
Meeting Report

FOURTH INTERNATIONAL CONGRESS ON TUMOUR NECROSIS FACTOR AND RELATED CYTOKINES

Meeting at Koningshof, Veldhoven, The Netherlands
2–6 May 1992

A. Meager

Tumour necrosis factor (TNF) describes two cytokines produced by activated monocytes/macrophages, lymphocytes and other cell types. Two genes encode two distinct forms of TNF, TNF- α and TNF- β , which despite being only 36% related in their amino acid sequences exhibit similar tertiary and quaternary structure, bind to common cell surface receptors, and have wide overlapping spectra of regulatory biological activities. Both TNF- α and TNF- β have cytotoxic activity against a range of tumour cells *in vitro* and induce haemorrhagic necrosis of a number of transplantable tumours in mice. These activities have suggested that TNF may have potential therapeutic uses in the treatment of cancer. In addition, TNF has been associated with several aspects of inflammation, including its deleterious effects, leading to the idea that downregulation of TNF production and actions may be clinically beneficial in many patients with acute illness, e.g. Gram-negative bacterial septicaemia, in patients following bone marrow or organ transplantation, and in patients with chronic diseases where there are intervals of active inflammation, e.g. multiple sclerosis, rheumatoid arthritis, malaria.

Thus, there has been intense scientific interest in studying the biology and biochemistry of TNF from the mid-1980s, when these two cytokines were cloned and made available in large amounts for research

and clinical investigations. The meeting held at Koningshof, Veldhoven was the fourth in a series begun in 1987 and gave an opportunity for the 500 scientists attending to discuss the most recent progress in this rapidly developing TNF and related cytokines' field. Much of the meeting was given over to the molecular and biological characterization of TNF membrane receptors and their soluble counterparts. It is now well documented that there are two molecularly distinct cell membrane TNF receptors. These receptors have different molecular weight and are frequently referred to as p55 or p60 and p75 or p80 according to size. Regrettably, some confusion has arisen as different research groups have designated them type I and type II receptors referring to p55/60 and p75/80 or vice versa. The meeting endorsed a committee proposal that the TNF receptors be assigned a CD number, but as this information will not be available until next year, the designations p60 and p80 will be used herein. There is some structural homology in the *N*-terminal extracellular domains of p60 and p80, but their intracellular domains are unrelated suggesting they have different functions. The extracellular domains have four characteristic, cysteine-rich, repeat units and this structure places these TNF membrane receptors into a broad family of cytokine- and other cell surface-receptors. It was revealed by C. Smith (Immunex Corp., USA) that there are at least ten members of this receptor superfamily; CD27, CD30, CD40, nerve growth factor receptor (NGF-R), OX40 (in rat), 4-1BB (in mouse), Fas antigen, p60, p80, and T2 (Shope poxvirus). The last named member, T2, bears some structural similarity to p80 extracellular domain and acts as a soluble TNF receptor. Apart from p60, p80,

From the Division of Immunobiology, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Herts, EN6 3QG.
© 1992 Academic Press Limited
1043-4666/92/060413+05 \$08.00/0

T2 and NGF-R, the ligands for the other receptors are unknown. However, C. Smith described the cloning of a 35 kDa glycoprotein present on the surface of B-cells which is the cognate ligand of CD40 and which functions to induce B-cell proliferation.

The Fas antigen also has interesting properties (S. Nagata, Osaka Bioscience Institute, Japan). It appears to function like TNF receptors in that, when crosslinked by anti-Fas antibodies, cytotoxicity (apoptosis) is induced in sensitive cells. In fact, it was revealed that the Fas antigen intracellular domain shows strong homology with the intracellular domain of p60. Mouse Fas, 49% homologous with human Fas, is coded for by a gene on chromosome 19 located within the lymphoproliferation (*lpr*) locus, and mice that are *lpr*⁻ either lack Fas mRNA due to a rearrangement in the second intron of the Fas gene or express a non-functional Fas due to a point mutation converting ileu to asp in the middle of the intracellular domain. In normal mice Fas antigen is found in the thymus, heart, lung and ovary, but in *lpr*⁻ mice lacking functional Fas antigen there is an accumulation of malignant CD4⁻ CD8⁻ T cells suggesting Fas is important in the thymic selection/development of T lymphocytes. The ligand for Fas, which is probably present in the thymic stroma, remains to be identified.

