TUMOR NECROSIS FACTOR-α AND TUMOR TARGETING

Regional and systemic administration of TNF-α in the rat for treatment of solid tumors

TUMOR NECROSE FACTOR-α EN TUMOR TARGETING

Regionale en systemische toepassing van TNF-α in de rat ter behandeling van solide tumoren

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Cover illustration: Structure of TNF-α molecule showing the three TNF subunits in blue, red and yellow. (From: Tumor Necrosis Factor: Molecular and Cellular Biology and Clinical Relevance; Ed. W.Fiers, W.A.Buurman, Karger, Basel, 1993)

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Chapter 1

General introduction and aim of the thesis

TUMOR NECROSIS FACTOR-α

Since the purification of Tumor Necrosis Factor-α (TNF-α) in the mid 1980's the anti-tumor capacity of TNF-α has received considerable attention. Acute softening of the tumor, hemorrhagic necrosis and occlusion of the neo-vasculature led to tumor necrosis. The mechanism behind this process was not well understood, until recently. The initial enthusiasm of the anti-tumor capacity of TNF-α in rodent tumor models was tempered by the severe toxicity encountered in clinical phase I/II trials. Only 1/50 to 1/20 of the dose required for anti-tumor effect in human xenograft tumor models in mice, can be administered in man. In 1992 Lejeune et al combined TNF-α, melphalan and Interferon γ (IFN-γ) to achieve impressive results in anticancer therapy. The combination proved to be very successful in the isolated perfusion setting in the treatment of extensively metastasized melanoma patients. Complete response (CR) rates of 80-90 % were achieved. In isolated limb perfusion (ILP) the concentration gap between animal studies and clinical trials could be overcome. Investigations were expanded to other forms of cancer, and Eggermont et al proved the same regimen to be as successful in locally advanced extremity soft tissue sarcomas, with a limb salvage percentage of over 80 %, followed by a much easier and less mutilating resection of the tumor remnants. Currently, TNF-α based isolated limb perfusion has become the standard of treatment for patients with multiple in transit melanoma metastases or non-resectable extremity sarcomas. This led to the approval and registration of TNF-α in Europe for clinical use in patients with locally advanced extremity soft tissue sarcomas treated by ILP with TNF-α and melphalan (Beromun®).

Angiographic studies revealed that the main target of TNF-α was the tumor associated vasculature (TAV). The immediate reaction and softening of the tumor in ILP treated patients were associated with selective occlusion of the TAV. Blood flow decreased and metabolic activity in tumors was arrested. Morphological and immunohistochemical studies of tumor biopsies from patients after TNF-α based ILP showed early damage to the TAV, as shown by perivascular release of von Willebrand Factor (vWF). Whether damage to the vascular endothelium equally plays a pivotal role is a matter of debate.

Previous findings in our laboratory with TNF-α based isolated limb perfusion.

After the initial success of isolated limb perfusion in patients with advanced disease a preclinical program was started to unravel the mechanism behind the observed impressive results obtained with TNF-α and melphalan.

A rat model for isolated limb perfusion was developed. In this model perfusion of a soft tissue sarcoma with TNF-α alone did not result in a measurable tumor

response, nor did perfusion with melphalan as the sole agent. The combination of TNF-α and melphalan however, resulted in a synergistic anti-tumor response. Surprisingly, in vitro no synergism between the two agents could be demonstrated. It was postulated that the main target of TNF-α-based isolated perfusion was the tumor associated vasculature. Non tumor vessels were relatively spared. The explanation for the susceptibility of the newly formed tumor blood vessels for TNF-α may be found in the vascular endothelium. Human endothelial cells in vitro showed growth arrest and cell death after treatment with TNF-α and IFN-γ (as shown by others). The endothelial cell lining was harmed after TNF-α-based ILP, with massive permeability changes. Platelet aggregation followed these initial changes. Two types of reaction of the TAV can be discerned: 1) the immediate-type and 2) the delayed-type. The immediate-type may result from upregulation of endothelial adhesion molecules, followed by influx of ploymorphonuclear cells (PMN's), another explanation being found in acute and marked hyperpermeability, followed by platelet aggregation, congestion of blood, edema and tumor necrosis. In contrast, the delayed-type is associated with mild hyperpermeability, interstitial edema, scattered tumor necrosis, possibly leading to increased concentration of melphalan in the tumor or prolongation of its effect. Tumor cells will thus be exposed to the direct toxicity of melphalan.

Previous findings with liposomes.

In contrast to isolated perfusion, systemic treatment with TNF-α either alone or in combination with melphalan does not result in a measurable response. The severe toxicity of TNF-α allows only low dosages and disseminated disease cannot be controlled. Furthermore, TNF-α is cleared rapidly from the circulation and demonstrates poor tumor localization.

Encapsulation of TNF-α in liposomes would not only lower the toxicity of the cytokine, but could also increase localization in tumor tissue and reduce clearance.

In studies with liposomal encapsulation of other cytokines and immunomodulators an enhancement of activity was demonstrated. A major drawback however remained the fairly rapid clearance of the first generation conventional liposomes. The incorporation of hydrophilic chains, like polyethylene-glycol (PEG) coupled to the phospholipid phosphatidylethanolamine, resulted in increased blood residence times with a concurrent enhancement of the percentage of injected dose at the tumor site. Another cytokine, Interleukin 2 (IL-2); was extensively studied after encapsulation in these so called long-circulating or sterically stabilized liposomes. Encapsulation resulted in enhancement of the immunomodulatory activity of IL-2 such as generation of leukocytes and anti-tumor activity in metastasized carcinoma.

TNF-α has been encapsulated in conventional liposomes. Improved pharmacokinetics and biological activity in vivo as compared to TNF-α in the free form has been demonstrated, as well as reduced toxicity. In anti-tumor therapy the encapsulation of TNF-α in long circulating liposomes seemed particularly attractive in the combination with liposome encapsulated doxorubicin (DOXIL®).

Aim of the study:
The aim of this thesis was to study the applicability of the combination of TNF-α and cytostatics in isolated organ perfusion. Furthermore, encapsulation of TNF-α in liposomes was studied to achieve a mean to revive the systemic application of the cytokine. To this end several experimental set-ups were designed and elaborated.

1. A model for isolated organ perfusion was designed in which the combination of TNF-α and melphalan could be tested. Various combinations were tested to achieve a maximal anti-tumor effect, in vitro as well as in vivo.

2. Melphalan is the agent of choice in isolated limb perfusion, pre-clinically as well as in patients. Doxorubicin however, is a drug with well known anti-tumor effects on sarcomas. Therefore, doxorubicin in combination with TNF-α was used in ILP, to study the putative mechanism of synergy between TNF-α and doxorubicin.

3. It was shown by others that TNF-α could lead to cell cycle disturbance, resulting in a decrease of anti-tumor effects of doxorubicin. The effect of TNF-α on cell cycle events was studied.

4. To minimize the toxicity of TNF-α when administered systemically, the possibility to encapsulate TNF-α in sterically stabilized liposomes was studied. The distribution of liposome encapsulated TNF-α and change of pharmacokinetic profile of TNF-α was investigated.

5. From the above experimental set up the interest in systemic administration of TNF-α was renewed. In TNF-α-based ILP high dose TNF-α is used to achieve the well known fine results. Others proved that also low dose TNF-α could lead to anti-tumor activity in combination with chemotherapy. The administration and subsequent efficacy of low dose TNF-α in combination with liposome encapsulated doxorubicin (DOXIL®) was therefore studied.

Chapter 2

In vivo isolated kidney perfusion with Tumor Necrosis Factor-α (TNF-α) in tumor bearing rats.

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ABSTRACT

Isolated perfusion of the extremities with high dose Tumor Necrosis Factor-α (TNF-α) plus melphalan leads to dramatic tumor response in patients with irresectable soft tissue sarcoma or multiple melanoma in transit metastases. We developed in vivo isolated organ perfusion models to determine whether similar tumor responses in solid organ tumors can be obtained with this regimen. Here, we describe the technique of isolated kidney perfusion. We studied the feasibility of a perfusion with TNF-α and assessed its antitumor effects in tumor models differing in tumor vasculature. The maximal tolerated dose (MTD) proved to be only 1 μg TNF-α. Higher doses appeared to induce renal failure and a secondary cytokine release with fatal respiratory and septic shock like symptoms.

In vitro the combination of TNF-α and melphalan did not result in a synergistic growth inhibiting effect on CC531 colon adenocarcinoma cells, while an additive effect was observed on osteosarcoma ROS-1 cells. In vivo isolated kidney perfusion, with TNF-α alone or in combination with melphalan, did not result in a significant antitumor response in either tumor model in a subrenal capsule assay. We conclude that, due to the susceptibility of the kidney to perfusion with TNF-α, the minimal threshold concentration of TNF-α to exert its antitumor effects was not reached. The applicability of TNF-α in isolated kidney perfusion for human tumors seems, therefore, questionable.

INTRODUCTION

Tumor Necrosis Factor-α (TNF-α) in combination with melphalan with or without the addition of interferon-γ is presently used in the treatment of patients with 'in transit' metastases of malignant melanoma and patients with locally advanced soft tissue sarcoma (STS). In both groups of patients the cytokine TNF-α and the cytotoxic agent melphalan are used in isolated perfusion of the limb (ILP). The efficacy in the treatment of locally advanced soft tissue sarcomas, characterised by high response rates (>80%) resulting in limb salvage in about 80% of the patients, has now been well established by published reports of multicentre experiences in up to 200 perfusions *(Eggermont et al, 1993; Eggermont et al, 1996a; Eggermont et al, 1996b).* Initially the procedure was developed in the more traditional field of applying ILP, e.g. in the treatment of in transit melanoma metastases, and high complete remission rates in melanoma patients have been reported *(Liénard et al, 1992, 1994).*

The exact mechanism of TNF-α anti-tumor activity has not yet been fully elucidated, but a number of theories exist *(Sidhu and Bollon, 1993).* TNF-α has direct and indirect effects. It can induce tumor specific immunity *(Spriggs and Yates, 1992)* and is cytotoxic/cytostatic for some tumor cell lines *in vitro (Dealtry et al, 1987).* Its direct effect on tumor cells was proven shortly after the discovery of the cytokine *(Watanabe et al, 1988),* but the indirect effects probably play a more important role. The detrimental effects on the tumor-associated vasculature is mediated by endothelial cells *(Shimomura, 1988),* however, the effect on the microvasculature seems to be dose dependent *(Fajardo et al, 1992).*

In contrast, high dosages of TNF-α exert a number of undesirable effects. The maximum tolerated dose (MTD) in humans is 350 $\mu g/m^2$ *(Brouckaert et al, 1986; Asher et al, 1987)* which is 10-50 times lower than the desired anti-tumor dose when given intravenously. Because of severe toxicity, observed already at relatively low doses of TNF-α after systemic administration, in phase I/II clinical trials virtually no tumor responses were observed *(Spriggs et al, 1988; Feinberg et al, 1988).* This is not surprising as TNF-α was never administered systemically at doses that might have anti-tumor activity. This led *Lejeune et al (1993)* to the development of the isolated perfusion with TNF-α together with melphalan and γ-interferon. The successful application of TNF-α in this setting warrants that the applicability of TNF-α in isolated organ perfusion setting is investigated. Isolated single lung perfusion with TNF-α proved to be safe *(Weksler et al, 1994; Pogrebniak et al, 1994; Pass et al, 1996).* Reports on the use of TNF-α in isolated liver perfusion at the National Cancer Institute in the USA *(Fraker et al, 1994)* as well as by our group *(Borel Rinkes et al, 1997)* have appeared very recently.

Here, we report on the development of *in vivo* isolated kidney perfusion and its anti-tumor effects in two tumor models.

MATERIALS AND METHODS

Animals

Male rats of the inbred WAG-Rij strain (Harlan CPB, Austerlitz, the Netherlands), weighing 250-300 grams were used. Rats were kept under standard laboratory conditions. All rats 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). The specific protocol was approved by the "Committee on Animal Research" of the Erasmus University Rotterdam, the Netherlands.

Tumor Necrosis Factor-α

Recombinant human TNF-α (rhTNF-α) was kindly provided by Boehringer Ingelheim GmbH, Ingelheim/Rhein, Germany. RhTNF-α had a specific activity of 5.8 x 10 U/mg as determined in the murine L-M cell assay *(Kramer and Carver, 1986)*. Endotoxin levels were less than 1.25 endotoxin units/mg protein. TNF-α was delivered in 0.5 ml vials in a concentration of 2.4 mg/ml.

Melphalan

Melphalan (Alkeran, 50 mg per vial, Wellcome, Beckenham, UK) was diluted in 10 ml diluent solvent. Further dilutions were made in 0.9 % NaCl to give a volume of 0.5 ml in the perfusion circuit.

Unilateral nephrectomy

It is probable that when two kidneys are perfused *in vivo*, kidney function as measured by blood urea nitrogen (BUN), creatinine and electrolytes remains stable after isolation and perfusion of one kidney. To analyse to what extent renal function is compromised by unilateral nephrectomy five rats underwent a unilateral nephrectomy and at regular intervals BUN, creatinine and electrolytes were recorded. The right kidney was chosen for anatomic reasons. The left kidney shows compensatory hypertrophy: the size of the kidney is larger, there is a gain in cell volume and diameter of the glomerulus as well as a larger volume of the tubule *(Fine 1986)*.

Operative procedures were carried out under clean conditions. Anaesthesia was induced with ether (Diethylether p.a. Merck, Darmstadt, Germany). The abdomen was shaved and prepped with Ethanol 70 %. Through a lumbotomy the right kidney was exposed and freed from its surrounding fat. Ureter, renal artery and vein were dissected and tied with 4-0 silk sutures (B Braun Melsungen AG, Germany). The right kidney was removed and the lumbotomy closed with 2-0 silk in a running way. After three weeks the animals were sacrificed.

Perfusion fluid

In our experiments a modified Krebs-Henseleit solution (NaCl 118.00 mmol/l, $CaCl_2$ 2.52 mmol/l, $MgSO_4$ 1.66 mmol/l, $NaHCO_3$ 24.88 mmol/l, KH_2PO_4 1,18 mmol/l, +D-glucose 5.55 mmol/l, Na-pyruvaat 2.00 mmol/l, Mannitol 33.00 mmol/l) was used as perfusate.

Operative technique: Isolated Kidney Perfusion (IKP)

Anaesthesia was induced with ether. A Zeiss operative microscope (Carl Zeiss, Germany) was used. The abdomen was shaved and prepped with Ethanol 70 %. Through a median laparotomy the left kidney and vessels were exposed in the retroperitoneum by sharp and blunt dissection. Branches of the renal artery and vein (adrenal and spermatic) were dissected when needed and temporarily occluded with ligatures. A tobacco-pouch ligature was placed in the vein with Nylon 9-0 (SSC, B Braun, Melsungen AG, Germany). A bolus of 50 IE Heparin (Heparin Leo, Weesp, the Netherlands) was injected intravenously. Isolation of the renal artery and vein was carried out by means of micro vessel clamps. Via a venotomy and arteriotomy the vessels were cannulated with Silastic tubing (.025 in. ID, .047 in. OD and .012 in. ID, .025 in OD respectively, Dow Corning, Michigan, U.S.A.). Another bolus of 50 IE Heparin was added to the perfusion circuit.

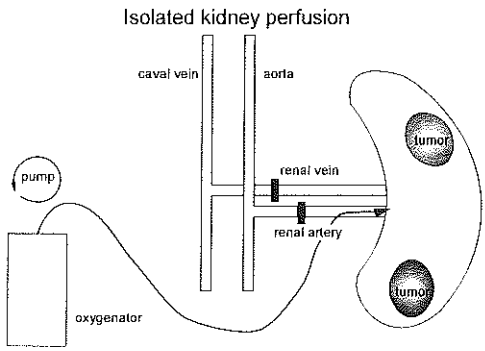

Figure 1. Schematic representation of isolated kidney perfusion

Perfusion model

Isolated kidney perfusion was performed to design a model resembling isolated limb perfusion where TNF-α is administered to the oxygenated extracorporeal perfusion circuit. In this model 1, 2 or 5 μg TNF-α was added to the perfusion circuit. Perfusion with TNF-α was carried out for 15 minutes. Thus TNF-α was allowed to pass the kidney multiple times.

Flow through the kidney was regulated by a non-pulsatile roller pump (Watson-Marlow 505U, Falmouth, UK). Perfusion pressure was recorded on a Datex AS/3 monitor and kept between 100 and 120 mmHg, adjusting the flow generated by the roller pump accordingly.

Perfusion fluid was warmed to approximately 37° C by countercurrent (Polyscience 210, Merck, Amsterdam, the Netherlands). Temperature was recorded (Thermodig KJ-11, Mera, Benelux).

Flow through the kidney was approximately 1 ml/min. The reservoir was gassed with Carbogen (95% O_2, 5% CO_2 gas mixture), to keep the oxygen pressure of the perfusate at 350-400 mmHg and the saturation at 99.5%.

When TNF-α and/or melphalan was added to the perfusion circuit a washout is carried out with 6 ml perfusion fluid (about 4 times the intravascular kidney volume).

At the end of the perfusion period the venotomy was closed by tightening the tobacco-pouch ligature. Arteriotomy was closed with Nylon 10-0 (SSC, B Braun, Melsungen AG, Germany). The laparotomy was closed with silk 2-0 (B Braun) in one layer in a running way. Total operation time varied between 90 and 120 min. Blood loss was kept to a minimum.

During the recovery period the animal was kept warm with a 200 W lamp and then returned to his cage.

Parameters and Histology

Weight loss was recorded after operation. In the post operative period, the animals were observed at regular intervals for signs of toxicity, and deaths were recorded. Clinical condition (skin, eyes, stools and behaviour) was judged. Renal function was assessed through blood urea nitrogen and creatinine levels as well as electrolytes in plasma.

For histopathological analysis 2 rats for each TNF-α concentration were sacrificed 24 hours after treatment. Kidneys were fixed (emersion method) in 4% formaldehyde solution and embedded in paraffin. Care was taken to keep the time of perfusion fixed, the warm ischaemia period as short as possible and time between nephrectomy and fixation constant. Sections of the kidneys were stained with haematoxylin and eosin.

For analysis of the vasculature of the tumors, the same procedure was followed.

Tumor models

Colon carcinoma CC531

The 1,2 dimethylhydrazine-induced, moderately differentiated colon adenocarcinoma CC531 *(Marquet et al, 1984)*, transplantable in syngeneic Wag-Rij rats, was used. The tumor is weakly immunogenic as determined by the immunization-challenge method of Prehn and Main (1957). The tumor was maintained in vitro in RPMI 1640 medium supplemented with 5% fetal calf serum (virus and mycoplasma screened), 1% penicillin (5000 U/ml), 1% streptomycin (5000 U/ml) and 1% L-glutamine (200mM). All supplementaries were obtained from Gibco (UK). Before usage the cells were trypsinized (5 min, 37° C), centrifuged (5 min, 700g), resuspended in RPMI 1640 and counted. Viability was measured with trypan blue (0.3% in a 0.9% NaCl solution). Viability always exceeded 95%.

For *in vivo* studies the tumor was inoculated in the flank of syngeneic Wag-Rij rats, where it was allowed to grow until the time of the experiment.

Osteosarcoma ROS-1

The ROS-1 osteosarcoma (transplantable to Wag-Rij rats) was used in the second series of experiments. This tumor originated spontaneously in the tibia of a rat. The ROS-1 cells grow as a monolayer in Dulbecco's modified Eagle's medium. To this medium 5% foetal calf serum and glutamic acid (Gibco, Paisley, UK) was added. Cells were maintained in a humidified atmosphere of CO_2/air (5/95) at 37°. From the tissue cultures new solid tumors were produced by serial inoculation in the flank where it was allowed to grow until the time of the experiment.

Experimental design

In vitro testing of tumor cell lines for susceptibility to TNF-α and melphalan: tumor cells were seeded at $1x10^4$ cells per well in a flat bottomed 96-well microtiter plates (Costar, USA) in a final volume of 0.2 ml medium per well, and incubated at 37°C in 5% CO_2 for 48 h in the presence of different concentrations of rHuTNF-α and melphalan. Concentrations of TNF-α used were 0.001, 0.01, 0.1, 1, 10 and 100 μmol/ml. Concentrations of melphalan used were 0.04, 0.1, 0.9, 5 and 8 μmol/ml.

Growth of tumor cells was measured using the Sulphorhodamine-B assay according to the method of *Skehan et al (1990)*. Eight replicate experiments were performed. Tumor growth was calculated using the formula: tumor growth = (test well/control) x 100 percent. The drug concentration reducing the absorbance to 50 percent of control (IC_{50}) was determined from the growth curves.

In vivo tumor model: for the *in vivo* isolated kidney perfusion the Sub-Renal Capsule Assay (SRCA) was used. Recipient animals were anaesthetised with ether, a midline incision was made and two small tumor fragments of 6-7 mg were placed under the renal capsule of either kidney, one in the upper pole and one in the lower pole of the kidney. To exclude perfusion defects of the kidneys the location of inoculation of CC531 and ROS-1 was varied between the upper or lower pole of the kidney. After seven days the animals were used for the experiments. At 14 days after inoculation the animals were sacrificed, the tumors were enucleated and weighed. The tumors in the right kidney were used as an internal control. Nine replicate experiments were performed.

Statistical significance was assessed using the Mann Whitney U test.

RESULTS

Unilateral nephrectomy

Unilateral nephrectomy was very well sustained. There was a minor increase in creatinine shortly after operation, but all animals showed a quick

recovery. Kidney functions fluctuated within a normal range.

Sham perfusion

In five animals isolated kidney perfusion with perfusate only was performed (figure 2). BUN and creatinine levels were elevated for 3 days, but returned to normal within 1 week. Blood levels were followed for three weeks, but showed only fluctuation. After perfusion kidneys showed slight oedema, depending on perfusion pressure. Perfusion pressure greater than 120 mm Hg resulted in some oedema, kidneys perfused with less than 120 mm Hg showed no oedema; thus all perfusions with TNF-α were performed with a pressure less than 120 mm Hg. At sacrifice no macroscopic abnormalities were seen. All rats survived the procedure.

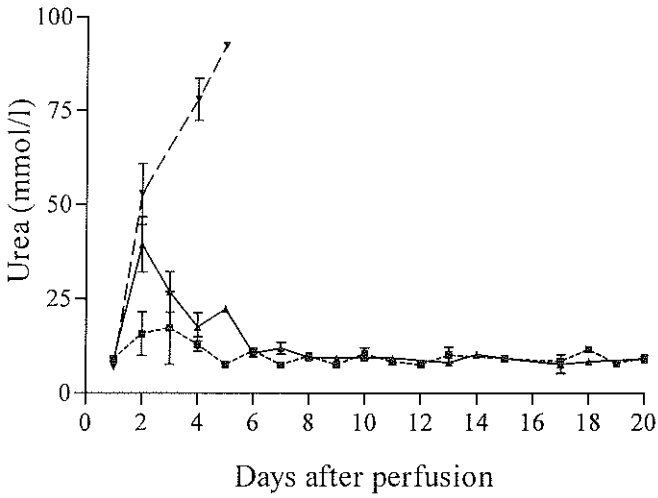

Figure 2. Course of kidney function parameters as a function of time (days) following isolated kidney perfusion with 1 μg TNF-α in a roller-pump regulated perfusion setting. After 15 minutes perfusion urea and creatinine levels returned to normal in sham (■) and 1 μg TNF-α groups (▲), after 2 μg TNF-α (▼) rats were sacrificed after 2-3 days in bad clinical condition (values depicted are a mean of 6 rats ± SD).

TNF-α perfusion

After perfusion with 1 μg TNF-α a rise in blood urea nitrogen (BUN) and creatinine levels were observed during the first four days after operation (figure 2). In spite of this initial toxicity all rats survived the procedure and kidney functions returned to normal after 6 days and remained within normal range. Animals recovered their preoperative weight after a median of 25 days after isolated kidney perfusion. Perfusion with 2 μg TNF-α resulted in a continuous rise of BUN and creatinine (figure 2), and rats were sacrificed in bad clinical condition. After two days rats were lethargic and had bloody diarrhoea.

After isolated kidney perfusions with 5 μg added to the perfusion circuit, rats died very quickly due to shock and respiratory failure within 24 hours (data not shown).

Histology

Kidneys perfused with perfusate only did not show any major changes in histology (figure 3a).

In kidneys perfused with 1 μg TNF-α no severe abnormalities could be seen, but signs of focal tubular necrosis and bleeding were seen in the 2 μg (figure 3b) and 5 μg groups. Scattered mononuclear inflammatory cells are present in the interstitium. The glomeruli appeared to be relatively unaffected.

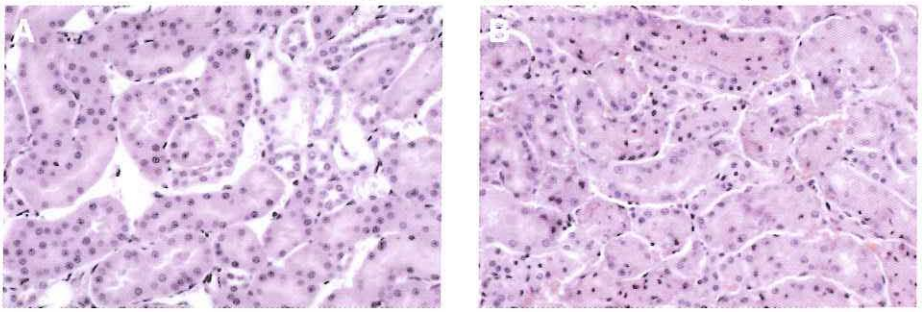

Figure 3. A: HE stained section (250 x) of a kidney 24 hours after perfusion with perfusate only. See text for explanation. B. HE stained section of a kidney 24 hours after 15 minute perfusion with 2 μg TNF-α. Focal tubular necrosis is indicated (arrow).

Animal weight

All animals showed a decrease in weight after the perfusion. The first week weight gain was minimal, but 21 days after isolated kidney perfusion the animals recovered their preoperative weight.

In vitro sensitivity of the colon adenocarcinoma CC531 to TNF-α and Melphalan

Cells of the CC531 tumor showed only a minor response to increasing dosages of rHuTNF-α as determined by the Sulphorodamine-B assay. The IC_{50} of cells treated with more than 10 μg/ml TNF-α was just reached, which means a significant reduction in the number of tumor cells after 48 hours of incubation ($p < 0.05$).

The dose/response curve of CC531 cells to melphalan alone (0 μg/ml TNF-α) showed sensitivity in vitro at dosages higher as 1 μg/ml (figure 4a). The IC_{50} of melphalan is reached with a concentration of >10 μg/ml. The cell line proved to be relatively resistant to the cytotoxic effects of melphalan.

The IC_{50} of the adenocarcinoma cells to treatment with melphalan was only slightly reduced in the presence of incrementing dosages of TNF-α (figure

4a, for clarity only 0.5 and 10 μg TNF-α is shown). The maximal growth of CC531 is reduced in the presence of TNF-α. Because the dose/response curves all bend towards total growth inhibition, irrespective of the concentration of TNF-α, a synergy between the cytokine and the cytotoxic drug in this tumor system in vitro could not be demonstrated.

In vitro sensitivity of the osteosarcoma ROS-1 to TNF-α and Melphalan

The dose-response curves of the ROS-1 osteosarcoma cell line to TNF-α and melphalan are depicted in figure 4b. The osteosarcoma cell line shows relative minor sensitivity to TNF-α alone *(Manusama et al, 1996b)*. The IC_{50} for melphalan is reached at 6 μg/ml.

Maximum growth of the osteosarcoma *in vitro* at lower dosages of melphalan is reduced in the presence of TNF-α at the various concentrations used (for clarity only 0.5 and 10 μg/ml TNF-α curves are shown). Total growth inhibition is reached with increasing dosages of melphalan, almost independent of TNF-α. These experiments therefore could not reveal synergism of TNF-α and melphalan in the tumor cytotoxic effects, but an additive effect at best.

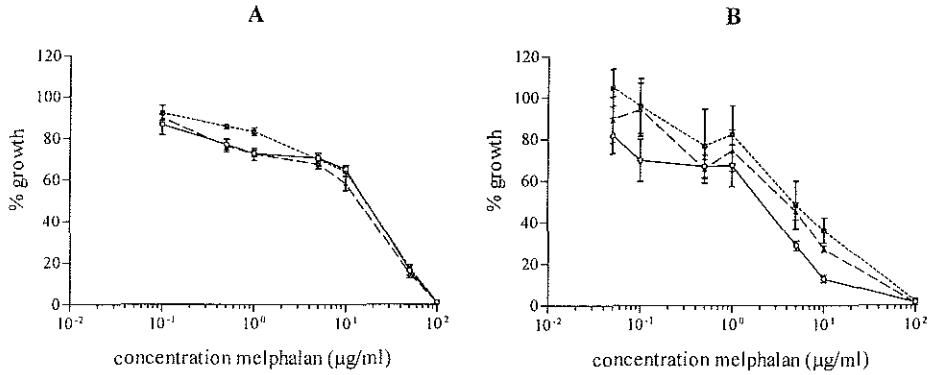

Figure 4. A: Dose/response curve of CC531 colon adenocarcinoma to melphalan in the absence or presence of various concentrations TNF-α, as determined in the Sulphorhodamine B assay (■ = 0 μg/ml TNF-α, ▲ = 0.5 μg/ml TNF-α, ○= 10 μg/ml TNF-α; for clarity concentrations between 0.5 and 10 μg/ml TNF have been omitted).
B: Dose/response curve of ROS-1 osteosarcoma to melphalan in the absence or presence of various concentrations TNF-α, determined in the Sulphorhodamine B assay (■ = 0 μg/ml TNF-α, ▲ = 0.5 μg/ml TNF-α, ○= 10 μg/ml TNF-α).

Tumor response of CC531

In the SRCA the relative low concentration of 0.2 μg/ml TNF-α had to be used. Kidney functions were severely disturbed at higher dosages. Sham perfusion and isolated kidney perfusion with 0.2 μg/ml TNF-α under oxygenated conditions showed no significant inhibition of tumor growth (data not shown).

There was minor growth inhibition of the solid tumor in this location, but none of the tumors showed regression.
A combination of the MTD of TNF-α (1 μg) and the dose used in isolated limb perfusions of 40 μg melphalan was chosen at first to investigate whether there were any synergistic or additive effects in vivo. We could not prove significant growth inhibition with this combination.

In subsequent experiments we tested the combination of a high dose melphalan (500 μg) with the MTD of TNF-α (1 μg). These experiments showed growth inhibition, but no significance was reached (figure 5, n=9; treated versus control; mean 57 mg versus 81.11 mg, s.d. 12.59 versus 13.22, p=0.1999).

Tumor response of osteosarcoma

In this tumor model only minor sensitivity was shown to the maximally tolerated dosages of TNF-α and melphalan. Mean tumor weight (n=9; treated 76.0±11.91 mg versus control 105.8±12.76 mg; p=0.157) was only slightly reduced, again without significance (figure 5).

