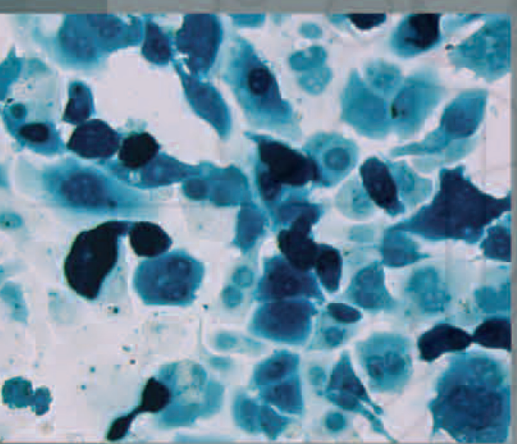
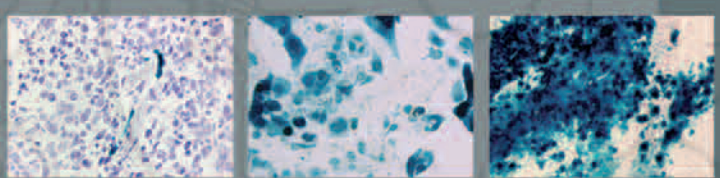
Isolated Liver and Limb Perfusion in Preclinical and Clinical Studies

Gene therapy and biochemotherapeutic strategies





B. van Etten

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Isolated Liver and Limb Perfusion in Preclinical and Clinical Studies

Gene therapy and biochemotherapeutic strategies

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Gentherapie en biochemotherapie strategieën

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I

1

Introduction and Aims of the Thesis.

Liver metastases.

The liver is the major site of metastatic spread of primary colorectal cancer, whereas 3% of all patients with colorectal cancer will develop resectable liver metastases (1). If a resection with curative intent is done, a five year survival rate of 25-30% has been demonstrated in a large number of studies (2-7). The natural history of untreated patients with comparable liver involvement shows a five year survival rate of 0-3% (8). As noted above, the majority of patients with evidence of liver metastases are irresectable, because of extra-hepatic disease or excessive liver involvement (2).

There is no standard treatment for unresectable hepatic metastases confined to the liver, so novel treatment modalities have to be developed. In order to achieve a better control of intrahepatic disease and to reduce systemic toxicity of the applied therapy, locoregional therapies have been developed. These therapies include hepatic arterial embolization (9), intratumoral injections of ethanol (10), acetic acid, biological agents (11,12), stereotactic or intra-arterial radiotherapy, intralesional laser therapy, cryotherapy, radiofrequency ablation (13) and regional infusion or perfusion of chemotherapeutic drugs (14).

Locoregional strategies.

Although response rates with novel systemic chemotherapeutic agents such as oxaliplatin and irinotecan in combination with 5-FU are promising, overall survival remains poor (15-17). In order to improve responses and survival loco-regional chemotherapeutic regiments have been developed such as hepatic artery infusion (HAI), chemoembolization and isolated hepatic perfusion (IHP). For most chemotherapeutic agents a steep dose response curve can be demonstrated and exposure of the liver metastases to higher drug concentrations by means of loco-regional treatment, might result in improved control of hepatic metastases. HAI exploits the first pass effect of the liver, resulting in high local, but low systemic drug exposure. Several repeated hepatic artery infusions regimens produced higher response rates, compared to systemic chemotherapy, with a 2 –year survival of 50-60% (18-23).

Introduction of TNF in isolated perfusion: the beginning of biochemotherapy.

The technique of isolated limb perfusion (ILP) was pioneered in the 1950's by Creech, Krementz and coworkers at Tulane University in New Orleans (24). It is a technique of cancer treatment which delivers high doses of cytostatic drugs to a tumor-bearing extremity that is isolated from the systemic circulation and connected to a heart-lung machine. By ILP regional concentrations of chemotherapeutic agents 15-20 times higher than those reached after

systemic administration can be achieved without systemic toxicity. The use of tumor necrosis factor-α (TNF) in ILP was pioneered by Lejeune et al. in 1992 (25). In patients with in-transit melanoma metastases the addition of TNF and interferon-gamma to Melphalan in ILP resulted in impressive response rates from 80-90%.

These promising results have prompted us to investigate possible application of this combination of agents in isolated perfusion settings of organs such as liver and kidney (26-29). Experience with addition of TNF to IHP with Melphalan, however, is limited. Therefore we developed an IHP rat model to study the applicability and efficacy of TNF in this setting. Hereafter, we investigated if IHP with TNF and Melphalan in rats results in similar synergistic anti-tumor effects between these agents as we previously observed in ILP.

Previous studies led to the hypothesis that the use of TNF in isolated limb perfusion causes specific destruction of tumor endothelial cells and thereby induces an increased permeability of tumor vasculature. However, whether TNF contributes to the therapeutic efficacy in IHP still remains unclear. Based on our findings in the ILP studies, it is indicated to study whether TNF can improve tumor response in different tumors after IHP and, if so, to investigate the capability of TNF to augment drug accumulation in this perfusion setting. By addressing this issue, the usefulness of TNF in IHP might be become clear. Clinical IHP for irresectable metastases is still an experimental and non-curative treatment modality. With respect to this magnitude, costs and non-repeatability of the procedure are its major drawbacks. We developed a less invasive, less costly and potentially repeatable balloon-catheter mediated isolated hypoxic hepatic perfusion (IHHP). A phase I-II study in patients is described in this thesis.

Gene therapy strategies.

Gene therapy is the introduction of foreign DNA into a cell. Agents that facilitate the transfer of genes to recipient cells are called vectors. Much progress has been made on the use of viral vectors for gene transfer. Several viruses can be used for this purpose such as retroviruses, adenoviruses and herpes viruses. In cancer gene therapy the transfer of DNA into the target cell is to eliminate or to reduce tumor burden by direct cell killing, immunomodulation or correcting genetic alterations.

Cancer is believed to be associated with multiple genetic alterations especially involving normal mechanisms that control growth. Colorectal carcinogenesis is well studied, this because most carcinomas arise from adenomas. Above all ras-oncogene mutations occur in an early stage of progression from adenoma to carcinoma. Ras mutations are present in 40 to

50% of human colorectal tumors (30). Inhibition of expression of mutated ras has been shown to cause tumor growth inhibition and apoptosis in human and murine tumor cell lines (31-35). Gene therapy offers possibilities to correct malignant cells by replacement or inhibition of mutated genes. In this thesis adenovirus mediated gene therapy is studied in pre-clinical in vitro and in vivo models. By locoregional administration of the genetic construct, tumor targeting may be improved and consequently result in more favorable transfection efficacy responses. Delivery of viral vectors via intratumoral injections is an established method to reach transduction but its clinical applicability is limited. A transvascular approach could increase the therapeutic window for gene therapy in clinical trials. Previously, we have demonstrated successful transduction and anti-tumor activity in a rat sarcoma model with isolated limb perfusion (36,37). We hypothesized that the route of delivery might play a crucial role in optimizing transduction efficacy and more importantly in anti-tumor activity in liver metastases. A transduction efficacy and tumor response study comparing different routes of administration of an adenoviral vector carrying the DNA for an intracellular antibody against ras. The study explores the pre-requisites for successful liver directed ras-gene therapy. Moreover an additional ras-gene therapy study was performed in an ILP model to evaluate anti-tumor activity of this particular vector on a different tumor and at a different site.

Limb threatening tumors.

In the management of locally advanced soft tissue sarcoma (STS) limb salvage is a major challenge. In contrast to the inefficacy of ILP with Melphalan alone in the management of irresectable extremity STS, with response rates between 10 and 35% only (38,39), the introduction of tumor necrosis factor-alpha (TNF) in combination with Melphalan in ILP has been reported in a European multicenter trial to result in response rates greater than 80% and limb salvage rates above 70% in STS (40,41). These results led to the approval of TNF in Europe (42). Also in the management of in-transit metastases TNF seems to improve CR rates (43). Since the experience with TNF-based ILP is still growing we now are able to analyse subgroups of patients from a single-institution database that is among the largest in the world. This allows us to report on new and unknown possibilities of the clinical utility of TNF-based ILP.

Recurrent melanoma of the limb has been a challenge for treatment over the years. Some patients however, qualify for re-treatment of the limb by ILP as they show to fail only locally without signs of systemic metastases. It is for these patients that repeat ILP might provide

prolonged local control (44-45). The use of TNF in the repeat ILP is thought to improve response rates in patients who do not (or insufficiently) respond to Melphalan-only-ILPs, but definite prove is still lacking (46).

As a consequence of epidemiological features we are confronted with a large group of elderly patients who might need a limb saving procedure such as ILP. Especially these patients will be severely limited in their mobility and loose independence when amputation of a limb has to be performed. Because of fear for TNF associated toxicity, ILP with TNF is not offered to old patients in some cancer centers. Here we report on a series of older patients with limb threatening sarcomas or multiple in-transit melanoma metastases, which demonstrates the safe and efficient use of TNF -based ILP.

Aims of the Thesis.

As described in the Introduction section several studies were performed in isolated liver and limb perfusion models in a pre-clinical and clinical setting.

In summary the Aims of the studies described in this Thesis are:

- The development of a pre-clinical IHP model in which the combination of TNF and Melphalan could be tested. Feasibility, efficacy and drug uptake studies were performed.
- To determine the utility of recombinant adenovirus gene therapy in experimental liver metastases models. Does loco-regional transvascular delivery offer therapeutic possibilities? What are the immunological effects?
- To study efficacy of recombinant adenovirus gene therapy in tumor-bearing rats by administration via ILP.
- To address new clinical options in the treatment of limb threatening soft tissue sarcoma or melanoma metastases in selective patient groups.
- To evaluate a new less invasive IHP technique using balloon catheters in a prospective phase I-II study.

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II

Pre-clinical biochemotherapeutic studies.

2

TNFα augments anti-tumor efficacy in isolated hepatic perfusion with Melphalan in a rat sarcoma model.

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Summary

Isolated hepatic perfusion (IHP) is an attractive approach towards treating irresectable liver tumors as the effects of systemic chemotherapy are poor and its application is hampered by severe general toxicity. In clinical and experimental settings the efficacy of isolated limb perfusion (ILP) with tumor necrosis factor-alpha (TNF) in combination with Melphalan for treatment of melanoma in-transit and soft tissue sarcoma has now been well established. In an ILP model in rats we previously observed synergistic anti-tumor effects of TNF and Melphalan on BN 175 soft tissue sarcoma extremity tumors. The aim of the present study was to investigate if similar synergy in anti-tumor effect could be achieved by treating experimental BN 175 soft tissue sarcoma liver tumors by IHP with these agents.

We demonstrated that IHP with TNF and Melphalan results in a dramatic increase in regional concentrations of perfused agents with virtually no concomitant systemic leakage. IHP with only carrier solution resulted in a significantly diminished growth rate of BN 175 liver tumors compared to the growth rate of tumors in non-perfused rats. Perfusing with Melphalan alone resulted in minimal anti-tumor effects. Perfusion with only TNF had a slight growth stimulatory effect on the BN 175 liver tumors, in any case no negative effect on tumor growth was observed. When TNF was added to Melphalan a dramatic anti-tumor effect was observed. Thus, like in the rat ILP setting, augmentation of anti-tumor effect is observed when adding TNF to IHP with Melphalan for treatment of BN 175 soft tissue sarcoma tumor-bearing rats. Strikingly, potentiation of tumor response occurred at relatively low concentrations of TNF compared to concentrations eliciting synergy with Melphalan in ILP.

Introduction

The clinical success of addition of high dose tumor necrosis factor-alpha (TNF) to isolated limb perfusion (ILP) with Melphalan has renewed the interest in this cytokine as an anti-tumor agent. Systemic administration of TNF in effective doses is restricted by severe general toxicity (1,2). ILP, however, allows addition of high dose TNF in combination with Melphalan for treatment of melanoma in-transit-metastases and soft tissue sarcoma and has resulted in tumor responses in over 80 % of cases (3,4,5). These promising results have prompted us to investigate possible application of this combination of agents in isolated perfusion settings of organs such as liver and kidney (6,7,8,9).

In clinical and experimental settings IHP with high dose Melphalan has resulted in significant

anti-tumor effects (10,11,12). Clinical experience with addition of TNF to IHP with Melphalan, however, is limited (7,13,14,15,16). Although first reports regarding tumor responses have been promising, it is clear that, as the liver is a vital organ and much more responsive to TNF, more than is the case in ILP, in IHP regional toxicity will dictate if adequate dosages of TNF can be administered.

In order to gain insight into the mechanisms by which TNF elicits its anti-tumor effects and for investigating by which agents and manipulations TNF efficacy can be enhanced and it's toxicity reduced, a pre-clinical model IHP is essential. Therefore we developed an IHP rat model using the highly vascularized BN 175 soft tissue sarcoma to study the applicability and efficacy of TNF in this setting. This tumor was chosen because it previously demonstrated synergistic anti-tumor effects between TNF and Melphalan in a rat ILP model, closely mimicking clinical observations regarding tumor responses and histopathology (17,18,19).

In this study we first defined the IHP rat model regarding degree of isolation of the hepatic vascular compartment during perfusion and characterized the effect of the IHP procedure itself on growth of experimental BN 175 liver tumors. Hereafter, we investigated if IHP with TNF and Melphalan in rats results in similar synergistic anti-tumor effects between these agents as we previously observed in ILP when treating BN 175 soft tissue sarcoma liver tumors.

Materials and Methods

Animals

Male inbred BN strain rats, weighing 250-300 g obtained from Harlan-CPB (Austerlitz, The Netherlands) were used. The rats were fed a standard laboratory diet. All animals were housed under standard conditions of light and accommodation. The experimental protocols adhered to the rules outlined in the Dutch Animal Experimentation Act 1977 and the published Guidelines on the Protection of Experimental Animals by the Council of the European Community 1986. The protocol was approved by the committee on animal research of the Erasmus University, Rotterdam, the Netherlands.

Tumor model

BN 175 soft tissue sarcoma (transplantable to BN rats) was used. BN 175 is a rapidly growing and metastasizing tumor and is highly vascularized. The tumor is non-immunogenic and can be maintained in tissue culture. From culture new tumors were produced by subcutaneous

inoculation in the flank of rats. Tumors were subsequentially passaged serially.

Small viable tumor fragments of 1-2 mm were implanted under the liver capsule in the left liver lobe with a 19 G Luer lock needle in a standardized manner. IHP was performed 6 days after implantation of BN 175 soft tissue sarcoma at which time the tumors had reached a diameter of approximately 6 mm. During follow-up tumor diameters were assessed through a small midline incission by calliper measurement. Tumor volume was calculated by using the equation 0.4 (A^2xB) where B is the largest tumor diameter measured and A the diameter perpendicular to A. Animals were sacrificed when tumor diameter exceeded 20 mm or when abdominal adhesions made further assessment of tumor size impossible.

Drugs

Melphalan (Alkeran, 50 mg per vial, Wellcome, Beckenham, UK) was diluted in 10 ml diluent solvent. Further dilutions were made in 0.9% NaCl to give a concentration of 0.2 mg/ml and stored at -20 °C for further use.

Recombinant human TNF was provided by Boehringer (Ingelheim, Germany) with a specific activity of $5.8x10^7$ U/mg as determined in the murine L-M cell assay (20). Endotoxin levels were <1.25 EU/mg protein.

Perfusion system

The perfusion circuit consisted of arterial and portal inflow limbs, a venous outflow limb and a collection reservoir/oxygenator. The circuit was primed with 30 ml Haemaccel (Behring Pharma, Amsterdam, Netherlands) containing 50 IU of Heparine. The perfusate was oxygenized in the reservoir with a mixture of O_2/CO_2 (95%:5%) and kept at 38-39 °C by means of a heat exchanger connected to a warm water bath. A temperature probe was positioned in the lumen of the portal catheter 5 cm from the catheter tip. Arterial and portal flow was maintained with two low-flow roller pumps (Watson Marlow type 505 U, Falmouth, UK) and kept at 2.5 ml/min and 10 ml/min respectively. Rats were perfused for 10 minutes with Haemaccel and dissolved agents, whereafter, a wash out of agents was performed with oxygenized Haemaccel for 2 minutes.

Surgical Procedure

The procedure was a modification of the IHP technique described by de Brauw et al (21). Anesthesia was induced and maintained with ether. During the surgical procedure, which on average took 60-80 minutes, rats were kept at constant temperature with a warmed mattress.

A mid-line laparotomy was performed and the hepatic ligament exposed. The pyloric side branch of the portal vein and the gastroduodenal side branch of the common hepatic artery were cannulated positioning the tips of the silastic cannulas (0.025 inch outer diameter (OD), 0.012 inch inner diameter (ID) (Dow Corning, Michigan, USA)) in the hepatic artery and portal vein. Through an inguinal incission the femoral vein was exposed. To collect hepatic venous outflow a silicon cannule (0.025 inch ID and 0.047 OD (Dow Corning, Michigan, USA)) was femorally introduced and retrogradely inserted in the caval vein with the tip positioned at the level of the hepatic veins.

Isolation of the hepatic vascular bed was obtained by clamping the hepatic artery and the portal vein. The venous outflow limb was isolated by clamping the supra hepatic caval vein and by applying a temporary ligature around the infra-hepatic caval vein containing the canule, cranial to the right adrenal vein. During isolation the mesenteric artery was clamped to reduce splanchnic bloodpressure and the risk for translocation of intestinal bacteria.

After the IHP procedure clamps on caval vein, portal vein, hepatic artery and mesenteric artery were released. Gastroduodenal artery, pyloric vein and femoral vein were ligated and gastroduodenal and pyloric canules removed.

Assessment of TNF concentrations in perfusate and systemic blood compartment during IHP

In order to validate the leakage free quality of the IHP model, TNF concentrations in regional and systemic blood compartments throughout and after perfusion were determined. Hereto three BN-strain rats underwent IHP with 20 µg TNF and 200 µg Melphalan added to the perfusate. Samples were obtained from the perfusate at 0, 5 and 10 minutes after start of perfusion and drawn from the iliac artery at 0, 5, 10, 12.5, and 15 minutes after start of perfusion. Samples were centrifuged at 2600 r.p.m. for 6 minutes whereafter the obtained plasma/carrier solution was stored at -70 °C until analysis. Plasma and perfusate TNF concentrations were determined using ELISA for rhTNF as described by Engelberts et al (22). The detection limit for human TNF was 20 pg/ml.

Treatment schedule

BN strain rats underwent IHP six days after implantation of the tumor providing tumor diameter was approximately 6 mm. Five rats served as untreated controls. Rats were perfused in random order with 200 µg Melphalan (n=7), 20 µg TNF (n=8), a combination of 20 µg TNF and 200 µg Melphalan (n=8), or underwent a sham perfusion with Haemaccel (n=5).

The administered Melphalan dose was extrapolated from effective dosages in ILP with TNF and Melphalan. The administered TNF dose was the maximum tolerated dose TNF for IHP in BN rats.

Statistics

Differences in mean tumor volumes of treatment groups at day 10 after IHP were evaluated for statistical significance with the Mann-Whitney U-test using SPSS® for Windows™ software. P-values < 0,05 were considered as significant.

Results

Over 85 % of IHP's were technically succesfull. All succesfully perfused animals survived after the IHP procedures until they had to be sacrificed due to tumor size or abdominal adhesions. IHP with carrier solution was well taken by perfused rats and had no apparent effect on their weights. Perfusing with either Melphalan or TNF alone or with a combination of both agents in some cases resulted in transient weight reduction or stagnation. However, weight reduction was never more than 10% *(data not shown).*

Distribution of TNF during IHP over hepatic and systemic blood compartments

In order to assess the leakage quality of this IHP rat model TNF plasma concentrations in the regional and systemic compartment during IHP were determined. Figure 1 shows the mean regional and systemic TNF concentrations during and after IHP of three rats. Throughout isolation TNF concentrations in the perfusate remained stable at approximately 550 ng/ml. Virtually no concomitant leakage of TNF to the systemic compartment was observed. The efficacy of the washout procedure was apparent from the fact that after isolation was terminated only a minor transient elevation of systemic TNF levels was observed (max 60 ng/ml).

