ISOLATED LIMB PERFUSION AS A TREATMENT MODALITY IN CANCER

From TNF to genetherapy

GEISOLEERDE EXTREMITEITSPERFUSIE ALS EEN BEHANDELINGSMODALITEIT BIJ KANKER

Van TNF tot gentherapie

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CHAPTER 1

INTRODUCTION AND AIMS OF THE THESIS

ISOLATED LIMB PERFUSION

The technique of isolated limb perfusion (ILP), was first described by Creech *et al.* in 1958 for the treatment of a patient with multiple in-transit metastasised melanoma who refused amputation.[1] A complete response and thus limb salvage was achieved using melphalan in an extra-corporeal circulation system. The procedure involved cannulation of the major vessels of the diseased limb in combination with the use of a tourniquet and a heart-lung machine. This treatment is based on the advantage of a leakage free regional perfusion system achieving high local drug concentrations without systemic contamination. Moreover, at the end of a perfusion a wash-out procedure is performed preventing systemic exposure of toxic drugs. After the first perfusion with melphalan, different combinations of cytostatic drugs have been used in ILP with various response rates. With melphalan response rates around 50% were achieved accompanied by mild regional toxicity.[2-4] Therefore, this drug has been used for many years as a single drug treatment reaching local drug concentrations 15-20 times higher than after systemic treatment.[5]

Besides application of different cytostatic agents, various strategies have been developed to improve response rates including hyperthermia, multiple perfusions and the introduction of biological response modifiers in combination with melphalan. Lejeune and Liénard pioneered the use of tumour necrosis factor alpha (TNF) and interferon gamma (IFN) in combination with melphalan and reached 80-100% complete response rates.[6-8] These excellent results in patients with in-transit metastasised melanoma were confirmed by several other authors.[9-11]

ILP with melphalan was also used for patients with locally advanced extremity soft tissue sarcoma, however, with marginal responses. Perfusions with other cytostatics were accompanied with high morbidity compared to perfusions with melphalan.[12] In a multi-center study Eggermont *et al.* demonstrated high response rates when TNF and IFN were used in combination with melphalan for this category of patients.[13,14] With the combination therapy tumour shrinkage occurred often which made irresectable tumours resectable and limb salvage was achieved in about 80% of patients. These significant therapeutic improvements have recently resulted in the approval of TNF by the European Medicine Evaluation Agency (EMEA) for advanced sarcomas.[15]

ANIMAL ILP MODELS

Because of the positive results obtained with ILP in humans with solid tumours of the extremities, animal models were developed to elucidate mechanisms of action, and explore ways to

further improve efficacy. Several authors used ILP models in dogs for pharmacokinetic studies using melphalan, doxorubicin or cisplatin.[16-18] In our laboratory the technique of ILP in rats originally described by Benckhuijsen *et al.*[19] was modified by Manusama *et al.*[20] Rapidly growing, spontaneously metastasising soft tissue sarcomas (BN-175) were used in Brown Norway rats for ILP. Moreover, in WAG-Rij rats a similar model was developed using a ROS-1 osteosarcoma.[21] In both syngeneic models small fragments of tumour tissue (3-5 mm) were implanted in the hind limb of rats and perfusions were performed when these tumours were around 10-15 mm in diameter. This tumour diameter was reached approximately 10 days after implantation. (Figure 1)

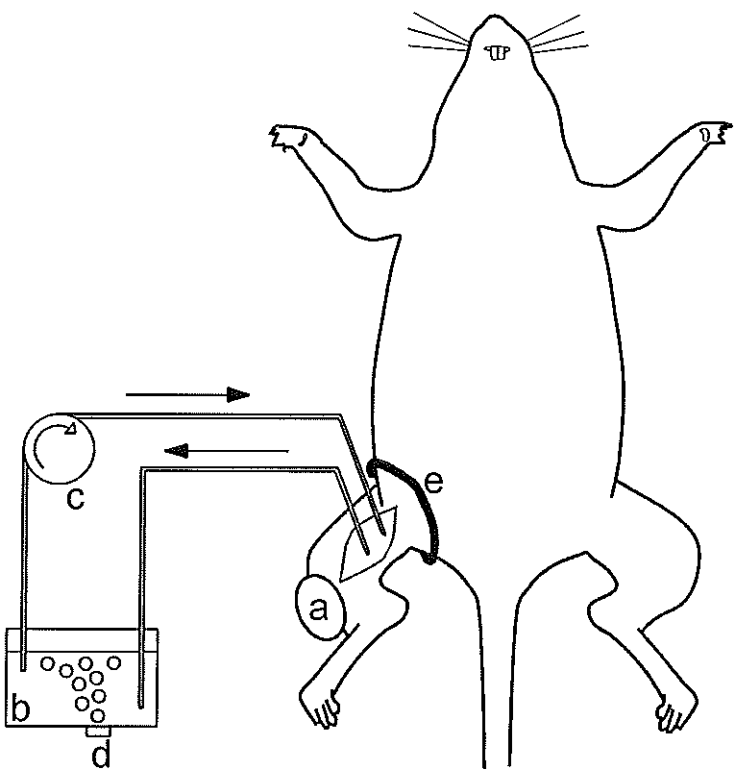

Figure 1. Schematic representation of an isolated limb perfusion setting in the rat: a) BN-175 soft tissue sarcoma or ROS-1 osteosarcoma, b) perfusion reservoir, c) roller pump, d) oxygenation of the perfusate, e) tourniquet.

Before perfusion rats were anesthetized and heparinized. The femoral artery and vein were cannulated and connected to an oxygenation reservoir and a roller pump. The perfusate consisted of Haemaccel and drugs were added as a bolus in this perfusate solution. Collaterals were occluded with a tourniquet and perfusion time started when the tourniquet was tightened. To keep the hind limb at a constant temperature a warm water mattress was applied. After a 30 min perfusion a wash-out procedure was performed and vessels were ligated. Tumour responses and limb toxicity was daily measured after perfusion.

PREVIOUS RESULTS

Responses after ILP in these rat models closely resemble observations in patients where TNF alone did not results in an antitumour response [20-22] and perfusions with melphalan are only marginally active in soft tissue sarcoma.[12-23] The combination of melphalan and TNF, however, results in very high response rates in these rat models just as in soft tissue sarcoma patients.[13,14,24,25]

Early endothelial damage, erythrostasis and platelet aggregation were observed in tumour vessels after perfusions with TNF and melphalan,[26] which is also in line with observation in patients.[27-29] These effects are possibly induced by the selective destruction of the tumour associated vascular bed by TNF which was previously demonstrated to lead to haemorrhagic necrosis in other *in vivo* models.[30,31] This vascular destruction was confirmed in angiographic and NMR studies in patients.[32,33] Manusama *et al.* demonstrated that the TNF-based antitumour effect in this ILP model is neutrophil dependent, since in neutropenic animals no antitumour responses were observed.[34] Other findings with this pre-clinical model were the lack of improved antitumour effects when IFN was added to the TNF/melphalan perfusions.[35] Despite synergistic antitumour effects of TNF and IFN in several other tumour models this could not be demonstrated in this ILP model as well as in patients.[36]

Several other groups used similar ILP models in rats to study drug kinetics of melphalan.[37,38] Wu *et al.* used a rat ILP model to study the effectiveness of different flow rates and perfusate solutions on perfusions with melphalan alone.[39] Addition of TNF in combination with melphalan in a fibrosarcoma model in rats demonstrated synergistic antitumour effects similar as in our model.[24] Recently, Walchenbach *et al.* used a DS rat sarcoma model in which a tumour growth delay was demonstrated using hyperlactacidaemia in hyperthermic ILP.[40]

AIMS OF THE THESIS

We used our rat isolated limb perfusion models with tumours that differ in histology and tumour vascular bed density to identify ways to further improve efficacy, to elucidate mechanisms that are responsible for the TNF-based antitumour responses, and to test new methodologies such as gene therapy.

Prerequisites

In *Chapter 2* we studied several prerequisites for an effective ILP using TNF and melphalan in BN-175 sarcoma-bearing rats. Both hypoxia and hyperthermia are known to potentiate antitumour activity of melphalan and TNF in vitro and were compared to oxygenated and normothermic perfusions not only for antitumour responses but also for their local toxicity.[41-44] Moreover, perfusion duration and optimal TNF concentration were determined in order to optimise perfusion protocols. Since these items have never been studied in a direct comparative fashion in the clinic they are addressed in our rat ILP model.

Tumour drug uptake

Several mechanism of TNF have been postulated to cause synergism with cytostatics like melphalan. It has been demonstrated that TNF increases permeability of tumour vasculature[45-46] and decreases interstitial pressure in tumour tissue.[47] Both these mechanisms could increase leakage of melphalan in tumour tissue and explain the observed synergy between TNF and melphalan. To demonstrate this hypothesis we analysed melphalan concentrations in tumour and limb tissue after melphalan ILPs with and without the addition of TNF in *Chapter 3*.

Doxorubicin combined with TNF

Melphalan is used in the majority of isolated perfusions for the treatment of melanoma and sarcoma, although other agents might be successful as well. The anthracycline doxorubicin has shown antitumour activity in clinical perfusion settings for the treatment of high-grade or non-resectable soft tissue sarcomas.[12,48] Moreover, Pfeiffer *et al.* studied pharmacokinetics and tissue toxicity in an experimental ILP model using single and double perfusions with doxorubicin.[17,49] In *Chapter 4* doxorubicin was used in combination with TNF in soft tissue- and osteosarcoma-bearing rats to examine whether doxorubicin can be a good alternative for melphalan in TNF-based ILPs. Moreover, possible in vitro and in vivo mechanisms by which TNF potentiates the antitumour activity of doxorubicin were studied.

TNF-mutant

Despite the successful use of TNF in a leakage-free ILP setting, systemic use is limited by severe systemic toxicity.[50] TNF mutants were developed to reduce this toxicicity and expand the applicability of TNF for other use than extremity perfusions. TNF-SAM2, is such a mutant that demonstrated to have both a two-fold higher cytotoxic activity in vitro and up to 20-fold lower acute toxicity in a murine model, compared to conventional TNF.[51-53] In *Chapter 5* antitumour effects of TNF-SAM2 were studied in our ILP model in combination with melphalan and doxorubicin. Clinical and histopathological responses were compared to those with conventional TNF.

Role of NO-inhibition

The inducible form of nitric oxide synthase (iNOS) is demonstrated in high concentrations in tumour cells.[54-55] The production of nitric oxide (NO) by iNOS is important to maintain the vascular tone of tumour vessels. Inhibition of NOS was demonstrated to reduce tumour blood flow and thereby reduce oxygen and nutrients to reach tumour cells.[56] To study the potential antitumour effect a NO–inhibitor (L-NAME) subcapsular renal tumours were treated systemically with L-NAME in *Chapter 6.* Moreover, the effect of L-NAME was studied in our ILP model alone and in combination with melphalan and TNF.

New methodology: gene therapy

Gene therapy is a new field in anticancer therapies using genetic materials introduced in cells by a variety of techniques with the ultimate goal of selective killing tumour cells. A major concern in gene therapy is the transfer of genes to organs other than the tumour, especially organs with a rapid cell turn-over. Therefore, tumour-specific gene delivery must be achieved which can be reached by tissue-specific administration of viral vectors.[57] *Chapter 7* describes, the efficiency and tumour-specificity of adenovirus-mediated gene transfer using ILP as an administration method. Adenoviral vectors carrying the luciferase marker gene were used to study luciferase expression after ILP which was compared to other administration routes such as systemic, regional and intratumoural injections. Moreover, adenoviral vectors carrying the LacZ marker gene were used to determine the intratumoural localisation of transfected cells after both ILP and IT administration.

Cytokine gene delivery

Several strategies for gene therapy in cancer treatment can be obtained using genes to correct a defect or aberrant expression of a control gene, to stimulate antitumour immunity, to

activate a transduced prodrug gene or to protect normal cells from damage by antitumour agents. Cytokine gene therapy to stimulate antitumour immunity is described in *Chapter 8* using the recombinant IL-3β gene. This gene has previously demonstrated tumour growth retardation after multiple intratumoural injections in L42 lung tumours in rats.[58] Antitumour activity of the IL-3β gene after ILP was studied using BN-175 and ROS-1 sarcoma-bearing rats and was compared to intratumoural injections.

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CHAPTER 2

PREREQUISITES FOR EFFECTIVE ISOLATED LIMB PERFUSION USING TUMOUR NECROSIS FACTOR ALPHA AND MELPHALAN IN RATS

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SUMMARY

An isolated limb perfusion (ILP) model using soft tissue sarcoma-bearing rats was used to study prerequisites for an effective ILP, such as oxygenation of the perfusate, temperature of the limb, duration of the perfusion and concentration of tumour necrosis factor alpha (TNF). Combination of 50 μg TNF and 40 μg melphalan demonstrated synergistic activity leading to a partial and complete response rate of 71%. In comparison to oxygenated ILP, hypoxia was shown to enhance activity of melphalan alone and TNF alone but not of their combined use. Shorter perfusion times decreased responses. At a temperature of 24-26°C, anti-tumour effects were lost, whereas temperatures of 38-39°C or 42-43°C resulted in higher response rates. However, at 42-43°C, local toxicity impaired limb function dramatically. Synergy between TNF and melphalan was lost at a dose of TNF below 10 μg in 5 ml perfusate.

We conclude that the combination of TNF and melphalan has strong synergistic anti-tumour effects in our model, just as in the clinical setting. Hypoxia enhanced activity of melphalan and TNF alone but not the efficacy of their combined use. For an optimal ILP, minimal perfusion time of 30 min and minimal temperature of 38°C was mandatory. Moreover, the dose of TNF could be lowered to 10 μg per 5 ml perfusate, which might allow the use of TNF in less leakage-free or less inert perfusion settings.

INTRODUCTION

Isolated limb perfusion (ILP) is considered the method of choice for the treatment of patients with multiple in-transit melanoma metastases confined to an extremity.[1] Melphalan has been the standard drug for this regional treatment because of low regional toxicity.[2] ILPs with melphalan or other cytostatic drugs has also been used in the treatment of patients with extremity soft tissue sarcomas, although with little success.[3,4] Therefore, Liénard *et al.* pioneered the application of high-dose tumour necrosis factor alpha (TNF) and interferon gamma with melphalan, which was reported to result in very high complete response rates in melanoma patients.[5] The impact of using TNF in this setting has been greatest, however, in the treatment of patients with irresectable extremity soft tissue sarcomas, as response rates and limb salvage rates of more than 80% have been reported in large series of patients destined for amputation of the limb.[6,7] The selective destruction of tumour vasculature, resulting in haemorrhagic necrosis of the tumour, has been shown in angiographic and histopathological studies.[6,8]

Yet many questions regarding mechanisms or conditional requirements by which ILP with TNF and melphalan are mediated, are not solved. Therefore, a tumour model with a highly aggressive non-immunogenic soft tissue sarcoma in BN rats was developed in our laboratory to

address these questions.[9] Response after ILP with melphalan and TNF in this model correspond well to what is observed in sarcoma patients in terms of synergy between TNF and melphalan, response rate, and histopathological observations.[9,10] This rat model could therefore serve as a credible model to study mechanisms and determine ways to optimize ILP efficacy for the clinical setting. Here we address requirements for an effective ILP setting, such as temperature of the perfusate and limb, duration of the perfusion, oxygenation of the perfusate and concentration of TNF.

MATERIAL AND METHODS

Animals

Male inbred BN rats, weighing 250-300 g, obtained from Harlan-CPB (Austerlitz, the Netherlands) were used for isolated limb perfusions. Rats were fed a standard laboratory diet ad libitum (Hope Farms Woerden, the Netherlands) and were housed under standard 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.

Melphalan

Melphalan (Alkeran, 50 mg per vial, Wellcome, Beckenham, United Kingdom) was diluted in 10 ml solvent. Further dilutions were made in 0.9% sodium chloride to give a concentration of 1 μg/μl. A volume of 40 μl (= 40 μg) was added to the perfusion circuit.

Tumour necrosis factor alpha

Recombinant human TNF (rHuTNF) 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 (Kramer and Carver, 1986). Endotoxin levels were < 1.25 endotoxin units (EU) per mg protein. TNF concentrations used were 2, 10 and 50 μg in 5 ml perfusate.

Isolated limb perfusion (ILP) model

The perfusion technique was performed as described previously.[9] Briefly, small fragments (3-5 mm) of the rapidly growing and metastasizing BN-175 soft tissue sarcoma were implanted

subcutaneously into the right hind limb. Perfusion was performed at a tumour diameter of 13 mm ± 3 mm at least 7 days after implantation. Animals were anaesthetized with Hypnorm (Janssen Pharmaceutica, Tilburg, the Netherlands) and 50 IU of heparin were injected intravenously to prevent coagulation. To keep the rat's hind limb at a constant temperature, a warm water mattress was applied. Temperature was measured with a temperature probe on the skin covering the tumour and was varied between room temperature (24-26°C), 'mild' hyperthermia (38-39°C) and 'true' hyperthermia (42-43°C). The femoral artery and vein were cannulated with silastic tubing (0.012 inch inner diameter (ID), 0.025 inch outer diameter (OD); 0.025 inch ID, 0.047 inch OD respectively, Dow Corning, Michigan, USA). Collaterals were occluded by a groin tourniquet, and isolation time started when the tourniquet was tightened. An oxygenation reservoir and a roller pump were included into the circuit. The perfusion solution was 5 ml Haemaccel (Behring Pharma, Amsterdam, the Netherlands) and the haemoglobin (Hb) content of the perfusate was 0.9 mmol/l. Melphalan and TNF 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 5 ml oxygenated Haemaccel was performed at the end of the perfusion. Subsequent tumour growth after perfusion was daily recorded by caliper measurement. Tumour volume was calculated as $0.4(A^2B)$, where A is the minimal tumour diameter and B the diameter perpendicular to A.

Assessment of TNF concentrations in perfusate

During ILP samples for determination of TNF concentrations were collected from the oxygen reservoir at 0.5, 5, 15 and 30 min. Samples were centrifuged and an aliquot of the supernatant was used for analysis. Enzyme-linked immunosorbent assay (ELISA) for rHuTNF was performed as described by Engelberts *et al.*[11] In short, a 96-well Immuno-Maxisorp plate was coated with murine anti-human TNF monoclonal antibody (mAb) 61E71. A standard titration curve was obtained by making serial dilutions of a known sample of rHuTNF in normal rat serum. Standards and samples were added to the wells and, after washing, the plates were incubated with a polyclonal rabbit antihuman TNF antiserum, followed by addition of an enzyme-labelled anti-rabbit reagent and enzyme reaction. The detection limit for human TNF is 20 pg/ml.

Assessment of melphalan concentrations in perfusate

During ILP, samples for determination of melphalan concentrations were collected from the oxygen reservoir at 0.5, 5, 15 and 30 minutes. Melphalan was measured by gas chromatography-mass spectrometry (GC-MS).[12] P-[Bis(2-chloroethyl)amino]-phenylacetic acid methyl ester was

used as an internal standard. Samples were extracted over trifunctional C18 silica columns. After elution with methanol and evaporation, the compounds were derivatized with trifluoroacetic anhydride and diazomethane in ether. The stable derivates were separated on a methyl phenyl siloxane GC capillary column and measured selectively by single ion monitoring GC-MS in the positive EI mode.

Assessment of tumour response

The classification of tumour response was: progressive disease (PD) = increase of tumour volume (> 25 %) within 5 days; no change (NC) = tumour volume equal to volume during perfusion (in a range of -25 % and + 25 %); partial remission (PR) = decrease of tumour volume (-25 and -90 %); complete remission (CR) = tumour volume 0-10% of volume during perfusion or skin necrosis.

Assessment of limb function

Limb function was a 'clinical' observation in which the rat's ability to walk and stand on the perfused limb was scored 5 days after ILP. On this scale a severe impaired function (grade 0) means that the rat drags its hindlimb without any function; a slightly impaired function (grade 1) means the rat does not use its hindlimb in a usual matter, but stands on it when rising; an intact function of the hindlimb (grade 2) means a normal walking pattern.

Statistical analysis

Mann-Whitney U-test was used to compare tumour volumes in different animal groups and to compare different tumour responses in different groups. Calculations were performed on a personal computer using Graph PadPrism and SPSS for Windows 95.

RESULTS

Synergy between TNF and melphalan

In the present study comprising experiments in 167 rats, the efficacy of ILP with TNF and melphalan as reported previously was confirmed (Figure 1).[9] Synergy was demonstrated for the combination of 50 μg TNF and 40 μg melphalan. At five days after ILP a significant difference was observed in mean tumour volume as compared to sham perfusions ($p<0.001$), TNF perfusions alone ($p<0.001$) and melphalan perfusion alone ($p<0.001$). No significant difference was found between all other groups.

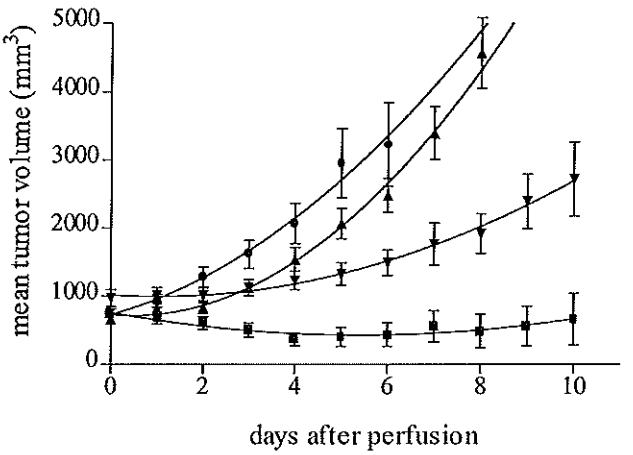

Figure 1. Growth curves of BN-175 sarcoma after 50 µg TNF (▲; n=11), 40 µg melphalan (▼; n=13), TNF plus melphalan (■; n=28) and sham isolated limb perfusion (●; n=12). Mean (± S.E.M) of tumour volumes are shown.

Oxygenation/hypoxia

Differences in tumour response in oxygenated versus hypoxic perfusions are summarized in Table 1. Sham oxygenated and hypoxic perfusions resulted in progressive disease in all animals. In oxygenated TNF perfusions, progressive disease occurred in all animals as well; however, significant anti-tumour effect was observed in hypoxic perfusions with TNF in comparison with TNF alone ($p<0.001$). Hypoxia also significantly increased the anti-tumour response after ILP with melphalan ($p=0.03$) as compared to oxygenated perfusions with melphalan alone. Oxygenated ILP with melphalan and TNF resulted in an overall response rate of 71%. No further improvement of this effect was demonstrated in hypoxic perfusions with the combination of melphalan and TNF. In all hypoxic perfusions, no additional limb toxicity was observed as compared with oxygenated perfusions (data not shown).

TNF and melphalan concentrations during perfusion

Pharmacokinetic studies of TNF and melphalan in the perfusate were performed, which shows a minimal decrease during perfusion of TNF concentrations, indicating a leakage-free perfusion system and a continuous exposure of the vasculature of high levels of TNF (Figure 2).

Melphalan concentrations, on the other hand, decreased dramatically in the first minutes of ILP, indicating rapid uptake by the tissues of the limb (Figure 2).

Table 1. Tumour responses of BN-175 sarcoma after isolated limb perfusion with or without hypoxia.

Tumour Response	Sham n=12	Sham+ hypoxia n=10	TNF n=11	TNF+ hypoxia n=11	Mel n=13	Mel+ hypoxia n=10	Mel+TNF n=28	Mel+TNF +hypoxia n=18
PD	12	10	11	3	3		2	2
NC				2	10	7	6	3
PR				5		3	3	9
CR				1			17	4
Response Rate (%)	-	-	-	**55**	-	**30**	**71**	**72**

Perfusions were performed with 50 µg TNF and 40 µg melphalan under constant temperature (38-39°C) for 30 min.

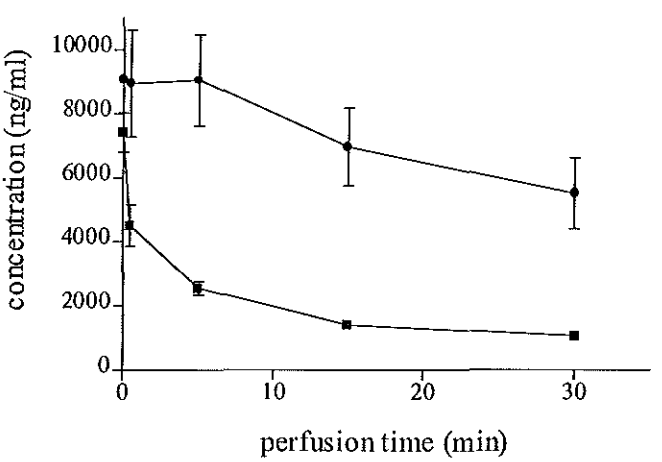

Figure 2. Concentration (ng /ml) of TNF (● ; n=5) and melphalan (■ ; n=5) in the perfusate as a function of time in rats. During isolated limb perfusion 50 µg TNF with 40 µg melphalan was added to 5 ml perfusate as a bolus.

Table 2. Tumour responses of BN-175 sarcoma after isolated limb perfusion with different temperature conditions.

Tumour Response	Melphalan+TNF 24-26°C n=12	Melphalan+TNF 38-39°C n=28	Melphalan+TNF 42-43°C n=10
PD	6	2	
NC	6	6	
PR		3	7
CR		17	3
Response rate (%)	-	71	100
Grade 0 Limb function	0/12 (0%)	6/28 (21%)	8/10 (80%)

Perfusions were performed with 50 µg TNF and 40 µg melphalan with oxygenation for 30 min.

Table 3. Tumour responses of BN-175 sarcoma after isolated limb perfusion with variable perfusion times.

Tumour Response	Melphalan+TNF 10 min n=11	Melphalan+TNF 20 min n=10	Melphalan+TNF 30 min n=28
PD	3	1	2
NC	3	3	6
PR	5	3	3
CR		3	17
Response rate (%)	45	60	71

Perfusions were performed with 50 µg TNF and 40 µg Melphalan under constant temperature (38-39°C) and with oxygenation.

Hyperthermia

Standard perfusions with TNF and melphalan were performed with 'mild' hyperthermia (38-39°C). At day five after perfusion, rats perfused at room temperature (24-26°C) show almost no anti-tumour effects versus the high response rates seen with 'mild' and 'true' hyperthermic conditions (both $p<0.001$). Quality of response (percentage of CR) was not further increased by 'true' hyperthermia in comparison with 'mild' hyperthermia, whereas toxicity to the normal tissues was significantly enhanced leading to increased grade 0 function of the limb (=severe impairment) in this group (Table 2).

Perfusion time

A gradual and almost complete loss of efficacy was observed when perfusion time was reduced from 30 min to 10 min. Table 3 demonstrates the effect of different perfusion times on tumour response of TNF and melphalan. Response rates decreased from 71% to 45% ($p<0.005$). Moreover, complete responses decreased from 17 out of 28 animals at 30 min to 0 out of 11 animals at 10 min.

Table 4. Tumour responses of BN-175 sarcoma after isolated limb perfusion with variable TNF concentrations.

Tumour Response	**2 µg TNF + 40 µg melphalan** n=10	**10 µg TNF + 40 µg melphalan** n=10	**50 µg TNF + 40 µg melphalan** n=28
PD	3	1	2
NC	6	2	6
PR	1	3	3
CR		4	17
Response rate (%)	**10**	**70**	**71**

Perfusions were performed under constant temperature (38-39°C), with oxygenation and for 30 minutes. TNF and melphalan doses are total doses added to 5 ml perfusate as a bolus.

Minimally required TNF dose

Standard perfusions were performed with 50 μg TNF in 5 ml perfusate on rats that weighed approximately 250 g. A de-escalation study demonstrated similar overall response rates of >70% at 10 μg (=40 μg/kg) and 50 μg TNF (=200 μg/kg) (Table 4). A small (but not significant) drop in complete responses was observed at 10 μg TNF. At 2 μg TNF (=8 μg/kg) no synergy with melphalan was observed, this anti-tumour response differed significantly with 10 μg ($p=0.01$) and 50 μg ($p<0.001$). No complete remissions, and only one partial response, were observed, which corresponds to what is usually observed after an ILP with melphalan alone. These findings indicate that the dose of TNF can be reduced five- to tenfold without effecting response rates, which might facilitate the use of TNF in less leakage-free settings or at sites more responsive to the toxic effect of TNF.