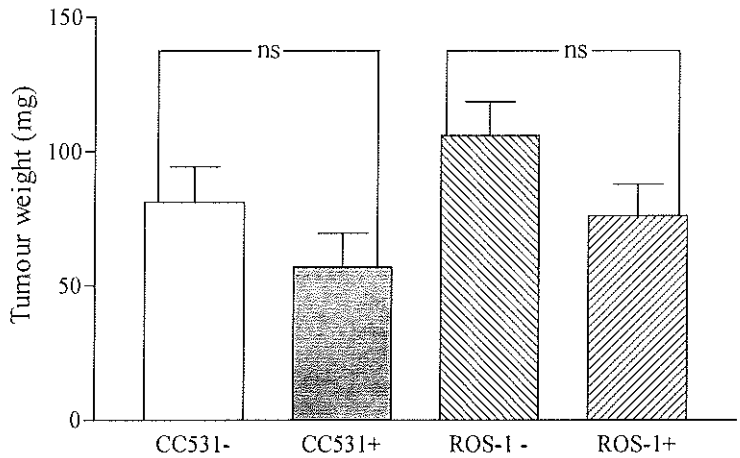

Figure 5. Growth inhibition of CC 531 adenocarcinoma and ROS-1 osteosarcoma in the Subrenal Capsule Assay after isolated kidney perfusion with 1 μg TNF-α combined with 500 μg melphalan (CC531- = sham, CC531+ = treated, ROS-1- = sham, ROS-1+ = treated; data shown are mean values of 9 rats ± SD, p=n.s.)

DISCUSSION

Isolated perfusion with Tumor Necrosis Factor-α in combination with ischaemia *(Manusama et al, 1994)* or with melphalan *(Manusama et al, 1996a,b)* has been reported to result in complete tumor regression in preclinical tumor models. Isolated limb perfusion in patients with in-transit melanoma with TNF-α

in combination with melphalan and γ-interferon resulted in high complete remission rates *(Liénard et al, 1992; 1994)*. Also high limb salvage rates have been reported with the same treatment protocol in patients with non resectable soft tissue sarcomas *(Eggermont et al, 1993; 1996a,b)*.

Isolated organ perfusion with TNF-α is clearly a more complicated matter than isolated limb perfusion. Different models have been developed to evaluate the efficacy of TNF-α in organ perfusion, such as in lung *(Weksler et al, 1993; 1994; Pogrebniak et al, 1994)*, and in liver *(Fraker et al, 1994; Borel Rinkes et al, 1997)*. TNF-α in isolated lung perfusion has been shown to be safe and in a phase I study tumor responses have been observed in patients with lung metastases *(Pass et al, 1996)*.

The isolated perfused kidney model has been used to study the effects of cytotoxic agents *(Asbach and Bersch, 1980)*. Here we demonstrate that analogous to the isolated limb perfusion (ILP) isolated perfusion of the kidney (IKP) is technically feasible.

In the current model the MTD was reached at 1 μg TNF-α, showing only a transient renal toxicity. At 2 μg TNF-α fatal renal toxicity was seen. This involved acute renal failure leading to death by day 4. An acute fatal shock syndrome was noted at 5 μg.

It is known that TNF-α may have direct toxic effects to the kidney *(Kahky et al, 1990; Gaskill, 1988; Tracey et al, 1986)*. Acute tubular necrosis was seen with portal infusion of sublethal doses of TNF-α with relative sparing of the glomeruli. In surviving animals a decrease in kidney function was noted. The serious toxicity seen in our experiments with higher dosages of TNF-α may be partially explained by the production of TNF-α by glomerular macrophages, mesangial cells and renal tubular cells upon stimulation *(Affres et al, 1991; Tipping et al, 1991; Baud and Ardaillou 1995)*. Analogous to the production of TNF-α and interleukin-1 by Kupffer cells *(Kahky et al, 1990)*, we hypothesise that a potent second cytokine release is responsible for the increased toxicity. In rats perfused with 5 μg the secondary cytokine response was so extreme that the rats died of acute respiratory distress. Similar observations have been reported by *Fraker et al (1994)* in pigs, shown to be due to a secondary cytokine response that could not be prevented by anti-TNF-α antibody treatment. Toxicity to the lung is manifested by pulmonary oedema and adult respiratory distress syndrome *(Pogrebniak et al, 1994)*. Thus, in contrast to the very high concentration of TNF-α that can be applied in the ILP setting, the kidney proves to be a very susceptible organ which only tolerates $1/50^{th}$ of the TNF-α dose used in ILP.

In vitro synergy between TNF-α and melphalan for the rat colon adenocarcinoma cell line CC531 was not observed. Also, synergy could not be proven for the osteosarcoma cell line ROS-1. Here an additive effect at best is reached.

For in vivo studies two solid tumor systems were chosen with a different

vascularization pattern. The rational for this choice is based on our previous work with the highly vascularized soft tissue sarcoma BN175 where the synergistic anti-tumor effects with the combination of TNF-α with melphalan results were shown to induce vascular changes accompanied by increased vascular permeability and platelet aggregation *(Manusama et al, 1996b; Nooijen et al, 1996).*

The observations in our *in vivo* experiments made clear, however, that no strong synergistic antitumor effects existed in either tumor. Instead of the high concentration of 10 μg/ml TNF-α as used in isolated limb perfusion with ROS-1, the relatively low dose of 0.2 μg/ml had to be used because of dose limiting toxicity. Thus, the minimal threshold concentration of TNF-α was not reached and therefore the crucial vascular effects in vivo described previously *(Manusama et al, 1996b; Nooijen et al, 1996)* are not observed.

Since isolated kidney perfusion in the rat allowed only low dosages of TNF-α, the dual role of TNF-α on the tumor vasculature may be an explanation for the discrepancies between in vitro and in vivo results. It has been demonstrated that low TNF-α concentrations are promoting angiogenesis while high concentrations of TNF-α are toxic to the vessels *(Fajardo et al, 1992).* The concentration of TNF-α used in isolated kidney perfusion is only 0.2 μg/ml. At this concentration a promotion of angiogenesis might even be more plausible than the vascular destruction seen with higher dosages. Thus, the typical effects of TNF-α are not seen which is an explanation for the absence of growth retardation in the tumor models used in our experiments.

While the model of isolated kidney perfusion was developed to evaluate the effect of TNF-α in isolated organ perfusion, it is also possible to treat kidney tumors with this regimen. Because the main target of TNF-α is the vascular endothelium, well vascularized renal tumors could potentially be responsive. In a recently published study isolated perfusion of the kidney in a miniature swine it appeared possible to perfuse the kidney with 1 mg/ml *(Walther et al, 1996).*

We conclude from our studies with the isolated kidney perfusion model that since only $1/50^{th}$ of the TNF-α concentration was tolerated the advantage of regional application is lost and perspectives for efficacy in tumor bearing species is much reduced. If the dose needed for anti-tumor effect is 50 times higher than the maximal tolerated dose in isolated kidney perfusion *(Asher et al, 1987; Liénard et al, 1992, 1994; Eggermont et al, 1996a,b)* the outlook for clinical applicability seems to be poor.

ACKNOWLEDGEMENT

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Chapter 3

TNF-α augments intratumoral concentrations of doxorubicin in TNF-α-based isolated limb perfusion in rat sarcoma models and enhances anti-tumor effects.

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ABSTRACT

We have shown previously that isolated limb perfusion (ILP) in sarcoma-bearing rats results in high response rates when melphalan is used in combination with TNF-α. This is in line with observations in patients. Here we show that ILP with doxorubicin in combination with TNF-α has comparable effects in two different rat sarcoma tumor models. The addition of TNF-α exhibits a synergistic anti-tumor effect, resulting in regression of the tumor in 54% and 100% of the cases for the BN175-fibrosarcoma and the ROS-1 osteosarcoma respectively. The combination is shown to be mandatory for optimal tumor response. The effect of high dose TNF-α on the activity of cytotoxic agents in ILP is still unclear. We investigated possible modes by which TNF-α could modulate the activity of doxorubicin. In both tumor models increased accumulation of doxorubicin in tumor tissue was found: 3.1-fold in the BN175 and 1.8-fold in the ROS-1 sarcoma after ILP with doxorubicin combined with TNF-α in comparison with an ILP with doxorubicin alone. This increase in local drug concentration may explain the synergistic anti-tumor responses after ILP with the combination. In vitro TNF-α fails to augment drug uptake in tumor cells or to increase cytotoxicity of the drug. These findings make it unlikely that TNF-α directly modulates the activity of doxorubicin in vivo. As TNF-α by itself has no or only minimal effect on tumor growth, an increase in local concentrations of chemotherapeutic drugs might well be the main mechanism for the synergistic antitumor effects.

INTRODUCTION

Low concentrations at the tumor site and dose limiting systemic toxicity are common causes for failure of solid tumor treatment with anti-tumor agents. As cytotoxic drugs typically exhibit a steep dose response-curve, increasing local concentration should favour tumor response. In isolated limb perfusions local drug concentrations are increased while systemic exposure to the drugs is minimal. In isolated limb perfusions (ILP) melphalan is used most commonly, but also other agents (e.g doxorubicin and cisplatin) are applied with varying success in perfusion of limb or organ (e.g lung) *(Abolhoda et al, 1997; Klaase et al, 1989; Rossi et al, 1992; Tonak et al, 1979; Weksler et al, 1994).* Tumor Necrosis Factor alpha (TNF-α), a cytokine with known anti-tumor activity, can not be used systemically in dosages high enough to obtain a tumor response *(Asher et al, 1987; Fajardo et al, 1992).* However, in ILP with TNF-α tumors are exposed to concentrations of up to 50 times higher than those reached after systemic administration of the maximum tolerated dose (MTD), without major side effects *(Benckhuijsen et al, 1988).* Previously it was demonstrated that the addition of TNF-α to melphalan in ILP could improve response rates in patients with multiple melanoma in transit

metastases or irresectable soft tissue extremity sarcomas *(Eggermont et al, 1993; Eggermont et al, 1996a; Eggermont et al, 1996b; Lejeune et al, 1993; Liénard et al, 1994; Liénard et al, 1992)*. In both patient groups very high response rates of above 85% have been reported, with a limb salvage rate of more than 85%. In Europe TNF-α was recently approved and registered for clinical use in patients for the treatment of locally advanced extremity soft tissue sarcomas by ILP with TNF-α and melphalan. Comparable results have been reported by us for ILP with the combination of TNF-α and melphalan in soft tissue sarcoma and osteosarcoma-bearing rats *(Manusama et al, 1996a; Manusama et al, 1996b)(de Wilt et al, 2000)*. ILP with TNF-α alone or melphalan alone at concentrations used in the clinical setting had negligible antitumor effects, whereas the combination showed strong synergistic anti-tumor efficacy.

TNF-α may potentiate the effects of chemotherapy in ILP in various ways. TNF-α has a broad spectrum of activities, which range from enhancement of proliferation to direct cytotoxicity on tumor cells, activation of inflammation and effects on endothelium *(Fajardo et al, 1992; Watanabe et al, 1988)*. The tumor associated vasculature (TAV) responds to TNF-α with rounding of the endothelial cells resulting in increased gaps, allowing easy passage of soluble materials and even cells *(Folli et al, 1993; Renard et al, 1995; Smyth et al, 1988)*. Moreover, intravenous injection of TNF-α in human melanoma xenograft-bearing mice resulted in significant reduction of the interstitial fluid pressure (IFP) of the tumors *(Kristensen et al, 1996)*. This phenomenon could increase localization of cytotoxic drugs in the tumor interstitium and explain improved tumor response. Secondly, clinical and experimental results demonstrating massive destruction of the endothelial cells, which has also been shown in vitro and on angiograms in patients after ILP, suggest that the TAV is the primary target for TNF-α and therefore that destruction of the endothelial lining might be responsible for the anti tumor response *(Olieman et al, 1997; Sato et al, 1986; Watanabe et al, 1988)*. This process is accompanied by inflammatory responses and seemed to be dependent on infiltrating leukocytes *(Manusama et al, 1998)*. Coagulative and haemorrhagic necrosis and destruction of the endothelial lining was also seen when TNF-α was used as a single agent in ILP, however without significant effect on tumor growth in rats. This indicates that the direct TNF-α effect is most likely playing a minor role in the anti-tumor capacity *(Manusama et al, 1996a; Nooijen et al, 1996a)*.

Although in the majority of the perfusions, especially for the treatment of melanoma, melphalan is used, also other agents might be successful. Anthracyclines are among the most active agents against solid tumors and doxorubicin is the most widely used agent of this class *(Bielack et al, 1996; Budd, 1995)*. Moreover, doxorubicin is the agent of choice for the treatment of sarcoma, and has shown good anti-tumor activity in clinical and experimental perfusion settings for the treatment of lung metastasises *(Abolhoda et al, 1997; Weksler et al, 1994)*, and could therefore be a suitable cytotoxic agent for ILP in sarcoma-

bearing patients.

In this study we undertook isolated limb perfusions with doxorubicin and TNF-α in soft tissue sarcoma- and osteosarcoma-bearing rats to examine the effect of TNF-α on the anti tumor activity of doxorubicin and secondly an attempt was made to unravel possible mechanisms by which TNF-α potentiates the anti-tumor activity of doxorubicin.

MATERIALS AND METHODS

Chemicals

Human recombinant Tumor Necrosis Factor-α (specific activity $5x10^7$ IU/mg) was kindly provided by Dr G. Adolf (Bender Wien GmbH, Wien, Austria) and stored at a concentration of 2 mg/mL. Endotoxin levels (LAL) were below 0.624 EU/mg. Doxorubicin (Adriablastina®) was purchased from Farmitalia Carlo Erba (Brussels, Belgium).

Animals and tumor model

Male inbred BN rats were used for the soft tissue sarcoma model (BN175) and WAG/RIJ rats for the osteosarcoma model (ROS-1). Rats were obtained from Harlan-CPB (Austerlitz, the Netherlands) and weighing 250 - 300 g. Small fragments (3 mm) of the syngeneic BN175 or ROS-1 sarcoma were implanted subcutaneously in the right hindleg as previously described *(Manusama et al, 1996a,b)*. Tumor growth was recorded by calliper measurements and tumor volume calculated using the formula $0.4(A^2xB)$ (where B represents the largest diameter and A the diameter perpendicular to B). All animal studies were done in accordance with protocols approved by the Animal Care Committee of the Erasmus University Rotterdam, the Netherlands.

The classification of tumor response was: progressive disease (PD): increase of tumor volume (> 25 %) within 5 days; no change (NC): tumor volume equal to volume during perfusion in a range of -25 % and + 25 %; partial remission (PR): decrease of tumor volume between -25% and -90 %; complete remission (CR): tumor volume less than 10% of initial volume.

Isolated limb perfusion protocol

Rats were perfused according to the isolated limb perfusion technique originally described by Benckhuijsen et al, and adapted for the rat by Manusama et al, *(Benckhuijsen et al, 1982a; Manusama et al, 1996a)*. Briefly, the femoral artery and vein of anaesthetized rats were cannulated with silastic tubing. Collaterals were occluded by a groin tourniquet and perfusion started when the tourniquet was tightened. The extracorporeal circuit included an oxygenation reservoir and a roller pump (Watson Marlow, Falmouth, UK). The perfusion was

performed with 5 mL Haemaccel (Behring Pharma, Amsterdam, Netherlands) and TNF-α (50 μg) and/or doxorubicin (400 μg BN175, and 200 μg ROS-1) were added as boluses to the oxygenation reservoir. Control rats (sham) were perfused with Haemaccel alone. The concentration of TNF-α was adapted from previous animal studies and doxorubicin concentrations were chosen which had no local toxicity and induced maximally stable disease after single perfusion *(Manusama et al, 1996a)*. Perfusion was maintained for 30 min at a flow rate of 2.4 mL/min. During the perfusion the hindleg of the rat was kept at a temperature of 38-39°C with a warm water mattress. A washout with 2 mL oxygenated Haemaccel was performed at the end of the perfusion. Perfusion was performed at a tumor diameter of 12-15 mm, which is around 7 or 10 days after implantation for BN175 and ROS-1 respectively.

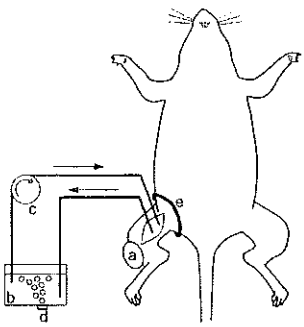

Figure 1. Schematic representation of isolated limb perfusion

In vitro assessment of anti-tumor activity

BN175 soft tissue sarcoma cells or ROS-1 osteosarcoma cells were added in 100 μl aliquots to 96-well plates at a final concentration of 10^5 cells per well and allowed to grow as a monolayer in DMEM supplemented with 10% FCS. Doxorubicin and/or TNF-α were diluted in DMEM supplemented with 10% FCS, added to the wells and allowed for three days to incubate. The range of final drugs in the wells was 0.0005 - 100 μg/mL for doxorubicin and 0 - 10 μg/mL for TNF-α. A total of 5 to 6 separate assays were performed in triple and the percentage of growth inhibition calculated according to the formula: percentage of tumor cell growth = (test well/control well)x100%. Percentage of tumor cell cytotoxicity was measured using the sulphorhodamine B assay *(Keepers et al, 1991)*.

In vitro assessment of doxorubicin uptake in tumor cells

To determine if the observed anti-tumor response after ILP and cytotoxicity in vitro correlated with cellular uptake of doxorubicin, cells were exposed to doxorubicin with and without TNF-α and intracellular doxorubicin levels determined by flowcytometry as previously described *(Luk and Tannock, 1989)*. Briefly, BN175 soft tissue sarcoma cells or ROS-1 osteosarcoma cells were added in 500 μl aliquots to 24-well plates at a final concentration of $5x10^4$ cells per

well and allowed to grow as a monolayer in DMEM supplemented with 10% FCS. Doxorubicin and TNF-α were diluted in DMEM supplemented with 10% FCS and added to the wells, after which cells were incubated for 0, 10, 30, 60, and 120 min. The final drug concentration in the wells was 0, 0.1, 1.0 and 10 μg/mL for both doxorubicin and TNF-α. Thereafter monolayers were treated with trypsin-EDTA for 2 min and the cell suspensions were washed two times in complete medium and resuspended in PBS. Cellular uptake was measured on a Becton Dickinson FACScan using Cell Quest software on Apple Macintosh computer. Excitation was set at 488 nm and emission at 530 nm. Fluorescence was corrected for cell size using the forward scatter (FSC) with the formula corrected fluorescence (FLcor) = fluorescence at 530 nm (FL530) / FSC - $FL530_c$ / FSC_c ($FL530_c$ and FSC_c are fluorescence and forward scatter with no drug added to the cells).

Assessment of doxorubicin accumulation in solid tumor and concentration in perfusate during ILP

To determine the influence of TNF-α on doxorubicin accumulation in tumors during ILP, tumors (and muscle) were surgically removed after ILP and total doxorubicin content determined as previously described *(Mayer et al, 1989).* As the ILP included a thorough washout there is no intravascular doxorubicin present. Briefly, after incubation in acidified isopropanol (0.075 N HCl in 90% isopropanol) for 24 h at 4°C, the tumors were homogenized (PRO200 homogenizer with 10 mm generator, Pro Scientific, CT, USA), centrifuged for 30 min at 2500 rpm and supernatants harvested. Samples were measured in a Hitachi F4500 fluorescence spectrometer (excitation 472 nm and emission 590 nm) and compared with a standard curve prepared with known concentrations of doxorubicin diluted in acidified isopropanol. Measurements were repeated after addition of an internal doxorubicin standard. Detection limit for doxorubicin in tissue was 0.1 μg per gram tissue.

For perfusate measurements samples were drawn from the perfusion vial at 0.5, 5, 15 and 30 min after ILP was started. Samples were centrifuged for 30 min at 2500 rpm and supernatant measured for doxorubicin content as described above. Cell pellets were incubated in acidified isopropanol and doxorubicin content determined as described above.

Statistical analysis

The in vivo and in vitro results were evaluated for statistical significance using the Mann Whitney U test with SPSS for windows. In vitro data was analysed by curve fitting using GraphPad Prism. P values below 0.05 where considered statistically significant.

RESULTS

In vivo tumor response to doxorubicin and TNF-α

To evaluate the anti-tumor activity of doxorubicin when combined with TNF-α in an isolated limb perfusion setting, soft tissue sarcoma and osteosarcoma-bearing rats were perfused with the agents combined or alone. Figure 2 shows the tumor responses of soft tissue sarcoma (BN175) in rats after ILP. Perfusion with buffer or TNF-α alone resulted in progressive disease in all animals. Although ILP with doxorubicin (400 μg) alone resulted in a slight inhibition of the BN175 tumor growth when compared with the sham control, none of the rats showed a tumor response (Table 1). ILP with 400 μg doxorubicin combined with 50 μg TNF-α resulted in increased anti tumor activity with a response rate of 54% (PR and CR combined)($p < 0.01$ compared with doxorubicin alone).

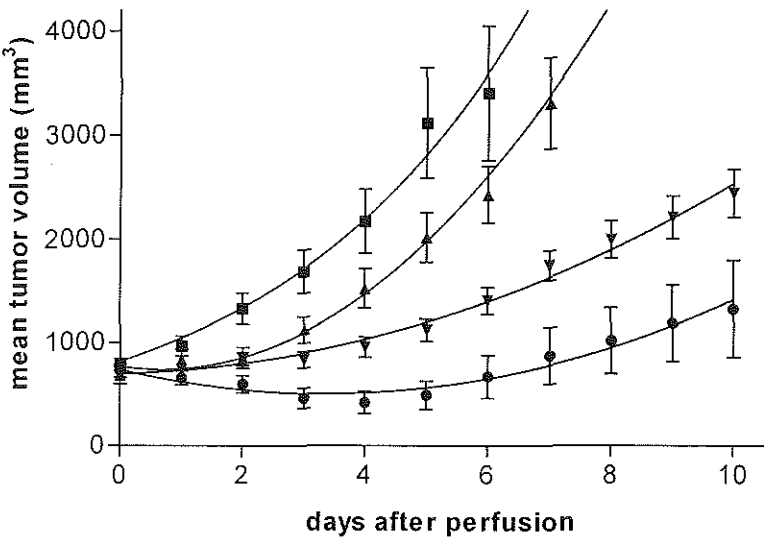

Figure 2. Growth curves of subcutaneous implanted soft tissue sarcoma BN175 after isolated limb perfusion with medium alone (■), 50 μg TNF-α (▲), 400 μg doxorubicin (▼), or combination of TNF-α and doxorubicin (●). Mean tumor volumes are shown ± SEM. Number of rats per group is shown in table 1.

In osteosarcoma (ROS1)-bearing rats ILP with buffer or doxorubicin (200 μg) alone had no significant effect on tumor growth (Figure 2). ILP with TNF-α alone resulted in significant inhibition of tumor growth as compared with the sham perfusion and a response rate of 33% was observed (Table 2). ILP with 200 μg doxorubicin combined with 50 μg TNF-α further increased the anti tumor response with a response rate of 100% (PR and CR combined) ($p < 0.05$ compared with TNF-α alone).

Table 1. Tumor response of BN-175 after Isolated Limb Perfusion with doxorubicin and Tumor Necrosis Factor-α (TNF-α) five days after treatment.

Tumor response[a]	Sham	TNF-α	Doxorubicin	TNF+Doxorubicin[b]
	n=12	n=10	n=10	n=13
PD[c]	12	10	6	2
NC			4	4
PR				6
CR				1
Response rate(%)	-	-	-	54

a responses were scored as described in Materials and Methods

b TNF-α and doxorubicin, 50 and 400 μg respectively, were added to the perfusate (5 mL) as boluses.

c PD: progressive disease, NC: no change, PR: partial remission, CR: complete remission.

Table 2. Tumor response of ROS-1 after Isolated Limb Perfusion with doxorubicin and Tumor Necrosis Factor-α(TNF-α) during five days after treatment.

Tumor response[a]	Sham	TNF-α	Doxorubicin	TNF+Doxorubicin[b]
	n=8	n=11	n=8	n=10
PD[c]	8	3	2	
NC		3	6	
PR		1		6
CR		2		1
Response rate(%)	-	**33**	-	**100**

a responses were scored as described in Materials and Methods

b TNF-α and doxorubicin, 50 and 400 μg respectively, were added to the perfusate (5 mL) as boluses.

c PD: progressive disease, NC: no change, PR: partial remission, CR: complete remission.

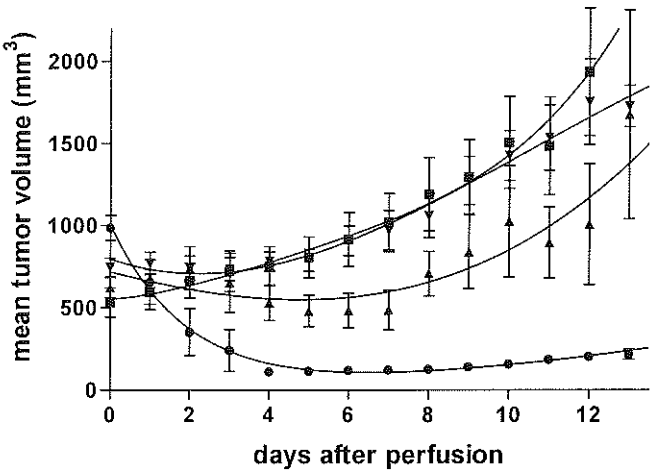

Figure 3. Growth curves of subcutaneous implanted osteosarcoma ROS-1 after isolated limb perfusion with medium alone (■), 50 μg TNF-α (▲), 200 μg doxorubicin (▼), or combination of TNF-α and doxorubicin (●). Mean tumor volumes are shown ± SEM. Number of rats per group is shown in table 2.

In vitro assessment of anti-tumor activity of doxorubicin and TNF-α

The in vivo experiments clearly demonstrate pronounced improvement of tumor response when doxorubicin was used in combination with TNF-α. In vitro experiments were performed to further study the nature of this interaction. Exposure of soft tissue sarcoma BN175 or osteosarcoma ROS-1 tumor cells to doxorubicin resulted in a response curve with an IC50 of 0.1 and 2.0 μg/mL respectively (figure 4). No significant cellular toxicity could be observed when BN175 cells were exposed to TNF-α alone, however a dose depended growth reduction was observed when ROS-1 cell were exposed to TNF-α with a maximum reduction of 38% at 10 μg/mL. Addition of TNF-α to doxorubicin did not significantly alter the IC50 of doxorubicin in the BN-175 cell cultures, indicating that addition of TNF-α in vitro did not influence the sensitivity of the cells to doxorubicin significantly. On ROS-1 cells only an additive effect of TNF-α and doxorubicin was observed. The curve only shifted downwards and not to a lower doxorubicin concentration, which indicates that the drugs do not influence each other but have separate effects.

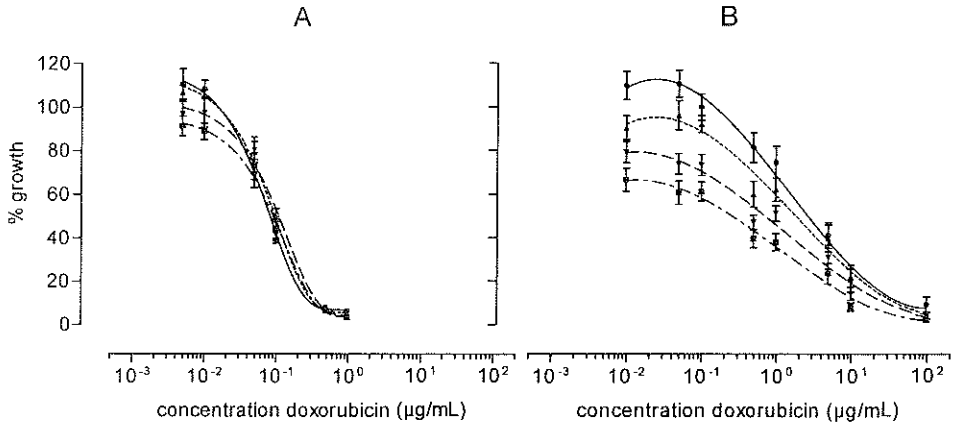

Figure 4. In vitro growth of (a) the BN175 and (b) ROS-1 tumor cells as function of the doxorubicin concentration in combination with 0 µg (●), 0.1 µg (▲), 1.0 µg (▼) or 10 µg TNF-α per mL (■). The mean of 5 to 6 individual experiments performed in triple is shown ± SEM.

In vitro uptake of doxorubicin in tumor cells

Figure 5 shows that increased intracellular concentrations of doxorubicin are observed in both cell types when cells were incubated with increasing concentrations of doxorubicin. A 10-fold higher doxorubicin concentration in culture supernatant (ranging from 1.0 to 10 µg/mL) resulted in 4.5-fold and 3.9-fold augmented cellular uptake for BN175 and ROS-1 respectively ($p < 0.01$ and $p < 0.05$). Addition however of TNF-α to the culture medium did not influence intracellular doxorubicin content significantly for all the TNF-α concentrations tested, or even a slight but not significant reduction in uptake was noticed with increasing concentrations of TNF-α (figure 6).

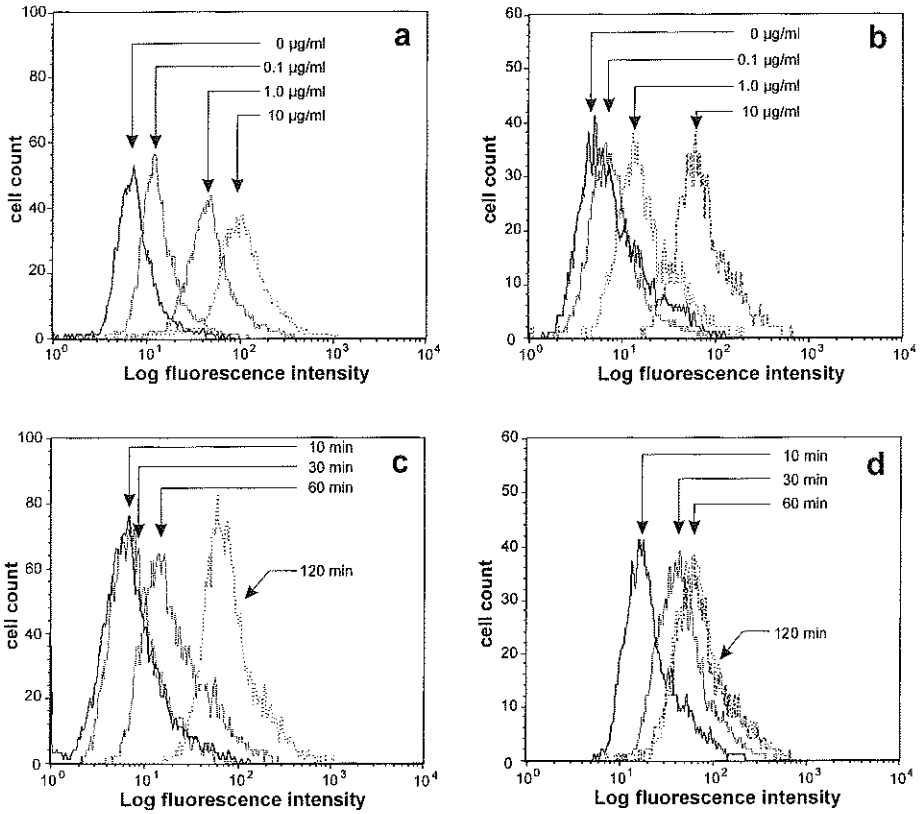

Figure 5. Uptake of doxorubicin by (a and c) BN175 tumor cells, or (b and d) ROS-1 tumor cells in vitro as determined by flowcytometry after exposure of the cells to 0, 0.1, 1.0 or 10 µg per ml doxorubicin for 2 hrs (a and b) or for various durations of time at a fixed doxorubicin concentration of 10 µg per ml c and d). The graphs are good representatives of the experiments performed.

