

# TNF and cancer: good or bad?

## Review Article

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**Key Words:** TNF, cancer, Apoptosis, Activation, anti-cancer therapy, gene polymorphism, carcinogenesis, neovascularisation, angiogenesis, extra cellular matrix, vasculature, lymphatics

**Abbreviations:** bacillus Calmette-Guérin, (BCG); basal cell carcinoma, (BCC); cervical intraepithelial neoplasia, (CIN); containing recombinant human TNF, (rhTNF); Death receptor 3, (DR3); epidermal growth factor, (EGF); germinal centres kinase, (GCK); I $\kappa$ B kinase, (IKK); inducible nitric oxide synthase, (iNOS); MAPK activate protein kinase, (MAPKAP); MAPK kinases, (MKK); matrix metalloproteinases, (MMP); monoclonal antibody, (mAb); TNF converting enzyme, (TACE); natural killer, (NK); osteoprotegerin, (OPG); reactive oxygen species, (ROS); Tissue inhibitors of MMP, (TIMP); TNF receptor associated Death domain protein., (TRADD)

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## Summary

Tumour necrosis factor (TNF) is a pro inflammatory cytokine whose role is established in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis and Crohn's disease. It is a 17KD molecule that exists as a trimer, dimer and monomer in equilibrium and as a membrane bound 26KD molecule. It binds to two receptors the 55KD and 75KD proteins. These receptors on binding aggregate and set up a number of signal transducing mechanisms that lead to cell apoptosis or gene upregulation. The latter often occurs via the MAPKinase and NF  $\kappa$ B pathways. TNF in large amounts can induce haemorrhagic necrosis of tumours. This anti-cancer effect is multi-factorial as TNF can cause vascular necrosis, a direct apoptotic effect on the cells and also free radical induced cell death. A number of studies have examined the anticancer effects of TNF in combination with other cytokines or chemotherapy agents. However the only use of TNF alone in clinical trials has been in limb perfusion studies for sarcoma and melanoma. More recently, TNF has been found to have a pro-cancerous effect. In a mouse skin model TNF induces carcinogenesis. Furthermore, gene polymorphisms that increase or decrease TNF production confer either an increased risk or protective effect on a number of different cancers and precancerous diseases including gastric cancer, lymphoma and cervical cancer as well as cervical intraepithelial neoplasia. Moreover, in murine models TNF promotes metastasis, tumour angiogenesis and cachexia. Trials with anti-TNF therapies are awaited to see the effects of blocking this cytokine in patients with cancer.

## I. Introduction

TNF was initially identified in two laboratories and alternatively named cachectin, for the wasting effect induced in mice (Beutler et al, 1985), and tumour necrosis factor, because it caused haemorrhagic necrosis of various murine tumours *in vivo* (Carswell et al, 1975). TNF has been identified as a key mediator in the pathogenesis of both acute and chronic diseases. Successful strategies have been developed to block its action in animal models of shock (Beutler et al, 1985; Tracey et al, 1987), collagen induced arthritis (Thorbecke et al, 1992; Williams et al, 1992) and experimental autoimmune encephalomyelitis (Baker et al, 1994). Anti-TNF monoclonal antibody (mAb) therapy in human clinical trials has had success in some but not all diseases. (Elliot et al, 1994, Abraham et al, 1995, D'Haens et al, 1999, 2001; Lipsky et al, 2000). Anti-TNF mAbs are licensed in the USA and Europe for the treatment of rheumatoid arthritis and Crohn's disease.

However the role of TNF in cancer is less clear with both anti-cancer and pro-cancerous effects.

### A. TNF structure

TNF is found as a 26kd membrane bound molecule which, when cleaved by the TNF converting enzyme (TACE), forms soluble TNF consisting of the 76 amino-terminal residues with a molecular weight of 17kd (Black et al, 1997). Under native conditions bound and soluble TNF exist as a monomer, dimer and trimer in equilibrium, with the trimer being the biologically active form. TNF belongs to the TNF superfamily, which includes Lymphotoxin and , Fas ligand, CD40 ligand, and two apoptosis inducing ligands, TRAIL/Apo-2 ligand (Wiley et al, 1995; Pitti et al, 1996) and LIGHT, which is also involved in T cell activation (Mauri et al, 1998; Zhai et al, 1998). These proteins are all ligands for the TNF receptor superfamily.

## B. TNF receptors

TNF binds with high affinity to two cell surface receptors, a 55kd protein (p55TNF-R) and a 75kd protein (p75TNF-R), both are expressed by most cell lines and primary tissues. However, the level of receptor expression varies with cell type. The p55TNF-R expression is dominant on most cells, except for haemopoetic cells, and is relatively constant, while the p75TNF-R expression fluctuates. The binding of trimeric TNF to membrane anchored receptors causes cross-linking and aggregation of the homologous receptors. The cytoplasmic portion of the receptors interacts with signal transducing molecules initiating down stream intracellular signalling events. It is thought that p55TNF-R is the major signal transducer of soluble TNF responses, due to the abundance and binding avidity of this receptor; while p75TNF-R is preferentially activated by membrane bound TNF (Grell et al, 1995). Both receptors belong to the TNF receptor superfamily, which include among others Fas, CD40, (Smith et al, 1994), the Death receptor 3 (DR3) (Chinnaiyan et al, 1996), the TRAIL receptors DR4 (Pan et al, 1997b), DR5 (Pan et al, 1997a), TRID (Sheridan et al, 1997), the LIGHT receptor TR2/HVEM (Kwon et al, 1997; Montgomery et al, 1996) and osteoprotegerin (OPG) which inhibits osteoclastic bone resorption (Simonet et al, 1997). All these receptors are membrane glycoproteins with sequence homology in the extra-cellular cysteine rich region. The p75TNF-R expression is controlled by extra cellular stimuli acting at the transcriptional and post-transcriptional level (Brockhaus et al, 1990; Thoma et al, 1990), and by receptor shedding. The extra cellular portion of both the p55 and p75 TNF receptors can be cleaved and released into serum as soluble forms. Soluble TNF receptors bind to soluble TNF, inhibiting systemic effects

of TNF and enhancing clearance by the kidneys (Olsson et al, 1989; Porteu and Nathan, 1990; Bemelmans et al, 1993).

## C. TNF function

The major sources of TNF are macrophages and to a lesser extent T lymphocytes, proliferating B cells, natural killer (NK) cells, mast cells and stimulated neutrophils (Gemlo et al, 1988; Sung et al, 1988; Djeu et al, 1990; Dubravec et al, 1990; Gordon and Gallis, 1990; Kinkhabwala et al, 1990; English et al, 1991; Stein and Gordan, 1991). Non-immune cells such as keratinocytes, smooth muscle cells, astrocytes and microglial cells have all been shown to produce TNF upon LPS stimulation *in vitro* (Sawada et al, 1989; Warner and Libby, 1989; Kock et al, 1990). TNF is a pleiotropic cytokine, which acts on a large variety of cells with wide ranging effects on individual cells (**Table 1**). Amongst the haemopoetic actions of TNF, are the activation of macrophages/monocytes (Trinchieri et al, 1986; Drapier et al, 1987; Kirchheimaer et al, 1988; Wang et al, 1990), lymphocytes (Jerlinek and Lipsky, 1987; Scheurich et al, 1987; Yokota et al, 1988), neutrophils (Schleiffenbaum and Fehr, 1990) and the promotion of coagulation (Lentz et al, 1991). It has a dual role in NK cells depending on the target cell. A subset of NK cells, lacking CD16, undergo TNF-induced apoptosis (Jewett et al, 1997), while IL-2 with TNF causes activation and increases NK cytolytic function (Ostensen et al, 1987). TNF induces bone resorption, important in bone metastasis formation (Bertolini et al, 1986; Johnson et al, 1989) and inhibits adipocyte proliferation which may contribute to cachexia (Kawakami et al, 1989).