DISCUSSION

The non-immunogenic BN-175 soft tissue sarcoma model in BN rats has previously been shown to be an adequate model to address questions for the clinical situation as response patterns to TNF, melphalan and their combination.[9] Moreover, histopathologic observations closely resemble observations in patients.[8,10,13] TNF alone is not active[14], melphalan is only marginally active in soft tissue sarcoma,[3,4] whereas the combination results in very high response rates in this rat model just as in melanoma patients[5,15] or soft tissue sarcoma patients.[6,7] Here we report on 167 ILPs to determine ways to optimize ILP efficacy for the clinical setting. Wu *et al.* previously demonstrated that high flow rate and protein-free perfusate may enhance the effectiveness of ILP with melphalan alone in nude rats.[16] The requirements for a successful ILP such as oxygenation of the perfusate, temperature of the limb, duration of the perfusion, and concentration of TNF are addressed in this study. Regarding the factors studied here, results are in line with clinical observations, although these elements have never been studied in a direct comparative fashion in the clinic.

Hypoxia can increase the sensitivity of tumour cells to chemotherapeutic agents since it can cause dividing cells to halt their progression through the cell cycle, by allowing them to progress to and then remain in a G1-like susceptible state.[17] Since Thompson *et al.* published results of hypoxic ILP, the question whether the much more expensive oxygenated ILPs using a heart-lung machine are really necessary for the treatment of patients with multiple melanoma metastases remains unanswered.[18] We observed that hypoxia enhanced the effects of TNF alone. Similarly hypoxia enhanced effects of melphalan alone, in line with Skarsgards *et al.* who reported increased

cytotoxicity of melphalan with hypoxia both in vitro and in vivo.[19] Part of the enhancement of the anti-tumour effects by hypoxia could be mediated by the reperfusion injury associated with hypoxic perfusions and absent during oxygenated perfusions. This reperfusion injury apparently would have a preferential effect on the tumour as no impairment of limb function, as a measure of local toxicity, is observed in hypoxic perfusions. Hypoxia did not further increase the efficacy of the combination of TNF and melphalan. Presumably the potential enhancing effect was overshadowed by the synergism of the combination of TNF and melphalan, which again seems to be the central and crucial phenomenon. For the clinical situation it remains an interesting questions whether the use of the oxygenator can be abandoned without causing an increase in regional toxicity as has been observed in the hypoxic setting in our model.

The application of hyperthermia in ILP is advocated since it has been shown that the in vivo drug uptake by in-transit metastases is higher at 39.5°C than at 37°C [20] and that hyperthermia enhance anti-tumour effects of melphalan dramatically in vitro.[21,22] Hyperthermia also enhances anti-tumour effects of TNF, as was demonstrated in different tumour models in vitro and in vivo.[23-25] We observed that the results in our animal model run parallel to the observations in the clinic. Only perfusions at 'mild' or 'true' hyperthermia resulted in a synergistic anti-tumour response of TNF and melphalan, whereas after ILP at 24-26°C all anti-tumour effects were lost. Hyperthermia not only demonstrated to potentiate anti-tumour responses in our animal model, but also increased regional toxicity dramatically when temperatures were above 42°C, which is in line with our clinical experience.[26]

There are no clinical or preclinical studies that compare different perfusion times in ILP. Traditionally a perfusion time of one hour has been adopted for ILPs in patients with melphalan based on pharmacokinetic patterns that request a duration of at least 30 min. The pharmacokinetic profile of melphalan in the perfusate in our model showed a similar rapid decrease in melphalan concentrations as was previously demonstrated by others.[27,28] We therefore did not study perfusions longer than 30 min and were more interested if identical results could be obtained after shorter perfusions. It was shown that 30 min is optimal and that lesser efficacy was observed after ILPs of 20 or 10 min. This is probably due to the fact that exposure times over 20 min are needed to get the vascular occlusive and destructive effects of TNF, needed for adequate tumour responses.

TNF is the crucial element in the observed synergy with melphalan. Low-dose TNF has a proliferative effect on angiogenesis, whereas higher doses can cause destruction of newly formed blood vessels.[29] This destruction of blood vessels may lead to thrombocyte aggregation, erythrostasis and haemorragic necrosis found in tumours after treatment with TNF.[8,13,30,31] In clinical ILP treatment schedules TNF is used in high doses (4 mg for a lower extremity) to induce the above

described effects. This dose is 10- to 50-fold higher than the maximum tolerated dose after intravenously administration in cancer patients.[32] It was shown that increasing TNF administration did not result in higher response rates but induced regional toxicity to the perfused limb.[15] It will obviously add to the safety of the procedure if one can reduce the dose of TNF, while retaining its anti-tumour effects. Pharmacokinetic observations of TNF in the perfusate in this animal model are similar to the clinical situation, in which TNF concentrations remain stable during perfusions.[33] Since there is a plateau in TNF levels well above saturation and thus well above threshold level, lower TNF concentrations seems to point to a reasonable action. However, it is not easy to perform a dose de-escalation study in the clinical setting because of the large number of patients needed for such a study. The only publication from Hill *et al.* in which TNF was used in about five-to sixfold lower concentrations, demonstrated similar high response rates for soft tissue tumors.[34] With a de-escalation study we demonstrated the minimally required TNF dose in our rat model to obtain synergy with melphalan to be 10 μg (=40 μg/kg).

Since recombinant human TNF (rHuTNF) in mice binds only to the p55 receptor and not to the p75 receptor its activity and toxicity is 5-10 times less than murine TNF (MuTNF).[35] Also in rats, rHuTNF is at least 5 times more toxic than MuTNF as we established that an intravenous dose of 40 μg MuTNF is lethal in rats,[36] whereas doses of 200 μg HuTNF are not lethal (own observations). This phenomenon can be explained if we assume that the same receptor binding pattern in rats and mice exists and that activity and toxicity of rHuTNF would be 5-10 times less in rats as well. Therefore, 40 μg/kg rHuTNF in the rat corresponds roughly with 4-8 μg/kg rHuTNF in the human setting and thus indicates that the dose of TNF currently used in the clinical setting (approximately 50 μg/kg) may well be reduced five- to tenfold while retaining synergy. Our experiments show that further reduction leads to the complete loss of TNF activity and thus to complete loss of synergy with melphalan. These findings might be of clinical relevance in more than one way. First, it suggests that the very high doses used presently in the clinical setting may well be reduced while retaining efficacy, but even more importantly this observation increases the chances that TNF may become applicable in other settings such as isolated hepatic perfusions.[37-40] These settings are clearly less ideal, due to local toxicity, than the setting of the 'inert' limb perfusion.

In conclusion, our ILP rat model demonstrates strong synergistic anti-tumour effects when TNF is combined with melphalan. We identified as basic requirements for an effective ILP a duration of 30 min and temperature of above 38°C, while hyperthermia above 42°C resulted in unacceptable damage to the normal tissues. Hypoxia was shown to enhance activity of melphalan and TNF alone but did not further improve results of their combined use. The minimally required

dose of TNF to induce synergy with melphalan was 10 μg (=40 μg/kg). These findings may serve as important guidelines for further developments in ILP in the clinical setting.

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CHAPTER 3

TUMOUR NECROSIS FACTOR ALPHA INCREASES MELPHALAN CONCENTRATION IN TUMOUR TISSUE AFTER ISOLATED LIMB PERFUSION

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SUMMARY

Several possible mechanisms for the synergistic antitumour effects between tumour necrosis factor alpha (TNF) and melphalan after isolated limb perfusion (ILP) have been presented. We found a significant sixfold increase in melphalan tumour tissue concentration after ILP when TNF was added to the perfusate, which provides a straightforward explanation for the observed synergism between melphalan and TNF in ILP.

INTRODUCTION

With isolated limb perfusions (ILP) high drug concentrations can be achieved in the vasculature of a limb with no or negligible leakage into the systemic circulation. With the addition of high dose tumour necrosis factor alpha (TNF) to melphalan high response rates were demonstrated in patients with melanoma and irresectable soft tissue sarcomas.[1-3] Similarly, in rat sarcoma models synergy has been demonstrated between melphalan and TNF.[4,5]

The exact mechanisms for synergistic anti-tumour effects between melphalan and TNF, however, are not clear. Several possible mechanisms have been suggested such as selective destruction of tumour vasculature, which is accompanied by thrombus formation and haemorrhagic necrosis of the tumour.[6,7] This process is accompanied by an inflammatory response that seems to be leukocyte dependent.[8-10] Moreover, TNF increases the permeability of tumour vasculature[11,12] and has been reported to lower interstitial pressure in the tumour,[13] which could both increase leakage of melphalan in tumour tissue and explain the observed synergy.

To demonstrate this hypothesis we analysed melphalan concentrations in tumour and limb tissue after melphalan isolated limb perfusions with and without the addition of TNF.

MATERIAL AND METHODS

Chemicals

Melphalan (Alkeran, Wellcome, Beckenham, UK) was diluted in 0.9% sodium chloride and stored at –20°C. Recombinant human TNF alpha was provided by Boehringer (Ingelheim, Germany), with specific activity of 5.8×10^7 U/mg and endotoxin levels < 1.25 endotoxin units (EU) per mg protein and stored at –80°C. During perfusion 40 µg melphalan with or without 50 µg TNF were added to the perforate as boluses.

Animal tumour model and perfusion setting

Male inbred BN rats, weighing 250-300 g, obtained from Harlan-CPB (Austerlitz, the Netherlands) were used. The perfusion technique was performed as described previously.[4] Briefly, small tumour fragments of the non-immunogenic BN-175 soft tissue sarcoma were implanted in the right hind limb. ILP was performed at a tumour diameter of 13 mm ± 3 mm at least 7 days after implantation. Animals received 50 IU of heparin and the hind limb was kept at a constant temperature of 38-39°C. The femoral artery and vein were cannulated and collaterals were occluded by a groin tourniquet. An oxygenation reservoir was included into the circuit and melphalan and TNF were added as boluses herein. A roller pump recirculated the perfusate at a flow rate of 2.4 ml/min for 30 min. A washout with 5 ml oxygenated Haemaccel was performed at the end of the perfusion. The committee on Animal Research of the Erasmus University Rotterdam, the Netherlands, approved the experimental protocol. Tumour growth after perfusion was daily recorded by calliper measurement. Tumour volume was calculated as $0.4(A^2B)$, where A is the minimal tumour diameter and B the diameter perpendicular to A. Tumour volumes were compared five days after perfusion.

Assessment of melphalan concentrations in tissue

Immediately after ILP the perfused tumour and hind limb tissues were excised, homogenised in 2 ml acetonitrile (PRO 200 homogenizer, Pro Scientific, CT, USA), centrifuged at 2500g and stored at –80°C. Melphalan was measured by gas chromatography-mass spectrometry (GC-MS), as described previously.[14] P-[Bis(2-chloroethyl)amino]-phenylacetic acid methyl ester was used as an internal standard. Samples were extracted over trifunctional C18 silica columns. After elution with methanol and evaporation, the compounds were derivatised with trifluoroacetic anhydride and diazomethane in ether. The stable derivatives were separated on a methyl phenyl siloxane GC capillary column and measured selectively by single ion monitoring GC-MS in the positive EI mode.

Statistical analysis

Mann-Whitney U-test was used to compare tumour volumes in different animal groups and to compare melphalan concentrations in different groups.

RESULTS

Tumour response after ILP

Mean tumour volumes were compared to demonstrate the efficacy of ILP with TNF and melphalan. Four groups of rats were perfused with sham (n=10), TNF alone (n=10), melphalan alone

(n=10) and the combination of TNF and melphalan (n=10). A synergistic anti-tumour response was observed with the combination of melphalan and TNF as demonstrated before (Figure 1).[4,5] A significant decrease in mean tumour volume after perfusions with the combination of melphalan and TNF was observed ($p<0.001$), whereas in all other perfusions tumour volume increased.

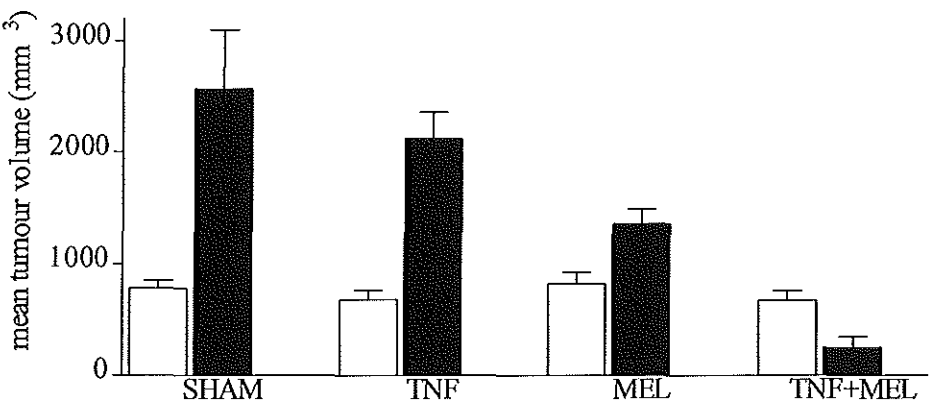

Figure 1. Mean tumour volumes (± SEM) of BN-175 sarcoma before (□) and five days after (■) ILP.

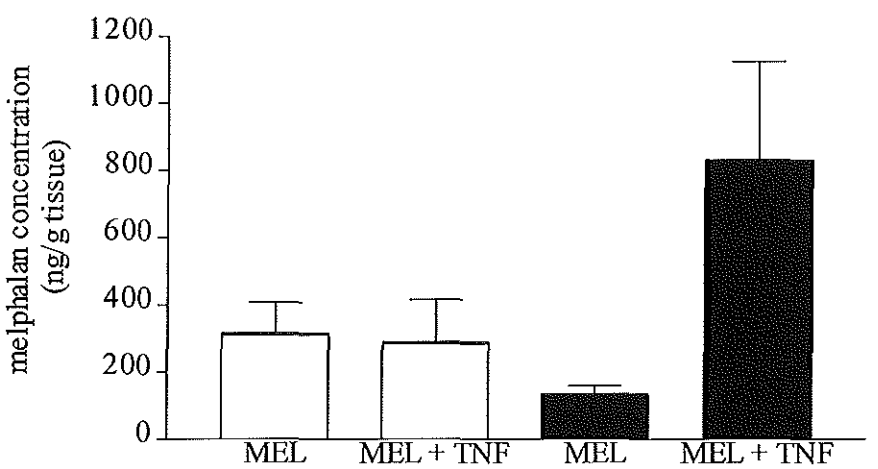

Figure 2. Melphalan concentration (± SEM) in skin/muscle tissue (□) and tumour tissue (■) immediately excised after ILP with melphalan with or without TNF.

Tissue concentrations of melphalan

Figure 2 demonstrates a sixfold increased melphalan concentration found in tumour tissue after perfusion with the combination of TNF and melphalan (n=6) in comparison with perfusions with melphalan alone (n=6)(p=0.01). TNF had no effect on skin and muscle tissue since melphalan concentrations after ILP were comparable with or without the addition of TNF.

DISCUSSION

In the present study we demonstrate an increased accumulation of melphalan in tumour tissue after ILP with the combination of melphalan and TNF as compared to melphalan alone. The increased melphalan accumulation could not be demonstrated in skin and muscle tissue, suggesting that TNF has no effect on normal tissue. The increased melphalan concentration in tumour tissue correlates very well with the observed tumour response.

The observed responses in this rat soft tissue sarcoma model are comparable to patients, where TNF or melphalan alone is not or only marginally active.[15-16] The combination of TNF and melphalan, however, results in high response rates.[1-3] Addition of TNF seems crucial in the observed synergy with melphalan and several mechanisms could be responsible for this. A direct effect of TNF on the anti-tumour activity of melphalan on BN-175 tumour cells was previously ruled out *in vitro*.[4]

Fajardo *et al.* previously demonstrated that low-dose TNF has a proliferative effect on angiogenesis, whereas higher doses can cause destruction of newly formed blood vessels.[17] It has been demonstrated that this destruction of blood vessels is the result of apoptosis and detachment of angiogenic endothelial cells[18] which may lead to thrombocyte aggregation, erythrostasis and haemorrhagic necrosis.[6,7,9,19]

Whereas previous studies focus on tumour regression resulting from TNF-mediated destruction of the vasculature we show that augmented melphalan concentrations in tumour tissue after ILP with TNF correlates very well with tumour response and provides an elegant and straight forward explanation for the observed responses. Similarly, drug accumulation in tumour tissue has been shown after systemic pre-treatment with TNF in mice treated with liposomal doxorubicin.[20,21] An explanation for this phenomenon can be the increased vascular permeability or decreased interstitial pressure that was demonstrated after administration of TNF.[11-13] Alexander *et al.* demonstrated an increased capillary leakage during isolated hepatic perfusions (IHP) and an increased uptake of I-131 albumin in tumour tissue compared to liver tissue.[22] However, addition of TNF did not affect melphalan concentrations in tumour tissue after IHP. Several reasons for this discrepancy are possible such as concentration of TNF used, sampling method and duration of perfusion. Another reason can be the difference in

tumourvasculature, since colorectal metastases are usually hypovascular and largely necrotic, whereas soft tissue sarcoma are usually hypervascular.

In conclusion, we hypothesise that increased tumour concentration of melphalan could very well be the main mechanism by which TNF enhances the antitumour response. This finding is not only important for further TNF-based limb perfusions using melphalan or other cytostatic agents, but also for other perfusions settings such as isolated liver,[22,23] lung[24] or kidney perfusions.[25]

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CHAPTER 4

TNF AUGMENTS INTRATUMOURAL CONCENTRATIONS OF DOXORUBICIN IN TNF-BASED ISOLATED LIMB PERFUSION IN RAT SARCOMA MODELS AND ENHANCES ANTITUMOUR EFFECTS

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SUMMARY

We have previously shown that isolated limb perfusion (ILP) in sarcoma-bearing rats results in high response rates when melphalan is used in combination with TNF. This is in line with observations in patients. Here we show that ILP with doxorubicin in combination with TNF has comparable effects in two different rat sarcoma tumour models. The addition of TNF exhibits a synergistic antitumour effect, resulting in regression of the tumour in 54% and 100% of the cases for the BN-175 fibrosarcoma and the ROS-1 osteosarcoma respectively. The combination is shown to be mandatory for optimal tumour response. The effect of high dose TNF on the activity of cytotoxic agents in ILP is still unclear. We investigated possible modes by which TNF could modulate the activity of doxorubicin. In both tumour models increased accumulation of doxorubicin in tumour tissue was found: 3.1-fold in the BN-175 and 1.8-fold in the ROS-1 sarcoma after ILP with doxorubicin combined with TNF in comparison with an ILP with doxorubicin alone. This increase in local drug concentration may explain the synergistic antitumour responses after ILP with the combination. *In vitro* TNF fails to augment drug uptake in tumour cells or to increase cytotoxicity of the drug. These findings make it unlikely that TNF directly modulates the activity of doxorubicin *in vivo*. As TNF by itself has no or only minimal effect on tumour growth, an increase in local concentrations of chemotherapeutic drugs might well be the main mechanism for the synergistic antitumour effects.

INTRODUCTION

Low concentrations at the tumour site and dose limiting systemic toxicity are common causes for failure of solid tumour treatment with anti-tumour agents. As cytotoxic drugs typically exhibit a steep dose response-curve, increasing local concentration should favour tumour response. In isolated limb perfusions local drug concentrations are increased while systemic exposure to the drugs is minimal. In isolated limb perfusions (ILP) melphalan is used most commonly, but also other agents (e.g doxorubicin and cisplatin) are applied with varying success in perfusion of limb or organ (e.g lung).[1-5] Tumour necrosis factor alpha (TNF), a cytokine with known antitumour activity, can not be used systemically in dosages high enough to obtain a tumour response.[6,7] However, in ILP with TNF tumours are exposed to concentrations of up to 50 times higher than those reached after systemic administration of the maximum tolerated dose (MTD), without major side effects.[8] Previously it was demonstrated that the addition of TNF to melphalan in ILP could improve response rates in patients with multiple melanoma in transit metastasises or irresectable soft tissue extremity sarcomas.[9-14] In both patient groups very high response rates of above 85% have been reported, with a limb salvage rate of more than 85%. In Europe TNF was recently approved and registered for clinical use in patients for the treatment of locally advanced extremity soft tissue sarcomas by ILP with TNF and melphalan. Comparable results have been reported by us for ILP with the combination of TNF and melphalan in soft tissue sarcoma

and osteosarcoma-bearing rats.[15-17] ILP with TNF alone or melphalan alone at concentrations used in the clinical setting had negligible antitumour effects, whereas the combination showed strong synergistic antitumour efficacy.

TNF may potentiate the effects of chemotherapy in ILP in various ways. TNF has a broad spectrum of activities, which range from enhancement of proliferation to direct cytotoxicity on tumour cells, activation of inflammation and effects on endothelium.[7,18] The tumour associated vasculature (TAV) responds to TNF with rounding of the endothelial cells resulting in increased gaps, allowing easy passage of soluble materials and even cells.[19-21] Moreover, intravenous injection of TNF in human melanoma xenograft-bearing mice resulted in significant reduction of the interstitial fluid pressure (IFP) of the tumours.[22] This phenomenon could increase localization of cytotoxic drugs in the tumour interstitium and explain improved tumour response. Secondly, clinical and experimental results demonstrating massive destruction of the endothelial cells, which has also been shown *in vitro* and on angiograms in patients after ILP, suggest that the TAV is the primary target for TNF and therefore that destruction of the endothelial lining might be responsible for the antitumour response.[18,23,24] This process is accompanied by inflammatory responses and seemed to be dependent on infiltrating leukocytes.[25] Coagulative and haemorrhagic necrosis and destruction of the endothelial lining was also seen when TNF was used as a single agent in ILP, however without significant effect on tumour growth in rats. This indicates that the direct TNF effect is most likely playing a minor role in the anti-tumour capacity.[15,26]

Although in the majority of the perfusions, especially for the treatment of melanoma, melphalan is used, also other agents might be successful. Anthracyclines are among the most active agents against solid tumours and doxorubicin is the most widely used agent of this class.[27,28] Moreover, doxorubicin is the agent of choice for the treatment of sarcoma, and has shown good anti-tumour activity in clinical and experimental perfusion settings for the treatment of lung metastasises, and could therefore be a suitable cytotoxic agent for ILP in sarcoma-bearing patients.[1,5]

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

MATERIAL AND METHODS

Chemicals

Human recombinant Tumour Necrosis Factor alpha (specific activity $5x10^7$ IU/mg) was kindly provided by Dr G. Adolf (Bender Wien GmbH, Wien, Austria) and stored at a concentration of 2 mg/mL at –80°C. Endotoxin levels (LAL) were below 0.624 EU/mg. Doxorubicin (Adriablastina®) was purchased from Farmitalia Carlo Erba (Brussels, Belgium).

Animals and tumour model

Male inbred BN rats were used for the soft tissue sarcoma model (BN-175) and WAG/RIJ rats for the osteosarcoma model (ROS-1). Rats were obtained from Harlan-CPB (Austerlitz, the Netherlands) and weighing 250-300 g. Small fragments (3 mm) of the syngeneic BN-175 or ROS-1 sarcoma were implanted subcutaneously in the right hindleg as previously described.[15,16] Tumour growth was recorded by calliper measurements and tumour volume calculated using the formula $0.4(A^2XB)$ (where B represents the largest diameter and A the diameter perpendicular to B). All animal studies were done in accordance with protocols approve by the Animal Care Committee of the Erasmus University Rotterdam, the Netherlands.

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

Isolated limb perfusion protocol

Rats were perfused according to the isolated limb perfusion technique originally described by Benckhuijsen *et al.*,[29] and adapted for the rat by Manusama *et al.*[15] Briefly, the femoral artery and vein of anaesthetized rats were cannulated with silastic tubing. Collaterals were occluded by a groin tourniquet and perfusion started when the tourniquet was tightened. The extracorporeal circuit included an oxygenation reservoir and a roller pump (Watson Marlow, Falmouth, UK). The perfusion was performed with 5 mL Haemaccel (Behring Pharma, Amsterdam, Netherlands) and TNF (50 µg) and/or doxorubicin (400 µg BN-175, and 200 µg ROS-1) were added as boluses to the oxygenation reservoir. Control rats (sham) were perfused with Haemaccel alone. The concentration of TNF was adapted from previous animal studies and doxorubicin concentrations were chosen which had no local toxicity and induced maximally stable disease after single perfusion.[15] Perfusion was maintained for 30 min at a flow rate of 2.4 mL/min. During the perfusion the hindleg of the rat was kept at a temperature of 38-

39°C with a warm water mattress. A washout with 2 ml oxygenated Haemaccel was performed at the end of the perfusion. Perfusion was performed at a tumour diameter of 12-15 mm, which is around 7 or 10 days after implantation for BN-175 and ROS-1 respectively.

In vitro assessment of antitumour activity

BN-175 soft tissue sarcoma cells or ROS-1 osteosarcoma cells were added in 100 µl aliquots to 96-well plates at a final concentration of 10^4 cells per well and allowed to grow as a monolayer in DMEM supplemented with 10% FCS. Doxorubicin and/or TNF were diluted in DMEM supplemented with 10% FCS, added to the wells and allowed to incubate for three days. The range of final drugs in the wells was 0.0005 - 100 µg/mL for doxorubicin and 0 - 10 µg/mL for TNF. A total of 5 to 6 separate assays were performed in triplicate and the percentage of growth inhibition calculated according to the formula: percentage of tumour cell growth = (test well/control well) X 100%. Percentage of tumour cell cytotoxicity was measured using the sulphorhodamine B assay.[30]

In vitro assessment of doxorubicin uptake in tumour cells

To determine if the observed antitumour response after ILP and cytotoxicity *in vitro* correlated with cellular uptake of doxorubicin, cells were exposed to doxorubicin with and without TNF and intracellular doxorubicin levels determined by flowcytometry as previously described.[31] Briefly, BN-175 soft tissue sarcoma cells or ROS-1 osteosarcoma cells were added in 500 µl aliquots to 24-well plates at a final concentration of $5x10^4$ cells per well and allowed to grow as a monolayer in DMEM supplemented with 10% FCS. Doxorubicin and TNF were diluted in DMEM supplemented with 10% FCS and added to the wells, after which cells were incubated for 0, 10, 30, 60, and 120 min. The final drug concentration in the wells was 0, 0.1, 1.0 and 10 µg/mL for both doxorubicin and TNF. Thereafter monolayers were treated with trypsin-EDTA for 2 min and the cell suspensions were washed two times in complete medium and resuspended in PBS. Cellular uptake was measured on a Becton Dickinson FACScan using Cell Quest software on Apple Macintosh computer. Excitation was set at 488 nm and emission at 530 nm. Fluorescence was corrected for cell size using the forward scatter (FSC) with the formula corrected fluorescence (FLcor) = fluorescence at 530 nm (FL530) / FSC - $FL530_c$ / FSC_c ($FL530_c$ and FSC_c are fluorescence and forward scatter with no drug added to the cells).

Assessment of doxorubicin accumulation in tumour and concentration in perfusate during ILP

To determine the influence of TNF on doxorubicin accumulation in tumours during ILP, tumours (and muscle) were surgically removed after ILP and total doxorubicin content determined as previously described.[32] As the ILP included a thorough washout there is no intravascular doxorubicin

present. Briefly, after incubation in acidified isopropanol (0.075 N HCl in 90% isopropanol) for 24 h at 4°C, the tumours were homogenized (PRO200 homogenizer with 10 mm generator, Pro Scientific, CT, USA), centrifuged for 30 min at 2500 rpm and supernatants harvested. Samples were measured in a Hitachi F4500 fluorescence spectrometer (excitation 472 nm and emission 590 nm) and compared with a standard curve prepared with known concentrations of doxorubicin diluted in acidified isopropanol. Measurements were repeated after addition of an internal doxorubicin standard. Detection limit for doxorubicin in tissue was 0.1 μg per gram tissue.

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

Statistical analysis

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

RESULTS

In vivo tumour response to doxorubicin and TNF

To evaluate the antitumour activity of doxorubicin when combined with TNF in an isolated limb perfusion setting, soft tissue sarcoma and osteosarcoma-bearing rats were perfused with the agents combined or alone. Figure 1 shows the tumour responses of soft tissue sarcoma (BN-175) in rats after ILP. Perfusion with buffer or TNF alone resulted in progressive disease in all animals. Although ILP with doxorubicin (400 μg) alone resulted in a slight inhibition of the BN-175 tumour growth when compared with the sham control, none of the rats showed a tumour response (Table 1). ILP with 400 μg doxorubicin combined with 50 μg TNF resulted in increased antitumour activity with a response rate of 54% (PR and CR combined) ($p< 0.01$ compared with doxorubicin alone).

In osteosarcoma (ROS-1)-bearing rats ILP with buffer or doxorubicin (200 μg) alone had no significant effect on tumour growth (Figure 2). ILP with TNF alone resulted in significant inhibition of tumour growth as compared with the sham perfusion and a response rate of 33% was observed (Table 2). ILP with 200 μg doxorubicin combined with 50 μg TNF further increased the antitumour response with a response rate of 100% (PR and CR combined) ($p <0.05$ compared with TNF alone).