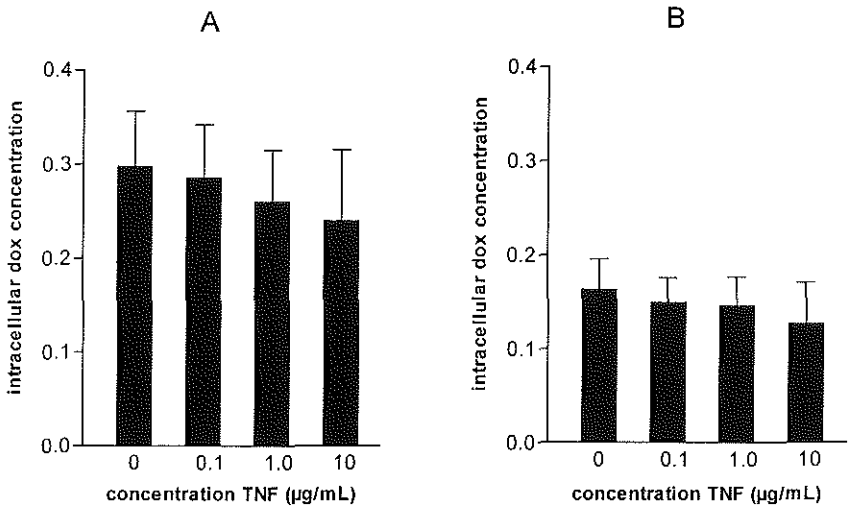

Figure 6. Uptake of doxorubicin in (a) BN175 or (b) ROS-1 tumor cells in vitro at respectively 120 and 60 min of exposure to the agent in the presence of 0, 0.1, 1.0 or 10 µg TNF-α per mL. The mean of 5 experiments is shown ± SD.

Doxorubicin accumulation in solid tumor after ILP

Possibly the observed beneficial effect of TNF-α in vivo could be explained by an increased extravasation of doxorubicin into the tumor interstitium, resulting in a higher local concentration and accordingly in an improved anti tumor activity. Therefore, concentrations of doxorubicin in tumor and surrounded tissue after ILP were determined. Figure 7 shows that measurable amounts of doxorubicin localized both in BN175 and ROS-1 tumors after ILP, which correlates with an observed decline of the drug concentration in the perfusate (data not shown). Moreover, addition of TNF-α to the perfusate resulted in significantly enhanced accumulation of doxorubicin in both these tumors, 3.1-fold in the BN175 and 1.8-fold in the ROS-1 sarcoma, when compared with ILP with doxorubicin alone. Addition of TNF-α had no significant effect on doxorubicin accumulation in muscle of the leg ($p > 0.4$). Strikingly a significant discrepancy in drug levels was observed between BN175 and ROS-1 tumors.

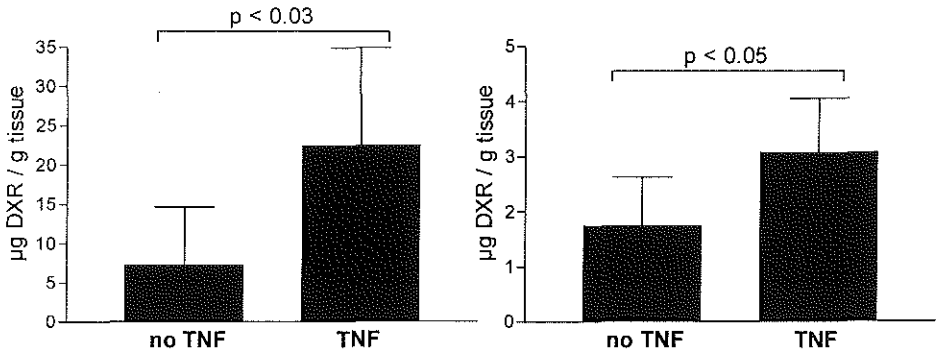

Figure 7. Accumulation of doxorubicin in (a) soft tissue sarcoma BN175 or (b) osteosarcoma ROS-1 in vivo during isolated perfusion. Rats were perfused with doxorubicin (400 µg BN175 and 200 µg ROS-1) with 50 µg TNF-α or without TNF-α, after which tumors and muscle were excised and total doxorubicin content determined as described in materials and methods. The mean of 6 rats are shown ± SD

DISCUSSION

In the present study we demonstrate that isolated limb perfusion in sarcoma-bearing rats with doxorubicin in combination with TNF-α results in high response rates in two different tumor models. These findings are in close agreement with our previous work using melphalan *(Manusama et al, 1996a; Manusama et al, 1996b)*. Secondly, it is demonstrated for the first time that TNF-α enhances intratumoral accumulation of doxorubicin, which is an attractive explanation for the augmented tumor response in TNF-α-based ILP. We speculate that TNF-α increases interstitial drug levels in the tumor as intravascular doxorubicin is washed out at the end of the ILP procedure and intracellular uptake of doxorubicin is not affected by TNF-α as was shown in vitro.

Doxorubicin has been shown to be the most effective drug in treatment of sarcomas and therefore put forward as the drug of choice in the treatment of these malignancies *(Bielack et al, 1996; Budd, 1995)*. Here we demonstrate that perfusion with doxorubicin alone is not or only partial effective, which however is also observed when melphalan is used as a single agent in the perfusion setting.

A striking observation is the augmentation of the doxorubicin-induced anti-tumor response by TNF-α in vivo, which has also been shown for melphalan and TNF-α in these tumor models *(Manusama et al, 1996a; Manusama et al, 1996b)*. Strong tumor responses were observed in both models after ILP with the combination therapy, which cannot be explained by just adding up the responses after ILP with the single agents. An important observation is that chemotherapy by itself is not or partially effective as shown here and by others *(Klaase et al, 1989)*. Secondly, it was previously shown in our rat tumor model as well as in the clinic

that ILP with TNF-α alone had no effect on tumor growth although massive haemorrhagic necrosis and pathology was observed *(Manusama et al, 1996a; Nooijen et al, 1996; Posner et al, 1994)*. These observations indicate that other mechanisms have to be identified to explain the interaction between TNF-α and chemotherapy.

Several specific activities of TNF-α could potentiate the anti tumor activity of chemotherapy. It has been postulated that the increased tumor response observed after ILP with melphalan and TNF-α is due to destruction of the TAV, resulting in haemorrhagic necrosis, platelet aggregation and erythrostasis *(Nooijen et al, 1996; Watanabe et al, 1988)*. Moreover, recently is has been shown that perfusion with melphalan in combination with TNF-α and IFN-γ resulted in apoptosis of endothelial cells of the TAV *(Ruegg et al, 1998)*. Also inflammatory events such as granulocyte infiltration were suggested to play a role *(Manusama et al, 1998; Nooijen et al, 1996)*. These findings led to the speculation that destruction of the TAV is the mechanism by which TNF-α potentiates cytotoxic agents. Watanabe et al demonstrated toxic effects of TNF-α on newly formed tumor vasculature in mice resulting in haemorrhage, congestion and blood circulation blockage *(Watanabe et al, 1988)*. Others suggested that TNF-α-induced thrombus formation played an important role *(Shimomura et al, 1988)*. However, these effects are also observed after perfusion with TNF-α alone *(Nooijen et al, 1996)*.

Recent studies show that perfusion of melanoma-bearing patients with melphalan in combination with TNF-α and IFN-γ results in detachment and apoptosis of endothelial cells of the tumor (Ruegg et al, 1998). Moreover, the vitro experiments demonstrated an important role for TNF-α and IFN-γ mediated down modulation of the αVβ3 function, which is speculated to play an prominent role in the in vivo observations. These finding would argue in favour for a TNF-α mediated destruction of the vasculature. The in vitro observations also demonstrated the necessity of IFN-γ for the induction of endothelial apoptosis. In our model, as well as in various clinical trails, on the other hand it has been shown that tumor responses are only slightly improved by the addition of IFN-γ *(Eggermont et al, 1996b; Liénard et al, 1992b)*. This would argue against an important role for TNF-α-mediated destruction of the TAV in the tumor response, or on the other hand it indicates that endogenous produced IFN-γ is of major importance.

A consistent finding in our two models is the augmented accumulation of doxorubicin in tumor tissue when TNF-α is added to the perfusate. In both models this increase could very well explain the improved efficacy. On the other hand, TNF-α may increase the uptake of doxorubicin by the tumor cells. However, intracellular concentration of doxorubicin in vitro was not enhanced when TNF-α was added in vitro. Moreover, TNF-α did not seem to affect the in vitro cytotoxic

activity of doxorubicin significantly. In contradiction to these findings, synergy between TNF-α and doxorubicin in vitro has been shown in previous studies depending on sensitivity of the cells to TNF-α, presence of multi-drug resistance or order of exposure *(Alexander et al, 1987; Bonavida et al, 1990; Fruehauf et al, 1991; Soranzo et al, 1990).* This effect has also been shown without an increased intracellular accumulation of doxorubicin *(Safrit et al, 1993).* Others demonstrated that exposure of tumor cells to TNF-α resulted in a reduced sensitivity of these cells to doxorubicin *(Prewitt et al, 1994).* It is suggested that arrest of the cells in the G1/0 phase by TNF-α turns them insensitive to doxorubicin, which is a cell cycle dependent cytotoxic agent. In our study we did not observe such phenomenon when the tumor cells were exposed to doxorubicin and TNF-α. These observations suggest that in vivo TNF-α has an indirect effect on the anti-tumor activity of doxorubicin. Therefore, we postulate that TNF-α augments the accumulation of doxorubicin in the tumor by increasing the leakiness of the tumor associated vasculature (TAV), and by doing so increases the local drug level. Previously an increased leakiness of the TAV as well as a reduction of the interstitial fluid pressure in tumor has been shown by others after systemic administration of TNF-α *(Folli et al, 1993; Kristensen et al, 1996; Renard et al, 1995; Smyth et al, 1988).* Moreover, increased drug accumulation in tumor has previously been shown after systemic treatment with TNF-α when a liposomal doxorubicin preparation was injected *(Suzuki et al, 1990).* Preliminary results from a clinical phase I-II trail with doxorubicin and TNF-α in hyperthermic ILP demonstrated comparable favourable outcome as is obtained with Melphalan and TNF-α *(Di Filippo et al, 1998).*

From our study we propose that the observed augmentation of the anti-tumor activity of doxorubicin by TNF-α is mainly due to an increased accumulation of doxorubicin in the tumor during ILP as is shown in both models. A direct effect of TNF-α on the sensitivity of the tumor cells to doxorubicin was ruled out by in vitro examinations.

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Chapter 4

Lack of cell cycle specific effects of Tumor Necrosis Factor-α on tumor cells in vitro: implications for combination tumor therapy with doxorubicin.

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ABSTRACT

Addition of Tumor Necrosis Factor–α (TNF–α) to chemotherapy enhances tumor response in several treatment modalities. However, it has been shown that TNF–α, and several other cytokines, exert inhibitory effects on cell cycle progression and by doing so may attenuate sensitivity of these cells to cell cycle dependent cytotoxic drugs (e.g. doxorubicin). Here we determined the cytotoxic effect of TNF–α on several tumor cell lines in vitro in combination with doxorubicin (cell cycle dependent) or melphalan (cell cycle independent), and its effect on cell cycle progression. The rat cell lines were prepared from tumors which were used previously in animals studies, in which synergy was shown between TNF–α and the cytotoxic drugs.

Here we demonstrate that addition of TNF–α to doxorubicin or melphalan in vitro had no attenuating effect on the cytotoxic drugs. Depending on the cell type used, addition of TNF–α induced no or only an additive cytotoxic effect. Moreover, also pre-incubation of the cells, prior to doxorubicin or melphalan, induced no or only an additive cytotoxic effect. Also in the TNF–α sensitive WEHI-164 cell line, which was used as a positive control, addition resulted in enhanced cytotoxic effect which was of an additive kind. Examination of the effect of TNF–α on cell cycle progression revealed that exposure of BN-175 or CC531 rat tumor cells or WEHI-164 tumor cells was without effect, independent of sensitivity of the cells to TNF–α. Only ROS-1 tumor cells demonstrated a cell arrest in the G2 phase, which did not result in attenuation of the cytotoxicity of doxorubicin towards these cells.

TNF–α has recently been registered in Europe for combination therapy of soft tissue sarcoma with melphalan by isolated perfusion. The presented findings warrant further exploration of the usefulness of TNF–α in combination with cytotoxic drugs for the treatment of solid tumors.

INTRODUCTION

Tumor Necrosis Factor-α (TNF-α) has impressive anti-tumor activity in clinical and preclinical setting. Especially isolated limb perfusion (ILP) with high dose TNF-α and melphalan (with or without interferon-γ (IFN-γ)) proved to be very successful in patients with large irresectable soft tissue sarcomas achieving limb salvage in over 80% of patients *(Eggermont et al., 1996b; Eggermont et al., 1996a)*. The same regimen was also highly effective as induction biochemotherapy in ILP for in transit melanoma metastases *(Liénard et al., 1992)*. We have previously shown in various tumor models in vivo that synergism between TNF-α and melphalan is responsible for the anti-tumor effect *(Manusama et al., 1996a; Manusama et al., 1996b; de Wilt et al., 1999)*.

From these studies it becomes clear that TNF–α enhances the anti-tumor activity of melphalan in ILP. Moreover, we demonstrated recently that TNF–α could also enhance the activity of doxorubicin, an anthracycline, in an ILP setting *(van der Veen, 2000)*. Anthracyclines are among the most active agents against solid tumors and doxorubicin is the most widely used agent of this class *(Bielack et al., 1996; Bramwell, 1988; Budd, 1995)*. Moreover, doxorubicin is the agent of choice for the treatment of sarcoma, and has shown good anti-tumor activity in clinical and experimental perfusions settings *(Weksler et al., 1994; Abolhoda et al., 1997; Wang et al., 1995)*. Clinically however, ILP with doxorubicin was reported to cause considerable regional toxicity and conflicting reports with regard to efficacy have been published *(Klaase et al., 1989; Rossi et al., 1994)*. Addition of TNF–α in such a setting could facilitate lower dosages of doxorubicin with concomitant diminished side-effects.

The success of application of TNF–α in an ILP setting has resulted in registration of TNF–α for the treatment of soft tissue sarcoma confined to the limb in combination with melphalan in an ILP setting. Extension of the modes of application of TNF–α was hereby initiated, including organ perfusion and systemic application of low dose TNF–α in combination with formulated drugs (such as liposomal doxorubicin). TNF–α is speculated to have a destructive effect on the tumor associated vasculature at high dosages, but on the other hand, TNF–α has growth promoting activities when only low levels are present, which could negatively influence tumor therapy *(Fajardo, et al. 1992)*. However, at relatively low dosages TNF–α was also found to increase the permeability of the vasculature for cytotoxic drugs which benefits local drug levels and therefore treatment outcome *(Brett et al., 1989; Folli et al., 1993)*.

Important however are the observations that TNF-α causes resistance in vitro to cell cycle specific agents *(Prewitt et al., 1994)*. It is shown that cytokines, like TNF-α, IL-1β and IL-6 cause cell cycle arrest acting at major control points in G0/G1 *(Prewitt et al., 1994; Sgagias et al., 1991; Belizario and Dinarello, 1991; Vieira et al., 1996)*. This may lead to a decreased effect of certain cell cycle specific chemotherapeutic drugs such as doxorubicin, an antibiotic that targets DNA topoisomerase II. Melphalan on the other hand exerts its effects throughout the cell cycle. It may therefore well be that pre-treatment with TNF-α attenuates the growth inhibiting effects of doxorubicin, but has no effect on melphalan activity. On the other hand, synergistic activity of doxorubicin and TNF-α has been shown in vitro, although the synergistic interaction between cytokines and cytotoxic agents seems to be sequence dependent *(Ehrke et al., 1998; Kreuser et al., 1995; Watanabe et al., 1995; D'Alessandro and Borsellino, 1996)*. We demonstrated, next to enhancement of the anti-tumor activity of doxorubicin by high dose TNF–α in ILP, that low dose TNF–α acts synergistically on solid tumors when combined with a long circulating liposomal formulation of doxorubicin (DOXIL*) (ten Hagen et al.,*

1999)(ten Hagen et al., in press). Preliminary clinical studies demonstrated beneficial effect of TNF–α when combined with doxorubicin in ILP *(Di Filippo et al., 1998).*

The recent registration of TNF–α for high dose anti-tumor therapy in ILP, and the demonstrated positive effects of TNF–α in other treatment modalities when combined with doxorubicin formulations, warrant further expansion of the application of this cytokine for the treatment of solid tumors. In this study we examined whether TNF–α is capable of inducing cell arrest in tumor cells and by doing so negatively influence the anti-tumor activity of doxorubicin. The tumor cells were extracted from experimental tumors which were shown to be sensitive to the combination treatment of TNF–α with melphalan or doxorubicin in vivo.

MATERIAL AND METHODS

Chemicals

Recombinant human TNF-α (TNF-α) was kindly provided by Boehringer Ingelheim GmbH, Ingelheim/Rhein, Germany. TNF-α had a specific activity of 5.8×10^7 U/mg as determined in the murine L-M cell assay. Endotoxin levels were less than 1.25 endotoxin units/mg protein. Melphalan (Alkeran, 50 mg per vial) was purchased from Wellcome (Beckenham, UK). It was diluted in 10 mL diluent solvent and further dilutions were made in 0.9 % NaCl. Doxorubicin (Adriablastina, 2 mg/mL) was purchased from Farmitalia Carlo Erba (Brussels, Belgium).

Tumor cell lines

Colon carcinoma CC531: The dimethylhydrazine-induced, moderately differentiated, weakly immunogenic rat colon adenocarcinoma CC531 was used *(Marquet et al., 1984).* CC531 tumor cells were maintained in vitro in RPMI medium supplemented with 10% fetal calf serum. To all cell cultures 1% penicillin (5000 U/mL), 1% streptomycin (5000 U/mL) and 1% L-glutamine (200 mM) was added. All media were obtained from Gibco (UK).

Osteosarcoma ROS-1: The ROS-1 rat osteosarcoma was used in the second series of experiments. This tumor originated spontaneously in the tibia of a rat *(Barendsen and Janse, 1978).* ROS-1 tumor cells were maintained in modified Eagle's medium, supplemented with 10% fetal calf serum.

Soft Tissue Sarcoma BN-175: The spontaneous rat soft tissue sarcoma BN-175 was used *(Kort et al., 1984).* BN-175 is a rapidly growing and metastasizing tumor. BN-175 tumor cells were maintained in RPMI supplemented with 5% fetal calf serum.

Fibrosarcoma WEHI-164: The highly TNF–α sensitive WEHI-164 murine tumor cell line was used and served as a positive control. WEHI-164 tumor cells were maintained in RPMI medium supplemented with 10% fetal calf serum.

Before usage the cells were trypsinized (5 min, 37°C), centrifuged (7 min, 2000 rpm), resuspended in complete medium and counted. Viability was measured with trypan blue (0.3% in a 0.9% NaCl solution), and always exceeded 95%. All tumor cells were incubated overnight, maintained in a humidified atmosphere of 5% CO_2 at 37°C.

In vitro assessment of anti-tumor activity of doxorubicin or melphalan on tumor cell lines when co-incubated with TNF-α.

Tumor cells were plated at $1x10^4$ cells per well in a flat bottomed 96-well microtiter plates (Costar, Cambridge, MA, USA) in a final volume of 0.1 mL of medium per well. Cells were incubated at 37°C in 5% CO_2 for 72 h in the presence of various concentrations of TNF-α, doxorubicin and melphalan. Concentrations of TNF-α used were between 0 and 0.05 μg/mL for the WEHI-164 cells and between 0 and 10 μg/mL for all other tumor cell lines. Concentrations of melphalan used were between 10^{-1}-10^2 μg/mL and concentrations of doxorubicin used were between 10^{-2}-10^2 μg/mL.

Growth of tumor cells was measured using the Sulphorhodamine-B (SRB) assay according to the method of Skehan et al (1990) *(Skehan et al., 1990)*. In short, cells were washed with phosphate buffered saline, incubated with 10% trichloric acetic acid (1h, 4°C) and washed again. Cells were then stained with SRB (15-30 min), washed with 1% acetic acid and were allowed to dry. Protein bound SRB was dissolved in TRIS (10 mM). Extinction was measured at 540 nm. Six replicate experiments were performed. Tumor growth was calculated using the formula: tumor growth = (test well/control) x 100 percent. The drug concentration reducing the absorbance to 50 percent of control (IC_{50}) was determined from the growth curves.

In vitro assessment of the effect of pretreatment with TNF-α on the drug sensitivity of the tumor cell lines.

Cells were cultured in 96-well microtiter plates as described above for 24 h. Cells were washed and exposed for 24 h to medium containing various concentrations of TNF-α, which were also used in the previous experiments. Subsequently, medium containing TNF-α was removed, cells were washed and exposed to doxorubicin or melphalan in various concentrations as described above for 72 h. Growth of tumor cells was measured using the SRB assay.

Effect of TNF–α on cell cycle and apoptosis of tumor cells.

The various tumor cells were plated in 24 well plates in a concentration of 10^5 cells/mL, 0.4 mL/well, and incubated overnight in various concentrations TNF-α (between 0 and 0.05 μg/mL for WEHI-164 and between 0 and 10 μg/mL for all other tumor cell lines), centrifuged (1 min, 1100 rpm), and washed with PBS. Thereafter, cells were trypsinized with trypsin-EDTA for 2 min, washed, and fixed in 1 mL ethanol (80%, 1h). Cells were centrifuged and washed again with PBS and 1 mL propidiumiodide buffer (0.12% triton-X100 and 0.12 mM EDTA), followed by incubation for 45 min with 1 mL Ribonuclease A (100 μg/mL). Nuclei were then stained with propidiumiodide solution and analyzed by flow cytometry in a FACScan (Beckton Dickinson using Cell Quest software on an Apple Mackintosh Computer). Positive control on cell cycle arrest was performed by incubation of the cells in minimal medium containing 0.5% FCS.

Statistical analysis

The in vitro results were analysed using SPSS 8.0 and GraphPad Prism 3.0 for windows. Data was evaluated for statistical significance using the Kruskal-Wallis statistic test and Mann-Whitney U 2-tailed significance test. Results were considered statistically significant for $p<0.05$.

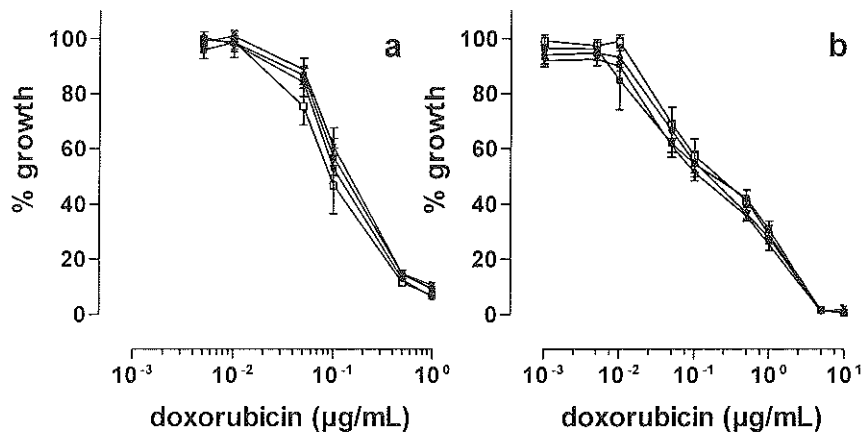

Figure 1. In vitro cytotoxicity of doxorubicin on (a) BN-175 soft tissue sarcoma or (b) ROS-1 osteosarcoma rat tumor cells after 72 h of co-incubation with TNF–α. The cytotoxicity is expressed as percentage growth compared to cells treated with medium alone as described in the materials and methods. Cells were exposed to 0 (□), 0.1 μg/mL (●), 1.0 μg/mL (▼) or 10 μg/mL TNF–α (■). Mean values of 6 to 8 bioassays are shown ± SEM.

RESULTS

Effects of TNF-α combined with cytotoxic drugs on tumor cell *in vitro*.

We investigated the effect of TNF-α on tumor cell sensitivity to either doxorubicin or melphalan on four tumor cell lines: the slowly growing adenocarcinoma CC531, the moderately growing osteosarcoma ROS1, and the fast growing soft tissue sarcoma BN-175. As a positive control the TNF-α sensitive cell line WEHI-164 was used. To examine the effect of TNF-α on doxorubicin or melphalan, the cell lines were exposed to the drugs *in vitro*.

In each of these cell lines a distinctive difference between the two chemotherapeutic agents was found. For all cell lines the IC50 of doxorubicin was significant lower as compared with melphalan (table 1). Thus, the various cell lines proved to be more sensitive for the cell cycle specific drug doxorubicin. Addition of TNF-α to doxorubicin or melphalan had no or marginal effect on the growth inhibiting activity of these agents on CC531 and BN-175 tumor cells. In figure 1 the dose response curves for doxorubicin in combination with TNF–α on these cells is depicted. With melphalan comparable lack of activity of TNF–α was observed (data not shown); the IC50 of doxorubicin or melphalan were relatively unchanged in the presence of TNF-α (table 1, $p>0.50$ for both drugs and both cell lines comparing IC50 of culture with and without TNF–α). The osteosarcoma cell line ROS-1 appeared

Table 1. Drug sensitivity of various tumor cell lines in vitro to doxorubicin or melphalan in combination with TNF–α.

	IC50[1]					
	Doxorubicin		Melphalan			
	TNF-α[2]		TNF-α			
Cell line	no	yes	no	yes	% dead[3]	TNF-α(μg/ml)
CC531	0.21	0.11	13.33	6.07	3	10
BN 175	0.15	0.09	2.87	2.03	0	10
ROS 1	1.39	0.17	4.30	1.14	34	10
WEHI 164	0.24	0.02	6.19	1.18	20	0.001

1) IC50 is expressed as concentration of cytotoxic drug in μg/mL.

2) IC50 is shown, when TNF–α is added to the cells, only at the TNF–α concentrations depicted in the last column.

3) percentage of dead cells as scored in the bioassay with TNF–α alone.

note: mean values of 6 to 8 bioassays are shown, which were calculated from the dose response curves individually as described in materials and methods.

to be modestly sensitive to treatment with TNF-α alone. A dose dependent growth reduction was observed when ROS-1 was exposed to TNF-α at an ineffective concentration of doxorubicin with a maximum reduction of 38% at

10 μg/mL (fig. 2). Addition of TNF–α to doxorubicin reduced the IC50 of the cells 7.5-fold as compared to doxorubicin alone ($p<0.005$), whereas the IC50 of melphalan was reduced 3.3-fold by TNF–α ($p<0.005$). In spite of the diminished IC50 found for both drugs after combination with TNF–α no objective cytotoxic synergy was found, as the maximum and minimum growth inhibition of the cells were found at the same drug concentrations for all TNF-α levels tested (fig. 2).

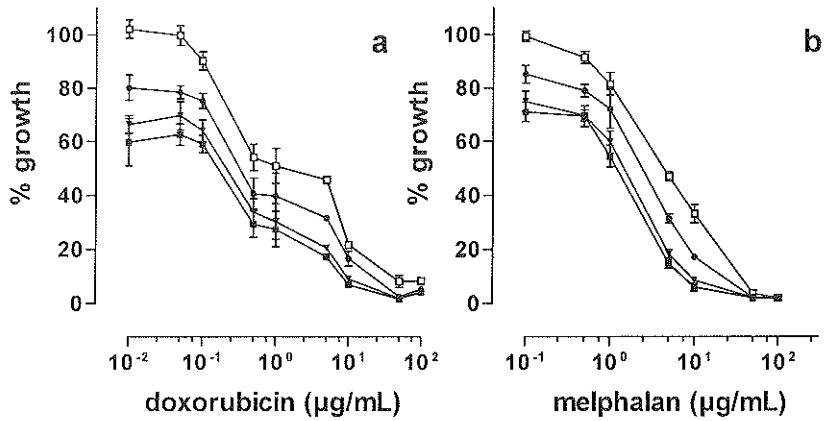

Figure 2. In vitro cytotoxicity of (a) doxorubicin or (b) melphalan in combination with TNF–α on ROS-1 rat osteosarcoma tumor cells after 72 h of co-incubation. The cytotoxicity is expressed as percentage growth compared to cells treated with medium alone as described in the materials and methods. Cells were exposed to 0 (□), 0.1 μg/mL (●), 1.0 μg/mL (▼) or 10 μg/mL TNF–α (■). Mean values of 6 to 8 bioassays are shown ± SEM.

As a control group the TNF-α sensitive cell line WEHI-164 was used. As depicted in figure 3 growth reduction between 50 and 70% is reached already after the addition of 0.001 μg/mL TNF-α. Without the addition of TNF-α the IC50 for doxorubicin is reached with 0.24 μg/mL and for melphalan with 6.19 μg/mL (table 1). Co-incubation of the WEHI-164 tumor cells with TNF–α and doxorubicin or melphalan resulted in strongly reduced IC50 for both drugs; respectively 0.02 ($p<0.001$) and 1.88 μg/mL ($p<0.005$ when culture with and without TNF–α was compared). However, as was also found for ROS-1 cells, the increased tumor cell killing resulted from additive effects between the drugs used rather than from synergy between TNF–α and the cytotoxic agents.

From the co-incubation studies we conclude that TNF–α has no or only an additive anti-tumor effect in vitro on the tumor cells which depends on cell line used. However, in non of the cell lines tested a TNF–α inflicted reduction of the sensitivity towards the cytotoxic drugs could be found. On the other

hand, initiation of cell cycle arrest followed by diminished sensitivity towards doxorubicin might require pre-exposure to TNF–α.

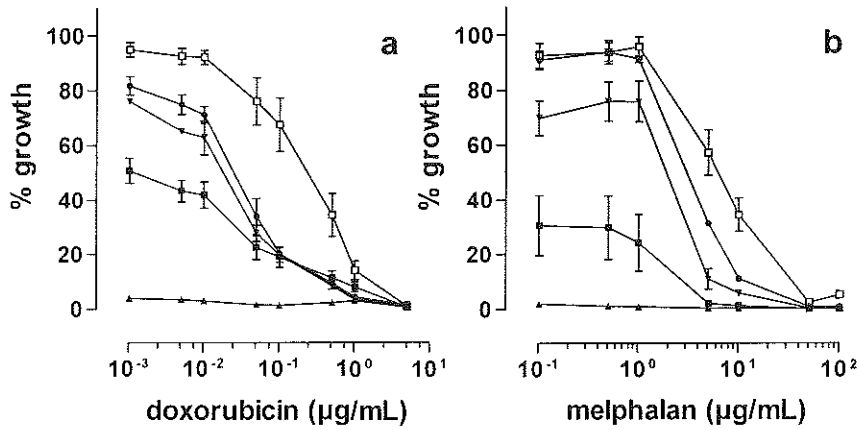

Figure 3. In vitro cytotoxicity of (a) doxorubicin or (b) melphalan in combination with TNF–α on WEHI-164 murine tumor cells after 72 h of co-incubation. The cytotoxicity is expressed as percentage growth compared to cells treated with medium alone as described in the materials and methods. Cells were exposed to 0 (□), 0.00001 µg/mL (●), 0.0001 µg/mL (▼), 0.001 µg/mL TNF–α (■) or 0.01 µg/mL TNF–α (▲). Mean values of 6 to 8 bioassays are shown ± SEM.

