**In vivo** targets of recombinant human tumour necrosis factor-α: blood flow, oxygen consumption and growth of isoinfused rat tumours

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Summary The impact of recombinant human tumour necrosis factor-alpha (1 μg kg⁻¹ to 1 mg kg⁻¹; 6.6 x 10⁻¹⁵ U mg protein⁻¹) on blood flow, oxygen consumption and growth of a moderately TNF-sensitive rat tumour (DS-carcinosarcoma) was studied. Tumour growth was stimulated at low TNF doses (1 and 10 μg kg⁻¹) and significantly retarded at higher TNF dose levels (0.1 and 1 mg kg⁻¹). Growth changes were concomitant with variations in oxygen consumption, lactate release and acidification of the metabolic micromilieu. Both single and repeated application of low TNF doses (1-10 μg kg⁻¹ i.v.) increased tumour perfusion whereas single administration of high TNF dose levels (0.1-1 mg kg⁻¹ i.v.) reduced tumour blood flow. After repeated application of high TNF doses tumours shrank to such small sizes that perfusion measurements could not be performed within the observation period of 2 weeks. It is concluded that TNF effects on solid tumours are at least partially mediated by changes in tumour perfusion. Thus, an altered tumour sensitivity towards other treatment modalities, e.g. irradiation, chemotherapy or hyperthermia, can be expected after TNF therapy. A beneficial TNF effect would critically depend on the dose level employed and on the sequence and timing of various combination regimes.

Tumour necrosis factor-α was discovered in tumour-bearing mice as an endotoxin-induceable serum factor causing ‘haemorrhagic’ tumour necrosis (Carrswell et al., 1975). Genetic sequence analysis indicated that TNF and cachectin, a serum factor which can cause wasting in chronic disease, are identical molecules (Beutler & Cerami, 1986). Considering antitumour mechanisms only, cytostatic or cytolytic activities are already evident in vitro (Sugarman et al., 1985; Creasey et al., 1987; Lewis et al., 1987). In vivo, TNF-α may indirectly enhance its antitumour effect via incompletely characterised vascular actions (Manda et al., 1987; Watanabe et al., 1988a). Probably, both mechanisms can lead to tumour regression (Shimomura et al., 1988). On the other hand, TNF-α can promote angiogenesis in living tissues even though it inhibits endothelial cell growth in vitro (Frater-Schroeder et al., 1987; Leibovich et al., 1990). These partly contradictory findings finally all imply changes of the nutritive tumour perfusion which in turn dictates therapeutically relevant parameters of the cellular micromilieu (Kallinowski et al., 1989). Thus, different TNF-related flow variations might at least partially explain an inconsistent effectiveness of TNF in the clinical situation (Spriggs et al., 1987). In a step towards further understanding TNF actions we have examined the response of tumour perfusion and proliferation to various TNF doses. Changes in the metabolism of tumour cells (O₂ and glucose uptake, lactate release, extracellular pH) were also followed during TNF treatment. A better knowledge of TNF effects might provide a rationale for combination with other treatment modalities including radiation, chemotherapy and hyperthermia (Selby et al., 1987).

Materials and methods

Animals

Sprague-Dawley rats were bred and maintained in the Department of Applied Physiology (University of Mainz, FRG). The animals were kept in pairs in Maclean cages with dust-free wood bedding. They fed on Altromin 1324 standard diet for rats (Altromin, Lage/Lippe, FRG) and water ad libitum. Room temperature was adjusted to 22 ± 1°C at a relative humidity of 55 ± 5% (12 h light/dark cycles).