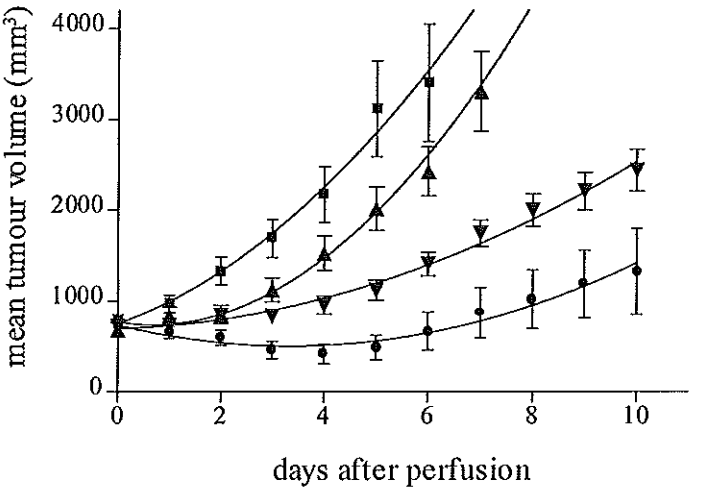


Figure 1. Growth curves of subcutaneous implanted soft tissue sarcoma BN-175 after isolated limb perfusion with medium alone (■), 50 µg TNF (▲), 400 µg doxorubicin (▼), or combination of TNF and doxorubicin (●). Mean tumour volumes are shown ± SEM. Number of rats per group is shown in table 1.

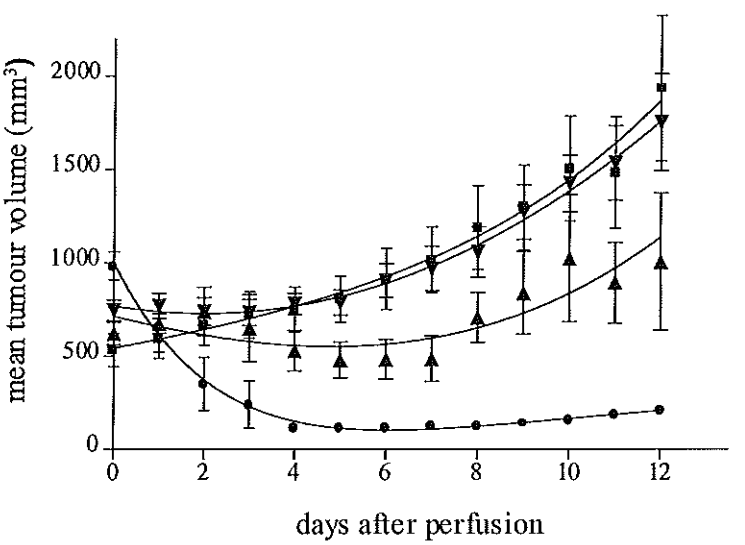


Figure 2. Growth curves of subcutaneous implanted osteosarcoma ROS-1 after isolated limb perfusion with medium alone (■), 50 µg TNF (▲), 200 µg doxorubicin (▼), or combination of TNF and doxorubicin (●) Mean tumour volumes are shown ± SEM. Number of rats per group is shown in table 2.

Table 1. Tumour response of BN-175 after isolated limb perfusion with doxorubicin and TNF five days after treatment.

Tumour Response	Sham n=12	TNF n=101	Doxorubicin n=10	TNF+ Doxorubicin n=13
PD	12	10	6	2
NC			4	4
PR				6
CR				1
Response rate (%)	-	-	-	**54**

Perfusions were performed with 50 μg TNF and 400 μg doxorubicin under constant temperature (38-39°C) for 30 min.

Table 2. Tumour response of ROS-1 after isolated limb perfusion with doxorubicin and TNF five days after treatment.

Tumour Response	Sham n=8	TNF n=11	Doxorubicin n=8	TNF+ Doxorubicin n=10
PD	8	3	2	
NC		3	6	
PR		1		6
CR		2		4
Response rate (%)	-	**33**	-	**100**

Perfusions were performed with 50 μg TNF and 200 μg doxorubicin under constant temperature (38-39°C) for 30 min.

In vitro assessment of antitumour activity of doxorubicin and TNF

The *in vivo* experiments clearly demonstrate pronounced improvement of tumour response when doxorubicin was used in combination with TNF. *In vitro* experiments were performed to further study the nature of this interaction. Exposure of soft tissue sarcoma BN-175 or osteosarcoma ROS-1 tumour cells to doxorubicin resulted in a response curve with an IC50 of 0.1 and 2.0 μg/ml respectively (Figure 3). No significant cellular toxicity could be observed when BN-175 cells were exposed to TNF alone, however a dose depended growth reduction was observed when ROS-1 cell were exposed to TNF with a maximum reduction of 38% at 10 μg/ml. Addition of TNF to doxorubicin did not significantly alter the IC50 of doxorubicin in the BN-175 cell cultures, indicating that addition of TNF *in vitro* did not influence the sensitivity of the cells to doxorubicin significantly. On ROS-1 cells only an additive effect of TNF and doxorubicin was observed. The curve only shifted downwards and not to a lower doxorubicin concentration, which indicates that the drugs do not influence each other but have separate effects.

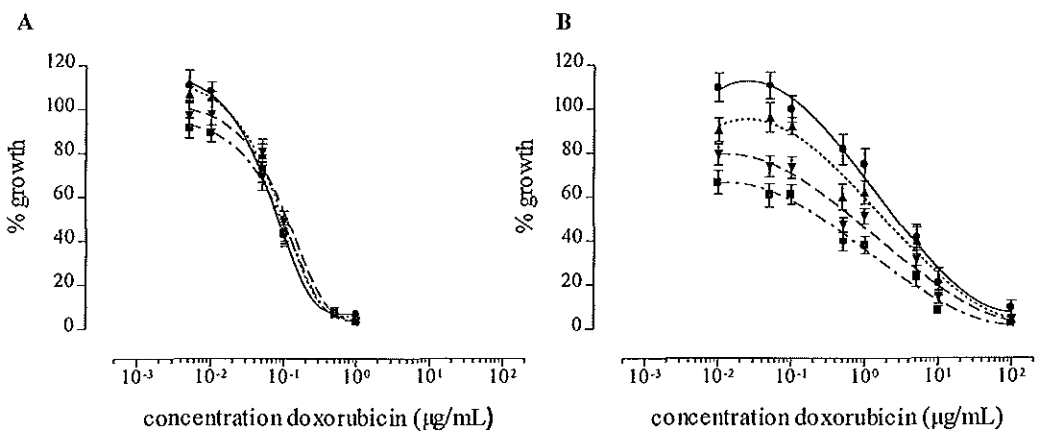


Figure 3. In vitro growth of (A) the BN-175 and (B) ROS-1 tumour cells as function of the doxorubicin concentration in combination with 0 μg (●), 0.1 μg (▲), 1.0 μg (▼) or 10 μg TNF per ml (■). The mean of 5 to 6 individual experiments performed in triplicate is shown ± SEM.

In vitro uptake of doxorubicin in tumour cells

Figure 4 shows that increased intracellular concentrations of doxorubicin are observed in both cell types when cells were incubated with increasing concentrations of doxorubicin. A 10-fold higher

doxorubicin concentration in culture supernatant (ranging from 1.0 to 10 µg/mL) resulted in 4.5-fold and 3.9-fold augmented cellular uptake for BN-175 and ROS-1 respectively ($p < 0.01$ and $p < 0.05$). Addition however of TNF to the culture medium did not influence intracellular doxorubicin content significantly for all the TNF concentrations tested, or even a slight but not significant reduction in uptake was noticed with increasing concentrations of TNF (Figure 5).

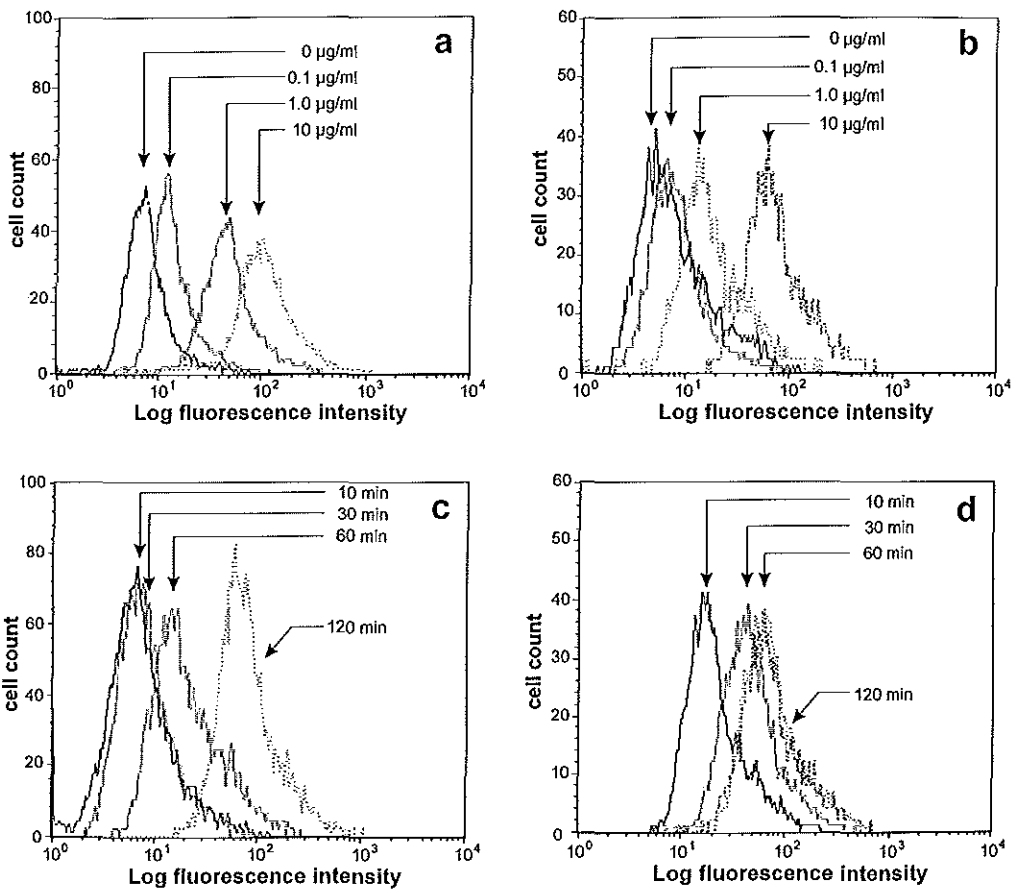


Figure 4. Uptake of doxorubicin by (a and c) BN-175 tumour cells, or (b and d) ROS-1 tumour cells in vitro as determined by flowcytometry after exposure of the cells to 0, 0.1, 1.0 or 10 µg per ml doxorubicin for 2 hrs (a and b) or for various durations of time at a fixed doxorubicin concentration of 10 µg per ml c and d). The graphs are good representatives of the experiments performed.

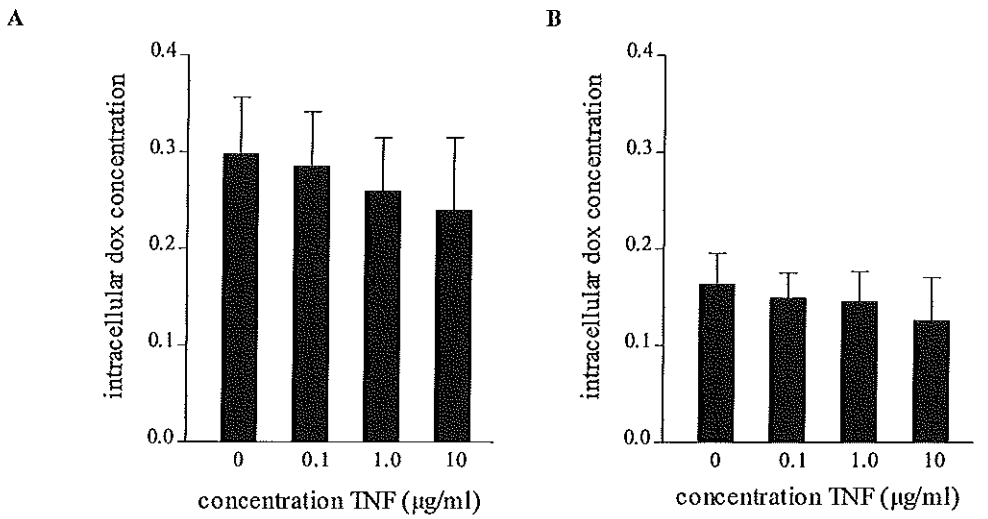
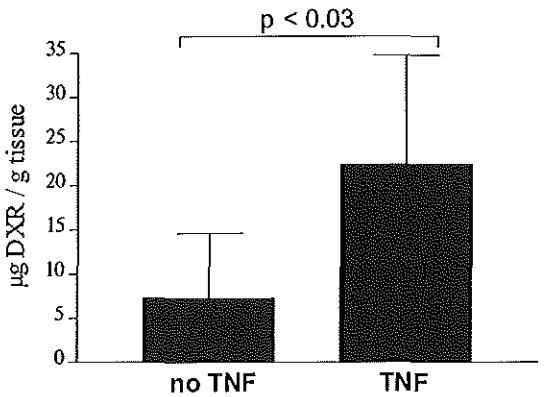


Figure 5. Uptake of doxorubicin in (A) BN-175 or (B) ROS-1 tumour cells in vitro at respectively 120 and 60 min of exposure to the agent in the presence of 0, 0.1, 1.0 or 10 µg TNF per ml. The mean of 5 experiments is shown ± SD.

Doxorubicin accumulation in solid tumour after ILP

Possibly the observed beneficial effect of TNF *in vivo* could be explained by an increased extravasation of doxorubicin into the tumour interstitium, resulting in a higher local concentration and accordingly in an improved antitumour activity. Therefore, concentrations of doxorubicin in tumour and surrounded tissue after ILP were determined. Figure 6 shows that measurable amounts of doxorubicin localized both in BN-175 and ROS-1 tumours after ILP, which correlates with an observed decline of the drug concentration in the perfusate (data not shown). Moreover, addition of TNF to the perfusate resulted in significantly enhanced accumulation of doxorubicin in both these tumours, 3.1-fold in the BN-175 and 1.8-fold in the ROS-1 sarcoma, when compared with ILP with doxorubicin alone. Addition of TNF had no significant effect on doxorubicin accumulation in muscle of the leg ($p > 0.4$). Strikingly a significant discrepancy in drug levels was observed between BN-175 and ROS-1 tumours.

A



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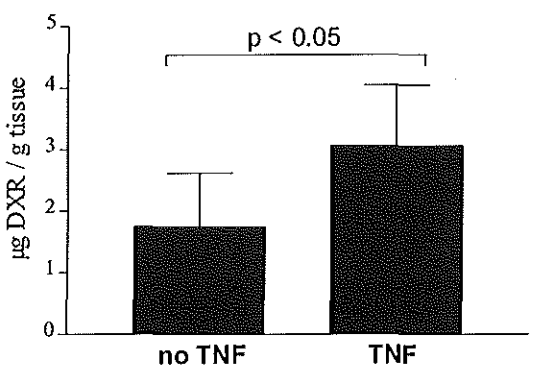

Figure 6. Accumulation of doxorubicin in (a) soft tissue sarcoma BN-175 or (b) osteosarcoma ROS-1 in vivo during isolated perfusion. Rats were perfused with doxorubicin (400 µg BN-175 and 200 µg ROS-1) with 50 µg TNF or without TNF, after which tumours and muscle were excised and total doxorubicin content determined as described in materials and methods. The mean of 6 rats are shown ± SD.

DISCUSSION

In the present study we demonstrate that isolated limb perfusion in sarcoma-bearing rats with doxorubicin in combination with TNF results in high response rates in two different tumour models. These findings are in close agreement with our previous work using melphalan.[15,16] Secondly, it is demonstrated for the first time that TNF enhances intratumoural accumulation of doxorubicin, which

is an attractive explanation for the augmented tumour response in TNF-based ILP. We speculate that TNF increases interstitial drug levels in the tumour as intravascular doxorubicin is washed out at the end of the ILP procedure and intracellular uptake of doxorubicin is not affected by TNF as was shown *in vitro*. Doxorubicin has been shown to be the most effective drug in treatment of sarcomas and therefore put forward as the drug of choice in the treatment of these malignancies.[27,28] Here we demonstrate that perfusion with doxorubicin alone is not or only partial effective, which however is also observed when melphalan is used as a single agent in the perfusion setting.

A striking observation is the augmentation of the doxorubicin-induced anti-tumour response by TNF *in vivo*, which has also been shown for melphalan and TNF in these tumour models.[15,16] Strong tumour responses were observed in both models after ILP with the combination therapy, which cannot be explained by just adding up the responses after ILP with the single agents. An important observation is that chemotherapy by itself is not or partially effective as shown here and by others.[2] Secondly, it was previously shown in our rat tumour model as well as in the clinic that ILP with TNF alone had no effect on tumour growth although massive haemorrhagic necrosis and pathology was observed.[15,26,33] These observations indicate that other mechanisms have to be identified to explain the interaction between TNF and chemotherapy.

Several specific activities of TNF could potentiate the antitumour activity of chemotherapy. It has been postulated that the increased tumour response observed after ILP with melphalan and TNF is due to destruction of the TAV, resulting in haemorrhagic necrosis, platelet aggregation and erythrostasis.[18,26] Moreover, recently is has been shown that perfusion with melphalan in combination with TNF and IFN resulted in apoptosis of endothelial cells of the TAV.[34] Also inflammatory events such as granulocyte infiltration were suggested to play a role.[25,26] These findings led to the speculation that destruction of the TAV is the mechanism by which TNF potentiates cytotoxic agents. Watanabe *et al.* demonstrated toxic effects of TNF on newly formed tumour vasculature in mice resulting in haemorrhage, congestion and blood circulation blockage.[18] Others suggested that TNF induced thrombus formation played an important role.[35] However, these effects are also observed after perfusion with TNF alone.[26]

Recent studies show that perfusion of melanoma-bearing patients with melphalan in combination with TNF and IFN results in detachment and apoptosis of endothelial cells of the tumour.[34] Moreover, the vitro experiments demonstrated an important role for TNF and IFN mediated down modulation of the $\alpha V\beta 3$ function, which is speculated to play an prominent role in the *in vivo* observations. These finding would argue in favour for a TNF mediated destruction of the vasculature. The *in vitro* observations also demonstrated the necessity of IFN for the induction of endothelial apoptosis. In our model, as well as in various clinical trails, on the other hand it has been shown that

tumour responses are only slightly improved by the addition of IFN.[11,14] This would argue against an important role for TNF mediated destruction of the TAV in the tumour response, or on the other hand it indicates that endogenous produced IFN is of major importance.

A consistent finding in our two models is the augmented accumulation of doxorubicin in tumour tissue when TNF is added to the perfusate. In both models this increase could very well explain the improved efficacy. On the other hand, may increase the uptake of doxorubicin by the tumour cells. However, intracellular concentration of doxorubicin *in vitro* was not enhanced when TNF was added *in vitro*. Moreover, TNF did not seem to affect the *in vitro* cytotoxic activity of doxorubicin significantly. In contradiction to these findings, synergy between TNF and doxorubicin *in vitro* has been shown in previous studies depending on sensitivity of the cells to TNF, presence of multi-drug resistance or order of exposure.[36-39] This effect has also been shown without an increased intracellular accumulation of doxorubicin.[40] Others demonstrated that exposure of tumour cells to TNF resulted in a reduced sensitivity of these cells to doxorubicin.[41] It is suggested that arrest of the cells in the G1/0 phase by TNF turns them insensitive to doxorubicin, which is a cell cycle dependent cytotoxic agent. In our study we did not observe such phenomenon when the tumour cells were exposed to doxorubicin and TNF. These observations suggest that *in vivo* TNF has an indirect effect on the anti-tumour activity of doxorubicin. Therefore, we postulate that TNF augments the accumulation of doxorubicin in the tumour by increasing the leakiness of the tumour associated vasculature (TAV), and by doing so increases the local drug level. Previously an increased leakiness of the TAV as well as a reduction of the interstitial fluid pressure in tumour has been shown by others after systemic administration of TNF.[19-22] Moreover, increased drug accumulation in tumour has previously been shown after systemic treatment with TNF when a liposomal doxorubicin preparation was injected.[42] Preliminary results from a clinical phase I-II trail with doxorubicin and TNF in hyperthermic ILP demonstrated comparable favourable outcome as is obtained with Melphalan and TNF.[43]

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

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CHAPTER 5

SYNERGISTIC ANTITUMOUR EFFECT OF TNF-MUTANT (TNF-SAM2) WITH MELPHALAN AND DOXORUBICIN IN ISOLATED LIMB PERFUSION IN RATS

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Submitted

SUMMARY

An isolated limb perfusion model (ILP) using soft tissue sarcoma bearing rats (BN-175) was used to study antitumour activity of a tumour necrosis factor alpha mutant (TNF-SAM2) in combination with melphalan and doxorubicin. On a TNF sensitive cell line (WEHI) we demonstrated that the concentration of TNF-SAM2 used in the perfusions was similar as in previous studies with recombinant human TNF (rHuTNF). Progressive disease was demonstrated in all animals after perfusions with sham or 50 μg TNF-SAM2. ILP with 40 μg melphalan or 400 μg doxorubicin resulted both in no change of tumour volume or progressive disease five days after perfusion. A synergistic anti-tumour effect was demonstrated using the combination of 50 μg TNF-SAM2 with 40 μg melphalan, leading to a partial and complete response rates of 76%. The combination of 50 μg TNF-SAM2 and 400 μg doxorubicin was synergistic as well as with a 70% response rate. Histopathologically this response consisted of hemorrhagic necrosis of the coagulative type, which is comparable to what has been demonstrated with rHuTNF.

In conclusion, TNF-SAM2 has similar anti-tumour activity in combination with melphalan or doxorubicin as rHuTNF in sarcoma-bearing rats. Because of its potential decreased toxicity it is eligible to be tested in clinical isolated limb perfusion settings.

INTRODUCTION

From experimental and clinical studies it appears that high local human recombinant tumour necrosis factor alpha (rHuTNF) concentrations is an important determinant of response. However, the use of rHuTNF systemically is limited by severe systemic toxicity. In a leakage free isolated limb perfusion (ILP) setting high concentrations of rHuTNF can be obtained without systemic toxicity and the use of rHuTNF in this setting in combination with melphalan is now well established.[1] Not only in patients with 'in transit' metastasized melanoma[2,3] but also with advanced soft tissue sarcoma high response rates are observed with this treatment.[4,5] For the treatment of locally advanced extremity soft tissue sarcomas rHuTNF has recently been approved by the EMEA in Europe.

In our laboratory we developed a soft tissue as well as an osteosarcoma bearing rat tumour models with response rates and histopathological characteristics similar to patients after ILP with rHuTNF and melphalan.[6-9] Recently, we also demonstrated that ILP with doxorubicin in combination with rHuTNF results in a synergistic antitumour response in both tumour models.[10] The observations with doxorubicin have been similar to what is seen with rHuTNF in combination with melphalan.

Although high doses of rHuTNF can be used with success in a leakage-free ILP-setting, its use systemically is limited by severe systemic toxicity that has prevented the use of effective doses

in patients.[11] Moreover, perfusion of vital organs such as liver,[12-15] lung[16] or kidney[17] are limited by toxicity of the perfused organ.

Therefore, TNF mutants have been developed to reduce toxic side effects and make it applicable for other use than ILP.[18-26] One such mutant, TNF-SAM2, has increased N-terminal basicity and has been shown to have both a two-fold higher cytotoxic activity in vitro and up to 20-fold lower acute toxicity in a murine model, compared to conventional TNF.[19,27] The present study is to determine whether the antitumour effects of TNF-SAM2 in vitro and in our preclinical ILP models in the rat in combination with melphalan and doxorubicin demonstrate similar efficacy in as has been observed with rHuTNF. If similar efficacy is observed TNF-SAM2 may be considered to be tested in the clinical setting to determine its toxic profile which if reduced in comparison to rHuTNF would make the agent eligible to be tested for efficacy in a number of clinical settings.

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 ad libitum (Hope Farms Woerden, the Netherlands) and were housed under standard 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.

Agents

TNF-SAM2 was provided by Prof. Soma having a specific activity of 5.7×10^6 U/mg as determined in the L929 cells assay.[19] Endotoxin levels were < 1.25 endotoxin units (EU) per mg protein. rHuTNF 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 (28). Endotoxin levels were < 1.25 endotoxin units (EU) per mg protein. 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. Doxorubicin (Adriblastina©, 50 mg/25 ml, Farmitalia Carlo Erba, Brussels, Belgium) was used. No further dilutions were made and 200 µl doxorubicin was added to the perfusate as a bolus.

Cytotoxic assay of TNF and TNF-SAM2 in vitro

rHuTNF and TNF-SAM2 levels were determined using the WEHI 164 bioassay. WEHI cells were allowed to grow as a monolayer in Dulbecco's modified Eagle's medium containing 5 % FCS and 0.3 mmol/l glutamic acid in a 96 well plate. Cells were exposed to TNF and TNF-SAM2 concentrations for three days and washes two times. Viable cells were stained using the MTT stain assay and tumour growth was calculated using the formula: tumour growth = (growth exposed cells/control growth) x 100 %.

Isolated Limb Perfusion Model (ILP)

The model and perfusion technique we used has been published previously.[6] Briefly, the non-immunogenic BN-175 sarcoma was used implanted subcutaneously into the right hind limb just above the ankle. Perfusion was performed at a tumour diameter of 13 mm ± 3 mm at least 7 days after implantation. Subsequent tumour growth was daily recorded by caliper measurement. Tumour volume was calculated as $0.4(A^2B)$, where A represents the smallest diameter and B the diameter perpendicular to A.

During perfusion animals were anaesthetized with Hypnorm® (Janssen Pharmaceutica, Tilburg, the Netherlands) and 50 iu of heparin were injected intravenously to prevent coagulation in the collateral circulation and in the perfusion circuit. To keep the rat's hind limb 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 started when the tourniquet was tightened. An oxygenation reservoir filled with 5 ml Haemaccel (Behring Pharma, Amsterdam, the Netherlands) and a roller pump were included into the circuit. Drugs 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.

Assessment of tumour response

The classification of tumour response was: progressive disease (PD) = increase of tumour volume (> 25 %) within 4 days; no change (NC) = tumour volume equal to volume during perfusion (in a range of -25 % and + 25 %); partial remission (PR) = decrease of tumour volume (-25 and -90%); complete remission (CR) = tumour volume 0-10% of volume during perfusion or necrosis.

Histology

The histopathological techniques and changes observed in tumours was assessed after perfusions as described earlier.[8] Briefly, tumours were excised with a rim of skin whereas the muscle layer formed the deep resection margin, fixed in 4% formaldehyde solution and embedded in paraffin. Histological sections were hematoxylin-eosin stained. Tumour samples were taken after 2 hours, 2 days and 5 days after perfusion.

Statistical analysis

Mann Whitney U test was used to compare tumour volumes in different animal groups and to compare different tumour responses in different groups. Calculations were performed on a personal computer using GraphPad Prism and SPSS for Windows 95.

RESULTS

In vitro results

To compare the specific activity of rHuTNF with TNF-SAM2 under controlled circumstances, *in vitro* cellular toxicity was examined using the TNF sensitive WEHI 164 (clone 13) cell line. Figure 1 shows the *in vitro* dose/response curves of the WEHI cell line, demonstrating similar in vitro toxicity of TNF-SAM2 against WEHI cells as compared to conventional TNF.

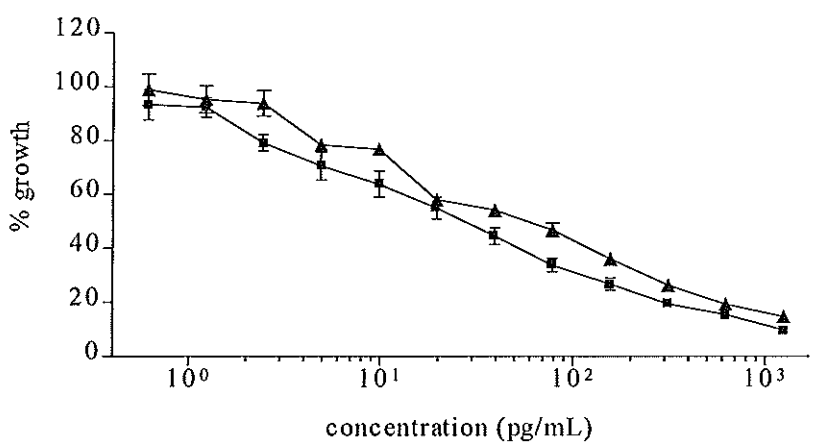

Figure 1. Dose-response curves of WEHI 164 cells to rHuTNF (■) and TNF-SAM2 (▲) as determined using the MTT stain assay.