Effects of 24 h pretreatment with TNF-α on tumor cell sensitivity to cytotoxic drugs.

According to the work of Prewitt et al (1994) TNF-α exposure shifts cells to the G_0/G_1 phase of the cell cycle *(Prewitt et al., 1994)*. As doxorubicin is a cell cycle dependent chemotherapeutic drug, an increase in survival might be expected when cells are pretreated with TNF-α before exposure to doxorubicin, an effect not to be expected with exposure to melphalan. The above mentioned tumor cell lines were exposed to TNF–α followed by incubation with doxorubicin or melphalan. Twenty-four hour pretreatment with TNF-α did not result in an attenuation of the sensitivity to doxorubicin (or melphalan) in CC531, ROS-1 or WEHI tumor cell lines (table 2). A slight, but not significant increase in IC50 was seen when BN-175 were incubated with TNF–α prior to doxorubicin. Moreover, as was observed in the co-incubation study, pre-treatment of CC531 and BN-175 cells with TNF-α influenced had only a marginal effect on the sensitivity of the cells to the drugs, comparable to the co-incubation. Likewise, pre-treatment of the ROS-1 cells with TNF–α resulted in a lower IC50 for both doxorubicin ($p<0.05$ when compared to culture without TNF–α) and melphalan ($p<0.01$ when compared to culture without TNF–α) comparable to the co-incubation study (table 2). Pre-treatment of WEHI with TNF–α resulted in strongly reduced IC50 for both drugs

($p<0.02$ when compared to culture without TNF–α in combination with doxorubicin and $p<0.001$ when compared to culture without TNF–α in combination with melphalan), and no attenuation of sensitivity of the cell line to the drugs was observed (table 2).

Table 2. Drug sensitivity of various tumor cell lines in vitro to doxorubicin or melphalan after 24h pre-treatment with TNF–α.

	IC50[1]					
	Doxorubicin		Melphalan			
	TNF-α		TNF-α			
Cell line	no	yes	no	yes	% dead[3]	TNF-α(μg/ml)
CC531	0.14	0.07	12.52	9.91	8	10
BN 175	0.12	0.20	4.82	3.04	0	10
ROS 1	5.85	0.32	5.73	1.71	27	10
WEHI 164	0.14	0.04	7.95	1.17	28	0.001

1) IC50 is expressed as concentration of cytotoxic drug in μg/mL.
2) IC50 is shown, when TNF–α is added to the cells, only at the TNF–α concentrations depicted in the last column.
3) percentage of dead cells as scored in the bioassay with TNF–α alone.

note: IC50 of doxorubicin or melphalan alone is not the same as in the combination study (table 1) which is due to the expanded incubation time of the cells in the pre-treatment experiment.
Mean values of 6 to 8 bioassays are shown, which were calculated from the dose response curves individually as described in materials and methods.

Effects of TNF-α on cell cycle kinetics and induction of apoptosis of various tumor cell lines.

As mentioned above, it is believed that TNF–α induces cell cycle arrest and by doing so influences the activity of cell cycle specific drugs. As we did not observe any counter-active effect of TNF–α pre-treatment or co-incubation on the cytotoxicity of doxorubicin towards the tumor cell lines tested we examined the effect of TNF–α on the cell cycle of these cells.

In figure 4 typical cell distributions are depicted as measured by flowcytometry in which the region for apoptotic cells is shown, as well as the regions for cells in G0/1 and G2 phase. No significant shift in G0/1:G2 ratio was observed after exposure of the BN-175 or CC531 cells to TNF–α (G0/1:G2 ratios at 0 μg/mL and maximum TNF–α concentration resp. BN-175: 1.5±0.2 and 1.6±0.1, CC531: 2.0±0.5 and 1.9±0.4).

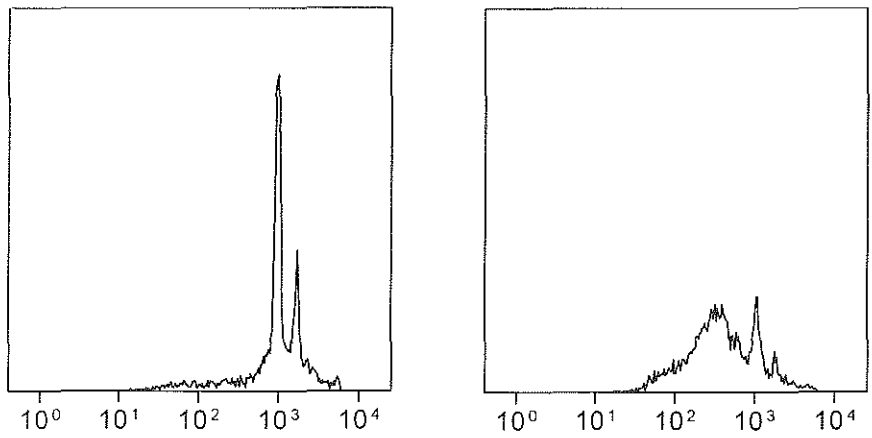

Figure 4. Representation of the analysis of apoptotic cells and cell-cycle progression by flowcytometry using propidium iodide (PI) as described in materials and methods. Fluorescence is presented in this figure on a log scale and represents the amount of DNA bound PI. The regions for apoptotic cells (M1), cells in G0/1 phase (M2) and G2 phase (M3) are indicated. Graphs are good representations of cell analysis as performed on TNF–α sensitive cell-lines when treated with (a) medium alone or (b) with TNF–α.

Moreover, incubation of the TNF–α sensitive cell line WEHI 164 had no effect on progression of the cells through the cell cycle (G0/1:G2 ratios at 0 µg/mL and maximum TNF–α concentration resp. 1.5±0.2 and 1.3±0.2). Exposure of ROS-1 cells to TNF–α exerted a significant effect on the G0/1:G2 ratio of these cells. Strikingly, exposure to TNF–α shifted the cell cycle to a higher mitotic index after exposure to TNF–α, indicating an accelerated progression through the G1 phase and a cell cycle arrest in G2 (fig. 5). Control experiments, in which the cells were depleted from vital nutrients by lowering the percentage of fetal calf serum to 0.5%, demonstrated an increased G1/0:G2 ratio in all cell lines as was expected (data not shown). Exposure of the ROS-1 and WEHI-164 cells to TNF–α resulted in an augmented percentage of apoptotic cells (fig. 6), whereas TNF–α failed to induce apoptosis in the BN-175 or CC531 cells (data not shown). These observations correlate very well with the effect of TNF–α on the growth rate of the cells in vitro.

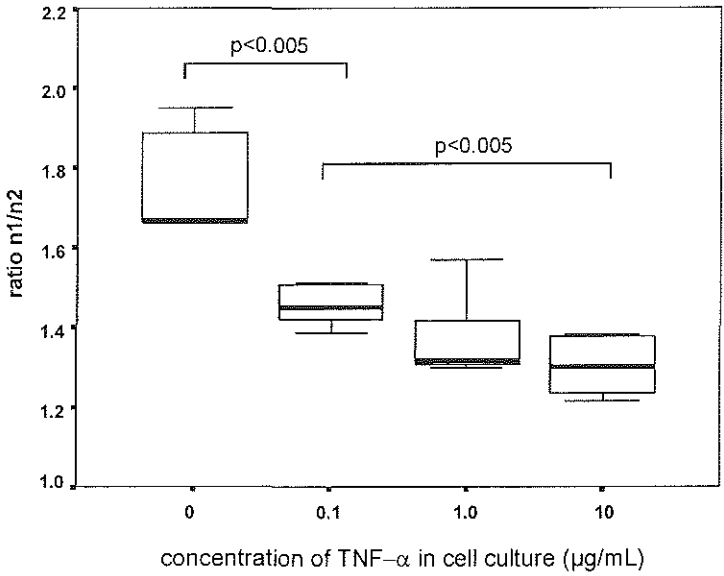

Figure 5. Effect of TNF-α on the mitotic index of ROS-1 cells in vitro. The mitotic index was calculated by division of the percentage of cells in G0/1 phase (n1) by the percentage of cells in G2 phase (n2). The median value is presented (horizontal bar) with the 50% limits (box) and the minimum and maximum values (whiskers). A total of 6 to11 independent tests were performed per TNF-α concentration.

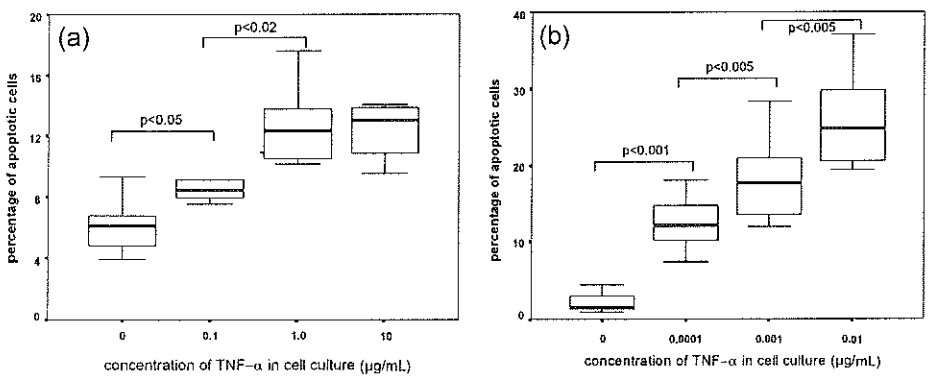

Figure 6. The effect of TNF-α on induction of apoptosis in (a) ROS-1 rat osteosarcoma cells or (b) WEHI-164 murine fibrosarcoma cells in vitro. The median value is presented (horizontal bar) with the 50% limits (box) and the minimum and maximum values (whiskers). A total of 6 to 11 independent tests were performed per TNF-α concentration.

DISCUSSION

In this work we tried to answer the question whether the addition of TNF-α to doxorubicin results in attenuation of the anti-tumor activity of doxorubicin in the light of the claimed inhibitory effect of TNF-α on cell cycle specific drugs *(Prewitt et al., 1994).* Various chemotherapeutic drugs are used in local (e.g. ILP) or systemic therapy, and possible adverse interactions between TNF-α and these cytotoxic drugs would have implications for anti-tumor therapy.

For various cell lines TNF-α was reported to protect against the detrimental effects of cycle specific chemotherapeutic agents through cell cycle arrest in the G_0/G_1 phase *(Prewitt et al., 1994; Darzynkiewicz et al., 1984; Vieira et al., 1996).* We however, demonstrated in the presented study that addition of TNF–α had no inhibitory effect on the anti-tumor activity of the cell cycle specific drug doxorubicin in several tumor cell lines. Moreover, we found that TNF–α had no or only an additive effect on the cytotoxicity of doxorubicin or melphalan, whereas a direct effect of TNF–α on the cells was absent or only marginal. Only the TNF–α sensitive cell line WEHI-164 demonstrated high response to TNF–α, but also in this cell line no effect of TNF–α on cell cycle progression was found.

In the literature conflicting data is presented concerning the combined activity of TNF–α and doxorubicin or other cytotoxic drugs in vitro on tumor cells. Comparable to our results synergism between TNF-α and topoisomerase II targeted drugs was absent on lung cancer cell lines or demonstrated only an additive effect *(Giaccone et al., 1990; Soranzo et al., 1990).* On the other hand synergistic enhancement of in vitro cytotoxicity of doxorubicin by TNF–α has been described by others *(Alexander et al., 1987; Safrit and Bonavida, 1992).* However, also a reduced activity of doxorubicin has been reported when cells were pre-treated with TNF–α *(Prewitt et al., 1994).* A proposed mechanism of the enhanced cytotoxicity, when TNF–α is combined with doxorubicin, is the down-regulation of constitutive levels of mRNA for TNF–α and a concomitant reduced production of TNF–α protein *(Safrit and Bonavida, 1992).* A positive correlation was found between production of TNF–α mRNA and protein by tumor cells and resistance to TNF–α. Strikingly, others demonstrated that the relationship between sensitivity to TNF-α and to doxorubicin was proven to be inverse *(Sleijfer et al., 1998).* The close proximity of topoisomerase-II gene and the gene encoding for c-erbB2 played a role in the enhanced sensitivity to TNF-α. Due to loss of c-erbB2 gene copies, doxorubicin resistant cell lines with decreased topoisomerase II gene copies can become sensitive to TNF-α. TNF-α can modify the topoisomerase activity, however the observed increase

was not required for the synergistic interaction between TNF-α and topoisomerase directed agents *(Baloch et al., 1995).*

In comparison to our results TNF-α has generally a low cytotoxicity by itself *(Haranaka et al., 1984),* although enhanced effect on varying cell lines is seen when TNF-α is combined with certain cytotoxic drugs. Several studies suggest that synergistic interaction between cytokines and cytotoxic drugs are sequence dependent *(Kreuser et al., 1995).* In some studies synergism was reported to be achieved only when TNF–α was applied after or simultaneously with doxorubicin *(Borsellino et al., 1994; Ehrke et al., 1998),* whereas others found in vivo synergism in a chemosensitive melanoma when TNF-α application preceded doxorubicin *(D'Alessandro and Borsellino, 1996).* Here we demonstrate that the administration of TNF-α concomitantly with or 24 hours before doxorubicin exposure did not influence the growth inhibiting effect of doxorubicin on various tumor cell lines.

As stated above also an inhibiting effect of TNF–α on the cytotoxic activity of doxorubicin has been described. It is proposed that TNF–α alters the cell cycle of tumor cells, and by doing so affects the activity of the cell cycle dependent drug doxorubicin. We demonstrated only for one tumor cell line (ROS-1) a shift in the cell cycle, which however did not result in an attenuation of the cytotoxicity of doxorubicin. The other three cell lines did not experience a shift in the cell cycle when exposed to TNF–α. Also in the literature conflicting data is presented. TNF–α, among other cytokines, is known to exert radioprotective and chemoprotective effect on bone marrow precursor cells, which is believed to result from the cell cycle-specific effects of this cytokine *(Dalmau et al., 1997).* It is already striking that TNF–α is able to exert both chemo- as well as radio-protection as it is suggested that cycling increases radioprotection, while arrest reduces chemical damage.

In most studies an arrest of tumor cells in the G0/1 phase has been shown *(Prewitt et al., 1994; Vieira et al., 1996; Pusztai et al., 1993; Belizario and Dinarello, 1991; Cheng et al., 1994; Jeoung et al., 1995).* For a breast cancer cell line, T47D, it was reported that the cytostatic effect of TNF-α occurs at the G_1/S phase transition of the cell cycle *(Pusztai et al., 1993).* L929 fibroblast cells, which are highly susceptible to TNF-α, cultured in the presence of TNF-α (and other cytokines like TGF-β, IL-1β, IL-6) show a growth arrest in G1 phase of the cell cycle, making them less vulnerable against subsequent TNF-α cytotoxicity *(Belizario and Dinarello, 1991).* Vieira et al demonstrated a TNF–α-induced dramatic decrease in Cyclin A, Cyclin B and $p34^{cdc2}$, which are involved in control of cell progression through the cell cycle, in cells in which TNF–α exerts cell cycle arrest *(Vieira et al., 1996).* Secondly, a correlation in accumulation of hypophosphorylated pRb and growth arrest of keratinocytes in the G0/1 phase after exposure to TNF–α was found. The TNF–α inflicted cell cycle arrest was also shown to be dependent

on cell type, showing growth arrest (and concomitant cell progression blockage in the G0/1 phase) for neuroblastoma cells, whereas in glioma cells growth rates were increased and higher cell numbers where found in the G2 phase *(Munoz-Fernandez et al., 1991)*. Others again demonstrated that addition of TNF–α resulted in cytostasis in cultures of L-cells, manifested as cell arrest in the G2 phase *(Darzynkiewicz et al., 1984)*. In comparison to our study, in which we found no effect of TNF–α on cell cycle progression in most tumor cell lines tested, also exposure of B-lymphoid cells to TNF–α had no effect on cell cycle distribution *(Lomo et al., 1987)*.

Direct interactions of TNF-α with tumor cells apparently play a minor role in the therapeutic outcome under in vivo conditions. The effect on the cell cycle of TNF-α seems therefore equivocal. Other mechanisms are likely to be responsible for the observed synergism between TNF-α and either doxorubicin or melphalan in vivo *(Manusama et al., 1996a; Manusama et al., 1996b; de Wilt et al., 1999; Eggermont et al., 1996b; Eggermont et al., 1996a)*. The major target organ of TNF-α remains the tumor vascular bed. In TNF-α based anti-tumor therapy therefore the vascular effects are key and may lead to ischaemic necrosis, immediate permeability changes, hyperpermeability of the tumor vascular bed and a drop in interstitial pressure, which in itself leads to a better penetration of the drug *(Renard et al., 1995; Nooijen et al., 1996; Kristensen et al., 1996; Folli et al., 1993; Yi and Ulich, 1992)*. We showed an increased accumulation of doxorubicin and melphalan in isolated perfusion setting with the addition of TNF-α *(van der Veen et al., 2000, de Wilt et al., 2000)*. Next to that, a significant increase in accumulation of liposome-formulated doxorubicin (Stealth liposomal doxorubicin, DOXIL) was observed after systemic administration of DOXIL in combination with low dosages of TNF–α, resulting in improved tumor response in a rat soft tissue sacroma model *(ten Hagen et al., 1999)(ten Hagen et al, in press)*. This phenomenon could be confirmed in a mouse melanoma model showing identical improved drug accumulation and tumor response when DOXIL was combined with TNF–α *(Takahashi et al, submitted)*. These results indicate that TNF–α at low local dosages has no attenuating effect on doxorubicin but improves drug penetration and performance. Furthermore, vasculotoxic effects of TNF-α lead to endothelial cell damage and suppression of adhesion receptor expressed by angiogenic endothelial cells *(Ruegg et al., 1998; Fajardo et al., 1992)*.

The effect of TNF–α on cytotoxicity of chemotherapeutic drugs appears more complicated than just a direct effect of the cytokine on the activity of cytotoxic drug. Although TNF–α appeared in our hands not or only marginally active towards tumor cells in vitro, and only an additive effect was shown when combined with doxorubicin or melphalan, in vivo (animal models or patients) strong synergy was observed. Isolated limb perfusion (ILP) or isolated perfusion of the liver (IHP) of tumor-bearing rats (BN-175 or ROS-1)

demonstrated strongly enhanced tumor response when TNF–α was added to doxorubicin or melphalan *(Manusama et al., 1996a; Manusama et al., 1996b; de Wilt et al., 1999)(van der Veen et al., 2000; van IJken et al. in press, J. Immunother.)*. Moreover, addition of high dose TNF–α to ILP with melphalan in patients with sarcoma or melanoma improved tumor response and limb salvage significantly *(Eggermont et al., 1996b; Eggermont et al., 1996a; Liénard et al., 1992)*. As stated above also low dose systemic TNF–α increases tumor response when combined with stealth liposomal doxorubicin with no adverse effect on cytotoxicity of the drug. These results indicate that it is at least difficult, if not impossible, to predict outcome of combination anti-tumor therapy in vivo on in vitro data. In agreement with this are the observations by Ehrke et al., who demonstrated that survival of mice from lymphoma was independent of the sensitivity of these tumor cells to TNF–α *(Ehrke et al., 1998)*.

To conclude, the major target of TNF-α is believed to be the neovascular bed, whereas cytotoxic drugs act primarily on the tumor cells itself explaining the synergy observed in vivo by this dual targeting. Direct interaction between TNF-α and cytotoxic drugs however does not seem to play a significant role as shown by us. A disadvantageous effect of TNF–α on the sensitivity of tumor cells to doxorubicin or melphalan could not be found. The results clearly demonstrate that TNF–α can be used in high dose isolated perfusion protocols in combination with doxorubicin and also warrant the exploration of the use of low dose TNF–α in systemic treatment with doxorubicin formulations.

ACKNOWLEDGEMENTS

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Chapter 5

Biodistribution and tumor localization of Stealth® liposomal Tumor Necrosis Factor-α in soft tissue sarcoma bearing rats.

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ABSTRACT

In this study blood residence half-life and organ distribution of recombinant human TNF-α encapsulated in sterically stabilized liposomes was investigated in rats bearing a soft tissue sarcoma in the hind leg. We studied the decay in blood concentration of 'empty' liposomes using the aqueous marker ^{67}Gallium-desferal, as well as the blood concentration of soluble TNF-α and liposome encapsulated TNF-α using ^{125}I. Encapsulation efficacy of TNF-α was 24%. The pharmacokinetics of TNF-α were dramatically altered after encapsulation in liposomes, with a 33-fold increase in mean residence time of TNF-α in the blood, and a concomitant 14-fold increase in the area under the plasma concentration versus time curve for liposomal TNF-α. Although the liposomes exhibit Stealth® characteristics, uptake by mononuclear phagocyte rich organs (e.g. liver and spleen) was noticeable, especially at later time points. Encapsulation of TNF-α in sterically stabilized liposomes resulted in a dramatic increase in localization of the cytokine in tumor measured as total uptake over time. However, peak TNF-α concentration levels in tumor were not significantly enhanced as compared with free TNF-α.

Besides the augmented localization of Tumor Necrosis Factor-α after encapsulation in sterically stabilized liposomes a diminished toxicity was observed.

INTRODUCTION

Tumor Necrosis Factor-α has impressive antitumor activity in clinical and preclinical settings. Isolated limb perfusion (ILP) with high dose TNF-α, melphalan and mild hyperthermia proved to be very successful in patients with in transit melanoma metastases or large nonresectable soft tissue sarcomas *(Eggermont et al., 1996a, 1996b)*. This was confirmed in our laboratory in a rat soft tissue sarcoma model *(Manusama et al., 1996)*. The high response rates reached with isolated limb perfusion prompted us and others to use TNF-α also in regional perfusion settings in treatment of solid tumors of organs such as liver, lung and kidney *(reviewed by Eggermont et al., 1996b)*. Isolated perfusion of organs appeared feasible, but proved to be technically demanding. As metastatic disease is usually widespread, treatment requires systemic application. However, systemic treatment with TNF-α is complicated by its dose limiting toxicity at already relatively low concentrations *(Tracey et al., 1986)*, as well as rapid plasma clearance and distribution to nonrelevant tissues *(Kedar et al., 1997)*. Thus, to obtain vasculo-toxic effects that require high concentrations of TNF-α

at the tumor site *(Fajardo et al., 1992),* that are easily achieved in isolated perfusion systems, a TNF-α targeting device is necessary to create potential efficacy after systemic administration.

In our quest to expand the application of TNF-α, we investigated the pharmacokinetic behavior and tumor localization of TNF-α encapsulated in so called sterically stabilized, long circulating (Stealth®) liposomes as TNF-α targeting device.

Liposomes can be given systemically or regionally. A drawback of so called classical liposomes however, is the rapid uptake by cells of the mononuclear phagocytic system (MPS). MPS recognition can be diminished by incorporation of the hydrophilic polymer polyethylene glycol (PEG) in liposomes *(Woodle et al., 1994).* It was shown that sterical stabilization of liposomes could increase blood residence time drastically *(Woodle and Lasic, 1992a),* resulting in enhanced tumor localization. These liposomes have been named Stealth® liposomes. Recent studies showed therapeutic advantages of encapsulation of drugs in sterically stabilized liposomes, and clinical trials with these liposomes have shown similar pharmacokinetic advantages as in pre-clinical studies *(Gabizon et al., 1994).*

It was previously shown that encapsulation of TNF-α in conventional liposomes reduced toxicity, allowed targeting to selected organs after intravenous administration, and immunomodulatory activity of TNF-α was retained *(Debs et al., 1989; 1990).* Encapsulation of TNF-α in long circulating liposomes may augment the therapeutic index of the cytokine not only by lowering toxicity, but also by increasing concentration in tumor and lengthening of bioavailability. Equally important, liposomal encapsulation might diminish clearance of TNF-α, which normally has a half-life in serum of minutes.

In the present study a solid rat tumor model has been used to investigate toxicity, biodistribution and tumor localization of TNF-α containing sterically stabilized liposomes after intravenous administration. We explored whether there is an alteration in blood circulation time, uptake of TNF-α in tissues, and systemic toxicity after encapsulation in sterically stabilized liposomes.

MATERIALS AND METHODS

Animals and tumor model

Male inbred Brown Norway rats, weighing 250-300 g, obtained from Harlan-CPB (Austerlitz, the Netherlands), were used. The spontaneous BN 175 sarcoma (transplantable to Brown Norway rats) was used *(Marquet et al., 1983).* The BN 175 is a rapidly growing and metastasizing soft tissue sarcoma, which is non-immunogenic as determined by the challenge method of Prehn and Main *(Prehn & Main, 1957),* and can be maintained in tissue culture. This well

vascularized solid tumor is maintained by serial passage *in vivo*. As previously described, viable fragments of the BN 175 sarcoma with a diameter of 2-3 mm were implanted subcutaneously in the right hind limb of the rat *(Manusama et al., 1996)*. Tumors were allowed to grow for approximately 10 days, when tumor diameter reached 10-15 mm, with a tumor weight between 500-1000 mg.

Tumor Necrosis Factor-α

Recombinant human Tumor Necrosis Factor-α (rHuTNF-α) was provided by Boehringer (Ingelheim, Germany) with a specific activity of 5.8 x 10^7 units per mg as determined in the murine L-M cell assay. Endotoxin levels were less than 1.25 endotoxin units (EU) per mg protein.

Preparation of sterically stabilized long circulating liposomes (SL)
Materials

The various chemicals were obtained as follows: Partially Hydrogenated Egg Phosphatidyl Choline (PHEPC) was kindly provided by Lipoid GMbH and Cholesterol (Chol) was obtained from Sigma (St. Louis, USA). Distearoyl phosphatidylethanolamine (DSPE) is derivatized at its amino position with a MW 1900 segment of poly-(ethyleneglycol) (PEG). PEG-DSPE was kindly provided by Dr. P. Working (SEQUUS Pharmaceuticals Inc., Menlo Park, California, USA). Gallium^{67} citrate was from Nordian (Montreal, Canada), Iodide^{125} from Amersham (Buckinghamshire, England), and deferoxamine mesylate (DF) was from CIBA-GEIGY (Basel, Switzerland).

A mixture of PHEPC (242.5 mg), Cholesterol (64.5 mg) and PEG-DSPE (67.5 mg) in a molar ratio of 1.85 : 1 : 0.15, suspended in chloroform/methanol, was evaporated to dryness in a rotary evaporator. The dried lipid film was resuspended with HEPES buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) as described previously *(Gabizon and Papahadjopoulos, 1988a)*. The hydrated lipidfilm was vortexed and liposomes were brought to specific size by sonication during 10 to 15 minutes at amplitude 9 using an ultrasonic disintegrator supplied with an exponential microprobe (diameter 3.5 mm) (Soniprep 150, Sanyo, UK). The liposomes were sterilized through a 0.2 μm filter. Liposome size was determined by dynamic light scattering (DLS, Malvern 4700 system, Malvern, UK). The liposomes were stored at 4°C until further use after determination of total lipid concentration.

Labeling of the liposomes with ^{67}Gallium

For *in vivo* biodistribution studies, liposomes were labeled with ^{67}Ga, using the high affinity ^{67}Ga-DF complex (deferoxamine mesylate DF) as aqueous liposomal marker, as described by Gabizon et al *(Gabizon et al., 1988b)*. ^{67}Ga-labeled deferoxamine complex was entrapped in the aqueous interior of liposomes. This label proved to be an excellent tracer of intact liposomes because

of minimal translocation of label to plasma proteins and rapid renal clearance when label is released from liposomes. Unencapsulated ^{67}Ga-DF, ^{67}Ga-oxine and excess oxine sulfate were removed by gel filtration on a Sephadex G-50 column eluted with HEPES buffer.

Encapsulation of TNF-α in sterically stabilized liposomes

TNF-α was incorporated into liposomes by addition of 2.5 ml TNF-α dissolved in HEPES buffer (400 μg/ml) to the freeze-dried lipidfilm (1000 μmol) for approximately 1 hour at 37 °C and vortexed. Liposomes were prepared as described above. After sonication, the liposomes were washed twice with HEPES-buffer by ultra-centrifugation for 2 hr at 60.000 rpm after which supernatant was discarded, to remove the remaining free TNF-α. The amount of TNF-α associated with the liposomes was determined by using ^{125}I-labeled TNF-α. Before usage, the TNF-α liposomes were filtered through a 0.2 μm filter. Loss of activity of TNF-α was assessed using the TNF-α sensitive WEHI-164 tumor cell line. Briefly, WEHI-164 cells were exposed to TNF-α in the presence of actinomycin D for 3 days after which cell death was determined using the MTT test. Samples were taken before and after every step in the procedure. Using this procedure TNF–α is encapsulated in the aqueous phase, but also association with the liposomal membrane occurs, as at a very low TNF–α concentration (1 μg/ml) 60% of the TNF–α is encapsulated.

Labeling of TNF-α with ^{125}I

Fate of free or liposomal TNF-α *in vivo* was followed by labeling TNF-α with ^{125}I. ^{125}I was coupled to TNF-α using the IODO-BEAD® method (Pierce, Rockford, IL, USA). Briefly, TNF-α (400 μg/ml), iodo-beads and ^{125}I (15 MBq) were diluted in iodination buffer. The reaction was allowed to proceed for 15 minutes at room temperature. Hereafter the beads were discarded and free label removed by gel filtration. A trace amount of ^{125}I-TNF-α was added to the TNF-α before injection or before encapsulation in liposomes.

Pharmacokinetics of sterically stabilized liposomes

Rats (6 at each time point) were injected via the penile vein with liposome-encapsulated ^{67}Ga. In a separate experiment, ^{125}I-TNF-α (15 μg/kg) containing sterically stabilized liposomes (60 μmol lipid/kg) were given. Rats were sacrificed at 0.08, 4, 12, 24, 48 and 72 hours after injection and tissues were excised, weighed, and counted in a Beckmann 8000 gamma counter. Tissues sampled included tumor, liver, spleen, kidneys, lungs, muscle, bone-marrow and synovia. Subcutaneous tumors could easily be separated from non-tumor tissue. Blood (0.5 ml) was taken from the heart under anesthesia, before sacrifice.

When free TNF-α labeled with ^{125}I was injected (15 μg/kg), rats were

sacrificed at 0, 1, 4, and 8 hours after injection and same tissues as above were excised and counted for specific radioactivity as described above.

