TNF as they suggest that novel TNF molecules can be constructed which retain full antitumour activity but have reduced ability to produce proinflammatory side-effects.

Results

TNF receptor expression on HEp-2 cells, U937 cells, neutrophils and HUVEC

The expression of TNFR55 and TNFR75 on these cells was examined by flow cytometry using the monoclonal antibodies htr-9 (TNFR55) and utr-1 (TNFR75) (Figure 1). HEp-2 cells expressed only TNFR55 providing a unique setting in which to examine the role of TNFR55 in cytotoxicity. In contrast, neutrophils, HUVEC and U937 cells expressed approximately equal numbers of both receptors consistent with previous reports (Hohmann *et al.*, 1989, 1990; Brockhaus *et al.*, 1990; Shalaby *et al.*, 1990; Porteu *et al.*, 1991; Mackay *et al.*, 1993).

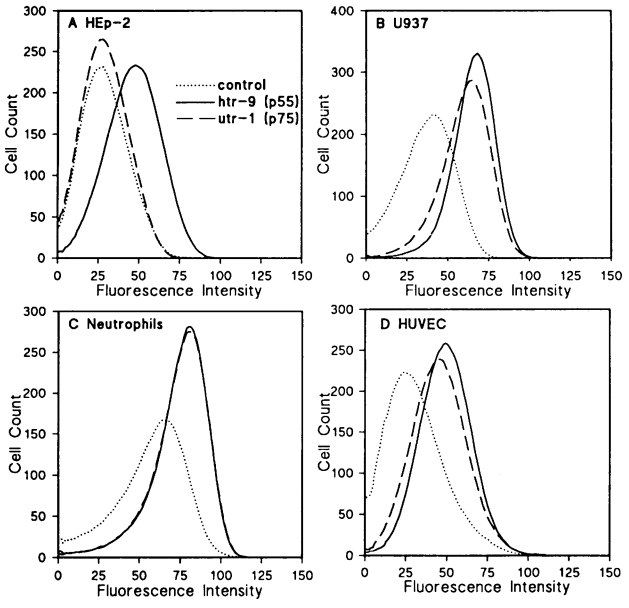

Fig. 1. Flow cytometry demonstrating the presence of TNF receptors on HEp-2 cells (**A**), U937 cells (**B**), neutrophils (**C**) and HUVEC (**D**). Negative control antibody (dotted line), htr-9 monoclonal antibody against TNFR55 (solid line) and utr-1 monoclonal antibody against TNFR75 (dashed line).

The relative binding affinities of the TNF mutants

Microtitre plates coated with recombinant human TNFR55 and TNFR75 were incubated with radiolabelled wild-type TNF (wtTNF) in the presence of different concentrations of competing wtTNF or mutant TNF. R32W (Van Ostade *et al.*, 1993), E146K (Van Ostade *et al.*, 1994) and R32W-S86T (Loetscher *et al.*, 1993) are TNFR55-selective mutants which have significantly decreased affinity for TNFR75, with 500-, 3300- and 5000-fold decrease respectively compared with wtTNF-α. These mutants have little, if any, reduction in binding to TNFR55, with 1.4-, 2- and 2.2-fold decrease for R32W, E146K and R32W-S86T respectively. D143F (Van Ostade *et al.*, 1994) is a TNFR75-selective mutant which has no demonstrable binding to TNFR55 but also exhibits a 30-fold decrease in binding to TNFR75.

Cytotoxicity and cytostasis of tumour cells

TNF-α, in the presence of cycloheximide (CHX), was directly cytotoxic, in a dose-dependent manner, to the human laryngeal carcinoma cell line, HEp-2. In experiments comparing the relative potencies of TNFR55-selective mutants R32W and E146K to wtTNF, we found that R32W maintained full cytotoxic potential while E146K displayed a 4-fold reduction in activity in keeping with the 2-fold decrease in binding affinity to TNFR55 seen with this mutant (Figure 2A). R32W-S86T demonstrated a $<$2-fold reduction in cytotoxicity. In addition, TNF-α inhibited proliferation of the monoblastoid leukaemic cell line U937 in a dose-dependent manner. We found that these mutants were also capable of inhibiting U937 cell growth with R32W being as potent as wtTNF and E146K exhibiting a 4-fold reduction in potency (Figure 2B). A summary of the cytotoxic and cytostatic activities and relative potencies of the TNFR55-selective mutants is shown in Table I. The same

Table I. Summary of the relative cytotoxic and cytostatic activities of the TNF mutants R32W, E146K and R32W-S86T

