THE MECHANISM OF ACTION OF TUMOUR NECROSIS FACTOR - α

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ABSTRACT

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The *in vivo* administration of Tumour Necrosis Factor - alpha (TNF-α) as an antineoplastic agent has been severely restricted by dose-limiting side effects. Neutrophils, monocytes and endothelium are believed to be involved in the generation of these unwanted side effects. In the mouse, the administration of human TNF, which binds only to the murine p55 TNF receptor (TNFR55), is much less toxic than murine TNF, which binds to both murine TNF receptors. In view of this species specificity, human TNF mutants with selective binding to the human TNF receptors were employed to examine the role of these receptors in the mediation of TNF's cytotoxic and proinflammatory activities. The TNFR55-selective mutants stimulated proinflammatory activity which was markedly less than wild-type TNF. TNF-α's priming of human neutrophils for superoxide production and antibody-dependent cell-mediated cytotoxicity, platelet-activating factor (PAF) synthesis and adhesion to endothelium were reduced by up to 170-fold. Activation of human endothelial functions represented by adhesiveness for neutrophils, E-selectin expression, neutrophil transmigration and IL-8 secretion were also reduced by up to 280-fold. The TNFR75-selective mutant did not stimulate any proinflammatory activity implying that TNFR75 facilitates the role of TNFR55 in mediating these activities. However, the TNFR55-selective mutants exhibited similar potency to wild-type TNF in causing cytotoxicity of a human laryngeal carcinoma-derived cell line and cytostasis in a human leukaemic cell line. Therefore, the *in vivo* use of TNFR55-selective mutants may result in reduced side effects whilst maintaining the antitumour activity of wild-type TNF.

The signal transduction mechanisms by which TNF-α elicits proinflammatory activity were examined in neutrophils and monocytes. Cytosolic phospholipase A_2 ($cPLA_2$), the rate-limiting enzyme in the production of eicosanoids and PAF, was selected for investigation. The rapid phosphorylation of $cPLA_2$ on serine residues by TNF-α was demonstrated and found to be coupled to the production of PAF in human monocytes. The TNFR55-selective mutants stimulated less $cPLA_2$ phosphorylation and PAF production than wild-type TNF and this is in keeping with the neutrophil and endothelial proinflammatory effects described.

The life span of the mature neutrophil *in vivo* is relatively brief (24 hours) and it is shown here that TNF-α shortens this time markedly. Apoptosis is induced by TNF-α in the majority of neutrophils within 3 hours as demonstrated by microscopy (light and fluorescent), DNA fragmentation gels and propidium iodide binding. The TNFR55-selective mutants induce significantly less apoptosis than wild-type TNF and implies similar TNF receptor biology for neutrophil proinflammation and apoptosis. Thus TNF-α, in stimulating neutrophil proinflammatory activities, induces an early noninflammatory death.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this thesis being made available for loan and photocopying if accepted for the award of the degree.

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ABBREVIATIONS

^{32}P	^{32}-Phosphorus
A23187	Calcium Ionophore
AA	arachidonic acid
Act D	Actinomycin D
ADCC	antibody-dependent cell-mediated cytotoxicity
AIDS	acquired immunodeficiency syndrome
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
CD	cluster determinant
CHX	cycloheximide
CHO	chinese hamster ovary
$cPLA_2$	cytosolic phospholipase A_2
cpm	counts per minute
CR1	complement receptor 1
CSF	colony-stimulating factor
DAG	diacylglycerol
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorter
Fas	Fas antigen
FasL	Fas ligand
FCS	foetal calf serum
FMLP	*N*-formylmethionylleucylphenylalanine
GM-CSF	granulocyte-macrophage colony-stimulating factor

h	human
H_2O_2	hydrogen peroxide
HBSS	Hanks balanced salt solution
HEPES	*N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HIV	human immunodeficiency virus
HUVEC	human umbilical vein endothelial cells
ICE	interleukin-1 converting enzyme
IFNγ	interferon gamma
IL	interleukin
kD	kilodalton
Kd	apparent dissociation constant
LPS	lipopolysaccharide
LT	leukotriene
LT-α	lymphotoxin-alpha (TNF-β)
LT-β	lymphotoxin-beta
m	murine
MAP kinase	mitogen-activated or microtubule-associated protein kinase
mAb	monoclonal antibody
MFI	mean fluorescence intensity
MHC	major histocompatibilty complex
M_r	molecular weight
n	number of experiments
O_2^-	superoxide anion
PAF	platelet-activating factor
PBS	phosphate-buffered saline
PC	phosphatidylcholine
PG	prostaglandin

PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
r	recombinant
R	receptor
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SLE	systemic lupus erythematosus
SMase	sphingomyelinase
TNF-α	tumour necrosis factor-alpha
TNF-β	tumour necrosis factor-beta (LT-α)
TNFR55	55 kD TNF receptor
TNFR75	75 kD TNF receptor
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TRAF	TNF receptor-associated factor
TRAK	TNF receptor-associated kinase
TRAP	TNF receptor-associated protein

PUBLICATIONS ARISING

Barbara JAJ, Smith WB, Gamble JR, Van Ostade X, Vandenabeele P, Tavernier J, Fiers W, Vadas MA and Lopez AF: Dissociation of TNF-α cytotoxic and proinflammatory activities by p55 receptor- and p75 receptor-selective TNF-α mutants. EMBO J 13(4):843-850, 1994.

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INTRODUCTION

Chapter 1

1.1 The Historical Background of TNF-α

Tumour Necrosis Factor - alpha (TNF-α) is a 17 kD protein produced mainly by activated macrophages which, despite its long history, is only now reaching major clinical prominence. In the previous century physicians noted that patients with malignancy who experienced life-threatening infection would sometimes have a reduction in tumour bulk. At the turn of the century a surgeon at the Memorial Hospital in New York, William B. Coley, used bacterial extracts with some success in the treatment of cancer in terminally ill patients (Coley, 1893). However, the clinical results were inconsistent and the toxic effects led to the decline of bacterial therapy. Surprisingly, it was not until 1975 that the responsible agent was discovered. The protein was coined Tumour Necrosis Factor by Elizabeth Carswell et al (1975), located in New York at the Memorial Sloan-Kettering Cancer Centre. Mice or rabbits were treated initially with Bacille Calmette-Guérin (BCG) for 10-14 days, followed by lipopolysaccharide (LPS), with the resultant release of TNF. When mice carrying methylcholanthrene-induced sarcoma were treated with TNF-containing serum, the tumours underwent haemorrhagic necrosis and regression. Studies on the wasting syndrome (cachexia) associated with parasitic infections and malignancy also led to the parallel discovery of TNF-α. Rabbits infected with *Trypanosoma brucei* became cachectic, losing more than half their original body weight. This wasting was associated with elevated triglycerides caused by a suppression of the enzyme lipoprotein lipase (LPL). It was discovered that LPS-treated animals also suppressed LPL and that this was due to a transferable serum factor (Kawakami and Cerami, 1981). Beutler et al (1985, 1985b) purified this 17 kD polypeptide and named it cachectin, and it soon became apparent that TNF-α and cachectin were one and the same molecule.

Pennica et al (1984) and others subsequently cloned the human TNF cDNA gene and found that it was well expressed in *Escherichia coli*. Therefore large quantities of TNF protein became available for research and clinical application. The gene is located on the short arm of

chromosome 6 near the HLA-B locus in the major histocompatibility complex (MHC) (Spies et al, 1986). The position of TNF-α within the MHC has led to speculation about the role of the TNF-α gene in the aetiology of MHC-linked diseases, especially those with an inflammatory or autoimmune component such as insulin-dependent diabetes mellitus and systemic lupus erythematosus (Pociot et al, 1993; Wilson et al, 1994).

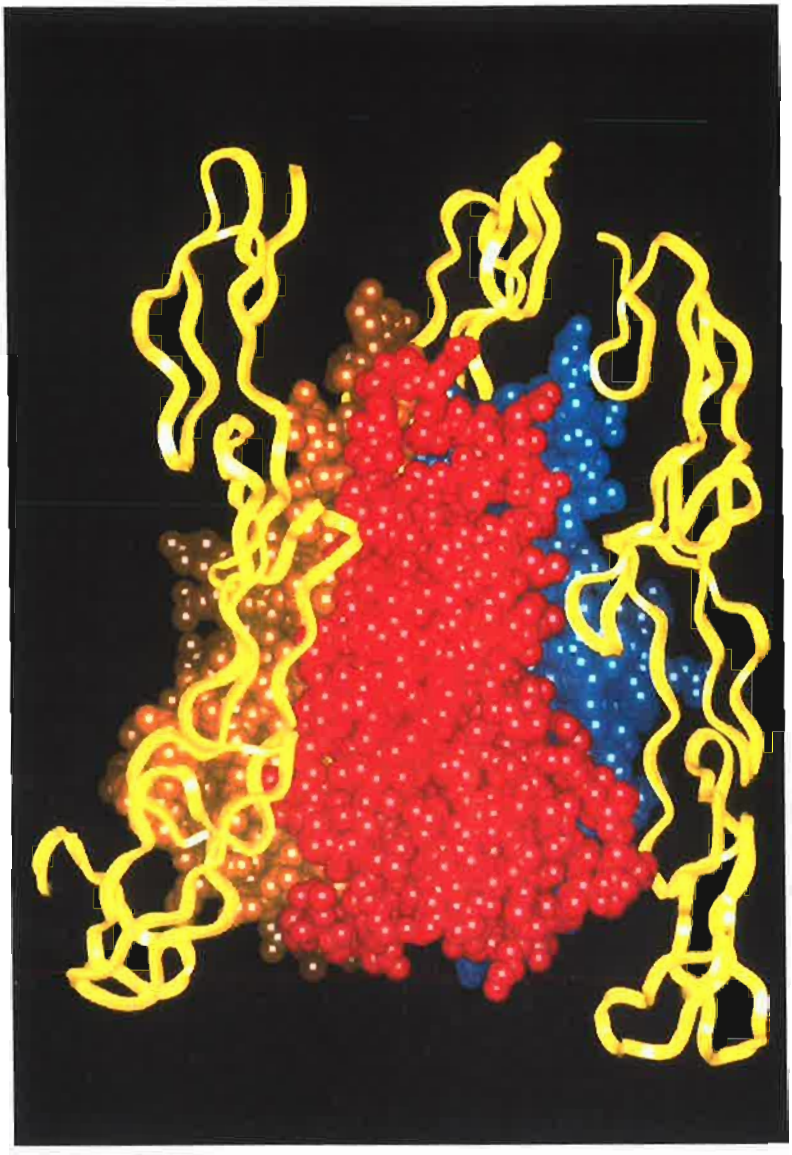
1.2 The TNF-α Molecule

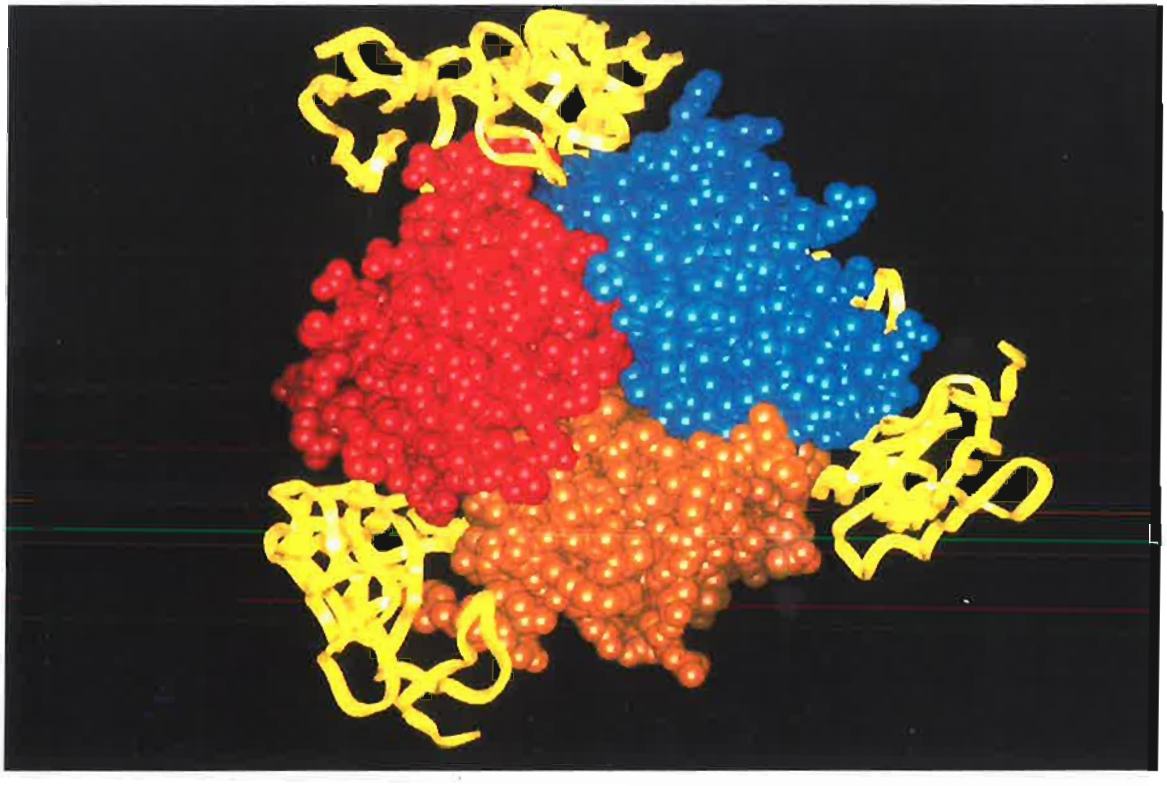
TNF-α is a 17 kD cytokine consisting of 157 amino acids with a presequence of 76 amino acids (Fiers et al, 1986) and it can exist in an unprocessed, membrane-bound form (233 amino acids, 26 kD). The processing of the TNF-α precursor by metalloproteinases has recently become an area of intense investigation and inhibitors of metalloproteinases may have therapeutic potential in diseases in which TNF-α is known to have a pathophysiological role (Mohler et al, 1994). TNF-α exists in the biologically active, physiological form as a homotrimer with a molecular mass of 52 kD. The trimeric structure was determined chemically by cross-linking experiments and also physicochemically by analytical ultracentrifugation and X-ray solution scattering (Smith and Baglioni, 1987; Wingfield et al, 1987; Lewit-Bentley et al, 1988). Well-diffracting crystals of TNF-α allowed the three-dimensional structure to be determined at 2.6 Å resolution (Eck and Sprang, 1989; Jones et al, 1989). The shape of the TNF-α homotrimer has the appearance of a triangular cone or bell in which each of the three subunits has a typical jelly roll-β structure and the three subunits are arranged edge to face (figure 1.1).

In order to establish the location of active sites on the TNF molecule, random mutagenesis of the gene was performed and inactive molecules were selected on the basis of their cytotoxic activity against murine L-M cells (Yamagishi et al, 1990) or murine L929 cells (Van Ostade et al, 1991). These independent studies demonstrated that the active sites of TNF-α were located in the lower half of the triangular pyramid in the groove between two subunits, and

Figure 1.1 The TNF-α molecule

The TNF-α homotrimer is shown binding to three TNFR55. The individual TNF monomers are coloured red, blue and brown, and TNFR55 is represented in yellow. (Top) Side view of TNF-α with the cell surface out of picture at the top of the page. (Bottom) Basal view of TNF-α (view from beneath its pyramidal base) showing TNFR55 in the binding pockets. The TNF-α computer-generated images were constructed using SiliconGraphics computer-ware and the coordinates of Eck and Sprang (1989), and the binding of TNFR55 to TNF-α was derived from Banner et al (1993). The balls represent all atoms other than hydrogen.





corresponds to the receptor binding sites. In addition, this mutational analysis gave rise to TNF mutants with selective binding to the TNF receptors (Loetscher et al, 1993; Van Ostade et al, 1993; Van Ostade et al, 1994).

1.3 Biological Activities of TNF-α

TNF-α is a cytokine produced mainly by monocytes and macrophages, however other cell sources include lymphocytes, mast cells, neutrophils, keratinocytes, astrocytes and microglial cells, smooth muscle cells, intestinal paneth cells and tumour cells (Matthews, 1981; Vassalli, 1992). It is a hormone-like peptide that enters the bloodstream to alter the biology of distant tissues or it can behave as a paracrine mediator that acts locally. Many stimuli induce the production of TNF-α including lipopolysaccharide, cytokines (IL-1, GM-CSF and TNF-α itself) and phorbol esters (Spriggs et al, 1992; Tracey and Cerami, 1994). TNF-α is truly a pleiotropic cytokine with numerous biological activities. The ability of TNF-α to kill tumour cells was the activity which led to its discovery in 1975 by Carswell et al but it has since acquired many other properties. In view of its cytotoxic potency, the ability of TNF-α to act as a growth factor on some cells - especially normal human fibroblasts - was indeed unexpected (Sugarman et al, 1985; Vilcek et al, 1986). In addition, Beutler et al (1985) found that TNF-α was responsible for the cachexia (wasting syndrome) associated with terminal malignancy and major parasitic infections. However, the interest in the above activities has to some extent been overshadowed by TNF-α's ability to incite an inflammatory response and act as an immunomodulatory agent. It is principally the cytotoxic and proinflammatory activities of TNF-α which have been examined in this thesis.

1.3.1 Cytotoxicity

TNF-α's ability to be cytotoxic to a range of human and murine malignant cells is the property which has held the greatest therapeutic potential over the last decade (Sugarman et al, 1985; Balkwill et al, 1986; Fransen et al, 1986). However, the cellular mode of action of TNF

cytotoxicity is still not well understood. TNF-α's cytotoxic activity does not appear to depend on RNA or protein synthesis and in fact is often enhanced up to 100-fold by inhibitors of transcription and translation (Ruff and Gifford, 1981). This indicates that certain cells may resist the toxic effects of TNF-α by the synthesis of protective factors (Wallach et al, 1988). Manganous superoxide dismutase, in some cell types, is one such protective factor (Wong et al, 1989) as shown by the increased resistance to TNF-α that occurs with overexpression of this mitochondrial enzyme.

TNF-mediated cytotoxicity, for reasons which are not clear, can take the form of either necrosis or apoptosis (Laster et al, 1988). In necrosis, the cell membrane integrity is lost, cells swell and lyse, and a significant inflammatory response results. The process has no energy requirements, there is no new gene transcription or protein synthesis and DNA is randomly digested (Wyllie et al, 1980; Allen et al, 1993). Apoptosis is a form of cell death which is morphologically and biochemically distinct from necrosis. The cell and nucleus shrinks without inciting an inflammatory response. The process is energy-dependent, requires protein synthesis and classically exhibits nonrandom DNA fragmentation (Kerr et al, 1972; Allen et al, 1993). This DNA fragmentation is mediated by endonucleases which cut between nucleosomes (Wyllie, 1980; Duke et al, 1983). Nucleosomes consist of two copies of each of the histones H3, H2A, H2B and H4 around which DNA is wound twice with each nucleosome linked by a short stretch of DNA to form a "beads on a string" formation (Richmond et al, 1984). The activation of endonucleases is not just an accompanying effect but an essential step in TNF-mediated tumor cell destruction (Flieger et al, 1989).

1.3.2 Proinflammation

The neutrophil is a specialized leukocyte which engulfs and kills invading microorganisms and provides the first line of immune defence against these invading pathogens. It is also the primary cell present in the cellular phase of the acute inflammatory response and is capable of

inflicting host tissue damage. The stimulation of neutrophils by TNF-α leads to increased phagocytosis, degranulation and respiratory burst activity (Klebanoff et al, 1986; Atkinson et al, 1988). In the respiratory burst, oxygen is converted to several toxic metabolites - superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH·). NADPH oxidase is the enzyme responsible for converting oxygen to toxic products:

$$NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^- + H^+$$

Superoxide anion can then be converted into H_2O_2, either through spontaneous or superoxide dismutase (SOD)-catalysed dismutation. The hydrogen peroxide formed reacts via the metal-catalysed Haber-Weiss reaction to produce the highly reactive hydroxyl radical (OH·). TNF-α also results in increased platelet-activating factor (PAF) formation in neutrophils (Camussi et al, 1987) and stimulates these cells to release arachidonic acid (AA) (Atkinson et al, 1990), enhances their antibody-dependent cell-mediated cytotoxicity (ADCC) (Shalaby et al, 1985), and increases the adherence of stimulated neutrophils to endothelium (Gamble et al, 1985). As can be seen from the aforementioned, many effects of TNF-α as a mediator of inflammation are due to its ability to stimulate neutrophil functions. In addition, the monocyte/macrophage, a long-lived phagocyte which presents processed antigen to T lymphocytes and is capable of many of the functions of neutrophils, is also stimulated to increased activity by TNF-α (Vassalli, 1992).

Vital to any effective inflammatory or immune response is the ability of leukocytes to adhere to and migrate through the endothelium to reach the site of action. TNF-α activates endothelium leading to enhanced neutrophil adherence (Gamble et al, 1985) and this is primarily due to the upregulated expression of the endothelial adhesion molecule E-selectin (Bevilacqua et al, 1989). TNF-α also increases IL-8 secretion by endothelium and increases the transmigration of neutrophils (Strieter et al, 1989; Huber et al, 1991; Smith WB et al, 1991; Kuijpers et al, 1992). Interleukin-8 (IL-8), previously known as neutrophil-activating peptide, induces shape change and degranulation in neutrophils, and is a powerful

chemoattractant for these cells (Baggiolini et al, 1989). TNF-α also increases PAF production by endothelium with resultant increase in neutrophil transmigration (Kuijpers et al, 1992).

1.4 TNF Receptors

The multiple activities of TNF-α are mediated through two distinct, high affinity receptors which have recently been identified (Hohmann et al, 1989; Brockhaus et al, 1990) and the cDNAs corresponding to both receptor types have been isolated and characterized (Dembic et al, 1990; Loetscher et al, 1990; Schall et al, 1990; Smith et al, 1990). TNFR55 (R1, type B, CD120a; Kd 0.5 nM) is a 55 kD receptor for TNF-α which is ubiquitous - except erythrocytes and unstimulated T cells - and TNFR75 (R2, type A, CD120b; Kd 0.1 nM) is a 75 kD receptor which is often more abundant on cells of haemopoietic 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; Slowik et al, 1993). These high affinity receptors share 28% homology in their extracellular domains but have a complete absence of homology intracellularly.

The expression of two different TNF receptors with unrelated intracellular domains suggests that they function independently via divergent signalling pathways to mediate different TNF-α activities. In an attempt to define the roles of the TNF receptors, several groups have used specific receptor antibodies and transfected cell lines expressing the cloned receptors (appendix 1). However, far from providing definite answers, the results, for example regarding cytotoxicity, have been highly controversial (Heller et al, 1992; Heller et al, 1993; Tartaglia et al, 1993). In addition, the role of these two receptors in human neutrophil priming, mediator release and adhesion to endothelium has remained entirely unexplored.

The trimeric structure of TNF-α with its three receptor-binding pockets indicated that receptor clustering occurred as part of receptor activation and signal transduction. This was

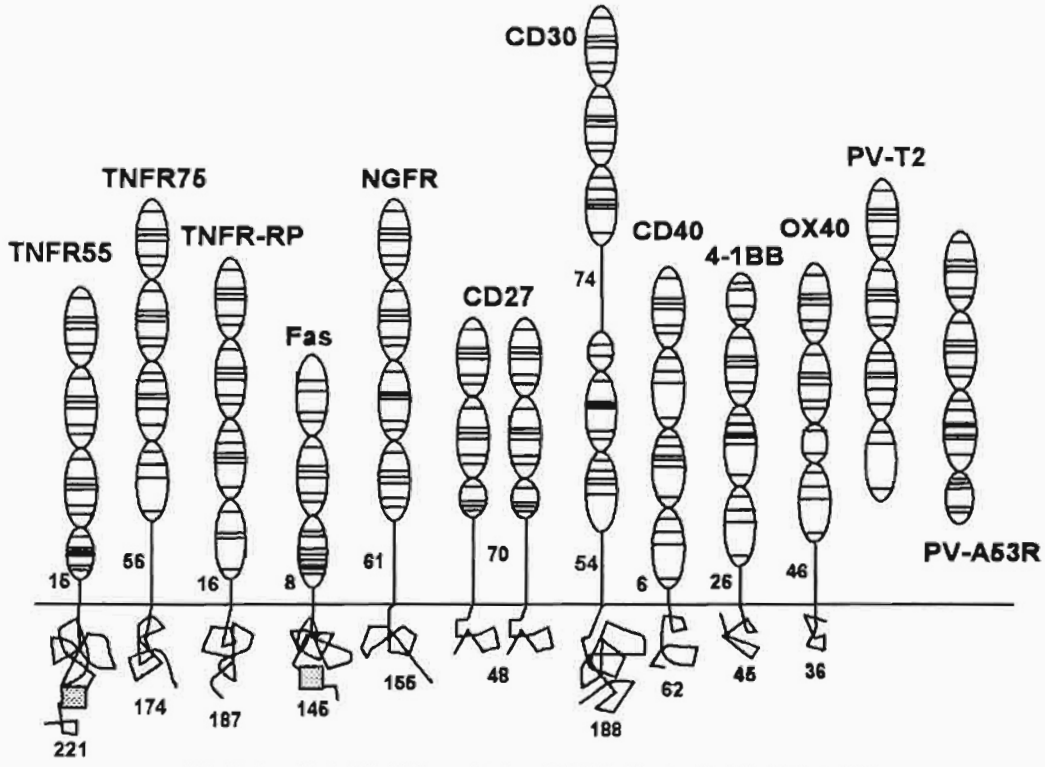
initially supported by antibody studies in which monovalent Fab fragments did not produce agonistic activity but when cross-linked with a second antibody activity resulted (Engelmann et al, 1990). The clustering phenomenon was subsequently confirmed for TNFR55 (Loetscher et al, 1991; Pennica et al, 1992) and TNFR75 (Tartaglia et al, 1991; Vandenabeele et al, 1992), and experiments performed by Loetscher et al (1992) using mixtures of the two TNF receptors revealed the formation of homocomplexes only. The aggregation of homocomplexes, and not heterocomplexes, is supported by the knowledge that the ligand-binding domain of TNFR75 is further from the cell surface than the ligand-binding domain of TNFR55 and this makes it unlikely that TNF-α could simultaneously interact with both receptors (Banner et al, 1993).

1.5 The TNF and TNF Receptor Superfamilies

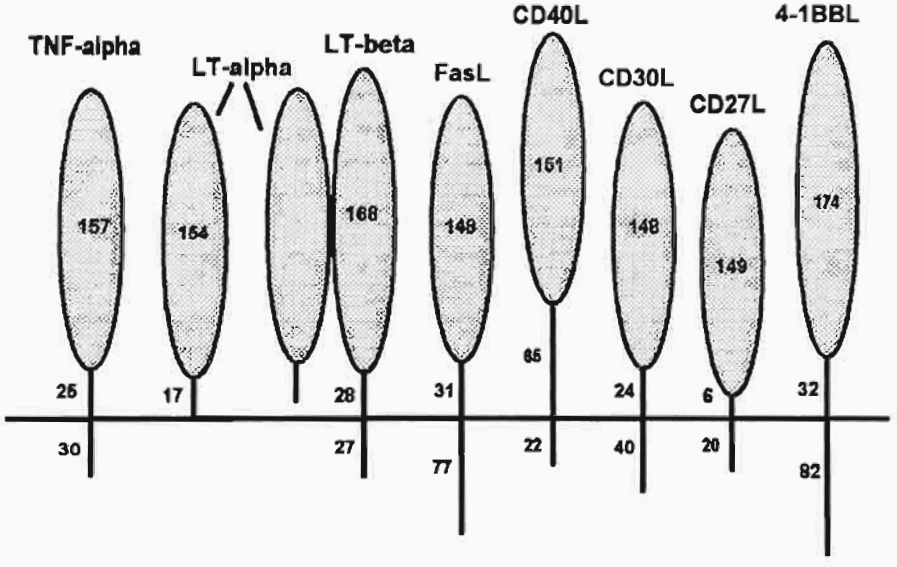
TNF-α and its receptors (TNFR55 and TNFR75) are the prototype members of two superfamilies (Beutler and van Huffel, 1994; Cosman, 1994; Smith et al, 1994). Twelve receptors have been identified in the TNF receptor superfamily and eight cognate ligands have been discovered thus far in the TNF superfamily (figure 1.2). The receptors are mainly type I membrane proteins (N-terminus extracellular, C-terminus intracellular and a single transmembrane segment) with sequence homology restricted to the extracellular region in the form of cysteine-rich pseudorepeats. The exceptions are the poxvirus gene products, T2 and A53R, which comprise soluble secreted proteins (Smith CA et al, 1991). The ligand family members, except LT-α (lymphotoxin-α, ≡ TNF-β), are type II membrane proteins (C-terminus extracellular, N-terminus intracellular and a single transmembrane component). Lymphotoxin-α (LT-α), which is produced by lymphocytes, is structurally and functionally similar to TNF-α and binds to both TNFR55 and TNFR75 but does not exist in a membrane bound form. TNF-α also exists in the proteolytically released form like LT-α but this has yet to be confirmed for other family members.

Figure 1.2 The TNF receptor and ligand superfamilies

(Top) The twelve members of the TNF receptor superfamily characterized by extracellular cysteine pseudorepeats (oval shapes ≡ homologous domains). The intracellular regions do not exhibit homology, except for the "death domain" of TNFR55 and fas (boxes), and are shown as random lines. The number of amino acids in the extracellular linker regions and cytoplasmic domains are indicated. (Bottom) The eight members of the TNF ligand superfamily. The homologous C-terminal domains are indicated by the oval shapes. The number of amino acids in each region is shown. TNF-α, LT-α and LT-β form oligomers. LT-α has secreted and membrane-associated forms. The figures were adapted from Smith et al (1994).



TNF RECEPTOR SUPERFAMILY



TNF LIGAND SUPERFAMILY

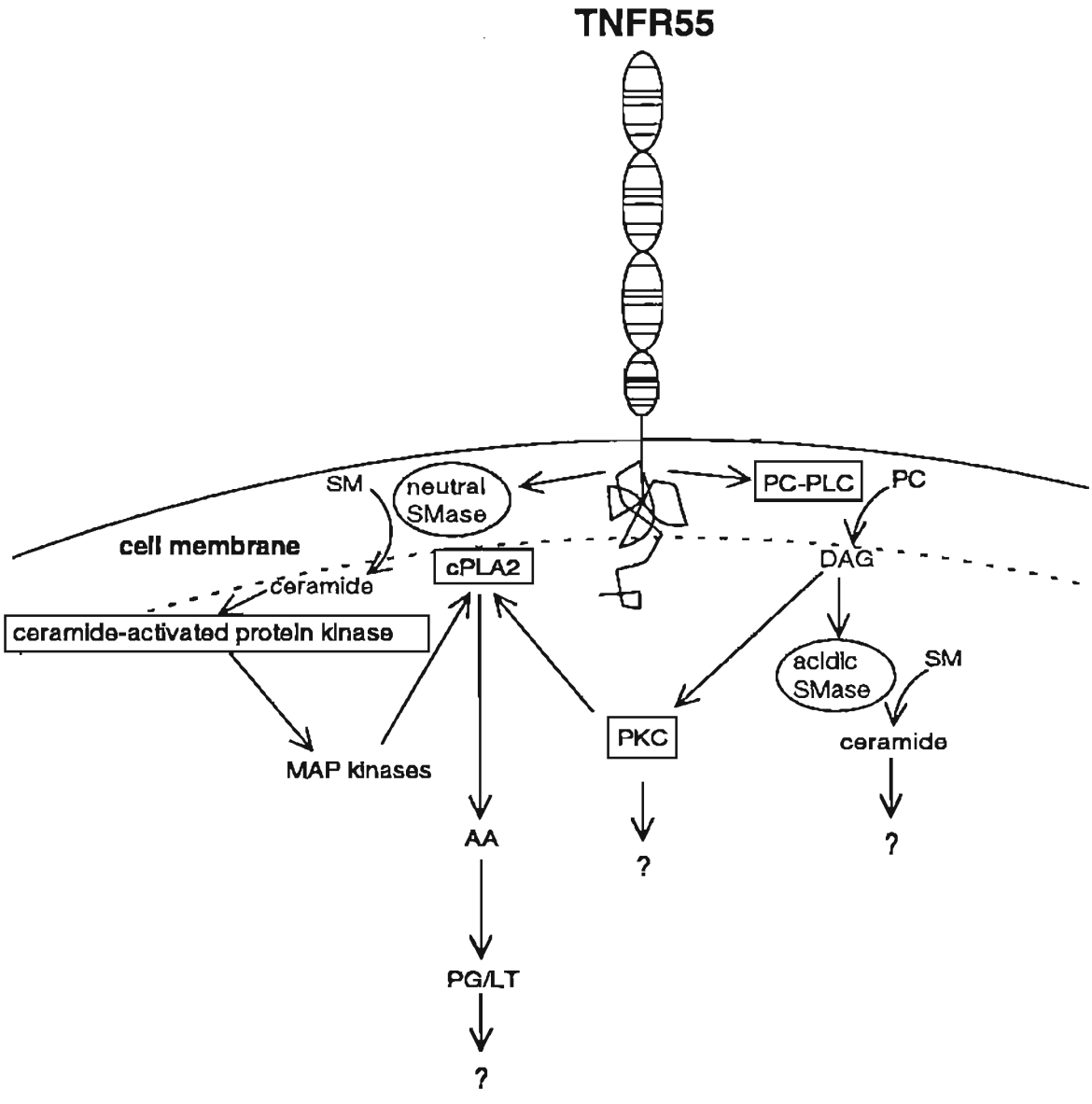
An interesting member of this fledgling group of receptors is the Fas antigen (Fas) which is known to mediate apoptosis (Trauth et al, 1989; Itoh et al, 1991). The recent cloning and expression of its cognate ligand (Fas Ligand; FasL) by Suda et al (1993) will allow a more complete examination of the Fas system to be undertaken. The clinical importance of the Fas system first became apparent in murine studies, where mice homozygous for lpr (lymphproliferation) or gld (generalised lymphoproliferative disease) mutations develop lymphadenopathy and suffer from an autoimmune disease similar to systemic lupus erythematosus (SLE). Lpr and gld are mutations in Fas antigen and Fas ligand respectively (Watanabe-Fukunaga et al, 1992; Takahashi et al, 1994). The lymphadenopathy and autoimmune disease observed in these mice suggest that the Fas system plays an important role in the apoptotic process that takes place during the development of T cells. Fas and TNFR55 are unique in being the only receptors known to mediate apoptosis. The intracellular region of Fas has a domain which is weakly homologous to the intracellular region of TNFR55 (27% identity over 66 amino acids) and this "death domain" is known to be required for apoptosis in both receptors (Itoh and Nagata, 1993; Tartaglia et al, 1993b). In view of this shared "death domain" it has been proposed that these receptors may trigger apoptosis by similar mechanisms. Significantly, the intracellular region of TNFR75 bears no such domain.