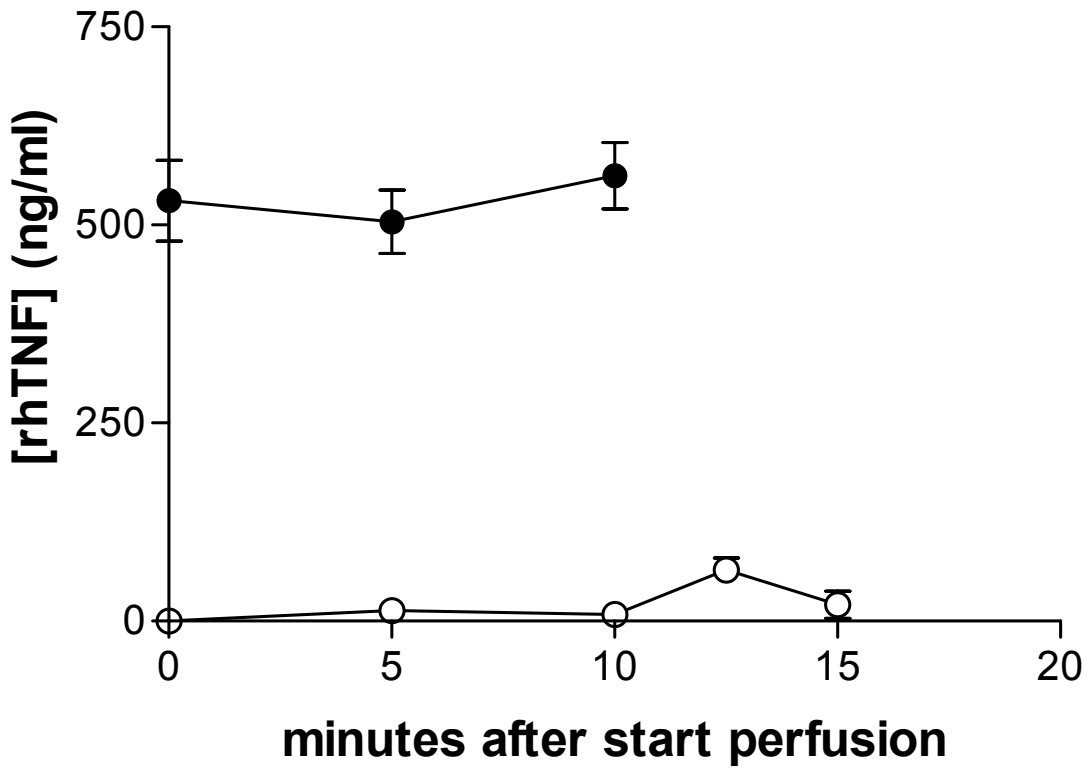

Fig.1 TNF plasma and perfusate concentrations in regional (●) and systemic (○) blood compartments during and after IHP with 20 µg TNF and 200 µg Melphalan in rats. Rats were perfused for 10 minutes whereafter during two minutes agents were washed out with carrier solution. Mean values are given +/- SEM (n = 3).

Tumor response after IHP

In figure 2 the growth curve of BN 175 soft tissue sarcoma in non perfused animals is compared with the growth curve of tumors in animals having undergone a sham perfusion with carrier solution. Strikingly, IHP with only carrier solution resulted in a significantly diminished growth rate of tumors compared to tumors in non-perfused animals.

Figure 2 also demonstrates the effect of IHP with various agents on tumor volume. IHP with 200 µg Melphalan (n=7) significantly reduced tumor growth rates compared to sham treated animals (n=5) ($p<0.02$), but failed to induce a reduction of mean tumor volume as all rats demonstrated progressive tumor growth. Perfusing with 20 µg/ml TNF alone (n=8) resulted in a slight growth stimulatory effect on BN 175 liver tumors, as compared to tumors in sham treated rats ($p<0.01$). No negative effect on tumor growth was observed in any animal. When IHP was performed with a combination of 20 µg TNF and 200 µg Melphalan (n=8) a dramatically enhanced tumor response was observed in all animals, resulting in a significant reduction of mean tumor volume compared to mean tumor volumes in rats perfused with either TNF or Melphalan alone ($p<0.005$ and $p<0.01$ respectively). At day 10 after IHP mean tumor volume was reduced by more than 50 % of its value before IHP. No animals

demonstrated tumor progression, while 4/8 animals demonstrated a reduction in tumor volume of more than 90 %. At day 14 3/8 animals demonstrated progression of tumor growth, while other animals showed a decline in tumor volume. Five of eight animals treated with TNF and Melphalan were sacrificed due to multiple abdominal adhesions. The remaining animals all demonstrated regrowth of tumors when followed up for a longer period of time *(data not shown).*

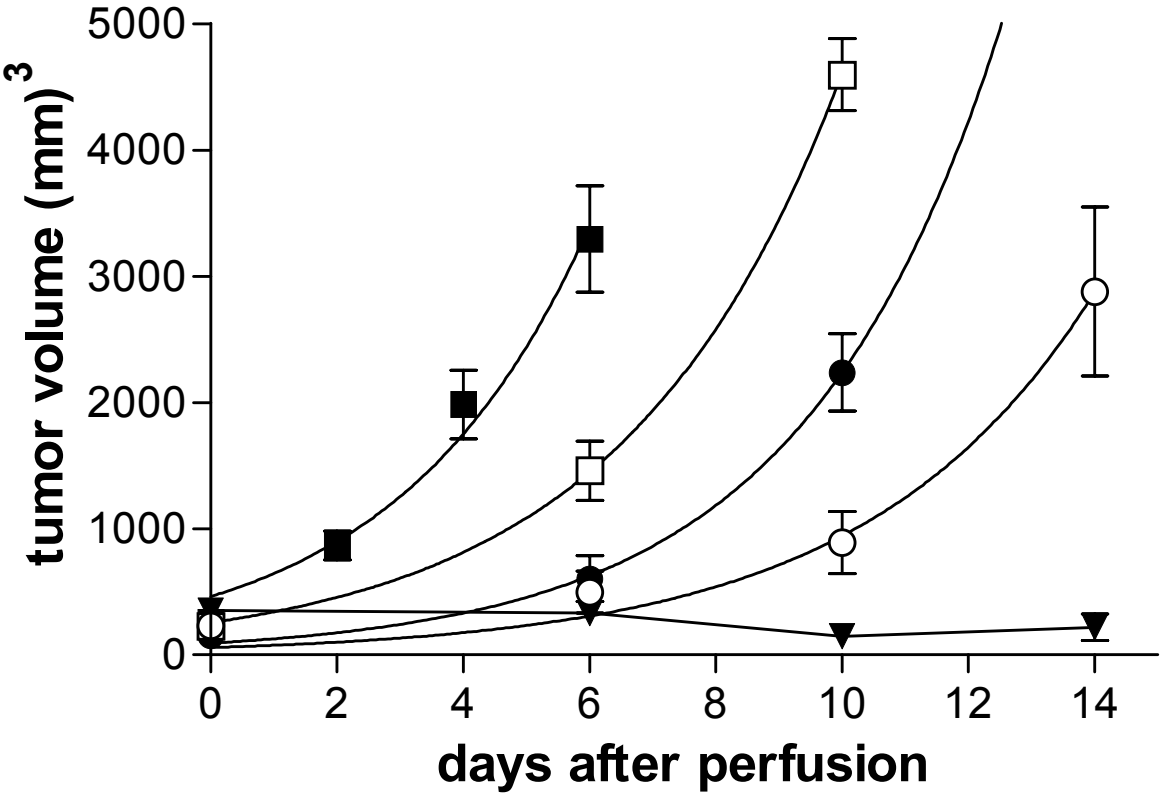

Fig.3 Mean tumor volumes of BN 175 soft tissue sarcoma liver tumors in rats after isolated hepatic perfusion with TNF and/or Melphalan and in non-perfused control rats (■, n = 5). Rats were perfused with 20 µg TNF (□, n = 8), 200 µg Melphalan (○, n=7), or a combination of 20 µg TNF and 200 µg Melphalan (▼, n = 8) or they underwent a sham perfusion (●, n = 5). Mean tumor volumes are given +/- SEM.

Discussion

In the present study we demonstrated a leakage free IHP rat model. We observed that IHP without addition of anti-tumor agents had an anti tumor growth effect in BN 175 soft tissue sarcoma bearing rats. When performing IHP with either TNF or Melphalan alone no tumor responses were observed. However, perfusing with a combination of TNF and Melphalan resulted in dramatically enhanced anti-tumor effects. Strikingly, this potentiation by TNF was observed at lower TNF concentrations than those that are necessary to elicit synergistic anti-

tumor effects between these agents in ILP when treating BN 175 soft tissue sarcoma extremity tumors.

Regional chemotherapy of liver tumors yields higher response rates than systemic therapy (23,24,25). Following the success of addition of TNF to ILP with Melphalan for the treatment of soft tissue sarcoma of the extremities (3,4), we set ourselves to investigate application of this cytokine in regional chemotherapy settings of the liver.

Hepatic artery infusion (HAI) exploits the high tissue extraction ratios that many chemotherapeutic agents demonstrate, leading to higher tumor concentrations and less systemic side effects (26,27). However, for an agent like TNF, which at high concentrations demonstrates relatively little hepatic uptake (8), subsequent systemic exposure is potentially fatal. In addition to HAI, IHP has been developed as a treatment modality that not only maximizes drug concentrations in the target organ, but at the same time also shields the organism from systemic toxicity (28,29,30). While TNF can be used in very high doses in the clinical ILP setting, theoretically similar doses could be used in IHP, as in both cases a complete wash out of agents can be achieved, thus limiting systemic exposure. Furthermore, temporary isolation of the hepatic vascular bed during IHP allows for the modulation of perfusate parameters like temperature, oxygenation and pH, thus creating optimal conditions for the anti-tumor agents to have an effect (19). Previously, it was demonstrated that IHP in rats leads to significantly higher tumor concentrations of Melphalan compared to HAI (12). In this study we perfused over both arterial and portal hepatic inflow limbs. Although hepatic metastases mainly derive their blood supply from the hepatic artery, connections with the portal circulation do exist at the periphery of the tumor (31). It seems rational therefore to perfuse both hepatic circulations if one aims its therapy at the viable, vascularized rim of the tumor. Additionally, in the clinical situation non-vascularized ‘micro-metastases’ can be expected to totally depend on the portal circulation.

TNF *in vivo* has the ability to induce tumor necrosis with acute softening of the tumor, believed to be brought about by a selective destruction of the tumor micro-vasculature, causing acute hemorrhagic necrosis of the tumor (3,18,32,33). This effect seems to be dose dependent as low dose TNF has been reported to have an angioproliferative effect, whereas higher concentrations cause destruction of newly formed vessels (34). Similar to clinical results, ILP in rats with high dose Melphalan and TNF yields tumor responses of BN 175 soft tissue sarcoma extremity tumors in over 80% of animals treated (17,19). These are believed to be predominantly indirect effects as previously, in *in vitro* studies, no direct cytotoxic effect and no synergistic potentiation by TNF of Melphalan were found using the BN 175 tumor

cells (17).

High concentrations TNF are necessary only for a short period as in clinical ILP an immediate increase in vascular permeability was observed resulting in an increased accumulation of the drugs (35). Likewise, experimental ILP in rats with TNF in combination with Melphalan or Doxorubicin clearly resulted in an enhanced accumulation of these agents in BN 175 soft tissue sarcoma extremity tumors, corresponding with observed augmented tumor responses (36,37). Having observed similar augmentation of anti-tumor effect on BN 175 soft tissue sarcoma after addition of TNF to Melphalan in IHP as in ILP one could postulate that endothelium mediated TNF effects are responsible in both settings.

A recent report by Alexander et al (38), however, questions the role of TNF in enhancing capillary endothelial permeability in tumor associated vasculature in hyperthermic IHP, as addition of TNF did not effect melphalan concentrations in liver tumors. Apart from the possibility that the hyperthermia related capillary leakage may have 'masked' a TNF effect, these contradicting observations may reflect the importance of tumor characteristics, like for instance the degree of tumor vascularization, in determining if TNF mediated effects can come about. Kuppen et al (39) demonstrated that although IHP in patients resulted in relatively high levels of TNF in liver tissue compared to systemic administration, TNF did not accumulate preferentially in the tissue of colorectal metastases. Interestingly, in a recent clinical IHP study using TNF and Melphalan markedly better results were observed in tumors of mesenchymal origin compared to colorectal metastases (16).

It is clear that isolated perfusion of organs is accompanied by a higher risk of regional toxic side effects than ILP. In IHP liver toxicity rather than systemic toxicity is dose-limiting (12,21). This has been shown to be particularly the case when perfusing with TNF (7,13,14). In an isolated kidney perfusion model in the rat we previously found that the maximum tolerated dose of TNF (0.2 μg/ml) was ineffective, which clearly demonstrated the restriction of TNF dose in perfusion settings due to local toxicity (9). The current IHP model, however, seems to allow above threshold levels of TNF regionally to elicit augmentation of anti-tumor effects.

In our experiments effective treatment of BN 175 liver tumors with IHP was achieved at TNF concentrations (550 ng/ml) approximately four times lower than the minimal required dose for treating this tumor in ILP (2 μg/ml) (19). Possibly, the IHP procedure itself, also contributes to the anti-tumor effects observed. This could not only explain the observed reduction in tumor growth in sham treated rats, but may also be in accordance with reports of tumor responses after IHP despite low tumor concentrations of perfused agents (11,39).

Explanations could be the secondary release of cytokines by the liver which is associated with IHP (40,41,42) or the mild hyperthermia of the perfusate as hyperthermic IHP by itself has been shown to have a tumoricidal effect (43). Other mechanisms could be (temporary) relative ischaemia during the procedure or transient changes in local hemodynamics as a result of IHP.

With the dawning of minimally invasive IHP techniques (8,44), the reduction in procedure associated risks enhances the clinical application potential of TNF in this setting. An efficient pre-clinical IHP model is extremely useful to further investigate by what means the agents-associated risks can be reduced and their efficacy improved. Next to investigating the possibility of dose reduction, it may be of use in investigating the application potential of TNF-mutants, which have shown to be less toxic than wild type TNF, but at the same time have retained their anti-tumor effects (45). An IHP model may also be of use in addressing questions concerning the concomitant systemic administration of agents during IHP that reduce the toxic side effects of TNF (46,47).

In this study we have shown that, like in ILP, addition of TNF to IHP with Melphalan for treatment of hepatic soft tissue sarcoma results in augmentation of anti tumor effects. Thus, as is the case for the ILP setting, we have a pre-clinical model rat model to study pre-requisites for optimal IHP with TNF and Melphalan.

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3

Degree of tumor vascularity correlates with drug accumulation and tumor response upon TNF-α based isolated hepatic perfusion.

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Summary

Isolated hepatic perfusion (IHP) with Melphalan with or without TNF is currently performed in clinical trials in patients with hepatic metastases. Previous studies led to the hypothesis that the use of TNF in isolated limb perfusion causes specific destruction of tumor endothelial cells and thereby induces an increased permeability of tumor vasculature. However, whether TNF contributes to the therapeutic efficacy in IHP still remains unclear. In an *in vivo* rat liver metastases model we studied three different tumors: colon carcinoma CC531, ROS-1 osteosarcoma and BN-175 soft tissue sarcoma which exhibit different degrees of vascularization. IHP was performed with Melphalan with or without addition of TNF. IHP with Melphalan alone resulted in all tumor types in a decreased growth rate. However in the BN-175 tumor addition of TNF resulted in a strong synergistic effect. In the majority of the BN-175 tumor bearing rats a complete response was achieved. *In vitro* cytoxicity studies showed no sensitivity (CC531 and BN-175) or only minor sensitivity (ROS-1) to TNF, ruling out a direct interaction of TNF with tumor cells. The response rate in BN-175 tumor bearing rats when TNF was co-administrated with Melphalan, was strongly correlated with drug accumulation in tumor tissue, as only in these rats a 5-fold increased Melphalan concentration was observed. Secondly, immunohistochemical analysis of microvascular density (MVD) of the tumor showed a significantly higher MVD for BN-175 tumor compared to CC531 and ROS-1. These results indicate a direct relationship between vascularity of the tumor and TNF-mediated effects. Assessment of the tumor vasculature of liver metastases would be a way of establishing an indication for the utility of TNF in this setting.

Introduction

Tumor necrosis factor alpha (TNF) is a cytokine with an interesting potential in the treatment of cancer (1). When administered systemically it is accompanied with severe toxicity, however especially when TNF in combination with chemotherapy is used locoregionally without systemic exposure it has very potent antitumor effects. Clinical trials of isolated limb perfusion (ILP) with recombinant human tumor necrosis factor alpha (TNF) and Melphalan resulted in high complete response rates of 75-90 % in patients with in transit melanoma and unresectable sarcoma of the extremities (2-5). This is in contrast to ILP with Melphalan alone

which is relatively effective against small in transit melanoma metastases (6) but achieves very poor results against large tumors such as soft tissue sarcomas (7-9).

In order to elucidate the mechanism of TNF several studies have been performed. In our pre-clinical ILP model we observed drastic alterations in tumor microvasculature integrity (10). Rüegg et al. demonstrated elegantly that TNF in combination with IFN-γ induced functional down regulation of αvβ3, resulting in detachment of the endothelial cells of the tumor vasculature (11). Moreover, angiographic studies performed in patients pre and post TNF perfusion showed selective destruction of tumor associated vasculature and histologic studies demonstrated hemorrhagic necrosis of the tumor (12). Recently we demonstrated, what we consider a key explanation for the potent synergy between TNF and chemotherapy, an up to six-fold increased intratumoral Melphalan or doxorubicin concentration in rat sarcomas after ILP when high dose TNF was co-administrated (13,14). These findings led to the hypothesis that TNF causes specific destruction of tumor endothelial cells and thereby induces an increased permeability of tumor vasculature.

As a result of the favourable experience with the ILP system, other isolated perfusion settings have been developed (15,16). Especially the liver offers superb opportunities for isolated perfusion. Irresectable liver metastases are a significant clinical problem. Isolated hepatic perfusion (IHP) with Melphalan with or without TNF is technically feasible and is currently performed in clinical trials in patients with hepatic metastases (17,18). Whether TNF contributes to the therapeutic efficacy in IHP still remains unclear.

Based on our findings in the ILP studies, it is indicated to study whether TNF can improve tumor response in different tumors after IHP and, if so, to investigate the capability of TNF to augment drug accumulation in this perfusion setting. By addressing this issue, the usefulness of TNF in IHP might be become clear. Since the tumor associated vasculature is the target of TNF, we expect that tumor microvessel density (MVD) is a predictor of the potentiating effect of TNF during isolated perfusions. Here we present data that indicate that the anti-tumor effect of TNF is correlated with the tumor microvessel density.