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Tumour DS-carcinosarcoma cells were serially passaged in the peritoneal cavity of SD-rats. TNF-sensitivity in *vivo* was tested using DS-carcinosarcoma cells in suspension culture (RPMI 1640 medium, Sigma Chemicals, St Louis, MO, USA; 5% CO₂ in air; 10% fetal calf serum, Gibco BRL, Bethesda, MD, USA). 10⁶ cells were plated into microtitre wells 12 h before various TNF concentrations were added. The specific activity of rhTNF-α was 6.6 x 10⁻¹⁵ U mg protein⁻¹ as determined by Limulus amoebocyte lysate assay. After 48 h, cell density was evaluated. Reduction of growth by 50% was found at 100 ng rhTNF-α ml⁻¹ (L929 cells; approx. 1 ng TNF ml⁻¹). Consequently, DS-carcinosarcoma cells were classified as moderately TNF-sensitive according to Creasey et al. (1987).

Investigations on ascites tumours

Tumours were implanted by i.p. injection of 0.7 ml ascites (approx. 10⁶ cells ml⁻¹). rhTNF-α (1-1000 μg kg⁻¹ body weight in 1 ml PBS) was given every 12 h i.p. beginning 8 h after tumour implantation. Injections of PBS served as control. After a growth period of 6 days, i.e. at the end of the exponential growth period of control tumours, the animals were killed by cervical dislocation and weighed before and after complete removal of the ascites from the peritoneal cavity. The total amount of ascites was obtained as the difference of both measurements. The percentage of cells in the ascitic fluid was determined by microcapillary centrifugation, and the cellular wet weight was calculated. The number of cells per unit volume of ascitic fluid as well as the diameters of tumour cells were assessed using a calibrated reticule in a microscope with 400 fold magnification. The ascites cells were differentiated into intact tumour cells, tumour cell ghosts, erythrocytes, monocytes, lymphocytes and polymorphonuclear leucocytes using four smears per animal (100-200 cells per smear). The smears were stained with Gugol Blue Wright-Giema stain (Gugol Stain Co., Long Island City, NY, USA). The cellular oxygen consumption was measured according to Mueller-Klieser et al. (1986). Glucose and lactate concentration in the ascitic fluid were determined using enzymatic tests (glucoquant glucose; monocell lactate; Boehringer Mannheim, Mannheim, FRG). pH values in the ascitic fluid were measured with a blood gas analyser (type MT 33, Eschweiler, Kiel, FRG).
Investigations on solid tumours

Volume growth curves of DS-carcinosaoma, implanted into the subcutis of the hind foot dorsum (s.c. injection of 0.4 ml ascites; approx. 10^7 cells ml^-1) were obtained by daily measurements of the three orthogonal diameters and subsequent calculation of an ellipsoid. Tumour-bearing animals were treated with daily injections of rhTNF-α (1-1000 µg kg^-1 body weight) into the tail vein starting 24 h after tumour implantation. PBS injections were used as controls.

In a separate series, tumour blood flow was determined using the krypton-85 clearance technique (Vaupel et al., 1977). Here, the animals were anaesthetised with Na-pentobarbital (35-40 mg kg^-1 i.p., Nembutal, Ceva, Paris, France). A catheter in the left carotid artery permitted the continuous monitoring of the mean arterial blood pressure and the applicability of the radioactive tracer. A Geiger-Mueller counting tube was placed over the tumours without compressing the tumour tissue. After i.a. injection of ^85Kr dissolved in 0.9% NaCl solution (injection of 0.1 ml; 37 MBq ml^-1; Amersham Buchler, Braunschweig, FRG) the subsequent washout was recorded. Blood flow was calculated from the washout curves (Vaupel et al., 1977).

In a first step, blood flow changes were evaluated 4 h after single TNF doses (1 µg to 1 mg rhTNF-α kg^-1 i.v.; average tumour size approx. 1.0 g). Then, possible influences of tumour size on flow reduction after high TNF doses (1 mg rhTNF-α kg^-1 i.v.) were investigated using small and larger DS-carcinosaomas (tumour sizes around 0.6 and 1.3 g, respectively). Next, the time course of a possible flow decrease was evaluated. Here, blood flow was measured before and in 30 min intervals up to 4 h after i.v. injection of rhTNF-α (1 mg kg^-1 in 1 ml PBS) or PBS (1 ml kg^-1). In a final series, the effect of repeated treatment with rhTNF-α on tumour blood flow was assessed (mean ttw: 0.6–1.0 g; growth period: 6–8 days). Since tumour blood flow critically depends on tumour size, PBS-treated tumours of similar sizes (tumour age: 7.0 ± 0.3 days, ttw: 0.8 ± 0.1 g) were used as controls.

