

The role of TNF in tumour growth and metastasis

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The capacity of TNF to induce necrosis of solid tumours *in vivo* and its cytostatic and cytotoxic activities toward tumour cells *in vitro* promoted intensive research on this molecule and is very well established (Carswell *et al.*, 1975; Haranaka *et al.*, 1984; Old, 1985; Palladino *et al.*, 1987). Application of high-dose recombinant TNF in a combination treatment protocol using isolation limb perfusion clearly demonstrates the antitumoral activity of TNF by selectively destroying the tumour vasculature (Lienard *et al.*, 1992). However, TNF, in addition to the antitumour effects, has a broad spectrum of biological effects that can complicate its use as an antitumor agent. The induction of IL1 (Dinarelli *et al.*, 1986) and IFN- β (Kohase *et al.*, 1986), the enhanced expression of HLA genes in tumour cells (Pfizenmaier *et al.*, 1987), or the supportive effect on NK (Männel *et al.*, 1989) and LAK (McIntosh *et al.*, 1989) activity might contribute to the beneficial antitumoral activity of TNF.

The role of endogenous TNF in the pathophysiology of cancer and metastasis is controversially discussed. Only limited information is available demonstrating enhanced levels in serum or tumour specimens of cancer patients (Naylor *et al.*, 1990; Balkwill *et al.*, 1987) and in tumour-bearing animals (Rakhmilevich and North, 1991; Männel *et al.*, 1990). Such endogenous TNF might also contribute to tumour progression. TNF is able to activate enzyme cascades that may enhance the tumour spread, e.g. proteases (Opdenakker and Van Damme, 1992; Dayer *et al.*, 1985), to stimulate angiogenesis *in vivo* (Frater Schroder *et al.*, 1987) and to increase the adherence of tumour cells to endothelium *in vitro* (Rice *et al.*, 1988; Dejana *et al.*, 1988; Bereta *et al.*, 1991). It has also been shown that TNF promotes the invasive growth of tumour cells in the peritoneum and their establishment as tumour nodules below the mesothelial surface (Malik *et al.*, 1989, 1990).

TNF and endothelial adhesion

Experiments concerning the influence of exogenous TNF on metastasis had rather controversial

results. In a murine fibrosarcoma model (Tomazic *et al.*, 1988), a lung cancer model (Schultz and Altom, 1990) and in melanoma models (Sylvester *et al.*, 1990; Lollini *et al.*, 1990), inhibition of formation of metastases by rhTNF has been reported when rhTNF was given within 3 days of tumour cell inoculation. On the other hand, administration of rhTNF led to an increase in pulmonary metastasis when given 1 h prior to i.v. inoculation of a human melanoma cell line into nude mice (Giavazzi *et al.*, 1990) or promoted the implantation of human ovarian cancer xenografts in the peritoneal cavity of nude mice (Malik *et al.*, 1989).

In an experimental metastasis model, mice received syngeneic fibrosarcoma cells (CFS1) i.v. The injection of either rmTNF or rhTNF 5 h prior to tumour cell inoculation led to a dose-dependent augmentation in the number of tumour colonies counted on the surface of the lungs after 12 days (fig. 1) (Orosz *et al.*, 1993). The stronger metastasis-promoting activity of rmTNF compared to rhTNF demonstrated the described species preference of TNF (Kramer *et al.*, 1988; Brouckaert *et al.*, 1992) and might be explained by species-specific activities mediated via the mouse p75 TNF receptor. Application of bacterial endotoxin (LPS) also augments metastasis, possibly by inducing endogenous TNF. Therefore, LPS contamination of the rmTNF and rhTNF preparation, respectively, was excluded by heating the TNF to 100°C for 15 min. This treatment destroyed the metastasis-enhancing capacity completely. A significant increase in the colonies on the lungs compared to control mice was observed only when rmTNF was given 5 h prior to or 1 h after tumour cells. Application of TNF 24 h before or 24 h and up to 4 days after tumour cell injection had no significant effect on pulmonary metastasis. Also, *in vitro* pretreatment of tumour cells with TNF had no influence on metastasis, in contrast to results described in a melanoma model (Lollini *et al.*, 1990). These results suggest that the observed metastasis-promoting effect might reside in an influence of TNF on the interaction of circulating tumour cells with the endothelium supporting the arrest, diapedesis and extravasation of the tumour cells.

