



# **Understanding and improving TNF-based cancer therapy**

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# **Understanding and improving TNF-based cancer therapy**

*Inzicht in en verbetering van  
TNF tumor therapie*

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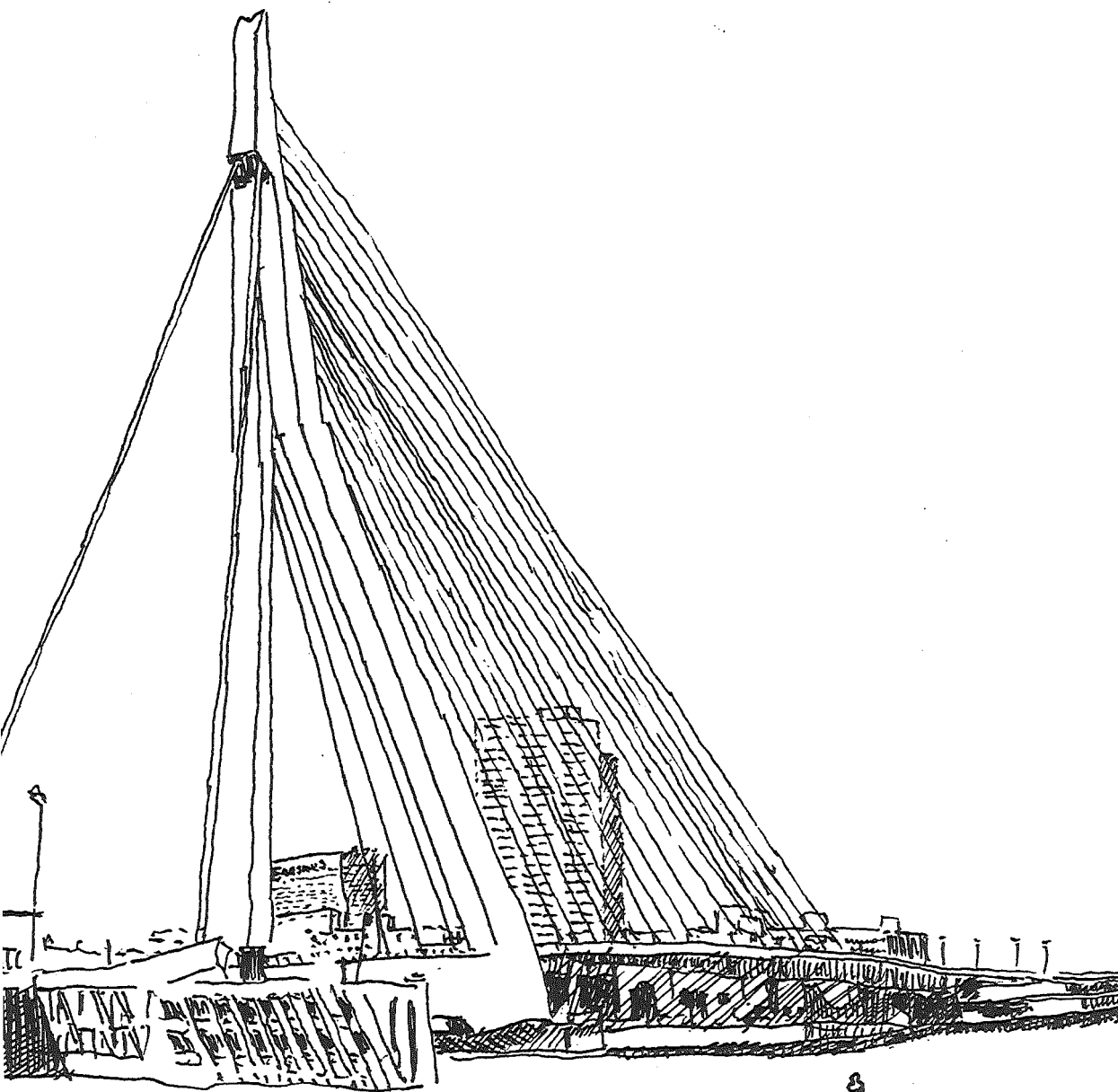






# 1

## INTRODUCTION



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## **SARCOMA**

Sarcomas are relatively rare mesenchymal tumors with an estimated incidence of 8100 in soft tissue and 2500 in bones and joints per year in the USA<sup>1</sup>, and approximately 5000 cases per year in Europe. Just over 50% of these patients will eventually die of the disease.

In general sarcomas do not originate from preexisting benign lesions unlike typical carcinoma progression. In that respect sarcomas are more similar to the de novo high-grade carcinomas. Gaining a thorough understanding of these disease entities is extremely difficult due to the low incidence and the great diversity in histopathologic presentation, anatomic site, and biologic behavior. It is nevertheless clear that most soft tissue sarcomas are curable when diagnosed at an early stage<sup>2</sup>. The major predictive parameters of regional control are size and location of the primary lesion, pathological grading, previous treatments and quality of surgical margins. When diagnosed at the time of extensive local or metastatic disease, soft tissue sarcomas are rarely curable. In spite of their diversity in histopathologic characteristics, the different types of sarcoma have many clinical and pathologic features in common. They are characterized by local invasiveness and the pattern of metastasis of most sarcomas is hematogenous, lymph node metastasis being uncommon. The clinical behavior of most types of sarcoma is also similar, determined more by anatomic localization, grade and size than by histologic markings.

## **MANAGEMENT OF SOFT TISSUE SARCOMA**

The fundamental treatment for all soft tissue sarcomas of the extremity and trunk is surgical excision<sup>3,4</sup>. Controversy exists on how extensive that surgical excision should be and on whether it should be preceded or succeeded by adjuvant therapy<sup>5</sup>. Radiation therapy is generally used as a surgical adjuvant. External beam radiation has been the most commonly applied treatment, although there is an increasing interest in adjuvant brachytherapy<sup>6,7</sup>. Radiation therapy can also be delivered in preoperative fashion<sup>8</sup>. Radiation alone is rarely used for unresectable tumors or for a medically inoperable patient.

To explore the role of adjuvant chemotherapy many randomized studies with different regimens of chemotherapeutics have been conducted<sup>9-13</sup>. A large number of these trials showed no detectable difference in outcome; some showed an advantage in disease-free survival, but no difference in overall survival<sup>14-17</sup>. Doxorubicin and Ifosfamide are the two most active agents<sup>18-20</sup>. As could be expected, these adjuvant therapies were associated with myelosuppression and neutropenic fever and toxic deaths from infection or heart failure were occasionally encountered. To overcome these problems different routes of administration have been pursued. Improvements in the efficacy of medical treatments for soft tissue sarcomas may derive from old strategies by aiming to counteract the side effects of standard chemotherapy regimes and from new, less toxic, anticancer drugs.

## **ISOLATED LIMB PERFUSION; COMBINATION THERAPY**

The technique of isolated limb perfusion (ILP) was first described by Creech *et al.* in 1958 at the Tulane University of New Orleans. It was used for the treatment of a patient with multiple in-transit metastasis of a melanoma, who refused amputation<sup>21</sup>. A complete response and thus limb salvage was achieved using melphalan in an extra corporeal circulation system. With this technique isolation of the blood circuit of a limb is achieved by clamping the major artery and vein, by ligating the collateral vessels, and by applying a tourniquet around the base of the limb to compress the remaining minor vessels in the muscles, subcutaneous tissues, and skin. After cannulation of the vessels, the isolated extremity is provided with an artificial circulation by means of an oxygenated extra corporeal circuit, into which cytostatic drugs can be administered. This way high local concentrations of the drug can be established, with an opportunity to apply local heat to improve the local uptake of the drug by tumor cells<sup>22,23</sup>. After the perfusion a washout procedure is performed to ensure no systemic exposure to the active substance used.

The advantage of this treatment modality is found in the fact that high local drug concentrations can be established while systemic side effects of high toxic doses can be avoided. Regarding soft tissue sarcoma, numerous combinations of cytostatic drugs have been utilized<sup>24</sup>. Isolated limb perfusion with cytostatic drugs

alone uniformly failed because these large soft tissue sarcomas showed a lack of response. This is in contrast to the usually small in transit-melanoma metastases for which an ILP with melphalan alone results in complete response rates in approximately 50% of patients, with an additional 25-30% of patients showing partial responses<sup>25,26</sup>. The application of Tumor Necrosis Factor (TNF) in combination with melphalan in the setting of ILP would completely change the situation for soft tissue sarcomas. Eggermont *et al.* used a combination regimen consisting of TNF, interferon gamma (IFN) and melphalan, which resulted in major tumor responses in 82 to 87% of all patients<sup>27,28</sup>. Moreover this rendered these large sarcomas resectable in most cases and limb salvage could be achieved in 82 to 84%. Although in melanoma the administration of melphalan alone is effective to reach adequate response rates, in soft tissue sarcoma TNF needs to be added too in order to achieve this. The synergizing role between TNF and IFN did not prove to be of benefit in these series, which eventuated in the continuation of the treatment with TNF and melphalan<sup>29</sup>.

## TUMOR NECROSIS FACTOR

TNF belongs to the family of cytokines, and is predominantly produced by activated cells of the immune system (monocytes, macrophages, natural killer cells, B-cells and T-cells). Many years went by since the observation of spontaneous regression of cancer in patients with a concurrent bacterial infection in 1891 by Coley<sup>30</sup>. It was not until 1975 that a factor causing hemorrhagic necrosis in experimental tumors, later named Tumor Necrosis Factor- $\alpha$ , was discovered by Carswell<sup>31</sup> and Old<sup>32</sup>. It took almost a century (in 1984) before the protein was isolated and cDNA was cloned, by Pennica and co-workers<sup>33</sup>. It turned out to be a 17kDa glycoprotein, which consists of 3 molecules. This tri-mer is the biologically active form of TNF and binds to the membrane-bound TNF receptor complex. Two well-defined receptors belong to this complex: a 55 kDa TNF-R1 and a 75 kDa TNF-R2 receptor<sup>34</sup>. Biological activity mediated by these two receptors is different, leading to cytotoxicity, apoptosis and fibroblast proliferation by TNF-R1, while T- and B-cell proliferation is predominantly mediated by activation of TNF-R2<sup>35</sup>.

TNF is released systemically in small amounts when the healthy immune system is triggered, leading to inflammation, fever, and an acute phase reaction thus protecting the individual from possible pathogens. Systemic TNF in large quantities may activate neutrophils, modify the anticoagulant properties of endothelial cells and induce the release of other inflammatory cytokines such as IL-6, IL-8 or IL-1 $\beta$  resulting in cardiovascular collapse, sepsis or shock. Locally administered high doses of TNF (e.g. by means of ILP) however mainly target the tumor associated vasculature, resulting in upregulation of endothelial adhesion molecules, a marked hyper permeability, platelet aggregation and hemorrhagic necrosis due to selective occlusion of the neo-vasculature.

The vasodilatation of tumor vessels and changes in perfusion patterns and vascular permeability enhances significantly drug uptake of cytostatic agents into the tumor<sup>36,37</sup> resulting in synergistic antitumor effects. Melphalan, an alkylating cytostatic that will interfere with mitosis and cell division in replicating cells irrespective of cycle stage, is an ideal cytotoxic agent for the ILP setting.

## **ANGIOGENESIS; BALANCE IN CYTOKINES**

The interactions between malignant cells and the surrounding tissue are complex and involve a large variety of mechanisms and factors. Tumors exert an effect on tumor stroma and potential on newly formed tumor vasculature using a complex web of signals that they can secrete consisting of interleukins, CAM's (cellular adhesion molecules), cytokines, integrines, proteases and receptors. Many studies by Folkman for instance have produced evidence that tumor growth is dependent on the capability to already form neovasculature in an early stadium<sup>38</sup>. Without this capability a tumor will not be able to grow over 0.2 mm in diameter in mice lung metastases, till a maximum of 2 mm diameter in case of avascular chondrosarcoma in a rat. The lethal power of a tumor, which is determined by the pace of growth and metastasis velocity, is also dependent on neovascularisation. Successful angiogenesis requires an increased permeability of the basal membrane and a migration of endothelium cells in one defined direction. Also formation of peri-endothelial stroma needs to take place. A fast expanding list of pro- and antiangiogenic factors secreted by tumors can serve as a proof that tumors themselves are responsible for this process of angiogenesis in their

environment<sup>39, 40</sup>. An extensive group of cytokines belongs to this list<sup>41</sup> and these may play a crucial role in the future of combined anticancer strategies.

## ENDOTHELIAL MONOCYTE ACTIVATING POLYPEPTIDE II

Infusing TNF into mice-bearing methylcholantrene A-induced (MethA) fibrosarcomas causes rapid induction of intravascular coagulation at the tumor site, resulting in a major drop in tumor blood flow. In this model that is used to study host-tumor interactions, the MethA tumor shows a rapid induction of thrombohemorrhage resulting in hemorrhagic necrosis and subsequent tumor regression after treatment with TNF. Since this feature did not appear in other mice tumors it was hypothesized that tumor-derived factors produced by this MethA tumor made this tumor more likely to respond to TNF with thrombotic events<sup>42</sup>. A number of polypeptides have been isolated from its supernatant, including a 44-kDa polypeptide endothelial monocyte activating polypeptide I (EMAP I) and the 22-kDa EMAP II<sup>43</sup>, the latter being the most potent of all polypeptides found. EMAP II was named after just one of its many effects since it influences also endothelial cells, neutrophils, and monocytes<sup>44</sup>.

It has also been shown that EMAP II enhances the induction of the procoagulant tissue factor (TF) on endothelial cells<sup>44</sup>, and is able to increase the cellular receptors for TNF on endothelial cells, which is likely to enhance the predisposition of tumors to undergo thrombosis and hemorrhagic necrosis once challenged with TNF<sup>45</sup>. Wu *et al.* demonstrated that this constitutive over-expression of EMAP II in a TNF-resistant melanoma cell line by retroviral-mediated transfer of EMAP II cDNA rendered this tumor sensitive to the effects of systemic TNF<sup>46,47</sup>. This feature sparked our recent interest in this new cytokine.

## CLINICAL IMPLICATIONS

The characteristic of EMAP II to sensitize tumors for the impact of TNF implies a promising improvement of TNF treatment in patients with disseminated disease. Moreover, recent studies also demonstrate an antiangiogenic effect of EMAP II by inhibiting corneal neovascularisation in mice<sup>48</sup> and by preventing vessel outgrowth in a rat aortic ring assay<sup>49</sup>.

This data suggests an important role of EMAP II in the treatment against cancer. Not only will EMAP II enhance the effect that TNF exerts on certain tumor types, it can also play a role in maintaining dormancy of tumor outgrowth by preventing vessel formation.

If the level of EMAP II expression in a tumor can be determined before TNF treatment is started, a response can be predicted and maybe even improved by upregulating the EMAP II expression. In the group of patients with a low EMAP II expressing tumor the perfusion could be preceded by a therapy to upregulate EMAP II, for instance by gene therapy as has been demonstrated in mice with a recombinant Vaccinia virus encoding the human EMAP II gene<sup>45</sup>.

EMAP II has the potential to become a potent anticancer agent since it can contribute in several important ways: diminishing local toxicity of TNF making a lower dose sufficient, improving anticancer effects, preventing formation of metastasis by the antiangiogenic effect and predicting outcome of TNF based therapy. Resistance to EMAP II is unlikely as its effects are mainly at endothelial cell level, which is a constantly renewing vessel lining. Tumors will be destructed after EMAP II-mediated sensitization to the vasotoxic effects of TNF in combination with chemotoxic agents.

## **AIM OF THE THESIS**

The aim of this thesis is to gain greater insight into how TNF produces its effect in current cancer treatment with the overall goal of improving TNF-based cancer treatment. TNF response as well as toxicity takes place at the vascular level where cytokines can have effect on the endothelial cells. We describe matters concerning this interaction as well as related experiments that have been conducted in our laboratory.

**Chapter 2** gives the results of a clinical trial in which we investigated if TNF based perfusion has additional toxic effect in humans, and if so, how much damage this evoked in the treated patient. In a unique setting we had the opportunity to compare two groups of patients. One group received a hepatic perfusion with TNF, the other group received a similar perfusion but without TNF. Clinical and pharmacologic results are presented of this retrospective study.



Since it is known that TNF exerts its effect on the tumor vasculature, one could speculate on the response rates in highly vascularized tumors. In **chapter 3** data of limb perfusions with TNF and melphalan in highly vascularized Stewart Treves angiosarcoma is presented. In a retrospective study clinical records of 10 patients with Stewart Treves angiosarcoma were reviewed.

Since radiotherapy influences vascularisation in the radiated field, we conducted a study in a group of patients who received radiotherapy for their primary tumor and we are now experiencing a relapse of their disease. In **chapter 4** we demonstrate that it is possible to perform perfusion in radiated tissue, and clinical data and response rates are presented. These clinical observations raise questions about the antitumor effect of TNF.

In **chapter 5** we demonstrate that TNF is the agent responsible for the increased intratumoral melphalan concentration in isolated perfusions with TNF and melphalan in a liver perfusion model performed in rats. When TNF was left out of the perfusate a 4- to 6-fold lower intratumoral melphalan concentration was measured.

On the cellular level improvement of the TNF response is pursued in **chapter 6**. EMAP II increases cellular receptors for TNF on endothelial cells, this feature enables EMAP II to confer TNF sensitivity of TNF-resistant tumors. In our lab we retrovirally transduced a rat soft tissue sarcoma with the EMAP II gene in order to create a tumor with an up regulated EMAP II expression. This tumor was injected into rats that received a TNF-based ILP after 10 days.

**Chapter 7** describes experiments with the chemokine Interferon-inducible Protein-10, IP-10, which has antiangiogenic properties *in vivo*. In our study we evaluate the effects of retroviral IP-10 gene delivery and protein expression on human melanoma xenografts in nude mice. We expect that inhibition of tumor angiogenesis reduces the growth rate of the tumor.

In **chapter 8** contemporary experiments and current controversies regarding EMAP II are reviewed. Considering our own experiments we describe the predictive value of EMAP II in patients with sarcoma and the problems that must be solved to achieve this. EMAP II is likely to be of importance in developing new approaches aimed at improving the efficacy of the use of TNF as an anticancer treatment. Finally, in **chapter 9**, the conclusions are summarized.

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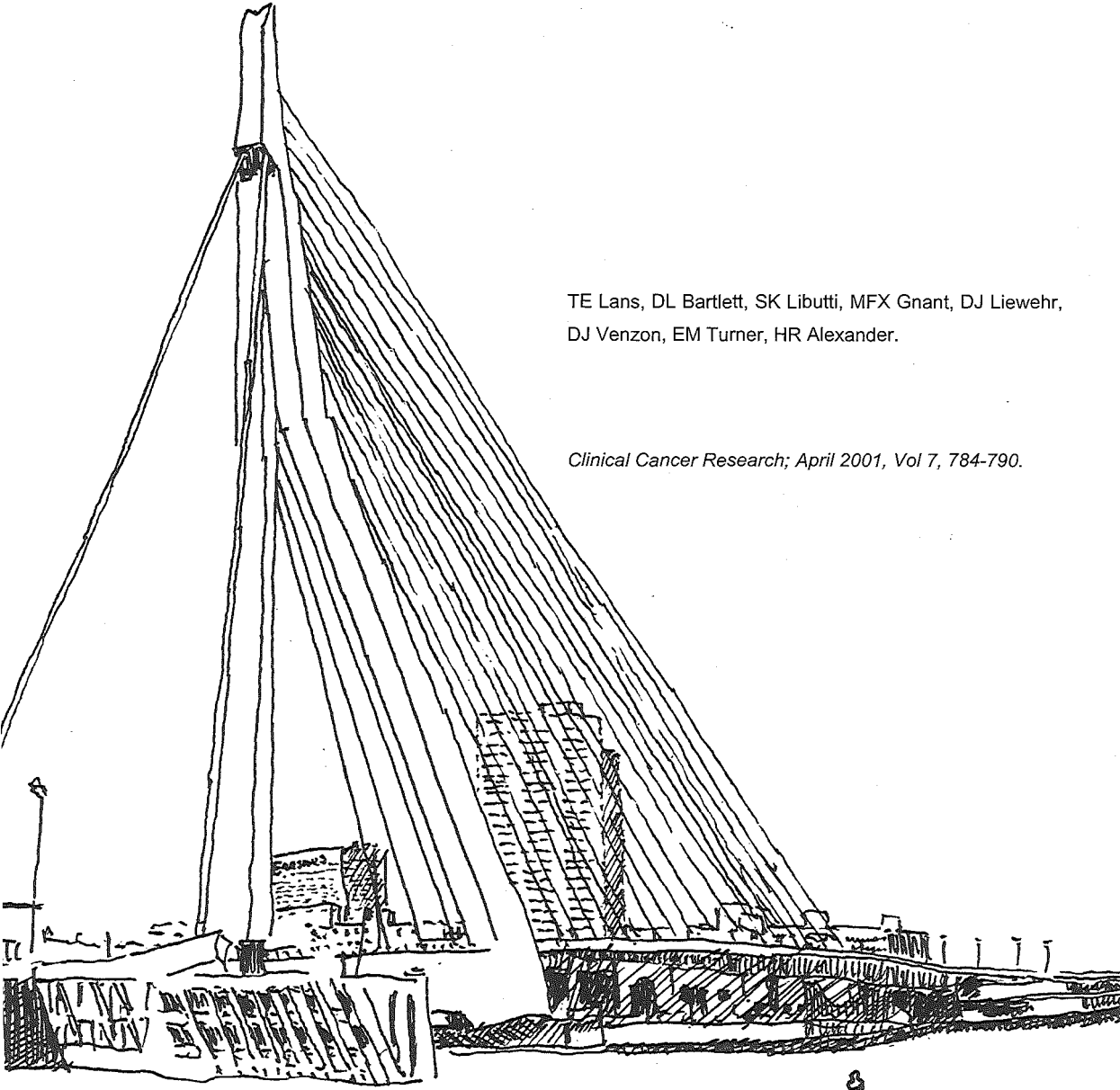
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# 2

## ROLE OF TUMOR NECROSIS FACTOR ON TOXICITY AND CYTOKINE PRODUCTION FOLLOWING ISOLATED HEPATIC PERFUSION

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DJ Venzon, EM Turner, HR Alexander.

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## ABSTRACT

**Introduction:** Isolated limb (ILP) or liver perfusion (IHP) with tumor necrosis factor (TNF) and melphalan results in regression of advanced cancers in the majority of treated patients. However, the contribution of TNF to the efficacy of isolation perfusion with melphalan has not been conclusively demonstrated in random assignment trials. Furthermore, TNF is an inflammatory cytokine and may be associated with significant systemic and regional toxicity. This study was conducted to characterize the toxicity and secondary cytokine production attributable to TNF by comparing these parameters in patients undergoing IHP using melphalan with or without TNF.

**Methods:** Thirty-two patients with unresectable colorectal cancer confined to the liver underwent a 60-minute hyperthermic IHP using 1.5 mg/kg melphalan alone ( $n=17$ ) or with 1.0 mg of TNF ( $n=15$ ). Post-IHP parameters of hepatic and systemic toxicity and cytokine levels (TNF, IL-6 and IL-8) in perfusate and serum were measured.

**Results:** Levels of IL-6 and IL-8 in perfusate at the end of the 60 minute IHP were significantly higher in TNF treated patients. Peak systemic IL-6 and IL-8 levels post-IHP were also significantly higher in TNF treated compared to non-TNF treated patients by 28 and 268 fold, respectively. The peak levels of these cytokines were associated with significantly lower systolic blood pressure and higher heart rate and mean pulmonary artery blood pressure in TNF treated patients during the first 48 h post-IHP. Serum bilirubin levels were significantly higher and platelets lower in TNF treated compared to non-TNF treated patients. However, elevations in AST, ALT, and alkaline phosphatase were not significantly different between groups and returned towards baseline within 1 week after IHP.

**Conclusions:** Addition of TNF to melphalan during IHP results in significant differences in post-IHP production of IL-6 and IL-8 with associated changes in mean arterial blood pressure and greater regional toxicity as reflected in higher levels of serum bilirubin. However, these measurable differences were transient and did not appear to be of major clinical consequence. The benefit of TNF in isolation perfusion should be demonstrated in random assignment trials prior to its routine use.

## INTRODUCTION

TNF, a secreted 17 kD protein with a range of physiological activities, was originally identified in the sera of bacillus Calmette-Guérin (BCG)-primed endotoxin-treated mice as a circulating factor resulting in remarkable hemorrhagic necrosis of tumors when administered to tumor bearing mice<sup>1</sup>. After it became available in recombinant form a number of clinical trials were performed with the expectation that it could produce similar results in patients with advanced cancer. However, it was shown that humans are exceedingly sensitive to the toxic effects of TNF and at the maximum tolerated systemic doses there was no meaningful antitumor activity<sup>2</sup>. Furthermore, there was contemporaneous evidence that TNF was the primary endogenous mediator of acute inflammatory conditions such as endotoxic shock and it appeared that inhibiting its effects might have broader clinical application than the protein itself<sup>3,4</sup>.

However, in 1992 Drs. Lienard and Lejeune reported their initial experience using TNF in isolated limb perfusion in combination with melphalan, interferon, and hyperthermia for in transit extremity melanoma or high grade unresectable extremity sarcoma<sup>5</sup>. The complete response rate in 29 evaluable patients was 90% and rekindled considerable interest in the use of TNF with melphalan in isolation perfusion. Subsequent reports have confirmed complete response rates of greater than 78% after ILP for patients with in transit extremity melanoma, a limb salvage rate of 84% for patients with unresectable high grade extremity sarcoma, and an overall response rate of 74% in patients with unresectable hepatic malignancies treated with isolated hepatic perfusion<sup>6-8</sup>. However, no reports have conclusively established a benefit of adding TNF to melphalan compared to melphalan alone in isolation perfusion. A single small prospective random assignment trial of ILP with melphalan, TNF, and IFN, compared with melphalan alone, showed no difference in overall or complete response rates between groups<sup>9</sup>. During ILP with TNF a perfusate leak of less than 5% into the systemic circulation is associated with significant hemodynamic effects, primarily hypotension<sup>10,11</sup>.

Recently various institutions have reported results using TNF and melphalan in IHP, with large variations in antitumor response as well as toxicity<sup>8,12-14</sup>. This



study characterizes the profile of inflammatory cytokine production (IL-6 and IL-8) and regional and systemic toxicities observed in a cohort of patients with metastatic unresectable adenocarcinoma of the colon confined to liver who underwent IHP with or without TNF and had otherwise identical treatment parameters. Moreover, because all patients had complete vascular isolation of the liver with no measurable leak of perfusate, differences between groups are not confounded by systemic exposure to the TNF or melphalan.

## **PATIENTS AND METHODS**

### *Patient Population*

Between March 1996 and November 1998, 32 patients were treated with a 60 minute hyperthermic IHP. In 15 patients the combination of TNF (1.0 mg) and melphalan (1.5 mg/kg) was given and in 17 patients melphalan alone (1.5 mg/kg) was used. The protocols were approved by the Institutional Review Board and the Cancer Therapy Evaluation Program (for the use of TNF) of the National Cancer Institute. Patients were treated on 2 consecutive Phase II protocols using identical IHP treatment parameters; the latter without TNF. All patients had unresectable biopsy-proven bilobar metastatic colorectal cancer confined to the liver. Standard staging studies including computed tomography scan of the chest, abdomen and pelvis, magnetic resonance imaging of the liver and, when clinically indicated, brain imaging or bone scan were performed. Eligibility criteria included Eastern Cooperative Oncology Group performance status of 0 or 1, a serum bilirubin <2.0 mg/dL, a platelet count >150,000 /mL, and a serum creatinine <1.5 mg/dL. All patients were evaluated 6 weeks after treatment and at 3 to 4 month intervals thereafter. Responses were scored by comparing gadolinium-enhanced T-1 weighted images on MRI scans during follow-up with pre-treatment images. All lesions were measured and a partial response was defined as a reduction of 50% or greater in the product of the perpendicular diameters of the lesions on MRI for a period of at least one month without the development of new lesions or progression of existing lesions.

*IHP*

The technique of IHP was performed as described previously<sup>8,15</sup>. Briefly, via a laparotomy the liver is extensively mobilized by dividing the falciform ligament and the right and left triangular ligaments. The duodenum is mobilized and reflected medially to expose the inferior vena cava (IVC). The right lobe of the liver is reflected anteriorly and medially and the IVC from the level of the renal veins to the diaphragm is completely dissected from the retroperitoneum. The porta hepatis structures are completely dissected and skeletonized and a cholecystectomy is performed. A 2 cm segment of the gastroduodenal artery (GDA) is dissected and serves as the arterial cannulation site during IHP. The portal vein and common bile duct are dissected from the head of the pancreas to the inferior border of the liver. All lymph node-bearing tissues around the porta hepatis structures are resected. A saphenous vein and left axillary cutdown are performed.

The patient is systemically heparinized with 200 units/kg and after 5 minutes a cannula is inserted into the saphenous vein and advanced into the IVC just below the renal veins. A second venous cannula is inserted into the axillary vein and both are connected to a veno-veno bypass circuit. The IVC is occluded above the renal veins and infra hepatic IVC blood flow is shunted to the axillary vein using a centrifugal pump. A short segment of infra hepatic IVC is isolated between vascular occluding clamps and a 20-24 French cannula is inserted through a venotomy and positioned behind the retrohepatic IVC just beneath the hepatic veins. This cannula is connected to the venous outflow line of the extra corporeal bypass circuit. A cannula is positioned in the portal vein and connected to the veno-veno bypass circuit to shunt portal vein blood flow systemically. The GDA is ligated, the common hepatic artery is occluded and a 3-4 mm GDA cannula is positioned at the orifice of the common hepatic artery. Finally, the supra hepatic IVC is cross-clamped just below the diaphragm and isolated hepatic perfusion is initiated.

The extra corporeal bypass circuit consists of a roller pump, membrane oxygenator, and heat exchanger. The perfusate consists of 700 ml of balanced salt solution primed with 300 ml of packed red blood cells and 2000 units of heparin. Arterial and venous perfusate blood gases are obtained at regular

intervals and arterial perfusate pH is maintained between 7.2 and 7.3 with sodium bicarbonate. Hepatic parenchymal temperature probes are placed and perfusate warmed using a Hemotherm water heater model #4 (Cincinnati SubZero Products, Cincinnati, Ohio). Flow rates are adjusted upward while monitoring for a stable reservoir volume and acceptable line pressures. There is typically rapid and uniform heating of the liver to target temperatures of 39.5 to 40°C. Melphalan, 1.5 mg/kg with or without TNF, 1.0 mg, are added sequentially to the arterial inflow line of the perfusion circuit at time 0 and IHP continued for 60 minutes. At the conclusion of IHP, the liver is flushed through the arterial inflow cannula with 1500 ml of crystalloid followed by 1500 ml of colloid and through the portal vein with 1 liter of normal saline. After decannulation and repair of the IVC and portal venotomies, normal physiological blood flow is reestablished promptly to the liver.

### *Drugs*

Melphalan was obtained from Glaxo-Wellcome, Research Triangle Park, NC and recombinant human TNF from Knoll Pharmaceuticals, Whippany, NJ.

### *Blood sampling and Hemodynamic Monitoring*

Perfusate was sampled at time 0, 15, 30 and 60 min during IHP. Blood samples were obtained at regular intervals beginning before and after IHP for clinical laboratory parameters; serum samples for cytokine determination were collected at the same intervals as perfusate samples during IHP and at regular intervals for 24 hours after IHP. Hemodynamic, pulmonary and other systemic endpoints were measured at intervals as clinically indicated but no less than every 8 hours after IHP for up to 60 hours.

### *Cytokine assays*

All blood and perfusate samples for cytokine determination were immediately refrigerated and serum separated and frozen until analyzed. TNF, IL-6 and IL-8 in perfusate and serum were measured by an enzyme linked immunosorbent assay (ELISA) using the Quantikine Immunoassay kits (R&D Systems Inc.). In these assays monoclonal antibodies raised against the cytokines were used according to procedures similar to those described earlier. Results for IL-6 and IL-8 are

expressed as picogram per milliliter; the lower limit of detection was 0.0312 ng/mL. Results for TNF are expressed as microgram per milliliter; the lower detection limit for the assay was 0.016 ng/mL.