To evaluate whether incorporation of TNF-α in Stealth® liposomes had any effect on the pharmacokinetic behavior of the liposomes, double labeled liposomes were prepared. Liposomes were labeled with ^{67}Ga liposomes and at the same time loaded with ^{125}I labeled TNF-α. The short decay of ^{67}Ga (half-life 72 hr) enabled detection of ^{67}Ga and ^{125}I separately. ^{67}Gallium was detected directly after collection of the samples, whereas ^{125}I was detected 30 days later. Different energy windows were used; 15 LL - 60 UL for ^{125}I and 80 LL - 600 UL for ^{67}Ga.

Toxicity of free and liposomal TNF-α

Free (60 μg/kg) and liposomal TNF-α (60 μg/kg) was injected intravenously into Brown Norway rats bearing the BN 175 soft tissue sarcoma in the right hind leg (n=6). Rats were inspected for morbidity and weighed daily.

Statistics

The pharmacokinetics of the liposomes and free TNF-α were analyzed using a two phase exponential decay curve fitting by Prism (GraphPad Software, San Diego, USA; Table 1)

RESULTS

Preparation of TNF-α encapsulated in sterically stabilized long circulating liposomes

Liposomes (average diameter of 97 ± 5 nm, polydispersity <0.2; measured by dynamic light scattering) did not degrade or aggregate in HEPES buffer for a period of 8 weeks.

Encapsulation of TNF-α at 400 μg/ml, resulted in a 24 % encapsulation of the cytokine, determined by using ^{125}I-labeled TNF-α. Free TNF-α was effectively removed from the encapsulated TNF-α by ultra-centrifugation. A 24 % incorporation means that 15 μg of TNF-α is associated with 60 μmol lipid. During the process of liposome production the activity of TNF–α was assessed using the TNF–α sensitive WEHI-164 cells. Significant loss of activity was not measured. It appeared that heating of TNF-α to 37°C resulted in 132±13 % of the initial activity, whereas sonication at 37°C for 20 minutes resulted in 104±27% of the initial activity.

The total procedure (heating to 37°C, sonication, etc.) seemed to slightly increase the activity of TNF-α to 109±26% of the initial activity. These results are in close agreement with previous publications on TNF-α activity after

manipulation *(Kedar et al., 1997).*

Release of TNF-α from liposomes was examined *in vitro*. Incubation of TNF-α associated to Stealth liposomes in 90% serum at 37°C resulted in a release of 15.5 ± 1.5% in the first 12 h. Thereafter no detectable release of TNF-α from the liposomes into the serum could be measured.

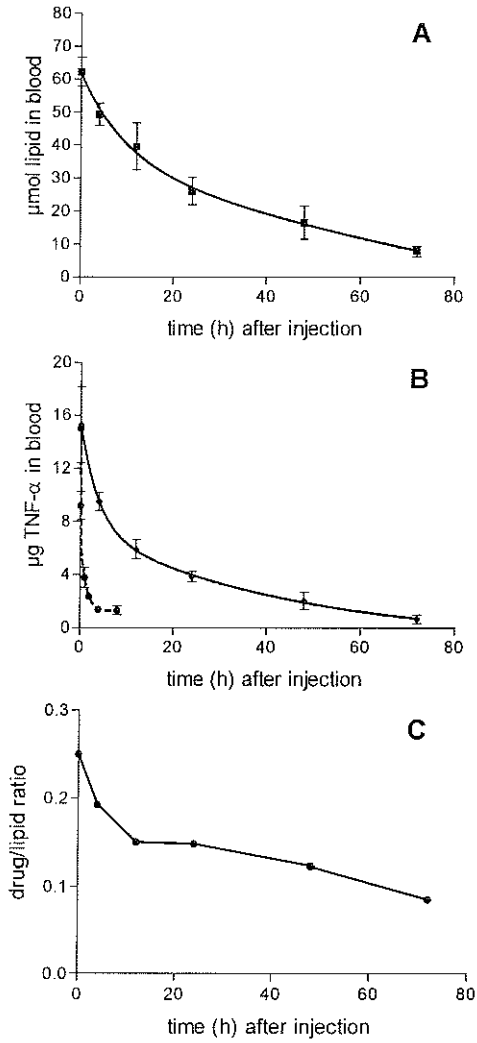

FIGURE 1. Plasma clearance of placebo sterically stabilized liposomes (A) and liposome-encapsulated Tumor Necrosis Factor-α (TNF-α) or free TNF-α (B). Rats were injected intravenously with 60 µmol/kg ^{67}Ga-labeled placebo liposomes (■), ^{125}I-labeled TNF-α encapsulated in long circulating liposomes (◆) and ^{125}I-labeled free TNF-α (●). Activity in blood samples (6 rats at each time point) was determined by gamma radiation. Mean values ± SD are presented. TNF-α to lipid ratio at the given time points (C). Ratio in original preparation was 2.5.

Pharmacokinetics of liposome-encapsulated TNF-α

Encapsulation of TNF-α in sterically stabilized liposomes might increase blood residence time and by doing so promote uptake of liposomes in tumor. Therefore we examined the biodistribution of TNF-α encapsulated in these liposomes from time of injection up to 72 hours.

TABLE 1. Pharmacokinetics of empty sterically stabilized liposomes, and free or liposome-encapsulated Tumor Necrosis Factor-α (TNF-α) in rat plasma after intravenous administration.

Parameters	Treatment group		
	Liposomes	TNF-α	
	60 μmol/kg	liposomal	free
$t_{½}\alpha$[1] (h)	6.01	2.64	0.05
$t_{½}\beta$ (h)	65.63	31.4	0.80
MRT (h)	33.56	13.38	0.4
AUC (U x h/ml)	102.5	17.7	1.27
Cl_{tot} (ml/h)	0.59	0.85	11.81
V_{DSS}(ml)	19.9	21.2	21.7

[1] -$t_{½}\alpha$, distribution half-life; $t_{½}\beta$, elimination half-life; MRT, mean residence time (the time required for 63.2% of the administered dose to be eliminated); AUC, total area under the plasma concentration versus time curve; Cl_{tot}, clearance of liposomes from plasma; V_{DSS}, volume of distribution at steady state.

Note: AUC of liposomes and AUC of TNF-α (free or encapsulated) can not be compared as the AUC is expressed in μmol for liposomes and in μg for TNF-α.

The values are the means of six experiments for each preparation.

First, placebo ^{67}Ga-labeled liposomes (PHEPC:Chol:PEG-DSPE) were injected into the penile vein of rats bearing the BN 175 soft tissue sarcoma implanted subcutaneously in the right hind leg. Liposome blood circulation time is shown in Figure 1 and Table 1. Typical Stealth® characteristics of the liposomes are shown by their clearance from the blood with a MRT (mean residence time in blood) of 33 h. The exponential decay of the liposomes in the blood is represented by the curve. The clearance of liposomes from the blood seems to be biphasic, with a rapid decay in the first hours ($t_2\alpha$=6.0 h) and a slower phase ($t_2\beta$=65.6 h) thereafter. Approximately 88 % of the injected dose was present in the blood after 4 h, whereas after 24 h almost 50 % was recovered. After 72 h 10-15 % of the injected dose was still circulating. This was not altered when the dose was changed from 60 to 240 μmol/kg (data not shown). Injection of fluorescently labeled liposomes demonstrated comparable pharmacokinetic behavior, indicating that decline in blood residence time was not due to release of ^{67}Ga as the fluorescent label is integrated with high stability in the lipid bilayer (data not shown).

Incorporation of TNF-α within the long circulating liposomes did not influence circulation time of the liposomes themselves, in spite of the possible presence of TNF-α at the membrane surface, as was shown with double labeled liposomes.

Encapsulation of TNF-α results in a MRT of 13.38 h for TNF-α, whereas intravenously injected free TNF-α is cleared rapidly, with a MRT in blood of approximately 0.4 hr. As already stated, 'empty' liposomes circulated with a MRT of 33 h, indicating a rapid release of a fraction of TNF-α from the liposomes, which can also be determined from the drug to lipid ratio, as expressed in Figure 1C. Calculated by comparing the AUCs of the different liposomal preparations, at 4 h 85 % of the TNF-α is still associated with the liposomes, whereas at 12 h 76 % is retained in the liposomes. Clearance of TNF-α from blood (Cl_{tot}) is decreased 14-fold (0.85 ml/h and 11.81 ml/h for encapsulated and soluble TNF-α, respectively), when TNF-α is encapsulated in liposomes. The possible bioavailability of TNF-α is thus increased considerably, as can be concluded from the calculated plasma AUC for TNF-α which is 14-fold enhanced for encapsulated TNF-α when compared to soluble TNF-α: 17.7 and 1.27 respectively.

Tissue distribution of TNF-α encapsulated in sterically stabilized liposomes

Figure 2 shows the distribution of respectively 'empty' sterically stabilized liposomes, liposomes with encapsulated TNF-α and intravenously injected soluble TNF-α in various tissues.

The tumor localized a considerable amount of the injected dose of liposomes. 24 h after injection 8 % of the injected dose localized in the tumor when 60 μmol/kg was administered. Around 5 % of the injected dose was recovered after 12 h when the 240 μmol/kg was used (data not shown). The percentage of injected dose recovered at later time points was lower, possibly expressing degradation of liposomes and rapid clearance of the marker.

The pharmacokinetic profile of TNF-α was dramatically changed after encapsulation in long circulating liposomes (Table 2). The localization of TNF-α in tumor was increased 13-fold (AUC in tumor 5.5 for free TNF-α and 69.9 after encapsulation). At 12 and 24 h after injection still a considerable amount of TNF-α could be measured in the tumor. Free TNF-α however was cleared rapidly by the kidney (peak at 1 h after injection), this in contradiction to TNF-α encapsulated in long circulating liposomes (Fig. 2E).

Liposome uptake by the liver and spleen, organs with an active MPS, occurred to be prolonged, as they took up an increasing dose of liposomes in the time span used in these experiments. Still, a relatively low dose accumulated in these organs, almost equal amounts of liposomes localized in spleen and liver,

reaching 14% after 72 h. Per gram tissue on the other hand, the spleen was shown to be very active whereas the liver took up only small amounts of liposomes (data not shown). The uptake in organs as lung, kidney, bone-marrow and skeletal muscle was low, or below detection level.

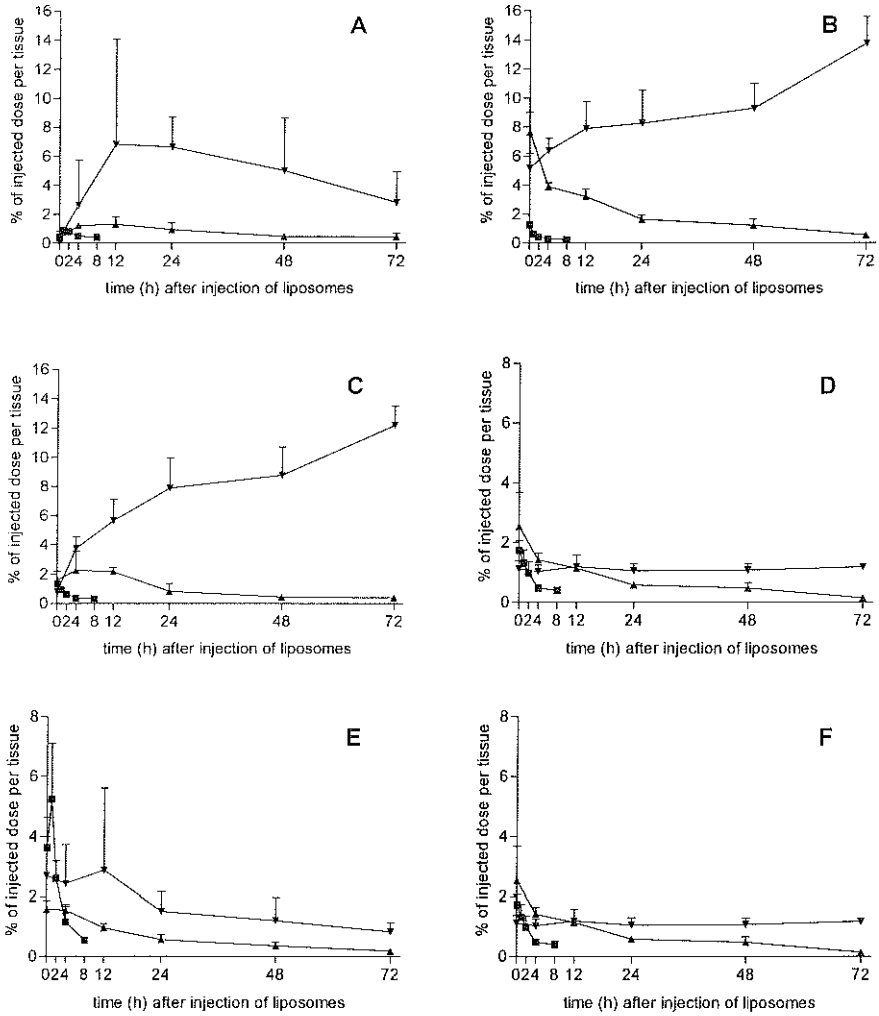

FIGURE 2. Tissue distribution of sterically stabilized liposomes and free or liposome-encapsulated Tumor Necrosis Factor-α. Rats were injected intravenously with ^{67}Ga-labeled placebo liposomes (▼), ^{125}I-labeled TNF-α encapsulated in sterically stabilized liposomes (▲) and ^{125}I-labeled free TNF-α (■). Biodistribution of preparations was assessed in tumor (A), liver (B), spleen (C), lung (D), kidney (E) and bone marrow (F). Activity in tissues (6 rats at each time point) is expressed as % of injected dose per tissue. Mean values + SD are presented.

TABLE 2. Pharmacokinetics of empty sterically stabilized liposomes,and free or liposome-encapsulated Tumor Necrosis Factor-α (TNF-α) in rat tissues after intravenous administration.

Parameters	Treatment group		
	Liposomes	TNF-α	
	60 μmol/kg	liposomal	free
$AUC_{hepatic}$ (U x h/ml)[1]	677.8	140.4	20.98
AUC_{spleen}	623.7	75.73	2.24
AUC_{tumor}	267.7	69.85	5.48
$Cl_{hepatic}$ (ml/h)	0.15	0.71	4.77
Cl_{spleen} (ml/h)	0.16	1.32	44.72
Cl_{tumor} (ml/h)	0.22	0.21	2.73
C_{max}	4.5 (μmol)	0.20 (μg)	0.22 (μg)
T_{max} (h)	12-24	12	1

[1]AUC, area under the concentration in tissue (liver, spleen, tumor) versus time curve; Cl, clearance in tissue (liver, spleen, tumor); C_{max}, maximum concentration at certain time point in tumor; T_{max}, time point at which maximum concentration in tumor tissue is reached.

Note: AUC of liposomes and AUC of TNF-α (free or encapsulated) can not be compared as the AUC is expressed in μmol for liposomes and in μg for TNF-α.

The values are the means of six experiments for each preparation.

Toxicity of free and sterically stabilized liposome-encapsulated TNF-α

Weight loss of rats upon injection of TNF-α is a sensitive parameter for systemic toxicity of this cytokine. After intravenous injection of 60 μg/kg free TNF-α all rats showed diarrhea as well as a decrease in weight of 17% as compared to control rats (mean of 6 rats). The weight loss is totally abolished after encapsulation of TNF-α in sterically stabilized liposomes. No adverse effects on clinical condition, nor mortality was observed.

DISCUSSION

Here we demonstrate that encapsulation of TNF-α in sterically stabilized, long circulating (Stealth®) liposomes results in a dramatic increase in blood circulation time and tumor localization of the cytokine. The area under the plasma concentration versus time curve for TNF-α increased 14-fold, suggesting a favorable pharmacokinetic profile. As toxicity is reduced, liposomal encapsulation may well be the means of systemic application of TNF-α and also allows multiple dosing.

The soluble cytokine TNF-α is cleared rapidly from the circulation, and has serious side effects when administered systemically. Therapeutic tumor effects with TNF-α were only seen when administered by means of intralesional injection or isolated perfusion *(Eggermont et al., 1996a)*. In studies performed in our laboratory with isolated perfusion of the extremity in an animal model, we showed that TNF-α can be used successfully in the treatment of solid tumors, when applied regionally in high concentration combined with other modalities, such as ischemia and cytotoxic drugs *(Manusama et al., 1994; 1996)*. To reach the same results in treating solid organ tumors and metastases, a sustained delivery system for TNF-α such as liposomes, which could increase exposure time or peak concentration level, seems attractive when combined with liposomal encapsulated cytotoxic agents.

In papers describing pharmacokinetics of long circulating liposomes a consistent finding is the prolonged residence time in blood *(Gabizon and Papahadjopoulos., 1988a; Woodle et al., 1992b)*, which was confirmed in this study. Prolonged circulation is generally considered as the most essential requirement for therapeutic application. The *in vivo* behavior of liposome encapsulated TNF-α is substantially different from intravenously injected free TNF-α, which has a MRT of only 0.4 h. Liposomal encapsulated TNF-α demonstrated a MRT of 13.4 h, resulting in a considerable enhanced pharmacokinetic profile as can be concluded from the increase of AUC in blood, and tumor uptake. This suggests an augmentation of the therapeutic index of TNF-α after encapsulation. The placebo liposomes used in the present study circulated with a MRT of 33 h, which is considerably longer than the liposomal TNF-α. The *in vitro* experiments demonstrated an initial release of TNF-α from liposomes of 15.5% in the first 12 h. The shorter plasma half-life of liposomal TNF-α as compared to placebo liposomes could therefore partially be explained by an initial release of liposome-associated TNF-α, as demonstrated *in vitro*, which is also shown by the drug to lipid ratio curve. The fact that the curves of liposomes itself and liposomal TNF-α are parallel in the second phase of the decline demonstrates that clearance of TNF-α is at those time points dictated by the pharmacokinetic behavior of the liposomes. Also *in vitro* no significant additional release of TNF-α was measured after 12 h of incubation. Although the release of TNF-α appeared to be more pronounced *in vivo*, we speculate that the rapid release observed in the first 12 h is responsible for most of the loss of TNF-α. This fraction might be associated on the outside of the liposome.

The increased blood circulation time results in enhancement of localization of liposomes and thus of liposomal TNF-α in tissue. In tumor an increased localization of TNF-α encapsulated in sterically stabilized liposomes was observed. After encapsulation uptake of TNF-α in tumor increased 12.7-fold as compared to the free cytokine. Free TNF-α is rapidly cleared by the kidney,

already at 1 h a peak in localization is observed. The spleen as well as the liver are exposed to high levels of liposomes for extended periods of time, thus increasing the uptake of these liposomes by the MPS. Although hepatic uptake of liposomes expressed per organ is substantial, uptake per gram tissue is much lower. The spleen on the other hand shows a dramatic uptake of sterically stabilized liposomes when expressed per gram tissue, especially at later time points. Others *(Gabizon et al., 1997)* did not find a splenic uptake of this magnitude in mice. A possible explanation could be that opsonins in rat plasma, like complement and fibronectin play a more active role in uptake of liposomes by the spleen in rats as compared to mice. It is also suggested that tumor-bearing animals, which were used by us, have an increased splenic clearance *(Moghimi and Patel 1996).*

Increased permeability of tumor associated microvasculature, together with the longer circulation times of sterically stabilized liposomes, is considered responsible for improved accumulation of these liposomes in tumor tissues *(Papahadjopoulos et al., 1991, Wu et al., 1993).* The leakiness of tumor associated vessels *(Jain et al., 1994)* allow extravasation of small diameter liposomes through the endothelial gaps *(Huang et al., 1992, Yuan et al., 1994).* The presence of intact liposomes in the tumor interstitium surrounding the blood vessels would provide a local depot for release of TNF-α for prolonged periods. We hypothesize that optimal anti-tumor activity with liposomal TNF-α will be reached when TNF-α is released after extravasation in tumor tissue. By using liposomes, which start to disintegrate after 12-24 h, the accumulation of TNF-α at the tumor site will be large, whereas toxicity is kept at a minimum. Moreover, liposomal delivered TNF-α is present in tumor tissue for an extended period. Thus, liposomes serve as a sustained delivery system. This may make the therapeutic application of systemic TNF-α again possible, not only as a single bolus injection, but multiple injections as well.

The liposomal preparations of TNF-α described until now *(Debs et al., 1989; 1990; Nii et al., 1991, Kedar et al., 1997)* were of the conventional type with diameters larger than 200 nm. Although reduction of toxicity of TNF-α has been demonstrated, these liposomes are not suitable for systemic disease, nor for the treatment of large solid tumors, as they are cleared relatively rapid and mainly target to MPS cells. The present study also shows decreased toxicity of TNF-α after incorporation in sterically stabilized liposomes. Therefore, incorporation of TNF-α in sterically stabilized liposomes seems to be promising, especially when combined with cytotoxic agents encapsulated within liposomes *(ten Hagen et al., 1997).*

The results shown in this paper clearly demonstrate the usefulness of encapsulation of TNF-α in sterically stabilized liposomes. As TNF-α alone was shown to be inactive during isolated limb perfusion of sarcoma *(Manusama et al., 1996)*, but demonstrated a strong synergistic antitumor activity when combined with doxorubicin (own observations), studies are conducted to determine the effect of Stealth® liposomal TNF-α in combination with Stealth® liposomal doxorubicin (DOXIL®).

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Chapter 6

Low dose Tumor Necrosis Factor-α augments anti-tumor activity of Stealth liposomal doxorubicin (DOXIL®) in soft tissue sarcoma-bearing rats.

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ABSTRACT

It has previously been demonstrated in the setting of an isolated limb perfusion that application of high dose TNF-α in combination with chemotherapy (melphalan, doxorubicin), results in strong synergistic anti-tumor effects in both the clinical and the preclinical setting. In this paper we demonstrate that systemic administration of low dose TNF-α augments the anti-tumor activity of a liposomal formulation of doxorubicin (DOXIL®). Addition of TNF-α to a DOXIL® regimen, which by itself induced some tumor growth delay, resulted in massive necrosis and regression of tumors. Furthermore, we could demonstrate a significant increase of liposomal drug in the tumor tissue when TNF-α had been co-administered. Administration of TNF-α augmented DOXIL® accumulation only after repeated injections, whereas accumulation of free doxorubicin was not affected by TNF-α. Drug levels in the tumor interstitium appeared crucial as intracellular levels of free or liposome-associated doxorubicin were not increased by TNF-α. Therefore, we hypothesize that low dose TNF-α augments leakage of liposomal-drug into the tumor interstitium, explaining the observed improved anti-tumor effects. Regarding the effects of systemic administration of low doses of TNF-α, these findings may be important for enhanced tumor targeting of various liposomal drug formulations.

INTRODUCTION

It has previously been shown that anti-tumor effects of TNF-α require high concentrations of this agent to be effective *(Asher et al., 1987; Fajardo et al., 1992)*. Pre-clinical and clinical studies demonstrated impressive anti-tumor activity of melphalan when combined with TNF-α in local regional treatment. Isolated perfusion of the limb with high dose TNF-α in combination with melphalan has been reported to result in high response rates in patients with irresectable sarcomas or melanoma in-transit metastases *(Eggermont et al., 1996a; Eggermont et al., 1996b; Liénard et al., 1992)*. Recently TNF-α has been approved and registered in Europe to be applied in the isolated limb perfusion setting for advanced soft tissue sarcomas. In syngeneic sarcoma rat models we have demonstrated that an isolated limb perfusion (ILP) with TNF-α in combination with melphalan or doxorubicin resulted in synergistic anti-tumor effects *(Manusama et al., 1996; van der Veen et al., 2000)*. These observations clearly demonstrated synergy between TNF-α and the cytotoxic drugs used. A possible mechanism is that TNF-α increases accumulation of the cytotoxic drug in the tumor, which could be demonstrated by us in ILP with melphalan or doxorubicin combined with TNF-α *(de Wilt, et al., 2000; van der Veen et al., 2000)*. Unfortunately, the systemic application of high dosages of TNF-α is hampered by severe toxicity, indicating

that only low dosages of TNF-α can be applied *(Feinberg et al., 1988)*. TNF-α however could also possibly increase accumulation of drugs when applied systemically in a low dose which could be translated to the human setting.

Although melphalan is the drug of choice in the majority of the isolated limb perfusions, especially for the treatment of melanoma and sarcoma, other agents might be useful too. Anthracyclines are among the most active agents against solid tumors and doxorubicin is the most widely used agent of this class. It is the agent of choice for the treatment of sarcoma. However, free doxorubicin is cardiotoxic, and induces bone marrow depletion and stomatitis *(Herman et al., 1983)*. These side effects can be diminished by using a liposome-encapsulated formulation of doxorubicin, DOXIL® *(Coukell and Spencer, 1997; Gabizon et al., 1986)*. In this preparation doxorubicin is encapsulated in high concentrations in so-called Stealth or long circulating liposomes. Stealth liposomes exhibit characteristics which prolong blood residence time and result in increased tumor localization *(Vaage et al., 1997; Papahadjopoulos et al., 1991)*. Treatment of refractory AIDS related Kaposi's sarcoma with DOXIL® demonstrated favorable responses *(Coukell and Spencer, 1997)*. This was accompanied by diminished toxicity in preclinical models *(Vail et al., 1997; Gabizon et al., 1989)*. Based on our observation, concerning the effects of TNF-α on the tumor vasculature and its permeability, we hypothesized that TNF-α could, by augmenting vascular leakage in the tumor, increase accumulation of liposomal-doxorubicin and so further potentiate the anti-tumor activity of this formulation.

In this study we examined the effect of systemically applied low dose TNF-α on the anti-tumor activity of DOXIL® in solid soft tissue sarcoma-bearing rats.

MATERIALS AND METHODS

Chemicals.

Human recombinant Tumor Necrosis Factor-α (specific activity $5x10^7$ IU/mg) was kindly provided by Dr. G. Adolf (Bender Wien GmbH, Wien, Austria) and stored at a concentration of 2 mg/mL. Endotoxin levels (LAL) were below 0.624 EU/mg. DOXIL® was kindly provided by Dr P. Working (SEQUUS Pharmaceuticals, Inc. Menlo Park, CA). Doxorubicin (Adriablastina®) was purchased from Farmitalia Carlo Erba (Brussels, Belgium).

Animals and tumor model.

Male inbred BN rats, weighing 250 - 300 g (Harlan-CPB, Austerlitz, the Netherlands) were used. Small fragments (3 mm) of the syngeneic BN175 soft tissue sarcoma were implanted subcutaneously in the right hindleg *(Manusama et al., 1996)*. Tumor growth was recorded by calliper measurements and tumor

volume calculated using the formula $0.4(A^2xB)$ (where B represents the largest diameter and A the diameter perpendicular to B). Rats were sacrificed if tumor diameter exceeded 25 mm or 4 weeks after treatment was started. All animal studies were done in accordance with protocols approved by the Animal Care Committee of the Erasmus University Rotterdam, the Netherlands.

Treatment protocol.

About 7-8 days after implantation tumors reached an average diameter of 8-12 mm when treatment was started. Rats were injected five times intravenously with an interval of 4 days between injections. First injection with DOXIL® was 4.5 mg/kg followed by four injections with 1mg/kg. Per injection 15 μg/kg TNF-α was administered to the rats in 0.2 mL volumes. When rats received both DOXIL® and TNF-α these agents were injected separately. Control rats were treated with equivalent amounts of placebo liposomes (i.e. drug free liposomes) or buffer.

Histology.

At 5 days (placebo or TNF-α treated rats) or 10 days (DOXIL® or DOXIL®+TNF-α treated rats) after start of treatment, tumors were excised with a rim of skin and muscle from three rats per group. After removal, tumors were cut in two equal parts, fixed in formalin and embedded in paraffin. The 4 μm sections were hematoxylin-eosin stained and assessed by conventional histological criteria. Video images of the slides were produced on a Leica microscope supplied with a Sony 3CCD DXC 950 video camera connected to a frame grabber.

In vitro cytotoxicity assay.

BN175 soft tissue sarcoma cells were added in 100 μl aliquots to 96-well plates at a final concentration of 10^4 cells per well and allowed to grow as a monolayer in DMEM supplemented with 10% FCS. Free doxorubicin, liposomal doxorubicin (DOXIL®) or TNF-α were diluted in DMEM supplemented with 10% FCS, added to the wells and allowed for three days to incubate. The range of final drug in the wells was 0.005 - 100 μg/mL for doxorubicin or DOXIL® and 0-10 μg/mL for TNF-α. Growth of tumor cells was measured using the Sulphorhodamine-B (SRB) assay. In short, cells were washed with phosphate buffered saline, incubated with 10% trichloric acetic acid (1h, 4°C) and washed again. Cells were then stained with SRB (15-30 min), washed with 1% acetic acid and were allowed to dry. Protein bound SRB was dissolved in TRIS (10 mM). Extinction was measured at 540 nm. A total of 3 separate assays were performed in triplicate and the percentage of growth inhibition calculated according to the formula: percentage of tumor cell growth = (test well/control well)x100%.

In vitro assessment of doxorubicin uptake in tumor cells.

To determine whether the in vitro observed toxicity correlated with cellular uptake of doxorubicin, the intracellular doxorubicin levels were determined by flowcytometry as previously described *(Luk and Tannock, 1989).* Briefly, BN175 soft tissue sarcoma cells or ROS-1 osteosarcoma cells were added in 500 µl aliquots to 24-well plates at a final concentration of $5x10^4$ cells per well and allowed to grow as a monolayer in DMEM supplemented with 10% FCS. Doxorubicin, DOXIL® and TNF-α were diluted in DMEM supplemented with 10% FCS and added to the wells, after which cells were incubated for 0, 10, 30, 60, and 120 min. The final drug concentration in the wells was 0, 0.1, 1.0 and 10 µg/mL for both doxorubicin or DOXIL® and TNF-α. Thereafter monolayers were treated with trypsin-EDTA for 2 min and the cell suspensions were washed two times in complete medium and resuspended in PBS. Cellular uptake was measured on a Becton Dickinson FACScan using Cell Quest software on Apple Macintosh computer. Excitation was set at 488 nm and emission at 530 nm. Fluorescence was corrected for cell size using the forward scatter (FSC) with the formula: corrected fluorescence (FLcor) = fluorescence at 530 nm (FL530) / FSC - $FL530_c$ / FSC_c ($FL530_c$ and FSC_c are fluorescence and forward scatter with no drug added to the cells).

Assessment of doxorubicin uptake in solid tumor and tissue distribution.

The effect of TNF-α on DOXIL® accumulation in tumors was examined. Since the tumor size of rats in the DOXIL® plus TNF-α group started to deviate significantly after three injections from the tumor size of rats in the group treated with DOXIL® alone, we decided to compare uptake not later than this time point. Rats received 1 or 3 injections of doxorubicin or DOXIL® with or without TNF-α with an interval of 4 days between injections as described in the treatment protocol. Tumor and muscle were excised 24 h after last injection, at which time maximal tumor localisation by Stealth liposomes was found *(van der Veen et al., 1998).* Tissues were analysed for doxorubicin and its fluorescent metabolites as previously described *(Mayer et al., 1989).* Briefly, after incubation of tissue in acidified isopropanol (0.075 N HCl in 90% isopropanol) for 24 h at 4°C, the tumors were homogenized (PRO200 homogenizer with 10 mm generator, Pro Scientific, CT, USA), centrifuged for 30 min at 2500 rpm and supernatants harvested. Samples were measured in a Hitachi F4500 fluorescence spectrometer (excitation 472 nm and emission 590 nm) and compared with a standard curve prepared with known concentration of doxorubicin diluted in acidified isopropanol. Measurements were repeated after addition of an internal doxorubicin standard. The detection limit for doxorubicin in tissue was 0.1 µg per g tissue.