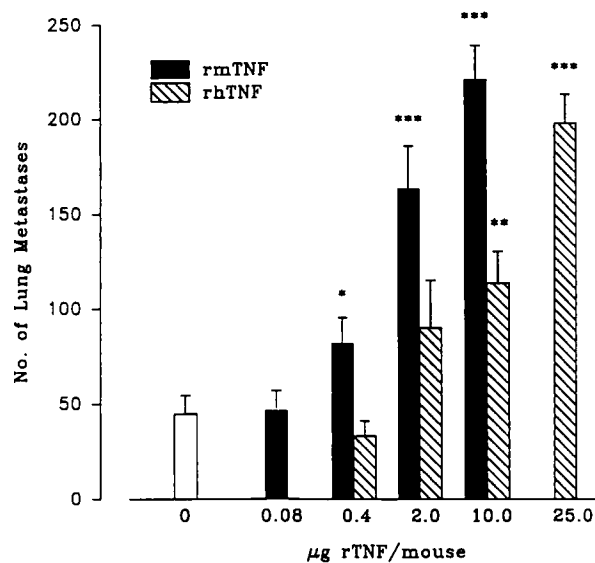


Fig. 1. Increase in experimental lung metastasis by different concentrations of rmTNF and rhTNF.

Mice were given i.p. injections of different concentrations of rmTNF or rhTNF 5 h prior to i.v. CFS1 tumor cell injection (3×10^5). Control mice received the same volume of PBS. Results are expressed as mean \pm SEM. $n = 12$ for control group; $n = 6$ for TNF treated groups. *** $p < 0.001$; ** $p < 0.005$; * $p < 0.05$.

Since tumour-bearing mice showed an enhanced capacity for TNF production, the influence of endogenous TNF on the formation of lung metastases was investigated by injecting i.v. neutralizing anti-mouse TNF monoclonal antibodies (V1q) (Echtenacher *et al.*, 1990) 5 h before tumour cell inoculation. The neutralization of endogenous TNF induced a significant decrease in metastasis (table I), demonstrating a supportive effect of endogenously produced TNF for metastasis. Whether this TNF effect is due to enhanced expression of adhesion molecules on endothelial cells and/or tumour cells

by a direct action of TNF on these cells or whether it is mediated via additional cells, *e.g.* granulocytes or platelets, is currently being investigated. A recent report demonstrating abolishment of TNF-induced enhanced metastasis in a similar model by blocking adhesion strongly supports the hypothesis of adhesion promotion by TNF. It has been reported that synthetic peptides containing recognition sequences of cell adhesion molecules (*i.e.*, Arg-Gly-Asp (RGD) in fibronectin and Tyr-Ile-Gly-Ser-Arg (YIGSR) in laminin) inhibit the development of tumour metastasis (Iwamoto *et al.*, 1987; Saiki *et al.*, 1989). A modified TNF molecule which carried the laminin-derived peptide YIGSR on the N-terminus did not enhance experimental metastasis, in contrast to unmodified TNF (Miyata *et al.*, 1992).

Tumour cell metastasis could be regulated in a similar way as lymphocyte migration from blood into tissue. Activation of integrins that mediate adhesion of T cells to the endothelium can be achieved by proteoglycan-immobilized cytokines, *e.g.* MIP-1 β (Tanaka *et al.*, 1993). A cellular proteoglycan that could serve the function of anchoring the activating cytokines is CD44, which has also been shown to be involved in regulation of metastasis (Arch *et al.*, 1992). It could be speculated that enhancement of expression of such activating cytokines by TNF might be one possible mechanism by which TNF increases the adherence of tumour cells to the endothelium (Rice *et al.*, 1988; Dejana *et al.*, 1988; Bereta *et al.*, 1991).

Preliminary evidence from our experiments using thrombocytopenic mice also indicates an involvement of platelets in the extravasation steps of metastasis. Ample data showing a correlation of metastasis with platelet aggregation can be found in the literature (Mogi *et al.*, 1991; Tzanakakis *et al.*, 1991; Nierodzick *et al.*, 1991; Sugimoto *et al.*, 1991; Okoshi *et al.*, 1991; Ordinas *et al.*, 1990). TNF leads to platelet activation via stimulation of granulocytes (Renesto and Chignard, 1991). The exact mechanisms, cells, and molecules involved in such a metastasis-provoking situation have yet to be analysed.

Table I. Reduction of experimental metastasis by neutralization of endogenous TNF.

Pretreatment	n	No. of lung metastases (mean \pm SEM)	Range	p
PBS control	8	58.5 \pm 7.9	22-82	
Rat anti-mTNF (V1q)	6	9.6 \pm 5.6	1-31	< 0.005
Rat IgG	6	66.9 \pm 18.0	23-114	

Mice were treated i.p. with the indicated substances 5 h before i.v. CFS1 tumour cell injection (3×10^5). Animals were killed on day 11, lungs were removed and number of surface colonies was counted.

Table II. Enhancement of tumour cell proliferation rate *in vivo* by rmTNF.

