**Advances in Brief**

**Effect of Tumor Necrosis Factor α on Vascular Resistance, Nitric Oxide Production, and Glucose and Oxygen Consumption in Perfused Tissue-isolated Human Melanoma Xenografts**

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**Abstract**

The effect of tumor necrosis factor α (TNF-α) on vascular resistance, nitric oxide production, and consumption of oxygen and glucose was examined in a perfused tissue-isolated tumor model in nude mice. One experimental group was perfused with heparinized Krebs-Henseleit buffer, a second one was perfused with TNF-α (2 μg/ml), and a third one was given an i.v. injection of TNF-α (500 μg/kg) 5 h before perfusion. The vascular resistance increased significantly 5 h after TNF-α injection. The increase in vascular resistance did not seem to be mediated by a decrease in tumor nitric oxide production, as determined by perfusate nitrate/nitrite concentrations, but may be due to aggregation of leukocytes, platelets, and erythrocytes and/or endothelial swelling. There was no difference in oxygen and glucose consumption among the three experimental groups. The oxygen consumption was linearly dependent on the amount of available oxygen in the perfusate, whereas the glucose consumption was constant and independent of the glucose delivery rate. The present experiments provide new insights into physiological and metabolic mechanisms of action of TNF-α for optimization of future treatment schedules involving TNF-α.

**Introduction**

TNF-α (also known as cachectin) was originally proposed as an anticancer drug because of its effect on tumors in mice (1). The clinical effect of TNF-α in doses close to dose-limiting toxicity (mainly hypotension) has, however, been disappointing (2). Isolated limb perfusion in patients with peripheral limb tumors (melanomas and sarcomas) enables regional delivery of anticancer drugs in doses up to 100 times above systemic doses. Recent studies of isolated limb perfusion with high-dose TNF-α, in combination with melphalan and γ-IFN, have shown impressive response rates (2, 3). The mechanism of action of TNF-α in this context has been thoroughly investigated, and it seems that the effect of TNF-α after limb perfusion is not due to the increased dosage of TNF-α in itself (4) but probably to an effect of TNF-α on drug delivery (2). Several studies corroborate this hypothesis; TNF-α has been proposed to increase vascular permeability (5, 6), decrease interstitial fluid pressure (7), and decrease tumor blood flow in high doses (above 100 μg/ml; Ref. 8). Also, TNF-α seems to induce NO release from the systemic circulation, thereby mediating the general vasodilatation associated with septic shock (9–13). Studies of tumor vascular resistance are sparse, although this parameter is important for regulation of tumor blood flow and drug delivery to tumor tissue (14). Previously, determination of vascular resistance from the relationship between perfusion flow and pressure drop across the tumor vessels was performed by perfusion of tissue-isolated tumors in rats (15). A recently developed tissue-isolated tumor model in nude mice allows perfusion of human tumor xenografts transplanted to the ovarian fat pad (16). In the present investigation, we used this model to test the hypothesis that TNF-α changes tumor vascular resistance by regulating NO production, and that the resulting change in blood flow will alter the consumption of glucose and oxygen. The melanoma line (S-MEL) used here was established from a patient tumor, which responded to isolated limb perfusion with TNF-α, melphalan, and IFN-γ, thus providing an excellent model for the study of physiological effects of TNF-α.

**Materials and Methods**

**Animals and Tumors.** For all experiments, 8–10-week old NCr/Sed-nu/nu female athymic mice bred in the Edwin L. Steele Laboratory’s animal facility were used. Before each surgical procedure, the mice were anesthetized with ketamine/xylazine (100/10 mg/kg BW s.c.). The human melanoma cell line S-MEL was kindly supplied by Dr. Douglas Fraker (NIH, Bethesda, MD) and was originally isolated from a peripheral melanoma of a patient responding to isolated limb perfusion with TNF-α, melphalan, and IFN-γ. Tumor tissue was serially minced and transplanted to the flank of immune-deficient mice to maintain a stock of donor tissue. For tissue-isolated tumors, a slurry of minced tumor tissue in Hanks' balanced salt solution was injected into the left ovarian fat pad of the mice as described in detail by Kristjansen et al. (16). The fat pad with the injected tumor slurry was wrapped in stretched Parafilm, and the pedicle containing the ovarian vessels and the tumor tissue was positioned in the s.c. space in the flank of the mouse. The Parafilm bag was changed every week during tumor growth. When the...
tumors reached a size of approximately 8 mm in diameter, the perfusion was performed.

