TNFα-BASED ISOLATED LIMB PERFUSION IN THE RAT

Development of a model and analysis of efficacy determining factors

GEISOLEERDE EXTREMITEITS PERFUSIE MET TNFα IN DE RAT

Ontwikkeling van een model en analyse van effectiviteits bepalende factoren

PROEFSCHRIFT

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To my parents

To Cornelieke

Contents

GENERAL INTRODUCTION AND AIM OF THE THESIS

1.1 Introduction

Isolated limb perfusion (ILP) with high dose TNFα in combination with IFNγ and melphalan in patients with melanoma in transit metastases confined to the limb has recently been reported to result in much higher complete tumor response rates than after the standard therapy of ILP with melphalan alone: 90 % *vs* 54 % complete remission[1,2]. Moreover the same protocol of ILP when applied as an induction bio-chemotherapy in patients with irresectable extremity soft tissue sarcomas, was reported to result in about 85 % response rates rendering most tumors resectable and resulting in a > 80% limb salvage rate[3,4]. The tumor response in many patients in both patient groups was characterized by an immediate (within 3 days) and grossly visible reaction to treatment, which shows a remarkable similarity to that observed in animal tumor models after systemic administration of TNFα.
ILP became the first setting, in which effective concentrations of TNFα could be reached and a reproducible antitumor effect could be measured. In patients i.v. administration of TNFα is limited to much lower doses than the effective doses in mice, since TNFα causes severe hypotension in man and is known to play a key role as a mediator in septic shock. Pathophysiologically, TNFα is a paracrine (and autocrine) mediator that is released at the inflammation site, with severe hypotensive effects when it is released systemically. Therefore the trials of systemic administration of TNFα, either alone or in combination with other cytokines or chemotherapy had marginal results.

1.2 Isolated limb perfusion

Creech et al. pioneered the Isolated Limb Perfusion (ILP) procedure in 1957 in the treatment of in transit metastasized melanoma[5]. In ILP regional cytostatic concentrations can be reached which are 15-20 times higher than those after systemic administration of the maximal tolerated dose (MTD)[6]. In this procedure the vascular circulation of a limb is isolated from the rest of the body by clamping the major artery and vein and tightening a tourniquet around the root of the limb. The major artery and vein are connected with a heart-lung machine and the drug is administered in the extra-corporeal circuit. At the end of the procedure the drug is washed out by an electrolyte solution to remove the drug from the vascular space and

tissues. ILP with melphalan is performed under hyper-thermic conditions, associated with a higher tumor response as compared to normo-thermia. Heating of the limb should be well controlled in the mild hyperthermia range of 39-40 C because true hyperthermia (> 41,5 C) is related to serious regional toxicity[7]. Continuous monitoring of leakage is very important to guaranty safety of ILP. Therefore a probe is placed in the pre-cordial region, which detects radio-labeled albumin, injected in the extra-corporeal circuit.

ILP with melphalan, the established mode of therapy for melanoma in-transit metastases shows a complete remission rate of about 50 % [1,2]. In the treatment of extremity soft tissue sarcoma (STS) rather poor results are obtained with ILP containing melphalan[8-12] (<20% CR+ PRs) or doxurubicin and other drugs[13-15]. It is clear that the lack of efficacy validates further investigations to improve ILP therapy.

1.3 TNFα

1.3.1 History of TNFα

At the end of last century William B. Coley was struck by the phenomenon of regression of advanced cancers in patients suffering from severe infections. To imitate this situation, Coley developed toxin's composed of a mixture of gram-negative and gram-positive bacteria[16], which led to tumor regressions in some of his patients. This therapy became not accepted as a standard therapy, since not surprisingly, severe complications occurred. Shear continued Coley's concept, not in patients but in an animal tumor model, in which he reported induction of hemorrhage in murine sarcoma after administration of a factor, derived from gram-negative organisms[17,18] and later reported that the antitumor response was evoked by an endogenous host factor[19]. In 1975 Carswell isolated an endogenous factor from sera of BCG-primed, endotoxin treated mice with tumor necrotizing potential and named this factor therefore "tumor necrosis factor". In 1984 TNFα became available on a large scale by recombinant techniques[20], which opened the way to investigate the antitumor effects of TNFα not only *in vitro* but also *in vivo*. The expectations of TNFα as therapeutic agent were very high, since recombinant TNFα was quite effective in various animal models and appeared to exert its antitumor effect on a different mechanism as conventional chemotherapeutic

agents.

Results of phase I/II trials, however were very disappointing. An overall response rate of 1-2 % was obtained[21-26]. The lack of antitumor effect appeared to be due to the fact that TNFα is highly toxic in man. The maximal tolerable dose (MTD) of TNFα in patients is about 1/50 of the effective dose in mice models (human tumors xenografted in nude mice). TNFα is a mediator in septic shock and causes apart from an acute drop of the vascular resistance and concurrent drop in blood pressure symptoms such as rigors, fever and fatigue[27-30]. Although febrile reactions could be reduced by paracetamol or indomethacin, at higher doses hypotension was the dose limiting factor.

TNFα, first known to be a factor with tumor necrotizing ability, later appeared to be an important cytokine which plays a central role in inflammation. The molecule is mainly produced by activated macrophages[31-32] and can activate immunocompetent cells and endothelial cells to induce an inflammatory infiltrate.

1.3.2 *In vitro* effects of TNFα

Early studies suggested that TNFα has specific cytostatic or cytotoxic effects on transformed cell lines[33,34]. The direct cytotoxic effects were therefore extensively studied on various cancer cell lines[35-38]. During these studies a lack of correlation between the number of TNFα-receptors and TNFα cytotoxicity was noticed[39-40]. Effects of TNFα on tumor cell lines can be growth-stimulating, cytostatic or cytocidal, which indicates that a number of events along the pathways of signal transduction are not well understood. The poor correlation between *in vitro* and *in vivo* observations also emphasize that direct cytotoxicity of TNFα may be of less relevance in the antitumor effect of TNFα *in vivo* and that other effects and interactions may be crucial:

1. Several experiments revealed a lack of correlation between the *in vitro* sensitivity and the *in vivo* sensitivity using the same tumor[42-44];

2. There exists a lag period of about 10-72 hours before any cytotoxicity can be noted *in vitro*[45], whereas morphological changes in a tumor can generally be observed at 4 hours after

administration;

3. Involvement of host related factors as the vasculature of the tumor and immune cells have been reported to play an important role *in vivo*.

1.3.3 *In vivo* effects of TNFα

TNFα antitumor effects may be mediated by TNFα effects on endothelial cells and hence on tumor vasculature. TNFα has dual activity on endothelial cells (ECs): In low concentrations TNFα stimulates proliferation of ECs and is a strong inducer of angiogenesis *in vivo*[46]. But, in higher concentrations TNFα may have destructive effects on micro-vasculature. Tumor associated vasculature shows an enhanced sensitivity for these effects of TNFα as compared to normal vessels, probably explained by a different micro-milieu of tumor and continuous growth stimulation of tumor associated vessels by angiogenetic factors. TNFα may increase permeability of the endothelial cell layer leading to edema and eventually hemorrhage and TNFα enhances pro-coagulant activity of EC resulting in the formation of micro-thrombi. In some tumor models the formation of fibrin -deposits has been described as an important event in the TNFα-mediated tumor response[47].

In murine tumor models TNFα response is characterized by immediate hemorrhagic necrosis. A substantial reduction of tumor blood flow within 24 h leads to massive necrosis of tumor cells. Darkening of skin at the tumor site and weakening or liquefaction of tumor is typical for TNFα treatment and is generally not found after conventional chemotherapy. Hemorrhagic necrosis has been reported at 4-6 h after TNFα treatment in experimental tumors, based on histological findings[42,48-51]. In preclinical models 1-2 h after TNFα treatment a considerable reduction of tumor blood flow was observed[52-54], as demonstrated by isotope washout and laser Doppler techniques[55,56]. In 24 h after TNFα treatment, metabolic and oxygenation state deteriorated in cells of solid tumors[52,56,57].

The importance of vascular injury within tumor after TNFα therapy may be confirmed by the fact that TNFα is more effective in larger tumors, in contrast with other biological response modifiers and chemotherapy, which are most effective in small tumors. TNFα antitumor activity depends on a developed capillary bed, which is a need for larger tumors[42,43,50,58-60]. In immunogenic tumors, an alternative explanation for critical tumor size

for TNFα/endotoxin effect could be production of an adequate amount of antigens[61].
Except from the aforementioned effects of TNFα on tumor associated vessels also immune mediated effects may cause tumor regression. As a broad pro-inflammatory cytokine it is easy to understand that TNFα anti tumor activity correlates with tumor immunogenicity. The more immune competent cells are evoked by the immunogenicity of tumor, the more anti tumor activity of TNFα may be mediated by immune competent cells. Expression of adhesion molecules on endothelial cells and white blood cells plays an important role in the initiation and stimulation of the inflammatory response against the tumor, since TNFα is strongly inducing or up-regulating the expression of several adhesion molecules[62]. In highly immunogenic tumor models in rodents generally complete regression was found after TNFα administration, while in less immunogenic tumor models only partial regression was observed[63]. Also in models of immune suppressed mice, the antitumor effect of TNFα on immunogenic tumors was decreased to only a partial response[63]. Histologically, this partial response exists of hemorrhagic necrosis in the tumor center with an outer ring of tumor cells remaining viable, while in immunogenic tumors in normal mice, this outer ring seems to be destructed by the presence of surrounding immune competent cells[63]. Since human soft tissue sarcoma is in most cases nonimmunogenic and melanoma only weakly immunogenic, the most realistic tumor models in rodents are nonimmunogenic or human xenograft in nude mice models.

1.3.4 Regional TNFα administration to avoid systemic toxicity

From animal data[65] and clinical reports[1-4] on ILP with TNFα, we may conclude that high local concentrations of TNFα, even after a single and short exposure time may result in immediate and massive tumor necrosis, due to specific alterations in tumor microvasculature. Apparently, there exist a specific sensitivity of endothelium of tumor vasculature to TNFα. From this perspective it is reasonable to design protocols of loco-regional administration.

Intratumoral administration
Poor results have been reported after intratumoral injection of TNFα in palpable tumors, lymph nodes or intrahepatic tumors, leading to approximately 15 % partial remissions (PRs)

and 20 % minimal responses (MRs)[66,67] and for hepatic metastases, 53 % stable disease (SD)[68]. Better results, 19 % CR, 35 % PR and 35 % MR, were achieved with i.t. TNFα administration in Kaposi sarcoma[69]. Nevertheless, these trials have never led to a standard therapy, since TNFα is extremely toxic and leakage of TNFα from tumor after i.t. administration leads to maximally tolerated doses (MTD) in the same range or slightly higher than MTD of i.v. administered TNFα. Intratumoral injections do not differ significantly from subcutaneous or systemical administration in their toxicity profile and have been abandoned.

Intracavitary administration

Intraperitoneal administration of TNFα in gynaecological cancer did not result in any tumor regression but unexpectedly resolution of ascites was observed[70,71] Since intraperitoneal administration of TNFα has been reported to stimulate micro-metastatic implantation in the peritoneum in an i.p. human xenograft models[72], this mode of TNFα administration risks enhancement of the cancer process.

Intraarterial infusion

Increase of therapeutic index may be obtained by intraarterial administration of TNFα, which should be based on "the first-pass effect". In hepatic arterial infusion (HA) another improvement of the therapeutic index may be theoretically expected since the hepatic artery is the main source of blood supply to liver metastases[73]. In HA of TNFα a maximal tolerable daily dose of six times the systemically administered maximal tolerable daily dose could be achieved. However, this MTD enhancement is not big enough to achieve effective doses (still approximately 1/10 of the effective dose). Not surprisingly, this approach led to moderate results: 14 % PRs and 21 % MRs[74], resembling results with intratumoral administration of TNFα. Similarly, moderate results were obtained in patients with malignant glioma in brain, treated with repeated intraarterial administration of TNFα[75].

Isolated limb perfusion

As discussed previously the crucial elements of the ILP system are: (1) a leakage free perfusion can be performed and thus very high doses of TNFα can be used safely. This means not only that very high local concentrations can be achieved but also that at the end of the procedure an effective washout procedure can be performed, thus truly preventing

systemic exposure to TNFα. At this point in time it seems to be the only methodology which allows for the effective use of TNFα in the clinical setting.

1.4 The aim of the thesis

The antitumor effect of TNFα is well documented in rodent tumor models. For the first time the high antitumor efficacy was consistently translated in cancer patients by its application in the ILP setting in combination with melphalan and IFNγ. As the standard cytostatic drug used in ILP for the treatment of patients with in transit metastasized melanoma, melphalan was used. IFNγ was applied, because of its reported *in vitro* and *in vivo* synergy with TNFα. Since this triple therapy was highly effective in patients with melanoma and sarcoma the contribution of the individual agents has not been further assessed. To mimic the clinical situation, an ILP model in a rodent tumor model should be developed to investigate the prerequisites for efficacy as well as the mechanisms by which the antitumor effects are achieved.

The aim of this thesis is to investigate:

1) whether realistic rat extremity tumor models can be developed, which mimics the clinical ILP setting

2) which factors determine the efficacy of TNFα in ILP

3) whether there exists synergy between TNFα, melphalan or IFNγ in ILP

4) which sequence of events as determined by histopathological studies is brought about by TNFα and melphalan that underlie the antitumor effects.

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CHAPTER 2

DEVELOPMENT OF THE ILP MODEL IN THE RAT

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Ischemia promotes the antitumor effect of tumor necrosis factor alpha (TNFα) in isolated limb perfusion in the rat
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Introduction

Tumor necrosis factor α (TNFα) is a broad spectrum pro-inflammatory cytokine, a major mediator of septic shock and is found to be able to induce tumor regression in animal studies comparable to the best chemotherapeutic agents[3]. However, its clinical use is limited by severe side effects and therefore only disappointing results have been reported in phase I/II trials[8,22]. The problem of systemic toxicity can be circumvented to a minor degree by local (intratumoral) administration of TNFα but significantly in the setting of isolated organ or limb perfusion (ILP), allowing for a 10 fold increase in dose. Treatment of stage III melanoma and irresectable soft tissue sarcoma with TNFα and INFγ in combination with melphalan has revealed an extraordinary antitumor effectivity[15,7,10]. This tumor response is associated with the characteristic TNFα antitumor features as observed in murine models: acute softening with hemorrhagic necrosis of the tumor and occlusion of tumor associated vessels.

These clinical findings together with data of studies in mice[12,16] indicate that tumor vasculature is a primary target of TNFα. Hyperemia, coagulation and endothelial damage have been, reported[9,29,18] and have been suggested to be responsible for vascular occlusion and hemorrhagic necrosis.

The center of the tumor is the most susceptible to TNFα-induced necrosis[28], this may be related to the relatively ischemic conditions in this part of the tumor[11,24].

Artificially induced ischemia might therefore be a means of obtaining necrosis in the entire tumor. This notion is supported by previous studies reporting a selective vulnerability of tumors to ischemia by vascular occlusion[31,5]. To evaluate the role of ischemia during ILP on the antitumor effect of TNFα, we compared the results, obtained in two different ILP models in the rat. In model I, 15 minutes ischemia of the hind leg occurred during vascular isolation, whereas in model II oxygenation of the hind leg was performed by bubbling the perfusate with a mixture of O_2/CO_2. In both models we used the nonimmunogenic BN 175 sarcoma. In contrast to many reports on TNFα antitumor effects obtained in immunogenic animal tumor models, we feel this model may mimic the clinical situations quite well.

Material and methods

Animals

Male rats of the inbred BN strain, weighing 250-300 g were used. Rats were obtained from Harlan-CPB (Austerlitz, the Netherlands). The rats were fed a standard laboratory diet delivered by Hope farms (Woerden, the Netherlands) and kept under standard laboratory conditions: housed with 3 in a cage, 12 h light/ 12 h dark. 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 E.C. (1986). The specific protocol was approved by the "Committee on Animal Research" of the Erasmus University Rotterdam, the Netherlands.

Tumor

We used BN 175 sarcoma[14,13,17] which is syngeneic to BN rats. The tumor was implanted s.c. in the flank of the BN rat and serially passaged. BN 175 is a high grade, metastasizing and rapidly growing sarcoma. As determined by the immunization-challenge method of Prehn and Main[19] BN 175 is nonimmunogenic.

Recombinant tumor necrosis factor alpha (TNFα)

Recombinant human tumor necrosis factor alpha was kindly provided by Boehringer (Ingelheim, Germany). TNFα had a specific activity of 5.8×10^7 u/mg as determined in the murine L-M cell assay. Endotoxin levels were < 1.25 endotoxin units per mg protein. TNFα was delivered in 0.5 ml vials in a concentration of 2.4 mg/ml.

Tumor model

Small fragments of 3-5 mm were implanted s.c. in the right hindlimb just above the ankle. At least 7 days after implantation, perfusion was performed at a tumor diameter of 15 mm $\pm$ 5 mm. Tumor growth was recorded by caliper measurement in 3 dimensions and tumor volume was estimated from these measurements, as $TV = \pi/6 \times a \times b \times c$, where a, b and c are 3 perpendicular diameters of the tumor[6]. Tumor diameters were measured at least three times weekly.

Classifications for tumor response were: progressive disease (PD), growth i.e. increase of

tumor volume ≥ 100 % (= relative tumor volume ≥ 2) within 4 days after perfusion; partial remission (PR), ≥ 50 % decrease of tumor volume (= relative tumor volume ≤ 0.5) after treatment and complete remission (CR), no tumor palpable.

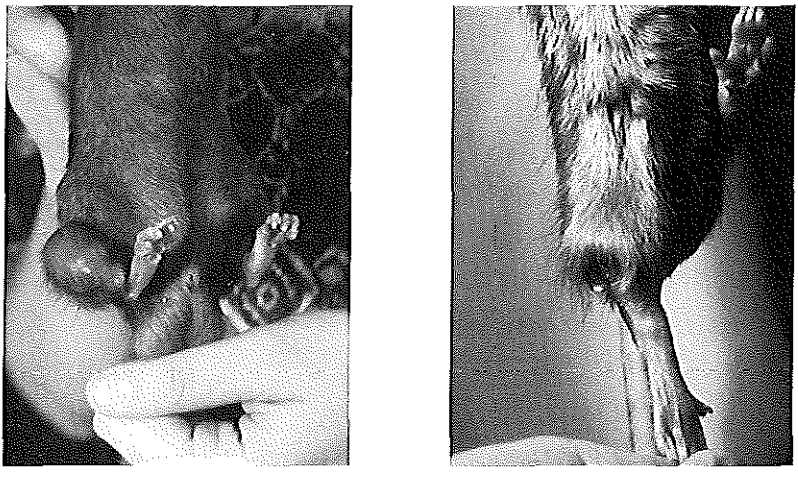

Figure 1. A. tumor at the hindleg of the rat before treatment. **B.** hindleg of the rat after ILP treatment showing complete remission (CR).

Isolated Limb Perfusion (ILP)

In both models Hypnorm (Janssen, Tilburg, the Netherlands) was used for anesthesia which was administered 10 minutes before operation. 50 i.u. of heparin was injected i.v. In order to keep the rat's hind leg at a constant temperature of 38-39°C, we applied a warm water mattress around the leg. The mattress consisted of 0.5 m silastic tube, fixed in adhesive tape and was connected to a warm water circuit. Temperature was monitored by a temperature probe (Ellab, Copenhagen, type DU-3) which was fixed at the convexity of the tumor. We used a perfusion technique originally described by Benckhuijsen, *et al.*[2] with some modifications. Briefly, before the operation was started the right groin of the rat was shaved and treated with alcohol. The femoral artery and vein were approached through an incision parallel to the inguinal ligament. Collaterals were temporarily occluded by the application of a tourniquet in the groin. The tourniquet was fixed at the inguinal ligament. The femoral artery and vein were cannulated by silastic tubing (0.30 mm inner diameter, 0.64 mm outer diameter; 0.64 mm inner diameter, 1.19 mm outer diameter respectively, Dow Corning, Michigan, USA). Isolation time was running at the moment the tourniquet was tightened.

Model I

Before cannulating the femoral artery and vein the tourniquet was pulled tight; 5 minutes later the vessels were cannulated and infusion was started. At a flow rate of 1 ml/min, 5 ml Haemaccel either with or without 100 μg TNFα was infused. A wash out was performed with 1 ml Haemaccel. Afterwards the femoral artery and vein were ligated, since in the BN rat collaterals can provide sufficient blood flow to the leg. In this model isolation time is 15 minutes and total infusion time 6 minutes.

Experimental groups were divided in two groups: group A, sham treated rats and group B, 100 μg TNFα treated rats.

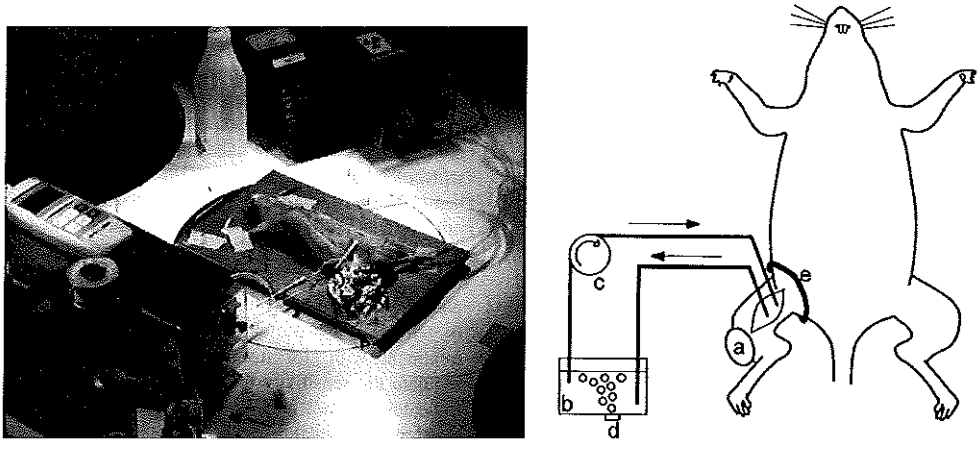

Figure 2. A. Setup for an ILP in the rat. **B.** Schematic drawing of an ILP: A tumor is implanted in the hindleg subcutaneously (a). The perfusion circuit consists of a reservoir with Haemaccel (b), and a roller pump (c). Oxygenation of the perfusate prevents hypoxia in the limb (d). A tight tourniquet is applied in the groin to occlude collaterals. During ILP a warm water mattress is wrapped around the leg to keep the hindlimb at a constant temperature (38-39 C) in order to get vasodilatation in all compartments of the limb.

Model II

In contrast to above described model, cannulation took place before tightening the tourniquet. Immediate after isolation the perfusion was started by circulating 5 ml Haemaccel. An oxygenation reservoir and a roller pump were included into the circuit. Oxygenation of the perfusate was realized by gassing a mixture of O_2/CO_2 (95:5) at a flow rate of 0.15 l/min. A roller pump (Watson Marlow, Falmouth, UK; type 505 U) recirculated the perfusate at

a flow rate of 2.4 ml/min. Isolation time was 30 minutes. Perfusion was started immediately after isolation and stopped just before the end of isolation, therefore the ischemia time was limited to a maximum of 30 seconds. Hb content of the perfusate was 0.9 mmol/l. A washout of 2 ml oxygenated Haemaccel was performed at the end of the perfusion.
In order to get insight in the oxygen pressure in the tumor before, during and after the ILP-procedure, we monitored oxygen pressure at the tumor site by the Pd-Porphyrin phosphorescence technique[20].

Leakage of TNFα and TNFα concentrations during ILP (Model II)
Bleedings were collected from the tail vein during the perfusion and perfusate samples were collected immediately after administration of TNFα. TNFα concentrations were measured by ELISA (in collaboration with CLB, Amsterdam, the Netherlands). Leakage was expressed as systemic concentration of TNFα divided through the concentration of TNFα in the perfusate.

Histology
For histopathological studies rats were sacrificed 24 h after treatment. Tumors were excised and fixed in phosphate-buffered 4 % formaldehyde solution and embedded in paraffin. Sections of the central part of the tumor were stained with hematoxylin and eosin.

Results

Ischemic model
Model I: Tumor response by ILP with TNFα was observed in this model. We noted 6 complete remissions (CRs) and 3 partial remissions (PRs) in TNFα perfused rat limbs. In all rats an acute weakening or liquefaction of tumor and necrosis of the skin was noted. Tumor regression was short lived: at the edge of the tumor site recurrences appeared within 3-11 days. Sham perfused rats did not respond, i.e. progressive disease (PDs) was noted (n=6). All results are summarized in Figure 1. Histological examination showed evidence for ischemic necrosis of the tumor.

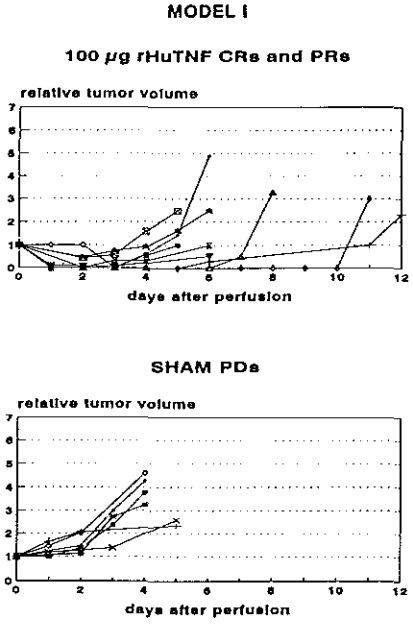

Figure 3. Relative tumor volume after ischemic perfusion (model I) with 100 μg TNFα: 6 complete remissions (CR) and 3 partial remissions (PR). After 3-11 days recurrences occurred at the edge of the tumor site. In sham perfused rats progressive disease was noted (n=6). Relative tumor volume is tumor volume (TV)/ tumor volume at the day of perfusion (TVp).

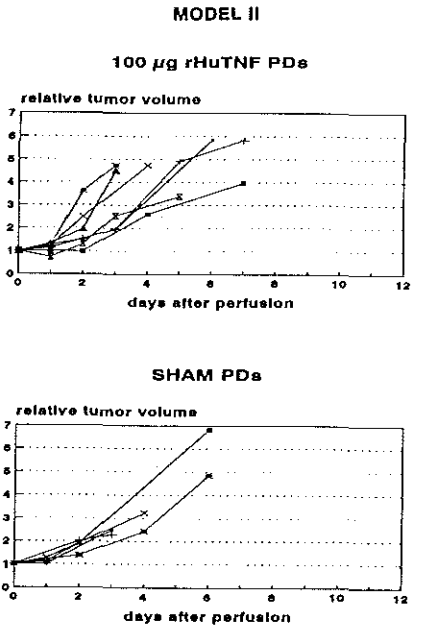

Figure 4. Relative tumor volume after perfusion in the oxygenated model (model II). No tumor response was observed in this model, neither in TNFα nor in sham perfused rats. Progressive disease (PD) was noted in 7 and 5 rats respectively.