**Table 1.** A list of TNF target cells

TARGET CELL	ACTION	REFERENCE
Macrophages/ Monocytes	Activation, differentiation, chemotaxis	(Trinchieri et al, 1986; Drapier et al, 1987; Kirchheimaer et al, 1988; Wang et al, 1990)
Neutrophils	Activation, chemotaxis	(Schleiffenbaum and Fehr, 1990)
T lymphocytes	Proliferation, activation	(Scheurich et al, 1987; Yokota et al, 1988)
B Lymphocytes	Proliferation, differentiation, activation	(Jerlinek and Lipsky, 1987)
NK and LAK lymphocytes	Proliferation, activation, apoptosis	(Ostensen et al, 1987; Jewett et al, 1997)
Endothelial cells	Promotes clotting, haemopoetic growth factors and cytokine production	(Seelentag et al, 1987; Gimbrone et al, 1989; Osborn, 1990)
Adipocytes	Inhibition	(Kawakami et al, 1989)
Myocytes	Inhibition	(Miller et al, 1988)
Fibroblasts	Proliferation, cytokine production	(Butler et al, 1988)
Cartilage	Inhibits proteoglycan synthesis, resorption	(Saklatvala et al, 1985; Saklatvala, 1986;)
Osteoclasts	Activation	(Bertolini et al, 1986; Johnson et al, 1989)
Oligodendrocytes	Cytotoxic	(Selmaj et al, 1990)
Astrocytes	Proliferation	(Selmaj et al, 1990)
Keratinocytes	Differentiation, inhibits proliferation, cytokine production	(Nickoloff et al, 1991)

TNF has multiple effects on endothelial cells *in vitro*, such as promoting cytokine production that increases angiogenesis *in vivo* (Yoshida et al, 1997). TNF promotes the pro-inflammatory cascade, by inducing the release of pro-inflammatory cytokines such as the chemokine IL-8 (Gimbrone et al, 1989; Schroder et al, 1990; Nickoloff et al, 1991), IL-6 (Jirik et al, 1989), Gro (Dong et al, 1999), haemopoietic growth factors including G-CSF (Seelentag et al, 1987) and adhesion molecules such as VCAM important in metastasis (Osborn, 1990).

TNF also induces increased matrix metalloproteinase expression in a number of cell types and integrin expression. As shown by TNF knockout mice studies, this cytokine is necessary for normal splenic organisation in foetal growth (Keffer et al, 1991). In general, TNF exerts a similar range of effects as IL-1, except that it is able to induce apoptosis (Wallach et al, 1998) and is less efficient in inducing cartilage resorption (Saklatvala, 1986; Saklatvala et al, 1985). The cellular effects of TNF occur through binding to its receptor, which leads to secondary signalling events. These signalling events cause either apoptosis or gene regulation.