	Cytotoxicity (HEp-2)			Cytostasis (U937)	
	n[a]	Biological activity (U/mg)	EC_{50}[b] (ng/ml)	n	EC_{50} (ng/ml)
wt-TNF		$9.95 \times 10^6 \pm 1.74$[c] (100%)[d]	0.65 ± 0.09		1.24 ± 0.15
R32W	8	$1.72 \times 10^7 \pm 0.45$ (166.8%)	0.43 ± 0.09	3	0.65 ± 0.02 (236.9%)
E146K	3	$3.03 \times 10^6 \pm 0.66$ (24.6%)	1.82 ± 0.40	3	3.97 ± 0.05 (23.7%)
R32W-S86T	2	$3.35 \times 10^6 \pm 1.35$ (66.3%)	1.77 ± 0.70		

[a]n = number of experiments performed.
[b]Effective concentration producing 50% activity.
[c]Standard error of the mean.
[d]Potency % = (wtTNF EC_{50}/mutant TNF EC_{50}) × 100.

relative potencies were obtained with the TNFR55-selective mutants in HEp2 cytotoxicity when a shorter treatment time of 90 min with TNF and CHX, followed by an 18 h incubation in the presence of CHX alone, was examined (wtTNF 100%, R32W 177.8%, E146K 22% and R32W-S86T 56.9%).

Stimulation of function and inflammatory mediator production by human neutrophils

In order to compare the direct antitumour cell activities of the TNFR55-selective mutants with those that may be responsible for at least some of the TNF side-effects, we examined those activities most likely to be important in exacerbating systemic inflammation. Since TNF-α is known to stimulate the function and production of inflammatory mediators from neutrophils (Klebanoff *et al.*, 1986) we next examined the TNFR55-selective mutants for their ability to activate human neutrophils and stimulate mediator synthesis. TNF-α primed human neutrophils for superoxide anion generation and antibody-dependent cell-mediated cytotoxicity in a dose-dependent manner. In experiments comparing the relative potencies of the TNFR55-selective mutants we found that stimulation with E146K, R32W-S86T and R32W resulted in superoxide anion generation in response to FMLP which was 30-fold, 35-fold and 3-fold less than wtTNF respectively. Similarly, when the TNFR55-selective mutants were examined in antibody-dependent cell-mediated cytotoxicity of P815 cells, the stimulation by E146K and R32W was 50-fold and 5-fold less than wtTNF respectively. TNF-α induced platelet-activating factor (PAF) production in human neutrophils in a dose-dependent manner (Camussi *et al.*, 1987). In experiments comparing the TNF mutants with the wild-type molecule, E146K and R32W stimulated 40-fold and 2-fold less PAF synthesis respectively. TNF-α enhanced, in a dose-dependent manner, the adherence of neutrophils to unstimulated HUVEC (Gamble *et al.*, 1985). The TNF mutants E146K and R32W exhibited 170-fold and 4-fold less activity than wild-type respectively. The comparative activity of E146K to wild-type is shown in Figure 3 and a summary of the relative potencies of the TNFR55-selective mutants is listed in Table II. The relative potency of R32W-S86T was very similar to that of the more widely examined E146K (Table II).

Regulation of endothelial function

We examined the TNFR55-selective mutants for their ability to upregulate E-selectin expression, enhance the adhesiveness

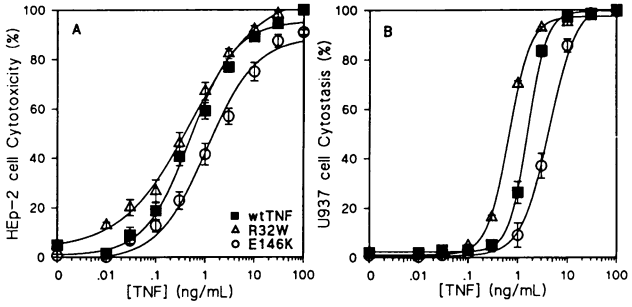

Fig. 2. The antitumour activities of the TNFR55-selective mutants. (**A**) The cytotoxic activity of R32W (eight pooled experiments) and E146K (three pooled experiments) compared with wtTNF on HEp-2 cells in the presence of 50 μg/ml cycloheximide. (**B**) The cytostatic activity of R32W (three pooled experiments) and E146K (three pooled experiments) relative to wtTNF on U937 cells. Data points represent means ± SEM. Wild-type TNF, closed square; R32W, open triangle; E146K, open circle.