Perfusion. The mouse was anesthetized with ketamine/xyazine (100/10 mg/kg BW) and placed on a heating pad adjusted to 37°C. After opening of the abdominal wall by electrocauterization, the viscera were drawn away from the abdominal cavity so that the retroperitoneum was exposed. All vascular branches to and from the aorta and vena cava were ligated, except for those feeding and draining the tumor. Finally, a PE-10 cannula was inserted in the aorta, and a PE-50 cannula was inserted in the vena cava; then the aorta and vena cava were ligated with a cranial and caudal suture. During the whole procedure, the surgical field was continuously irrigated with 37°C 0.9% NaCl. In most clinical studies, TNF-α perfusion is combined with hyperthermia, but because the present experiments were set up to isolate the effect of TNF-α, we chose 37°C perfusion for these experiments. Perfusion was established by connection of silicone tubing to the aortic cannula. The perfusate was a Krebs-Henseleit solution composed of 118 mm NaCl, 4.7 mm KCl, 1.2 mm KH₂PO₄, 1.2 mm MgSO₄, 2.55 mm CaCl₂, 11.1 mm glucose, 7000 units/ml sodium heparin, and 2.5% BSA (A7030; Sigma Chemical Co., St. Louis, MO). The buffer was oxygenated, and pH was equilibrated in a gas exchanger, in which the perfusate was led through 16 feet of Silastic tubing (Dow-Corning, Midland, MI), while equilibrating with a warm humidified mixture of 95% O₂ and 5% CO₂. A pressure transducer was introduced in the perfusion system close to the arterial inlet.

Experimental Setup. The tumors were split into three experimental groups. The control group was perfused with modified Krebs-Henseleit buffer as described above. The second group was perfused with Krebs-Henseleit buffer containing 2 μg/ml TNF-α, and the third group was given a tail vein injection of 500 μg/ml TNF-α and perfused with Krebs-Henseleit buffer 5 h later. The perfusate TNF-α concentration of 2 μg/ml was chosen because this concentration is comparable to the concentration used for clinical perfusions, and a comparable peak serum concentration can be reached in mice by systemic injection of 500 μg/kg BW (2).

Perfusions were initiated with a low perfusion rate of approximately 60 μl/min and stepwise increased to approximately 150 μl/min during 1 h. At each perfusion level, the pressure drop across the tumor vessels was determined at steady-state, the outlet flow rate was determined by collection of outlet perfusate for 2 min and weighing of the collected samples, and samples of perfusate were collected in glass capillary tubes from the inlet and outlet line for determination of oxygen partial pressure. When a maximal perfusion rate was reached, as determined by visible ballooning of the tumor vein, the flow rate was lowered to previous levels to check the reproducibility of the flow/pressure relationship. At the end of the experiment, tumors were given an arterial injection of Evan’s blue to check the distribution of perfusate in the tumor tissue. The tumors were cut in half, and tumors with less than 75% blue staining of the tumor tissue were excluded from the data analysis. Small nonstained areas were allowed, because central necrotic areas were expected in almost all tumors of this age and size.

Vascular Resistance. For each tumor, the pressure drop from the pressure transducer to the venous outlet of the tumor was plotted as a function of inlet perfusate flow rate, and the total resistance was calculated from the slope of the linear regression function. The resistance of the tubing leading from the pressure transducer to the arterial inlet was subtracted in each experiment as described by Kristjansen et al. (17).

NO Production. Inlet and outlet perfusate samples were analyzed for nitrate and nitrite concentration by the Griess method (18). Two slightly different assays were used, but for each experiment, the standard curves for varying concentrations of nitrate and nitrite were linear (r² > 0.99), and analysis of five different perfusate dilutions showed strict linearity of the relationship between sample dilution and light absorbance in both assays (r² = 0.99). In the first assay, 50 μl of each perfusate sample was pipetted into a 96-well microtiter plate with 50 μl of nitrate reductase (0.5 units/ml in 1.2 mM ammonium formate and 0.5 mM HEPES, pH = 7.25). The plate was incubated at 37°C for 6 h; at this time, more than 90% of the nitrate was reduced to nitrite. After 10 min centrifugation at 1000 × g, 50 μl of 1% sulfanilamide in 30% acetic acid and 50 μl of 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in 60% acetic acid was added. The plate was read at 540 nm in a Bio-Rad microplate reader. The second assay was a commercially available nitrate/nitrite assay kit (Cayman Chemical, Ann Arbor, MI) using the same Griess method. Similar results were obtained from the same sample when tested in the two assays.