Oxygenated model

Model II: In this model neither liquefaction nor skin necrosis was observed in TNFα and sham perfused rats (n=7 and n=5). Tumor growth was hardly influenced by the procedure (summarized in Figure 2). Histological examinations showed in tumors of sham treated rats: edema, dilated vessels and lymphocytes, but no ischemic necrosis. In the TNFα treated rats, on top of the findings described above hemorrhagic necrosis was consistently noted in the center of the tumor.

Leakage of TNFα and TNFα concentrations during ILP

Leakage was 1.36 % (sd 1.25 %). Furthermore, in none of the perfused rats, systemic

toxicity of TNFα has been observed. Figure 3 shows the TNFα concentrations during perfusion and after the wash out procedure. There exist no statistical difference between TNFα concentrations 5 minutes and 20 or 29 minutes after adding the boluses of TNFα. (n=11, p= 0.99, paired *student t-test*).

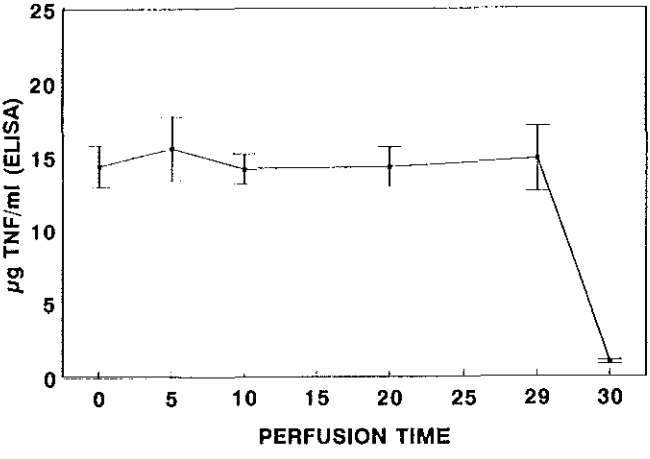

Figure 5. TNFα concentrations, as determined by ELISA, in de perfusion circuit. TNFα concentrations remain stable during the ILP (p= 0.99, between 5 min vs 20 or 29 min, paired *student t-test*). Between 29 and 30 minutes washout of TNFα with Haemaccel was performed.

Check of adequacy of oxygenation

Pd-porphyrin oxygen pressure: During perfusion (at a flow rate of 2.4 ml/min and an oxygen flow of 0.15 l/min) oxygen pressure at the tumor was nearly the same as before the operation (110 %). Likewise, so was oxygen pressure after the perfusion without reconstruction (92 %) and after reconstruction (101 %). Reconstruction did not contribute to an important increase of oxygen pressure.

Discussion

To investigate whether there is a potentiating role of ischemia in the TNFα antitumor effect, we applied an ischemic ILP model (model I) and an ILP model in which the perfusate is adequately oxygenated (model II) in the rat BN 175 limb sarcoma model.

Tumor regression in TNFα treated rats was only observed in model I. In model II, tumors showed some histological changes, characteristic for TNFα, but no tumor regression was observed. Therefore, we suggest an additive or synergistic role of ischemia during ILP to TNFα antitumor activity.

Ischemia during 15 minutes without TNFα treatment in model I did not lead to tumor regression or hemorrhage. However, after 75 minutes of mechanically-induced ischemia, hemorrhage and decrease of functional blood vessels in a mouse sarcoma has been described, while normal tissue was not affected[31]. This indicates that ischemia alone is able to induce vascular hyperpermeability or destruction of tumor endothelium. Since TNFα exerts most of its antitumor effect by destructing tumor associated vasculature, it is plausible that even a short time of ischemic anoxia can potentiate TNFα response.

In critical care medicine the role of TNFα in ischemia-reperfusion injury has been extensively studied. In an *in vivo* model of rat hepatic ischemia-reperfusion injury pulmonary pathologic alterations occurred: hyperpermeability of lung vasculature and sequestration of neutrophils. These findings were attributed to systemic TNFα release[4]. Also after bilateral hindlimb tourniquet ischemia, TNFα played a central role in the development of lung injury[30]. Hypoxia has been found to induce release of TNFα by macrophages in an *in vitro* study[20]. These data indicate a role in antitumor activity of endogenous TNFα, induced by ischemia in ILP in addition to exogenous administered TNFα.

Lowered pH in tumor is correlated with a higher sensitivity for cytotoxic agents as melphalan[21] and hyperthermia[27,23]. Likewise a dramatic increase in TNFα-mediated cytolysis was found at pH 5.3 when compared to cells exposed to TNFα at pH 7.4[1]. Ischemia by vascular occlusion is a method to lower pH in order to enhance tumor response. In a recent clinical study a selective decrease of pH in tumor by arterial clamping before and after ILP was reported[25]. Therefore, it is conceivable that short and controlled extension of clamping time before and after ILP with TNFα enhances tumor response.

In conclusion, TNFα in high concentrations did not induce tumor regression in the nonimmunogenic BN 175 sarcoma when administered in a well oxygenated ILP. Short ischemic anoxia combined with even shorter exposure to TNFα resulted in impressive tumor response, characteristic for TNFα.

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CHAPTER 3

SYNERGISTIC ANTITUMOR EFFECT OF RECOMBINANT HUMAN TUMOR NECROSIS FACTOR α (TNFα) WITH MELPHALAN IN ISOLATED LIMB PERFUSION IN THE RAT

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Abstract

The efficacy of isolated limb perfusion (ILP) for in " in transit " metastases from malignant melanoma and irresectable soft tissue sarcoma has been improved considerably by the addition of tumor necrosis factor α (TNFα). A rat sarcoma tumor model was, therefore, developed to evaluate the effects of TNFα, melphalan and the combination of these drugs in the treatment of sarcoma. In BN rats bearing the nonimmunogenic BN 175 sarcoma isolated limb perfusions were performed with perfusate only, or TNFα, or melphalan alone or in combination when tumors had grown to $\pm$ 1.5 cm in diameter. All rats, treated with sham perfusion or perfusion with 50 μg TNFα showed progressive disease (PD). After perfusion with 40 μg melphalan no change (NC) of tumor diameter was observed in all rats at 4 days. After a combined perfusion with 40 μg melphalan and 50 μg TNFα complete remission was noted in 12/16 rats (75 %). This synergistic effect *in vivo* between two relatively ineffective doses of TNFα and melphalan was not observed *in vitro*.

Introduction

Tumor necrosis factor α (TNFα) in combination with Interferon γ (IFNγ) and melphalan in isolated limb perfusion (ILP) results in a high complete remission rate in patients with in-transit metastases from malignant melanoma[1,2]. Identical treatment has been applied to patients with irresectable soft tissue sarcoma (STS) of the limbs in an attempt to render the tumors resectable and thus avoid amputation or disarticulation. An 88 % overall response rate with a limb salvage rate of 87 % has been reported[3]. Isolated limb perfusion was first described by Creech, *et al.*[4] and provides high regional drug concentrations with minimal systemic toxicity. Response rates obtained with ILP with conventional cytostatic agents, such as melphalan and doxorubicin, have been disappointing in patients with soft tissue sarcomas[5,6]. This prompted Lejeune and coworkers to investigate the addition of high doses of TNFα to the treatment of irresectable sarcomas[1].

During ILP tumors may be exposed to extremely high levels of TNFα, but the disappointing results from systemic administration of TNFα in phase I-II trials[7-11] may be due to the fact that toxicity in humans allows only for the administration of about 1/20 - 1/50 of the dose required for antitumor effects in murine experimental tumor models[12]. Since ILP can achieve a 20 fold increase in concentration of the drug[13] this may be the best way of determining whether TNFα can be used effectively.

TNFα as a single agent has only been administered by ILP in a small number of patients in a feasibility study. In this study, therefore, the efficacy of TNFα, melphalan and combination thereof were investigated in a rat model of isolated limb perfusion.

Material and methods

Animals

Male inbred BN strain rats, weighing 250-300 g, obtained from Harlan-CPB (Austerlitz, the Netherlands) were used. The rats were fed a standard laboratory diet (Hope Farms Woerden, the Netherlands). They were housed under standard condition of light and accommodation. The experimental protocols adhered to the rules outlined in the "Dutch Animal Experimentation Act" (1977) and the published "Guidelines on the protection of Experimental

Animals" by the council of the E.C. (1986). The protocol was approved by the committee on Animal Research" of the Erasmus University, Rotterdam, The Netherlands.

Tumor

The spontaneous BN 175 sarcoma (transplantable to BN rats) was used[14,15] and implanted subcutaneously in the flank and passaged serially. BN 175 sarcoma is a rapidly growing and metastasizing tumor. As determined by the immunization-challenge method of Prehn and Main[16], the BN 175 sarcoma is nonimmunogenic and can be maintained in tissue culture.

Melphalan

Melphalan (Alkeran, 50 mg per vial, Wellcome, Beckenham, United Kingdom) was diluted in 10 ml diluent solvent. Further dilutions were made in 0.9 % NaCl to give a volume of 0.2 ml in the perfusion circuit.

TNFα

Recombinant human TNFα was provided by Boehringer (Ingelheim, Germany) with a specific activity of 5.8×10^7 u/mg as determined in the murine L-M cell assay[17]. Endotoxin levels were < 1.25 endotoxin units per mg protein.

Tumor model

Fragments (3-5 mm) of the BN 175 sarcoma were implanted into the right hind limb subcutaneously just above the ankle. Perfusion was performed at a tumor diameter of approximately 15 mm at least 7 days after implantation. Subsequent tumor growth was recorded by caliper measurement. The mean of two perpendicular diameters was obtained. Tumor diameters were measured at least three times weekly.

The classification of tumor response was: progressive disease (PD) (increase in tumor diameter more than 25 % within 4 days; no change (NC) (tumor diameter equal to diameter during perfusion range -50 % to + 25 %)); partial remission (PR) (decrease in tumor diameter of less than 50 %; complete remission (CR) (no tumor palpable)). When complete remission was associated with skin necrosis at the tumor site this response was classified as CR_{SN}.

Isolated Limb Perfusion (ILP)

Modification of the perfusion technique originally described by Benckhuijsen, *et al.* was used[18,19]. Briefly, animals were anaesthetized with Hypnorm (Janssen Pharmaceutica, Tilburg, the Netherlands) and 50 i.u. of heparin was injected i.v. To keep the rat's hind leg at a constant temperature of 38-39°C, a warm water mattress was applied. The femoral artery and vein were cannulated with silastic tubing (0.30 mm inner diameter, 0.64 mm outer diameter; 0.64 mm inner diameter, 1.19 mm outer diameter respectively, Dow Corning, Michigan, USA). Collaterals were occluded by a groin tourniquet and isolation time commenced when the tourniquet was tightened. An oxygenation reservoir and a roller pump were included into the circuit. The perfusion commenced with 5 ml Haemaccel (Behring Pharma, Amsterdam, The Netherlands) and the haemoglobin (Hb) content of the perfusate was 0.9 mmol/l. Melphalan and TNFα were added as boluses to the oxygenation reservoir. A roller pump (Watson Marlow, Falmouth, UK; type 505 U) recirculated the perfusate at a flow rate of 2.4 ml/min. A washout with 2 ml oxygenated Haemaccel was performed at the end of the perfusion. In the rat collateral circulation via the internal iliac artery to the leg is so extensive that it allows ligation of the femoral vessels without detrimental effects and after ligation of the femoral artery backflow from the femoral vein was seen in all rats immediately after release of the tourniquet. The partial pressure of oxygen (PaO_2) was similar after ligation of the femoral vessels[19].

In vivo tumor response studies

Experiments were performed in four groups of rats: group 1, sham perfusion (n=9); group 2, melphalan perfusion with total doses of 40 μg (n=4), 0.2 mg (n=6) and 1 mg (n=4); group 3, perfusion with 40 μg melphalan (n=5); group 4, perfusion with 50 μg TNFα (n=9); group 5, perfusion with 40 μg melphalan and 50 μg TNFα (n=16). (Previous studies have shown that perfusions with even 100 μg TNFα had no antitumor effects[19]).

Histology

For histopathological studies an additional 16 rats were used: 4 rats per group were sacrificed 24 h after treatment. Tumors were excised and fixed in 4 % formaldehyde solution and embedded in paraffin. Sections of the tumor were stained with hematoxylin and eosin.

In vitro assessment of antitumor activity

BN sarcoma 175 grows as a monolayer in Dulbecco's modified Eagle's medium containing 5 % fetal calf serum and glutamic acid (0.3 mmol/l)(Gibco, Paisley, UK). The sulphorhodamine B (SRB) protein stain assay was used according to the method of Skehan, *et al*[20]. Eight replicate experiments were performed. Tumor growth was calculated using the formula: tumor growth = (test well/control) x 100 %. The drug concentration reducing the absorbance to 50 % of control (IC_{50}) was determined from the growth curves. Sensitization ratios of combinations of melphalan with TNFα were determined by dividing the IC_{50} in the presence of the single agent by the IC_{50} in the presence of the additional agent.

Statistical analysis

To compare tumor diameters in different animal groups Mann Whitney *U* test (MW) and Student's *t*-test (ST) were used.

Results

Dose ranging for melphalan

Perfusions were performed with 1 mg, 0.2 mg and 40 μg melphalan. Perfusions with 1 mg melphalan resulted in tumor regression consisting of 2/4 partial remissions (PR) and 2/4 complete remissions (CR). Remission was characterized by a gradual disappearance of tumor without darkening of the skin. Atrophy of the musculature occurred. In only one rat

Group	PD	NC	PR	CR	CRsn	Total
Sham perfusion	9					9
50 μg TNFα	9					9
40 μg melph.		9				9
50 μg TNFα en 40 μg melph.		4		3	9	16

Table 1. Antitumor effects of isolated limb perfusion in the BN 175 sarcoma model

was a longlasting CR obtained (sacrificed at day 105). Perfusion with 0.2 mg melphalan resulted in no change for four of six tumors and two of six complete remissions without marked atrophic changes in the hind leg. Perfusion with 40 μg melphalan inhibited tumor growth for at least 4 days in 4/4 rats (table 1).

Tumor response study

Results are summarized in table 1. Sham perfusion and perfusion with TNFα did not inhibit tumor growth and progressive disease was observed in all rats in both groups. After perfusion with 40 μg melphalan tumor growth was arrested in 5/5 animals for at least 4 days. Complete tumor remission occurred after combined perfusion with 50 μg TNFα and 40 μg melphalan in 12 of 16 rats; nine of the complete remissions were classified as CR_{SN}. Recurrences were noted in 8/16 rats. Eight rats did not have recurrences, but these rats had to be killed, as they developed skin necrosis at the tumor site. Regrowth of tumor was usually seen about 11 days after perfusion (mean (s.d.) 10.9(2.8) days). In figure 1. growth curves are shown of all treatment groups.

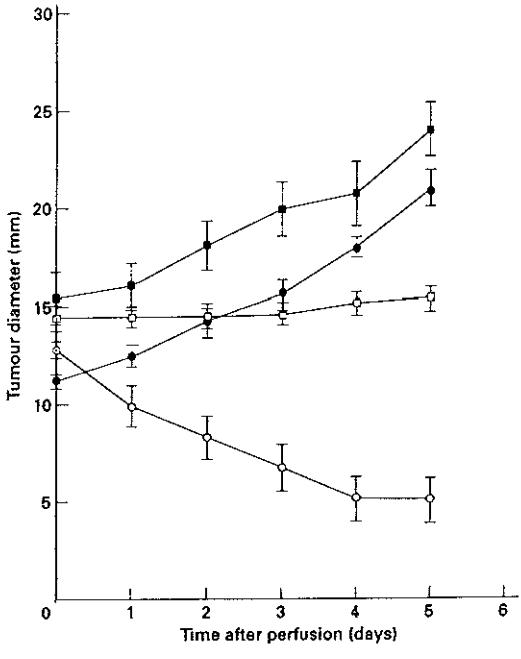


Figure 1. Growth curves of hindlimb BN 175 sarcoma after sham perfusion (■; n=9), 50 μg tumor necrosis factor (TNF) α (●; n=9), 40 μg melphalan (□; n=9) and TNFα + melphalan (○; n=16). Values are the mean (s.e.m.) of tumor diameters. At day 5 after perfusion: combined *versus* melphalan group, combined *versus* TNFα group, and melphalan *versus* TNFα group show significant differences ($P<0.001$, Mann-Withney U test and Student's t test). No significant difference was apparent between the TNFα and sham groups ($P>0.05$, Mann-Withney U test and Student's t test).

Histology

In non-perfused tumors and in tumors harvested 24 h after sham and melphalan ILP edema, dilated vessels and lymphocytes, scattered through the tumor, were found. Hemorrhagic and ischemic necrosis was absent or limited to < 5 %. In addition, rats treated with TNFα and the combination of melphalan and TNFα demonstrated hemorrhagic necrosis in the central part of the tumor. In those treated with TNFα and melphalan hemorrhagic necrosis was much more extensive than in rats receiving ILP with TNFα alone. In two rats margination of polymorphonuclear cells in the tumor vessels with increased influx into the tumor was observed.

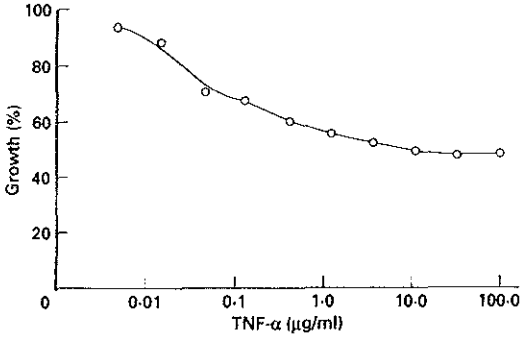
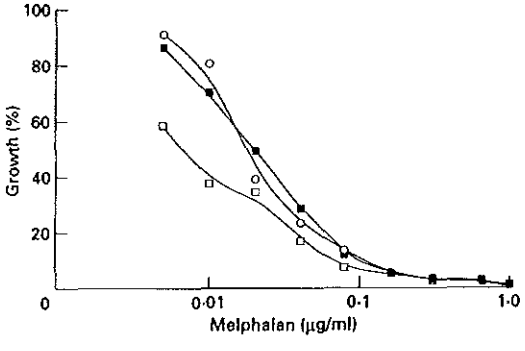


Figure 2. Dose/response curve of BN 175 sarcoma to tumor necrosis factor (TNF)α determined in the sulphorhodamine B assay.



In vitro cytotoxicity assay

The *in vitro* dose/response curve of BN 175 sarcoma cells to TNFα is depicted in Figure 2. In Figure 3 the dose/response curves with melphalan alone or combined with 0.1 and 1.0 μg/ml TNFα are depicted. The IC_{50}s were equivalent for 0 and 0.1 μg/ml TNFα, but attenuated in the presence of 1.0 μg/ml TNFα. The mean (s.d.) sensitization ratio in the presence of 1.0 μg TNFα was 2.9 (0.5) calculated from three different experiments. As 1.0 μg/ml TNFα alone can reduce tumor growth to 55 %, the enhanced cytotoxicity is probably due to additive effects.

Figure 3. Dose/response curve of BN 175 sarcoma to melphalan in the absence of tumor necrosis factor (TNF)α (■), in the presence of 0.1 μg/ml TNFα (○), and 1.0 μg/ml TNFα (□), determined in the sulphorhodamine B assay.

Discussion

These experiments have shown that TNFα is only effective, when administered in combination with melphalan. Isolated limb perfusion with perfusate alone or 50 μg TNFα alone had no impact on tumor growth and 40 μg melphalan only temporarily inhibited tumor growth. In the groups perfused with the combination of both complete remission occurred in 12 of 16 rats. Histologically, hemorrhagic necrosis was observed after ILP with TNFα alone but this was much more extensive with the combined treatment.

Interaction of the two agents on direct tumor cytotoxicity could be one explanation for the *in vivo* synergism, seen *in vivo*. *In vitro* studies demonstrated no synergy, but an additive effect at best. All observations therefore indicate that the *in vivo* antitumor effects are mediated by indirect mechanisms such as: (i) effects on the tumor-associated vasculature (TAV); (ii) increased penetration of the cytostatic agent by these effects on TAV and (iii); and the immune system.

Disappearance of the tumor within 2-3 days together with the histological observation of hemorrhagic necrosis are typical of TNFα treatment based on the vascular effects reported in many experimental studies[21-23]. Increased endothelial permeability, erythrostasis and thrombocyte aggregation, followed by vascular occlusion and hemorrhagic necrosis have been described in tumors treated with ILP and TNFα in patients[24]. Moreover, preferential occlusion of all the TAV has been demonstrated angiographically in patients after ILP[25]. Combination of TNFα with melphalan resulted in remission, which was associated with extensive hemorrhagic necrosis of the tumor, within 24 h of ILP. This suggests a potentiating role of melphalan on the effects of TNFα on the TAV. For extensive hemorrhagic necrosis to occur, another detrimental agent in addition to TNFα was apparently necessary. As with the alkylating agent cyclophosphamide, melphalan may well induce damage to endothelial cells[26].

Better penetration of the therapeutic agents may result from increased permeability of TAV induced by TNFα. The synergism of specific immune therapy and cyclophosphamide in a guinea-pig metastasis model was also found to be associated with vascular leakage within tumors[27]. In recent studies the accessibility of monoclonal antibodies was increased after the administration of TNFα[28]. Based on these reports, melphalan delivery to the tumor may be enhanced by TNFα.

For sequential biopsies in patients, changes in TAV are associated with leucocyte infiltration in the tumor[29]. By contrast, in the present rat model increased polymorphonuclear cells margination and influx was not uniformly present in the areas of hemorrhagic necrosis. In previous studies in mice polymorphonuclear cells and macrophages have been implicated in the induction of hemorrhagic necrosis by TNFα[30,31]. As distinct from their role in early TNFα effects such as hemorrhagic necrosis, inflammatory cells are probably of crucial importance in tumor regression in later phases[32]. However, this has only been shown to be the case in immunogenic tumors, and not in nonimmunogenic tumors[12]. As a nonimmunogenic tumor was used, it is likely that a second step immune reaction following hemorrhagic necrosis may not be relevant in this model. In nonimmunogenic tumors, a viable rim of tumor is suggested to replace the central hemorrhagic necrosis in the tumor[12]. This explains the lack of tumor regression, despite hemorrhagic necrosis, found after ILP with TNFα alone in this model.

In conclusion, these experiments have revealed synergism between TNFα and melphalan with a 75 % complete response rate. The model appears, therefore, to be clinically relevant. The synergy observed is mainly mediated by indirect antitumor mechanisms such as the destruction of TAV and consequently an enhanced efficacy of melphalan. Further elucidation of the mechanisms involved will be necessary to allow development of treatment programs and to extend isolated perfusion with TNFα to the treatment of other tumors such as those in the liver and lung[33,34].

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CHAPTER 4

SYNERGISTIC EFFECTS OF TNFα AND MELPHALAN IN AN ISOLATED LIMB PERFUSION MODEL OF RAT SARCOMA. A HISTOPATHOLOGICAL, IMMUNOHISTOCHEMICAL AND ELECTRON MICROSCOPICAL STUDY

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Abstract

Isolated limb perfusion (ILP) with tumor necrosis factor alpha (TNFα) and melphalan has shown impressive results in patients with irresectable soft tissue sarcomas and stage III melanoma of the extremities. The mechanisms of the reported *in vivo* synergistic antitumor effects of TNFα and melphalan are not precisely understood. We have developed an ILP model in the rat using a nonimmunogenic sarcoma where similar *in vivo* synergy is observed. The aim of the present study was to analyze the morphological substrate for this synergistic response of TNFα in combination with melphalan in order to shed more light on the pathomechanisms involved. Histology of the tumors from saline (n=14) and melphalan-treated (n=11) rats revealed apparently vital tumor cells over 80 % of the cross-sections. Interstitial edema and coagulation necrosis were observed in the remaining part of the tumor. Hemorrhage was virtually absent. TNFα (n=22) induced marked edema, hyperemia, vascular congestion, extravasation of erythrocytes and haemorrhagia necrosis (20-60 % of the cross sections). Edema and hemorrhage suggested drastic alterations of permeability and integrity of the microvasculature. Using light and electron microscopy, we observed that hemorrhage preceded generalized platelet aggregation. Therefore we suggest that the observed platelet aggregation was rather the result of the microvascular damage than its initiator. Remarkably, these events hardly influenced tumor growth. However, perfusion with the combination of TNFα and melphalan (n=24) showed more extensive haemorrhagia necrosis (80-90 % of the cross sections) and revealed a prolonged remission (mean 11 days) in comparison to the other groups of rats. Electron microscopical analysis revealed similar findings as described after TNFα alone, although the effects were more prominent at all time points after perfusion. In conclusion, our findings suggest that the enhanced antitumor effect after the combination of TNFα with melphalan results from potentiation of the TNFα-induced vascular changes accompanied by increased vascular permeability and platelet aggregation. This may result in additive cytotoxicity or inhibition of growth of residual tumor cells.

Introduction

Isolated limb perfusion (ILP) with TNFα and melphalan has shown impressive results in patients with irresectable soft tissue sarcomas and stage III melanoma of the extremities[1,2]. ILP involves isolation of the diseased limb, its connection to a heart-lung machine, and the administration of a triple drug regimen at 39-40°C (mild hyperthermia), based on the reported synergism of TNFα with melphalan and interferon γ (IFNγ)[3]. The rationale of ILP is to improve the response rate by increasing the drug concentration, while avoiding systemic toxicity. Morphological and immunohistochemical analysis of biopsies from patients after ILP suggested that the tumor microvasculature is a major target for TNFα and melphalan. We and others showed previously that events such as von Willebrand factor release, platelet aggregation and congestion concentrated on the tumor vasculature, leaving the normal tissues largely unaffected[4,5]. However, the mechanisms of *in vivo* synergistic antitumor effects of TNFα and melphalan are still not understood precisely.

As the drug regimen in patients contains at least two experimental drugs, it does not allow us to reach definitive conclusions about the relative contribution of TNFα and melphalan, respectively. Therefore, an experimental isolated perfusion model on sarcoma in the rat was set up. Rats, transplanted with a nonimmunogenic BN sarcoma in the hind leg were treated by isolated limb perfusion[6,7,8]. Using this model, we recently showed *in vivo* synergism between TNFα and melphalan in ILP with a tumor response resembling the clinical results[9]. ILP in the rat with saline or 50 μg TNFα alone had no impact on tumor growth and 40 μg melphalan only temporarily inhibited tumor growth. In the group perfused with the combination of both TNFα and melphalan, complete regression occurred in 75 % of the rats. The aim of the present work was to study the histopathological changes after ILP with TNFα alone, melphalan alone and the combination of both drugs and so shed more light on the pathomechanisms involved. We hereby present evidence, at the light and electron-microscopical level that the histopathological changes observed in the tumors after perfusion with TNFα alone were augmented by addition of the chemotherapeutic agent.

Material and methods

Animals

Male rats of the inbred BN strain, weighing 250-300 g, were obtained from Harlan-CPB (Austerlitz, The Netherlands) and kept under standard laboratory conditions (Hope Farms Woerden; The Netherlands) The specific protocol was approved by the committee on animal research of The Erasmus University, Rotterdam, The Netherlands.