In vivo results of TNF-SAM2, melphalan and doxorubicin.

Previously, synergy between rHuTNF and melphalan has been shown by us in ILP in BN rats.[6] In Figure 2 results are shown after ILP with the combination of 50 μg TNF-SAM2 and 40 μg melphalan, which are in close agreement with results found with rHuTNF and melphalan (dotted line). At five days after ILP a significantly decreased tumour growth was observed as compared to sham perfusions ($p<0.001$), TNF-SAM2 perfusions alone ($p<0.001$) and melphalan perfusion alone ($p=0.001$). No significant difference was found between all other groups.

ILP using doxorubicin and rHuTNF demonstrated that similar results can be obtained as compared with melphalan when 400 μg doxorubicin is used.[10] Here we demonstrate likewise synergy between doxorubicin in combination with the mutant TNF-SAM2 (Figure 3). A significantly decreased tumour growth was observed as compared to sham perfusions ($p<0.0001$), TNF-SAM2 perfusions alone ($p=0.0001$) and doxorubicin perfusion alone ($p=0.02$).

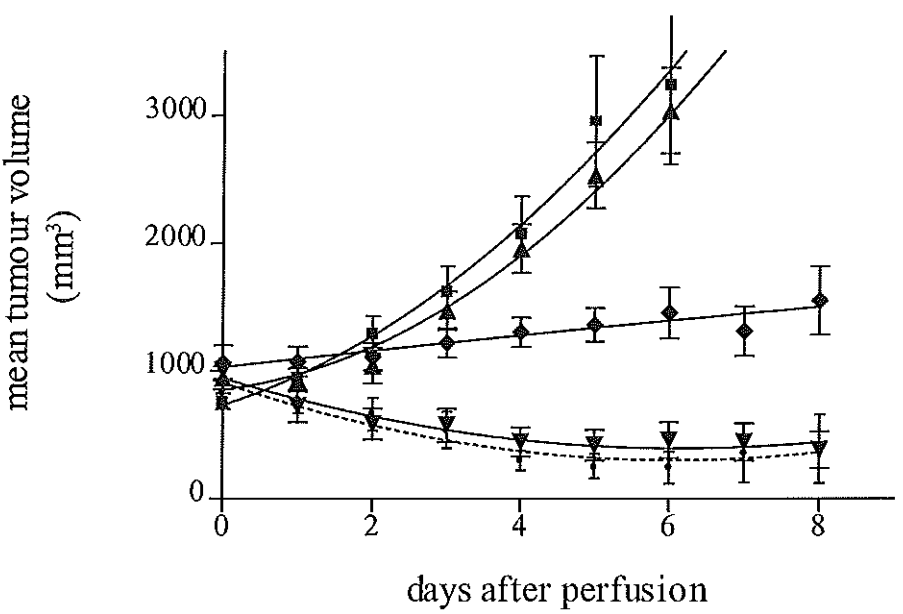

Figure 2. Growth curves of BN-175 sarcoma after sham (■; n=10), 50 μg TNF-SAM2 (▲; n=9), 40 μg melphalan (◆; n=10) and TNF-SAM2 plus melphalan isolated limb perfusion (▼; n=29). The dotted line is the growth curve of 50 μg rHuTHF in combination with 40 μg melphalan. Mean (± SEM) of tumour volumes are shown.

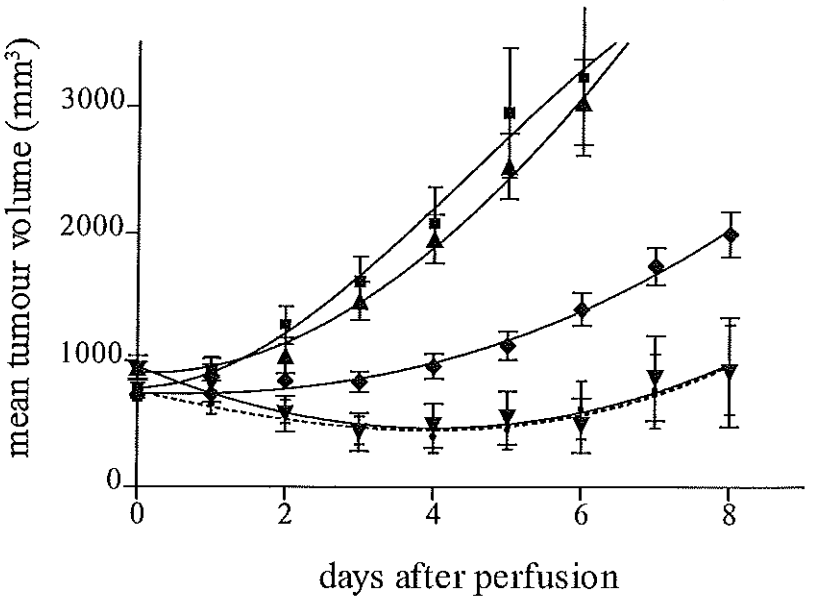

Figure 3. Growth curves of BN-175 sarcoma after sham (■; n=10), 50 µg TNF-SAM2 (▲; n=9), 400 µg doxorubicin (◆; n=10) and TNF-SAM2 plus doxorubicin isolated limb perfusion (▼; n=10). The dotted line is the growth curve of 50 µg rHuTHF in combination with 400 µg doxorubicin. Mean (± SEM) of tumour volumes are shown.

Table 1. Tumour response of BN-175 after isolated limb perfusion

Tumour Response	Sham n=10	TNF-SAM2 n=9	Melphalan n=10	TNF-SAM2 +Mel n=29	Doxorubicin n=10	TNF-SAM2 +Dox n=10
PD	10	9	2	3	7	1
NC			8	4	3	2
PR				9		6
CR				13		1
Response Rate (%)	-	-	-	**76**	-	**70**

Perfusions were performed with 50 µg TNF-SAM2, 40 µg melphalan and 400 µg doxorubicin under constant temperature (38-39°C) for 30 min.

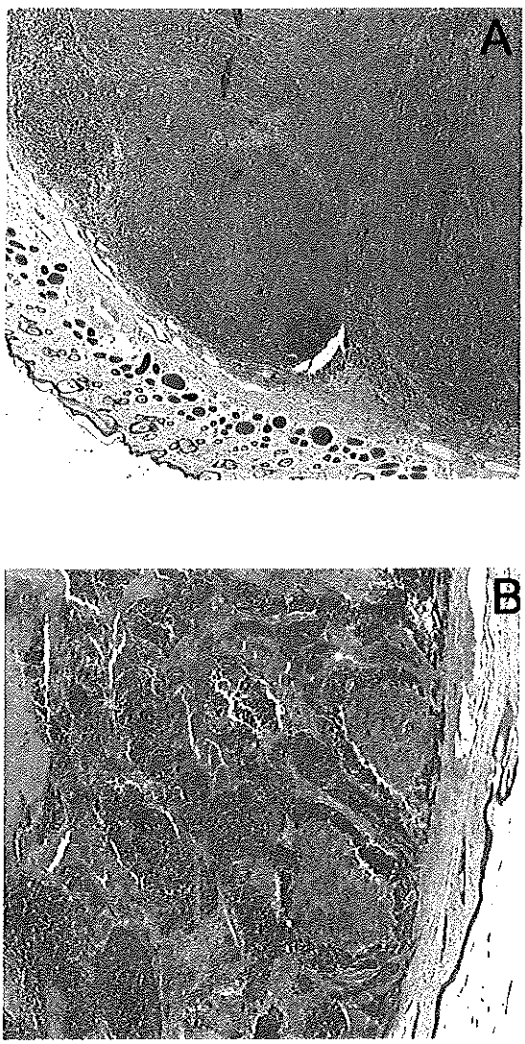

Figure 4A+B.
Hematoxylin and eosin (HE) stained tumour sections two days after ILP with A) haemacell (control) shows viable tumourcells and B) the combination of 50 μg TNF-SAM2 and 40 μg melphalan shows the aspect of hemorrhagic necrosis.

Tumour responses were determined five days after perfusion and summarized in Table 1 for melphalan and doxorubicin. Perfusions with sham or TNF-SAM2 alone resulted in progressive disease in all animals. ILP with melphalan or doxorubicin alone resulted both in no change or progressive disease. Addition of TNF-SAM2 to melphalan or doxorubicin resulted in an overall response rate of respectively 76% and 70%. These responses were statistically significant different from perfusions with sham ($p<0.0001$), TNF ($p<0.001$), melphalan ($p<0.001$) or doxorubicin alone ($p<0.001$).

Histological observations

Sham treated rats showed individual cell necrosis, both of the coagulative type. Over 80% of the tumour consisted of apparently vital tumour cells two days after perfusion (Figure 4a). Slices of tumours treated with melphalan or TNF-SAM2 showed apparently vital tumour cells over 80% of the cut surface of the tumour sections (data not shown). Slices of tumours treated with the combination of melphalan and TNF-SAM2 showed 80-90% necrosis with the aspect of haemorrhagic necrosis two days after perfusion (Figure 4b). Cell debris, oedema, thrombi and mononuclear inflammatory cells were observed in these tumours. The results two days after perfusion were in comparison with observations made with rHuTNF and melphalan.[8] Observations two hours and five days after perfusion (data not shown) were also similar as in previous studies with rHuTNF.

DISCUSSION

The pleiotropic cytokine tumour necrosis factor alpha (TNF) is not only an important mediator of host defense in infections, it has also direct cytotoxic activity on some cell lines in vitro (20). Moreover, TNF can induce haemorrhagic necrosis in experimental tumours in animal models most likely due to effects on the tumour neovasculature. However, its use appeared limited in phase I and II trials due to several toxic side effects. Hypotension has been the major dose-limiting toxicity observed in trials involving systemic administration of human recombinant TNF (rHuTNF). Because of the inherent toxicity it is impossible to administer rHuTNF at a dose that has antitumour effects in humans. However, isolated limb perfusion (ILP) allows administration of high dosages of rHuTNF without major side effects and has resulted in high response rates as part of a multimodality therapy for melanomas[2,3] and soft tissue sarcomas.[4,5] During these perfusions systemic monitoring is important because hypotension is a significant complication due to unexpected leakage. Since rHuTNF can only be used in the isolated perfusion setting, TNF-mutants

were developed to reduce the systemic toxicity without loosing antitumour activity.

Series of TNF-mutants were shown to have retained the antitumour effects of rHuTNF but caused much less toxicity.[18-25] TNF-SAM2 is such a mutant, that has been developed by Soma *et al.*[19,27] and its reduced systemic toxicity was demonstrated in a canine model.[29] Moreover, an (unexpected) increase in tumour-cytotoxicity on several different tumours *in vitro* was demonstrated when compared to conventional TNF.[30]

In the present study we demonstrated synergy between TNF-SAM2 and melphalan in sarcoma bearing rats after ILP. Moreover, synergistic antitumour activity was shown after ILP with TNF-SAM2 combined with doxorubicin. These results are similar to those obtained with conventional TNF in combination with these cytostatic drugs as published previously.[6,9,10] Complete and partial response rates of 76% were achieved with TNF-SAM2 and melphalan five days after perfusion. The combination of TNF-SAM2 and doxorubicin resulted in 70% partial and complete responses. Histopathologically the response consisted of individual cell necrosis with the aspect of haemorrhagic necrosis. These observations are similar to the results found both in rats[8] and humans after ILP.[31,32]

A direct cytotoxic effect on the endothelial cells, as well as polymorphonuclear cell infiltrations might be responsible for the antitumour effect of TNF in these tumours.[33] The potentiation of the TNF-SAM2 antitumour effect by doxorubicin and melphalan is thought to work through a dual targeting system. TNF has its antitumour effect by effecting the tumour vasculature, while doxorubicin and/or melphalan have direct cytotoxic activity. Hyperthermia, used in these perfusions might further potentiate the antitumour effects of both TNF-SAM2[34] and melphalan.[35]

Most likely the mutant used in this study has full antitumour activity as compared to rHuTNF. In the event of unexpected massive leakage during ILP it is obvious that the reduced toxicity of TNF-SAM2 can make ILP a safer procedure when used in combination with cytostatic drugs. More importantly with the demonstrated antitumour effects in tumour models that have yielded virtually identical observation as those observed in patients treated by rHuTNF-based ILPs for soft tissue sarcomas or melanomas, it may open the possibility to test TNF-SAM2 in other clinical settings because of its potential inherent reduced toxic profile. Thus it becomes an interesting agent to be tested in the regional treatment modalities for liver metastases such as isolated hepatic perfusion as well as repeated hepatic artery infusion. Furthermore this mutant will make the administration of TNF applicable in other regional perfusions which are not leakage free (e.g. pelvis or abdomen). Moreover, it would allow the evaluation of repeated low dose treatments which have been shown to enhance significantly the homing of doxorubicin-long circulation liposomes to the tumour site and enhance intratumoural doxorubicin concentration and thus yield

important tumour responses not obtained without the systemic administration of TNF (ten Hagen, unpublished observation).

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CHAPTER 6

INHIBITION OF NITRIC OXIDE SYNTHESIS BY L-NAME RESULTS IN SYNERGISTIC ANTITUMOUR ACTIVITY WITH MELPHALAN AND TUMOUR NECROSIS FACTOR ALPHA-BASED ISOLATED LIMB PERFUSION

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Submitted

SUMMARY

NO is an important molecule in regulating tumour blood flow and stimulating tumour angiogenesis. Inhibition of NO synthase might induce an antitumour effect by limiting nutrients and oxygen to reach tumour tissue by reducing blood flow and inhibiting neovascularisation. Wag-Rij rats bearing a subcapsular CC531 adenocarcinoma in both kidneys were used for L-NAME systemic treatment and tumour weight was compared with controls ten days after treatment. ILP was performed on BN-175 soft tissue sarcoma-bearing rats using L-NAME alone or in combination with TNF and melphalan. Tumour volumes and responses were measured daily and compared to control treatment. Systemic treatment with L-NAME inhibited growth of subrenal CC531 adenocarcinoma significantly but was accompanied by impaired renal function. Reduced tumour growth was observed when L-NAME was used alone in ILP. In combination with TNF or melphalan L-NAME increased response rates significantly compared to perfusions without L-NAME (0 to 64% and 0 to 63% respectively). An additional antitumour effect was demonstrated when L-NAME was added to the synergistic combination of melphalan and TNF (responses increased from 70 to 100%).

In conclusion, systemic inhibition of NO synthase inhibits tumour growth, however is accompanied by systemic toxicity. A synergistic antitumour effect of L-NAME in combination with melphalan and/or TNF is observed in rats using ILP. These results might improve future clinical regional perfusion strategies for patients with advanced soft tissue sarcoma in which response rates are currently around 70% using TNF and melphalan.

INTRODUCTION

Nitric oxide (NO) is a multi-functional messenger molecule derived from the amino acid, L-arginine, in a reaction catalysed by NO synthase (NOS). There are three isoforms of NOS: the calcium-dependent endothelial (eNOS) and neuronal (nNOS) and the calcium-independent inducible (iNOS=NOS2). High levels of NOS activity are present in several tumour cell lines as well as in human cancer.[1,2] An important function of NO is to maintain or increase tumour blood flow via dilatation of arteriolar vessels in some tumours.[3] This effect on tumour vasculature by NO enables vital nutrients and oxygen to reach tumour cells and can result in a promoted tumour growth in tumour cells that constantly released NO.[4] Moreover, recent studies demonstrated another important effect of NO in stimulating tumour angiogenesis.[5-7] Inhibition of NO synthase might inhibit tumour neovascularisation and in this way reduce tumour growth.

Several authors demonstrated a selectively reduced tumour blood flow in rodents treated with NO inhibitors, such as L-NAME.[8-10] Orucevic and Lala demonstrated a concentration dependent antitumour effect of L-NAME in adenocarcinoma-bearing mice.[11] With other NO-inhibitors used as a single agent antitumour effects have been demonstrated as well.[12] The reduction

in tumour blood flow leads to hypoxia in tumourtissue and might thus be a useful strategy in antitumour therapy in combination with other agents. For alkylating agents such as melphalan and for cytokines such as tumour necrosis factor alpha (TNF) it has been demonstrated that hypoxia can potentiate the cytotoxic effects.[13,14]

To study the potential antitumour effects of systemic administration of L-NAME we used a renal subcapsular tumour model, using a coloncarcinoma in WAG/Rij rats. Secondly, we examined whether addition of L-NAME to melphalan and/or TNF in an isolated limb perfusion (ILP) model could further improve response rates. For this we used a well established perfusion model developed in our laboratory which is based on the successful treatment of patients with in-transit metastasis from malignant melanoma[15-17] and irresectable or locally advanced soft tissue sarcoma.[18,19] For the last group of patients TNF has recently been approved by the EMEA (European Medicine Evaluation Agency) in the ILP setting in combination with melphalan.[20] In our ILP model strong synergistic antitumour effects were previously demonstrated when TNF was used in combination with two different chemotherapeutics (melphalan or doxorubicin).[14,21,22] The synergistic antitumour effects were accompanied by higher intratumoural melphalan concentrations after perfusion with TNF compared to perfusions with melphalan alone.[23] The observed effects in rats corresponded well to ILP in patients in terms of response rate and histopatholigical observations.[21,24] Therefore, this rat model is applied to study usefulness of additional agents in ILP to improve response rates or find synergy between agents which allow lower dosages of toxic agents like TNF.

MATERIAL AND METHODS

Animals

Male inbred BN and Wag/Rij rats, weighing 250-300 g, obtained from Harlan-CPB (Austerlitz, the Netherlands) were used. The rats were fed a standard laboratory diet ad libitum (Hope Farms, Woerden, the Netherlands) and were housed under standard 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.

Drugs

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 (= 40μg). Recombinant human TNF alpha (TNF) 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.[25] Endotoxin levels were < 1.25 endotoxin units (EU) per mg protein. N_ω-nitro-L-arginine methyl ester (L-NAME)(10 g per vial, Sigma, the Netherlands) was dissolved in 0.9% NaCl and administered intraperitoneal at a concentration of 80 mg/kg or it was dissolved in Haemaccel and added to the perfusate to provide a concentration of 2 mg/ml.

Western Blot Analysis for detection of inducible Nitric Oxide Synthesis (iNOS).

Protein extracts were prepared from tissue pieces crushed under liquid nitrogen and homogenised on ice in RIPA buffer [50 mM Tris-HCl, 150 mM NaCl, 1%Triton X-100, 1% deoxycholate, and 0.1% SDS] containing 1 mM DTT, 0.1 mM PMSF and 10 mg/l aprotinin. Supernatant was prepared by centrifugation at 120,000 g for 10 min, and protein concentrations were determined with the Coomassie Plus Protien Reagent (Pierce, IL). One-dimensional Western blot analysis was carried out, for detection of iNOS.[26] Briefly, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in the Biorad minigel system with 7% polyacrylamide gel using 300 μg of soluble protein extracts. Electrophoresed proteines were transfered to a PVDF membrane (Millipore Corp., MA) and unspecific binding was blocked by incubation of the membrane in TBST [10 mM Tris, 150 mM NaCl, and 0.05% Tween 20] plus 2% BSA for 1h at room temperature. The membranes were probed with a polyclonal rabbit anti-rat iNOS antibody (N-20, Santa Cruz Biotechnology Inc.,CA), diluted 1:40.000 in TBST. iNOS antibody was detected using a secondary mouse antibody to rabbit which was alkaline phosphatase labelled (Sigma). Colour development was performed using the alkaline phosphate substrate nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) in AF-buffer until colour was fully developed (Boehringer Mannheim, Mannheim, FRG).

Renal sub-capsular tumour model

Renal sub-capsular tumour model was established in male rats of the inbred WAG-Rij strain introducing 8 mg of solid CC531 coloncarcinoma under the capsule of both kidneys under microscopic vision, according to a previously described method.[27] Treatment was started one day after implantation by intraperitoneal injection of 80 mg/kg L-NAME twice daily. Control rats were treated with a phosphate-buffered saline (PBS) solution. After 10 days of treatment rats were

sacrificed and kidney tumours weighed. Both groups consisted of eight rats and all animals were evaluable. With respect to systemic toxicity of L-NAME, body weights of the rats were measured 4 and 10 days after treatment and creatinine and urea levels were determined at sacrifice.

Isolated Limb Perfusion model

The technique we used has been published previously.[21] Briefly, a spontaneous, nonimmunogenic BN-175 sarcoma was used and implanted subcutaneously in the right hind limb in BN rats.[28] Perfusion was performed at a tumour diameter of 13 mm ± 3 mm at least 7 days after implantation. Animals were anaesthetised with Hypnorm (Janssen Pharmaceutica, Tilburg, the Netherlands) and 50 IU of heparin were injected intravenously to prevent coagulation in the perfusion circuit. A warm water mattress was applied to maintain a constant temperature of 38-39°C in the hind limb during perfusion. 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 started 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. L-NAME was dissolved in the perfusate, melphalan and TNF were added as boluses to the oxygenation reservoir. A roller pump (type 505 U; Watson Marlow, Falmouth, UK) 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. After ligation of the femoral artery back-flow from the femoral vein was seen in all rats immediately after release of the tourniquet.

Subsequent tumour growth was daily recorded by caliper measurement. Tumour volume was calculated as 0.4 (A^2B), where B represents the longest diameter and A the diameter perpendicular to B.

Assessment of tumour response

The classification of tumour response was: progressive disease (PD) = increase of tumour volume (> 25%) within 4 days; no change (NC) = tumour volume equal to volume during perfusion (in a range of -25% and + 25%); partial remission (PR) = decrease of tumour volume (-25 and -90%); complete remission (CR) = tumour volume 0-10% of volume during perfusion or necrosis.

Statistical analysis

Mann Whitney U test was used to compare tumour volumes in different animal groups and to compare different tumour responses in different groups. Calculations were performed on a personal computer using GraphPad Prism and SPSS for Windows 95.

RESULTS

iNOS western blot

Western blot analysis of tumour extracts demonstrated distinct iNOS bands at approximately M_r 125.000-138.000.[29] iNOS was demonstrated in tumour tissue but not in muscle tissue (Figure 1). The results suggest that iNOS is more abundant in tumour tissue than in normal muscle tissue in the rat and suggest an important role for iNOS in tumour tissue.

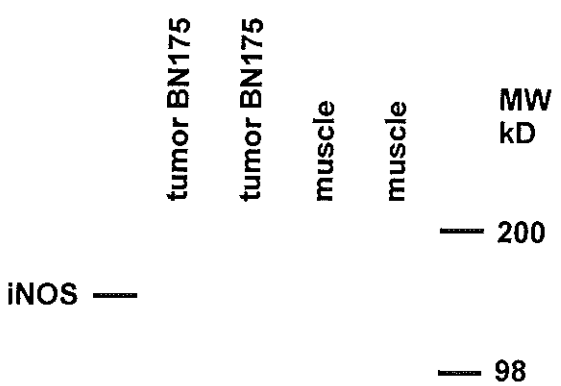


Figure 1. Western blot analysis showing a distinct band at approximately 125.00-138.00 M_r.

Renal sub-capsular tumour model

Systemic treatment with L-NAME resulted in a statistically significant growth inhibition of CC531 colon carcinoma, growing under de capsules of kidneys compared to untreated rats ($p < 0.005$)(Figure 2). Ten days after treatment body weight of rats were statistically not significantly different between both groups (-13.0 ± 5.4 g after L-NAME treatment versus -5.5 ± 3.1 g after sham treatment). At day 10 after intraperitoneal administration creatinine and urea levels were statistically significantly different from the control group (creatinine 28.5 ± 4.3 versus 68.5 ± 8.5 µmol/l ($p<0.005$) and urea 5.6 ± 0.4 versus 8.1 ± 1.3 mmol/l ($p<0.05$) in the L-NAME and control group respectively). This increase in urea and creatinine levels indicate a decrease in renal function that might be the results of impaired renal blood flow.

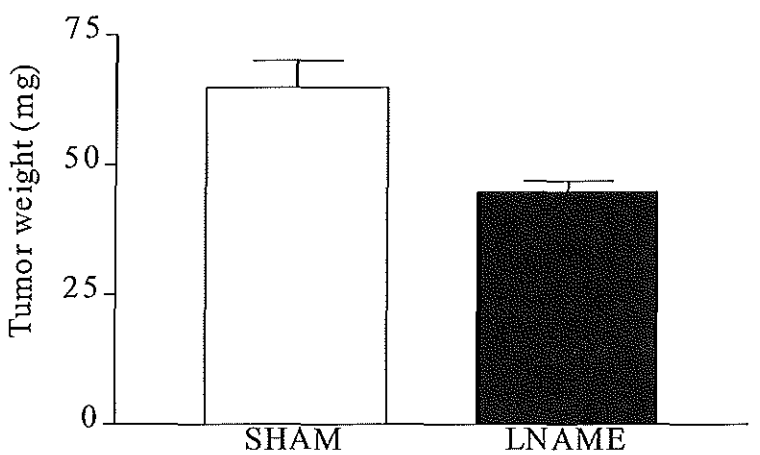


Figure 2. Tumour weight (mean ± S.E.M.) of renal subcapsular CC531 adenocarcinoma after ten days treatment with sham (□;n=8) or 80 mg/kg L-NAME intraperitoneal injection (■;n=8).

Tumour response after isolated limb perfusions with L-NAME, TNF and/or melphalan

We studied the possible benificial role of L-NAME on tumour response in ILP. Synergy between melphalan and TNF in ILP was previously demonstrated in our laboratory and could be confirmed in this study for which we used 10 rats in each study group (Table 1).[14,21] Sham perfusion did not inhibit tumour growth and progressive disease was observed in all rats. Perfusions with L-NAME as a single agent, however, resulted in tumour growth arrest after five days in 4 out of 11

rats which was statistically significant different from sham ILP (p=0.02), resulting in a growth delay as is shown in figure 3A.

ILP with TNF alone resulted in progressive disease in all animals, similar as in sham perfused rats. Addition of L-NAME to TNF improved tumour responses from 0 to 64% which was significantly differenct from TNF alone (p<0.001) (figure 3A).

After perfusion with melphalan tumour growth was arrested in 8 out of 10 animals (no change) and progressive tumour growth was observed in 2 out of 10 animals. Melphalan in combination with L-NAME showed a 63 % partial and complete response rate, which was statistically significant different from melphalan alone (p=0.001) (figure 3B).

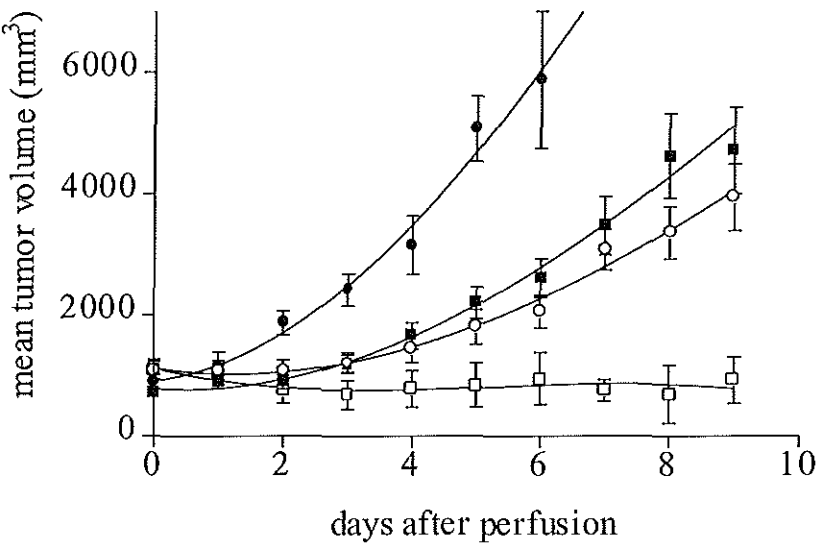
TNF and melphalan have a synergistic antitumour effect and is highly effective with a 70% partial and complete response rate. Addition of L-NAME to the combination of TNF and melphalan, however, could further improve tumour responses to 100% five days after treatment but this was not statistically significant (p=0.3) (figure 3C). After perfusion with TNF and melphalan recurrent tumour growth was demonstrated in all animals after a mean of 9±2 days. When L-NAME was added to the perforate one animal did not show tumour growth 50 days after ILP, whereas recurrent tumour growth occurred in 9 out of 10 animals after a mean of 20±7 days (data not shown). The observed response rate was therefore not only more pronounced but the antitumour effect extended for a longer period after perfusion.

Table 1. Responses five days after isolated limb perfusions with or without L-NAME.