To investigate the effect of TNF-α on tissue distribution of DOXIL® and free doxorubicin levels in lung, liver, hart, kidney and spleen, these organs were harvested at the time point described above. Doxorubicin extraction and measurements were performed according to tumor druglevel determinations.

Statistical analysis. Results were evaluated for statistical significance with the Mann Whitney U test. P-values below 0.05 were considered statistically significant.

RESULTS

Effect of low dose TNF-α on anti-tumor activity of DOXIL®.

Intravenous administration of a total of 5 injections of DOXIL® in combination with TNF-α (15 μg/kg) with an interval of 4 days between injections resulted in significant tumor response of the soft tissue sarcoma (figure 1). Responses lasted for more than three weeks. In contrast, treatment of the tumor-bearing rats with doxorubicin or DOXIL® alone did not induce a significant tumor regression, only a slight delay in growth was observed. Administration of TNF-α in the free form alone, also had no significant anti-tumor effect.

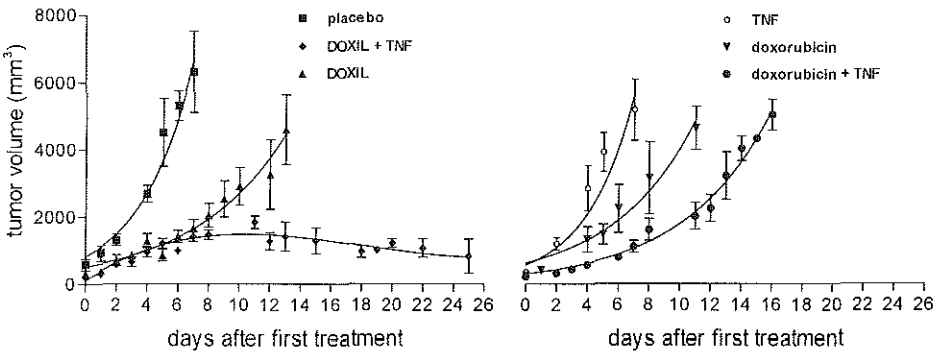

FIGURE 1. Anti-tumor activity of Stealth® liposome-encapsulated doxorubicin (DOXIL®) in combination with low dose TNF-α on soft tissue sarcoma in rats. Rats were injected five times with DOXIL® alone (4.5 mg/kg first dose and 1.0 mg/kg for consecutive doses) (▲), TNF-α (15 μg/kg per injection) plus DOXIL® (same dosages) (◆), or placebo liposomes (■) or five times with TNF-α alone (○), free doxorubicin alone (same dosages as DOXIL®)(▼), or free doxorubicin plus TNF-α (●), after which tumor growth was recorded. Mean tumor volume of 6 to 14 rats +/- SEM is expressed.

Combining low dose TNF-α with free doxorubicin slightly enhanced tumor growth delay, however, no tumor regression was observed. Administration of higher dosages of TNF-α (e.g. 200 μg/kg) resulted in an anti-tumor response comparable

with 15 μg/kg TNF-α, which was however accompanied by severe systemic toxicity such as extreme weight loss and high mortality. Treatment with low dose TNF-α combined with DOXIL® had no significant toxic effect on the rats as measured by body weight changes (table 1), diarrhea and behavior of the animals.

Table 1. Body weight changes in tumor-bearing rats treated with multiple administration of DOXIL® or free doxorubicin in combination with low dose TNF-α.

		TNF-α (μg/kg)			
Drug	Day	0	5	10	15
DXR	8	94.7 (2.7)[1]	93.6 (9.5)	95.4 (2.1)	91.1 (2.7)
	16	-[2]	-	-	-
DOXIL®	8	94.6 (3.1)	98.2 (2.9)	94.8 (1.8)	105.8 (3.3)
	16	-	110.8 (1.1)	111.6 (5.1)	109.0 (4.2)
PL	8	99.1 (2.0)	104.5 (0.3)	107.6 (5.6)	92.3 (7.6)
	16	-	-	-	-

[1] Values are weight changes 8 and 16 days after begin of treatment with free doxorubicin (DXR), Stealth liposomal doxorubicin (DOXIL®) or placebo liposomes (PL) with or without TNF-α, and expressed as percentage of initial body weight.

[2] Rats were sacrificed before this time point because of excessive tumor growth.

Note. Untreated rats with no tumor implanted normally gain around 5-10% in a period

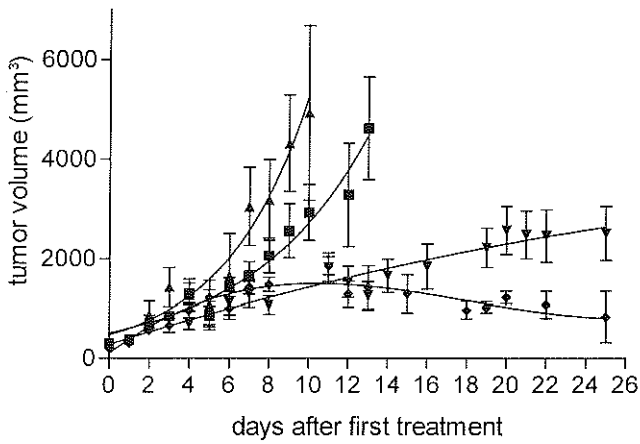

FIGURE 2. Anti-tumor activity of Stealth® liposome-encapsulated doxorubicin (DOXIL®) in combination with decreasing concentrations of TNF-α on soft tissue sarcoma in rats. Rats were injected five times with DOXIL® (4.5 mg/kg first dose and 1.0 mg/kg for consecutive doses) combined with 15 μg/kg (◆), 10 μg/kg (▼), 5 μg/kg (▲) or 0 μg/kg TNF-α (■). Mean tumor volume of 6 to 14 rats +/- SEM is expressed.

A clear dose response curve was observed when declining dosages of TNF-α were combined with DOXIL® (figure 2). Whereas a dose of 10 μg/kg TNF-α to the DOXIL® treatment still resulted in significant enhancement of the tumor response, a dose of 5 μg/kg had no effect anymore on DOXIL® activity. Although not significant, a slight increased growth rate was observed when 5 μg/kg TNF-α was administered in combination with DOXIL® when compared to DOXIL® alone. This might be explained by the growth stimulating activity of TNF-α at very low concentrations.

Histologic examination of anti-tumor activity of DOXIL® combined with TNF-α.

Treatment of the rats with the combination of DOXIL® and TNF-α resulted in severe necrosis of the tumor (figure 3). Massive coagulative necrosis of at least 60% of the area and extensive cell death was seen, accompanied by infiltrates and irregular hyperaemia. Percentage of (central) necrosis as observed in cross section of rats treated with placebo liposomes, TNF-α, or DOXIL® alone was at most 30%, occasionally accompanied by infiltrates.

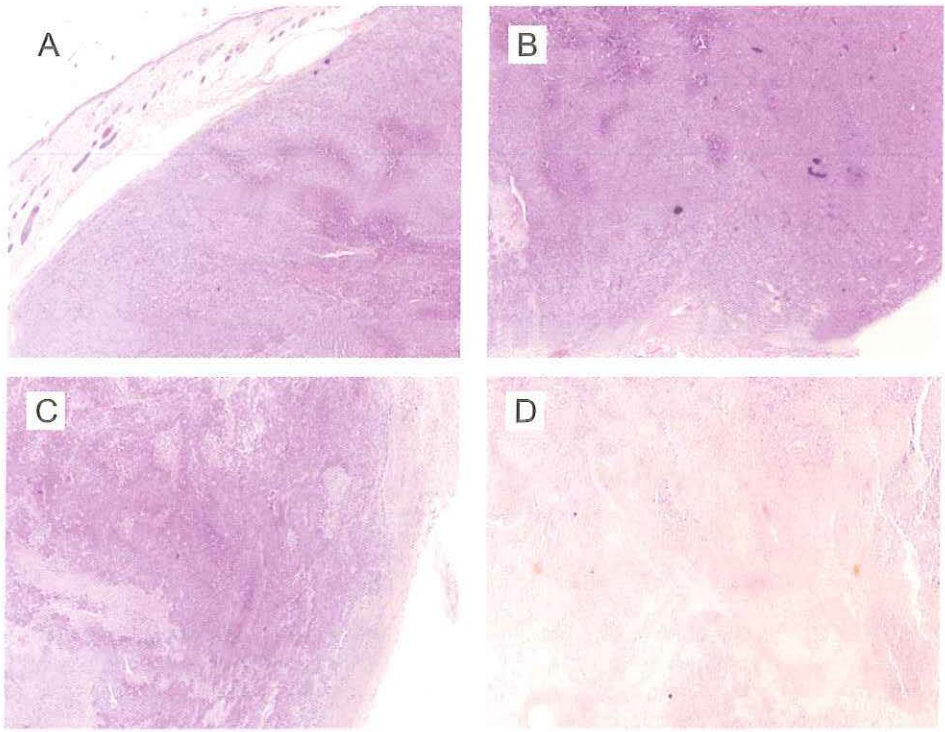

FIGURE 3. Paraffin sections of rat BN175 soft tissue sarcoma from systemically treated rats, haematoxylin-eosin stained. Overview of tumor after five fold treatment with (a) placebo liposomes, (b) free TNF-α (15 μg/kg), (c) DOXIL® (4.5 mg/kg first dose and 1 mg/kg dose 2 to 5) or (d) TNF-α combined with DOXIL®. Original magnification 2.5X.

In vitro activity of DOXIL® combined with TNF-α on BN175 tumor cells.

To evaluate whether the observed synergistic anti-tumor activity of DOXIL® and TNF-α *in vivo* was due to a direct effect on the cytotoxicity of DOXIL®, an in vitro bio-assay was conducted.

Figure 4 shows that exposure of BN175 tumor cells to doxorubicin resulted in a dose dependent cell growth inhibition, with an IC50 of 0.1 μg/mL. DOXIL® however appeared in vitro much less active (IC50 2.5 μg/mL). Incubation of the tumor cells with TNF-α alone had no effect on cell growth. Moreover, addition of TNF-α to doxorubicin or DOXIL® had no effect on the cytotoxicity of the anti-tumor agents. Comparable IC50 values were found with or without the addition of TNF-α.

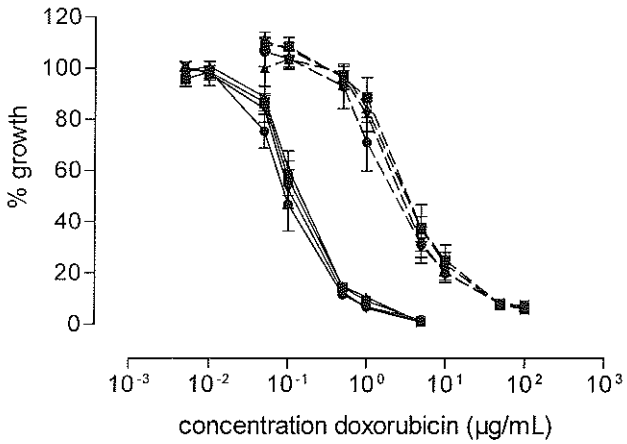

FIGURE 4. In vitro growth of the BN175 tumor cells as function of the added amount of DOXIL® (dotted line) and doxorubicin (continuous line) in combination with 0 μg (●), 0.1 μg (▼), 1.0 μg (▲) or 10 μg TNF-α per mL (■). The mean of 5 individual experiments performed of 16 days.in triplo is shown ± SEM.

In vitro uptake of DOXIL® or doxorubicin in tumor cells.

We examined if TNF-α augmented the intracellular accumulation of DOXIL®, which could explain the improved tumor responses. We observed that the addition of TNF-α, up to 10 μg/mL, had no effect on intracellular doxorubicin levels of DOXIL®. TNF-α also had no effect on uptake of free doxorubicin in vitro (data not shown).

DOXIL® and doxorubicin accumulation in solid tumor and tissue distribution.

To investigate whether the observed beneficial effect of TNF-α *in vivo* could be explained by an increased extravasation of DOXIL® into the tumor interstitium, concentrations of doxorubicin in tumor and surrounding tissue were determined. Figure 5A shows measurable amounts of doxorubicin in the tumors after single intravenous administration of free doxorubicin. Moreover, doxorubicin levels in tumors increased 3 to 3.5-fold when doxorubicin encapsulated in stealth liposomes was injected ($p < 0.02$ when compared to free doxorubicin). Addition of TNF-α induced no further accumulation of DOXIL® at this time point. However, when three injections of DOXIL® were given, addition of TNF-α augmented tissue concentrations of stealth liposome-encapsulated doxorubicin 3-fold when compared to three administrations of DOXIL® alone ($p = 0.02$)(figure 5B). A significant decline in tumor accumulation of DOXIL® was observed after the third injection as compared to a single injection with the liposomal formulation when no TNF-α was added. When three injections of free doxorubicin were given tissue levels remained comparable to levels after a single injection.

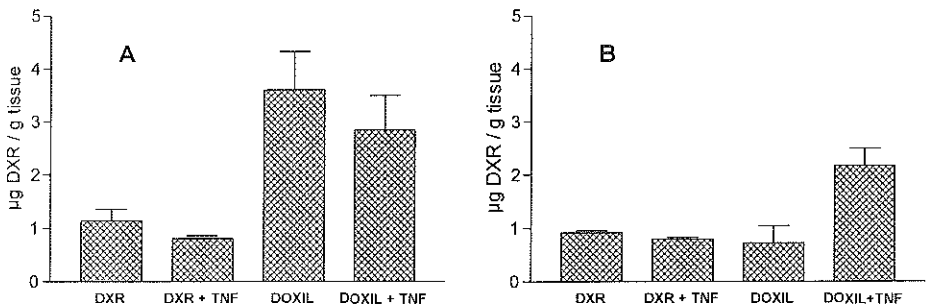

FIGURE 5. Concentrations of doxorubicin in soft tissue sarcoma BN175 after systemic administration of doxorubicin (DXR) or stealth liposome-encapsulated doxorubicin (DOXIL®) with or without TNF-α. Tumors were excised 24 h after end of treatment and total doxorubicin content was determined as described in materials and methods. Treatment consisted of (A) a single injection of free doxorubicin or DOXIL® with or without TNF-α or (B) three injections of free doxorubicin or DOXIL® with or without TNF-α as described in materials and methods. The mean of 4 to 6 rats are shown ± SEM.

Accumulation of free doxorubicin or DOXIL® in healthy muscle after single or multiple injections remained below 1.0 µg/mL (data not shown). Also in heart accumulation of doxorubicin remained below 1.0 µg/mL and was not significantly affected by addition of TNF-α (figure 6). In lung, liver and kidney a slight increase of doxorubicin was observed after multiple treatments for both free and liposomal doxorubicin. The liposomal formulation however, accumulated significantly better in spleen than free doxorubicin. In all organs no improved uptake of doxorubicin was observed when TNF-α was added to the treatment.

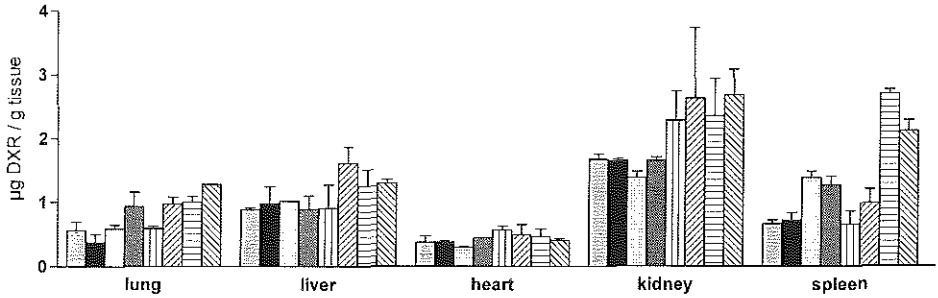

FIGURE 6. Concentrations of doxorubicin is organs of BN rats after administration of doxorubicin (DXR) or stealth liposome-encapsulated doxorubicin (DOXIL®) with or without TNF-α. Tumors were excised 24 h after end of treatment and total doxorubicin content was determined as described in materials and methods. Treatment consisted of a single injection (1x) or three injections (3x) of free doxorubicin or DOXIL® with or without TNF-α as described in materials and methods. The mean of 3 rats are shown ± SEM.

DISCUSSION

Anti-tumor activity in animals and in man has been obtained with TNF-α when used in an isolated perfusion setting combined with melphalan *(Eggermont et al., 1996b; Liénard et al., 1992).* Previously we reported on the anti-tumor activity of melphalan plus TNF-α in soft tissue sarcoma-bearing rats, which were in close agreement with the clinical findings *(Manusama et al., 1996).* A consistent finding in these *in vivo* studies is the strong synergy between melphalan and TNF-α, which was also demonstrated in an ILP model in rats and in humans with doxorubicin *(Di Filippo et al., 1998)(van der Veen et al., 2000).* Here we demonstrate that TNF-α also augments the anti-tumor activity of liposomal formulated doxorubicin (DOXIL®) when administered systemically. Secondly, repeated injection of DOXIL® combined with TNF-α results in augmented accumulation of the doxorubicin which could explain the observed synergistic anti-tumor effect.

A major draw back in the application of TNF-α is the severe toxicity which accompanies its systemic use. Application of this drug in isolated perfusion is likely to reduce toxic side effects. It has been shown that isolated limb perfusion results in very low systemic exposure levels due to extreme low leakage during perfusion and an effective wash out procedure thereafter *(Barker et al., 1995).* Therefore, in ILP dosages can be used which are up to 50 times the MTD resulting in strong anti-tumor effects. Systemic treatment with TNF-α however is only possible at very low dosages. Undesirable side effects are also noted when doxorubicin is used in the free form. Therefore a liposomal formulation has been developed which lowers toxicity. Moreover, with the application of the Stealth technology the efficacy of the preparation, ie DOXIL®, is increased when

compared to free doxorubicin *(Gabizon, 1992; Vaage et al., 1997).* The application of Stealth liposomes, relatively small liposomes which are coated with polyethylene glycol, results in prolonged circulation time of the encapsulated drug with enhanced accumulation in solid tumors *(Papahadjopoulos et al., 1991; Yuan et al., 1994; Gabizon et al., 1989; Gabizon, 1992)* This mechanism is believed to be responsible for the improved tumor responses of the liposomal formulation DOXIL® when compared with free doxorubicin. DOXIL® is effective in the treatment of AIDS-related Kaposi's sarcoma, and in some studies appears to be better than standard doxorubicin, bleomycin and vincristine (ABV) combination therapy *(Coukell and Spencer, 1997).*

Although doxorubicin is the drug of choice for the treatment of sarcoma, in ILP mostly melphalan is used. We and others demonstrated that strong anti-tumor effects can be obtained when doxorubicin is used in this setting when combined with high dose TNF-α *(Di Filippo et al., 1998., van der Veen et al., 2000).* These results indicate that the synergistic activity of TNF-α is independent of the chemotherapeutic drug used. In the present study, it is shown that systemic treatment with free doxorubicin or DOXIL® resulted in delay of tumor growth, with better results when DOXIL® is used. In vitro on the other hand free doxorubicin appeared much more potent than DOXIL®. The improved anti-tumor activity of DOXIL® *in vivo* is explained by an augmented accumulation of the drug in the tumor as shown here by us and previously by others *(Gabizon et al., 1989; Gabizon, 1992; Vaage et al., 1994; Papahadjopoulos et al., 1991; Vaage et al., 1997).* However, addition of TNF-α to a single injection of DOXIL® did not further improve accumulation, which is in discrepancy with findings by others *(Suzuki et al., 1990).* They observed an increased accumulation of liposome-encapsulated doxorubicin when combined with TNF-α after one single injection. In this study doxorubicin was encapsulated in liposomes which were not very well defined and probably have a large size distribution. The large liposomes not only contain a relatively large quantity of the drug, but also do not pass the endothelial lining in the tumor, as the gaps exhibit a cut off of 400 and 600 nm *(Yuan et al., 1995).* TNF-α increases the leakiness of the vasculature by increasing the gaps between the endothelial cells possibly explaining the augmented accumulation after extravazation of the large liposomes *(Brett et al., 1989; Smyth et al., 1988).* In another study the addition of TNF-α was shown to increase the anti-tumor activity of doxorubicin encapsulated in large liposomes (with sizes varying in the μm range), which was however not accompanied by a significant increase in tumor accumulation of the drug *(Maruo et al., 1992).* Stealth liposomes on the other hand already extravazate relatively easily as they are small (with a very small size distribution) and experience little resistance due to the lubricating activity of PEG *(Yuan et al., 1994).* This most likely explains the high accumulation after a single injection. We speculate that any additive effect of TNF-α at this stage is superseded by the observed initial high localization of DOXIL®. We could however

demonstrate an improved accumulation of DOXIL® in tumor tissue after three injections when combined with TNF-α. This is in line with the efficacy results, as significant anti-tumor effect is observed after three injections when the DOXIL® and DOXIL® plus TNF-α groups start to deviate from each other. Before that, the tumor growth curves of the DOXIL® plus TNF-α treated rats are comparable to tumor growth curves from rats treated with DOXIL® alone. Clearly a continuation of doxorubicin accumulation has to take place to induce a significant anti-tumor response, which does not take place in the rats treated with DOXIL® alone. No significant effect of TNF-α on drug accumulation however could be observed in all organs tested independent of drug formulation used, indicating a more or less tumor vasculature specific activity of TNF-α.

To explain this discrepancy, an important observation is the decline in DOXIL® accumulation after three administrations. For a tumor to grow new vessels have to be formed. As the neo-vascularization is characterized by leaky vessels, especially in this region extravazation of liposomes is expected *(Wu et al., 1993)*. Due to administration of DOXIL® a growth inhibition is observed, which is most likely accompanied by an diminished demand for new vessels, resulting in a diminished accumulation of DOXIL®. We speculate that administration of TNF-α apparently compensates for this phenomenon: it increases the leakiness of the endothelial lining, irrespective of maturation state and thus inducing an ongoing accumulation of DOXIL®.

In conclusion, we demonstrate that administration of DOXIL® results in improved drug accumulation in soft tissue sarcoma when compared to free doxorubicin. However, in all of these rats co-administration of TNF-α results in a dramatic enhancement of tumor reponse, which is shown to be due to an ongoing improved accumulation of DOXIL®. In rats receiving DOXIL® alone a decline in tumor accumulation of doxorubicin is observed. These findings therefore also indicate that multiple administrations are mandatory to receive a good tumor response.

ACKNOWLEDGEMENTS

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Chapter 7

Successful regional administration of TNF-α and developments towards new opportunities for systemic application

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Submitted.

ABSTRACT

Tumor Necrosis Factor-α is a multifunctional cytokine which use has been extensively investigated in the treatment of cancer. Initial enthusiasm was tempered when systemic treatment with TNF-α was found to cause severe toxic side effects in phase I/II studies. Other modalities for treatment were sought and a revival of the drug was the application of high dose TNF-α in combination with the cytotoxic drug melphalan in isolated limb perfusion (ILP). This review covers the preclinical and clinical studies which led to the success in treatment of patients with irresectable extremity soft tissue sarcoma and multiple melanoma in-transit metastases confined to the limb. TNF-α is now approved for registration in Europe for ILP in combination with melphalan for locally advanced extremity sarcoma. This achievement may lead to the development of new applications of TNF-α in isolated organ perfusion settings. An extension of the use of TNF-α may be found in the application of the cytokine in sterically stabilized liposomes, which are also considered here. The review concludes with the possible application of low dose TNF-α, which can be given systemically to enhance the anti-tumor potency of solid tumor targeting formulations, e.g. doxorubicin encapsulated in sterically stabilized liposomes for treatment of cancer. TNF-α remains a highly interesting vasculotoxic anticancer drug with a number of settings for its application to be explored.

INTRODUCTION

Since the observation of spontaneous regression of cancer in patients with concurrent bacterial infection *(Coley, 1896)* many years have passed. It was not until 1975 that a factor causing hemorrhagic necrosis in experimental tumors, later named Tumor Necrosis Factor-α (TNF-α), was discovered *(Carswell, 75, Old, 85).*

The common structure of human TNF-α protein is a homotrimeric complex of 52 kD that is biologically active *(Pennica, 84).* TNF-α is produced by many cell types, but is mainly secreted by activated monocytes/macrophages *(Matthews, 81, Aggarwal, 85, Sidhu, 93).* Its expression and regulation is affected by a variety of other cytokines, as interferon-γ (IFN-γ), interleukines (IL-1, IL-2, IL-12), GM-CSF, Platelet Aggregating Factor (PAF) as well as TNF-α itself *(Sidhu, 93).*

TNF-α has pleiotropic effects, which may depend on its concentration. It has been shown to have vasculotoxic effects at high concentrations *(Fajardo,*

92), while at low concentrations it may promote DNA synthesis and angiogenesis *(Beyer, 90, Fajardo, 92).* High concentrations of TNF-α have anti-tumor activity in murine tumor models *(Nawroth, 86).*

The effects of TNF-α are exerted by binding to two types of receptor, with molecular weights of 55 kDa (TNF-R1) and 75 kDa (TNF-R2) respectively *(Brockhaus, 90, Tartaglia, 92),* which are present on nearly all mammalian cells *(Aggarwal, 85).* Apart from these two distinct types of receptors, also soluble forms consisting of the extracellular domain of the receptors have been described *(Engelmann, 90).* The number of receptors on the cell does not predict the magnitude of response to TNF *(Creasey, 87),* but upregulation (IFN-γ) and downregulation (IL-1) of TNF receptors have been reported *(Hieber, 94).*

It was not until the purification of Tumor Necrosis Factor-α (TNF-α), using recombinant DNA techniques *(Pennica, 84),* that clinical application became feasible.

The introduction of TNF-α in clinical trials commenced with systemic administration. These phase I/II trials proved to be disappointing, mainly caused by the deleterious side effects of TNF-α *(Chapman, 87, Sherman, 88 Spriggs, 88).* Locoregional administration of TNF-α seemed the only option for successful application of TNF-α. Intralesional *(Bartsch, 89, Kahn, 89)* and intraperitoneal *(Raeth, 91)* administration were reported to increase response rates somewhat but proved no essential improvement of the effective use of TNF-α. This all changed when TNF-based isolated extremity perfusion in melanoma *(Liénard, 92)* and in soft tissue sarcoma *(Eggermont 93, 96a, 96b)* were reported to yield impressive response rates.

This led to the exploration of its use in the isolated organ perfusion setting. It was attempted in lungs *(Weksler, 94, Pass 96),* livers *(Fraker 94, Borel Rinkes 97),* and in kidneys *(van der Veen, 99).* In general, the isolation procedures proved to be technically difficult and organ toxicity limited the dose of TNF-α that could be used. Moreover, disseminated disease still remained an untreatable entity. Therefore, ways to find a reappraisal of systemic treatment with TNF-α were sought. For instance less toxic mutants of TNF-α *(Soma, 88, Kuroda, 95)* have been developed and their use has been explored *(Lodato, 95, Van Ostade, 93, Miyata, 92, Shikama, 95, De Wilt, submitted).*

Yet another possibility for systemic application is the incorporation of TNF-α in liposomes. Drug-delivery systems, like conventional liposomes, were used to incorporate TNF-α with some success *(Debs, 89,90, Düzgünes, 89).* The main drawback of these multilamellar vesicles however, is the rapid recognition and uptake by the mononuclear phagocyte system (MPS). We therefore tried to incorporate TNF-α in new generation (long circulating)

liposomes as will be described below *(van der Veen, 98).*

In this review we will discuss the role of TNF-α in anti-tumor therapy, from preclinical studies to a reappraisal of systemic therapy with low dose TNF-α .

PRECLINICAL STUDIES WITH TNF-α

In vitro studies

For a small minority of cancer cell lines TNF-α is directly cytostatic or cytotoxic *(Haranaka, 81).* On other cell types TNF-α shows a growth inhibitory or even a growth stimulatory effect *(reviewed by Sidhu, 93, Hieber, 94).* The mechanism by which TNF-α exerts its cytotoxic effects are not yet fully understood. The number of receptors on the tumor cell are probably of less importance than the role of oxygen free radicals in TNF-α cytotoxicity, and activation of lysosomal enzymes *(Fiers, 95, Sidhu, 93).*

In vitro, synergism between TNF-α and a number of cytotoxic agents may be present. In our laboratory we investigated several cell lines on susceptibility to TNF-α and certain cytotoxic drugs, like the alkylating agent melphalan or the topoisomerase-II inhibitor doxorubicin. In contrast to what was expected, in two sarcoma nor in an adenocarcinoma cell line thus far investigated, no direct cytotoxic effects of TNF-α, nor synergism with melphalan or doxorubicin in vitro could be observed *(Manusama, 96a,b, van der Veen, 2000).* Additive anti-tumor effects however were noted.

Furthermore, cytotoxic effects of TNF-α can be enhanced by a number of other biological response modifiers like IFN-γ *(Salmon, 87, Schiller, 87, Dealtry, 87)* or IL-1 *(Ruggiero, 87),* hyperthermia *(Watanabe, 88a)* and irradiation *(Matsunaga, 92).*

In vivo studies

Many animal studies have demonstrated the anti-tumor effect of TNF-α in vivo. In fact Carswell, in her original work on describing tumor necrosis factor, studied the Meth A sarcoma in mice *(Carswell, 75).* This tumor showed the classical hemorrhagic necrosis after application of TNF-α, but eventually recurred.

Making use of the rapidly growing, spontaneously metastasizing, non-immunogenic, grade III fibrosarcoma (BN 175), which originated spontaneously in the Brown Norway rat *(Kort, 84),* we developed an isolated limb perfusion model in our laboratory *(Manusama, 96a).* Moreover, in a wistar-derived WAG rat strain a similar tumor model *(Manusama, 96b)* with an osteosarcoma ROS-1, which originated spontaneously in the tibia of a WAG rat, was developed *(Barendsen, 78).* In these non-immunogenic and syngeneic tumor systems both tumors grow in fully immunocompetent rats, and the models resemble the

clinical situation closely. In both tumor systems highly synergistic anti-tumor effects are observed when TNF-α is combined with melphalan, resulting in a complete remission rate of 70-80% *(Manusama, 96a,b, De Wilt, 99)*. Histopathologically extensive haemorrhagic necrosis is observed after ILP with the combination and not after ILP with melphalan alone *(Nooijen, 96)*. Early endothelial damage and platelet aggregation in the tumor vessels are observed after ILP with TNF-α and melphalan and this is believed to lead to ischemic (coagulative) necrosis, which is in line with observations in patients *(Renard, 95, Nooijen, 96, Fraker, 96, Manusama, 98)*. We also performed isolated limb perfusions with TNF-α and doxorubicin in solid tumor bearing rats with comparable outcome *(van der Veen, 2000)*. Both solid tumor models showed progressive disease after perfusion with doxorubicin alone. Addition of 50 μg of TNF-α however resulted in a 54% response (partial and complete) in the soft tissue sarcoma and a 100% response in the osteosarcoma. An increased accumulation of doxorubicin was found in tumor tissue after ILP with a combination of doxorubicin and TNF-α in comparison with an ILP with doxorubicin alone. Synergistic anti-tumor response in vivo could be explained by this enhancement of local drug concentration. In vitro TNF-α fails to augment drug uptake in tumor cells or to increase cytotoxicity of the drug. These findings make it unlikely that TNF-α directly modulates the activity of doxorubicin in vivo.