1.6 TNF Signal Transduction

The mechanisms through which TNF-α mediates its numerous activities after binding to its cell-surface receptors are poorly understood. The intracellular domains of the two TNF receptors have no sequence homology to any other receptor nor to any known catalytic domains of protein kinases. However, those aspects of TNF signal transduction that are known have been summarized in figure 1.3. When TNF binds to TNFR55, diacylglycerol (DAG) is rapidly produced from membrane phospholipids by the activation of a phospholipase C (PLC). DAG then activates two signalling enzymes, a calcium-independent protein kinase C (PKC) and acidic sphingomyelinase (SMase). SMase results in the breakdown of

Figure 1.3 The signal transduction pathways of TNF-α

The pathways associated with TNFR55 have been more intensively studied than TNFR75 and are shown in this diagram adapted from Heller and Krönke (1994). AA, arachidonic acid; $cPLA_2$, cytosolic phospholipase A_2; DAG, diacylglycerol; LT, leukotriene; MAP kinase, mitogen-activated/microtubule-associated protein kinase; PC-PLC, phosphatidylcholine-specific phospholipase C; PG, prostaglandin; PKC, protein kinase C; SM, sphingomyelin; SMase, sphingomyelinase.


TNFR55
SM
neutral SMase
PC-PLC
PC
cell membrane
cPLA2
DAG
ceramide
ceramide-activated protein kinase
acidic SMase
SM
PKC
ceramide
MAP kinases
AA
?
?
PG/LT
?

sphingomyelin to ceramide, a second messenger known to stimulate a number of cellular responses including TNF-stimulated apoptosis (Obeid et al, 1993; Hannun, 1994).

TNF-α also activates a neutral SMase which hydrolyses membrane sphingomyelin to ceramide and this results in the activation of a ceramide-activated protein kinase that is able to phosphorylate MAP kinase (Liu et al, 1994). The activation of MAP kinase and PKC has recently been shown to be relevant to the functional status of phospholipase A_2 (PLA_2) (Lin et al, 1993; Nemenoff et al, 1993). The importance of PLA_2 in the signal transduction pathway of TNF-α has been recognized for some years. PLA_2 is essential to the release of arachidonic acid from membrane phospholipids with resultant leukotriene and prostaglandin formation, and is necessary for the formation of PAF (Irvine, 1982; Hanahan, 1986).

1.6.1 Cytosolic Phospholipase A_2 ($cPLA_2$)

The recent purification of a high molecular weight form of PLA_2 (110 kD) from the human monocytic cell line U937 has renewed interest in the role of PLA_2 as a signal transduction molecule. This enzyme, cytosolic PLA_2, is distinct from the relatively small (14 kD) secretory phospholipases A_2 ($sPLA_2$) in being activated and translocated to the membrane by physiological/submicromolar levels of ionised calcium, and is resistant to the reducing conditions of the cell (Clark et al, 1990; Clark et al, 1991; Kramer et al, 1991; Sharp et al, 1991). $cPLA_2$ selectively hydrolyzes arachidonic acid (AA) from the 2-acyl ester bond of membrane phospholipids and this represents the rate-limiting step in the production of eicosanoids and platelet-activating factor (PAF). Therefore, it would seem that $cPLA_2$ may play a central role in the mediation of TNF's inflammatory activity and its functioning in myeloid cells has been examined in this thesis.

1.6.2 Platelet-activating Factor

PAF is a lipid mediator of inflammation with a wide range of biological activities (Braquet et al, 1987; Zimmerman et al, 1992). PAF was initially discovered as a product of IgE-sensitized rabbit basophils (Benveniste et al, 1972) and was found to be equivalent to 1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine (Demopolous et al, 1979). PAF activates human neutrophils in a number of ways: priming, β_2 integrin activation, shape change, chemotaxis, degranulation and oxygen radical generation and stimulates monocytes to aggregate, release TNF-α, undergo complement-dependent phagocytosis and adhere to endothelium (Zimmerman et al, 1992). TNF-α induces the synthesis and release of PAF from neutrophils, macrophages and endothelial cells (Camussi et al, 1987), however most of the PAF synthesized by endothelium remains cell-associated. The synthesis of PAF in response to TNF-α is rapid and occurs in inflammatory cells via two enzymatic steps: (i) hydrolysis of arachidonic acid from 1-*O*-alkyl-2-acyl-*sn*-glyceryl-3-phosphorylcholine by phospholipase A_2 and (ii) acetylation of the resultant lyso-PAF at position 2 by an acetyl transferase. The synthesis of PAF in response to TNF-α appears to be a specialized response of cells involved in inflammation, since it was shown by Camussi et al (1987) that TNF-α does not stimulate PAF production in cells such as fibroblasts.

1.7 *In Vivo* TNF-α (Antitumour agent)

The antineoplastic effect of TNF-α in mice has led to the exciting prospect of its use as a therapeutic agent in humans. Numerous phase I and II trials with TNF-α have now been reported (Jones and Selby, 1989; Spriggs et al, 1990a; Alexander and Rosenberg, 1991; Taguchi and Sohmura, 1991; Saks and Rosenblum, 1992; Spriggs and Yates, 1992) with TNF-α being administered intravenously (bolus or infusion), intramuscularly, subcutaneously or intratumorally. In clinical trials to date the doses of TNF-α have been limited by major side effects with the maximum tolerated dose ranging between 150-300 $\mu g/m^2$/day. The most frequent dose-limiting side effect is hypotension, but other side effects included fatigue, fever,

chills, anorexia, headaches, diarrhoea, nausea, vomiting, myalgias, hepatotoxicity, respiratory insufficiency and thrombocytopenia. These side effects are believed to be due in major part to the proinflammatory effects of TNF-α (Fiers, 1993). However, the local administration of TNF-α has been used with success. Lienard et al (1992) reported the treatment of patients with melanoma or sarcoma who received high-dose TNF-α, in combination with IFNγ and melphalan, by isolated perfusion of the involved limbs. 21 of 23 patients underwent a complete response and the remaining two showed a partial response.

Several lines of experimental evidence exist to support the role of TNF-induced inflammation in the mediation of *in vivo* side effects: (i) A single injection of indomethacin or ibuprofen prior to treatment with TNF-α completely prevents rapid killing and reduces eventual lethality in mice by 70% (Kettlehut et al, 1987). These drugs are cyclooxygenase inhibitors which prevent the formation of AA metabolites (prostaglandins and thromboxane). (ii) Manipulation of the oxygen radical-scavenging capacity in mice alters host sensitivity to tumour necrosis factor toxicity but does not interfere with its antitumour efficacy (Hauser et al, 1990). (iii) Superoxide dismutase (SOD), a superoxide anion scavenger, was able to reduce the mortality rate of mice challenged with endotoxin (Broner et al, 1988). (iv) Bismuth subnitrate, an oxygen radical scavenger, was able to protect mice from TNF-induced toxicity while not interfering with the antitumour effect of this cytokine (Satomi et al, 1988). (v) Necropsy of rats treated with lethal doses of TNF revealed sequestration of neutrophils in the pulmonary vessels, with intense margination of these cells along the vessel walls (Tracey et al, 1986). (vi) The release of PAF from neutrophils and monocyte/macrophages results in significant hypotension (Braquet et al, 1987). These proinflammatory side effects have restricted the dosage of TNF-α in clinical trials to such an extent that no consistent antineoplastic effect has been seen with TNF-α. Thus, TNF-α would seem to have a limited role in the treatment of human malignancy unless the side effects could be minimized. This may be achieved by utilizing the knowledge contained in the species specificity of TNF-α.

1.8 Species Specificity

The murine TNFR55 (mTNFR55) is 64% identical to the human TNFR55 (hTNFR55) and the mTNFR75 is 62% homologous to its human counterpart. However, the homology lies in different domains for the two receptor types. The mTNFR55 has an extracellular domain of 183 amino acids with 70% identity to the hTNFR55 and a cytoplasmic domain of 219 amino acids with 59% identity to hTNFR55. In contrast, the extracellular domain of mTNFR75 has 235 amino acids with 58% identity to hTNFR75 and a 188 amino acid domain with 73% identity to hTNFR75. In view of the different homology patterns, it is not surprising that in the murine system mTNFR55 has a similar affinity for both mTNF-α and hTNF-α, whereas mTNFR75 shows strong specificty for recombinant mTNF-α (Lewis et al, 1991). *In vivo*, this is reflected by mTNF-α being 50 times more potent than hTNF-α in causing murine lethality implying a specific role for TNFR75 in TNF toxicity (Brouckaert et al, 1992).

1.9 TNF Mutants

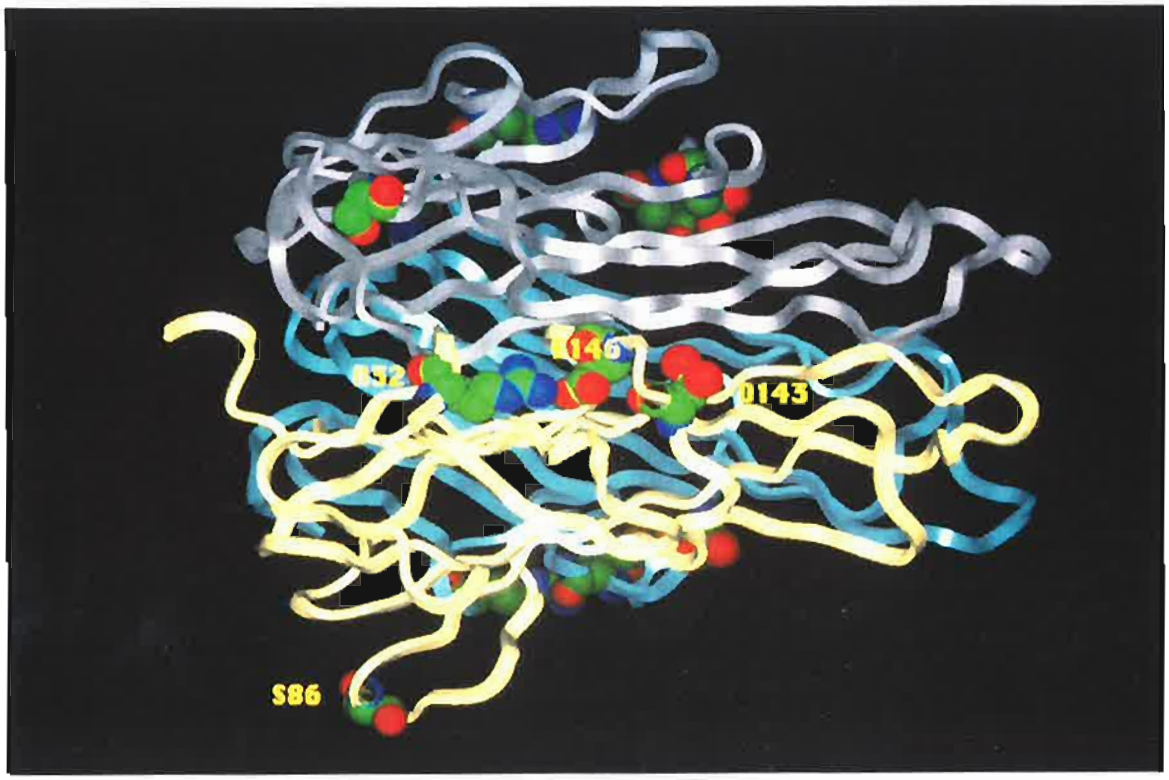
In the successful attempt to locate the active sites on TNF-α, random mutagenesis of the TNF gene was performed and inactive molecules were selected on the basis of their cytotoxic activity against murine L-M cells (Yamagishi et al, 1990) or murine L929 cells (Van Ostade et al, 1991). A considerable decrease in TNF activity was seen when alterations were introduced in amino acid regions 29-36, 84-91 and 143-148, without marked changes in immunoreactivity or physicochemical characteristics. Some of these human TNF mutants were subsequently shown to exhibit selective binding to the TNF receptors (table 1.1; figure 1.4) (Loetscher et al, 1993; Van Ostade et al, 1993; Van Ostade et al, 1994). In view of the likely role of mTNFR75 in the TNF-induced lethality which occurs in mice, human TNF mutants which selectively bind to TNFR55 may reduce unwanted side effects *in vivo*. The first step in the evaluation of this hypothesis is to critically examine the functional roles of the TNF receptors in the mediation of TNF-induced proinflammation and cytotoxicity *in vitro*. The use of TNF mutants with selective binding characteristics enables such an examination to be undertaken.

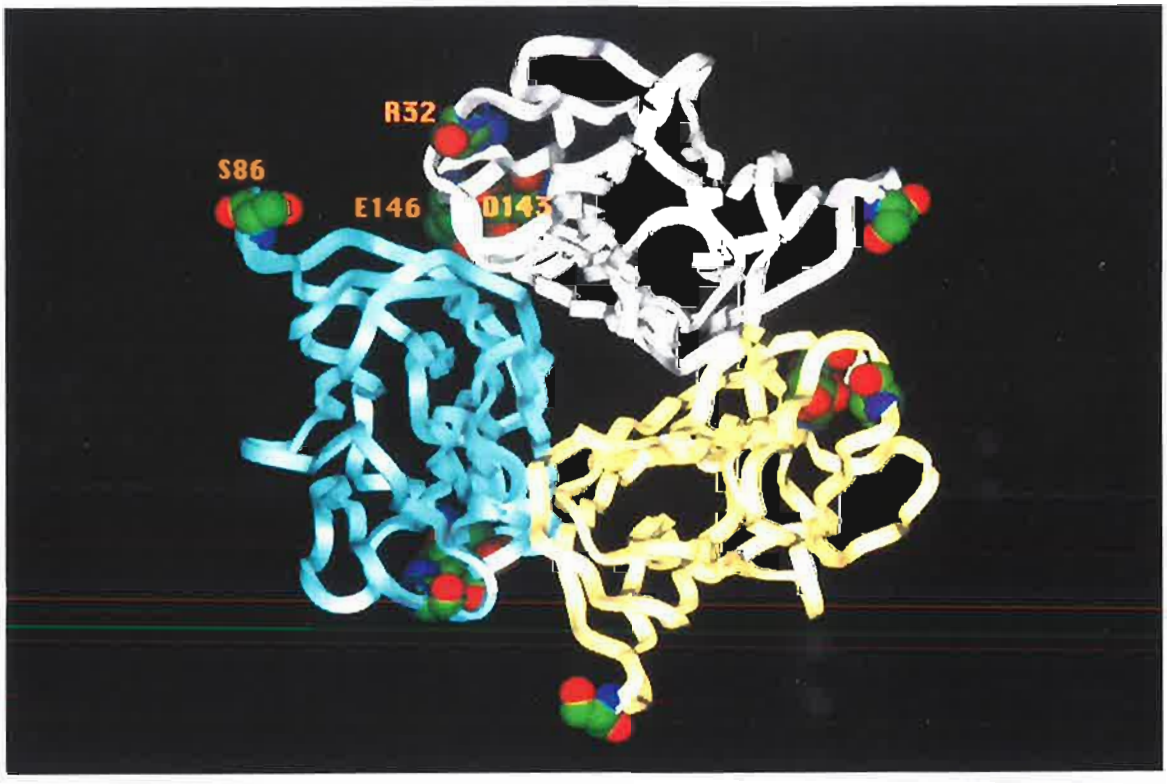
Table 1.1 The relative binding affinities of the TNF mutants

TNF mutant	Relative TNFR55 affinity	Relative TNFR75 affinity	Specificity
Wild-Type	1	1	R55 = R75
R32W arginine to tryptophan	0.69	0.002	R55 > R75
E146K glutamate to lysine	0.5	0.0003	R55 >> R75
R32W-S86T arginine to tryptophan serine to threonine	0.45	<0.0002	R55 >> R75
D143F aspartate to phenylalanine	<0.0002	0.03	R75

Figure 1.4 TNF-α structure highlighting the amino acids which are substituted in the TNF mutants (R32W, E146K, R32W-S86T and D143F)

The ribbon backbone of the TNF homotrimer is demonstrated and was generated using SiliconGraphics computer-ware and the coordinates of Eck and Sprang (1989). The highlighted amino acids are in close proximity to the receptor binding pocket and represent those residues which have been substituted to produce the TNF receptor-selective mutants used in this thesis. The specific amino acids are shown in ball structure (green = neutral components, red = acidic, blue = basic).


E146
D143
S86


R32
S86
E146
D143

MATERIALS and METHODS

Chapter 2

2.1 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 sulphate fractionation and polyethylenimine precipitation followed by purification on a MONO-Q chromatography column (Pharmacia, Sweden). Only the wild-type TNF 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 Biorad protein dye reagent (Van Ostade et al, 1991). The content of endotoxin was <2.5 ng/mg as determined by the limulus assay.

2.2 Cell Lines

HEp-2 cells, U937 cells and P815 cells were cultured in RPMI-1640 with 10% foetal 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 β-mercaptoethanol (2-ME; 5 x 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.

2.3 Cell Purification

2.3.1 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 450g 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 (bovine serum albumin; fraction V, fatty acid-free, Boehringer Mannheim, Sydney, Australia). This method yielded cells which were >98% viable by trypan blue exclusion and 96% identifiable as neutrophils (neutrophils 96.0 ± 0.18%, eosinophils 2.4 ± 0.31%, basophils 0.3 ± 0.25%, monocytes 0.4 ± 0.13%, lymphocytes 1.0 ± 0.35%).

2.3.2 Peripheral Blood Mononuclear Cell Preparation

Twenty ml of venous blood is diluted 1:1 with Hanks Buffered Salt Solution (HBSS: KCl 5.4 mM, KH_2PO_4 0.4 mM, NaCl 136.9 mM, $NaHCO_3$ 4.2 mM, Na_2HPO_4 0.1 mM). 10 ml of Lymphoprep (Nycomed, Oslo, Norway) was underlayed and the samples centrifuged for 25 min at 1600 rpm in the Beckman J-6 M/E centrifuge (Beckman Instruments, Palo Alto, CA). The layer containing monocytes and lymphocytes was removed, washed in HBSS, centrifuged for 5 min at 1500 rpm and resuspended in RPMI-1640 medium containing 10% FCS. This method yielded cells which were >98% viable by trypan blue exclusion and >31% identifiable as monocytes and >68% as lymphocytes (monocytes 31.2 ± 1.80%, lymphocytes 68.2 ± 1.81%).

2.3.3 Purification of Human Monocytes

The method of monocyte purification is based on the protocol of Sanderson et al (1977). Fresh blood obtained from healthy donors at the Red Cross blood bank (Pirie street, Adelaide) was diluted 1:1 with HBSS. This was underlayed with Lymphoprep and centrifuged in the Beckman J-6M/E at 1800 rpm for 25 min at room temperature (no brakes on deceleration). Supernatant is aspirated off above the layer containing mainly monocytes and lymphocytes. This layer is pooled and subsequently washed with HBSS. After spinning at 1500 rpm for 5 min the cells are resuspended in HBSS. The next spin at 1000 rpm for 5 min is to remove platelets. The cells are then resuspended in Elutriation Buffer (HBSS, FCS 0.1%, EDTA 0.01%, glucose 0.1%) and placed in a Beckman JE-6B elutriator using the Sanderson chamber, with a rotor speed of 2050 rpm and a flow rate of 12 ml/min. The monocytes remain

confined to the elutriation chamber and the lymphocytes are eluted. To collect the monocytes the rotor is stopped and the flow pump is increased. The monocytes are then centrifuged (1500 rpm, 5 minutes) and resuspended in RPMI-1640 medium/10% FCS. This method yielded cells which were >98% viable by trypan blue exclusion and >85% identifiable as monocytes. The differential comprised monocytes 85.9 ± 2.10%, lymphocytes 5.8 ± 0.98%, neutrophils 5.5 ± 1.07%, eosinophils 1.4 ± 0.42%, basophils 1.6 ± 0.6%.

2.3.4 Eosinophil Purification

Eosinophils were purified on a hypertonic metrizamide gradient as previously described (Vadas et al, 1979). Venous blood is collected from a donor with an elevated peripheral eosinophil count and sedimented using dextran (Dextran T-500, Pharmacia, Uppsala, Sweden). The buffy coat is washed to remove platelets, the cells are resuspended in RPMI-1640 medium and DNAse II is added. The cell suspension is placed on top of a metrizamide gradient (Nyegaard, Oslo, Norway; gradient from top to bottom: 20%, 22%, 23% and 24% metrizamide). The gradients are centrifuged at 2400 rpm for 45 min (Beckman J-6 M/E) and the cell layers are carefully separated. The metrizamide is diluted in Tyrodes buffer containing gelatin (0.1%).

2.3.5 Endothelial Cell Preparation

Human umbilical venular endothelial cells (HUVEC) were isolated by collagenase treatment of human umbilical cord veins obtained within 24 hours of delivery (Wall et al, 1978). After flushing the veins they are filled with collagenase and incubated in a 37°C waterbath for 10 min. The veins are then washed with the washings being retained and centrifuged, and the cells are resuspended in endotoxin-free RPMI-1640 medium with 10% FCS and endothelial cell growth supplement which contains basic FGF. Endothelial cells were cultured in 25 cm^2 or 75 cm^2 gelatin coated plastic flasks and when confluency was reached they were harvested

by detachment with trypsin/EDTA and passaged (1 in 2 split). Cells between passages 2 - 6 were used for the experiments. Endotoxin levels in media were < 50 pg/ml by limulus assay.

2.4 Microscopy

2.4.1 Light Microscopy

Cells were centrifuged onto glass slides at 1000 rpm for 5 minutes using the Cytospin 2 (Shandon Southern Products Ltd, Cheshire, England). Following staining with Jenner-Giemsa (Merck, Darmstadt, Germany), the cells were viewed with a light microscope (Olympus BH-2, Tokyo, Japan).

2.4.2 Fluorescent Microscopy

At the conclusion of the experiment the neutrophils (1 x 10^6 per sample) are centrifuged at 1200 rpm for 2 minutes (Beckman J-6 M/E). The supernatant is removed and the cells are gently resuspended in the residual volume. 20 µl of cell suspension is placed on a glass slide to which is added an equal volume of dye solution containing acridine orange (8 µg/ml; BDH Chemicals Ltd, Poole, England) and ethidium bromide (4 µg/ml; Boehringer Mannheim, Germany). After mixing, a glass coverslip is placed on the slide and the cells are examined without delay using the fluorescent microscope (blue filter; Olympus BH-2 RFL, Tokyo, Japan).

2.4.3 Transmission Electron Microscopy of Neutrophils

The neutrophils are purified and exposed to TNF-α. At the completion of the experiment, the cells are fixed in 2.5% glutaraldehyde in cacodylate buffer (Probing and Strucutre, Thuringowa Central, Queensland) and spun at 500 rpm for 5 minutes. The cells are placed in Osmium tetroxide (2%; BDH Ltd, Poole, England) and spun again. This is followed by two washes in absolute ethanol to dehydrate the samples and then the neutrophils are placed in Spurr's epoxy resin (Probing and Structure, Thuringowa Central, Queensland). The cells are

spun in a plastic capsule followed by 15 hours in an oven at 60°C. A 0.5 μm slice stained with toluidine blue is used as a survey section and then an ultrathin section is placed on a copper grid (100 μm; Taab Laboratories, England) and stained with uranyl acetate (Ajax chemicals, Auburn, NSW) and lead citrate (K & K Laboratories, Plainview, New York). The cells are now ready for transmission electron microscopy (JEOL 1200EX; print magnification 7800x).

2.5 Flow Cytometry

The monoclonal antibodies htr-9 (recognises TNFR55) and utr-1 (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 x 10^5 cells at 10 μg/ml (45 minutes, 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 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). Using the same technique, Fas antigen was detected with the monoclonal antibodies M3 and M38 (IgG1; neutralizing antibodies; gift from Mark Alderson, Immunex, Seattle). Frequency histograms of fluorescence intensity for each cell population were constructed by the Profile software and the MFI (mean fluorescence intensity) of each population was calculated. Histograms were mathematically smoothed, without changing the shape of the curve, using a program written by Mr Alan Bishop (Flow Cytometry supervisor, Hanson Centre for Cancer Research, IMVS)

2.6 TNF Cytotoxicity Assay

Target cells (HEp-2) at 4 x 10^4/well were incubated at 37°C for 2 hours. Serial dilutions of wild-type or mutant TNF were prepared with 100 μg/ml cycloheximide (CHX; not serially diluted) and added to each well. The cells were incubated for 18 hours at which time the supernatant was removed and the residual cells were stained with 0.2% crystal violet (BDH Chemicals, Kilsyth, Vic., Australia) in 25% (v/v) methanol for 15 minutes (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).

2.7 Cytostasis of a Human Monoblastoid Leukaemic Cell Line U937

Leukaemic cells ($5x10^4$/well) were incubated with wild-type or mutant TNF at 37°C for three 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 hours 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).

2.8 Superoxide Anion Generation

Superoxide anion release was measured in a colorimetric assay based on the reduction of ferrocytochrome C. Neutrophils were incubated at 37°C for 45 minutes with ½log dilutions of wild-type or mutant TNF. These cells, at a final concentration of 10^6/ml, were incubated at 37 °C for 15 minutes with 10^{-7} M FMLP (N-formylmethionylleucylphenylalanine; Sigma, St.Louis, MO) and Cytochrome C (type VI, 12.4 mg/ml; Sigma). After incubation, the cells were rapidly cooled and pelleted at 4°C, and supernatants were transferred to plastic disposable cuvettes (Kartelle Plastics, Adelaide, South Australia). Superoxide production was measured by the reduction of Cytochrome C monitored at 550 nm using a DU-50 spectrophotometer (Beckman, Palo Alto, CA). Levels of superoxide were quantitated in nmoles per 10^6 neutrophils as described previously (Lopez et al, 1986) and converted to a percentage.

2.9 PAF Synthesis and Bioassay

After stimulation with wild-type or mutant TNF for 45 min (dose response experiments) the neutrophils/monocytes were extracted into ice-cold ethanol (80%) overnight, evaporated to dryness under reduced pressure and reconstituted in Tyrode's solution (HEPES 10 mM, NaCl

137 mM, $NaHCO_3$ 11.9 mM, NaH_2PO_4 0.4 mM, KCl 2.7 mM, $MgCl_2$ 0.26 mM, D-glucose 11 mM and BSA 0.25% [fraction V, fatty acid-free] 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 femtomoles/10^7 cells. Biologically active material extracted from cells was characterised 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).

2.10 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 Cr^{51} (Du Pont, North Ryde, Australia) at 37°C for 1 hour. The cells were washed with PBS and resuspended in 500 μl PBS, to which 100 μl trinitrophenyl was added (10 mg/ml in PBS) followed by an incubation period of 20 mins at room temperature. After washing twice with medium containing FCS to remove uncoupled DNP, the P815 cells were resuspended in RPMI-1640/0.1% BSA at $1x10^5$/ml. Neutrophils were resuspended to $1.5x10^6$/ml. The final reaction mixture was set up in 96-well V-bottom microtitre plates (Costar, Cambridge, MA) and comprised the following: 16 μl wild-type or mutant TNF, 24 μl anti-TNP antibody (polyclonal, ICN Biomedicals, CA), 40 μl P815 ($4x10^3$/well), 80 μl neutrophils ($1.25x10^5$/well), resulting in a killer to target cell ratio of 30:1. The mixture was incubated at 37°C for 2½ hours. Without disturbing the cell pellet, 80 μl was removed and counted on the gamma counter (Cobra 5010, Packard Instrument Company).

2.11 Neutrophil Adherence Assay

Neutrophil adherence was determined as previously described (Gamble et al, 1985). HUVE cells were plated at 10^5 cells per cm^2 and grown to confluence. To each well ^{51}Cr-labelled neutrophils ($5x10^5$) and wild-type or mutant TNF was added for 30 minutes, however in the case of primary HUVEC stimulation TNF was added to the HUVE monolayer 4 hours 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 cpm in NH_4OH lysate/total ^{51}Cr cpm added) x 100.

2.12 E-selectin (Endothelial Leucocyte Adhesion Molecule-1) Expression

Endothelial cells were stimulated with wild-type or mutant TNF for 4 hours. Antibody directed to E-selectin (mAb 9D9 raised in the laboratory) was added for 30 minutes at 37°C, followed by sheep anti-IgG Fab_2-FITC labelled antibody (Silenus, Vic., 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.

2.13 Neutrophil Transmigration

Transwells (Costar, Cambridge, MA) (6.5 mm diameter, polycarbonate membrane with 3 μm pores) were prepared with confluent endothelial monolayers as described (Smith WB et al, 1991). Monolayers were preincubated with wild-type or mutant TNF for 4 hours prior to the assay. Monolayers were washed with medium then 10^6 neutrophils were added and the transwells placed in 24-well cluster dishes for 45 minutes. 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 taken for counting of migrated neutrophils

in a Coulter Counter (model ZF; Harpenden, Herts, U.K.). Counts were expressed as a percentage of the cells added.

2.14 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 hours, the medium was changed and 1 hour 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 Kunkel SL, Ann Arbor, Michigan. Samples were added and IL-8 was detected with biotinylated polyclonal anti-IL-8 (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) and read at 490 nm on the spectrophotometer.

2.15 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 x 10^4 cells/well were exposed to wild-type TNF, D143F or E146K in the presence of hIL-1β (1 ng/ml). After 24 hours, the supernatants were tested for rat GM-CSF activity in an FDCp1 proliferation assay.

2.16 Radioiodination of $cPLA_2$

$cPLA_2$ was iodinated by reaction with iodine monochloride (ICl; Sigma, St. Louis, MO) and ^{125}I (Du Pont, Sydney, Australia) as previously described (Contreras et al, 1983). Briefly, $cPLA_2$ (2 mg) is diluted in 90 ml of PBS pH 7.0, and 1 mCi (37 MBq) ^{125}I.Na added in 10 µl

The ICl solution (0.02 M) is diluted 1:100 in 2 M NaCl and 2 ml is added to the protein solution. After incubating for one minute at room temperature a further 5 ml of ICl solution is added for another minute. This is applied immediately to a Sephadex G-25 PD10 column (Pharmacia, Uppsala, Sweden) which has been pre-equilibrated with elution buffer (PBS with 0.02% tween20) to separate the protein from free $^{125}I^-$. Fractions are collected and the specific radioactivity based on the known mass of reacted protein is calculated.