Tumour Response	Sham n=10	Sham+ LNAME n=11	TNF n=10	TNF+ LNAME n=11	Mel n=10	Mel+ LNAME n=16	Mel+TNF n=10	Mel+TNF +LNAME n=10
PD	10	7	10	3	2	1	1	
NC		4		1	8	5	3	
PR				6		5	1	3
CR				1		5	6	7
Response rate (%)	-	-	-	**64**	-	**63**	**70**	**100**

30 Min perfusions were performed with 50 µg TNF, 40 µg melphalan and/or 10 mg L-NAME at 38-39°C.

A



B

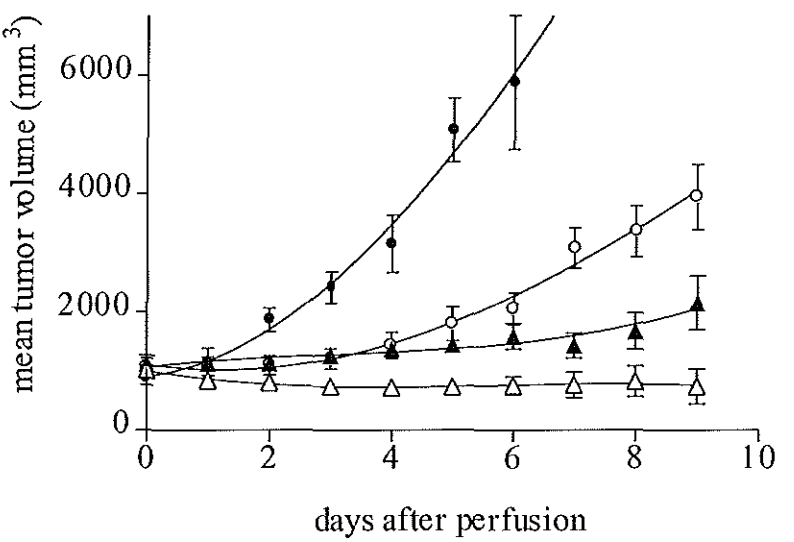


Figure 3A,B. Growth curves of BN-175 sarcoma after isolated limb perfusion with sham (●;n=10), 10 mg L-NAME (○;n=11), 50 μg TNF (■;n=10), 50 μg TNF with 10 mg L-NAME (□;n=11), 40 μg melphalan (▲;n=10), 40 μg melphalan with 10 mg L-NAME (△;n=16). Mean (± S.E.M.) of tumour volumes are shown.

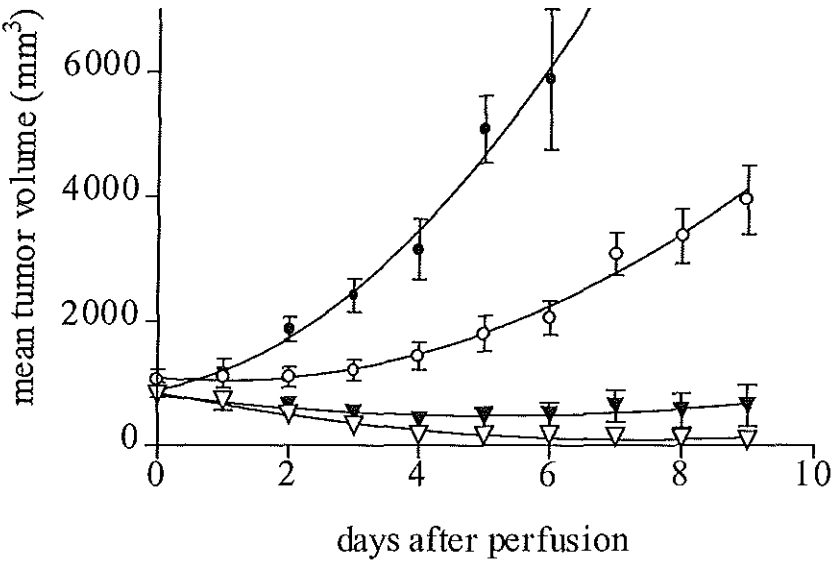


Figure 3C. Growth curves of BN-175 sarcoma after isolated limb perfusion with sham (●;n=10), 10 mg L-NAME (O;n=11), 50 μg TNF in combination with 40 μg melphalan (▼;n=10) and 50 μg TNF in combination with 40 μg melphalan and 10mg L-NAME (V;n=10). Mean (± S.E.M.) of tumour volumes are shown.

DISCUSSION

The results of the present study show a statistically significant reduced tumour growth after intraperitoneal administration of the nitric oxide (NO) inhibitor L-NAME in tumour bearing rats. The growth inhibition of L-NAME in the renal sub-capsular assay, may be partially due to a decrease in renal blood flow. Kassab *et al.* previously demonstrated a decreased renal blood flow after administration of NO inhibitors which is confirmed in our study by elevated creatinine and urea levels.[30] Therefore conclusions concerning antitumour effect of NO inhibitors from the data obtained in de renal sub-capsular assay can not be drawn, but these data strongly suggest a growth inhibitory effect on the tumour by NO inhibitors.

In the experiments in which L-NAME was used in an isolated perfusion setting in sarcoma-

bearing rats a decreased tumour growth was demonstrated when L-NAME was used alone. Moreover, strong synergy was observed when L-NAME was used in combination with either TNF (response rates improved from 0% to 64%) or melphalan (response rates improved from 0 to 63%). Even in the setting of the strongly synergistic combination of melphalan and TNF (response rates: 70%) the addition of L-NAME enhanced response rates to 100%. Moreover, when L-NAME was added to the perfusion tumour growth recurrences occurred at approximately 20 days after ILP, whereas tumours recur after a mean of 9 days after ILP with TNF and melphalan alone.

The antitumour effects of L-NAME in the highly vascularized BN-175 soft tissue sarcoma found in this study are similar to previously demonstrated effects of NO inhibitors in mice.[11,12] The function of NO inhibition in tumour biology however, is not clear since NO has a multifactorial role in the vascular, nervous and immune system and is demonstrated in many different cells lines and tissues. High concentrations of NO synthase (NOS) are present in different tumour cell lines, where the enzyme activity correlates with the tumour grade.[2,29] We demonstrated iNOS to be present in the BN-175 soft tissue sarcoma and not in the surrounding muscle tissue using a western blott analysis. Jenkins *et al.* found that a promotion of tumour growth in tumour cells that constantly produce NO.[4] Recently others demonstrated that iNOS activity was higher in metastazising head and neck cancer tissue compared to normal tissue suggesting an important role for NO in tumour biology.[7]

One mechanism of NO is to increase or maintain tumour blood flow and therefore supply nutrients and oxygen to the tumour.[31] In studies in which rodents were treated with NO inhibitors a selectively reduced tumour blood flow was demonstrated.[8,9] This decreased flow is initiated by a decreased central tumour perfusion in some tumours[10] or a decreased peripheral perfusion in others.[3] Localisation of NOS is cell and tumour dependent and as a result the response to NO inhibitor is likely to be heterogeneous and tumour dependent. Horsman *et al.* did not find a decrease in oxygenation status of tumours treated with NO inhibitors despite a significantly reduced tumour blood flow.[32] However, Wood *et al.* demonstrated that reduced flow by a NO inhibitor decreased the energy status of several murine tumours, whereas the normal skin was unaffected. Moreover, they found evidence for an increase in tumour sensitivity to a level sufficient to enhance the efficacy of cytostatic agents.[33] In vivo studies have shown that reduction of tumour blood flow with agents such as hydralazine can enhance the tumouricidal effect of melphalan. In vitro studies on human tumour cells also demonstrated a potentiation of melphalan cytotoxicity by both hypoxia and acidic pH.[13] We previously demonstrated promotion of TNF as well as melphalan antitumour effects with hypoxia in soft tissue sarcoma bearing rats in ILP.[14] The enhanced antitumour effect of L-NAME with Melphalan and/or TNF as we describe in this study might thus be explained by hypoxia that is induced by the reduced tumour blood flow.

More recently an important role of NO in tumour angiogenesis was suggested.[5,6] Gallo *et al.* demonstrated that inhibition of NO by L-NAME in squamous cell carcinoma transplanted in the rabbit cornea decreased tumour-induced angiogensis.[7] Since BN-175 is a highly vascularized and fast growing tumour, inhibition of neovascularisation in this tumour might well be a good explanation for the significant tumour responses and later re-growth after perfusion with L-NAME in combination with melphalan and TNF.

Leukocyte-endothelial interactions in tumour vessels is a major limitation of immune therapy or host immune response against tumours. NO has a possible role in down-regulating these actions and inhibition of NO was demonstrated to increase leukocyte rolling and adhesion to the vessel wall in tumours significantly.[34] Lejeune *et al.* found an enhanced tumour-infiltrating lymphocyte proliferation in rat colon adenocarcinoma when NO production was inhibited with L-NAME.[35] Previous work in our laboratory and by others demonstrated that TNF induced antitumour effect might be leukocyte dependent.[34,36,37] Increased leukocyte-endothelial interactions induced by L-NAME might therefore be another reason for the additional antitumour effect of L-NAME to TNF. Meyer *et al.* suggested that the vascular effects they observed in tumours treated with L-NAME was not only due to leukocyte adhesion but also by the development of microthrombi resulting from platelet aggregation.[10] Since platelet aggregation is an important event in the TNF antitumour response as well, this mechanism might be another reason for the enhanced effect of L-NAME with TNF.[24,38]

In conclusion we demonstrate that L-NAME leads to a reduction in tumour growth both after systemic administration as well as in ILP against a non-immunogenic soft tissue sarcoma in the rat. The observed high response rates with the addition of L-NAME to melphalan and TNF are promising for the use of L-NAME in the clinical setting. Since the dose limiting toxic effect of TNF in cancer therapy is mainly hypotension induced by NO production in endothelial cells, systemic L-NAME may favourably alter this toxicity which can result in a higher maximum tolerated doses.[39,40] This might open therapeutical options for TNF cancer treatment in other settings than in ILP. Optimisation of NOS inhibitor concentrations and kinetics will be necessary to fully exploit this potential therapy.

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CHAPTER 7

ISOLATED LIMB PERFUSION FOR LOCAL GENE DELIVERY: EFFICIENT AND TARGETED ADENOVIRUS-MEDIATED GENE TRANSFER INTO SOFT TISSUE SARCOMAS

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SUMMARY

Tumour-specificity of therapeutic gene expression can be accomplished by adjustment of the route of vector delivery. The potential of isolated limb perfusion (ILP) for efficient and tumour-specific adenoviral-mediated gene transfer was evaluated in sarcoma-bearing rats. Luciferase activity was determined in the tumour and several other organs after ILP with $1x10^9$ iu recombinant adenovirus carrying the luciferase marker gene and compared to systemic administration (SYS), regional infusion (REG) or intratumoural injection (IT). Localisation studies using adenoviral vectors carrying the LacZ gene were performed to evaluate the intratumoural location of transfected cells after both ILP and IT. Gene delivery via ILP or IT administration resulted in an efficient intratumoural gene transfer with a significantly higher mean luciferase activity compared to REG and SYS administration. Luciferase gene expression in extratumoural organs lying either outside or within the isolated circuit was minimal after ILP. Localization studies demonstrated that IT transfection was confined to tumour cells lying along the needle tract, whereas after ILP gene transfer was found in viable tumour cells as well as in the tumour-associated vasculature. In conclusion, ILP can accomplish efficient and tumour-specific marker gene transfer and might be used to target suicide or cytokine-encoding genes in anti-cancer gene therapy.

INTRODUCTION

Recent advances in molecular engineering have enabled gene therapy to become a promising therapeutic entity for an ever increasing number of clinical applications. Among the potentially applicable viral and non-viral vector systems, that are considered crucial for the transfer of therapeutic genes into target cells, recombinant retroviruses and adenoviruses have been most widely used in both pre-clinical studies and clinical trials.[1] Virus-mediated gene transfer can be accomplished by either *ex vivo* or *in vivo* approaches. The *ex vivo* strategy involves the harvesting of the target cells that are, subsequently, genetically modified *in vitro*, after which they are reimplanted into the patient. The *in vivo* approach involves the direct transfection of target cells with recombinant viruses with transgene in vivo by either systemic, regional or tissue-specific administration.[2]

Although gene therapy has originally been developed for correction of genetic deficiencies of inherited disorders of metabolism, current interest is mainly focussing on its potential therapeutic role for cardiovascular disease and cancer. Among the various approaches of cancer gene therapy, which include genetic marking, cancer vaccination, inhibition of oncogene expression, restoration of tumour suppressor genes and the use of suicide genes, the last strategy may be one of the most successful therapeutic strategies of anti-cancer gene transfer to date. The suicide gene strategy aims at the induction of drug sensitivity by introducing genes such as the herpes simplex thymidine

kinase (HSV-TK) gene into the tumour cells, whose expression initiates the formation of prodrug metabolising enzymes. HSV-TK converts Ganciclovir into phospholyrated metabolites that act as chain terminators during DNA synthesis and, in this way, cause selective cell death.[3,4]

A major concern in the enzyme/prodrug approach is the transfer of suicide genes to organs other than the tumour, especially organs with a rapid cell turn-over. The risk of infecting cell types other than target cells is negligible in strategies involving the *ex vivo* suicide gene transfer.[5,6] However, apart from a possible role in cancer vaccination, *ex vivo* gene transfer is clearly not applicable in anti-cancer gene therapy. *In vivo* gene delivery, on the other hand, should be targeted to tumour cells to avoid complications due to leakage of genes to other cells in the body. Tumour-specific *in vivo* gene delivery can, among others, be achieved by tissue-specific administration of viral vectors to tumour cells.[2]

In surgical oncology trials, isolated limb perfusion (ILP) is successfully used for administration of chemotherapeutics and cytokines to locally advanced soft tissue extremity sarcomas and in-transit melanoma metastases.[7-10] ILP involves the recirculation of high drug concentrations within a vascularly isolated extremity resulting in minimal exposure of this drug to organs lying outside the closed circuit.

In the present study, the efficiency and tumour-specificity of adenovirus-mediated gene transfer using ILP was evaluated in an established sarcoma-bearing rat model.[11-13] We quantified the activity of a marker gene in limb sarcomas after ILP with adenoviral vectors carrying the luciferase marker gene. The intratumoural luciferase gene expression was compared to the luciferase activity in other organs either in- or outside the isolated vascular circuit. The efficiency and tumour-specificity of ILP-mediated gene transfer was compared to other delivery routes, including SYS and REG infusion and IT injection. Moreover, adenoviral vectors carrying the LacZ marker gene were used to determine the intratumoural localisation of transfected cells after both ILP and IT administration.

MATERIAL AND METHODS

Adenoviral Vectors

All adenoviral vectors used in this study were derived from human adenovirus type 5 and were deleted for the E1 region in which the transgenes were cloned. The E3 region was retained in all vectors. The cytomegalovirus promoter (CMV) and adenoviral major late promoter (MLP) were used to drive the LacZ and luciferase (Luc) marker genes, respectively. The construction and

production of IG.Ad.MLP.Luc and IG.Ad.CMV.LacZ recombinant adenoviruses is described in detail elsewhere.[13,14] Briefly, recombinant adenoviral vectors were plaque purified twice, propagated on 293 or PER.C6 cells, purified by CsCl density centrifugation, dialysed and stored in buffer containing 13 mM Na_2HPO_4 (pH 7.4), 140 mM NaCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 5% (m/v) sucrose at -80°C. The virus titers (infectious units (iu)/ml) were determined by end-point cytopathogenic effect (CPE) titrations using 911 cells.[4] All recombinant adenoviral vectors were produced at IntroGene, Leiden, the Netherlands.

Animals

Inbred male Brown Norway rats, weighing 200-300 grams, were obtained from Harlan (Zeist, The Netherlands). Animals were kept at standard laboratory conditions and were fed a standard laboratory diet (Hope Farms, Woerden, The Netherlands). The experimental protocols adhered to the rules described in the 'Dutch Animal Experimentation Act' and the 'Guidelines on the Protection of Experimental Animals' by the Council of the European Community. Prior to initiation of the experiments the protocols were approved by the 'committee of animal research' of the Erasmus University in Rotterdam and the University of Leiden, The Netherlands.

Tumour model

The spontaneous BN-175 sarcoma was implanted in the flank of donors and passaged serially. BN-175 is a non-immunogenic, rapidly growing and metastasising tumour with a tumour doubling time of approximately 5 days.[15] For the present study, small tumour fragments were subcutaneously implanted into the right hind limb just above the ankle. All surgical interventions were performed at a tumour diameter between 5 and 10 mm at least 7 days after implantation.

Administration techniques

All surgical procedures were performed under Hypnorm anaesthesia (Janssen Pharmaceutica, Tilburg, The Netherlands). For ILP the technique described by Manusama *et al.* was used.[11] Briefly, the femoral vessels were approached through an incision parallel to the inguinal ligament after systemic heparin administration (50 IU). Subsequently, 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 temporarily occluded by the application of a tourniquet around the groin. Perfusion was performed with recombinant adenoviral vectors ($1x10^9$ iu IG.Ad.MLP.Luc or $1x10^9$ iu IG.Ad.CMV.LacZ) added as a bolus in 5 ml Haemaccel (Behring Pharma, Amsterdam, the

Netherlands). An oxygenation reservoir and a roller pump were included in the isolated circuit. The perfusate was circulated at a flow speed of 2 ml/min for a time period ranging from 5 till 30 minutes. Following ILP, the isolated circuit was perfused with haemaccel for another 5 minutes to wash out the non-bound viruses. During ILP and wash out the rat hind leg was kept at a constant temperature of 38-39°C with a warm water mattress applied around the leg. After wash out, the isolated circuit was discontinued and, after tube removal, the femoral vessels were ligated. Previous experiments have shown that the collateral circulation to the leg is so extensive that ligation of the femoral vessels can be performed without detrimental effects.[11]

For IT injection the same amount of recombinant adenoviral vectors ($1x10^9$ iu IG.Ad.MLP.Luc or $1x10^9$ iu IG.Ad.CMV.LacZ) was injected into the centre of the BN-175 tumour using a 25 gauge needle. Leakage of virus was minimised by tamponade of the injection site with a cotton tip. For SYS administration adenoviral vectors ($1x10^9$ iu IG.Ad.MLP.Luc) were injected into the penile vein using a 25 gauge needle followed by tamponade to prevent virus and blood leakage. For REG administration a silastic tube (0.30 mm inner diameter, 0.64 mm outer diameter) was implanted into the femoral artery. Recombinant adenoviruses ($1x10^9$ iu IG.Ad.MLP.Luc), dissolved in 1 ml of haemaccel, were infused through the implanted tube, followed by 1 ml of haemaccel to wash out the adenoviral vectors from the silastic tube. Also in these animals the femoral artery was ligated after tube removal .

Luciferase Assay

Two days after administration of Ad.MLP.Luc the experimental animals were sacrificed and tumour and quadriceps muscle, lying within the isolated circuit, were removed. In addition, liver, spleen, heart, lung, kidney, intestine, gonads and aorta, all lying outside the isolated limb, were harvested for luciferase activity measurement. Cross contamination of the tissue samples was avoided by cleaning the operation equipment thoroughly with 1% SDS, H_2O and ethanol between dissection of each sample. Removed tissues were weighed, frozen in liquid N_2 and stored at –20°C. Later, samples were thawed in 2 ml ice cold lysis buffer (8 mM $MgCl_2$, 1 mM EDTA, 1 mMDTT, 1% Triton X-100 and 15% Glycerol in PBS) and homogenised. Lysed cells were collected and centrifuged (14000 rpm for 7 min) at 2-6°C to remove cell debris. Luciferase activity present in 20 µl lysate was determined by addition of 100 µl of Luciferase-Assay Reagent (Promega, Madison, WI, USA). After 10 sec preincubation the produced light was measured for 30 sec in a Lumat LB9501 luminometer (Berthold, Wildbad, Germany). Protein concentrations of different tissues were determined using the Biorad Protein Assay kit and luciferase activity was hereafter calculated as relatively light units per mg protein.

β-Galactosidase histochemistry

Two days after either ILP or intratumoural injection the experimental animals that were exposed to Ad.CMV.LacZ were sacrificed and the tumour was dissected. Tumour tissues were fixed in ice-cold 2% paraformaldehyde/0.25% glutaraldehyde solution for 1 hour. After incubation the tissues were washed with PBS and freshly prepared X-gal staining solution (Boehringer) was added for 1 day. Hereafter, the tissue was washed again with PBS and fixed in 10% buffered formalin solution (40 g NaH_2PO_4, 81.5 g $Na_2H_2PO_4$ and 1lit 100% formalin in 10 lit demi H_2O). Subsequently, histological slices were prepared for qualitative analysis.

Statistical Analysis

The Mann-Whitney U test was used to statistically compare luciferase activity in the various organs after different routes of administration (ILP, IT, SYS and REG).

RESULTS

Optimal duration of ILP using recombinant adenoviral vectors

To determine optimal duration of ILP for maximal gene transfer into tumour tissue luciferase activity was determined and statistically compared after 5, 15 or 30 min perfusion with $1x10^9$ iu IG.Ad.MLP.Luc.

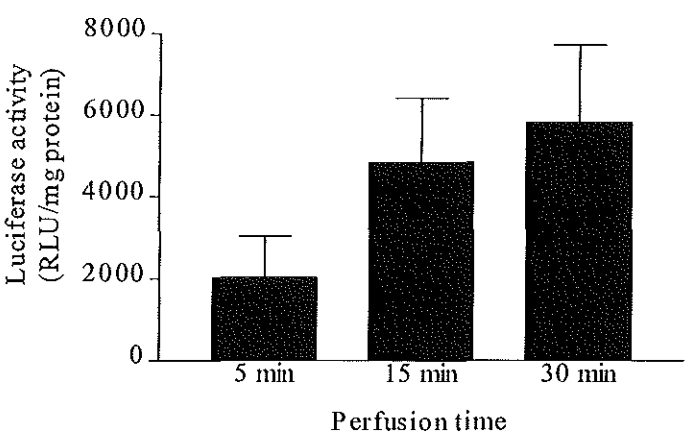

Figure 1. Mean luciferase activity (± SEM) in BN-175 tumours 48 h after adenovirus-mediated gene transfer using ILP. Limbs were perfused with $1x10^9$ iu IG.Ad.MLP.Luc for 5 min (n=6), 15 min (n=6) or 15 min (n=6).

As is shown in Figure 1 luciferase activity in tumour tissue increased with longer perfusion times. However, increments in luciferase gene expression demonstrated a tendency to decrease with longer perfusion time. Luciferase activity in tumour tissue after 30 min ILP did not increase statistically significant in comparison with 15 min (p=1.0), therefore 15 min was used for further experiments. Systemic leakage of adenoviral vectors to other organs did not increase with longer perfusions, indicating a rather leakage free perfusion system (data not shown).

Efficiency of gene transfer in tumour tissue using different administration methods

The efficiency of luciferase gene transfer in tumours after 15 min perfusion with $1x10^9$ iu IG.Ad.MLP.Luc in an isolated limb was compared with SYS, REG and IT administration with the same amount of recombinant adenovirus (Figure 2). Both ILP and IT injections resulted in a significantly higher mean intratumoural luciferase activity when compared to SYS administration (both $p<0.005$) and REG infusion (both $p<0.005$). IT injection resulted in higher gene expression in tumour tissue in comparison with ILP but the difference was statistically not significant (p=0.7). In the IT group, a larger standard error of the mean (SEM) was observed when compared to the other routes of administration, indicating a large variance in gene transfer between the different injections of adenoviral vectors in the tumour.

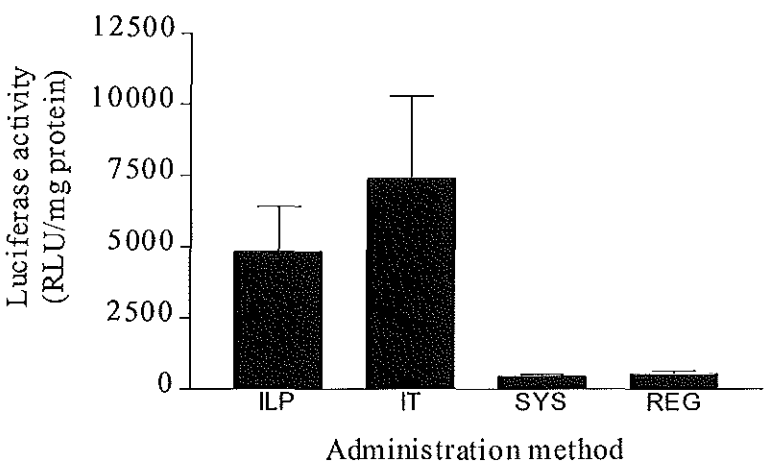

Figure 2. Efficacy of gene transfer in BN-175 tumours after administration of $1x10^9$ iu IG.Ad.MLP.Luc using isolated limb perfusion (ILP, n=6), intratumoural injection (IT, n=6), regional administration (REG, n=6) or systemic administration (SYS, n=6). Mean luciferase activity (± SEM) 48 h after ILP and IT administration is statistically significantly different from SYS (both $p<0.005$) and REG administration (both $p<0.005$).

Systemic leakage of adenoviral vectors

Luciferase activity after ILP was measured in various organs outside and inside the isolated circuit during perfusion with $1x10^9$ iu IG.Ad.MLP.Luc. The analysed organs included aorta, heart, lungs, liver, spleen, kidney, intestine, gonads and in addition, the quadriceps muscle of the right hind limb, located within the perfused circuit. Figure 3 shows negligible luciferase activity in organs outside the isolated circuit. Luciferase gene expression fluctuated around detection level (100 RLU/mg protein), indicating that the isolated limb perfusion is leakage free. Mean luciferase activity in the tumour was significantly higher ($p<0.005$) when compared to its activity in quadriceps muscles suggesting a preference for tumour cell transfection.

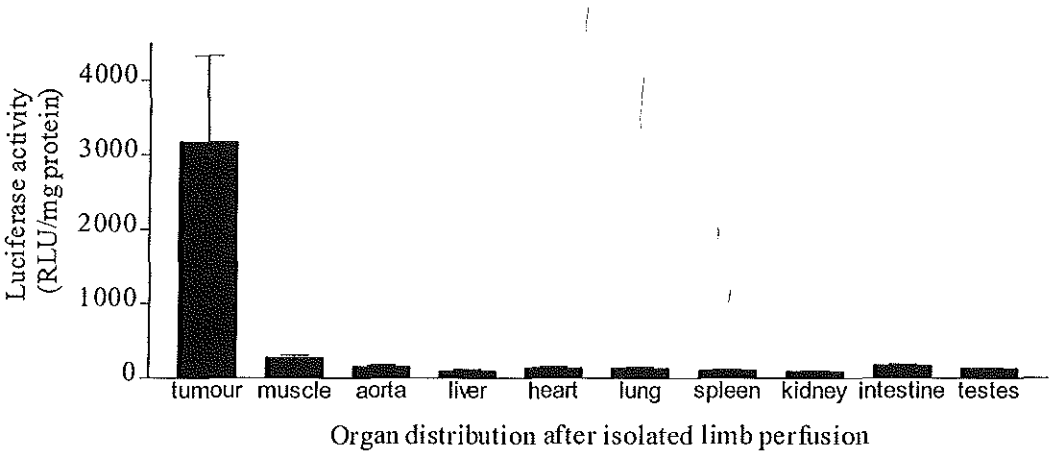

Figure 3. Organ specificity of gene delivery 48 h after ILP using $1x10^9$ iu IG.Ad.MLP.Luc (n=6). Mean luciferase activity (± SEM) is statistically significantly higher in tumour tissue as compared to all other organs in- or outside the isolated circuit ($p<0.005$).

Intratumoural location of transfected marker genes after ILP and IT

Twelve animals underwent either ILP or IT administration with $1x10^9$ iu IG.Ad.CMV.LacZ and were sacrificed after 48 hours. Tumours were harvested and histological slides were prepared with staining for ß-galactosidase. In these slides LacZ-positive cells were identified and qualified for cell type and location within the tumour. After IT LacZ-positive cells were found along the needle tract, without staining tumour cells in other parts of the tumour (Figure 4a). After ILP LacZ expression was observed around tumour associated vessels (Figure 4b). Moreover, in different areas of the tumour LacZ positive cells were found with a preferential location in the (viable) rim of the tumour (Figure 4c).