Materials and Methods

Rat Liver Metastases Model

We used male inbred WAG/RIJ or Brown-Norway (BN) strain rats, weighing 250-300 g, obtained from Harlan-CPB (Austerlitz, The Netherlands). The rats were fed a standard

laboratory diet. All animals were housed under standard conditions of light and accommodation. The protocol was approved by the committee for animal research of the Erasmus University, Rotterdam, the Netherlands.The experimental protocols adhered to the rules outlined in the Dutch Animal Experimentation Act of 1977 and the published Guidelines of the UKCCCR for the Welfare of Animals in Experimental Neoplasia (UKCCCR, 1998).
Three different tumors were used in this study. The weakly immunogenic colon carcinoma CC531 is an 1,2-dimethylhydrazine-induced, moderately differentiated adenocarcinoma transplantable in syngeneic WAG/RIJ rats. The estimated doubling in vivo is about 6-8 days. The spontaneously originated nonimmunogenic osteosarcoma ROS-1 is also transplantable in the WAG-RIJ rat and in the liver metastases model it has a mean doubling time of about 4-5 days. The spontaneously originated nonimmunogenic soft tissue sarcoma BN-175 is the fastest growing tumor of the tumors tested, with an estimated doubling time in vivo of about 2-3 days and is transplantable in syngeneic BN rats. Following a standardized protocol, small viable tumor fragments of CC531, ROS-1 or BN-175 tumor fragments of 1 by 2 mm were implanted under the liver capsule, one in the left and one in the right side of the left liver lobe, using a 19 G Luerlock needle. Experiments started at a fixed tumor diameter between 5 and 6 mm. When tumors reached a size of 20 mm in diameter or animals showed obvious signs of discomfort the animals were sacrificed.

Drugs

Recombinant human TNF-alpha (TNF, 4.9-5.8 x 10^7 units/mg) was provided as a kind gift by Boehringer Ingelheim GmbH, Ingelheim/Rhein, Germany. Melphalan (L-pam, Alkeran, Wellcome Ltd., London, United Kingdom) was obtained as a sterile powder (100 mg) that was dissolved aseptically using solvent and diluent provided by Burroughs Wellcome (London, United Kingdom).

Isolated Hepatic Perfusion

This rat isolated liver perfusion model has been described in detail earlier by van IJken et al (15). A schematic representation is shown in Figure 1. Anaesthesia was induced and maintained with ether (Merck, Darmstadt, Germany). During the surgical procedure, with an average duration of 60-75 minutes, rats were kept at a constant temperature using a warmed mattress. A mid-line laparotomy was performed and the hepatic ligament exposed. The gastroduodenal side branch of the common hepatic artery was cannulated, positioning the tips of the cannula (0.025 outer diameter (OD), 0.012 inch inner diameter (ID), (Dow Corning,

Michigan, USA)) in the proper hepatic artery. Through a small inguinal incision the femoral vein was exposed. To collect hepatic venous outflow a silicon cannula (0.047 OD, 0.025 inch ID), (Dow Corning, Michigan, USA) was introduced in the femoral vein and moved up into the caval vein positioning the tip of the cannula at the level of the hepatic veins.

Isolation of the hepatic vascular bed was obtained by temporarily ligating the common hepatic artery and the portal vein. The venous outflow limb was isolated by temporarily clamping the supra hepatic caval vein and by applying a temporary ligature around the infra-hepatic caval vein containing the cannule, cranial to the right adrenal vein. The mesenteric artery was temporarily clamped in order to reduce splanchnic blood pressure. The circuit was primed with 10 ml Haemaccel (Behring Pharma, Amsterdam, Netherlands). Arterial flow of 5 ml/min was maintained with a low-flow roller pump (Watson Marlow type 505 U, Falmouth, UK). Rats were perfused for ten minutes with oxygenated Haemaccel in which Melphalan and/or TNF was dissolved. Afterwards a washout was performed by perfusing with 10 ml of oxygenated Haemaccel. 50 IU of Heparin (Heparine Leo, The Netherlands) was added to the perfusate. The perfusate was oxygenated in a reservoir with a mixture of O_2/CO_2 (95%:5%) and was kept at 38-39 °C by means of a heat exchanger and a warm water bath. A temperature probe was positioned in the lumen of the arterial catheter, 5 cm from the catheter tip. Following the washout-procedure, the clamps on caval vein, portal vein, hepatic artery and mesenteric artery were released. The gastroduodenal artery and femoral vein were ligated and the gastroduodenal and femoral cannulas were removed.

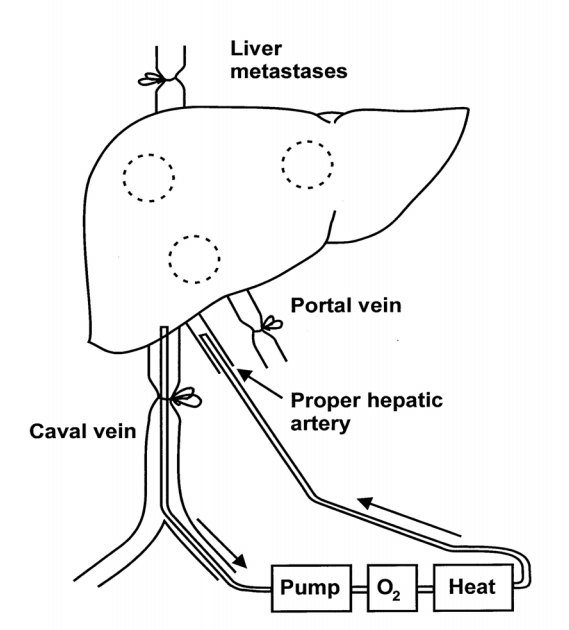

Fig. 1 Schematic representation of an Isolated Hepatic Perfusion

In Vivo Anti-tumor Efficacy Study

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

In Vitro Cytotoxicity Assay

CC531 and BN-175 cells were grown in RPMI 1640 and ROS-1 cells in modified Eagle's medium (Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum (Harlan/Sera-Lab, UK), 1% penicillin (5000 IU/ml), 1% streptomycin (5000 IU/ml) and 1% L-glutamine (200mM) (all Gibco BRL) in a humidified incubator at 37 °C and 5% CO_2. Before usage, the cells were trypsinised (1 min, 37 °C), centrifuged (5 min, 700 g), resuspended and the viability measured by trypan blue exclusion. For *in vitro* testing of proliferation inhibition, 1.0 x 10^4 viable cells were seeded in flat bottomed 96-well microtiter plates (Costar, USA). After 24 hours the cells were incubated with different concentrations of TNF for 72 hours ranging from 0 to 10 μg/ml . Afterwards, cells were washed with PBS and fixed for 1 hour with 10% trichloro-acetic acid at 4 °C. Growth of tumor cells was measured using the sulpharhodamine-B assay according to the method of Skehan et al (19). Tumor cell proliferation was measured using the formula: tumor growth = (test well/control) x 100%. Five independent tests were performed for each point on the line.

Measurement of Melphalan in Tissue

Five minutes after the restoration of the circulation the perfused tumor and part of the liver were excised. The tissues were immediately frozen in liquid nitrogen to stop metabolism of

Melphalan and stored at –80°C. Tumor and liver tissues were homogenized in 2 ml acetonitrile (Pro 200 homogenizer, Pro scientific, CT, USA) and centrifuged at 2500g. Melphalan was measured in the supernatant by gas chromatography-mass spectrometry (GC-MS). P-[Bis(2-chloroethyl)amino]-phenylacetic acid methyl ester was used as an internal standard. Samples were extracted over 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 described earlier by Tjaden et al (20).

Assessment of Tumor Microvessel Density by Immunohistochemistry

Cryosections of tumors were fixed for 15 minutes with 4% formaldehyde. After rinsing with PBS, sections were incubated for 1 hour with 1:10 PBS diluted, mouse-anti-rat-endothelial cell antibody (RECA-1, Instruchemie, Hilversum, The Netherlands). For the negative control an aspecific mouse IgG was used (SantaCruz Biotechnology, Santa Cruz, California, USA). Thereafter sections were rinsed with PBS and incubated for 1 hour with 1:100 diluted, in 5% normal rat serum in PBS, goat-anti-mouse peroxidase labeled antibody (DAKO, Carpinteria, CA, USA). After rinsing with PBS, positive cells were revealed by immunoperoxidase reaction with DAB solution (DAB-kit, DAKO) and counterstained with hematoxylin. For microvessel quantification two independent persons performed a blinded analysis. Positive cells were counted in 3 different high power fields (magnification 160x) in each slide according to the method of Bosari et al (21). In total 3 slides per tumor and 3 tumors per tumor type were evaluated.

Statistical Analysis

In vitro bioassays and in vivo tumor response results were evaluated for statistical significance with the Mann-Whitney-U tests with SPSS8.0 for Windows 98. Mann Whitney U test was used to compare Melphalan concentrations in different groups and Kruskal-Wallis test to compare number of positive cells in different tumors. A significance level of $p<0.05$ was used in all analyses.

Results

Tumor Response after Isolated Hepatic Perfusion

The anti-tumor efficacy of IHP with Melphalan with or without TNF was evaluated for the CC531, ROS-1 and BN-175 tumor starting at an equal size of 5-6 mm in diameter. In all groups sham IHP's with only perfusion medium were performed. The graphs in Figure 2 show the growth curves of CC531 tumor (A) , ROS-1 (B) and BN-175 (C) after IHP with Melphalan, TNF, both, or after sham perfused rats. Perfusion with Melphalan alone significantly reduced tumor growth rates compared with sham perfused animals in all tumor types. When IHP was performed in BN-175 bearing rats with the combination of Melphalan and TNF a dramatically enhanced tumor response was observed in all animals. This is a significant reduction of mean tumor volume compared with rats perfused with either TNF only or Melphalan alone ($p<0.005$ and $p<0.01$ respectively). In the CC531 or ROS-1 tumors this effect was not observed.

In Vitro Cytotoxicity Assay

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

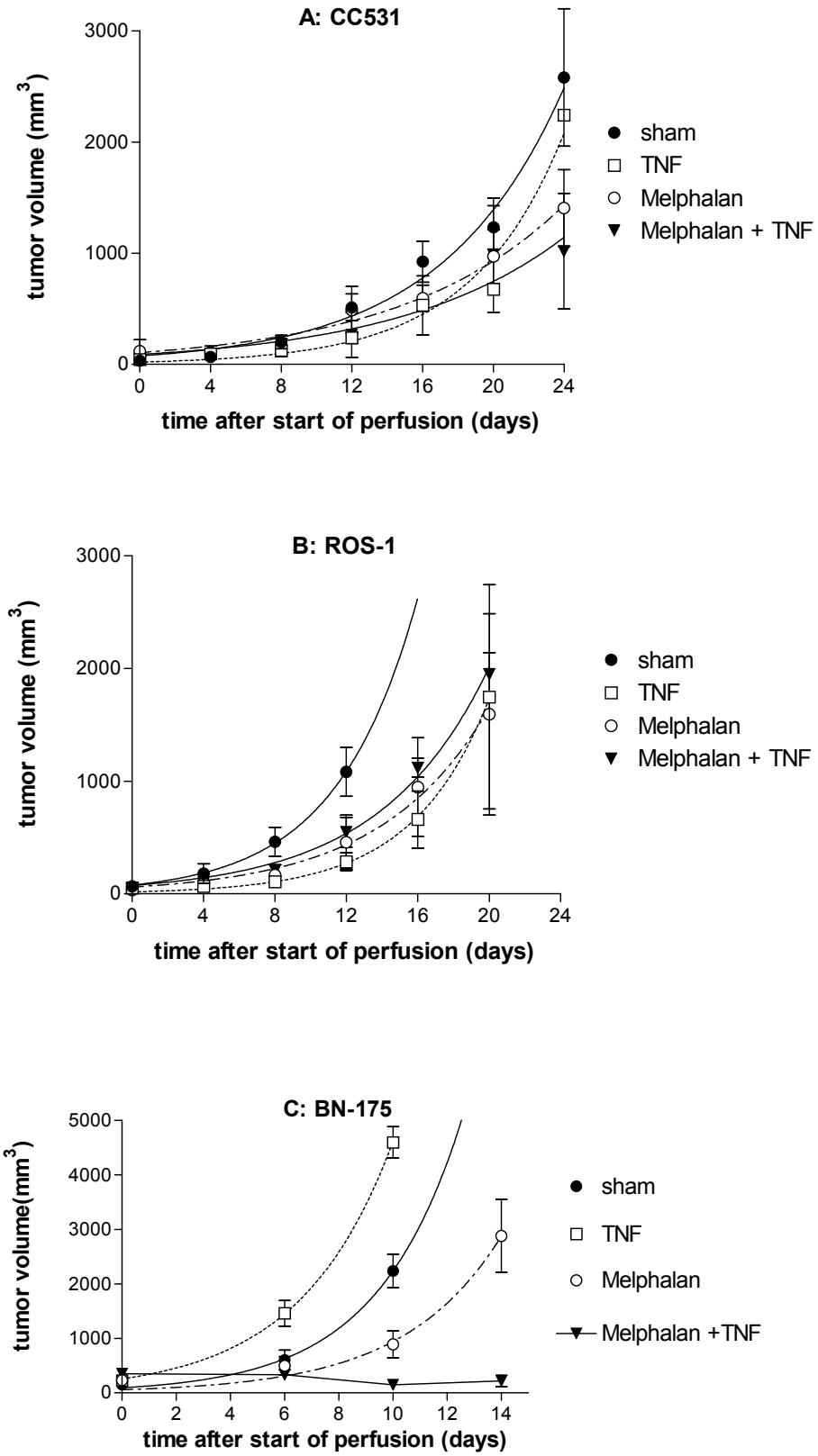

Fig. 2 Growth curves of in vivo tumors after IHP. Each group contains at least 6 animals. Mean values (±SEM) are shown.

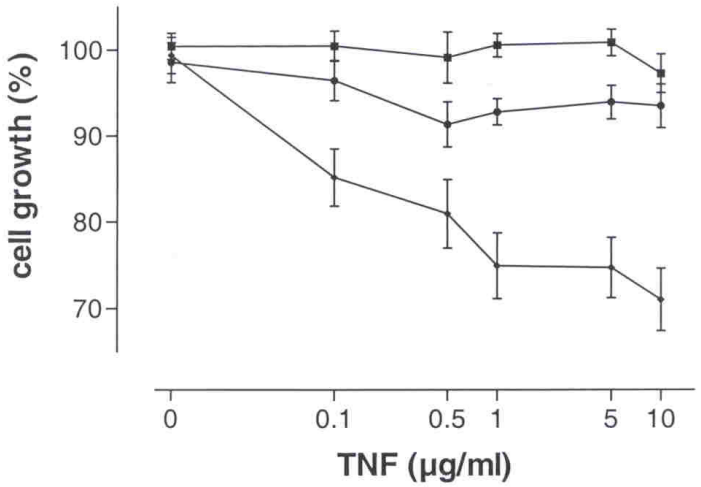

Fig. 3 In vitro growth curves of tumor cells upon exposure to TNF; CC531 (●), ROS-1 (♦), BN-175(■). Six independent assays were performed in duplicate for each point on the line. Mean values (± SEM) are shown.

Melphalan Concentration in Tumor and Liver Tissue

In this perfusion setting, in which the dose of TNF is 20% of the dose used in ILP, an enhanced drug accumulation in tumor tissue might take place as well, as observed after TNF based ILP. In order to investigate this mechanism, Melphalan concentrations were measured in tumor and liver tissues after IHP with Melphalan with and without TNF. In the CC531 and ROS-1 tumors Melphalan concentration did not increase significantly after IHP with Melphalan and TNF (Figure 4A en 4B). After IHP with Melphalan alone in the BN175 tumor bearing rats the Melphalan concentration in tumor and liver tissue was equal (Figure 4C). After IHP with TNF however a more than 5-fold increase of Melphalan in tumor tissue is measured compared to tumor tissue after IHP without TNF; ($p<0.05$). So an augmented drug accumulation can also be achieved in the IHP setting when TNF is co-administered.

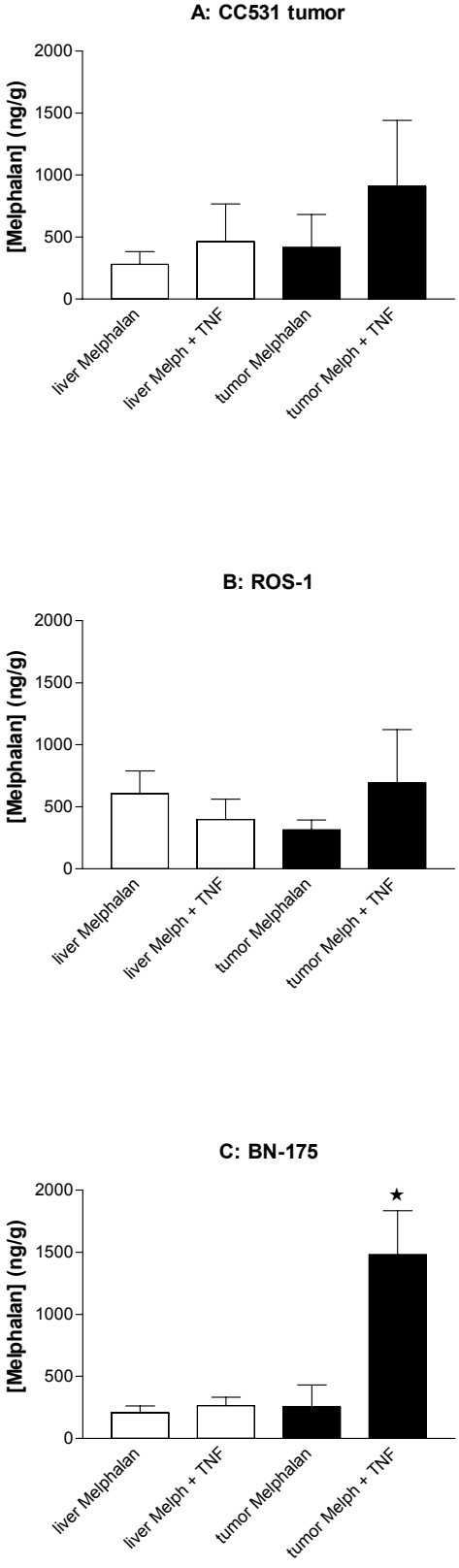

Fig. 4 Melphalan concentrations in liver and tumor tissue after IHP with Melphalan with or without TNF. Six IHPs were performed per tumor type. Mean values (±SD) are shown.
*****=P<0.05 BN-175 tumor after IHP with Melphalan + TNF vs. Melphalan alone.

Assessment of Tumor Microvessel Density

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

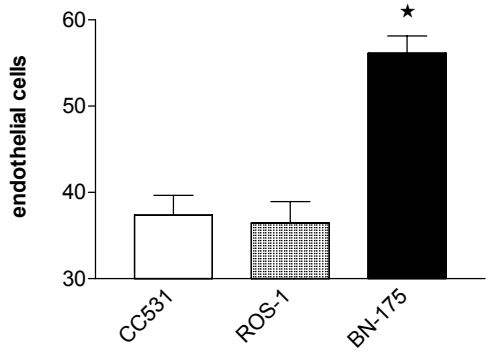

Fig. 5 Microvessel count of CC531, ROS-1 and BN-175 tumors. Mean values (SEM) are shown. *=P<0.001 BN-175 vs. ROS-1 and vs. CC531.