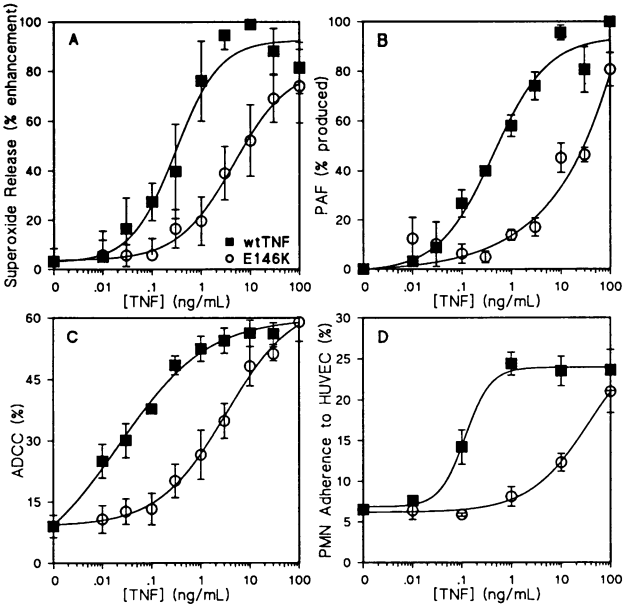

Fig. 3. The activation of neutrophils by the TNFR55-selective mutant E146K. (**A**) Superoxide production (O_2^-) showing six pooled experiments, $P < 0.05$. (**B**) Platelet-activating factor synthesis with three pooled experiments, $P < 0.05$. (**C**) Antibody-dependent cell-mediated cytotoxicity with four pooled experiments, $P < 0.05$. (**D**) Adhesion of neutrophils to unstimulated HUVEC; representative experiment of three performed with triplicates. Data points represent means ± SEM. Wild-type TNF, closed square; E146K, open circle.

Table II. Summary of the relative activities of the TNF mutants R32W, E146K and R32W-S86T on neutrophil functions

	Superoxide		ADCC		PAF		Adherence	
	n[a]	EC_{50}[b] (ng/ml)	n	EC_{50} (ng/ml)	n	EC_{50} (ng/ml)	n	EC_{50} (ng/ml)
wt-TNF		0.25 ± 0.04[c] (100%)[d]		0.07 ± 0.03		1.13 ± 0.29		0.10 ± 0.03
R32W	10	0.68 ± 0.15 (32.4%)	8	0.29 ± 0.08 (20.7%)	9	2.86 ± 0.88 (46.5%)	5	0.46 ± 0.14 (21.7%)
E146K	6	9.27 ± 3.15 (3.6%)	4	3.70 ± 1.60 (1.9%)	3	23.20 ± 5.03 (2.4%)	3	10.34 ± 6.70 (0.6%)
R32W-S86T	6	9.24 ± 5.34 (2.9%)						

[a]n = number of experiments performed.
[b]Effective concentration producing 50% activity.
[c]Standard error of the mean.
[d]% Potency = (wtTNF EC_{50}/mutant TNF EC_{50}) × 100.

of endothelium for neutrophils, stimulate the transmigration of neutrophils across endothelium and production of IL-8. TNF-α upregulated E-selectin expression in endothelial cells in a dose-dependent manner (Bevilacqua *et al.*, 1989). E-selectin (endothelial leucocyte adhesion molecule) enables neutrophils to adhere to endothelium. E146K, R32W-S86T and R32W exhibited 125-fold, 170-fold and 3-fold less stimulation of expression of E-selectin respectively when compared with wild-type. In HUVEC adhesiveness for neutrophils (Gamble *et al.*, 1985) we found that E146K and R32W could both stimulate this activity but their potencies were 280-fold and 7-fold less than wtTNF respectively. TNF-α also increased neutrophil transendothelial migration in a dose-dependent fashion (Smith *et al.*, 1991). E146K and R32W stimulated 15-fold and 2-fold less transmigration of neutrophils than wtTNF respectively. IL-8 is a recently characterized cytokine which activates neutrophils and is a powerful chemotactic agent (Bagglioni *et al.*, 1989). E146K and R32W stimulated 50-fold and 4-fold less secretion of IL-8 by HUVEC than wtTNF respectively. The relative activity of E146K compared with wild-type is displayed in Figure 4 and a summary of the relative potencies of the TNFR55-selective mutants in regulating endothelial cell interactions with neutrophils is shown in Table III.

Lack of proinflammatory activity of the TNFR75-selective mutant

D143F, a TNFR75-selective mutant, did not produce any proinflammatory activity when tested for its ability to stimulate neutrophil superoxide production, adherence of neutrophils to stimulated HUVEC, E-selectin expression and IL-8 production by HUVEC (Figure 5). In addition, when selected concentrations of D143F were combined with E146K and examined for superoxide production and E-selectin expression, no increase in activity was observed when compared with E146K alone (Figure 5A and B).

Induction of GM-CSF in PC60-hTNFR75⁺ cells

PC60-hTNFR75⁺ cells which bear the human TNFR75 but not human TNFR55 are a suitable system for testing hTNFR75 mediated activity. Although D143F showed no activity in selected neutrophil and endothelial activities this TNFR75-specific mutant did stimulate rat granulocyte-macrophage colony stimulating factor (GM-CSF) production by PC60 cells transfected with the human TNFR75 (Table IV). The amount of GM-CSF produced was approximately one-third that of wild-type in keeping with the reduced binding of D143F to TNFR75. The TNFR55-selective mutant E146K did not result in any significant increase in GM-CSF production over baseline.