### *Statistical analysis*

Values are expressed as mean  $\pm$  standard error of the mean (SEM). For the regional toxicity variables, a Wilcoxon rank sum test was performed on the data collected one day before IHP to determine whether the two groups were the same at baseline. AST, ALT alkaline phosphatase and systemic vascular resistance were log10 transformed and serum bilirubin levels were square root transformed to reduce skewness in distributions. A repeated measured analysis of variance (ANOVA) was then performed on each variable starting with time 0. To account for correlation between repeated measurements, we tested autoregressive, heterogeneous autoregressive, and Toeplitz covariance matrices for each variable and chose the best fitting matrix according to Schwarz's Information Criteria<sup>16</sup>. For variables in which the interaction term was significant, the p values from the combined test of the treatment effects over time is reported. The assumption of normally distributed residuals was satisfied. P values  $p < 0.05$  were considered to be statistically significant.

## RESULTS

Patient demographics and tumor characteristics are shown in Table 1.

**Table 1 - Patient demographics and tumor characteristics**

	Total	TNF	No TNF
N	32	15	17
Male:Female	22:10	9:6	13:4
Age (years)			
Mean	54.5 $\pm$ 2.2	51.1 $\pm$ 3.5	57.4 $\pm$ 2.6
Range	25-74	25-72	33-74
Previous treatment (%)	23 (72%)	10 (67%)	13 (76%)
Median no. metastases (range)	--	6 (2-38)	6 (1-19)
Size largest lesion (cm)*	--	9.1 $\pm$ 4.1	8.2 $\pm$ 4.7
Median PHR	--	25 (10-70)	20 (5-65)
Pre-operative CEA level (mean $\pm$ SD in ng/mL)*	--	603 $\pm$ 722	1479 $\pm$ 3316

\* $p > 0.3$ , unpaired t-test

PHR= percent hepatic replacement

There was a similar mean age, a roughly 2:1 male/female ratio and the percent of patients who had been previously treated was comparable between groups. In

addition, the tumor burden, as reflected by number of liver metastases, size of largest lesion, percent hepatic replacement, and pre-operative CEA level were similar between groups. The overall radiographic response rate was 72% and not different between those receiving and not receiving TNF (Table 1). Fifteen patients were treated with a 60-minute hyperthermic IHP using 1.5 mg/kg melphalan; seventeen patients also received 1 mg of TNF. IHP perfusion parameters are listed in Table 2.

**Table 2: IHP parameters**

	Total	TNF	No TNF
<i>n</i>	32	15	17
Melphalan dose			
Mean (mg)	109.3±2.0	110±3.7	108.5±2.1
Range (mg)	90-130	90-130	92-122
Duration (min)	60	60	60
Flow rate			
Mean (ml/min)	882.3±24.6	833.7±27.0	925.3±37.3
Range (ml/min)	600-1350	600-1050	650-1350
pH perfusate			
Mean	7.21±0.03	7.18±0.04	7.23±0.05
Range	6.92-7.64	6.92-7.51	6.96-7.64
Mean central hepatic T (°C)	40.0±0.04	39.9±0.04	40.0±0.06
Mean perfusion pressure (mm Hg)	162.3±5.3	160.1±9.0	164.2±6.3
Perfusion pressure Range (mm Hg)	103-240	103-240	120-202
Mean change reservoir volume (ml)	64.8±13.4	96.7±22.5	36.6±12.7

All parameters were comparable between the two groups except for flow rate that was higher, but not significantly ( $p = 0.06$ ) in the no TNF group. The reason for this is not clear and not due to any bias or attempt to achieve higher flow rates in patients not receiving TNF. Because there was no difference in the pH of the perfusate between groups, we presume that the differences in flow rates were physiologically inconsequential and did not influence hepatic toxicity. Using a radiolabeled I-131 albumin continuous leak monitoring system, there was no identifiable leakage of perfusate into the circulation in any patient. Because of the unique vascular anatomy of the liver it our experience that complete vascular isolation with no leak of perfusate can be routinely achieved during IHP<sup>8</sup>. This is further supported by the fact that the mean change in reservoir volume in each group was minimal, less than 100 mL, indicating no leak of systemic blood into the perfusion circuit. Small changes in reservoir volume that occur during IHP most likely reflect changes in passive filling or emptying of the hepatic vascular bed.

A number of hemodynamic parameters were compared between groups during the first 48 hours after IHP. There were no significant differences in systemic vascular resistance, cardiac output or temperature. However, patients who received TNF had a significantly higher mean heart rate, lower systolic blood pressure and higher peak mean pulmonary artery blood pressure compared to melphalan alone (Figure 1). However these differences were gone by 48 to 60 hours and did not appear to be of major clinical consequence. Hypotension was treated with fluid resuscitation and no patient in the TNF group required circulatory support with cardiopressor agents.

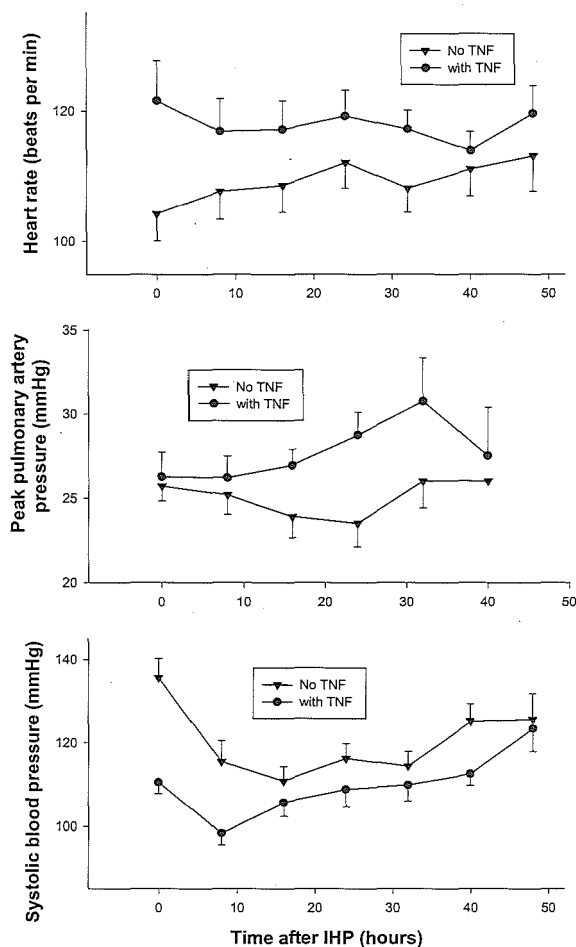


Figure 1 Mean heart rate, peak pulmonary artery pressure, and systolic blood pressure in patients following IHP with ( $n=15$ ) or without ( $n=17$ ) TNF. In patients receiving TNF heart rate ( $p<0.03$ ) and peak mean pulmonary artery pressure ( $p=0.033$ ) were higher and systolic blood pressure lower ( $p<0.01$  at 0 and 8 hours and  $p<0.05$  at 40 hours) in patients receiving TNF compared to those that did not.

In patients receiving TNF initial mean perfusate levels determined 15 minutes after beginning IHP were  $637\pm333$   $\mu\text{g/mL}$  and decreased to  $520\pm185$   $\mu\text{g/mL}$  at the end of IHP consistent with our previously reported findings<sup>17</sup>. Perfusate IL-6 and IL-8 levels are shown in Figure 2.

Baseline levels of each cytokine were comparable between groups and significantly higher after IHP in the TNF group compared to melphalan alone. In

patients receiving TNF, systemic levels were consistently measurable only at 1.5 hours after IHP ( $7.5 \pm 20$   $\mu\text{g/mL}$ , range: 1- 83) whereas in those not receiving TNF there was no detectable systemic TNF in any sample at any time point after IHP. Systemic IL-6 and IL-8 levels are shown in Table 3.

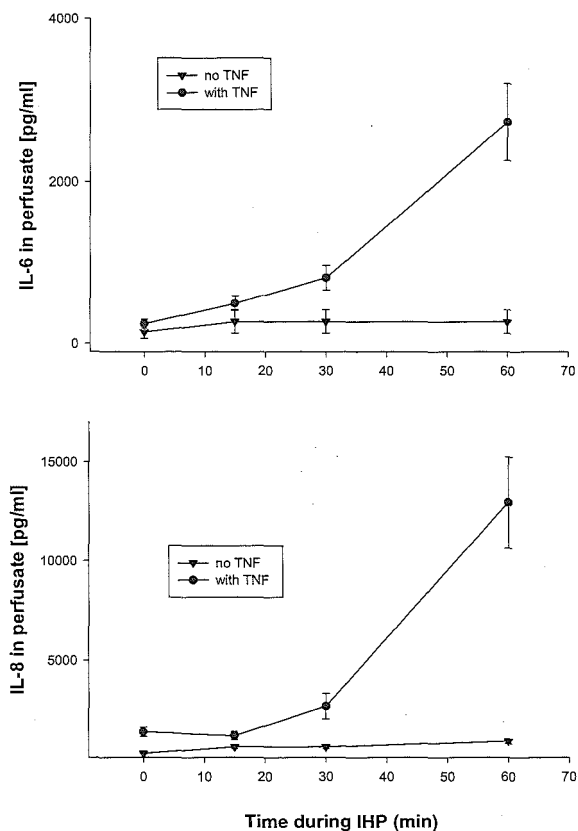


Figure 2 Mean IL-6 and IL-8 levels in perfusate during IHP in patients treated with ( $n=15$ ) or without ( $n=17$ ) TNF. Perfusate cytokine levels were significantly higher in those treated with TNF ( $p<0.0001$ ).



**Table 3 - Systemic cytokine concentrations starting immediately after IHP**

	0 hours after IHP*	4-6 hours	24 hours
a. Serum IL-6 (pg/ml)			
With TNF	312±43	23543±4144	478±95
Range	60-670	4661-60033	15-1312
No TNF	287±31	841±335	264±77
Range	44-451	96-4763	31-1035
b. Serum IL-8 (pg/ml)			
With TNF	250±73	7333±1113	53±14
Range	48-1190	672-12492	9-221
No TNF	65±13	274±42	52±7
Range	23-195	71-580	22-112

\*Time starting just after IHP when native blood flow to liver had been established.

Time 0 represents the time immediately after IHP just after the native hepatic vascular blood flow had been re-established. In patients receiving TNF, serum levels of IL-6 increased rapidly to a peak value of 23543±4144 pg/mL and IL-8 increased to 7333±1113 pg/mL between 4 to 6 hours after IHP and returned towards baseline within 24 hours. The peak systemic levels of cytokines were significantly correlated (Spearman rank correlation coefficient  $r=0.68$ ,  $p=0.006$ ). The peak values were significantly greater than those observed with melphalan alone and occurred coincident with the maximum changes in systolic blood pressure.

All patients had significant elevations in AST and ALT that returned towards baseline within the first week after IHP as previously reported for patients undergoing IHP with melphalan and TNF<sup>8</sup>. However, after IHP there is a transient marked elevation in serum bilirubin in patients receiving TNF that was not observed in those receiving melphalan alone (Figure 3).

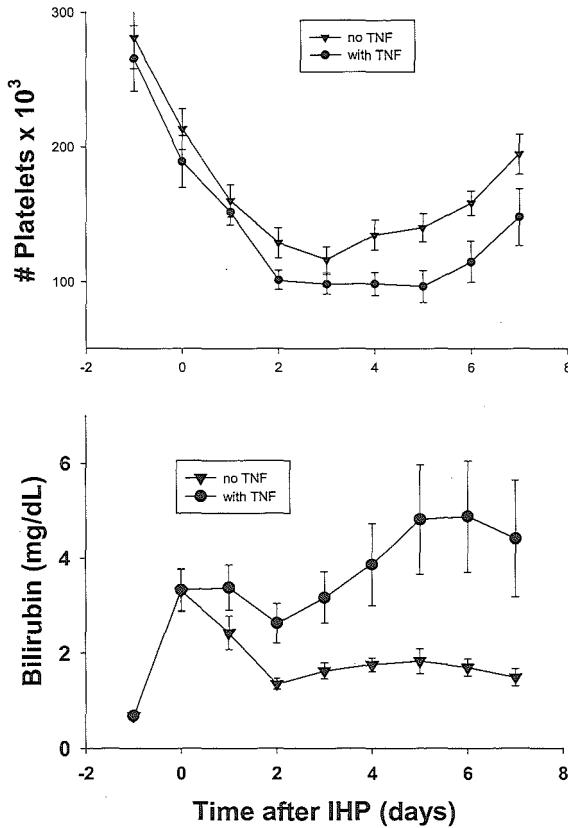


Figure 3 Mean platelet count and serum bilirubin levels in patients following IHP with ( $n=15$ ) or without ( $n = 17$ ) TNF. Platelet levels were significantly lower in patients treated with TNF on days 4 through 7 ( $p<0.05$ ) and serum bilirubin levels averaged over days 1 through 7 were significantly higher ( $p=0.017$ ) in patients receiving TNF compared to those that did not. There were no differences in baseline values between groups

Platelets decreased after perfusion with a significant difference observed between the groups after day 3. However, in both groups the level of thrombocytopenia did not require any intervention and started to normalize after one week. Mean pre-IHP creatinine levels were significantly different ( $p=0.002$ ) for reasons that are not clear. Creatinine levels were not different between groups after IHP following correction for differences in baseline levels. There were no differences in post-IHP alkaline phosphatase, white blood cell count or prothrombin time.

## DISCUSSION

The data presented in this study show that in patients undergoing IHP with virtually all major treatment variables equal and without any measurable leak of perfusate into the systemic circulation, the addition of TNF to melphalan in the perfusate is associated with significant regional and systemic toxicity compared to those receiving melphalan alone. Interestingly, the significantly lower mean systolic blood pressure in patients treated with TNF was coincident with significantly higher peak levels of IL-6 and IL-8 occurring 3 to 4 hours after IHP. Other studies have reported systemic toxicities associated with the use of TNF in ILP or IHP that were due largely, if not exclusively, to systemic exposure of TNF secondary to perfusate leak during treatment<sup>10,14,18,19</sup>. Thom *et al.*<sup>10</sup> observed that patients experiencing a perfusate leak of greater than 1% during ILP with TNF and melphalan had significantly greater systemic toxicity associated with higher circulating levels of IL-6 and IL-8. Activation of the fibrinolytic system as reflected by significant increases in circulating tissue plasminogen activator activity has been observed following systemic TNF leak in ILP patients<sup>19</sup>. Similarly, increases in circulating levels of the soluble TNF receptor are observed following systemic TNF leak during ILP in amounts proportional to the serum TNF up to levels of 1.5 ng/ml. TNF induced hypotension appears to occur when the capacity of released soluble TNF receptors to neutralize circulating TNF levels is exceeded. Under conditions in which serum TNF levels are considerably greater than 1.5 ng/ml following ILP, refractory hypotension with associated hematological, hepatic and pulmonary toxicities can occur<sup>20,21</sup>. Lidner *et al.*<sup>18</sup> reported considerable toxicity when performing IHP with melphalan and 30 to 200 µg of TNF which resulted in a 22% mortality that was most likely due to systemic exposure of TNF and melphalan. These previous data highlight the importance of controlling perfusate leak of TNF during isolation perfusion. The data in this study are consistent with a previous report of 6 patients undergoing IHP using melphalan with or without TNF<sup>22</sup>. In that study data from 3 patients who received 0.4 mg of TNF and 1 mg/kg of melphalan and 3 patients who received melphalan alone showed that systemic TNF levels peaked soon after IHP in the former group and were not detectable in the latter. In addition, perfusate levels of IL-6 increased during IHP only in those receiving TNF and systemic levels were significantly higher between

2 and 9 hours after IHP compared to those who received melphalan alone. The current study demonstrates that with complete vascular isolation of the liver and virtually all other treatment parameters equal, the production of secondary mediators in the liver after IHP with TNF and melphalan may result in subsequent transient hemodynamic alterations not observed with melphalan alone.

IL-6 and IL-8 are inflammatory cytokines with known effects on the cardiovascular system<sup>23-26</sup>; whether they are the direct cause of the relatively greater degree of hypotension following IHP in this study is not known. Of note, the differences in blood pressure between those who did or did not receive TNF were clearly transient and did not appear to be a major clinical consequence. No patient treated with TNF required cardiopressor support because of hypotension. Elevated plasma levels of IL-6 have been reported in patients with sepsis and shown to correlate with heart rate, hypotension, thrombocytopenia, and death<sup>23; 24,27</sup>. The mean peak systemic IL-6 level in patients treated with TNF and IHP was over 23,000 pg/mL (maximum > 60,000 pg/mL) which is higher than the reported mean levels of patients in septic shock of approximately 10,000 pg/mL<sup>23,24</sup>. Similarly, elevated plasma IL-8 levels have been reported in patients with sepsis and shown to correlate with hypotension and death<sup>25,28</sup>. The mean peak systemic levels in TNF treated IHP patients (>7,000 pg/mL) is about 2-fold greater than plasma levels in patients with lethal septic shock. The correlation between systemic IL-6 and IL-8 levels in this study have also been observed in septic patients<sup>28</sup>. The fact that the high circulating levels of IL-6 and IL-8 observed in this study were associated with only transient hemodynamic changes is most likely due to the limited and controlled exposure to TNF during IHP compared to septic individuals with uncontrolled bacteremia and ongoing cytokine production. Our data indicate that hepatic synthesis and release of pro-inflammatory cytokines may be a major source of endogenous mediators during sepsis and that TNF can stimulate significant hepatic pro-inflammatory cytokine production clinically.

Although there are limited clinical data, during ILP for in transit melanoma of the extremity, the use of TNF alone is associated with minimal regional toxicity<sup>29</sup>. However, because of the metabolic nature of the liver the use of TNF alone in IHP has significant toxic effects. In a previous phase I study of escalating dose TNF administered with 0.2 mg interferon-gamma via IHP, dose-limiting

coagulopathy was encountered at 2.0 mg of TNF and did not result in any meaningful antitumor activity<sup>12</sup>. A subsequent phase I study of alternating dose escalations of TNF and melphalan was conducted<sup>15</sup> and dose-limiting melphalan toxicity was observed at 2.0 mg/kg (renal and liver) and TNF toxicity at 1.5 mg (coagulopathy). The maximum safe-tolerated dose of 1.0 mg of TNF when used with melphalan in IHP determined in our clinical trials is considerably less than to 3-4 mg dose used in ILP at various institutions and highlights the different tissue tolerances of the protein. In a previous phase II trial of IHP using TNF and melphalan significant transient elevations in hepatic transaminases and bilirubin levels were observed<sup>8</sup>. Hepatic failure from veno-occlusive disease (VOD) is a known complication of high dose chemotherapy with a grave prognosis manifested initially by progressive elevation in serum bilirubin followed by ascites and progressive liver failure<sup>30</sup>. Although serum bilirubin were significantly higher in patients treated with TNF in this series, there were no other clinical or laboratory indications of hepatic VOD in any patient treated in this cohort. Liver biopsies were not routinely obtained so the pathological changes responsible for the elevations in bilirubin are not known. However, because of the transient nature of the hyperbilirubinemia, it was presumed to be related more to cholestasis than VOD.

In summary, the data in this study provide additional insights of the pathophysiology of TNF toxicity during isolation perfusion and suggest that the liver may be a significant source of proinflammatory cytokine production secondary to TNF. As in animal models of endotoxin or TNF-induced shock<sup>31,32</sup>, strategies to neutralize the IL-6 or IL-8 may ameliorate the hemodynamic or other consequences of TNF when perfused directly into the liver or when systemic leak occurs during ILP with this protein. The data indicate that if there is clinical benefit in terms of efficacy that TNF can be safely administered in isolation perfusion.

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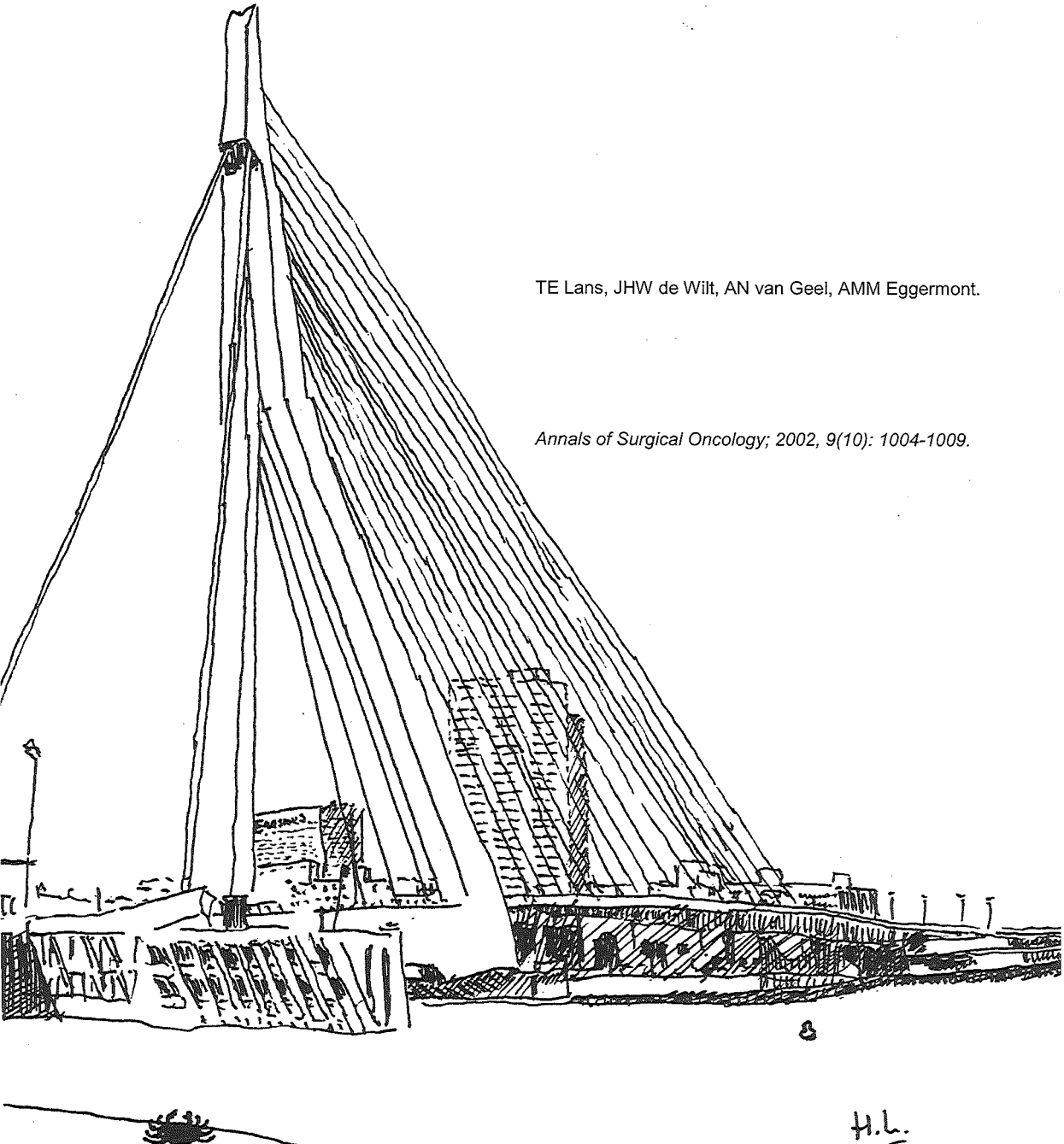
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# 3

## ISOLATED LIMB PERFUSION WITH TNF AND MELPHALAN FOR NONRESECTABLE STEWART TREVES LYMPHANGIOSARCOMA



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## ABSTRACT

**Introduction:** Cutaneous Stewart Treves lymphangiosarcoma represent a rare group of tumors. They are characterized by a high grade of vascularisation and by localization in an extremity with iatrogenic or congenital lymphedema. The multifocality and the localization makes these tumors eligible for treatment with isolated limb perfusion (ILP). ILP with melphalan and Tumor Necrose Factor (TNF) is a safe and highly effective biochemotherapy procedure that can achieve limb salvage in 80% or more of all patients with non-resectable extremity soft tissue sarcoma or melanoma.

**Methods:** Here we present the outcome of 16 ILPs with TNF plus melphalan in 10 patients with the rare condition of multifocal Stewart Treves lymphangiosarcoma of the extremities. All patients would have been candidates for exarticulation of the extremity.

**Results:** After ILP we observed an 87% overall response rate, 1 patient had a mixed response and 1 patient failed to respond to the therapy. In 9 perfusions (56%) a complete response (CR) was achieved and 5 perfusions (31%) resulted in a partial response (PR). Limb salvage was achieved in 8 patients (80%) with a mean follow-up duration of 34.8 (3-115+) months. Regional toxicity was limited and systemic toxicity minimal to moderate with no toxic deaths.

**Conclusions:** Our results demonstrate that a combined treatment with melphalan and TNF can eventuate in good clinical response rates in patients with extensive disease who were candidates for amputation. This implies that with the ILP we have a new treatment option in the management of Stewart Treves angiosarcoma.

## INTRODUCTION

Sarcomas are malignant tumors arising from skeletal and extra skeletal connective tissues. The cellular elements of these anatomic structures are diverse and as a consequence so is the natural history of both bone and soft tissue sarcomas. Soft tissue sarcomas (STS) have been classified histogenetically according to their morphological resemblance to normal congenate tissues. The best predictor of STS development is the grade, a measure of differentiation and aggressivity. In Europe, the incidence of STS is approximately 5000 cases per year. Little over 50% of these new patients will eventually die of the disease. Most STS do not have a clearly defined etiology and a great diversity of histopathologic presentation, anatomic site, and biologic behavior exists.

Angiosarcomas constitute one of the many categories of STS. The generic term 'angiosarcoma' includes the histologic subtypes of sarcoma originating from either lymphatic or capillary endothelium, comprising lymphangiosarcoma and haemangiosarcoma respectively. Together these lesions account for approximately 4% of all STS. In this report we limit ourselves to one histological type of highly vascularised sarcoma with lymphedema of the extremity as the common etiologic cause.

Lymphedema has long been an established factor in the multifocal development of lymphangiosarcoma. In 1906, Löwenstein was the first to report this entity in a patient with chronic post-traumatic upper extremity lymphedema. It was not until Stewart and Treves in 1948 described the syndrome of multifocal lymphangiosarcoma<sup>1</sup>, which carries their name, that this feature became more widely recognized. They described six cases of lymphangiosarcoma in chronically lymphedematous arms following radical mastectomy. Since their report over 300 cases have been reported in literature. This patient group will develop lymphangiosarcoma in edematous sites in the arm, with no correlation to areas that have been subjected to radiation therapy<sup>2</sup>. Lymphangiosarcoma may also arise in a chronic and severely edematous extremity (elephantiasis), usually of congenital origin without a preceding tumor<sup>3-5</sup>, rarely in idiopathic lymphedema of delayed onset<sup>6,7</sup>, and even more rarely in chronic lymphedema secondary to the parasitic disease filariasis<sup>8,9</sup>. Although the relative odds of

lymphangiosarcoma of the upper extremity following breast cancer are high, the absolute risk is low and estimated to be 0,001%<sup>10</sup>.

Previous reports on the prognosis of lymphangiosarcoma in post mastectomy lymphedema are very poor. Sordillo *et al.*<sup>11</sup> report an overall survival 19 months after appearance of the first lesions of 50%, regardless of the type of therapy. The 5-year survival was as low as 13.6%. Management strategies of this multifocal disease so far have included amputation or exarticulation of the limb or limb sparing attempts utilizing radiation therapy and/or chemotherapy<sup>12</sup>.

With the introduction of the hyperthermic isolated limb perfusion (HILP) by Creech in 1958<sup>13</sup> a new technique was initiated to approach extremity malignancies. In 1992, Lienard *et al.*<sup>14</sup> published their first report of the efficacy of ILP using TNF, melphalan and interferon gamma in patients with in transit metastasized melanoma in the extremity. Their promising results encouraged other investigators to use this treatment regimen, sparking the modern era of clinical TNF application. After disappointing results for the local treatment of STS with various chemotherapeutics, including the failure of treating locally advanced STS with ILP with melphalan alone<sup>15</sup>, the combination of TNF and melphalan emerged as a very promising option for the limb-saving management of locally advanced STS<sup>16</sup>. With this multi-drug approach a high response rate in patients with STS of the extremities was reached; an 82% limb salvage rate at a median follow-up of almost 2 years. We have demonstrated both in clinical<sup>17</sup> and in experimental studies<sup>18</sup> that TNF in combination with melphalan in the setting of ILP exerts its antitumor effects primarily on the tumor-associated neovasculature with hardly any direct cytotoxic effect on cancer cells<sup>19; 20</sup>. This makes the group of patients with Stewart Treves angiosarcoma especially eligible to be treated with TNF based therapy. Here we report on our unique experience in addressing this.

## PATIENTS AND METHODS

### *Patient Population*

Clinical records of 10 patients with Stewart Treves angiosarcoma were reviewed. All but two patients were post-mastectomy patients with a sarcoma in the arm after a prolonged period of lymphedema. Two patients suffered from congenital

lymphedema in their leg and developed a sarcoma in this area. They were treated between April 1994 and October 2001 with melphalan and TNF via hyperthermic ILP. Patient characteristics, treatment, results and follow-up data are listed in Table 1. All patients are women, with an age range of 45 to 82 years (mean age 64.0 years), and a total of 16 ILPs is described. Previous radiotherapy had failed in 2 patients and chemotherapy in 1 patient, with either a short-lived response or no response at all. At time of referral to us all patients were considered to be candidates for amputation by their referring specialists.

### *ILP*

The technique of the hyperthermic ILP with recombinant TNF and melphalan is described in detail elsewhere<sup>16</sup>. Briefly, recombinant human TNF (Boehringer Ingelheim GmbH, Ingelheim/Rhein, Germany) and the cytostatic drug melphalan were obtained as sterile powder (100 mg) dissolved aseptically using solvent and diluents (Burroughs Wellcome, London, UK). ILPs were performed under general anesthesia and normally took 3 to 5 hours. Isolation of the blood circuit of a limb was achieved by clamping the major artery and vein and by applying a tourniquet to compress the remaining collateral vessels. Perfusion was performed at the axillary, brachial, iliac, or femoral level. ILP consisted of a 90-minutes perfusion with 1 to 4 mg TNF, and a 10 mg/l (leg) or 13 mg/l (arm) volume of melphalan (25 to 110 mg) at mild hyperthermia (39 to 40°C). In 2 patients 0.2 mg Interferon was used according to the protocol that was in effect at the moment of perfusion. Composition of the perfusate was as follows: the priming volume of 700 to 850 ml consisted of 400 to 500 ml blood (50% RBCs, 50% plasma), 200 to 400 ml 5% dextran 40 in glucose 5% (Isodex; Pharmacia, Uppsala, Sweden), 10 to 30 ml 8.4% sodium bicarbonate, and 0.5 ml of 2500 to 5000 IU heparin. TNF was injected as bolus into the arterial line, provided limb tissue temperature was above 38°C. Melphalan was administered 30 minutes later, at limb temperatures between 39 and 40°C. At the end of the ILP, the limb was washed with at least 2 l of 6% dextran 70 (Macrodex; Pharmacia, Uppsala, Sweden).

### *Response evaluation*

CR was defined as the disappearance of all measurable disease in the limb for longer than 4 weeks, PR as regression of the tumor size by greater than 50% for

more than 4 weeks, no change (NC) as regression of less than 50% or progression of less than 25% for longer than 4 weeks, and progressive disease (PD) as greater than 25% disease progression. Responses were standardized according to World Health Organization criteria.

#### *Assessment of toxicity*

Regional toxicity was graded according to Wieberdink *et al.*<sup>21</sup> as follows: 1 = no toxicity, 2 = redness and slight edema, 3 = considerable edema or erythema with some blistering, 4 = extensive epidermolysis or obvious damage to the deep tissues, causing definite functional disturbances, or threatening, or manifest compartmental syndrome, 5 = reaction requiring amputation. Systemic toxicity was graded according to Eastern Cooperative Oncology Group (ECOG)-WHO criteria.