Discussion

In the present study we demonstrated that addition of TNF in IHP results in strongly improved response rates of rat liver sarcoma. In vitro no or only minor sensitivity of tumor cells to TNF was found. Even in ROS-1 tumors, which are moderately sensitive to TNF in vitro, IHP with TNF alone showed no tumor response. These data indicate strongly that *in vivo* indirect mechanisms mediated by TNF in combination with Melphalan determine anti-tumor effects in IHP. Our data support the notion that this indirect mechanism is the selectively destructive effect of TNF on the tumor associated vessels and thereby increasing vascular permeability (10,11). To investigate this hypothesis the Melphalan uptake in liver and tumor tissue was measured after IHP with or without TNF. Tumor Melphalan

concentrations were increased in all tumors but varied significantly in a tumor type dependent way. Moreover enhanced uptake of Melphalan by healthy liver was not observed. With TNF alone no tumor response was found in any of the tumor types. Only the combination of TNF and Melphalan resulted in a complete tumor response in the BN175 tumor. To elucidate this tumor type dependent response, the MVD of the tumors was determined. We expected a higher tumor vascularity in this tumor. Indeed a significantly higher MVD compared to the CC531 and ROS-1 tumors could be demonstrated. This indicates that TNF has specific tumor vascular mediating capacity in this perfusion model, which results in enhanced tumor responses in highly vascularized tumors. As a result of our findings in ILP and now also in IHP we know that TNF is able to augment the accumulation of Melphalan. The presence of lack of TNF mediated synergy appeared to be independent of tumor size as also in smaller (diameter 3-4 mm) or bigger (7-8 mm) tumors comparable tumor responses were observed (data not shown). We are of the opinion that this observation is essential in understanding and explaining the impressive responses demonstrated.

Changes in vascular permeability in patients which underwent IHP with TNF was studied by Alexander and coworkers (22). Vascular permeability was measured by diffusion of radiolabeled I^{131} albumin in liver and tumor tissue. A significant increase of the I^{131} albumin post-perfusion could be demonstrated compared to levels I^{131} albumin measured before perfusion. However, this rise was equal in tumors perfused with or without TNF. A TNF independent mechanism of the increased endothelial permeability was suggested by the authors. However in the present study we demonstrated that TNF is effective in increasing vascular permeability for Melphalan selectively in tumor tissue. A more important finding however, is that this effect could only be found in the highly vascularized BN-175 tumor. The results of Alexander et al. reported on intra-tumoral I^{131} albumin concentrations were mainly based on colorectal carcinoma liver metastases. In hypovascular rat colon carcinoma we also could not find an increase of Melphalan intratumorly. We therefore hypothesize that the usual hypovascularity of colorectal metastases in patients explains the lack of TNF-benefit in the experience as described by Alexander in patients, which correlates closely to our observations in our hypovascular colon cancer liver metastases model in rats.

IHP with Melphalan and TNF performed in patients with metastases of ocular melanoma or leiomyosarcoma showed overall response rates of 50-52% (23,24). Both tumor types are highly vascularized. A prolonged duration of response was found in melanoma patients: 14 months after IHP with TNF versus 6 months after IHP without TNF (23). After IHP with Melphalan with or without TNF in patients with colorectal liver metastases the mean duration

of response was in both groups 8-10 months (17,25). The data we now present and the first reports of IHP in melanoma and sarcoma liver metastases strongly indicate that in these patients TNF has therapeutic potential in IHP. In patients with colorectal liver metastases however, IHP with Melphalan alone may well be just as effective as combined with TNF. Assessment of the degree of tumor vasculature of liver metastases would be a way of establishing an indication for the utility of TNF in this setting.

Acknowledgements

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III

Pre-clinical gene therapy studies.

4

Prerequisites for effective adenovirus mediated gene therapy of colorectal liver metastases in the rat using an intracellular neutralizing antibody fragment to p21-Ras

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Summary

Ras mutations are present in 40 to 50% of colorectal cancers. Inactivating this oncogene may therefore reduce proliferation capacity. In order to target ras we studied the transduction efficacy and anti tumor activity of an adenoviral vector expressing a intracellular, neutralizing single chain antibody to p21-ras (Y28). In *in vitro* studies transfection levels of the K-ras mutated rat coloncarcinoma cell line CC531 were studied using the LacZ marker gene. In our *in vivo* liver metastases model different routes of administration were evaluated to determine which regimen resulted in the best transfection levels and tumor responses: intravenous injection (IV), intratumoral injection (IT), isolated liver perfusion (IHP), or hepatic artery infusion (HAI).

CC531 cells are readily transfected *in vitro,* resulting in significant inhibition of tumor cell proliferation by the Y28 construct. Intravenous injection did not result in any measurable transfection. Intratumoral injection resulted only in the transfection of tumor cells along the needle track. IHP as well as single HAI achieved low transfection levels of tumor tissue. Expression of Y28 was demonstrated in tumors after IT injection, HAI and IHP. Whereas, repeated HAI's clearly achieved expression in and around tumor associated vessels. Only five times repeated HAI's with Y28 resulted in a tumor response: in all animals tumor growth was inhibited, and in 3 rats out of 8 a complete regression of the liver tumors was observed.

Introduction

After resection of the primary tumor recurrent colorectal carcinoma occurs in about 50% of patients. In these patients the liver is the major site of metastatic disease (1). Patients with resectable liver metastases may have a partial hepatectomy with a five year survival rate of 25-30% (2-4). On the other hand the natural history of patients with untreated liver metastases shows a five-year survival rate of 0-3 % (5).

Colorectal carcinogenesis is associated with multiple genetic alterations. Ras mutations occur in an early stage of progression from adenoma to carcinoma. Ras mutations are present in 40 to 50% of human colorectal tumors (6). Physiologically, the ras gene leads to the production of p21–ras, a protein that catalyses the hydrolysis of guanosine triphosphate to guanosine diphosphate, and in this way controls cell proliferation by regulating signal transduction

pathways (7). Inhibition of expression of mutated ras has been shown to cause tumor growth inhibition and apoptosis in human and murine tumor cell lines (8-12).

In the development of gene therapy protocols it has been often shown that data from *in vitro* experiments do not always predict anti-tumor effects *in vivo*. The main reason for this may be insufficient tumor targeting. By locoregional administration of the genetic construct, tumor targeting may be improved and consequently result in more favorable responses. Previously, we have demonstrated successful transfection and anti-tumor activity in a rat sarcoma model with isolated limb perfusion (13,14)

Isolated liver perfusion and hepatic artery infusion are used in surgical oncology trials for the administration of chemotherapeutics and cytokines in patients with liver tumors (15-20). High local drug concentrations can be achieved at the tumor site by means of isolated hepatic perfusion (IHP), a technique with minimal systemic exposure. Effective administration of adenoviral vectors via IHP has already been demonstrated by de Roos an coworkers (21-23).

Repetitive locoregional administration of drugs can be achieved by regional infusions via the hepatic artery (17, 24, 25). Thus repeated delivery of adenoviral vectors via the hepatic artery may further increase the efficacy of transfection.

We anticipated that the route of delivery plays a crucial role in optimizing transduction efficacy and more importantly in anti-tumor activity. We report here on a transduction efficacy study of an adenoviral vector encoding the LacZ marker gene, administrated to the liver via systemic and different locoregional routes of administration, and subsequently on the antitumor activity of an adenoviral vector expressing a single chain antibody to p21-ras (Y28) *in vitro* and *in vivo* using the rat colon carcinoma CC531.

Materials and methods

Recombinant adenovirus constructs

AV1.0CMV.Y28 is a recombinant replication-deficient adenovirus vector expressing the Y28 gene. It encodes the hypervariable regions of an anti-p21-ras single chain antibody driven by the human cytomegalovirus (CMV) promoter. It is derived from the rat Y13-259 monoclonal antibody to p21-ras (11, 26, 27). The Y28 expression unit, which also contains the bovine Growth Hormone polyadenylation signal (bGH polyA), replaces the E1 adenovirus region. The AV1.0CMV.Y28 backbone is an E1/E3 deleted human adenovirus serotype 5. This construct was subjected to multiple plaque purification and produced in the 293 cell line

(human transformed primary embryonal kidney cell line) trans-complementing for E1 gene products. Adenovirus was recovered from cell culture supernatant and purified by two rounds of cesium chloride ultracentrifugation. Purified virus was then gel-filtered on a PD10 column with a PBS buffer containing 0.5 mM MgCl2, 0.5 mM CaCl2 and 10% glycerol. Virus stock was aliquoted and stored at -80°C until used. The batch used, met the preclinical grade specifications (Quality Control analyses), in regard to sterility, endotoxins, mycoplasma, viral particles and plaque forming unit titers. AV1.0CMV.LacZ and AV1.0CMV are also recombinant replication-deficient adenovirus vectors constructed on the same basis of an E1 and E3 deleted human adenovirus type 5 backbone and produced in 293 packaging cell line. The former expresses the E. coli derived ß-galactosidase protein that can be detected by histochemistry in order to access the transduction efficacy of the vector. The latter contains only the CMV promoter and SV40 signal without any transgene inserted. This "empty" construct has been used as the control vector in all experiments.

Tumor

The colon carcinoma cell line CC531, a 1,2-dimethylhydrazine-induced, moderately differentiated adenocarcinoma was used (28). The cell line is transplantable in syngeneic WAG/RIJ rats. It exhibits a mutated K-ras gene in codon 12 (GGT to GAT), changing glycine to aspartic acid (unpublished data, laboratory of Dr. R. Koesters, DKFZ-Heidelberg, Germany). The tumor is weakly immunogenic, as determined by the immunisation method of Prehn and Main (29). The tumor can also be maintained in tissue culture. New tumor was produced from this culture by subcapsularly implantation in the liver. It is a relatively slowly progressing and poorly vascularized tumor. In vivo tumors were subsequently passaged serially.

In vitro bioassay

CC531 cells were grown in RPMI 1640 (Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum (Harlan/Sera-Lab, UK), 1% penicillin (5000 IU/ml), 1% streptomycin (5000 IU/ml) and 1% L-glutamin (200mM) (all Gibco BRL) in a humidified incubator at 37 °C and 5% CO_2. Before usage, the cells were trypsinized (1 min, 37 °C), centrifuged (5 min, 700 g), resuspended and the viability measured by trypan blue exclusion. Viability always exceeded 85%. For *in vitro* testing of proliferation inhibition, 1.0 x 10^4 cells were seeded in flat-bottomed 96-wells microtiter plates (Costar, USA). After 24 hours the cells were incubated with different concentrations of the Y28 or empty construct for 48 hours ranging from a

multiplicity of infection (MOI) of 1 to 2.0 x 10^5. Afterwards, cells were washed with PBS and fixed for 30 minutes with 10% trichloro-acetic acid at 4 °C . Growth of tumor cells was measured using the sulpharhodamine-B assay according to the method of Shekan et al (30). Tumor cell proliferation was measured using the formula: tumor growth = (test well/control) x 100%. Five independent tests were performed for each point on the line.

Animals

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

In vivo colorectal liver metastases model

Following a standardized protocol, small viable CC531 tumor fragments of 1 by 2 mm were implanted under the liver capsule, one in the left and one in the right side of the left liver lobe, using a 19 G Luerlock needle. Experiments started at a tumor diameter of 5-6 mm, which was reached about 14 days after implantation of the tumor.

Isolated hepatic perfusion

This rat isolated liver perfusion model has been described in detail earlier by van IJken et al (31). Briefly, the perfusion circuit consisted of an arterial inflow limb, a venous outflow limb and a collection reservoir/oxygenator. The circuit was primed with 10 ml Haemaccel (Behring Pharma, Amsterdam, Netherlands). Arterial flow of 5 ml/min was maintained with a low-flow roller pump (Watson Marlow type 505 U, Falmouth, UK). Rats were perfused for ten minutes with oxygenated Haemaccel and 1.0 x 10^{11} virus particles (vp), which was determined as the Maximum Tolerated Dose (MTD), in a pilot study performed previously. 50 IU of Heparin (Heparine Leo, The Netherlands) was added to the perfusate. The perfusate was oxygenated in the reservoir with a mixture of O_2/CO_2 (95%:5%) and was kept at 38-39 °C by means of a heat exchanger and a warm water bath. A temperature probe was positioned in the lumen of the arterial catheter, 5 cm away from the catheter tip. Afterwards, a wash out to remove all left viruses was performed by perfusing with 10 ml of oxygenated Haemaccel.

Surgical procedure of the isolated hepatic perfusion.

Anesthesia was induced and maintained with ether (Merck, Darmstadt, Germany). During the surgical procedure, with an average duration of 60-75 minutes, rats were kept at a constant temperature using a warmed mattress. A mid-line laparotomy was performed and the hepatic ligament exposed. The gastroduodenal side branch of the common hepatic artery was cannulated, positioning the tips of the cannula (0.025 inch outer diameter (OD), 0.012 inner diameter (ID), (Dow Corning, Michigan, USA)) in the proper hepatic artery. Through a small inguinal incision the femoral vein was exposed. To collect hepatic venous outflow a silicon cannula (0.047 inch OD, 0.025 ID), (Dow Corning, Michigan, USA) was introduced in the femoral vein and moved up into the caval vein positioning the tip of the cannula at the level of the hepatic veins.

Isolation of the hepatic vascular bed was obtained by temporarily ligating the common hepatic artery and the portal vein. The venous outflow limb was isolated by temporarily clamping the supra hepatic caval vein and by applying a temporary ligature around the infra-hepatic caval vein containing the cannule, cranial to the right adrenal vein. The mesenteric artery was temporarily clamped in order to reduce splanchnic blood pressure.

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

Single time and repeated hepatic artery infusion

Single time hepatic artery infusion.

Anesthesia was carried out with Hypnorm (Janssen Animal Health, Beerse, Belgium) and Ketamine (Apharmo B.V., Arnhem, The Netherlands). During the total procedure, which took on average 2 hours and 20 minutes, rats were kept at a constant temperature with a warmed mattress and a heat producing lightbulb. A mid-line laparotomy was performed and the hepatic ligament exposed. The gastroduodenal side branch of the common hepatic artery was then cannulated, positioning the tips of the cannula in the proper hepatic artery. The cannula was connected to the infusion pump (B.Braun, Melsungen AG, Germany). 2.5×10^{11} vp (MTD) were dissolved in 1.5 ml of 0.9% NaCl/fractionated heparin solution (Fragmin, Pharmacia & Upjohn, Woerden, The Netherlands) (2500IU Fragmin per 100 ml 0.9% NaCl). The virusses were infused in one hour. Afterwards the cannula was flushed with 0.9% NaCl in order to infuse the remaining viruses in the cannula. During the infusion the arterial hepatic

blood supply was maintained. At the end of the procedure the gastroduodenal artery was ligated and the cannula removed.

Repeated hepatic artery infusion.

After positioning of the canula in the gastroduodenal side branch of the hepatic artery as describred above, the cannula was led through a flexible tube and connected via a bubbletrap to a continuous 0.9% NaCl/Fragmin solution infusion pump which was set at a continuous infusion rate of 0.5 ml/hr. Treatment with 2.5 x 10^{11} vp (MTD) started at the day of operation and was repeated with 2.5 x 10^{11} vp every other day. The animals stayed in an adjusted filtertop cage during the treatment schedule.

Intravenous injection

Anesthesia was induced and maintained with ether. A volume of 200 µl of PBS containing 2.5 x 10^{11} vp (MTD) was slowly injected into the penile vein using a syringe with a 25 G needle.

Intratumoral injection

Anesthesia was induced and maintained with ether. A mid-line laparotomy was performed and the tumors were exposed. Using a syringe with a 28 G needle, 5.0 x 10^{10} vp (MTD) in 50 µl sterile PBS were injected centrally in each tumor.

In vivo transduction efficacy study

Experiments started at a diameter between 5 and 6 mm. In order to determine the transduction efficacy, tumor-bearing rats were treated with the AV1.0CMV.LacZ construct intravenously, by intratumoral injection, via a single time hepatic artery infusion, isolated hepatic perfusion and hepatic artery infusion repeated five times. PBS solution was used as a control. 24 hours after treatment the animals were sacrificed. Tumors, liver and spleen were taken out, snapfrozen in liquid nitrogen and stored at –80 °C until further usage.

X-Gal staining on cultured cells and cryosections

Staining of cultured cells.

1.0 x 10^4 CC531 cells were seeded in flat-bottomed 96-wells microtiter plates. After 24 hours the cells were incubated for 48 hours with various concentrations of the LacZ construct ranging from a MOI of 1 up to 2.0 x 10^5. Then, cells were washed with PBS and fixed for 30 minutes with 4% paraformaldehyde at 4°C . The cells were washed three times with PBS and stained with X-gal staining solution overnight at 37°C. This solution is a mixture of solution

A: $K_4Fe(CN)_6.3H_20$ 5 mM, $K_3Fe(CN)_6$ 5mM in wash buffer ($MgCl_2$ 2mM, deoxychlate 0.01%, NP-40 0.02% in 0.1 M sodium phosphate buffer pH 7.8) and solution **B**: 5-bromo, 4-chloro, 3-indolyl β-d-galactopyranoside 50 mg/ml in dimethyl formamide) at ratio of 50 : 1. The cells were then washed once with PBS and stored at 4°C.

Staining of cryosections.

Cryosections of snapfrozen tissue samples were fixed in 4% paraformaldehyde for 30 minutes at 4°C. After three washes with phosphate buffered saline (PBS) pH 7.4, the sections were incubated overnight with X-gal staining solution at 37°C. Then, the sections were washed twice in PBS, counter-stained with hematoxylin, dehydrated with ethanol and xylene and coverslipped with entalan.

Endothelial immunohistochemistry

In order to investigate transfected cells in relationship to the tumor vasculature cryosections were first stained by the X-gal method (see above) and secondly counter stained using a mouse-anti-rat antibody against rat endothelial cell antigen (RECA-1, Instruchemie, Hilversum, The Netherlands). After overnight X-gal staining sections were thoroughly rinsed with PBS. RECA-1 was diluted 1:10 in PBS and cryosections were incubated for 1 hour. Thereafter sections were rinsed with PBS and incubated for 1 hour with 1:100 diluted, in 5% normal rat serum in PBS, goat-anti-mouse peroxidase labeled antibody (DAKO, Carpenteria, CA, USA). After rinsing with PBS, positive cells were revealed by immunoperoxidase reaction with DAB-solution (DAB-kit, DAKO) and counter stained with hematoxylin.

In vivo anti-tumor efficacy study

The various treatment modalities were started at a tumor size of 5-6 mm in diameter about 14 days after implantation of the tumor. After start of treatment tumor size was measured via a small midline laparotomy every fourth day. Tumor volume was calculated by using the following formula: tumor volume = A^2 x B x 0.4. In which B is the largest diameter and A the diameter perpendicular to B, measured with a standardized calliper. In every treatment group, control rats were included. The AV1.0CMV construct was used as negative control in the efficacy study. In each treatment group, except for the 5xHAI treated rats, 2 animals were sacrificed 24 hours after start of treatment in order to collect tumor and liver tissue. Tissue were snapfrozen in liquid nitrogen and stored at –80 °C until usage for immunohistochemistry.

Y28 immunohistochemistry

Cryosections of snapfrozen tissue samples were fixed in aceton for a few seconds. After three washes with PBS/Tween for 15 minutes the sections were incubated for 60 minutes with the 1:2000 diluted polyclonal rabbit anti-Y28 antibody (kindly provided by M. Janicot, Institut Curie, Paris, France) at room temperature. After incubation the sections were washed twice in PBS/0.5% BSA, and incubated for 60 minutes with 1:20 diluted FITC-conjugated $F(ab')_2$ fragment of swine anti-rabbit immunoglobuline. (Dako, Glostrup, Denmark). The sections were again washed with PBS/0.5% BSA. Afterwards slides were coverslipped with 90% PBS/glycerol and immediately analysed and photographed with a Leica DM-RXA fluoresence microscope equiped with a Sony DXC950 digital camera.

Toxicity study

In order to determine possible toxicity of the virus, rats were weighed every four days after start of treatment. Four days after treatment blood samples were taken via the tail vein. Serum was collected after centrifugation (14,000 rpm) and stored at -80°C until further analysis. Liver functions (alkaline phosphatase, alaline aminotransferase, aspartate aminotransferase, total bilirubin and γ-glutamyl transpeptidase) and renal functions (creatinin and urea) were measured by spectophotometric analysis (ELAN-analyzer; Eppendorf-Merck, Hamburg, Germany). Thrombocyte, leukocyte, and erythrocyte numerations were also determined in these samples (Sysmex; Kyoto, Japan).