Tumor

The spontaneous BN 175 sarcoma transplantable to BN rats was used[6]. This BN 175 sarcoma is a rapidly growing and metastasizing tumor. Immunogenicity of the tumor was determined by inoculating rats with tumor, excising the tumor, reinoculating the rats with the same tumor and measure the percentage of rats that show tumor take. According to this method, described by Prehn and Main[10], BN 175 sarcoma is nonimmunogenic.

Isolated limb perfusion (ILP)

The tumor model and perfusion procedure were described previously by Manusama, *et al.*[8,9]. Briefly, small fragments of BN 175 sarcoma of 3-5 mm were implanted in the right hind limb subcutaneously. Perfusion was performed in rats with established tumors with a mean diameter of 13.6 $\pm$ 3.0 mm at 10.3 $\pm$ 3.0 days after transplantation. Hypnorm (10 mg/ml fluanisone, 0.315 mg/ml fentanylcitrate, Janssen Pharmaceutica, Tilburg, The Netherlands) was given i.v. for anaesthesia. A warm water mattress was applied around the leg to maintain the temperature of the leg at 38-39 °C. The temperature of the leg was measured during the perfusion by a naked bead type K probe fixed with its tip at the convexity of the tumor and connected with a digital thermometer (Mera Benelux, Berkel-Enschot, The Netherlands). The femoral artery and vein were approached by a parainguinal incision and cannulated in distal direction with silastic tubing (0.30 mm inner diameter, 0.64 mm outer diameter; 0.64 mm inner diameter, 1.19 mm outer diameter respectively; Dow. Corning, Michigan, USA). Collaterals of the femoral vessels were occluded during the perfusion by the application of a tourniquet in the groin. The tourniquet was fixed at the inguinal ligament. Isolation time commenced when the tourniquet was tightened. The circuit included an oxygenation chamber (5 ml syringe, Braun Melsungen Germany) and a roller pump (type 505

U; Watson Marlow, Falmouth, UK). The perfusion was started by circulating 5 ml Haemaccel (Behring Pharma, Amsterdam, The Netherlands) resulting in a haemoglobin content of approximately 1 mmol/l (mean 0.94 SD $\pm$ 0.16). Melphalan (Wellcome, London, U.K.) and TNFα (recombinant human TNFα, Boehringer Ingelheim, Germany) were added as boluses to the oxygenation reservoir. The treatment modalities are specified in table 1.

Treatment	Sacrifice (hours after perfusion)	Number of rats
Controls (without perfusion)	0	5
saline	2	4
	4	5
	12	1
	24	3
	72	1
40 μm melphalan	2	3
	4	4
	12	1
	24	3
50 μm TNFα	2	5
	4	6
	12	3
	24	6
	72	2
40 μm melphalan and 50 ug TNFα	2	4
	4	6
	12	3
	24	8
	72	3

Table 1. Specification of the treatment modalities

The roller pump recirculated the perfusate at a flow rate of 2.4 ml/minute, which was

sufficient to maintain the partial pressure of oxygen (p_{aO2}) in the tumor[8]. A washout was done at the end of the perfusion with 2.0 ml oxygenated Haemaccel. The perfusion time was 30 minutes, including the washout. After the procedure the femoral vessels of the perfused limb were ligated, which was allowed by restoration of the collateral circulation. In the BN rat, the presence of collateral circulation was demonstrated by a continuous venous return when the femoral artery was ligated. Furthermore, oxygen pressure in the tumor after ligation of the femoral vessels was equivalent to that before ligation as described in a previous study[8].

Histological procedure

The tumors were excised with a rim of skin whereas the muscle layer formed the deep resection margin. After removal, the tumors were carefully cut in two almost equal parts in dorsoventral direction. In 42 out of 76 rats one half of the specimen was directly frozen in liquid nitrogen and stored at -80°C until further processing and the other half was divided in a peripheral part (containing the margin tumor/pre-existing tissues) and a central part (containing tumor from the center of the lesion) and prepared for ultrastructural analysis. In 34 rats, both parts of the specimen were formalin-fixed and embedded in paraffin. Cryostat and paraffin sections (4 μm) were hematoxylin-eosin stained. Tumor necrosis was assessed by conventional histological criteria; and the percentage of non-viable tumor was estimated in a representative histological section that included both the central and the peripheral borders of the tumor bed and expressed as percentage of the cross-section and scored in the following categories: 0-20 %, 21-50 %, 51-80 %, 81-100 %. The extent and character of the inflammatory infiltrate was evaluated. The slides were read by two different observers. In case of disagreement, consensus was reached during joined re-examination.

Immunohistochemistry

Platelet aggregation was visualized by immunohistochemistry on representative cryostat sections that included both the central and the peripheral border of the tumor bed, 4, 12 and 24 h after perfusion with saline (n=4), melphalan (n= 4), TNFα (n= 6) and TNFα in combination with melphalan (n=6). Cryostat sections (4 μm) were stained using a two-step immunoperoxidase procedure, as described previously[11], using Mab PL1-1/Er 21[12] directed against rat platelets, kindly provided by Dr. E. De Heer (University Hospital Leiden, Leiden, The Netherlands), and using PAb RaHu FVIII, recognizing von Willebrand factor (VWF)

from the CLB[1]. As secondary antibodies peroxidase-conjugated swine anti-rabbit Ig and rabbit anti-mouse Ig were used (Dako, 1:100), preabsorbed with 5 % normal rat serum. The peroxidase label was visualized by incubation with 3-amino-9-ethylcarbazole as a substrate. The semiquantitative grading used was as follows: no change (o), sporadic event (±); focal event (+); generalized event (++).

Electron microscopy

Tumor material was carefully cut into two parts to separate the central part from the peripheral part of the tumor. Small tissue fragments were immediately fixed for 24 h in 2.5 % glutaraldehyde with 0.1 mol osmium sodium cacodylate. The material was postfixed in 1% osmium sodium cacodylate buffer for 1 h at room temperature (RT), dehydrated and embedded in epon 812. One micron sections were cut and stained with toluidine blue for light microscopy. Ultrathin sections were cut with a diamond knife (Drukker, Cuijk, The Netherlands) on an ultra microtome (Reichert Jung, Vienna, Austria). The ultrathin sections were contrasted for 15 minutes with uranyl 3 % followed by a three minutes treatment with lead citrate and examined and photographed with a JEOL 1200 EX/II electron microscope (Tokyo, Japan) at 60 kV. The electron microscopical analysis focused on the microvascular changes.

Results

Histology

The semiquantitative assessment of tumor necrosis in a representative histological section after the different treatment modalities at various time points after the perfusion is shown in Figure 1.

Controls

Slices of tumors from saline-treated and untreated control rats showed the presence of a solid and grey tumor mass between the epidermis and the muscle of the right hind limb. Histology revealed individual cell necrosis and scattered areas of confluent necrosis, both of the coagulative type. Over 80 % of the tumor consisted of apparently vital tumor tissue with several mitotic figures and showing a high vascularity (Figure 2A, B). Apoptotic bodies were seen incidentally. Hyperemia and edema were observed locally. Scattered mononuclear inflammatory cells were noted in the tumor and at the interface of tumor with dermal and subcutaneous tissues (not shown). Margination of polymorphonuclear cells was apparent in dilated vessels next to the tumor (not shown).

Melphalan

Slices of tumors treated with melphalan generally appeared solid and grey. Microscopical examination 24 h after melphalan showed a highly cellular tumor mass (Figure 2C, D). Over 80 % of the cut surface of the tumor sections consisted

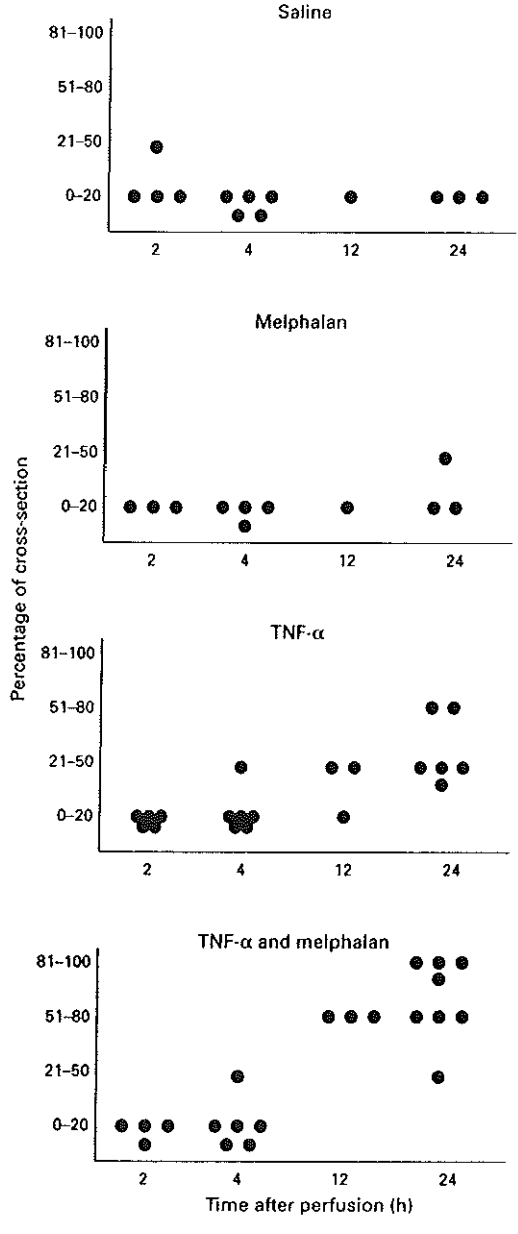

Figure 1. Degree of tumor necrosis after various treatment regimes, expressed as percentage of the cross-section.

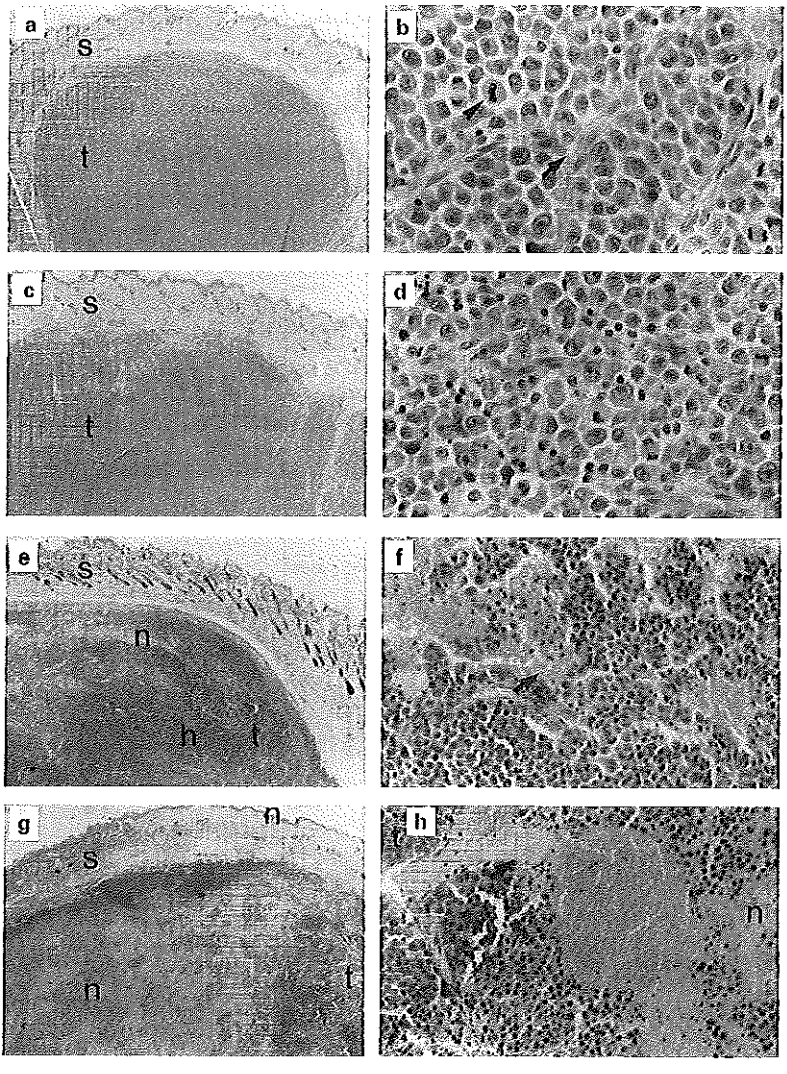

Figure 2. Paraffin sections of ILP-treated rat BN sarcoma, hematoxylin-eosin (HE) stained. **A.** Overview of tumor and skin, 24 h after ILP with saline (magnification 40x). **B.** Tumor, 24 h after ILP with saline (magnification 200x, detail from 1A). A blood vessel is marked by an arrow and a mitotic figure by an arrowhead.**C.** Overview of tumor and skin, 24 h after ILP with melphalan (magnification 40x). **D.** Tumor, 24 h after ILP with melphalan (magnification 200x, detail from 1C). **E.** Overview of tumor and skin, 24 h after ILP with TNFα (magnification 40x). **F.** Tumor, 24 h after ILP with TNFα (magnification 100x, detail from 1E). Hemorrhage is marked by an arrow. **G.** Overview of tumor and skin, 24 h after ILP with the combination of TNFα and melphalan (magnification 40x). **H.** Tumor, 24 h after ILP with the combination of TNFα and melphalan (magnification 100x, detail from 1G). A blood vessel with a thrombus is marked by an arrow. **s**, skin; **t**, tumor; **n**, necrosis; **h**, hemorrhage.

of apparently vital tumor tissue. Interstitial edema, scattered areas of necrosis and individual cell necrosis were observed in the remaining part of the tumor. Necrosis was of the coagulative type. Tumor cells with fragmented nuclei were encountered, compatible with apoptosis. Hemorrhage was virtually absent. The tumor mass was hardly infiltrated by polymorphonuclear cells, but scattered mononuclear inflammatory cells were observed in the center and at the margin.

TNFα

Slices of tumors from animals treated with TNFα generally appeared red and soft in comparison with the solid grey tumors in the untreated and saline-treated controls. Histology of the material harvested 2 h after ILP with TNFα revealed vascular congestion, marked interstitial edema and focal extravasation of erythrocytes (not shown). These vascular effects were most obvious in the tumor margins and in the adjacent connective tissue. 4 h after ILP, TNFα-induced a red discoloration with diffuse hemorrhage and marked vascular congestion (defined as: dilated vessels compacted with erythrocytes as a sign of hyperemia and/or hemostasis) as compared to the controls, both in the central parts of the tumor and at the interface of the tumor with dermal tissues (not shown). 24 h after TNFα treatment, hemorrhage and tumor cell necrosis could be observed centrally and constituted 20-60 % of the tumor (Figure 2E, F). Vascular congestion and thrombi were often seen. Histologically vital-appearing tumor cells were situated at the margins next to normal skin. In and around areas with hemorrhage, scattered mononuclear inflammatory cells could be observed. Infiltration of the tumor by polymorphonuclear cells was seen in 4 rats (two rats 4 h after ILP and two rats 24 h after ILP) (not shown).

Combination of TNFα and melphalan

Slices of tumors from animals treated with the combination of drugs generally appeared red and soft. Microscopical examination 2 h after ILP revealed marked vascular congestion and interstitial edema along with extravasation of erythrocytes (not shown). Scattered mononuclear inflammatory cells were present in the tumor and at the periphery. Margination of polymorphonuclear cells was observed in a few cases in some dermal vessels next to the tumor. At 4 h after perfusion, vascular congestion and hemorrhage were generally seen (not shown). 12 h after ILP these effects were intensified with increased

disintegration of tumor cells showing apparent nuclear pycnosis or fragmentation. These effects were more prominent centrally in the tumor. By 24 h approximately 80-90 % of the tumor had undergone extensive necrosis. Cell debris, edema, hemorrhage, thrombi and mononuclear inflammatory cells were observed (Figure 2G, H). In 3 rats (out of 24) a moderate infiltration of the tumor by polymorphonuclear cells was observed (not shown). The epidermis overlying the area of central necrosis also was necrotic (Figure 2G). A rim of viable tumor cells persisted at the margins next to the dermis (Figure 2G, H) and seemed to be responsible for the outgrowth of the tumor over the subsequent days.

Immunohistochemistry

PL1-1 (platelets) The semiquantitative assessment of platelet aggregation in a representative histological section following various treatment modalities at various time points after perfusion is shown in Figure 3. Sections of the salinetreated rats 4, 12 and 24 h after perfusion showed sporadical (±) intravascular PL1-1 staining in the tumor (Figure 4A). PL1-1 staining 4,

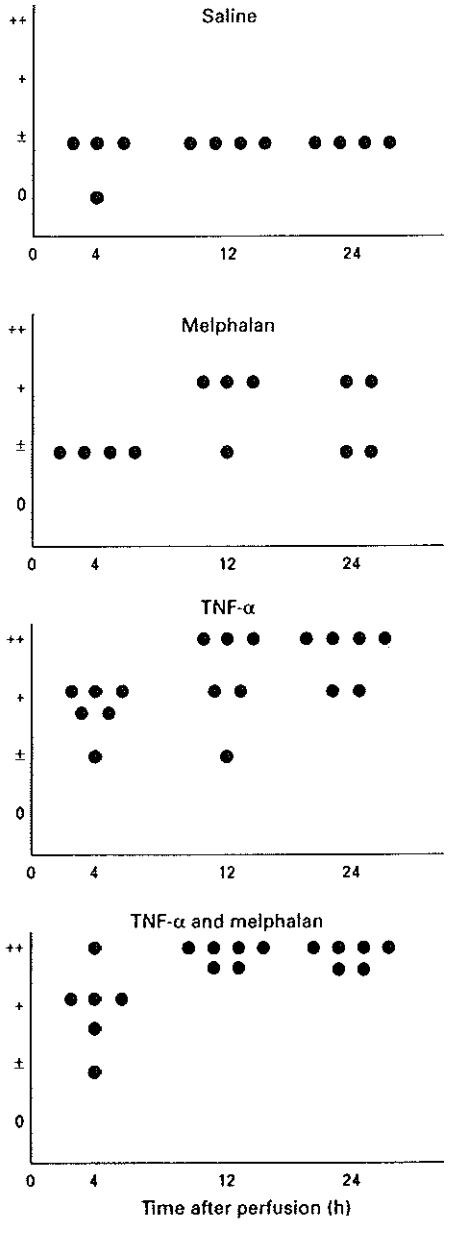

Figure 3. Semiquantitative assessment of platelet aggregation after various treatment regimes, in a representative cryostat section. O, No change; ±, sporadic event; +, focal event; ++, generalized event.

12 and 24 h after melphalan-treatment varied from sporadic (±) to focal (+) (Figure 4B). PL1-1 staining in the vessels outside the tumor was sporadically observed. TNFα-treated rats showed intravascular PL1-1 staining focally (+) in the tumor 4 h after treatment (not shown). Generalized (++) PL1-1 staining was observed 12 and 24 h after TNFα-perfusion (Figure 4C). PL1-1 staining was also observed in vessels adjacent to the tumor. Perfusion treatment with TNFα in combination with melphalan showed intravascular PL1-1 staining focally (+) in the tumor in sections 4 h after perfusion (not shown). In all sections 12 and 24 h after perfusion with TNFα in combination with melphalan marked generalized (++) PL1-1 staining was observed (Figure 4D), and also in vessels adjacent to the tumor (Figure 4E).

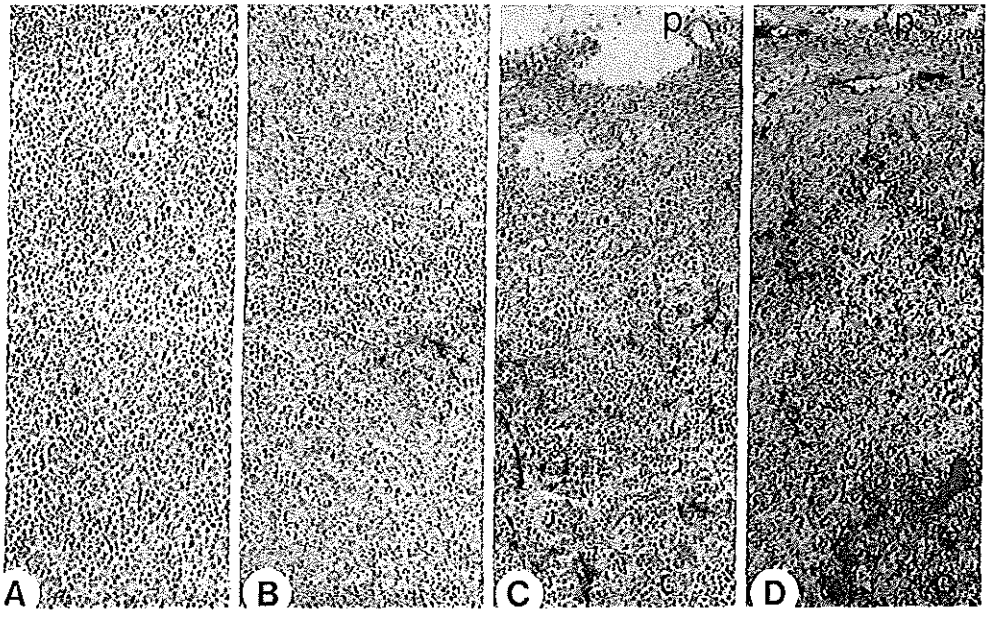

Figure 4. Immunohistochemical staining with the platelet marker PL1-1 on frozen sections of rat BN sarcoma. A. Overview of tumor, 12 h after ILP with saline (magnification 50x) showing sporadic (±) PL1-1 staining. B. Overview of tumor, 12 h after ILP with melphalan (magnification 50x) showing focal (+) PL1-1 staining. C. Overview of tumor, 12 h after ILP with TNFα (magnification 50x) showing generalized (++) PL1-1 staining. D. Overview of tumor, 12 h after ILP with TNFα and melphalan (magnification 50x) showing marked generalized (++) PL1-1 staining. c, Tumor center; p, periphery of the tumor.

rHu FVIII (von Willebrand factor) We found that this antibody was not suitable for the analysis as possible leakage of von Willebrand factor by the endothelium after perfusion treatment in our animal model, as the intensity of the endothelial staining was low in combination with a diffuse background staining.

Electron microscopy

The tumors contained numerous small vessels. Intratumoral vessels at the periphery of the control tumors and after saline perfusion revealed a continuous endothelial cell lining, often surrounded by pericytes. Smooth muscle cells were only occasionally found. Centrally in

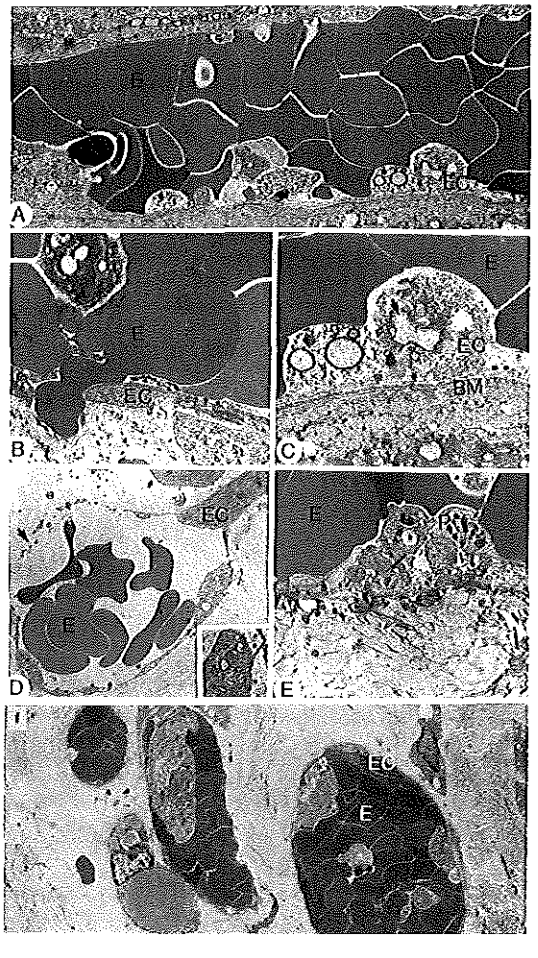

Figure 5.

Ultrastructural microvascular changes after ILP with TNFα. A. Blood vessel at the periphery of the tumor, 12 h after ILP, loaded with erythrocytes and lined by degenerated endothelial cells (magnification 4000x). B. Erythrocyte extravasation in the periphery of the tumor, 4 h after ILP (magnification 5000x). C. Detail of the endothelial cell in figure 3A, showing signs of degeneration. A swollen mitochondrion is marked by an arrow (magnification 6000x). D. Blood vessel in the tumor center, 12 h after ILP showing disturbed integrity of the endothelial cell (marked by an arrow) (magnification 2500x). Inset, high power image of part of the cytoplasm of the endothelial cell showing merely intact mitochondria. E. Platelet aggregation in a blood vessel in the periphery of the tumor, 12 h after ILP (magnification 6000x). F. Blood vessel in the dermis adjacent to the tumor, 12 h after ILP, loaded with erythrocytes. E, erythrocytes; EC, endothelial cell; BM, basement membrane; P, platelet.

the tumor, few degenerated endothelial cells were observed in areas with edema and hemorrhage, suggestive of vascular leakage. These findings were not accompanied by a marked inflammatory cell infiltrate. Platelets were found adherent to the vessel wall. The ultrastructural findings after ILP with melphalan (40 μg) resembled those described after saline perfusion.

After perfusion with TNFα (50 μg) marked intercellular edema was seen. Increased hemorrhage, both centrally and at the periphery (2, 4 and 12 h after ILP), suggested vascular leakage (Figure 5B). Intratumoral and peritumoral vessels showed marked erythrostasis (Figure. 5A, F). At the periphery of the tumors the vessels loaded with erythrocytes revealed signs of endothelial cell degeneration, i.e. electron-lucent cytoplasm and swollen mitochondria (Figure 5A, C). Extensive aggregation of platelets was observed both in the intratumoral vessels and just outside the tumor, but merely mural and not occlusive (Figure 5E). Increased hemorrhage and intravascular platelet aggregation were observed 24 h after perfusion with TNFα in the tumor, with disintegration of the endothelial cells (Figure 5D), only in the tumor center, with extensive edema and tumor cell necrosis. These phenomena were not accompanied by a marked inflammatory cell infiltrate.

Ultrastructural analysis of sections after perfusions with TNFα and melphalan revealed similar findings as described after TNFα alone, although the findings were more prominent at all time points after perfusion. Because of this similarity, only the ultrastructural changes after TNFα are shown.