2.17 $cPLA_2$ Phosphorylation

The monocytes are resuspended in phosphate free buffer (pH 7.4, filter sterilised; HEPES 30 mM, NaCl 110 mM, KCl 10 mM, glucose 10 mM, $CaCl_2$ 1 mM, $MgCl_2$ 1 mM, L-glutamine 2 mM, BSA 0.2%; glutamine and BSA added fresh) at 10^7 per ml for 60 min at 37°C. ^{32}Phosphorus (^{32}P; in HCl-free water; Bresatec, Adelaide, Australia) is then added at 1 mCi/ml for 90 min at 37°C with the monocytes at approximately $3x10^7$/ml. The cells are then washed twice, resuspended in buffer, and subjected to TNF-α or TNF mutants. At the end of the stimulation period the cells are placed on ice and an equal volume of lysis buffer (RIPA x2) is added (RIPA buffer: NP40 2%, SDS 0.2%, NaCl 0.15 M, NaH_2PO_4 10 mM, Na_2HPO_4 10 mM, sucrose 0.68 M, EDTA 5 mM, NaF 10 mM, sodium pyrophosphate 5 mM, sodium orthovanadate 1 mM, ATP 1 mM, PMSF 1 mM, leupeptin 1 µg/ml, pepstatin 1 µg/ml, chymostatin 1 µg/ml, antipain 1 µg/ml, DNAse 100 µg/ml, FCS 5%, pH 7.2). After 30 min, the samples are centrifuged at 25,000 rpm for 20 min at 4°C and the supernatants are transfered to new tubes. Preclearance with protein A sepharose beads (CL-4B, Pharmacia Biotech, Uppsala, Sweden) overnight at 4°C is followed by immunoprecipitation of $cPLA_2$ protein with monoclonal antibody to $cPLA_2$ (1 mg/ml; Genetics Institute, Cambridge, MA), rabbit anti-mouse polyclonal antibody (2 ml, Dako Corporation, Carpinteria, CA) and protein A sepharose beads, each step occurring for 60 min at 4°C. In the initial phosphorylation experiments polyclonal $cPLA_2$ antibody was used in a volume ratio of 1:400. The supernatant is then removed, the beads are washed 5 times with ice-cold RIPA buffer and 40 µl of

reducing buffer added. After heating for 2 min at 100°C the samples are centrifuged and the supernatants are loaded onto a 10% SDS-polyacrylamide gel. The gel is dried and exposed to radiograph film and phosporimager plate. Quantitation of $cPLA_2$ bands was carried out on the Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

2.18 Western Blotting

$cPLA_2$ was immunoprecipitated as described above and electrophoresed on a 10% SDS-polyacrylamide gel. The gel was placed in transfer buffer (tris-Cl 20 mM, glycine 150 mM, methanol 20% and SDS 0.1%) for equilibration and a transfer sandwich was set up with equilibrated sponges, filter paper and nitrocellulose paper. The transfer to nitrocellulose occurred at 25 V overnight in a trans-blot cell (Bio-Rad, North Ryde, Australia). The nitrocellulose is placed in TNT (tris-Cl 1 mM, NaCl 15 mM, tween20 0.005%, pH 8) with 3% BSA (Sigma A0281 - fatty acid and globulin free), sealed in plastic and put on a shaker for 1.5 hr. After washing three times with TNT, the nitrocellulose is incubated with either the anti-phosphotyrosine antibody (mAb PY20, 1 µg/ml, IgG2b, ICN, Costa Mesa, CA) or $cPLA_2$ antibody (mAb, 1 µg/ml) in 5 ml of TNT with 1% BSA, sealed in plastic and shaken for 1.5 hr. Incubation with ^{125}I-labelled Protein A (80 µCi/ml, Du Pont NEN products, Boston, MA) follows four washes with TNT. 40 µl ^{125}I-labelled Protein A is added to 40ml of TNT containing 1% BSA. After 30 minutes on a shaker the nitrocellulose is washed four times with TNT and then quantified on a Phosphorimager or autoradiographed. With the $cPLA_2$ antibody an interim step with rabbit anti-mouse polyclonal antibody (2 ml, Dako, Denmark) is required before the ^{125}I-labelled Protein A is applied. Reuse of western blots was achieved by washing the filter for 30 minutes at 60°C in a mixture of sodium phosphate 0.005 M (pH 7.5), β-mercaptoethanol 0.002 M and SDS 2%.

2.19 Phosphoamino acid Analysis

The method for two-dimensional phosphoamino acid analysis has been adapted from the published method of Cooper et al (1983). ^{32}P-labelled cPLA$_2$ was separated on a 10% SDS-polyacrylamide gel and dried onto cellophane. After locating the cPLA$_2$ bands using the Phosphorimager they were removed and placed in eppendorf tubes. 1 ml fresh NH_4HCO_3 (50 mM) is added for 5 minutes at room temperature to allow rehydration to occur. After grinding, 50 µl of β-mercaptoethanol (14.3 M) and 10 µl of SDS (10%) are added and the samples are boiled for 5 min followed by >90 min at room temperature. The eluate is placed on ice and 20 µg of heat-treated RNAse A (pancreatic; Sigma, St Louis, MO) is added, followed by 250 µl ice-cold trichloroacetic acid (100%). The mixture is left on ice for 60 min and then microfuged for 10 min at 4°C. The supernatant is removed, the pellet is washed with 500 µl ice-cold absolute ethanol and air dried. The pellet is resuspended using 75 µl constant boiling HCl (5.7 M). After 60 min at 110°C the samples are dried in a Speed Vac (SVC 100, Savant Instruments, Farmingdale, NY) and resuspended in 5µl of pH 1.9 buffer (formic acid 100%:acetic acid:water; 22:78:900 v:v:v) which contains 15 parts of buffer to 1 part of cold phosphoamino acid standards (phosphoserine, phosphotyrosine and phosphothreonine at 1 mg/ml). The ^{32}P-labelled phosphoamino acids were analysed by two-dimensional electrophoresis on thin-layer cellulose plates using an HTLE-7000 (C.B.S Scientific Company, Del Mar, CA). The first stage was carried out in pH 1.9 buffer at 1.5 kV for 20 minutes. After drying, the buffer was changed to pH 3.5 buffer (acetic acid:pyridine:water; 50:5:945 v:v:v) and the plate was loaded onto the apparatus at 90° counterclockwise. Electrophoresis in the second stage occurred for 16 minutes at 1.3 kV followed by drying of the plate. The phosphoamino acid standards were visualised by spraying with 0.25% ninhydrin in acetone (w/v) and the ^{32}P-labelled amino acids were identified by analysis on Phosphorimager and autoradiography.

2.20 Extraction of Genomic DNA from Neutrophils and DNA Laddering Gels

The preparation of genomic DNA from neutrophils requires cell lysis and deproteinization followed by the recovery of DNA. After stimulating the neutrophils with TNF-α and/or TNF mutant, the cells are pelletized followed by resuspension in 2 mls of solution containing sodium acetate (150 mM) and EDTA (5 mM, pH 7). A further 2 mls of sodium acetate/EDTA is added with proteinase K (0.2 mg/ml; Merck, Darmstadt, Germany) and SDS (1%). The tubes are inverted several times and incubated at 55°C overnight. Fresh proteinase K is added (0.1 mg/ml), mixed for 10 minutes then incubated at 55°C for a further hour. After cooling to room temperature 4 ml of phenol is added, mixed for 30 minutes then centrifuged at 3700 rpm for 10 minutes (Beckman J-6 M/E). The DNA phase is carefully removed with a short Pasteur pipette and extracted in an equal volume of phenol/chloroform. After centrifuging at 3700 rpm for 10 minutes the top layer is removed and extracted in an equal volume of chloroform. Two volumes of absolute ethanol are added and, after slow mixing, the DNA precipitates. The precipitate is washed in ethanol (75%) followed by absolute ethanol and then dried. The pellet is dissolved in 250 µl TE (TrisCl 10 mM, EDTA 1 mM, pH 8) at room temperature and quantified by reading the absorbance at 260 nm on the DU-50 Spectrophotometer (Beckman, Palo Alto, CA). Loading buffer (bromophenol blue 0.25%, xylene cyanol FF 0.25%, 30% glycerol in water) containing RNAse A (1:100 dilution of 10 mg/ml; Sigma, St Louis, MO) is added to 3-10 µg of DNA and placed in a 1.2% agarose gel. The gel is electrophoresed at 40 V in TAE buffer (Tris-acetate 0.04 M, EDTA 0.001 M). Markers used include the 1 kb ladder (Gibco BRL, Glen Waverley, Victoria) and SPP1 (Bresatec, Adelaide, South Australia). The gel is placed in ethidium bromide and photographed using a Polaroid camera and UVP Transilluminator (UVP, San Gabriel, CA).

2.21 Propidium Iodide Binding to DNA

3 x 10^6 neutrophils are pelletted and resuspended in 1.5 mls of propidium iodide solution (propidium iodide 50 µg/ml [Sigma, St. Louis, MO], 0.1% Triton X-100, 0.1% sodium citrate, pH 7.5). The samples are stored at 4°C, in the dark, overnight. Analysis by flow cytometry using the Coulter EPICS Profile II Flow Cytometer follows.

2.22 Statistical Analysis

All results are shown as mean ± standard error of the mean (SEM) unless otherwise indicated. *P* values were calculated by the Student's *t*-test or analysis of variance (ANOVA) as indicated. The potencies of the TNF mutants were calculated relative to the wild-type TNF according to the formula: (TNF wild-type EC_{50} / TNF mutant EC_{50}) x 100.

DISSOCIATION OF THE CYTOTOXIC AND PROINFLAMMATORY ACTIVITIES OF TNF-α

Chapter 3

INTRODUCTION

Tumour necrosis factor-alpha (TNF-α), a 17kD protein which exists in the biologically active, physiological form as a homotrimer, is produced primarily by activated macrophages and was originally characterised as a protein that induced necrosis of Meth A sarcomas *in vivo* (Carswell et al, 1975). TNF-α 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; Balkwill et al, 1986; 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 selectively kill 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 proinflammatory effects which have been manifested in human clinical trials by dose-limiting hypotension, hepatotoxicity, pulmonary oedema, thrombocytopenia, intravascular thrombosis and haemorrhage (Kilbourn et al, 1990; Van Der Poll et al, 1992). 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 haemopoietic 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; Slowik 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 pleiotropic 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; Heller et al, 1993; Tartaglia et al, 1993). In addition, the role of these two receptors in neutrophil priming, mediator release and adhesion to endothelium remains unknown. In order to evaluate the functions of the two receptors in the human system, hTNF mutants were constructed which interact selectively with the TNF receptors.

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 >6666-fold decrease respectively compared to wild-type TNF. 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. The physical location of the substituted amino acids is shown in figure 1.4 and the relative binding affinities of the TNF mutants are summarized in table 1.1.

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 (Lewis et al, 1991), it was concluded that TNFR75 contributes specifically to the severe toxicity (Brouckaert et al, 1993; Fiers, 1993). Therefore TNF mutants which selectively bind to TNFR55 might result in less toxicity when administered as an antineoplastic agent to humans. In this chapter the roles of the two TNF receptors in the mediation of TNF's cytotoxic and proinflammatory activities were examined. It was 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 tumouricidal and tumouristatic potency. However, the TNFR75-selective mutant D143F was unable to stimulate neutrophils or endothelium. The 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 elicit proinflammatory side effects.

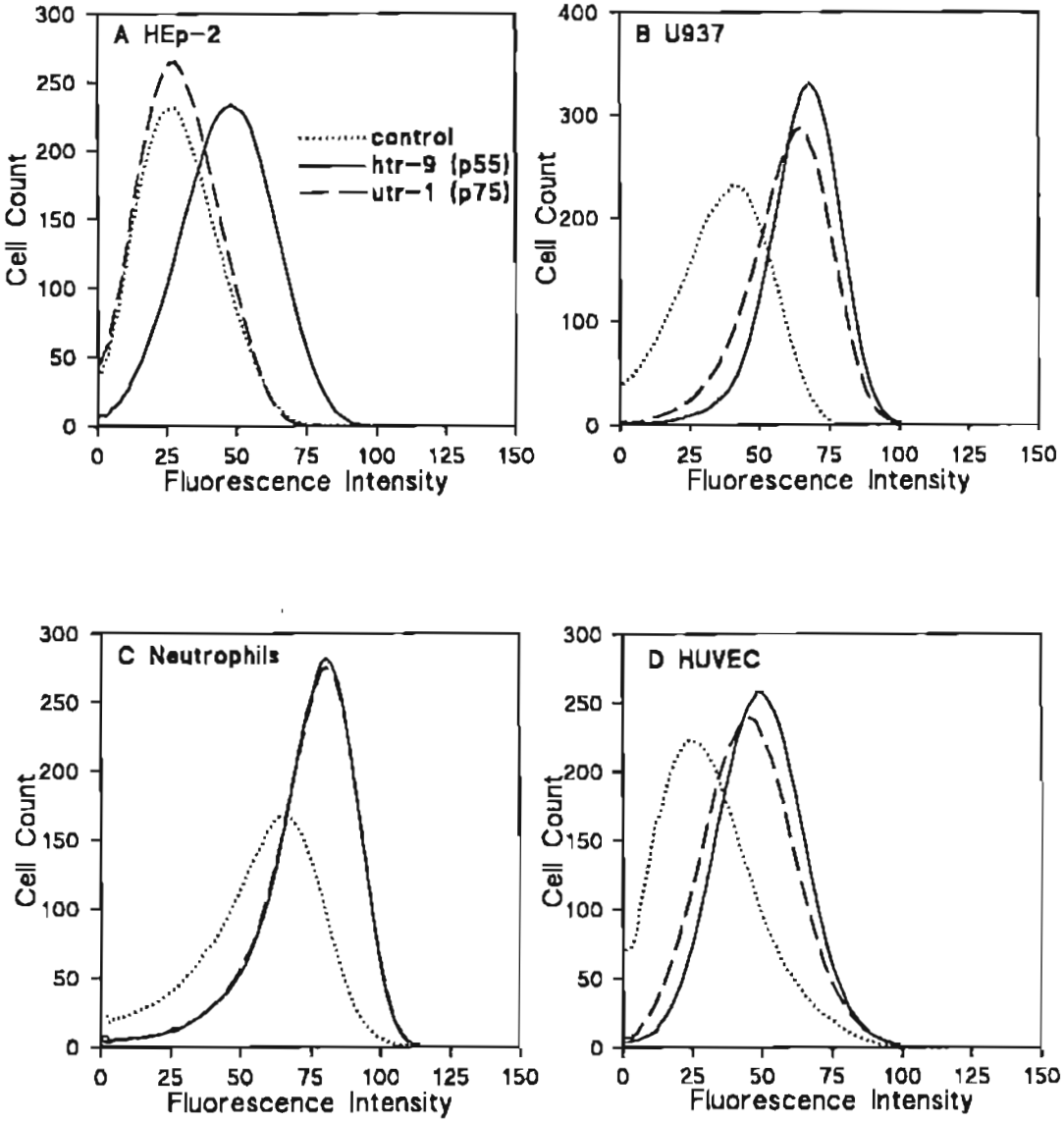
RESULTS

3.1 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 3.1). HEp-2 cells express only TNFR55 providing a unique setting in which to examine the role of TNFR55 in cytotoxicity. In contrast, neutrophils, HUVEC and U937 cells express approximately equal numbers of both receptors consistent with previous reports (Hohmann et

Figure 3.1 Flow cytometry demonstrating the presence of TNF receptors on cells

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 to TNFR55 (solid line) and utr-1 monoclonal antibody to TNFR75 (dashed line).


A HEp-2
control
htr-9 (p55)
utr-1 (p75)
Cell Count
Fluorescence Intensity
B U937
C Neutrophils
D HUVEC

al, 1989; Brockhaus et al, 1990; Hohmann et al, 1990; Shalaby et al, 1990; Porteu et al, 1991; Mackay et al, 1993; Slowik et al, 1993).

3.2 Cytotoxicity and Cytostasis of Tumour Cells

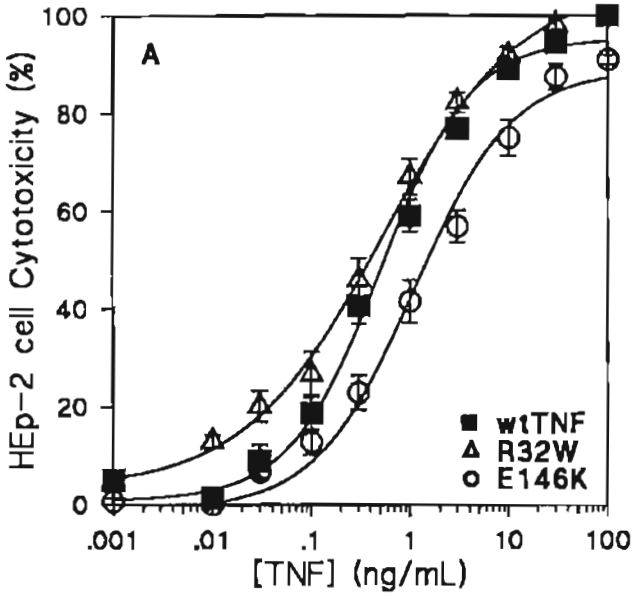
TNF-α, in the presence of cycloheximide (a protein synthesis inhibitor), was directly cytotoxic, in a dose-dependent manner, to the human laryngeal carcinoma cell line HEp-2. In experiments comparing the relative potencies of TNFR55-selective mutants R32W and E146K to wild-type TNF, it was found that 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 3.2A). R32W-S86T demonstrated a less than 2-fold reduction in cytotoxicity. In addition, TNF-α inhibited proliferation of the monoblastoid leukaemic cell line U937 in a dose-dependent manner. These mutants were also capable of inhibiting U937 cell growth with R32W being as potent as wild-type TNF, and E146K exhibiting a 4-fold reduction in potency (figure 3.2B). A summary of the cytotoxic and cytostatic activities and relative potencies of the TNFR55-selective mutants is shown in table 3.1. The same relative potencies were obtained with the TNFR55-selective mutants in HEp-2 cytotoxicity when a shorter incubation time of 90 minutes was examined (wtTNF 100%, R32W 177.8%, E146K 22% and R32W-S86T 56.9%).

3.3 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, those activities most likely to be important in exacerbating systemic inflammation were examined. Since TNF-α is known to stimulate the function and production of inflammatory mediators from neutrophils (Shalaby et al, 1985; Klebanoff et al, 1986; Atkinson et al, 1988) the TNFR55-selective mutants were examined for their ability to activate human neutrophils and stimulate

Figure 3.2 The antitumour activities of the TNFR55-selective mutants

(A) The cytotoxic activity of R32W (8 pooled experiments) and E146K (3 pooled experiments) compared to wild-type TNF on HEp-2 cells in the presence of 50 µg/ml cycloheximide. (B) The cytostatic activity of R32W (3 pooled experiments) and E146K (3 pooled experiments) relative to wild-type TNF on U937 cells. Data points represent means ± SEM. Wild-type TNF - closed square, R32W - open triangle and E146K - open circle.


A
HEp-2 cell Cytotoxicity (%)
100
80
60
40
20
0
.001
.01
.1
1
10
100
[TNF] (ng/mL)
wtTNF
R32W
E146K

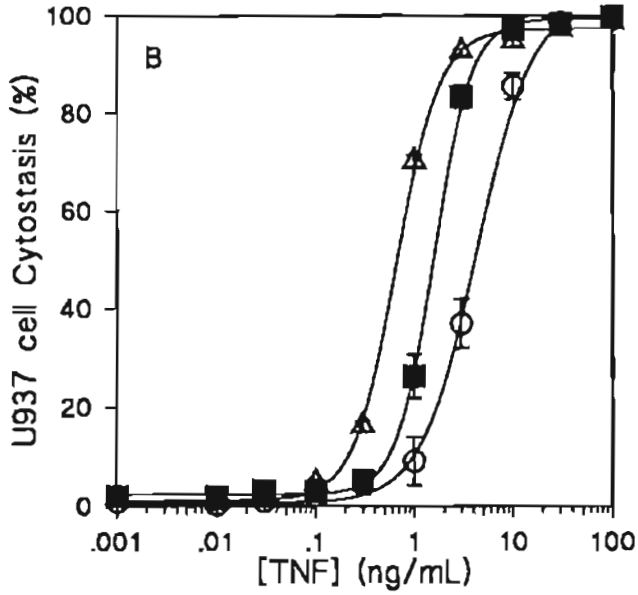

B
U937 cell Cytostasis (%)
100
80
60
40
20
0
.001
.01
.1
1
10
100
[TNF] (ng/mL)

Table 3.1 The relative cytotoxic and cytostatic activities of the TNF mutants

	Cytotoxicity		Cytostasis
	HEp-2		U937
	na Biological Activity (U/mg)	EC50b (ng/mL)	n EC50 (ng/mL)
wt-TNF	9.95 x 106 ± 1.74c **(100%)**d	0.65 ± 0.09	1.24 ± 0.15
R32W	8 1.72 x 107 ± 0.45 **(166.8%)**	0.43 ± 0.09	3 0.65 ± 0.02 **(236.9%)**
E146K	3 3.03 x 106 ± 0.66 **(24.6%)**	1.82 ± 0.40	3 3.97 ± 0.05 **(23.7%)**
R32W-S86T	2 3.35 x 106 ± 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 % = wild-type TNF EC50 / TNF mutant EC50) x 100.

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 it was 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 wild-type TNF 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 wild-type TNF respectively. TNF-α induced PAF production in human neutrophils in a dose-dependent manner (Camussi et al, 1987). In experiments comparing the TNF mutants to 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 TNF is shown in figure 3.3 and a summary of the relative potencies of the TNFR55-selective mutants are listed in table 3.2. The relative potency of R32W-S86T was very similar to that of the more widely examined E146K (table 3.2).

3.4 Regulation of Endothelial Cell Function

The TNFR55-selective mutants were examined for their ability to upregulate E-selectin expression, enhance the adhesiveness of endothelium for neutrophils, stimulate the transmigration of neutrophils across endothelium, secrete IL-8 and synthesize PAF. 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 to wild-type. In HUVEC adhesiveness for neutrophils (Gamble et al, 1985) it was found that

Figure 3.3 The activation of neutrophils by the TNFR55-selective mutant E146K

(A) Superoxide production (O_2^-) showing 6 pooled experiments, $p<0.01$. (B) Platelet-activating factor synthesis with 3 pooled experiments, $p<0.0001$. (C) Antibody-dependent cell-mediated cytotoxicity with 4 pooled experiments, $p<0.005$. (D) Adhesion of neutrophils to unstimulated HUVEC; representative experiment of 3 performed with triplicates, $p<0.0001$. Data points represent means ± SEM. Wild-type TNF - closed square, E146K - open circle. Data analysed by ANOVA.

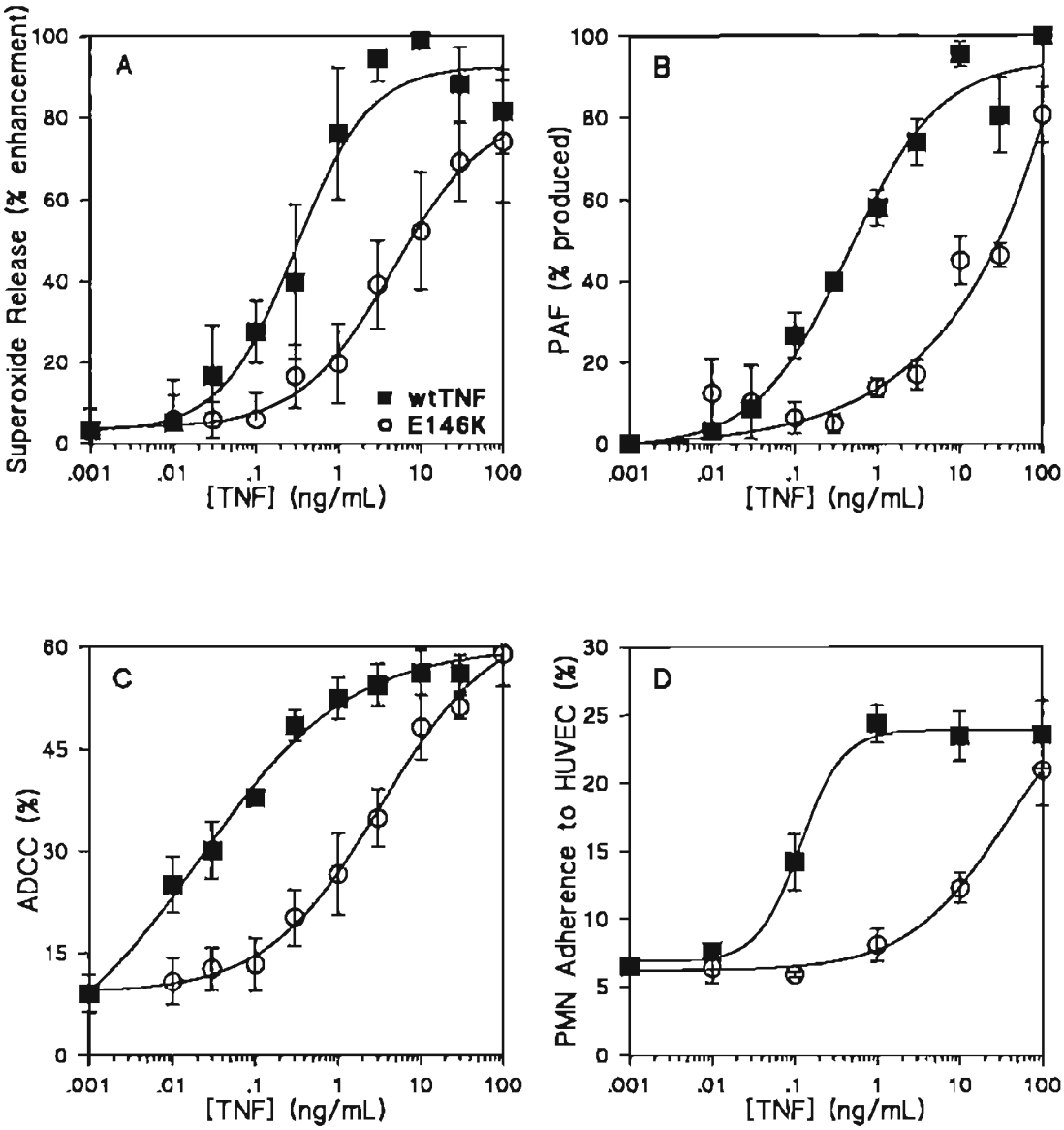

A
Superoxide Release (% enhancement)
100
80
60
40
20
0
.001
.01
.1
1
10
100
[TNF] (ng/mL)
wtTNF
E146K
B
PAF (% produced)
100
80
60
40
20
0
.001
.01
.1
1
10
100
[TNF] (ng/mL)
C
ADCC (%)
60
45
30
15
0
.001
.01
.1
1
10
100
[TNF] (ng/mL)
D
PMN Adherence to HUVEC (%)
30
25
20
15
10
5
0
.001
.01
.1
1
10
100
[TNF] (ng/mL)

Table 3.2 The relative activities of the TNF mutants on neutrophil functions

	Superoxide		ADCC		PAF		Adherence	
	na	EC50b (ng/mL)	n	EC50 (ng/mL)	n	EC50 (ng/mL)	n	EC50 (ng/mL)
wt-TNF		0.25 ± 0.04c (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 % = wild-type TNF EC50 / TNF mutant EC50) x 100.

E146K and R32W could both stimulate this activity but their potencies were 280-fold and 7-fold less than wild-type TNF respectively. TNF-α also increased neutrophil transendothelial migration in a dose-dependent fashion (Smith WB et al, 1991). E146K and R32W stimulated 15-fold and 2-fold less transmigration of neutrophils than wild-type TNF respectively. IL-8 is a recently characterised 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 wild-type TNF respectively. Surprisingly, the synthesis of PAF (cell-associated and secreted) by TNF-α in endothelium was not evident (3-10 ng/ml, 4 hr). This is contrary to the studies of Camussi et al (1987) and Kuijpers et al (1992). In the four experiments performed, histamine (10^{-5}M, 10 min) and thrombin (1U/ml, 10 min) induced PAF synthesis as previously shown by Lorant et al (1991). The relative activity of E146K compared to wild-type TNF is displayed in figure 3.4 and a summary of the relative potencies of the TNFR55-selective mutants in regulating endothelial cell interactions with neutrophils is shown in table 3.3.

3.5 Lack of Proinflammatory Activity by 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 neutrophil to stimulated HUVEC, E-selectin expression and IL-8 production by HUVEC (figure 3.5). 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 to E146K alone (figure 3.5A and C).

3.6 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

Figure 3.4 The activation of HUVEC by the TNFR55-selective mutant E146K

(A) E-selectin expression showing 7 pooled experiments, $p<0.001$. (B) Adhesion of neutrophils to stimulated HUVEC showing a representative of 3 experiments performed with triplicates; data points are means ± SEM. (C) Transendothelial migration with a representative of 3 experiments performed, $p<0.001$. (D) IL-8 secretion with a representative experiment of 4 performed with duplicates, $p<0.001$. Wild-type TNF - closed square, E146K - open circle. Data analysed by ANOVA.

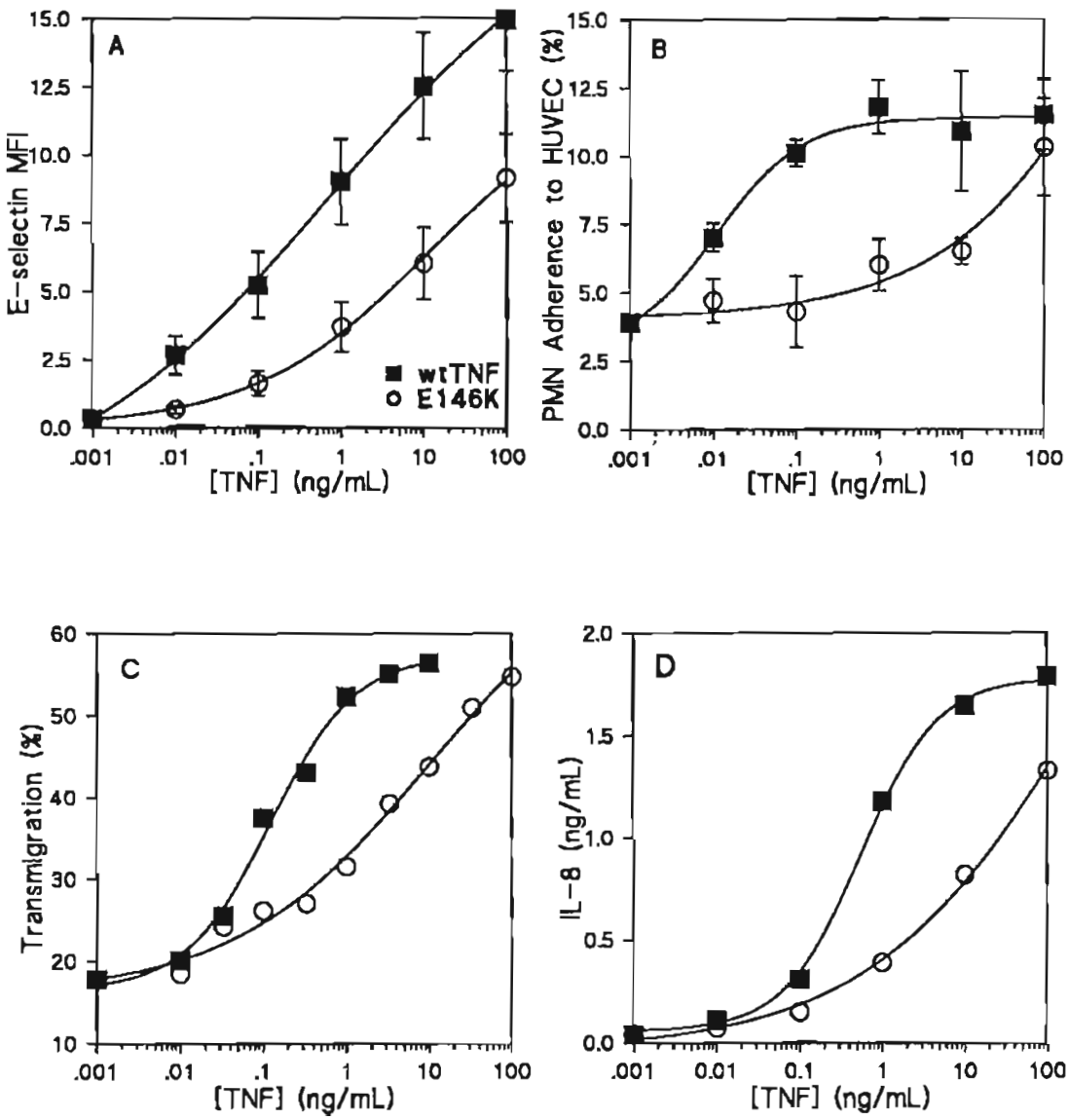

A
E-selectin MFI
15.0
12.5
10.0
7.5
5.0
2.5
0.0
.001
.01
.1
1
10
100
[TNF] (ng/mL)
wtTNF
E146K
B
PMN Adherence to HUVEC (%)
[TNF] (ng/mL)
C
Transmigration (%)
60
50
40
30
20
10
[TNF] (ng/mL)
D
IL-8 (ng/mL)
2.0
1.5
1.0
0.5
0.0
.001
0.01
0.1
1
10
100
[TNF] (ng/mL)

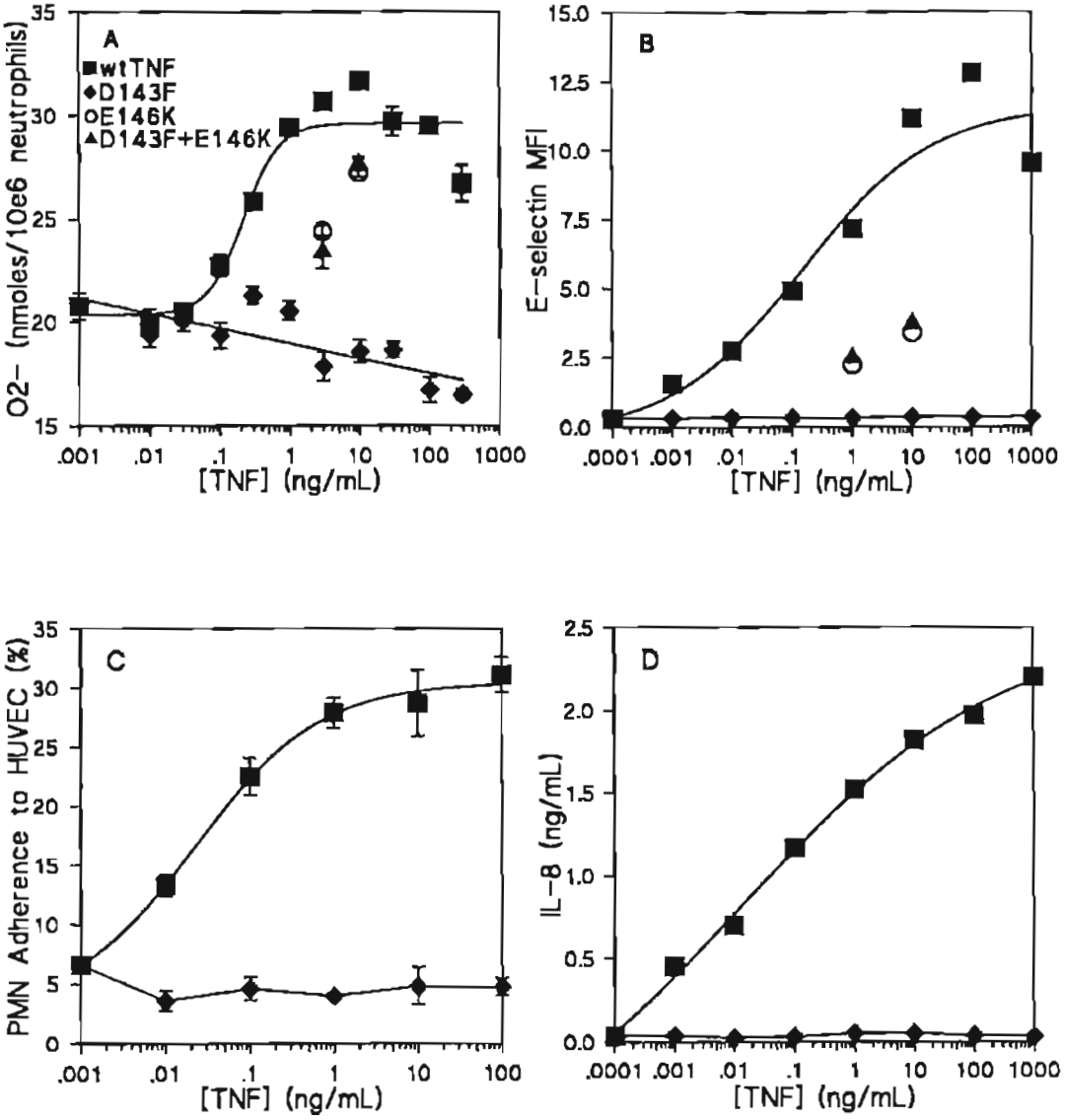
Table 3.3 The relative activities of the TNF mutants on endothelial cell functions

	E-selectin	Adherence	Transmigration	IL-8
	na EC50b (ng/mL)	n EC50 (ng/mL)	n EC50 (ng/mL)	n EC50 (ng/mL)
wt-TNF	0.58 ± 0.16c **(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 % = wild-type TNF EC50 / TNF mutant EC50) x 100.