Returning to TNF receptors, W. Lesslauer (Hoffman-La Roche, Switzerland) described the construction of fusion proteins comprising the extracellular domains of p60 linked to the Fc portions of IgG₃ heavy chains. These form dimers which bind well to TNF α and have enabled X-ray crystallography to be applied to the complexes. This showed that a TNF- α trimer in its native active state binds three extracellular domains of p60-IgG₃ molecules with the cysteine-rich repeat units aligned in an up-down-up-down configuration. The contact points on the TNF- α molecule are at the external interfaces between the monomeric subunits close to the base of the trimer, but the head or tip of this trimer is orientated down towards what would be the cell membrane if the p60 extracellular domains were anchored to the membrane. The team from Hoffman-La Roche also demonstrated by using soluble forms of p60 and p80 that TNF- α binding involves segregation to form complexes containing only three p60s or three p80s; there was no evidence for the formation of complexes containing two p60s and one p80 or one p60 and two p80s.

Much was presented regarding which TNF receptor, p60 or p80, mediated which particular TNF activity (P. Scheurich, University of Stuttgart, Germany; D. Wallach, Weizmann Institute, Israel; W. Fiers, Laboratory of Molecular Biology, Gent, Belgium; D. Goeddel, Genentech, California, USA; H. Loetscher, Hoffman-La Roche, Basel, Switzerland). The overall impression was that p60

was probably responsible for mediating most of TNF's actions including cytotoxicity, antiviral activity and induction of mRNA (manganous superoxide dismutase, MnSOD; IL-6; c-fos; NF- κ B; plasminogen activator inhibitor-2, PAI-2; etc.) whereas the function of p80 was probably mainly related to lymphocytic proliferation and maturation including increased expression of interleukin 2 receptor α chain (Tac), granulocyte-macrophage colony stimulating factor (GM-CSF) and HLA-DR. However, the idea that there was some cooperativity between p60 and p80 was gaining ground. D. Goeddel (Genentech) suggested that p80 acted as the front line receptor to concentrate TNF- α from solutions containing low levels of TNF- α and then pass this to the more functionally active p60. How this could occur remains, however, to be elucidated.

Studies with specific monoclonal and polyclonal antibodies to p60 and p80 have clearly demonstrated that these receptors need to be crosslinked before they will function in signal transduction. However, early intracellular events following signal transduction across the cell membrane remain difficult to elucidate. Although several pathways could be involved and different protein kinases are activated, it appears that one of the initial second messengers, diacylglycerol (DAG) is generated not from phosphatidylinositol, but by phospholipase cleavage of phosphatidylcholine, a step that is blocked by the xanthogenate D609 inhibitor (S. Schütze, Technical University of Munich, Germany). DAG can activate protein kinase C (PKC), but activation of NF- κ B occurs in TNF-stimulated cells by a PKC-independent pathway. It was found that DAG could also activate sphingomyelinase (SMase) and this in turn could generate ceramide from sphingomyelin. Exogenous ceramide was shown to induce NF- κ B activation indicating that ceramide is an important second messenger in TNF-stimulated cells. Subsequent to nuclear NF- κ B activation it is apparent that several genes are derepressed leading to the transcription of mRNA and their translation. From human fibroblasts many cDNAs corresponding to TNF-upregulated mRNAs have been isolated and characterized, e.g. for IL-8, monocyte chemoattractant protein-1 (MCP-1), collagenase, stromelysin, etc. One of these cDNAs (TSG-6), however, corresponded to a previously unknown protein whose predicted amino acid sequence shows some homology to the lymphocyte homing receptor, CD44 (J. Vilcek, New York University Medical Center, USA). The TSG-6 protein is glycosylated and has a hyaluronic acid binding domain. It is secreted and is found in association with an 80 kDa binding factor, but its function remains unknown. Another early TNF-stimulated gene, A20, was reported by V.M. Dixit (University of Michigan, Ann Arbor, USA), the

product of which is a DNA-binding zinc-finger protein. The function of this protein appears to be associated with resistance to TNF-mediated cytotoxicity. NIH 3T3 cells transfected with A20 become more resistant to TNF, but if adenovirus E1a is co-transfected into these cells, they become sensitive again due to E1a down-modulation of A20.

The cytotoxic action of TNF does not depend on protein synthesis, although resistance to cytotoxicity appears to a large extent to do so (see above). Multiple intracellular pathways are probably involved in TNF-mediated cell killing, but evidence is accumulating that early damage of mitochondrial functions is strongly associated (W. Fiers, Laboratory of Molecular Biology, Gent, Belgium). Evidence was presented that mitochondrial production of oxygen radicals mainly generated at the ubisemiquinone site is a causal mechanism of TNF cytotoxicity. The phosphorylation of the small heat shock protein hsp28, which occurs via a PKC-independent pathway in TNF-stimulated cells may, on the other hand, be linked to enhanced resistance to TNF cytotoxicity (A-P. Arrigo, CNRS, Claude Bernard University, Lyon, France). De novo synthesis of hsp28 is not required for this protection, but it appears that synthesis of the large heat shock protein hsp72, which can also increase resistance to TNF in some cells, is required (M. Jäättelä, Fibiger Institute, Copenhagen, Denmark).