## **RESULTS**

#### *Treatment outcome*

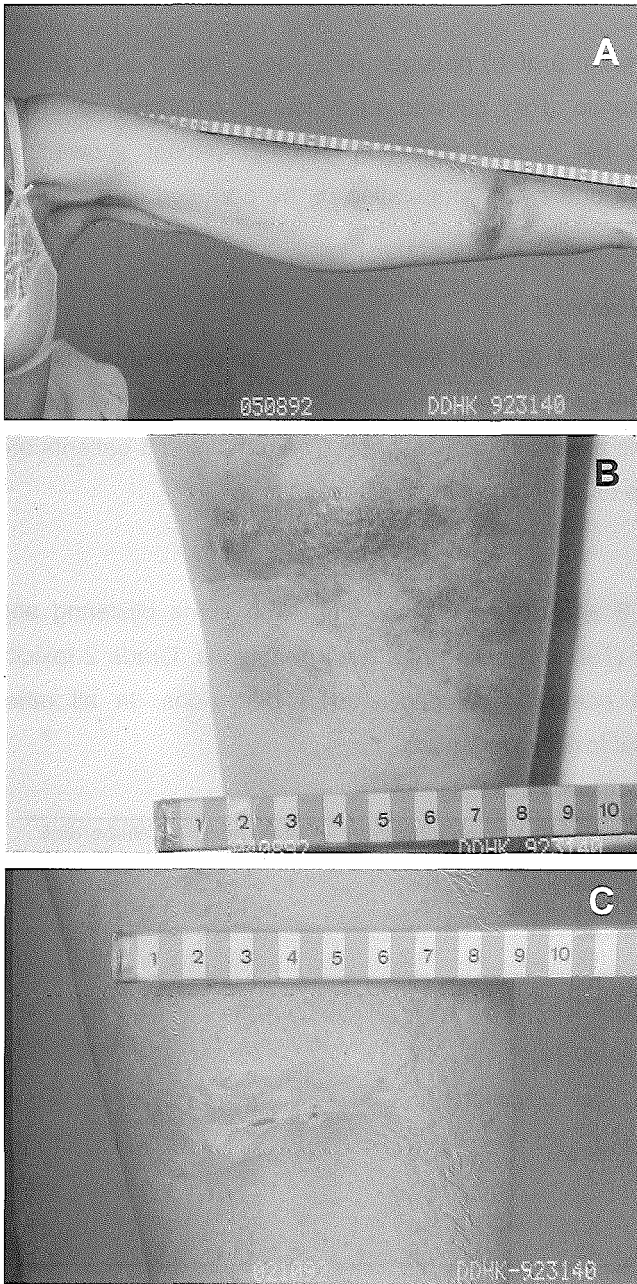
Results are shown in Table 1. Sixteen ILPs with TNF plus melphalan were performed in 10 patients. All patients would have been candidates for exarticulation of the extremity. After ILP we observed an 87% overall response rate (CR+PR), 1 patient had a mixed response and 1 patient failed to respond to the therapy. In 9 perfusions (56%) a complete response was achieved and 5 perfusions (31%) resulted in a partial response. Limb salvage was achieved in 8 patients (80%) with a mean follow-up duration of 34.8 (3-115+) months. 4 patients received a second or even third ILP upon recurrent disease after a period of CR or PR achieved by the previous ILP. Figure 1 A/B/C shows clinical pictures of patient number 2 who is still alive with no evidence of disease 115 months since her perfusion.

Table 1: Characteristics of 16 ILPs in 10 lymphangiosarcoma patients

Patient No.	Sex/Age (years)	No. of tumors	Site	Size (cm)	P / R	Grade	Stage	Type ILP	Final outcome	Duration response (months)	Limb Salvage	Dead or Alive	Cause of death	Follow-up in months
1 1 <sup>st</sup>	F / 78	>100	Lo Arm	1x1	R	2	2	Brach	CR	20				
2 <sup>nd</sup>	F / 80	>100	Lo Arm	3x2	R	1	2	Axil	CR	13				
3 <sup>rd</sup>	F / 82	>100	Lo Arm	1x1	R	1	2	Axil	PR	2	Yes	D	CVA	47
2	F / 67	>100	Total arm	1x2	P	1	1	Axil	CR	>115	Yes	A, NED	-	115+
3 1 <sup>st</sup>	F / 45	>100	Up Leg	max 5x5	P	2	1	Iliac	PR	3				
2 <sup>nd</sup>	F / 47	>100	Up Leg	max 5x5	R	2	2	Iliac	PR	4	No	A, NED	-	82+
4	F / 52	>30	Up Arm	3x4	P	1	1	Axil	PR	2	Yes	D	PD, lung mets	3
5 1 <sup>st</sup>	F / 67	>20	Lo Arm/ hand	8x4	P	1	1	Axil	CR	18				
2 <sup>nd</sup>	F / 69	>20	Up Arm	2x3	R	2	2	Axil	CR	>12	Yes	A, NED	-	34+
6	F / 60	>50	Up Arm/ elbow	7x14	P	1	1	Axil	NC	NC	No	A, NED	-	15+
7	F / 65	4	Up + Lo Arm	6x4	R	1	2	Axil	MR	3	Yes	D	PD, lung mets	8
8 1 <sup>st</sup>	F / 77	>30	Lo Arm	max 5x4	R	2	2	Brach	CR	19				
2 <sup>nd</sup>	F / 78	>20	Up + Lo Arm	max 3x3	R	1	2	Axil	CR	13	Yes	A, NED	-	46+
3 <sup>rd</sup>	F / 80	>20	Up + Lo Arm	max 8x4	R	1	2	Axil	CR	>11				
9	F / 78	>20	Up Arm	1 x 1	P	1	1	Axil	CR	>6	Yes	A, NED	-	6+
10	F / 46	>20	Lo Leg	8x4	P	1	1	Fem	PR	>5	Yes	A	-	5+

Abbreviations : F = female ; M = male ; Lo Arm = lower arm ; Up arm = upper arm ; Up leg = upper leg ; Lo Leg = lower leg ; P/R = primary /recurrence ; Brach = brachial ; Axil = axillary ; Iliac = iliac ; Fem = Femoral ; CR = complete response ; PR = partial response ; MR = Mixed Response consisting of 2x CR and 2x NC ; NC = no change. NED = no evidence of disease.





*Figure 1* Stewart Treves angiosarcoma in left arm; A pre-perfusion overview, B pre-perfusion detail, C post-perfusion.

*Limb salvage*

Limb salvage was achieved in 8 of 10 patients (80%), see Table 1. One patient with an insufficient response (PR) showed evidence of extensive recurrent disease in her leg after her second perfusion. Another patient showed no response at all. They both underwent amputation of the perfused limb within 4 months of perfusion.

*Systemic metastases and survival*

At a median follow-up of 34.8 (3-115) months only 2 patients have died of metastatic disease and one patient died of a cerebrovascular accident (Table 1). At this moment 1 patient is alive with local disease. The remaining 6 patients are alive without any evidence of local or systemic disease.

*Toxicity*

Regional edema (grade 3 according to Wieberdink) with some blistering and slightly disturbed mobility (Table 2) occurred after 4 perfusions. Grade 2 toxicity with redness and slight edema developed after 6 perfusions. In all other perfusions no signs of local toxicity were seen.

**Table 2 - Leakage and Regional Toxicity**

Patient No.	mg TNF	mg melphalan	mg interferon	Leakage %	Wieberdink classification
1 1 <sup>st</sup>	3	36	0,2	<1	2
2 <sup>nd</sup>	3	33	0,2	<1	2
3 <sup>rd</sup>	3	45	0	2	3
2 1 <sup>st</sup>	3	42	0,2	<1	3
3 1 <sup>st</sup>	4	100	0	6	1
2 <sup>nd</sup>	4	110	0	<1	1
4 1 <sup>st</sup>	3	50	0	0	3
5 1 <sup>st</sup>	3	40	0	10	2
2 <sup>nd</sup>	2	32.5	0	0	2
6 1 <sup>st</sup>	2	40	0	0	1
7 1 <sup>st</sup>	3	25	0	0	2
8 1 <sup>st</sup>	3	32.5	0	1,5	3
2 <sup>nd</sup>	1	33	0	0	2
3 <sup>rd</sup>	3	33	0	0	1
9 1 <sup>st</sup>	1	40	0	0	1
10 1 <sup>st</sup>	4	45	0	0	1

Transient motor neurapraxia of less than 6 months was seen after 2 perfusions. Overall no severe regional toxicity was observed in spite of lymphedema being present in all extremities prior to the (first) ILP.

Systemic toxicity was absent to mild and easily manageable. There have been no cases of toxic shock, liver toxicity, haematotoxicity or renal dysfunction. In 3 perfusions a transient period of fever between 39 and 40°C followed ILP as at the time of the program no NSAIDs were administered immediately after the ILP. Total days of admission varied between 4 and 14 with a mean of 8.1 days.

## DISCUSSION

In this unique patient population of 10 patients with this rare, limb threatening vascular sarcoma we demonstrate that amputation can be avoided in the majority of patients by treating the condition with an ILP with TNF plus melphalan. Also survival has been remarkably high compared to the other reports in the literature. 7 patients are still alive, 6 with no evidence of disease, at a mean follow up of 35 months and a maximum follow-up of almost 10 years in a patient with over 100 tumors before treatment. In literature there is no comparable patient group treated in this fashion. Various treatment regiments have been tried; radiation therapy, amputation or disarticulation, intravenous chemotherapy or combinations of these modalities<sup>12</sup>. While there have been anecdotal reports of long-term survivors following systemic chemotherapy<sup>22,23</sup>, overall there has been limited success at treating Stewart Treves with chemotherapy. The 5-years survival was as low as 13.6% in a report by Sordillo *et al*<sup>11</sup>. He reviewed 44 cases seen at the Memorial Hospital, New York City. He reported a median survival of 48 months in 11 patients treated with amputation compared to a 20 months median survival in those patients treated with radiation therapy or chemotherapy. They conclude that surgical treatment should consist of either wide excision or amputation. A 5-year survival of only 8.5% was reported by Woodward *et al*<sup>24</sup>. Yap *et al.* reviewed 22 patients seen at M.D. Anderson Hospital for post-mastectomy lymphangiosarcoma over a 20-year period. 13 patients were treated regionally, systemically, or both, with a variety of single or combination chemotherapeutic agents; the overall response rate was 42%<sup>23</sup>. 5 patients who failed to respond to chemotherapy had a median survival of 4 months, whereas the 6 chemotherapy responders had a median survival of 26.5 months. 2 cases described by Kaufmann *et al.* comprehend long-term survivals after combined modality treatment with Actinomycin-D chemotherapy and high dose radiation therapy<sup>25</sup>.

They survived at least 13 and 19 years respectively; nonetheless in the first case an amputation of the arm was performed. Overall, even in the setting of initial surgical treatment, survival was poor (<40%) in a collective comparison of 160 cases reported in literature<sup>12</sup>.

Length of survival in untreated patients is only 5-8 months<sup>24</sup>. Planning of appropriate therapy for these patients is dependent on the presence or absence of metastatic disease at the time of presentation. Death from Stewart Treves Syndrome usually occurs as a result of pulmonary metastasis, while spread of disease to bone and liver may also occur.

The rationale of the combination of TNF and melphalan delivered locally is to make use of targeting both the stromal neovascular compartment and the cancer cell compartment of the tumor<sup>26-28</sup>. The permeability enhancing effect of TNF has also been demonstrated in a rat ILP model to significantly increase intra-tumor melphalan concentrations as a key explanation for the synergistic antitumor effects observed<sup>29</sup>. The resulting effect is a rapid and profound necrosis. The selective destructive effects of TNF-ILP on tumor-associated vessels have been illustrated in previous publications by means of pre and post perfusion angiographies<sup>17</sup>. In sarcoma patients we have clearly shown with magnetic resonance spectrometry studies that the metabolic shut down of tumor is virtually complete within 16 hours after the perfusion, confirming the likelihood of TNF mediating its most important effect on the tumor vasculature<sup>30</sup>. Our results demonstrate that a combined treatment with melphalan and TNF can lead to good clinical response rates in patients with extensive disease who were candidates for amputation. This implies that with the ILP we have a new treatment option in the management of Stewart Treves angiosarcoma.

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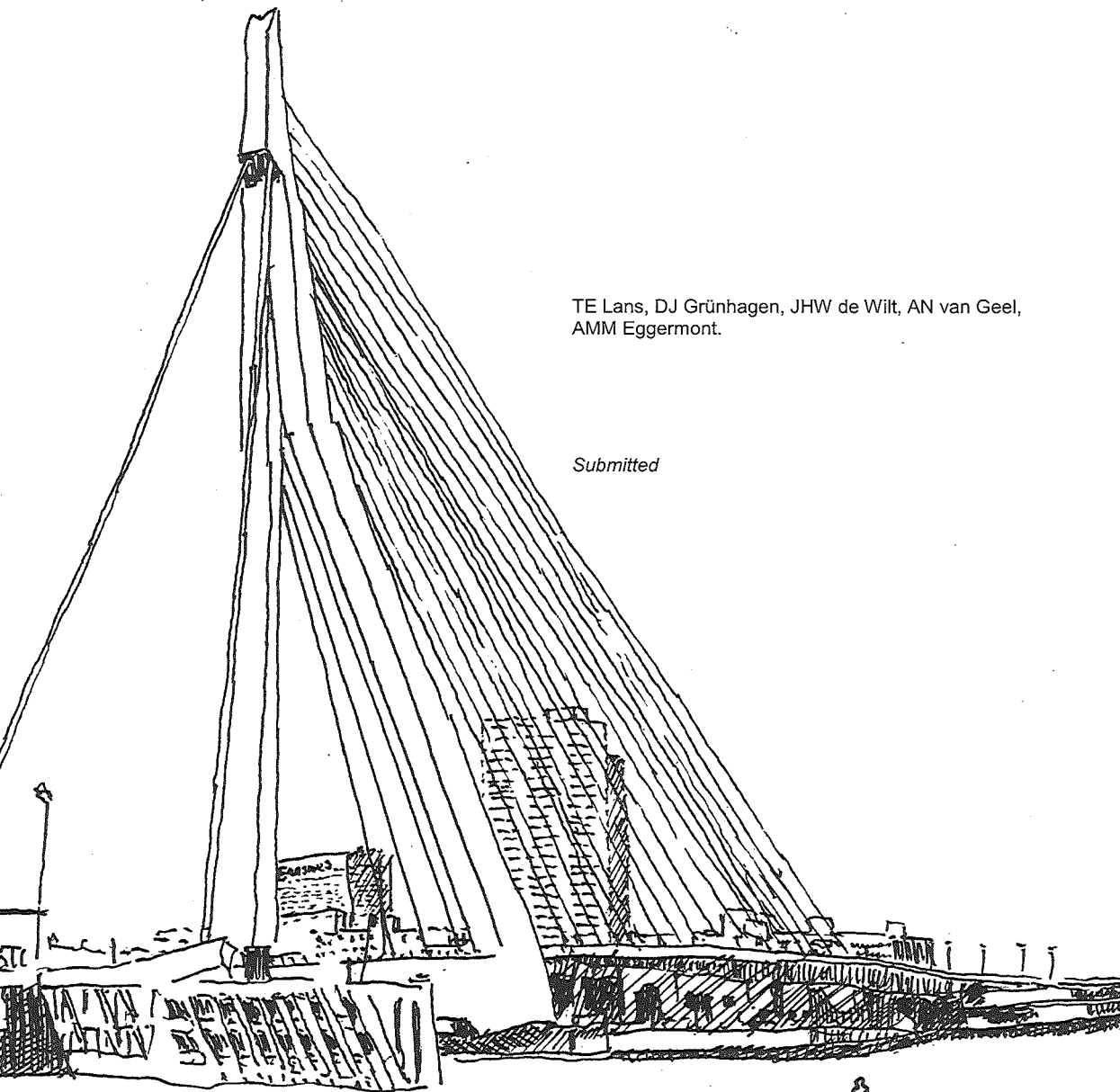
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# 4

## ISOLATED LIMB PERFUSIONS WITH TNF AND MELPHALAN FOR LOCALLY RECURRENT SOFT TISSUE SARCOMA IN PREVIOUSLY IRRADIATED LIMBS

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*Submitted*







## ABSTRACT

**Background:** Recurrent soft tissue sarcoma (STS) in a previously operated and irradiated area can usually only be managed by amputation. Tumor Necrosis Factor (TNF) based isolated limb perfusion (ILP) is an established alternative to achieve limb salvage but is assumed to require sufficient vasculature. Since radiotherapy is known to destruct vasculature, we wanted to evaluate retrospectively whether the outcome of ILP in patients previously treated with radiotherapy for their primary tumor nonetheless benefited from TNF treatment.

**Methods:** A database of TNF-based ILPs at the Rotterdam Cancer Center was used. Out of 342 TNF-based ILPs done between 1991-2003, 30 ILPs were performed in 26 patients with recurrent STS in the irradiated field after prior surgery and radiotherapy. 11 patients (42%) had multiple tumors (2->20). All patients were candidates for amputation.

**Results:** We observed 6 complete responses (20%), 15 partial responses (50%), no change in 8 patients (27%) and progressive disease in 1 patient (3%). Median duration of response was 16 months (3->56), at a median follow-up of 22 (3->67) months. The local recurrence rate was 45% in patients with multiple tumors and 27% in patients with single tumors. 10 patients (35%) died of systemic metastases. Limb salvage was achieved in 17 patients (65%). Regional toxicity was limited and systemic toxicity minimal.

**Conclusions:** TNF-based ILP can avoid amputations in the majority of patients with recurrent extremity STS in a prior operated and irradiated field.

## INTRODUCTION

The approval by the European Medicine Evaluation Agency (EMA) of Tumor Necrosis Factor (TNF) in combination with melphalan in the setting of isolated limb perfusion (ILP) for the treatment of locally advanced soft tissue extremity sarcomas (STS), based on the excellent limb salvage results of multicenter trials in Europe, has added an important treatment modality to avoid amputations of the limb<sup>1-4</sup>. Patients with local recurrences in the limb after prior surgery and high dose radiotherapy and patients with multiple sarcomas are usually all candidates for amputation of the extremity.

In the early the TNF-based ILP program for irresectable extremity STS much attention was given to the post-ILP necrosis of the foot a particular patient treated by TNF-based ILP after prior resections, high dose radiotherapy and prior ILP with Cisplatin<sup>5</sup>. It was speculated that the high dose radiotherapy might have resulted in damage to the vasculature of the foot. This might have rendered it susceptible to the toxic effects of the combination of high dose TNF and melphalan leading to complete necrosis of the “healthy tissues” in contrast to the usually selective toxic effects on the tumor vasculature only. This observation led to hesitation to offer a TNF-based ILP to patients with a local recurrence presented in a high dose irradiated area. In an attempt to avoid amputation we decided to offer a TNF-based ILP in all cases where amputation was the only option regardless of prior treatments. We performed 30 ILPs in 26 patients with tumor recurrences in irradiated limbs. Here we report on this unique experience.

## PATIENTS AND METHODS

Between 1992 and 2003, 26 patients with recurrent STS initial treated with surgery and radiotherapy, were treated with an isolated limb perfusion (ILP) with melphalan and TNF. All patients were considered candidates for amputation at the time of referral because of extensive and/or irresectable disease.

The technique of the ILP with recombinant TNF and melphalan is in detail described elsewhere<sup>2</sup>. Briefly, recombinant human TNF (Boehringer Ingelheim GmbH, Ingelheim/Rhein, Germany) and the cytostatic drug melphalan, obtained as a sterile powder (100 mg) were dissolved aseptically using solvent and

diluents (Burroughs Wellcome, London, UK). ILPs were performed under general anesthesia and normally took 2.5 to 4 hours. Isolation of the blood circuit of a limb was achieved by clamping the major artery and vein and by applying a tourniquet to compress the remaining collateral vessels. Perfusion was performed at the axillary, brachial, iliac, femoral, or popliteal level. ILP consisted of a 90-minutes perfusion with 1.5 to 3 mg (arm) or 2-4 mg (leg) TNF and a 10 mg/l (leg) or 13 mg/l (arm) volume of melphalan at mild hyperthermia. Maximum tissue temperatures were 39.5°C in the leg and 38.5°C in the arm. Composition of the perfusate was as follows: the priming volume of 700 to 850 ml consisted of 400 to 500 ml blood (50% RBCs, 50% plasma), 200 to 400 ml 5% dextran 40 in glucose 5% (Isodex; Pharmacia, Uppsala, Sweden), 10 to 30 ml 8.4% sodium bicarbonate, and 0.5 ml of 2500 to 5000 IU heparin. TNF was injected as bolus into the arterial line, provided limb tissue temperature was higher than 38°C. Melphalan was administered 30 minutes later at limb temperatures between 38 and 39.5°C. Directly after the ILP, the limb was washed with between 1 l (axillary) and 4 l (iliac perfusion) of physiologic saline solution and 6% dextran 70 (Macrodex; Pharmacia, Uppsala, Sweden).

## EVALUATION OF RESPONSE AND TOXICITY

An assessment of the tumor response was performed at least twice between four and twelve weeks after perfusion. Complete response (CR) was defined as the disappearance of all measurable disease in the limb for more than 4 weeks, a partial response (PR) as tumor size regression by greater than 50% for more than 4 weeks, no change (NC) as regression of less than 50% or progression of less than 25% for longer than 4 weeks, and progressive disease (PD) as more than 25% disease progression. In those patients where a resection of residual tumor and/or necrotic tissue mass was performed after ILP, a histological response rate was assessed by determining the percentage of necrosis. Clinical responses were standardized according to World Health Organization (WHO) criteria.

Regional toxicity was graded according to Wieberdink *et al.*<sup>6</sup> In this Grade 1 = no toxicity, Grade 2 = redness and slight oedema, Grade 3 = considerable oedema or erythema with some blistering, Grade 4 = extensive epidermolysis or obvious

damage to the deep tissues causing definite functional disturbances, or threatening or manifest compartmental syndrome and Grade 5 = reaction requiring amputation. Systemic toxicity was graded according to Eastern Cooperative Oncology Group (ECOG)-WHO criteria.

## RESULTS

Over the past 10 years more than 340 patients with advanced melanoma or sarcoma were treated with an ILP using melphalan in combination with TNF in the department of surgical oncology at the Erasmus MC - Daniel den Hoed Cancer Center. 26 of these patients were previously treated with surgery and high dose radiotherapy (50-70 Gy) for their primary sarcoma and developed one or more irresectable local recurrences. One patient had known distant metastases at the time of treatment. Patient and perfusion characteristics are shown in Table 1. The group consisted of 15 men and 11 women, with a median age of 50 (range 21-84, mean 52) years. Median interval between treatment of primary tumor and recurrence of disease was 19 months and ranged between 0 and 156 months.

In all patients systemic toxicity after ILP was mild to moderate and easily manageable. More than 10% leakage was measured in five patients during perfusion, but this was without significant toxicity. When postoperative hypotension occurred it responded immediately to fluid administration and none of the patients required circulatory support with vasopressors. One patient experienced a fever over 40°C after the ILP, this however was reversible within 24 hours. Mean Wieberdink toxicity was 2, and no patients required amputation due to perfusion damage. Mean stay in hospital after ILP was 9 days (range 3-42).

Table 1: Characteristics of 26 recurrent sarcoma patients treated with ILP (N=30) after radiotherapy treatment.

Pt no	sex	age	no of tu-mors	site	histology	grade	1st-2nd rec	p/r interval (months)	type ILP	Local Toxicity (Wieberdink)	Clinical outcome	Histology (% necrosis)	Final outcome	Duration of response (months)	Limb salvage	D/A	Follow-up (months)
1	M	72	1	Up leg	Fibro	2	R	19	Iliac	3	NC	-	NC	-	Yes	DOD	17
2	F	46	2	Wrist	Synovio	1	RR	156	Brach	3	PR	-	PR	2	No	A, NED	59+
3	F	63	1	Lo leg	MFH	3	RR	22	Pop	2	NC	<50 (NC)	NC	-	No	A, NED	49+
4	M	44	1	Lo arm	MFH	3	R	39	Brach	2	NC	10 (NC)	NC	-	No	A, NED	52+
5	M	74	1	Lo arm	MFH	3	R	33	Brach	2	CR	-	CR	43	Yes	DU	56
6	F	27	2	Lo leg	Kaposi	2	RR	14	Pop	2	PR	-	PR	19 (+)	Yes	DOD	19
7	M	48	1	Up leg	MFH	3	R	156	Iliac	2	PR	-	PR	9	Yes	DOD	30
8	F	81	4	Lo leg	MFH	2	R	28	Pop	2	NC	-	NC	9			22
		82	6	Lo leg + Ankle	MFH	3	RR	9	Fem	2	CR	NA	CR	2	No	DOD	
9	M	72	3	Fos pop	MFH	3	R	12	Iliac	2	PR	-	PR	9	Yes	DOD	22
10	F	50	1	Lo arm	MFH	2	R	38	Brach	3	NC	-	NC	-			67+
		50	1	Lo arm	MFH	2	R	-	Brach	2	PR	>50 (PR)	PR	5	No	A, NED	
11	F	33	6	Up leg	Leio	3	RR	9	Brach	2	PR	100 (CR)	CR	8			44
		34	2	Up leg	Leio	3	RR	6	Iliac	2	PR	NA	PR	17	Yes	DOD	
12	M	22	1	Up arm	Schwan	1	R	24	Axil	1	NC	<10 (NC)	NC	-	Yes	DOD	20
13	F	84	3	Lo leg	MFH	3	R	19	Pop	2	PR	>50 (PR)	PR	9	No	DU	26
14	M	41	1	Lo leg	Synovio	2	RR	24	Fem	1	PR	>50 (PR)	PR	57+	Yes	A, NED	57+
15	M	72	1	Up leg	Schwan	2	R	26	Iliac	1	CR	-	CR	44+			44+
		75	1	Up leg	Schwan	3	RR	30	Iliac	1	PR	-	PR	10+	Yes	A, NED	10+
16	M	25	1	Fos cub	Lipo	3	R	14	Brach	1	PR	100 (CR)	CR	12+	Yes	AWD	12+
17	F	21	10	Up + Lo leg	Ag fibro	1	RR	9	Iliac	3	PR	-	PR	30+	Yes	A, NED	30+
18	M	72	3	Up leg + Fos pop	MFH	2	RR	0	Fem	1	PR	-	PR	21+	Yes	A, NED	21+
19	M	33	3	Up leg + Fos pop	Desmo	1	RR	12	Iliac	2	PR	-	PR	16+	Yes	A, NED	16+
20	F	33	1	Fos pop Up + Lo leg	Desmo	1	RR	14	Fem	2	NC	NA	NC	15+	Yes	A, NED	15+
21	M	20	8	leg	Ag fibro	1	RR	0	Iliac	3	PR	-	PR	9+	Yes	A, NED	9+
22	M	40	1	Fos cub	Synovio	3	R	32	Brach	2	NC	<50 (NC)	NC	-	No	DOD	22
23	F	54	3	Up leg + Fos pop	Neuro	2	R	98	Iliac	1	CR	100	CR	22+	Yes	A, NED	22+
24	F	53	1	Up arm	MFH	3	RR	6	Axil	2	PD	-	PD	-	No	A	3+
25	M	72	1	Lo arm	Fibro	3	RR	93	Brach	1	PR	-	PR	26+	Yes	A, NED	26+
26	M	79	1	Lo arm	Pleio	3	RR	2	Brach	1	NC	>50 (PR)	PR	3	No	A, NED	14+

Abbreviations : F = female ; M = male ; Histology : explained in tekst ; Lo Arm = lower arm ; Up arm = upper arm ; Up leg = upper leg ; P/R = primary /recurrence ; R = recurrence ; RR = re-recurrence; Brach = brachial ; Axil = axillary ; Iliac = iliacal ; Fem = femoral ; Pop = poplital ; CR = complete response ; PR = partial response ; NC = no change; PD = progressive disease ; NA = not available ; NED = no evidence of disease; DOD = died of disease ; DU = death unrelated to disease ; AWD = alive with disease

A major tumor response was seen in 21 of 30 perfusions (70%), with a partial response in 15 patients (50%) and a complete response in 6 patients (20%). 9 perfusions (30%) were not followed by any significant tumor response; 4 of these patients (n° 3,4,22,24) underwent an amputation of the limb. Two patients died of systemic disease with tumor present in their limb (n° 1,12). Patient n° 8 had a mixed response to the first perfusion, with 4 out of 6 tumors showing a CR but 2 tumors not responding at all, resulting in a NC score. The two remaining tumors responded well to the repeated perfusion scheduled 13 months after the first ILP. However, she relapsed within 3 months after the second perfusion and eventually underwent an amputation. Patient n° 10 responded insufficiently to the first perfusion but showed a partial response after a second ILP 7 months later. Due to a local recurrence, an amputation had to be performed after 10 months. In patient n° 20, the clinical response was insufficient for a PR score. However, the tumor had shrunk enough to allow for a resection. Histological responses could be established in 11 patients who were treated with an additional tumor resection, which in 3 patients showed much more necrosis in the tumor remnant than clinically expected (patient n° 11,16,26).

In 17 out of 26 (65%) patients limb salvage could be achieved. Duration of response was defined as the time from ILP until first evidence of local recurrent disease, and varied from 2 to 57 (and ongoing) months with a median of 12 months (mean 17). Median follow-up of patients was 22 months (range 3 to >61 months, mean 29).

In 9 patients (11 perfusions) the tumor recurred locally after ILP, in 4 patients (4 ILPs) with a single tumor (24%) and in 5 patients (7 ILPs) with multiple tumors (54%). An amputation had to be performed to achieve local control in 5 of the patients, while in 3 patients no amputation was performed because of the poor short-term prognosis due to systemic metastases. In one patient (n° 5) a local recurrence occurred 43 months after the perfusion, which could primarily be resected. This patient died one year later due to lung carcinoma. Nine patients developed systemic metastases after ILP. There was no significant difference in response between low-grade or high-grade tumors. There was no correlation between tumor size and subsequent tumor response.

## DISCUSSION

Our experience with 30 TNF-based ILPs in 26 patients with irresectable extremity STS recurrences in limbs previously treated with surgery in combination with high dose radiotherapy shows that even in this patient population limb salvage can be achieved in a majority of patients. Moreover, limb salvage was achieved in 65% of the patients in spite of multiple recurrences being present in 42% of the patients. Furthermore, we have shown that TNF-based ILP in previously highly irradiated limbs is not associated with an increased local toxicity or complication rate.

In general, the management of extremity sarcomas has moved away from ablative surgical procedures towards function preserving surgery and is often combined with radiotherapy. As a result an increasing proportion of patients now incur locally recurrent sarcoma arising in previously irradiated areas. Amputation might be the most effective treatment option in this selected group of patients with local recurrences, but although this will improve local disease control it does not affect overall survival rates<sup>7-11</sup>. Since amputation implies a significant decrease in quality of life, treatment modalities that guarantee preservation of the extremity as well as good limb function have become more important. Surgical resection of recurrent tumor in a previously irradiated field is often impossible as it usually requires the resection of all tissue exposed to a high radiation dose of 60-70 Gy. This can occasionally be dealt with by free transfer of vascularised tissue, but in most cases will require an amputation.

To compare our experience with data from the literature is not easy since comparable series of patients with recurrent soft tissue sarcomas in an irradiated field are not at hand. Moreover, 42% of patients in our series had multiple tumors, and these cases are usually not present in series that discuss the application of single use or combinations of re-surgery and/or re-irradiation of the recurrent sarcoma. Thus, comparison to data from the literature is limited to cases with single tumor recurrences after prior surgery in combination with radiotherapy.