Figure 3.5 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 104 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 and D143F+E146K - closed triangle.


A
wtTNF
D143F
E146K
D143F+E146K
O2- (nmoles/10e6 neutrophils)
[TNF] (ng/mL)
B
E-selectin MFI
[TNF] (ng/mL)
C
PMN Adherence to HUVEC (%)
[TNF] (ng/mL)
D
IL-8 (ng/mL)
[TNF] (ng/mL)

GM-CSF production by PC60 cells transfected with the human TNFR75 (table 3.4). The GM-CSF produced was approximately one third that of wild-type TNF 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.

DISCUSSION

TNF-α has diverse biological activities, of which the antitumour effect has clinical desirability and 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 (Fiers, 1993). Therefore, ameliorating these proinflammatory side effects whilst maintaining full antitumour activity may pave the way for the successful clinical application of TNF-α. The results presented here 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. These results suggest 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 less than 2-fold reduction in cytotoxicity. The HEp-2 cells express only TNFR55 (figure 3.1;

Table 3.4 GM-CSF induction in PC60-hTNFR75^{+} cells by wild-type TNF and TNF mutants

TNFa	GM-CSF (ng/ml)	SDb
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.

Brockhaus et al, 1990; Hohmann et al, 1990) and this, combined with the established poor binding of these TNF mutants to TNFR75 (Loetscher et al, 1993; Van Ostade et al, 1993; Van Ostade et al, 1994) 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 3.1; 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 (Loetscher et al, 1993; 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 (Brakebusch et al, 1992; 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.

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. The data shown here with the TNFR55-selective mutants revealed up to 170-fold decrease in activity when compared to wild-type TNF, 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. This mutant is not without activity as evidenced by the secretion of GM-CSF from PC60-hTNFR75$^+$ cells (table 3.4). A second important component of the inflammatory activities of TNF-α is at the endothelial cell barrier. The activities examined pertain directly to the

mechanisms involved in the adherence and transmigration of neutrophils through this barrier. The results, which demonstrated up to 280-fold decrease in biological activity with the TNFR55-selective mutants relative to wild-type TNF, 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. 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; Brouckaert et al, 1993). It is not yet known which cellular systems are involved in this effect but the leukocyte/endothelial system is likely to play a significant 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.

The results seen 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 effects, whereas only TNFR55 seems involved in mediating cytotoxicity and cytostasis. To study the role of TNFR75 in the mediation of TNF's effects, the TNF mutant D143F which selectively binds to TNFR75 was employed (Van Ostade et al, 1994). This mutant did not stimulate neutrophil and HUVEC activities in the dose response experiments performed (figure 3.5) 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 to 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 described here. This model suggests that at lower concentrations of TNF, binding initially occurs to the higher affinity (approximately 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 be occurring at the intracellular domain level or further down the signal transduction pathway.

In conclusion, the TNF mutants with their selective receptor-binding properties have enabled the dissociation of the two principle activities of TNF-α (cytotoxicity and inflammation) and have allowed the formulation of possible roles for TNFR55 and TNFR75 in these functions. The data supports the notion that TNFR55 mediates cytotoxicity and cytostasis, while TNFR75 facilitates the mediation of neutrophil and endothelial cell activation through TNFR55 especially at lower TNF doses. 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 realised.

SUMMARY

TNF-α is a pleiotropic cytokine capable of killing mammalian tumour cells *in vitro* and *in vivo*, and of enhancing the proinflammatory activity of leukocytes and endothelium, the latter effects limiting its usage as an antitumour agent in humans. Using TNF mutants with a selective capacity to bind to TNFR55 or TNFR75 it is shown 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 reduced proinflammatory activity when compared to 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.

TUMOUR NECROSIS FACTOR - α INDUCES CYTOSOLIC PHOSPHOLIPASE A_2 PHOSPHORYLATION IN HUMAN MONOCYTES AND IS COUPLED TO INFLAMMATORY MEDIATOR RELEASE

Chapter 4

INTRODUCTION

TNF-α, a pleiotropic cytokine with inflammatory and cytotoxic activities, is known to stimulate the release of arachidonic acid (AA) from a variety of different cell types (Clark et al 1988; Atkinson et al, 1990; Spriggs et al, 1990b) and also results in the production of platelet-activating factor (PAF) (Camussi et al, 1987). Since AA and PAF are potent proinflammatory agents the possibilty arises that TNF-α's inflammatory activity could be due principally to the mechanisms of formation of these inflammatory substances and to this end phospholipase A_2 (PLA_2) is the rate-limiting enzyme critical to their formation. The recent isolation of a cytosolic PLA_2 has resulted in a renewed interest in this area. Cytosolic phospholipase A_2 ($cPLA_2$) is an 110 kD / 750 amino acid protein (85 kD by sequence and cloning) which was purified from the human monocytic cell line U937 and is known to be distributed in a variety of cell types including macrophages (Leslie et al, 1988; Wijkander and Sundler, 1989), platelets (Kramer et al, 1988) and kidney (Gronich et al, 1990).

$cPLA_2$ is distinct from the relatively small (14 kD) secretory phospholipases A_2 ($sPLA_2$) in being activated and translocated to the membrane by physiological/submicromolar levels of ionised calcium, and is resistant to the reducing conditions of the cell (Clark et al, 1990; Clark et al, 1991; Kramer et al, 1991; Sharp et al, 1991). This Group IV phospholipase A_2 has no detectable sequence homology to the 68 known secretory PLA_2's ($sPLA_2$; Groups I-III phospholipase A_2) present in snake and bee venom, mammalian pancreas, synovial fluid, platelets, spleen and liver (Clark et al, 1991). Unlike the $sPLA_2$'s, $cPLA_2$ selectively hydrolyzes AA from the 2-acyl ester bond of membrane phospholipids and this represents the rate-limiting step in the production of eicosanoids and PAF (Irvine, 1982; Hanahan, 1986). Therefore, agents which modulate $cPLA_2$ activity regulate the production of these potent inflammatory mediators.

The mechanism by which $cPLA_2$ becomes activated has been under intense investigation and it would seem that phosphorylation of this enzyme plays a significant role in the regulation of its activity. Lin et al (1992a) described how several agents (ATP, thrombin, PMA and A23187 [calcium ionophore]) increased arachidonic acid release in CHO (Chinese Hamster Ovary) cells overexpressing $cPLA_2$ (but not $sPLA_2$) demonstrating hormonal coupling of $cPLA_2$ and that these same agents all phosphorylated $cPLA_2$ as demonstrated in gel shift assays. In addition, ATP was shown to increase $cPLA_2$ activity in a liposome assay. Importantly, the phosphorylation of $cPLA_2$ resulting from these agonists occurs on serine residues and this has led to the investigation of kinases which could be involved in the $cPLA_2$ signal transduction pathway.

One such kinase is mitogen-activated protein 2 (MAP) kinase (also known as p42 microtubule-associated protein 2 kinase) which is a member of a family of kinases designated ERKs (extracellular signal-regulated kinases) (Ray and Sturgill, 1988; Boulton et al, 1991; Cobb et al, 1991). MAP kinase has a consensus sequence for substrate phosphorylation that is found within the primary sequence of $cPLA_2$ (Pro-Leu-Ser505-Pro) (Alvarez et al, 1991; Clark et al, 1991; Gonzalez et al, 1991). Lin et al (1993) and Nemenoff et al (1993) have recently demonstrated that $cPLA_2$ is a substrate for MAP kinase with phosphorylation occurring on the serine residue 505. Mutating serine 505 to alanine results in a protein which is no longer a substrate for MAP kinase and does not result in AA release. Another kinase which has recently been shown to phosphorylate and activate $cPLA_2$ is protein kinase C (PKC; Lin et al, 1993; Nemenoff et al, 1993). PMA (phorbol 12-myristate 13-acetate), a known activator of PKC, can increase $cPLA_2$ activity suggesting that $cPLA_2$ is regulated by protein phosphorylation via PKC.

In HeLa cells (fibroblasts) TNF-α alone induces a modest increase in $cPLA_2$ activity (AA release) and TNF-α treatment (50 ng/ml) of HeLa cells for 20 min caused approximately 1.5-2 fold

increase in $cPLA_2$ phosphorylation (Hoeck et al, 1993). In addition, TNF-α was shown to increase $cPLA_2$ expression after several hours and this was inhibited by dexamethasone, a potent anti-inflammatory agent. In this chapter the role of $cPLA_2$ in the signal transduction pathway of TNF-induced inflammation has been examined in human myeloid cells. In addition, the receptor biology of TNF-α in this system was investigated with TNF mutants that selectively bind to the TNF receptors. As stated in chapter 3, TNFR75 facilitates TNFR55 in the mediation of TNF-α's proinflammatory activities in neutrophils and endothelium. It is hypothesized here that $cPLA_2$ phosphorylation in human monocytes in response to TNF-α is also mediated in the same fashion. Monocytes were chosen for investigation because they play a central role in inflammation and immune reactivity. Not only do they present processed antigen to T lymphocytes but they also secrete many inflammatory products including PAF (Camussi et al, 1987) and TNF-α itself (Matthews, 1981).

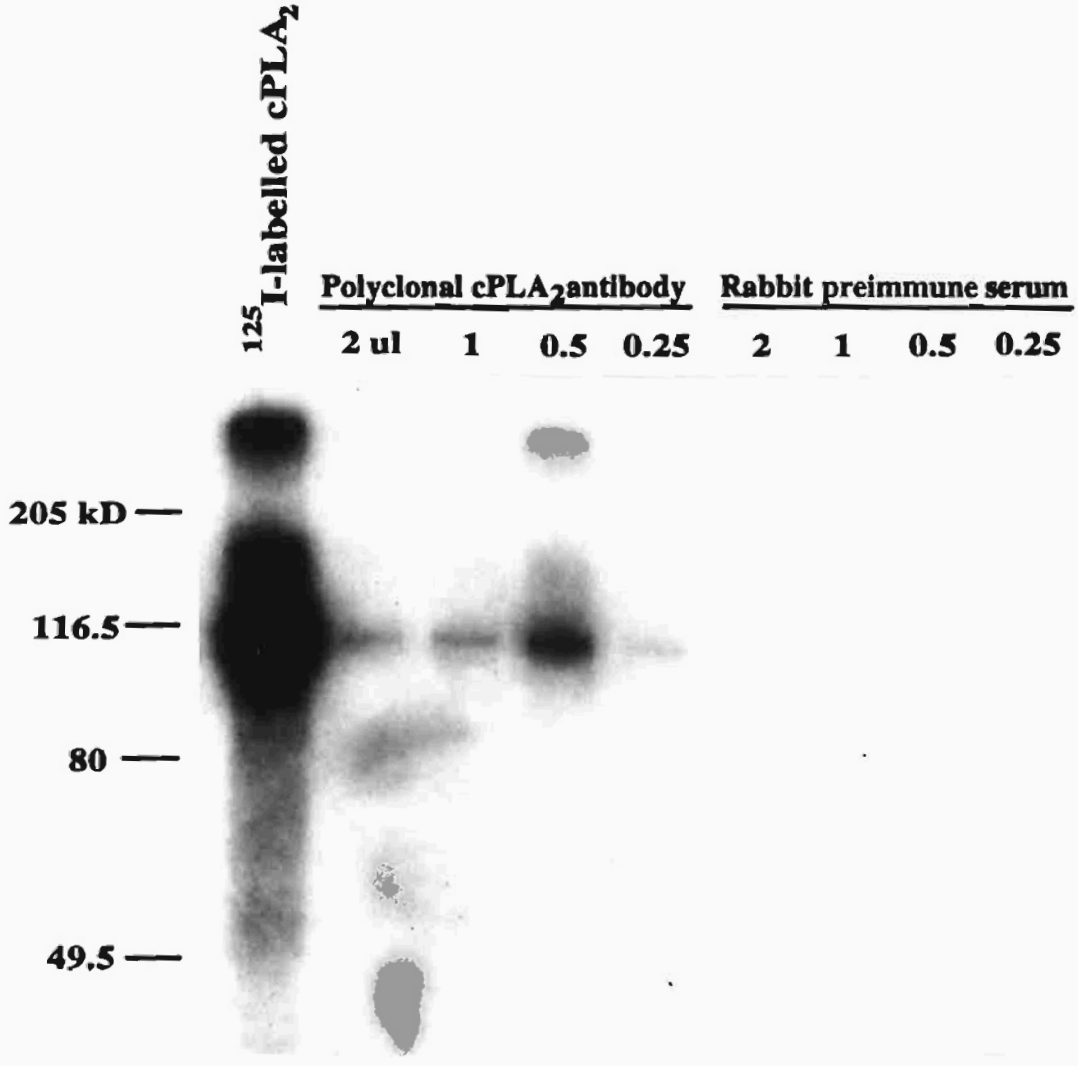
RESULTS

4.1 Titration of Polyclonal $cPLA_2$ Antibody and Initial Phosphorylation Experiments

The phosphorylation of $cPLA_2$ in myeloid cells was examined using a method adapted from Changelian and Fearon (1986). The cells (≈5 x 10^7/sample) were initially depleted of phosphate and then loaded with ^{32}P. After stimulation, the cells were lysed and immunoprecipitation of $cPLA_2$ was performed the next day (refer to methods 2.17). In order to establish the quantity of polyclonal $cPLA_2$ antibody required to immunoprecipitate $cPLA_2$ in the phosphorylation experiments a titration of antibody to ^{125}I-labelled $cPLA_2$ was performed (figure 4.1). It was determined that polyclonal antibody added to $cPLA_2$ in a ratio of 1:400 (ie 0.5 µl : 200 µl) resulted in the best immunoprecipitation of $cPLA_2$ protein. Rabbit preimmune serum behaved as

Figure 4.1 Titration of polyclonal $cPLA_2$ antibody against ^{125}I-labelled $cPLA_2$ protein

Rabbit preimmune serum was included as a negative control. The various volumes of antibody were added to a total volume of 200 µl. The immunoreactive $cPLA_2$ is seen at 110 kD.


^{125}I-labelled cPLA$_2$
Polyclonal cPLA$_2$antibody
Rabbit preimmune serum
2 ul
1
0.5
0.25
2
1
0.5
0.25
205 kD
116.5
80
49.5

the negative control, immunoprecipitating negligible amounts of $cPLA_2$ protein. As shown in figure 4.1, $cPLA_2$ displays a molecular weight on SDS-polyacrylamide gel electrophoresis of 110 kD (Clark et al, 1990, Kramer et al, 1991). Monoclonal antibody to $cPLA_2$ was not available at the time that the first phosphorylation experiments were performed but was later provided by John Knopf (Genetics Institute, Cambridge, MA) and used at a concentration of 1 μg/ml.

The $cPLA_2$ protein was first purified from the human promonocytic cell line U937 (Clark et al, 1990; Kramer et al, 1991) and subsequently the polyclonal antibody was raised to this protein. In view of this, U937 cells were chosen for the initial $cPLA_2$ phosphorylation experiments so as to establish the technique. These cells were stimulated with PMA, a phorbol diester and co-carcinogen, which is a potent activator of PKC. In CHO cells overexpressing $cPLA_2$, stimulation with PMA resulted in serine phosphorylation of $cPLA_2$ (Lin et al, 1992a). A representative experiment shown here also demonstrates that $cPLA_2$ is phosphorylated in response to PMA (figure 4.2) and this was 2.2-fold greater than control (Phosphorimager quantitation).

4.2 Absence of $cPLA_2$ Phosphorylation in Human Neutrophils

Having established that $cPLA_2$ in U937 cells could be detected by immunoprecipitation with polyclonal antibody to $cPLA_2$ and that this protein was phosphorylated in response to PMA, mature human myeloid cells (neutrophils and monocytes) were examined. The presence of $cPLA_2$ in neutrophils at the time of investigation had not been reported in the literature. Several experiments were performed using the agonists GM-CSF, PMA and FMLP, however no $cPLA_2$ could be elicited (figure 4.3). The phosphorylation of complement receptor 1 (CR1) by PMA in neutrophils was included in these experiments as a positive control (Changelian and Fearon, 1986) and indicated that the phosphorylation technique was functional in this system. CR1 is the

Figure 4.2 Phosphorylation of $cPLA_2$ in U937 cells

Phosphorylation of $cPLA_2$ in U937 cells in response to PMA (100 ng/ml) applied for 10 min at 37°C. The control was also placed at 37°C for 10 min. ^{125}I-labelled $cPLA_2$ confirms the presence of immunoreactive $cPLA_2$ at 110 kD (marker). 5 x 10^7 U937 cells were present in each sample.

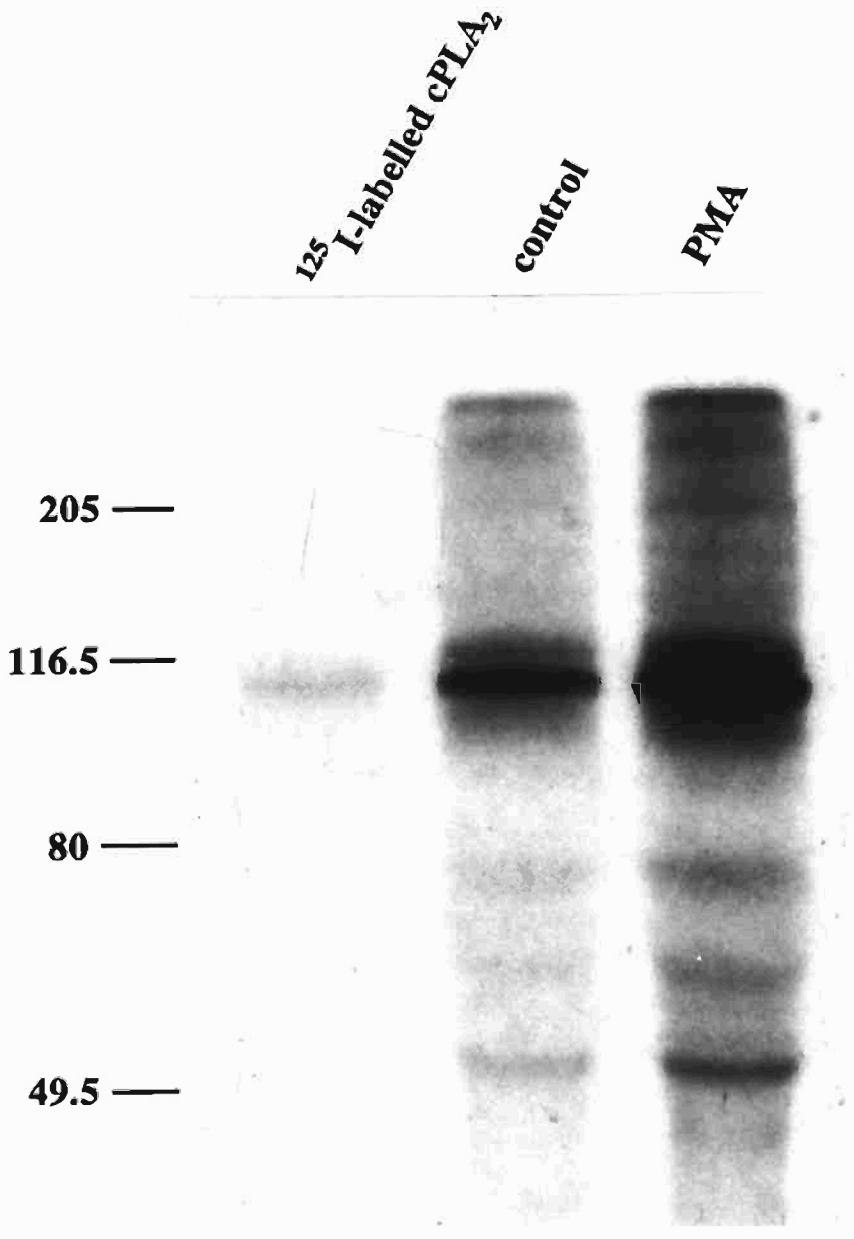
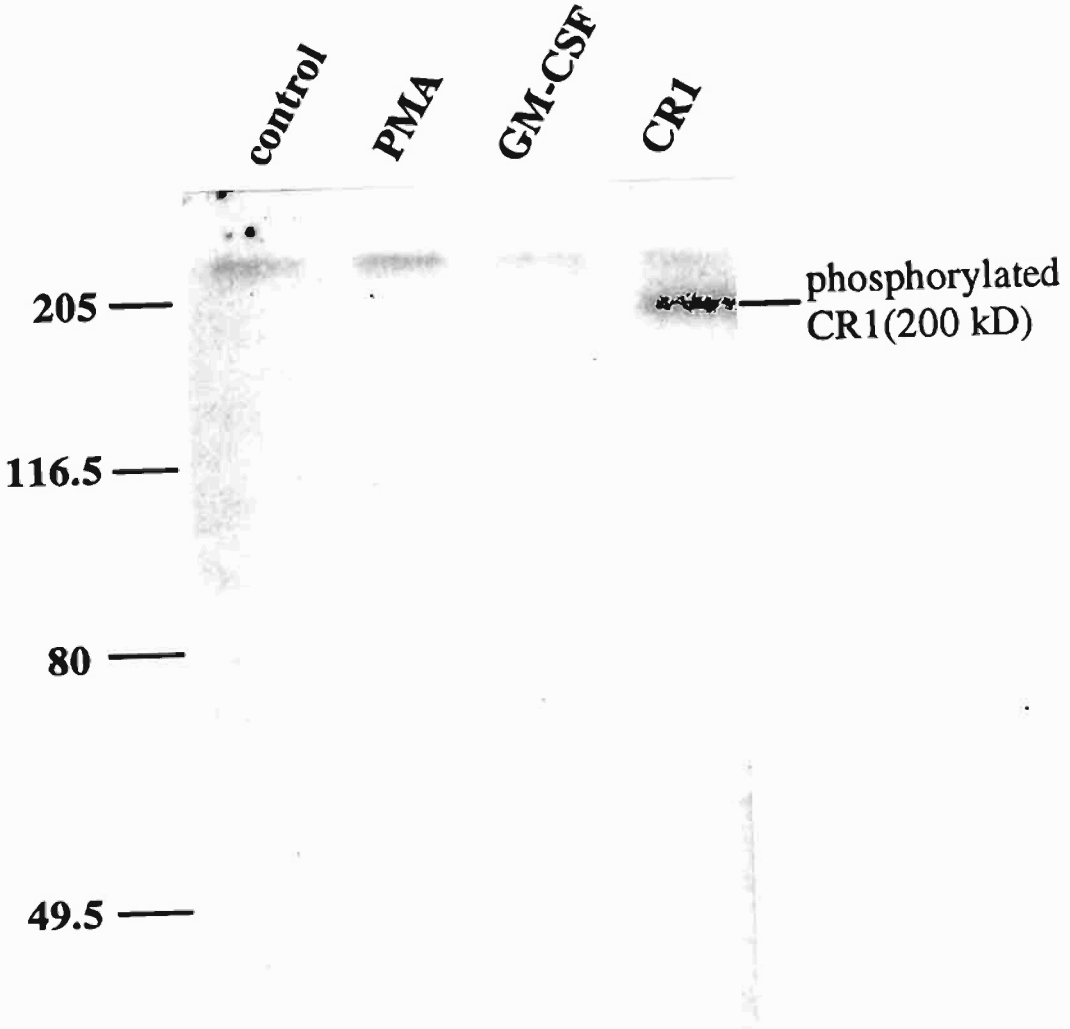

^{125}I-labelled cPLA$_2$
control
PMA
205
116.5
80
49.5

Figure 4.3 The absence of $cPLA_2$ phosphorylation in human neutrophils

This experiment is representative of 4 experiments performed and demonstrates the absence of $cPLA_2$ phosphorylation with PMA 100 ng/ml and GM-CSF 100 ng/ml at 37°C for 10 min. CR1 positive control: neutrophils were stimulated with PMA 100 ng/ml for 3 min at 37°C and immunoprecipitated with monoclonal CR1 antibody (200 ml = 5 mg). ^{125}I-labelled $cPLA_2$ acted as a marker and 4×10^7 neutrophils were used for each sample.


control
PMA
GM-CSF
CR1
205
116.5
80
49.5
phosphorylated
CR1(200 kD)

C3b/C4b complement receptor (M_r 200 kD) which has been shown to be involved in the phagocytosis of particles bearing the cleavage fragments of complement C3.

4.3 PMA-induced $cPLA_2$ Phosphorylation in Human Monocytes

Human monocytes, in view of the results obtained with the U937 promonocytic cell line, were likely to express a $cPLA_2$ species that could be identified by the polyclonal antibody. Experiments stimulating monocytes with PMA were performed on seven occasions and resulted in increased $cPLA_2$ phosphorylation but this was less than 2-fold when compared to control (figure 4.4A and B), and was also shown to occur in a time-dependent fashion, rapidly peaking at 10 minutes (2.5-fold increase). The differences in phosphorylation observed are not large and this is reflected in the literature by the use of cell lines overexpressing $cPLA_2$ (Lin et al, 1992a) and the qualitative study of $cPLA_2$ phosphorylation by electrophoretic mobility (Nakamura et al, 1992; Hoeck et al, 1993). In addition, the constitutive phosphorylation seen in the control monocyte population probably reflects activation resulting from the abnormal *ex vivo* environment to which these cells are subjected (Elliott et al, 1990). Also included in figure 4.4A is the phosphorylation of CR1 protein which also occurs in monocytes in response to PMA. Thus phagocytes (monocytes and neutrophils) both possess CR1 protein which is phosphorylated in response to stimulation with PMA. This phosphorylation may be important for the active phagocytosis of particles bearing C3 fragments.

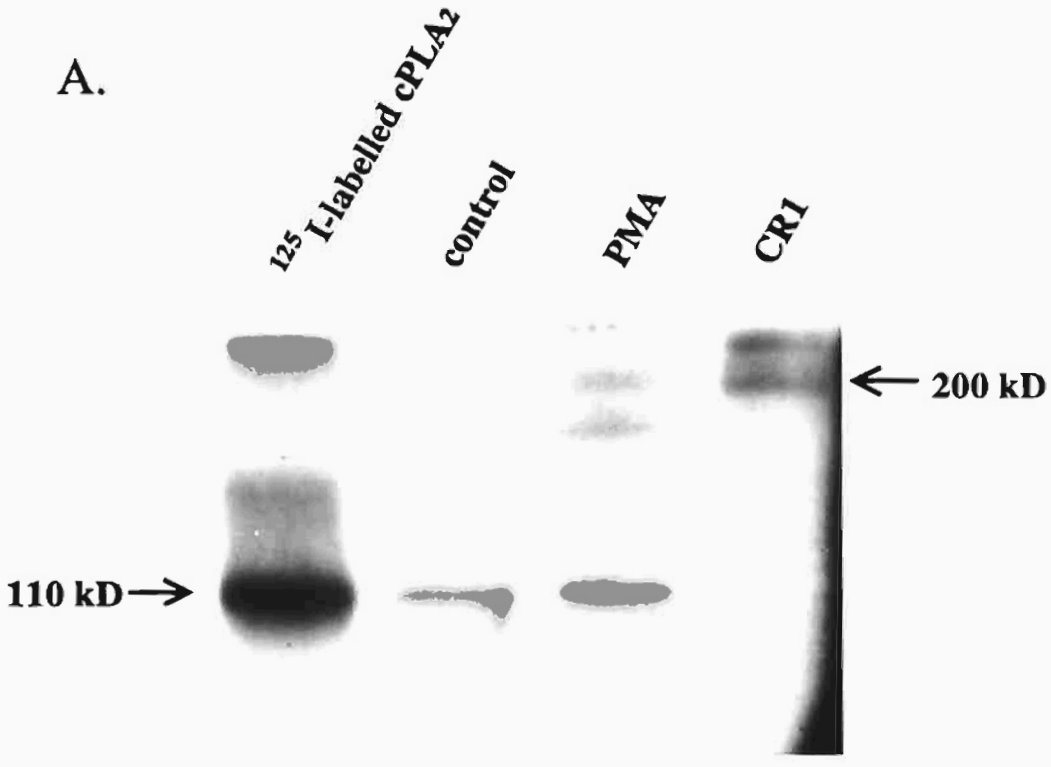
4.4 TNF-induced $cPLA_2$ Phosphorylation in Monocytes and Coupling to PAF Synthesis

TNF-α, a proinflammatory cytokine released principally from monocytes/macrophages, is here hypothesized to activate $cPLA_2$ as evidenced by the release of AA and PAF from various cell types (Camussi et al, 1987; Clark et al 1988; Atkinson et al, 1990; Spriggs et al, 1990b). In view of the central role of monocytes in inflammation and TNF biology, the ability of TNF-α to induce

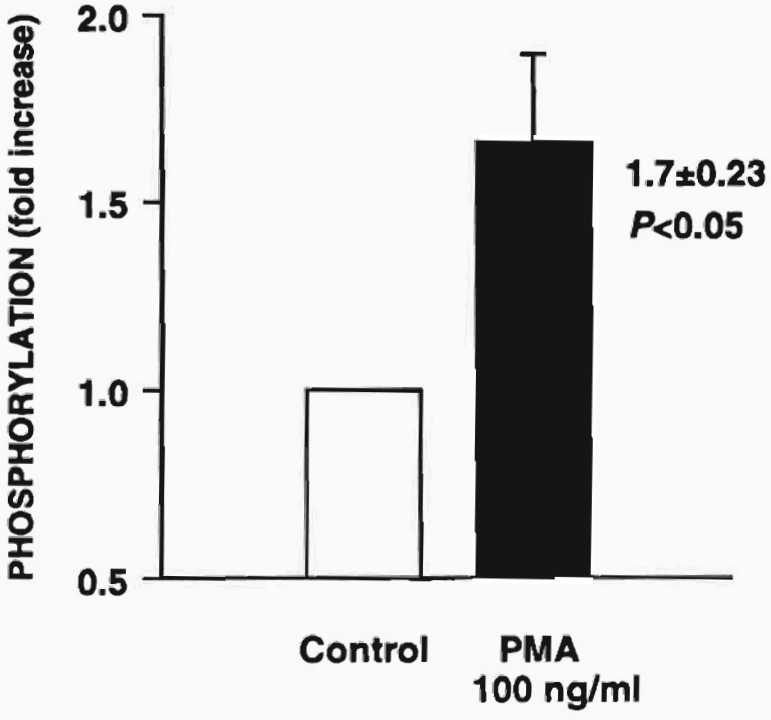
Figure 4.4 The phosphorylation of $cPLA_2$ by PMA in human monocytes

(A) The experiment is representative of 6 others in which monocytes (6 x 10^7/sample) were stimulated with PMA 100 ng/ml for 10 min at 37°C. The phosphorylation of CR1 is included as a positive control (PMA 100 ng/ml, 3 min at 37°C) and ^{125}I-labelled $cPLA_2$ as a marker. (B) Quantitation of cPLA2 phosphorylation induced by PMA. The data is derived from 7 experiments using PMA at 100 ng/ml for 10 min at 37°C. The error bars represents the SEM and the data was analysed by the Student's *t*-test. The amount of phosphorylation was quantified using the Phosphorimager.

A.


^{125}I-labelled cPLA2
control
PMA
CR1
200 kD
110 kD

B.