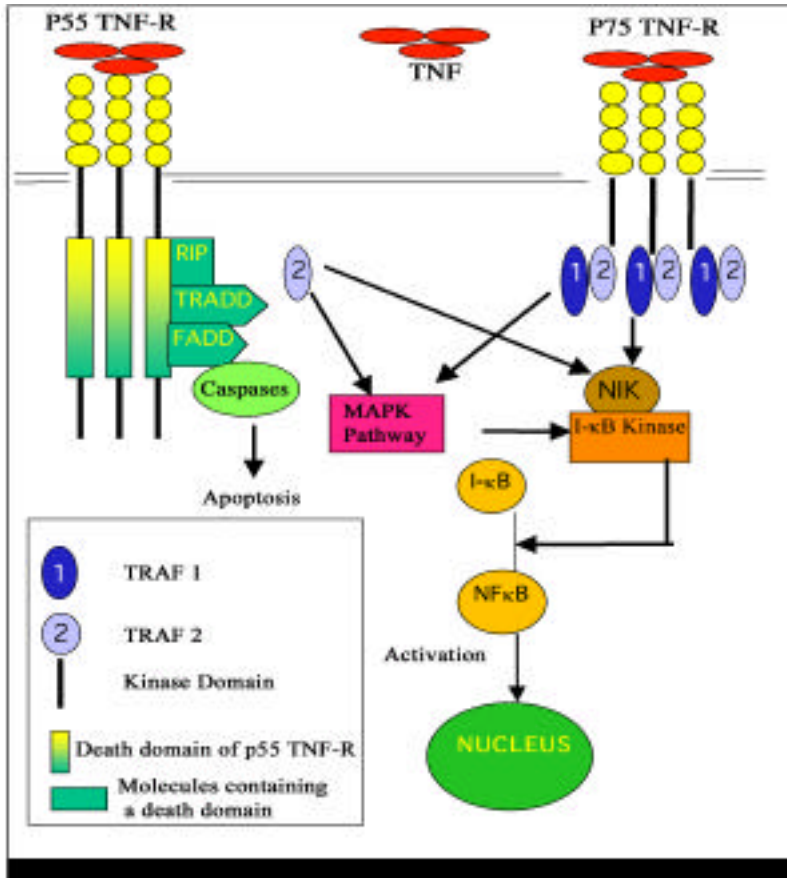
## D. TNF receptor signalling

### 1. Apoptosis

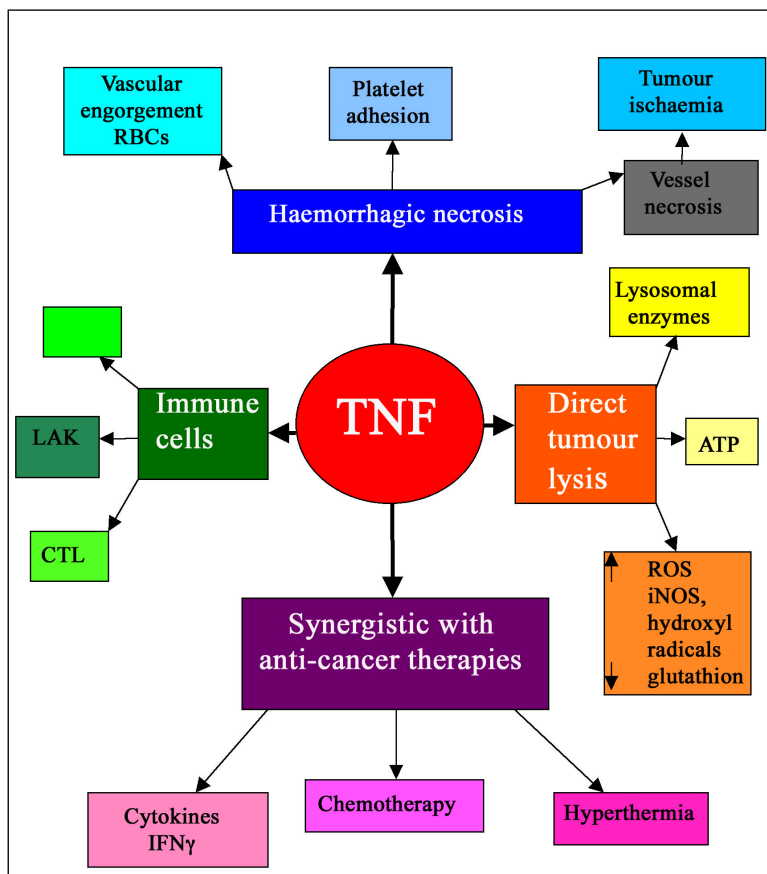
In recent years, there has been significant progress in unravelling the TNF cell signalling pathways following receptor ligation, although this complex area is still under investigation (**Figure 1**). Upon TNF binding and aggregation of p55TNF-R, a portion of the intracellular domain of the receptor, known as the death domain, binds to an intracellular signalling moiety TRADD (TNF receptor associated Death domain protein) (Hsu et al, 1995). The death domains consist of six-amphipathic  $\alpha$ -helices, in an anti-parallel arrangement that can interact with other death domains. TRADD, in turn, through its own death domain-like region interacts with MORT1/FADD (Varfolomeev et al, 1997), RIP and RAIDD, sequentially. This complex then recruits caspases 8 and 10, which belong to a family of enzymes essential in apoptosis (Boldin et al, 1996; Muzio et al, 1996). Caspase 2 is also recruited by the p55TNF-R via its N-terminal recruitment domain CARD that interacts with the CARD domain on RAIDD/CRADD using the RIP-RAIDD axis instead of MORT-1/FADD, to induce apoptosis (Ahmad et al, 1997; Duan and Dixit, 1997). The caspase cascade leads to the cleavage and disruption of proteins such as I-CAD, which acts as an inhibitor of CAD, a DNase that degrades nuclear DNA into fragments characteristic of apoptosis (Enari et al, 1998). Apoptosis, however, only occurs when a cell is stressed, for example, by exposure to UV radiation or a protein/RNase synthesis inhibitor, such as actinomycin D. Normally apoptosis is prevented from occurring through the recruitment of TRAF molecules (TNF receptor associated factor) (Rothe et al, 1995; Kelliher et al, 1998). The recruitment of TRAF molecules by TNF leads to up-regulation of genes and cellular activation.

### 2. Activation

TNF proliferative and stimulatory responses occur by induction of a number of genes such as other cytokines as well as cell cycling mechanisms. For this to occur the ligation and aggregation of p55TNF-R recruits TRADD and RIP, as previously described. However, TRADD and RIP can also act via alternative signalling pathways by recruiting TRAFs. To date six TRAF molecules have been identified that all have a conserved C terminal protein-protein interacting domain known as the TRAF domain, which interacts with members of the TNF-R superfamily (Hu et al, 1994; Rothe et al, 1994; Cheng et al, 1995; Mosialos et al, 1995; Regnier et al, 1995; Sato et al, 1995; Cao et al, 1996; Ishida et al, 1996; Nakano et al, 1996). TRAF 1, 2, 5 and 6 activate the NF- $\kappa$ B and JNK pathways, and TRAF 1 and 2 are associated with TNF signalling (Rothe et al, 1995; Song et al, 1997). TRAF 2 recruits TRAF 1, which then interacts with MAPK (Mitogen Activated Phosphorylation Kinase) pathways, proteins belonging to the MAPKKK superfamily that phosphorylates I $\kappa$ B kinase (IKK). This kinase then degrades cytoplasmic I $\kappa$ B (Regnier et al, 1997), which releases NF- $\kappa$ B to translocate into the nucleus. This prevents apoptosis and activates other cellular responses. TRAF 2 is recruited directly via the p75TNF-R to activate the NF- $\kappa$ B and JNK pathways, explaining the overlapping actions of both receptors (Natoli et al, 1998). The best studied of the signalling pathways is the MAPKinase pathway (**Figure 2**). It involves a signalling cascade, which upon TRAF 2 recruitment, leads to phosphorylation of a serine/threonine kinase known as MAPK kinase (MKKK). The process by which TRAF 2 leads to activation of MKKKs remains unclear but may involve small G-proteins and further MKKKs. The MKKKs in turn phosphorylate other serine/threonine kinases known as MAPK kinases (MKK). There are a number of MKKs activated by cytokines and other environmental factors. The TNF receptor is thought to activate MKK3 leading to p38 MAPK phosphorylation (Winston et al, 1997). Other kinases such as ASK 1 and MEKK, a MKKK, may also phosphorylate MKK3, although they have been implicated to have a major role in the phosphorylation of p54 MAPK (JNK/SAPK) (Nishitoh et al, 1998; Yujiri et al, 1998). p38 MAPK phosphorylates targets downstream that affect the transcription factor ATF2 and cytosolic proteins cPLA<sub>2</sub> and Hsp27. cPLA<sub>2</sub> and MAPK activate protein kinase (MAPKAP), another cytosolic protein, along with the transcription factor Elk1 can also be activated by TNF via p42/44 MAPK (ERK). In murine macrophages this involves phosphorylation of MEKK (Winston et al, 1997) and in HL-60 and Cos cells this involves cRaf1 (Berra et al, 1995; Yao et al, 1995). Upon TNF receptor ligation, TRAF2 can also activate p54 MAPK, via a number of pathways involving ASK1, that in turn activates MKK7 (Ichijo et al, 1997) or MEKK-1 that interacts with germinal centres kinase (GCK), a MAP4K (Shi and Kehrl, 1997). p54 MAPK activation can also occur via the tyrosine protein kinase Pyk2 and the small G proteins PAK, Rac and cdc42 (Tokiwa et al, 1996).



**Figure 1.** TNF signalling through the p75 and p55 TNF receptor. This is a simplified diagram to show the main components of TNF signalling through its aggregated receptors. This process can either lead to apoptosis via the death effector molecules, such as TRADD, and the caspases or cell activation via the TRAF molecules to protein kinases such as MAPK and NF- $\kappa$ B..



**Figure 2.** Schematic diagram to show the anti-cancer effects of TNF. TNF causes haemorrhagic necrosis *in vivo* with destruction of tumour vasculature and ischaemia. It also promotes tumour lysis by activating the anti-tumour immune response and can lead to direct tumour lysis via hydroxyl radicals and lysosomal enzymes. TNF can act synergistically with variety of other agents such as cytokines, chemotherapy and hyperthermia to induce tumour killing.

In summary, TNF on receptor ligation leads to activation of TRAF molecules which causes phosphorylation of a cascade of serine threonine kinases known as the MAP kinases pathway leading to activation of a number of cytosolic proteins which eventually lead to activation of transcription molecules and gene regulation.