Discussion

Tumor necrosis factor α (TNFα) in combination with IFNγ and melphalan in an isolated limb perfusion setting resulted in a high remission rate in patients with irresectable soft tissue sarcomas[1]. An overall response rate of 88 % with a limb salvage of 87 % was reported[3]. However, perfusion with TNFα alone proved to be ineffective[13]. The mechanism of tumor regression by the combination of TNFα and the chemotherapeutic agent melphalan, *in vivo,* is not precisely understood but was proposed to follow a dual targeting pathway[4,14]. The first target is represented by the tumor microvasculature. TNFα was assumed to induce endothelial cell damage, leading to von Willebrand factor release (VWF). Released VWF

may play a role in the adhesion between platelet and the damaged endothelium or the denuded vessel wall. As a consequence, the blood flow is impaired, leading to congestion and edema. The second target is represented by the tumor cells themselves, which are increasingly subjected to the cytotoxic effects of melphalan in a hypoxic environment.

In order to study the impact of the individual drugs on tumor regression we used an experimental model of sarcoma in the rat. We previously showed an *in vivo* synergism between two relatively ineffective doses of TNFα (50 μg) and melphalan (40 μg) in this rat model, with a tumor response resembling the clinical response[9]. The present study was set up to analyze the morphologic substrate for this synergistic response employing light microscopic, immunohistochemical and electron microscopical methods. Histopathological analysis of the transplanted nonimmunogenic BN sarcoma after ILP with TNFα alone showed similar findings as reported by Asher, et al[15]. in a nonimmunogenic MCA-102 murine sarcoma after TNFα i.v. injection and closely resembled the effects described for endotoxin-induced histopathological features[16-18]. TNFα-induced edema, hyperemia, vascular congestion, extravasation of erythrocytes and hemorrhagic necrosis (20-60 %)[19-21]. Remarkably, these events hardly influenced tumor diameter and tumor growth in our rat ILP model. Increased doses of only TNFα did not lead to an increased antitumor effect: even with doses of 100 μg in all seven rats progressive disease was observed (unpublished results). Furthermore, when using TNFα intravenously in toxic doses, no effect on tumor growth was noted in the same model (unpublished results). Therefore, the combination with melphalan is necessary to obtain an effective antitumor response. On the other hand, at higher doses of melphalan alone, regression occurred with a gradual disappearance of the tumor without hemorrhagic necrosis[9]. Combination with TNFα lowered the effective dose of melphalan and introduced a vascular component into the mechanism of action, characterized by extensive hemorrhagic necrosis.

Edema and hemorrhage suggested dramatic alterations of permeability and integrity of microvascular endothelial cells. The cause of the vascular stasis and congestion is not clear, but it may be related to a direct cytotoxic effect of TNFα on endothelial cells[21]. A direct toxic effect and activation of procoagulant activity in tumor endothelial cells, with subsequent thrombus formation might be responsible for hemorrhagic necrosis[22-25]. Using light and electron microscopy, we observed, however, that hemorrhage preceded generalized platelet aggregation without fusion of platelets with endothelial cells[26]. Therefore, we suggest that the

observed platelet aggregation was the result of the endothelial damage rather than the initiator. These findings are consistent with those observed in sequential biopsies of human lesions after ILP[4]. In most cases, the center of the tumor seemed to be more vulnerable to the treatment than the periphery. Aberrant branching and twisting of the vasculature and abnormal high pressure in the interstitial matrix of the tumor center, with pre-existing increased vascular permeability, may have lead to an uneven distribution of the drugs and uneven distribution of the vascular changes observed[27,28]. The vascular changes in the tumor margins, accompanied with stasis, may have contributed to a decreased blood flow out of the tumor with development of central hemorrhagic necrosis.

The antitumor activity and necrosis induced by the combination of intratumorally injected TNFα and IFNγ systemically were studied by de Kossodo, *et al*[29]. in a breast cancer xenograft model. Recent study in an experimental ILP model revealed almost similar findings using the combination of TNFα and melphalan. Similar potentiation of the antitumor activity were observed, associated with vascular congestion and accumulation of platelets in areas of vascular damage. Whether polymorphonuclear leukocytes contributed to the vascular damage in our rat ILP model is not clear. A polymorphonuclear cell infiltrate in the tumor after perfusion with TNFα and with the combination generally was not observed, whereas hemorrhage and necrosis were a consistent finding. The exact role of polymorphonuclear leukocytes in the antitumor effect has to be analyzed in future studies by perfusions of granulocytopenic rats. Although it is difficult to derive the dynamics of vascular damage and tumor regression from static images, our observations support the interpretation of Regenass[19] that the TNFα-induced increase in permeability of the endothelial cells leads to increased blood viscosity. In this view, the decreasing tumor blood flow is further impaired by intravascular platelet aggregation, resulting in a sustained hemostasis and finally in hemorrhagic infarction. The increased vascular permeability together with a reduced blood flow out of the tumor may have lead to increased intratumoral concentrations of melphalan or prolongation of its effect. Our findings indicate that it is important to measure the effects on vascular permeability. Experiments to determine vascular permeability changes and melphalan concentrations in the tumors are in progress.

Perfusion with the combination of TNFα and melphalan increased both the occurrence and extent of the hemorrhagic necrosis. Necrosis was not confined to the tumor cells but also involved microvascular cells, both in the tumor and in the adjacent dermis. The potentiation

of the TNFα-induced vascular effects by melphalan might be explained by additive cytotoxicity directed to the vascular endothelium[19,30,31] and/or increased endothelial cell reactivity to platelets[32]. Our (histo)pathological findings support the proposition by Lejeune[14] that the combination of TNFα and melphalan works through a dual targeting system. In this view, the first target is represented by the tumor vasculature and the second one by the tumor cells themselves. However, the proposed granulocyte-mediated endothelial damage does not seem to be a *conditio sine qua non* and therefore should be studied further. In conclusion, our findings suggest that the enhanced antitumor effect after the combination of TNFα with melphalan results from potentiation of the TNFα-induced vascular changes accompanied by increased permeability. This may result in additive cytotoxicity or inhibition of growth of residual tumor cells.

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CHAPTER 5

ASSESSMENT OF ROLE OF NEUTROPHILS ON THE ANTITUMOR EFFECT OF TNFα IN AN *IN VIVO* ISOLATED LIMB PERFUSION MODEL IN SARCOMA BEARING BROWN NORWAY RATS

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Abstract

Isolated limb perfusion (ILP) with TNFα in combination with melphalan and IFNγ has resulted in an immediate and dramatic tumor response in patients. Such an effect was also noted following ILP in a rat sarcoma model. This model enables us to investigate several factors responsible for the TNFα-induced tumor responses. We applied total body irradiation (TBI) to reduce white blood cell count, to investigate the contribution of leukocytes to the antitumor effect of TNFα.

Small fragments of the nonimmunogenic BN 175 sarcoma were implanted s.c. in the lower hind leg. 5 Gy TBI was performed before ILP at a tumor diameter of approximately 15 mm. The hind limbs of 63 rats were perfused and were divided into 6 groups: group 1, sham perfusion (n=9); group 2, TBI + sham perfusion, (n=6); group 3, TNFα 50 μg (n=9); group 4, melphalan 40 μg (n=9); group 5; TNFα 50 μg + melphalan 40 μg (n=22) and group 6; TBI + TNFα + melphalan ILP (n=8). In addition, 10 rats were perfused for histological analysis at 24 h post ILP.

We observed in group 1, 9/9 progressive disease (PD); group 2, 6/6 PD; group 3, 9/9 PD; group 4, 9/9 no change (NC) of tumor diameter for at least 4 days; group 5, 6/22 NC, 16/22 complete remission (CR), 12/16 of which showed skin necrosis at the tumor site; and group 6, 7/8 NC and 1/8 CR (without skin necrosis). After TBI, WBC reduction of 80-95 % was observed, while the number of platelets was not significantly reduced and platelet aggregation was maintained at 72 %. Histological analysis revealed decreased hemorrhagic necrosis associated with the absence of PMN infiltration at the tumor margins in the TBI rats.

TBI and the associated reduction in WBC count decreased the tumor response by TNFα and melphalan significantly and abrogated the immediate response of skin necrosis at the tumor site, as found in rats treated with TNFα and melphalan without TBI. These data strongly suggest that leukocytes play an important role in the hemorrhagic effects of TNFα.

Introduction

In patients, isolated perfusion of the extremities has been proven an excellent means to administer effective doses of the highly toxic TNFα in combination with IFNγ and melphalan, resulting in high tumor response rates. Complete remission (CR) was achieved in > 80 % of patients with in-transit melanoma metastases compared to 52 % CR after ILP with melphalan alone[1,2]. In the management of locally advanced extremity soft tissue sarcomas the same treatment has now established itself as highly effective, with response and limb salvage rates in over 80 % of the patients[3,4]. The selective disruption of the tumor vasculature, indirectly resulting in tumor necrosis has been shown in histopathological and immunohistochemical studies on the vasculature of the tumors of patients treated with TNFα triple combination ILP[5]. Furthermore, on angiography the tumor vasculature, visible before ILP, totally disappeared within 10 days after ILP[6].

We developed in BN rats a limb perfusion model that correlates well in terms of response rate with the histopathological observations on the responding soft tissue sarcomas in patients[7,8,9].

Endothelial injury of the tumor microvasculature after isolated limb perfusion with TNFα and melphalan is considered to play an important role in the pathogenesis of tumor necrosis. Intravascular thrombus formation and PMN-mediated damage to the endothelium have been postulated as important aspects of the typical TNFα-mediated antitumor effects[10-13]. We addressed this question in our limb perfusion model by comparing the outcome of ILP with TNFα + melphalan in rats under neutropenic conditions with those under normal conditions. In the present study we investigated whether irradiation as a means to reduce circulating white blood cells can attenuate the tumor response of TNFα and melphalan in our ILP model in the rat. Furthermore, the number and function of circulating platelets have been assessed, since platelets have a potential role in TNF-mediated antitumor effect.

Material and methods

Animals

Male inbred BN strain rats, weighing 250-300 g, obtained from Harlan-CPB (Austerlitz, the Netherlands) were used. The rats were fed a standard laboratory diet (Hope Farms Woerden, the Netherlands). They were housed under standard light accommodation conditions. The experimental protocols adhered to the rules outlined in the "Dutch Animal Experimentation Act" (1977) and the published "Guidelines on the Protection of Experimental Animals" by the council of the E.C. (1986). The protocol was approved by the committee on "Animal Research" of the Erasmus University Rotterdam, The Netherlands.

Irradiation

Total body irradiation (TBI) with doses of 5 Gy was performed with a Gammacell 40 Caesium 137 irradiation unit (Atomic Energy of Canada Ltd, Ottawa, Canada), 24-48 h before ILP or before blood was taken for *in vitro* aggregation assays.

Tumor

The spontaneous BN 175 sarcoma (transplantable to BN rats) was used[14,15] and implanted subcutaneously in the flank and passaged serially. BN 175 sarcoma is a rapidly growing and metastasizing tumor. As determined by the immunization-challenge method of Prehn and Main, the BN 175 sarcoma is nonimmunogenic.

Melphalan

Melphalan (Alkeran, 50 mg per vial, Wellcome, Beckenham, United Kingdom) was diluted in 10 ml diluent solvent. Further dilutions were made in 0.9 % NaCl to give a volume of 0.2 ml in the perfusion circuit.

TNFα

Recombinant human TNFα was provided by Boehringer (Ingelheim, Germany) with a specific activity of 5.8×10^7 u/mg as determined in the murine L-M cell assay[16]. Endotoxin levels were < 1.25 endotoxin units (EU)/mg protein.

Tumor model

Fragments (3-5 mm) of the BN 175 sarcoma were implanted into the right hind limb subcutaneously just above the ankle. Perfusion was performed at a tumor diameter of 15 mm $\pm$ 5mm at least 7 days after implantation. Subsequent tumor growth was recorded by caliper measurement. The mean of two perpendicular diameters was obtained. Tumor diameters were measured at least three times weekly.

The classification of tumor response was: progressive disease (PD), increase of tumor diameter (>25 %) within 4 days; no change (NC), tumor diameter equal to diameter during perfusion (in a range of -50 % to +25 %); partial remission (PR), decrease of tumor diameter (< -50 %); complete remission (CR), no tumor palpable. When complete remission was associated with skin necrosis at the tumor site this response was classified as CR_{SN}.

Isolated Limb Perfusion

The perfusion technique we used has been published previously[8]. Briefly, animals were anaesthetized with Hypnorm (Janssen Pharmaceutica, Tilburg, the Netherlands) and 50 i.u. of heparin was injected iv to prevent coagulation in the collateral circulation and in the perfusion circuit. To keep the rat's hind leg at a constant temperature of 38-39°C, a warm water mattress was applied. The femoral artery and vein were cannulated with Silastic tubing (0.012 inch ID, 0.025 inch OD; 0.025 inch ID, 0.047 inch OD, respectively, Dow Corning, Michigan, USA). Collaterals were occluded by a groin tourniquet and isolation time commenced when the tourniquet was tightened. An oxygenation reservoir and a roller pump were included into the circuit. The perfusion commenced with 5 ml Haemaccel (Behring Pharma, Amsterdam, the Netherlands) and the haemoglobin (Hb) content of the perfusate was 0.9 mmol/l. Melphalan and TNFα were added as boluses to the oxygenation reservoir. A roller pump (Watson Marlow, Falmouth, UK; type 505 U) recirculated the perfusate at a flow rate of 2.4 ml/min. A washout with 2 ml oxygenated Haemaccel was performed at the end of the perfusion. In the rat collateral circulation via the internal iliac artery to the leg is so extensive that it allows ligation of the femoral vessels without detrimental effects and after ligation of the femoral artery backflow from the femoral vein was seen in all rats immediately after release of the tourniquet. The partial pressure of oxygen (PaO_2) was similar after ligation of the femoral vessels to that prior to ligation[7].

In vivo tumor response studies

Six groups of rats were used: group 1, sham perfusion (n=9); group 2, TBI + sham perfusion, n=6; group 3, perfusion with 50 μg TNFα (n=9); group 4, melphalan perfusion with total doses of 40 μg; group 5, perfusion with 50 μg TNFα and 40 μg melphalan (n=22) and group 6, TBI rats that got the combination therapy similar to group 5 (n=8).

Histology

The histopathological techniques and histopathological changes observed in tumors after sham perfusion, TNFα perfusion alone, and the augmentation of the antitumor effect by the addition of melphalan were described earlier[9]. For histopathological studies an additional 10 rats were used in the present study, divided into a nonirradiated group (n=5) that underwent ILP with a combination of TNFα and melphalan and a TBI group (n=5) that underwent the same therapy. Tumors were excised and fixed in 4 % formaldehyde solution and embedded in paraffin. Histological sections were Hematoxylin-eosin stained. The tumor were excised with a rim of skin whereas the muscle layer formed the deep resection margin. Tumor necrosis was assessed by conventional histological criteria: the percentage of nonviable tumor was estimated in a representative histological section that included both peripheral borders of the tumor bed and are expressed as a percentage of the cross-section. The extend and character of the inflammatory infiltrate were evaluated. Enzymehistochemical detection of chloroacetate-esterase for the localization of PMN's and mast cells was performed by a standard method[17]. The semiquantitative grading of the tumor-infiltrating leukocytes was as follows: none, any leukocytes up to small perivascular foci, distinct perivascular foci up to leukocytes in the tumor, many leukocytes focally among tumor cells, and many leukocytes diffusely distributed among tumor cells. The slides were read by two different observers. In case of disagreement consensus was reached during joined reexamination.

Hematologic studies

Complete blood counts including platelet counts were obtained from venous bleedings collected in EDTA, using a Coulter counter (Sysmex AD-260, TOA Medical Electronics, Kobe, Japan). Samples were taken before ILP, 0.5 h, 1 day and 5-8 days after ILP.

In vitro platelet aggregation assay

platelet aggregation function was measured in two groups of (nonperfused) rats: group 1, sham group (n=6) and group 2, TBI rats (n=6). Under ether anaesthesia the animals received iv 50 iu heparin, after which laparotomy took place. At sacrifice the abdominal aorta was dissected at its bifurcation, and blood was collected by aortic puncture. Platelet aggregation was carried out using the Chronolog-Whole Blood aggrometer (Chronolog Corp., U.K.). Collagen was added (2.5 μg/ml) to induce platelet aggregation (n=6 per group) to heparinized (10 iu/ml) whole blood samples, which were diluted 1:1 with 0.9 % saline. Ten-minute recordings of impedance in Ohms were analyzed.

Statistical analysis

To compare tumor diameters in different animal groups the Mann Whitney *U* test (MW) was used.

Group	PD	NC	PR	CR	CR_{sn}	Total
Sham perfusion	9					9
50 μg TNFα	9					9
40 μg melph.		9				9
TNFα + melph.		6		4	12	22
TBI + TNFα + melph.		7		1		8

Table 1. Antitumor effects of isolated limb perfusion in the BN 175 sarcoma model.

Results

Tumor response study

Results are summarized in Table 1. In rats that underwent sham perfusion, TBI + sham perfusion and perfusion with TNFα alone progressive disease was observed. Complete tumor regression occurred after combined perfusion with 50 μg TNFα and 40 μg melphalan in 16/22 rats with 4/22 CR and 12/22 CR_{SN}. Between tumor diameters of group 5 and all other groups a highly significant difference exists ($p < 0.001$). TBI rats receiving the TNFα +

melphalan therapy responded with 7/8 NC and 1 CR. No skin necrosis could be noted in the TBI rats. A slight difference exists between tumor diameters of group 4 and 6 ($0.01 < p < 0.05$)(Fig. 1).

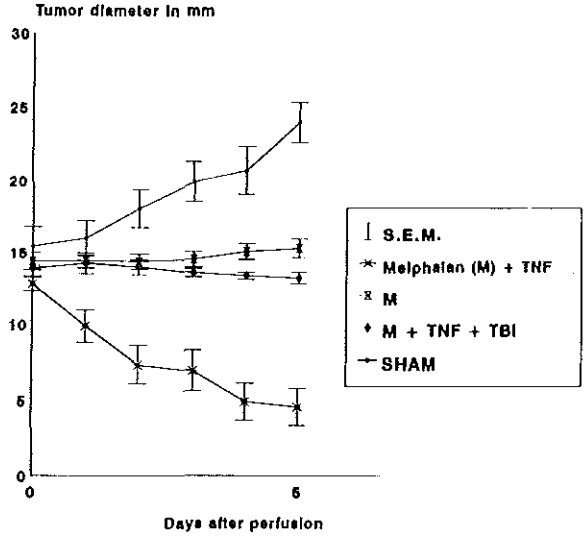
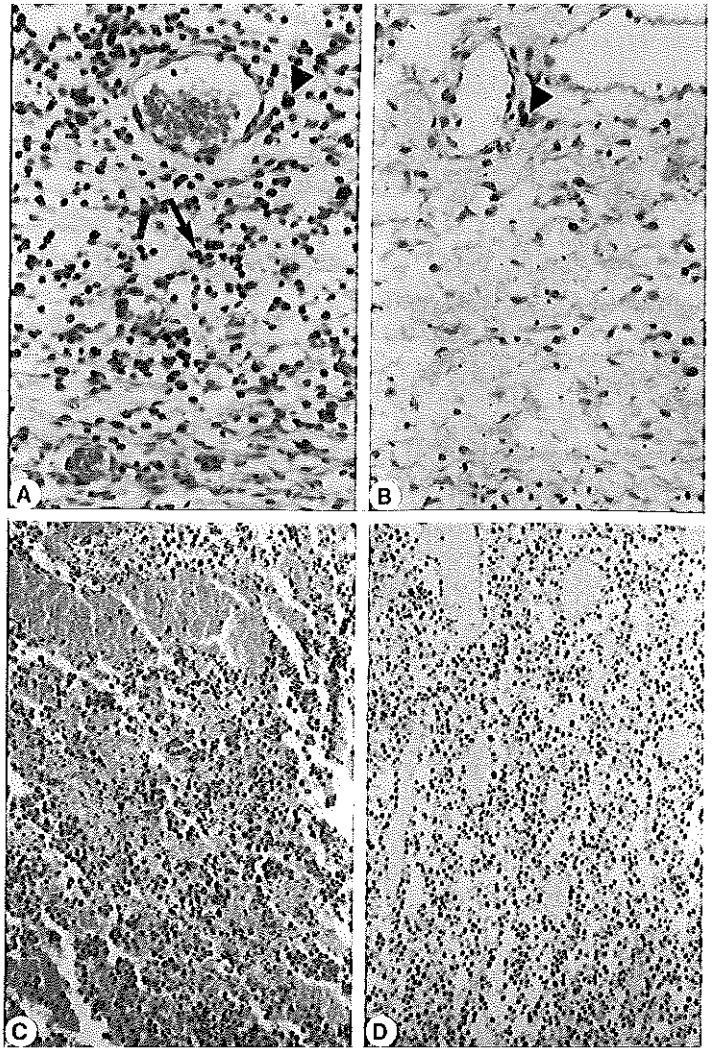


Figure 1. Growth curves of hindlimb BN 175 sarcoma after sham (n=9), melphalan (n=9) TNFα + melphalan treatment in nonirradiated rats (n=22), and the same treatment in TBI rats (n=8). Values are mean (± SEM) of the tumor diameters. At day 5 after perfusion: combined *versus* M + TNFα + TBI group, Sham *versus* M + TNFα + TBI group show highly significant differences ($P<0.001$, Mann-Whitney *U* test). Significant difference was found between M only and M + TNFα + TBI group ($0.01<P<0.05$, Mann-Whitney *U* test).

Histology

Slices of tumors from saline-treated control rats showed individual cell necrosis and scattered areas of confluent necrosis, both of the coagulative type. Over 80 % of the tumor consisted of apparently vital tumor cells. Margination of PMNs was apparent in dilated vessels next to the tumor. Slices of tumors treated with melphalan showed apparently vital tumor cells over 80 % of the cut surface of the tumor section; scattered areas of necrosis and individual cell necrosis were observed in the remaining part. Slices of tumors after the combination of TNFα and melphalan showed 80-90 % necrosis with the aspect of hemorrhagic necrosis. In addition the epidermis overlying the area of central necrosis was necrotic. PMN infiltration was located at the periphery of the tumors. Slices of TNFα and melphalan after total body irradiation lacked the PMN infiltrates at the tumor margins (see figure 2). In the absence of PMNs (numbers were lower than the PMN infiltrates in the sham-perfused group) necrosis was observed, but over 70 % of the cut surface of the tumor sections consisted of vital tumor cells, and necrosis was of the coagulative type (see figure 2). Hemorrhage was virtually absent and comparable to the sham-perfused rats.

Figure 2. Enzyme-histochemical detection of chloroacetate-esterase for the localization of neutrophils and mast cells in paraffin sections of rat skin (dermis) and tumor, 24 h after ILP with the combination of TNFα and melphalan with and without TBI. (200 x)



A. The skin near the tumor after ILP without TBI is infiltrated by neutrophils (arrow). (A mast cell is indicated with an arrowhead).

B. The skin near the tumor after ILP *with* TBI is not infiltrated by neutrophils. (A mast cell, positively stained as an internal control for the staining, is indicated with an arrowhead).

C. The tumor after ILP *without* TBI shows hemorrhagic necrosis in the absence of neutrophils.

D. The tumor after ILP *with* TBI shows coagulative necrosis in the absence of neutrophils.

Hematology

In Figure 3 and 4 the counts of white blood cells and platelets, respectively, are depicted. A 80-95 % reduction of white blood count was observed in TBI rats as compared to the nonirradiated rats. No difference between TBI and nonirradiated rats was found for the platelet count.

A marked drop was noted for the white blood cells 0.5 h after ILP, most likely due to leukocyte margination in the treated limb. This (statistically significant) drop could also be observed in the TBI rats. Also platelet count diminished after ILP.

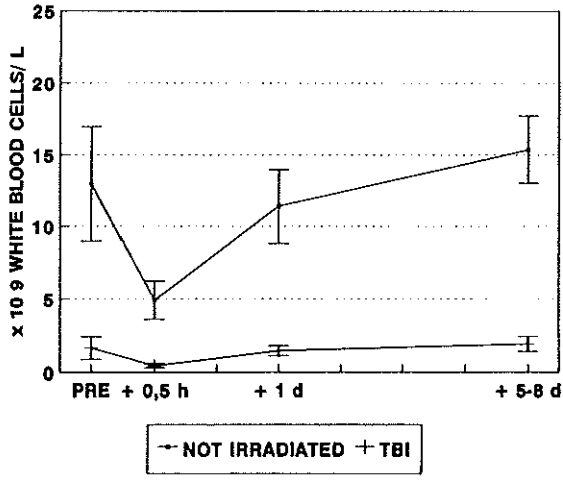


Figure 3. 80 - 95 % reduction of white blood cell count was found in the bleedings of TBI rats, before and after ILP at different time points.

In vitro platelet aggregation assay

After induction of platelet aggregation with a concentration of 2.5 µg/ml collagen 28 % reduction of platelet aggregation could be observed (12.9 +/- 2.4 Ω in the TBI group *versus* 17.97 +/- 3.87 Ω in the Sham group; P=0.021).

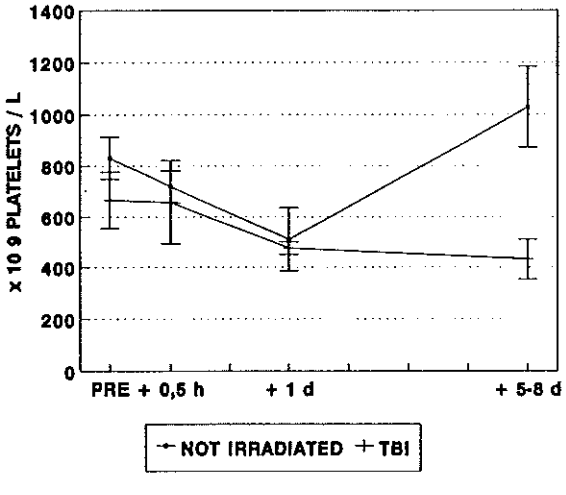


Figure 4. No significant differences were found in platelet count before perfusion and at 0,5 h and 1 day after perfusion. In TBI rats no restoration of platelet count occurred in 5-8 days after perfusion.

Discussion

This study revealed an attenuated tumor response in rats that were irradiated (TBI) before ILP with TNFα and melphalan. In this group of rats neither remissions with skin necrosis nor pronounced hemorrhagic necrosis (HN) in the tumors were observed. Tumor response in the TBI rats is nearly the same as that observed in the rats that were perfused with melphalan only (Table 1.), although there exists a slight but significant difference between both groups (Figure 1.). Thus the TNFα-related characteristics of the tumor response were significantly attenuated, if not abrogated, by irradiating the rats before ILP.

An important role of PMNs is suggested by the observation that a 80-95 % reduction of the WBC count in the TBI rats was associated with an attenuated tumor response. Furthermore in both TBI and nonirradiated rats a significant drop in the WBC count was noted 30 minutes after ILP, which may be explained by margination of leukocytes in the perfused limb. Accordingly, in patients that were treated iv with TNFα[17] in a phase I anticancer trial, leukocytopenia has been reported 30 minutes after administration, due to leukocyte adherence to the endothelial cells. Another indication that PMNs may play an important role in the acute tumor response by TNFα is the difference between TBI and nonirradiated rats with respect to absence and presence of PMNs in the skin adjacent to the tumor. Remarkably, no PMNs were found in the tumors of both the nonirradiated or the TBI rats; as described in other tumor models only the adjacent tissue was infiltrated with PMNs. We assume that cytotoxic products released from PMNs at the margins of the tumor have a deleterious effect on the vasculature of the whole tumor, since most penetrating tumor vessels originate (and are downstream) from the perpendicular vessels in the tumor margins[19].