Mechanism of action on tumor associated vascular bed

Data obtained in experimental animal studies as well as in human studies strongly indicate that TNF-α targets the vascular bed of the tumor. The tumor vasculature consists of pre-existent vessels in normal tissues in which the tumor has invaded as well as microvessels as a result of angiogenesis, resulting in neovascularization *(Skinner, 90, Brown, 98)*. These vessels are characterized by an inherent high permeability caused by incomplete endothelial linings and basement membranes, and the vasculature is not well organized. The vessels run a tortuous and convoluted path, with venous lakes, regurgitant flow and stasis *(Dvorak, 88, Dewhirst, 89)*. Blood flow through the neovasculature is therefore irregular and sluggish. In addition, tumor stroma is characterized by absence of lymphatics, increased permeability of endothelial cells and high interstitial pressure. Drug delivery to solid tumor therefore is influenced by the microvasculature and tumor stroma *(reviewed by Jain, 96b)*.

The majority of TNF-α anti-tumor effects are believed to be indirectly mediated through damage of the tumor associated vasculature (TAV). Thus, activation of procoagulant activity in vascular endothelium appeared to be responsible for hemorrhagic necrosis *(Bevilacqua, 86, Watanabe, 88b)*, also thrombus formation and negative influence on tumor blood flow seemed to play a pivotal role *(Nawroth, 88, Shimomura, 88, Kallinowski, 89, Renard, 95,*

Nooijen, 96). Vasculotoxic effects lead to anoxic necrosis. It is suggested that TNF-α induces endothelial cell damage, leading to congestion, hemorrhage and edema as representatives of an impaired blood flow *(Renard, 95 Nooijen, 96, Manusama, 96, Fraker, 95, Dvorak, 88, Denekamp, 83).* Furthermore, anti-vascular effects lead to immediate permeability changes *(Vargas, 96, Kristensen, 96).* Endothelial damage, proven by a change in distribution of von Willebrand factor, occurred already three hours after onset of TNF-α based perfusion during ILP *(Renard, 95).* However, also a delayed type of hyperpermeability may be present, explaining the fact that complete tumor regression frequently requires longer periods after TNF-α based isolated perfusion *(Nooijen, 98).* Vasculotoxic effects of TNF-α lead to a significant drop in tumor interstitial pressure and permeability changes *(Kristensen, 96)* which leads to better penetration of cytotoxic drugs into tumor tissue *(Suzuki, 90).* We showed an augmented accumulation of doxorubicin as well as melphalan in tumor tissue when TNF-α was added to the perfusate *(Van Der Veen, 2000, De Wilt, 2000).* Moreover, vasculotoxic effects lead to endothelial cell activation, upregulation of adhesion molecules and infiltrate formation through influx of polymorphonuclears, more specifically granulocytes. Subsequently injury to the endothelial cells may be the beginning of a cascade of events *(Yi, 92, Renard 94, Manusama, 98).*

Recently, more insight in the mechanism of disruption of tumor associated vasculature was obtained *(Ruegg, 98).* Suppression of integrin αVβ3, an adhesion receptor expressed by angiogenic endothelial cells and that binds to provisional extracellular matrix components, is the putative mechanism by which TNF-α and interferon-γ causes disruption of the vasculature. Synergistic or additive anti-tumor effect between TNF-α and IFN-γ were observed earlier *(Brouckaert, 86, Balkwill, 87, Marquet, 87),* probably based on receptor upregulation, which is also shown in vitro. However, conflicting results have been reported *(Manusama, 99),* with IFN-γ slightly enhancing anti-tumor efficacy of TNF-α in combination with melphalan, whereas toxicity was considerably increased. Moreover, we recently demonstrated that an increase in drug accumulation due to the addition of TNF-α could very well explain the improved tumor response by itself *(van der Veen, 2000).*

Synergism with other cytokines like IL-1 *(Okhura, 90), Il-2 (Nishimura, 87, Winkelhake, 87, Mcintosh, 88)* and il-6 *(Mule, 90)* have also been described.

In vivo, synergism of TNF-α with drugs like alkylating agents (melphalan) is the crucial element that determines the success of isolated limb perfusion *(Manusama, 96a,b),* whereas also synergism with topoisomerase II inhibitors (doxorubicin) *(Hieber, 94, Fiers, 95, van der Veen, 2000),* or

temperature *(Yamaguchi, 92)* have been reported. Prerequisites for effective isolated limb perfusion with TNF-α and melphalan have been described by our group. A 30 min perfusion under mild hyperthermic conditions (38-39°C) proved to be optimal to achieve a synergistic effect *(de Wilt et al 1999).* Synergism between TNF-α and melphalan in high dose perfusion setting may be explained by dual targeting, where TNF-α (and IFN-γ) is suggested to be responsible for disruption of the neovasculature, while melphalan causes a non-specific necrosis of cancer cells in vivo *(Lejeune, 95).* Also, for another chemotherapeutic drug, 5-fluorouracil (5-FU), it was observed that the in vivo anti-tumor effects of the combined treatment of 5-FU and TNF-α depend upon the development of capillaries in tumors *(Manda, 90)*

Moreover, immune factors are seemingly important in determining the anti-tumor effect of TNF-α. Immunogenic tumors showed more regression than non-immunogenic tumors *(Asher, 87),* and the viable rim of tumor left after administration of TNF-α disappeared only in immune competent hosts.

To lower systemic toxicity of native TNF-α, it was proposed to couple TNF-α to polyethylene glycol (PEG), thereby increasing their molecular size and steric hindrance, resulting in augmented plasma half lives, increased bioavailability and stability *(Tsutsumi, 95, 96).* In vitro, a reduction in bioactivity was noted, in vivo however, PEGylated TNF-α showed markedly higher percentages of hemorrhagic necrosis in solid tumor (S-180 sarcoma) than native TNF-α as well as an abrogation of its toxic side effects.

The success of isolated limb perfusion and the indication that the common denominator of the anti-tumor effect is the tumor vascular bed led to expansion of the field of isolated perfusion with TNF-α to isolated organ perfusions as lung *(Weksler, 94, Pogrebniak, 94),* kidney *(van der Veen, 99)* and liver *(Fraker, 94, Borel Rinkes, 97, de Vries, 98, van IJken, 98).* In each of these organs isolated perfusion with TNF-α was feasible, with marked differences in systemic toxicity, which probably accounts for the reported variance in tumor responses.

CLINICAL STUDIES WITH TNF-α

Systemic application

Systemic application of TNF-α, whether as a bolus or continuous intravenous (i.v.) injection, proved to be deleterious to the organism and clinical phase I/II studies yielded disappointing results with severe toxicity. The maximal tolerated dose (MTD) varied between 200 and 400 $\mu g/m^2$, depending on treatment schedule *(Brouckaert, 86, Feinberg, 88, Spriggs & Yates, 92).* A variety of side effects was noted after i.v. injection of TNF-α *(Hieber, 94, Fiers, 95),* hypotension being the dose-limiting toxicity *(Feinberg, 88, Spriggs, 88).* In patients systemic toxicity prevents the administration of

adequate doses of TNF as only 1/50 of the effective dose in murine tumor models could be given in humans. Due to these low concentrations of TNF-α when given systemically, only low response rates were achieved. Phase II studies revealed a meager 1-2% complete remission rate after intravenous administration of TNF-α as a single agent *(Figlin, 88, Kemeny, 90, Feldman, 92)* or combination therapy with chemotherapeutic drugs *(Jones, 92).* The addition of IFN-γ or IL-2 did not enhance anti-tumor efficacy appreciably *(Fiedler, 91, Negrier, 92),* but it did further increase toxicity.

Locoregional application
Intratumoral injection

Since the MTD of TNF-α in clinical trials was 50 fold lower than the TNF-α levels necessary to obtain responses in animal models *(Asher, 87),* other routes of injection were explored to achieve a high local concentration of TNF-α in tumor tissue. Intratumoral injection revealed only slightly better responses than intravenous injection with similar side effects at the same MTD *(Bartsch, 89, Kahn, 89, Ijzermans, 91).* Hepatic artery infusion *(Mavligit, 92)* and intraperitoneal *(Raeth, 91)* administration of TNF-α revealed only modest results.

Isolated limb perfusion (ILP)

The first consistent anti-tumor results with TNF-α were obtained after isolated perfusion of the limbs (ILP). In this setting TNF-α is combined with melphalan and interferon-γ to treat high grade melanoma and irresectable sarcoma *(Liénard, 92, Eggermont, 93).* A major advantage of ILP is the achievement of high local concentrations: 15-20 times higher than those reached after systemic administration *(Benckhuijsen, 88).*

Isolated perfusion of the extremities was developed by Creech over 40 years ago *(Creech, 58).* Isolation of the blood circuit of a limb is achieved by clamping the artery and vein, by ligating collateral vessels and by applying a tourniquet around the base of the limb to compress the remaining minor vessels. After cannulation of the vessels, the isolated extremity is connected to an oxygenated extracorporeal circuit, into which the cytostatic agent is injected. After perfusion a thorough rinsing procedure to wash out the drugs is performed, after which the cannulas are removed and the circulation is restored.

In combination with hyperthermia, isolated limb perfusion with melphalan, an alkylating agent, yielded a 54% complete response rate for patients with melanoma in transit metastases confined to the limb *(Eggermont, 96d).* In the late eighties ILP with melphalan, high dose Tumor Necrosis Factor-α and interferon-γ was undertaken for multiple melanoma in transit metastases and irresectable high grade soft tissue sarcomas of the Limb

(Liénard, 92, Lejeune, 94, Eggermont, 93, 96a,b). The rationale for this strategy was dose escalation of a cytotoxic agent with virtually absent systemic toxicity while determining the superior effect of addition of TNF-α. The choice for melphalan as the cytotoxic drug in isolated limb perfusions was the result of the vast experience with this drug in the standard isolated perfusion for melanoma. For irresectable soft tissue sarcomas confined to the limb, ILP was applied as induction treatment to render these sarcomas resectable. It became clear that high complete response rates were feasible, with a 82% limb salvage rate *(Eggermont, 96b,c, 97)*.

As previously mentioned in animal studies, also in clinical setting the tumor vascular bed appears to be the selective target for TNF-α. The effects on the tumor associated vasculature after ILP with TNF-α were described as early endothelium activation, upregulation of adhesion molecules and invasion of polymorphonuclear cells, leading to coagulative necrosis with or without hemorrhagic necrosis *(Renard, 94,95)*. However, examination of melanomas and sarcomas of patients treated with the triple regimen in ILP did not show differences in expression of adhesion molecules as ICAM-1, E-selectin (ECAM-1), VCAM-1 or PECAM-1 in tumors compared with healthy tissue *(Nooijen, 96)*. Recently, in patients treated with isolated limb perfusion with TNF-α, IFN-γ and melphalan, detachment and apoptosis of the integrin αvβ3 positive endothelial cells was demonstrated in vivo in melanoma metastases *(Ruegg, 98)*, again pointing to the importance of selective disruption of tumor associated blood vessels. This was further indicated by angiography studies which clearly showed the disappearance of tumor associated vessels after TNF-based ILP *(Eggermont, 94, Olieman, 97)*.

Anthracyclines are among the most active agents against solid tumors and doxorubicin is the most widely used agent of this class *(Bielack, 96, Bramwell, 88, Budd, 95)*. Moreover, doxorubicin is the agent of choice for the treatment of sarcoma, and has shown good anti-tumor activity in clinical and experimental perfusion settings for the treatment of lung metastases *(Weksler, 94, Abolhoda, 97, Wang, 95)*. Therefore, doxorubicin has also been used in isolated perfusion of the extremity *(Rossi, 94)*. However, in most series results were inferior to melphalan. When comparable results were obtained this was often at the cost of increased regional toxicity *(Thompson, 92, Krementz, 94)*.

Isolated organ perfusion

The indications that the tumor vascular bed is the common denominator for success of isolated perfusions with TNF-α, opened gates to the application of TNF-α in other settings, as isolated organ perfusion. Various organs which are amenable to isolated perfusion have been described in which the applicability and anti-tumor efficacy of TNF-α was evaluated. Isolated

lung perfusion proved to be relatively safe *(Pass, 96)*, phase I studies for isolated renal perfusion are underway *(Walther, 96)* and recent reports described the efficacy of isolated liver perfusion with TNF-α *(Fraker, 94, Oldhafer, 98, Alexander, 98)*.

Our own experiences in pigs showed that drug levels comparable to those achieved in isolated limb perfusion can be obtained in a leakage free isolated hepatic perfusion setting *(Borel Rinkes, 97)*. In a phase I study in patients with irresectable colorectal cancer liver metastases isolated hepatic perfusion with TNF-α in patients with irresectable colorectal metastases in the liver are summarized here. One histologically confirmed complete remission (CR) and 4 partial remissions (PR) were found in 6 evaluable patients. Two patients died due to acute coagulation disorders after substantial blood loss, and protracted hepatic toxicity related dysfunction was observed *(De Vries, 98)*. Toxicity was less dramatic in the series reported by Alexander et al. *(Alexander, 98)*. In our patients the liver was perfused via the portal vein and the hepatic artery simultaneously. This may in part account for the unexpected high toxicity, as it has been shown that infusion via the portal vein is more toxic than when given systemically *(Khaky, 90)*. Anti-tumor effects may be due to activation of inflammatory and coagulation cascades, resulting in induction of secondary cytokines and a prolonged period of high levels TNF-α. However, TNF-α does not preferentially accumulate in tumor tissue *(Kuppen, 97)*.

NEW STRATEGIES FOR SYSTEMIC APPLICATION OF TUMOR NECROSIS FACTOR-α

Mutants of Tumor Necrosis Factor-α

Because of the systemic toxicity associated with TNF-α, less toxic but equally effective recombinant TNF-α molecules were sought. In one of these mutants of conventional TNF-α the N-terminal amino acid sequences were altered to achieve higher basicity (TNF-S_{AM}). Various types of TNF-S_{AM}, differing in amino acid species and position in the N-terminus were described *(Soma, 88)* Toxic side effects seemed to be lower with these molecules, whereas TNF-S_{AM1} and TNF-S_{AM2} revealed a stronger cytocidal activity than conventional TNF-α on various murine tumors *(Gatanaga, 89)*. Isolated limb perfusion with TNF-S_{AM2} and either melphalan or doxorubicin resulted in a synergistic anti-tumor response in BN175 soft tissue sarcoma in rats (de Wilt, submitted). Other muteins, in which amino acids were changed (F4236, F4168, F4614), showed less lethal toxicity and increased anti-tumor activity *(Miyata, 92, Miyata, 95, Kuroda, 95)*. Low inducibility of nitric oxide and prostaglandin E_2 correlated with a reduced hypotensive effect, consequently an increased therapeutically effective dose compared to native TNF-α could be given *(Shikama, 95)*. New TNF-α analogues as LK 801 and LK 805 confirmed that

modification of the parental molecule leads to lower systemic toxicity and comparable anti-tumor effect *(Novakovic, 97)*. These novel muteins were thought to have greater potential for clinical application.

Liposomes

Conventional liposomes

Liposomes were originally described some 30 years ago by Bangham *(Bangham, 65)*. He described a concentric arrangement of lipids, which were called liposomes for the first time in 1968 *(Bangham, 65, Sessa and Weissmann, 68)*. These liposomes were composed of several layers of phospholipids, which enclosed an aqueous center.

These multilamellar vesicles were first used in pharmaceutical experiments by Gregoriadis and Ryman *(Gregoriadis And Ryman, 72)*. They concluded that liposomes are captured by the liver macrophages (Kupffer cells), and are degraded in lysosomes. Later, investigators discovered that by encapsulating drugs in liposomes the pharmacokinetics were altered, which could mean a broadening of the therapeutic window *(Forssen And Tokes, 79, 81)*. Hence, it was foreseen that potentially toxic drugs could be used in cancer therapy *(Gabizon, 97)*.

In the early eighties it became clear that liposomes were not the magic bullet they once were thought to be. Unexpected problems in large scale production and long-term stability arose, and major drawbacks with the use of conventional liposomal formulations were the retention of the drug while in circulation, the inability to extravasate in tumor tissue, as well as the problem of rapid clearance by the mononuclear phagocyte system (MPS), formerly called the reticulo-endothelial system (RES). Consequently, other approaches were tried. Temperature-sensitive liposomes were manufactured as well as pH-sensitive liposomes *(Yatvin, 78, 80)*, but these liposomes were also not the answer to the major problem in human cancer: the metastatic lesion.

Thus, new strategies were sought to make liposomes more resistant to factors responsible for clearance. Initially, incorporation in the lipid bilayer of small amounts of natural glycolipids, primarily monosialoganglioside GM_1 and hydrogenated phosphatidylinositol (HPI), enhanced circulation time of liposomes *(Woodle, 95)*.

Sterically stabilized liposomes

In the late eighties, a new kind of liposome surface modification was introduced *(Allen, 87, Klibanov, 90)*. These liposomes are coated with a polyethyleneglycol (PEG) of 1900 to 5000 daltons linked to the distearoylphospatidylethanolamine (DSPE) *(Gabizon 88, Papahadjopoulos, 91, Woodle and Lasic, 92)*, which increases hydrophilicity of the liposomal

surface, thereby reducing adherence of plasma proteins and opsonins, and prolonging blood circulation. Unlike conventional liposomes, the new generation of sterically stabilized liposomes provide a means to avoid rapid uptake by the MPS and to alter drug kinetics and tissue distribution for therapeutic advantage *(Gabizon, 92, 97, Lasic, 95)*.

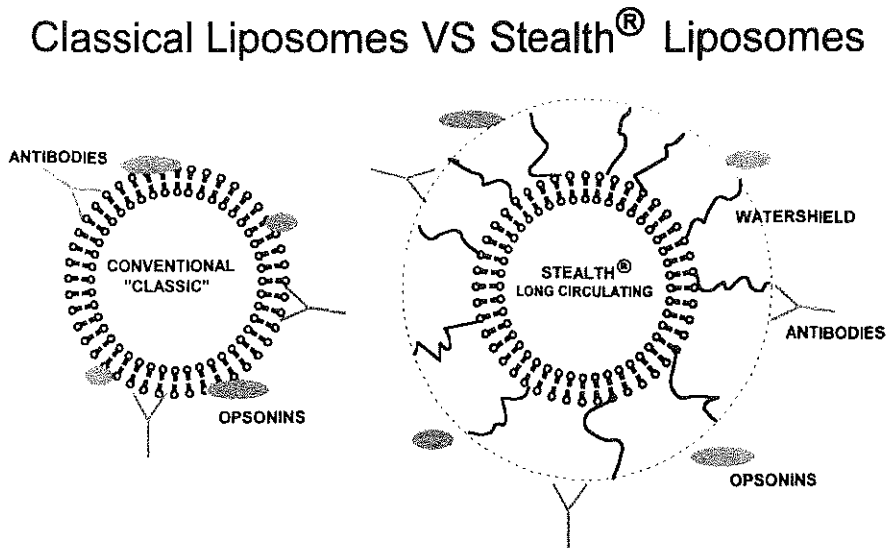

Figure 1. Schematic representation of conventional liposomes and Stealth® liposomes (courtesy of dr. T.L.M. ten Hagen)

The optimum level of PEG in the bilayer appears to be between 3 and 10 mol% *(Allen, 94a)*. It was also proved to be possible to insert polyethylene glycol derivatized phospholipid into pre-formed liposomes, which showed the same pharmacokinetic advantages as sterically stabilized liposomes *(Uster, 96)*. The longer circulation times were largely dose independent *(Allen, 91, Lasic, 91)*, and the longer life plasma half-life of these sterically stabilized (Stealth®) liposomes in circulation allow accumulation in the tumor tissue *(Gabizon, 92)*. This is thought to facilitate passive targeting, whereas active targeting by coupling antibodies to these liposomes also proved to be feasible *(Allen, 94b, Hansen, 95)*.

Stability and clearance of liposomes

One important factor to consider when using liposomes is their stability in circulation. Because liposomes are injected intravenously, plasma proteins play a major role in liposomal clearance. The stability of the vesicle in plasma depends on size, lipid composition, lamellarity and temperature *(Jones,*

91, Scherphof, 84, Lasic, 91). Several groups have elaborated on the importance of opsonization and subsequent elimination of liposomes *(Patel, 92, Chonn 92, Harashima, 93).* Here, attention will be focused upon the sterically stabilized liposomes.

The presence of the hydrophilic, flexible polymer poly-ethylene glycol (PEG), extending from the liposomal membrane for a distance of 5-6-nm *(Needham, 92),* alters the interaction between plasma proteins and the liposomes by steric hindrance *(Senior, 91, Allen, 94).* PEG-coated liposomes still accumulate in the MPS, mainly comprised of Kupffer cells in the liver and fixed macrophages in the spleen, although uptake also takes place in bone marrow and lymph nodes *(Allen, 97),* but the exact mechanism of elimination of sterically stabilized liposomes is not clear. It is suggested that the removal from circulation of PEG-liposomes occurs by the same mechanism as the removal of conventional liposomes, as a result of the gradual removal of the PEG from the liposomal membrane and their subsequent opsonization *(Allen, 94).* Complement is likely to play an important role in liposomal clearance, influenced by cholesterol *(Ishida, 97),* animal species *(Liu, 95, Harashima, 96)* and liposome size *(Litzinger, 94, Devine, 94).* Larger (ie diameter >300 nm) liposomes show an increased accumulation into spleen, whereas PEG-liposomes of smaller diameter (below 90 nm) are preferentially taken up by the liver *(Allen, 94).* Enhancement of uptake of pegylated liposomes by the spleen was noted before *(Gabizon, 92, Woodle, 94),* probably resulting from high blood concentration. In our sarcoma model we found an equal amount of sterically stabilized liposomes in liver and spleen, however per gram tissue the spleen was shown to be very active *(van der Veen, 98).* Non-immunogenic opsonins in rat plasma, like fibronectin, may also play a role in the uptake of liposomes by the spleen *(Aramaki, 95),* furthermore, it is suggested that tumor-bearing animals have an increased splenic clearance, possibly related to an altered opsonic activity of serum in these animals *(Moghimi, 96).*

Application of liposomes in anticancer therapy

The advantages of liposomes as an effective delivery system are most obvious in anticancer therapy. Cytotoxic drugs are characterized by their low therapeutic index (TI) due to (i) inability to achieve therapeutic concentrations at the site of the tumor, (ii) nonspecific cytotoxicity to normal tissues or (iii) problems arising with the formulation of the drug *(Sharma, 97).*

In general, the purpose of encapsulation of cytotoxic drugs will be a reduction of toxicity, while at the same time enhancement of therapeutic efficacy (e.d. reversal of multidrug resistance mechanisms, slow release system) and selective delivery of the encapsulated drug to tumor tissue is pursued *(Ranson, 96).*

For cytotoxic drugs to reach their target site it is mandatory that the

carrier system leaves the circulation. Solid tumors show a disorganized microvasculature, with increased capillary permeability due to wider endothelial gaps and depleted endothelial layers and basal lamina *(Dvorak, 88)*. In addition, physiological parameters as the increased interstitial fluid pressure and the absence of an adequate lymphatic barrier play a role in the deposition of liposomes in the tumor *(Jain, 87, 94)*.

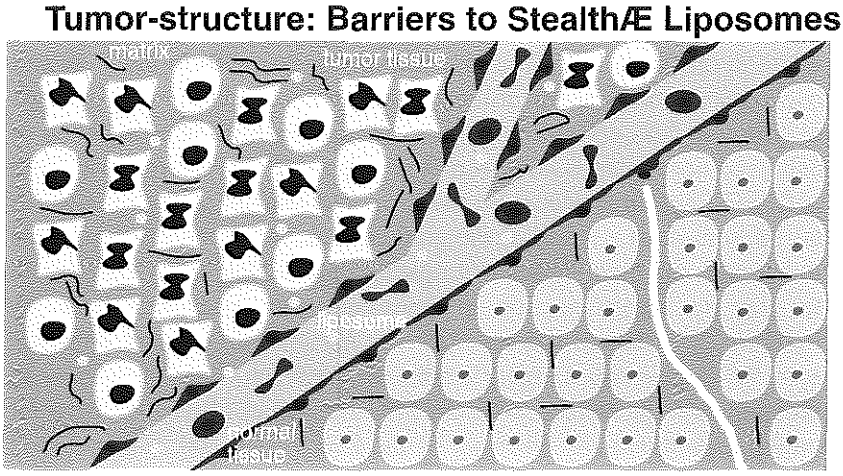

Figure 2. Schematic representation of tumor tissue vs normal healthy tissue (courtesy of dr. T.L.M. ten Hagen)

Sterically stabilized liposomes, with their extended circulation times, have a high probability of extravasation in the tumor interstitial space *(Jain, 96a, Gabizon, 97)*, and with light and electron microscopy in vivo distribution was studied *(Huang, 92a)*. It appeared that the PEG-liposomes were located around blood vessels in the tumor *(Litzinger, 94)* and in areas with angiogenesis. The perivascular distribution of intact liposomes was heterogeneous *(Brown, 98)*. The difference between intravascular hydrostatic and interstitial pressure serves as a driving force for liposomes to leave the bloodstream *(Yuan, 94, Kristensen, 96)*, and with fluorescence studies it was proven that sterically stabilized liposomes, which show a higher vascular permeability than conventional liposomes *(Yuan, 95)*, preferentially are taken up in tumor tissue as opposed to non-tumor tissue with relative impermeable capillaries *(Wu, 93, Dewhirst, 95, Wu, 97)*.

The encapsulation of anthracyclines, especially doxorubicin, epirubicin and daunorubicin, in liposomes has been recently reviewed *(Gabizon, 94, 97, Ranson, 96)*. In this review we will discuss the preclinical and clinical studies with anticancer drugs in sterically stabilized liposomes, focussed on encapsulated doxorubicin.

The pharmacokinetics of anticancer drugs in pegylated liposomes are

characterized by a prolonged blood residence time, slow plasma clearance and a small volume of distribution, as well as a reduced leakage rate of the encapsulated drug. The altered pharmacokinetics and efficacy of anthracyclines encapsulated in sterically stabilized liposomes have been described for a variety of animal tumor models *(Papahadjopoulos, 91, Huang, 92b, Mayhew, 92, Gabizon, 92, 93, Siegal, 95, Vaage, 92,93).* All showed an increased accumulation of the encapsulated drug versus the non-encapsulated drug, with peak levels in tumor tissue reached at a later timepoint. In a xenografted human pancreatic carcinoma, drug levels remained high for a prolonged period of time *(Vaage, 97).* The longevity in circulation, characteristic of steric stabilization, with resultant selective extravasation at the tumor site, was suggested to be responsible for improvement of therapeutic efficacy in an ascitic tumor model in mice (J-6456) as well as a solid mouse lung carcinoma (M-109) *(Gabizon, 96).*

Not only for solid tumors, also in a liver metastasis model the deposition of doxorubicin loaded sterically stabilized liposomes (Doxil®) was followed *(Cay, 97).* Intravital fluorescence microscopy of the liver revealed homogeneous distribution of the liposomes to metastatic liver tumors after intraarterial and intraportal administration, where a sustained release of encapsulated cytotoxic drug was observed *(Unezaki, 96).* Electron microscopy confirmed intracytoplasmic, perinuclear uptake of liposomes in tumor cells. Intravenous injection of the liposomes did not seem as effective as locoregional administration, therefore this last study opens new perspectives in the field of sterically stabilized liposomal anticancer drugs.

In human studies the alteration of pharmacokinetics of doxorubicin after encapsulation in sterically stabilized liposomes was confirmed *(Gabizon, 94).* The plasma elimination of Doxil® was even slower than in animal studies *(Gabizon, 93),* following a biexponential curve with median values of 2 and 45 h. Doxil®, tested at two dose levels, 25 and 50 mg/m^2, showed a slow clearance and small volume of distribution (V_d). When the V_d is decreased as opposed to free drug, the drug concentration in normal tissues will be reduced. Therefore the toxicity profile of the free drug is altered, sometimes at the cost of new toxicities. In a subsequent phase I study, patients with a variety of malignant tumors were treated with escalating doses between 25 and 60 mg/m^2 every 3 to 4 weeks *(Gabizon, 94, Gordon, 95).* The efficacy of PEG-liposomal doxorubicin is most obvious in the management of AIDS-related Kaposi's sarcoma, where augmented tumor site concentration of doxorubicin was measured *(Northfelt, 96)* and overall response rates were impressive *(Coukell, 97).* Higher response rates were achieved with pegylated liposomal doxorubicin (20 mg/m^2) than with the standard regimen containing doxorubicin, bleomycine and vincristine given systemically *(Northfelt, 98).* The drug formulation is generally well tolerated. In a minority of patients dose

limiting skin toxicity (hand-foot syndrome; palmar-plantar erythrodysaesthesia) occurred, which is characterized by skin eruptions on the palms and soles, with pain and inflammation Other, major, toxicities are myelosuppression, neutropenia in particular, nausea, vomiting and alopecia *(Alberts, 97).*

These studies led to the approval of doxorubicin (Doxil®, Caelyx®) for clinical use. Several hundreds of patients with HIV-related refractory Kaposi sarcoma have been treated up to now. At present multiple phase I/II and III studies are being performed for a variety of tumors, like (metastatic or recurrent) breast cancer, refractory ovarian cancer and prostate cancer as well as non-small cell lung cancer and soft tissue sarcomas *(Lasic 97, Sharma, 97, Muggia, 97).*

Application of TNF-α in liposomes

Typically, cytokines exhibit short plasma half-lives, biodistribution to non-relevant tissues, and high toxicity, which hampers clinical applicability. Delivery of cytokines via liposomes may extend the therapeutic index of the encapsulated agent. Indeed, improvement of pharmacokinetics and biologic ability of a wide spectrum of cytokines after encapsulation in liposomes has been recently reviewed *(Kedar, 97b).*

To address the main adverse effect of Tumor Necrosis Factor-α, being its severe toxicity, liposomal encapsulation was proposed. Preparation of TNF-α in conventional, multilamellar liposomes (MLV) has been described by several authors *(Debs, 89, 90, Düzgünes, 89, Nii, 91, Utsumi, 91, Weber, 94, Lodato, 95, Kedar, 97).*

Encapsulated TNF–α retained immunomodulatory and anti-tumor activity in vivo, at the same time reducing toxicity compared to free TNF-α in rats *(Debs, 90).* For alteration of the biological activity of TNF-α, stable association on or within the liposomal membrane appears mandatory. This was further proven by the fact that liposomal TNF-α retains bioactivity in vitro in the presence of anti-TNF-α antibodies *(Debs, 89).* Sensitive target cells, like the human melanoma A375 and the murine fibroblasts L929, were effectively lysed by TNF-α entrapped in liposomes *(Nii, 91).* Others found that liposomes loaded with TNF–α had a growth inhibitory effect on human glioma cells, a cell line which is resistant to free TNF-α *(Weber, 94).*

It was soon recognized that encapsulation of TNF-α in liposomes was hampered by the low hydrophobicity of the cytokine. The preparation of a more lipophilic variety of the native TNF-α was therefore attempted. Incorporation of TNF-α coupled to long chain fatty acids proved to be successful *(Utsumi, 91, Klostergaard, 92)* in small unilamellar vesicles. The biological activity however, was negatively influenced by modification of the

amino residues in TNF-α, thought to be essential for its cytolytic effect. Nevertheless, the binding efficiency was markedly improved (>50%), and a possible usage was foreseen in treating tumors.
Encapsulation of TNF–α in liposomes was also enhanced by varying the calcium concentration in the membrane *(Saito, 95)*. Supposedly, negatively influencing the stability of the phospholipid membrane played a role in the process.