Statistical analysis

In vitro and in vivo results were evaluated for statistical significance with the Kruskal-Wallis and Mann-Whitney-U tests with SPSS8.0 for Windows 98. A significance level of $p<0.05$ was used.

Results

In vitro transfection efficacy and cytotoxicity study

Cells were incubated with different concentrations of the AV.1.0CMV.LacZ construct. After X-gal staining the percentage of transfected cells was calculated by scoring upon light microscopy. The concentration of virus required to achieve a 50% transfection rate was determined as TD 50 (Transfection Dose). Our estimated TD 50 in this experiment was

determined at a MOI of 10,000. The maximum percentage of transfected cells was 64% at a MOI of 20,000. Above this concentration incubation of CC531 cells with LacZ virus resulted in cell death.

Inhibition of proliferation of CC531 cellls was observed with both constructs at a MOI higher than 2000. However, the Y28 construct showed a much stronger inhibition (Figure 1). The MOI resulting in 50% growth inhibition (ID 50) was 15,000 for the Y28 construct and 89,000 for the empty construct. Therefore next to a direct cytotoxic effect of the adenovirus, an additive inhibitory effect of Y28 on CC531 cells *in vitro* was demonstrated.

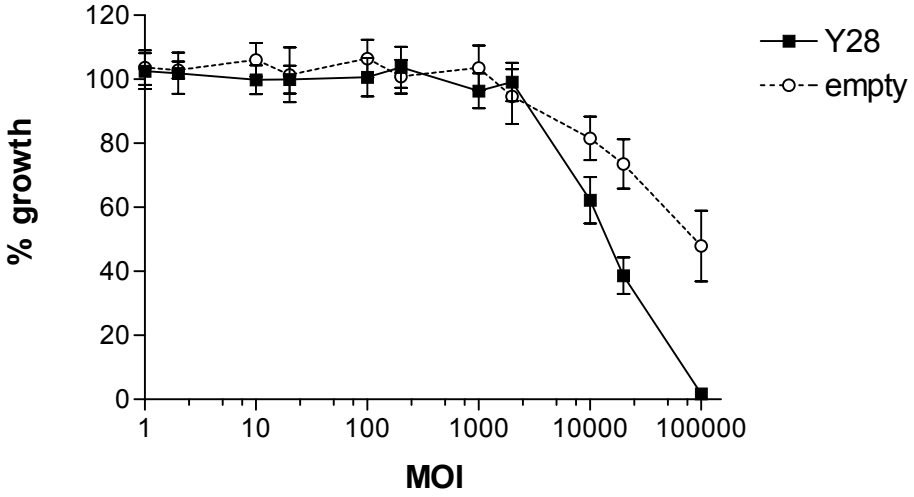

Fig. 1 Growth of CC531 colon carcinoma cells *in vitro* after exposure to increasing concentrations of AV.1.0CMV.Y28 (Y28) or AV1.0CMV (empty). Five independent assays were performed in duplicate for each point on the line. Mean values (+/- SEM) are shown. (MOI: multiplicity of infection).

In vivo transduction efficacy study

X-gal staining of cryosections and the Y28 immunohistochemistry studies revealed a needle track transfection pattern of tumor cells after intratumoral injection, with an estimated percentage of positive cells 5% (Figure 2, panel A). After IHP scattered foci of positive tumor cells per frozen section could be observed, 2-3 % positive cells (Figure 2, panel B). Almost no transfected tumor cells could be detected after single time intravenous or repeated (5 times) intravenous or a single time hepatic artery infusion. After repeated HAI (5xHAI) however clear foci of transfection of tumor vasculature and peri-tumor vasculature transfection were observed, estimated 2-3% positive cells (Figure 2, panel C and Figure 3). Very low transfection levels were observed in liver tissue in all groups.

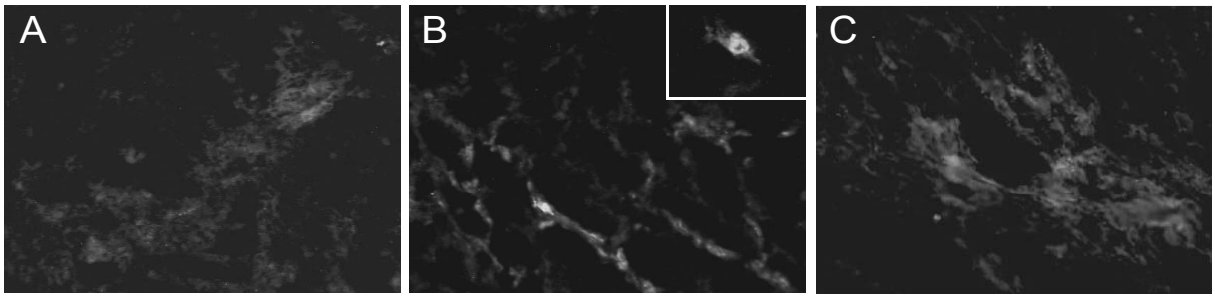

Fig. 2 Y28 fluorescence immunohistochemistry on cryosections of tumours collected 24 hours after treatment *in vivo* with AV.1.0CMV.Y28 (Y28). **A**: tumour after IT, transfection around the needle track. **B:** foci of Y28 expression in tumour after IHP. **C:** expression in tumour after HAI.
Original magnification: A, B and C: 16 x, insert 40 x. No staining was found in case of treatment with AV1.0CMV.

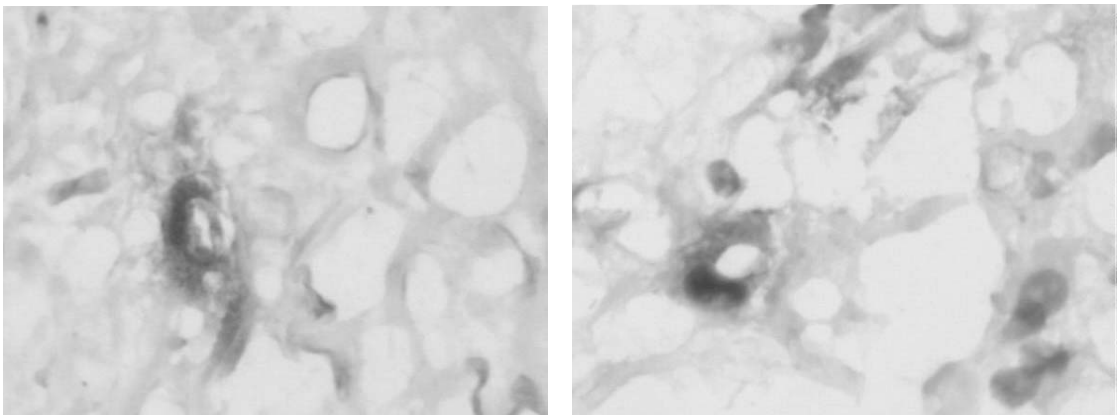

Fig. 3 Two examples of X-gal stained and RECA stained cryosections of tumours of different animals after HAI treatment with AV.1.0CMV.LacZ (left and right). Tumour vessel (brown) and perivascular orientation of transfected cells (dark blue) are clearly visible. Original magnification: 40 x. No staining was found in case of treatment with AV1.0CMV.

In vivo anti-tumor efficacy study

In correspondence with the findings of the transduction efficacy study intratumoral injection, intravenous injection, single time hepatic artery infusion and isolated hepatic perfusion with the Y28 construct did not result in any tumor response. All of the treated rats demonstrated progressive disease (Table 1). Only the tumors injected IT with Y28 showed a slight, but non significant decrease in growth rate compared to empty construct. Strikingly, five times repeated hepatic artery infusion showed a complete response in 3 out of 8 rats, a partial response in 1 rat and a growth inhibition in the other 4 tumors, resulting in an overall response rate of 50% ($p<0.02$ on day 12 and $p<0.05$ on day 16, compared to controls). All rats treated by 5xHAI with the empty construct demonstrated progressive disease (Figure 4, Table 1). As a consequence of these results we performed additional five times repeated intravenous injections with Y28 as an additive control group. This repeated treatment resulted in progressive disease in all animals (Table 1).

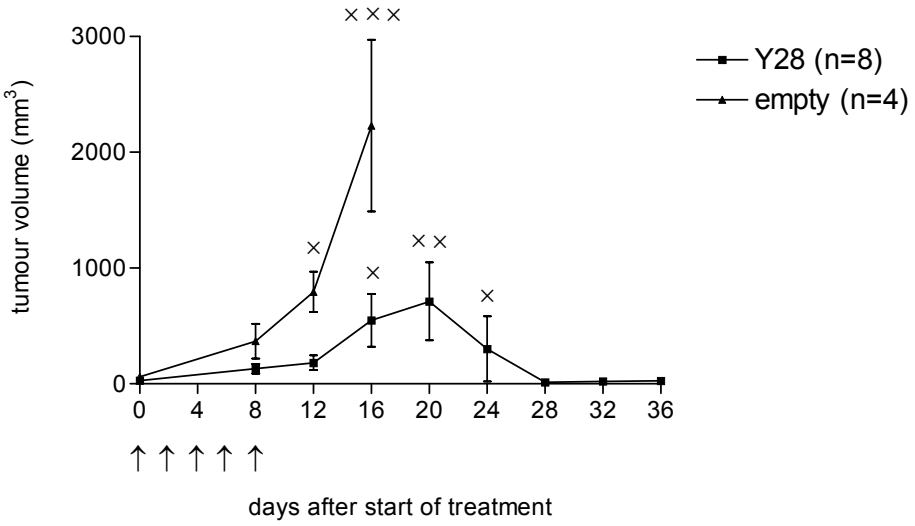

Fig. 4 Tumour response of CC531 tumours *in vivo* after 5xHAI with AV.1.0CMV.Y28 (n=8) or AV1.0CMV (n=4) treated rats. On day 0, 2, 4, 6, and 8 rats were infused (treatment schedule indicated by: ↑). Mean values (+/- SEM) are shown ($p<0.02$ on day 12 and $p<0.05$ on day 16). At day 12-24 animals with progressive disease had to be sacrificed because of bulky tumour growth (indicated by: ×), so only the partial and complete responders are depicted after that time point.

Table 1: Responses of CC531 tumors *in vivo* on day 20 after start of treatment with AV1.0.CMV.Y28 (Y28) or AV1.0CMV (empty)

		Responses*				
Treatment model[§]	rats (n)	PD	SD	PR	CR	RR[†] (%)
IT Y28	4	4	-	-	-	0
IT empty	4	4	-	-	-	0
IHP Y28	4	4	-	-	-	0
IHP empty	4	4	-	-	-	0
1xHAI Y28	4	4	-	-	-	0
1xHAI empty	4	4	-	-	-	0
5xHAI Y28	8	4	-	1	3	50
5xHAI empty	4	4	-	-	-	0
IV Y28	4	4	-	-	-	0
IV empty	4	4	-	-	-	0

*Response were scored by measurement of tumor size as described in the Materials and methods section; PD: progressive disease growth= >25%, SD: stable disease= growth <25% increase or <25% decrease of tumor size, PR: partial response= >50% growth reduction, CR: complete response= disappearance of all detectable disease.
[§]IT= intratumoral injection, IHP= isolated hepatic perfusion, 1xHAI= single time hepatic artery infusion, 5xHAI= five times repeated hepatic artery infusion, IV= intravenous injection.
[†]RR= response rate: percentage of rats with PR or CR.

Toxicity study

No severe hepatic, renal or hematological toxicity could be detected in any group after treatment with the Y28 construct. Sera from rats treated by repeated hepatic artery infusion were collected at day 8 after start of treatment. Levels of toxicity parameters measured in sera varied in an range of + and – 25% of the control values. The only abnormality observed was a doubling of γ-glutamyl transpeptidase (γGT) levels, which was detected in all groups and was equal after AV.1.0CMV.Y28 and AV.1.0CMV administration. Most changes in toxicity parameters seemed to be due to the surgical procedure or the viral constructs rather than the encoded gene. Hematological parameters showed a normal leucocyte count after IHP with Y28, but in all other groups there was a rise in leukocytes. Only in rats who underwent IT this leucocytosis was not statistically significant. The course of animal weights showed a decrease of at maximum 5 %, except for IHP and 5xHAI treated rats, where a transient weight loss of about 10% was observed in most of the animals.

Discussion

For successful cancer gene therapy, tumor targeting is essential. This can be achieved by adjusting the vector, but also by adjusting vector delivery (32). In the present study, we describe several loco-regional delivery routes for treatment of tumors confined to the liver. The aim was to target a poorly vascularized colorectal tumor located in liver tissue and to study anti-tumor efficacy by targeting p21-ras. Delivery via intratumoral injection is an established method to achieve transfection but its clinical applicability is limited. We consider a transvascular approach a prerequisite for testing the real therapeutic window of gene therapy. IHP and HAI are transvascular administration modalities, which give rise to high local concentrations of the drug through out the liver. De Wilt et al. successfully performed isolated limb perfusion in sarcoma-bearing rats for the transfer of the IL-3β-gene (14). The IL-3β cytokine was excreted upon transfection of the cells and thereby induced a major bystander effect in this tumor model, which resulted in excelent tumor responses. The BN-175 tumor used in the IL-3β study is a high grade, rapidly growing tumor with an extensive vascularization. These characteristics offer superior possibilities for transfection. Fast growing tumors are more susceptible for genes driven by a CMV promotor (33). In contrast to the BN175 tumor, CC531 tumors have a slow growth rate and are poorly vascularized. Apart

from that, the immunologic activity of the liver, in comparison with a limb, is enormous. In vitro transfection studies demonstrated that transfection of CC531 cells is possible. The in vitro cytotoxicity assay of AV.1.0CMV.Y28 construct showed effective tumor growth inhibition and cytotoxicity on CC531 cells. The TD 50 in the transfection efficacy study is about equal to the ID 50 of AV.1.0CMV.Y28 in the cytotoxicity assay. This suggests that transfection of a CC531 cell with Y28 anti-ras antibody results in proliferation inhibition. Next to this we performed in vitro bioassays on human umbilical vein derived endothelial cells (HUVECs) which do not harbor a ras mutation. On HUVECs AV.1.0CMV.Y28 hardly results in growth inhibition compared with the CC531 cell line (data not shown).

In order to study transfection efficacy in vivo we targeted the tumor by various locoregional delivery routes. We initiated treatment when the tumor size was about 5-6 mm in diameter. In patients, a liver tumor of 5 mm diameter is the minimal size of metastases detectable with the current imaging techniques. Therefore, in our model we used clinically relevant tumors that are locally established and have their own blood supply. Obviously, the tumor-associated vasculature is essential for the delivery of vectors.

In vivo IT injection shows significant transfection of tumor cells on X-gal stained cryosections and immunohistochemistry. Strikingly, a single administration of AV.1.0CMV.Y28 intratumorally did not result in a significant tumor response. Only minimal growth inhibition was seen, which corelates with previous observations (16). This might be explained by the fact that IT injection results in transfection only around the needle track. A trans-endothelial route causes a more homogeneous distribution of transfection of the tumor. We have shown this previously in a highly vascularized rat sarcoma model and particular in the viable rim of the tumor (34).

The internalization of adenoviral vectors into a cell is receptor mediated. This occurs predominantly via the Coxsackievirus-adenovirus receptor (CAR) and via αvβ3-integrin (35-41). αvβ3 is upregulated in response to vascular damage, during angiogenesis and in certain types of malignancy (42). Isolated perfusion of a limb or an organ causes minor vascular damage. As a consequence of this, an increase of αvβ3 may occur, which could improve internalization of adenoviruses into cells in the perfused region. CC531 however, is a poorly vascularized tumor and we speculate that upregulation of αvβ3 may not effectively influence the transfection, which may contribute to the low transfection efficacy we found after IHP.

Bilbao et al. found that a blood-tumor barrier in hepato-cellular carcinoma in rats limits the gene transfer in tumors greater than 5 mm in diameter (43). Moreover, they also concluded that tumors beween 2 and 5 mm could only be transfected by hepatic artery infusion. As

already mentioned we started treatment at a tumor diameter of about 5 mm, so a blood-tumor barrier may well play a role in the limited transvascular transfection we observed. After multiple HAI, the transfection rate of tumor tissue still remains low, however in the AV1.0CMV.LacZ and Y28 immunohisochemistry experiments more transfection could be clearly detected around tumor vessels.

It is known that down regulation of p21-ras causes a decrease of vascular endothelial growth factor (VEGF) production (44, 45). A bystander effect caused by down regulation of VEGF after p53-gene therapy in vivo has already been described (46). Transfection of the tumor associated endothelial cells by the Y28 construct may cause an anti-angiogenic effect by this VEGF-pathway. A higher transfection of tumor endothelial cells and, as a consequence of this, a reduction of VEGF levels around tumor vessels upon 5xHAI could explain the 50% tumor response observed in this treatment group.

Moreover, a direct cytotoxic effect of Y28 on endothelial cells and peri vascular tumor cells could contribute to the anti-tumor effect as well. Regions of the tumor supplied by affected vessels could have a deficiency of oxygen and nutrients and as a result will become necrotic.

Our experiments indicate that indirect immunological anti-tumor effect by repeated adenovirus administration can be ruled out, because not only five times repeated HAI with the empty control vector, but also five times repeated systemic treatment with AV1.0.CMV.Y28 did not result in any anti-tumor efficacy. Both of these treatment modalities resulted in a leucocytosis at least as high as 5xHAI with Y28 (data not shown). It seems to be a prerequisite to deliver this vector loco-regionally and in a repetitive way.

Targeted gene therapy is a major issue in the development of gene therapy towards clinical trials. Furthermore, gene therapy has to be safe. In this study we report on a successful repeated administration of adenoviral vectors without significant toxicity. We demonstrated that loco-regional gene therapy of slowly progressing, poorly vascularized colon carcinoma liver metastases is feasible and that repeated treatment might offer possibilities for future gene therapy trials.

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5

Impaired neutralizing antibody formation and high transduction efficacy after isolated hepatic perfusion with adenoviral vectors.

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Submitted

Summary

Isolated hepatic perfusion (IHP) is a methodology that offers possibilities to deliver high concentrations of viral vectors locally without systemic exposure and washout possibilities for non-bound viruses. By means of IHP a very high transduction efficacy is achieved in vivo without significant toxicity using an E1-deficient adenoviral vector in fully immunocompetent rats. Moreover a remarkable decreased immune response to the adenoviral vector after IHP was observed. Significantly impaired neutralizing antibody formation and decreased leukocytes proliferation was demonstrated. These findings are a strong argument for further development of gene therapy in pre-clinical isolated perfusion settings.

Introduction

Adenoviruses are widely used in gene therapy protocols as they proved among the most active in transfecting cells and gene expression. However promising in vitro results are often not predictive for good in vivo activity. The main reason for this is insufficient transduction efficacy in vivo. A method, which improves in vivo efficacy, is locoregional administration of the genetic construct. By augmenting local vector concentration while abrogating systemic spread targeting is improved and results in more favorable transduction efficacy.

Previously, we and others demonstrated successful transduction and subsequent tumor response in rat gene therapy studies after isolated limb perfusion (ILP) and isolated hepatic perfusion (IHP) (1-4). These studies indicate that IHP could be an attractive modality for hepatic disorders and malignancies.