Glucose Consumption. Measurement of glucose concentration in perfusate samples was performed using an enzymatic/colorimetric glucose kit (Sigma Diagnostics).

Oxygen Consumption. Samples were collected in glass capillary tubes and immediately analyzed in an ABL 300 gas analyzer (Radiometer, Copenhagen, Denmark). Inlet perfusate was sampled from a 3-way stopcock inserted in the perfusate tubing right before the arterial cannula. Outlet perfusate was sampled directly from the venous outlet. The oxygen consumption rate was calculated from the arterio-venous difference in partial pressure and converted to milliliters O₂ per min according to the following equation:

\[ V_{O2} = q \Delta P O_2 \times S \]

where \( V_{O2} \) is oxygen consumption and \( q \) is the perfusate flow rate, using an \( S \) (solubility constant) value of 0.003 ml O₂ x 100 ml⁻¹ x mmHg⁻¹ (19).

Statistical Analysis. Vascular resistance in the two TNF-α treated groups (pretreatment and perfusion) were compared to the control group by a two-tailed Mann-Whitney U test. The glucose and oxygen consumption and NO production of each tumor was determined as the mean of all measurements from this particular tumor, and mean values in the pretreatment and perfusion groups were similarly compared to controls by a two-tailed Mann-Whitney U test.

Results
Twenty-four perfusions were performed. In two tumors, there was no linearity between pressure and flow rate. Also, in one tumor, the nonperfused area as determined by Evan’s blue perfusion was less than 60%, and in one tumor, the arterial contribution to the tumor vascularization was accidentally derived from the left epigastric artery. These were excluded from...
the study. Thus, 20 perfusions were included in the data analysis. Table 1 shows the tumor weight and time of tumor growth in each of the three groups.

The median vascular resistance was higher in both experimental groups (68 and 120 mmHg·min·g⁻¹·ml⁻¹) as compared to controls (25 mmHg·min·g⁻¹·ml⁻¹; Fig. 1). This increase was significant when TNF-α was injected 5 h before perfusion (P < 0.05). There was no correlation between tumor size and vascular resistance (r² = 0.15).

NO production as a function of perfusion time is shown in Fig. 2. There was no consistent change in NO production with time, and no significant difference between controls and TNF-α-treated tumors. The median NO production in controls and TNF-α-perfused and -pretreated tumors was 2.7, 1.8, and 2.1 nmol/min/g tumor wet weight, respectively.

There was a linear relationship between the rate of oxygen delivery and oxygen consumption rate (r² = 0.96) as demonstrated in Fig. 3. Furthermore, the fraction of delivered oxygen extracted by the tumor tissue did not change with time, and there was no difference in oxygen extraction fraction between the TNF-α-treated tumors (medians, 66 and 60%) and the control group (63%; Fig. 2).

The glucose consumption was constant with time and independent of the amount of available glucose (r² = 0.24). Median glucose consumption in the control group was 0.41 μmol/min/g and did not differ significantly from the glucose consumption in the TNF-α-perfused tumors (0.66 μmol/min/g) or the TNF-α-pretreated tumors (0.39 μmol/min/g).

H&E-stained tissue sections from tumors fixed in formaldehyde 5 h after TNF-α injection showed areas of coagulative necrosis with erythrostasis and intravascular cell aggregates involving mainly polymorphonuclear cells (Fig. 4).

**Discussion**

The demonstrated increase in vascular resistance 5 h after i.v. injection of TNF-α indicates that either the 5-h time point and/or the presence of blood constituents as leukocytes (for example) are necessary for induction of this vascular effect. Fukumura et al. (20) have shown an increase in leukocyte adhesion to tumor vessels 4–5 h after TNF-α injection. Blood constituents are not present in the tumor tissue during TNF-α treatment of the TNF-α-perfused group, and although not significant, an increase in the vascular resistance in these tumors was found (Fig. 1), indicating that leukocyte-endothelial interaction may not be necessary for eliciting TNF-αs vascular effect. Pathological studies of patient tumor biopsies after TNF-α treatment have demonstrated considerable swelling of endothelial cells (21), significant intravascular recruitment of polymorphonuclear cells (21), and platelet aggregation secondary to endothelial damage (22). The endothelial swelling can substantially decrease the lumen of tumor vessels, and this phenomenon in combination with thrombocyte aggregation, concomitant with or secondary to leukocyte-endothelial interaction, may very well be the cause of the increase in vascular resistance. Swelling of the endothelium may also increase the vascular permeability.