The cascade of events starting with activation of endothelial cells with expression of adhesion molecules, influx of activated PMNs, and subsequent injury to the endothelial cells by their products, is suggested by Renard, *et al*[5]. This was based on the finding that the expression of adhesion molecules peaked as early as at 3 h after ILP in sequential biopsies of melanoma and sarcoma in patients. However, Nooijen, et al. found that the expression of the adhesion molecule E selectin was not selective for the tumor vascular endothelial cells and was also observed in the vessels of normal skin distant to the tumor[20]. The induction of E selectin expression is therefore unlikely to be the decisive mechanism behind the selective disruption of tumor vessels. This absence of selectivity is not unexpected since TNFα induces over-

expression of adhesion molecules in normal tissue as shown by others in nontumor bearing animals[21-23]. Correspondingly, in normal tissue sensitivity to TNFα in the induction of HN has been described, provided that the tissue is presensitized by local injection of endotoxin (Local Schwartzmann reaction)[24-26].

We are alluded to the idea that the tumor is such a sensitized site, possibly due to the angioneogenic and inflammatory events at the tumor site[27].

North, *et al.* showed in an immunogenic tumor model, using T-cell-depleted mice, that the progression of HN was partially blocked, which indicates that T cells at least partially mediate HN[28]. In contrast, we used a nonimmunogenic tumor model, closer to the clinical situation. In our model the role of a T-cell-mediated response is expected to be less relevant, which is confirmed by the presence of an infiltrate, predominantly consisting of granulocytes.

Since thrombus formation and vascular injury are common findings in tumors treated with TNFα, we investigated the number and function of platelets in rats that underwent TBI versus nonirradiated rats. Thrombus formation with fibrin deposition is observed selectively in the tumor at 4 h after TNFα administration in several transplantable tumor models in rodents[11-13,29]. This is suggested to be due to the selective induction of procoagulant factors by TNFα on the angioneogenic endothelium[29,30]. However, in some studies no abrogated TNFα-induced tumor regression was found when thrombus formation was suppressed by heparin[13,31]. Accordingly, in our ILP model, tumor regression after TNFα + melphalan ILP could be found despite the administration of heparin and the absence of fibrin deposition.

Within 24 h after ILP, thrombocyte aggregation could be observed in our model, similar to the findings of Renard, *et al*[32]. Well-known clinical syndromes as thrombocytopenic purpura and hemolytic uremic syndrome are characterized by vascular damage due to platelet aggregation[33].

Furthermore, in a cerebral malaria mouse model platelet interaction with the endothelium accounts for the vascular injury, as evidenced by platelet depletion experiments, using monoclonal antibodies[34].

However, in both patients as *in vivo* experiments after TNFα containing ILP, thrombocyte aggregation was preceded by hemorrhage and edema, suggesting that thrombocyte aggregation is rather a result than a cause of endothelial injury. Together with the fact that we found no significant platelet depletion with slight thrombocytopathy after TBI, it is unlikely that the decreased tumor response after TBI is mediated by platelets.

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CHAPTER 6

TOXICITY AND ANTITUMOR ACTIVITY OF INTERFERONγ ALONE AND IN COMBINATIONS WITH TNFα AND MELPHALAN IN ISOLATED LIMB PERFUSION IN THE BN175 SARCOMA TUMOR MODEL IN RATS

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Abstract

Isolated limb perfusion (ILP) with TNFα, melphalan (M), and IFNγ results in high tumor response rates in patients with soft tissue sarcomas, melanomas and other tumors. IFNγ can act synergistically in combination with TNFα but in clinical studies this has not been properly investigated. In the BN175 rat sarcoma limb perfusion model we investigated the role of IFNγ. There were 8 different treatment groups: (I) sham ILP (n=9); (II) IFNγ alone (n=10); (III) TNFα 50 μg (n=9); (IV) TNFα + IFNγ (n=6); (V) melphalan 40 μg (n=11); (VI) M + IFNγ (n=6); (VII) TNFα+M (n=27); (VIII) TNFα + M + IFNγ (n=9). Tumor response and hindlimb function were analyzed. In group I-VI no tumor regressions were observed at 5 days after ILP. ILP with TNFα+M was highly effective response rate (RR) of 73%; Complete Response (CR) rate 55%), very similar to RR in patients. Addition of IFNγ increased the RR by 16% to 89% and the CR rate by 23% to 78%. This difference was not statistically significant. When IFNγ was added to TNFα or TNFα+M it increased limb toxicity significantly ($p < 0.05$ and $p < 0.005$). Since such regional toxicity has not been observed in patients while similar increases in tumor response rates have been reported with IFNγ it is of importance to define the role of IFNγ in the clinical setting.

Introduction

When Liénard and Lejeune reported on the initial experiences with isolated limb perfusion (ILP) with TNFα + IFNγ + melphalan[1,2] it was clear that the 91% complete response (CR) rate obtained in patients with multiple in transit melanoma metastases indicated a considerable improvement over the 50% CR rate that is reported with ILP with melphalan alone[3]. The application of TNFα in ILP in patients with locally advanced soft tissue sarcoma represents a major improvement in the management of patients normally deemed to undergo amputation of the limb. The large experience with TNFα-containing ILPs has clearly demonstrated to result in major tumor response and limb salvage rates of over 80 %[4,5].

IFNγ may enhance the TNFα antitumor effect since it increases the number of TNFα-receptors on malignant cells[6,7]. Both *in vitro* and *in vivo* murine tumor models as well as human tumor xenograft models in nude mice have shown synergistic antitumor effects of TNFα and IFNγ[8-10]. The relatively low dose of 0.2mg of IFNγ used in the early clinical protocols was based on the assumption that synergy with TNFα might be achieved through low dose priming with IFNγ possibly as a result of the upregulation of TNFα-receptor expression by IFNγ[1]. Pretreatment with IFNγ may prime the vascular bed of the tumor and enhance it's sensitivity to TNFα. The neo-vasculature of the tumor is a major target for TNFα[11-13]. TNFα is vasculotoxic at high concentrations[14]. Asher and coworkers already pointed out that in murine tumor models up to 50-fold of the maximum tolerated dose of recombinant human TNFα in humans was needed to observe consistently tumor regressions in mice[15]. This kind of a gap can be overcome successfully in the isolated limb perfusion setting, where concentrations can be achieved more than 50 fold the maximum tolerated dose after systemic administration[16]. This dramatic vasculotoxic effect is clearly demonstrated in patients with highly vascularized soft tissue sarcomas where angiographies before and after ILP clearly show the selective destruction of the tumor associated vascular bed[17]. In the clinical situation it has not been possible so far to properly investigate the role IFNγ. We recently demonstrated synergy between TNFα and melphalan in ILP models with the BN175 soft tissue sarcoma in BN rats as well as with the ROS-1 osteosarcoma in WAG rats[18-20]. The aim of the present study was to investigate the role of IFNγ in combination with TNFα and melphalan in ILP.

Material and methods

Animals

Male inbred BN rats, weighing 250-300 g, obtained from Harlan-CPB (Austerlitz, the Netherlands) were used. The rats were fed a standard laboratory diet (Hope Farms Woerden, the Netherlands). They were housed under standard conditions of light and accommodation. The experimental protocols adhered to the rules outlined in the "Dutch Animal Experimentation Act" (1977) and the published "Guidelines on the protection of Experimental Animals" by the council of the E.C. (1986). The protocol was approved by the committee on Animal Research" of the Erasmus University Rotterdam, the Netherlands.

Tumor

The spontaneous BN 175 sarcoma (transplantable to BN rats) was used[21,22] and implanted subcutaneously in the flank and passaged serially. BN 175 sarcoma is a rapidly growing and metastasizing tumor. As determined by the immunization-challenge method of Prehn and Main[23], the BN 175 sarcoma is nonimmunogenic.

Drugs

Melphalan: melphalan (Alkeran, 50 mg per vial, Wellcome, Beckenham, United Kingdom) was diluted in 10 ml diluent solvent. Further dilutions were made in 0.9 % NaCl to give a volume of 0.2 ml in the perfusion circuit. In all experiments a dose of 40 μg melphalan was used, since this dose when given alone inhibits tumor growth, but induces tumor regression when combined with TNFα, making it the appropriate dose in synergy studies.

TNFα: Recombinant human TNFα was provided by Boehringer (Ingelheim, Germany) having a specific activity of 5.8×10^7 u/mg as determined in the murine L-M cell assay[24]. Endotoxin levels were < 1.25 endotoxin units (EU) per mg protein. In all ILP experiments a dose of 50 μg of TNFα was used, since this dose is not effective when used alone, but shows tumor regression when combined with melphalan in ILP.

IFNγ: Recombinant rat IFNγ (rRIFNγ) was provided by ITRI-TNO, Rijswijk, The Netherlands). The protein was prepared from cultures of a transformed chinese hamster ovary cell line carrying the relevant gene encoding for rat IFNγ. Details about the methods of production and purification have been described elsewhere[25]. In clinical ILP trials, patients

received 0.2 mg or 1.5 x 10^6 u (= 2 x 10^4 u/kg) recombinant human IFNγ s.c. two and one day prior to the ILP. During perfusion, IFNγ was added in the same dose as a bolus. In the rat ILP model, the same regimen was performed, using a dose of 5 x 10^4 (= 2 x10^5 u/kg). A dose of 5 x 10^5 u/kg inhibited tumor growth in a rat tumor model, when given daily systemically[26].

Tumor model

Fragments (3-5 mm) of the BN 175 sarcoma were implanted into the right hind limb subcutaneously just above the ankle. Perfusion was performed at a tumor diameter approximately of 15 mm at about 7 days after implantation. Subsequent tumor growth was recorded by caliper measurement. The mean of two perpendicular diameters was obtained. Tumor diameters were measured at least three times weekly.

The classification of tumor response was: progressive disease (PD) (increase in tumor diameter more than 25 % within 4 days; no change (NC) (tumor diameter equal to diameter during perfusion range - 50 % to + 25 %)); partial remission (PR) (decrease in tumor diameter of less than - 50 %; complete remission (CR) (no tumor palpable)).

Isolated Limb Perfusion (ILP)

The perfusion technique we used has been published previously[17]. Briefly, animals were anaesthetized with Hypnorm (Janssen Pharmaceutica, Tilburg, the Netherlands) and 50 i.u. of heparin was injected i.v. to prevent coagulation in the collateral circulation and in the perfusion circuit. To keep the rat's hind leg at a constant temperature of 38 - 39°C, a warm water mattress was applied. The femoral artery and vein were cannulated with silastic tubing (0.30 mm inner diameter, 0.64 mm outer diameter; 0.64 inner diameter, 1.19 mm outer diameter respectively, Dow Corning, Michigan, USA). Collaterals were occluded by a groin tourniquet and isolation time commenced when the tourniquet was tightened. An oxygenation reservoir and a roller pump were included into the circuit. The perfusion commenced with 5 ml Haemaccel (Behring Pharma, Amsterdam, the Netherlands) and the haemoglobin (Hb) content of the perfusate was 0.9 mmol/l. Melphalan and TNFα were added as boluses to the oxygenation reservoir. A roller pump (Watson Marlow, Falmouth, UK; type 505 U) recirculated the perfusate at a flow rate of 2.4 ml/min. A washout with 2 ml oxygenated Haemaccel was performed at the end of the perfusion. In the rat, collateral circulation via

the internal iliac artery to the leg is so extensive that it allows ligation of the femoral vessels without detrimental effects and after ligation of the femoral artery backflow from the femoral vein was seen in all rats immediately after release of the tourniquet. The partial pressure of oxygen (PaO_2) was similar after ligation of the femoral vessels[26].

In vivo tumor response studies

Eight treatment groups were studied: group I, sham perfused rats (n= 9); group II, pre-treatment and perfusion with IFNγ only (n=5); group III, TNFα perfusion (n=9); group IV, TNFα combined with IFNγ (n=6); group V, perfusion with melphalan (n=11); group VI, melphalan-IFNγ treatment (n=6); group VII, TNFα + melphalan combination (n=27); group VIII: triple combination of TNFα + melphalan + IFNγ (n=9).

Hind-limb function score

The gait of treated rat was " clinically " observed 5 days after perfusion and a score on a scale 0-3 was used to quantify the rat's ability to walk. On this scale 0 = the rats drags its hindlimb without any function; 1 = heavily impaired function of the leg; 2 = slightly impaired function; 3 = intact function of the hind-leg (when walking).

Statistical analysis

To compare tumor diameters in different animal groups Student's *t*-test (ST) were used. Mann Whitney *U* test (MW) was performed in the evaluation of limb function score of the different treatment groups.

Results

Tumor response study after ILP with TNFα and/or melphalan without IFNγ

Results are summarized in table 1 (uneven groups). Sham perfusion (group I) and perfusion with TNFα (group III) did not inhibit tumor growth and PD was observed in all rats in both groups. After perfusion with melphalan (group V) tumor growth was arrested in 11/11 animals (NC). Conform our previous study which demonstrated the synergism between TNFα and melphalan, this combination treatment was highly effective in this study with and

overall response rate of 73% and a complete response rate of 55%.

Tumor response after IFNγ containing ILPs

Results are summarized in table 1 (even groups). Perfusion with IFNγ (group II) as a single agent induced PD in 3/10 and NC in 7/10 rats. In contrast with sham or TNFα groups, IFNγ arrested tumor growth ($p < 0.001$ with sham group). This growth inhibiting ability seemed less strong and of shorter duration than what is observed after an ILP with melphalan alone, although no statistical significance existed between both groups (n.s. between group II and V). The TNFα-IFNγ group (group IV), showed 1/6 PD and 5/6 NC, (n.s. with group 2 and $p < 0.05$ with group III). The melphalan-IFNγ group (group VI) showed 6/6 NC (n.s with group 2 and 5).

Group	PD	NC	PR	CR	Total	T.D. (sem) mm
I. Sham ILP	9				9	24.0 (1.4)
II. IFNγ (I)	3	7			10	17.6 (0.8)
III. TNFα (T)	9				9	21.0 (0.9)
IV. T + I	1	5			6	17.7 (0.9)
V. Melph. (M)		11			11	16.0 (0.8)
VI. M + I		6			6	16.0 (0.9)
VII. T + M		3	2	6	11	4.3 (2.2)
VIII. T + M + I		1	1	7	9	3.1 (2.1)

Table 1. Tumor response and mean Tumor Diameter (T.D.) and Standard Error of the Mean (sem) in millimeters (mm) at 5 days after ILP.

Figure 1 shows the nearly similar growth curves of group V and VI. Addition of IFNγ to the combination of TNFα-melphalan did improve the response rates (RR:89%; CR78%) but the difference was not statistically significant : 1/9 NC, 1/9 PR and 7/9 CR. In Figure 1 the growth curves of group VII and VIII are shown (Figure 1).

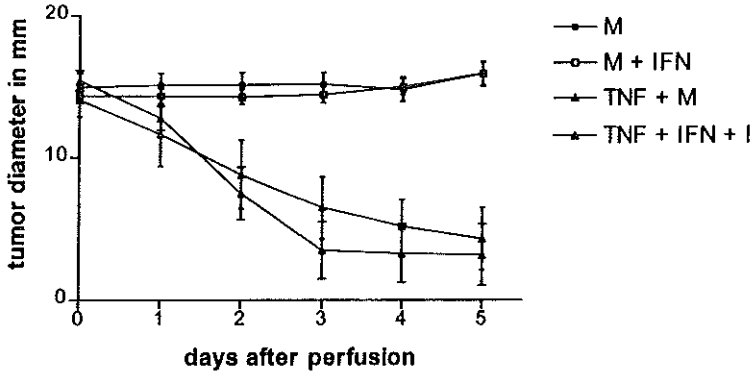

Figure 1. Tumor growth curves (± s.e.m.) of groups (V) melphalan; (VI) melphalan + IFNγ; (VII) TNFα + melphalan and (VIII) TNFα + IFNγ + melphalan. In the groups I-IV no tumor regressions were observed (not depicted).

Hind-limb function score

Results of hind limb function are shown in table 2. Treatment with IFNγ alone only slightly reduced hind-limb function (n.s with group I). Toxic effects of IFNγ were not observed in the combination melphalan-IFNγ (n.s. with group V). Combining IFNγ with TNFα (Group IV), resulted in significant enhancement of regional toxicity compared to ILP with TNFα alone ($p < 0.05$, with group III). Addition of IFNγ to the combination of TNFα-melphalan (Group VIII) resulted in even more toxicity ($p < 0.005$ with group VII).

Group	Limb function	Median of limb function
I Sham	0,2,3,3,3,3,3,3,3	3
II IFNγ (I)	0,0,2,2,2,3,3,3,3,3	2.5
III TNFα (T)	1,2,3,3,3,3,3,3,3	3
IV T+I	0,0,0,1,3,3	0.5
V Mel (M)	1,3,3,3,3,3,3,3,3,3	3
VI M+I	3,3,3,3,3,3	3
VII T+M	0,0,1,1,2,2,3,3,3,3,3	2
VIII T+M+I	0,0,0,0,0,0,0,1	0

Table 2. Limb function in different groups on a scale of 0-3: 0 = no function of the limb, 1= heavily impaired limb function, 2= slightly impaired function and 3 = no impairment of limb function.

Discussion

In this series of experiments in the BN175 soft tissue sarcoma model, in which we have previously demonstrated similar responses after TNFα-based ILPs as are observed in the clinical setting in sarcoma patients[4,5], both with respect to response rates as well as with respect to histologic observations[18,28,29], we confirmed the synergistic activity between TNFα and melphalan as the dominating and crucial element that determines the efficacy of this procedure. This requirement for melphalan and the potentiation of its effects we believe to be mediated in part by the immediated increase of permeability caused by TNFα[29] and by the TNFα mediated drop in intratumoral interstitial pressure, as reported by Kristensen and coworkers[30]. These mechanisms result in a better penetration of the tumor by melphalan and we recently did find proof of this as we could demonstrate significantly increased intratumoral concentrations of doxorubicine after ILP with TNFα + doxorubicin as compared to ILP with doxorubine alone in our BN175 as well as our ROS-1 ILP models in rats (Ten Hagen, manuscript in preparation).

In our experiments presented here we tested whether IFNγ plays a significant role in the possibility to further enhance antitumor effects of this therapy with TNFα+M based ILP. We found that treatment with IFNγ alone induced significant retardation of tumor growth with a slight but not significant decrease in hind limb function. This observation is of some interest as IFNγ alone has never been tested in the clinical ILP setting. Two important observations are made in this study in rats: 1) Once IFNγ is added to TNFα significant and severy regional toxicity occurs with major impairment of limb function. This also occurs without any clear increase in response rate when melphalan is left out; 2) Addition of IFNγ does increase the response rates as observed with the combination of TNFα + melphalan. The increase in local toxicity however is far greater (and highly significant) than the small increase in response rate (overall response T+M 73% vs TIM 89%; CR rate TM 55% vs TIM 78%). The combination IFNγ-melphalan did not show enhanced toxicity.

The fact that IFNγ only aggravated toxicity in combination with TNFα is highly interesting and are reminiscent of synergistic toxic effects between TNFα and IFNγ, when administered systemically as previously reported by Talmedge and collegues[30]. The mechanism of toxicity was partly thought to be due to the induction of arachidon metabolites, which induces multi-focal thrombi, leading to ischemic necrosis. This increase in regional toxicity however has

not been observed in patients treated bij the triple combination of T+I+M[1,2,4]. Furthermore the observation published very recently by Ruegg and coworkers is of considerable interest. They have shown in vitro as well as in vivo that the vasculotoxic effect of TNFα is significantly enhanced by IFNγ. The combination of TNFα + IFNγ leading to apoptosis of the endothelial cells and detachtment of the endothelial cells from the vessel wall[32].

Where the interaction of TNFα + melphalan is the dominant factor in obtaining the antitumor effects it is of interest however that when one analyzes the reports on T+I+M vs T+M in patients with melanoma or sarcoma that a roughly 10% higher complete response rate and overall response rate is reported for the T+I+M combination. Liénard recently reported the outcome of a randomized phase II trial in 64 melanoma patients with multiple in transit metastases. A 78% CR rate was observed with the T.I.M. regimen versus a 69% CR rate after ILP with T+M. Moreover a similar 10% difference has been observed with soft tissue sarcomas. Eggermont and coworkers reported a 36% CR rate in the first 55 sarcoma patients, all treated with T+I+M ILPs whereas in the 131 patients treated with T+M ILPS a 26% CR rate was observed. Again a drop of 10% was noted. This is in line with the results of the study reported here in rats bearing the nonimmunogenic BN175 soft tissue sarcoma. What is of considerable importance here is that high dose IFNγ in combination with TNFα has not been tested, not in the rat model because of restricted availability of rat rIFNγ, nor in the clinical setting. In our rat model it seems of little interest as regional toxicity of even low dose of IFNγ was already very severe. But given the fact that IFNγ alone did have some antitumor activity (NC in 7/10 rats) and given the fact that IFNγ in the clinical setting was not associated with increased regional toxicity it must be concluded that the role of IFNγ merits further studies in the clinical setting. A 10% increase in complete remissions is in the absence of increased toxicity is obviously of clinical importance. Moreover the effects of higher doses of IFNγ should be investigated.

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CHAPTER 7

ISOLATED LIMB PERFUSION WITH TNFα AND MELPHALAN IN A RAT OSTEOSARCOMA MODEL: A NEW ANTITUMOR APPROACH

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Abstract

Isolated limb perfusion (ILP) with TNFα, IFNγ and melphalan causes impressive tumor reduction in patients with irresectable soft tissue sarcomas with a high limb salvage rate. Since this therapy could be of value in patients with progressive osteosarcoma, we performed a study in an osteosarcoma tumor model in the rat.

The ROS-1 osteosarcoma was implanted s.c. in the hind leg of WAG rats. Rats were divided in four groups: Rats that underwent ILP with perfusate alone, TNFα alone, melphalan alone or their combination.

Almost all rats, treated with a sham ILP or a perfusion with 40 μg melphalan showed progressive disease (PD) (6/6 and 5/6). After perfusion with 50 μg TNFα alone a varied response was observed: 2/6 PD, 2/6 no change (NC) and 2/6 a complete remission (CR). After combined perfusion: 3/6 rats had a partial remission and 3/6 a CR.

The best and most consistent responses are obtained by combining TNFα and melphalan. The discrepancy with the *in vitro* sensitivity of ROS-1 indicates that indirect effects are important in this tumor model.

Introduction

Osteosarcoma is a rare tumor (1 to 2 cases per million each year) and occurs mainly in patients in the second decade of life. Despite its rarity, osteosarcoma has attracted much attention, since pre- and post-operative chemotherapy increases survival rate considerably in patients with primary osteosarcoma[1,2]. Tumor control of the primary tumor by preoperative chemotherapy allows more conservative surgery[3] and the degree of tumor necrosis is an important prognostic factor[4]. Progressive disease in spite of chemotherapy is associated with a poor prognosis both with respect to local tumor control as well as with respect to survival. In most of these "lost cases" ablative surgery is needed with no hope for cure.

In these patients isolated limb perfusion (ILP) with TNFα, IFNγ and melphalan could be very beneficial, since this therapy often converts large tumors into necrotic, shrunken tumor remnants that can be resected at little functional cost of the extremity. This has been demonstrated for irresectable extremity soft tissue sarcomas (STS)[5-7]. Rendering large tumors of the extremities resectable by loco-regional therapy has not only been described for STS but also for osteosarcoma: Vaglini, *et al.* reported that large osteosarcoma became resectable after hyperthermic-antiblastic perfusion in combination with intraarterial and intravenous chemotherapy in 11/18 of the patients[8]. ILP with TNFα, IFNγ and melphalan is less complex and avoids systemic administration of cytostatic agents and therefore needs to be evaluated in patients with irresectable osteosarcoma.

In a previous study in rats we found that ILP with a combination of TNFα and melphalan was effective against an aggressive soft tissue sarcoma in the Brown Norway ILP-rat model[9]. The aim of the present study is to investigate whether these effects can be found in the ROS-1 osteosarcoma tumor model. In addition, we are interested in the role of direct cytotoxicity of the agents, either alone or combined. The presence of indirect effects of both agents in this tumor model may provide a rationale to use this combination therapy in clinic, despite chemoresistance, which is principally based on direct cytotoxic effects only.

Material and methods

Animals

Male inbred WAG-Rij strain rats, weighing 250-300 g obtained from Harlan-CPB (Austerlitz, the Netherlands) were used. The rats were fed a standard laboratory diet delivered by Hope Farms (Woerden, The Netherlands) and kept under standard laboratory conditions of light and accommodation. 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 E.C. (1986). The protocol was approved by the "Committee on Animal Research" of the Erasmus University Rotterdam, The Netherlands.

Tumor

The ROS-1 osteosarcoma (transplantable to WAG/Rij rats) was used. This osteosarcoma originated spontaneously in the tibia of a rat[10]. Cells from this tumor were maintained in tissue culture and from these cultures new tumors were produced by inoculation in the flank.

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 0.2 ml in the perfusion circuit.

TNFα

Recombinant human TNFα was provided by Boehringer (Ingelheim, Germany) with a specific activity of 5.8×10^7 u/mg as determined in the murine L-M cell assay[11]. Endotoxin levels were < 1.25 endotoxin unit per mg protein.

Tumor model

Fragments of 3-5 mm were implanted in the right hind limb s.c. just above the ankle. Perfusion was performed at a tumor diameter approximately of 15 mm at least 7 days after implantation. Tumor growth was recorded by calliper measurement. The mean of two perpendicular diameters was obtained. Tumor diameters were measured at least three times a week.

Isolated limb perfusion

We used a perfusion technique originally described by Benckhuijsen, *et al.*[12] with some modifications[13]. Briefly, Hypnorm (Janssen Pharmaceutica B.V., Tilburg, the Netherlands) was given for anesthesia and 50 i.u. of heparin was injected i.v. To keep the rat's hind leg at a constant temperature of 38-39°C, a warm water mattress was applied around the leg. Temperature was monitored by temperature probe (Ellab, Copenhagen, type DU-3) fixed at the convexity of the tumor. The femoral artery and vein were approached through an incision parallel to the inguinal ligament. Collaterals were temporarily occluded by the application of a tourniquet in the groin. The tourniquet was fixed at the inguinal ligament. The femoral artery and vein were cannulated with silastic tubing (0.30 mm inner diameter, 0.64 mm outer diameter; 0.64 mm inner diameter, 1.19 mm outer diameter respectively, Degania Silicone, Degania Bet, Israel). Isolation time of 30 minutes commenced when the tourniquet was tightened. An oxygenation reservoir and a roller pump were included into the circuit. The perfusion commenced with 5 ml Haemaccel (Behring Pharma, Amsterdam, the Netherlands) and the haemoglobin (Hb) content of the perfusate was 1.45 g/dl (0.9 mmol/l). Melphalan and TNFα were added as boluses to the oxygenation reservoir. The roller pump (Watson Marlow, Falmouth, UK; type 505 U) recirculated the perfusate at a flow rate of 2.4 ml/min. A washout of 2 ml oxygenated Haemaccel was performed at the end of the perfusion. The collateral circulation via the internal iliac artery towards the leg is so extensive that it allows ligation of the femoral vessels without detrimental effects. Despite ligation of the femoral artery, backflow from the femoral vein is seen in all rats immediately after release of the tourniquet. Moreover, in a previous study we measured the partial oxygen pressure (PaO_2) which was similar after ligation of the femoral vessels to that prior to perfusion[13].