However adenoviral mediated gene transfer results in transient the gene expression and is accompanied by an immune response towards the vector. The loss of the therapeutic gene requires readministration to achieve prolonged gene expression. After systemic treatment however, neutralizing antibodies formation towards viral antigens preclude secondary gene transfer (5,6). Upon a non-systemic route of administration like IHP it is unknown to which extend neutralizing antibodies are produced by the host. Since IHP is a technique with minimal systemic exposure and washout possibilities after the perfusion immune response after adenoviral treatment may be impaired. We performed IHP in rats with a recombinant adenoviral vector and investigated the production of neutralizing antibodies and leukocytes compared to systemic intravenous (IV) treatment.

Materials and methods

Animals

Male inbred immunocompetent WAG/RIJ rats, weighing 250-300 g (Harlan-CPB, Austerlitz, The Netherlands) were used. The rats were fed a standard laboratory diet and were housed under standard conditions of light and accommodation. The protocol was approved by the committee for animal research of the Erasmus University Medical Center, Rotterdam, the Netherlands. The experimental protocols adhered to the rules outlined in the Dutch Animal Experimentation Act of 1977 and the published Guidelines on the Protection of Experimental Animals by the Council of the European Community 1986.

Recombinant adenovirus construct

The viral constructs were provided by Aventis-Pharma (Vitry-sur-Seine, France) and are described previously by us in detail (4). AV1.0CMV is a recombinant replication-deficient adenovirus vector constructed on the basis of an E1 and E3 deleted human adenovirus type 5 backbone. It contains the human cytomegalovirus (CMV) promoter and SV40 signal without a transgene inserted. AV1.0CMV.LacZ expresses the E. coli derived ß-galactosidase protein that can be detected by histochemistry in order to access the transduction efficacy of the vector.

Routes of administration

Isolated hepatic perfusion.

We have described the rat isolated liver perfusion model in detail earlier (7). Briefly, the perfusion circuit consisted of an arterial inflow limb in the hepatic artery, a venous outflow limb in the caval vein and a collection reservoir/oxygenator. Isolation of the hepatic vascular bed was obtained by temporarily ligating the common hepatic artery and the portal vein. The venous outflow limb was isolated by temporarily clamping the supra-hepatic caval vein and by applying a temporary ligature around the infra-hepatic caval vein containing the cannula, cranial to the right adrenal vein. The mesenteric artery was temporarily clamped in order to reduce splanchnic blood pressure. The circuit was primed with 10 ml colloid fluid (Haemaccel, Behring Pharma, Amsterdam, Netherlands). Arterial flow of 5 ml/min was maintained with a low-flow roller pump. Rats were perfused for ten minutes with oxygenated and heated (38-39 °C) Haemaccel with 1.0×10^{11} virus particles (vp). This dose was determined as the Maximum Tolerated Dose (MTD), in a previously performed pilot study.

50 IU of heparin was added to the perfusate. Afterwards, a washout procedure was performed to remove all non-bound viruses by perfusing with 10 ml Haemaccel.

Intravenous injection.

A volume of 200 µl of PBS containing 2.5 x 10^{11} vp (MTD) was slowly injected into the penile vein using a syringe with a 25 G needle.

Blood sampling

In order to determine toxicity and neutralizing antibody production blood samples were taken via the tail vein at day 0, 3, 6, 9,16, 23, and 30 after treatment. Serum was collected after centrifugation (14,000 rpm) and stored at -80°C until further analysis. Moreover rats were weighed every four days after start of treatment.

Tissue sampling and X-Gal staining

In order to determine the transduction efficacy rats were treated with the AV1.0CMV.LacZ construct IV or IHP. PBS solution was used as a control. 24 hours after treatment the animals were sacrificed. Liver was taken out, snap frozen in liquid nitrogen.

Cryosections of snap frozen tissue samples were stained according to the X-gal staining protocol we previously described (4).

Measurement of neutralizing antibodies

Adenovirus type 5 specific neutralizing antibodies were measured by the virus neutralization (VN) test as previously described (8). The VN test was conducted with Hep-2 cells growing in 96-well microtiter plates. Serum samples were inactivated for 30 min at 56 °C (9). 50 µl of serial serum dilutions were incubated with equal amounts of adenovirus with a concentration of 100 TCID50 (tissue culture infective dose when 50% of cells are infected with a virus titer of 10^2). After 60 min at 37 °C 100 µl Hep-2 cells were added with a concentration of $2x10^5$/ml. After 5 days the presence of cytopathic effects caused by the virus were scored under the microscope. Neutralizing antibody titers were expressed as the highest serum dilution showing no cytopathological effects.

Measurement of leukocyte count

Leukocyte numerations were determined with a microcell counter (Sysmex; Kyoto, Japan). Liver functions (alkaline phosphatase, alaline aminotransferase, aspartate aminotransferase, total bilirubin and γ-glutamyl transpeptidase) and renal functions (creatinin and urea) were

measured by spectophotometric analysis (ELAN-analyzer; Eppendorf-Merck, Hamburg, Germany).

Statistical analysis

Results were evaluated for statistical significance with the Kruskal-Wallis and Mann-Whitney-U tests with SPSSv10.0 for Windows 2000. A significance level of $p<0.05$ was used.

Results

Firstly we tested whether we could repeat previously reported results and show augmented transfection of cells after an isolated perfusion compared to systemic therapy. IHP resulted in a high transduction efficacy of near 80-90 % (Figure **1-A**). After IV injection low transduction efficacy (±5%) of hepatocytes after IV injection was observed (Figure **1-B**). All animals survived the surgical procedures.

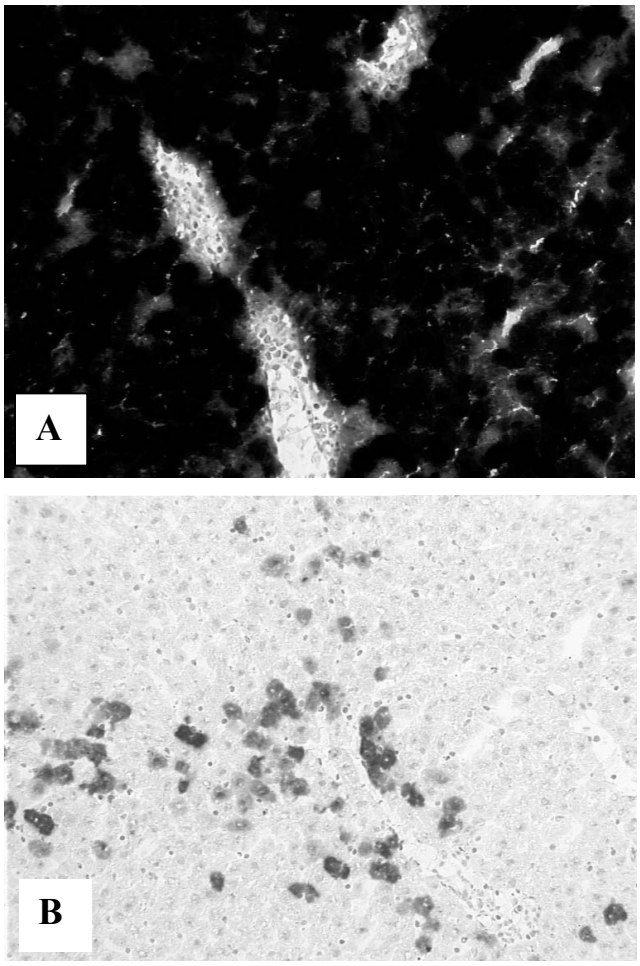

Fig. 1

X-gal staining of liver tissue after treatment with AV1.0CMV.LacZ.
A After IHP, estimated transduction of 80-90 % **B** After IV injection, estimated transduction of about 5 %.

Secondly we studied the production of neutralizing antibodies towards adenovirus after IHP compared to systemic injection. Antibody titers after IHP were significantly lower compared to IV from day 3 up to day 23 (Figure 2). On day 6 after treatment a mean peak titer could be measured: 352 after IV injection versus 27 after IHP (p= 0.03). During follow-up, performed up to 30 days after treatment the levels decreased and were equalized approximately as from day 23.

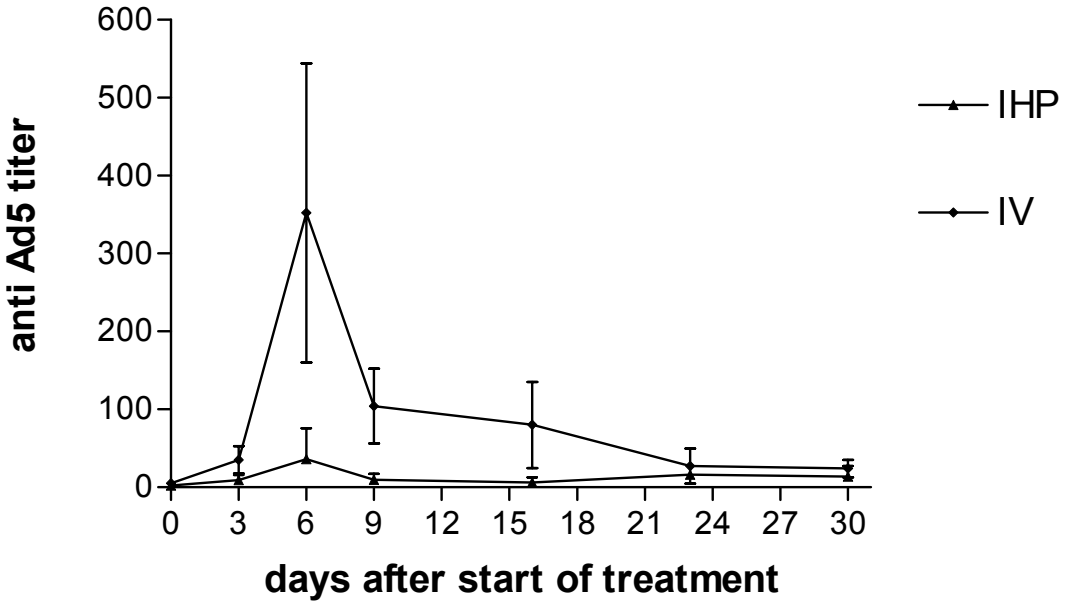

Fig. 2 Neutralizing antibody titer after IVor IHP with AV1.0CMV determined as described in materials and methods section. Antibody titers after IHP were significantly lower compared to IV from day 3 up to day 23 ($p< 0.05$). On day 6 after treatment a mean peak titer could be measured: 352 after IV injection versus 27 after IHP ($p= 0.03$). Mean values of 5 animals ± SD are shown.

Leukocyte count was determined at day 6 after treatment (Figure 3). Numerations of leukocytes were significantly higher after IV compared to IHP or the untreated control group whereas leukocyte count after IHP was not increased compared to control values. The increased leukocyte count following IV administration correlates directly with the rise of titers measured after IV treatment.

No severe hepatic or renal toxicity could be detected in the IV or IHP group after treatment. Levels of toxicity parameters measured in sera varied in a range of + and – 25% of the control values (data not shown). After IV injection no weight loss was observed. Following IHP a maximum transient weight loss of 10% was observed, which could not be related to the viral vector as similar weight loss was observed upon sham IHP.

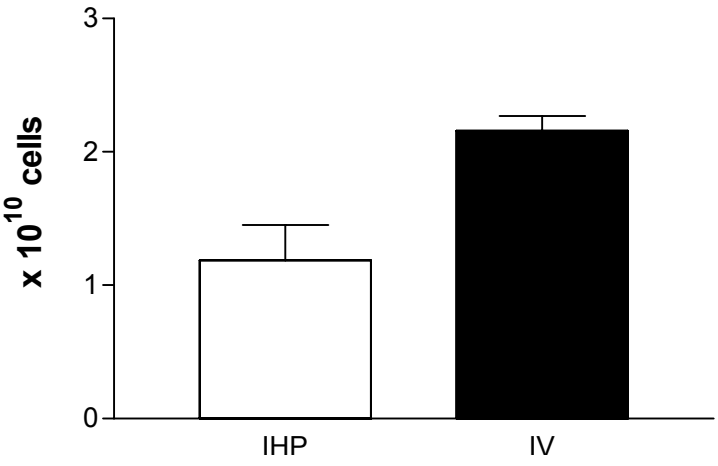

Fig. 3 Leukocyte count after IHP and IV with AV1.0CMV measured on day 6 after treatment. Controls are untreated rats. Numerations of leukocytes were significantly higher after IV than after IHP (IV vs. IHP: $p< 0.05$) and also compared to the untreated control group and (IV vs. control: $p< 0.05$). Leukocyte level after IHP is not increased compared to control values. Mean values of 3 animals ± SD are shown.

Discussion

Isolated hepatic perfusion is an effective and safe method to deliver adenoviral vectors towards liver parenchyma and liver tumors in experimental rodent models (1,4). A transduction efficacy up to 45% of hepatocytes by means of IHP with Ad.RSV.βgal combined with a chelating agent has been described by de Roos et al.(1). We here demonstrate that this highly selective delivery method can result in even higher transduction rates and importantly, is accompanied by an impaired neutralizing antibody formation and leukocyte proliferation. Several studies have been conducted to influence immune response upon adenoviral gene therapy, including incorporation of immunosuppressive genes into the vector or manipulation of the immune system during administration (10-12). To our knowledge this is the first report demonstrating an impaired immune response using isolated perfusion methodology with a "regular" adenoviral vector in an immunocompetent animal model.

The liver is known for its ability to induce immune tolerance. Already since the beginning of liver transplantation the liver is a fascinating organ for immunologists (13). There are several reports of patients that received a graft liver with long-term survival without the need for immunosuppression (14). This host-to-graft tolerance was also commonly observed in experiments with pigs and rats (15). The liver locally controls the immune response when antigens from the systemic circulation enter the liver. Limmer et al. investigated the role of

the liver sinusoidal endothelial cells (LSECs), which clear the antigen from the blood and can function as an antigen presenting cell (16). The naïve T cells, which are activated by the LSECs, do not differentiate into effector T-cells thereby inducing antigen specific T cell tolerance (17). This mechanism might play a role in the lesser immune response we observed.
At the end of the IHP we perform a thorough washout until the effluent is completely clear. Exposure to viral vectors is strongly diminished to a short period with high local concentrations of the virus. It is known that the height of neutralizing antibodies titers is correlated with the dose of virus administrated (18). Since most circulating viruses are washed away at the end the perfusion, only a relative low viral load is left behind in the liver. In this respect acute innate immune mechanisms may contribute in the low titers we observed. Worgall et al. demonstrated this innate immune component dominates elimination of adenoviral vectors in vivo (19). They reported that 90 % of circulating viruses are cleared within 24 hours due to this mechanism and the remnant viruses are eliminated by the normal immune response. So if hardly any circulating viruses are left after the IHP and the majority is cleared rapidly, only an extremely low amount of viruses can induce the humoral immune response and thereby results in impaired neutralizing antibody production.
Peeters et al. performed biliary infusion of adenoviruses in mice that resulted in increased hepatic restricted gene transfer, and was also combined with lower neutralizing antibody titers (20). Another report showed that the route of administration strongly determines the humoral immunity to the transgene in experiments with adeno-associated virus vectors in mice. Loco-regional delivery via the hepatic artery resulted in higher transgene expression and an absent immune response to the transgene product (21). These results and our current findings suggest that loco-regional liver directed gene therapy offers advantages at gene transfer efficacy level as well as at immune response level.
In conclusion this remarkable finding of impaired neutralizing antibody formation after IHP with an adenoviral vector is a strong argument for further research on delivery of viral vectors in isolated perfusion settings and could widen the therapeutic index for adenovirus based gene therapy.

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6

Isolated limb perfusion based anti-p21*ras* gene therapy in a rat rhabdomyosarcoma

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Summary

Inhibition of ras oncogene is a promising new strategy. Gene therapy against ras proved successful in human and murine tumor cell lines. Previously we demonstrated effective targeted transfection of tumor in a rat model by using an isolated limb perfusion (ILP) for the delivery of adenoviral vectors.

This study explores the anti tumor activity of an adenoviral construct encoding an intracellular single-chain antibody (scFv) against p21*ras* (Y28). In order to determine the influence of the ras status on the efficacy of the scFv we used a wild type rat rhabdomyosarcoma and its ras–oncogene transfectant, for in vitro studies. In vivo we used the ILP delivery method to study anti-tumor activity on established limb tumors.

In vitro studies demonstrated an inhibition of growth caused by the Y28 construct. No significant difference between transfected and wild type cell lines could be demonstrated. Upon ILP homogeneous transduction in the tumor with on estimate 5 % transduction of tumor cells was observed. Perfusion with the Y28 construct however, did not result in any additional anti-tumor activity compared to controls.

Despite in vitro activity and in vivo transfection no significant tumor response could be detected using anti-p21ras gene therapy in this ILP-tumor model.

Introduction

The wildtype ras gene leads to the production of p21–ras, a protein that catalysis the hydrolysis of guanosine diphosphate to guanosine triphosphate. Through this pathway cell proliferation is controlled by interfering with signal transduction (1). Inhibition of ras oncogene expression is a promising new strategy in anti-tumor therapy. Gene therapy against ras using ribozymes, anti-sense RNA or single chain antibodies (scFv) proved successful in causing growth inhibition and apoptosis in human and murine tumor cell lines (2-13).

However, data obtained in in vitro gene therapy experiments often do not correlate with in vivo results. In vivo transfection of established tumors remains a major problem and as a consequence limits the possibilities of clinical cancer gene therapy. Isolated limb perfusion (ILP) proved to be a successful method for the administration of chemotherapeutics and cytokines in patients with locally advanced soft tissue sarcomas and in-transit melanoma metastases (14-17). We were able to replicate this way of administration in our rat ILP model

(18-21). High concentrations of anti tumor agents can be reached at the tumor site with negligible systemic exposure. Recently others and we demonstrated effective targeted transfection of tumor in a rat model by using an ILP for the delivery of adenoviral vector (22, 23).

We now report on a study that explores the anti tumor activity of an adenoviral construct encoding an intracellular scFv against p21-ras. Delivery of viral vectors via intratumoral injections is an established method to reach transfection but its clinical applicability is limited. A transvascular approach could increase the therapeutic window for gene therapy in clinical trials. Considering this advantage we used the ILP delivery method to study anti-tumor activity of an anti-ras construct on established tumors in the hind limb of a rat.

In order to study the influence of the ras status on the efficacy of the scFv we used a wild type rat rhabdomyosarcoma and its ras–oncogene transfectant, expressing six copies of the ras gene, during in vitro studies. In experiments performed in vivo we only used the ras oncogene transfected rhabdomyosarcoma.

Materials and methods

Recombinant adenovirus constructs

AV1.0CMV.Y28 is a recombinant, replication-deficient adenoviral vector expressing the Y28 gene. It encodes for the hypervariable regions of an anti-p21-ras single chain antibody driven by the human cytomegalovirus (CMV) promoter. It is derived from the rat Y13-259 monoclonal antibody to p21-ras (3, 24, 25). The Y28 expression unit, which contains also the bovine Growth Hormone polyadenylation signal (bGH polyA), replaces the E1 adenovirus region. The AV1.0CMV.Y28 backbone is an E1 deleted and E3 partially deleted human adenovirus serotype 5. This construct was subjected to multiple plaque purification and produced in the 293 cell line (human transformed primary embryonal kidney cell line) trans-complementing for E1 gene products.

AV1.0CMV.LacZ and AV1.0CMV are also recombinant replication-deficient adenovirus vectors constructed on the same basis of an E1 and partially E3 deleted human adenovirus type 5 backbone and produced in 293 packaging cell line. The former express the E. coli derived ß-galactosidase protein that can be detected by histochemistry in order to access the transduction efficacy of the vector. The latter contains only the CMV promoter and bGH polyA signal without any transgene inserted. This "empty" construct has been used as the

control vector in all experiments. A pilot study revealed a Maximum Tolerated Dose (M.T.D.) of 2,5 x 10^{11} vp for systemic treatment in vivo for each of the constructs.