## II. TNF as an anti-cancer agent

Initially TNF was isolated from the serum of mice infected with bacillus Calmette-Guérin (BCG) treated with endotoxin. It was found to mimic the action of endotoxin by inducing tumour necrosis *in vivo* when given directly to a range of transplanted tumours including Meth A sarcoma (Carswell et al, 1975). Furthermore, *in vitro* it was cytotoxic to L293 cells and cytostatic to Meth A sarcoma cells (Carswell et al, 1975). A number of studies using syngeneic cancer models, particularly the transplantable methylcholanthrene induced sarcoma model, have shown tumour regression with either direct intra-tumoural TNF injection or systemic intravenous TNF injections (Creasey et al, 1986; Watanabe et al, 1988). Animal xenograft models have also shown that intra-tumoural injection of recombinant TNF can lead to tumour regression (Balkwill et al, 1986; Creasey et al, 1986).

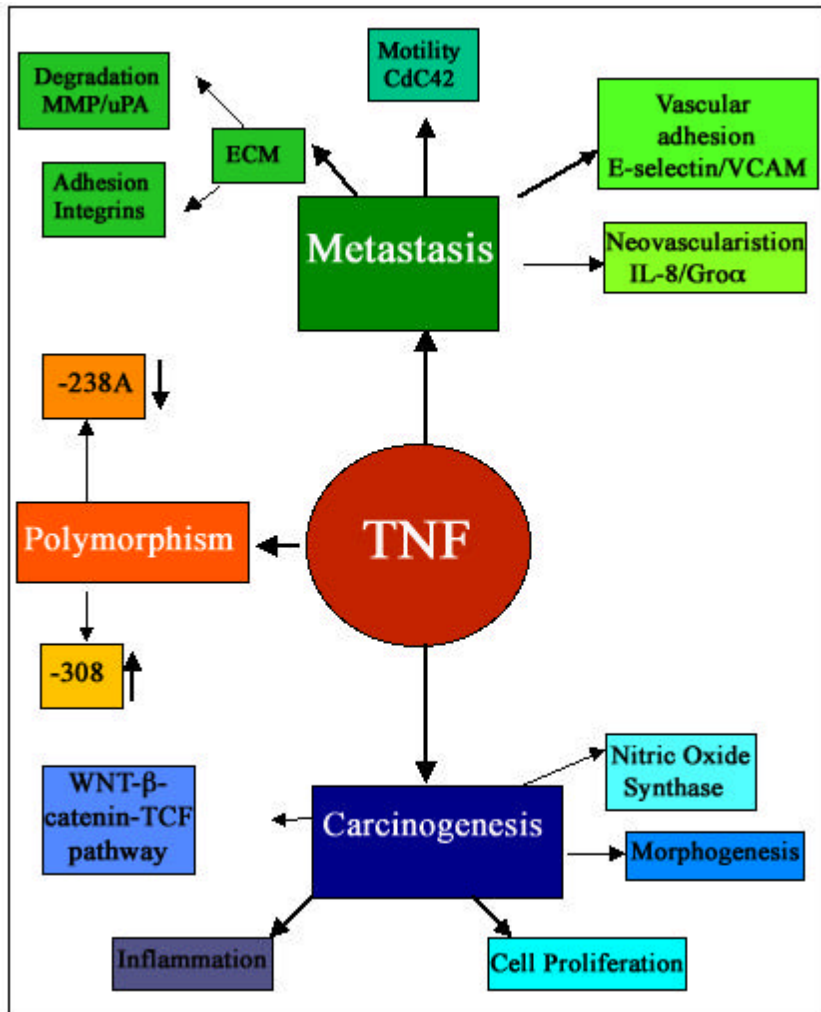
### A. Mechanisms of anti-cancer action

There appear to be a number of mechanisms by which TNF induces an anti-cancer effect (**Figure 3**). *In vivo* recombinant TNF directly injected into tumours destroys the tumour vasculature. It blocks blood flow, inducing congestion and haemorrhage of tumour vasculature (Watanabe et al, 1988a). On close examination of the tumours there are multiple petechial haemorrhages in the tumour-vascular bed causing ischaemia to the centre of the tumours due to the loss of blood supply (Havell et al, 1988). This however, only leads to 75% destruction of the tumour with a small rim of viable tissue remaining (Havell et al, 1988). TNF has also been shown to cause haemorrhagic necrosis in conjunction with IFN  $\gamma$ , inducing vascular engorgement by erythrocytes and adhesion of platelets to tumour vascular endothelium. This then leads to destruction of the tumour vasculature with necrosis and apoptosis of tumour cells (de Kossodo et al, 1995). In isolated limb perfusion studies with TNF, within hours of TNF perfusion, the tumour endothelial cells appear swollen with increased VCAM and ELAM I adhesion molecules and tumour destruction due to a coagulative necrosis. Within 3 days there was significant polymorphonuclear cell colonisation of tumours, followed by T cells and macrophages 4 days later and B cells in the second week (Renard et al, 1994).

TNF may induce killing of tumours by immune cells. Genetically engineered tumour cells producing high levels of TNF have been implanted into tumours and, although they do not kill the tumours, they inhibit growth through the activation of macrophages and NK cells (Blankenstein et al, 1991). Using TNF knockout mice the ability of NK and LAK cells to induce cytotoxicity in a variety of tumour cell targets were found to be impaired. This cytotoxicity in the knockout mice required Fas-ligand and

perforin, while the infusion of recombinant TNF in the knockout mice restored TNF induced cytotoxicity by these cells, similar to wild type mice. *In vivo* the TNF knockout mice were unable to reject MC57X syngeneic fibrosarcomas but did so if injected with recombinant TNF. Clearly, this shows, both *in vitro* and *in vivo*, TNF is required for NK and LAK induced tumour killing and tumour rejection *in vivo* (Baxevanis et al, 2000). It is likely that the NK cells themselves produce TNF along with FasL and other cytokines to induce apoptosis of tumours (Kashii et al, 1999). CTL tumour elimination and immunity also appears to be TNF dependant. In a Lewis lung carcinoma model (A9) injection of tumour cells containing a CD8 T cell epitope transgene GP33 leads to tumour elimination. This immunity was also perforin, IFN  $\gamma$  and TNF dependant, providing evidence for the crucial role of TNF in CD8 directed tumour elimination *in vivo* (Prevost-Blondel et al, 2000). Conversely, tumours grown in T cell deficient mice had impaired tumour eradication, therefore, to achieve complete TNF-induced intra tumoural haemorrhagic necrosis an adequate host T cell immunity is required (Havell et al, 1988). Dendritic cells also have a potent anti-tumour effect against breast cancer cells *in vitro*, which is mediated by TNF and blocked by the addition of anti-TNF monoclonal antibodies (Manna and Mohanakumar, 2002). Furthermore, immature dendritic cells induced apoptosis of tumour cells by TNF, FasL, lymphotoxin and TRAIL through the corresponding death receptors in a range of cancer cells (Lu et al, 2002).