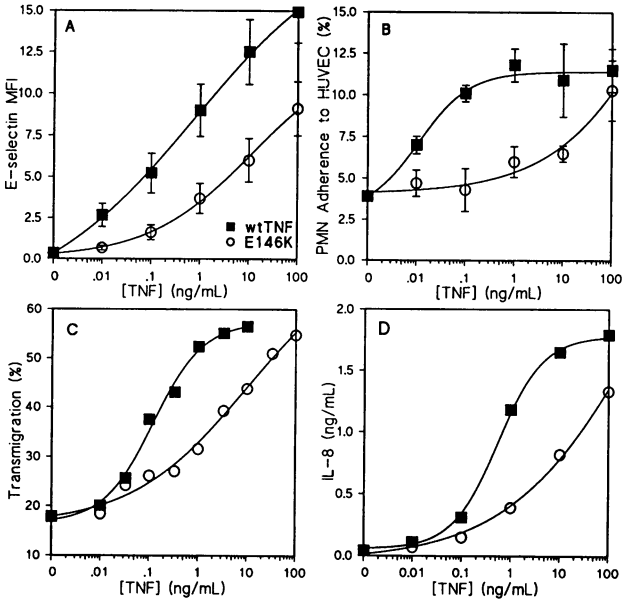

Fig. 4. The activation of HUVEC by the TNFR55-selective mutant E146K. (**A**) E-selectin expression showing seven pooled experiments, $P < 0.05$. (**B**) Adhesion of neutrophils to stimulated HUVEC showing a representative of three experiments performed with triplicates, data points are means ± SEM. (**C**) Transendothelial migration with a representative of three experiments performed. (**D**) IL-8 secretion with a representative experiment of four performed with duplicates. Wild-type TNF, closed square; E146K, open circle.

Discussion

TNF-α has diverse biological activities, of which the anti-tumour effect has clinical desirability and great therapeutic potential. However, the efficacy of TNF-α as an antitumour agent has been restricted by dose-limiting side-effects which are believed to be the result of the proinflammatory activities of TNF-α (Hauser *et al.*, 1990; Kilbourn *et al.*, 1990; Brouckaert *et al.*, 1992; Van Der Poll *et al.*, 1992). Therefore, ameliorating these proinflammatory side-effects whilst maintaining full antitumour activity may pave the way for the successful clinical application of TNF-α. Our results with the TNFR55-selective mutants (E146K, R32W-S86T and R32W) indicate that this dissociation of TNF activities can be achieved *in vitro* and this argues well for the clinical application of TNFR55-selective mutants. Our results suggest

Table III. Summary of the relative activities of the TNF mutants R32W, E146K and R32W-S86T on human endothelial cell function

	E-selectin		Adherence		Transmigration		IL-8	
	n[a]	EC_{50}[b] (ng/ml)	n	EC_{50} (ng/ml)	n	EC_{50} (ng/ml)	n	EC_{50} (ng/ml)
wt-TNF		0.58 ± 0.16[c] (100%)[d]		0.05 ± 0.02		0.19 ± 0.07		0.18 ± 0.13
R32W	4	1.25 ± 0.48 (38.4%)	4	0.33 ± 0.10 (15.2%)	3	0.16 ± 0.06 (62.5%)	3	0.73 ± 0.46 (27.4%)
E146K	7	87.18 ± 42.86 (0.8%)	3	8.52 ± 7.30 (0.4%)	3	4.28 ± 1.99 (6.5%)	4	9.07 ± 4.14 (2.0%)
R32W-S86T	3	207.0 ± 165.3 (0.6%)						

[a]n = number of experiments performed.
[b]Effective concentration producing 50% activity.
[c]Standard error of the mean.
[d]% Potency = (wtTNF EC_{50}/mutant TNF EC_{50}) × 100.

that TNFR55 is the sole mediator of TNF cytotoxicity and cytostasis and a major mediator of neutrophil and endothelial activation, while TNFR75 potentiates neutrophil and endothelial cell activation but by itself does not seem to be sufficient to stimulate these functions.