Effect of rhTNF-α on liver and kidney blood flow

Acute changes of normal organ blood flow were evaluated 4 h after i.v. injection of high TNF doses (1 mg kg^-1). Liver perfusion was determined by ^85Kr clearance (Vaupel et al., 1978). Global kidney perfusion was measured after cannulation of the renal vein by timed collection of the venous outflow (Guenther et al., 1974; Arendshorst et al., 1975). As controls, both untreated and PBS-treated animals in (1 ml kg^-1 bw i.v.) were used.

Statistical evaluation

Descriptive statistical parameters were routinely calculated. Two-tailed *t* test and Mann–Whitney *U* test were used for evaluation of statistically significant differences between various treatment groups. In the following, means ± s.e. are given if not indicated otherwise.

Results

Effect of TNF on ascites tumours

Tumour growth was markedly reduced after treatment with high TNF doses (0.1 and 1.0 mg kg^-1 i.p.). Administration of low TNF doses (1 and 10 µg kg^-1 i.p.) was followed by a slight increase both in the ascitic volume and in the total weight of the ascites cells (Figure 1). Similarly, the absolute number of ascites cells was significantly decreased after treatment with high TNF doses (*P<0.01*, Table 1). After treatment with small TNF doses, cell numbers varied only slightly compared to control counts (Table 1).

The growth changes were concomitant with an immigration of host cells into the ascites and an accumulation of tumour cell debris (Table 1, Figure 2). Hereby, small TNF doses already led to morphological changes and the disruption of tumour cell membranes. The pronounced invasion of micro- and macrophages into the peritoneal cavity at high TNF doses probably contributed to the destruction of tumour cells. Cellular TNF effects were further evidenced by monitoring the diameters of malignant cells. During control conditions, the mean cell diameter is 28.7 ± 0.7 µm. The intraperitoneal application of small TNF doses reduced this value to 26.3 ± 0.9, to 25.3 ± 0.7 and to 23.0 ± 0.9 µm at 1, 10 and 100 µg kg^-1 TNF (*P<0.005*). Higher TNF levels were followed by an even more marked reduction to values of 21.0 ± 0.5 µm (*P<0.0001*).

![Figure 1](image1.png)

**Figure 1** Influence of TNF treatment on total ascites weight (circles) and total cellular wet weight (dots) 6 days after implantation, i.e. at the end of the exponential growth period. TNF doses were administered i.p. every 12 h. PBS application served as control. Numbers in parentheses indicate the number of tumours investigated. Values are means ± s.e. *P<0.05; **P<0.01; ***P<0.001.

| TNF concentration | 0 | 1 | 10 | 100 | 1000 |
|-------------------|---|---|----|-----|------|
| Total cell count  | 8.19 | 8.51 | 7.60 | 4.50 | 1.30 |
| Tumour cells      | 7.01 | 6.25 | 5.07 | 2.72 | 0.74 |
| Tumour cell ghosts| 0.52 | 1.10 | 1.07 | 0.54 | 0.15 |
| Granulocytes      | 0.39 | 0.70 | 1.00 | 0.83 | 0.30 |
| Lymphocytes       | 0.19 | 0.29 | 0.33 | 0.31 | 0.07 |
| Monocytes         | 0.08 | 0.17 | 0.13 | 0.10 | 0.04 |

*rhTNF-α (µg kg^-1 in 1 ml kg^-1 PBS)* was injected every 12 h into the peritoneal cavity. Values are means from 3–4 animals.