Finally, TNF can have a direct effect on the tumour cells. Using inhibitors to lysosomal enzymes, hydroxyl radicals and mitochondrial respiratory inhibitors Watanabe et al found that there was a reduction in TNF induced tumour death. This study indicates that for TNF induced cell destruction, lysosomal enzymes, hydroxyl radicals and ATP may be needed (Watanabe et al, 1988b). TNF also leads to tumour cell death by inducing cytochrome c release from mitochondria and mitochondrial membrane permeabilisation leading to apoptosis (Partheniou et al, 2001). One of the mechanisms tumours use to generate a growth advantage is reducing TNF induced apoptosis through mutations in p53. Mutations in p53 were found to reduce caspase 8 activation and mitochondrial membrane permeabilisation and infection with adenovirus containing wildtype p53 restored caspase cleavage and mitochondrial permeabilisation and apoptosis due to TNF (Ameyar-Zazoua et al, 2002). TNF induced apoptosis may not always be p53 dependant. In a non-small cell lung cancer cell line the combination of TNF and IFN  $\gamma$  induced apoptosis, without altering the expression levels of p53, indicating this was p53 independent. However, the addition of c-myc anti-sense oligonucleotides did reduce the combined TNF/IFN  $\gamma$  induced apoptosis indicating that c-myc may contribute to TNF induced apoptosis of this lung cancer cell. Another mechanism by which TNF induces tumour cell apoptosis or resistance to apoptosis, is via the inhibition or activation of NF- $\kappa$ B. In lung adenocarcinoma cells the constitutive activation of NF- $\kappa$ B leads to resistance to apoptosis, however, in the presence



**Figure 3.** Schematic diagram to show the role of TNF in the up-regulation of Cancer. TNF can induce cancer by affecting tumour proliferation, altering the cell structure and appears to act early to promote carcinogenesis. Furthermore, TNF also helps tumours to metastasise by inducing extracellular matrix (ECM) adhesion and degradation as well as promoting adhesion of tumour cells to endothelial cells and neovascularisation. TNF also contributes to cancer cachexia by increasing proteolysis and lipid metabolism. Polymorphisms in the TNF promoter region regulate TNF production and may affect prognosis.

of TNF the blocking of NF- $\kappa$ B by proteasome inhibitors induces apoptosis (Milligan and Nopajaroonsri, 2001). Therefore, TNF induced apoptosis of tumour cells is very much dependant on which cell signalling pathways are constitutively active in tumour cells. IFN $\gamma$  leads to sensitisation of ovarian tumour cells to TNF induced apoptosis by down regulating NF- $\kappa$ B. This occurs by IFN $\gamma$  inducing inducible nitric oxide synthase (iNOS), which generates nitric oxide. The nitric oxide can then react with oxygen reducing the production of hydrogen peroxide an activator of NF- $\kappa$ B (Garban and Bonavida, 2001). Studies with MCF 7 breast cancer cells have shown that TNF alone also up-regulates iNOS, thereby leading to cell apoptosis (Binder et al, 1999). In Erlich ascitic tumours, TNF increased reactive oxygen species (ROS), which led to a reduction in mitochondrial glutathione and caused apoptosis in mice already depleted of glutathione by eating a glutamate-enriched diet. Glutamate is an inhibitor of glutathione (Obrador et al, 2001). A recent study has shown a direct link between increased ROS due to TNF and the reduction in mitochondrial ATPase protein subunits, cytochrome c oxidase subunit II and increased protein levels of phosphofructokinase; these changes were associated with an increase in L929 cell apoptosis (Sanchez-Alcazar et al, 2002). TNF induces tumour cell apoptosis by generating ROS at the mitochondrial

membrane. Oxidative substrates, electron-transport inhibitors, caspase inhibitors, glutathione and thiol-reactive agents modulate the ROS production induced by TNF (Goossens et al, 1999).

### B. TNF in combination with other anti-cancer therapies

To enhance the ability of TNF to kill tumours, a number of studies have examined the synergistic effects of TNF in combination with chemotherapy agents. Using L-M cells the addition of a number of commonly used chemotherapy agents to cultures containing recombinant human TNF (rhTNF) produced a 4-347-fold decrease in the IC50 (the concentration required for 50% inhibition of cell growth) compared to rhTNF alone (Watanabe et al, 1988c). A similar study examined the cytotoxicity of TNF with hyperthermia in L-M cells and found a 500-fold increase in toxicity at 40°C compared to 37°C. The combination of TNF and hyperthermia *in vivo* with transplantation of Meth A fibrosarcoma cells in mice also produced cures in 5 mice compared to a partial response with TNF alone (Watanabe et al, 1988c). However, due to the high toxicity profile of TNF, its systemic use is limited. To circumvent this problem Curnis et al have coupled TNF to CNGRC, a peptide that targets tumour neovasculature. This allows a 10-fold decrease in the dose

of TNF required. They have used TNF to alter the endothelial barrier within tumour vasculature and thereby increase the efficacy of doxorubicin by 8-10 fold without increasing toxicity (Curnis et al, 2002). The other way to reduce systemic toxicity of TNF has been by isolated limb perfusion of TNF; this has been used in rat models and patients (Eggermont et al, 1996; de Wilt et al, 1999).

### C. Clinical trials of recombinant TNF

TNF has been administered intravenously to a wide range of tumours in Phase I and II clinical trials with none or limited tumour responses and was associated with severe toxicity particularly hypotension, rigors, fever and hepatotoxicity (Selby et al, 1987; Creagan et al, 1988; Brown et al, 1991; Furman et al, 1993). The use of TNF and IFN $\gamma$ , which had been shown *in vitro* to act synergistically, has also been evaluated in clinical trials, again the toxicity produced was unacceptable and the tumour responses disappointing (Abbruzzese et al, 1990; Fiedler et al, 1991). TNF however, has been shown to be useful in limb perfusion studies for patients with melanoma and soft tissue sarcomas. In these studies, the limb vasculature was isolated from the body and large amounts of systemically toxic TNF were infused into these tumour-bearing limbs to necrose the tumour (Eggermont et al, 1996). This strategy is licensed in Europe in combination with melphalan, since the addition of TNF to melphalan increased the response rates considerably.

The use of TNF as an anti-cancer agent has clear limitations due to its toxicity and may even be deleterious in the long term, as it can lead to re-growth of resistant tumours and, in the case of melanoma, more aggressive strains (Zouboulis et al, 1990). There is significant evidence pointing to TNF as an agent promoting different aspects of cancer (Figure 3).

## III. TNF as a carcinogen

### A. TNF in human tumours

TNF has been detected in a number of different tumour types such as ovarian and breast tissue as well as haematological malignancies (Naylor et al, 1993; Miles et al, 1994; Sati et al, 1999; Warzocha et al, 2000). Both mRNA expression and TNF protein has been found in human epithelial ovarian tumour cells as well as within the infiltrating macrophages. The p55 TNFR has also been detected within ovarian tumour cells and infiltrating macrophages but not stromal macrophages whilst the p75 TNFR has only been found within the infiltrating macrophages (Naylor et al, 1993). In 49 biopsies taken from patients with breast cancer, 43 expressed TNF mRNA and protein compared to 4/11 biopsies from patients with benign breast disease. The TNF was localised to tumour stroma and infiltrating macrophages. Furthermore, though the number of macrophages did not increase with tumour grade, the expression of TNF within the macrophages increased with tumour grade (Miles et al, 1994). A similar picture of increased production of TNF correlating with worse prognosis has been identified in patients with prostrate cancer (Nakashima et al, 1998). In

these patients, raised serum TNF levels were associated with a reduction in body mass index and other factors associated with cachexia as well as a significantly increased mortality (Nakashima et al, 1998). In keeping with this, TNF has been shown to inhibit androgen receptor sensitivity, a poor prognostic indicator, and hence induce androgen independent proliferation in the LNCaP cell line (Mizokami et al, 2000). In chronic B cell lymphocytic leukaemia, increased TNF levels were found at all stages with a progressive increase in serum TNF levels in relation to the disease (Adami et al, 1994). Patients with other haematological malignancies such as lymphoma have also been examined: correlating the production of TNF with histology revealed higher levels of TNF, p55TNFR, Lymphotoxin (LT) and LT-R mRNA in follicular NHL than other histological entities (Warzocha et al, 2000).