PHOSPHORYLATION (fold increase)
2.0
1.5
1.0
0.5
1.7±0.23
P<0.05
Control
PMA
100 ng/ml

cytosolic PLA_2 phosphorylation in human monocytes was investigated. Prior to determining whether TNF-α could induce $cPLA_2$ phosphorylation in human monocytes the TNF receptor population on the surface of peripheral blood mononuclear cells was examined. In addition, the ability of TNF-α to induce the synthesis of cell-associated PAF was studied. Using FACS analysis the presence of both TNF receptors on monocytes was demonstrated (figure 4.5). Peripheral blood mononuclear cells were separated into lymphocytes and monocytes by FACS using size separation and the monocyte specific antibody MO-2 (anti-CD14, IgMκ, Coulter Immunology, Hialeah, Florida). The TNF receptors were identified using the monoclonal antibodies utr-1 (TNFR75) and htr-9 (TNFR55). The peripheral blood mononuclear cell preparations gave the following percentage of cells when studied morphologically: >31% monocytes and >68% lymphocytes.

Given the presence of both TNF receptors on human monocytes, TNF mutants with selectivity for TNFR55 were used to examine the functional roles of the TNF receptors in the production of PAF, a phospholipid with potent proinflammatory activities (Braquet et al, 1987). The ability of TNF-α to stimulate PAF production in macrophages in a time and dose-dependent manner has been illustrated by Camussi et al (1987). However, in addition to demonstrating this to be the case, the TNFR55-selective mutant R32W stimulated elutriated monocytes to produce less PAF than wild-type TNF in both time course and dose response experiments (figure 4.6). The implication here is that binding to TNFR75 is required for full PAF production in monocytes as shown previously for neutrophils (Barbara et al, 1994).

The production of the proinflammatory mediator PAF is dependent on the activity of $cPLA_2$ and this has been shown to be regulated in part by phosphorylation of the enzyme (Lin et al, 1992a). In addition, TNF-α results in $cPLA_2$ phosphorylation in HeLa cells in a time and dose-dependent

Figure 4.5 Flow cytometry demonstrating the presence of TNF receptors on monocytes

These results are representative of 4 such determinations and the TNF receptors were identified using the monoclonal antibodies htr-9 (TNFR55) and utr-1 (TNFR75). (B) The mean fluorescence intensity for the two TNF receptors on monocytes are represented. There were 4 separate determinations as in (A). Error bars represent the SEM and the data was analysed by the Student's *t*-test.

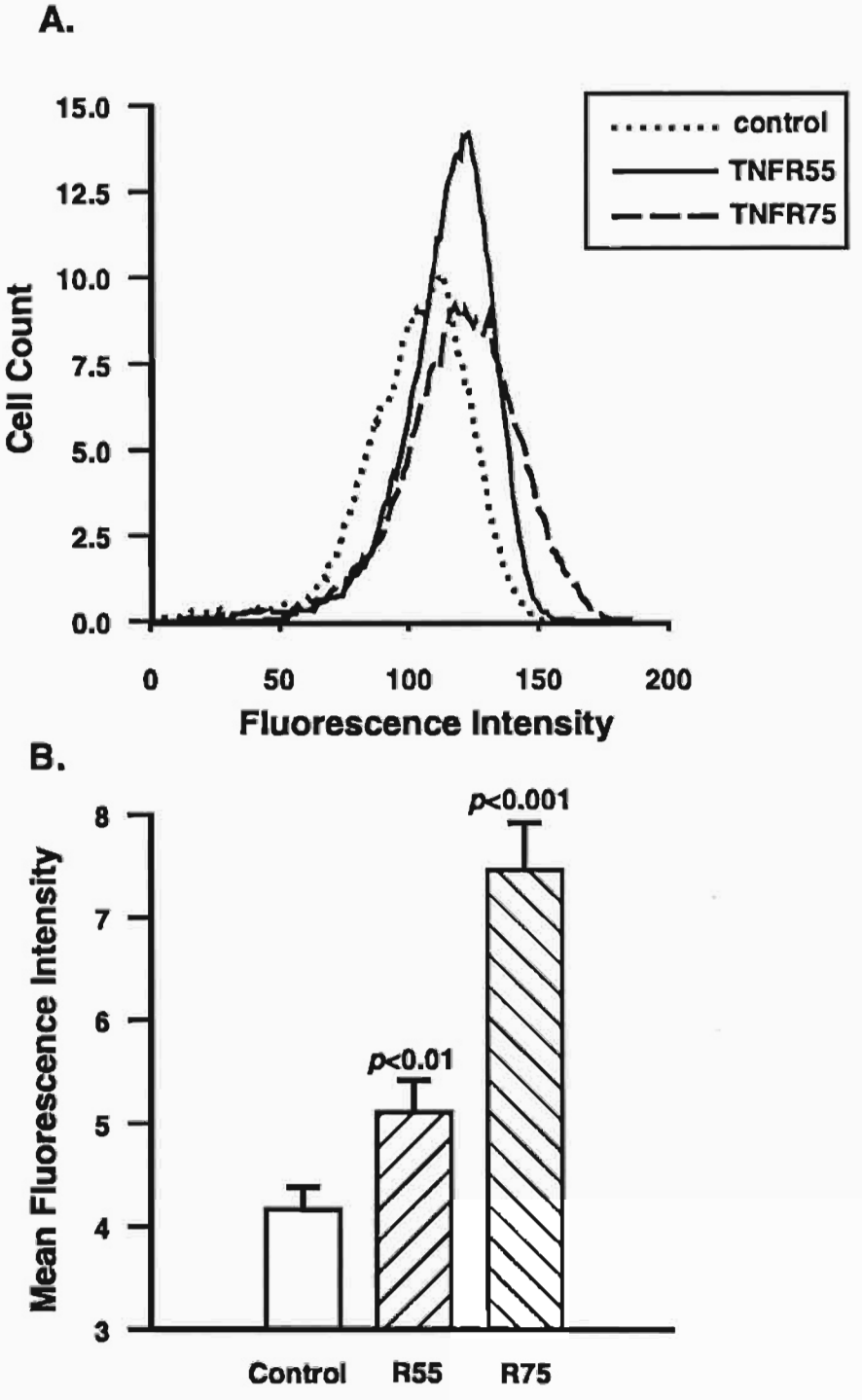
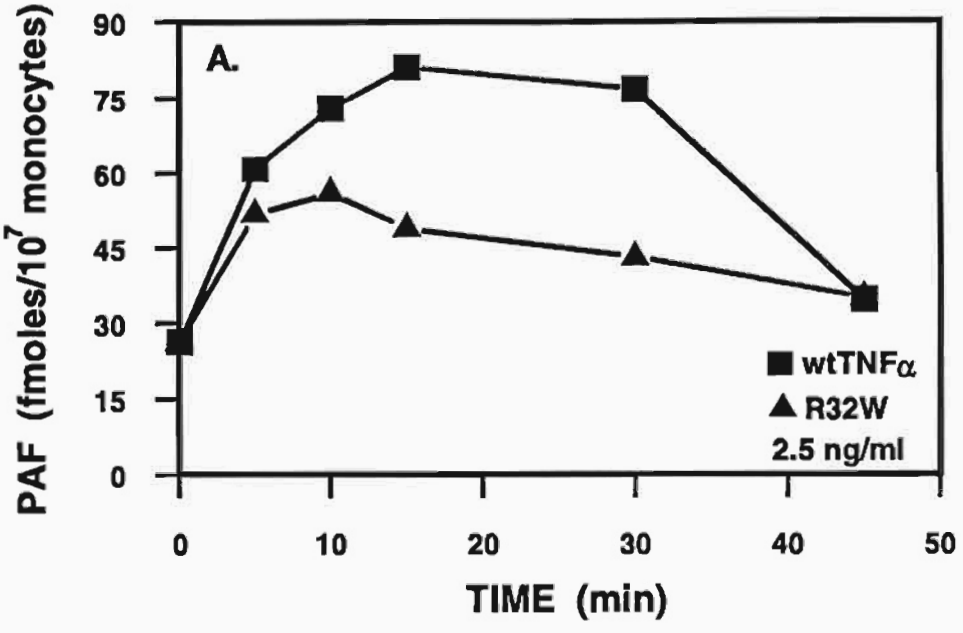
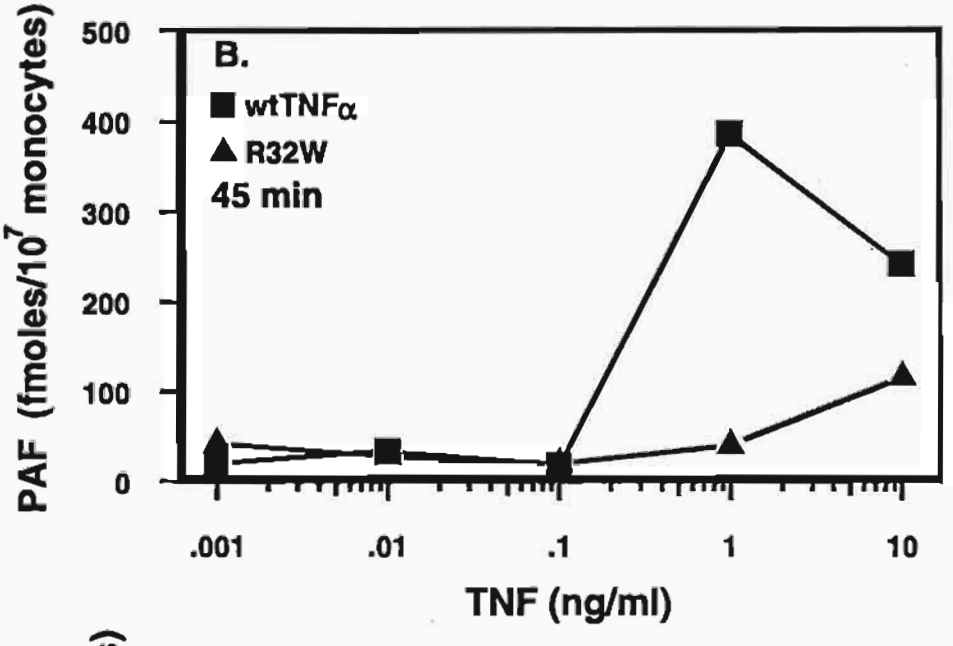
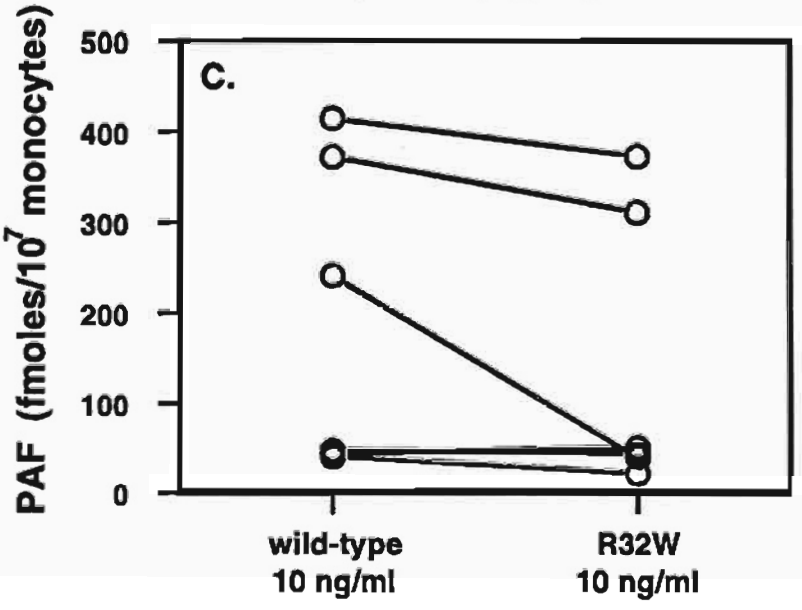

A.
15.0
12.5
10.0
7.5
5.0
2.5
0.0
Cell Count
0
50
100
150
200
Fluorescence Intensity
control
TNFR55
TNFR75
B.
8
7
6
5
4
3
Mean Fluorescence Intensity
p<0.001
p<0.01
Control
R55
R75

Figure 4.6 The production of cell-associated PAF in human monocytes by wild-type TNF and TNF mutant

The synthesis of cell-associated PAF in monocytes by wild-type TNF and R32W are represented in these time course (A) and dose response (B) experiments. The concentration of wild-type TNF and R32W in the time course experiment shown was 2.5 ng/ml and the duration of exposure to stimulus in the dose response experiment was 45 minutes. (C) The data from six different donors is shown with R32W producing less cell-associated PAF than wild-type TNF, $p<0.07$ (approaching significance, Wilcoxon's Rank Sum test).


A.
PAF (fmoles/10^7 monocytes)
90
75
60
45
30
15
0
0
10
20
30
40
50
TIME (min)
wtTNFα
R32W
2.5 ng/ml


B.
PAF (fmoles/10^7 monocytes)
500
400
300
200
100
0
.001
.01
.1
1
10
TNF (ng/ml)
wtTNFα
R32W
45 min


C.
PAF (fmoles/10^7 monocytes)
500
400
300
200
100
0
wild-type
10 ng/ml
R32W
10 ng/ml

manner (Hoeck et al, 1993). For the first time it is demonstrated here that $cPLA_2$ phosphorylation occurs in human monocytes in response to TNF-α stimulation, and that this occurs in a time-dependent manner using a dose which produces full stimulation of PAF production in monocytes (2.5 ng/ml) (figure 4.7). Also, the TNFR55-selective mutant (R32W) did not phosphorylate $cPLA_2$ to the same extent as wild-type TNF indicating a role for TNFR75 in $cPLA_2$ phosphorylation. Similar results were obtained with the other TNFR55-selective mutants E146K and R32W-S86T. The reduction in $cPLA_2$ phosphorylation seen with E146K was 46% at 10 minutes and 59% at 30 minutes; R32W-S86T produced similar reductions in phosphorylation with 26% at 10 minutes and 59% at 30 minutes. Of particular interest was the relationship between the time of maximal $cPLA_2$ phosphorylation and maximal PAF production. As seen in figures 4.6 and 4.7 both occur at approximately 10 minutes with TNF stimulation which strengthens the argument that phosphorylation of $cPLA_2$ results in increased activity.

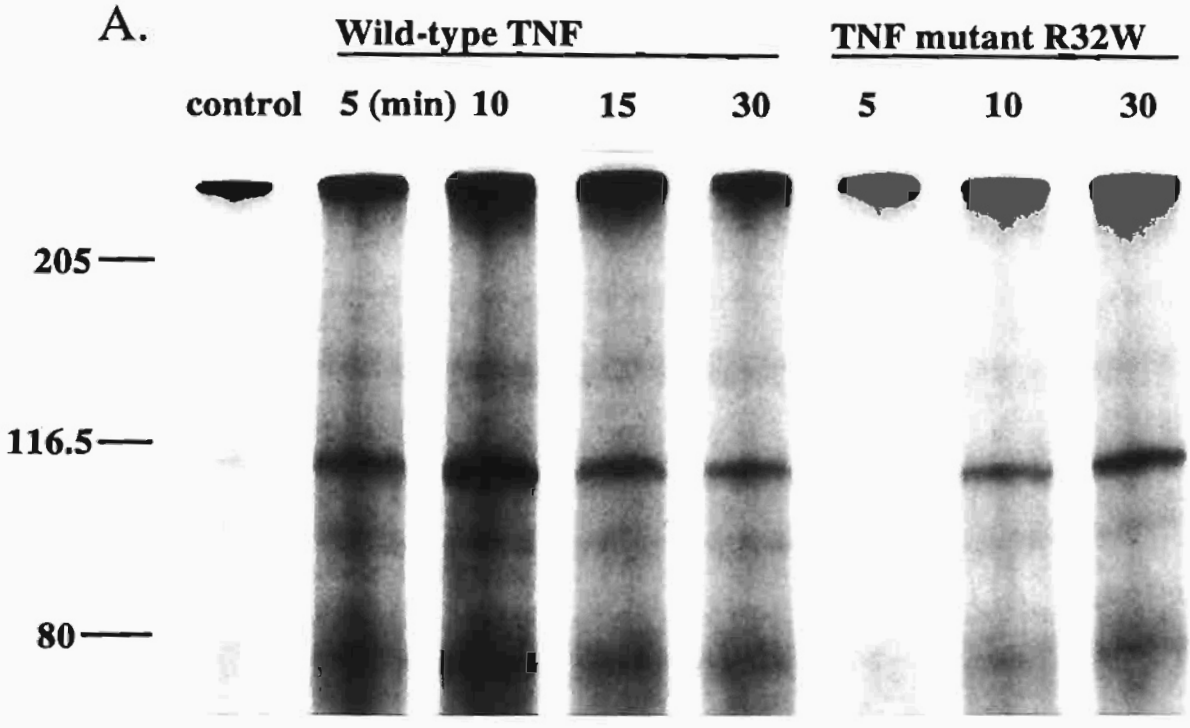
4.5 TNF-induced Serine Phosphorylation of $cPLA_2$ in Monocytes

It has been demonstrated that $cPLA_2$ is phosphorylated in response to TNF-α and to a lesser extent by the TNFR55-selective mutants implying a functional role for TNFR75. However, what is the molecular mechanism by which TNF-α induces $cPLA_2$ phosphorylation? By determining which residue(s) are phosphorylated we may gain insights into the signal transduction mechanisms involved. Lin et al (1993) recently demonstrated that $cPLA_2$ is phosphorylated at serine residue 505 by MAP kinase however EGF and PDGF, which bind to receptor tyrosine kinases, are also known to activate PLA_2 (Bonventre et al, 1990; Domin and Rozengurt, 1993). Western blot analysis with phosphotyrosine antibody (PY20) after immunoprecipitating TNF-stimulated monocytes with monoclonal antibody to $cPLA_2$ did not reveal phosphorylation on tyrosine residues (figure 4.8A). However, the presence of $cPLA_2$ protein was verified by western blotting with $cPLA_2$ antibody (figure 4.8B). In view of the negative phosphotyrosine results

Figure 4.7 The induction of $cPLA_2$ phosphorylation in human monocytes by wild-type TNF and TNF mutant

The phosphorylation of $cPLA_2$ in monocytes by wild-type TNF and R32W. (A) Time course experiment representative of 5 others using wild-type TNF and TNF mutant at a concentration of 2.5 ng/ml. Monoclonal antibody to $cPLA_2$ was used at a concentration of 1 μg/ml. 3×10^7 monocytes were included per sample. (B) The $cPLA_2$ bands shown in (A) were quantified by Phosphorimager analysis and displayed.

A.


Wild-type TNF
TNF mutant R32W
control
5 (min)
10
15
30
5
10
30
205
116.5
80

B.

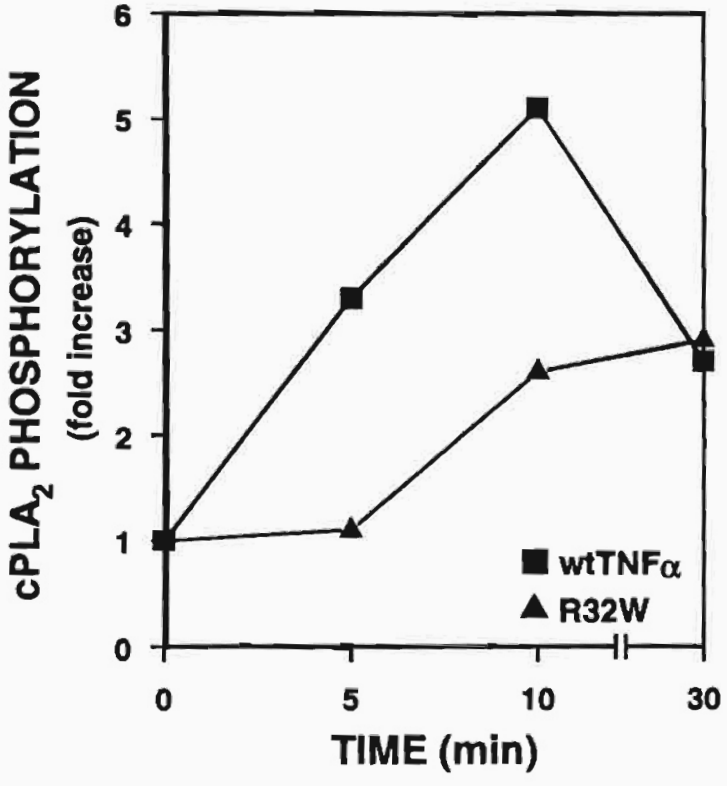
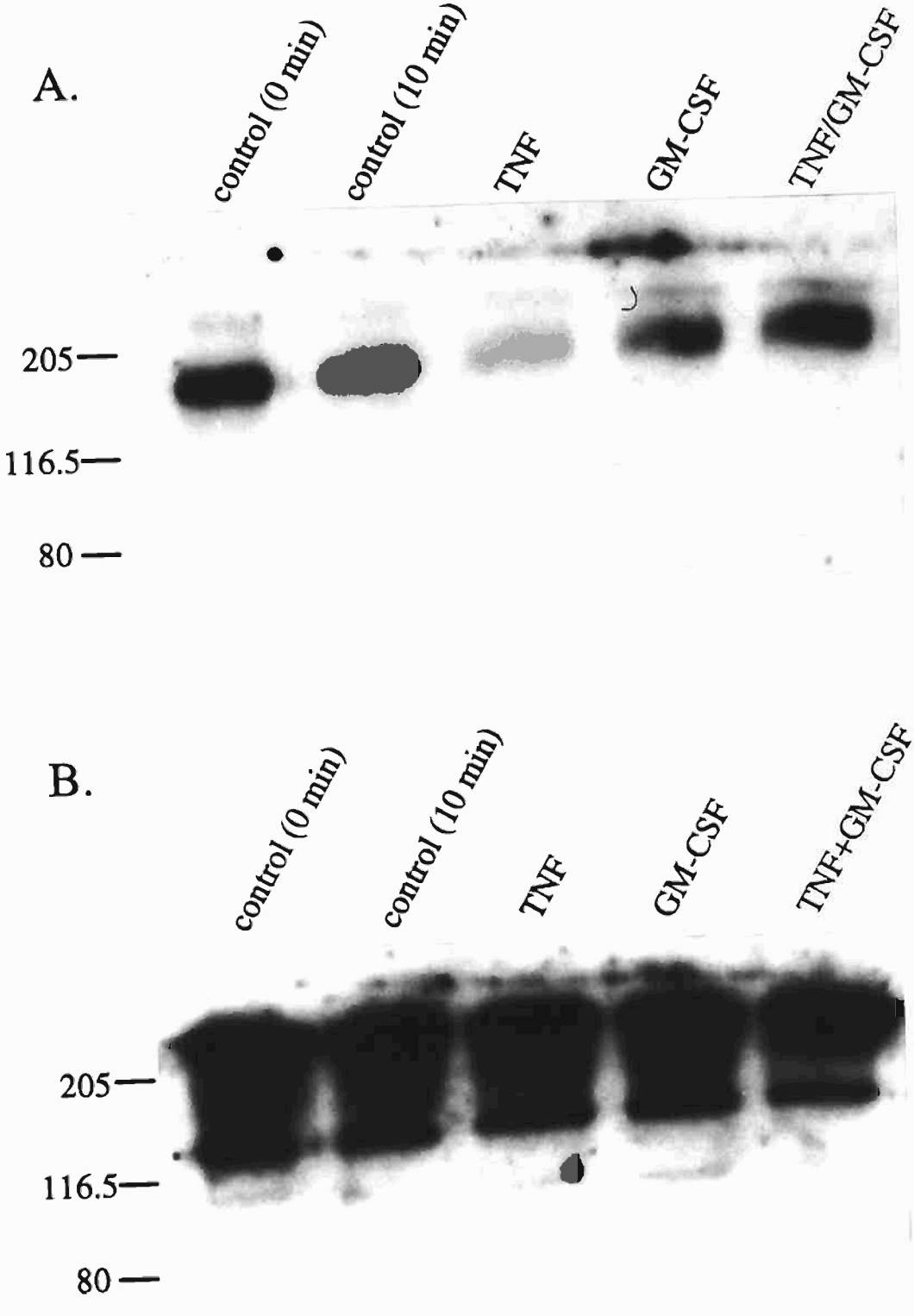

cPLA2 PHOSPHORYLATION
(fold increase)
0
1
2
3
4
5
6
wtTNFα
R32W
0
5
10
30
TIME (min)

Figure 4.8 Western blot analysis of $cPLA_2$ protein derived from human monocytes

(A) Monocytes (2×10^7/sample) were treated with TNF-α (2.5 ng/ml), GM-CSF (100 ng/ml) or unstimulated. The stimulus was applied for 10 min at 37°C. The nitrocellulose was probed with PY20 (antiphosphotyrosine antibody) and the result demonstrates that no phosphorylation of tyrosine residues occurred on $cPLA_2$ (no bands evident at 110 kD). The high molecular weight bands present are due to the nonspecific detection of immunoglobulin by ^{125}I-labelled Protein A. (B) After stripping the nitrocellulose from (A), the western blot was probed with $cPLA_2$ antibody and the presence of $cPLA_2$ protein is verified at 110 kD.


A.
control (0 min)
control (10 min)
TNF
GM-CSF
TNF/GM-CSF
205
116.5
80
B.
control (0 min)
control (10 min)
TNF
GM-CSF
TNF+GM-CSF
205
116.5
80

phosphoamino acid analysis was undertaken and this revealed that only serine phosphorylation of $cPLA_2$ occurs in human monocytes in response to TNF-α (figure 4.9). In addition, it was shown that GM-CSF also phosphorylates $cPLA_2$ on serine residues in human monocytes.

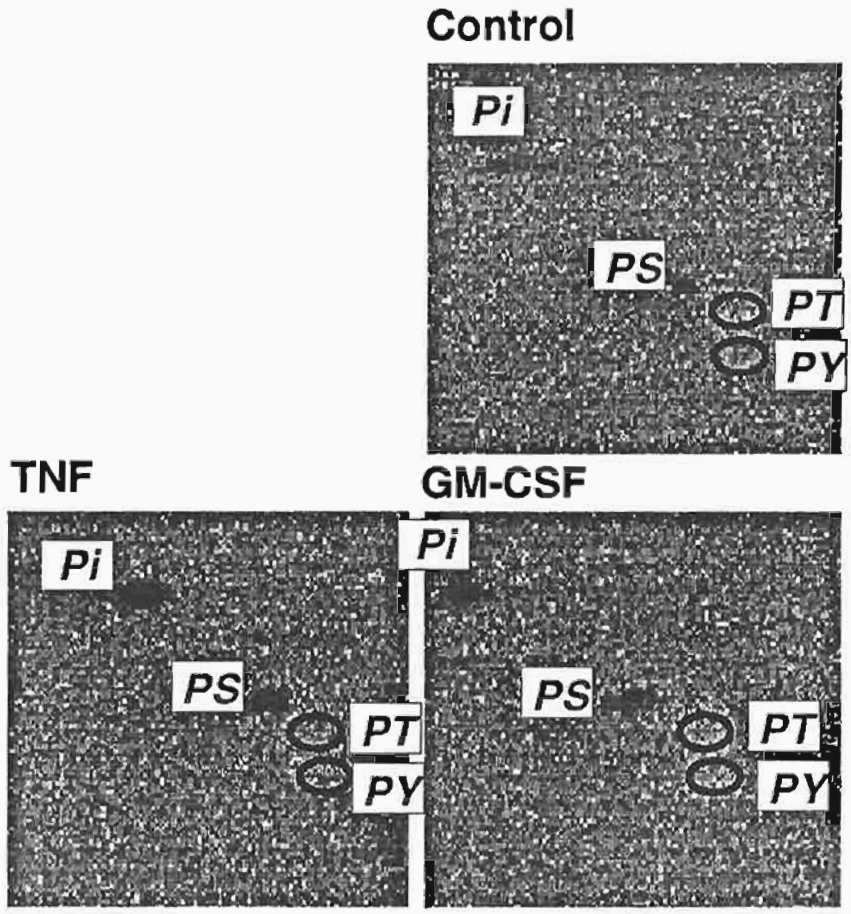
DISCUSSION

$cPLA_2$ is a recently discovered enzyme which functions in the cytosol and is selective for membrane phospholipids possessing arachidonic acid. This enzyme is the rate-limiting step in the production of potent proinflammatory mediators (eicosanoids and PAF) whose formation results from TNF stimulation of inflammatory cells. In this chapter the role of $cPLA_2$ in the signal transduction pathway of TNF-α in myeloid cells was examined. The phosphorylation of $cPLA_2$ is known to be responsible in part for the functional activation of the enzyme. When CHO cells overexpressing $cPLA_2$ were stimulated with ATP, thrombin, PMA and A23187, increased AA release resulted (Lin et al, 1992a). These agents also phosphorylated $cPLA_2$ but the inhibition of phosphorylation by staurosporine (a protein kinase inhibitor) also reduced AA release further correlating $cPLA_2$ phosphorylation with activation. It has been illustrated here that in human monocytes stimulated with TNF-α the enzyme $cPLA_2$ is phosphorylated in a time-dependent manner and this is coupled to the production of PAF. In addition, TNF-α resulted in a dose-dependent production of PAF in human monocytes.

The $cPLA_2$ phosphorylation experiments proved to be difficult for a number of reasons. Monocytes contain proteolytic enzymes and phosphatases which need to be neutralised by an effective RIPA buffer. The RIPA buffer used contained multiple inhibitors (refer to methods 2.17) but in certain monocyte populations this was inadequate as evidenced by either

Figure 4.9 Two-dimensional phosphoamino acid analysis of ^{32}P-labelled $cPLA_2$ from human monocytes

(Top right) Unstimulated monocytes. (Bottom left) TNF-α 2.5 ng/ml. (Bottom right) GM-CSF 100 ng/ml. 2.8 x 10^7 monocytes were used per sample and placed at 37°C for 10 minutes in the presence or absence of stimulus. The location of the phosphoamino acid standards - phosphoserine (PS), phosphothreonine (PT) and phosphotyrosine (PY) - was determined by staining with ninhydrin.


Control
Pi
PS
PT
PY
TNF
Pi
PS
PT
PY
GM-CSF
Pi
PS
PT
PY

fragmentation of $cPLA_2$ or by the minimal amount of phosphorylation which occurred. In addition, as previously shown by other investigators (Hoeck et al, 1993), the amount of phosphorylation which occurs in response to various stimuli is not much greater than basal values, unless the cells are overexpressed with $cPLA_2$ protein (Lin et al, 1992a). This, in the case of human monocytes, is explained in part by the limitation of monocytes numbers used in these experiments: 500 ml of venous blood will yield, on average, approximately 7×10^7 monocytes. Also, similar phosphorylation experiments were performed in neutrophils however the antibody to $cPLA_2$ did not immunoprecipitate the $cPLA_2$ species in these cells despite having a functional positive control to the complement receptor 1 (CR1). It is possible that the $cPLA_2$ species which exists in neutrophils is not recognised by the antibody or that $cPLA_2$ in neutrophils is present in amounts which escape the sensitivity of the technique used. Additionally, the protease and phosphatase inhibitors in the lysis buffer may have been inadequate in preserving phosphorylated protein. Of interest is the recent demonstration by Doerfler et al (1994) of $cPLA_2$ phosphorylation in neutrophils in response to bacterial lipopolysaccharide and PMA. Using the same $cPLA_2$ antibodies and cell numbers but a different buffering solution and technique (immunoprecipitation followed by immunoblotting; no ^{32}P incorporation), phosphorylated protein was apparent by its reduced gel migration however the examples published were not convincing. In the neutrophil experiments presented in this chapter, it could be reasoned that the technique was the limiting factor or the buffering solution used was inadequate.

Despite these difficulties the results suggest a role for $cPLA_2$ in the inflammatory signal transduction pathway of TNF-α. The phosphorylation of $cPLA_2$ by TNF-α was time-dependent and coupled to the release of PAF in human monocytes. The recent demonstration by Hoeck et al (1993) that $cPLA_2$ mRNA is also increased in response to TNF-α indicates that the activation of $cPLA_2$ by TNF-α is rather complex and involves both enzyme phosphorylation and gene

transcription. In monocytes stimulated with M-CSF (macrophage-colony stimulating factor) (Nakamura et al, 1992) and in human fibroblasts stimulated with IL-1 (Lin et al, 1992b) $cPLA_2$ activity increased and was related to $cPLA_2$ phosphorylation and expression. In addition, it has recently been shown that $cPLA_2$ is phosphorylated in macrophages by zymosan, PMA, okadaic acid and A23187, and treatment of cells with phosphatase reduced stimulated $cPLA_2$ activity to near control levels (Qiu et al, 1993).

The mechanism of phosphorylation of $cPLA_2$ by TNF-α will allow insights to be gained into the signal transduction pathways employed by this cytokine. $cPLA_2$ is phosphorylated on serine residues by a number of different stimuli (ATP, thrombin, PMA, A23187, PDGF, EGF, zymosan and okadaic acid) and MAP kinase specifically phosphorylates serine residue 505 of $cPLA_2$ (Lin et al, 1993; Nemenoff et al, 1993). For the first time, it has been reported here that $cPLA_2$ in human monocytes is phosphorylated on serine residue(s) in response to TNF-α. This was established by phosphoamino acid analysis and western blots (PY20 phosphotyrosine antibody). In addition, PMA also serine phosphorylated $cPLA_2$ in monocytes. Therefore, kinases such as PKC and MAP kinase may well be involved in the signal transduction pathway of TNF-α, especially those processes dealing with the proinflammatory activities of TNF-α.

PKC and MAP kinase increase $cPLA_2$ phosphorylation/activity with MAP kinase phosphorylating the middle domain of $cPLA_2$ whereas PKC phosphorylates all 3 domains (Nemenoff et al, 1993). Phosphorylation of $cPLA_2$ by MAP kinase is markedly reduced by pretreatment of cells with PMA implying that PMA phosphorylates $cPLA_2$ at the same site in the middle domain. PKC may phosphorylate $cPLA_2$ through the activation of MAP kinase (Kazlauskas and Cooper, 1988), through the activation of other kinases, or directly. MAP kinase can also be activated by polypeptide growth factors and hormones acting through G-protein coupled receptors (Huckle et

al, 1990; Ahn et al, 1991); activation requires phosphorylation of the enzyme on tyrosine and threonine residues. The phosphorylation of multiple sites in $cPLA_2$ may be required for the full activation of $cPLA_2$. Alternatively, phosphorylation of $cPLA_2$ at some sites may be required for processes other than releasing AA from membrane phospholipids or may not be functionally relevant.