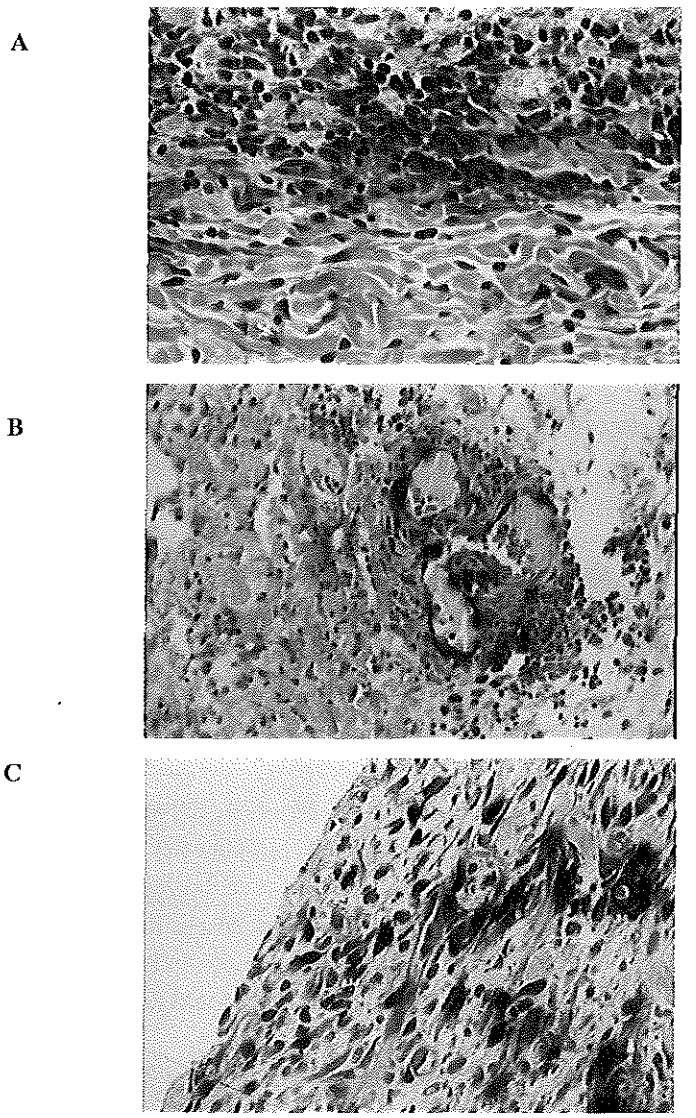

Figure 4. Adenovirus-mediated LacZ gene transfer to BN-175 soft tissue sarcoma in rats. Blue staining represents cells actively expressing β-gal 48 h after treatment with 1x10^9 iu IG.Ad.CMV.LacZ. After intratumoural injection blue staining was only found around the needle tract and not in other parts of the tumour (4A). After isolated limb perfusion gene transfer was demonstrated in various parts of the tumour including tumour-associated vessels (4B) and in the viable tumour cells in the rim of the tumour (4C).

Chapter 7

DISCUSSION

In order to compete with the conventional therapeutics anti-cancer gene therapy should be both effective and safe.[2] The first requirement implies the use of either viral or non-viral vector systems that guarantee efficient gene transfer in addition to the application of promoters that offer appropriate expression of the desired genes. For the safety of anti-cancer gene therapy tissue specificity of gene expression is essential since expression of transfected genes in organs other than tumour tissue may cause potentially dangerous complications. Tissue-specificity may be accomplished at the level of gene transfer by vector targeting which necessitates the use of ligands or antibodies that can be conjugated to both viral and non-viral vector systems.[2] Ligands are capable of targeting a vector system to specific (tumour) cell types by interaction with receptors that are exclusively present on the surface of these target cells. Various ligands have been used for vector targeting to tumour cells including folate, asialoorosomucoid and epidermal growth factor allowing for tumour-specific gene delivery in ovarian cancer, hepatocellular carcinoma and lung cancer respectively.[16,17] Apart from targeted vector delivery, tumour-specific gene expression can be achieved with the use of tumour-specific promoters, such as carcinoembryonic antigen (CEA) and human surfactant protein A, that are only activated in tumour cells containing these substances in their nucleus.[18,19]

The most widely applied gene transfer vectors, derived from either retroviruses or adenoviruses as well as standard promoters do not generate tumour-specific gene expression. In the present study, systemic administration of adenoviral vectors carrying the luciferase marker gene did not result in significant tumour tissue expression, as was expected. Recent experiments in our laboratory using another promoter driving the luciferase gene (CMV) in other animals (Wag/Rij rats bearing a ROS-1 osteosarcoma in the hindlimb) demonstrated a predominant expression of luciferase in liver tissue (van der Kaaden, unpublished observation). Also in these experiments there was no preferential gene uptake in tumour tissue. This observation confirms recommendations of other authors that advise against the use of systemically administered anti-cancer gene therapy for clinical trials unless tissue-specific vector systems are included.[2]

Apart from systemic delivery recombinant viruses can be administered by a catheter into the tumour vasculature. Regional infusion of a target organ has previously been explored for lung[20], liver[21] and brain[22] and demonstrated effective viral-mediated gene transfer. In the present study, we observed a slight, but not significant, increase in luciferase gene expression in tumour tissue after regional infusion when compared to systemic administration. Gene transfer of adenoviral vectors

after intraarterial infusion was remarkably ineffective and not superior to systemic intravenous administration.

Theoretically, the simplest route of tumour-specific gene delivery is clearly local administration of vectors into tumours by direct injection which is successfully performed in the case of subcutaneous malignancies[23] and brain gliomas[24], the later with the help of stereotactic guidance. The present experiments demonstrated IT injection of adenoviral vectors to result in an efficient transfer of luciferase genes to tumour cells. However, localisation studies demonstrated that the distribution of LacZ-positive tumour cells after intratumoural injection was confined to the injection site in the tumour (needle tract staining).

With the ILP technique an extremity can be exposed to high drug concentrations for various periods of time, which may result in a higher tissue uptake as has been demonstrated for melphalan.[25,26] Moreover, the ILP technique allows for a wash-out procedure to remove non-bound drugs and hereby minimise systemic contamination after recirculation. In previous studies using TNF and melphalan we have demonstrated an almost leakage free isolated system in the hind limb of the rat.[13] Since this sarcoma bearing rat model excellently mimics the clinical situation it can be used as a pre-clinical model for pharmacokinetic studies and antitumour responses. In the present study, ILP of adenoviral vectors carrying the luciferase marker gene resulted in a significantly higher luciferase activity in the tumour than after systemic or regional administration, indicating an efficient gene transfer using this technique. Moreover, gene delivery using ILP showed LacZ gene transfer around tumour-associated vessels and in the viable rim of the tumour compared to expression only around the needle tract as seen after IT administration.

Efficacy of adenoviral-mediated gene delivery has previously been demonstrated in other isolated perfusion settings including isolated liver perfusion and isolated lung perfusion.[27,28] In these studies no tumour was included in the isolated circuit so it is unknown whether there is preferential transfection of tumour cells in relation to other cell types lying within the perfused circuit. The current quantitative analysis of luciferase activity in the perfused limb clearly demonstrates a significantly higher uptake of luciferase genes by tumour cells compared to muscle tissue. Moreover, ILP prevents systemic leakage of marker genes since luciferase activities in organs lying outside the isolated circuit were minimal.

The concept of using ILP for tumour-specific gene transfer has recently been explored by Milas *et al.* who in analogy to our findings demonstrated efficient gene delivery in tumour tissue via an isolated limb perfusion model in the rat using an adenovirus Ad.LacZ.[29] Systemic leakage was, however, not directly quantified by measuring marker gene activity as in the present study but with the help of radioactive-labelled red blood cells. Moreover, the efficiency and tumour-specificity of

adenoviral-mediated gene delivery via ILP were not quantitatively compared to other methods of administration.

In conclusion, our results indicate that in sarcoma-bearing rats delivery of adenoviral vectors via ILP is efficient, reproducible and, above all, safe. Consequently, ILP might be useful for efficient and tumour-specific delivery of recombinant adenoviruses carrying various therapeutic gene constructs, including genes encoding for cytokines, angiogenesis inhibitors and suicide genes to enhance tumour control. Pre-clinical studies are presently conducted to explore these possibilities in limb and organ perfusion settings that may ultimately prove beneficial to cancer patients.

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CHAPTER 8

ADENOVIRUS-MEDIATED IL-3β GENE TRANSFER USING ISOLATED LIMB PERFUSION INHIBITS GROWTH OF LIMB SARCOMA IN RATS

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Human Gene Therapy; in press

SUMMARY

Cytokine gene transfer using (multiple) intratumoural injections can induce tumour regression in several animal models, but this administration technique limits the use for human gene therapy. In the present studies we describe tumour growth inhibition of established limb sarcomas after a single isolated limb perfusion (ILP) with recombinant adenoviral vectors harbouring the rat IL-3β gene (IG.Ad.CMV.rIL-3β). In contrast, a single intratumoural injection or intravenous administration did not effect tumour growth. Dose finding studies demonstrated a dose dependent response with a loss of antitumour effect below $1x10^9$ iu IG.Ad.CMV.rIL-3β. Perfusions with adenoviral vectors bearing a weaker promoter (MLP promoter) driving the rIL-3β gene did not result in antitumour responses, suggesting that the rIL-3β mediated antitumour effect depends on the amount of rIL-3β protein expressed by the infected cells. Furthermore, it was shown by direct comparison that ILP with IG.Ad.CMV.rIL-3β in the ROS-1 osteosarcoma model is at least as efficient as the established therapy with the combination of TNFα and melphalan. Treatment with IG.Ad.CMV.rIL-3β induced a transient dose dependent leucocytosis accompanied with an increase in peripheral blood levels of histamine.

These results demonstrate that ILP with recombinant adenoviral vectors carrying the IL-3β transgene inhibits tumour growth in rats and suggest that cytokine gene therapy using this administration technique might be beneficial for clinical cancer treatment.

INTRODUCTION

Gene therapy using cytokines has been proposed as a promising new approach in antitumour therapy, because of the observed impressive tumour responses in experimental animals.[1-3] Gene transfer has traditionally relied upon genetic modification of cells *in vitro*, but introduction of genes directly *in vivo* could eliminate the need to construct cell lines from each patient and could therefore reduce treatment time. Moreover, direct *in vivo* transfection of established tumours with cytokine genes may induce immune responses against a broader array of tumour antigens than immunization with *in vitro*-cultured tumour cells.[4] Several studies have shown that implantation of tumour cells genetically engineered to produce cytokines evoked tumour specific immunity, as evidenced by rejection of subsequently injected parental tumour cells. Such effects have been observed with transduced tumour cells expressing IL-1,[5] IL-2,[6] IL-3,[7] IL-4,[8] IL-7,[9] IL-12,[10] interferon-γ,[11] GM-CSF[12] and TNF.[13] Less information is present of antitumour responses in established tumours by direct transfection with adenoviral vectors harbouring cytokines. However, recently significant antitumour responses have been described with IL-1α,[14] IL-2,[15,16] IL-12[17] and TNF.[18] With adenoviruses harbouring the rat interleukin-3β (rIL-3β) transgene antitumour effects were described as well after multiple direct intratumoural injections in tumour bearing rats.[14] Chiang *et al.*

previously demonstrated an important role of IL-3 gene expression on tumour response in combination with irradiation in a fibrosarcoma model.[19]

IL-3 is a hematopoietic growth factor that has a wide range of target cells, including hemopoietic stem cells and multiple types of hemopoietic progenitor cells of every lineage.[20] Activated T lymphocytes are the major natural source of IL-3 which can functionally activate mature blood cells including monocytes, basophils and eosinophils which is suggestive for a role of IL-3 in the inflammatory response.[21-23]

IL-3 was initially purified from medium of cultured WEHI-3 mouse cells.[24] Esandi *et al.* described the presence of two different mRNA isoforms of rat IL-3 of which IL-3β mRNA is more abundantly present (> 90 %) than the rat IL-3α mRNA and rat IL-3β is the predominant if not exclusive variant protein present in white blood cells *in vivo*.[25] For the action of IL-3 a strong species specificity has been found,[26] therefore the study of IL-3 activity requires *in vitro* and *in vivo* models where the appropriate homology between target tissue and cytokine is respected. IL-3 exerts its biologic activities through binding to a specific high-affinity receptor on the cell surface. Whether this IL-3 receptor is present on tumour cells is unknown, but its presence was demonstrated on vascular endothelium [27,28] Intravascular delivery of IL-3 could therefore generate a specific antitumour effect on the tumour vasculature which might not be present using direct intratumoural injections.

With isolated limb perfusion (ILP) first described by Creech *et al.* high regional drug concentrations with minimal leakage and concomitant systemic toxicity can be achieved.[29] In irresectable sarcoma and 'in transit' metastasized melanoma it has been shown that ILP with the combination of tumour necrosis factor alpha (TNF), interferon gamma (IFN) and melphalan results in high response rates.[30-33] Several studies performed in rats bearing non-immunogenic aggressively growing hind limb soft tissue sarcomas or osteosarcomas showed a similar histological and overall antitumour response with the combination of TNF and melphalan as in patients.[34-38] Using this animal model we recently demonstrated that ILP with adenoviral vectors harbouring the luciferase or LacZ gene leads to effective tumour transfection without systemic contamination.[39]

The objective of this study was to explore the antitumour effect of a recombinant adenoviral vector carrying the IL-3 transgene on two different rat tumours (ROS-1 osteosarcoma and BN-175 sarcoma) using different treatment modalities. Moreover, different promoters driving the rIL-3β gene were compared and IL-3β gene therapy was compared with the established and highly effective combination therapy with TNF and melphalan.

MATERIAL AND METHODS

Recombinant adenoviral vectors

The adenoviral vectors used in this study are all derived from human adenovirus type 5 and are deleted for the E1-gene in which the transgenes are cloned, thereby rendering it replication defective. The E3 region is retained in all vectors. The cytomegalovirus promoter (CMV) and adenoviral major late promoter (MLP) were used to drive the rat interleukin-3β transgene (rIL-3β). Ad.RR was used as a control adenoviral vector and does not contain a transgene but only the CMV promoter. The construction and production of IG.Ad.CMV.rIL-3β, IG.Ad.MLP.rIL-3β and Ad.RR recombinant adenoviral vectors, is described in detail elsewhere.[25,40,41] Briefly, the recombinant adenoviral vectors were plaque purified twice, propagated on 293 or PER.C6 cells[42] purified by CsCl density centrifugation, dialysed and stored in buffer containing 13 mM Na_2HPO_4 (pH 7.4), 140 mM NaCl, 0.9 mM $CaCl_2$, 0.5 $MgCl_2$ and 5% (m/v) sucrose at –80°C. The virus titers (infectious units (iu)/ml) were determined by end-point cytopathogenic effect (CPE) titrations using 911 cells.[43] All recombinant adenoviral vectors were produced at IntroGene.

Drugs

Melphalan (Alkeran®, L-phenyl-alanine-mustard.hydrochloride 50 mg per vial, GlaxoWellcome, UK) was diluted in 10 ml dilution buffer. Recombinant human TNFα (2 mg/ml) was obtained from Boehringer Ingelheim (Germany). Both drugs were added to the perfusion medium as a single bolus in the oxygenation chamber (dose: 50 μg TNFα, 40 μg melphalan).

Tumour models

The ROS-1 osteosarcoma originated spontaneously in the tibia of a Wag/Rij rat.[44] The rapidly growing BN-175 sarcoma originated as a spontaneous tumour in the retroperitoneal region of a BN rat.[45] Cells from both tumours were maintained in tissue culture and new tumours were produced by inoculation in the flank. The ROS-1 has a tumour doubling time of 5 days, the BN-175 sarcoma is a rapidly growing tumour and has a tumour doubling time of 2 days. Using the immunisation challenge method of Prehn and Main the BN-175 sarcoma was found to be non-immunogenic.[46]

Animals

For the studies male Wag/Rij and Brown Norway (BN) inbred rats (weight 220-270 g) were used (Harlan, Zeist, The Netherlands). 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' and the 'Guidelines on the protection of Experimental Animals' published by the council of the EC. The protocols were approved by the 'Committee on Animal Research' of the University of Leiden, The Netherlands.

Experimental procedures

Fragments (3x3x3 mm) of ROS-1 or BN-175 tumours were implanted subcutaneously just above the ankle in the right hind leg of Wag/Rij or BN rats, respectively. When the tumour reached a diameter between 7 and 10 mm, a direct intratumoural injection (IT) was given or a 15 minutes ILP was performed as described previously by Manusama *et al.*[34] Briefly, animals were anaesthetized with Hypnorm (Jansen Pharmaceutica, Tilburg, The Netherlands) and 50 IU of heparin were injected intravenously. To keep the rat's hind limb at a constant temperature of 38-39 °C a warm water mattress was applied. The femoral artery and vein were cannulated with silastic tubing. Collaterals were occluded by a groin tourniquet and isolation of the circulation of the hind limb started when the tourniquet was tightened. An oxygenation reservoir and a roller pump (Watson Marlow, Falmouth, UK; type 505U) were included in the circuit. The perfusion was performed by using a perfusion volume of 5 ml Haemaccel (Behring Pharma, Amsterdam, The Netherlands) and a flow of 2.4 ml/min. The perfusion time started when the vector/drug was added as a bolus to the Haemaccel present in the oxygenation reservoir. After 15 minutes the perfusion was ended and a washout was performed with 5 ml of Haemaccel. After the procedure, the cannulas were removed and the femoral vessels of the perfused limb were ligated. The blood supply in the perfused leg is restored in the animals by extensive collateral circulation.

Tumour growth was measured by calliper measurement in two dimensions. The tumour volume was calculated using the formula: $^4/_3 x \Pi x (0.5 x D_{avg})^3$, in which D_{avg} is the mean of the two diameters measured.

Determination of leucocyte and histamine concentration in blood

For determination of leucocyte concentration rats were anaesthetised by using ethrane. Ten µl blood was collected from the rat tail in chilled K_2.EDTA cups and was mixed with 90 µl of Turk solution. The mixture was pipetted in a Bürker chamber and the amount of leucocytes were counted. For determination of histamine concentrations blood was centrifuged at 1250 rpm at 4 °C and plasma collected and frozen until analysed. The histamine concentration was determined by using a histamine EIA kit (Immunotech).

Statistical analysis

The growth curves were subject to analysis of variance on the log of the tumour volume searching for effects of treatment and of time after treatment. All available data were used for the calculations. To evaluate differences between individual treatments the Scheffé a posteriori test was employed. P values below 0.05 were considered significant.

RESULTS

Antitumour activity of IG.Ad.CMV.rIL-3β on ROS-1 osteosarcoma

To investigate the effect of ILP on tumour growth, ROS-1 tumour bearing rats underwent a sham ILP (mock perfusion without addition of adenoviral vector or drugs to the perfusion medium). Tumour growth of sham treated tumours was compared with tumour growth of tumours that were untreated (Figure 1A).

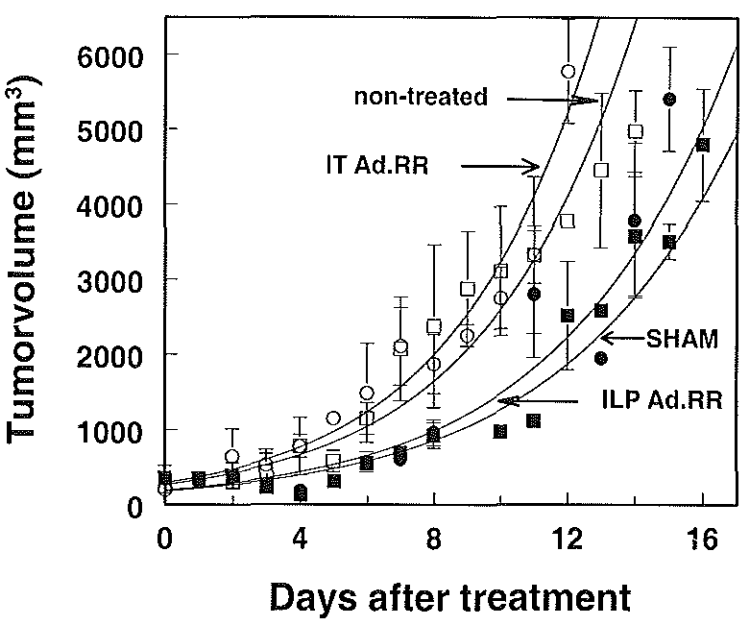

*Figure 1A. Growth curves of ROS-1 hind limb osteosarcomas following treatment with sham ILP (■, n=6), ILP with 1x10⁹ iu Ad.RR (●, n=4), intratumoural injection (IT) with 1x10⁹ iu Ad.RR (○, n=4) and untreated (□, n=8). Data are expressed as tumour volume (mm³) against days after treatment and represent average ± STD. Statistically significant effects (P≤0.05) are indicated by * when compared to sham ILP.*

Sham perfusion resulted in a small delay of tumour growth, which has been observed before by other investigators and is characteristic for ROS-1 tumours after ILP (Eggermont, unpublished observation). Statistical analysis of sham ILP and untreated groups showed no statistically significant difference in tumour growth of both groups. The effect of treatment on ROS-1 tumour cells with control adenoviral vector without a transgene was studied by administration of $1x10^9$ iu Ad5.RR. No effect on the tumour growth was observed when ROS-1 tumours were treated via ILP or direct intratumoural injection (IT)(Figure 1A).

Antitumour activity of IL-3 was compared between direct IT injection and ILP by administration of $1x10^9$ iu IG.Ad.CMV.rIL-3β. Figure 1B shows that IT administration did not result in an antitumour response since tumour growth was similar to non-treated tumours. With i.v. administration of the same dose adenoviral vectors no responses on tumour growth were seen as well (data not shown). However, ILP with $1x10^9$ iu IG.Ad.CMV.rIL-3β resulted in a statistically significant ($p<0.05$) tumour growth inhibition in all animals when compared to sham ILP treated tumours (Figure 1B).

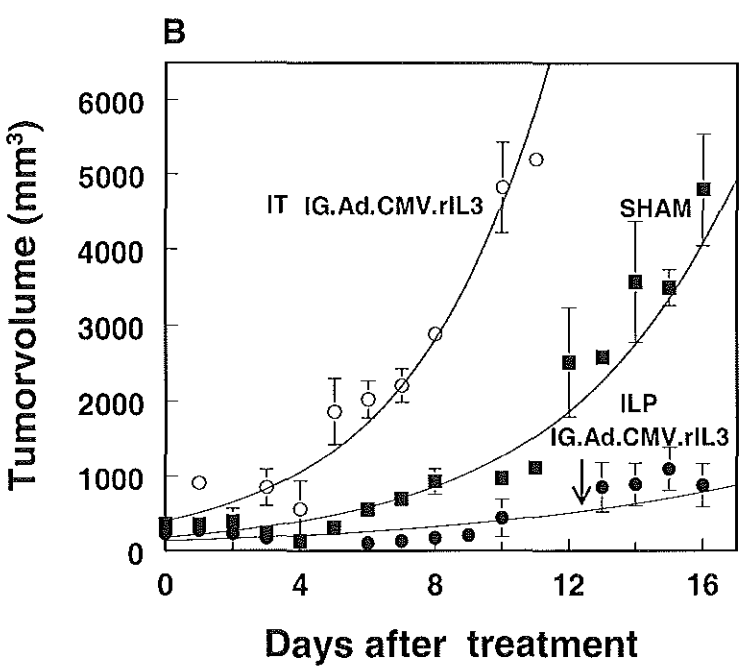

Figure 1B shows growth curves of ROS-1 osteosarcomas treated with ILP with $1x10^9$ iu IG.Ad.CMV.rIL-3β (●, n=9), IT of $1x10^9$ iu IG.Ad.CMV.rIL-3β (○, n=6) or sham ILP (■, n=6).*

Nine ROS-1 tumour bearing rats were treated with $1x10^9$ iu IG.Ad.CMV.rIL-3β. Two rats showed a complete remission and two tumours showed complete remission until day 9, after that the tumours slowly started to grow. Five animals showed no change of tumour size until day 10 after treatment, after that the tumours started to grow out. In conclusion, IG.Ad.CMV.rIL-3β results in a significant antitumour response after ILP, which can not be demonstrated after intratumoural administration.

Antitumour activity of IG.Ad.CMV.rIL-3β on BN-175 soft-tissue sarcoma

Isolated limb perfusion with $1x10^9$ iu IG.Ad.CMV.rIL-3β on a second rat tumour (BN-175 soft tissue sarcoma) resulted in a 89 % tumour response rate 15 days after perfusion. One rat showed progressive disease of the BN-175 tumour, two rats showed no change of tumour size and 6 rats showed a delayed tumour growth. Again no effect on tumour growth was demonstrated after IT administration of the same viral vector dose (Figure 2).

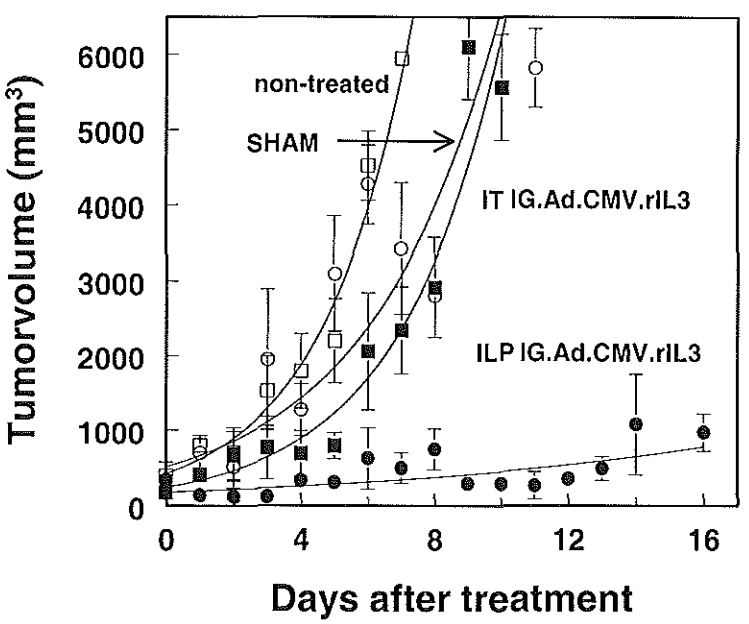

Figure 2. Growth curves of hind limb BN-175 sarcomas following treatment with a sham ILP (○, n=5), ILP with $1x10^9$ iu IG.Ad.CMV.rIL-3β (●, n=8), IT injection of $1x10^9$ iu IG.Ad.CMV.rIL-3β (■, n=11) or no treatment (□, n=4). Data are expressed as tumour volume (mm^3) against days after treatment and represent the average ± STD. Statistically significant effects ($P \leq 0.05$) are indicated by * when compared to sham ILP.*

Statistical analysis of the growth curves demonstrated a statistically significant ($p<0.05$) delay in tumour growth after ILP with IG.Ad.CMV.rIL-3β when compared to sham perfusions. There was no statistical significant difference between untreated, sham ILP and IT IG.Ad.CMV.rIL-3β treatment groups. Thus, in BN-175 tumours rIL-3β gene therapy can only induce a significant antitumour response when administered via ILP.

Antitumour effect of IG.Ad.CMV.rIL-3β after ILP: dose finding

In both models $1x10^9$ iu is shown to result in significant anti-tumour responses. Reduction of the dose of viral vectors resulted in a likewise decrease in tumour response. No statistically significant antitumour effect was observed in ROS-1 tumours after ILP with concentrations of $1x10^7$ iu and $1x10^5$ iu IG.Ad.CMV.rIL-3β (Figure 3A). Similar results were obtained with the BN-175 tumour model (Figure 3B). It is concluded that the optimal dose of IG.Ad.CMV.rIL-3β for ILP tested so far is $1x10^9$ iu both for ROS-1 and BN-175 tumours.