The antitumour effect of the TNFR55-selective mutants was examined by their ability to cause direct cytotoxicity in HEp-2 cells (human laryngeal carcinoma-derived cell line) and direct cytostasis in U937 cells (human monoblastoid leukaemic cell line). R32W maintained full activity in these systems, E146K displayed a 4-fold decrease in activity in keeping with the 2-fold decrease in binding affinity to TNFR55 and R32W-S86T demonstrated a <2-fold reduction in cytotoxicity. The HEp-2 cells express only TNFR55 (Figure 1; Brockhaus *et al.*, 1990; Hohmann *et al.*, 1990) and this, combined with the established poor binding of these TNF mutants to TNFR75 (Van Ostade *et al.*, 1993; Van Ostade *et al.*, 1994; Loetscher *et al.*, 1993) implies that, in this system, cytotoxicity is mediated through TNFR55. U937 cells have approximately equal numbers of both TNF receptors present on the cell surface (Figure 1; Brockhaus *et al.*, 1990) and in these cytostatic or growth inhibition assays R32W and E146K produced similar results to those seen in HEp-2 cytotoxicity, further implicating TNFR55 as the mediator of cytotoxicity and cytostasis. These results support the notion that TNFR55 is the receptor involved in cytotoxicity as suggested by previous studies using TNF mutants (Van Ostade *et al.*, 1993), agonistic and antagonistic antibodies to TNFR55 (Engelmann *et al.*, 1990; Espevik *et al.*, 1990; Thoma *et al.*, 1990; Tartaglia *et al.*, 1991) and mutagenesis studies of the TNFR55 intracellular domain (Tartaglia and Goeddel, 1992a). In contrast, Heller *et al.* (1992) concluded that TNFR75 mediated the cytotoxicity; however, both TNF receptors were present whenever cell death was demonstrated raising doubts as to the significance of these findings (Tartaglia *et al.*, 1993).

The priming and activation of neutrophils is likely to be important in host defence but is also likely to play a major role in the inflammatory effects of TNF-α. To date there have been no reports of the relative roles of each receptor in human neutrophil functioning. Our data with the TNFR55-selective mutants revealed a decrease of up to 170-fold in activity when compared with wtTNF, implying a major role for TNFR75 in these proinflammatory activities. When the priming of superoxide release by neutrophils was examined by the TNFR75-selective mutant D143F no activity resulted, suggesting that although TNFR75 is involved in neutrophil activation it does not directly signal this activity by itself. This mutant is not without activity as evidenced by the secretion of GM-CSF from PC60-hTNFR75$^+$ cells (Table IV). A second important component of the inflammatory activities of TNF-α is at the

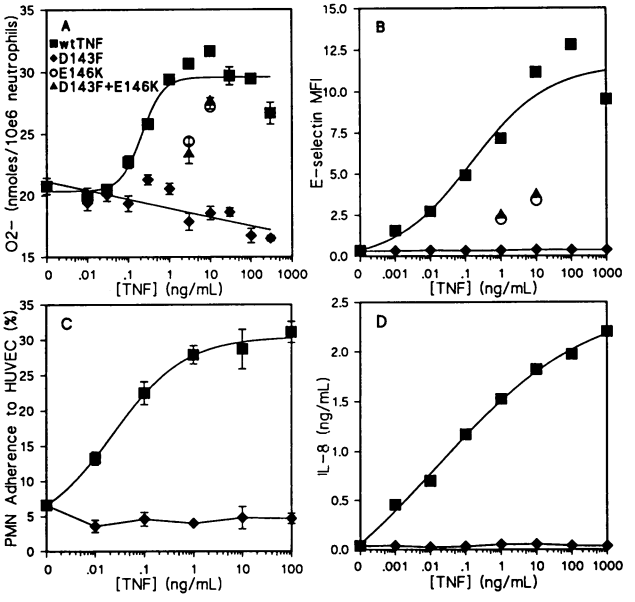

Fig. 5. The proinflammatory activity of D143F, a TNFR75-selective mutant, alone or in combination with E146K. Representative experiments of at least two performed in each case. (**A**) Superoxide production, data points representing the mean ± SEM of triplicates. (**B**) E-selectin expression with each point representing the mean of 10^4 determinations. (**C**) Neutrophil adhesion to stimulated HUVEC with the mean ± SEM of triplicates at each point. (**D**) IL-8 secretion with each point representing duplicates. Wild-type TNF, closed square; D143F, closed diamond; E146K, open circle; D143F+E146K, closed triangle.

Table IV. GM-CSF induction in PC60-hTNFR75$^+$ by TNF and mutants

TNF[a]	GM-CSF (ng/ml)	SD[b]
wtTNF	98.96	17.77
D143F	27.50	2.17
E146K	0.83	0.04
Nil	0.53	0.12

[a]TNF at 500 ng/ml
[b]Standard deviation

endothelial cell barrier. The activities we have examined pertain directly to the mechanisms involved in the adherence and transmigration of neutrophils through this barrier. Our results, which demonstrated up to 280-fold decrease in biological activity with the TNFR55-selective mutants relative to wtTNF imply that both receptors are involved in the mediation of these effects. However, when several of these activities were examined with the TNFR75-selective mutant D143F no activation was seen implying an indirect role only for TNFR75. Mackay *et al.* (1993) also measured E-selectin expression in endothelial cells treated with TNFR55-specific mutants. They used a high concentration of TNF mutant and under these conditions no role for TNFR75 was observed.