Human monocytes express both TNF-α receptors and the function of these receptors in the phosphorylation of $cPLA_2$ was examined by stimulating the monocytes with the TNFR55-selective mutants. The experimental data reported here indicate that both receptors are required for full $cPLA_2$ phosphorylation. When only binding to TNFR55 occurred, the phosphorylation of $cPLA_2$ was reduced compared to wild-type TNF, implying a role for TNFR75 in the phosphorylation of $cPLA_2$. This data correlates well with the conclusions reached earlier regarding the functional roles of the TNF receptors in the mediation of TNF's proinflammatory activity in human neutrophils and endothelium. These data suggest that TNFR75 facilitates the full proinflammatory effect of TNF-α in human myeloid cells and thus TNF mutants with selective binding to TNFR55 may result in improved antineoplastic efficacy in the clinical setting.

The role of $cPLA_2$ in the mediation of TNF cytotoxicity has also attracted considerable interest in recent times. TNF-α triggers two distinct intracellular responses in relation to cytotoxicity, one toxic and the other protective. Activation of the protective response, which relies primarily on TNF-induced expression of manganese superoxide dismutase, protects the mitochondria against oxygen radicals generated by the toxic response (Wong and Goeddel, 1988; Wong et al, 1989). Data presented by Hollenbach et al (1992) provide evidence for an additional mechanism of resistance to TNF-α. C3HA (murine 3T3-like cell line), SK-MEL-28 (human melanoma-derived cell line) and pVBETK-1cl15.2 (SV40-transformed murine L cell line) are resistant cell lines

which are unable to induce the activity of PLA_2. However in the presence of cycloheximide or actinomycin D the cells became sensitive to the cytotoxic action of TNF-α and PLA_2 was activated. In addition to preventing the TNF-induced expression of superoxide dismutase, cycloheximide and actinomycin D inhibit the expression of a short half-life protein which prevents the TNF-induced activation of PLA_2; the identity of this protein and its mechanism of action are not known.

Further corroborating evidence has come from Hayakawa et al (1993). They isolated a TNF-resistant subline (C12) from the TNF-sensitive cell line L929 (murine fibrosarcoma) and the cell line displayed a significant decrease in $cPLA_2$ activity. The expression of a cloned murine $cPLA_2$ cDNA in C12 cells increased the sensitivity of cells to TNF cytotoxicity. However, several cell lines that do not suffer TNF cytotoxicity release AA in response to TNF (Clark et al, 1988). In addition, the independent investigation of TNF cytotoxicity by two groups reached similar conclusions stating that TNF-mediated cytotoxicity is correlated with PLA_2 activity but not with AA or its metabolites (Neale et al, 1988; Suffys et al, 1991).

SUMMARY

Cytosolic phospholipase A_2 is an enzyme which mediates the release of eicosanoids and platelet-activating factor, both potent inflammatory mediators, and phosphorylation of $cPLA_2$ has been correlated to the increased activity of this enzyme. The phorbol ester PMA resulted in increased phosphorylation of $cPLA_2$ in U937 cells and human monocytes, indicating the involvement of PKC and serine phosphorylation in the activation of $cPLA_2$. The 110 kD $cPLA_2$ was not detected in human neutrophils and this may indicate the presence of an alternative form. In

monocytes, which possess both TNF receptors, $cPLA_2$ was rapidly phosphorylated by TNF-α (peak phosphorylation at 10 minutes). The phosphorylation of $cPLA_2$ induced by TNFR55-selective mutants (R32W, E146K and R32W-S86T) was reduced in comparison to wild-type TNF, implying the involvement of both receptors in this process. The production of PAF in monocytes by TNF-α was coupled in a time-dependent manner to the phosphorylation of $cPLA_2$, and was similarly less when TNFR55-selective mutants were used. Interestingly, the phosphorylation of $cPLA_2$ occurred on serine residue(s) as evidenced by phosphoamino acid analysis and negative phosphotyrosine western blots. These results demonstrate an important role for $cPLA_2$ in TNF signal transduction and the serine phosphorylation of this enzyme implies that MAP kinase and/or PKC are also involved in the mediation of TNF's proinflammatory activities.

TNF-α INDUCED NEUTROPHIL APOPTOSIS

Chapter 5

INTRODUCTION

Apoptosis (programmed cell death) is a form of cell death which is morphologically and biochemically distinct from necrosis. This process was first described by Kerr et al in 1972 and was based on observed morphological changes in normal physical situations. Shrinkage necrosis was the term originally used (Kerr et al, 1971) but when its widespread incidence and significance in health and disease were recognised the name apoptosis was proposed. The term apoptosis was suggested to Kerr et al by Professor James Cormack (University of Aberdeen) as it is the Greek term used to describe the "dropping off" or "falling off" of petals from flowers, or leaves from trees.

Numerous morphological and biochemical differences distinguish apoptosis from necrosis and the most outstanding changes occur in the nucleus. In apoptosis, single cells are deleted within tissues, there is cell-surface membrane blebbing with no loss of integrity and the cells shrink ultimately forming apoptotic bodies. These are phagocytosed by adjacent normal cells and macrophages without an inflammatory response. The nucleus is the location of much of the drama in apoptosis. The exact pattern is different from cell type to cell type but in general the nucleus shrinks and its chromatin becomes very dense, collapsing into patches, then into crescents in tight apposition to the nuclear envelope, and finally in many cells into one or several dense spheres. Biochemically, apoptosis is induced by physiological stimuli and is a tightly regulated process with synthetic and activation steps. The process requires energy and macromolecular synthesis, there is associated *de novo* gene transcription and nonrandom oligonucleosomal-length fragmentation of DNA. The DNA fragmentation results from random double-stranded DNA breaks in the linker regions between nucleosomal cores and can be detected as a ladder pattern on agarose gel electrophoresis (Kerr et al, 1972; Allen et al, 1993; Ueda and Shah, 1994).

Necrosis is invariably associated with a gross departure from physiological conditions such as severe hypoxia and ischaemia, major changes in environmental temperature, disruption of cell membranes by complement and exposure to toxins. The critical event leading to the development of necrosis is loss of cellular volume homeostasis; gross degrees of increased permeability of the plasmalemma are reflected in the failure to exclude vital dyes such as trypan blue. Morphologically, there is death of cell groups, membrane integrity is lost, cells swell and lyse, and a significant inflammatory response ensues. The necrotic process is energy independent, there are no requirements for protein synthesis, no new gene transcription, and there is random digestion of DNA (Wyllie et al, 1980; Allen et al, 1993).

The biological importance of apoptosis is highlighted by its participation in numerous processes. Apoptosis is involved in cell turnover in many healthy adult tissues (bone marrow, skin and gastrointestinal tract), the physiological involution and atrophy of various tissues, and is responsible for the focal elimination of cells during normal embryonic development (Glücksmann, 1951). It occurs spontaneously in untreated malignant neoplasms, and participates in at least some types of therapeutically induced tumour regression. In addition, it is implicated in the maturation of the immune system and is important in the pathophysiology of some viral infections (Kerr et al, 1972; Ueda and Shah, 1994).

TNF-α is well known for its induction of cytotoxicty in malignant cells and this can take the form of either apoptosis or necrosis (Sugarman et al, 1985; Balkwill et al, 1986; Fransen et al, 1986; Laster et al, 1988). However, the ability of TNF-α to induce cytotoxicty in normal cells has not been generally recognised but it is plausable that this may play an important homeostatic role in the noninflammatory apoptotic removal of certain cells. Neutrophils, which are stimulated by TNF-α to produce an array of proinflammatory effects, have the shortest half-life amongst the leukocytes, dying by apoptosis in approximately 24 hours (Begley et al, 1986; Lopez et al, 1986; Colotta et al, 1992). TNF-α may affect this apoptotic

process in neutrophils providing clues as to the true functioning of this pleiotropic cytokine. It is hypothesized here that TNF-α, after stimulating neutrophil activation in an attempt to rid the body of unwanted invaders, will induce apoptosis in these cells and thus aid in their removal in a noninflammatory manner. In addition, through the use of TNF mutants with selective TNF receptor-binding characteristics, the mechanism of action of TNF-α through its receptors has been explored in this process.

The TNF receptors (TNFR55 and TNFR75) are members of a family of receptors known as the TNF receptor superfamily (Smith et al, 1994). Prominent amongst this receptor family is the Fas antigen (Fas), or Apo-1, a 45 kD protein which is known for its ability to mediate apoptosis (Trauth et al, 1989; Itoh et al, 1991; Oehm et al, 1992). Interestingly, the intracellular region of Fas has a domain which is weakly homologous to the intracellular region of TNFR55 (27% identity over 66 amino acids) and this domain is known to be required for apoptosis (Itoh and Nagata, 1993; Tartaglia et al, 1993b). Thus, it has been proposed that both receptors may trigger apoptosis by similar mechanisms. Notably, the intracellular region of TNFR75 has no such "death domain". In view of the above, the possible role of Fas in TNF-induced neutrophil apoptosis has also been investigated in this chapter.

RESULTS

5.1 Neutrophil Apoptosis Examined by Microscopy

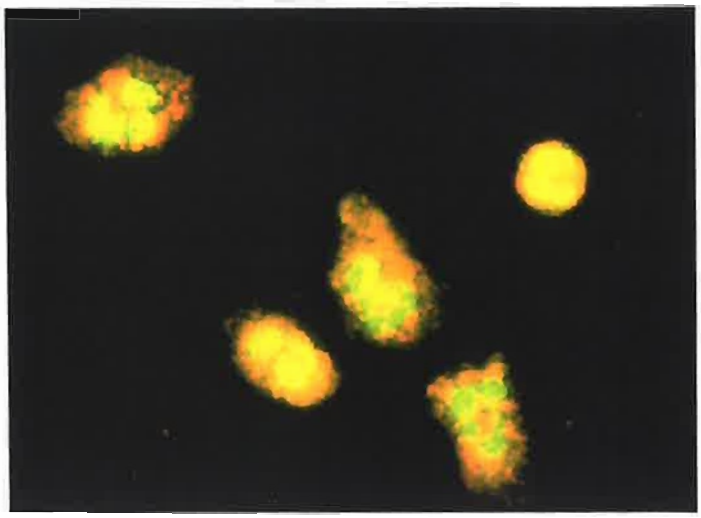
The apoptosis of human neutrophils obtained from normal healthy volunteers was examined by various techniques: fluorescent microscopy, light microscopy, transmission electron microscopy, DNA fragmentation gels and propidium iodide binding to DNA. Acridine orange (a cell-permeant DNA stain) and ethidium bromide were used to examine apoptosing neutrophils by fluorescent microscopy. The benefits of this method are easy visualization of

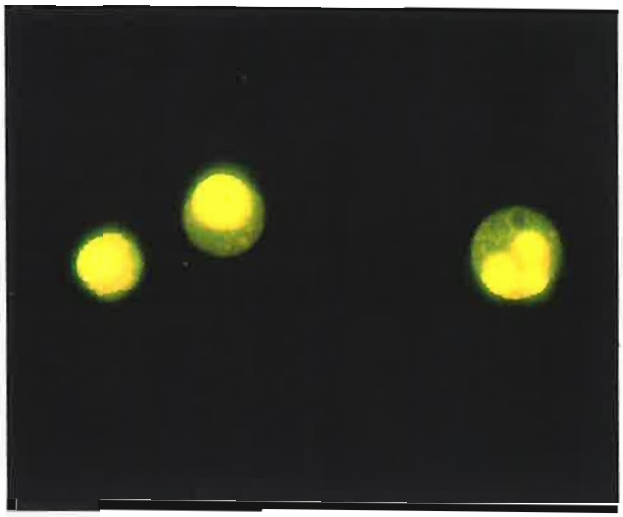
nuclear morphology by acridine orange (apple green staining) and the recognition of necrotic neutrophils by their ethidium bromide uptake (the entire cell stains bright orange). The necrotic neutrophils, as evidenced by ethidium bromide uptake, are also the same cells which allow entry of trypan blue. It is apparent by fluorescent micoscopy that the apoptosing neutrophils become smaller and spherical, and the nucleus becomes shrunken, dense and round (figure 5.1 top). As the cell shrinks it is likely that the cell-surface protrusions assume a spherical configuration, detach, and float away in the medium as discrete globules (Wyllie et al, 1980). During the neutrophil apoptosis experiments the cells were placed in RPMI-1640 medium with 0.1% BSA and were kept shaking gently in a 37°C waterbath. Almost all neutrophils underwent death by apoptosis in the time course and dose response experiments performed, with <2% becoming necrotic as evidenced by ethidium bromide and/or trypan blue uptake.

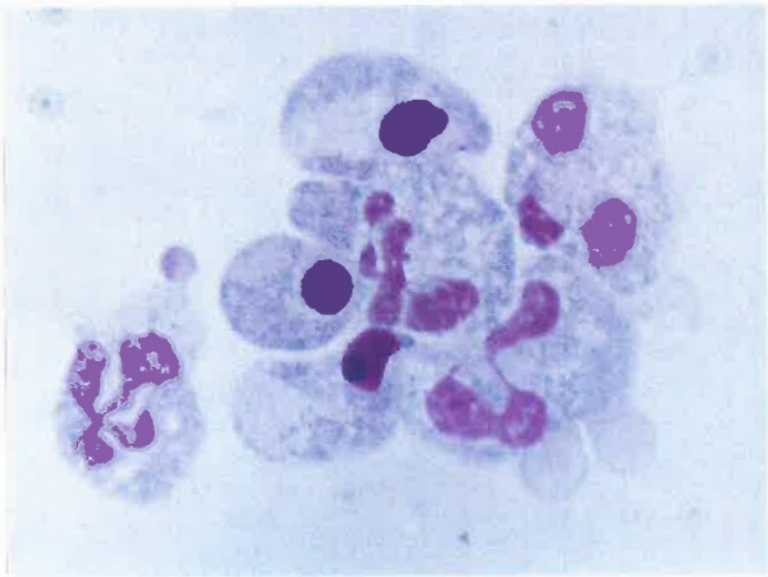
By light microscopy (Jenner-Giemsa stain), apoptosing neutrophils possessed intensely staining, shrunken nuclei but the overall size was not reduced (figure 5.1 middle). This is most likely to be the result of cells colliding with the glass slide during slide preparation (1000 rpm for 5 minutes) as the cytoplasmic membranes of apoptotic neutrophils are probably not as rigid and this leads to splaying of these cells. Transmission electron microscopy also revealed apoptotic neutrophils to be as large as normal nonapoptotic cells and this would also seem to be an artifactual result of the preparative process. As seen in figure 5.1 (bottom), the apoptosing neutrophils show a condensed spherical nucleus (no longer multinucleated/polymorphonuclear) and a cytoplasmic membrane which has lost its projections. In the fluorescent microscopy technique, the neutrophils are pipetted onto the surface of the slide and a coverslip is gently placed on top of the cells. Under these conditions the small apoptotic cells are observed.

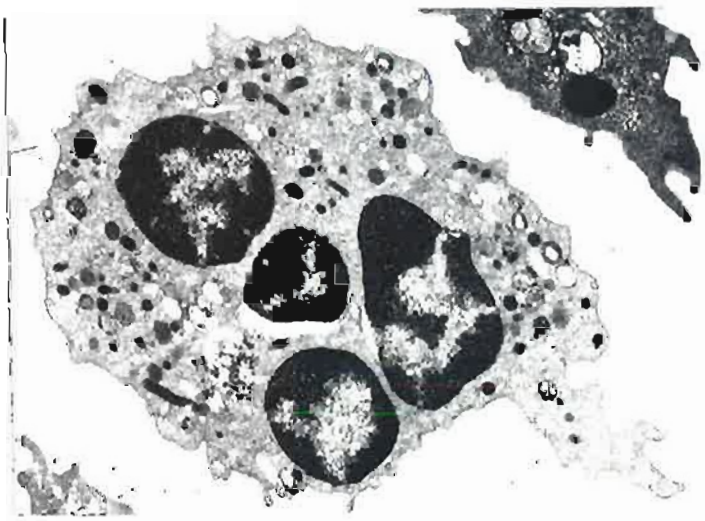
Figure 5.1 Apoptotic and normal neutrophils examined by microscopy

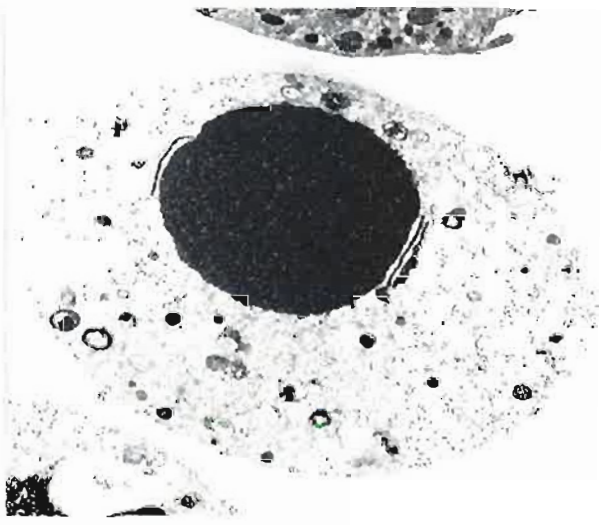
Apoptotic and normal neutrophils as examined by (Top) fluorescent microscopy - the apoptotic neutrophils (top right) are easily distinguished from normal neutrophils (top left) by their shrunken cell size and small spherical nuclei (600x, acridine orange stain); (Middle) light microscopy - normal neutrophils are polymorphonuclear and apoptotic neutrophils have small spherical, intensely staining nuclei (600x, Jenner-Giemsa stain); (Bottom) Transmission electron microscopy - apoptotic neutrophils (bottom right) have lost their cell-surface projections and the nuclei are spherical and condensed compared to normal neutrophils (bottom left) (7800x).











5.2 TNF-induced Neutrophil Apoptosis

TNF-α activates neutrophils resulting in inflammatory sequelae (Klebanoff et al, 1986; Barbara et al, 1994). It was hypothesized that this activation of neutrophils by TNF-α would result in the accelerated death of neutrophils but in a manner which would not incite further inflammation i.e. death by apoptosis. In this way, TNF-α could be seen as a biological agent which responds to infection in a controlled, homeostatic manner. It is established here that TNF-α induces rapid apoptosis of human neutrophils, as examined by fluorescent microscopy (figure 5.2). Within 3 hours, 50.3 ± 3.5% of neutrophils exposed to TNF-α (100 ng/ml) are apoptosed. This percentage gradually increased to 92.0 ± 3.8% at 24 hours with statistical significance reached at all time points except the last. At the same dose of TNF-α, no effect on human eosinophil apoptosis was demonstrated when compared to unstimulated eosinophils (appendix 2). In addition, GM-CSF did not enhance neutrophil survival as has been previously reported using other methods (Begley et al, 1986; Lopez et al, 1986; Colotta et al, 1992), however doses of GM-CSF used were much greater in the experiments reported here. The ability of TNF-α to induce neutrophil apoptosis also occurred in a dose-dependent manner (figure 5.3). Concentrations of TNF-α as low as 1 ng/ml induces a perceptible increase in neutrophil apoptosis and there is a plateauing effect at higher concentrations with 58.4% of cells apoptosed at 300 ng/ml compared to 53.1% at 100 ng/ml of wild-type TNF. As the three hour incubation with TNF-α (100 ng/ml) produced significant neutrophil apoptosis in the time course experiments (50.3%), this time point was chosen for the dose response experiments.

5.3 Confirmation of TNF-induced Neutrophil Apoptosis by DNA Fragmentation Gels and Propidium Iodide Binding to DNA

The hallmark of apoptosis is considered by many to be DNA fragmentation, in which endonuclease activity fragments DNA at linker sites between nucleosomes, generating a population of DNA fragments of varying sizes. The DNA fragments are multimers of approximately 180 base pair nucleosomal units, resulting in the appearance of "DNA

Figure 5.2 TNF-α induces rapid apoptosis in human neutrophils

The results are the pooled data from 3 experiments using different neutrophil donors and examined by fluorescent microscopy. TNF-α 100 ng/ml (solid bars), GM-CSF 100 ng/ml (cross-hatched bars) and control (open bars). The error bars represent the SEM and the results were analysed by the paired Student's *t*-test.

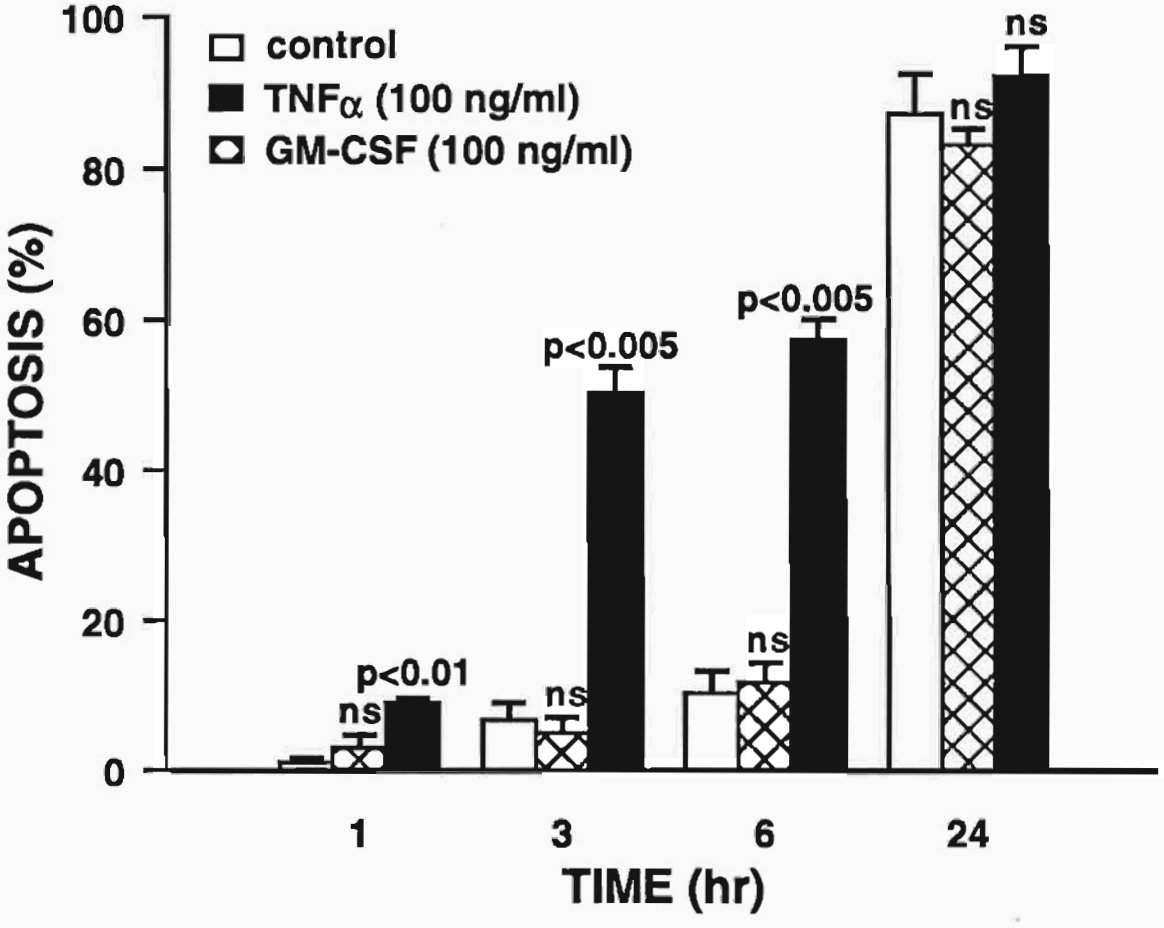
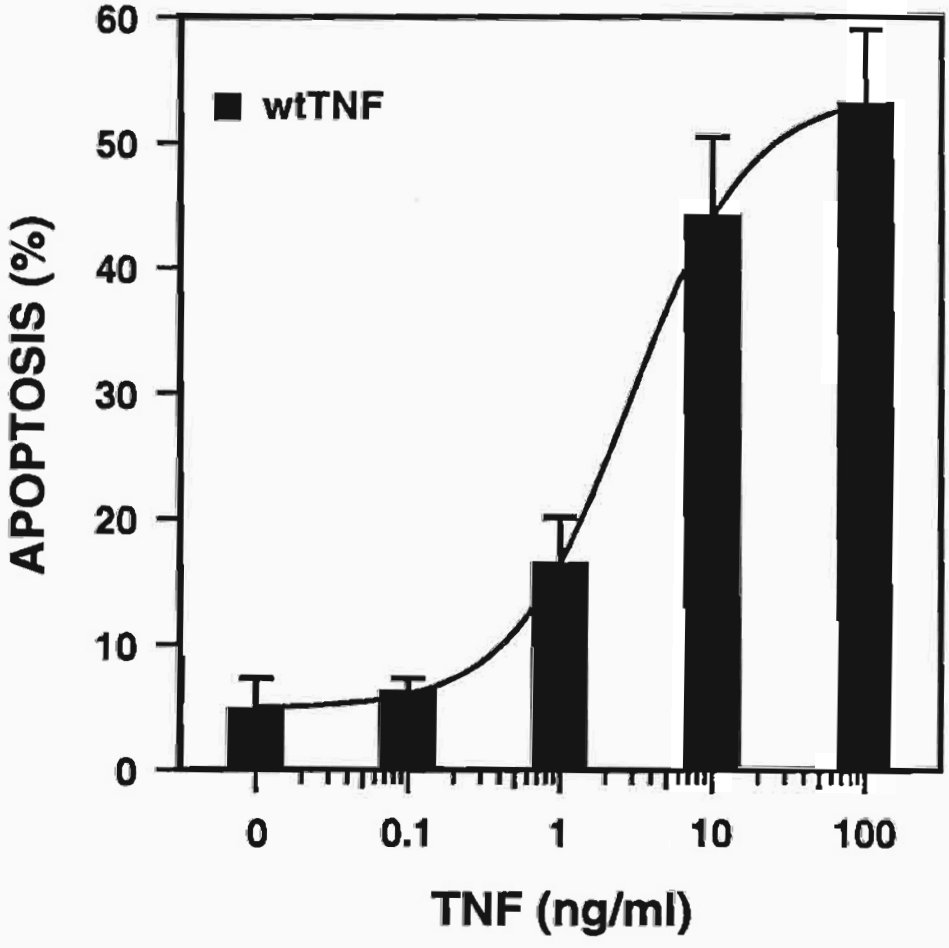

100
80
60
40
20
0
APOPTOSIS (%)
control
TNFα (100 ng/ml)
GM-CSF (100 ng/ml)
p<0.01
ns
ns
p<0.005
ns
p<0.005
ns
ns
1
3
6
24
TIME (hr)

Figure 5.3 TNF-α induces neutrophil apoptosis in a dose-dependent fashion

The results of 4 experiments using neutrophils from different donors, each performed in triplicate, are shown in a pooled manner. The neutrophils were examined by fluorescent microscopy. The incubation time for these experiments was 3 hours. Error bars represent the SEM.


60
50
40
30
20
10
0
wtTNF
APOPTOSIS (%)
0
0.1
1
10
100
TNF (ng/ml)

laddering" when DNA extracted from apoptotic cells is electrophoresed on agarose gels (Wyllie, 1980). The morphological findings described above, as determined by fluorescent microscopy, were confirmed by DNA fragmentation gel experiments. It was found that TNF-α induces nonrandom DNA fragmentation in neutrophils in a time and dose-dependent manner (figure 5.4) with DNA fragmentation evident as early as two hours and occurring with TNF concentrations of 1 ng/ml and above. 10 μg of DNA was loaded per lane and run on a 1.2% agarose gel. In addition to the microscopic and DNA fragmentation findings, the apoptosis of neutrophils by TNF-α was examined using propidium iodide. As a cell apoptoses and its DNA fragments, less propidium iodide binds to the DNA and this can be analysed using a flow cytometer (Nicoletti et al, 1991). As shown in figure 5.5, TNF-α (100 ng/ml) stimulation of neutrophils for 3 hours results in a marked decrease in propidium iodide binding compared to control samples.

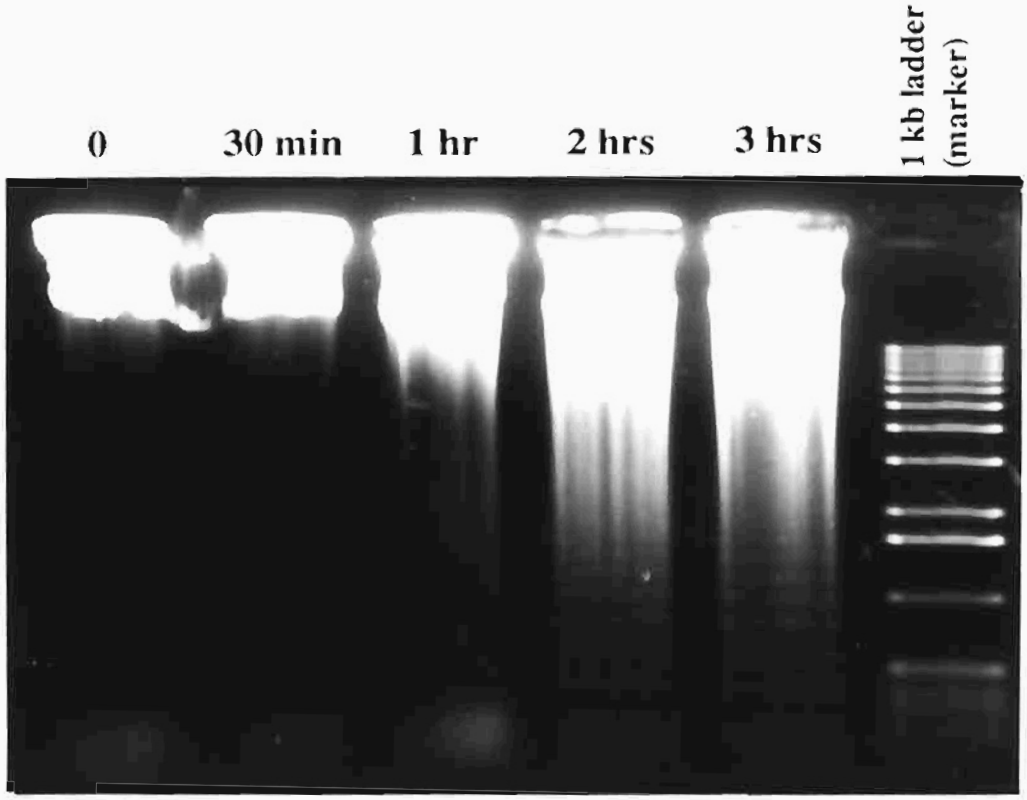
5.4 The Roles of the TNF Receptors in TNF-induced Neutrophil Apoptosis

Having established that TNF-α induces neutrophil apoptosis in a time and dose-dependent manner, the receptor-selective TNF mutants were examined (using fluorescent microscopy) for their ability to induce neutrophil apoptosis. It was hypothesized that the roles of the TNF receptors as determined for neutrophil proinflammatory activities (chapter 3) may similarly apply to the apoptotic death of neutrophils since these cells die in an accelerated manner whilst being stimulated to produce proinflammatory activity. If these processes are coordinated as part of a homeostatic function of TNF-α then it would seem reasonable to suggest that the same TNF receptor biology may apply. It was found that the TNFR55-selective mutants, E146K and R32W-S86T, resulted in 16-fold and 14-fold less neutrophil apoptosis than wild-type TNF respectively (figure 5.6). The potency (%) / ED50 for E146K (6.1%; 82.7 ± 4.3 ng/ml) and R32W-S86T (7.2%; 69.5 ± 5.4 ng/ml) was significantly different to wild-type TNF (100%; 5.0 ± 1.7 ng/ml) with $p<0.005$ and $p<0.01$ respectively as determined by the paired Student's *t*-test. These results indicate a role for both receptors in neutrophil apoptosis. The

Figure 5.4 Nonrandom DNA fragmentation induced in human neutrophils by TNF-α

(A) Time course: 10 μg DNA loaded per lane on a 1.2% agarose gel. TNF-α (100 ng/ml) was used as the stimulus. DNA laddering is evident as early as 2 hours. (B) Dose response: 10 μg DNA loaded per lane. Incubation time for the dose response experiments was 3 hours. E146K and D143F were also examined at 10 and 100 ng/ml. E146K resulted in less DNA fragmentation compared to wild-type TNF and D143F induced little, if any, DNA fragmentation. The marker used in the time course and dose response experiments was the 1 kb ladder (Gibco BRL).

A.


0
30 min
1 hr
2 hrs
3 hrs
1 kb ladder (marker)

B.