The results after re-irradiation are reported by Essner *et al.* in a group of 32 patients who received a second course of radiation for soft tissue sarcoma<sup>12</sup>. In this group 84% of patients showed benefit from pre-operative radiotherapy in combination with subsequent surgery. Local excision of recurrent tumors followed

by a second course of post-operative radiation resulted in a local failure in 8 of 14 patients (57%) and could not be recommended as a valuable therapy.

The use of external beam therapy is restricted to patients with large tumors lying at least partially outside the previous treatment volume. When soft tissue sarcomas recur in a previously irradiated area, further external beam radiation is often not possible. Here brachytherapy allows a radiotherapeutic alternative in an attempt to reduce the risk of further local recurrence<sup>13</sup>. Pearlstone *et al.* reported on 26 patients who underwent resection and peri-operative brachytherapy in conjunction for recurrent STS<sup>14</sup>. At a median follow-up of 16 months, they reported a 5-year local recurrence-free survival rate of 52%, and a 33% disease-free survival. This experience shows that in a series with single tumor recurrences the local control rate is still far from optimal. In addition, 15% of all patients experienced major wound complications that warranted re-operations. Another study by Nori *et al.* describes 40 patients treated with brachytherapy, with a 5-year local control rate of 68% and a 12.5% severe wound complication rate<sup>15</sup>. Catton *et al.* advocate combined conservative surgery with re-irradiation to be the primary salvage therapy for patients who fail combined therapy and who are suitable for conservative re-excision<sup>16</sup>. In this highly selective patient population local control for patients treated with conservative excision without radiation was only 36%, compared to 100% for conservative surgery with re-irradiation. A very high proportion of patients (60%) experienced post-radiation complications. Obviously this patient population, eligible for conservative surgery does not even resemble the patient population with single tumor recurrences that we have treated with TNF-based ILP, let alone the patients with multiple tumors.

Up to now trials with systemic neoadjuvant chemotherapy have failed to achieve any significant improvement in the survival of patients with primary or recurrent STS<sup>17</sup>. According to our findings there are no data available in the literature that describe the use of chemotherapy alone or in combination with the above mentioned therapies for recurrent sarcoma in previously irradiated areas.

Regarding locoregional toxicity, no enhanced toxicity was observed compared to TNF-based ILP in patients without prior surgery in combination with radiotherapy. In essence TNF-based ILP has no increased regional toxicity over ILP with melphalan alone<sup>18</sup>. Regarding systemic toxicity, no toxicity of importance was observed in this patient population. This was the case in patients without



significant leakage as well in the few patients with significant leakage during the perfusion and is in line with earlier reports on our experience with these patients<sup>19,20</sup>. This underscores our opinion that TNF-based ILP is a safe procedure and should be considered in all patients with limb threatening tumors, irrespective of age, number of tumors or prior therapies<sup>4,21-23</sup>.

In conclusion, we want to state that our experience clearly demonstrates that extremities should not be amputated without considering a TNF-based ILP for limb salvage. In the described patient population with extremely unfavorable characteristics of (multiple) limb threatening sarcoma recurrences in an irradiated field after prior surgery and radiotherapy, the achievement of a 65% limb salvage rate shows the efficacy of the TNF-based ILP approach to avoid amputations in what commonly are considered "lost cases" .

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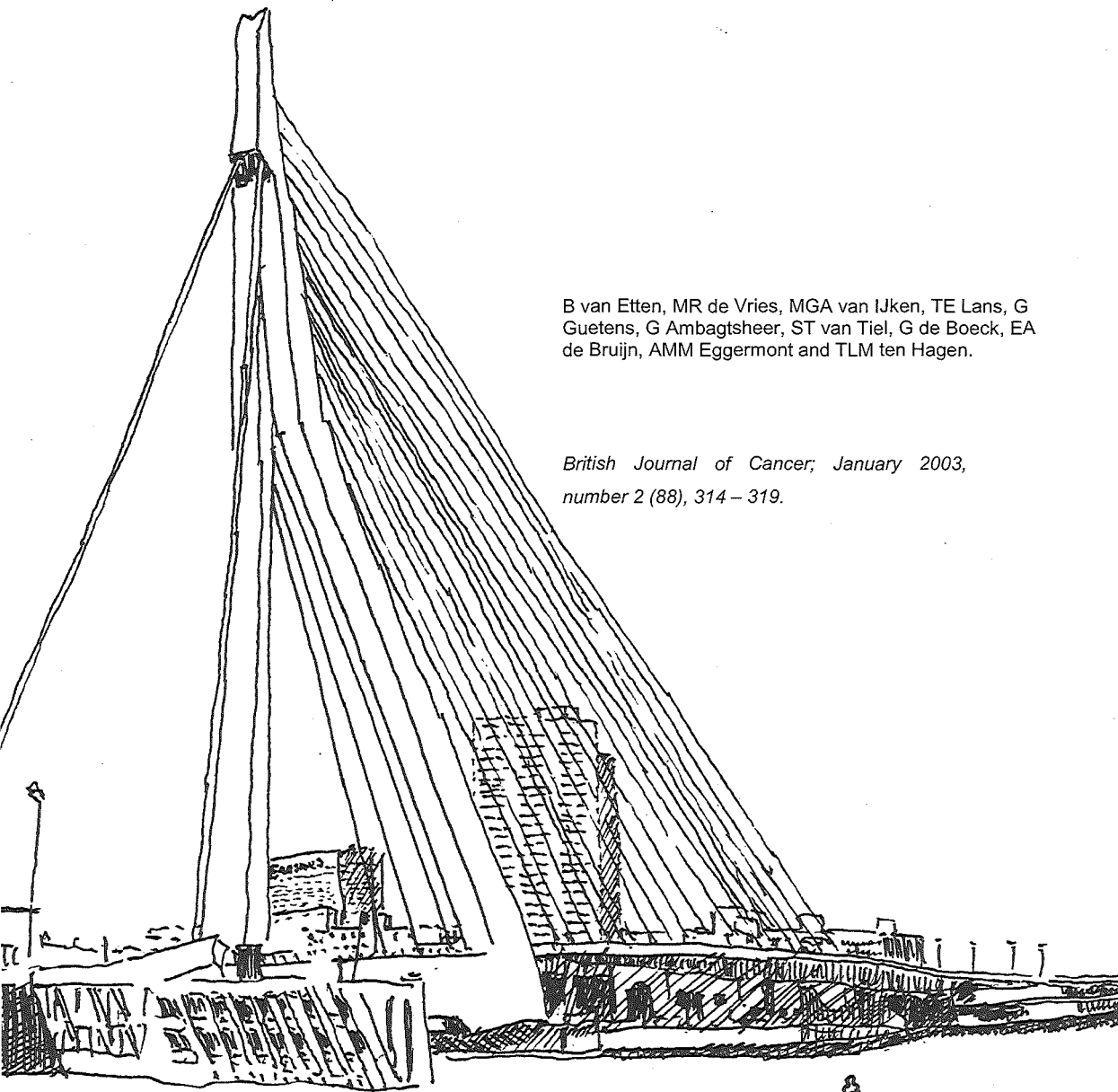


# 5

## DEGREE OF TUMOR VASCULARITY CORRELATES WITH DRUG ACCUMULATION AND TUMOR RESPONSE UPON TNF-BASED ISOLATED HEPATIC PERFUSION

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## ABSTRACT

**Introduction:** Isolated hepatic perfusion (IHP) with melphalan is currently performed both with and without Tumor Necrosis Factor (TNF) in clinical trials in patients with hepatic metastases. Previous studies led to the hypothesis that the use of TNF in isolated limb perfusion causes specific destruction of tumor endothelial cells and thereby induces an increased permeability of tumor vasculature. However, whether TNF contributes to the therapeutic efficacy in IHP remains unclear.

**Methods:** We studied three different tumor types in an *in vivo* rat liver metastases model. These tumors, colon carcinoma CC531, ROS-1 osteosarcoma and BN-175 soft-tissue sarcoma exhibit different degrees of vascularisation. IHP was performed with melphalan both with and without the addition of TNF.

**Results:** IHP with melphalan alone resulted in a decreased growth rate in all tumor types. The addition of TNF resulted in a strong synergistic effect in the BN-175 tumor. A complete response was achieved in the majority of the BN-175 tumor-bearing rats. *In vitro* cytotoxicity studies showed no sensitivity (CC531 and BN-175) or only minor sensitivity (ROS-1) to TNF, ruling out a direct interaction of TNF with tumor cells. The response rate in BN-175 tumor-bearing rats when TNF was co administrated with melphalan was strongly correlated with drug accumulation in tumor tissue, as only in these rats a five-fold increase in melphalan concentration was observed. Secondly, immunohistochemical analysis of microvascular density (MVD) of the tumor showed a significantly higher MVD for BN-175 tumor compared to CC531 and ROS-1.

**Conclusions:** These results indicate a direct relation between vascularity of the tumor and TNF-mediated effects. Therefore we conclude that the level of tumor vasculature of liver metastases forms an indication for the usability of TNF in this setting.

## INTRODUCTION

Tumor Necrosis Factor (TNF) is a cytokine with interesting potential in the treatment of cancer<sup>1</sup>. When administered systemically it is accompanied with severe toxicity. However when TNF is used loco regionally without systemic exposure and in combination with chemotherapy, it has very potent antitumor effects. Clinical trials of isolated limb perfusion (ILP) with recombinant human TNF and melphalan resulted in high complete response rates of 75-90% in patients with in-transit melanoma and unresectable sarcoma of the extremities<sup>2,3,4</sup>. This is in contrast to ILP with melphalan alone, which is relatively effective against small in-transit melanoma metastases<sup>5</sup>, but achieves very poor results against large tumors such as soft-tissue sarcomas<sup>6,7,8</sup>.

Several studies have been performed to elucidate the mechanism of TNF. In our preclinical ILP model, we observed drastic alterations in tumor microvasculature integrity<sup>9</sup>. Others elegantly demonstrated that TNF in combination with IFN induced functional down regulation of  $\alpha v \beta 3$ , resulting in detachment of the endothelial cells of the tumor vasculature<sup>10</sup>. Moreover, angiographic studies performed in patients pre- and post-TNF perfusion show selective destruction of tumor-associated vasculature and histologic studies demonstrate haemorrhagic necrosis of the tumor<sup>11</sup>. Recently we demonstrated what we consider a key explanation for the potent synergy between TNF and chemotherapy: an up to 6-fold increase of intratumoral melphalan or doxorubicin concentration in rat sarcomas after ILP when high-dose TNF was co administrated<sup>12,13</sup>. These findings led to the hypothesis that TNF causes specific destruction of tumor endothelial cells and thereby induces an increased permeability of tumor vasculature.

As a result of the favorable experience with the ILP system, other isolated perfusion settings have been developed<sup>14,15</sup>. Especially the liver offers excellent opportunities for isolated perfusion. Irresectable liver metastases form a significant clinical challenge. Isolated hepatic perfusion (IHP) with melphalan with or without TNF is technically feasible and is currently performed in clinical trials in



patients with hepatic metastases<sup>16,17,18</sup>. Whether TNF contributes to the therapeutic efficacy in IHP still remains unclear.

Our findings in previous ILP studies led us to study whether TNF can improve tumor response in different tumors after IHP and, if so, to investigate the capability of TNF to augment drug accumulation in this perfusion setting. By addressing this question the usefulness of TNF in IHP might be clarified. Since the tumor-associated vasculature is the target of TNF, we expect that tumor microvessel density (MVD) is a predictor of the potentiating effect of TNF during isolated perfusions. Here we present data that indicate that the antitumor effect of TNF is correlated with the tumor microvessel density.

## MATERIALS AND METHODS

### *Rat liver metastases model*

We used male inbred WAG/RIJ or Brown-Norway (BN) strain rats, weighing 250-300 g, obtained from Harlan-CPB (Austerlitz, the Netherlands). The rats were fed a standard laboratory diet. All animals were housed under standard conditions of light and accommodation. The protocol was approved by the committee for animal research of the Erasmus University, Rotterdam, the Netherlands. The experimental protocols adhered to the rules outlined in the Dutch Animal Experimentation Act of 1977 and the published Guidelines of the UKCCCR for the Welfare of Animals in Experimental Neoplasia (UKCCCR, 1998).

Three different tumors were used in this study. The weakly immunogenic colon carcinoma CC531 is a 1,2-dimethylhydrazine-induced, moderately differentiated adenocarcinoma transplantable in syngeneic WAG/RIJ rats. The estimated doubling time *in vivo* is about 6-8 days. The spontaneously originated non-immunogenic osteosarcoma ROS-1 is also transplantable in the WAG-RIJ rat and in the liver metastases model it has a mean doubling time of about 4-5 days. The spontaneously originated non-immunogenic soft-tissue sarcoma BN-175 is the fastest growing tumor of the tumors tested, with an estimated doubling time *in vivo* of about 2-3 days and is transplantable in syngeneic BN rats. Following a standardized protocol, small viable tumor fragments of CC531, ROS-1 or BN-175

tumor fragments of 1x2 mm<sup>2</sup> were implanted under the liver capsule, one on the left and one on the right side of the left liver lobe, using a 19 G Luerlock needle. Experiments started at a fixed tumor diameter between 5 and 6 mm. When tumors reached a size of 20 mm in diameter or animals showed obvious signs of discomfort the animals were sacrificed.

### *Drugs*

Recombinant human TNF ( $4.9\text{--}5.8 \times 10^7$  U/mg) was provided as a kind gift by Boehringer Ingelheim GmbH, Ingelheim/Rhein, Germany. Melfalan (L-pam, Alkeran, Wellcome Ltd, London, UK) was obtained as a sterile powder (100 mg) that was dissolved aseptically using solvent and diluent provided by Burroughs Wellcome (London, UK).

### *Isolated hepatic perfusion*

A rat isolated liver perfusion model has been described in detail elsewhere<sup>17</sup>. A high-level schematic representation is shown in Figure 1. Anaesthesia was induced and maintained with ether (Merck, Darmstadt, Germany). During the surgical procedure, with an average duration of 60–75 min, rats were kept at a constant temperature using a warmed mattress. A mid-line laparotomy was performed and the hepatic ligament was exposed. The gastroduodenal side branch of the common hepatic artery was cannulated, positioning the tips of the cannula (0.025 outer diameter (OD), 0.012 in inner diameter (ID)) (Dow Corning, MI, USA) in the proper hepatic artery. Through a small inguinal incision the femoral vein was exposed. To collect hepatic venous outflow a silicon cannula (0.047 OD, 0.025 in ID) (Dow Corning, MI, USA) was introduced in the femoral vein and moved up into the caval vein positioning the tip of the cannula at the level of the hepatic veins.

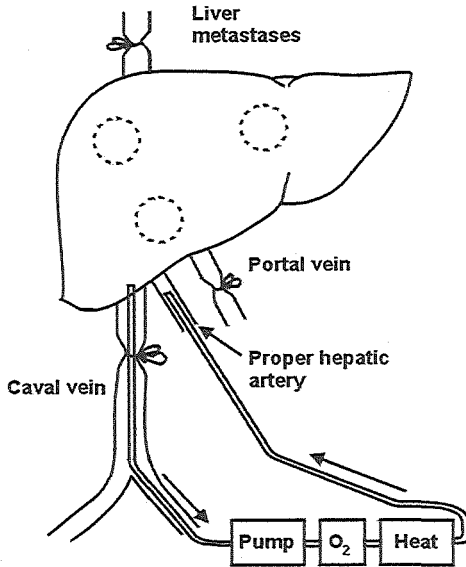


Figure 1 Schematic representation of an IHP.

Isolation of the hepatic vascular bed was obtained by temporarily ligating the common hepatic artery and the portal vein. The venous outflow limb was isolated by temporarily clamping the supra-hepatic caval vein and by applying a temporary ligature around the infra-hepatic caval vein containing the cannule, cranial to the right adrenal vein. The mesenteric artery was temporarily clamped in order to reduce splanchnic blood pressure. The circuit was primed with 10 ml Haemaccel (Behring Pharma, Amsterdam, the Netherlands). Arterial flow of 5 ml/min was maintained with a low-flow roller pump (Watson Marlow type 505 U, Falmouth, UK). Rats were perfused for ten min with oxygenated Haemaccel in which melphalan and/or TNF was dissolved. This short perfusion time was used as we observed rapid clearance of melphalan from the perfusate in this time frame. Secondly, perfusion of the liver beyond 10 min may increase the risk of tissue damage to the liver, but also to the gut as blood flow to the gut is impaired during the perfusion. Afterwards a washout was performed by perfusing with 10 ml of oxygenated Haemaccel. Heparin (50 IU) (Heparine Leo, the Netherlands) was added to the perfusate. The perfusate was oxygenated in a reservoir with a mixture of  $O_2/CO_2$  (95%:5%) and was kept at 38-39°C by means of a heat

exchanger and a warm water bath. A temperature probe was positioned in the lumen of the arterial catheter, 5 cm from the catheter tip.

Following the washout procedure, the clamps on caval vein, portal vein, hepatic artery and mesenteric artery were released. The gastroduodenal artery and femoral vein were ligated and the gastroduodenal and femoral cannulas were removed.

#### *In vivo antitumor efficacy study*

Treatment started at a fixed tumor size of 5-6 mm in diameter. Rats were perfused in random order. For this study we chose the melphalan dose which inflicted a partial tumor response during a pilot-dose finding study performed for each tumor type. So in the case of additive or synergistic effect of TNF on melphalan this could still be demonstrated in the growth curves of the tumors. All animals underwent IHP only once. CC531-bearing rats were treated with 50 µg melphalan ( $n=6$ ), with 20 µg TNF ( $n=6$ ) or with a combination of 50 µg melphalan and 20 µg TNF ( $n=6$ ). ROS-1-bearing rats were perfused with 50 µg melphalan ( $n=6$ ), with 20 µg TNF ( $n=8$ ), or with a combination of 50 µg melphalan and 20 µg TNF ( $n=6$ ). In the BN-175-bearing rats perfusions were carried out with 200 µg melphalan ( $n=6$ ), with 20 µg TNF ( $n=6$ ), or with a combination of 200 µg melphalan and 20 µg TNF ( $n=6$ ). After IHP tumor size was measured via a small midline laparotomy every fourth day. Tumor volume was calculated by using the following formula: tumor volume =  $A^2 \times B \times 0.4$ , where B is the largest diameter and A the diameter perpendicular to B, measured with a standardized calliper. In every treatment group, sham perfused rats ( $n=6$ ) and untreated control rats ( $n=5$ ) were included.

#### *In vitro cytotoxicity assay*

CC531 and BN-175 cells were grown in RPMI 1640 and ROS-1 cells in modified Eagle's medium (Gibco BRL, Paisley, UK) supplemented with 10% foetal calf serum (Harlan/Sera-Lab, UK), 1% penicillin (5000 IU/ml), 1% streptomycin (5000 IU/ml) and 1% L-glutamine (200 mM) (all Gibco BRL) in a humidified incubator at 37°C and 5% CO<sub>2</sub>. Before usage, the cells were trypsinised (1 min, 37°C), centrifuged (5 min, 700 G), resuspended and the viability measured by trypan

blue exclusion. For *in vitro* testing of proliferation inhibition,  $1.0 \times 10^4$  viable cells were seeded in flat-bottomed 96-well microtiter plates (Costar, USA). After 24 h the cells were incubated with different concentrations of TNF for 72 h ranging from 0 to 10  $\mu\text{g/ml}$ . Afterwards cells were washed with PBS and fixed for 1 h with 10% trichloroacetic acid at 4°C. Growth of tumor cells was measured using the sulpharhodamine-B assay according to the method of Skehan *et al*<sup>19</sup>. Tumor cell proliferation was measured using the formula: tumor growth = (test well/control)  $\times 100\%$ . Five independent tests were performed for each point on the line.

#### *Measurement of melphalan in tissue*

After 5 min of the restoration of the circulation the perfused tumor and part of the liver were excised. The tissues were immediately frozen in liquid nitrogen to stop metabolism of melphalan and were stored at -80°C. Tumor and liver tissues were homogenised in 2 ml acetonitrile (Pro 200 homogenizer, Pro Scientific, CT, USA) and centrifuged at 2500 G. Melphalan was measured in the supernatant by gas chromatography-mass spectrometry (GC-MS). *p*-[Bis(2-chloroethyl)amino]-phenylacetic acid methyl ester was used as an internal standard. Samples were extracted over tri-functional C18 silica columns. After elution with methanol and evaporation, the compounds were derivatised with trifluoroacetic anhydride and diazomethane in ether. The stable derivatives were separated on a methyl phenyl siloxane GC capillary column and measured selectively by single-ion monitoring GC-MS in the positive EI mode<sup>20</sup>.

#### *Assessment of tumor microvessel density by immunohistochemistry*

Cryosections of tumors were fixed for 15 min with 4% formaldehyde. After rinsing with PBS, sections were incubated for 1 h with 1:10 PBS diluted, mouse-anti-rat-endothelial cell antibody (RECA-1, Instruchemie, Hilversum, the Netherlands). For the negative control an aspecific mouse IgG was used (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Thereafter, sections were rinsed with PBS and incubated for 1 h with 1:100 diluted, in 5% normal rat serum in PBS, goat-anti-mouse peroxidase-labeled antibody (DAKO, Carpinteria, CA, USA). After rinsing with PBS, positive cells were revealed by immunoperoxidase reaction with DAB solution (DAB-kit, DAKO) and counterstained with haematoxylin. For

microvessel quantification two independent persons performed a blinded analysis. Positive cells were counted in three different high-power fields (magnification x160) in each slide according to the method of Bosari *et al*<sup>21</sup>. In total, three slides per tumor and three tumors per tumor type were evaluated.

#### *Statistical analysis*

*In vitro* bioassays and *in vivo* tumor response results were evaluated for statistical significance with the Mann-Whitney *U*-tests with SPSS8.0. The Mann-Whitney *U*-test was used to compare melphalan concentrations in different groups and the Kruskal-Wallis test to compare the number of positive cells in different tumors. A significance level of  $P < 0.05$  was used in all analyses.

## **RESULTS**

#### *Tumor response after isolated hepatic perfusion*

The antitumor efficacy of IHP with melphalan both with and without TNF was evaluated for the CC531, ROS-1 and BN-175 tumor starting at an equal size of 5-6 mm in diameter. In all groups, sham IHPs with perfusion medium only were performed. The graphs in Figure 2 show the growth curves of CC531 tumor (A), ROS-1 (B) and BN-175 (C) after IHP with melphalan, with TNF, with both and after IHP in sham perfused rats. Perfusion with melphalan alone significantly reduced tumor growth rates compared with sham perfused animals in all tumor types. When IHP was performed in BN-175-bearing rats with the combination of melphalan and TNF, a dramatically enhanced tumor response was observed in all animals. This is a significant reduction of mean tumor volume compared with rats perfused with either TNF only or melphalan alone ( $P < 0.005$  and  $< 0.01$ , respectively). In the CC531 or ROS-1 tumors no synergy between TNF and melphalan was observed.

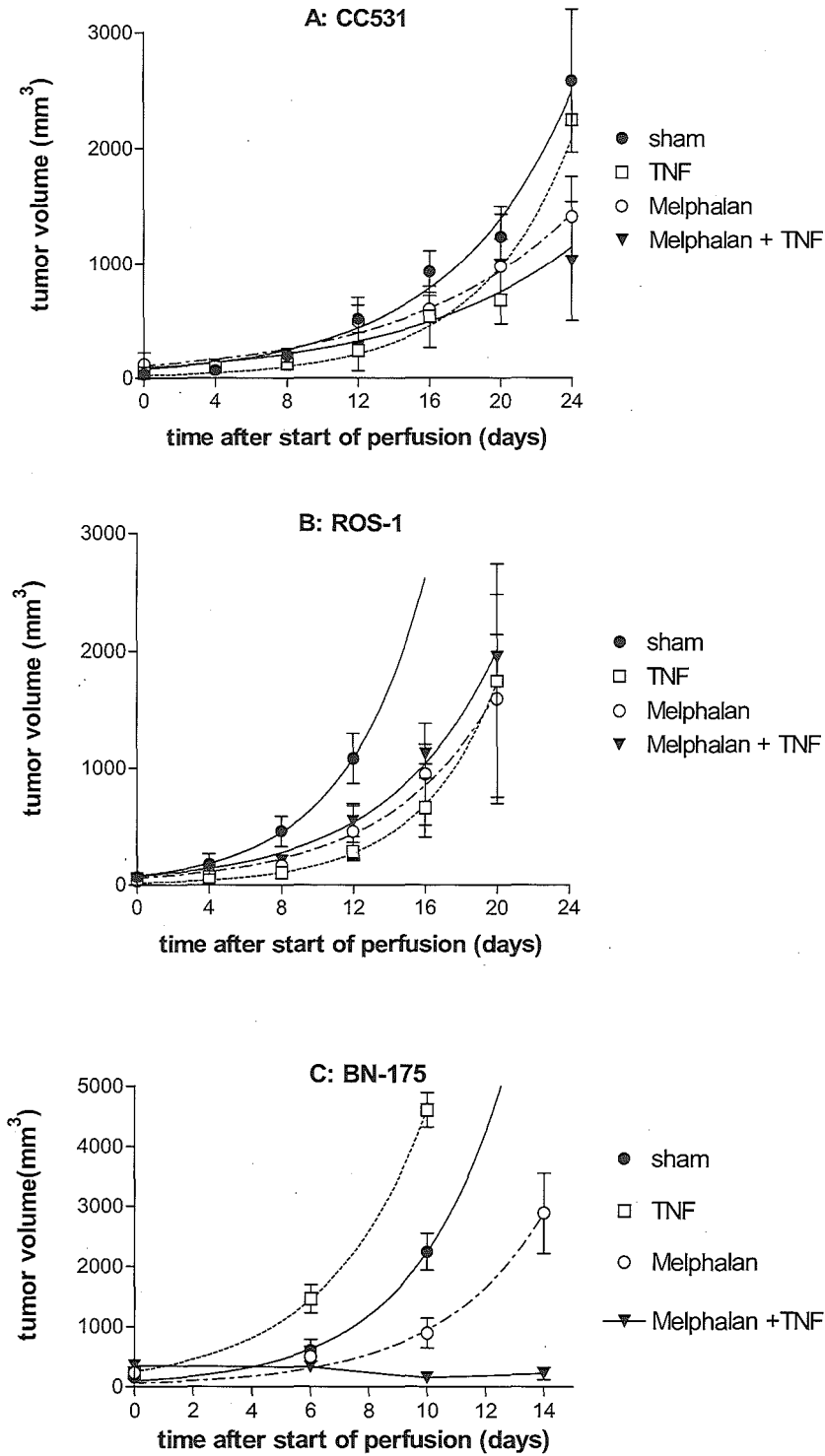


Figure 2 Growth curves of *in vivo* tumors after IHP. Each group contained at least 6 animals. Mean values ( $\pm$ s.e.m.) are shown; (A) CC531, (B) ROS-1, (C) BN-175.

*In vitro cytotoxicity assay*

The effect of TNF on the growth of tumor cells *in vitro* was determined to evaluate whether the synergistic effect of TNF could be related to direct tumor cell toxicity. The calculated concentration of TNF in the perfusate during IHP *in vivo* is approximately 1.5  $\mu\text{g/ml}$ . *In vitro* tumor cells were exposed to a range of TNF concentrations varying from 0 to 10  $\mu\text{g/ml}$ . The growth curves are shown in Figure 3. It is demonstrated that the BN-175 and the CC531 tumor cell line did not show significant sensitivity to TNF. Only the ROS-1 tumor cells were moderately sensitive to TNF, as a growth inhibition of up to 30% at 10  $\mu\text{g/ml}$  was observed.

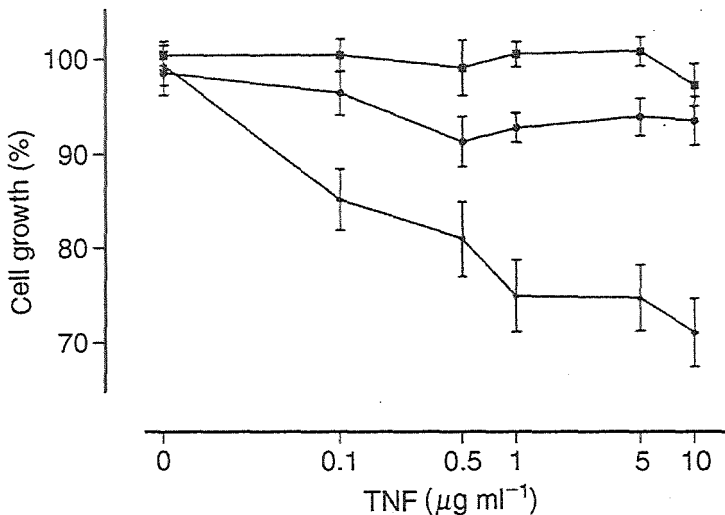


Figure 3 *In vitro* growth curves of tumor cells upon exposure to TNF; CC531 (●), ROS-1 (◆), BN-175 (■). Six independent assays were performed in duplicate for each point on the line. Mean values ( $\pm$ s.e.m.) are shown.

*Melphalan concentration in tumor and liver tissue*

In this perfusion setting, in which the dose of TNF is 20% of the dose used in ILP, an enhanced drug accumulation in tumor tissue might take place as well, as observed after TNF based ILP. In order to investigate this mechanism, melphalan concentrations were measured in tumor and liver tissues after IHP with melphalan both with and without TNF. In the CC531 and ROS-1, tumors, melphalan concentration did not increase significantly after IHP with melphalan and TNF (Figure 4A,B). After IHP with melphalan alone in the BN175 tumor-bearing rats the melphalan concentration in tumor and liver tissue were identical



(Figure 4C). After IHP with TNF however a more than 5-fold increase of melphalan in tumor tissue is measured compared to tumor tissue after IHP without TNF; ( $P < 0.05$ ). This demonstrates that an augmented drug accumulation can also be achieved in the IHP setting when TNF is co administered.

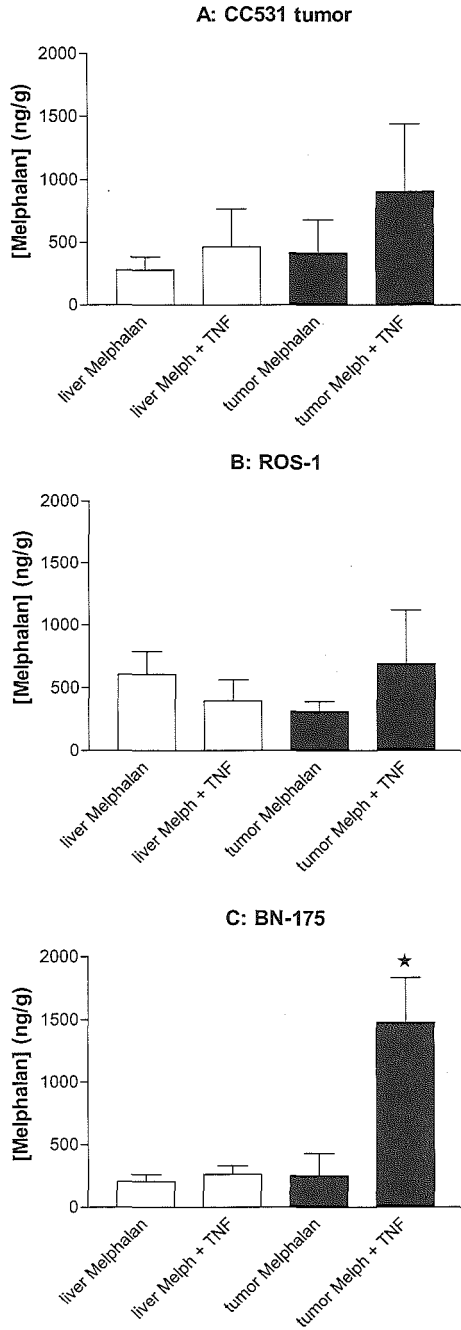


Figure 4 Melphalan concentrations in liver and tumor tissue after IHP with melphalan with or without TNF. Six IHPs were performed per tumor type. Mean values ( $\pm$ s.d.) are shown. (\*= $P < 0.05$  vs tumor melphalan concentration after IHP with melphalan alone); (A) CC531, (B) ROS-1, (C) BN-175.