Our results with the TNFR55-selective mutants (E146K, R32W-S86T and to a lesser extent R32W) indicate that both TNF receptors are required to achieve a full range of TNF-α effects, whereas only TNFR55 seems involved in mediating cytotoxicity and cytostasis. To study the role of TNFR75 in the mediation of TNF-α's effects we used the TNF mutant D143F, a molecule which selectively binds to TNFR75 (Van Ostade *et al.*, 1994). This mutant did not stimulate neutrophil and HUVEC activities in the dose–response experiments performed (Figure 5) suggesting that TNFR75 on its own was not sufficient for activation and that it must somehow interact with TNFR55 to elicit full neutrophil and HUVEC activities. However, it should be noted that D143F has a 30-fold reduction in affinity for TNFR75 raising the possibility that clustering of TNFR75 by D143F is suboptimal, especially at low receptor numbers per cell. With higher receptor expression, as in the case of the PC60-hTNFR75$^+$ cells, D143F did result in activity in the form of GM-CSF production. Nevertheless, the results seen with D143F emphasize the major role played by TNFR55 and the facilitative role for TNFR75 in neutrophil and endothelial cell activation.

The exact mechanisms by which TNFR75 interacts with TNFR55 to mediate neutrophil and HUVEC activation by TNF-α is not known. This interaction may be occurring extracellularly or intracellularly. A recent report suggested that both TNF receptors bind to TNF using similar interaction sites (Banner *et al.*, 1993). This, combined with the knowledge that TNFR75 is much larger and its combining site further away from the cell membrane than the TNFR55 site, suggests that a single TNF trimer cannot bind TNFR75 at the same time as TNFR55. Therefore a direct interaction between the two different TNF receptors via a single TNF homotrimer at the extracellular level would seem unlikely to occur. Similarly, the extracellular 'passing on' model of Tartaglia and Goeddel (1992b) is not applicable to the results we have described. This model suggests that at lower concentrations of TNF, binding initially occurs to the higher affinity ($\sim$5-fold) and larger TNFR75 which captures the TNF and passes it onto TNFR55, which is then activated. With the TNFR55-selective mutants, which bind poorly or not at all to TNFR75, this effect cannot explain the large decreases in activity which have occurred. Alternatively, synergism of the two TNF receptors may well be occurring at the intracellular domain level or further down the signal transduction pathway. Previous research has shown that in mice, TNFR75 triggering (in addition to TNFR55 interaction) enhances up to 50-fold the lethality of TNF (Brouckaert *et al.*, 1992, 1993). It is not yet known which cellular systems are involved in this effect but the leucocyte/endothelial system is likely to play a predominant role. Therefore, the much reduced 'inflammatory responses' of human neutrophils and endothelial cells following treatment with TNFR55-selective mutants may well be directly relevant to the clinical situation.

In conclusion, the TNF mutants with their selective receptor-binding properties have enabled us to dissociate two principal activities of TNF-α, cytotoxicity and inflammation, and have allowed us to suggest possible roles for TNFR55 and TNFR75 in these functions. Our data support the notion that TNFR55 mediates cytotoxicity and cytostasis, while TNFR75 facilitates the mediation of neutrophil and endothelial cell activation through TNFR55. Through the employment of TNFR55-selective mutants such as E146K or R32W-S86T, which maintain full direct cytotoxicity but reduced proinflammatory effects, the efficacious clinical application of TNF-α as an antitumour agent may be realized. In addition, the use of synergistic combinations such as TNFR55-selective mutant and interferon may enable further efficacy to be attained.

Materials and methods

TNF-α

The recombinant human TNF-α (rhTNF) and the TNF mutants were purified to >90% homogeneity according to a previously described method using ammonium sulfate fractionation and polyethylenimine precipitation followed by purification on a Mono-Q chromatography column (Pharmacia, Sweden). Only the wild-type was further purified by an additional Mono-S (Pharmacia, Sweden) chromatography step (Tavernier *et al.*, 1990). Concentrations of wild-type and mutant proteins were determined using the Bio-Rad protein dye reagent (Van Ostade *et al.*, 1991). The content of endotoxin was <2.5 ng/mg as determined by the Limulus assay.

Cell lines

HEp-2 cells, U937 cells and P815 cells were cultured in RPMI-1640 with 10% fetal calf serum (FCS; Flow, North Ryde, NSW, Australia), 120 μg/ml penicillin G, 160 μg/ml gentamicin, 2 mM L-glutamine and 0.2% sodium bicarbonate at 37°C in a CO_2 incubator. All these cell lines were originally obtained from the American Type Culture Collection (Rockville, MD). PC60-hTNFR75$^+$(clone 26) were also cultured in medium as above, supplemented with sodium pyruvate (1 mM) and 2-ME (5×10^{-5} M), and the factor dependent FDCp1 cells were grown in the same medium but supplemented with 10% WEHI-3 supernatant as a source of mIL-3.