It is becoming evident that the soluble TNF receptors play an important role in the modulation of TNF activities. The soluble receptors are derived by proteolytic cleavage of p60 and p80; there is no evidence that alternative splicing of receptor mRNAs occurs giving rise to truncated receptors that are secreted. Cleavage of p60 occurs close to the transmembrane domain (D. Wallach, Weizmann Institute, Rehovot, Israel), but it is hard to pinpoint precisely the C-terminal amino acid. It is also apparent that ten N-terminal amino acids are clipped off compared to the membrane-bound extracellular domain. The cleavage process, both for p60 and p80, is independent of either receptor-signalling or receptor-uptake. However, one of the actions of TNF appears to be stimulation of soluble receptor shedding. Measurement of circulating levels of soluble TNF receptors following LPS stimulation *in vivo* indicates that they rise substantially 1–2 hours following increases in TNF- α levels (D. Radoux, Medgenix, Belgium; M. Brockhaus, Hoffman-La Roche, Basel, Switzerland). It is also clear that both p60- and p80-derived soluble receptors are raised in cancer patients and in particular the ascitic fluids of ovarian cancer patients contain high levels, up to 50 ng/ml (C. Granger, University of California at Irvine, USA). It is likely that the tumour cells themselves contribute much of these soluble receptors; *in vitro*,

ovarian tumour cells could be stimulated with a range of cytokines, including IL-1, IL-2, IL-4, IL-6 and IFN- γ , as well as PMA, to shed high levels of soluble receptors into the medium. This suggests that tumour cells attempt to counteract the antitumour effects of TNF- α by shedding receptors which annul its activity. Nevertheless, effector cells such as macrophages and lymphocytes also shed receptors suggesting the role of soluble receptors in cancer is more complex.

Elevated soluble TNF receptor levels were also reported in chronic lymphocytic leukaemia (B-CLL) and hairy cell leukaemia (HCL) (M. Brockhaus, Hoffman-La Roche, Basel, Switzerland), and in general levels appear to reflect disease activity in cancer patients and correlate well with levels of carbohydrate antigen-125 (C. Granger, University of California at Irvine, USA). It was therefore proposed that assays to measure soluble TNF receptors could be useful to monitor for recurrence of active disease. Such an application could also be relevant to diseases other than cancer (see below).

Levels of soluble TNF receptors are also raised in bacterial sepsis, malaria (particularly cerebral malaria; G. Grau, University of Geneva, Switzerland), chronic and acute hepatitis (M. Brockhaus, Hoffman-La Roche, Switzerland) and in chronic inflammatory diseases such as arthritis, multiple sclerosis (MS) and systemic lupus erythromatosis (SLE). In rheumatoid arthritis patients, bioactive TNF- α can still be detected in synovial fluids despite markedly elevated levels of soluble receptors (M. Feldmann, Sunley Research Institute, London, UK; D. Wallach, Weizmann Institute, Rehovot, Israel), whereas in osteoarthritis patients, where soluble TNF receptors are also present in synovial fluids, bioactive TNF- α is rarely detectable. Such observations suggest that soluble TNF receptors are functional inhibitors of TNF, and results from an animal model system where p60-derived soluble receptor protected galactosamine-treated mice from toxicity induced by TNF- α (V. Hornik, Interpharm Laboratories, Ness Ziona, Israel) support this hypothesis. The kidney appears to be important in the clearance of soluble TNF receptor-TNF complexes and in the mouse, p80-derived soluble receptors appear to be mainly responsible for clearing TNF from the circulation (W. Buurman, University of Limburg, Maastricht, The Netherlands).

Excess levels of TNF- α appear in the circulation, synovial fluids and other anatomical locations in virtually all acute and chronic inflammatory diseases and have adverse effects. In contrast, TNF is probably protective at certain levels and is involved in the resolution of infections and disease. Studies in transgenic mice carrying the TNF- α gene under specific controls suggest that LPS-stimulated TNF- α production is far wider in terms of types of