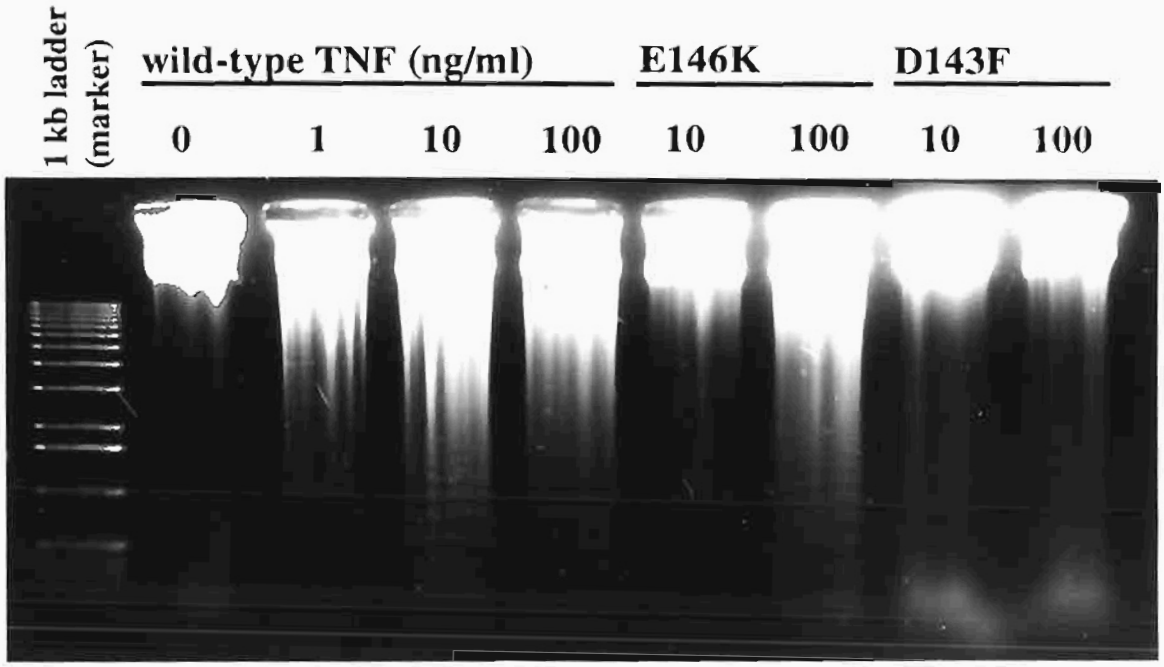

1 kb ladder (marker)
wild-type TNF (ng/ml)
E146K
D143F
0
1
10
100
10
100
10
100

Figure 5.5 TNF-induced DNA fragmentation in neutrophils demonstrated by reduced propidium iodide binding

(A) Unstimulated neutrophils at 37°C for 3 hours. (B) TNF-stimulated neutrophils (100 ng/ml) at 37°C for 3 hours. The left-sided shoulder in (A) represents the population of neutrophils which has reduced propidium iodide uptake and equates with the much larger population of cells in (B) [linear fluorescent intensity range 125-150].

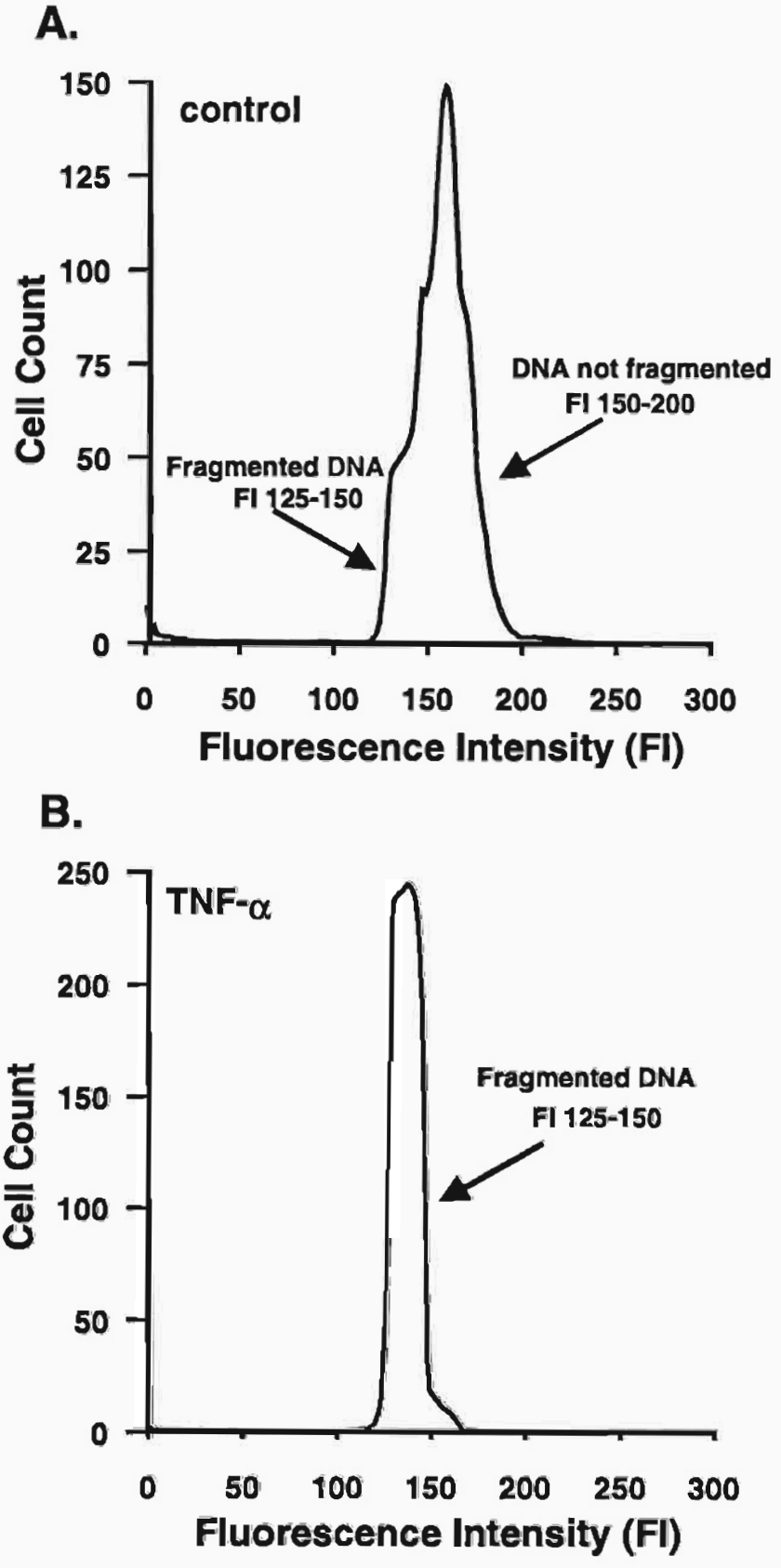
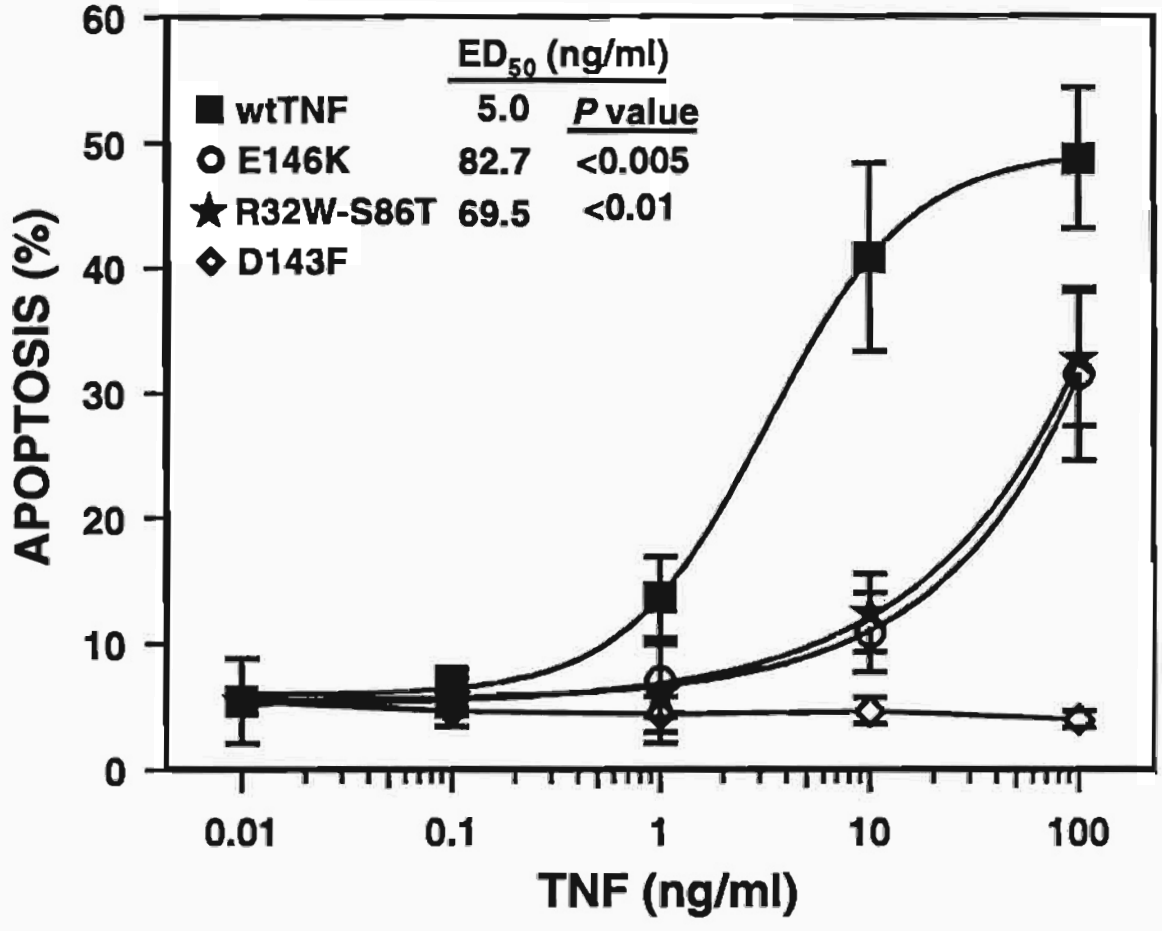

A.
control
150
125
100
75
50
25
0
Cell Count
DNA not fragmented
FI 150-200
Fragmented DNA
FI 125-150
0
50
100
150
200
250
300
Fluorescence Intensity (FI)
B.
TNF-α
250
200
150
100
50
0
Cell Count
Fragmented DNA
FI 125-150
0
50
100
150
200
250
300
Fluorescence Intensity (FI)

Figure 5.6 Neutrophil apoptosis induced by the TNF receptor-selective mutants

Three pooled dose response experiments are shown with each experiment performed in triplicate. The neutrophils were exposed to wild-type TNF and TNF mutants for 3 hours. Error bars represent the SEM. ED_{50}'s for E146K and R32W-S86T are shown and are significantly greater than wild-type TNF as determined by the paired Student's *t*-test. Wild-type TNF (closed squares), E146K (open circles), R32W-S86T (closed star) and D143F (open diamond).


60
50
40
30
20
10
0
APOPTOSIS (%)
ED50 (ng/ml)
P value
wtTNF 5.0
E146K 82.7 <0.005
R32W-S86T 69.5 <0.01
D143F
0.01
0.1
1
10
100
TNF (ng/ml)

TNFR75-selective mutant D143F did not induce neutrophil apoptosis and indicates that TNFR75 on its own is not sufficient. These TNF mutant results were also confirmed in DNA fragmentation experiments (figure 5.4B) and reflect those observed with the neutrophil and endothelial proinflammatory activities examined in chapter 3. Therefore, TNFR75 would also seem to have a facilitative role in apoptotic signalling through TNFR55.

5.5 Fas Antigen Expression on Leukocytes and Lack of Modulation by TNF-α

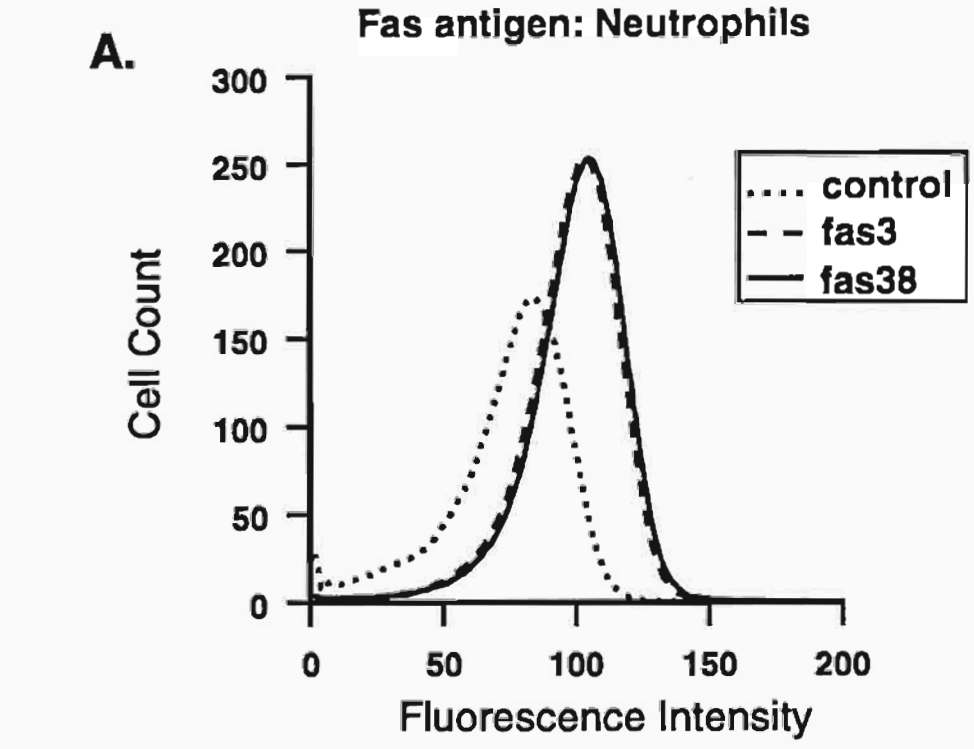
In view of the established role of Fas in mediating apoptosis (Trauth et al, 1989; Itoh et al, 1991), the expression of Fas on human neutrophils and its possible regulation by TNF-α was investigated. Using antibodies recognising the Fas antigen (gift from Mark Alderson, Immunex, Seattle) it was determined here that the Fas antigen is a cell-surface antigen on human neutrophils (figure 5.7), contrary to the unpublished data of Takeda et al (1993) who indicated that Fas could not be identified on neutrophils. Fas antigen was also found to be present on human monocytes, eosinophils and lymphocytes (figure 5.7B). It was hypothesized that the rapid apoptosis of neutrophils by TNF-α might in part be explained by its upregulation of Fas. However, as seen in figure 5.8, the upregulation of Fas in response to TNF-α was not significantly greater than that observed with control or GM-CSF.

DISCUSSION

Apoptosis is an important physiological process, morphologically and biochemically distinct from necrosis, which is only now receiving the same interest as the other basic cellular processes, proliferation and differentiation. In this chapter the apoptosis of human neutrophils was examined and in particular, the role of TNF-α and its receptors in the mediation of this process was investigated. It is shown here that TNF-α reduces neutrophil survival by enhancing apoptosis in a time and dose-dependent manner as determined by microscopy, DNA

Figure 5.7 Flow cytometry demonstrating the presence of Fas antigen on human leukocytes

(A) A representative experiment of 4 determinations with neutrophils using Fas antibodies M3 and M38. The negative control antibody was 3D3.3. (B) The presence of Fas antigen is illustrated on neutrophils (n=4), monocytes (n=4), eosinophils (n=2) and lymphocytes (n=4) using M38 antibody.


A.
Fas antigen: Neutrophils
Cell Count
300
250
200
150
100
50
0
0
50
100
150
200
Fluorescence Intensity
control
fas3
fas38

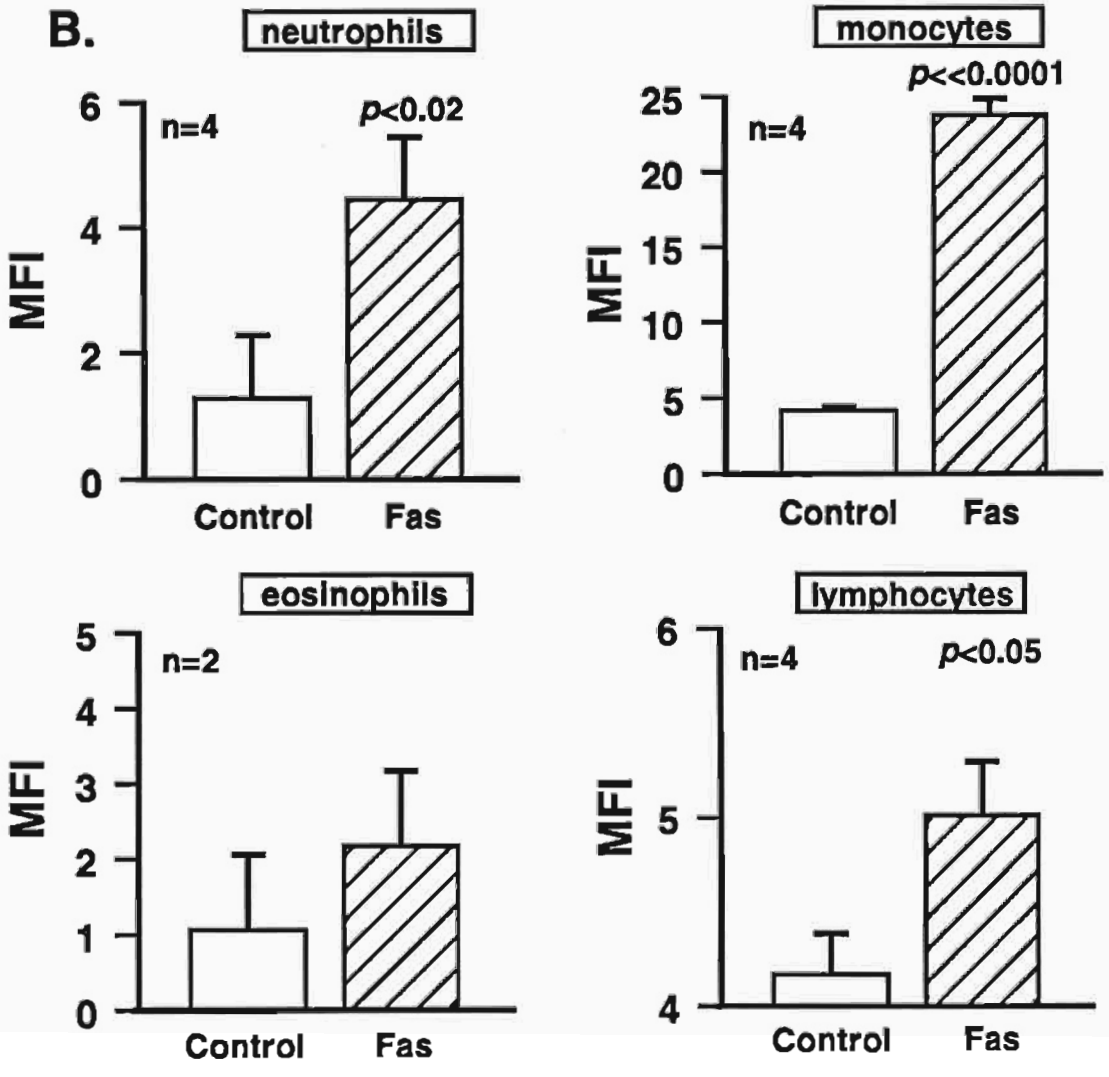
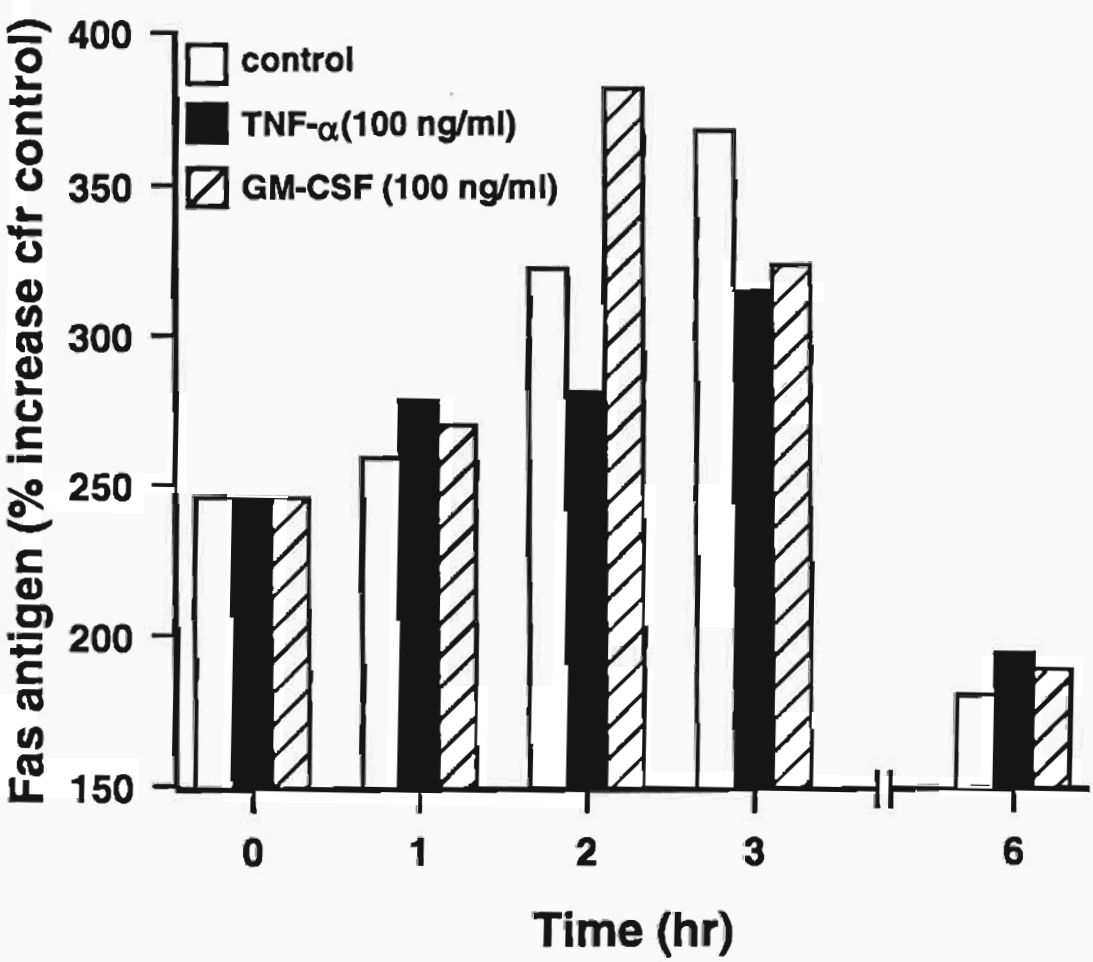

B.
neutrophils
n=4
p<0.02
MFI
6
4
2
0
Control
Fas
monocytes
p<<0.0001
n=4
MFI
25
20
15
10
5
0
Control
Fas
eosinophils
n=2
MFI
5
4
3
2
1
0
Control
Fas
lymphocytes
n=4
p<0.05
MFI
6
5
4
Control
Fas

Figure 5.8 The expression of Fas antigen on neutrophils in response to TNF-α

This time course experiment is representative of 3 performed and samples were prepared in triplicate each time. Control (open bars), TNF-α (solid bars), and GM-CSF (diagonal lines). Each determination by flow cytometry is the average of 10,000 analysed cells.


Fas antigen (% increase cfr control)
400
350
300
250
200
150
control
TNF-α(100 ng/ml)
GM-CSF (100 ng/ml)
0
1
2
3
6
Time (hr)

fragmentation gels and propidium iodide binding. Also, the results obtained with the TNF mutants indicate that, as with the neutrophil and endothelial proinflammatory activities, TNF55 is the dominant receptor in the mediation of this activity. However, TNFR75 appears to facilitate the process but on its own is insufficient.

The apoptotic process is especially pertinent to haemopoiesis and the overall maintenance of a functional immune system. The first line of defence in the immune system is occupied by the neutrophil which incidentally has the shortest half-life among the leukocytes (Begley et al, 1986; Lopez et al, 1986; Colotta et al, 1992). Neutrophils die rapidly in culture with percentage survival at 24, 48, 72 and 96 hours of 97.3, 36.8, 14.5 and 4.2 respectively (Colotta et al, 1992). It was hypothesized here that neutrophils stimulated with TNF-α to produce inflammatory activity would experience an accelerated death by apoptosis, resulting in the noninflammatory removal of these cells. Indeed, this was shown to be the case, with the majority of neutrophils apoptosing within the first few hours of exposure to TNF-α. This time frame is similar to TNF's stimulation of neutrophil inflammatory activities (Atkinson et al, 1988). Therefore, TNF-α activates neutrophils to protect the body from foreign invaders and simultaneously sets in motion the noninflammatory removal of these cells so as to keep the immune response homeostatically regulated. However, in the case of septic shock, it is postulated that this homeostatic function of TNF-α is overwhelmed by other processes (Standiford and Strieter, 1992).

In contrast to the above findings, Colotta et al (1992) determined that TNF-α, as well as IL-1 β, GM-CSF, G-CSF and IFNγ, resulted in a marked increase in viability of neutrophils as determined by trypan blue exclusion. Apoptotic morphology was studied, however data regarding TNF-α was not included in the paper. DNA fragmentation was quantified using diphenylamine (qualitative DNA fragmentation gels were not illustrated) and this indicated

that TNF-α produced less fragmentation than control, in keeping with the viability data. The findings of Colotta et al (1992) are at odds with those presented here and probably reflects the different methodologies used and illustrated. However, the independent study of Takeda et al (1993) is consistent with the results presented in chapter 5, indicating that TNF-α induces rapid apoptosis in neutrophils.

The role of the two TNF receptors in the mediation of neutrophil apoptosis was also investigated. The TNFR55-selective mutants, E146K and R32W-S86T, resulted in neutrophil apoptosis which was significantly decreased compared to wild-type TNF, however at higher concentrations the results approximated those of wild-type. The implication is that TNFR75, along with TNFR55, is involved in the mediation of such activity especially at lower TNF concentrations. However, as shown by the lack of apoptotic activity with the TNFR75-selective mutant D143F, TNFR75 on its own is insufficient and therefore must facilitate the dominant TNFR55. As mentioned, Itoh and Nagata (1993) and Tartaglia et al (1993b) have demonstrated that the intracellular domain of TNFR55 has weak homology to Fas (27% identity over 66 amino acids; known as the "death domain") whereas TNFR75 has no such domain and this molecular knowledge is consistent with the results obtained.

In view of the demonstrated ability of Fas to mediate apoptosis in a variety of cells (Trauth et al, 1989; Itoh et al, 1991) and the shared "death domain" in Fas and TNFR55, the presence of Fas on human neutrophils (and other leukocytes) and the effect of TNF-α on Fas expression was investigated. When the expression of Fas on neutrophils was verified it was postulated that upregulation of Fas by TNF-α in neutrophils might assist in the apoptotic death of these cells, in addition to as yet unidentified direct apoptotic pathways induced by TNF-α. However, TNF-α does not appear to alter the expression of Fas in neutrophils relative to the control. This is in contrast to that reported by Yonehara et al (1989) in which co-

downregulation of both TNF receptor and Fas was shown in response to TNF-α, but is consistent with the findings of Grell et al (1994b) where no co-modulation of Fas and TNF receptors was observed. It is possible that TNF-α may affect the Fas system through the Fas ligand and not the Fas antigen, however the production of Fas ligand by neutrophils has not yet been determined.

The importance of apoptosis is highlighted by the therapeutic benefits that a full understanding of this process will bring. Despite the lack of knowledge concerning the cellular mechanisms involved, there already exists great therapeutic potential in the treatment of malignancies and viruses such as the Epstein-Barr virus (EBV) and the Human Immunodeficiency virus (HIV). Anti-APO-1 (antibody to Fas) has been shown to induce apoptosis in adult T-cell leukaemic cell lines (Debatin et al, 1990). In acquired immunodeficiency syndrome (AIDS) a drug that inhibits apoptosis might be useful in preventing T helper cell loss, allowing time for other forms of therapy to clear the infection. Alternatively, with other viruses one may be able to prevent virus propagation by inducing apoptosis of potential host cells. However, viruses such as EBV have already developed mechanisms to prevent apoptosis (Henderson et al, 1991). In addition, EBV has a protein (BHRF1) which is homologous to the anti-apoptotic cellular protein bcl-2 (Harrington et al, 1994). Only a full understanding of the intracellular pathways of apoptosis will allow appropriate therapies to be developed.

SUMMARY

The life span of the human neutrophil is relatively short (approximately 24 hours) with the cell dying by apoptosis. In the presence of TNF-α neutrophils rapidly apoptose with 50% dying within 3 hours. The apoptotic death of neutrophils induced by TNF-α was found to occur in a time and dose-dependent manner, as determined by fluorescent microscopy, DNA laddering

gels and propidium iodide binding. The TNF receptor-selective mutants revealed that TNF-induced neutrophil apoptosis is primarily mediated by TNFR55, with TNFR75 facilitating the full mediation of this activity especially at lower doses. Stimulation of TNFR75 on its own is insufficient to mediate neutrophil apoptosis. The functional role of the two TNF receptors in neutrophil apoptosis parallels that seen with TNF-induced neutrophil and endothelial proinflammatory activity and indicates that once neutrophils are activated by TNF-α they are also induced to undergo apoptosis with resultant noninflammatory removal. In addition, Fas was present on the cell surface of neutrophils but its expression does not appear to be regulated by TNF-α.

DISCUSSION

Chapter 6

Since the cloning of the TNF-α gene in 1984, the availability of this cytokine has allowed intensive research to be undertaken and we are now starting to enjoy the benefits. For example, the use of a chimeric monoclonal antibody to TNF-α has resulted in improvement of the autoimmune disorder, rheumatoid arthritis (Elliott et al, 1994). TNF-α is also implicated in the pathophysiology of many other disorders including septic shock, AIDS, malaria, diabetes mellitus, systemic lupus erythematosis and allograft rejection (Fiers, 1991). A greater understanding of the biological interaction of TNF-α with its receptors and a more comprehensive knowledge of associated intracellular signal transduction pathways will permit the development of appropriate therapeutic strategies for the above disorders.

In this thesis, the functional roles of the TNF receptors in the mediation of TNF's cytotoxic and proinflammatory activities were examined by using TNF mutants which selectively bind to the TNF receptors. TNFR75 mediates significant toxicity/lethality in the murine model and it was postulated here that TNFR75 may have an important role in the mediation of TNF's inflammatory activities in myeloid cells and endothelium. The signal transduction mechanisms of TNF-α were explored by investigating the expression and functional activation of phospholipase A_2, an enzyme which is critical in the formation of eicosanoids and PAF, both potent inflammatory mediators. It was hypothesized that PLA_2 functioning may correlate with the mediation of proinflammatory activities in myeloid cells stimulated by TNF-α and the TNF mutants. In addition to examining a range of neutrophil activities in response to TNF-α and TNF mutant stimulation, the demise of these inflammatory cells was also investigated. It was proposed that TNF-α would induce apoptotic cell death in activated neutrophils thus allowing removal of these cells in a noninflammatory manner. This would be consistent with TNF-α behaving as an homeostatic cytokine which only becomes harmful if the system is overwhelmed, as in septic shock.

The role of the TNF receptors in TNF cytotoxicity, whether it be by necrosis or apoptosis, is controversial. Several groups have argued as to the inclusion or exclusion of TNFR75 in TNF-mediated cytotoxicity. In chapter three it was established that only TNFR55 is involved in the cytotoxicity and cytostasis of the malignant cell lines HEp-2 (human carcinoma of the larynx) and U937 (human monoblastoid leukaemic cell line) respectively. Van Ostade et al (1993) using the TNFR55-selective mutant R32W also demonstrated an important role for TNFR55 in the cytotoxicity of HT-29 (human colonic carcinoma cell line) cells *in vivo*. Independently, Loetscher et al (1993) using the TNF mutant R32W-S86T showed that this TNFR55-selective mutant produced a cytotoxic response in KYM-1 cells (human rhabdomyosarcoma) indistinguishable from that of wild-type TNF. These studies are supported by those of other groups using 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 (Brakebusch et al, 1992; Tartaglia and Goeddel, 1992a).

However, Heller et al (1992) provided several lines of evidence which strongly implicated TNFR75 in TNF-mediated cytotoxicity: (i) Murine TA1 (adipocyte) cells which were shown to be sensitive to murine TNF and not human TNF implying that, in accordance with the species specificity of TNF-α, TNFR75 is important in cytotoxicity. (ii) Human HeLa (epithelial) cells transfected with TNFR75 cDNA became sensitive to TNF-α. (iii) Murine NIH 3T3 (fibroblasts) cells are not sensitive to hTNF but when transfected with the human TNFR75 the cells become sensitive to hTNF. Tartaglia et al (1993a) responded to this work by performing studies with similar experimental design and refuted the findings of Heller et al (1992). Whether TNFR75 alone could cause cytotoxicity was not addressed in the published work of Heller et al (1992) because TNFR55 and TNFR75 were present whenever cell death occurred. Recently, Bigda et al (1994), using TNF receptor antibodies, concluded that TNFR75 could contribute to the cytotoxicity of TNF by its own signaling and also by

regulating the access of TNF-α to TNFR55 as per the "passing on" model of Tartaglia and Goeddel (1992b). It would also seem that TNFR75 is involved in the mediation of TNF cytotoxicity in a cell-specific manner (Medvedev et al, 1994). In chapter 5 it was also shown that TNFR75 was involved in the apoptotic death of neutrophils through the facilitation of TNFR55. In view of the above, it would seem reasonable to suggest that TNFR75 has a role, albeit unclear, in the mediation of cytotoxicity in certain cell systems.