A NO-mediated effect of TNF-α on the tumor vascular tone comparable to what occurs in the systemic (11, 13), muscular (9), pulmonary (23), and cerebral circulation (24) is also a possible explanation of the increase in vascular resistance. In fact, NO seems to be able to regulate vascular resistance in tumors (25, 26). However, in the present experiments and in studies of patient serum after isolated limb perfusion with TNF-α, it was not possible to demonstrate a TNF-α-induced release of NO. Although the applied Griess method may be too insensitive to detect tracer amounts of NO, a more probable explanation is that tumor cells seem to suppress TNF-α-induced endothelial NO production (27). Consequently, the induction of NO synthesis by TNF-α in the systemic circulation (9–13) may not occur in tumor vasculature.

Increased vascular resistance probably decreases tumor blood flow, as others have previously detected in s.c. rat tumors by laser Doppler technique, using comparable concentrations of TNF-α (8, 28). The decrease in blood flow was found to occur between 1 h (28, 29) and 4 h (8) after TNF-α injection, corroborating the present increase in vascular resistance after 5 h.

The implication of an increase in vascular resistance for drug delivery to the tumor tissue is complex. Because it has been shown that a selective increase in tumor blood flow leads to an increase in tumor uptake of low molecular weight substances (30), it is most likely that the decrease in tumor blood flow

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**Table 1** Tumor characteristics before perfusion

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>TNF-α perfusion</th>
<th>TNF-α pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>7</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Weight (mg)</td>
<td>(170-275)</td>
<td>(175-374)</td>
<td>(184-255)</td>
</tr>
<tr>
<td>Time after transplantation (days)</td>
<td>42</td>
<td>36</td>
<td>32</td>
</tr>
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There was no statistically significant difference between the control group and any of the TNF-α-treated groups.

**Fig. 1.** Median vascular resistance in the three experimental groups. Bars, interquartile range. *, P < 0.05

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4 H. Dvorak, personal communication.
5 D. Lienard and F. Lejeune, personal communication.
occurring in response to increased vascular resistance after TNF-α treatment will lead to a decrease in uptake of melphalan (for example). However, this effect may be counteracted by increased diffusion of melphalan across the vascular wall because of the previously demonstrated increase in permeability after TNF-α treatment (5, 6), although there was no TNF-α induced increase in fluid leakage in the present experiments. Alternatively, a decrease in blood flow after melphalan injection may decrease the clearance of drug from the tumor.

The oxygen consumption of perfused S-MEL xenografts was linearly dependent on the amount of oxygen available, as has been demonstrated previously in perfused rodent (31) and human (17) tumor lines. With the amount of oxygen available in the present experiments, we did not reach a level of oxygen tension where the tumor oxygen consumption became saturated (Fig. 3). There was no change in oxygen consumption after TNF-α treatment, in contrast to what has been found previously in the cerebral circulation of rats by Tureen (24), who found a reduced oxygen uptake and increased cerebrospinal fluid lactate concentration 2–6 h after intracisternal injection.

With the inlet glucose concentration applied to the present experiments (200 mg/dl or 11.1 mmol/liter), the glucose consumption was relatively constant at a level of 0.4–0.7 μmol/min/g during all of the perfusion experiments. These values are directly comparable to the values obtained by Kristjansen et al. (17) in small cell lung cancer xenografts and slightly lower than the maximum glucose consumption rate obtained by Gullino et al. (32) in Walker Carcinoma 256 in rats (11 mmol/h/100 g corresponding to 1.8 μmol/min/g). In contrast to the data from the Walker Carcinoma 256 in rats (32), we found no increase in glucose consumption with increasing glucose availability. Consequently, the glucose consumption in S-MEL tumors may be saturated at an inlet concentration of 11.1 mmol/liter.

The lack of difference in both oxygen and glucose consumption rates between the TNF-α pretreated and the control group is surprising, considering the decrease in bioenergetic status (31) detected by Kluge et al. (28) 1–3 h after injection of TNF-α. However, their doses of 1–2 mg/kg were 2–4 times higher than the dose level used in the present study (500 μg/kg). Furthermore, the data by Kluge et al. (28) were

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**Fig. 2** NO production (A–C) and fraction of available oxygen extracted in the tumor tissue (D–F) during perfusion. Control tumors were perfused with Krebs-Henseleit buffer with heparin and albumin (A and D); TNF-α-perfused tumors were perfused with Krebs-Henseleit buffer containing 2 μg/ml TNF-α (B and E); and pretreated mice were given an i.v. injection of 500 μg/kg TNF-α 5 h before perfusion with Krebs-Henseleit buffer (C and F). Each symbol indicates data from one separate perfusion experiment. There was no significant difference in NO production or oxygen consumption between any of the TNF-α-treated groups and the control group.