Tumor response studies

The limbs of 24 rats were perfused. There were 4 experimental groups: sham perfusion (n=6), perfusion with 50 μg TNFα (n=6), 40 μg melphalan perfusion (n=6) and a perfusion with both 40 μg melphalan and 50 μg TNFα (n=6). The concentrations used are equivalent to those used in an efficacy study against the BN 175 fibrosarcoma in the BN rat[9]. Since systemic administration of TNFα in clinic is limited by severe toxicity, it makes no sense to perform i.v. dose-effect studies in the rat.

The classification for tumor response was: progressive disease (PD), increase of tumor

diameter > 25 % within 10 days; no change (NC), tumor diameter equal to diameter during perfusion ± 25 %; partial remission (PR), > 25 % decrease of tumor diameter; complete remission (CR), no palpable tumor.

In vitro assessment of antitumor activity

We determined the *in vitro* sensitivity of ROS-1 osteosarcoma for melphalan and TNFα. This cell line grows as a monolayer in Dulbecco's modified Eagle's medium supplemented with 5 % fetal calf serum and glutamic acid (0.3 Mm), all obtained from Gibco (Paisley, UK), in a humidified atmosphere of CO_2/ air (5:95) at 37°C. We used the sulphorhodamine B (SRB) protein stain assay according to the method of Skehan, *et al*[14]. Briefly, cells were isolated from cultures in exponential growth phase by trypsinization, and counted and plated in 96-well micro titre plates (Costar, Cambridge Mass). Each well contained 100 µl. 24 h after plating, 100 µl culture medium, or culture medium containing drug, was added to the wells. 72 h after drug addition cells were incubated with trichloroacetic acid (200 µl/well) at 4°C for 1 h by means of protein precipitation and washed five times with tap water. The cells were stained for at least 15 minutes with 0.4 % SRB dissolved in 1 % acetic acid and subsequently washed thoroughly with 1 % acetic acid to remove superfluous dye. After drying the plates, the bound protein stain was solubilized with 150 µl 10 mM unbuffered TRIS. The optical density was read at 540 nm. All experiments were performed eight times. Tumor growth was calculated using the formula: tumor growth = (test well/control) x 100 %.

Statistical analysis

Tumor diameters are given as means ± SEM. Differences in results between the treatment groups were tested by Student Newman-Keul's (SNK) test, after one-way analysis of variance.

Results

ILP response studies

In Table 1 the tumor responses of the different groups are summarized. Only in rats that were perfused with TNFα alone or with TNFα in combination with melphalan, was tumor regression observed in 33 % and 100 % respectively. Sham perfusions or perfusions with melphalan alone did not result in tumor regression. In Figure 1 the curves of the mean tumor diameters in the different groups are depicted. Only the group that underwent TNFα + melphalan perfusion showed a statistically significant (SNK test: $p < 0.05$) difference to the group that received sham perfusion at 3-15 days after perfusion.

The recurrence rate was 100 %. Tumors reappeared 7-13 days after perfusion. After recurrence tumors grew as fast as tumors in rats that had received sham perfusion.

Group	PD	NC	PR	CR	CR_{SN}	Total
Sham perfusion	6					6
40 μg melph. (M)	5	1				6
50 μg TNFα	2	2		2		6
M + TNFα			3	3		6

Table 1. tumor response of ROS-1 osteosarcoma after ILP.

In vitro cytotoxicity assay

The dose/response curves of the ROS-1 osteosarcoma cell line to TNFα and melphalan are depicted in Figure 2. ROS-1 appeared to be relatively resistant to TNFα, as evidenced by 60 % growth at even very high concentrations (50 μg/ml) of TNFα. However, ROS-1 was sensitive to melphalan with an IC_{50} at 0.009 μg/ml.

In Figure 3 the dose/response curves of ROS-1 to melphalan is shown in the presence or

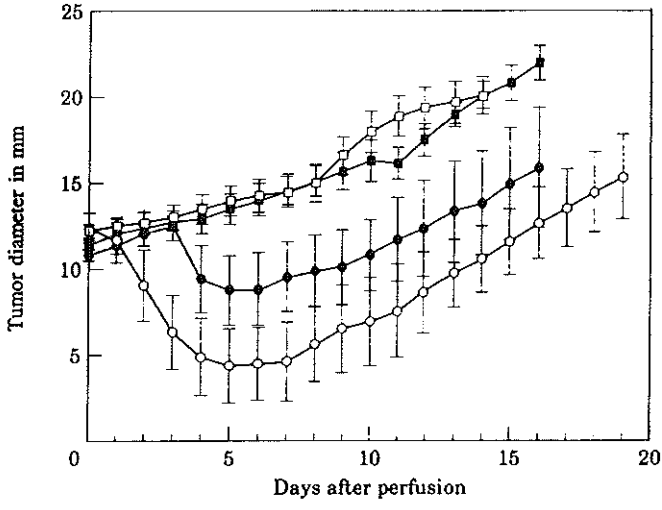

Figure 1. Growth curves of ROS-1 osteosarcoma in the hind limb after sham (n=6, -■-), melphalan 40 μg (n=6, -□-), TNFα 50 μg (n=6, -●-) and melphalan + TNFα treatment (n=6, -○-). The mean (± s.e.m.) of the tumor diameters are depicted. Only statistically significant differences exist between the combined and sham group at day 3-15 after perfusion (SNK test: $p < 0.05$).

absence of different concentrations of TNFα. The maximal growth of ROS-1, shown as a plateau at the lower concentrations melphalan, is reduced in the presence of TNFα in a concentration-dependant manner, which can be explained by addition of effects. The dose/response curves bend towards total growth inhibition at the same dose of melphalan independent of what concentration of TNFα is used. Thus, these experiments could not reveal synergism in direct tumor cytotoxic effects between both agents.

Discussion

The present study demonstrated that an experimental osteosarcoma responded in all rats treated with the combination of TNFα and melphalan in an ILP model. No statistically significant tumor response was noted in groups that were sham perfused or perfused with melphalan alone or TNFα alone. Perfusion with melphalan alone and sham perfusion was

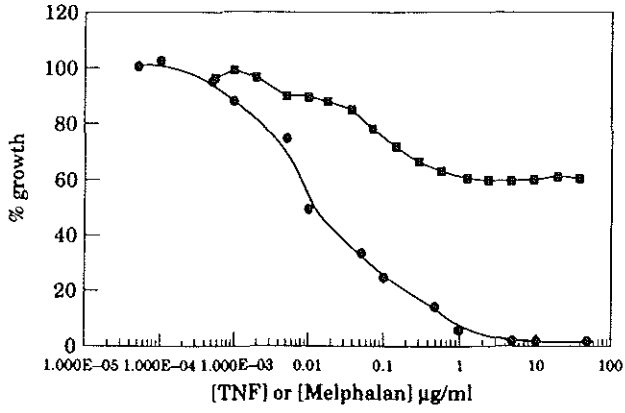

Figure 2. Dose/response curves of ROS-1 osteosarcoma to TNFα (-■-) and melphalan (-●-) determined in the sulphorhodamine B assay. Cell number measured as absorbance in the colorimetric assay is represented as a percentage of the control cell growth.

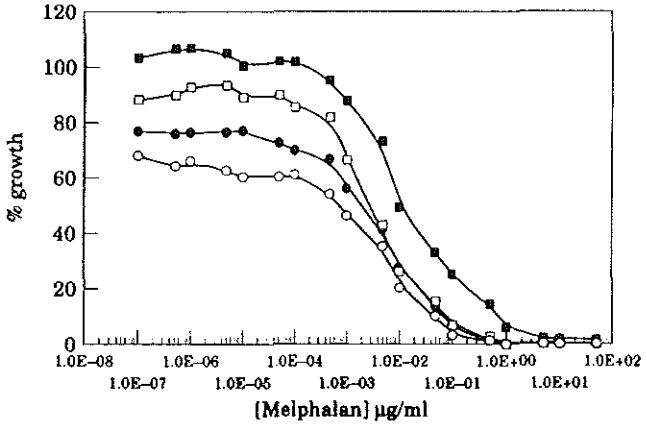

Figure 3. Dose/response curve of ROS-1 osteosarcoma to melphalan in the absence or presence of various concentrations TNFα, determined in the sulphorhodamine B assay (-■- = melphalan (M) only; -□- = M + 0.1 µg/ml TNFα; -●- = M + 1 µg/ml TNFα; -○- = M + 10 µg/ml TNFα).

followed by progressive disease. After ILP with TNFα alone the response varied, but no consistent antitumor activity was observed. In spite of the absence of consistent antitumor effects of TNFα, the combination of TNFα with melphalan showed clear synergistic antitumor activity resulting in a 100 % response rate. These observations are in line with the synergy observed between TNFα and melphalan in the Brown Norway soft tissue sarcoma model[9]. Regarding the varied response to TNFα alone it should be kept in mind that anoxia

in the tumor may be a critical determinant for its propensity to respond to TNFα alone[13,15,16], and thus a variation in size or structure may explain why responses after TNFα alone may vary.

In contrast to the *in vivo* data are the observations *in vitro* show the relative resistance of ROS-1 in culture to TNFα and its sensitivity to melphalan. Furthermore, no synergistic effects were observed in the *in vitro* experiments. Also, in previous studies the lack of correlation between direct tumor-cytotoxicity of TNFα and the *in vivo* tumor response has been shown[17,18]. The opposite picture of the *in vivo* results to the *in vitro* results obtained in the present study makes it clear that indirect, host mediated effects must be important in the tumor response of ROS-1, observed after ILP with TNFα + melphalan.

The effect of TNFα on the neo-vasculature of the tumor has been subject of many preclinical studies[19-21]. The tumor response with evident hemorrhagic necrosis within 24 h is characteristic for TNFα[23,24]. Also in man vascular effects have been associated with the response on TNFα: in patients with soft tissue sarcoma, treated with an ILP with TNFα, IFNγ and melphalan, the tumor response was associated with the angiographic disappearance of the tumor's neo-vasculature[25] together with the histopathologic findings of vascular occlusion and hemorrhagic necrosis[26,27]. In the present study, the tumor regression within 3 days is typical for the TNFα tumor response with a target role of the tumor's neo-vasculature. Also the involvement of the immune system is considered to be important in the TNFα tumor response and believed to be executed by the following mechanisms: i) invasion of PMNs in the acute phase enhances the detrimental effects on the neo-vasculature[27-29] and ii) stimulation of inflammatory cells e.g. lymphocytes results in tumoricidal activity. The latter effect can result in a prolonged tumor control and is only described in immunogenic tumors[30]. Since recurrences were found in all rats that showed tumor regression, we believe that the immune-mediated responses may not play an important role in our ILP model.

The synergism between TNFα and melphalan, observed in our tumor model, first can be explained by an enhancement by melphalan of the TNFα-induced vascular effects, since melphalan may well induce vascular damage, similar as reported for the alkylating agent cyclophosphamide[31]. A second underlying mechanism of the synergism could be an improved penetration of the cytostatic agent in the tumor by the TNFα effects on the tumor's neo-vasculature. In previous studies vascular leakage and an enhanced accessibility of the therapeutical agent has been associated with a better tumor response[32,33]. Both mechanisms

of synergism may well be relevant in the chemoresistant osteosarcoma in man and therefore it seems justified that in a setting of ILP TNFα is combined with melphalan.

To mimic the clinical situation of advanced osteosarcoma, we waited to treat tumors until they were relatively large. In the presence of an ineffective dose of melphalan, TNFα-induced its typical quick tumor response, based on vascular effects. Thus, the concept of targeting neo-vasculature seems also valid for experimental osteosarcoma. Since all large tumors are dependent on their neo-vasculature, irrespective of their histological type, we anticipate that ILP with TNFα and melphalan will be effective in all advanced osteosarcomas and herewith may overcome the problem of chemoresistance. However, the early recurrences observed in all rats is a warning against too high expectations of this combination therapy on the long term, and indicate that ILP with TNFα and melphalan should always be followed by dissection of the shrunken tumor remnants.

Thus, in line with the experience in patients with locally advanced soft tissue sarcomas, ILP with TNFα and melphalan could be of great therapeutical benefit in patients with chemoresistant osteosarcoma to obtain local tumor control.

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CHAPTER 8

ISOLATED LIMB PERFUSION WITH TUMOR NECROSIS FACTORα AND MELPHALAN FOR UNRESECTABLE BONE SARCOMAS OF THE LOWER EXTREMITY

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Abstract

Isolated limb perfusion (ILP) with recombinant tumor necrosis factor -alpha (rTNFα) and melphalan has recently been reported to induce major tumor responses and results in limb salvage in over 80% of patients with unresectable soft tissue sarcomas of the extremities. We investigated whether TNFα-based ILP could avoid amputation of limbs in patients with primary or recurrent sarcomas of osteogenic origin that had failed chemotherapy or where chemotherapy was refused and where amputation of the limb was imminent.

From August 1992 to December 1997, we employed this technique in 13 patients with unresectable bone sarcoma of the lower extremity, who were candidates for amputation. The aim was to reduce tumor size and allow the performance of limb-sparing surgery (LSS) or to just obtain local control in the limb in the presence of metastatic disease at visceral sites. There were no surgical complications. Following ILP, only 1 patient experienced significant systemic side effects related to the procedure, and LSS was subsequently performed in 7/13 patients. Three patients were not operated due to advanced metastatic disease and three patients underwent an amputation due to local disease progression.

ILP with rTNFα and melphalan represents an option that can lead to limb salvage in patients with locally advanced sarcomas of osteogenic origin that have failed standard treatment options. It merits further evaluated to determine its potential role in the treatment of unresectable bone sarcomas of the (lower) extremities.

Introduction

The treatment of primary bone sarcomas consists of two elements: systemic (intravenous or intraarterial) neoadjuvant chemotherapy, and wide resection of the tumor with free margins achieved by LSS or amputation[1-3]. Bone sarcomas are considered unresectable, thus necessitating amputation, according to the following criteria: major neurovascular bundle involvement; extensive multicompartmental involvement; displaced pathologic fracture; inappropriate biopsy site; significant infection at the planned resection site and immature skeletal age[2].

When a bone sarcoma is considered unresectable due to its dimensions or involvement of the neurovascular bundle, intraarterial chemotherapy may induce further tumor necrosis and reduction in size, thus increasing the chance of limb salvage[4,5]. Intraarterial administration of chemotherapy allows a higher concentration of the cytotoxic agent to be delivered to the primary tumor site. Although chemotherapeutic agents are injected regionally, the modality is considered systemic, since after reaching the tumoral mass the drugs are drained via the venous system and distributed systemically[6,7]. To achieve even higher tumoral drug concentration, intraarterial tourniquet infusion and chemoembolization have been employed, albeit with limited success[8,9].

ILP using an extracorporeal heart-lung machine can achieve near total isolation of the affected extremity, and allows the infusion of high doses of cytotoxic drugs that are 6 to 20 times higher than the maximally tolerated systemic dose, with minimal systemic leak and virtually no systemic toxicity[10]. ILP with melphalan alone results in a complete response rate of about 50% in the treatment multiple in transit malignant melanoma metastases[10] and since its combined use with tumor necrosis factor-alpha (TNFα) complete response rates as high as 80-90% have been reported[12]. The use of TNFα has had a much greater impact in the management of soft tissue sarcoma because of its efficacy to achieve limb salvage. Major tumor responses and limb salvage rates of 80%-90% have been reported in large series of patients in which the large majority were originally scheduled for an amputation[13-15]. For this indication TNFα is now submitted for registration in Europe as in locally advanced soft tissue sarcoma the addition of TNFα has brought about a new situation and has changed the poor results of ILP with cytostatic drugs alone which yield reponse rates below 30%[16,17]. The objective of the perfusion, which is a local adjuvant with minimal to no effect on metastatic

disease, is to enable LSS in patients with an unresectable tumor that could otherwise be treated only by amputation or at the cost of unacceptable functional morbidity. We report thirteen patients with bone sarcomas of the lower extremity who met the criteria for immediate amputation and underwent ILP with rTNFα and melphalan in order to allow LSS.

Materials and Methods

From August 1992 to December 1997, thirteen patients with bone sarcomas of the lower extremity who were candidates for immediate major amputation underwent hyperthermic ILP with rTNFα and melphalan. Patients were treated at the Tel-Aviv Sourasky Medical Center, Tel-Aviv, Israel and the University Hospital Rotterdam - Dr Den Hoed Cancer Center , The Netherlands. The treatment protocol which was written for limb salvage indication in patients with locally advanced soft tissue sarcomas was approved by institutions' Helsinki Ethics Committees. In these patients with imminent amputations because of failing treatments of their sarcomas of bone origin the soft tissue protocol was used for its limb salvage indication on a compassionate basis and informed consent was obtained from each patient. There were 7 males and 6 females, ranging in age from 14 to 83 years (average, 44.4 years). Of the 13 tumors, 6 were primary bone sarcomas, four were metastatic bone sarcoma to the lower extremity, and three were locally recurrent lesions. The only option to achieve local control in these patients were amputations as an alternative to TNFα-based ILP. Table 1 summarizes the histopathological diagnosis, anatomical location, surgical stage[18], prior and subsequent treatments of the 13 patients.

All patients in whom the diagnosis was made correctly at the initial presentation received standard chemotherapy except for three patients (patients # 6, 7 and 12). These (elder) patients refused chemotherapy and were only interested in a local procedure to get local control. In patients number 5 and 13 the initial diagnosis and treatments were done outside the center. In order to avoid imminent amputation an ILP was performed for recurrent disease. Upon revision of the diagnosis chemotherapy after ILP was offered in addition to these patients. The patients with metastatic disease from osteosarcomas (patients # 2,3,4) all had multiple chemotherapies, amputations of the limb where the tumor originated as well as resections of lung metastases (patient #2).

Pat. number	Age	Sex	Diagnosis	Anatomical location	Surg. Stage	Prior Therapies	Subsequent Therapies
1	29	F	Osteosarcoma	distal femur	IIB[3]	Chemotherapy	LSS
2	20	M	Osteosarcoma	whole leg mult metastases	III [2]	chemotherapy resection lung mets	LSS
3	23	F	Osteosarcoma	mid thigh metastases	III [2]	chemotherapy amputation other leg	LSS Chemotherapy
4	20	F	Osteosarcoma	metastases prox thigh /hip	III [2]	chemotherapy amputation lower leg	hemipelvectomy
5	69	M	Osteosarcoma	foot	IIIB [1]	initial diagnosis MFH	No Surgery Chemotherapy Radiation Th.
6	83	M	Extraskeletal osteosarcoma	proximal thigh	IIB [3]	initial diagnosis MFH	LSS refused chemotherapy
7	74	F	Osteosarcoma	distal femur	IIIB [1]	refused chemotherapy	none
8	50	M	Extraskeletal osteosarcoma	calf	IIB [1]	chemotherapy	LSS
9	14	F	Ewing's sarcoma	proximal femur	IIB [1]	chemotherapy	LSS Chemotherapy
10	30	M	Ewing's sarcoma	midshaft femur	III [2]	chemotherapy	Amputation
11	49	F	Malignant fibrous histiocytoma of bone	distal femur	IIB [3]	chemotherapy	LSS Amputation
12	63	M	Malignant fibrous histiocytoma of bone	distal femur	IIB [1]	refused chemotherapy	LSS
13	54	M	Chondrosarcoma	fibula	IIB [3]	resection + irradiation	chemotherapy

[1] Primary bone sarcoma
[2] Metastatic bone sarcoma
[3] Local recurrence after previous resection of a primary bone sarcoma

Table 1: Diagnosis, anatomical Location and surgical Staging of the thirteen patients treated for unresectable bone sarcomas

ILP with rTNFα and Melphalan

The ILP technique has been described in detail elsewhere[13]; isolation of the blood circuit of the lower extremity was achieved by clamping the major artery and vein, ligating the collateral vessels, and by applying a tourniquet to compress the minor vessels in cutis, subcutis, and muscles. The arterial and venous cannulae were connected to a heart-lung machine, and after being placed in a thermic cover, the limb was perfused with oxygenated

heparinized blood.

While limb temperatures being maintained between 39-40°C, rTNFα (Boehringer, Ingelheim, Germany) was given as a 4 mg bolus injection into the arterial line proximal to the oxygenator. Thirty minutes later, melphalan (Alkeran®, Burroughs Wellcome, London, UK) 10 mg / L leg volume were administered and the limb was perfused for additional 60 minutes. Overall, the limb was perfused for 90 minutes, with flow rates 40 ml / min x L leg volume.

To assess systemic leakage, Tc^{99m} human serum albumin (0.5 m Bq/kg) was injected into the systemic circulation and a 10-fold higher dose of the same isotope was infused into the isolated extremity. Continuous monitoring was performed with a precordial scintillation probe. The extent of systemic leakage was expressed as a percentage (100% leakage representing a homogeneous distribution of the isotope in the body).

At the end of ILP, the limb was washed with at least 1 L of polygeline (Haemaccel®, Marburg, Germany) and 1 L of 6% dextran (Macrodex®, Pharmacia, Uppsala, Sweden). Postoperatively, early mobilization was encouraged, and patients were asked to wear elastic stockings for one month to minimize swelling.

Systemic toxicity was graded using system established by the Eastern Cooperation Oncology Group - World Health Organization[19]. Regional toxicity was graded according to the system developed by Wieberdink et al.[20]: 1 = No toxicity; 2 = Redness and slight edema; 3 = Considerable erythema or edema (some blistering, slightly disturbed mobility possible); 4 = Extensive epidermolysis or obvious damage to the deep tissues, causing definitive functional disturbances; threatening or manifested compartment syndrome; and 5 = Reaction requiring amputation).

Follow-Up and Definitive Surgery

Tumor response was evaluated clinically according to the WHO guidelines[19]: (1) *Complete response* : Disappearance of all clinically detectable disease for at least four consecutive weeks, (2) *Partial response* : A 50% or greater reduction in the sum of the products of the two greatest perpendicular diameters of all measurable lesions for a period of at least four consecutive weeks, and (3) *No change* or stable disease: A < 25% increase or <50% decrease in tumor size as defined above throughout the period of treatment, and the absence of new lesions. Tumor response was assessed by MRI comparing pre and post-ILP MRI's.

When a resection could be performed this was done six to 16 weeks after the ILP depending on speed of tumor regression and full recovery of the normal tissue from regional ILP-related toxicity. The surgical specimen was sectioned along the longitudinal and transverse planes. The mean percentage of tumor necrosis was calculated on the basis of macroscopic evaluation of necrotized tissue and histologic examination of tumor tissue surrounding the necrotic area. All the patients underwent follow-up visits consisting of a physical examination, CT scan and/or MRI of the affected limb, and CT scan of the chest, every three months following resection.

Results

Tumor necrosis

In 13 patients 7 ILPs were performed at the iliac level because of tumors in the upper leg or multiple tumors throughout the leg and 6 ILPs were performed at the femoropopliteal level for tumors situated at the level of the knee or in the lower leg. There were no peroperative technical complications and no serious postoperative complications. There were no treatment related deaths. Table 2 summarizes the type of perfusion performed, leakage, systemic and locoregional toxicity, clinical response, percentage of tumor resection specimen(s), type of definitive surgery, and patient status an follow up.

Response to ILP and Limb Salvage

Complete response was observed on sequential MRI scans in 1 patient, a partial response was observed in 9 patients; the remaining 3 patients showed no change in tumor size following ILP. Two of the latter three patients, in which no change in tumor size was observed, showed a remarkable softening of the tumor. Therefore, LSS was considered feasible in 11 patients. Nine patients were operated while the other three were not because of the emergence of diffused metastatic disease (patients # 5,7,13). Three patients (patients # 4,10,11) underwent amputation for a progressive local disease; in one patient it was performed after the execution of LSS, while in the other two (patient # 4 and 10) an early amputation was indicated due to rapid tumor progression.

Patient No.	Type ILP	Leakage (%)	Systemic Toxicity (WHO)	Regional Toxicity (Wieberdink)	Clin. resp.	Tumor Necrosis (%)	Post ILP Surgery	Patient Status (follow-up months)
1	Iliac	0	0	2	PR	80-90	LSS	NED 20 months
2	Iliac	2	2	2	PR	> 90	LSS	Dead 4 months
3	Iliac	0	0	3	NC	50	LSS	Dead 12 months
4	Iliac	0	0	3	NC	< 50	LSS → Amputation	NED 32 months
5	Femoral	0	0	3	CR	Not evaluated	No surgery	AWD (lung mets) 26 months
6	Iliac	0	0	2	PR	80-90	LSS	Dead 10 months
7	Femoral	0	0	3	PR	Not evaluated	No surgery	Dead 12 months
8	Femoral	0	0	3	PR	50-60	LSS	NED 4 months
9	Iliac	4	4	3	PR	80-90	LSS	Dead 6 months
10	Femoral	0	0	3	NC	Not evaluated	Amputation	Dead 3 months
11	Femoral	2	2	3	PR	< 50	LSS → Amputation	Dead 6 months
12	Iliac	0	0	3	PR	80-90	LSS	NED 41 months
13	Popliteal	0	0	2	PR	Not evaluated	No surgery	Dead 10 months

PR - Partial response, CR - Complete response, NC - No change
LSS - Limb sparing surgery
NED - No evidence of disease, AWD - Alive with disease, D - Dead

Table 2: anatomical approach to the perfused limb, leakage and ILP-related complications, clinical response, rate of tumor necrosis, type of definitive surgery, and patient status.

Histologic evaluation of the surgical specimens revealed $\geq$ 80% necrosis in 5 patients, 50%-80% in 1 patient, and $\leq$ 50% in 3 patients (two of which developed progressive local disease that necessitated amputation). Specimen was not evaluated in 4 patients, three of which were not operated (patients # 5,7,13), while the fourth (patient # 10) underwent amputation surgery in an other hospital and the surgical specimen was not evaluated for necrosis.

Complications

Only one patient (patient 9), in which a 4% leak was documented during the ILP, had a significant systemic side effect, related to the use of TNFα. That patient experienced a septic shock-like syndrome, that resolved after 2 days. Two patients developed transient bone marrow depression, as evidenced by anemia, leukopenia, and thrombocytopenia. One patient had transient mild elevation of liver transaminases. All patients did experience a considerable edema of the limb following perfusion; however, the swelling spontaneously regressed after few weeks.