### Assessment of tumor microvessel density

We already hypothesized that TNF enhances intratumoral concentrations of chemotherapeutics by increasing leakage of tumor vessels. The increased uptake of melphalan might therefore be correlated with the microvessel density (MVD) of the tumor. Quantification of the MVD was performed by immunohistochemical staining of endothelial cells. The microvessel count of the colon carcinoma CC531 and the osteosarcoma ROS-1 were equal (Figure 5). The soft-tissue sarcoma BN-175 however showed a significantly higher MVD than CC531 and ROS-1. These results indicate a relation between vascularity of the tumor and TNF-mediated effects.

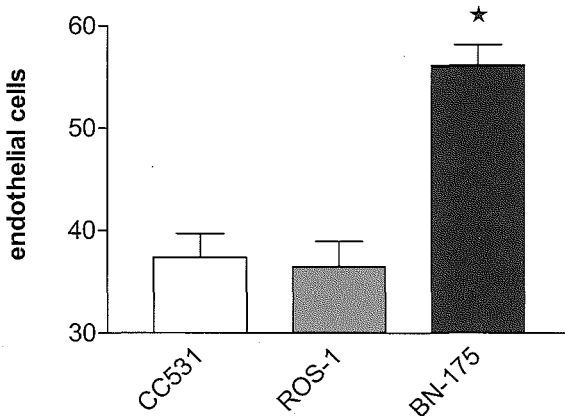


Figure 5 Microvessel count of CC531, ROS-1 and BN-175 tumors. Mean values ( $\pm$ s.e.m.) are shown (\*= $P < 0.001$  vs CC531 and vs ROS-1).

## DISCUSSION

In the present study, we demonstrated that addition of TNF to IHP with melphalan results in strongly improved response rates in a tumor with high vascular density. *In vitro*, no or only minor sensitivity of tumor cells to TNF was found. Even in ROS-1 tumors, which are moderately sensitive to TNF *in vitro*, IHP with TNF alone showed no tumor response. These results strongly indicate that *in vivo* indirect mechanisms mediated by TNF in combination with melphalan determine antitumor effects in IHP. Our results support the notion that this indirect mechanism is the selective destructive effect of TNF on the tumor-associated

vessels, thereby increasing vascular permeability<sup>9,10</sup>. To investigate this hypothesis, the melphalan uptake in liver and tumor tissue was measured after IHP both with and without TNF. Tumor melphalan concentrations were increased in all tumors but varied significantly in a tumor-type-dependent way. Moreover, enhanced uptake of melphalan by healthy liver was not observed. With TNF alone, at most some tumor growth was observed. Only the combination of TNF and melphalan resulted in a complete tumor response in the BN175 tumor. To elucidate this tumor-type-dependent response the MVD of the tumors was determined. We expected higher tumor vascularity in this tumor. Indeed a significantly higher MVD compared to the CC531 and ROS-1 tumors could be demonstrated. This indicates that TNF has specific tumor vascular mediating capacity in this perfusion model, which results in enhanced tumor responses in highly vascularised tumors. As a result of our findings in ILP and now also in IHP, we know that TNF is able to augment the accumulation of melphalan. The presence or lack of TNF-mediated synergy appeared to be independent of tumor size as also in smaller (diameter 3-4 mm) or bigger (7-8 mm) tumors comparable tumor responses were observed (data not shown). We are of the opinion that this observation is essential in understanding and explaining the impressive responses seen in the experiments.

Changes in vascular permeability in patients who underwent IHP with TNF was studied by Alexander *et al*<sup>16</sup>. Vascular permeability was measured by diffusion of radio-labeled <sup>131</sup>I albumin in liver and tumor tissue. A significant increase of the <sup>131</sup>I albumin post-perfusion could be demonstrated compared to <sup>131</sup>I albumin levels measured before perfusion. However, this rise was equal in tumors perfused with or without TNF. A TNF independent mechanism of the increased endothelial permeability was suggested by the authors. However, in the present study, we demonstrate that TNF is effective in increasing vascular permeability for melphalan selectively in tumor tissue. A more important finding is that this effect could only be found in the highly vascularised BN-175 tumor. The results of Alexander *et al*, reported on intratumoral <sup>131</sup>I albumin concentrations were mainly based on colorectal carcinoma liver metastases. In hypovascular rat colon carcinoma, we could also not find an increase of intratumoral melphalan. We therefore hypothesize that the usual hypovascularity of colorectal metastases in

patients explains the lack of TNF-benefit in the experience as described by Alexander in patients, which correlates closely to our observations in our hypovascular colon cancer liver metastases model in rats.

IHP with melphalan and TNF performed in patients with metastases of ocular melanoma or leiomyosarcoma showed overall response rates of 50-52%<sup>21,22</sup>. Both tumor types are highly vascularized. A prolonged duration of response was found in melanoma patients: 14 months after IHP with TNF vs. 6 months after IHP without TNF (Alexander et al, 2000). After IHP with melphalan both with and without TNF in patients with colorectal liver metastases the mean duration of response was 8-10 months in both groups<sup>16,23,24</sup>. The data we now present and the first reports of IHP in melanoma and sarcoma liver metastases strongly indicate that TNF has therapeutic potential in IHP in these patients. In patients with colorectal liver metastases however, IHP with melphalan alone may well be just as effective as combined with TNF. Therefore we conclude that the level of tumor vasculature of liver metastases forms an indication for the usability of TNF in this setting.

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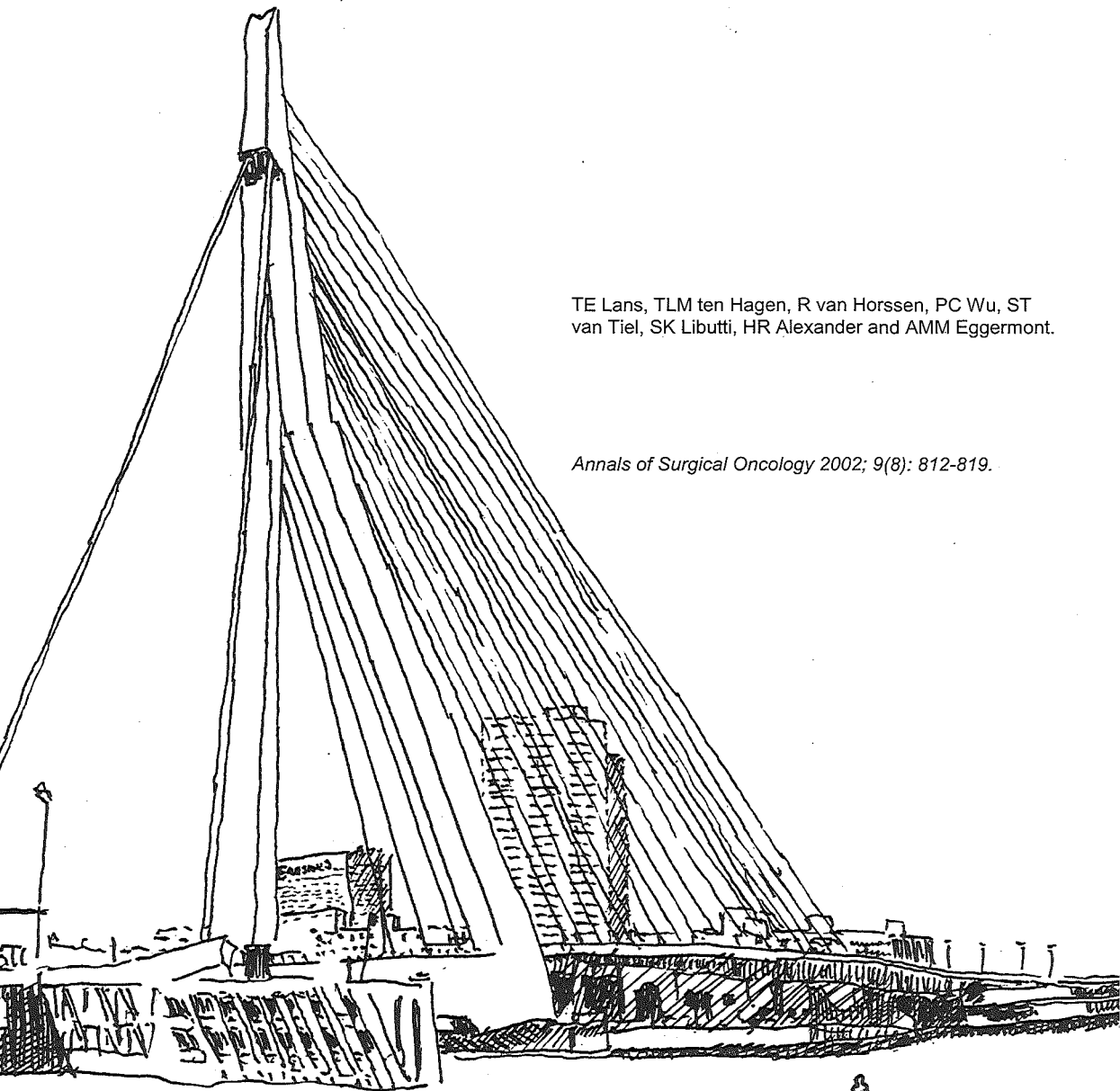


# 6

## IMPROVED ANTITUMOR RESPONSE TO ISOLATED LIMB PERFUSION WITH TNF AFTER UPREGULATION OF EMAP II IN SOFT TISSUE SARCOMA

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## ABSTRACT

**Introduction:** Tumor Necrosis Factor (TNF) is a proinflammatory cytokine with potent antitumor activity. Its clinical use in cancer treatment is limited by considerable toxicity after systemic administration and is currently confined to isolated limb and organ perfusion settings. A better understanding of the mechanisms of the TNF-induced antitumor effect may provide valuable insight into how its clinical use in cancer treatment could be expanded. The novel tumor-derived cytokine, Endothelial Monocyte-Activating Polypeptide II (EMAP II) has been demonstrated to render a TNF-resistant tumor sensitive to TNF therapy.

**Methods:** We demonstrate here that *in vitro* stable transfection of rat sarcoma cells leads to an upregulation of endogenous pro-EMAP II in these tumor cells. This gene transfer induced sensitivity to subsequent regional TNF treatment by means of an isolated limb perfusion (ILP) in rats. To confirm that this was indeed an EMAP II effect, tumor bearing rats were pre-treated with an intravenous injection of recombinant EMAP II followed by an ILP with TNF.

**Results:** These experiments resulted in a comparable tumor response as was observed in the rats undergoing EMAP II gene therapy followed by TNF ILP.

**Conclusions:** These results indicate that methods to introduce or upregulate EMAP II in tumors may lead to an increased applicability of TNF in clinical treatment strategies.

## INTRODUCTION

Studies of the antitumor activity of systemically administered TNF in humans have been hampered by severe toxicity at doses of the cytokine which are too low to cause a sufficient antitumor response<sup>1</sup>. This situation changed when TNF was applied in the isolated limb perfusion (ILP) setting. The efficacy of the application of TNF in combination with cytostatic agents in ILP for the treatment of patients with in transit melanoma metastases or locally advanced soft tissue sarcomas has now been well established<sup>1,2,3</sup>. This treatment can be used as a limb-sparing neoadjuvant therapy when followed by local excision of residual tumor. TNF became a registered cancer drug in Europe based on results obtained in multicenter studies in which clinical response rates of 75% and limb salvage rates of 71% were observed in 196 patients previously classified as having unresectable tumors manageable only by amputation. Approval for the use of TNF in the setting of an isolated limb perfusion for locally advanced extremity grade II-III soft tissue sarcomas (STS) was given by the EMEA<sup>4</sup>. However in 25% of all patients an antitumor effect sufficient for limb preservation can not be achieved. The answer as to why this group of patients is not responding is not known.

There is evidence that the mechanism of TNF does not act on tumor cells directly but acts indirectly by destruction of tumor associated endothelium<sup>5</sup>. This destruction may eventually lead to death of tumor cells when used in combination with an antitumor drug. The antitumor effects after TNF-containing ILPs can be extremely rapid, indicating that the TNF-mediated collapse of the tumor vascular bed may play an essential role in the antitumor mechanism. The selective destructive effects of a TNF ILP on tumor associated vessels have been illustrated in the clinic by means of pre- and post perfusion angiography<sup>1</sup>. Severe hemorrhagic necrosis accompanied with vascular destruction was observed after ILP with TNF in combination with melphalan.

Our laboratory has developed isolated limb perfusion models with different tumors in rats to study prerequisites for improving efficacy and to study mechanisms of synergy<sup>6,7</sup>. These models closely mimic the clinical setting with respect to response rate and histopathology. The crucial observation has been

that TNF enhances the uptake of melphalan and doxorubicin in the tumors selectively and highly significantly <sup>6</sup>.

EMAP II is a tumor-derived cytokine with pro-inflammatory properties. It induces procoagulant activity on the surface of endothelial cells and monocytes/macrophages *in vitro*, as well as upregulation of E- and P-selectin expression and the ability to upregulate TNF receptor-1 expression on tumor vasculature. Furthermore EMAP II induces apoptosis and has antiangiogenic effects<sup>1</sup>. These data led us to hypothesize that EMAP II could be of additional benefit in the TNF perfusion circuit. Wu *et al.*<sup>8</sup> defined a way to upregulate EMAP II production by means of retroviral transfection in a melanoma cell line. We used this method of transfection on a TNF-resistant soft tissue sarcoma. Here we describe the application of the sensitizing gene therapy and subsequent regional TNF treatment and its effects on *in vivo* tumor growth. Since EMAP II can have additional benefits in the long-term anticancer treatment through its antiangiogenic effect (e.g. by preventing neovasculature and outgrowth of metastases), and is toxic when injected systemically, a long lasting and local production of EMAP II in a tumor should be favorable. This can be maintained after retroviral transfection and subsequent upregulation of the protein synthesis by tumor cells.

It is our goal to identify factors of TNF resistance or sensitivity and the potential role of EMAP II in this.

## **MATERIALS AND METHODS**

### *Animals*

Inbred male Brown Norway rats, weighing 200 to 300 grams, were obtained from Harlan-CPB, Austerlitz, the Netherlands. All animals were kept at standard laboratory conditions and were fed a standard laboratory diet (Hope Farms, Woerden, the Netherlands). The experimental protocols adhered to the rules laid down in the "Dutch Animal Experimentation Act" (1977) and the published "Guidelines on the protection of Experimental Animals" by the council of the EC (1986). All animal studies were done in accordance to protocols approved by the Animal Care Committee of the Erasmus University of Rotterdam, the Netherlands.

### *Tumor cell lines*

BN-175 is a non-immunogenic, rapidly growing soft tissue sarcoma syngeneic in Brown Norway rats, with a tumor doubling time of approximately 2 to 3 days. This tumor was implanted into the flank of donor rats and passaged serially. For this study we used small tumor fragments ( $\pm 1 \text{ mm}^2$ ) that were implanted subcutaneously into the right hind limb just above the ankle. All surgical interventions were performed at a tumor diameter between 5 and 10 mm, at least 7 days after implantation. Rats were sacrificed when tumor diameter exceeded 25 mm.

The HUVECs used were obtained from BioWhittaker Europe and consisted of pooled cells by multiple donors.

### *Drugs*

Recombinant human Tumor Necrosis Factor- $\alpha$  (hTNF;  $4.9\text{-}5.8 \times 10^7$  units/mg), recombinant human EMAP II (hEMAP II;  $3.85 \mu\text{g/ml}$ ) and antibodies directed to hEMAP II were provided as a kind gift by Dr. G. Adolf (Boehringer Ingelheim GmbH, Vienna, Germany).

### *Transfection of BN-175 tumor cells with the EMAP II gene*

Viral supernatant from the Gibbon Ape ecotropic packaging cell line PG-WU was used to transfect the BN-175 tumor cell line. Tumors were grown in 6-well plates to approximately 40% confluence. The viral supernatant was then collected and filtered across a 0.45 micron low protein-binding membrane (Corning Costar, Cambridge, MA) and polybrene (Sequa Brene; Sigma) was added in a concentration of  $1 \mu\text{l/ml}$  supernatant. Tumor cells were washed twice with PBS and incubated for 6 hours on 3 subsequent days in an incubator at  $37^\circ \text{C}$  and 5%  $\text{CO}_2$ . In between incubation with viral supernatant cells were given normal medium to recover. After 5 to 7 days the transfected tumor cells (named BN-E) were placed into  $75 \text{ cm}^2$  flasks and expanded under neomycin selection at  $800 \mu\text{g/ml}$ .

### *Detection of incorporation of hEMAP- in genomic DNA of rat tumor cells by PCR*

Transfected tumor cells growing in  $800 \mu\text{g/ml}$  neomycin were trypsinated and pelleted. DNA was extracted using the Qlamp DNA mini kit (Qiagen Inc.) and

amplified by PCR using primers specific for both a part of the EMAP II insert and the downstream retroviral IRES region. For EMAP the following primers were used: 5'-AATCGGATGGTGATTTTACTTTGTA-3' (EF-1) as the both times as the forward primer and backward: 5'-CATTTTATTTGATTCCACTGTTGC-3' (ER-1) with a PCR product of 333 bp for the EMAP part, and backward for IRES and EMAP: 5'-GAATGCTCGTCAAGAAGACAG-3' (ER-2), providing a 577 bp PCR product. PCR amplification was performed under the following conditions: 5 minutes at 95°C, 35 cycles (94°C for 45 seconds, 55°C for 45 seconds, 72°C for 60 seconds), and 7 minutes at 72°C, soak at 4°C. Amplified PCR-products were subjected to gel electrophoresis on a 1% agarose gel containing ethidium bromide.

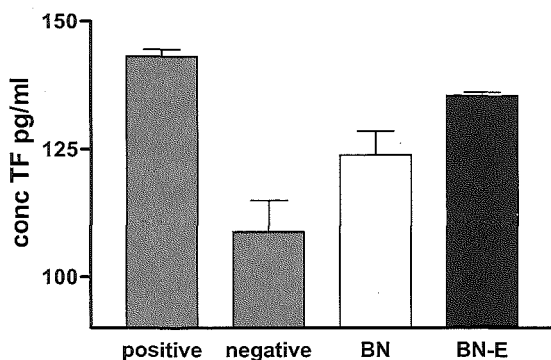
#### *Western blot analysis of transfected tumor cells for EMAP*

After transfection of the tumor cells, cells were harvested, washed and centrifuged. Cell pallets were lysed at 4°C in a buffer containing 50 mM TrisCl (pH 8.0); 150 mM NaCl; 0.02% Na-azide; 0.1% SDS; 1 µg/ml aprotinine; 5% Na-deoxycholate; 1 % Nonidet P-40; DNase I, 10 ng/ml and 100 µg/ml PMSF for 10 min. Samples were vortexed and debris was pelleted by centrifugation at 15000 G for 10 minutes. Supernatant protein content was determined by BCA protein assay (Pierce) and a final loading concentration of 120 µg per sample was used. Lysates were mixed with Laemmli buffer containing 5% β-mercaptoethanol, boiled for 5 minutes and electrophoresed on a 15% Acrylamide-gel using SDS-PAGE. The membranes were subsequently blocked for non-specific binding overnight at 4 °C with PBS/ 5% nonfat dry milk/ 0.05% tween and incubated for 2 hours with EMAP II polyclonal rabbit antiserum (diluted 1:2000 in PBS/ 5% nonfat dry milk/ 0.05% tween). After washing thoroughly, the membranes were incubated for 1 hour with biotinylated goat-anti-rabbit IgG antiserum (diluted 1:3000 in PBS/ 5% nonfat dry milk/ 0.05% tween). Membranes were washed again and incubated with alkaline-phosphatase labeled streptavidine and developed with enhanced chemiluminescence by adding BCIP/NBT.

#### *Coagulation Assay*

Conditioned medium from wild-type BN-175 and BN-E tumor cell lines was tested for procoagulant activity using a two-stage coagulation assay. HUVECs were

sarcoma, it was shown that only when TNF was combined with melphalan or doxorubicin a tumor response could be reached. In comparison to the clinical setting, our rat model demonstrated partial or complete regression of the tumor in approximately 75% of rats receiving an ILP with melphalan plus TNF. We used the BN-175 soft tissue sarcoma to set up an experimental environment where we could improve the response rate to TNF.

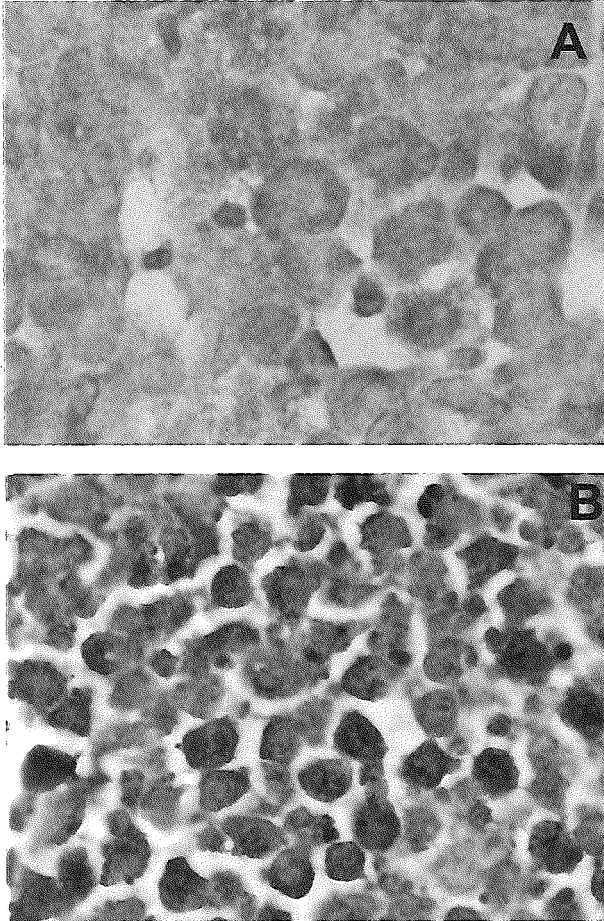


*Figure 2* Tissue Factor Assay. Lane 1 positive control, medium of cells treated with 1  $\mu$ g TNF- $\alpha$ ; Lane 2 medium of untreated cells; Lane 3 wild-type tumor medium; Lane 4 transfected tumor medium.

Briefly, small fragments (3-5 mm) of tumor were implanted subcutaneously into the right hind limb of the rat. About 7 to 8 days after implantation tumors reached an average diameter of 8-12 mm when treatment was started. Animals were anaesthetized with Hypnorm (Janssen Pharmaceutica, Tilburg, the Netherlands) and Ketamine (Apharmo BV, Arnhem, the Netherlands). Heparin (50 IU) was injected intravenously to prevent coagulation. To keep the rat's hind limb at a constant temperature, a warm water mattress was applied. Temperature was measured with a temperature probe on the skin covering the tumor and was maintained between 38 and 39 °C. The femoral artery and vein were cannulated with silastic tubing (0.012 inch ID, 0.025 inch OD; 0.025 inch ID, 0.047 inch OD respectively, Dow Corning, Michigan, USA). Collaterals were occluded by a groin tourniquet and time of isolation started when the tourniquet was tightened. An oxygenation reservoir and a roller pump were included into the circuit. The perfusion solution consisted of 5 ml Haemaccel (Behring Pharma, Amsterdam, the Netherlands) in the sham perfusions, and TNF (50  $\mu$ g) was added to the oxygenation reservoir as a bolus in the treatment group. A roller pump (Watson

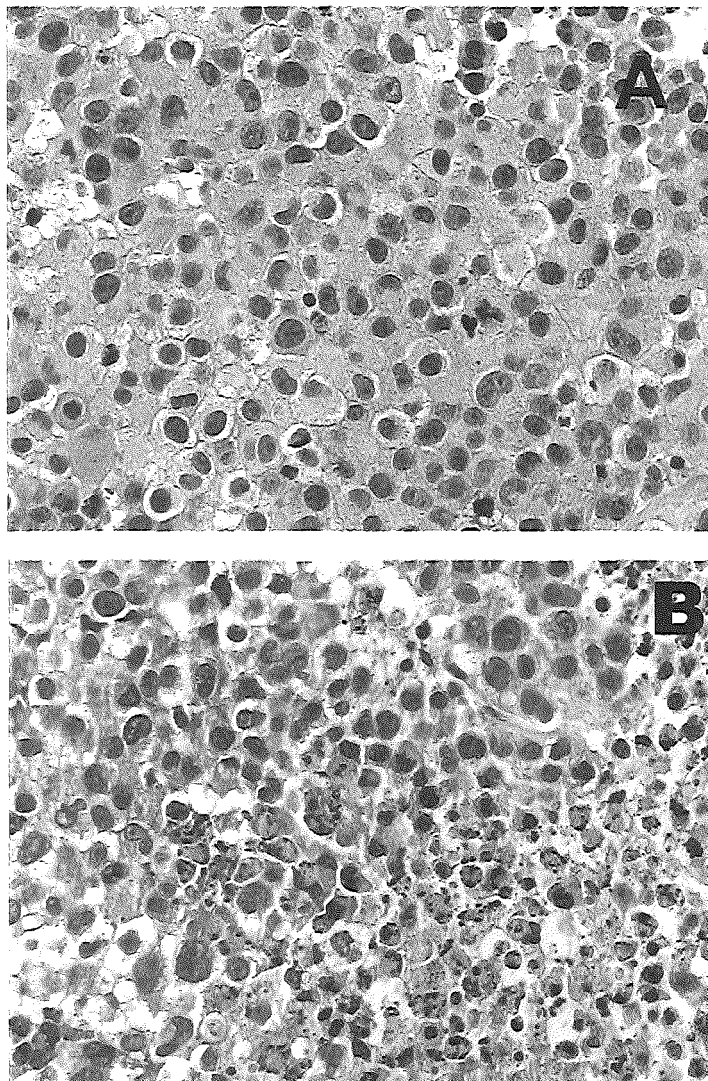


Marlow, Falmouth, UK; type 505 U) recirculated the perfusate at a flow rate of 2.4 ml/min. A washout with 5 ml oxygenated Haemaccel was performed at the end of the perfusion. After the perfusion, the cannulas were removed and the femoral vessels of the perfused limb were ligated. The extensive collateral circulation was capable to restore the blood supply of the perfused leg.



*Figure 3* Immunohistochemical staining with hEMAP II (100x). A) shows the non-transfected tumor. In B) we show a marked upregulation of the intracellular present hEMAP II

The perfusion with the wild-type tumor took place 16 hours after intravenous injection with rEMAP II. In these experiments 50  $\mu$ g recombinant EMAP II, dissolved in 200  $\mu$ l PBS, was injected intravenously. Rats were closely monitored



*Figure 4* Hematoxylin and eosin (HE) stained tumor sections (100 x). A) shows a normal morphology of wild-type (BN) viable tumor cells. In B) the transfected tumor is shown with an aspect of more profound necrosis, disruption of cell structure and smaller cells.

afterwards and moderate but reversible toxicity was observed after IV administration. Subsequent tumor growth was recorded daily by calliper measurement. Tumor volume was calculated as  $0.4 \times (A^2 \times B)$ , where B represents the longest diameter and A the diameter perpendicular to B.

## RESULTS

### *Transfection of EMAP II gene*

Rat soft tissue sarcoma cells were transfected *in vitro* with retroviral particles containing the P-WU construct (BN-E) and appeared resistant to continuous exposure with 800 µg/ml neomycin, demonstrating effective transfection with the construct (data not shown). After 2 weeks of growing in selection media, genomic DNA was extracted from the transfected tumor cell lines. PCR analysis of expression of the EMAP II gene proved positive for the BN-E cell line (data not shown). Western Blot analysis of 120 µg protein of cell lysate of wild-type and transfected tumor cells, compared to 100 ng rEMAP II, demonstrated a band corresponding to the size of the human precursor EMAP II, approximately 34 kDa (Figure 1). We only show the results with normal growing tumor cells. In our ongoing experiments we would like to demonstrate the generation of active EMAP II (~22 kDa) that was used as a control in these experiments and intermediate cleavage products by inducing apoptosis in the tumor cells. However this expression seems to be very unstable, transient and shows tremendous variation.

### *Coagulation Assay*

We wanted to test whether the EMAP II transfected cell line, BN-E, produced functional protein. We used a functional bioassay of EMAP II activity based on the induction of tissue factor expression on human endothelial cells. HUVECs were treated with conditioned medium of transfected tumor cells. As shown in Figure 2, a 6 hour exposure of HUVECs to medium conditioned by tumor transfected with EMAP II (BN-E) induced a 20 % increase in the production of tissue factor by these cells, as compared to treatment with conditioned medium from wild-type BN or medium alone.

### *Immunohistochemical staining of hEMAP in rat tumor tissue*

The tumor model used for our *in vivo* studies was generated by subcutaneous injection of  $2 \times 10^6$  transfected tumor cells in the hind limb of a syngeneic Brown Norway rat. Tumors were grown to an average diameter of 8-12 mm and we observed a growth rate corresponding to a wild-type tumor. As shown in Figure 3,

cells stained positive for EMAP II as compared to controls. On the H&E staining (Figure 4) the morphology of transfected tumor is altered with a tendency to more apoptotic cells. This however was not reflected in the growth rate of the BN-E. We regard this as apoptosis being induced by the increased EMAP II production of this tumor.

*Isolated limb perfusion in rats preceded by rEMAP II injection.*

50 µg rEMAP II was injected intravenously in rats 16 hours prior to TNF perfusion. Rats showed a moderate toxic response immediately after injection nevertheless this was reversible within 4 hours. Figure 6 shows the tumor volume of animals treated with TNF perfusion after pre-treatment with rEMAP II. Sham perfusions after EMAP II injection were also performed. From the second day a significant difference in tumor volume was observed in TNF treated animals bearing the BN-E versus wild-type tumor. Tumors treated with sham perfusion or TNF-ILP alone continued to grow, the rEMAP II pre treated animals showed a delay in tumor growth after TNF-ILP. This difference was observed until the ninth day, when both groups started to develop the same growth rate. Both wild-type and BN-E tumor showed the same growth rate in the presence of systemically administered rEMAP II alone.

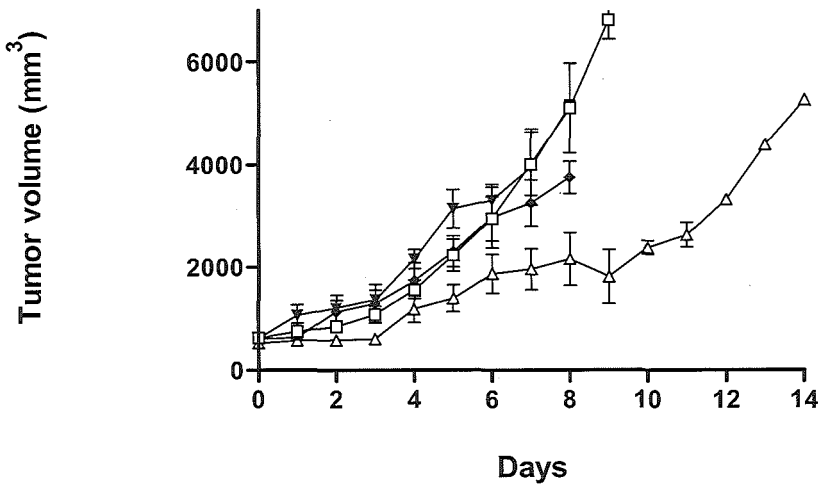


Figure 5 Growth curves of BN tumor after ILP with sham (■) n=5 and TNF (◆) n=7; BN-E tumor after ILP with sham (□) n=7 and TNF (Δ) n=5.

## DISCUSSION

The results presented in this study show that transfection with EMAP II of the TNF resistant tumor BN-175 will render this tumor TNF sensitive and responsive to ILP treatment with TNF. This approach could enhance efficacy in the clinical setting where 25% of patients with locally advanced limb threatening extremity soft tissue sarcoma do not respond to TNF + chemotherapy in the ILP setting for limb salvage to be achieved.