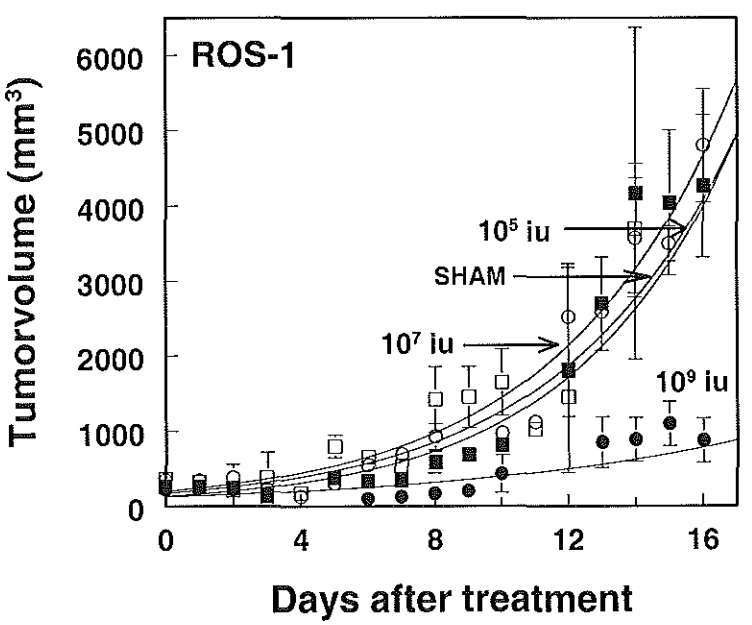

Figure 3A. Growth curves of ROS-1 hind limb tumours following ILP treatment with $1x10^9$ iu (●, n=9), $1x10^7$ iu (□, n= 6), $1x10^5$ iu (■, n=7) IG.Ad.CMV.rIL-3β and compared to sham ILP treatment (○, n=6). Data are expressed as tumour volume (mm^3) against days after treatment and represent the average ± STD. Statistically significant effects ($P\leq0.05$) are indicated by * when compared to sham ILP.*

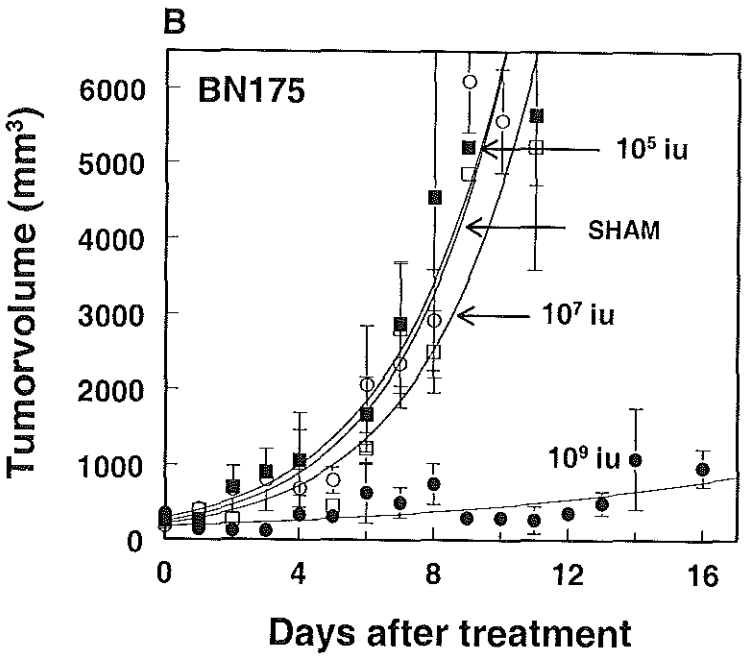

Figure 3B. BN-175sarcomas after ILP with 1x10^9 iu (●, n=9), 1x10^7 iu (□, n=5), 1x10^5 iu (■, n=5) IG.Ad.CMV.rIL-3β and compared to sham ILP treatment (○, n=6).*

Effect of different promoters driving the rIL-3β gene on antitumour response

Esandi *et al.* have demonstrated that cell lysates of 293 adenoproducer cells infected with IG.Ad.CMV.rIL-3β produced approximately 10 times more rIL-3β protein than cells infected with recombinant adenoviral vector in which the rIL-3β gene is driven by the MLP promoter (IG.Ad.MLP.rIL-3β).[14] To determine the importance of high IL-3β production per infected cell, isolated limb perfusions of ROS-1 tumours were performed using 1x10^9 iu IG.Ad.MLP.rIL-3β and compared with the 1x10^9 iu IG.Ad.CMV.rIL-3β ILP treated ROS-1 tumours (Figure 4). No antitumour response was observed with the weaker MLP promoter driving the rIL-3β gene. The CMV promoter itself is not cytostatic since it has been shown that treatment with control virus containing the CMV promoter did not evoke an antitumour response (Figure 1A). High production of rIL-3β in tumour cells driven by the CMV promoter seems therefore necessary to induce an antitumour response after ILP.

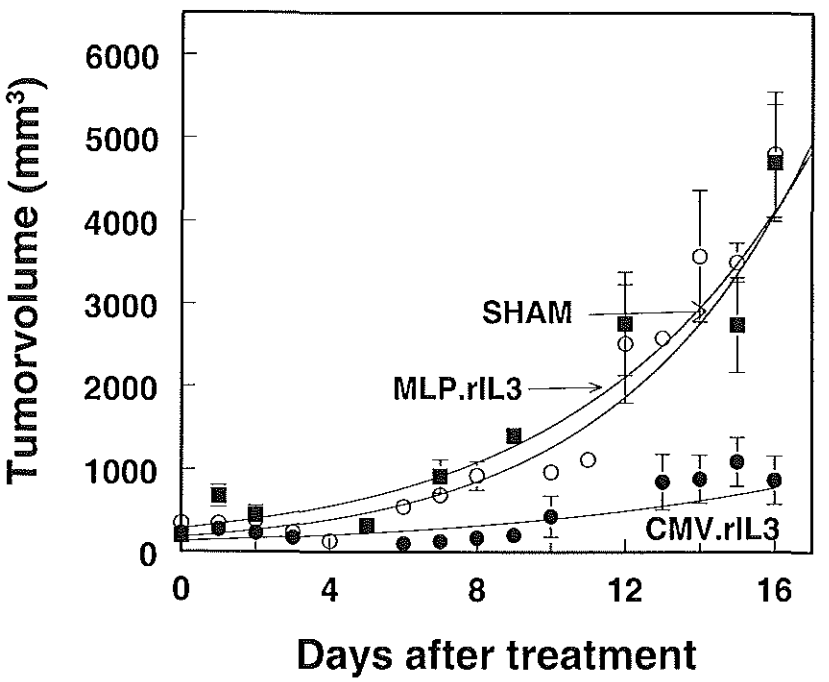

Figure 4. Effect of different promoters driving the rIL-3β gene on ROS-1 tumour growth following ILP with $1x10^9$ iu IG.Ad.MLP.rIL-3β (□, n=4) and compared with ILP treatment using $1x10^9$ iu IG.Ad.CMV.rIL-3β (●, n=9) or sham ILP (○, n=6). Data are expressed as tumour volume (mm^3) against days after treatment and represent the average ± STD. Statistically significant effects ($P \leq 0.05$) are indicated by * when compared to sham ILP.*

Comparison of IG.Ad.CMV.rIL-3β with the combination of TNF and melphalan

Manusama *et al.* previously demonstrated a significant antitumour response with the combination of melphalan and TNF in ROS-1 osteosarcoma bearing rats after ILP.[35] To compare this clinically established treatment with the IG.Ad.CMV.rIL-3β gene delivery therapy, ROS-1 tumours were treated with the combination of 50 μg TNF and 40 μg melphalan and compared rIL-3β transgene perfusions. Figure 5 shows that ILP with IG.Ad.CMV.rIL-3β resulted in an antitumour response similar to combination therapy with TNF and melphalan.

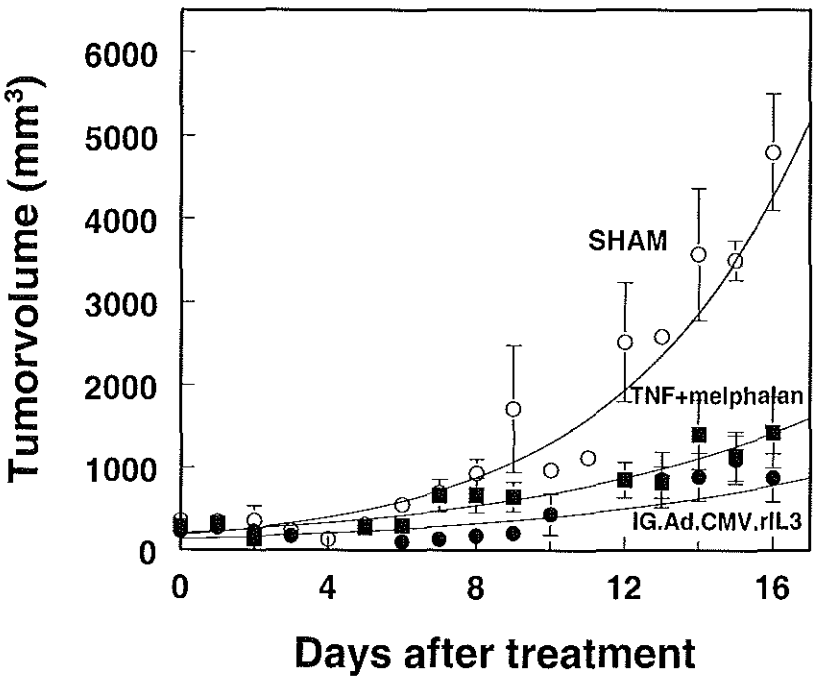

Figure 5. Growth curves of ROS-1 hind limb tumours following ILP treatment with $1x10^9$ iu IG.Ad.CMV.rIL-3β (●, n=9) or TNF (50 μg) melphalan (40 μg) combination therapy (■, n=5) and compared to sham ILP (○, n=6). Data are expressed as tumour volume (mm^3) against days after treatment and represent the average ± STD. Statistically significant effects (P≤0.05) are indicated by * when compared to sham ILP.*

Effect of IG.Ad.CMV.rIl-3β treatment on the blood leucocyte concentrations

In vivo experiments in rhesus monkeys[47] and mice[48] have shown that IL-3 stimulates the blood cell production from immature, multipotent progenitor cells. In particular, the amount of leucocytes increased after IL-3 administration, with a major increase of histamine containing basophils. To investigate the effect of IL-3β on leucocytes, blood samples were drawn from ROS-1 tumour bearing rats treated with 10^9 iu IG.Ad.CMV.rIL3β via ILP, direct intratumoural injection (IT) or intravenous injection (IV, via penile vein). As a control, blood was sampled from non-tumour bearing Wag/Rij rats and ROS-1 tumour bearing rats that were untreated or sham ILP treated. As is shown in figure 6 treatment with IG.Ad.CMV.rIL-3β resulted in a transient increase of leucocytes after ILP, IT and IV administration. Leucocytes increased from 14200 ± 2365

leucoytes/mm^3 to a maximum of approximately 95000 leucocytes/mm^3 7 days after administration. At day 21 basal level was reached in all situations (data not shown). Leucocytosis was caused by expression of IL-3 transgene since untreated or sham ILP treated tumour bearing animals did not show leucocytosis. Leucocytosis was accompanied with an increase in body temperature of approximately 2 °C and by redness of ears, footpads and scrotum of the animals (data not shown). Treatment with IG.Ad.CMV.rIL-3β leads to a transient leucocytosis independent of the route of administration of the recombinant adenoviral vector.

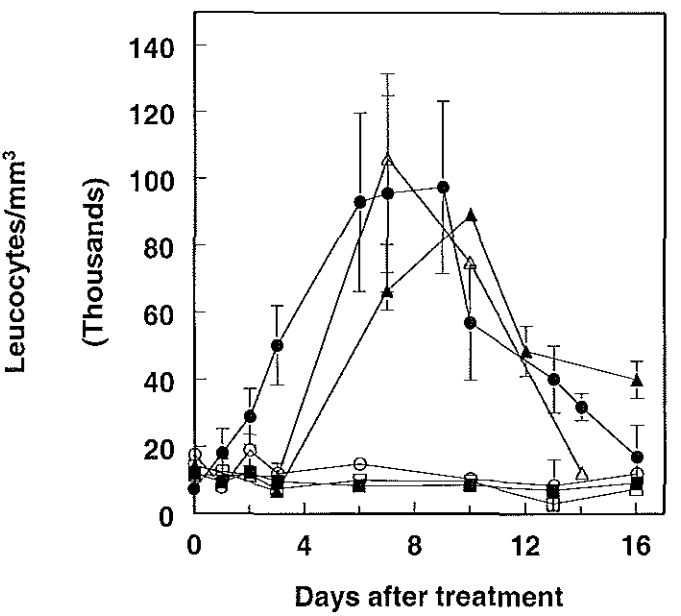

Figure 6. Effect of treatment with 1x10^9 iu IG.Ad.CMV.rIL-3β on blood leucocyte concentration. ROS-1 osteosarcoma bearing rats were treated with 1x10^9 iu IG.Ad.CMV.rIL-3β which was administered via ILP (●, n=3), direct IT injection (▲, n=3) or i.v. injection (Δ, n=3). Other tumour bearing animals were sham ILP treated (○, n=3) or untreated (■, n=3). Non tumour bearing animals were used as controls (□, n=3). Blood leucocyte concentration was determined in time. Data represent average ± STD.

Effect of ILP treatment with various doses of IG.Ad.CMV.rII-3β on blood leucocyte and histamine concentrations

In order to determine whether the observed leucocytosis was dose dependent, ROS-1 tumour bearing rats were perfused with various doses of IG.Ad.CMV.rIL-3β and the amount of

leucocytes and blood histamine concentration were measured. No increase in leucocyte concentration was observed after treatment with 10^5 iu IG.Ad.CMV.rIL-3β. Higher doses of IG.Ad.CMV.rIL-3β (10^7 iu and 10^8 iu) likewise increased leucocyte concentration (figure 7A).

To study whether histamine containing basophils are involved in the leucocytosis the amount of histamine in the blood was analysed. The increase in histamine concentration followed the leucocyte response (figure 7B). Treatment with 10^5 iu IG.Ad.CMV.rIL-3β did not resulted in a histamine increase. Peak levels of 6.7 ± 1.7 (56-fold) and 37.2 ± 20.9 (310-fold) μM were demonstrated after treatment with 10^8 and 10^9 IG.Ad.CMV.rIL-3β respectively. This demonstrates a dose dependent relationship between the administered IG.Ad.CMV.rIL-3β and both the leucocyte and histamine concentration.

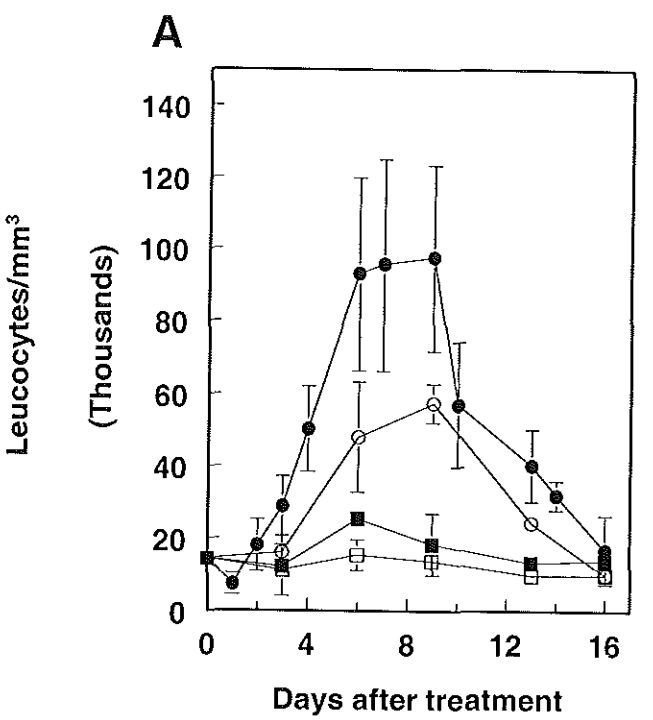

Figure 7A. Effect of treatment with various doses of IG.Ad.CMV.rIL-3β on blood leucocytes. ROS-1 osteosarcoma bearing rats were ILP treated with 1x10⁹ (●, n=3), 1x10⁸ (○, n=3), 1x10⁷ (■, n=3) or 1x10⁵ (□, n=3) iu IG.Ad.CMV.rIL-3β. Blood leucocyte concentration is expressed in time. Data represent average + STD.

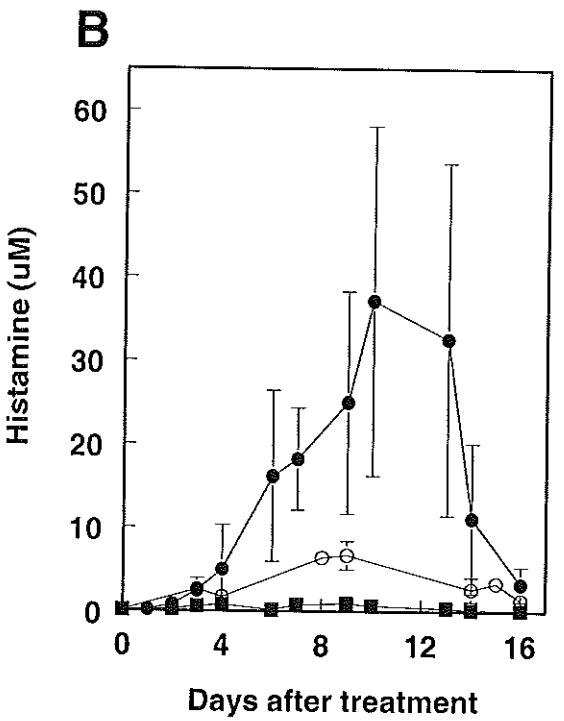

Figure 7B. Effect of treatment with various doses of IG.Ad.CMV.rIL-3β on and histamine concentration. ROS-1 osteosarcoma bearing rats were ILP treated with $1x10^9$ (●, n=3), $1x10^8$ (○, n=3), $1x10^7$ (■, n=3) or $1x10^5$ (□, n=3) in IG.Ad.CMV.rIL-3β. Blood histamine concentrations are demonstrated in time.

Effect of IL-3 mediated leucocytosis on tumour growth

To study whether the observed systemic leucocytosis could play an important role in the antitumour response ROS-1 tumour bearing rats were treated with sham ILP at the tumour-bearing leg after which leucocytosis was evoked with i.v. administration of 10^9 in IG.Ad.CMV.rIL-3β. Figure 8 demonstrates the tumour growth and leucocyte response curves of sham treated rats with or without systemic administration of IG.Ad.CMV.rIL-3β vector. No significant difference was measured in tumour volume between both treatment groups, indicating that IL-3β mediated leucocytosis is not responsible for the antitumour effect, but that intratumoural expression of IL-3β is responsible for the observed antitumour effect.

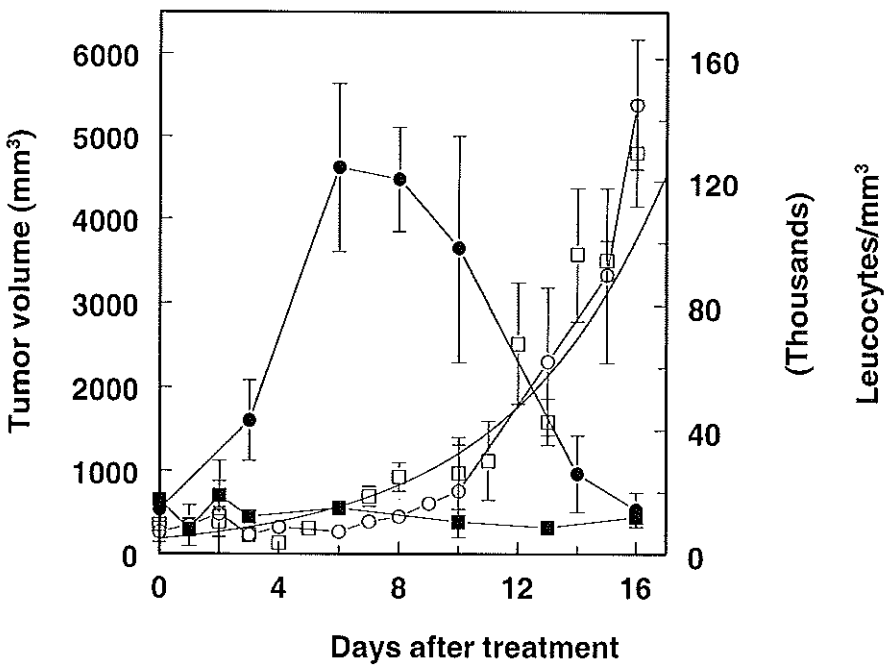

Figure 8. Effect of rIL-3β mediated leucocytosis on tumour growth in sham ILP treated ROS-1 hind limb tumours. Rats with ROS-1 hindlimb osteosarcoma were sham ILP treated after which 1x10^9 iu IG.Ad.CMV.rIL-3β was administered intravenously (n=3). Tumour growth (○) and leucocyte concentrations (●) were determined in time and compared with tumour growth (□) and leucocyte concentrations (■) of sham ILP treated tumours without IG.Ad.CMV.rIL-3β administration (n=3). Data represent average ± STD.

DISCUSSION

In the present study we describe a profound antitumour effect of IG.Ad.CMV.rIL-3β on two different tumours when administered using ILP. It is demonstrated that all but one animal with experimental osteosarcomas and soft tissue sarcomas responded, whereas a direct intratumoural injection with the same amount of IG.Ad.CMV.rIL-3β adenoviral vector (1x10^9 iu) did not result in an antitumour response. Effective gene transfer in tumour tissue using an ILP tumour model was recently demonstrated using recombinant adenoviral vectors harbouring the luciferase or lacZ

marker gene.[39,49] Moreover, ILP resulted in diffuse expression of the LacZ gene located in the tumour vasculature and viable tumour cells, whereas direct intratumoural injection resulted in expression only around the needle tract.[39] The more homogenous distribution and specific delivery of recombinant adenoviral vector via the tumour vasculature might be necessary for the observed antitumour effect observed in the present study. This phenomenon has recently been demonstrated by Mizuguchi *et al.* using TNF gene therapy, where a profound antitumour effect was demonstrated when it was administered in the artery leading to the tumour and no effect on tumour growth was found when the TNF gene was injected intratumoural.[18]

Previously, Esandi *et al.* demonstrated an IL-3β mediated antitumour effect in an established subcutaneous rat tumour model using multiple intratumoural injections of IG.Ad.CMV.rIL-3β transfected 'cracked' adenoproducer cells.[14] In the present study, no effect was found on tumourgrowth after a single intratumoural injection of both ROS-1 and BN-175 tumours. This might be due to the fact that Esandi *et al.* used a slower growing tumour (L42 non-small lung cancer) and that multiple intratumoural injections were necessary to induce an antitumour effect. Application of multiple intratumoural injections augments the risk of repeated toxicity which could be reduced with a single treatment using the ILP method.

The observed antitumour effect is caused by rIL-3β since no response was found in tumours that were sham perfused or perfused with an adenovirus lacking a therapeutic gene. Moreover, doses below $1x10^9$ iu IG.Ad.CMV.rIL-3β resulted in the loss of antitumour response and recombinant adenoviral vectors with the weaker MLP promoter driving the rIL-3β gene were not able to evoke an antitumour response.

IL-3 exerts its biologic activities through binding to a specific high-affinity receptor to the cell surface (IL-3R). Expression of the IL-3R was previously thought to be restricted to cells of haemopoietic origin. However, recently it has been demonstrated that several vascular and connective tissue-type cells, including human umbilical vein endothelial cells (HUVEC), smooth muscle cells (SMC) and foreskin fibroblasts (HFF) also express IL-3R suggesting a potentially much wider role for IL-3 than previously anticipated.[50-52] Korpelainen *et al.* demonstrated that IL-3 enhances the expression of G-CSF, IL-6, IL-8, E-selectin and MHC-II in endothelial cells.[28] These modulators and receptors have been shown to play a role in recruitment of neutrophils to inflammatory foci and transmigration of the neutrophils across endothelial monolayers. Whether or not this mechanism plays a role in our system (even without the additional IL-3R upregulation by TNFα) remains to be elucidated. We did however, demonstrate a transient increase in leucocytes five to ten days after perfusion, which was accompanied by an increase in histamine levels and body temperature. We showed that tumour growth inhibition is not induced by the increase in

leucocytes only, as inhibition in tumour growth could not be induced by systemic administration of IG.Ad.CMV.rIL-3β, which resulted in comparable leucocytosis. We therefore assume that transduction of IG.Ad.CMV.rIL-3β in vital tumour cells and in tumour associated vasculature leads to local IL-3β production that attracts inflammatory and immune cells to the tumour and induce a cytotoxic response.[53,54]

This study demonstrates that isolated limb perfusion with monotherapy of IG.Ad.CMV.rIL-3β is as efficient as the established combination therapy using TNF and melphalan.[35] In conclusion, isolated limb perfusion with recombinant adenoviral vectors carrying the IL-3 transgene at doses of $1x10^9$ iu showed antitumour efficacy and could be of great therapeutical benefit for patients with osteosarcoma and sarcoma to obtain local tumour control and finally may lead to preservation of the limb and limb function.

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CHAPTER 9

GENERAL DISCUSSION

Submitted in revised form:

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

A.H. van der Veen, T.L.M. ten Hagen, J.H.W.de Wilt, A.M.M. Eggermont.

INTRODUCTION

Isolated limb perfusion (ILP) is not any longer restricted to the treatment of multiple in-transit metastasised melanoma, but has now established itself as the new treatment option to manage limb threatening soft tissue sarcomas as a result of the synergistic combination of TNF and melphalan.[1-3] The development of animal ILP models provide ways to evaluate efficacy and mechanisms of new drugs and to introduce new treatment modalities such as gene therapy.

ILP of the extremities was first described by Creech *et al.* over 40 years ago for a patient with in transit metastasised melanoma.[4] Isolation of the blood circuit is achieved by cannulation of the femoral or iliac artery and vein followed by the application of a tourniquet around the base of the limb to compress the remaining collateral vessels. The isolated extremity is connected to an oxygenated extracorporeal circuit, into which the cytostatic agent is injected. After perfusion a thorough rinsing procedure to wash out the drugs is performed, after which the cannulas are removed and the circulation is restored. With ILP 15-20 times higher local drug concentrations can be obtained than those reached after systemic administration.[5] Since an almost leakage-free system is achieved, chemotherapeutics that would be very toxic or lethal if given systemically can thus be administered regionally in adequate dosages.

Several drugs such as melphalan, doxorubicin, actinomycin D, methotrexate or cisplatin have been used in ILP protocols alone or in combination. Melphalan has been used as the standard single drug in the treatment of in-transit metastasised melanoma because of its low regional toxicity and fairly good response rates.[6-9] For locally advanced extremity tumours such as soft-tissue sarcoma, osteosarcoma and carcinoma, results after ILP were modest since only few complete response rates have been reported.[10-15] Various strategies have been developed to improve responses including hyperthermia, multiple perfusions and introduction of biological response modifiers. Lejeune and Liénard were the first to combine melphalan with high dose tumour necrosis factor-α (TNF) and interferon-γ (IFN) for multiple in-transit metastasised melanoma.[16,17] High response rates were achieved with this combination and several other authors demonstrated similar good results.[1,8,18-22] The success of this treatment was explored to locally advanced soft tissue sarcoma. It became clear that high complete response rates were feasible, with a limb salvage rate of more than 80%.[2,3,23] For irresectable osteosarcoma and nonmelanoma skin carcinoma promising results have recently been reported using TNF in combination with melphalan as well.[24,25]

TNF IN ISOLATED LIMB PERFUSIONS

TNF in vitro

TNF is a homotrimeric complex of 52 kD which is produced by many cell types, but is mainly secreted by activated monocytes/macrophages.[26,27] Its expression and regulation is affected by a variety of other cytokines, as interferon-γ (IFN-γ), interleukines (IL-1, IL-2, IL-12), GM-CSF, Platelet Aggregating Factor (PAF) as well as TNF itself.[27] TNF is directly cytostatic or cytotoxic to only a few cancer cell lines.[28] On other cell types TNF shows a growth inhibitory or even a growth stimulatory effect.[27,29] The effects of TNF are exerted by binding to two types of receptor, with molecular weights of 55 kDa (TNF-R1) and 75 kDa (TNF-R2) respectively, which are present on nearly all mammalian cells.[26,30] The number of receptors on the cell does not predict the magnitude of response to TNF, but up-regulation (IFN-γ) and down-regulation (IL-1) of TNF receptors have been reported.[29]

In vitro, synergism between TNF and a number of cytotoxic agents may be present.[31] We investigated several cell lines on susceptibility to TNF and certain cytotoxic drugs (e.g. the alkylating agent melphalan or the topoisomerase-II inhibitor doxorubicin). No direct cytotoxic effects of TNF, nor synergism with melphalan or doxorubicin were observed in vitro but only additive antitumour effects were noted.[32-34] Cytotoxic effects of TNF can be enhanced by a number of other biological response modifiers like IFN-γ [35] or IL-1 [36], hyperthermia [31] and irradiation.[37] The mechanism by which TNF exerts its cytotoxic effects are not yet fully understood. The number of receptors on the tumour cell are probably of less importance than the role of oxygen free radicals in TNF cytotoxicity, and activation of lysosomal enzymes.[27]

TNF in vivo

Many animal studies have demonstrated antitumour effects of TNF in vivo, leading to hemorrhagic necrosis in tumours.[38,39] Systemic application of TNF in humans however, proved to be deleterious to patients and in phase I/II studies severe toxicity was reported. A variety of side effects was noted, hypotension being the dose-limiting factor.[40-42] The maximal tolerated dose (MTD) varied between 200 and 400 μg/m^2, which was only 1/50 of the effective dose in murine tumour models.[41,43] Due to these low concentrations of TNF when given systemically, only low response rates were achieved. Phase II studies revealed a 1-2% response rate after intravenous administration of TNF as a single agent or combination therapy with chemotherapeutic drugs.[44-46] Addition of IFN-γ or IL-2 did not enhance antitumour efficacy but further increased toxicity.[47,48]

Because of the severe toxicity after systemic use of TNF in clinical trials, other routes of

administration were explored to achieve high local concentration of TNF in tumour tissue. Intratumoural injection revealed only slightly better responses than intravenous injection with similar side effects.[49-51] Hepatic artery infusion[52] and intraperitoneal[53] administration of TNF revealed only modest results.