![Figure 2](image2.png)

**Figure 2** Proportion of different cell types in the DS-carcinosaoma ascites 6 days after tumour implantation. TNF was given i.p. every 12 h. The values are averages of nine ascites tumours in each group. In each tumour, several hundred cells were differentiated.
Growth changes upon TNF application were paralleled by alterations in oxygen consumption rates (Figure 3). At higher TNF doses, a marked reduction of the $O_2$ uptake was observed whereas the oxygen consumption was increased at low TNF doses. Only traces of glucose were found in the ascitic fluid in all treatment groups indicating an avid glucose consumption under all conditions. The production of lactate somewhat increased at low TNF dose levels. At higher TNF doses the reduction of growth rates was concomitant with a decreased lactate release leading to lower lactate levels in the ascitic fluid (Figure 4). Extracellular pH values generally followed changes of the ascites lactate concentrations (Figure 4).

**Effect of TNF on solid tumours**

The growth rate of solid tumours was markedly retarded upon application of high TNF doses. After application of low TNF doses, tumour volumes increased at faster rates (Figure 5).

These growth changes were concomitant with a modulation of tumour perfusion. Blood flow of s.c. DS-carcinosarcomas after repeated rhTNF-$\alpha$ administration, starting 24 h after tumour implantation, was assessed in order to evaluate a possible alteration of tumour neovascularisation by TNF treatment in vivo. Since the growth stage critically influences tumour perfusion, flow changes were evaluated at comparable tumour sizes (about 0.8 g). This necessarily implies different growth periods. Blood flow of PBS-treated tumours after a growth period of 7 days was $0.87 \pm 0.07$ ml g$^{-1}$ min$^{-1}$. After daily application of lower TNF doses, an increased perfusion was observed (growth period: 6 days). Here, TBF was $1.19 \pm 0.10$ ml g$^{-1}$ min$^{-1}$ after 1 $\mu$g kg$^{-1}$, and $1.17 \pm 0.08$ ml g$^{-1}$ min$^{-1}$ after 10 $\mu$g kg$^{-1}$. Higher TNF doses retarded tumour growth as described above. Considering sizes comparable to that of control tumours, a slight reduction of tumour blood flow was obvious after application of 0.1 mg kg$^{-1}$ (0.73 $\pm 0.04$ ml g$^{-1}$ min$^{-1}$; growth period: 8 days). Mean arterial blood pressures of tumour-bearing animals in all experimental groups were not significantly different (115–125 mmHg). Sizes of tumours treated with highest TNF doses (1 mg kg$^{-1}$ i.v.) were reduced to such an extent (<0.3 g) that flow measurements could not be performed within the observation period. This time span was limited to two weeks by the appearance of TNF-binding antibodies in rats after daily application of rhTNF-$\alpha$ (Keilhauer, BASF/Knoll AG, personal communication).

Modulation of tumour perfusion after single TNF treatment might critically influence sequence and timing of combination therapy. In this study, dose-dependent changes of tumour blood flow were observed after single i.v. administration of rhTNF-$\alpha$. Here, tumour perfusion was reduced 4 h after high TNF doses and elevated at the same time after low TNF dose levels (Figure 6). Mean arterial blood pressure (MABP) of control animals was 113 $\pm$ 3 mmHg. Compared to these values, low TNF doses were followed by an eleva-
ton of perfusion pressure (124 ± 3 mmHg, \( P < 0.05 \)) whereas high TNF doses led to a MAP reduction (101 ± 2 mmHg, \( P < 0.05 \)).