responding cells than was previously known and that spontaneous TNF- α production occurs at certain stages of development in normal animals, e.g. in thymic and trophoblastic development (B. Beutler, Howard Hughes Medical Institute, Texas, USA; G. Kollias, Hellenic Pasteur Institute, Athens, Greece). Overexpression of TNF- α in transgenic mice leads either to a lethal wasting syndrome or chronic inflammatory polyarthritis, further emphasizing the deleterious effects of an excess of TNF- α . Therefore, there is much clinical interest in either curbing TNF- α production or neutralizing TNF activity with specific antibodies or soluble TNF receptors. The drug pentoxifylline, which is used clinically for the treatment of vascular deficiencies, is an inhibitor of TNF- α production *in vivo* (P. Zabel, Borstel Medical Clinic, Germany) and was demonstrated to reduce TNF-dependent systemic symptoms in severe pulmonary tuberculosis. It may thus also be useful in reducing the risk of bone marrow rejection, in reducing HIV production and in preventing cachexia in cancer patients, all of which have been associated with TNF- α production. This potential wide therapeutic range remains to be established. Monoclonal antibodies (MAb) against TNF- α are in clinical trials to treat Gram-negative septicaemics and bone marrow graft recipients. Results using a humanized anti-TNF MAb in an animal model of septic shock indicate MAb treatment can dramatically reduce mortality (M. Bodmer, Celltech Ltd., Slough, UK). The humanized MAb has low immunogenicity and therefore offers certain advantages for patient treatment. Results from clinical trials have so far failed to indicate an overall therapeutic effect of anti-TNF MAb, but sepsis in man is heterogeneous and possibly only certain groups of patients will respond positively (J. Cohen, Royal Postgraduate Medical School, Hammersmith, London, UK). An anti-TNF MAb, B-C7, has also been used in bone marrow graft patients showing graft-vs-host disease, but while good clinical responses were observed in some patients, particularly in skin and gut, the overall mortality rate remained the same (P. Herve, Hospital Jean Minjot, Besancon, France). Other diseases such as rheumatoid arthritis, cerebral malaria and possibly MS are also indicated as targets for anti-TNF- α MAb or soluble TNF receptor therapy.

TNF- α itself was originally indicated as a cancer therapeutic agent because of its antitumour activity. However, several clinical trials with TNF- α have shown that TNF- α has very toxic side effects when given systemically and has poor efficacy against various solid tumours. It has been thought that the failure of TNF- α to have an antitumour effect is because the maximum tolerated dose, about 50 $\mu\text{g}/\text{kg}$, is too low to be effective. This appears to be borne out

by a phase II clinical trial involving isolated limb perfusion for the treatment of malignant melanoma where high dose (3-4 mg) TNF- α combined with IFN- γ , melphalan and hyperthermia has led to complete responses in 90% of patients (F. Lejeune, CPO, Lausanne, Switzerland). This treatment was shown to destroy tumour vasculature, without harming normal blood vessels, and induce rapid necrosis of malignant nodules. The main benefit of this treatment was that the patient did not have to undergo amputation of the affected limb, and, although relapses often occurred, the quality of life was markedly improved during remissions. Another use of TNF- α in the management of cancer was in fluid extravasation in malignant ascites (V. R ath, Ruprecht-Karl University, Heidelberg, Germany). Peritoneal involvement and ascitic fluid accumulation is a late phenomenon of advanced cancer and can cause discomfort and pain due to abdominal distension. It was found that injected TNF- α led to fluid extravasation and to a reduction of pain, although there were systemic side effects (controlled by paracetamol). This treatment, however, did not increase the survival of these patients. In the nude mouse with human ovarian tumour xenografts, TNF- α was shown to markedly increase the adhesion of ascites cells and probably augment tumour progression (F. Balkwill, Imperial Cancer Research Fund, London, UK). This, if true also for the human situation, strongly indicates that TNF- α should be used with extreme caution in certain cancer patients.

The measurement of TNF- α , especially in clinical samples, is beset with all sorts of problems. The now proven presence of soluble TNF receptors is probably one of the major causes of the wide discrepancies among TNF levels of similar biological fluids reported by different laboratories (J. Cohen, Royal Postgraduate Medical School, London, UK). Only certain immunoassays (e.g. Medgenix IRMA) can fully detect all TNF- α in such fluids because many antigenic determinants on the TNF- α molecule can be made inaccessible by the binding of soluble TNF receptors (I. Engelberts, University of Limburg, Maastricht, The Netherlands). However, it is not yet clear whether measurement of complexed TNF- α is more clinically relevant than determination of biologically active TNF- α . Measurement of soluble TNF receptors themselves has been made easier by the introduction of specific immunoassays and the use of MAbs that do not interfere with TNF binding forms the basis of a novel ELISA system, i.e. immobilized MAb-soluble TNF receptor- ^{125}I -TNF- α (M. Brockhaus, Hoffman-La Roche, Basel, Switzerland), that detects functionally-active soluble receptors.

A WHO International Standard for human TNF- α (87/650) has recently been established and is available from The National Institute for Biological Standards

and Control, South Mimms, Herts, EN6 3QG, UK. Reference reagents for mouse TNF- α and human TNF- β are similarly available. Requests should be addressed to the Director.