Sterically stabilized liposomes

It is likely that incorporation of certain biologic response modifiers in sterically stabilized liposomes opens possibilities for a wider range of anti-tumor therapy. The prolonged circulation time, extravasation and accumulation in tumor tissue and decreased toxicity of newer generation liposomes caused attention to become focused on encapsulation of cytokines in these long circulating liposomes. Especially the problem of disseminated disease seems to be rendered manageable.

Incorporation of IL-2 in sterically stabilized liposomes (SSL) enhanced immunomodulatory activity *(Kedar, 94a)*, and SSL-IL-2 were demonstrated to have superior therapeutic efficacy in mice with advanced metastatic tumors, either alone or in combination with cyclophosphamide pretreatment *(Kedar, 94b)*.

Our laboratory demonstrated that Tumor Necrosis Factor–α could be incorporated in sterically stabilized liposomes of 95 nm diameter with an encapsulation efficiency of 24%, meaning that 15 μg TNF-α is associated with 60 μmol lipid *(van der Veen, 98)*. No significant loss of activity was measured using the TNF-α sensitive cell-line WEHI 164.

These liposomes were therefore used to determine biodistribution and tumor localization in a soft tissue sarcoma bearing rat model. Earlier, we have shown that the blood circulation of TNF-α encapsulated in sterically stabilized liposomes (TNF-α-SSL) was comparable to the circulation times mentioned in the literature for other long circulating liposome formulations *(Gabizon, 94, Harashima, 96)*. With incorporation in the aqueous compartment of ^{67}Gallium-desferal, a mean residence time in blood of approximately 33 hours was demonstrated. TNF-α encapsulated in liposomes had a shorter residence half-life, probably caused by in vivo release of TNF-α from the liposomes *(Ten Hagen, 97)*. Initial release of entrapped drugs from the circulating liposomes is more rapid than the aqueous labels, producing a faster rate of clearance, followed by a slower release from the liposomes making the liposome clearance rate dominate at the latter time *(Woodle, 93)*. This phenomena is also known for IL-2 in sterically stabilized liposomes *(Kedar, 94a)*. Still, the possible bioavailability of TNF-α was increased considerably. The liposomes used showed a localization in a soft tissue sarcoma in the leg, a tumor model

also used in isolated limb perfusion studies, of almost 8% of the injected dose, 24 hours after injection. The localization of TNF-α in tumor was increased by 13-fold, but also uptake by the MPS appeared prolonged *(van der Veen, 98)*.

Comparable to the results shown with TNF-α entrapped in conventional liposomes, toxicity with TNF-α sterically stabilized liposomes was markedly reduced. No loss in body weight and no alteration in blood cell count was recorded.

Subsequently, in soft tissue sarcoma bearing rats we tested the anti-tumor efficacy of different combinations of TNF-α-SSL and doxorubicin encapsulated in Stealth® liposomes (Doxil®, kindly provided by Dr. Working, SEQUUS Pharmaceuticals, Menlo Park, CA, USA). With the combination of TNF-α-SSL (15μg/kg per injection) and Doxil® (1st dose 4.5 mg/kg, followed by 4 dosages of 1 mg/kg), injected five times over a 20 day period, we observed a tumor response which is improved compared with Doxil® alone *(van der Veen, 96)*. All the tumors showed at least a stable disease for a prolonged period of time. In a number of cases the tumor, but also the overlying skin, became necrotic, as is also observed in the isolated perfusion setting using TNF-α and melphalan. In histopathologic studies this region of necrosis was marked, albeit not a 100%. A viable rim was seen around the necrotic region *(Ten Hagen, 97)*.

Low dose TNF-α in combination with liposomal anti-tumor formulations

From the studies with liposomal encapsulated TNF-α it seems probable that to improve an anti-tumor response by augmenting homing of drug formulations only low local concentrations of TNF-α are needed. Although in vivo activity of TNF-α is preserved after encapsulation in liposomes, we therefore explored the possibility of combining low dose TNF-α, systemically injected in combination with Doxil® *(Ten Hagen, in press)*. In two solid tumor models (a soft tissue sarcoma and an osteosarcoma) addition of low dose (15 μg/kg) TNF-α to Doxil® (same regimen as above) resulted in an increased anti-tumor response, potentiating the activity of Doxil® alone. Whereas administration of doxorubicin liposomes alone had only a marginal effect, co-administration of TNF-α resulted in a strong tumor response and long duration of tumor control. It appeared that addition of TNF-α enhanced accumulation of doxorubicin in the tumor interstitium after repeated injections of the liposomal drug in combination with TNF-α. Similarly, augmented accumulation of less well defined liposomes by TNF-α has been shown before, however with minimal effect on tumor response *(Suzuki, 90)*. Drug levels in the tumor interstitium appeared to be crucial as intracellular levels of free or liposome encapsulated doxorubicin were not increased.

5. CONCLUSIONS

After the initial disappointing results of systemic application of Tumor Necrosis Factor-α in clinical trials, the demonstration of anti-tumor efficacy of TNF-α and melphalan in isolated perfusion of the extremities caused a revival in enthusiasm. Strong and consistent anti-tumor effects have been reported for TNF-α in animal and human studies. This led to the registration of TNF-α as an anticancer drug in Europe in the setting of isolated limb perfusion in combination with melphalan for advanced extremity sarcomas. The primary target for TNF-α seems to be the disorganized tumor vasculature, and especially the vascular endothelium. The interaction with cytostatic agents has been proven to be of paramount importance in the treatment of cancer.

New strategies have been sought to extend the therapeutic use of TNF-α. Pegylation of TNF-α to extend its circulation half-life, muteins of the parental cytokine to diminish its toxic side effects and increase the therapeutic index proved to be of value. Other regional treatment protocols with TNF-α, like organ perfusions and stop-flow infusion techniques are currently under investigation. These developments in the application of TNF-α have led to new perspectives in systemic application, namely encapsulation of TNF-α in sterically stabilized liposomes or treatment with low-dose TNF-α in combination with (liposomal) formulated drugs, in order to improve homing, resulting in increased tumor respons.

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Chapter 8

Summary and Conclusions

SUMMARY

Effective treatment of solid tumors is complicated by factors due to the nature of the tumor and by possible side effects which accompany treatment. The penetration of solid tumors and the heterogeneity with which this takes place is very much influenced by the vascularization of the tumor. It is known that in well vascularized tissue higher drug levels can be reached than in poorly perfused regions. Furthermore, in tumors the interstitial pressure tends to be higher, which may act as an extra barrier to penetration of a suitable drug and especially of formulated drugs, such as liposomes.

Solid tumors however also exhibit characteristics which favor the passage of drugs to and in the tumor tissue. Especially, the tumor associated vasculature (TAV) has characteristics differing from normal vasculature. These vessels are characterized by an inherent high permeability caused by incomplete endothelial linings and basement membranes, and the vasculature is not well organized. Blood flow through the neovasculature is irregular and sluggish. In addition, tumor stroma is characterized by absence of lymphatics. Drug delivery to solid tumor therefore is influenced by the microvasculature and tumor stroma.

The majority of Tumor Necrosis Factor-α (TNF-α) anti-tumor effects are believed to be indirectly mediated through damage of the tumor associated vasculature (TAV). It has been shown to have vasculotoxic effects at high concentrations, while at low concentrations it promotes DNA synthesis and angiogenesis. Systemic administration of TNF-α however resulted in serious side effects, hypotension being the dose-limiting toxicity. The maximal tolerated dose was reported to be 200 – 400 $\mu g/m^2$, which is only 1/50th of the doses that causes tumor regression in animal tumor models. Locoregional administration seemed the only option for successful clinical application of TNF-α. TNF-α-based isolated extremity perfusion (ILP) in combination with cytotoxic agents revived the clinical use of the cytokine. It was proposed that the application of TNF-α in isolated perfusion of organs would also be possible. Furthermore, an experimental program was started to make systemic administration of TNF-α again possible, avoiding the severe toxic side effects.

Chapter one describes the previous work with TNF-α and the applicability of isolation perfusion. Furthermore previous work with liposomes is described. The aim of the present study was the development of isolated organ perfusion models and the development of protocols to resurrect the systemic administration of TNF-α, thereby exploring possibilities to treat disseminated disease.

In *chapter two* we describe the development of an isolated organ perfusion model. For technical reasons the kidney was chosen. At first, the

technique of isolated kidney perfusion (IKP) is described, subsequently the feasibility of a perfusion with TNF-α is studied and the anti-tumor effects of IKP with TNF-α and melphalan is assessed. The maximal tolerated dose (MTD) of TNF-α proved to be 1 μg. Higher doses induced renal failure and fatal respiratory disease, a septic shock like syndrome allegedly caused by a secondary cytokine release. In vitro and in vivo experiments with TNF-α and melphalan failed to show the synergistic anti tumor response so characteristically for the combination in isolated limb perfusion (ILP). Apparently, the minimal threshold concentration of TNF-α to exert its anti-tumor effects was not reached, therefore the outlook for clinical applicability of isolated kidney perfusion with TNF-α seems low.

In *chapter three* the combination of high dose TNF-α and doxorubicin in ILP was explored to extend the preclinical-clinical anti-tumor program currently performed in Rotterdam with TNF-α and melphalan. Doxorubicin is one of the more potent drugs in the treatment of soft tissue sarcoma and therefore it was warranted that the efficacy of doxorubicin and TNF-α was explored in the preclinical model of ILP in two different rat sarcoma tumor models. It is shown that the anti-tumor effects of the combination are comparable to the synergistic response of TNF-α and melphalan in earlier studies. The addition of high dose TNF-α to doxorubicin results in regression of the tumor in 54% of the BN175-fibrosarcomas and in 100% of the ROS-1 osteosarcomas. The combination is shown to be mandatory for optimal tumor response. Secondly, it was demonstrated that TNF-α enhances intratumoral accumulation of doxorubicin: 3.1-fold in the BN175 and 1.8-fold in the ROS-1 sarcoma after ILP with doxorubicin combined with TNF-α in comparison with an ILP with doxorubicin alone. *In vitro* however, TNF-α fails to augment drug uptake in tumor cells or to increase cytotoxicity of the drug. Enhancement of intratumoral drug concentration is explained by increasing the leakiness of the tumor associated vasculature as well as a reduction in interstitial pressure by TNF-α. It is suggested from this study that TNF-α does not directly modulates the activity of doxorubicin *in vivo*. As TNF-α by itself has no or only minimal effect on tumor growth, an increase in local concentrations of chemotherapeutic drugs might well be the main mechanism for the synergistic anti-tumor effects.

In *chapter four* we explored whether TNF-α could play a regulatory role in the cell cycle. Doxorubicin is a well known cell cycle dependent drug, and TNF-α is claimed to have inhibitory effects on doxorubicin. This is not in agreement with our findings in ILP, where a clear synergism between the two drugs was proven *in vivo*. Cells were tested *in vitro* where no attenuating effect of TNF-α on doxorubicin cytotoxicity could be demonstrated in either of the

three cell lines tested. Also, preincubation of the cells with TNF-α, prior to incubation with doxorubicin or melphalan, did not induce a significant effect on cytotoxicity. To evaluate whether TNF-α has indeed effect on cell cycle kinetics, flow cytometry was used to determine the DNA content of nuclei isolated from cells exposed to TNF-α. It was demonstrated that TNF-α slightly increases the amount of cells in G_2 phase only in the osteosarcoma cell line, however this did not result in an attenuation of the cytotoxicity of doxorubicin towards these cells. In the other tumor cell lines tested no or marginal effect on cell cycle progression was found.

In *chapter five* the applicability of TNF-α in long circulating liposomes was explored. Liposomal encapsulation has several advantages: prolonged half-life time of encapsulated drugs, reduced toxicity, smaller volume of distribution and the possibility of reaching high concentrations at specific sites, more in particular in tumor tissue. Newer generation liposomes, sterically stabilized or Stealth® liposomes, tend to have longer circulation times in circulation and are therefore of special interest in anti-tumor therapy. Long circulating liposomes can be manufactured by various methods, however different methods can have more or less detrimental effects on the bioavailability of TNF-α. With these liposomes the blood residence half-life and organ distribution of TNF-α encapsulated in sterically stabilized liposomes were investigated in tumor-bearing rats. Pharmacokinetic profile of TNF-α was markedly different after encapsulation: 33-fold increase in blood residence time with a concomitant 14-fold increase in the area under the curve. Total uptake in tumor tissue of TNF-α after encapsulation also was enhanced, peak TNF-α concentration however, was not increased in tumor tissue as compared to free TNF-α. The toxicity of the product was diminished. We concluded that encapsulation of TNF-α was promising, the encapsulation efficacy however, was not as high as expected.

In *chapter six* the option of systemic administration of *low dose* TNF-α in combination with chemotherapeutics was explored. Again, the synergy between TNF-α and cytotoxic drugs found in isolated limb perfusions with *high dose* TNF-α can be explained by the enhanced accumulation of the cytotoxic drug in the tumor. It was therefore interesting to examine the effect of systemic administration of *low dose* TNF-α on the distribution of cytotoxic drugs. Doxorubicin however, has also toxic side effects, mainly on cardiac muscle. The combination of systemic low dose TNF-α and liposome encapsulated doxorubicin (Doxil®) seemed especially attractive. We demonstrated that not only TNF-α enhances the anti-tumor activity of liposomal doxorubicin, also three or more injections of Doxil® combined with TNF-α results in augmented accumulation of the cytotoxic drug in tumor tissue when compared to either the free drug or Doxil® alone. Therefore, the ongoing

accumulation of doxorubicin at the site of the tumor after co-administration of low dose TNF-α results in a synergistic response probably due to a permissive effect of TNF-α on the neo vascularization of the tumor. This effect is translated in positive anti-tumor responses after multiple administrations of Doxil® and low dose Tumor Necrosis Factor-α. Moreover, this enhanced tumor targeting of liposomal drug after systemic low dose TNF-α may also be seen with other liposomal formulations.

In *chapter 7* the above mentioned results are discussed in the light of the present knowledge about the applicability of TNF-α. With the registration of TNF-α in Europe for combination therapy of advanced extremity sarcoma in isolation limb perfusion, further exploration of the usefulness of TNF–α in combination with cytotoxic drug for the treatment of solid tumors is warranted.

CONCLUSIONS

Application of *high dose* TNF-α in anti-tumor therapy in conjunction with cytotoxic drugs results in high response rates in isolated limb perfusion. This led to the approval and registration of Tumor Necrosis Factor-α in Europe (Beromun®) for clinical use in patients with locally advanced extremity sarcomas treated by ILP with TNF-α and melphalan.
In this thesis other modalities to achieve the same impressive results are explored. We gained insight in the mechanism by which TNF-α exerts its effects and by means of a revival of systemic application of *low dose* TNF-α in combination with chemotherapeutics, paved the path for treatment of multiple forms of cancer as well as metastatic disease. More research is needed to extend the positive results of pre-clinical experiments with low dose TNF-α to the clinic, where successful anti-tumor therapy and duration of remission is all the more important. Conclusions made on the basis of the studies are:

1. IKP model: technically feasible, but not a suitable model to test efficacy of TNF-α due to high toxicity.
2. Synergistic anti-tumor response in *high dose* TNF-based isolated limb perfusion with doxorubicin in vivo.
3. Enhanced levels of doxorubicin in tumor tissue in *high dose* TNF-based ILP.
4. TNF-α has no measurable effect on cell cycle.
5. TNF-α can be encapsulated in long circulating liposomes with acceptable efficiency.

6. Pharmacokinetic profile of TNF-α is positively influenced after encapsulation.
7. Systemic application of *low dose* TNF-α in combination with liposome encapsulated Doxorubicin (Doxil®) results in enhanced accumulation of the cytotoxic drug in tumor tissue and results in long lasting anti-tumor effects.

Chapter 8

Samenvatting en Conclusies

SAMENVATTING

Effectieve behandeling van solide tumoren wordt bemoeilijkt door de eigenschappen van de tumor en bijwerkingen, welke de behandeling kunnen compliceren. De penetratie van geneesmiddelen in tumorweefsel en de heterogeniteit waarmee dit samengaat worden voor een belangrijk deel bepaald door de vascularisatie van de tumor. Het is bekend dat in goed gevasculariseerd weefsel een hogere spiegel van cytostatische geneesmiddelen wordt gevonden, dan in slecht geperfundeerde gebieden. Bovendien is de interstiële druk in het tumorweefsel hoger, wat een extra barrière vormt in de penetratie van geneesmiddelen of carriers hiervan, zoals bijvoorbeeld liposomen.

Solide tumoren hebben echter ook kenmerken die de passage van geneesmiddelen naar en in het tumorweefsel vergemakkelijken. Met name wijken de eigenschappen van de in de tumor aanwezige vaten af van normaal. De capillairen in het tumorweefsel worden gekarakteriseerd door een hoge permeabiliteitsfactor, veroorzaakt door de onderbroken endotheelcellaag van de tumorvaten en een afwezige basaalmembraan. Bovendien is de organisatie van het tumorvaatbed wanordelijk. De bloedstroom in het tumorvaatbed is traag en irregulair. Het tumorweefsel wordt voorts gekenmerkt door de afwezigheid van lymfebanen. De depositie van geneesmiddelen wordt derhalve voor een belangrijk deel bepaald door de microvasculatuur van de tumor en de eigenschappen van het tumorweefsel.

De anti-tumoreffecten van Tumor Necrose Factor-α worden voor een belangrijk deel veroorzaakt door directe beschadiging van het tumorvaatbed. Het is aangetoond dat TNF-α een vasculotoxische werking heeft indien toegediend in hoge doseringen, doch in lage doseringen DNA synthese en angiogenese kan bevorderen.

Systemische toediening van TNF-α bij patiënten resulteerde in ernstige bijwerkingen, waarbij met name het optreden van hypotensie de dosering beperkte. De maximaal getolereerde dosis (MTD) was 200 – 400 $\mu g/m^2$, wat maar $1/50^e$ is van de dosis, die tumor regressie veroorzaakt in diermodellen. Locoregionale toepassing van TNF-α leek de enige optie voor klinische toepassing. Door de veel effectievere combinatie van TNF-α met cytostatica onstond een hernieuwde klinische belangstelling voor de toepassing van geïsoleerde extremiteitsperfusie. Gezien het succes hiervan bij patiënten met melanoom en sarcoom, werd gedacht dat de toepassing van TNF-α en cytostatica ook mogelijk moest zijn in de vorm van geïsoleerde perfusie van organen. Daarnaast werd een experimenteel programma opgestart om na te gaan of zelfs systemische toepassing van TNF-α nu mogelijk was zonder ernstige toxische bijwerkingen.

Hoofdstuk 1 beschrijft eerder onderzoek met TNF-α en de toepasbaarheid in geïsoleerde perfusie. Bovendien zijn de resultaten met

liposomen als carrier vermeld. Het doel van de in dit proefschrift bewerkte studies wordt beschreven.

In *hoofdstuk 2* beschrijven we de ontwikkeling van een model voor orgaanperfusie. Hiervoor werd de nier gekozen. De techniek van geïsoleerde nierperfusie (IKP) met TNF-α alléén en in combinatie met melphalan wordt beschreven. De anti-tumoreffecten van deze combinatie werden onderzocht. De maximaal getolereerde dosis (MTD) van TNF-α in deze setting bleek maar 1 μg te zijn. Hogere doses veroorzaakten nierfalen en fatale respiratoire complicaties, zoals voorkomend bij septische shock, mogelijk veroorzaakt door in overmaat circulerende cytokines. Mede hierdoor kon het synergisme tussen TNF-α en melphalan, wat bij geïsoleerde extremiteitsperfusie wordt gevonden, bij geïsoleerde nierperfusie niet worden aangetoond. Door de slechte tolerantie werd de drempel voor het bereiken van anti-tumoreffecten middels TNF-α niet bereikt. Klinische toepassing van geïsoleerde nierperfusie van de combinatie TNF-α en melphalan lijkt dan ook nog niet tot de mogelijkheden te behoren.

Hoofdstuk 3. In het kader van het preklinisch-klinisch anti-tumor programma in Rotterdam betreffende de toepassing van TNF-α werd de combinatie van TNF-α en doxorubicine onderzocht. De resultaten van geïsoleerde extremiteitsperfusie met doxorubicine en TNF-α worden beschreven. Doxorubicine is werkzaam gebleken bij de behandeling van het weke delen sarcoom en het was daarom nuttig om de effectiviteit van de combinatie in twee verschillende sarcoma modellen in de rat te onderzoeken. Een zelfde synergistische werking werd gevonden als bij de toepassing van melphalan. Het fibrosarcoom BN 175 liet een tumorrespons zien van 54%, het osteosarcoom ROS-1 zelfs van 100%. Aangetoond werd dat voor een optimale tumorrespons TNF en doxorubicine inderdaad gecombineerd moeten worden. Ten eerste werd aangetoond dat TNF-α de concentratie van doxorubicine in het tumorweefsel verhoogde, 3,1-voudig bij het weke delen sarcoom en 1,8-voudig bij het osteosarcoom. *In vitro* echter werd geen toename van de concentratie doxorubicine in de tumorcellen zelf gevonden, evenmin werd de cytotoxische werking van doxorubicine gestimuleerd. De toename van de intratumorale concentratie van doxorubicine werd verklaard door een toename van het capillaire lek in de tumorvaten, alsmede door een verlaging van de interstiële druk in het tumorweefsel door TNF-α. Het is waarschijnlijk dat TNF-α *in vivo* de anti-tumoractiviteit van doxorubicine indirect beïnvloedt. Omdat TNF-α géén of maar een minimaal effect heeft op tumorgroei, is de toename van de concentratie van cytostatica in de tumor door TNF-α waarschijnlijk het belangrijkste mechanisme dat de synergistische anti-tumorrespons bewerkstelligt.

Hoofdstuk 4 behandelt de vraag of TNF-α een regulerende functie heeft in de celcyclus. De fase van de celcyclus speelt een rol in de cytotoxische werking van doxorubicine. TNF-α zou de voortgang van de celcyclus afremmen in de G_0/G_1 fase en zodoende de werking van doxorubicine verminderen. Onze bevindingen in geïsoleerde extremiteitsperfusie tonen synergisme aan tussen TNF-α en doxorubicine. In géén van de drie geteste cellijnen, werd een remmende werking van TNF-α op doxorubicine aangetoond. Ook voorbehandeling met TNF-α had geen significant effect op de cytotoxiciteit van melphalan of doxorubicine. Door middel van flowcytometrie werd de hoeveelheid DNA in de nucleï van aan TNF-α blootgestelde cellen bepaald. Er bleek een lichte toename te zijn van osteosarcoomcellen in de G_2 fase, hetgeen echter niet resulteerde in een afname van de cytotoxische werking van doxorubicine op deze cellen. In de andere geteste cellijnen werd géén of slechts een marginaal effect op de celcyclus gevonden.

In *hoofdstuk 5* wordt onderzoek beschreven naar systemische toepasbaarheid van TNF-α. Hiertoe werd TNF-α geïncorporeerd in liposomen. Liposomale inkapseling biedt een aantal voordelen: langere halfwaarde tijd van ingekapselde geneesmiddelen, afname van de toxiciteit, kleiner distributievolume en mogelijk het bereiken van hogere weefselspiegels in tumorweefsel. De nieuwe generatie Stealth® liposomen circuleren langer in de bloedbaan en zijn daarom interessant in het kader van anti-tumortherapie. Stealth® liposomen kunnen op verschillende manieren worden gemaakt, hetgeen hun biologische beschikbaarheid beïnvloedt. De halfwaardetijd in de circulatie alsmede de orgaandistributie, van in lang circulerende liposomen geïncorporeerd TNF-α, werden onderzocht in tumordragende ratten. Het farmacokinetische profiel van TNF-α veranderde evident: de verblijfsduur in de circulatie nam 33-voudig toe, gepaard gaande met een 14-voudige toename van de area onder the curve. Tumorweefsel toonde ook een hogere opname van TNF-α na inkapseling, hoewel de piekconcentratie van TNF-α in tumorweefsel in vergelijking met vrij TNF-α niet toenam. De toxiciteit van TNF-α was minimaal. Wij concludeerden dat inkapseling van TNF-α veelbelovend was, hoewel het percentage ingekapseld TNF-α niet zo hoog was als verwacht.

Hoofdstuk 6 beschrijft de systemische toepassing van *lage doses* TNF-α in combinatie met cytostatica. Zoals eerder gezegd wordt de synergie tussen *hoge doses* TNF-α en cytostatica in geïsoleerde extremiteitsperfusie verklaard door een toename in de concentratie van het cytostaticum in het tumorweefsel. Het effect van systemisch toegediende lage dosis TNF-α op de distributie van cytostatica was tot nu toe onduidelijk en daarom interessant om te onderzoeken. Een bijwerking van systemisch toegediend doxorubicine, in

werkzame doses, is cardiale toxiciteit. Daarom werd de combinatie van systemisch toegepast TNF-α in lage dosering en liposomaal ingekapseld doxorubicin (Doxil®) bestudeerd. Wij toonden aan dat TNF-α niet alleen de anti-tumoractiviteit van liposomaal doxorubicine verhoogde, maar dat, na drie of meer injecties van Doxil® en TNF-α, de concentratie van doxorubicine in het tumorweefsel toenam in vergelijking met hetzij de vrije vorm van doxorubicine als Doxil® alleen. De gevonden toename van doxorubicine in het tumorweefsel bij gelijktijdige toediening van TNF-α resulteert in een synergistische respons, waarschijnlijk veroorzaakt door de effecten van lage dosis TNF-α op het tumorvaatbed. Dit laat zich vertalen in een positief anti-tumoreffect na multipele toedieningen van Doxil® en lage doses TNF-α. Bovendien zou deze toegenomen tumor targeting ook kunnen gelden voor andere liposomaal ingekapselde medicijnen.

In *hoofdstuk 7* tenslotte worden de hierboven genoemde resultaten besproken in het licht van de huidige stand van zaken omtrent de toepassing van TNF-α. Met de registratie van TNF-α in Europa voor combinatietherapie van sarcomen in geïsoleerde extremiteitsperfusie, is verder onderzoek naar de bruikbaarheid van TNF-α in combinatie met chemotherapeutica in de behandeling van solide tumoren nuttig.

CONCLUSIES

Toepassing van hoge dosis TNF-α in anti-tumortherapie gecombineerd met cytostatica geeft een synergistische respons in geïsoleerde extremiteits-perfusie. Dit heeft geleid tot de registratie van Tumor Necrose Factor-α in Europa (Beromun®) voor toepassing in geïsoleerde extremiteitsperfusie in combinatie met melphalan bij patiënten met locaal irresectabele sarcomen.

In dit proefschrift worden andere modaliteiten onderzocht, die zouden kunnen leiden tot dezelfde indrukwekkende resultaten als bereikt in geïsoleerde perfusie van ledematen. Er werd inzicht verkregen in het werkingsmechanisme van TNF-α. Systemische toediening van *lage* doses TNF-α is mogelijk, waardoor in combinatie met al dan niet liposomaal ingekapselde cytostatica, verscheidene tumorsoorten en mogelijk ook metastasen behandelbaar worden. Verder onderzoek is echter noodzakelijk om de positieve resultaten van de toepassing van lage doses TNF-α in deze combinaties te vertalen naar de kliniek, waarin een hoge tumorespons en remissieduur van groot belang zijn.

Conclusies naar aanleiding van dit proefschrift:

1. Geïsoleerde nierperfusie is technisch mogelijk, doch gezien de hoge locale toxiciteit van TNF-α niet geschikt als tumormodel.
2. *Hoge* doses TNF-α in combinatie met doxorubicine in vivo geeft een synergistische anti-tumorrespons in geïsoleerde extremiteitsperfusie.
3. Bij toepassing van *hoge* doses TNF-α in geïsoleerde extremiteitsperfusie worden hogere concentraties doxorubicine gevonden in tumorweefsel.
4. Het effect van TNF-α op de celcyclus is verwaarloosbaar.
5. TNF-α kan met een acceptabele effectiviteit worden geïncorporeerd in lang circulerende liposomen.
6. Liposomale inkapseling van TNF-α leidt tot een verbeterd pharmacokinetisch profiel.
7. Systemische toepassing van *lage* doses TNF-α in combinatie met liposomaal doxorubicine (Doxil®) resulteert in een toegenomen accumulatie van het cytostaticum in tumorweefsel en veroorzaakt een langdurig anti-tumoreffect.

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CURRICULUM VITAE

Alexander van der Veen werd 21 juni 1959 geboren te Warnsveld. Na verscheidene omzwervingen werd Delft als domicilie gekozen, waar het ongedeeld VWO aan het Christelijk Lyceum Delft in 1977 werd afgesloten. Dankzij uitlotingen kon in Amsterdam studie-ervaring worden opgedaan aan de Vrije Universiteit. Nadat via de hardheidsclausule een opleidingsplaats was veroverd, werd in 1980 begonnen met de studie medicijnen aan de Erasmus Universiteit Rotterdam, waar in 1987 het artsexamen werd behaald. Tijdens zijn studie werd onderzoek gedaan in Gainesville, Florida op het laboratorium van Prof. Fulton Crews, en werd in Rotterdam onderzoek gedaan bij patiënten met een Barrett oesophagus onder begeleiding van de gastro-enterologen M. van Blankenstein en J. Dees.
In 1988 werd begonnen met het AGNIO-schap chirurgie in het Zuiderziekenhuis te Rotterdam, alwaar in januari 1990 ook de opleiding tot chirurg werd gestart (opleider: Dr. G.A.A. Olthuis, na diens pensionering: Dr. M.K.M. Salu en Dr. K.J. Brouwer). In januari 1993 werd de opleiding voortgezet in het Academisch Ziekenhuis Dijkzigt te Rotterdam (opleider: Prof. dr. H.A. Bruining). In maart 1994 werd de opleiding onderbroken voor een onderzoeksjaar voor arts-assistenten gefinancierd door het KWF/NKB, alwaar de basis voor dit proefschrift werd gelegd. In april en mei 1995 werkte hij in het Ikazia ziekenhuis (opleider Dr. H.F. Veen). Op 1 juli 1996 volgde inschrijving in het specialisten register als chirurg.
Van 1 juli 1996 tot 31 december 1998 was hij fellow traumatologie op de afdeling heelkunde van het Academisch Ziekenhuis Dijkzigt te Rotterdam. Sinds 1 januari 1999 werkt hij met veel plezier als maat in de maatschap chirurgie van het Catharina ziekenhuis te Eindhoven.
Schrijver dezes is getrouwd en heeft drie kinderen.