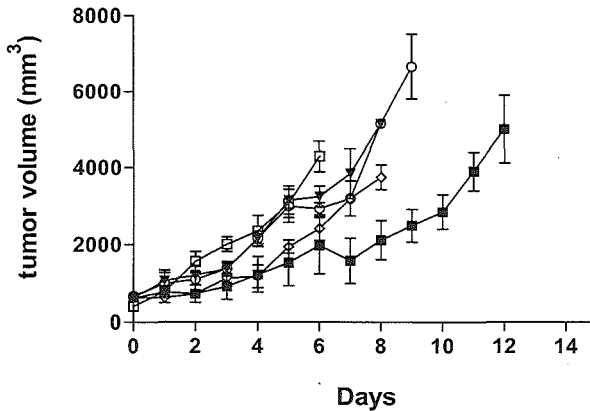


Figure 6 Results ILP in wild-type BN tumor. Control tumor (□) n=6, sham perfusion (▼) n=5, TNF (◇) n=7, EMAP II followed by sham perfusion (○), and ILP with EMAP II + TNF (■) n=6.

By introducing the isolated perfusion as a mode to apply TNF in patients we have been able to achieve effective concentrations of TNF in the tumor region. Antitumor effects after TNF containing ILPs occur extremely rapidly. This indicates that also in humans the TNF mediated collapse of the tumor vascular bed plays an essential role in the antitumor mechanism as previously demonstrated in clinical studies by pre and post perfusion angiography<sup>11</sup>. Moreover in studies with magnetic resonance spectrometry in sarcoma patients we have clearly shown that the metabolic shut down of the tumor is virtually complete within 16 hours after the perfusion, confirming the likelihood of TNF mediating its most important effects on the vasculature of the tumor<sup>11</sup>. At the histopathological level these intravascular effects such as thrombocyte aggregation, erythrosthesis, endothelial and vascular destruction have been described for the early stage changes after ILP which may resemble closely observations made in experimental tumor systems<sup>11</sup>.

Since TNF is known to act on the tumor endothelium a range of cytotoxic agents have been used in combination to cause an additional antitumor effect. After the initial destruction of tumor vessels these agents can exert their toxic effect on the tumor cells.

Here we introduce an approach based on a different mechanism to improve the effect of TNF therapy. After the observation that varying tumors respond to TNF in a distinct manner, a cytokine that contributes to this variation was isolated. This cytokine, EMAP II, is produced by tumors and influences the formation of neovasculature in a number of ways, one of them being the upregulation of TNF-R1 on tumor associated endothelial cells. This is an important finding in the context of the use of TNF in anticancer therapy.

Therefore we investigated whether we could enhance the TNF-sensitivity of a non-responding rat sarcoma *in vivo* by transfecting the tumor with EMAP II, in order to increase its endogenous EMAP II content. EMAP II is a tumor-derived cytokine with pro-inflammatory properties, inducing procoagulant activity on the surface of endothelial cells and monocytes/macrophages *in vitro* as well as up-regulating of E- and P-selectin expression and having the ability to upregulate TNF receptor-1 expression on tumor vasculature<sup>12</sup>. The distribution of EMAP II protein is relatively restricted and usually associated with tissues that display high turnover and high levels of protein synthesis, e.g. tumor tissue<sup>13</sup>. It was first isolated from the conditioned medium of the murine Meth-A fibrosarcoma (Meth. A). This tumor responds well to TNF therapy and investigators found that in the supernatant of this tumor the cytokine EMAP II was excreted. Studies have shown that EMAP II is capable of sensitizing resistant tumors to the antitumor effects of TNF<sup>14,15</sup>. In response to EMAP II exposure, endothelial cells are activated and the expression of TNF-R1 is upregulated, an effect which is known to take place between 16 and 24 hours after exposure to EMAP II<sup>15</sup>. It led us to hypothesize that EMAP II could be of additional benefit in the TNF perfusion circuit. This enabled us to create a more sensitive tumor to TNF therapy, without the toxic side effects of systemic EMAP II application. In our next experiments we injected recombinant hEMAP in rats that were subjected to an isolated limb perfusion system with TNF 16 hours later. In a dose of 50 µg rEMAP per rat combined with 50 µg TNF we saw a significant decrease of growth of the tumors

with an immediate, although moderate and reversible, toxic reaction to the injected EMAP II. These results were comparable with the effect seen in the transfected tumor and suggest that this effect is mediated by EMAP II rather than an effect created by transfection of the tumor or an effect seen because of varying levels of EMAP II receptors.

Isolated limb perfusion is an interesting method for new treatment modalities such as adenoviral-vector mediated gene therapy<sup>16, 17</sup>. In our laboratory we have shown that ILP is the best and most selective method for effective homogeneous transvascular local gene-delivery by using adenoviral vectors. Experiments in our soft tissue sarcoma and osteosarcoma isolated limb perfusion models have clearly shown this by making use of luciferase-marker gene and LacZ gene methodology<sup>18</sup>. Moreover we have shown that ILP with adenoviral-vector gene delivery of the cytokine IL-3 is the sole method in these tumor models to achieve adequate tumor responses, this in contrast other methods such as IV administration, intra-arterial administration or intratumor administration which fail to do so<sup>17</sup>. It demonstrates that ILP is a valuable method to treat advanced limb tumors and to develop new treatment modalities.

The possibility of manipulating the sensitivity for TNF in a tumor *in vivo* also creates an opportunity for systemic application of TNF in future settings. If non-invasive vectors can increase tumor sensitivity such that the TNF dose can be lowered we may be able to save patients the invasive perfusion procedure in the future, and may be able to administer TNF systemically in patients.

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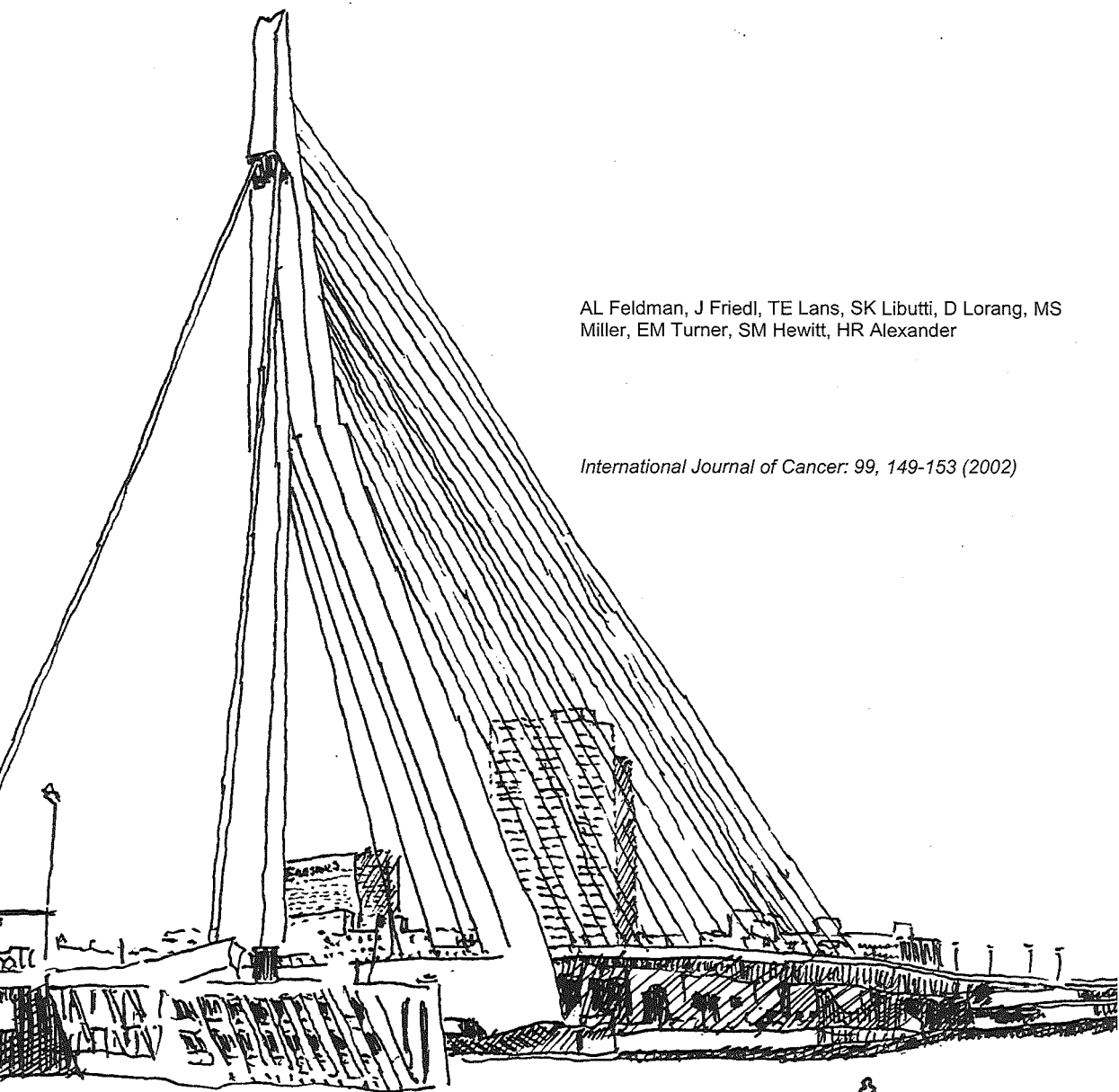


# 7

## RETROVIRAL GENE TRANSFER OF INTERFERON-INDUCIBLE PROTEIN 10 INHIBITS GROWTH OF HUMAN MELANOMA XENOGRAFTS

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## ABSTRACT

**Background:** Interferon-inducible protein 10 (IP-10) is an immunomodulatory chemokine recently recognized to have potent antiangiogenic activity *in vivo*. Due to difficulties in the stability, manufacture and chronic administration of recombinant forms of endogenous antiangiogenic proteins, antiangiogenic gene therapy has emerged as a promising new form of cancer treatment.

**Methods:** We retrovirally transduced A375 human melanoma cells with the human IP-10 gene and injected cells subcutaneously into nude mice. IP-10-transduced cells also were mixed with null-transduced cells in varying proportions before injection.

**Results:** *In vivo* growth of IP-10-transduced melanoma cells was markedly diminished compared to parental or null-transduced cells. This growth inhibition was associated with a marked reduction in microvessel density. The degree of growth inhibition of tumors following injection of a mixed population of null- and IP-10-transduced cells was directly associated with the fraction of IP-10-transduced cells present.

**Conclusions:** We conclude that retroviral transduction of human melanoma cells with the IP-10 gene leads to sufficient protein secretion to inhibit angiogenesis and tumor growth. These findings suggest that IP-10 gene therapy might be an effective therapy in patients with cancer.

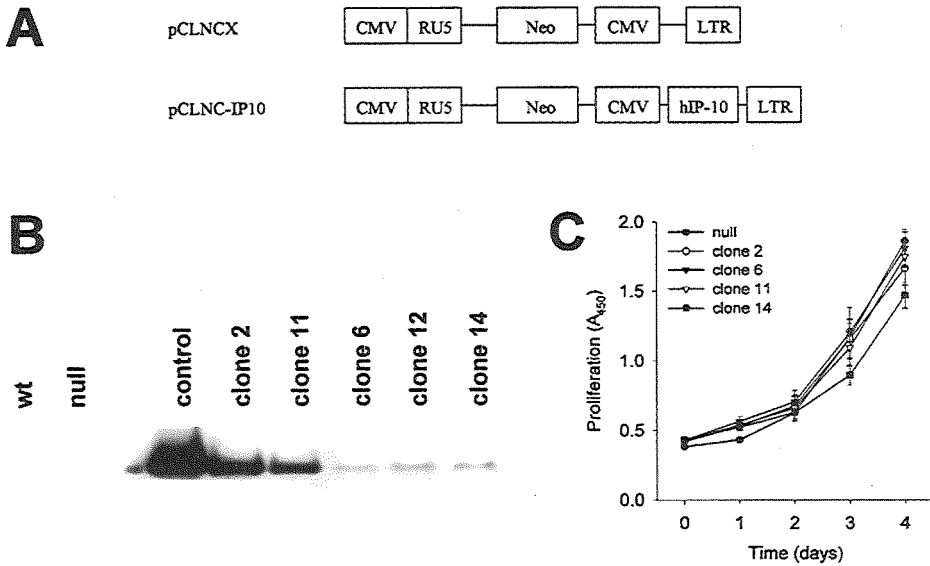
## INTRODUCTION

Because tumors require angiogenesis for sustained growth<sup>1</sup> inhibition of tumor angiogenesis is an attractive new strategy for treating cancer patients. However, *in vitro* instability, manufacturing constraints and pharmacokinetic properties of recombinant forms of endogenous antiangiogenic proteins have led to preclinical investigations of gene therapy approaches to the antiangiogenic treatment of cancer<sup>2</sup>. Interferon-inducible protein 10 (IP-10) is a chemokine that, in addition to being a chemoattractant for stimulated T lymphocytes<sup>3</sup>, has been demonstrated to have antiangiogenic properties *in vivo*<sup>4</sup>. In our study, we evaluated the effects of retroviral IP-10 gene delivery and protein expression on human melanoma xenografts in nude mice.

## MATERIALS AND METHODS

### *Cloning the human IP-10 gene*

Human umbilical vein endothelial cells (Clonetics, San Diego, CA) were plated overnight in EGM-2 medium (Clonetics). Cells then were stimulated with recombinant human interferon-gamma (100 IU/mL; R&D Systems, Minneapolis, MN) for 6 hr and total RNA was isolated using silica gel columns (RNeasy Mini Kit; Quiagen, Valencia, CA) according to the manufacturer's instructions. The RNA was treated with M-MLV reverse transcriptase (Life Technologies, Gaithersburg, MD) to obtain cDNA. The published sequence for human IP-10 (GenBank accession number X02530)<sup>5</sup> was used to derive PCR primers (sense, GATCAAGCTTCCACCATGAATCAAAGTGGGATTCTGAT; antisense, GATCGGATCCTTATTAAGGAGATCTTTTAGACATTTCTT), incorporating a Kozak consensus sequence<sup>6</sup> upstream of the start codon. The IP-10 gene was cloned into the TA cloning vector (TOPO TA Cloning Kit; Invitrogen, Carlsbad, CA) and sequenced (ABI Prism 310 autosequencer; PE Applied Biosystems, Foster City, CA).



**Figure 1** *In vitro* characteristics of IP-10-transduced tumor cells. (a) Human melanoma cells were transduced with retroviruses containing the null vector, pCLNCX, or the vector, pCLNC-IP10, containing the human IP-10 gene. (b) Western blot demonstrated the presence of IP-10 in cell supernatants from IP-10-transduced clones but not in cell supernatants from null-transduced or wild-type (wt) cells. Clones 2 and 11 demonstrated the highest IP-10 expression of those tested. The lane labeled control contained recombinant human IP-10. (c) *In vitro* proliferation was measured by WST-1 colorimetric assay. Most clones, including clones 2 and 11, demonstrated *in vitro* growth curves nearly identical to that of null-transduced cells.

#### *Generation of the pCLNC-IP10 retroviral construct and stable transduction of A375 human melanoma Cells*

The TA/IP-10 cloning vector was digested with BamHI (New England Biolabs, Beverly, MA), filled in using Klenow fragment (Life Technologies) and digested with HindIII. The IP-10 construct was gel purified and cloned into the multiple cloning site of the retroviral vector, pCLNCX<sup>7</sup>, (obtained from Dr. P. Robbins, National Cancer Institute) to generate the vector pCLNC-IP10 (Fig. 1a). Retrovirus production and target cell transduction was carried out as previously described<sup>8</sup>. Briefly, replication-incompetent pseudotyped retroviruses were generated by cotransfecting the packaging cell line 293GP (Dr. P. Robbins) with pCLNC-IP10 or pCLNCX and the plasmid pMD.G (Dr. P. Robbins), containing the G protein gene from vesicular stomatitis virus<sup>9</sup>. The A375 human melanoma cell line (American Type Culture Collection, Manassas, VA) was transduced with pCLNCX- or pCLNC-IP10-derived retrovirus in the presence of hexadimethrine

bromide (8 g/mL; Sigma, St. Louis, MO) and selected in G418 (400 g/mL; Life Technologies). IP-10-transduced clones were selected by plating transduced cells in limiting dilution.

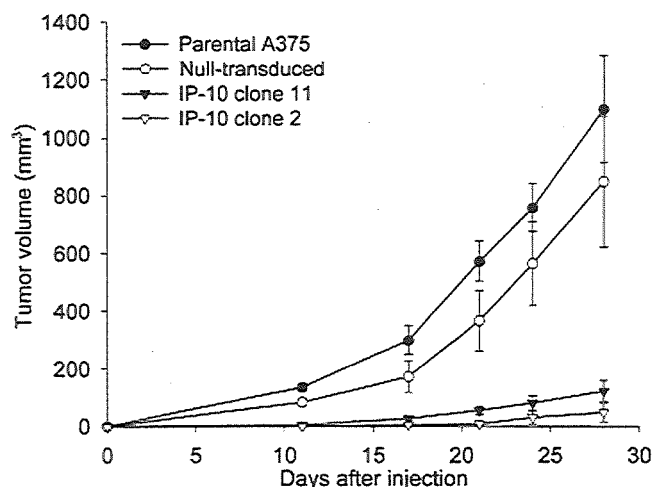


Figure 2 Subcutaneous tumor growth after injection of parental A375 cells, null-transduced cells and IP-10-transduced clones 2 and 11 ( $n=7$  mice per group). The growth of both clones was significantly inhibited compared to the control tumors ( $p=0.0002$ ).

### Western blotting

Clones were plated in 6-well plates at a density of 106/well in complete medium, consisting of Modified Eagle's Medium with 10% fetal calf serum, 4 mM glutamine, 4 mM pyruvate and supplemental vitamins (Biofluids, Rockville, MD). After overnight incubation at 37°C, the medium was replaced with 1.0 mL of fresh medium. After 24 hr incubation, supernatants were harvested and centrifuged at 860g. Equal volumes from the supernatants were mixed with loading buffer and separated by gel electrophoresis under reducing conditions (10% Bis-Tris; NuPAGE, Novex, San Diego, CA). Medium alone was used as a negative control and recombinant human IP-10 (R&D Systems) was used as a positive control. The gel was transferred to a nitrocellulose membrane (Novex) and the membrane was blocked with 5% milk in Tris-buffered saline, pH 7.4 (TBS) for 6 hr at 4°C. The membrane then was probed with goat anti-human IP-10 polyclonal antibody (0.2 g/mL, diluted in TBS with 5% milk; R&D Systems) overnight at 4°C. The membrane was washed in TBST and incubated with HRP-conjugated donkey



anti-goat secondary antibody (diluted 1:10,000 in TBS/1.5% BSA; R&D Systems) for 1 hr. After washing in TBST, detection was performed using the ECL chemoluminescence detection kit (Amersham Pharmacia Biotech, Uppsala Sweden).

#### *Proliferation assay*

IP-10- or null-transduced cells were plated in type I collagen-coated 96-well plates (Biocoat, Becton Dickinson) at a density of 1,000 cells/well and incubated at 37°C in complete medium. Proliferation was analyzed daily for 4 days by WST-1 assay (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. Eight samples in each group were tested. The degree of proliferation was expressed as the absorbance at 450 nm (A450) measured in a Multiskan MCC/340 plate reader (Titertek, Huntsville, AL).

#### *Tumor formation by retrovirally transduced cells*

Animal experiments were conducted according to protocols approved by the NIH Animal Care and Use Committee. Eight-week-old female nude mice (Charles River Laboratories, Wilmington, MA) were injected with  $2 \times 10^6$  parental A375 cells, null-transduced cells, IP-10 clone 2 cells or IP-10 clone 11 cells. Subcutaneous (SQ) injections were administered in the right flank in 100  $\mu$ L PBS. Each group consisted of 7 animals. Tumors were measured in 2 dimensions using calipers at regular intervals by a blinded observer (M.M.) and tumor volumes were calculated according to the formula: volume = width<sup>2</sup>  $\times$  length  $\times$  0.52.

To evaluate the ability of IP-10-transduced cells to inhibit the growth of neighboring non-IP-10-transduced cells (paracrine effect), IP-10 clone 2 cells were combined with null-transduced cells *in vitro* to yield mixtures containing 100%, 50%, 25%, 12.5% and 0% clone 2 cells. Mice were injected as described above with  $2 \times 10^6$  mixed cells. Each group consisted of 8 animals. Tumors were measured as described above.

#### *Immunohistochemistry and histopathologic evaluation*

Tumors were harvested immediately after animal sacrifice by cervical dislocation and fixed in 10% buffered formalin. Tissue blocks were paraffin embedded within

48 hr and sectioned at 5  $\mu$ m. Sections were stained with hematoxylin and eosin (H&E) or antibodies specific for von Willebrand factor (vWF; DAKO, Carpinteria, CA), proliferating cell nuclear antigen (PCNA; PharMingen, San Diego, CA) or caspase-3 (PharMingen). For immunostaining, sections were deparaffinized in xylene and hydrated in decreasing concentrations of ethanol. After endogenous peroxidase activity was blocked using 3% hydrogen peroxide in methanol for 10 min, sections were incubated for 1 hr in blocking solution containing 10% normal goat serum. Sections were incubated in primary antibody at 4°C overnight. Slides then were washed, incubated in biotinylated species-appropriate secondary antibody for 1 hr and exposed to avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). Sections were reacted with 0.06% 3,3 DAB (Sigma) and counterstained with hematoxylin.

H&E and immunostained sections from the tumors with the 4 median volume measurements in each group were analyzed by a pathologist (S.H.) blinded to the identity of the groups. Only good-quality sections with uniform, well-demarcated staining and low background were analyzed. Microvascular density of each tumor was calculated as the mean number of vWF-positive microvessels per high-power field (hpf, 600 $\times$  total magnification) based on a minimum of 5 hpfs. Mitoses similarly were counted as numbers of cells with nuclei staining positively for PCNA per hpf. Apoptosis was evaluated based on the presence of cells staining positively for caspase 3, according to the following scoring system: 1, no apoptotic cells; 2, rare, isolated apoptotic cells; 3, scattered apoptotic cells (1-2/hpf); and 4, clustered or numerous apoptotic cells (>2/hpf).

#### *Statistical analysis*

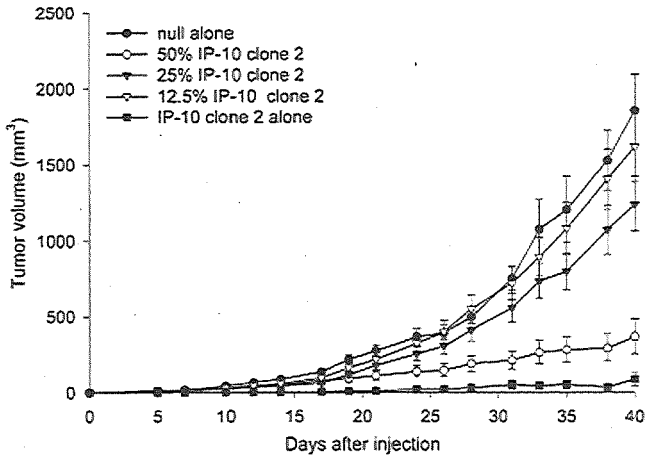
Data are presented as the mean  $\pm$  SE. Comparisons between groups were performed using the Mann-Whitney U test or Kruskal-Wallis test, where appropriate. Two-tailed p-values <0.05 were considered significant.

## **RESULTS**

#### *In vitro characteristics of parental and transduced A375 cells*

Western blotting of supernatants from IP-10-transduced clones revealed bands with electrophoretic mobility equal to that of recombinant human IP-10 (Fig. 1b).

These bands were absent in supernatant from parental or null-transduced A375 cells. Of the clones tested, supernatant samples from clones 2 and 11 demonstrated the densest bands on Western blot. These high-expressing clones demonstrated *in vitro* growth characteristics nearly identical to those of null-transduced cells (Fig. 1c).



**Figure 3** Growth of tumors resulting from injection of mixtures of IP-10-transduced clone 2 and null-transduced cells. Each group of animals received a total of  $2 \times 10^6$  cells, consisting of varying proportions of IP-10-transduced cells ( $n=8$  mice per group). Tumor growth correlated inversely with the fraction of IP-10-transduced cells injected. A mixture containing 50% IP-10-transduced cells demonstrated significant growth inhibition when compared to null-transduced tumors ( $p = 0.003$  at day 40).

#### *In vivo growth of parental and transduced A375 cells*

By 28 days after injection, animals bearing subcutaneous parental or null-transduced A375 melanoma had tumor volumes of  $1,100 \pm 185$  and  $848 \pm 227$  mm<sup>3</sup>, respectively (Fig. 2). Tumor volumes in animals injected with IP-10 clones 2 and 11 were  $47 \pm 31$  and  $124 \pm 37$  mm<sup>3</sup>, respectively ( $p = 0.0002$ , Kruskal-Wallis test). Similar differences in growth were observed in multiple, independent experiments. The difference in growth of clones 2 and 11 was not statistically significant ( $p = 0.09$  at day 28, Mann-Whitney U test).

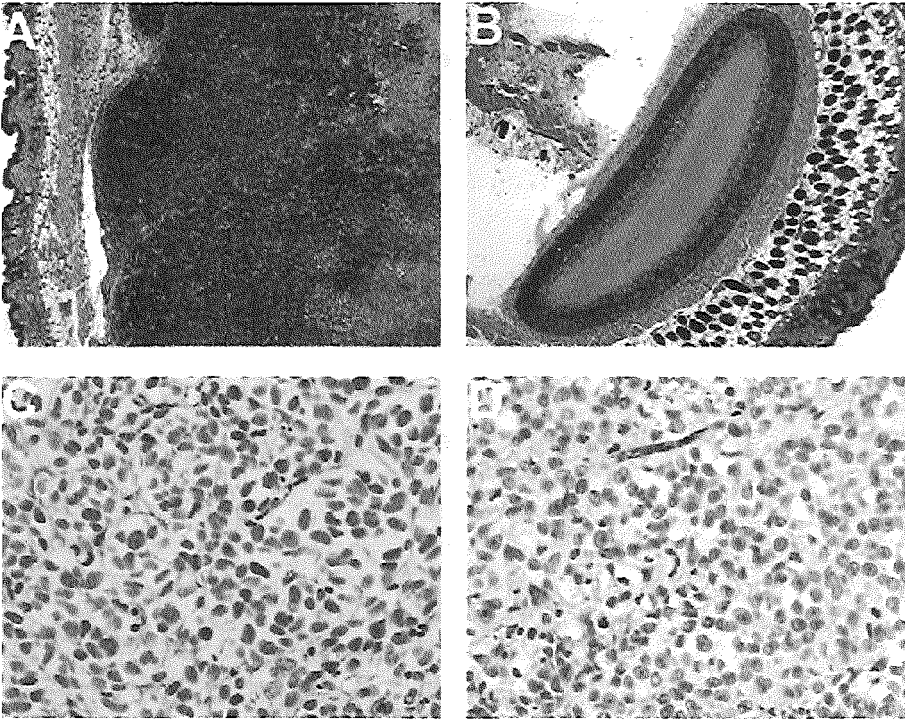
The size of subcutaneous tumors resulting from the injection of a mixed population of cells correlated inversely to the fraction of IP-10-transduced cells present (Fig. 3). By day 40 after injection, tumors consisting of 50% IP-10-transduced cells were significantly smaller than tumors consisting of null-

transduced cells only ( $371 \pm 112 \text{ mm}^3$  vs.  $1241 \pm 178 \text{ mm}^3$ , respectively;  $p = 0.003$ , Mann-Whitney U test). At this time, tumors consisting of IP-10-transduced cells only were  $88 \pm 45 \text{ mm}^3$ .

#### *Histopathologic findings*

H&E-stained sections of control (null-transduced) tumors demonstrated large areas of confluent tumor cells with zones of central necrosis (Fig. 4A). IP-10-transduced tumors were considerably smaller and had confluent central areas of necrosis surrounded by a thin rim of viable tumor cells and a fibrotic capsule (Fig. 4B). Immunohistochemical staining for von Willebrand factor demonstrated well-formed networks of capillaries surrounding nests of tumor cells in the control tumors (Fig. 4C). In contrast, the IP-10-transduced tumors demonstrated only rare, isolated microvessels (Fig. 4D).

Microvessel counts for both clone 2 and clone 11 tumors ( $5.7 \pm 1.6$  and  $4.2 \pm 0.4$  microvessels/hpf, respectively) were significantly lower than those for null-transduced tumors ( $65.6 \pm 9.2$  microvessels/hpf;  $p < 0.05$ ) (Fig. 5a). Similarly, mitotic counts for clone 2 and clone 11 tumors ( $3.1 \pm 0.5$  and  $2.1 \pm 1.2$  mitoses/hpf, respectively) were significantly lower than those for null-transduced tumors ( $6.3 \pm 0.9$  mitoses/hpf;  $p < 0.05$ ). Apoptosis scores based on caspase-3 immunostaining did not differ between IP-10-transduced and control tumors (mean score, 1.5 in each group).

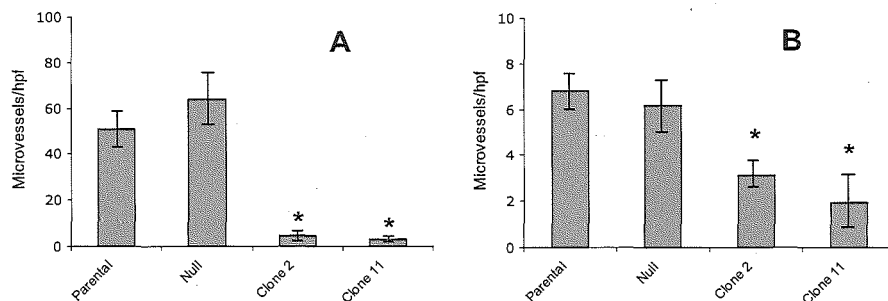


**Figure 4** Histologic characteristics of null-transduced and IP-10-transduced melanoma xenografts. (A) H&E-stained control tumors showed large areas of confluent tumor cells, with zones of necrosis. (B) At the same magnification, IP-10-transduced tumors appeared markedly smaller with central areas of necrosis surrounded by a thin rim of viable tumor cells. (C) Staining for von Willebrand factor (brown reaction product) revealed well-formed networks of capillaries surrounding nests of tumor cells in the control tumors. (D) At the same magnification, IP-10-transduced tumors revealed only occasional, isolated microvessels. Original magnification  $\times 50$  (A,B);  $\times 400$  (C,D). [Normal View 100K | Magnified View 230K]

## DISCUSSION

The demonstration that tumors require neoangiogenesis for sustained growth<sup>1</sup> has prompted intense investigation into the inhibition of tumor angiogenesis as a strategy for treating cancer patients. More than 40 endogenous inhibitors of angiogenesis have been described<sup>2</sup>. One of these, interferon-inducible protein 10 (IP-10), was first described as a chemokine induced by interferon- (IFN-) in U937 lymphoma cells<sup>5</sup>. IP-10 subsequently was shown to demonstrate thymus-dependent antitumor properties<sup>10</sup> and to be a chemoattractant for monocytes and T lymphocytes<sup>3</sup>. Studies in athymic mice<sup>4</sup> demonstrated that IP-10 inhibited

angiogenesis *in vivo*, despite lacking an effect in conventional *in vitro* assays of endothelial cell growth, attachment and migration.



**Figure 5** Microvessel and mitotic counts in control and IP-10-transduced tumors. (A) Microvessel counts in IP-10-transduced clone 2 and clone 11 tumors were markedly less than those in control tumors, based on von Willebrand factor immunostaining (Fig. 4C,D). (B) Similarly, mitotic counts in the IP-10-transduced tumors were significantly lower than in control tumors, based on immunostaining for proliferating cell nuclear antigen (PCNA). \*,  $p < 0.05$  vs. null-transduced tumors. [Normal View 100K | Magnified View 230K]

Previous preclinical studies of IP-10 gene therapy have investigated the use of adenoviral vectors. Narvaiza *et al.*<sup>11</sup> demonstrated that an adenovirus carrying the IP-10 gene had synergistic immunologic and antitumor effects when combined with an adenovirus carrying the interleukin (IL-) 12 gene; however, the IP-10 vector did not inhibit macroscopic tumor growth when administered alone. Regulier *et al.*<sup>12</sup> demonstrated rejection of B16 melanomas in immunocompetent mice after combined intravenous and intratumoral injection of an adenovirus containing the IP-10 gene but not after intratumoral treatment only.