AgNOR parameter	Pretreatment		p
	PBS control (mean \pm SEM)	rmTNF (mean \pm SEM)	
No. of AgNOR/cell	1.2 \pm 1.2	8.4 \pm 0.7	< 0.001
AgNOR area (μm^2)/AgNOR	0.02 \pm 0.02	0.13 \pm 0.01	< 0.001
AgNOR content/cell	9.4 \pm 9.4	65.8 \pm 5.6	< 0.001

Mice received 10 μg rmTNF or 100 μl PBS 5 h prior to i.v. tumour cell inoculation. Lungs were removed on day 5, fixed in formalin and AgNOR parameters were determined. n = 6 per group.

TNF and cell activation

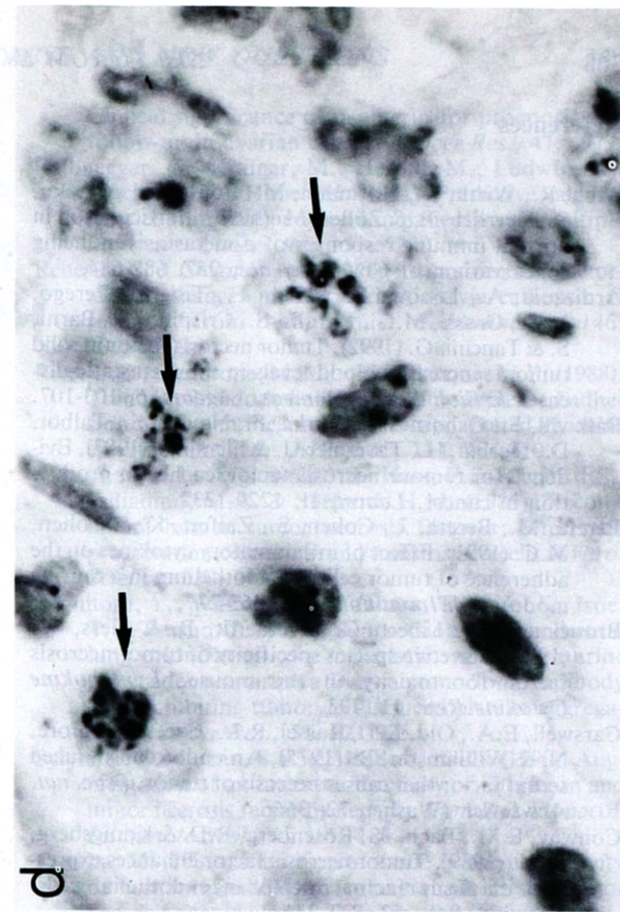
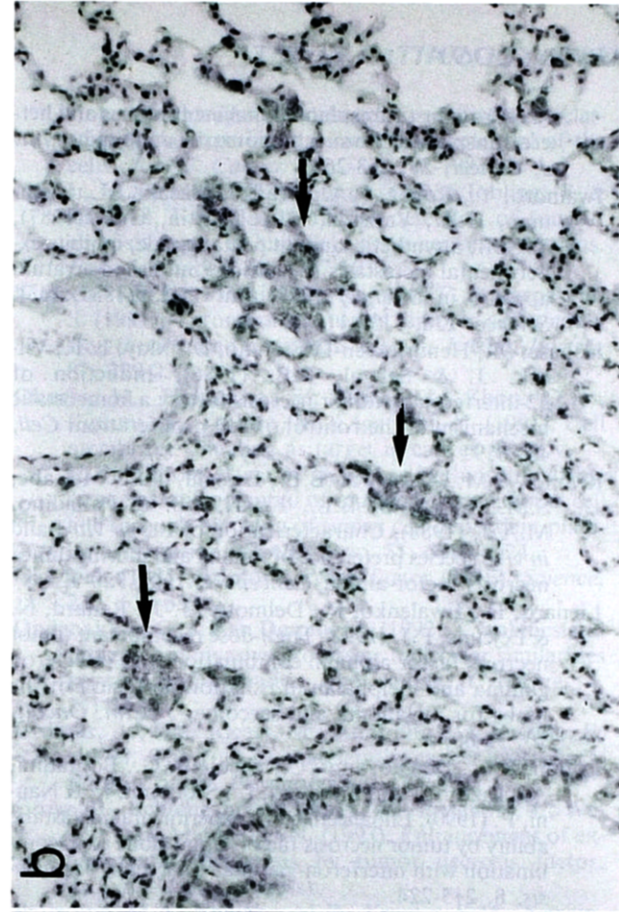
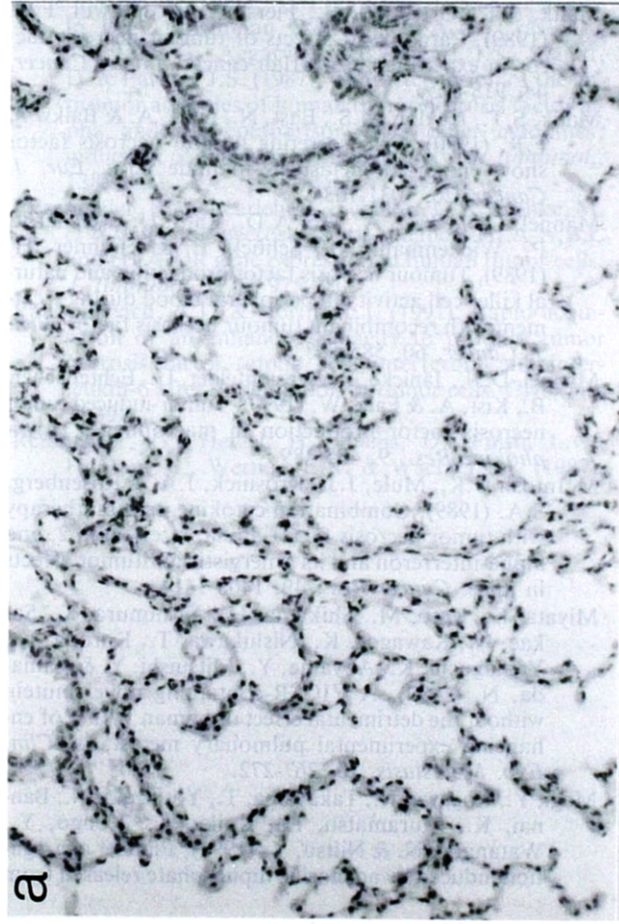
Histological analysis of lung sections from rmTNF-pretreated mice 5 days after tumour cell inoculation showed more and larger pulmonary metastases in the septae of the lung parenchyma (fig. 2). Number and size of silver-stained proteins associated with nuclear organizer regions (AgNOR) per cell were determined on paraffin sections by digital image analysis as a parameter for *in vivo* proliferation (Ruschoff *et al.*, 1990). Lung sections from rmTNF-pretreated mice showed a significantly enhanced number of AgNOR dots, AgNOR area and AgNOR content per cell, indicating an augmented proliferation rate compared to the control group (fig. 2, table II). *In vitro* treatment with TNF, however, did not influence the proliferation rate of the fibrosarcoma cells, indicating that the augmented metabolic and proliferative activity *in vivo* might be host-dependent. Preliminary data with tumour cells growing as spheroids in three-dimensional culture indicated opposing effects of TNF. At lower TNF concentrations, the size of the tumour spheroids was larger compared to TNF-free cultures, whereas at higher TNF concentrations, the tumour spheroids remained very small (unpublished results). Since TNF has been shown to exert growth stimulatory activities on some cell types (Sugarman *et al.*, 1985) growth-promoting and growth-inhibitory activities could be two overlapping functions of TNF. The mechanisms of the apparently opposing effects remain to be analysed.