Many of the effects of TNF-α as a mediator of inflammation are due to its ability to stimulate neutrophil activities, and in this thesis superoxide anion generation, PAF formation, ADCC activity and neutrophil adherence to endothelium was examined. For the first time, the roles of the TNF receptors in the mediation of neutrophil activation was investigated in this thesis and it was shown, using the TNF receptor-selective mutants, that TNFR55 is the dominant receptor associated with the signalling of all these activities. However, TNFR75 is also involved, especially at lower doses of TNF-α, but on its own is insufficient in signalling these activities. In contrast, Menegazzi et al (1994) recently demonstrated that the activation of the neutrophil respiratory burst by TNF-α was only mediated by TNFR55, stating that the agonistic acitivity of the anti-TNFR55 monoclonal antibodies was comparable to TNF-α. However, htr-9 (mAb to TNFR55) only induced a response which was 65% that of TNF-α, implying a role for TNFR75 as indicated by the TNF mutant data in this thesis. Utr-1 (mAb to TNFR75) did not exhibit any agonist activity on the neutrophil respiratory burst indicating that TNFR75 on its own is insufficient. This is consistent with the lack of activity seen with the TNFR75-selective mutant, D143F.

The endothelial cell constitutes another major site of inflammatory activity for TNF-α. The ability of TNF-α to stimulate neutrophil adherence to, and transmigration through, endothelium was examined along with the underlying mechanisms of E-selectin expression and IL-8 secretion. Interestingly, the roles of the TNF receptors in endothelial cell activation by

TNF-α were identical to that seen with the neutrophil activities. It is possible that the receptor biology described for neutrophils and endothelium is specific for the cell systems which participate in the inflammatory activities of TNF-α. As seen in chapter 4, the phosphorylation of $cPLA_2$ and the synthesis of PAF by TNF-α in monocytes would appear to follow the same dictum.

The functional roles of the TNF receptors in endothelial cell activation has also attracted keen interest from various groups (Loetscher et al, 1993; Mackay et al, 1993; Slowik et al, 1993). Using the TNFR55-selective mutant R32W, Slowik et al (1993) indicated that the mutant was equipotent to wild-type TNF in upregulating several different leukocyte adhesion molecules as well as class I MHC molecules, implying that only TNFR55 is involved in the mediation of these activities. However, dose response experiments comparing R32W to wild-type TNF were not shown. In contradiction, Slowik et al (1993) implied that a role for TNFR75 was demonstrated when induction of ELAM-1 (E-selectin) by low concentrations of TNF-α was partly inhibited by blocking antibodies to TNFR75. These antibody results reflect the TNF mutant data reported in chapter 3 which showed a role for TNFR75 especially at lower concentrations of TNF-α. As previously demonstrated, the TNF mutant R32W is not as selective for TNFR55 as E146K and R32W-S86T and its relatively greater binding to TNFR75 may explain the results obtained. In addition, Mackay et al (1993) concluded that the exclusive activity of TNFR55 was required for the expression of E-selectin, ICAM-1 and VCAM-1 on endothelium. However, only qualitative time course data was shown (cytofluorometric histograms; 10 ng/ml) and dose response experiments were not included.

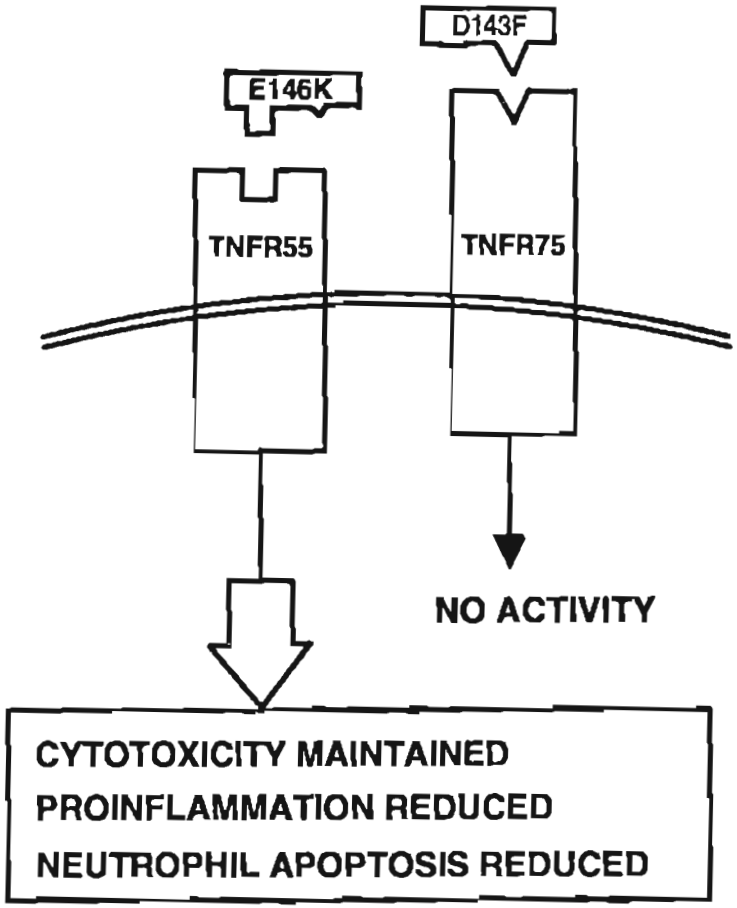
In addition, Loetscher et al (1993) reported that GM-CSF and IL-6 secretion from human endothelial cells was induced by R32W-S86T (10 ng/ml) at a similar specific activity to wild-type TNF, once again indicating exclusive involvement of TNFR55. However, results with lower concentrations of TNF-α and mutant were not shown and it is at these lower

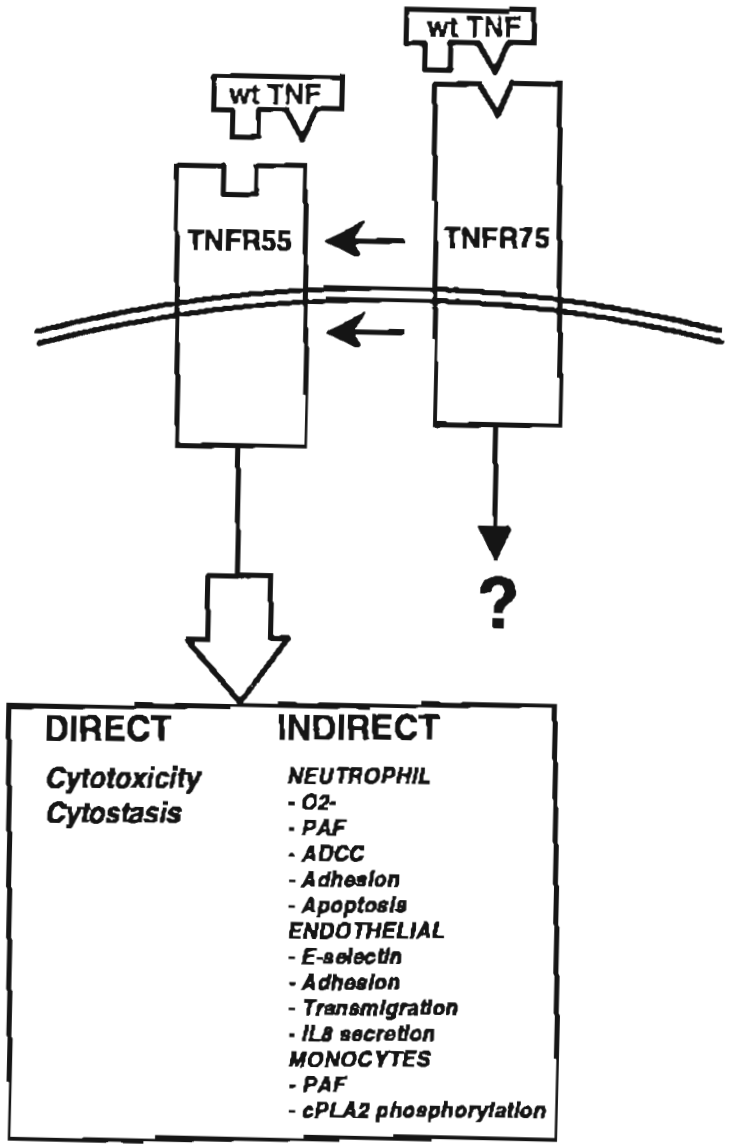
concentrations that more sizeable differences become apparent. At higher concentrations of TNF-α it would seem that full activity can be mediated solely through TNFR55 without TNFR75 facilitation. Stimulation of endothelium with the TNFR75-selective mutant D143N-A145R (>2500-fold decrease in binding to TNFR55 and only 6.7-fold reduced binding to TNFR75) induced no activity in keeping with the results presented here (chapter 3).

Paleolog et al (1994), using agonistic and antagonistic antibodies to TNFR55 and TNFR75, examined the endothelial expression of tissue factor antigen and adhesion molecules, and the secretion of IL-8 and GM-CSF from these cells. They reported that the TNFR55-agonistic antibody results in the activation of endothelium however the TNFR75-agonistic antibody produces very little or no endothelial cell responses. In addition, the antagonistic antibodies resulted in a reduction of these endothelial activities with the effect of the TNFR55-antagonistic antibody being much greater than the TNFR75-antagonistic antibody. These results, contrary to the above TNF mutant studies, correlate well with the TNF mutant data presented in chapter 3.

In the systems described, the TNF mutants with their selective binding characteristics have allowed a unique examination of TNF's mode of action through its receptors and a model has been proposed (figure 6.1). The TNFR55-selective mutants (R32W and E146K) resulted in the same, if not greater, cytotoxicity and cytostasis than wild-type TNF implying that TNFR55 is the sole receptor for cytotoxicity and cytostasis in these cell systems. In the proinflammatory activities examined, the TNFR55-selective mutants (E146K and R32W-S86T) resulted in significantly less activity than wild-type TNF indicating that TNFR75 may be involved in these neutrophil, monocyte and endothelial activities. The reduction in activity was less for R32W and this is probably explained by its reduced selectivity compared to the other TNFR55-selective mutants (table 1.1).

Figure 6.1 Proposed model of TNF's mechanism of action through the TNF receptors


D143F
E146K
TNFR55
TNFR75
NO ACTIVITY
CYTOTOXICITY MAINTAINED
PROINFLAMMATION REDUCED
NEUTROPHIL APOPTOSIS REDUCED


wt TNF
wt TNF
TNFR55
TNFR75
?
DIRECT
INDIRECT
Cytotoxicity
Cytostasis
NEUTROPHIL
- O2-
- PAF
- ADCC
- Adhesion
- Apoptosis
ENDOTHELIAL
- E-selectin
- Adhesion
- Transmigration
- IL8 secretion
MONOCYTES
- PAF
- cPLA2 phosphorylation

The TNFR75-selective mutant (D143F) was employed to investigate the role of TNFR75 in the proinflammatory activities of neutrophils and endothelium. Stimulation of TNFR75 by this mutant did not result in any proinflammatory activity and indicated that TNFR75 on its own was insufficient. Therefore, it has been concluded that TNFR75 facilitates TNFR55 in the mediation of TNF's proinflammatory activities, especially at lower doses. In addition, the combination of TNFR55-selective and TNFR75-selective mutants did not simulate the full activity of wild-type TNF (figure 3.5). This could suggest that the occupation and stimulation of TNFR55 by the TNFR55-selective mutant somehow inhibits interaction with TNFR75. The findings of chapter 5 represent a departure from the above model. Here it was shown that TNFR75 participates in the apoptotic death of neutrophils in much the same way as occurs in the activation of neutrophils by TNF-α. It is hypothesized here that in normal non-malignant cells, TNFR75 may facilitate TNFR55 in the mediation of TNF-induced apoptosis.

The mode of interaction of TNFR75 with TNFR55 in the mediation of neutrophil and endothelial activities remains obscure, but may occur extracellularly or intracellularly. In view of the structural characteristics of the extracellular domains of the TNF receptors it would seem unlikely that TNF-α would cross-link the two different receptors. TNFR75 projects itself further from the cell surface than TNFR55 making it physically difficult for the binding sites on the different receptors to engage the TNF homotrimer at the same time (figure 1.2; Banner et al, 1993). In addition, experiments demonstrating TNF-induced aggregation of multiple TNFR55 or TNFR75 in homocomplexes, have not detected heterocomplexes under the same conditions (Loetscher et al, 1992).

Tartaglia and Goeddel (1992b) proposed a model of TNF-α interaction with its receptors in which TNF-α, at low concentrations, binds initially with the higher affinity TNFR75 (5-fold higher affinity) which captures the molecule and subsequently passes it onto TNFR55 which mediates the activity. However, the results seen with the TNF mutants are not consistent with

this model. The TNFR55-selective mutants induced proinflammation which was up to 280-fold less than wild-type TNF. In view of the lack of binding to TNFR75 one would expect only a 5-fold reduction in activity if the passing on model of Tartaglia and Goeddel were applicable.

The intracellular facilitation of TNFR55 by TNFR75 is a view point which has been strengthened by the recent findings of Darnay et al (1994a, 1994b) and Rothe et al (1994). These groups describe the association of proteins and protein kinases with the TNF receptors. Darnay et al (1994a) describe a ligand-activated serine/threonine protein kinase that associates with the cytoplasmic domain of TNFR75 and an affiliated 59 kD protein. This novel kinase induces the phosphorylation of **both** TNF receptors. The TNFR75-associated protein (59 kD) and the kinase have been coined p80-TRAP (TNF receptor-associated protein; p80 referring to TNFR75) and p80-TRAK (TNF receptor-associated kinase). In addition, Darnay et al (1994b) have described a kinase which is specifically associated with the cytoplasmic domain of TNFR55 and causes its phosphorylation (p60-TRAK); the cytoplasmic domain of TNFR75 is not one of its substrates. A 52 kD protein (p60-TRAP) is also known to associate with the cytoplasmic domain of TNFR55. Rothe et al (1994) describes two TNF receptor-associated factors (TRAF 1 and 2) which are affiliated with TNFR75 and also identifies a C-terminal region of 78 amino acids within the cytoplasmic domain of TNFR75 which is required for signal transduction. The TNF receptor-associated factors (TRAF1 ~45 kD and TRAF2 ~56 kD) associate with the cytoplasmic domain of TNFR75 (C-terminal region of 78 amino acids) in a heterodimeric complex in which TRAF2 contacts the receptor directly. TRAF2 is a novel protein containing an N-terminal RING finger sequence motif which may imply a direct signal transduction pathway to the nucleus. Incidentally, TRAF2 (56 kD) may be the 59 kD protein (p80-TRAP) described by Darnay et al (1994a).

As mentioned, p80-TRAK phosphorylates both receptors whereas p60-TRAK can phosphorylate only TNFR55. Since p80-TRAK can phosphorylate TNFR55 it is plausible to suggest that intracellular facilitation of TNFR55 by TNFR75 may occur via this mechanism. In addition, these receptor-associated kinases may be involved in the signalling cascade which results in the phosphorylation of $cPLA_2$ or may even directly phosphorylate this enzyme. VanArsdale and Ware (1994) also describe the ligand-dependent stimulation of a serine protein kinase activity associated with TNFR55 which may regulate neutral sphingomyelinase or phospholipase C. Therefore the signalling mechanisms involved in the mediation of TNF activity are becoming better defined. The elucidation of TNF's signalling cascades will allow the development of inhibitors which can be used in a variety of diseases in which TNF-α is a known pathophysiological contributor.

It was hoped that the development of TNF receptor-deficient mice would resolve some of the controversy regarding the roles of the TNF receptors in mediating the biological activities of TNF-α. Pfeffer et al (1993) and Rothe et al (1993) have demonstrated that mice homozygous for a disrupted TNFR55 allele are resistant to the lethal effects of lipopolysaccharide and S. aureus enterotoxin B. This indicates that TNFR55 is crucial for the toxicity of TNF-α. However, this does not formally exclude TNFR75 from involvement in TNF-mediated toxicity as it has been suggested in this thesis that TNFR75 requires TNFR55 for mediation of its activity. These studies are consistent with the notion of TNFR75 facilitating TNFR55 in the mediation of TNF-α's toxic proinflammatory activities.

In an attempt to clarify the physiological role of TNFR75, mice deficient in TNFR75 were generated (Erickson et al, 1994). The $TNFR75^{-/-}$ mice are much less sensitive to TNF-α: 10 of 11 TNFR75-deficient mice survived injection of 10 μg mTNF, whereas all wild-type mice died at this dose. However, the $TNFR75^{-/-}$ mice are not completely resistant as they died when higher doses were administered. These results demonstrate that TNFR75 definitely

contributes to TNF toxicity in the murine model and confirms the earlier findings of Brouckaert et al (1992).

The results of chapter 3 have indicated that the TNFR55-selective mutants, which induce significantly less neutrophil and endothelial proinflammatory activity than wild-type TNF, would be less toxic *in vivo* and prove to be more effective than wild-type TNF as an antineoplastic agent. The first such trial *in vivo* has been reported by Van Zee et al (1994) however only toxic effects, and not antineoplastic activity, were examined. The TNFR55-selective mutant R32W-S86T and wild-type TNF were administered to healthy baboons (*Papio sp.*) at a dose of 100 μg/kg as a single bolus injection into the femoral vein. R32W-S86T was shown to bind selectively to the baboon TNFR55 as in humans. The wild-type and mutant TNF produced comparable cardiovascular disturbances (hypotension and tachycardia) and tissue injuries (elevated blood lactate, renal and hepatic dysfunction). It was therefore implied that TNFR75 had no significant role in systemic TNF toxicity. However, the single dose of 100 μg/kg is quite large and as seen in the dose response experiments examining neutrophil and endothelial proinflammatory activities (chapter 3) when large doses are used there is no difference in effect between wild-type TNF and the TNFR55-selective mutant but at lower doses there is indeed a significant difference. Of greater significance is the disparity in pharmacokinetics between wild-type TNF and the R32W-S86T mutant. The half-life of R32W-S86T was almost three-fold greater than wild-type TNF (169 versus 61 minutes) and probably reflects the ability of soluble TNFR75 to act as a clearing mechanism for TNF-α (Bemelmans et al, 1993). Therefore, after several hours the levels of mutant TNF become much greater than wild-type (approximately 30-fold in 8 hours) and yet the side effects are about the same. Thus, the inference should be that the mutant TNF is significantly less toxic than wild-type TNF. Further *in vivo* studies are eagerly awaited.

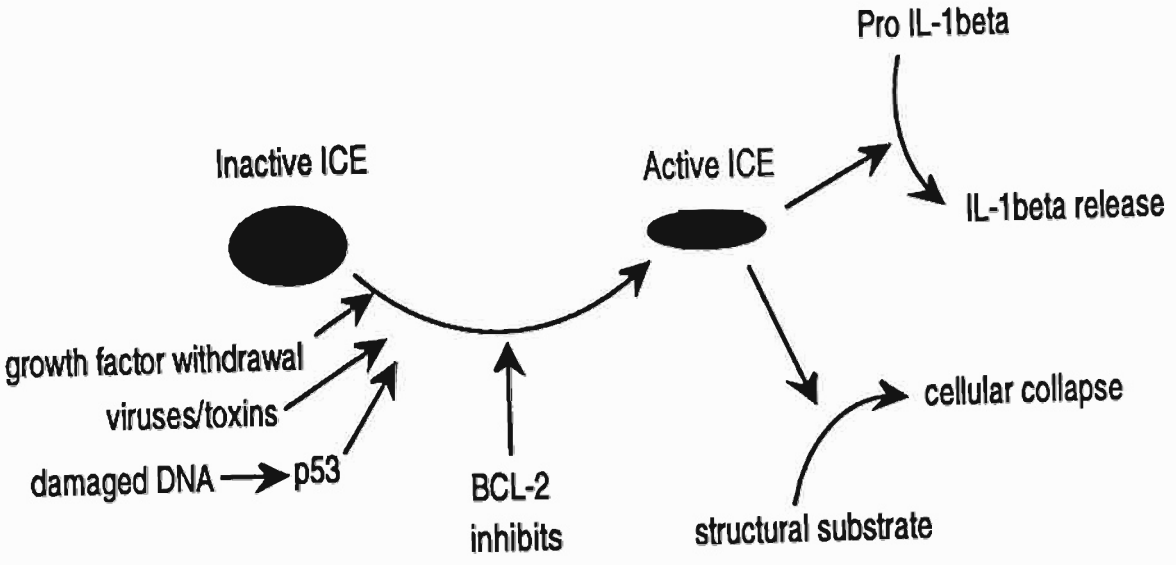
In the final section of this thesis TNF-α was investigated for its ability to induce apoptosis in human neutrophils. Apoptosis, as defined in chapter 5, is an active process leading to a noninflammatory form of cell death and is characterized by nonrandom DNA fragmentation. It was hypothesized that TNF-α, in activating neutrophils to repel invading microorganisms, would also activate apoptotic death in these cells leading to a noninflammatory removal. Therefore TNF-α would be seen as a homeostatic cytokine in addition to its many other biological properties. Indeed, TNF-α induced rapid apoptosis in neutrophils in a time frame similar to its induction of neutrophil proinflammatory activities. Also the use of TNF mutants with selective binding characteristics indicated that TNFR75 facilitated TNFR55 in the apoptosis of neutrophils in a manner identical to TNF's induction of neutrophil proinflammatory activities. These TNF mutant results are in keeping with the "homeostatic hypothesis of TNF-α" in that the same TNF receptor biology is employed by both neutrophil proinflammation and apoptosis.

Apoptosis participates in many fundamental processes such as the turnover of normal tissues, embryonic development and the maturation of the immnue system. It is now also recognised that a disturbance of this basic process is central to some disease states such as autoimmune disorders and AIDS. Autoimmune diseases demonstrate an imbalance between the production and removal of various cell types: lymphocytes (SLE), synovial cells (rheumatoid arthritis) and fibroblasts (scleroderma) (Mountz et al, 1994). Treatments currently used for autoimmune disease (steroids, azathioprine, cyclophosphamide and methotrexate) are potent inducers of apoptosis but result in troublesome and often major side effects, including the possibility of developing malignancy. However, therapies that could induce apoptosis of specific cell types may significantly improve the management of these disorders. In contrast, HIV results in T cell (CD4) apoptosis and onc potential treatment strategy against AIDS would be to block the apoptotic signal transduction pathway in these cells (Ameisen et al, 1995).

However, the intracellular pathways involved in signalling apoptosis are not well understood but information is being rapidly accrued on proteins such as bcl-2, c-myc, p53 and ICE (figure 6.2). In view of TNF's ability to induce apoptosis, it is reasonable to suggest that these proteins also participate in the signal transduction pathways of this cytokine thus adding to its complexity.

Figure 6.2 The intracellular pathways involved in the signalling of apoptosis

ICE (interleukin-1 converting enzyme) appears to be important in the apoptotic process and is modified by various stimuli. Activated ICE induces the release of IL-1 which may act to alert neighbouring cells of apoptosis occurring in the cell of origin. In addition, ICE also sets in motion processes which lead to cellular collapse. Figure adapted from Vaux et al (1994).


Pro IL-1beta
Inactive ICE
Active ICE
IL-1beta release
growth factor withdrawal
viruses/toxins
damaged DNA
p53
BCL-2
inhibits
cellular collapse
structural substrate

FUTURE WORK

One of the major conclusions of this thesis is that the TNFR55-selective mutants, with maintenance of cytotoxicity but reduced proinflammatory activity, could be used *in vivo* as an antineoplastic agent with the likelihood of reduced side effects. Primate studies (Van Zee et al, 1994) have already begun however, for reasons mentioned in the general discussion, others are eagerly awaited.

The recent discovery of the TNF receptor-associated kinases (TRAKs) (Darnay et al, 1994a and 1994b) has provided a molecular explanation for the model of TNF's mechanism of action presented in this thesis. The activation of these kinases by the TNF mutants and the ability of the TRAKs to phosphorylate other proteins eg MAP kinases, PKC and $cPLA_2$, would certainly warrant examination. An RNA probe for $cPLA_2$ constructed during the course of this candidature (appendix 3) will allow the investigation of $cPLA_2$ synthesis in addition to its phosphorylation.

Finally, as discussed in chapter 5, the role of Fas ligand in myeloid cell apoptosis and its interaction with TNF-α awaits investigation. It is plausible to suggest that neutrophils produce Fas ligand in response to TNF-α and this results in enhanced apoptosis by binding to Fas on the same cell in an autocrine loop.

APPENDICES

APPENDIX 1: TNF Receptor Functions

TNFR55

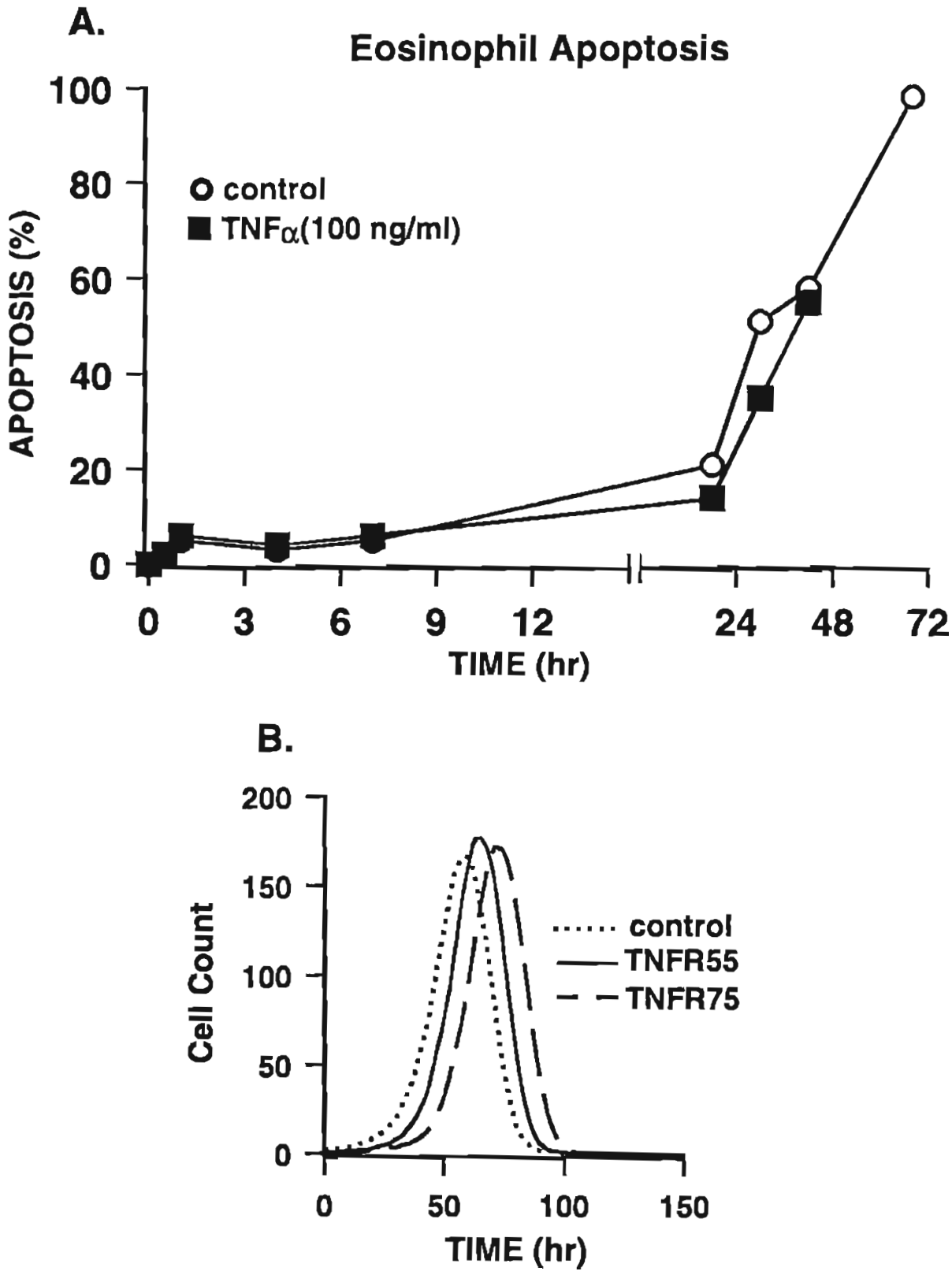
ACTIVITY	REFERENCE	METHOD
Cytotoxicity, fibroblast proliferation, chlamydial growth inhibition, prostaglandin E_2 synthesis	Engelmann et al, 1990	Antibody
Cytotoxicity, fibroblast proliferation, IL-6 production by endothelium, adherence of HL60 to endothelium	Espevik et al, 1990	Antibody
Activation of NF-κB	Hohmann et al, 1990	Antibody
Cytotoxicity, IL-6 production from endothelium, adherence of HL60 to endothelium	Shalaby et al, 1990	Antibody
Cytotoxicity/cytostasis, IL-2 receptor expression, HLA-A,B,C,DR expression	Thoma et al, 1990	Antibody
Cytotoxicity, induction of manganous superoxide dismutase	Tartaglia et al, 1991	Antibody
Proliferative signals in mononuclear cells	Gehr et al, 1992	Antibody
DNA fragmentation in HL60 and U937 cells, differentiation in HL60 cells	Greenblatt and Elias, 1992	Antibody
Cytotoxicity	Tartaglia and Goeddel, 1992a	Deletion mutagenesis
Stimulation of protein kinase C, sphingomyelinase and phospholipase A_2, production of diacylglycerol and ceramide	Wiegmann et al, 1992	Transfected TNFR55
Antiviral activity	Wong et al, 1992	Antibody Deletion mutagenesis
Apoptosis	Tartaglia et al, 1993b	Deletion mutagenesis
Apoptosis	Grell et al, 1994a	Antibody
Antiproliferative for mature haemopoietic cells	Jacobsen et al, 1994	Antibody
Protein phosphorylation	Mire-Sluis and Meager, 1994	Antibody
Apoptosis	Wong and Goeddel, 1994	Antibody

TNFR75

ACTIVITY	REFERENCE	METHOD
Activation of NF-κB	Hohmann et al, 1990	Antibody
Cytotoxicity, IL-6 production from endothelium, adherence of HL60 to endothelium	Shalaby et al, 1990	Antibody
Proliferation of thymocytes and cytotoxic T cells	Tartaglia et al, 1991	Antibody
Proliferative signals in mononuclear cells	Gehr et al, 1992	Antibody
Cytotoxicity	Heller et al, 1992	Transfected TNFR75
GM-CSF secretion from T cells	Vandenabeele et al, 1992	Transfected TNFR75
Apoptosis	Grell et al, 1994a	Antibody
Antiproliferative for primitive haemopoietic cells	Jacobsen et al, 1994	Antibody

Appendix 2 The lack of TNF-induced eosinophil apoptosis

(A) This time course experiment is indicative of 2 performed in which TNF-α (100 ng/ml) does not promote apoptosis of human eosinophils compared to control. The samples were incubated at 37°C in a shaking waterbath. (B) The presence of both TNF receptors on eosinophils is demonstrated. Antibodies used were htr-9 (TNFR55), utr-1 (TNFR75) and 3D3.3 (control).


A.
Eosinophil Apoptosis
APOPTOSIS (%)
100
80
60
40
20
0
control
TNFα(100 ng/ml)
0
3
6
9
12
24
48
72
TIME (hr)
B.
Cell Count
200
150
100
50
0
control
TNFR55
TNFR75
0
50
100
150
TIME (hr)

APPENDIX 3: Construction of an RNA probe for $cPLA_2$

The plasmid pMT.2 containing $cPLA_2$ (obtained from John Knopf, Genetics Institute, Cambridge, MA) was digested with the enzyme Sal I giving two fragments - pMT.2 and $cPLA_2$. Another plasmid ($pGEM_1$), also digested with Sal I, was ligated to the $cPLA_2$ fragment. The orientation of $cPLA_2$ within $pGEM_1$ was determined by digestion with EcoR I which results in fragments of either 4800/780 or 2000/3600 nucleotides. Digestion of the 2000/3600 orientation with Hinc II produces $pGEM_1$ with 837 nucleotides of $cPLA_2$ attached. When this was digested with Nco I, a 102 nucleotide $cPLA_2$ template was generated. Using this template, T7 polymerase and ^{32}P-UTP, an RNA probe can be prepared to examine the synthesis of $cPLA_2$.

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AMENDMENTS

Abstract:
pII (paragraph 2, line 1): ...it is shown here that TNF-α shortens the *in vitro* life span markedly.

Chapter 2:
p20 (paragraph 2): Eosinophil purity was 97.8% in the pellet. The remaining 2.2% were neutrophils.

p30 (line 3): pelleted not pelletized.

Chapter 3:
p36 (paragraph 2, line 9): ...with R32W being more potent than wild-type TNF...

p45 (figure 3.5 legend, line 3): 104 should read 10^4.

p47 (table 3.4): This experiment was representative of three performed in triplicate.

Chapter 4:
p55 (figure 4.1): The $cPLA_2$ protein used was derived from U937 cells.

p57 (figure 4.2): The other bands present are non-specific and presumably represent non-$cPLA_2$ proteins detected by the polyclonal antibody. The smaller sized bands may represent proteolytically cleaved $cPLA_2$. Basal phosphorylation indicates that either the enzyme is constitutively active or that the control conditions were sufficient to phosphorylate $cPLA_2$.

Chapter 5:
p87 (figure 5.7): MFI = mean fluorescence intensity.

p88 (figure 5.8): Untreated neutrophils still undergo apoptotic death and this may be associated with increased fas expression. The control antibody was 3D3.3 (IgG1 negative control).

Chapter 6:
p93 (paragraph 2, line 3): model should be replaced by system.