Advantages of using gene-therapy approaches in the antiangiogenic treatment of cancer include obviating difficulties with the manufacture and *in vitro* stability of recombinant proteins, reducing the need for chronic administration and possibly achieving greater efficacy through continuous elevation of circulating protein levels rather than the peak/trough kinetics of bolus protein administration<sup>2</sup>. Genes such as that for IP-10 are particularly attractive because they confer immunologic antitumor properties as well as antiangiogenic effects in preclinical models. The previously described adenoviral studies demonstrate the potent effects that might be achieved in a clinical setting of IP-10 gene therapy of cancer.

We carried out the present series of experiments with 3 goals. First, we wished to investigate the efficacy of retroviral IP-10 gene delivery, given concerns regarding toxicity associated with systemic delivery of adenovirus in humans. Second, we sought to determine whether antitumor effects could be directly correlated with evidence of antiangiogenic activity in the inhibited lesions, as the distinction between antiangiogenic and immunologic effects in previous gene therapy models has been difficult. Finally, since current gene delivery approaches cannot be expected to result in transduction of 100% of target cells, we investigated whether IP-10 gene delivery could affect neighboring, non-IP-10-transduced tumor growth (paracrine effect).

We selected a human melanoma xenograft model for this series of experiments. Because properties of multifunctional human and murine cytokines may differ, it is important to demonstrate efficacy of the delivery of human genes in human tumor models whenever possible. In addition, melanoma patients are among those most likely to benefit from immunologic antitumor effects in clinical trials<sup>13</sup>. Finally, the use of athymic mice allows the analysis of antiangiogenic effects of gene delivery independent of effects mediated by T lymphocytes<sup>4,10</sup>.

We first demonstrated successful transfer of the IP-10 gene into A375 melanoma cells and secretion of the protein product into the cell supernatant by Western blot. Two clones demonstrating high IP-10 expression were selected for *in vivo* experiments to lessen the probability that observed effects might be due to clonal variation rather than IP-10 itself. Both clones demonstrated marked growth inhibition *in vivo* when compared to parental or null-transduced cells. This effect could not be attributed to differences in *in vitro* growth characteristics. Furthermore, *in vivo* growth inhibition was associated with a potent antiangiogenic effect, assessed by analysis of microvascular density. We therefore conclude that retroviral IP-10 gene transfer has antitumor efficacy associated with inhibition of angiogenesis.

To adapt this model to the clinical setting, 2 general strategies might be used. One would be *in vivo* transduction of tumor cells, which has been demonstrated in preclinical antiangiogenic gene therapy models using injection of retroviral packaging cells<sup>14-17</sup>. This strategy presently cannot be expected to result in 100% transduction efficiency and thus some paracrine effect would be required to expect clinical benefit from such an approach. The other strategy would be to

transduce normal autologous cells *ex vivo*, then adoptively transfer them to the host. This strategy first was safely employed with T lymphocytes in humans<sup>18</sup> and has had antitumor efficacy using fibroblasts in an antiangiogenic model of peritoneal cancer in mice<sup>19</sup>. Success of this strategy would also depend on a paracrine effect to inhibit the growth of nearby disease.

To determine the effect of IP-10 gene transduction on neighboring cells, we injected mixtures of IP-10-transduced and null-transduced cells and observed the resultant tumor growth characteristics. Inhibition of tumor growth increased with increasing fractions of IP-10-transduced cells; tumors consisting of 50% IP-10-transduced cells demonstrated significant growth inhibition sufficient to conclude that gene delivery to every tumor cell is not required for efficacy. It should be noted, however, that the presence of a paracrine effect is not the only condition necessary for translation of retroviral antiangiogenic gene therapy to the clinical setting. For example, if *ex vivo* transduction of autologous cells were used, these transduced cells either would need to produce sufficient gene product to elevate circulating cytokine levels, or would need to be able to target the tumor specifically. In addition, our current understanding of tumor angiogenesis suggests that production of the therapeutic protein would need to persist for prolonged periods of time to be of clinical benefit. Clearly, ongoing investigation of retroviral gene-therapy approaches to improve the amount, duration and tumor specificity of gene expression is warranted.

In conclusion, our data demonstrate that retroviral delivery of the human IP-10 gene results in secretion of protein sufficient to inhibit angiogenesis and tumor growth *in vivo* and that the resultant antitumor effect is conferred to neighboring, non-IP-10-transduced cells. These results suggest that IP-10 gene therapy models utilizing *in vivo* retroviral transduction of tumor cells or adoptive transfer of autologous cells transduced *ex vivo* might be effective in the clinical setting.



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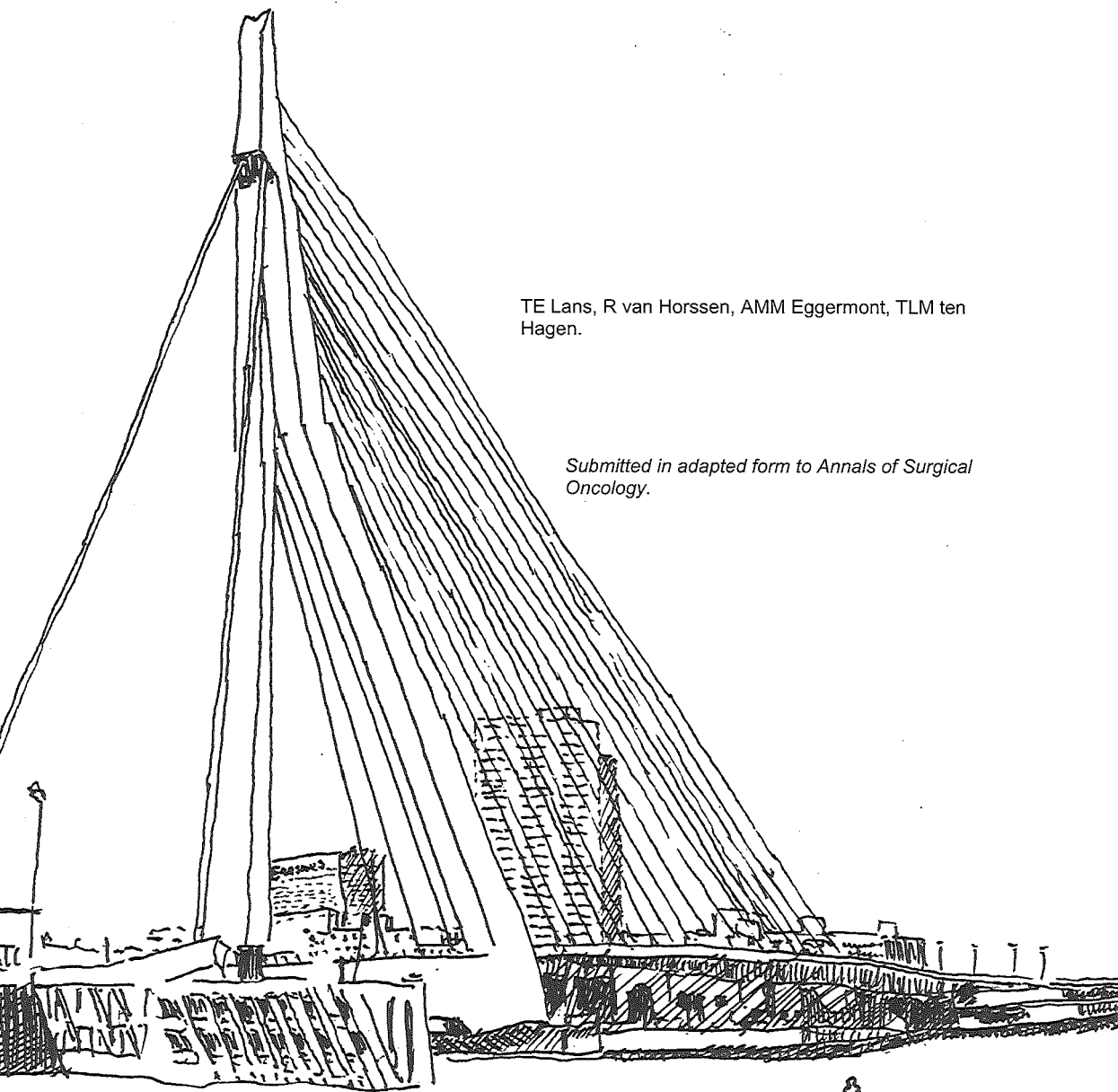


# 8

## INVOLVEMENT OF ENDOTHELIAL MONOCYTE ACTIVATING POLYPEPTIDE II IN TUMOR NECROSIS FACTOR-ALPHA BASED ANTICANCER THERAPY

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## ABSTRACT

In 1990 Clauss *et al.* first reported on a 44-kDa polypeptide, later called Endothelial Monocyte Activating Polypeptide II (EMAP II). This protein was discovered in the supernatant of Meth-A fibrosarcoma cells and had shown to enhance the induction of the procoagulant Tissue Factor (TF) on endothelial cells. Besides upregulation of TF mRNA, EMAP II increases cellular receptors for TNF on endothelial cells, which is likely to enhance the predisposition of tumors to undergo thrombosis and hemorrhagic necrosis, once challenged with TNF. This feature enables EMAP II to upregulate TNF sensitivity of TNF-resistant tumors, an observation of importance in developing new approaches aimed at improving the efficacy of the use of TNF as an anticancer treatment.

We describe the potential additional effects of EMAP II, when used in combination with TNF, with regards to antitumor activity in the Isolated Limb Perfusion (ILP) setting. In addition we describe our experimental data in human sarcoma, which also supports this hypothesis.

## INTRODUCTION

Angiogenesis, formation of new vessels from the endothelium of the preexisting vasculature, is a key element in a large number of normal and pathologic processes<sup>1</sup>. When focusing attention on pathologic processes the contribution of angiogenesis can be demonstrated to play a crucial role in tumor development. Studies from Folkman<sup>2</sup> and other groups<sup>3</sup> have proven that production of pro-angiogenic factors by tumor cells is a prerequisite for tumor growth beyond a certain size, ranging from 0,2 mm diameter (in murine lung metastasis models) to a maximum of 2 mm diameter (in an a-vascular chondrosarcoma in the rat)<sup>4</sup>. Along with this finding it is known that tumors with greater capability to develop neovasculature are more lethal by means of growth velocity and metastatic potential<sup>5-12</sup>. These observations have further strengthened the idea that antiangiogenic compounds may supply an additional treatment to slow down or inhibit the growth of primary solid tumors and their metastasis<sup>13-15</sup>.

Neovascularisation of a tumor requires that a tumor switches to the angiogenic phenotype by changing the balance of angiogenesis inducers and countervailing inhibitors<sup>16</sup>. At least two general mechanisms are recognized: (1) angiogenic activity arises from the tumor cells themselves by releasing angiogenic molecules; (2) angiogenic activity arises from host cells recruited by the tumor (e.g. macrophages) by mobilizing the extra-cellular matrix and requiring concomitant loss of physiological inhibition of endothelial cell proliferation. The mechanism underlying shifts in the balance between all angiogenic regulators remain not fully understood.

Malignant cells communicate with their surrounding tissue and matrix by means of a large variety of signals that they produce. Interleukines, CAM's (Cellular Adhesion Molecules), chemokines, integrines, proteases and receptors can all exert an effect on stroma, tumor matrix, tumor vasculature and pre-existing vasculature. This fast expanding list of pro- and antiangiogenic factors supports the hypothesis that the tumor itself is responsible for the process of angiogenesis. Cytokines from various sources are released in the vicinity of the tumor in response to hypoxia or ischaemia, and can rapidly initiate angiogenesis. Tumors can produce a large number of cytokines<sup>17</sup>, all of them having different functions in the regulatory pathway of angiogenesis. Some have mitogenic effects on

endothelial cells, others have chemotactic activity or induce tube formation or even have multiple functions. Some cytokines are chemotactic for macrophages, infiltrate the tumor and stimulate secretion of more angiogenic cytokines. There is hope that these cytokines will be of major importance in the next decade in the field of antiangiogenic therapy. Therapies based on antiangiogenic strategies aimed at malignancies continue to evolve and clinical trials are being conducted<sup>18</sup>. More than a decade has passed since Clauss<sup>19</sup> first described a unique cytokine that was excreted by certain tumors, called EMAP II by later authors<sup>20</sup>. Here we will discuss the role of this cytokine, its antiangiogenic properties and the possible co-existing impact on vascular damage when combined with TNF leading to destruction of established tumors and inhibition of tumor growth.

## **TNF AND THE ISOLATED LIMB PERFUSION**

TNF is one of the cytokines currently used as an anticancer therapy. The systemic administration of TNF in cancer patients is associated with dose-limiting toxicity already at low and ineffective doses without antitumor effects. The effective delivery of high concentrations of TNF in the clinic was pioneered by Lejeune and Lienard with the application of TNF in isolated limb perfusions in 1988, and led to a report of high complete responses in melanoma and sarcoma patients<sup>21</sup>. In a multi-center European trial, ILP with TNF and melphalan (with<sup>22,23</sup> or without<sup>22</sup> IFN- $\gamma$ ) resulted overall in a 76% response rate and a 71% limb salvage rate in patients with limb-threatening soft-tissue sarcomas<sup>24</sup>. TNF-based ILP has been established as a highly effective new method of induction biochemotherapy in extremity soft tissue sarcomas with a 20-30% complete remission (CR) rate and an approximate 50% Partial Remission (PR) rate. On the basis of these results in the multi-center program TNF was approved and registered in Europe in 1998. In the mean time TNF-based ILP programs for limb salvage have been commenced in more than 30 cancer centers in Europe<sup>22</sup>. High dose TNF destructs tumor vasculature and it increases tumor-selective drug uptake (e.g. melphalan, doxorubicin) 3- to 6-fold<sup>25,26</sup>. This latter observation makes it of critical importance to have as many TNF receptors as possible on the target tumor endothelium. Existing experimental data in animal studies suggest that EMAP II may add to this requirement.

## EMAP II: IDENTIFICATION AND BACKGROUND

Endothelial Monocyte Activating Polypeptide II (EMAP II) is a cytokine first identified as a tumor cell secreted protein when it was isolated from the supernatant of a murine methylcholantrene A (meth-A) fibrosarcoma<sup>27,28</sup>.

Based on the diversity in responses of tumors to TNF together with the fact that the TNF actions seem to be vasculature-mediated, the search for soluble mediators secreted by neoplastic cells was started. Kao *et al.*<sup>27</sup> focused on the Meth-A tumor cells, a tumor sensitive to TNF *in vivo*, but TNF-resistant *in vitro*<sup>29</sup>. By directing a pilot study to possible factors influencing this effect they found 2 polypeptides in the supernatant of Meth-A tumor cells that modulated monocyte function *in vivo*, leading to the acronym EMAP I and II. The latter soon proved to be the most potent in influencing the TNF-mediated thrombosis in tumors<sup>30</sup>.

EMAP II mRNA and the corresponding precursor protein, proEMAP, are constitutively expressed and produced by all cell types analyzed *in vitro*, whereas the mature cytokine is only present in the supernatant of apoptotic cells<sup>31</sup>. EMAP II (or Meth-A factor) is an approximately 18 kDa pro-inflammatory cytokine, synthesized as a precursor protein lacking a conventional secretion signal peptide<sup>28</sup>. Despite the cytokine activity of the mature EMAP II, no sequence homology is found based on amino-acid sequence with any other known cytokine<sup>32</sup>. The sequence of several proEMAP-related proteins however, suggests that the p43 component of the amino acryl-tRNA multienzyme synthetases complex is the precursor of the active mature cytokine<sup>33</sup>. Aminoacyl-tRNA synthetases are proteins that catalyze the activation their cognate amino acids and transfer to the relevant tRNA. This complex consists of a subset of 9 synthases all specific for their own amino acid, together with three auxiliary proteins<sup>34</sup>. One of those three auxiliary proteins turned out to be proEMAP<sup>35</sup>. Wakasugi has shown<sup>36</sup> that human full-length Tyrosyl-tRNA synthetase (TyrRS), which normally resides in the cell cytoplasm, was secreted by a human hematopoietic cell line that had been triggered by serum deprivation. TyrRS is inactive as a cell-signaling molecule but intracellular TyrRS has enzymatic activity and after cleavage into two fragments by extra cellular proteases both fragments surprisingly appear to have retained their cytokine activity. The carboxyl-terminal fragment happens to show some homology with EMAP II, and is able to stimulate



leukocyte and monocyte chemotaxis, to induce myeloperoxidase, and to excite tissue factor and tumor necrosis factor synthesis. The amino-terminal fragment behaves like the cytokine IL-8, yet still retains complete TyrRS activity. This and other findings established the fact that the precursor of EMAP II is a multi-functional protein that assists in protein synthesis in normal cells. Upon apoptosis it is released as functional cytokine, linking cell viability to programmed cell death<sup>37</sup>.

The mechanism of cleavage and secretion of EMAP II has been controversial. Knies *et al.* suggested that the coordinate program of cell death includes activation of a caspase-like activity that initiates the processing of a cytokine responsible for macrophage attraction to the sites of apoptosis<sup>38</sup>. This same group of researchers soon identified with *in vitro* experiments that caspase-7, and to a lesser degree caspase-3, is capable of cleaving proEMAP to a fragment corresponding to mature EMAP II<sup>39</sup>. However Zhang and Schwarz, who could not manage to get the proEMAP cleaved by caspases-3 or -7, speak against these results<sup>40</sup>. They suggest a more important function for EMAP II with regard to induction of apoptosis, and are currently focusing on the protease that can be held responsible for the cleavage. The presence of apoptosis seems to correlate with posttranslational processing of EMAP II, which may explain apoptosis-induced influx and sequestration of leukocytes in the reperfused kidney<sup>41</sup>. More research should be done to point out the exact relation between EMAP II and apoptosis.

## EMAP II: ACTIONS AND FUNCTIONS

Cells undergoing apoptosis are rapidly removed by monocyte-derived macrophages, suggesting a contemporaneous release of factors with leukocyte and monocyte chemotactic activity. EMAP II has shown to be a cytokine that recruits monocytes and macrophages in areas with apoptosis for scavenging apoptotic corpses. This tumor-derived cytokine has potent effects on endothelial cells<sup>42</sup>.

The interest in our lab for EMAP II was sparked by observations of Wu *et al.* that upregulation of EMAP II can render a previous non-responding tumor sensitive to TNF treatment<sup>43</sup>. This research group demonstrated that by over-expressing

EMAP II in a TNF-resistant human melanoma line by retroviral-mediated transfer of EMAP II cDNA, the tumor becomes TNF-sensitive *in vivo*, but not *in vitro*. A possible explanation for this is that TNF acts on the vasculature, which, after being primed by EMAP II, eventuates in an upregulation of TNF-R1 and TF expression on the endothelial cells. This mechanism will not be present *in vitro* cell cultures of tumor cells<sup>44</sup>. TNF acts through two distinct cell-surface receptors of 55 kDa (TNF-R1) and 75 kDa (TNF-R2), which are ubiquitously expressed<sup>45</sup>. The receptors utilize both dependent and shared intracellular signaling pathways to mediate a variety of effects on cells, some of which are receptor specific. Cytotoxic effects of TNF are mediated by TNFR-1, whilst TNF-R2 has proliferative effects, as for example in lymphoid cells<sup>46</sup>. TNF-R1 expression may be associated with the induction of endothelial cell apoptosis by TNF. Therefore, EMAP II produced by tumors may, at least in part, determine *in vivo* sensitivity to TNF by up-regulating TNF-R1 expression on endothelial cells, triggering cell death in the presence of TNF leading to eventual ischaemic necrosis of the tumor. *In vitro* studies of Liu<sup>47</sup> show an EMAP II-like mediated cytotoxicity and apoptosis of corneal endothelial cells. This effect was even more profound after adding cycloheximide, a protein synthesis inhibitor.

An increase of secretion of active EMAP II by a tumor leads also to an upregulation of TF on the surrounding endothelial cells, providing another link between EMAP II and TNF as used in an ILP, where it frequently results in hemorrhagic necrosis of the tumor. This upregulation will lead to an increased pro-coagulant activity, and also a more permeable vasculature. Friedl *et al.* have found another explanation for the effects on tumor vasculature<sup>48</sup>. They demonstrated that TNF only exerts its permeability and procoagulant activity when TF expression on cell surface is combined with extrinsic clotting factors in plasma.

## OUR DATA

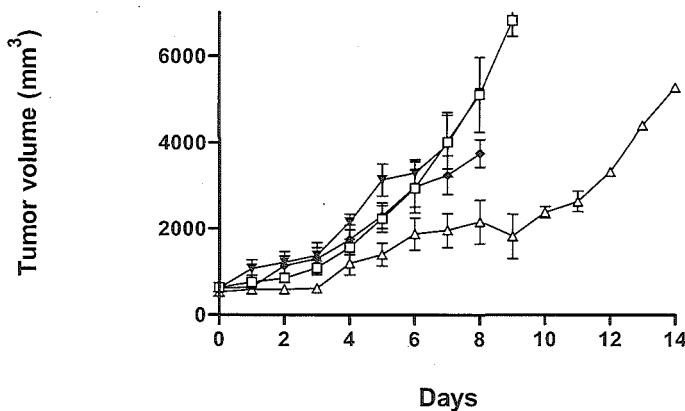
We have demonstrated that *in vitro* stable transfection of rat sarcoma cells led to an upregulation of endogenous pro-EMAP II in these tumor cells. This gene transfer induced sensitivity to subsequent regional TNF treatment by means of an isolated limb perfusion (ILP) in rats. To confirm that this was indeed an EMAP II

effect, tumor bearing rats were pre-treated with an intravenous injection of recombinant EMAP II followed by an ILP with TNF. These experiments resulted in a comparable tumor response as observed in the rats undergoing EMAP II gene therapy followed by TNF ILP<sup>49</sup>.

**Table 1 - Tumor volume (mm<sup>3</sup>) in rats on consecutive days after sham or TNF isolated perfusion.**

	0	2	4	6	8	10	12	14
<b>Wild type</b>								
Sham	622	1210	2156	3299				
TNF	617	1134	1740	2967	3752			
<b>Transfected</b>								
Sham	713	846	1569	2941	5107			
TNF	532	589	1184	1877	2159	2381	3322	5220

Table 1 and Figure 1 show results of TNF ILP in rats bearing the transfected tumor compared to the wild type tumor. Perfusion with Haemaccel alone resulted in progressive disease in all animals. Although ILP with TNF resulted in a slight inhibition of tumor growth of the wild type BN tumor compared with the sham control, there was no significant difference in tumor response. As can be seen in this graph the EMAP II transfected tumor BN-E responded significantly better to ILP with 50 µg TNF. We saw macroscopically a more profound necrosis in the BN-E tumor that led to a significant slower outgrowth of tumor after the perfusion. Even in animals with transfected tumors a small rim of viable tumor cells survived. However as can be seen at day 9, the overall observed response rate still implied a decline in tumor dimension, while profound recurrent tumor growth was demonstrated in all animals from day eleven.



**Figure 1** Growth curves of BN tumor after ILP with sham (■) *n*=5; or TNF (◆) *n*=7; BN-E tumor after ILP with sham (□) *n*=7; or TNF (△) *n*=5.

## EMAP II - POTENTIAL CLINICAL IMPLICATIONS

Pharmacological administration of EMAP II as a neo-adjuvant therapy prior to TNF will not take place before complete elucidation of the mechanism of activation of the mature protein. Since the relative expression of EMAP II likely may correlate with a tumor's sensitivity to TNF based on a dose-dependent increase of TNF receptor on endothelial cells, high EMAP II levels must be obtained before treatment with TNF. Intended effects will be the upregulation of TF on tumor endothelial cells leading to massive thrombosis at the place of the vascular damage. This combined with an increased amount of TNF receptors will intensify the action of administered TNF, and as a result to this necrosis of tumor vasculature an increased port d'entrée for melphalan or other chemokines.

At this moment the approved use of TNF in Europe is restricted to the Isolated Limb Perfusion setting where TNF is combined with melphalan for unresectable soft-tissue sarcomas to facilitate limb salvage<sup>50</sup>. Furthermore TNF is used successfully in this setting treating a great variety of tumors such as melanoma, drug resistant recurrences of bony sarcomas, squamous cell carcinomas, merkel cell carcinomas and others<sup>50</sup>.

All these studies have greatly increased the understanding of the way in which TNF works. As a consequence of its toxicity when administered systemically, techniques like the isolation of limbs and organs have been developed. At present we hope that EMAP II can be valuable in potentiating the effect of TNF. By hypothesizing that a higher EMAP II concentration in the surroundings of a tumor, either by systemic injection, gene therapy, or other modalities, will lead to a profound response to TNF treatment, perhaps a diminished dose of TNF might be enough to induce the same clinical responses. So far this hypothesis can not be validated by clinical data.

We have used immunohistochemistry to analyze the expression of EMAP II in sarcoma of 18 patients that had been treated with TNF and melphalan in an ILP, in order to estimate the predictive value of EMAP II expression. From a large database of patients treated with an ILP with TNF and melphalan we selected two patient groups with identical demographic characteristics and equal grade and histologic classification of their soft tissue sarcomas. Patients in the first group responded with a complete response or partial response, with over 50%

tumor necrosis; patients in the second group responded with progressive disease, no change, or a partial response with less than 25% tumor necrosis. Biopsies from defined morphologic areas of paraffin embedded tumors were analyzed by immunohistochemistry with EMAP II antibody, vascular endothelial growth factor (VEGF) antibody, CD34 antibody as a vascular marker and MIB-1 antibody to determine proliferative indices; these characteristics were compared to patient outcome concerning response rates. A promising good correlation was found between the extent of EMAP II expression in the tumors and the response of the patient. Table 2 demonstrates a showed great diversity between the tumors regarding EMAP II expression, with a preference to a high intensity in the group of patients with a CR, and a much lower expression of EMAP II in the NC group. However throughout these experiments data was published that immunohistochemistry with EMAP II would mark as well pro-EMAP as the mature and active form of EMAP II. This data implied that our initial conclusion could no longer be sustained. Currently we are conducting a prospective study to determine EMAP II expression in tumors by means of Western Blot. With this method we will be able to distinguish between pro- and mature EMAP II and we will be in the position to answer the question. Until then we have no rationale to reject the hypothesis, however clinical evidence still lies ahead of us.

**Table 2 - Score staining intensity**

	Score CR group (n=9)	Score NC Group (n=9)
VEGF	2.8	2.7
EMAP II	3.2	1.5
MIB-1	2.0	2.5
CD34	3.6	3.5

Currently studies are directed to the exact cleavage proteases, the profile of the receptor on endothelium, effects of EMAP II on normal cells and expression patterns of EMAP II in tissue of patients treated with TNF. Also more research needs to be done in order to define possible side effects of EMAP II, since cytokines have shown to provoke numerous.

## SUMMARY

The action of EMAP II in cancer is complex, just as is the case for cytokines such as IL-1 $\beta$  and TNF- $\alpha$ . Endogenous EMAP II chronically produced in the tumor microenvironment enhances the antitumor properties of TNF in an ILP. Moreover EMAP II is a crucial effector molecule in apoptosis, it shows chemotactic properties, and it is capable of upregulation proteins on endothelial cells like TF and TNF-R1. It seems that EMAP II plays a role in stabilizing vascular architecture by maintaining a balance in angiogenic pathways. The angiogenic response is governed by the interaction of angiogenic growth factors and cytokines with specific receptors on the endothelium, as well as the interaction of these cells with their surrounding matrix, which is regulated by matrix-degrading proteases and adhesion molecules such as integrines. A number of agents have been discovered and developed that aim to inhibit angiogenesis and to convert the tumor to a dormant state. EMAP II was identified in the process of unraveling the mechanism of action by which TNF exerts its tumor effect. It was discovered that it was produced in the TNF-sensitive Meth-A tumor. Successive experiments have shown that EMAP II can confer TNF resistant tumors to TNF sensitivity. When missing links are elucidated and some controversies about EMAP II are cleared future studies are justified where EMAP II will be administered as a neoadjuvant or priming therapy, concurrent with or prior to TNF administration. By exploring these routes of multiple-therapy an increased understanding of the balance between proangiogenic and antiangiogenic agents will be obtained. Therapies that have proven to be effective in animal studies will in future studies have to demonstrate whether this is also true in man.

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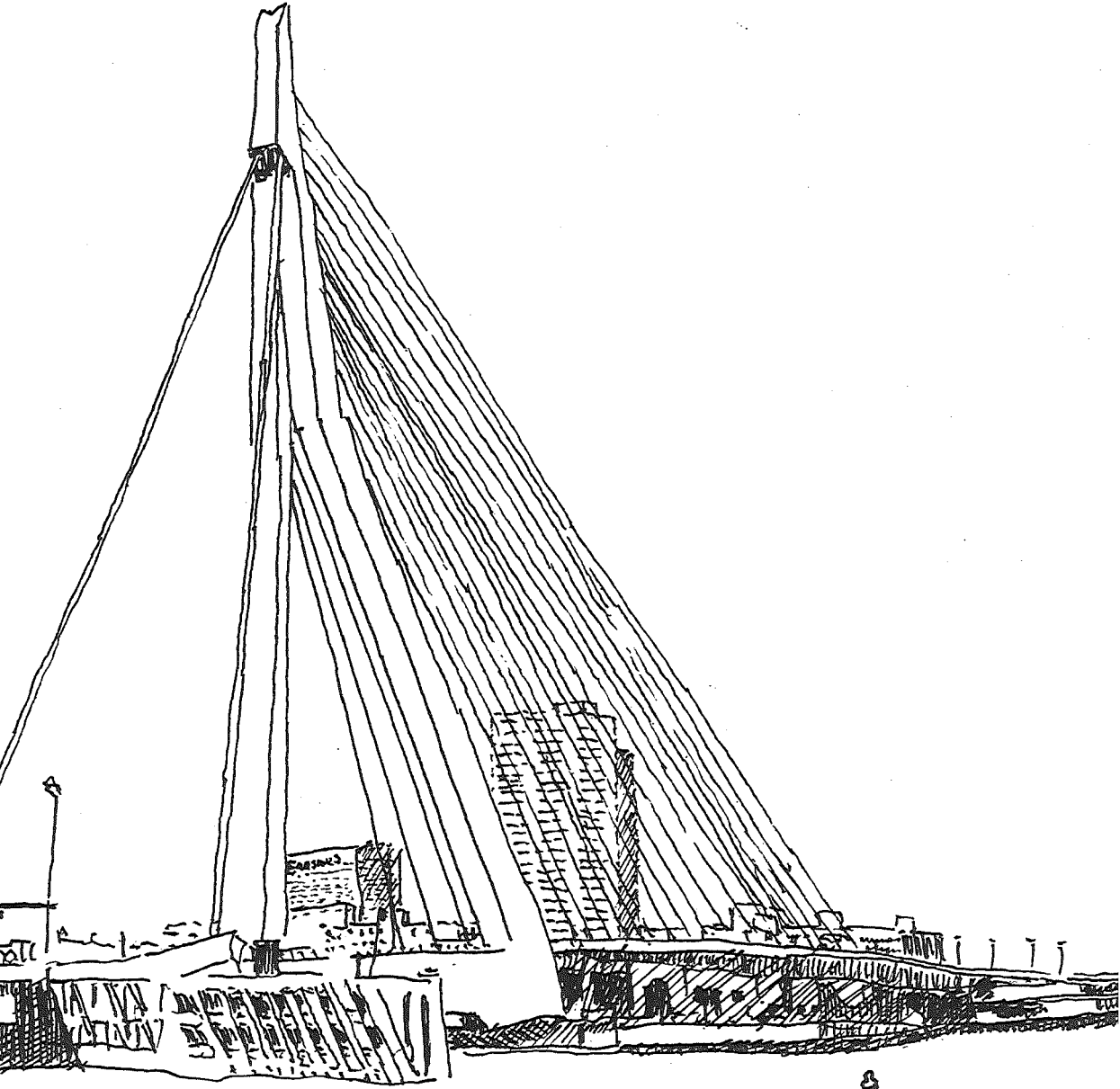


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# 9

## SUMMARY AND CONCLUSIONS



8

H.L.