### B. TNF gene polymorphism and cancer

A single gene polymorphism within the TNF locus (-308) has been identified using an allele-specific polymerase chain reaction. This together with a polymorphism on the LT locus has been measured in 273 lymphoma patients (Warzocha et al, 1997). The presence of the TNF allele involved in gene transcription was associated with higher plasma levels of TNF at the time of tumour diagnosis. Expression of the two alleles associated with increased TNF production were found to be a risk factor for failure of first-line chemotherapy, a shorter progression-free survival and a reduction in overall survival (Warzocha et al, 1997). A similar increase in risk of developing MGUS and myeloma has also been associated with high TNF producers (Davies et al, 2000).

TNF microsatellite polymorphisms have also been examined in gastrointestinal cancer. In 47 patients with gastric cancer there was an increase in frequency of TNFa3 allele and a decrease in frequency of TNFa10 allele compared to normal controls. In 77 patients with colorectal cancers there was an increase in frequency of TNFd7 allele compared to normal controls. No correlation with expression of the allele and TNF production were discussed in the paper (Saito et al 2001). Other studies have shown that the expression of the TNF-308A allele, which is known to up-regulate TNF did not increase the risk of gastric cancer but the expression of TNF-238A allele, which is known to down-regulate TNF transcription could be protective against gastric cancer, although the sample size was small (Gonzalez et al 2002).

Single nucleotide polymorphisms of the promoter region of TNF were examined in prostate cancer. The 488 locus was associated with a 17 fold increased incidence of prostate cancer and an increase in tumour staging was related to polymorphisms at the -308 locus (Oh et al, 2000). However, a more recent larger study looking at single nucleotide polymorphisms at the TNF-308 locus found no difference between patients and controls (MacCarron et al, 2000).

In two other cancers, microsatellite polymorphism studies have found a correlation with TNF polymorphism and the risk of cancer. In a study of Swedish women with

the HLA DR15-DQ6-haplotype there was an increased frequency of TNFa-11 polymorphism and an increase in HPV16 positivity. The TNF polymorphism was not associated with the pre-cancerous lesion cervical intraepithelial neoplasia (CIN) alone, however the relative risk of CIN conferred by the combination of TNFa-11, HLA-DQ6 and HPV 16 positivity was 15 (Ghaderi et al, 2001). In the same population, the risk of cervical cancer associated with the TNFa-11 polymorphism was also examined. The increased frequency of TNFa-11 was associated with HPV18 positivity but not HPV16 and TNFa-11 increased the risk of cancer in patients with the HLA DQ6 haplotype (Ghaderi et al, 2001). A further study in patients with cervical cancer has also shown under representation of the TNF-238 polymorphism, which is associated with a down regulation of TNF transcription (Calhoun et al, 2002). In cutaneous basal cell carcinoma (BCC), there was difference in the frequency of a1 and a7 polymorphisms in patients with BCC compared to controls. There was also an increase in the number of BCC in patients with alleles d4 and d6 alone or TNFa2-b4-d5 haplotype (Hajeer et al, 2000).

### C. The role of TNF in carcinogenesis

A number of studies attempted to establish a link between inflammation and carcinogenesis; including experiment to assess the ability of pro-inflammatory cytokines such as TNF, to induce tumours. TNF is a cytokine that is produced early in the inflammatory cascade and has been shown to promote carcinogenesis in murine skin tumours. Using TNF knockout mice the development of skin carcinomas by chemical carcinogen DMBA (7.12-dimethylbanz[a]-anthracene) and tumour promoter TPA (12-0-tetradecanoyl-phorbol-13-acetate) were decreased compared to wildtype mice (Moore et al, 1999, Suganuma et al, 1999). Using pentoxifylline, which was shown to inhibit TNF and IL-1 gene expression, the growth of DMBA/TPA induced papillomas were inhibited (Robertson et al, 1996). Pentoxifylline was also able to inhibit the inflammatory response and TNF production induced by cutaneous UV-B light exposure. Indicating that TNF may be involved in the mechanism by which long-term UV-B light exposure, can contribute to skin cancer (Oberyszyn et al, 1998). Earlier studies have shown that TNF is able to induce growth of v-Ha-ras transfected BALB/3T3 cells though not the non-transfected BalB3/T3 cells and that the chemical carcinogen okadaic acid induces mouse TNF- in the transfected and non-transfected tumours. These results suggest that a chemical tumour promoter can induce the secretion of TNF- from various cells and that TNF can then act as an endogenous tumour promoter *in vivo* (Komori et al, 1993). The mechanism and signalling events associated with this carcinogenesis are still being elucidated. In basal cell keratinocytes, the chemical promoter TPA induces PKC a process down-regulated in TNF knockout mice, as is the transcription factor AP-1. AP-1 induces GM-CSF, MMP 9 and MMP 3 proteins that are important in tumour development (Arnott et al, 2002). Using the epidermal JB6 murine model, AP-1, NF- B and nitric oxide synthetase have all been implicated in tumour promotion by TPA,

epidermal growth factor, TNF and oxidative stress. In this particular model, the effect of TNF was primarily in up-regulating NF- B (Dhar et al, 2002). Other groups, however, have shown that TNF, along with other pro-inflammatory cytokines, induces nitric oxide synthetase in a cholangiocarcinoma cell line (Jaiswal et al, 2000). This enzyme produces nitric oxide, which can increase DNA damage by inhibiting sensitive DNA repair enzymes, and thereby contributes to an increase in genetic mutations (Jaiswal et al 2000). Other studies have shown that the presence of iNOS in gynaecological tumours correlates with dedifferentiation (Thomsen et al, 1994). Therefore, the production of nitric oxide through TNF induction of iNOS may not only lead to tumour cell apoptosis, as described previously, but may also promote carcinogenesis. In a gastric carcinoma cell line the up-regulation of WNT10A and WNT10B by TNF and *Helicobacter pylori* may be an important pathway in carcinogenesis (Kirikoshi et al, 2001). The WNT 10A and 10B genes are human orthologues of the mouse proto-oncogene *Wnt-10b*, which activates the catenin-TCF signalling pathway. Deregulation of this pathway has been implicated in several forms of cancer such as colon cancer and melanoma (Brantjes et al, 2002). In liver tumour formation, Knight et al found that TNF was up-regulated by hepatic stem cells (oval cells) and contributed to their proliferation via p55 TNFR, as there was a reduction in proliferation and liver tumour formation in p55TNFR but not p75 TNFR knockout mice (Knight et al, 2000). TNF however, is not the only important cytokine in liver tumour formation, hepatocellular proliferation and tumour formation in rats exposed to a peroxisome proliferator can be induced via IL-1 and IL-6 (Anderson et al, 2001). It may be that different carcinogens require different cytokines to aid carcinogenesis. The signalling pathways induced by TNF have also been examined in rat mammary cells. TNF stimulated growth and morphogenesis of normal rat mammary epithelial cells as well as transformed mammary epithelial tumours. NF- B/p50 DNA binding was present in the tumour cells but absent in normal mammary epithelium, however, TNF stimulation of normal epithelia leads to an induction of NF- B/p50 DNA binding (Varela et al, 2001). Therefore, TNF may induce carcinogenesis by up-regulating NF- B leading to the up-regulation of other proteins that cause cell proliferation and morphogenesis.