The flow changes were found to be independent of the tumour size (0.5–1.3 g). Both in small and larger DS-carcinosarcomas, tumour blood flow decreased to 30–40% of control values after administration of high TNF doses (1 mg kg\(^{-1}\) i.v.). The time course of flow reductions at these dose levels was evaluated using tumours with wet weights between 0.8 and 1.0 g. PBS administration (1 ml kg\(^{-1}\) i.v.) served as control. In both groups, the first measurements were obtained 15 min after surgical procedures. At this time, without drug administration, the flow values were almost identical (PBS group: 0.81 ± 0.07 ml g\(^{-1}\) min\(^{-1}\); TNF group: 0.84 ± 0.06 ml g\(^{-1}\) min\(^{-1}\)). After measurement of baseline TBF, drug administration was performed. Thirty minutes later, blood flow was reduced by approx. 10% in both groups. Thereafter, a marked reduction of blood flow occurred in TNF-treated tumours reaching 50% of baseline values 90 min after injection of the drug. Between 90 and 240 min after TNF administration no further flow change was observed. In the control group, a maximum TBF drop of 25% was detected during the observation period, the actual flow values being not significantly different from baseline data. Mean arterial blood pressure was 100–120 mmHg in both groups without marked changes during the observation period.

Blood flow of liver and kidney was 1.3 ± 0.1 and 3.8 ± 0.5 ml g\(^{-1}\) min\(^{-1}\), respectively. Four hours after a single injection of high TNF doses (1.0 mg kg\(^{-1}\) i.v.), perfusion rates were not significantly different from control values.

**Side-effects of rhTNF-α treatment**

Significant side-effects were observed only in animals treated with the highest TNF dose used (1.0 mg kg\(^{-1}\)). Here, a haemorrhagic diarrhea developed after single TNF administration. Concomitantly, there was a mild drop of mean arterial blood pressure (100 vs 120 mmHg) and a weight loss of approx. 14%. The slight increase in mean arterial haematocrit (0.48 vs 0.44) has to be taken as evidence for an increased extravasation of plasma due to an enhanced vascular permeability after TNF treatment. The overall lethality observed at the highest TNF dose used was approx. 10% for animals treated with daily intravenous injections and 20% for animals with intraperitoneal TNF administration twice daily.

**Discussion**

DS-carcinosarcoma cells are moderately TNF sensitive *in vitro*. In *vivo*, tumour response depends on TNF dose. A somewhat increased volume growth was found at low TNF concentrations whereas a significant reduction occurred at high TNF doses. Enhanced proliferation rates upon TNF treatment were previously reported for normal cells in *vitro* (Sugarman *et al.*, 1985; Creasey *et al.*, 1987). Lewis *et al.* (1987) demonstrated a dose-dependent growth modulation of tumour cells *in vitro*. TNF further acts as an autocrine growth factor for chronic B-cell malignancies (Cordingley *et al.*, 1988). Based on the ascites data, it can be concluded that the apparent increase in tumour volume at low TNF doses observed in this study is probably due to a pronounced immigration of host cells, predominantly polymorphonuclear leukocytes. Here, TNF could be directly chemotactic (Ming *et al.*, 1987) or could lyse tumour cells leading to the release of chemotactic stimuli. The growth reduction at high TNF concentrations is caused by direct effects on tumour cells (Sugarman *et al.*, 1985; Creasey *et al.*, 1987), action of activated host cells (Shau, 1988), a modulation of tumour-specific immunity (Talmadge *et al.*, 1988) and metabolic alterations of host and tumour cells as demonstrated here.