Flow cytometry

The monoclonal antibodies htr-9 (against TNFR55) and utr-1 (against TNFR75) (Brockhaus *et al.*, 1990) and an IgG1 negative control (3D3.3) were used with all cells studied. Htr-9 and utr-1 were added to 5×10^5 cells at 10 μg/ml (45 min, 4°C). After washing, goat anti-mouse IgG labelled with phycoerythrin (Southern Biotechnology, Birmingham, AL) is diluted 1 in 50 and added. The cells were then washed and resuspended in FACS FIX (phosphate-buffered saline with 2% glucose, 1% formaldehyde and 0.02% sodium azide) and analysed on a Coulter EPICS Profile II Flow Cytometer (Coulter Electronics, Florida).

TNF cytotoxicity assay

Target cells (HEp-2) at 4×10^4/well were incubated at 37°C for 2 h. Serial dilutions of wild-type or mutant TNF-α were prepared with 100 μg/ml cycloheximide (not serially diluted) and added to each well. The cells were incubated for 18 h at which time the supernatant was removed and the residual cells were stained with 0.2% crystal violet (BDH Chemicals, Kilsyth, Victoria, Australia) in 25% (v/v) methanol for 15 min (50 μl/well). The wells were washed and the remaining cells lysed with 1% SDS. The released dye was measured spectrophotometrically at 570 nm (Fransen *et al.*, 1986).

Cytostasis of a human monoblastoid leukaemic cell line U937

Leukaemic cells (5×10^4/well) were incubated with wild-type or mutant TNF at 37°C for 3 days. Cell proliferation was determined by adding 1 μCi [^{3}H]thymidine (Du Pont, Boston, MA) to each well. The cells were

incubated for 5 h and harvested with a Skatron cell harvester (MS.5, Lier, Norway). The radioactivity of the samples was measured with a liquid scintillation analyser (Tri-Carb 2000CA, United Technologies Packard, Downers Grove, IL).

Purification of human neutrophils

Neutrophils were obtained from the peripheral blood of normal volunteers after dextran sedimentation (Dextran T-500, Pharmacia, Uppsala, Sweden) and density gradient centrifugation at 450 *g* on Lymphoprep (Nycomed, Oslo, Norway) followed by hypotonic lysis of erythrocytes using 0.2% sodium chloride solution. The solution was brought to the correct osmolarity using 1.6% sodium chloride. The cell preparations were resuspended in RPMI-1640 with 0.1% BSA (fraction V, fatty acid-free, Boehringer Mannheim, Sydney, Australia). This method yielded cells which were >99% viable by trypan blue exclusion and >98% identifiable as neutrophils.

Endothelial cells

Human umbilical venular endothelial cells (HUVEC) were isolated by collagenase treatment of human umbilical cord veins and maintained in endotoxin-free RPMI 1640 medium with 20% FCS and endothelial cell growth supplement which contains basic FGF (Wall *et al.*, 1978).

Superoxide anion generation

Neutrophils were incubated at 37°C for 45 min with wild-type or mutant TNF followed by 10^{-7} M FMLP (*N*-formylmethionylleucylphenylalanine, Sigma, St Louis, MO) and cytochrome *c* (type VI, 12.4 mg/ml; Sigma) for 15 min. Superoxide production was quantified in nmol/10^6 neutrophils as described previously (Lopez *et al.*, 1986) and converted to a percentage.

PAF synthesis and bioassay

After stimulation with wild-type or mutant TNF for 45 min the neutrophils were extracted into ice-cold 80% ethanol overnight, evaporated to dryness under reduced pressure and reconstituted in Tyrode's solution (10 mM HEPES, 137 mM NaCl, 11.9 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 2.7 mM KCl, 0.26 mM $MgCl_2$, 11 mM D-glucose and 0.25% BSA adjusted to pH 7.4). PAF was detected by the aggregation of washed horse platelets (Mustard *et al.*, 1972; Hanahan and Wientraub, 1985) and quantified by comparison with a standard curve constructed using known concentrations of hexadecyl PAF (Boehringer Mannheim, Sydney, Australia), expressed in fmol/10^7 neutrophils and converted to a percentage. Biologically active material extracted from cells was characterized as PAF on the basis of its ability to induce acetylsalicylate-resistant platelet aggregation and inhibition of bioactivity by the selective PAF receptor antagonist WEB 2086 (1 μM) (Boehringer Ingelheim, Ingelheim, Germany).