## SUMMARY

Surgery represents the principal treatment of all resectable tumors. In cases of wide spread local disease or metastasized sarcomas however, surgical treatment provides insufficient results. In those cases chemotherapeutics can be a useful alternative to palliate patients and in specific cases may even lead to full recovery. Tumor vascularisation plays a critical role in both the development and the treatment of cancer. High levels of vascularisation generally have a positive impact on chemotherapeutic results, while in the meantime this drives tumor growth and growth of metastases. The development of new blood vessels – angiogenesis - is regulated by a large number of cytokines. Cytokines are soluble proteins, which under normal circumstances are produced by the mononuclear cells of the immune system. They exert influence on the immune system and regulate it. When produced by tumor cells however, cytokine production can lead to harmful interactions, such as the intensification of angiogenesis, which can stimulate tumor growth.

Effective treatment of solid tumors like sarcomas, as described in this thesis, is hampered by specific tumor properties and the side effects of the treatment. Present chemotherapeutics do not seem to be very effective and lead to tumor resistance, while causing significant toxicity. Therefore alternative therapies are required for this group of patients. In this context the isolated limb perfusion (ILP) with tumor necrosis factor (TNF) and melphalan has proven to be a successful therapy for the treatment of melanoma and sarcoma. An isolated perfusion creates a closed system in which high dose chemotherapeutics can be administered locally, without risking systemic toxicity. TNF is a cytokine which can bind to tumor endothelium and cause local hemorrhagic necrosis of the tumor, provided that high enough concentrations are reached. This necrosis arises from extended damage of the endothelium through which cytostatics such as melphalan can enter the tumor cell.

To further improve the response to this therapy it is essential to gain a better understanding of how sarcoma growth takes place and to develop new chemotherapeutics.

This thesis describes our results with the partly experimental treatment of irresectable sarcoma based on the use of a combination of drugs. Our hypothesis

was that the addition of a new cytokine, Endothelial Monocyte Activating Polypeptide (EMAP II), to the existing combination of TNF and melphalan in the perfusion circuit would lead to an improved clinical response. The aim of the clinical studies and laboratory experiments in this thesis is to investigate the mechanisms around TNF and to develop methods to improve TNF-based therapy.

In **Chapter I.1** a short overview is given on previous results with TNF using the ILP technique. The aim of the present study is described, namely to obtain insight in the way in which TNF influences tumor endothelium. In this section we describe several clinical studies in patients with disseminated disease and the development of EMAP II as a possible new adjuvant chemotherapeutic in combination with TNF.

Since the contribution of TNF to the efficacy of isolated perfusion of organs has not yet been conclusively demonstrated, it is important to determine whether its use has any irreversible effects. Hence a study was conducted in patients undergoing an Isolated Hepatic Perfusion (IHP) to characterize the toxicity and secondary cytokine production attributable to TNF. The results of this study are described in the clinical part of this thesis, starting with **Chapter II.2**. Thirty-two patients with irresectable colorectal cancer confined to the liver were divided in two groups. One group received an IHP with melphalan alone, while the other group underwent an IHP with melphalan and TNF. The patients who did receive TNF had significant higher IL-6 and IL-8 (inflammatory cytokines) levels in serum and perfusate during the first 24 hours after the perfusion. In addition there was significant systemic and regional toxicity compared to those receiving melphalan alone. Significant systemic (low blood pressure, high heart frequency, and low pulmonary pressures) as well as local (increased bilirubin levels and lowered platelet counts) differences were observed during the first 48 hours post-perfusion. The most important observation however was that these effects were transient and did not appear to be of clinical consequence.

The demonstration of the limited nature of TNF's side effects leaves few arguments against its use in ILP treatment. As TNF exercises its effects via the

tumor endothelium, the expectation is that highly vascularized tumors in particular would benefit from treatment with TNF. **Chapter II.3** describes results of 16 limb perfusions using TNF and melphalan in 10 patients with Nonresectable Stewart Treves lymphangiosarcoma. Thus far amputation was the only available treatment for these patients, as chemo- and radiotherapy provided insufficient results. In our experiments we were able to prevent amputation in 80% of the patients, which indicates that we have developed a new treatment option.

TNF affects tumor growth at the TNF receptors on tumor vasculature. This gave rise to the question whether patients with soft tissue sarcoma that were previously subjected to radiotherapy, and thus displaying a scarred and disrupted vascular bed, would still respond to treatment with TNF. In **Chapter II.4** we present the results of ILP treatment with TNF and melphalan in 26 patients with recurrent Soft Tissue Sarcoma, whose primary tumor was initially treated by resection followed by radiotherapy. Limb salvage could be established in 17 (65%) patients, who at time of their referral were considered to be candidates for amputation. Regional toxicity was limited and systemic toxicity minimal to moderate, with no toxic deaths. We concluded that ILP with TNF and melphalan can be performed safely and with satisfying results in patients with locally nonresectable recurrent soft tissue sarcoma, previously treated with radiation therapy (and possibly resection).

The third section of this thesis focuses on the laboratory research conducted to clarify the molecular basis of the workings of TNF. Many studies have already demonstrated that TNF operates via the tumor endothelium. **Chapter III.5** explains why not all tumors react equally well to TNF treatment, once more establishing that this is tumor endothelium dependent. It appears that tumors with lower levels of vascularisation, such as the colon carcinoma metastases, are less sensitive to TNF treatment. Through animal experiments we demonstrate that the concentration of chemotherapeutics in the tumor depends on the tumor vascularisation levels. ILPs with TNF and melphalan were performed in rats with three different tumors, the CC531 colon carcinoma, the ROS-1 osteosarcoma, and the BN-175 soft tissue sarcoma. The levels of vascularisation were measured through immunohistology and the Hb-assay. High concentrations of

melphalan were detected in the highly vascularized BN-175 tumor, compared to low concentrations in the less vascularized ROS-1 and CC531 tumors. The high melphalan concentration in the BN-175 tumor resulted in full tumor regression in the majority of the rats with the BN-175 tumor.

Previous chapters already describe that tumor vascularisation is a cause of the large variation in patient response to isolated limb perfusion with TNF and melphalan. Another cause can be found by observing the cytokine production of the tumor itself. EMAP II is a tumor-secreted cytokine which is at least in part held responsible for the variations in response to TNF. EMAP II is a relatively newly discovered cytokine with a wide range of interactions in the area of angiogenesis.

It is also known that EMAP II secretion invokes an upregulation of TNF receptors on the tumor endothelium. In **Chapter III.6** the question is raised whether EMAP II production can be increased in tumors producing only low levels of EMAP II. Laboratory experiments have been conducted with rat soft tissue sarcoma which appeared to be relatively insensitive to TNF administration. We constructed a tumor clone by means of retroviral transfection in which we incorporated m-RNA of EMAP II, thus upregulating the EMAP II expression in this tumor. We then used this tumor in our existing TNF based limb perfusions in rats. It turned out that the TNF sensitivity of this tumor *in vivo* was significantly increased.

**Chapter III.7** demonstrates the feasibility of increasing cytokine production in a tumor by retroviral transfection. The chemokine Interferon-inducible Protein-10 (IP-10) has antiangiogenic characteristics. By transfecting a human melanoma cell line with an IP-10 gen construct we tested the hypothesis that by increasing production of an antiangiogenic protein in a tumor the tumor growth rate would be reduced. We found that the vasculature in these tumors was reduced such that the tumor growth in an *in vivo* nude mouse model had decreased significantly. The conclusion is that it is feasible to initiate the upregulation of a gen expression, which subsequently leads to increased secretion of a functioning cytokine *in vivo*. This experiment also demonstrates the sensitivity of tumor growth to cytokines with antiangiogenetic characteristics.



**Chapter IV.8** finally presents the general discussion of this thesis. EMAP II, a new cytokine with complex interactions in angiogenesis and tumor growth is reviewed and proposed as a cytokine with beneficial effects on tumor necrosis when used in combination therapy with TNF. Implications and possible clinical applications are discussed.

## CONCLUSIONS

- Addition of TNF to melphalan during an isolated hepatic perfusion (IHP) results in a significant difference in post-IHP production of IL-6 and IL-8 in serum and perfusate.
- Toxicity as a result of TNF administration after an isolated hepatic perfusion with TNF is mild and reversible, making TNF administration safe, provided that the dose does not exceed 1 mg. Above 1 mg severe toxicity can occur.
- ILP with TNF and melphalan can lead to good clinical response rates and can avoid amputation in 80% of the patients with extensive irresectable Stewart Treves angiosarcoma and should be considered as a treatment option for this group of patients accordingly.
- Recurrent disease of soft tissue sarcoma previously treated with radiation therapy responds virtually equally well to limb perfusion with TNF and melphalan as sarcomas in patients who did not receive radiotherapy. In 65% of the radiated patients this treatment led to the preservation of the limb with a good functional outcome and low morbidity.
- Retroviral transfection of human melanomas with the antiangiogenic chemokine IP-10 invokes a functional protein secretion causing a decrease in tumor growth.
- The level of tumor vascularisation predicts the intra-tumor concentration of a cytostatic after isolated perfusion with TNF and melphalan.
- A higher concentration of intra-tumor melphalan highly improves the tumor regression.
- The tumor sensitivity to TNF can be improved by increasing the EMAP II production of the tumor.
- Our results justify the broadening of research into clinical application of EMAP II.

## **SAMENVATTING**

Chirurgie is de basisbehandeling van alle resectabele tumoren. Indien er reeds sprake is van zeer uitgebreide lokale verspreiding of gemetastaseerde tumoren, dan is excisie ontoereikend. In dat geval kunnen cytostatische geneesmiddelen een waardevolle aanvulling op palliatie vormen of zelfs gericht zijn op het bereiken van curatie. Zowel bij het ontstaan als bij de behandeling van tumoren speelt de vasculatuur van de tumor een grote rol. Een hoge mate van tumorvascularisatie kan het effect van chemotherapie gunstig beïnvloeden, echter de tumor is voor zijn groei en metastasering ook afhankelijk van de ontwikkeling van voldoende nieuwe bloedvaten. Deze angiogenese staat onder regulatie van een groot aantal cytokinen. Cytokinen zijn oplosbare proteïnen die normaal gesproken door de mononucleaire cellen van het immuun systeem worden geproduceerd en op het immuun systeem een regulerend effect uitoefenen. Wanneer zij daarentegen door tumoren geproduceerd worden, kunnen zij tot zeer schadelijke interacties leiden, die bijvoorbeeld intensivering van de angiogenese veroorzaakt.

Effectieve behandeling van solide tumoren, zoals sarcomen beschreven in dit proefschrift, wordt bemoeilijkt door bepaalde eigenschappen van deze tumoren en de bijwerkingen van de behandeling. Bestaande chemotherapie blijkt veelal weinig effectief en kan leiden tot resistentie van de tumor, terwijl een aanzienlijke toxiciteit in de patiënt ontstaat. Voor deze groep van tumoren zijn alternatieve therapieën vereist. Zo is de geïsoleerde extremiteits perfusie (ILP) met tumor necrose factor (TNF) en melphalan een succesvolle therapie gebleken voor de behandeling van melanomen en sarcomen. Met de geïsoleerde perfusie kunnen geneesmiddelen die systemisch tot toxiciteit zouden leiden lokaal in hoge dosis toegediend worden. TNF is een cytokine dat, mits in hoge concentratie aanwezig, na binding aan TNF-receptoren op het endotheel aanleiding geeft tot hemorrhagische necrose van de tumor. Deze necrose is het resultaat van uitgebreide beschadiging van de endotheelcel waardoor chemotherapeutica (b.v. melphalan) de tumor kunnen binnendringen.

Een beter begrip van de groeiwijze van sarcomen, gecombineerd met de ontwikkeling van nieuwe chemotherapeutica, is essentieel om in de toekomst verbetering van de klinische respons in deze patiënten te bereiken.

Dit proefschrift beschrijft onze resultaten met de deels experimentele benadering van irresectabele sarcomen op basis van het toedienen van combinaties van geneesmiddelen. Onze hypothese is dat het toevoegen van het nieuwe cytokine Endotheel Monocyte Activating Polypeptide (EMAP II) aan de bestaande combinatie van TNF en melphalan in het perfusie circuit een verbeterde respons tot gevolg heeft. In klinische studies en laboratorium experimenten wordt de werking van TNF, deels in combinatie met EMAP II, onderzocht en worden methoden voor verbetering van deze therapie ontwikkeld.

In **hoofdstuk I.1** wordt in een korte inleiding een overzicht gegeven van de geschiedenis van de behandeling met TNF in de geïsoleerde perfusie. Vervolgens wordt de doelstelling van het proefschrift uiteengezet, te weten het verkrijgen van inzicht in de manier waarop TNF het tumor endotheel beïnvloedt. Dit wordt gedaan door middel van het beschrijven van een aantal experimentele behandelingen bij patiënten met gedissimineerde sarcomen en de ontwikkeling van EMAP II als een mogelijke nieuwe adjuvante therapie in combinatie met TNF.

Aangezien er nog steeds een controverse bestaat over de toegevoegde waarde van TNF met betrekking tot de respons bij de geïsoleerde perfusie is het van belang voorop te stellen dat het gebruik van TNF geen irreversibele bijwerkingen in de patient tot gevolg heeft. In het klinische deel van dit proefschrift wordt in **hoofdstuk II.2** de additionele systemische en lokale toxiciteit, en de cytokine productie bij het gebruik van TNF beschreven in een groep patiënten die een geïsoleerde lever perfusie onderging. 32 patiënten met irresectabele lever metastasen van een colon carcinoom werden in twee groepen verdeeld. De eerste groep kreeg een geïsoleerde lever perfusie met alleen melphalan, in de tweede groep ondergingen de patiënten een lever perfusie met melphalan en TNF. Het bleek dat in deze tweede groep de concentraties IL-6 en IL-8 (inflammatoire cytokinen) in het perfusaat en in het bloed de eerste 24 uur na de perfusie significant hoger waren. Dit ging gepaard met een toegenomen toxisch profiel in deze patiënten. Zowel systemisch, te weten lage bloeddruk, hoge hartfrequentie en verhoogde pulmonaire drukken, als op lokaal niveau met een bilirubine stijging en afgenomen aantal bloedplaatjes waren er gedurende 48 uur

postoperatief significante verschillen waarneembaar. Echter van groot belang is de constatering dat deze effecten tijdelijk en reversibel waren, en geen klinische consequentie hadden.

Met het aantonen van de gelimiteerde bijwerkingen van het gebruik van TNF in de geïsoleerde perfusie zijn er weinig argumenten tegen het gebruik van TNF. Aangezien bekend is dat TNF zijn werking uitoefent via het tumor endotheel, valt te verwachten dat met name zeer gevasculariseerde tumoren baat hebben bij behandeling met TNF.

In **hoofdstuk II.3** worden de resultaten beschreven van 16 extremitets perfusies met TNF en melphalan in een groep van 10 patiënten die gediagnosticeerd waren met een irresectabel Stewart Treves lymfangiosarcoom. Tot dusver was voor deze patiënten slechts een amputatie voorhanden als palliatieve therapie, aangezien chemo- en radiotherapie onvoldoende effect bewerkstelligden. Met onze resultaten bij 10 patiënten tonen we aan dat wij een nieuwe behandelingsstrategie hebben toegevoegd, met een preventie van amputatie in 80% in deze patiënten groep.

Het effect van TNF op tumoren wordt gemedieerd via de TNF receptoren op de tumor vasculatuur. Wanneer tumoren behandeld zijn met radiotherapie is er een verstoorde vascularisatie in het tumor gebied met een toegenomen fibrosering ter plaatse. Dit gaf aanleiding tot de vraag of bij patiënten die voor hun primaire tumor met radiotherapie behandeld waren nog een mogelijkheid bestond een tumor respons te verkrijgen met het gebruik van TNF.

In **hoofdstuk II.4** wordt het gebruik beschreven van TNF en melphalan bij 26 patiënten die een lokaal recidief van een weke delen sarcoom hadden, waarbij de primaire tumor met excisie en vervolgens radiotherapie behandeld was. Bij de initiële verwijzing kwamen allen in aanmerking voor een amputatie wegens de uitgebreidheid van het tumor recidief. Na perfusie kon de ledemaat echter in 17 patiënten behouden blijven (65%). Bij deze perfusie was de regionale toxiciteit beperkt, en de systemische toxiciteit minimaal, zonder mortaliteit. Hieruit hebben we geconcludeerd dat patiënten met een lokaal irresectabel recidief van een weke delen sarcoom, die primair behandeld zijn met radiotherapie en eventueel

chirurgische excisie, een ILP met TNF en melphalan veilig en met bevredigend resultaat kan worden uitgevoerd.

Het derde deel van dit proefschrift spitst zich toe op het laboratorium onderzoek dat wij hebben verricht om de moleculaire basis van het gebruik van TNF op te helderen. Vele studies hebben aangetoond dat TNF werkzaam is op het niveau van het endotheel in de tumor. In **hoofdstuk III.5** wordt een verklaring gegeven waarom niet alle tumoren in gelijke mate op TNF behandeling reageren, waarbij eens te meer gedemonstreerd wordt dat dit van tumor endotheel afhankelijk is. Het blijkt dat tumoren met een mindere mate van vascularisatie zoals bijvoorbeeld de colon carcinoom metastasen, minder gevoelig zijn voor behandeling met TNF. Door middel van dierexperimenten (geïsoleerde lever perfusie in ratten met TNF en melphalan) wordt gedemonstreerd dat de concentratie van chemotherapeutica in de tumor afhankelijk is van de mate van vascularisatie van de tumor. In drie verschillende tumoren werden perfusies uitgevoerd, het CC531 coloncarcinoom, het ROS-1 osteosarcoom en het BN-175 weke delen sarcoom. Hierbij werd de mate van vascularisatie bepaald met immunohistologie en het Hb-Assay. Hoge concentraties van melphalan werden gedetecteerd in de goed gevasculariseerde tumor, de BN-175, in vergelijking tot lage concentraties melphalan in de minder gevasculariseerde ROS-1 en CC531 tumoren. De hoge melphalan concentratie in de BN-175 tumoren resulteerde in de meerderheid van de ratten met de BN-175 tumor tot een complete regressie van deze tumor na de perfusie.

In eerdere hoofdstukken wordt reeds beschreven dat vascularisatie van een tumor een van de oorzaken is van de grote variatie tussen patiënten die met een geïsoleerde perfusie met TNF en melphalan behandeld zijn. Een andere oorzaak kan gevonden worden door te kijken naar de cytokine productie van de tumor zelf. EMAP II is een cytokine dat door tumoren geproduceerd wordt, en tenminste gedeeltelijk verantwoordelijk kan worden gehouden voor het verschil in respons op TNF. EMAP II is een relatief nieuw ontdekt cytokine, met vele interacties op het gebied van de angiogenese. Daarbij is aangetoond dat EMAP II secretie aanleiding geeft tot een opregulatie van de TNF receptoren op het tumor endotheel. In **hoofdstuk III.6** dient de vraag zich aan of in tumoren met weinig

EMAP II productie, deze door manipulatie van de tumor verhoogd kan worden. In het laboratorium hebben we experimenten verricht met een weke delen sarcoom in de rat dat relatief ongevoelig bleek te zijn voor de effecten van TNF. Nadat we van deze tumor retroviraal een kloon hadden geconstrueerd waarbij we in het genoom een EMAP II m-RNA konden incorporeren waarmee de expressie van EMAP II in deze tumor verhoogd werd, hebben we deze tumor gebruikt in de bestaande TNF perfusie modellen die we in de rat toepassen. De experimenten tonen een verbeterde tumor respons aan na een ILP met TNF als de expressie van EMAP II in de tumor hoger is. Het bleek dat de gevoeligheid van de tumor *in vivo* significant verhoogd was.

In **hoofdstuk III.7** wordt beschreven dat het mogelijk is door middel van een retrovirale transfectie de proteïne secretie in een tumor te verhogen. Het chemokine Interferon-inducible Protein-10 (IP-10) heeft anti-angiogene eigenschappen. Door retroviraal een humane melanomen cellijn te transfecteren met een IP-10 gen-construct werd de hypothese getest of door ophogen van een anti-angiogenetisch proteïne in een tumor zijn groeisnelheid gereduceerd kon worden. Het bleek dat de vasculatuur in deze tumoren zodanig was afgenomen dat de groei van de tumor in een *in vivo* naakte muis model significant afgenomen was. Concluderend is het mogelijk gebleken *in vitro* een opregulatie te veroorzaken van een expressie van een gen, dat tot een verhoogde secretie van een functionerend cytokine *in vivo* leidt. Dit experiment levert tevens het bewijs voor de afhankelijkheid van de groeisnelheid van tumoren van cytokinen met angiogenetische werking.

**Hoofdstuk IV.8** behelst als laatste onderdeel van dit proefschrift de discussie. EMAP II, een nieuw cytokine met complexe interactie op het gebied van de angiogenese en tumorgroei wordt beschreven als een cytokine met een positief effect op tumornecrose, indien het in combinatie met TNF gebruikt wordt. Implicaties en mogelijke klinische toepassingen worden uiteengezet.

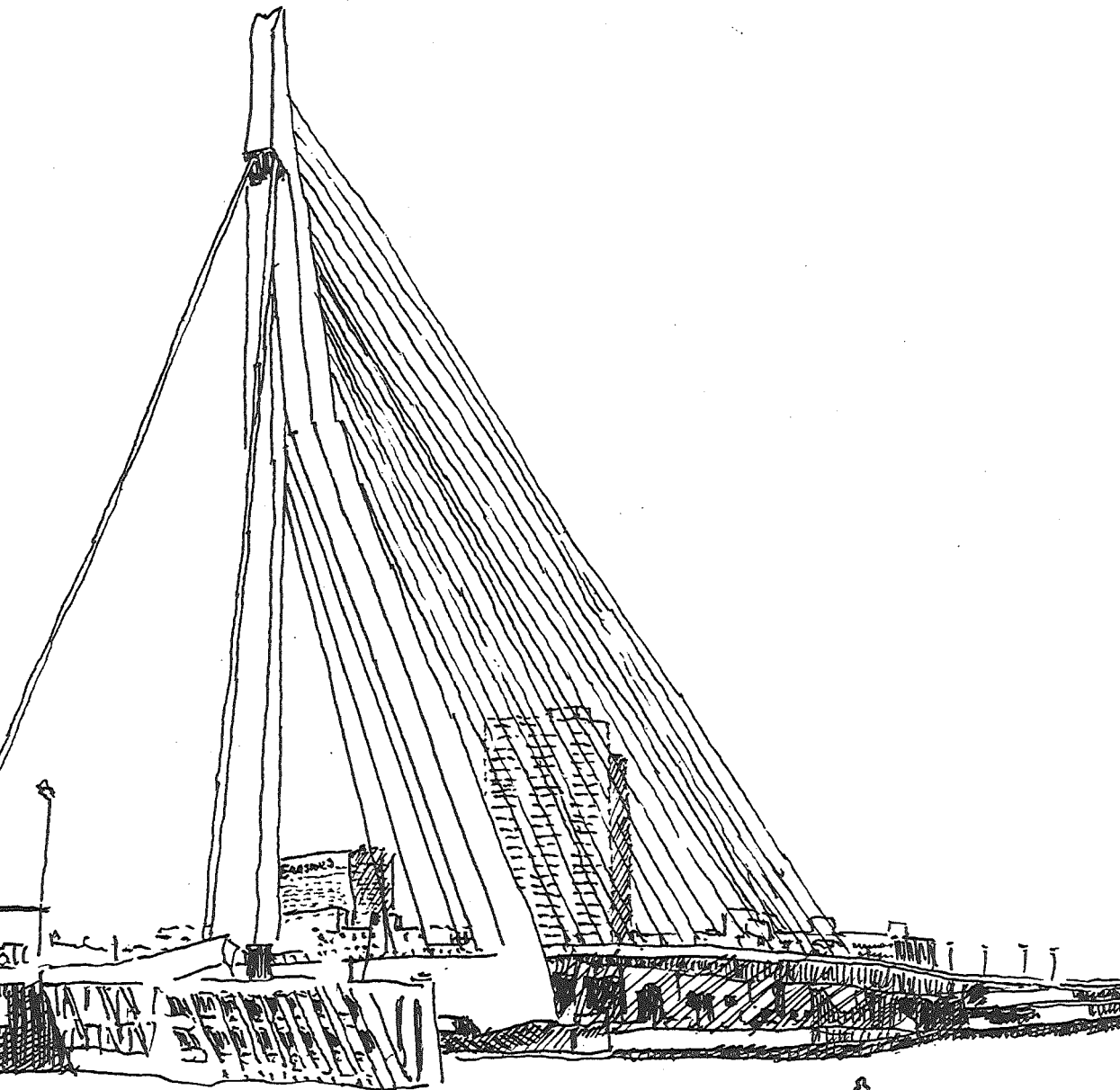
## CONCLUSIES

- Het toevoegen van TNF aan de geïsoleerde leverperfusie met melphalan resulteerde in een significant verschil in IL-6 en IL-8 productie in serum en perfusaat.
- De toxiciteit die ten gevolge van het gebruik van TNF in de patiënt optreedt na een geïsoleerde perfusie met TNF in de lever is mild en reversibel, waardoor TNF veilig kan worden toegediend mits deze dosis niet hoger is dan 1 mg. Hierboven kunnen ernstige stollingsstoornissen optreden.
- ILP met TNF en melphalan kan leiden tot een goede klinische respons en behoud van de ledemaat in 80% van de patiënten met irresectabele Stewart Treves angiosarcomen en moet als een behandelingsmodaliteit bij deze groep van patiënten overwogen worden als alternatief voor amputatie.
- Sarcoomrecidieven in bestraald gebied responderen nagenoeg even goed op extremiteitsperfusie met TNF en melphalan als niet eerder behandelde sarcomen. Deze behandeling leidt in 65% van de bestraalde patiënten tot behoud van de ledemaat met goede functionele uitkomst en lage morbiditeit.
- Retrovirale transfectie van humane melanomen met het anti-angiogenetisch chemokine IP-10 zorgt voor een functionele proteïne secretie leidend tot remming van de groei van de tumor.
- De mate waarin een tumor gevasculariseerd is, voorspelt mede de intra-tumorale concentratie van een cytostaticum in de tumor na perfusie met TNF en melphalan.
- Een hogere concentratie intra-tumorale melphalan verbetert in sterke mate de regressie van de tumor.
- De sensitiviteit van een tumor voor TNF verbetert door de EMAP II productie van deze tumor te verhogen.
- Onze resultaten rechtvaardigen verdieping van het onderzoek naar de klinische toepassing van EMAP II.





ADDENDUM





## ACKNOWLEDGEMENTS / DANKWOORD

Het schrijven van dit dankwoord geeft me een gevoel van opluchting, het betekent dat het proefschrift in de laatste fase is beland! Aan de andere kant is het dankwoord vaak het meest gelezen hoofdstuk en zou het als geen ander volledig moeten zijn. Terecht, want zonder onderstaande personen zou het boekje niet eens hebben bestaan. Zonder enigszins de illusie te hebben volledig te zijn wil ik graag een aantal van hen die hebben bijgedragen aan de totstandkoming van dit proefschrift bedanken.

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Prof.dr. H.J. Bonjer, opleider in het ErasmusMC. Beste Jaap, ik vind het bewonderenswaardig hoe je de veranderingen met betrekking tot de steeds inkrimpende assistentenwerktijden voor probeert te zijn en met grote betrokkenheid in Rotterdam een competitieve geest staande houdt. Dank dat je de secretariële verplichtingen van de promotiecommissie op je wilde nemen.

De overige leden van de promotiecommissie: Prof.dr. J. Verweij, Prof.dr. Th.H. van der Kwast, Prof.dr. I.H.M. Borel Rinkes, Prof.dr. B.B.R. Kroon. Dank voor het beoordelen van het manuscript, het waardevolle commentaar en uw bereidheid plaats te nemen in de promotiecommissie.

When I started my research at the NCI I was determined to gather as much data as I could in a very short time and I expected to publish my first paper within months. Ewa Turner, technician in the lab, tried to get me back to reality. Ewa, you helped me with all my experiments when I lost the courage to go on. I never forget your word 'spooky' when you described my blots... thanks for being there.

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Josef Friedl, dear friend. At the NCI we worked together and shared the good times after work. Always the quest for an answer: "Why is the tumor vasculature leaky??". Coffee didn't solve it but was sometimes needed more than the answer.

De meiden van het ECO-lab, Sandra van Tiel, Ann Seynhave, Gisela Ambagtsheer, Saske Hoving, alle stagiaires; dank voor alle gezelligheid en San, we weten inmiddels ècht allemaal dat het lab zonder jou niet draait. Dank voor alle rattenperfusies die je voor me uitvoerde. De mannen, Remco van Horssen en Joost Rens. Remco, ik ben jou vooral veel dank verschuldigd voor alle gezamenlijke besprekingen over EMAP, en het feit dat jij de fakkel van me hebt

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Ook de chirurgen van de Daniel den Hoed kliniek, met name Dr. Hans de Wilt en Dr. Bert van Geel, wil ik hartelijk danken voor hun bijdrage aan de klinische studies. Veel dank ben ik ook verschuldigd aan de secretaresses van de Daniel en in het bijzonder aan Marja van Wijngaarden. Ongelofelijk hoe jij het volhoudt de agenda van Lex te beheren. Altijd bereid een plekje in de agenda te zoeken waarop 'hij waarschijnlijk wel in Nederland is'...

In het IJsselland ziekenhuis is de chirurgie voor mij gaan leven, vooral dankzij de enthousiaste chirurgen en assistenten. Ik ben allen veel dank verschuldigd en dr. Dawson in de eerste plaats. U vertelde me 5 jaar geleden over het belang van wetenschappelijk onderzoek om evidence-based te leren beslissen in de kliniek. Dit boekje en mijn opleiding zijn mede het gevolg van uw motiverende woorden en van alle mooie kanten van de chirurgie die ik in het IJsselland heb leren kennen.

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Titia

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## **CURRICULUM VITAE**

Titia Evelien Lans werd geboren op 31 december 1970 te Brielle. Na het behalen van het VWO diploma aan de Rijksscholengemeenschap te Brielle begon zij, vanwege uitloting in Nederland, met de studie geneeskunde in België, aan de Vrije Universiteit te Brussel. Na het behalen van de propadeuse aldaar kon zij een jaar later in 1990 in Rotterdam aan de Erasmus Universiteit met de studie beginnen. In 1997 behaalde zij daar het artsexamen.

Haar eerste werkplek was in het IJsselland ziekenhuis te Capelle a/d IJssel (dr. I.Dawson), waar zij als AGNIO chirurgie een jaar gewerkt heeft. Via prof.dr. A.M.M. Eggermont kreeg ze de mogelijkheid onderzoek te gaan doen aan het National Institute of Health in Bethesda, Maryland (VS). Hier heeft ze een jaar lang onder leiding van dr. H.R.A. Alexander, MD., als arts-onderzoeker gewerkt bij de Surgery Branch van het National Cancer Institute, waar de basis voor dit proefschrift gelegd werd. Na terugkomst heeft zij dit onderzoek voortgezet in het Laboratorium voor Experimentele Chirurgie van het Erasmus MC te Rotterdam.

In december 2000 is zij begonnen aan de opleiding tot algemeen chirurg in het St. Clara ziekenhuis te Rotterdam, tegenwoordig het MCRZ, met opleiders dr. J.F. Lange en dr. K.J. Brouwer. Deze opleiding zal zij eind 2004 voortzetten in het Erasmus MC te Rotterdam met als opleider prof.dr. H.J. Bonjer.