A similar bimodal effect of TNF was also observed in angiogenesis. Whereas angiogenesis was inhibited at high concentrations of TNF, low concentrations induced it (Fajardo *et al.*, 1992). Thus, in a situation of low tissue concentration, TNF might support the growth of microvessels in neoplasms and therefore eventually promote tumour growth. Recent data seem to support this hypothesis. Fibrosarcoma cells overexpressing tissue factor and growing as solid tumours showed better *in vivo* growth than those expressing low amounts (P. Nawroth, personal communication). Since TNF is an inducer of tissue factor expression on some cell types (Conway *et al.*, 1989; Wiggins *et al.*, 1990; Tanaka, 1989) such indirect effector mechanisms might also be involved in the complex regulation of tumour growth.

A positive correlation of stimulated immune status with poor prognosis and enhanced metastasis has been found (Reibnegger *et al.*, 1987, 1991). The determination of enhanced levels of neopterin, a macrophage-derived product, and IL6 in tumour patients served as parameters for the stimulated immune system. Increased neopterin and IL6 production are possible consequences of enhanced TNF production. In support of this observation is a recent study that directly documents increased blood TNF levels in metastatic cancer patients over those without metastases (Ardizzoia *et al.*, 1992). This correlation of exaggerated TNF secretion with neoplastic metastatic disease supports the pathological role of TNF in metastasis.

Fig. 2. Histological sections of lung tissue from untreated mice (a,c) or TNF-treated mice (b,d) exhibiting small metastatic tumour nodules within alveolar septae (arrows).

Haematoxylin/eosin stain; a) original $\times 400$, b) original $\times 200$. Nodule of metastatic tumour cells showing multiple silver stained NOR dots (AgNOR) within nuclei (arrows). c,d) AgNOR stain, original $\times 2,500$.



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