Discussion

We describe here our first experience with isolated limb perfusion with tumor necrosis factor-alpha and melphalan in 13 patients with imminent amputations of their lower limb due to progression of their primary, locally recurrent or metastatic bone sarcoma after failing other treatments. Because of our excellent experience of this treatment option as a limb salvage procedure in locally advanced extremity soft tissue sarcomas[13-15] we tried to reverse a locally hopeless situation into a resectable one. This is the first report on ILP with TNFα and melphalan in this group of patients and represents to our knowledge the only experience anywhere until now. It represents an attempt to determine whether ILP with TNFα and melphalan can sometimes play a role to in addition to or after standard chemotherapy to avoid an amputation of the limb.

Experience in the use of ILP for treating bone sarcomas in humans is limited. Cavaliere et al.[21] performed ILP with *L*-PAM (melphalan) and Actinomycin D in 76 patients with osteosarcoma of the extremities. ILP was reported to induce a sclerotic rim around the tumor (termed stop effect) which facilitated resection and subsequently 17 of these patients underwent LSS. The degree of necrosis obtained by that protocol was not reported.

Vaglini et al.[22] performed ILP with cis-platinum in 18 patients with osteosarcoma of the extremities who were candidates for amputation. LSS was performed in 11 patients, 9 of whom had a good histologic response (>90% necrosis). None of these patients had local recurrence at follow-up, which averaged 28 months. Since 90% of local recurrences after resection of primary bone sarcoma occur within 24 months[23], this protocol is apparently

efficient in terms of local control. The extent of the role of ILP can not be determined because it was preceded by a complex neoadjuvant regimen composed of intravenous administration of cis-platinum and high-dose methotrexate. Following a similar neoadjuvant regimen, Takeyama et al.[24] performed ILP with cis-platinum in 12 patients with osteosarcoma of the lower extremities. LSS was performed in 8 of these 12 patients. The efficacy of ILP in that series is even harder to determine, because it was not clear whether the patients were candidates for amputation prior to treatment, and because the degree of tumor necrosis was examined in the surgical specimen of only seven patients.

TNFα has an antineoplastic effect, demonstrated in a variety of tumor- bearing mice and tumor cells cultures[25-28]. It has a direct cytolytic effect, probably due to its ability to elicit apoptosis [25,29]. The administration of TNFα induces the generation of many cytokines and mediators, such as interleukin (IL)-1, IL-6 and IL-8, interferon, oxygen free radicals and arachidonic acid metabolites[30]. The most prominent effect of TNFα *in vivo* is its effect on tumor's vasculature. TNFα induces thrombocyte aggregation, erythrostasis, and rapid migration of leukocytes to the tumor's capillary bed, adhesion to the endothelium, and release of oxygen free radicals and other cytokines that result in extensive local inflammatory reaction, obstruction, and destruction of the tumor's capillary bed[27,31-34].

Only anecdotal cases of partial response were described among several hundred cancer patients treated with systemic intravenous TNFα[35-37]. This failure is attributed to the inability to administer sufficient doses of TNFα. The dose required to achieve the anticancer effect is 10 times higher than the maximal tolerated systemic dose in humans, which appears to be around 200μg/m^2[38]. Upon it systemic administration, rTNFα immediately mimics the hemodynamic alterations typically associated with septic shock and multiorgan failure[39]. ILP enables to treat an extremity with a therapeutic level of rTNFα without exposing the treated patient to its systemic toxic effect. Nevertheless, administration of TNFα alone, which was carried out as a pilot study in few patients as well as an animal model with BN 175 sarcoma or ROS-1 osteosarcoma, led to far lower tumor response rates relative to the combination of TNFα and melphalan[40,41].

Melphalan has no proven efficacy in the treatment of either bone or soft-tissue sarcomas. It is effective only in combination with TNFα. It is assumed that the effect of TNFα on the tumor neovasculature improves penetration of concomitantly given chemotherapeutic agents[40-42]. On the basis of these findings, one may speculate that the effectiveness of TNFα + any

chemotherapeutic agent in treating a specific bone or soft tissue sarcoma may increase the efficacy of that particular drug. Recent results from the Rotterdam laboratory program in *in vivo* models of both soft tissue sarcomas and osteosarcomas give further credence to this TNFα enhancing effect of chemotherapeutic drugs as significantly higher concentrations of doxorubine intratumorally were observed after ILP with the combination of TNFα and doxorubicin as compared to ILP with doxorubicine alone. ([43], Veen van der AH et al, manuscript in preparation).

Osteosarcoma could be a model for such a drug selection process, since it is the most common primary bone sarcoma and there are extensive data concerning the efficacy and safety of various drug regimens. Cisplatinum was shown to increase the rate of limb sparing surgery when given as a single agent via the intraarterial route[4,5]. In light of the findings of these studies and those of Vaglini et al.[22], we suggest that ILP with TNFα and cisplatinum might be considered as part of the preoperative chemotherapeutic regimen in the treatment of large, unresectable osteosarcomas of the extremities. As for other bone sarcomas, an alternative drug (in addition to TNFα) should be chosen according to the specific tumor sensitivity to chemotherapy.

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CHAPTER 9

GENERAL DISCUSSION

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Isolated limb perfusion makes TNFα clinically applicable

In the 1980's many reports from murine tumor models were published concerning the ability of Tumor Necrosis Factor α (TNFα) to induce tumor necrosis with acute softening of the tumor, hemorrhagic necrosis and occlusion of the neo-vasculature. The possibility to administer an agent with a toxic effect on the neo-vasculature of the tumor made TNFα a promising agent and led to many phase I/II trials, only to encounter much systemic toxicity and very little antitumor activity. The extreme toxicity of TNFα in man already observed at relatively low systemic concentrations without evident antitumor effect limited its therapeutical use. Only 1/50 - 1/20 of the dose required for antitumor effect in human xenograft tumor models in mice, can be administered in man. This concentration gap can only be overcome by isolated limb perfusion (ILP) methodology. In 1992, Liénard and Lejeune reported[1] the first impressive results with TNFα in the ILP setting in combination with melphalan and IFNγ. Patients with in transit metastasized melanoma showed high complete response (CR) rates of 80-90 %[2-5]. These results should be seen against an average 50 % CR rate reported in the literature after ILP with melphalan alone[6]. Moreover, recent reports on TNFα-based ILP as induction therapy in patients with locally advanced extremity soft tissue sarcomas indicate that limbs can be saved in over 80 % of patients by shrinking the tumor volume (30 % CR and 52 % PR), followed by a much easier and less mutilating resection of the tumor remnants[3,7,8].

TNFα targets tumor associated vasculature (TAV)

Patients treated with TNFα containing ILP showed the typical antitumor features of TNFα, known from experimental models, with an immediate reaction and softening of the tumor. These observations were associated with the selective occlusion of TAV as demonstrated by pre- and post- perfusion angiographies from sarcoma patients[7-9]. Recent investigations with magnetic resonance spectroscopy (MRS) of sarcoma patients treated in Rotterdam revealed sudden arrest (completed within 16 hours) of metabolic activity in the tumor[10]. In rodent tumor models comparable measurements were made after i.v. administration of TNFα[11,12], and concurred with a decreased tumor blood flow, as shown by Doppler flow techniques.

Thus, these data strongly indicate that selective effects on TAV explain the immediate tumor response, both in experimental tumors and in man.

Morphological and immunohistochemical studies of tumor biopsies from patients after TNFα-containing ILP confirmed the effects of the treatment on TAV. Early damage at 3 hours post-ILP, as shown by perivascular release of Von Willebrand Factor (VWF), preceded erythrostasis and platelet aggregation found 24 hours after ILP[13-15].

The property of TNFα to induce tissue factor in TAV, resulting in fibrin deposition and occlusion of these vessels is assumed to be an important mechanism to explain TNFα antitumor activity[16]. Surprisingly, in human tumor biopsies, no indications can be found for the proposed pro-coagulant events. Not coagulation but leakage of the endothelial layer may cause sludging of erythrocytes and platelet aggregation eventually leading to disruption of the circulation in TAV[14].

Isolated limb perfusion model in the rat

Since many mechanistic questions can only be answered in preclinical models, we developed an ILP model in the rat. Furthermore, histopathological analysis may reveal the sequential mechanistic steps leading to the TNFα induced tumor response. A non immunogenic tumor model was used, since this is representative for the clinical situation. The non immunogenic tumor is a rapidly growing, spontaneously metastasizing, grade III sarcoma (BN 175), which originated in the BN rat [17]. Moreover, the ROS-1 osteosarcoma was used, which originated spontaneously in the tibia of a WAG rat (ROS-1)[18]. Fragments with a diameter of 3-5 mm of the BN 175 sarcoma[19,20] or of the ROS-1 osteosarcoma[21] were implanted into the right hind limb subcutaneously just above the ankle. Perfusions were performed at a tumor diameter of approximately 15 mm, which is relatively large, resembling advanced sarcoma in the clinical situation. Rats were anaesthetized and 50 units heparin were injected i.v.. To keep the rat's hind leg at a constant temperature of 38-39°C., a warm water mattress was applied. The femoral artery and vein were cannulated with silastic tubing and collaterals were occluded by a groin tourniquet. An oxygenation reservoir and a roller pump were included in the circuit. The perfusion started with 5 ml oxygenated Haemaccel. Melphalan and recombinant human TNFα were added as boluses to the oxygenation reservoir. A roller pump recirculated

the perfusate at a flow rate of 2.4 ml/ min and a washout with 2 ml oxygenated Haemaccel was performed at the end of the 30 minute perfusion. Tumor diameters were measured at least three times weekly.

Observations in the ILP rat model

Synergy with cytostatic agents

In BN 175 tumor and ROS-1 no direct cytotoxic effects were observed of TNFα *in vitro* and no synergistic potentiation by TNFα of melphalan was found[20,21]. *In vivo* after ILP with TNFα alone in BN 175 sarcoma hemorrhagic necrosis was observed in the center of the tumor encircled by a viable rim. Remarkably, this does not inhibit tumor-growth, in spite of the vascular effects in the tumor center[20]. Also in the clinical setting TNFα alone containing ILP rarely results in tumor regression[22] and when regression occurs, it is only short-lived, likely due to the rapid growth from the remaining viable ring of tumor. Asher et al. described similar insensitivity of non immunogenic tumors when treated with i.v. administered TNFα, but often found regression in immunogenic tumors, associated with a preexisting infiltrate in the periphery of the tumor[23].

Apparently, also in our tumor ILP model involvement of the outer tumor ring seems to be a prerequisite to obtain regression, since the clearly synergistic combination of TNFα and melphalan with a 75 % regression rate[20], was associated with the extension of hemorrhagic necrosis affecting the periphery of the tumor[24]. The synergism between TNFα and melphalan may be based on the effects of both agents on the endothelial cells of TAV. The assumption of melphalan related endothelial toxicity is supported by previous reports on chemotherapeutic toxic effects on endothelial cells[25,26]. Since melphalan alone does not induce hemorrhagic necrosis, it is likely that melphalan interacts synergistically with TNFα in the formation of hemorrhagic necrosis.

Another explanation for the reported synergism between TNFα and melphalan can be the ability of TNFα to modulate intratumoral distribution of melphalan. Due to the highly inefficient architecture of TAV, consisting of tortuous vessels with aberrant branching, the delivery of therapeutical agents may be significantly hampered by the heterogeneous blood supply[27] and the long transport distances in the interstitium[28]. Leaking TAV, most prominent

in the center of the tumor, sustains a high interstitial pressure resulting in a fluid stream towards the periphery of the tumor, where interstitial hypertension does not exist. Inward diffusion of the chemotherapeutic agents is opposed by outward convection and therefore therapeutical agents may penetrate the tumor center less than the tumor margins[29]. One may speculate that TNFα by its effects on TAV disturbs the outward convectional stream resulting in better penetration of the cytostatic agent in the tumor center. Moreover, recently the ability of TNFα was reported to reduce interstitial pressure in the tumor[30]. This requirement for melphalan and the potentiation of it's effects we believe to be mediated in part by the immediated increase of permeability caused by TNFα and by the TNFα mediated drop in intratumoral interstitial pressure, as reported by Kristensen and coworkers[30]. These mechanisms result in a better penetration of the tumor by melphalan and we recently did find proof of this as we could demonstrate significantly increased intratumoral concentrations of doxorubicine after ILP with TNFα + doxorubicin as compared to ILP with doxorubine alone in our BN175 as well as our ROS-1 ILP models in rats (Ten Hagen, manuscript in preparation). The mechanisms, intratumoral drug levels and homogeneity in distribution as potentially TNFα-related factors are presently under further investigation in our laboratory.

Light and electron microscopic analysis of tumors

In rats that underwent sham or melphalan ILP the tumor cells were vital in over 80 %, and hemorrhage was virtually absent, while after ILP with TNFα alone, hemorrhagic necrosis was found in 20-60 % of the cross sections[24]. Edema, hemorrhage, erythrostasis and degeneration of endothelial cells found early after ILP (at 2,4 and 12 hours) suggest vascular leakage as an initial event, followed by increased hemorrhage and intravascular platelet aggregation and endothelial cell disintegration, limited to the tumor center. ILP's with TNFα + melphalan resulted in similar findings but the vascular alterations were more prominent at all time points and affected approximately 80-90 % of the tumor. These findings are in line with those observed in patients[7-8,13-14].

Role of white blood cells (WBC)[31]

Because of the strong effects of TNFα on neutrophils in terms of adhesion to the vessel walls and the promotion of brisk infiltrates into tumors an these white blood cells have been hypothesized to mediate in part the antitumor effects of TNFα. This hypothesis was

investigated in the limb perfusion model in rats by comparing antitumor effects in normal rats versus neutropenic rats. Total body irradiation (5 Gy) to reduce WBC count to virtually zero was applied in the rat ILP model to investigate the contribution of leukocytes in the TNFα-melphalan tumor response. Total body irradiation (TBI) significantly decreased antitumor effects, and abrogated skin necrosis at the tumor site, a TNFα-related effect. TBI followed by TNFα-melphalan containing ILP resulted in a tumor response resembling that of rats that got ILP with melphalan only, i.e. temporary growth arrest. TBI rats showed partial tumor necrosis with a coagulative character and the absence of polymorphonuclear cell (PMN) consisting infiltrate. In nonirradiated rats that underwent the combination ILP, necrosis was hemorrhagic and more extended than in the TBI rats. Furthermore, PMN infiltration was found in the adjacent skin, but surprisingly not in the tumor. Since TAV sprouts from the tumor neighboring tissues, we assume that toxic products from PMN's may affect the vascular bed of the tumor.

Role of anoxia

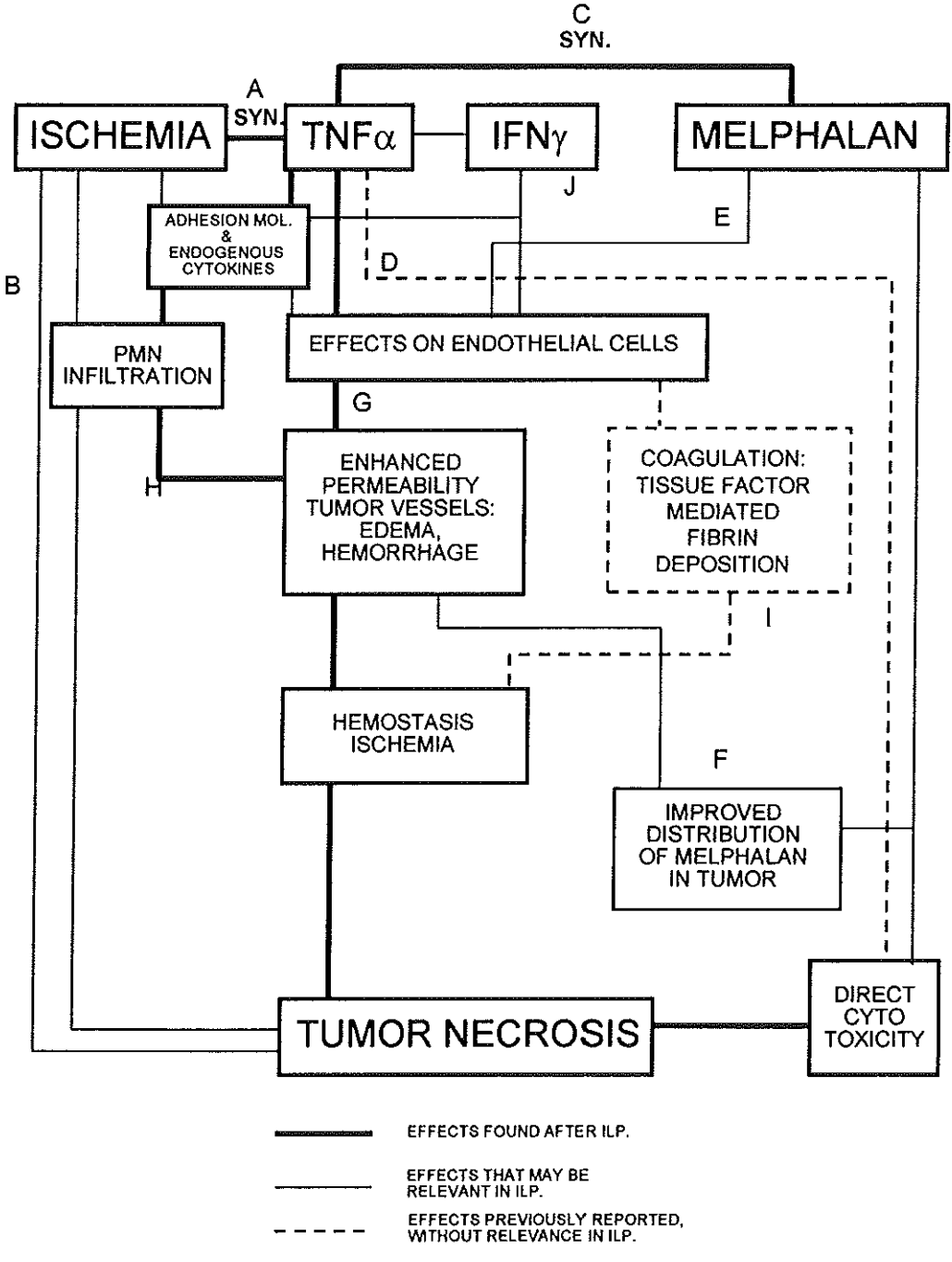
TNFα has important effects on the bloodflow of tumors, mostly by reducing it abruptly, by causing initial vasodilatation resulting in stasis and by causing massive leakage of tumor vessels and an important drop in interstitial pressure. Anoxia can also profoundly change bloodflow distribution as well as pH in tumors and may thus enhance the effects of TNFα. We have demonstrated[19] that nonoxygenated ILP with TNFα alone during 5 minutes, with 5 minutes ischemia before and after ILP, has remarkable antitumor effects, as opposed to the lack of tumor regression after a 30 minutes oxygenated ILP with TNFα alone. In most rats that got non oxygenated perfusions CR's were found, but rapid recurrences (within 5 days) were noted in all. In spite of the bad quality of the anoxia in combination with TNFα induced tumor response, and observed increased regional toxicity, the potentiating effect of anoxia needs to be further explored. In ILP ischemia/anoxia can be modulated by arterial clamping, which even may result in a significant drop of tissue pH, as van de Merwe and others demonstrated[32]. Moreover, in hypoxic isolated limb perfusion protocols[33] or stop-flow balloon catheter techniques[34], anoxia plays an essential part in the protocol design to achieve synergistic interactions with certain drugs such as mitomycin C and others. TNFα can now be added to the list of agents which activity can be enhanced by anoxia.

Role of Interferon-gamma (IFNγ)

IFNγ may enhance the antitumor effect of TNFα since it increases the number of TNFα-receptors on malignant cells. Both *in vitro* and *in vivo* murine tumor models as well as human tumor xenograft models in nude mice have shown synergistic antitumor effects of TNFα and IFNγ. The relatively low dose of 0.2mg of IFNγ used in the early clinical protocols was based on the assumption that synergy with TNFα might be achieved through low dose priming with IFNγ possibly as a result of the upregulation of TNFα-receptor expression by IFNγ. Pretreatment with IFNγ may prime the vascular bed of the tumor and enhance it's sensitivity to TNFα. The neo-vasculature of the tumor is a major target for TNFα. TNFα is vasculotoxic at high concentrations. We investigated the potential activity of low dose IFNγ in the BN175 rat ILP model. Treatment with IFNγ alone resulted in significant growth retardation, with a slight but not significant effect on hind limb function. When IFNγ was added to TNFα-ILP no increase of tumor response was noted, but significant ($p < 0.05$) increased regional toxicity was observed. Tumor response was increased when IFNγ was added to TNFα-melphalan-ILP (TIM), by an overall response of 89 % after TIM *vs* 72% after TM perfusions and a complete remission (CR) response of 78 % after TIM *vs* 55 % after TM perfusions. Severe regional toxicity was observed in the TIM combination ($p < 0.005$ *vs* TM combination ILP). Although, there exists no statistically significant difference with respect to tumor diameter between TIM-ILP and TM-ILP , the effect of IFNγ cannot be ruled out, since there exists a roughly 20 % higher CR-rate. Lienard also reported a (statistically not significant) higher complete response rate of 78 % in TIM-ILP *vs* 69 % in TM-ILP in the treatment of multiple in transit metastases melanoma[35]. Moreover, in the treatment of sarcoma patients a CR-rate of 36% in TIM-ILP vs 26 % in TM-ILP was observed[8]. Enhancement of regional toxicity by addition of IFNγ, as observed in the rat ILP model, was never found in patients and is therefore an extra argument to further investigate IFNγ in ILP in combination with TNFα and melphalan.

Local tumor control

The ILP protocols containing TNFα in the clinic are very effective with respect to percentage of tumor response (85 %) and limb salvage (84 %) for patients with the irresectable soft tissue sarcoma in the limbs[7]. After shrinkage of the tumor, the tumor remnant is resected, resulting in a low recurrence rate of 5/39 (13%)[7]. However, when resection was not per-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WITHOUT RELEVANCE IN ILP.

Figure 1. Flow diagram with the events following ischemia, exposure to TNFα, IFNγ and melphalan and their interactions potentially leading to tumor necrosis. In ILP in sarcoma bearing BN rats, immediate tumor regression with necrosis of the skin at the tumor site, was only found after anoxic TNFα-ILP and not after oxygenated TNFα-ILP (A). The observed synergistic effects between ischemia and TNFα may be mediated by expression of adhesion molecules, cytokines, infiltration of polymorphonuclear cells and direct effects of ischemia on tumor cells (B).
In ILP in the rat, synergism was also found between TNFα and melphalan (C). *In vitro* experiments showed relative insensitivity of tumor cells to TNFα and no enhancement of melphalan cytotoxicity in the presence of TNFα (D). Thus, the mechanism of synergism is likely due to indirect effects: Melphalan may have toxic effects on endothelium, increasing the TNFα induced vascular effects (E). Furthermore, immediate rheologic and structural changes induced by TNFα may alter the pharmaco-distribution of melphalan in the tumor (F). Morphological and electron-microscopical studies revealed enhanced permeability of the endothelium with subsequent edema and hemorrhage, eventually leading to hemostasis and ischemia in the tumor (G). Irradiation of rats prior to ILP with TNFα and melphalan, to reduce white blood cell count abrogated the tumor response. The absence of polymorphonuclear cell infiltration in the neighboring tissue was associated with the lack of hemorrhage in the tumor. This suggests a key role of PMNs in the occurrence of hemorrhagic necrosis (H). Coagulation in tumor vessels, described in many reports as an important TNFα effect leading to tumor necrosis, seems to be irrelevant, since fibrin deposition was not observed in tumor vessels after TNFα-ILP (I). IFNγ showed possible but statiscally not significant enhancement of the tumor response by TNFα, with increased toxicity. Further studies should be conducted to investigate the effects of IFNγ in combination with TNFα-melphalan ILP (J).

formed (for reasons of multiple tumors or distant metastases) recurrence was found in 5/16 (31 %)[7]. This percentage may be even higher when patients, who died of systemic disease had lived longer. These findings in patients correspond well with the observations in rats. In rats, receiving ILP with TNFα and melphalan, high complete response rates (75%) were observed, and in rats, that were not killed, rapid recurrence (11 days) was noted). In the ROS-1 tumor model recurrent tumor grew as fast as tumors in rats that underwent sham perfusion only. These observations in nonimmunogenic tumor models suggest that TNFα rather has tumor debulking than tumor growth inhibiting properties. Treatment of non immunogenic tumor as soft tissue sarcoma with ILP with TNFα and melphalan should therefore always be followed by resection of the tumor remnant or additive chemotherapy/radiotherapy.

Conclusions regarding ILP model(s)

In conclusion, in the ILP rat model many factors determining TNFα antitumor activity, such as the addition of other therapeutical agents or manipulation of normal physiology can be recognized. Furthermore, the ILP rat model provides answers on mechanistic questions, so strategies in the application of TNFα as a therapeutical agent in cancer patients can be improved. The use of TNFα in ILP protocols, however, can hardly be made more effective, but further insight in the regional application of TNFα is especially needed in ILP derived techniques such as organ perfusions and stop-flow infusions.

TNFα in organ perfusion models

A number of isolated organ perfusion models are available in which applicability and antitumor effect of TNFα can be assessed. Recently, the results of these investigations were reported for isolated lung[36], liver[37,38] and kidney[39,40] perfusion models. Here we summarize our experiences in an isolated hepatic perfusion (IHP) model in pigs in our laboratory. Pigs underwent an IHP with inflow catheters in both the hepatic artery and the portal vein. In all pigs a stable perfusion could be achieved with leakage < 0.02 % and perfusate levels of TNFα were comparable to TNFα levels in limb perfusions in the clinical setting. All pigs did well except for one, who died of intraabdominal bleeding. We observed transient liver enzyme elevations with peak levels on day 1, normalizing within 7 days, without functional or structural loss of liver, as documented 1 month after the perfusion.

These hopeful results opened the way for IHP in 9 patients with irresectable colorectal metastases in the liver. Two patients died due to acute coagulation disturbances after substantial blood loss, and protracted hepatic toxicity related dysfunction was observed. One histologically confirmed complete remission (CR) and 4 partial remissions (PRs) were found in 6 evaluable patients.

Clear activation of the coagulation and inflammatory cascades were found, resulting in extremely high levels of secondary cytokines (such as IL-6)[41] and a prolonged period of high levels of soluble TNFα receptors.[42] Remarkably, the level of TNFα in the liver tissue samples was about 7-8 times higher than in tumor tissue. These results demonstrate that TNFα in IHP does not preferentially accumulate in tumor tissue[43].

Fraker and others have encountered similar response rates but less toxicity in his more extended experience at the National Cancer Institute in the USA[5]. The difference in toxicity may be due to the fact that we perfused via the hepatic artery and the portal vein, whereas in their routine perfusion was performed via the hepatic artery only. Infusion of TNFα via the portal vein has been shown to be more toxic than when given systemically[44].