Antibody-dependent cell-mediated cytotoxicity

The murine mast cell line P815 is the target for the antibody-dependent neutrophil cytotoxicity assay as previously described (Vadas *et al.*, 1983). The P815 cells were labelled with 200 μCi ^{51}Cr (Du Pont, North Ryde, Australia) and coupled to trinitrophenyl (TNP). The reaction mixture consisted of 16 μl TNF (wild-type or mutant), 24 μl anti-TNP antibody (polyclonal, ICN Biomedicals, CA), 40 μl P815 (4×10^3/well), 80 μl neutrophils (1.25×10^5/well), resulting in a killer:target cell ratio of 30:1. After incubating for 2.5 h an aliquot was removed and counted on a gamma counter (Cobra 5010, Packard Instrument Company).

Neutrophil adherence assay

Neutrophil adherence was determined as previously described (Gamble *et al.*, 1985). HUVEC were plated at 10^5 cells per cm^2 and grown to confluence. To each well ^{51}Cr-labelled neutrophils (5×10^5) and wild-type or mutant TNF was added for 30 min; however, in the case of primary HUVEC stimulation TNF was added to the HUVE monolayer 4 h prior to the addition of neutrophils. Aspirated nonadherent neutrophils, incubation medium, and the wash medium from each well were pooled in individual counting tubes for measurement of radioactivity in a gamma counter. The HUVE monolayers and the adherent ^{51}Cr-labelled neutrophils were lysed with 1 M NH_4OH and measured in a gamma counter. % Adherence = (^{51}Cr c.p.m. in NH_4OH lysate/total ^{51}Cr c.p.m. added) $\times$ 100.

E-selectin expression

Endothelial cells were stimulated with wild-type or mutant TNF for 4 h. Antibody directed against E-selectin (endothelial leucocyte adhesion molecule-1) was added for 30 min at 4°C, followed by sheep anti-IgG Fab_2-FITC labelled antibody (Silenus, Victoria, Australia). The cells were harvested by trypsin-EDTA treatment, pelleted and resuspended in FACS FIX. The fluorescence profiles were analysed by flow cytometry using a Coulter EPICS Profile II Flow Cytometer. 10 000 cells per sample were analysed.

Neutrophil transmigration

Transwells (Costar, Cambridge, MA) (6.5 mm diameter, polycarbonate membrane with 3 μm pores) were prepared with confluent endothelial monolayers as described by Smith *et al.* (1991). Monolayers were preincubated with wild-type or mutant TNF for 4 h prior to the assay. Monolayers were washed with medium then 10^6 neutrophils were added and the Transwells were placed in 24-well cluster dishes for 45 min. At the end of this period the wells were shaken to dislodge neutrophils from the lower surface of the Transwells. The medium in the lower wells was thoroughly mixed and aliquots were taken for counting of migrated neutrophils in a Coulter Counter (model ZF; Coulter, Harpenden, UK). Counts were expressed as a percentage of the cells added.

IL-8 secretion by endothelium

IL-8 was measured in supernatants of stimulated endothelium by ELISA. Endothelial monolayers were stimulated with wild-type or mutant TNF for 4 h, the medium was changed and 1 h supernatants were collected. The ELISA was based on the method described by Van Zee *et al.* (1991). Vinyl assay plates (Costar, Cambridge, MA) were coated with purified antibody from a rabbit IL-8 antiserum provided by S.L.Kunkel, Ann Arbor, MI. Samples were added and IL8 was detected with a biotinylated polyclonal anti-IL-8 antibody (biotinylation kit, Amersham, UK), which was then incubated with streptavidin-biotinylated horseradish peroxidase complex [Vectastain ABC kit (HRP), Vector, Burlingame, CA] and developed with *o*-phenylenediamine (Sigma, St Louis, MO); the OD at 490 nm was then read.

Induction of GM-CSF in PC60-hTNFR75$^+$ cells

PC60-hTNFR75$^+$(clone 26) is a cell line derived from a fusion between an IL-2 dependent murine CTL line and a rat thymoma transfected with the human TNFR75, and induction experiments were performed as previously described (Vandenabeele *et al.*, 1992). Briefly, 3×10^4 cells/well were exposed to wtTNF, D143F or E146K in the presence of hIL-1β (1 ng/ml). After 24 h, the supernatants were tested for rat GM-CSF activity in an FDCp1 proliferation assay.

Statistical analysis

Data analysis was performed using a two-way analysis of variance incorporating replicates. All results are shown as mean ± standard error of the mean (SEM) unless otherwise indicated. The potencies of the TNF mutants were calculated relative to the wtTNF according to the formula: (wtTNF EC_{50}/mutant TNF EC_{50}) $\times$ 100.

Acknowledgements

Expert technical assistance was provided by Susan Beltrame and Leanne Noack. Typing assistance was kindly provided by Mari Walker. This work was supported by grants from the National Health and Medical Research Council (Australia) and Hoffmann-La Roche Ltd (Switzerland).

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Received on September 7, 1993; revised on November 15, 1993