Tumor

The rat rhabdomyosarcoma cell line R2 and the transfectants R2T24 and R2-neo, which can be maintained in tissue culture, have been described previously (26, 27). The in vivo tumor was produced by subcutaneous implantation of cells. The tumor is transplantable in syngeneic WAG/RIJ rats and was subsequently passaged serially. The R2T24 cell line was cotransfected with the plasmid pT24 carrying the c-H-ras oncogene (28) and the plasmid carrying the neo gene (29). The R2-neo cell line was transfected by the neo gene only. The R2T24 contains six copies of the 6.6 kb *Bam*HI fragment of the pT24 plasmid revealed by Southern blot analysis and exhibits constitutive expression of the c-H-ras oncogene determined by Northern blot assay (26). In the parental R2 cell line no H-ras oncogene could be detected (26).

In vitro transfection efficacy study and anti tumor activity study

R2T24, R2 and R2-neo cells were grown in Dulbecco's modified culture medium (Gibco BRL, Paisley, GB) supplemented with 10% fetal calf serum (Harlan/Sera-Lab, GB), 1% penicillin (5000 IU/ml), 1% streptomycin (5000 IU/ml) and 1% L-glutamin (200mM) (all Gibco BRL) in a humidified incubator at 37 °C and 5 % CO_2. Before usage, the cells were trypsinized (1 min, 37 °C), centrifuged (5 min, 700 g), resuspended and viability was measured by trypan blue exclusion. Viability always exceeded 85%. For in vitro tests, cells were seeded in flat-bottomed 96-wells microtiter plates (Costar, USA), in a final volume of 200 µl and 1.0 x 10^4 cells per well. After 24 hours the cells were incubated with different concentrations of adenovirus for 48 hours ranging from 1.0 x 10^4 virus particles (vp) /ml to 2.0 x 10^{10} vp/ml in a final volume of 200 µl per well (corresponding with a multiplicity of infection (M.O.I.) of 5 to 100,000).

Transfection efficacy study.

After 48 h incubation with the LacZ construct cells were washed with PBS and fixed for 30 minutes with 4% paraformaldehyde at 4 °C. Then cells were washed three times with PBS and stained with X-gal staining solution overnight at 37 °C. This solution is a mixture of solution *A*: $K_4Fe(CN)_6.3H_20$ 5 mM, $K_3Fe(CN)_6$ 5mM in wash buffer ($MgCl_2$ 2mM, deoxychlate 0.01%, NP-40 0.02% in 0.1 M sodium phosphate buffer pH 7.8) and solution *B*: 5-bromo, 4-chloro, 3-indolyl β-d-galactopyranoside 50 mg/ml in dimethyl formamide) in a

ratio of 50 : 1. The cells were then washed once with PBS and stored at 4 °C until further analysis.

Anti-tumor activity study.

After incubation with Y28 or empty construct, cells were washed with PBS and fixed for 30 minutes with 10% trichloro-acetic acid at 4 °C. Growth of tumor cells was measured using the sulpharhodamine-B assay according to the method of Shekan et al (30). Tumor cell proliferation was measured using the formula: tumor growth= (test well/control well) x 100%. At least five independent tests were performed for each time point.

Animals

Male inbred WAG/RIJ strain rats, weighing 250-300 g, obtained from Harlan-CPB (Austerlitz, The Netherlands) were used. The rats were fed a standard laboratory diet. All animals were housed under standard conditions of light and accommodation. The experimental protocols adhered to the rules outlined in the Dutch Animal Experimentation Act 1977 and the published Guidelines on the Protection of Experimental Animals by the Council of the European Community 1986. The protocol was approved by the committee on animal research of the Erasmus University Medical Centre, Rotterdam, the Netherlands.

Isolated Limb Perfusion (ILP) model

The perfusion technique was performed as described previously (18, 19) and a schematic representation is shown in Figure 1. Briefly, small fragments (3-5 mm) of the rhabdomyosarcoma were implanted subcutaneously into the right hind limb. Perfusion was performed at a tumor diameter between 12 and 15 diameter. Time between implantation and perfusion was about 14-16 days. Animals were anaesthetized with Hypnorm (Janssen Pharmaceutica, Tilburg, the Netherlands) and Ketamine (Apharmo B.V., Arnhem, The Netherlands). Heparin (50 IU) was injected intravenously to prevent coagulation. To keep the rat's hind limb at a constant temperature, a warm water mattress was applied. Temperature was measured with a temperature probe on the skin covering the tumor and was maintained between 38.0 and 39.0 ºC. 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 solution consisted of 5 ml Haemaccel (Behring Pharma,

Amsterdam, the Netherlands) and the adenoviruses 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. After the perfusion, the cannulas were removed and the femoral vesels of the perfused limb were ligated. The extensive collateral circulation restored the blood supply of the perfused leg.

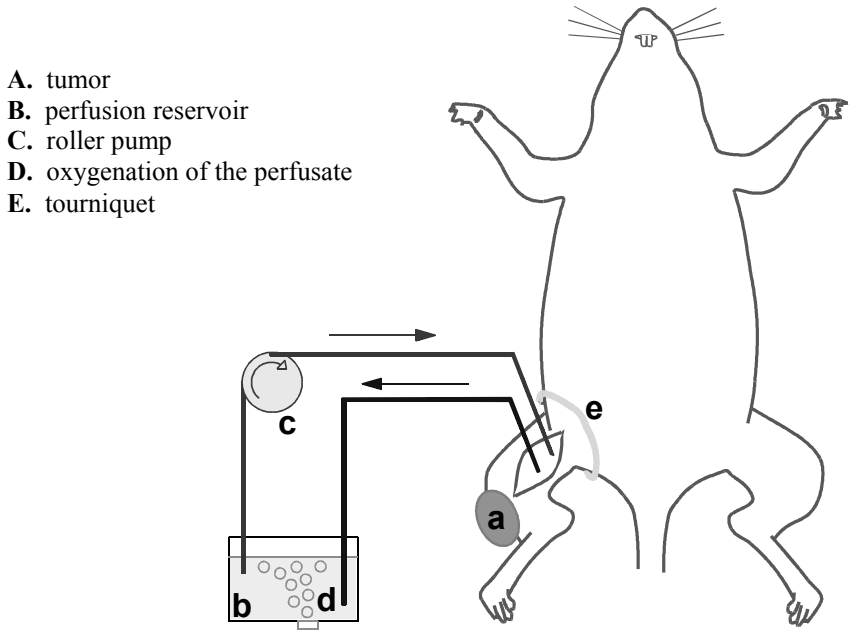

Fig.1 Schematic representation of an Isolated Limb Perfusion setting in the rat.

In vivo transfection efficacy study

In order to determine transfection efficacy, tumor-bearing rats (n=3) were treated with 2,5 x 10^{11} vp AV1.0CMV.LacZ construct by ILP. Animals were sacrificed 24 hours after treatment. Tumor, quadriceps muscle of the perfused limb, quadriceps muscle of the other hind limb, liver and spleen were taken out, snapfrozen in liquid nitrogen and stored at –80 °C until further analysis. Before X-gal staining cryosections were fixed in 4% paraformaldehyde for 30 minutes at 4 °C. After three times washing with phosphate buffered saline (PBS) pH 7.4, sections were incubated in with X-gal staining solution overnight at 37 °C. Thereafter sections were washed twice in PBS, counter-stained with haematoxylin, dehydrated with ethanol and xylene and coverslipped with entalan.

In vivo anti-tumor efficacy study

Four rats were left untreated to determine the normal growth pattern of the R2T24 tumor in vivo. Another four rats were sham perfused with Haemaccel only. ILP with 2,5 x 10^{11} vp of AV1.0CMV.Y28 (n=6) or the control vector AV1.0CMV (n=5) were performed. Tumor size was determined daily by calliper measurement in a standardised way. Tumor volume was calculated by using the formula: tumor volume = A^2 x B x 0.4. In which A is the smallest diameter and B the diameter perpendicular to A.

Statistical analysis

In vitro and in vivo results were evaluated for statistical significance with the Kruskal-Wallis test and Mann-Whitney test with SPSS v10.0 for Windows 2000. A significance level of p=0.05 was used.

Results

In vitro transfection efficacy is dose dependent

After X-gal staining the percentage of transfected cells was calculated by means of light microscopic scoring. Positive cells were counted in four different fields with magnification of 100 X. The results for the different cell lines are shown in Table 1. The wild type R2 is more sensitive to transfection by the LacZ construct then the transfected R2T24 and R2-neo cell line at concentrations from 2.0 x 10^5 to 2.0 x 10^7 vp/ml ($p<0.05$). There are no significant differences between R2T24 and R2neo. A clear dose-transfection relation is present in all cell lines ($p<0.05$).

Dose (vp/ml)	R2 % pos (±SD)	R2T24 % pos (±SD)	R2-neo % pos (±SD)
$2x10^8$	100 (28)	100 (19)	86 (36)
$1x10^8$	100 (33)	100 (21)	75 (26)
$2x10^7$	93 (19)	72 (39)	65 (30)
$1x10^7$	82 (27)	66 (29)	59 (38)
$2x10^6$	59 (17)	29 (11)	33 (16)
$1x10^6$	45 (30)	24 (18)	20 (7)
$2x10^5$	18 (7)	7 (12)	8 (9)

Table 1 In vitro transfection rate by means of X-gal staining on R2, R2T24 and R2-neo cells after 48-hour incubation with increasing concentrations of AV1.0CMV.LacZ. Wild type R2 is more sensitive to transfection in vitro by the LacZ construct then the transfected R2T24 and R2-neo cell line at concentrations from 2.0 x 10^5 to 2.0 x 10^7 vp/ml ($p<0.05$). A clear dose-transfection relation is present in all cell lines ($p<0.05$).

In vitro anti-tumor activity induced by the Y28 construct

The bioassay of R2, R2T24 and R2-neo incubated with the viral constructs demonstrated an inhibition of growth caused by the Y28 construct (Figure 2, panel A-C). Inhibition of growth occurred at a concentration of 1.0 x 10^8 vp/ml and higher for all cell lines. The dose causing 50% growth inhibition (ID 50) was 3.9 x 10^8 vp/ml in the wild type R2 cell line, 1.5 x 10^9 vp/ml in the R2T24 cell line, and 8,9 x 10^9 vp/ml in the R2-neo cell line. Statistical analysis showed no significant difference between the three cell lines (p=0.17).

Homogeneous in vivo transduction by ILP

X-gal staining of cryosections of tumors and organs (3 slides per tumor or organ) treated with AV.1.0CMV.LacZ were scored by light microscopy. Upon ILP tumors showed on estimate 5 % transduction of tumor cells (Figure 3). There was a homogeneous distribution of transduction in the tumor (3-A,B). Moreover around tumor-associated vessels transduced cells could often be detected (3-C). Muscle tissue of the perfused limb showed almost no transduced cells. In liver tissue a few positive cells could be detected, which can be caused by adenoviruses left in the limb after the ILP or by some leakage during the ILP (Figure 3-D). No transduced cells could be detected in spleen, kidney, heart, lung or intestine.

Lack of in vivo anti-tumor efficacy

All rats survived the surgical procedure and no systemic toxic side effects were observed as measured by weight changes. However, in each of the virus treated group one rat suffered impaired limb function and oedema. So this local toxicity determined the dosage of 2,5 x 10^{11} vp as the M.T.D. Sham ILP resulted in a temporary growth inhibition of R2T24 tumors compared to untreated tumors (Figure 4). We previously observed similar perfusion related growth inhibitory effect after sham ILP in the ROS-1 osteosarcoma that also grows in WAG-RIJ rats [31]. We hypothesize this may be a rat strain dependent effect, rather then a tumor dependent effect. Perfusion with the Y28 anti-ras construct however, did not result in any additional anti-tumor activity. The control vectors showed comparable results (Figure 4). In all groups rats demonstrated progressive disease 7 days after ILP.

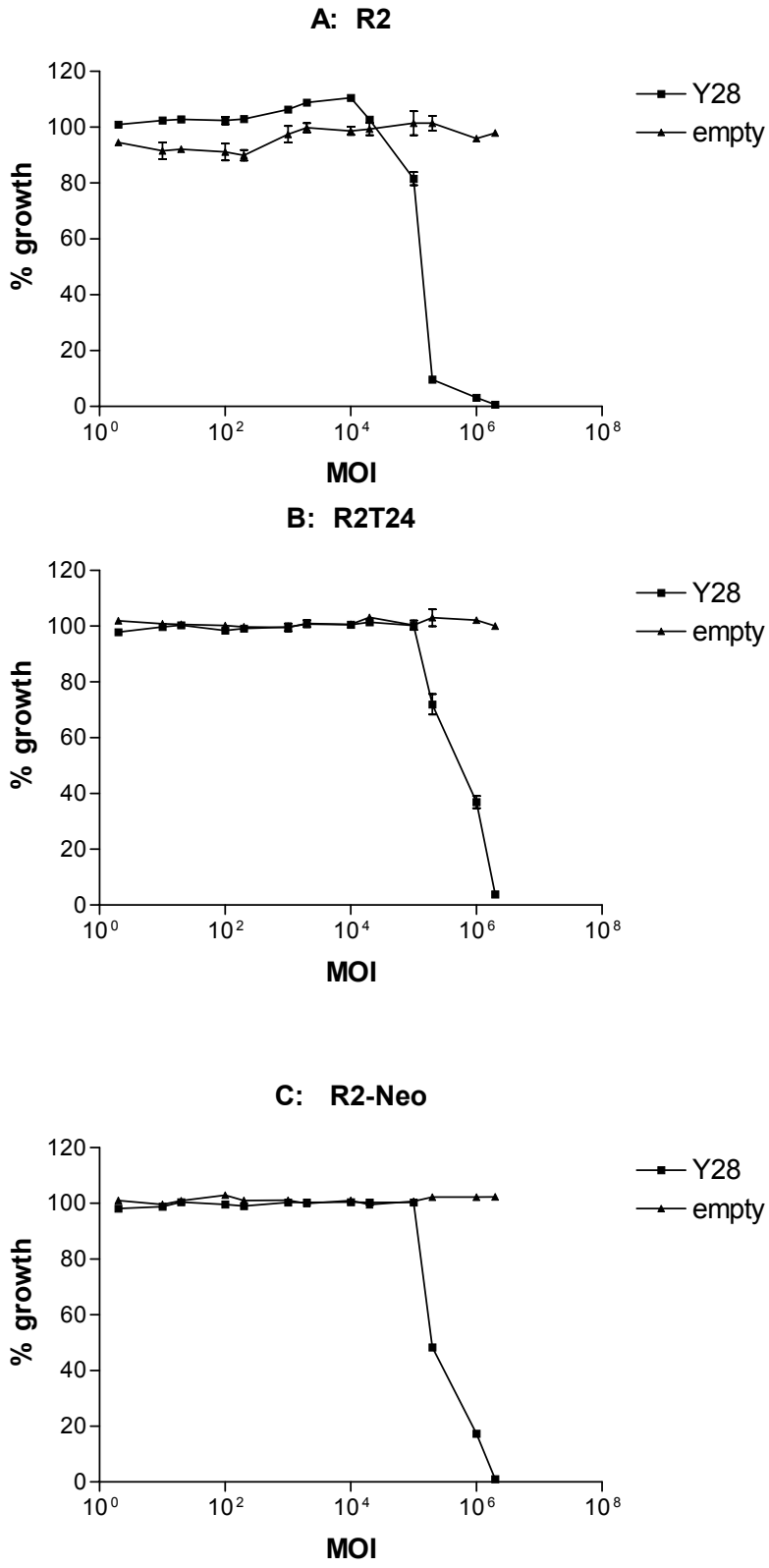

Fig. 2 In vitro bioassay of A: wild type R2 cells, B: R2T24 cells transfected with six copies of H-ras, C: R2-neo cells transfected with the neo gene only. Cells were incubated for 48 hours with increasing concentrations of AV.1.0CMV.Y28 (■) or AV1.0CMV (empty) (▲). Four independent bioassays were performed in duplicate for each point of the line. Mean values ± SEM are shown. MOI: multiplicity of infection.

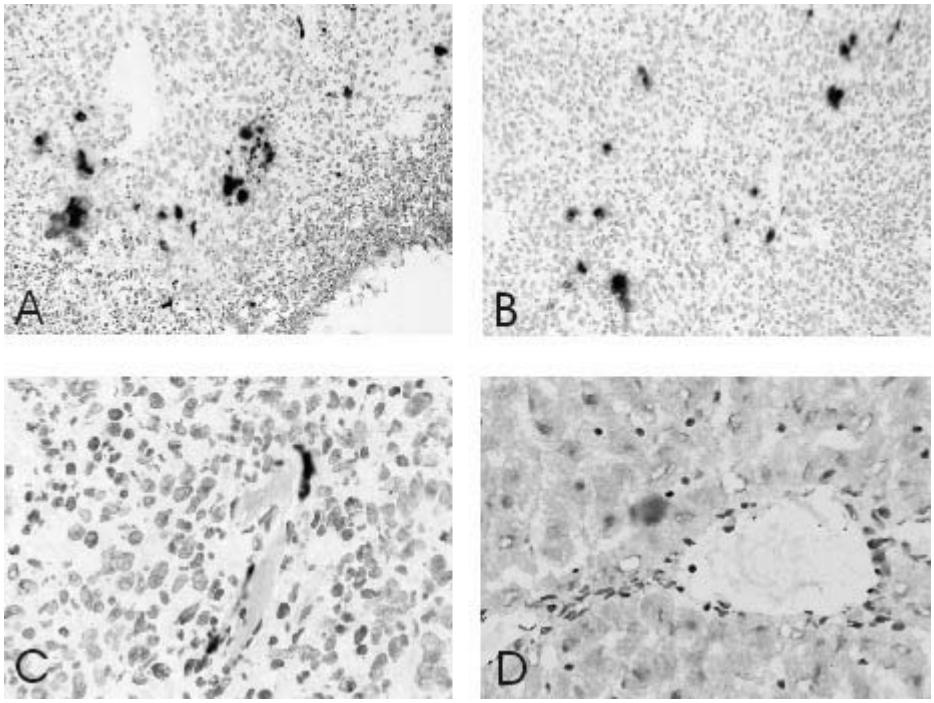

Fig. 3 Light microscopy of X-gal stained cryosections of tumor and liver 24 hours after in vivo treatment by ILP with 2.5 x 10^{11} vp AV1.0.CMV.LacZ. Showing: **A** R2T24 tumor, viable rim area **B** R2T24 tumor, central area, **C**: R2T24 tumor, detail: perivascular transduction , **D**: liver. Original magnification: A, B and C: 160 x, D: 400x.

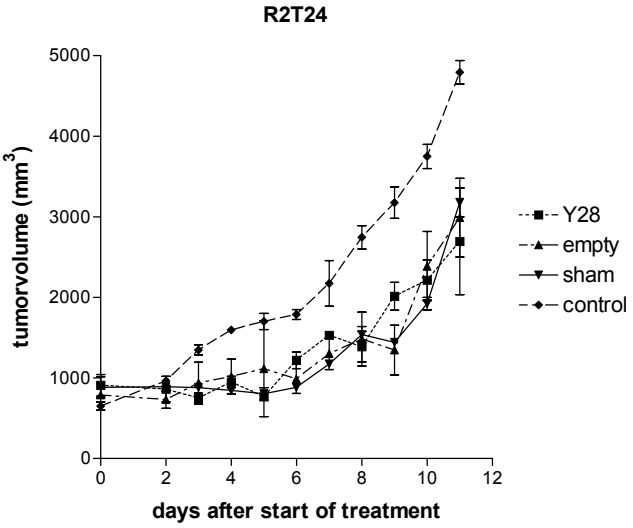

Fig. 4 Growth curve of R2T24 tumor in vivo: control (without treatment) (◆, n=4), after sham ILP with perfusion medium only (▼, n=4) after ILP with 2.5 x 10^{11} vp AV1.0CMV(empty) (▲, n=6) or 2.5 x 10^{11} vp AV.1.0CMV.Y28 (■, n=6). Mean values ± SEM are shown.