### D. The role of TNF in metastasis

During inflammation, a number of proteins can be up-regulated to allow immune cells to migrate to sites of inflammation. Tumours use these same processes to invade adjacent structures. TNF is a potent pro-inflammatory cytokine that can be utilised by tumours to induce other downstream molecules involved in the metastatic process. Recombinant TNF injected into mice inoculated with a methylcholanthrene-induced fibrosarcoma increased the number of lung metastases (Orosz et al, 1993). Cells transfected with the TNF gene were also found to increase metastatic potential. In Chinese hamster ovarian cells transfected with TNF there

was increased intraperitoneal invasion, compared to cells infected with vector alone, and furthermore, antibodies to TNF abrogated this ability. (Malik et al, 1990). Similarly, ESB tumour cells infected with a retrovirus carrying the TNF gene were found to have augmented metastatic tumour activity and this metastatic process could be reversed with anti-TNF mAbs (Quin et al, 1993). Blocking TNF using the human p55-IgG fusion protein in a murine B16-BL6 melanoma model reduced the number of metastatic lung tumours indicating that some tumours may intrinsically use TNF within their microenvironment to aid metastasis (Cubillos et al, 1997). The administration of intraperitoneal TNF in human ovarian xenograft models had a paradoxical effect on the tumours. The intraperitoneal administration of rhTNF had anti-tumour activity in two out of three xenografts with tumour clumps in the peritoneum being surrounded by host inflammatory cells and necrosis of the tumours in 4-7 days. The third xenograft however, continued to grow and rhTNF promoted adhesion of the tumour cells to the peritoneum and the establishment of tumour nodules on the mesothelial surface, phenomena noted in the other two xenografts as well (Malik et al, 1989). This suggests that human TNF may also promote metastasis in human tissue. Metastasis can be divided into a series of biological processes described below. TNF appears to be involved in the up-regulation of these pro-metastatic factors and hence contributes to the completion of each of these processes.

### **1. Neovascularisation, angiogenesis and the role of TNF**

In order for a primary tumour to expand, it requires nutrition and oxygen. When tumours are less than 200µm in diameter this occurs by diffusion, however larger tumours require vasculature. Chemokines such as IL-8 and Gro as well as other growth factors e.g. FGF, PDGF and thymidine phosphorylase are important in neovascularisation (Folkman, 1986, 1995; Folkman and Klagsbrun, 1987; Auerbach and Auerbach, 1994; Fidler and Ellis, 1994; Nagy et al, 1995; Leek et al, 1998). They attract endothelial cells and cause the migration of capillaries into the tumours. The endothelial cells proliferate and form vascular loops with new basement membranes with different cellular composition, permeability and stability as well as growth regulation compared to the host capillaries. TNF has been found to increase the expression of IL-8 and Gro in a number of different cell types (Strieter et al, 1995). In histological samples of malignant breast cancer, increased TNF staining correlated with increased thymidine phosphorylase an important enzyme in angiogenesis (Leek et al, 1998).

There also needs to be down-regulation of various inhibitors in order for angiogenesis to occur. These include inhibitors of matrix metalloproteinases (MMP), as they prevent migratory endothelial cells degrading basement membrane. A number of artificial MMP inhibitors are being used in anti-angiogenic trials to inhibit endothelial invasion (Nemunaitis et al, 1998; Shalinsky et al, 1999). TNF has been found to up-regulate MMP 9 and thereby

may contribute to angiogenesis (Shin et al, 2000). Therefore, inhibition of TNF may have a role in preventing angiogenesis by inhibiting MMPs. It is thought that the anti-angiogenic mechanism of thalidomide is in part due to the inhibition of pro-inflammatory cytokines such as TNF. Thalidomide is in Phase II trials for a number of tumours including renal cancer and melanoma (Stebbing et al, 2001).

### **2. TNF increases detachment from the primary site**

Cells within a tissue are retained within the structure by their adhesion to neighbouring cells and by the extra cellular matrix. Therefore, in order for invasion to occur tumour cells need to detach and become mobile. There are four groups of adhesion molecules important in this process. The first are the cadherins, which interact with other cadherins to form cell-to-cell attachments. The down-regulation of E-Cadherin in particular has been implicated in cancer invasion in a number of human malignancies (Shiozaki et al, 1991; Tohma et al, 1992; Dorudi et al, 1993). Stimulation of intestinal cells with TNF reduced E-Cadherin levels enhanced invasion, via a Src kinase dependant mechanism (Kawai et al, 2002).

The second group of important adhesion molecules are the integrins, which are made up of differing combinations of  $\alpha$  and  $\beta$  subunits. These molecules enable cells to adhere to components of the basement membrane and stroma such as collagen, vitronectin, laminin and fibronectin during migration (Hynes, 1992). In OST osteosarcoma cells, stimulation with TNF causes up-regulation of  $\alpha 2 1$  and  $\alpha 5 1$  with increased adhesion and migration through the extra cellular matrix (Kawashima et al, 2001).

The third group of adhesion molecules are members of the immunoglobulin superfamily including ICAM 1, 2 and 3, and other immunoglobulin superfamily members such as VCAM, which bind integrins and are important in cell-to-cell interactions. These molecules are up-regulated by pro-inflammatory cytokines such as TNF, IFN  $\gamma$ , and IL-1 and they have a major role in T cell and NK cells adhesion and migration. In a cancer setting, TNF appears to attenuate the basal expression of ICAM-1 in the presence of the extra cellular matrix in a thyroid cancer cell line (Miller et al, 2000).

The fourth major group of adhesion molecules are the selectins, which bind to carbohydrate groups on glycoproteins. E-selectin found on endothelial cells binds sialyl-Lewis X and G found on epithelial cells in colon and gastric carcinomas. TNF appeared to stimulate E-selectin expression on cultured human vascular endothelial cells to increase their adhesion to Sialyl-Lewis (a) on pancreatic cancer cells and thereby aid tumour entry into the vasculature (Nozawa et al, 2000).