**Fig. 3** Rate of oxygen consumption as a function of oxygen delivery rate in the control group. There was a close linear correlation ($r^2 = 0.96$).
consumption of oxygen and glucose occurred within the first 5 tumor specific. In tissue-isolated tumors from the human breast degree of fluid leakage in perfused tumors seems to be mainly a-perfused, and TNF-α-pretreated group, respectively. The de-

perfusate recoveries were 66, 63, and 66% in the control, TNF-

the arterial inlet. In fact, there was considerable fluid leakage performed assuming that the venous efflux of perfusate equals TNF-α. The calculations leading to the presented data were all control tumors must have been below this detection limit. We found increased areas of necrosis in histological sec-

tions of pretreated tumors. Because no significant change in between different groups of tumors.

We found increased areas of necrosis in histological sections of pretreated tumors. Because no significant change in consumption of oxygen and glucose occurred within the first 5 h after i.v. injection of TNF-α, these necrotic areas were probably not abundant enough to change the nutrient consumption significantly. We have estimated the lowest difference in necrotic fraction resulting in a significant decrease in nutrient consumption to be approximately 30% in our assays, and consequently the difference in necrotic fraction between the pretreated and control tumors must have been below this detection limit.

The perfused, tissue-isolated xenograft model in nude mice allowed us to study the vascular resistance, NO production, and nutrient consumption rate in a human melanoma treated with TNF-α. The calculations leading to the presented data were all performed assuming that the venous efflux of perfusate equals the arterial inlet. In fact, there was considerable fluid leakage from all tumors included in the present experiments; the median perfusate recoveries were 66, 63, and 66% in the control, TNF-

α-perfused, and TNF-α-pretreated group, respectively. The de-

gree of fluid leakage in perfused tumors seems to be mainly tumor specific. In tissue-isolated tumors from the human breast carcinoma ZR75-1, we found a perfusate recovery of 82%, whereas in the colon adenocarcinoma LS174T, it was 90–95%, which is comparable to the recovery in the small cell lung cancer lines CPH SCLC 54A and 54B (17). Other authors have reported a recovery of only 58% in tissue-isolated Walker 256 sarcomas in rats (33). In other perfusion studies, the perfusate recovery is not reported (25, 34, 35). Ohkouchi et al. (33) assumed that most of the fluid was leaking from the venous outlet, and the leakage is thus of less importance for the obtained pressure and perfusate concentration data. Fluid leakage from the tumor vein was occasionally observed in our experiments, and the amount of fluid oozing out from the tumor surface to the Parafilm bag was usually minimal. We are aware, though, that the less than 100% perfusate recovery may influence our results, but because the fluid leakage was of equal magnitude in the three experimental groups, the data should still be valid for comparisons between the three groups. Furthermore, the presented values are directly comparable to data from studies on vascular resistance (17, 25, 34) and glucose/oxygen consumption of tissue-isolated small cell lung cancer xenografts (17).

In conclusion, perfused tissue-isolated melanoma xenografts treated with TNF-α in concentrations comparable to what is used for perfusion of patient extremities induced a significantly increased tumor vascular resistance after 5 h. The increase in vascular resistance may be due to thrombocyte/erythrocyte aggregation, leukocyte-endothelial adhesion, and/or substantial endothelial swelling, and does not seem to be related to an induction of tumor NO synthesis. Glucose and oxygen consumption did not change significantly 5 h after TNF-α treatment. It would be worthwhile to measure the delivery of melphalan to the tumor tissue in the presence and absence of TNF-α. For this purpose, the perfused tissue-isolated tumor provides an excellent model.

Acknowledgments

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References


obtained from repeated measures of the same tumors before and after treatment, whereas we were limited to comparisons be-

between different groups of tumors.

Fig. 4 H&E-stained sections of perfused S-MEL tumor tissue 5 h after injection of 500 μg/kg TNF-α i.v. The vessels are plugged by cell aggregates (mainly polymorphonuclear cells). A, longitudinal cut through vascular network. The framed area in the bottom is magnified (×2) in the larger frame. The arrow shows a granulocyte plugging the vessel lumen. B, transverse cut through a larger vessel in the tumor periphery. The arrow indicates the vessel wall.
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