Future prospects: Reduction of TNFα-toxicity and development of systemic treatment with TNFα by tumor targeting

TNFα-related toxicity needs to be greatly reduced to make its systemic application possible. One way is using TNFα-mutants, that have retained antitumor activity but are significantly less toxic[45]. The other way is targeting TNFα to the tumor by encapsulation into liposomes resulting in putative high local levels and low systemic levels. As discussed earlier solid tumors have several characteristics which influence drug targeting to tumor cells negatively: heterogeneous structure, an uneven distribution of blood vessels, poorly perfused regions in the tumor, abnormally high interstitial pressure[46]. However, solid tumors also exhibit characteristics which benefit targeting and efficacy of antitumor therapy. Vascular permeability of tumors is in general higher than in various normal tissues. Tumor growth is accompanied by a process called angiogenesis resulting in new vessels which supply the tumor with essential nutrients and oxygen. The newly formed vessels are leaky and allow large particles to extravasate. The permeability of TAV allows particles such as liposomes to extravasate. ILP-like concentrations of drugs may be achieved after systemic administration by targeting these agents to the tumor by long circulating liposomes (i.e. Stealth® liposomes, SEQUUS Pharmaceuticals, Menlo Park, Ca, USA) as drug carriers. We have demonstrated that TNFα and melphalan can be encapsulated in Stealth® liposomes with good encapsulation efficiency[47]. A preferential localization of Stealth® liposomes in the BN175 soft tissue sarcoma was observed reaching almost 10% of the injected dose at 12 h after injection. Seventy-two hours after injection of the liposomes up to 15% of the injected dose could still be detected in the circulation, and a tumor localization of 4% at that time point. With the combination of Stealth® liposome-encapsulated TNFα and doxurubicin containing liposomes (DOX-SL) for the first time we observed a significant antitumor effect in our

model after systemic treatment[48]. Moreover much less toxicity was observed after the repeated administration of TNFα-liposomes than after repeated injections of equal doses of free TNFα. These findings will prompt research to the treatment of other tumors at other sites (lung, liver) than the extremities with a combination of liposomal agents.

Conclusions

After the frustrating results of systemic administration of TNFα, strong and consistent antitumor effects of TNFα have eventually been reported in humans when used in the setting of ILP. Interestingly, the vascular effects of TNFα, as extensively reported in animal tumor models, can also be found in human cancer. The interaction of TNFα with cytostatic agents have proved to be of great importance, validating further investigations on its mechanism in the ILP models in the rat. These models also serve to determine optimal combination therapy and efficacy-enhancing manipulations. The acquired knowledge is especially needed to set up other regional treatment protocols with TNFα, such as isolated organ perfusions and stop flow infusion techniques. Organ perfusion models were successfully developed in the form of isolated liver, lung and kidney perfusions. The experience in the regional application of TNFα may provide new perspectives to its systemic application: Tumor-targeting of TNFα by its encapsulation in Stealth® liposomes seems to be one of the options that merits further evaluation.

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CHAPTER 10

SUMMARY AND CONCLUSIONS

Chapter 1 presents the impressive results in patients with irresectable soft tissue extremity sarcomas and with in transit metastasized melanoma of the limb after treatment with TNFα + melphalan based isolated limb perfusion (ILP). To understand the antitumor characteristics of TNFα an overview of its history is given. TNFα is a pleiotropic cytokine with the endothelial cell as its prime target. Already at low doses it is very toxic when administered systemically, whereas high concentrations are required for optimal vasculotoxic and antitumor effects. Loco-regional application of TNFα may be the answer on the toxicity problem. Only in isolated limb perfusion adequate doses of TNFα can be reached to induce tumor regression. Other attempts to administer TNFα loco-regionally such as intratumoral, intraarterial or intraperitoneal applications of TNFα all failed.
Since many questions remained open about the antitumor behavior of TNFα in isolated perfusion models, the aim is to answer the question whether a realistic animal model can be developed, which mimics the clinical situation of ILP. Furthermore, we investigated which factors influence the efficacy of TNFα in ILP, we examined whether there exists synergy, and which sequential steps can be observed in the TNFα-mediated tumor response to elucidate the antitumor mechanism.

Chapter 2 describes the development of an ILP model in the rat. First a simple ischemic model was designed. In this model all rats responded to TNFα with tumor regression. In the non-ischemic, oxygenated model tumor regression did not occur after TNFα treatment. Since ischemia by itself can induce damage to tumor vessels, resulting in hemorrhagic necrosis of the tumor, it is conceivable that the combination of TNFα and ischemia may act synergistically. Furthermore, TNFα-ischemia synergism may be explained by the activation of macrophages by ischemia, resulting in local endogenous production of TNFα.
Oxygen pressure at the tumor site was checked to be sufficient during and after (non-ischemic) ILP by the Pd Porphyrine phosphorescence technique. Furthermore, in the ILP model leakage was very low, since rats did not show systemic toxicity to TNFα and TNFα collected from the tail vein during ILP, as measured by ELISA was about 1-2 % as compared to TNFα in the ILP-circuit.

Chapter 3 presents the synergistic antitumor effects of ILP with TNFα and melphalan *in vivo*, while *in vitro* no synergism between both agents could be demonstrated. TNFα was not

found to be cytotoxic *in vitro* and therefore it most likely interacts indirectly on the tumor cells by: 1) effects on tumor associated vasculature (TAV); 2) tumor distribution effects 3) effects mediated by the immune system.
TNFα alone induced hemorrhagic necrosis confined to the tumor center, while TNFα + mephalan-ILP showed much more extensive hemorrhagic necrosis. This suggests a potentiating role of melphalan on the effects of TNFα on TAV (melphalan alone does not induce hemorrhagic necrosis).
Vascular leakage induced by TNFα within the tumor may influence the bio-availibility of mephalan within the tumor, and therefore enhancing tumor necrosis.

Chapter 4 elaborates on the morphological and ultrastructural changes in tumors after saline, melphalan, TNFα and combination ILP in the rat at 2, 4, 12, 24 and 72 hours after treatment. Hemorrhage was virtually absent in tumors treated with sham or melphalan, while hemorrhage with edema was present already at 4 hours after ILP in tumors treated with TNFα or TNFα + melphalan. These findings are associated with dramatic alterations of permeability and integrity of microvascular endothelial wall. Platelet aggregation followed these initial changes, first at 12-h post-ILP only focally and at 24-h post-ILP as a generalized event. In other rodent tumor models activation of pro-coagulant activity of endothelial cells by TNFα, resulting in thrombus formation and eventually in hemorrhagic necrosis was supposed an important mechanism in tumor necrosis induced by TNFα. But, our data obtained with light and electron microscopy revealed that platelet aggregation was the result of endothelial damage rather than the initiator. In the tumors that were exposed to the combination of TNFα and melphalan the findings were more prominent, suggesting that melphalan potentiates the TNFα-induced vascular changes.

Chapter 5 discusses the role of neutrophils on the TNFα induced antitumor effect. Early leukocyte adherence to the endothelial wall is suggested by the observation of leukocytopenia in cancer patients at 30 minutes after intravenous TNFα administration. Early loss of endothelial wall integrity after TNFα administration as described in the previous chapter may well be explained by leukocyte margination. This hypothesis seems be hold true since tumor response was attenuated in leukocytopenic rats. Histologically, nonirradiated rats showed an infiltrate of polymorphonuclear cells (PMNs) adjacent to the tumor with nearly total tumor

necrosis with an aspect of hemorrhagic necrosis, while in preirradiated rats, the PMN-infiltrate was absent and only partial necrosis with a coagulative character was observed. The effect of irradiation on platelets seems not relevant for the TNFα-melphalan tumor response, since the number and aggregation activity of platelets was hardly affected in irradiated rats. In addition, microscopic and ultrastructural studies revealed that endothelial damage preceded platelet aggregation (Chapter 4).

Chapter 6 shows higher (but not statistically significant) overall response rate as well as complete response rate when low dose IFNγ was added to the combination of TNFα and melphalan. In contrast to the lack of a significant enhancement of the tumor response, a significant increase of regional toxicity was observed, when adding low dose IFNγ to TNFα ($p < 0.05$) and to TNFα + melphalan (< 0.005). The use of higher doses IFNγ seems not of interest in our model, since the regional toxicity problem and restricted availability of rat IFNγ. Since in the rat model a 20 % higher response rate was found in the TIM-ILP as compared to TM-ILP, an enhancing role for IFNγ cannot be excluded. Accordingly, Lienard reported a roughly 10 % higher CR rate in TIM-ILP vs TM-ILP's in the treatment of patients with in transit metastasized melanoma. Similarly, Eggermont et al. observed a 10 % higher CR rate in patients with soft tissue sarcoma. Although statistically not significant, these observed differences of response rate together with the absence of regional toxicity of IFNγ in man (in contrast to rats) justify further clinical investigations of the role of IFNγ.

Chapter 7 shows, in accordance with data from chapter 4, synergy between TNFα and melphalan in an ILP model using the osteosarcoma ROS-1 in WAG-Rij rats. Again no synergy between TNFα and melphalan could be found *in vitro*. Thus, similar observations could be reproduced in a different rat strain, bearing a histologically different tumor. Since all solid tumors depend on vessels nourishing their tumor cells it may be expected that all advanced human tumors, including osteosarcoma, will respond to the TNFα-melphalan combination in ILP. Thus ILP with TNFα and melphalan may be considered as a limb salvage procedure also in patients with chemoresistant osteosarcoma, in which prognosis is now extremely poor.

Chapter 8 reports on the first 13 patients with a skeletal or extraskeletal osteosarcoma who

were treated by TNFα-based ILP to avoid amputation of the limb. Most patients had received multiple chemotherapeutic regimens with or without radiation and were now confronted with a limb threatening local problem. Some patients refused chemotherapy (mostly because of old age) and opted for a TNFα-based ILP to achieve local control.
In the majority of the patients an amputation could be avoided. This, so far unique experience with this procedure in osteosarcoma, demonstrates that also in osteosarcoma TNFα-based perfusions can play a role in obtaining limb salvage in cases without other treatment options.

Chapter 9 is the general discussion of this thesis. According to the results of this thesis the putative mechanisms of TNFα and melphalan interaction are described in a flow diagram. In conclusion, TNFα-ischemia and TNFα-melphalan synergistic effects were found to be important in the rat ILP model, whereas IFNγ effects were overshadowed by the TNFα-melphalan synergism. Furthermore, evidence was found that indirect effects on the tumor are most relevant in the ILP model. Enhanced permeability of tumor vessels was followed by hemostasis and platelet aggregation. The presence of polymorphonuclear cells seems necessary for hemorrhagic changes in the tumor. TNFα-melphalan-ILP results in tumor reduction in patients with soft tissue sarcoma, but the recurrence rates are high, when resection of the tumor is not performed. Accordingly, in the rat TNFα-melphalan therapy induced tumor regression was followed by rapid recurrence. Thus, the TNFα-melphalan combination in ILP seems to have only strong tumor de-bulking capacity in nonimmunogenic tumors, and when possible should be followed by resection of the tumor remnant.

Conclusions

1. We have developed a rat ILP model, which resembles the clinical situation with respect to procedure and response pattern, both clinically as well as histopathologically.

2. Ischemia enhances the antitumor effects of TNFα in ILP.

3. There is no correlation between the *in vitro* cytotoxicity of TNFα (± melphalan) and their *in vivo* antitumor efficacy.

4. The earliest effects of TNFα (± melphalan) are hemorrhage and edema, followed by coagulative effects as platelet aggregation, eventually leading to hemostasis and hemorrhagic necrosis.

5. In the presence of melphalan, hemorrhagic effects induced by TNFα are more extensive, leading to tumor regression. This suggests synergistic activity of both agents on the endothelial lining of tumor vessels.

6. Hemorrhagic effects induced by TNFα depend on the presence of polymorphonuclear leukocytes (PMN).

7. Data obtained from the rat model suggests a possible therapeutical value of IFNγ in TNFα-based ILP triple combination therapy, but in the rat model IFNγ was associated with excessive locoregional toxicity.

8. TNFα-based ILP is not only effective in the highly vascularized BN 175 soft tissue sarcoma but also in the less vascular ROS-1 osteosarcoma.

9. TNFα-based ILP can be effective in patients with sarcomas of osteogenic origin which have failed standard treatment options and who are faced with imminent amputation of the limb.

HOOFDSTUK 10

SAMENVATTING EN CONCLUSIES

Hoofdstuk 1 geeft in een literatuuroverzicht de indrukwekkende resultaten weer die behaald zijn bij patiënten met niet reseceerbare weke delen sarcomen van de extremiteit of met multipele in transit melanoma metastasen na een geïsoleerde extremiteits perfusie (ILP) met TNFα en melphalan. Teneinde de antitumor werking van TNFα beter te begrijpen wordt achtergrondinformatie van TNFα besproken. TNFα is een cytokine met pleiotrope werking met de endotheelcel als belangrijk doelwit. TNFα is zeer toxisch, al bij lage doseringen indien aan de systemische circulatie toegediend, terwijl wij pas bij hoge doseringen optimale antitumor activiteit kunnen verwachten. Locale dan wel regionale toediening zou een oplossing kunnen betekenen voor het toxiciteits-probleem. Alleen in het geïsoleerde extremiteits-perfusie model kunnen voldoende hoge concentraties TNFα worden bereikt om tumorregressie te verkrijgen. Andere pogingen waarbij TNFα loco-regionaal werd toegediend, zoals intratumoraal, intraarteriel en intraperitoneaal, leverden geen goed resultaat op.
Omdat veel vragen over de eigenschappen van TNFα in het geïsoleerde extremiteits perfusie model open bleven, werd gestreefd naar de ontwikkeling van een representatief ILP proefdiermodel, waarin de klinische situatie goed zou kunnen worden nagebootst. Ten doel werd gesteld in dit model te onderzoeken welke factoren de effectiviteit van TNFα antitumor activiteit beïnvloeden, uit te zoeken of er synergisme bestaat en uit welke opeenvolgende stappen de TNFα tumor respons is opgebouwd, teneinde helderheid te verschaffen omtrent het antitumor mechanisme.

Hoofdstuk 2 beschrijft de ontwikkeling van het ILP model in de rat. Eerst werd een eenvoudig ischemisch model ontworpen. In dit model werd in alle ratten die met TNFα werden behandeld tumor teruggang gezien. In het niet ischemische, geoxygeneerde model vertoonde TNFα behandeling daarentegen geen tumor afname. Het TNFα-ischemie synergisme is eenvoudig te begrijpen gezien het feit dat ischemie zelf kan leiden tot tumorvaat-schade. Voorts kunnen wij het TNFα-ischemie synergisme verklaren door activering van macrofagen door ischemie, resulterend in de (endogene) productie van TNFα. Zoals aangetoond m.b.v. de Pd Porphyrine fosforescentie techniek, blijft de zuurstofspanning ter plaatse van de tumor nagenoeg hetzelfde tijdens en na de ILP (in vergelijking met preoperatief). Verder, was de lekkage uit het perfusie-circuit naar de systemische circulatie minimaal, er konden namelijk bij de behandelde ratten geen tekenen van TNFα toxiciteit worden waargenomen en de intraveneuze TNFα concentraties bedroegen ongeveer 1-2 % van

de concentraties TNFα in het perfusiecircuit.

Hoofdstuk 3 demonstreert het synergisme tussen TNFα en melphalan toegediend in ILP in de rat, zonder dat dit synergisme *in vitro* wordt waargenomen. Omdat TNFα *in vitro* geen cytotoxiciteit vertoonde is het zeer waarschijnlijk dat TNFα een indirecte werking op de tumorcel uitoefent: 1) via effecten op de tumorvaten; 2) effecten op de farmacologische verdeling in de tumor; 3) effecten door het immuunsysteem gemedieerd.
ILP met TNFα alleen resulteert in hemorrhagische necrose, die zich beperkt tot het centrale gedeelte van de tumor, terwijl de combinatie van TNFα + melphalan veel uitgebreidere hemorrhagische necrose laat zien. Aangezien melphalan zelf geen hemorrhagische necrose kan induceren lijkt het er derhalve op dat melphalan de TNFα effecten op tumorvaten versterkt. Door TNFα veroorzaakte vaatlekkage binnen de tumor zou de beschikbaarheid van melphalan aan het tumorweefsel ten positieve kunnen beïnvloeden, hetgeen resulteert in meer tumornecrose.

Hoofdstuk 4 bespreekt de veranderingen die optreden binnen de tumor op licht- en elektronen-microscopisch niveau na blanco, melphalan, TNFα en combinatie behandeling op verschillende tijdstippen van 2, 4, 12, 24 and 72 uur na de behandeling. Hemorrhagie werd bijna nooit gezien na blanco en melphalan behandeling, terwijl hemorrhagische veranderingen wel konden worden gezien na ILP met TNFα of TNFα + melphalan en wel reeds 4 uur na de behandeling. Deze bevindingen gingen gepaard met sterke veranderingen in de permeabiliteit en integriteit van de microvasculaire endotheelwand. Aansluitend werd plaatjesaggregatie waargenomen, eerst focaal in de tumor 12 uur en diffuus in de tumor 24 uur na ILP.
In andere proefdier tumor-modellen werd het belang van de stimulatie van de endotheelcel procoagulatoire activiteit door TNFα aannemelijk gemaakt. Deze verhoogde stollingsactiviteit leidt tot trombusvorming en uiteindelijk tot hemorrhagische necrose. Echter in ons model, toonde de histologische studie aan dat plaatjesaggregatie eerder het gevolg dan de oorzaak van de endotheelschade was. In de tumoren die waren blootgesteld aan de combinatie van TNFα en melphalan was hemorrhagische necrose veel meer uitgesproken, hetgeen wijst op de mogelijke versterking door melphalan van de door TNFα geïnduceerde vaatveranderingen.

Hoofdstuk 5 bespreekt de rol van neutrofiele witte bloedcellen in het TNFα antitumor effect. De daling van leukocyten onder het normale niveau 30 minuten na de toediening van TNFα in het bloed bij kanker patiënten zoals eerdere studies rapporteerden, wijst op een vroege adherentie van leukocyten aan de endotheelcelwand in de tumor. Vroege endotheel wand permeabiliteits-veranderingen, zoals in het vorige hoofdstuk beschreven, zouden kunnen worden veroorzaakt door leukocyten die zich aan de vaatwand hechten. Deze hypothese wordt gesteund door de waarneming dat de tumor respons duidelijk is afgenomen in ratten met leukocytopenie. In niet voorbestraalde, (derhalve normoleukocytaire) ratten werd naast de tumor een infiltraat bestaande uit neutrofiele witte bloedcellen gezien, terwijl in voorbestraalde (en derhalve leukocytopene) ratten dit infiltraat niet werd waargenomen. In deze laatste groep werd dan ook alleen partiële necrose gezien van een coagulatief karakter. Bloedplaatjes spelen waarschijnlijk geen cruciale rol in de ontstaanswijze van hemorrhagische necrose, want voorbestraling heeft weinig effect op de bloedplaatjes zowel qua aantal als qua aggregatie-functie. Daarenboven werd reeds gezien dat bloedplaatjes-aggregatie secundair optreedt aan endotheel-beschadiging (en niet andersom).

Hoofdstuk 6 laat een hoger tumor respons percentage zien (statistisch niet significant) wanneer IFNγ wordt toegevoegd aan de combinatie TNFα en melphalan. Er bestond echter toename van de toxiciteit, reeds bij toevoeging van een lage dosering IFNγ aan TNFα alleen ($p < 0,05$) en toevoeging van IFNγ aan TNFα + melphalan ($p < 0.005$). Wegens een beperkte beschikbaarheid ratten-IFNγ en het optreden van regionale toxiciteit bij lage doseringen, leken ons verdere experimenten met hogere doseringen IFNγ in ons model niet zinvol. Omdat het overall response percentage ongeveer 20 % hoger was in de TIM-ILP groep, vergeleken met de TM-ILP groep, kan een verhoogd antitumor effect door IFNγ niet worden uitgesloten. Overeenkomstig, beschreven Lienard et al. een ruwweg 10 % hoger CR percentage in TIM-ILP *versus* TM-ILPs bij de behandeling van patiënten met het in transit gemetastaseerde melanoom. Ook Eggermont et al. vonden een 10 % hoger CR percentage in patiënten met het weke delen sarcoom die IFNγ kregen bij ILP. Ondanks de afwezigheid van statistische significantie, zijn de hogere tumor response percentages in zowel de klinische studies als in de ratten studie en de afwezigheid van een versterking van de regionale toxiciteit door IFNγ in de mens voldoende aanleiding tot verder onderzoek naar de rol van IFNγ.

Hoofdstuk 7 beschrijft, zoals ook in hoofdstuk 4, synergie tussen TNFα en melphalan in een ILP model in WAG Rij ratten met het ROS-1 osteosarcoom model. Opnieuw kon er geen enkele aanwijzing worden gevonden voor *in vitro* synergie tussen TNFα en melphalan. Er werden derhalve vergelijkbare resultaten gevonden in een andere rattenstam met een tumor van een andere histologische oorsprong. Omdat alle solide tumoren afhankelijk zijn van een vasculatuur, waarmee de tumorcellen worden gevoed, is het te verwachten dat alle gevorderde tumoren, inclusief het osteosarcoom gevoelig zijn voor de melphalan-TNFα combinatie in ILP. Omdat ook het chemo-resistente osteosarcoom afhankelijk is van een vaatvoorziening, kan ILP met TNFα + melphalan overwogen worden als procedure om amputatie te voorkomen bij patiënten met een chemo-resistent osteosarcoom en een dreigende amputatie. De algemene prognose bij deze patienten is overigens uiterst slecht.

Hoofdstuk 8 presenteert de eerste 13 patiënten met een osteosarcoom, die behandeld werden met TNFα + melphalan behandeling in ILP met als doel amputatie van de extremiteit te voorkomen. De meeste van deze patiënten waren eerder behandeld met chemotherapie wel of niet gecombineerd met radiotherapie en werden nu geconfronteerd met het dreigend verlies van de aangedane extremiteit. Sommige patiënten weigerden chemotherapie (vaak om redenen van hoge leeftijd) en kozen voor TNFα-ILP teneinde het tumor proces lokaal te beheersen. In de meerderheid van de patiënten kon een amputatie worden vermeden. Deze tot nu toe enige ervaring met de behandeling van het osteosarcoom d.m.v. deze procedure, laat zien dat TNFα-ILP bij sommige patiënten, die in het eindstadium van hun ziekte verkeren, palliatie kan bieden in de vorm van het (vaak functionele) behoud van hun extremiteit.

Hoofdstuk 9 is de algemene discussie van dit proefschrift. Aan de hand van de resultaten van dit proefschrift worden de vermeende mechanismen betreffende de interactie van TNFα en melphalan weergegeven in een flow diagram. Concluderend, zijn synergie tussen TNFα en ischemie en tussen TNFα en melphalan belangrijk naar voren gekomen in het ILP model in de rat, terwijl de effectiviteit van IFNγ minder belangrijk leek. Voorts is gebleken dat indirecte effecten op de tumor het meest relevant waren in het ILP model. Toename van de permeabiliteit van tumorvaten, werd gevolgd door bloedstilstand in de vaten en plaatjesaggregatie. De aanwezigheid van neutrofiele witte bloedcellen leek noodzakelijk voor het ontstaan van hemorrhagische veranderingen binnen de tumor. ILP met TNFα en

melphalan resulteert in tumor reductie bij patiënten met het weke delen sarcoom, echter de locale recidief percentages zijn hoog, indien ILP niet wordt gevolgd door resectie van de tumor. Overeenkomstig werd er in het ratten model snel tumor recidief gezien na eerst tumor regressie na ILP met TNFα en melphalan. Derhalve lijkt de TNFα-melphalan combinatie in ILP zeer sterke tumor debulking eigenschappen te bevatten, echter in niet-immunogene tumoren is na deze therapie resectie van de tumor rest geïndiceerd.

Conclusies

1. We hebben een ratten model ontwikkeld, dat grote overeenkomsten vertoont met de klinische situatie voor wat betreft de procedure, het patroon van de tumor respons, zowel in klinisch opzicht als in histopathologisch opzicht.

2. Ischemie versterkt de antitumor effecten van TNFα in ILP

3. Er bestaat geen correlatie tussen de *in vitro* cytotoxicteit van TNFα +/- melphalan en de *in vivo* antitumor effectiviteit van ILP met TNFα en melphalan in het ratten sarcoom model.

4. De eerste effecten van TNFα +/- melphalan bestonden uit intratumorale hemorrhagie en oedeemvorming, gevolgd door stollings verschijnselen zoals plaatjes aggregatie, waarna uiteindelijk bloedstilstand en hemorrhagische necrose optrad.

5. De hemorrhagische effecten van TNFα waren uitgebreider in aanwezigheid van melphalan, leidend tot tumor afname. Dit wijst op synergistische activiteit van TNFα en melphalan op het endotheel van de tumor vaten.

6. Het optreden van hemorrhagische effecten, geïnduceerd door TNFα is afhankelijk van de aanwezigheid van neutrofiele witte bloedcellen in de circulatie en bij de tumor.

7. In het rattenmodel lijken lage doses Interferon-gamma het antitumor effect van een ILP met TNFα + M te versterken. In het rattenmodel gaat dit, in tegenstelling tot bij

patienten, gepaard met een toename van de regionale toxiciteit

8. In het ratten ILP model is ILP met TNFα en melphalan niet alleen effectief in het zeer goed gevascullariseerde BN 175 weke delen sarcoom, maar ook in het minder sterk gevascullariseerde ROS-1 osteosarcoom.

9. TNFα bevattende ILP kan effectief zijn in patiënten met osteosarcomen, bij wie standaard therapie geen uitkomst biedt en de extremiteit wordt bedreigd met de noodzaak tot amputatie.

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CURRICULUM VITAE AUCTORIS

Eric Robert Manusama was born on April 8th, 1965 in The Hague, The Netherlands. After finishing high school (Stedelijk Gymnasium, Johan van Oldenbarnevelt, Amersfoort) in 1983, he went to Medical School at the University of Utrecht. As part of the graduation program he spent a period in the Aberdeen Royal Infirmary/ City Hospital in Aberdeen (U.K.) (Internal Medicine; Dr C.C. Smith, FRCP) at the C.H.R.U. at Montpellier, France (Neurology; Prof. J.M. Blard) and the Diakonessenhuis, Parimaribo, Surinam (Gynecology; Dr R. van Kanten). He graduated in 1990 from Medical School and subsequently served in the Royal Dutch Navy as a resident in Surgery in the Gemini Hospital in den Helder, The Netherlands. He was also employed as a Medical Officer at a naval base and on a frigate. From september 1992 till february 1996 he joined a collaborative research program between the Dept. of Pathology of the University of Nijmegen and the Dept. of Surgery, University Hospital Rotterdam- Dr Daniel den Hoed Cancer Center, Rotterdam, the Netherlands (Prof. dr A.M.M. Eggermont, Prof. dr D.J. Ruiter and Dr R.L. Marquet). In march 1996 he started his training in Surgery at respectively the Surgical Departments of the Reinier de Graaf Gasthuis, Delft (Dr P.W. de Graaf) and the University Hospital Rotterdam, the Netherlands (Prof. dr H.A. Bruining).