### **3. Increased motility and the possible role of TNF**

Tumour invasion requires the cells to be motile. Autocrine motility factors and those due to stromal cytokine production are associated with changes in the

tumour cell cytoskeleton. TNF has been found to increase the motility of a number of cancer cells (Rosen et al, 1991; Dekker et al, 1994; Carpenter et al, 1997). In order for cells to move they undergo distinct events that are regulated by separate signalling pathways (Condeelis et al, 2001; Kassis et al, 2001; Price and Collard, 2001). Dividing the process into separate entities, the initial event is the extension of the lamellipodia, which is then stabilised by adhesion to the substratum. This is followed by the generation of contractile forces causing translocation of the body of the cell and finally the detachment of the trailing edge. To produce the lamellipodia there needs to be reorganisation of the cytoskeleton and the cyclic polymerisation and depolymerisation of actin. The Rho family of GTPases affects these processes. Cdc42, Rho and Rac1 have all been shown *in vitro* to lead to the formation of actin stress fibres, lamellipodia and filopodia respectively, which are all involved in motility. In fibroblasts, TNF and IL-1 stimulate Cdc42 causing filopodia formation (Puls et al, 1999) and via ceramide, increase stress fibre formation (Hanna et al, 2001). The effects of TNF on motility does, however, appear to be cell-dependant. In macrophages for example, TNF inhibits filopodia and reduces F-actin via the p55 TNFR death domain. Inhibition of the death domain by the synthetic compound D609 or TNFR mutants increases F actin with accumulation at the cell cortex and involves the FAN binding site of the receptor (Peppelenbosch et al, 1999). Therefore, the effect of TNF on cytoskeletal reorganisation may depend on the region of the TNFR that is activated. In tumour cells epidermal growth factor (EGF) has been shown to activate Rho-GTPases and induce cytoskeletal reorganisation and tumour invasion *in vitro*. The effect of TNF on cytoskeletal reorganisation in tumour cells remains to be elucidated. The adhesion of the lamellipodia and de-adhesion of the trailing edge involves the regulation of adhesion factors such as integrins. TNF has been shown to up-regulate integrins and aid invasion *in vitro* in specific tumour cells (Kawashima et al, 2001).

#### **4. TNF increases invasion of the extra cellular matrix**

In order for cells to migrate they need to degrade the basement membrane. The membrane primarily consists of type IV collagen and stroma, the latter is composed of types I, II, III collagen, proteoglycan and glycoprotein. To degrade the membrane the cancer cells produce matrix-degrading enzymes. The major family of degrading enzymes are the MMP, which contain a zinc-binding domain at their catalytic site. They are secreted in their inactive form and are activated by other proteases. The MMP can be divided into different groups based on their properties and substrates. MMP 2 and 9 are up-regulated in breast (Davies et al, 1993b), prostate (Stearns and Wang, 1993) ovarian (Davis et al, 1993a) and bladder cancer (Davies et al, 1993c). TNF appears to up-regulate MMP 2 and 9 in some bladder cancer cell lines (Shin et al, 2000). Host stromal cells also produce MMP and cancer cells may utilise them to facilitate invasion. TNF

production by stromal cells induces MMP 9 production in human giant cell tumours of bone (Rao et al, 1999). MMP have natural inhibitors known as TIMP (Tissue inhibitors of MMP), which control their activity. Tumour invasion depends in part on the balance of MMP with TIMP and pro inflammatory cytokines such as TNF can tip the balance in favour of MMP (Hajitou et al, 2001).

Other proteases that degrade the extra cellular matrix include serine proteases, which have a serine in their active site. An example of this is urokinase-plasminogen activator that catalyses the conversion of plasminogen to plasmin, which degrades components of the extra cellular matrix. TNF has been found to up regulate urokinase-plasminogen and thereby increase invasiveness of tumours (Wu et al, 1999).

#### **5. Entry into vasculature and lymphatics**

Once the tumour cells invade through the basement membrane they enter the lymphatic or vascular system and disseminate to the rest of the body. The lymphatics and blood stream are interlinked, so that tumours that pass into one system can readily pass into the other system. Due to the processes of neovascularisation the capillary vasculature lies close to the basement membrane, so that tumour cells, once they have invaded the basement membrane, are able to adhere to the endothelial cells and pass into the vasculature easily. The adhesion of tumour cells to endothelial cells occurs via endothelial adhesion molecules such as E-selectin and VCAM (Nozawa et al, 2000; Flugy et al, 2002; Simiantonaki et al, 2002) that bind to glycoproteins and integrins on the tumour cells (Voura et al, 2001). The capillaries tend to be more permeable than the normal physiological capillary vasculature, enabling tumour cells to squeeze between endothelial cells into the blood vessels.

#### **6. Extravasation**

The circulating tumours are able to adhere to the endothelium and using pseudopodial projections invade the surrounding tissue (Morris et al, 1997). The tumours adhere to components of the extra cellular matrix using integrins in a similar process to invasion from the primary site (Renkonen et al, 1999; Tanaka, 1999). Once they have penetrated the organ parenchyma, their proliferation depends on the environment.

#### **7. Proliferation of metastases**

Once tumours arrive at their sites of metastasis, they can manipulate the host environment to develop the tumour architecture. TNF may help in this by stimulating the proliferation of fibroblasts and collagen (Mauviel et al 1991, Bategay et al 1995). Tumours also use the host environment to aid proliferation by binding to growth factors released from the stroma. For example, in multiple myeloma, TNF induces bone marrow stromal cells to produce IL-6, a myeloma growth factor (Hideshima et al, 2001). Once metastatic tumours grow, they again need to develop a vasculature to increase beyond a certain size and do so in a similar way to the primary tumours. This in turn

aids metastasis, as they are then able to metastasise to other organs.

Since tumours are genetically unstable, often the metastatic tumours may have developed an advantage compared to the primary tumour aiding their survival. Parratto et al, (1989) found an inverse correlation between a strong antibody response and metastatic ability, so that the host's immune response may be selecting out the poorly metastatic clones and allowing the highly metastatic clones to proliferate. The production of TNF by the host immune cells may thereby contribute to the development of metastatic clones.

#### IV. Conclusion

TNF has a wide range of activities in cancer including cancer related cachexia that has not been covered in this review. It was initially thought that the majority of the effects of TNF on cancers were beneficial enhancing immunological rejection of cancers via NK and CTL responses. However, the clinical trials using TNF to treat cancer were disappointing due to the high toxicity caused by large amounts of cytokine. Indeed now the only therapeutic role that remains is for the treatment of melanoma in isolated limb perfusion. More recently, as is often seen with TNF, it has converse actions that induce a number of pro-inflammatory genes, which the tumours utilise to promote cancer such as cytokines, angiogenic factors and MMPs. These factors contribute to tumour formation, growth, invasion and metastasis to other sites. Many of the actions of TNF may occur by the stimulation of stromal tissue, tumour-associated macrophages and fibroblasts. These cells may then produce inflammatory cytokines including TNF itself, as well as some of the angiogenic factors described above, contributing to tumour proliferation and invasion. Anti-TNF mAbs have now been licensed in the USA and Europe and are widely used for the treatment of rheumatoid arthritis and Crohn's disease. We await with interest the long term follow up of these clinical trials which have specifically blocked to TNF as they may provide an indication of the role of this cytokine in promoting cancer.

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