Changes in haemodynamic and vascular functions further alter the response of solid tumours to TNF treatment. After single administration of low TNF doses, a rise in perfusion pressure led to an increased tumour blood flow. The elevation of blood pressure was probably caused by a rise in cardiac output as a sign of a hypercirculatory state (Tracey *et al.*, 1986). The flow reduction observed after single administration of high TNF doses can be caused by both systemic and tumour-specific mechanisms. A flow chart depicting possible pathways is given in Figure 7. Considering systemic effects, high TNF doses are followed by a reduction of perfusion pressure which could indicate the initiation of a 'septic shock syndrome' (Tracey *et al.*, 1986). In keeping with this syndrome, an increased vascular permeability leads to a systemic haemoconcentration, an elevated blood viscosity and a reduced tumour perfusion. As a further consequence, a reduction in total blood volume and a decreased cardiac output have to be expected. Due to the lack of functioning lymphatics, fluid leakage into the tumour interstitium causes a decreased perfusion pressure and thus, a drop in tumour blood flow. Considering relatively tumour-specific effects, thrombi formation in tumour vessels contributes to the reduction of tumour perfusion at high TNF doses (Nawroth *et al.*, 1986; Shimomura *et al.*, 1988). The stimulation of polymorphonuclear leukocytes and macrophages (Shau, 1988), the binding of activated neutrophils to endothelial cells (Gamble *et al.*, 1985), endothelial cell damage (Movat *et al.*, 1987), and the release of procoagulant activity (Nawroth *et al.*, 1986; Bevilacqua *et al.*, 1986) and of interleukin-1 (Locksley *et al.*, 1987; Kurt-Jones *et al.*, 1987) are pathophysiological mechanisms involved in vascular damage.

After repeated application of low TNF doses, an increased tumour perfusion concomitant with a rise in perfusion pressure was noted. After high TNF doses, tumours shrank to such small sizes that valid perfusion measurements could not be performed. The reduction in tumour volume might be due to sustained vascular damage or due to cellular effects discussed above.

So far, phase I clinical trials with TNF doses ranging from 1–7.5 μg kg\(^{-1}\) i.v. indicate only limited efficacy of TNF monotherapy (Conkling *et al.*, 1988; Herrmann & Mertelsmann, 1989). Dose-limiting side effects in patients include pyrexia and hypotension. In rodents, similar side effects of TNF were observed (Tracey *et al.*, 1986). In our system, single application of rhTNF-α at a dose of 1 mg kg\(^{-1}\) i.v. was lethal for about 10% of the animals. Histological examination after

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**Figure 7** Relevant pathophysiological mechanisms involved in reduction of tumour blood flow after TNF treatment.
TNF treatment suggests that the gastrointestinal tract is more TNF sensitive than other tissues (Remick et al., 1987). Partial tolerance to the gastrointestinal effects of high TNF-α doses developed when TNF application was repeated daily (Patton et al., 1987). In good agreement with these data haemorrhagic diarrhea with weight loss developed only after the first TNF application. Furthermore, blood flow of liver or kidney was not altered after single application of high TNF doses indicating that, at that time, severe vascular damage in these organs is unlikely.

The decrease of nutritive blood flow through malignant tumours leads to ischaemic changes, and to a distinct worsening of the supply of nutrients and of the energy status of these tumours, thus contributing to cell killing (Shine et al., 1989). Furthermore, the changes of the tumour perfusion and of the regional micromilieu might have sustained impact on possible combinations with other non-surgical treatment modalities. The reduction of tumour blood flow markedly influences the intratumour pharmacokinetics of anti proliferative agents and is thus critical for a possible combined treatment. On the other hand, hyperthermia applied either locally (Kallinowski et al., 1988) or as whole body treatment (Haranaka et al., 1987; Watanabe et al., 1988) may benefit from a preceding TNF application. It is most likely that an increased oxygen consumption at low TNF doses or a decreased oxygen supply after treatment with high TNF doses levels worsen tumour tissue oxygenation and thus induce radiation resistance in vivo. In vitro, no significant benefit of a combination of radiation therapy and TNF treatment is evident (Chang & Keng, 1988).

Similar to other lymphokines, there is some specificity of TNF actions in various species (Fransen et al., 1986). We chose rats as our tumour hosts since they permit the investigation of TNF effects in vivo over a wide dose range (Tracey et al., 1986). It has been demonstrated that species specificity can lead to an underestimation of the biological potency of TNF-α from heterologous sources (Kramer et al., 1988). Thus, use of recombinant TNF from rat rather than from human sources might alter quantitative values of the data presented here, but will probably not alter the qualitative mechanisms evaluated.

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