TNF in isolated limb perfusion

In ILP sufficient concentrations of TNF could be achieved leading to antitumour effects as observed in animal models. The tumour vascular bed appears to be the selective target for TNF in ILP. The effect of TNF on tumour vasculature demonstrated to be concentration dependent leading to vasculotoxic effects at high concentrations, while at low concentrations it may promote DNA synthesis and angiogenesis.[54] The effects on the tumour associated vasculature after ILP with TNF were described as early endothelium activation, upregulation of adhesion molecules and invasion of polymorphonuclear cells, leading to coagulative necrosis with or without hemorrhagic necrosis.[55,56] However, examination of melanomas and sarcomas of patients treated with the triple regimen in ILP did not show differences in expression of adhesion molecules as ICAM-1, E-selectin (ECAM-1), VCAM-1 or PECAM-1 in tumours compared with healthy tissue.[57] Recently, in patients treated with isolated limb perfusion with TNF, IFN-γ and melphalan, detachment and apoptosis of the integrin αvβ3 positive endothelial cells was demonstrated in vivo in melanoma metastases,[58] again pointing to the importance of selective disruption of tumour associated blood vessels. This was further indicated by angiography and NMR studies which clearly showed the disappearance of only tumour associated vessels after TNF-based ILP.[59-61]

ISOLATED LIMB PERFUSION IN ANIMAL MODELS

ILP with chemotherapeutics

Several pharmacokinetic studies have been reported with ILP models in dogs using cisplatin, doxorubicin and melphalan.[62-65] In general, histological evaluation showed a modest impact in terms of induction of necrosis, indicating the need for further research for better perfusion agents.[66] Rat ILP models were also used for pharmocokinetic studies demonstrating optimal tissue penetration of melphalan when pH was physiologic (between 7.3-7.7), temperature of the perfusate was between 40 to 41°C, and perfusion rate was low.[67] Wu *et al.* however, demonstrated a higher intratumoural uptake of melphalan when perfusion rate was high in a human melanoma xenograft model.[68] Moreover, it was demonstrated that the perfusate solution should have low melphalan binding capacity (no albumin) to

maximise melphalan uptake in tumour tissue. These prerequisites in perfusate conditions demonstrated to decrease tumour growth and rat survival.[69] Recently, Walgenbach *et al.* demonstrated hyperlactacidaemia to induce tumour necrosis and subsequently growth delay in hyperthermic perfusions in a DS-sarcoma model.[70] Several animal models are used to study drugkinetics and antitumour effects and results obtained from these studies might improve further clinical perfusion techniques.

TNF-based ILP

Since many mechanistic questions concerning TNF-based ILP can only be answered in preclinical models, a rat ILP model was developed in our laboratory. The technique described by Benckhuijsen *et al.* was modified using a rapidly growing, spontaneously metastasising, non-immunogenic, grade III fibrosarcoma (BN-175).[32,71] This tumour originated spontaneously in the Brown Norway rat.[72] Moreover, in a wistar-derived WAG/Rij rat strain a similar tumour model [33] with an osteosarcoma ROS-1, which originated spontaneously in the tibia of a WAG/Rij rat, was developed.[73] Both non-immunogenic and syngeneic tumours grow in fully immunocompetent rats, and the models resemble the clinical situation closely. In both tumour systems highly synergistic antitumour effects were observed when TNF is combined with melphalan, resulting in a complete remission rate of 70-100%.[32,33,74] Similar results have been reported by others using TNF in combination with melphalan in GF fibrosarcoma-bearing rats.[75] Histopathologically early endothelial damage and platelet aggregation in tumour vessels are observed after ILP with TNF and melphalan leading to ischemic (coagulative) necrosis, which is in line with observations in patients.[21,56,57,76,77] We identified as basic requirements for an effective ILP with TNF and melphalan a perfusion duration of 30 min and a minimal temperature of the perfused limb of 38°C, while hyperthermia above 42°C resulted in unacceptable damage to normal tissue. Regional toxicity after hyperthermia in ILP was previously demonstrated in humans as well.[78] Hypoxia enhanced antitumour activity of melphalan and TNF alone but did not further improve results of their combined use. However, it was feasible to perform hypoxic perfusions without increasing locoregional toxicity. This makes the application of hypoxic perfusions a reasonable option. The minimally required dose of TNF to induce synergy with melphalan demonstrated to be 10 μg (=40 μg/kg) in our model, which might indicate that a 10 fold dose reduction of TNF in the clinical situation may still be effective.[74]

We also performed ILPs with TNF and doxorubicin in solid tumour bearing rats. Anthracyclines are among the most active agents against solid tumours and doxorubicin is the most widely used agent of this class.[79-81] Moreover, doxorubicin is the agent of choice for the treatment of sarcoma, and has shown tolerability and antitumour activity in clinical and experimental perfusion

settings for the treatment of soft tissue sarcoma and its lung metastases.[82-84] Previously doxorubicin has been used in isolated perfusion of the extremities.[12,64] It was demonstrated that when comparable results were obtained this was often at the cost of increased regional toxicity.[6,7] In our rat model both solid tumour models showed progressive disease after perfusion with doxorubicin alone and a 50-100% response (partial and complete) was demonstrated when TNF was added to the perfusate.[34]

Effect of TNF on tumour vasculature

Synergism of TNF with cytostatic drugs (e.g. melphalan or doxorubicin) is the crucial element that determines the success of isolated limb perfusion.[29,32,33,40] It is probably based on dual targeting, where TNF (and IFN-γ) is suggested to be responsible for disruption of the neovasculature, while melphalan causes a non-specific necrosis of cancer cells in vivo.[19,75] In vivo antitumour effects of the combined treatment of 5-FU and TNF also depend upon the development of capillaries in tumours.[85] The endothelial cell damage induced by TNF leads to congestion, hemorrhage and oedema as representatives of an impaired blood flow.[39,56,76] The endothelial damage, proven by a change in distribution of von Willebrand factor, occurred already three hours after onset of TNF-based perfusion.[56] However, also a delayed type of hyperpermeability may be present, explaining the fact that complete tumour regression frequently requires longer periods after TNF-based isolated perfusion.[86] Vasculotoxic effects of TNF lead to a significant drop in tumour interstitial pressure and permeability changes which leads to better penetration of cytotoxic drugs into tumourtissue.[87-90] We showed an increased doxorubicin uptake in tumour tissue when TNF was added to the perfusate.[34] Similarly, a 4-6 fold increase of intratumoural melphalan was demonstrated after TNF-based isolated limb perfusion. These observations may well be the crucial mechanism behind the success of TNF in ILP.[91] Moreover, vasculotoxic effects lead to endothelial cell activation, upregulation adhesion molecules and infiltrate formation through influx of polymorphonuclears, more specifically granulocytes. Subsequently injury to the endothelial cells may be the beginning of a cascade of events.[55,92,93] Synergistic or additive antitumour effect between TNF and IFN-γ were observed earlier, probably based on receptor upregulation, which is also shown in vitro.[94-96] However, conflicting results have been reported with IFN-γ slightly enhancing antitumour efficacy of TNF in combination with melphalan, whereas toxicity was considerably increased.[97]

The observations made in our animal ILP model correspond well with observations made in the clinic, therefore this model can serve as a tool to investigate different ways to further improve ILP protocols or explore new antitumour modalities.

NEW STRATEGIES IN ISOLATED LIMB PERFUSIONS

Use of TNF mutants

Because of the systemic toxicity associated with TNF, less toxic but equally effective recombinant TNF molecules were sought. In one of these mutants of conventional TNF the N-terminal amino acid sequences were altered to achieve higher basicity (TNF-SAM2). Various types of TNF-SAM2, differing in amino acid species and position in the N-terminus were described.[98] Toxic side effects seemed to be lower with these molecules, whereas TNF-SAM1 and TNF-SAM2 revealed a stronger cytotoxic activity than conventional, recombinant human TNF on various murine tumours.[99] We demonstrated synergistic antitumour activity with melphalan and doxorubicin using TNF-SAM2 in our isolated limb perfusion model.[100] Clinical and histopathological responses were comparable to results observed with conventional TNF. Other mutants, in which amino acids were changed (F4236, F4168, F4614), also showed increased anti tumour activity and less lethal toxicity.[101-103] Low inducibility of nitric oxide and prostaglandin E_2 correlated with a reduced hypotensive effect, consequently an increased therapeutically effective dose compared to native TNF could be given.[104] New TNF analogues as LK 801 and LK 805 confirmed that modification of the parental molecule leads to lower systemic toxicity and comparable anti tumour effect.[105] These novel mutants are thought to have great potential for clinical application. Not only can they make perfusion a more safe procedure, but they can also expand the use of TNF in other perfusion settings which are less leakage free or in organs which are sensitive to the toxic effects of TNF (e.g. liver or lung).

Use of vasculotoxic agents

Nitric oxide (NO) is an important molecule in the maintenance of tumour blood flow by dilatating arteriolar vessels in tumours and is produced by NO synthase (NOS).[106] This effect on tumour vasculature by NO enables vital nutrients and oxygen to reach tumour cells. High levels of NOS activity are present in several tumour cell lines as well as in human cancer.[107,108] Recent studies demonstrated another important effect of NO in stimulating tumour angiogenesis.[109-111] Inhibition of NOS might thus inhibit tumour blood flow and neovascularisation and in this way reduce tumour growth. Several authors demonstrated a selectively reduced tumour blood flow in rodents treated with NO inhibitors.[112,113] Antitumour effects were also demonstrated with NO-inhibitors used as a single agent.[114,115] However, systemic treatment with these NO inhibitors can decrease renal blood flow and induce hypertension.[116] To reduce this systemic toxicity a NO inhibitor (L-NAME) was used in our ILP model. A decreased tumour growth was demonstrated in sarcoma-bearing rats when

L-NAME was used alone. Moreover, strong synergy was observed when L-NAME was used in combination with either TNF or melphalan. Even in the setting of the strongly synergistic combination of melphalan and TNF the addition of L-NAME enhanced response rates and tumour recurrences were delayed or not observed.[117] These results strongly suggest that L-NAME or other NO inhibitors are promising agents in the treatment of cancer and can rather safely be administered in the leakage free isolated perfusion setting. Further pre-clinical and clinical studies with L-NAME should optimise concentration and kinetics to exploit this potential antitumour drug.

Gene therapy in ILP

Gene therapy has been proposed as a promising new approach in antitumour therapy, because of the observed impressive tumour responses in experimental animals.[118] Among the several potentially applicable viral and non-viral vector systems, recombinant retroviruses and adenoviruses have been most widely used in cancer treatment.[119] One major challenge in cancer gene therapy is to transfect only tumour cells since transfection of other organs may cause potentially dangerous complications. Tumour-specificity may be accomplished at the level of gene transfer by vector targeting with the use of ligands, antibodies or the use of specific promoters.[118] However, most widely applied gene transfer vectors as well as standard promoters do not generate tumour-specific gene expression.

Another way to induce specific gene delivery is specific administration of viral vectors such as intratumoural injection or regional administration in the artery that give blood supply to the tumour. For lung,[120] liver[121] and brain[122] regional infusions have previously been demonstrated effective in virus-mediated gene transfer. Isolated perfusion settings have also been demonstrated effective in adenoviral-mediated gene delivery including isolated liver perfusion[123] and isolated lung perfusion.[124] Tumours were not included in these isolated circuits and it is therefore unknown whether tumour cells are preferentially transfected in the perfused circuit. In preclinical ILP models we and others demonstrated effective gene transfer in tumour tissue using recombinant adenoviral vectors harbouring the luciferase and lacZ marker gene.[125,126] Systemic leakage of luciferase marker gene after ILP was negligible demonstrating an almost leakage free perfusion system. Moreover, ILP resulted in a more diffuse expression of the LacZ gene located in the tumour vasculature and viable tumour cells, whereas direct intratumoural injection resulted in expression only around the needle tract.[126]

Endothelial cells are an attractive target for gene therapy because they are intimately involved in disease processes associated with inflammation and angiogenesis and because endothelial cells are readily accessible to gene vector therapy. Mizuguchi *et al.* demonstrated a

profound antitumour effect when TNF gene therapy was administered in the artery leading to the tumour. On the other hand no tumour growth was observed when the TNF gene was injected intratumourally.[127] We demonstrated a similar lack of antitumour responses with intratumoural injections using the rat interleukin-3β (rIL-3β) gene in two different tumour models. Intravascular administration using the ILP technique, however, demonstrated a significant growth retardation in both ROS-1 and BN-175 tumours.[128] We assume that transduction of rIL-3β in vital tumour cells and in tumour associated vasculature leads to local IL-3β production that attracts inflammatory and immune cells to the tumour and induce a cytotoxic response.[129,130] IL-3 exerts its biologic activities through binding to a specific high-affinity receptor on the cell surface. Whether this IL-3 receptor is present on tumour cells is unknown, but its presence was demonstrated on vascular endothelium.[131,132] Intravascular delivery of IL-3 might therefore generate a specific antitumour effect on the tumour vasculature.[129,130]

ILP might be useful for efficient and tumour-specific delivery of viral and non-viral therapeutic gene constructs, including genes encoding for cytokines, angiogenesis inhibitors and suicide genes to enhance tumour control. Moreover, ILP is an interesting screening model to test efficacy of various approaches in gene therapy. Preclinical studies are presently conducted to explore these possibilities in limb and organ perfusion settings. Although much research needs to be done, the possibility of specific gene targeting with cytokines (e.g. TNF or IL-3β) makes this area of gene therapy a promising one for future investigations.

CONCLUSIONS

Isolated limb perfusion is a technique which allows perfusion of high dosages of cytostatic agents without systemic toxicity. The combination of TNF and melphalan in ILP has yielded high response rates in patients with in transit metastasised melanoma and locally advanced soft-tissue sarcoma. The primary target for TNF is the tumour vasculature and the interaction with cytostatic agents has been proven to be of paramount importance in the treatment of cancer. Several ILP models have been developed to study drug kinetics and antitumour mechanisms. In our ILP models in the rat different mechanisms of TNF-based antitumour effects were demonstrated and new treatment options were developed and tested. An extension of the use of ILP as a treatment modality might be found in the application of less toxic TNF-mutants, the introduction of other vasculotoxic agents and the use of gene therapy. This hopefully will result in clinical applications in isolated perfusion protocols in limb or other organs that eventually may improve outcome of cancer patients.

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CHAPTER 10

SUMMARY AND CONCLUSIONS

During isolated limb perfusion (ILP) high intratumoural concentrations of chemotherapeutics are obtained with low systemic exposure to these drugs. ILP is achieved by isolating the vascular bed of a limb by cannulating the main bloodvessels. Impressive results are obtained when treating patients with irresectable soft tissue sarcoma and in transit metastasised melanoma with melphalan in combination with tumour necrosis factor alpha (TNF) using the ILP technique. These results were the basis of the development of an ILP model in the rat to study mechanisms and efficacy of established and new treatment modalities.

In *Chapter 1* previous results and observations made with this model are described and the aims of this thesis are outlined.

In *Chapter 2* prerequisites for an effective ILP, such as oxygenation of the perfusate, temperature of the limb, duration of perfusion and concentration of tumour necrosis factor alpha (TNF) are studied. The combination of 50 μg TNF and 40 μg melphalan demonstrates synergistic antitumour activity leading to a 71% partial and complete response rate in BN-175 soft tissue sarcoma-bearing rats. In comparison to oxygenated ILP, hypoxia shows to enhance activity of melphalan and TNF alone but not of their combined use. Hypoxic perfusions were feasible without an increase in regional toxicity. Perfusion duration less than 30 min or limb temperatures below 38-39°C decrease responses. At a temperature of 42-43°C higher response rates are found, but local toxicity impaired limb function dramatically. Synergy between TNF and melphalan is lost at a dose of TNF below 10 μg in 5 ml perfusate.

In *Chapter 3* a significant increase in melphalan tumour tissue concentration is demonstrated after ILP when TNF is added to melphalan in the perfusate. Melphalan concentration is not increased in muscle and skin tissue, which illustrates that TNF works only on the tumour vasculature.

In *Chapter 4* doxorubicin is used in combination with TNF and demonstrated a synergistic antitumour effect. In BN-175 soft tissue sarcoma and ROS-1 osteosarcoma 54% and 100% response rates are reported respectively. In both models an increased accumulation of doxorubicin in tumour tissue is found after ILP when TNF is added to doxorubicin. *In vitro* TNF fails to augment drug uptake in tumour cells or to increase cytotoxicity of the drug. These findings make it unlikely that TNF directly modulates doxorubicin activity *in vivo*. Increase in doxorubicin tissue accumulation after ILP with TNF seems an important mechanism in the observed synergistic antitumour response.

In *Chapter 5* synergistic antitumour activity of a TNF mutant (TNF-SAM2) is demonstrated in combination with melphalan and doxorubicin in the ILP model using BN-175 soft tissue sarcoma-bearing rats. Histopathologically the response consisted of hemorrhagic necrosis of the coagulative type, which is comparable to what has been demonstrated with recombinant human TNF in combination with melphalan.

In *Chapter 6* antitumour effects of a nitric oxide (NO) synthase inhibitor (L-NAME) are investigated. Systemic treatment with L-NAME inhibits growth of subrenal CC-531 adenocarcinoma significantly but is accompanied by impaired renal function. Reduced tumour growth is observed when L-NAME alone is used in the ILP model using BN-175 sarcoma-bearing rats. In combination with TNF or melphalan, L-NAME increases response rates significantly compared to perfusions without L-NAME (0 to 64% and 0 to 63% respectively). An additional antitumour effect is demonstrated when L-NAME is added to the synergistic combination of melphalan and TNF (responses increased from 70 to 100%).

In *Chapter 7* gene therapy is introduced in the ILP setting and intratumoural luciferase or LacZ gene expression is compared with other routes of adenoviral vector delivery. Gene delivery using ILP or intratumoural administration results in an efficient gene transfer with a significantly higher mean intratumoural luciferase activity compared to regional or systemic administration. Luciferase gene expression in extratumoural organs lying either outside or within the isolated circuit is minimal after ILP. LacZ expression studies demonstrates that intratumoural administration was confined to tumour cells lying along the needle tract, whereas after ILP gene transfer is found in viable tumour cells as well as in the tumour-associated vasculature.

In *Chapter 8* cytokine gene transfer using the IL-3β gene is studied in the ILP model and compared with intratumoural injections and systemic treatment. A dose dependent tumour growth inhibition is demonstrated after ILP with recombinant adenoviral vectors harbouring the rat IL-3β gene (IG.Ad.CMV.rIL-3β). In contrast, a single intratumoural injection or intravenous administration does not effect tumour growth. Perfusing with a weaker promoter (MLP promoter) driving the rIL-3β gene does not result in antitumour responses, suggesting that the rIL-3β mediated antitumour effect depends on the amount of rIL-3β protein expressed by the infected cells. ILP with IG.Ad.CMV.rIL-3β is at least as efficient as the established therapy with the combination of TNF and melphalan.

Chapter 10

In *Chapter 9* the results of the presented studies are described in a general discussion. Conclusions made on the basis of the studies are:

- The used isolated limb perfusion model demonstrates synergistic antitumour effects of TNF in combination with both melphalan and doxorubicin.
- Both hypoxia and hyperthermia increase TNF and melphalan antitumour responses after ILP.
- Increased drug concentration in tumours after TNF-based ILP provides a straightforward explanation for the observed synergy.
- Antitumour effects of the less toxic TNF-mutant (TNF-SAM2) in combination with melphalan and doxorubicin is comparable to recombinant human TNF.
- NO-inhibition improves antitumour responses after ILP using TNF in combination with melphalan.
- The use of adenoviral vectors in ILP results in effective and safe intratumoral gene expression.
- Cytokine gene therapy using the rIL-3β gene inhibits tumour growth after ILP and not after intratumoural administration.

CHAPTER 10

SAMENVATTING EN CONCLUSIES

Hoofdstuk 10

Met behulp van geïsoleerde extremiteitsperfusies kunnen in tumoren hoge concentraties chemotherapeutica worden bereikt, met tegelijkertijd lage systemische concentraties. Om dit te bewerkstelligen worden de aan- en afvoerende vaten van een extremiteit gecannuleerd en aangesloten op een pompsysteem. Hierna wordt de extremiteit geïsoleerd van de systemische circulatie door het aanleggen van een tourniquet. Patiënten met niet-resectabele weke delen tumoren en locaal gemetastaseerde melanomen worden met deze techniek behandeld. Zeer goede resultaten zijn beschreven met melphalan in combinatie met tumor necrosis factor alpha (TNF). Dit heeft geleid tot het ontwikkelen van een diermodel in ons laboratorium om de mechanismen en effecten van deze therapie te bestuderen.

In *Hoofdstuk 1* worden de verschillende resultaten beschreven die reeds eerder werden gevonden met dit model in de rat. Tevens worden in dit hoofdstuk de doelstellingen van dit proefschrift uiteengezet.

In *Hoofdstuk 2* worden voorwaarden bestudeerd voor een optimale perfusie zoals oxygenatie en temperatuur van het perfusaat, duur van de perfusie en TNF concentratie. De combinatie van 40 µg melphalan en 50 µg TNF resulteert bij 71% van de tumoren in een partiële of volledige respons. Met zowel melphalan als TNF wordt er met niet-geoxygeneerde perfusies een beter antitumor effect verkregen dan met geoxygeneerde perfusies. Na perfusies korter dan 30 minuten of kouder dan 38°C wordt een verminderd antitumor effect waargenomen. Komt de temperatuur van het perfusaat echter boven 42°C dan neemt de toxische schade aan de poot van de rat significant toe. Het synergisme tussen melphalan en TNF wordt nog gezien bij toediening van 10 µg TNF (in 5 ml perfusaat), lagere TNF concentraties resulteren echter niet in synergie met melphalan.

In *Hoofdstuk 3* wordt een significant hogere concentratie van melphalan in tumorweefsel gevonden wanneer TNF wordt toegevoegd aan de perfusies. In huid- en spierweefsel wordt deze stijging van melphalan concentratie wordt niet gevonden, hetgeen suggereert dat TNF vooral een effect heeft op tumorweefsel en nauwelijks op normaal weefsel. De aangetoonde stijging van melphalan is een goede verklaring voor de gevonden synergie tussen melphalan en TNF.

In *Hoofdstuk 4* wordt doxorubicine in combinatie met TNF gebruikt, hetgeen ook resulteert in een synergistisch antitumor effect. Zowel bij BN-175 als bij ROS-1 tumoren wordt respectievelijk in 54 en 100% een respons waargenomen. In beide tumormodellen wordt tevens een verhoogde doxorubicine concentratie in tumorweefsel gevonden wanneer TNF aan de perfusie is

toegevoegd. In vitro heeft TNF geen effect op de doxorubicine opname in de cel of op het antitumor effect. Dit toont aan dat TNF via een indirect verlopend mechanisme het effect van doxorubicine op tumorweefsel versterkt.

In *Hoofdstuk 5* wordt ook synergie in antitumor effect aangetoond tussen een TNF-mutant (TNF-SAM2) en cytostatica (melphalan en doxorubicine) in het perfusiemodel. Het antitumor effect met deze minder toxische TNF variant is histologisch en 'klinisch' vergelijkbaar met de resultaten die zijn beschreven met recombinant humaan TNF.

In *Hoofdstuk 6* worden antitumor effecten van L-NAME, een stikstofoxyde (NO) synthese remmer, bestudeerd. Systemische behandeling van CC-531 nierkapsel tumoren met L-NAME toont een tumorgroei vertraging hetgeen echter gepaard gaat met een verminderde nierfunctie. Met een geïsoleerde extremiteitsperfusie kan een groeivermindering worden aangetoond in BN-175 tumoren in de poot, zonder systemische bijwerkingen. L-NAME in combinatie met TNF en melphalan resulteert in een versterkt antitumor effect (respons percentages respectievelijk van 0 naar 64 en 61). Ook het effect van de combinatie van TNF en melphalan wordt na toevoeging van L-NAME verbeterd (van 70 naar 100%).

In *Hoofdstuk 7* wordt gentherapie toegepast in het perfusiemodel waarbij intratumorale expressie van luciferase en LacZ is vergeleken met andere toedieningsvormen. Zowel na een geïsoleerde extremiteitsperfusie als na intratumorale toediening is er een significant betere genexpressie dan na systemische of regionale (intraarteriële) toediening. Lekkage van adenovirale vectoren is na geïsoleerde extremiteitsperfusie minimaal. Na perfusie wordt een opvallend homogene verdeling van het LacZ gen gevonden in vergelijking met intratumorale toediening, waarbij slechts expressie van LacZ wordt gevonden in het traject van de naald. Tevens wordt na geïsoleerde extremiteitsperfusie expressie van het LacZ gen gevonden in het tumorvaatbed.

In *Hoofdstuk 8* worden tumoren behandeld met cytokine gentherapie. Perfusie, systemische en intratumorale toediening worden vergeleken met betrekking tot het antitumor effect. Er wordt een dosis afhankelijke remming van de tumorgroei aangetoond na geïsoleerde perfusie met het rIL-3β gen. Intratumorale toediening met dezelfde hoeveelheid virus daarentegen heeft geen effect op de tumorgroei. Perfusies met een zwakkere promotor laten geen remming van de tumorgroei zien, hetgeen suggereert dat de rIL-3β genexpressie in de tumor, daadwerkelijk verantwoordelijk is voor de groeiremming. Het antitumor effect na geïsoleerde extremiteitsperfusie met het rIL-3β gen was vergelijkbaar met perfusie met de combinatie van melphalan en TNF.

Hoofdstuk 10

In *Hoofdstuk 9* worden de resultaten van de verschillende studies besproken in de algemene discussie. Conclusies naar aanleiding van dit proefschrift:

- Het geïsoleerde extremiteitsperfusie model in de rat toont synergie in antitumor effect aan tussen TNF en de cytostatica melphalan en doxorubicine.
- Zowel hypoxie als hyperthermie versterken de antitumor effecten van TNF en melphalan.
- Een verhoogde intratumorale concentratie van doxorubicine en melphalan na toevoeging van TNF is een belangrijk mechanisme in de synergie tussen deze stoffen.
- De antitumor effecten van het minder toxische TNF-SAM2 in combinatie met melphalan en doxorubicine zijn vergelijkbaar met recombinant humaan TNF.
- Stikstofoxyde synthese remming door L-NAME versterkt de antitumor werking van TNF en melphalan.
- Gentherapie kan veilig en effectief worden toegepast in een geïsoleerde extremiteitsperfusie.
- Cytokine gentherapie met het rIL-3β gen remt de tumor groei na geïsoleerde extremiteitsperfusie en niet na intratumorale of systemische toediening.

NAWOORD

Nawoord

Vele mensen hebben de afgelopen tijd hun bijdrage geleverd aan de totstandkoming van dit proefschrift. Ik wil een ieder daarvoor hartelijk bedanken, maar een aantal mensen wil ik in het bijzonder noemen.

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CURRICULUM VITAE

De auteur van dit proefschrift werd 31 januari 1967 te Vlaardingen geboren. Het atheneum-B diploma werd in 1985 behaald aan het Groen van Prinsterer Lyceum te Vlaardingen. Hetzelfde jaar begon hij met de studie geneeskunde aan de Erasmus Universiteit te Rotterdam. Het doctoraal examen werd in 1990 behaald en het artsexamen in 1992 (*cum laude*). Tijdens zijn studie deed hij onderzoek op de afdeling algemene Heelkunde in het Academisch Ziekenhuis Dijkzigt te Rotterdam onder leiding van Prof.dr. J. Jeekel. Het afstudeeronderzoek werd verricht op de afdeling Perinatal Research aan de University of Colorado Health Sciences Center te Denver (V.S.) onder leiding van Prof. F.C. Battaglia.

Van november 1992 tot december 1993 vervulde hij zijn dienstplicht als onderdeelsarts van de 43^{e} afdeling veldartillerie te Havelte en als arts-assistent chirurgie in het militair perifeer team te Roosendaal. De opleiding tot algemeen chirurg werd in januari 1994 begonnen in het Zuiderziekenhuis te Rotterdam (opleider: Dr. K.J. Brouwer). In januari 1997 werd de opleiding vervolgd in het Academisch Ziekenhuis Dijkzigt te Rotterdam (opleider: Prof.dr. H.A. Bruining/ Dr. H.J. Bonjer). Van april 1997 tot april 1998 onderbrak hij de opleiding voor één jaar onderzoek in het kader van een KWF arts-assistenten beurs, hetgeen de basis vormde voor dit proefschrift.