Discussion

In the present study we explored in vitro and in vivo anti tumor activity of an anti-ras adenovirus construct. Significant growth inhibition of the Y28 scFv could be demonstrated in vitro, but was only noted when high concentrations were used indicating only modest activity at best. The empty control virus did not have any anti-tumor activity. Administration of an adenoviral LacZ construct in the ILP model resulted in homogeneous transfection of the tumor without significant systemic transfection or systemic toxicity. Despite these in vitro and in vivo results, the Y28 construct lacks anti tumor efficacy using ILP in this tumor model.

The in vitro results suggests that the status of the ras gene has no influence on the response of tumor cells after AV1.0CMV.Y28 transfection. The R2T24 cell line expressing six copies of H-ras did not show an increased or reduced sensitivity to the anti-p21ras scFv. Russell et al. found Y28 mediated enhancement of radiosensitivity in various tumor cell lines with N-ras, K-ras and wild type ras in vitro (31), confirming that the mechanism of Y28 is independent of the status of the ras-gene. The Y28 scFv has been derived from the neutralizing antibody Y13-259. This antibody recognizes the H-, N-, and K-ras proteins (24, 25). It seems that this aspecific inhibition of ras expression in tumor cells influences the cell function in a broad spectrum from enhancement of radiosensitivity to induction of apoptosis (4, 31).

In soft-tissue sarcoma, osteosarcoma and colon carcinoma rat tumor models we already demonstrated transfection efficacy of adenoviral vectors by ILP and isolated hepatic perfusion (IHP)(13, 22). Moreover, ILP for adenovirus mediated IL-3ß gene therapy resulted in a significant tumor respons in the soft tissue sarcoma (BN-175) as well as the osteosarcoma (ROS-1)(32). We currently performed ILP with an adenoviral vector harbouring the LacZ gene in order to determine the transduction efficacy in R2 rhabdomyosarcoma. Remarkable perivascular transduction was observed in the tumor with on estimate 5% transduction of tumor cells. On the basis of these results we wanted to study the in vivo anti-tumor activity study of the Y28 construct. In vivo we currently only investigated the R2T24 tumor because it has the fasted growth in vivo compared to the R2 and R2neo (26). This is of special importance because vectors driven by a CMV promoter are preferably active in dividing cells, since the promoter is activated by factors in the nucleus (33). Nevertheless, AV1.0CMV.Y28 could not induce a significant anti-tumor effect in this tumor by using the ILP. In spite of the fact that the R2T24 is fastest growing tumor among these it took about 2 weeks after implantation in the limb to obtain a tumor with the an adequate size for ILP. The above-mentioned BN-175 and ROS-1 tumors grow twice as fast. We previously demonstrated that

fast growing tumors are well vascularized (34). We speculate R2T24 is less vascularized in which individual tumor cells are difficult to reach by a transvascular delivery method.

Cochet et al. described significant growth inhibition of HCT116 human colon carcinoma cell line after intratumoral injection of AV1.0CMV.Y28 (4). They reported similar transduction efficacy after intratumoral injection. As a consequence some bystander effect of Y28 transduction in vivo in the HCT116 cell line was suggested. However, upon Y28 based ILP no additional anti tumor activity was present, indicating that Y28 transduction lacks a bystander effect in this tumor. We hypothesize that a higher transduction level is needed to achieve anti-tumor efficacy in this tumor model. Higher transduction rates can be obtained by using a higher virus dose or repeated administration. As previously demonstrated in our liver metastases model in which we achieved tumor response only upon five times repeated hepatic artery infusion (13). Since there is minimal systemic exposure in ILP of the vector we anticipated a dose escalation. However in this tumor model we were confronted with local toxicity of the limb in 2 out of the 11 perfusions. This result prohibited usage of higher dosages of the vector.

Furthermore internalisation of adenoviral vectors is receptor mediated. Pre-dominantly this occurs via the Coxsackie- and adenovirus receptor (CAR) (35). Absence of CAR on the surface of R2, R2-neo and R2T24 cells might be responsible for the relatively high M.O.I. necessary for inducing proliferation inhibition in vitro as well as growth inhibition in vivo.

For successful cancer gene therapy tumor targeting is essential. Adjusting vector delivery methods has a major influence on transduction efficacy (36). ILP proved to be a effective in reaching the tumor, however next to vector delivery, also tumor type, extent of tumor vascularisation, presence of receptors and type of gene to deliver are factors crucial in the process of achieving a tumor response in vivo.

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7

Gene therapy in *in vivo* isolated perfusion models.

A review.

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Summary

Locoregional administration of a genetic construct by means of isolated perfusion (IP) of a target organ or extremity is a method that may increase in vivo efficacy. Vascular isolation and perfusion minimizes systemic exposure and thereby reduces unwanted side effects. Isolated hepatic perfusion (IHP) is the most extensively studied IP model, especially in gene therapy protocols for inborn errors of metabolism. To achieve stable transduction most frequently retroviruses are used in IHP. IHP is combined with hepatectomy or vascular ligation of liver lobes to induce liver regeneration increasing transduction efficacy. When adenoviruses are used in IHP high transduction percentages of hepatocytes can be achieved without significant toxicity. In tumor models adenoviral IHP has been performed, but has not been very successful up till now.

Isolated limb perfusion (ILP) is a promising treatment modality in pre-clinical cancer gene therapy studies. After ILP a homogeneous distribution of transduced cells was demonstrated especially at the viable rim of the tumor and around tumor associated vessels. Moreover complete tumor responses have been observed. Isolated pulmonary perfusion (IPP) results in selective expression in the perfused lung and the duration of expression is longer than after systemic administration. In rats a significant decrease of tumor nodules upon IPP can be achieved. Furthermore other less studied perfusion models are discussed: isolated kidney perfusion (IKP), isolated spleen perfusion (ISP) and isolated cardiac perfusion (ICP).

IP is a methodology that delivers vectors highly selectively, with a long exposure time and high concentrations at the target side. This results in higher transduction rates and thereby may improve therapeutic effects.

Introduction

A method for safe and efficient introduction of foreign DNA into a target cell would allow further development of gene therapy protocols in the treatment of inborn errors of metabolism and cancer. Promising in vitro results are often faced by poor in vivo activity. The main reason for this is insufficient transduction efficacy in vivo. A method, which may improve in vivo efficacy, is locoregional administration of the genetic construct by means of isolated perfusion of the target organ or extremity. Isolated perfusion is a technique that uses the in vivo, in situ vasculature of the target organ or extremity for highly selective, mostly arterial, delivery and has controlled venous outflow of the perfusate. Vascular isolation of the target minimizes systemic exposure and connection to closed circuit with roller pumps offers the option to recirculate the perfusate to further increase the delivery of drugs or vectors. By augmenting local vector concentration while abrogating systemic spread, targeting is improved and may result in more favorable transduction efficacy. Isolated perfusion models administering cytostatic agents are already successfully used in clinical oncology trials. By isolated perfusion regional concentrations of chemotherapeutic agents 15-20 times higher than those reached after systemic administration can be achieved without systemic toxicity. Isolated limb perfusion (ILP) with Melphalan in combination with tumor necrosis factor-alpha (TNF) performed in patients with limb threatening soft tissue sarcoma or in-transit melanoma metastases results in impressive tumor responses and high limb salvage rates (1-3). Isolated hepatic perfusion (IHP) with a cytostatic agent is a treatment option for patients with unresectable primary or metastatic tumors confined to the liver and can mediate clinical regression of advanced liver metastases (4-8). This methodology of regional administration is also conducted in several pre-clinical gene therapy studies in various models and by using different type of viral and non-viral vectors. This review outlines the results of administration of vectors *in vivo* isolated perfusion models in a wide field of research on inborn genetic defects and cancer gene therapy.

Isolated hepatic perfusion (IHP)

The liver is an important target organ for gene therapy already for many years. As a consequence IHP is the most extensively studied isolated perfusion model in gene therapy protocols. All studies reported in literature are performed with viral vectors (Table 1).

IHP perfusion techniques and circumstances vary greatly in the studies published. With respect to the inflow site of the perfusion the portal vein is most commonly used. Only de Roos et al performed double infusion via the arterial as well as the portal vascularisation of the liver (9). Our group currently performs IHP via the hepatic artery only (10,11). Especially in liver metastases models the inflow site is of specific interest since the metastatic tumors are mainly vascularized by the hepatic artery (12,13).

Hepatocyte-targeted Gene Therapy for Inborn Errors of Metabolism.

The majority of reported IHP studies aim to transduce hepatocytes in order to develop treatment options for inborn errors of metabolism. Retroviral transfection ensures genomic integration and results in stable expression of the foreign gene (14-17). However, cell division is required to achieve retroviral transfection and most hepatocytes are quiescent cells in vivo. So as a consequence transduction efficacy will be low. Following partial liver resection many hepatocytes start dividing to regain complete liver volume (18,19). A peak in DNA synthesis occurs 20-24 hours after hepatectomy (20). This phenomenon of organ regeneration offers possibilities to increase retrovirus transfection efficacy. All hepatocyte-directed studies listed in Table 1 combine IHP with hepatectomy or vascular ligation of liver lobes. Already in 1991 Ferry et al. described a rat model in which they performed a partial hepatectomy followed by a retrovirus mediated gene transfer by IHP 24-48 hours later(21). This study showed that the time interval between perfusion and hepatectomy is an important factor. Significantly higher numbers of LacZ positive cells were counted in rats perfused 24 hours after hepatectomy compared to a time interval of 48 hours (2-5% vs. 0.05%). One year later Rozga et al. confirmed these findings with retroviral hygromycine-B delivery into hepatocytes (22). IHP performed 20 hours after anterior lobe portal vein ligation resulted in expression of hygromycine-B in 16% of harvested hepatocytes vs. 2.8 % in the negative controls in which IHP was performed without artificial DNA synthesis stimulation. When IHP was carried out during the same surgical procedure as the portal vein ligation 9% positive cells were found. De Godoy et al. perfused rat livers with retrovirus 24 hours after partial hepatectomy for 60 minutes followed by a 30-minute washout and high transduction rates were obtained. In up to 34 % of hepatocytes transgene expression could be detected. The very long exposure to the vector might be the explanation for this very high transduction efficacy in this study.

Adenoviruses are able to introduce genes in non-dividing cells and have a high affinity for liver cells when administrated intravenously. When adenoviruses are used in IHP it has been demonstrated that high transduction percentages can be achieved (9,10,23,24). Regarding toxicity following IHP a maximum transient weight loss of 10% was observed, which could

not be related to the viral vector as similar weight loss was observed upon sham IHP (10). Drazan et al. studied the influence of adenovirus mediate gene transfer on hepatic function in further detail by means histological, biochemical and functional studies (23). They demonstrated that even with high transduction rates the rat liver was not compromised regarding hepatic cell metabolism and integrity. In liver directed gene therapy for inborn errors of metabolism these vectors seem suitable, however, the major disadvantage is the transient expression of the foreign gene since it is not integrated in the genome of the target cell. Expression is typically detectable for a few weeks only. Moreover a strong immune response is induced against viral proteins and the transgene product precluding secondary gene transfer (25-27) In anti-cancer protocols adenoviruses are widely used because of their high transfection efficacy. Moreover stable integration is not needed in cancer directed gene therapy.

Comparability Problems.

It is hard to compare the results of transduction efficacy between the different studies, because of many interstudy variations. In this respect several factors are of importance: (1) type and doses of viral vector, (2) type of animal or rat strains, (3) specific perfusion conditions and (4) methods to determine the number of transduced cells. These detection techniques vary from histochemistry of cryosections of liver samples, to *ex vivo* harvesting of hepatocytes or luciferase activity of tissue samples. Regarding the technical aspects of the isolated perfusion many different techniques and conditions are used. The inflow site is already mentioned above. Also the flow rate can influence the transduction efficacy. Higher flow rates might, as an example, induces an increased perfusion pressure and as a consequence virus particles pass trough the fenestrae of the sinusoidal endothelial lining more easily and higher transduction rates might be reached. Hydrodynamic studies with increased injection pressure are already reported for plasmid based gene therapy in mice(28). After IHP in pigs Borel Rinkes et al. have reported mild transient sinusoidal dilatation as well as septal edema, without necrosis or severe hepatic dysfunction suggesting that the perfusion pressure is higher than the physiologic blood pressure in the hepatic circulation (29). Only in three IHP models recirculation of the perfusate is carried out. Recirculation is a technically more demanding procedure but has the advantage that after the first passage all non-bound viruses can be administered repeatedly making the whole procedure more efficient. Washout of the target organ is also an option in isolated perfusion methodology, however is not often used in IHP gene therapy protocols. Washout by rinsing the liver with clean perfusate directly after perfusion might decrease toxicity of non-bound viruses and increases the selectivity of the

transfection to the perfused organ only. De Godoy et al. performed a 30 minutes washout procedure and measured the viral load in the venous effluent (30). During the rinsing procedure a dramatic decrease in retrovirus concentration was measured. Oxygenation of the perfusate is also not frequently used. The liver is very tolerant to ischaemic conditions. From clinical experience it is known that the normothermic in situ liver can accept an anoxic period up to one hour. Godoy et al. perfused rat livers during 90 minutes but with oxygenation and heating of the perfusate, toxicity however was not determined (30). In hepatocyte directed gene therapy for inborn errors of metabolism stable transfection is warranted, however high transfection levels are not often indicated. Tada et al. demonstrated that only 1-5 % of hepatocytes have to be transduced to induce a decrease in bilirubin in Gunn rats (31). Following IHP with recombinant retrovirus encoding bilirubin uridine diphosphoglucoronate-glucoronosyl transferase (UGT) in 3 weeks time bilirubin levels decreased 20 to 25%. These levels remained during the study period (18 months), indicating a stable transfection.

Gene Therapy for Cancer.

In cancer directed gene therapy often higher transduction rates have to be reached. We previously reported adenoviral IHP transferring a single chain antibody to p21 *ras* in to colon carcinoma cells (10), but with only 1-3 % transduced tumor cells a tumor response could not be induced. In this tumor model there was no bystander effect, and probably higher transduction rates were necessary. Some viral constructs are known for their bystander effect inducing capacity, (32-36), but have not been described in isolated liver perfusion model up till now. Another option is combination therapy like Alves et al. showed by performing retroviral gene therapy with herpes simplex virus-thymidine kinase (HSV-TK) and interleukin-2 (IL-2) followed by ganciclovir administration (37). Monotherapy with these vectors failed to induce tumor regression, but combination therapy demonstrated a partial tumor response.

Author	Year	Animal	Virus	A/P	Time (min)	Flow (ml/min)	Re-circ.	W.O.	O_2	Tox.	Transd. (% + cells)	Resp
Ferry	1991	rat	RV	P[1]	10	5	-	-	-	-	2-5	NA
Rozga	1992	rat	RV	P	15	3	+	-	-	-	9-16[2]	NA
Moscioni	1993	rat	RV	P	15	3	-	-	-	-	21-26[3]	NA
Cardoso	1993	dog	RV	P[4]	20	20-80	-	+	-	-	0.15-0.6	NA
Fong	1995	rat	HSV	P[5]	20	0	-	-	-	-	1-3	NA
Drazan	1995	rat	AV	p	10	2.5	-	-	-	-	30	NA
de Roos	1997	rat	AV	P+A	25	20+5	+	+	+	ND	45[6]	NA
Tada	1998	rat	RV	P[7]	10	5	-	-	-	-	1-5%	Bili↓
Futagawa	2000	rat	AV	P	15	0.5	-	-	-	-	Variable[8]	NA
DeGodoy	2000	rat	RV	P[1]	60	2	+	+	+	ND	34	NA
Brooks	2001	rat	HSV	P[5]	20	0	-	-	-	-	1-3	TNF↑[9]
van Etten	2002	rat	AV	A	10	5	+	+	+	-	Tumor[10] 1-3	PD
Alves	2003	rat	RV	P[11]	5	0	-	-	-	-	Tumor +	PR[12]
van Etten	subm	rat	AV	A	10	5	+	+	+	-	80-90	NA

Tabel 1 Gene therapy by Isolated Hepatic Perfusion (IHP).

Abbreviations:
RV, retrovirus; HSV, herpes simplex virus; AV, adenovirus; P, portal inflow site; A, arterial inflow site; Recirc., recirculation; W.O., washout; O_2, oxygenation of perfusate; Tox, toxicity; Transd. Transduction; NA, not applicable; ND, not determined; bili, bilirubine; TNF, Tumor Necrosis Factor; PD, progressive disease; PR, partial response.

Notes:
1. IHP performed 24-48 hours after 66% partial hepatectomy.
2. Transduction efficacy determined ex vivo in isolated hepatocytes. IHP which was performed with portal branch ligation of the anterior liver lobes.
3. IHP combined with 70% partial hepatectomy. Transduction efficacy:see note 2. In situ β-galactosidase histochemistry showed 0.5% positive hepatocytes.
4. IHP performed 72-96 hours after 44-72% partial hepatectomy.
5. Portal vein infusion with total vascular outflow occlusion, followed by a 20 minutes stopflow period.
6. Determined after ex vivo IHP with chelating agent. In vivo IHP only with AV.Luc: after IHP 5 times more luciferase activity compared to portal vein infusion.
7. See note 1. Performed in hyperbilirubemea Gunn rats.
8. Variable transduction of liver different liver lobes.
9. IHP with HSV transduced cells expressed TNF. Elevated levels of TNF in liver tissue.
10. Transduced CC531 coloncarcinoma cells, transduction of hepatocytes not determined.
11. See note 5. Stopflow period 5 minutes.
12. All tumors showed transduction, quantification not reported. Partial response achieved after combination gene therapy with IL-2.

Isolated limb perfusion (ILP)

Isolated perfusion of an extremity is a treatment modality used successfully in pre-clinical and clinical studies (38). Several studies have already explored the possibilities for gene transfer by viral vectors in ILP models (Table 2). All of these studies were performed in tumor bearing rats. Since only two different groups worldwide performed the studies perfusion conditions and surgical aspects were almost equal. Perfusions were carried at out during 15 minutes with a flow a 2.5 ml/min. The perfusate consisted of saline or colloid solution and was heated and oxygenated. Milas et al. demonstrated efficient gene delivery in tumor tissue via ILP using an adenovirus (39). Leakage to the systemic circulation was measured by radioactive labeled red blood cells. We performed ILP studies in which we quantified the local and systemic transduction rate compared to other routes of administration by LacZ and luciferase methods (40). Upon ILP tumor tissue showed 10-30 times more luciferase activity compared to all others tissue including muscle tissue of the perfused limb. So highly selective tumor transduction is possible by adenovirus based ILP. Moreover, the histochemistry showed remarkable differences in transduction pattern in tumor tissue. After intratumoral injection (IT) a needle track was clearly visible but after ILP a more homogeneous distribution of transduced cells was demonstrated especially at the viable rim of the tumor and around tumor associated vessels. Two studies have been performed to investigate anti-tumor activity of adenovirus mediated gene transfer. We previously performed ILP with an adenoviral IL3β construct driven by a CMV promotor and observed strong tumor growth inhibition (41). In 50% of the animals complete remission was achieved. Tumor response was not observed after intratumoral injection or by viral constructs with a weaker promotor. Due to expression of IL3β transient leucocytosis and fever was observed. We also studied an adenovirus construct carrying the Y28 gene that encodes a single chain antibody, which intracellularly blocks p21-*ras* oncogene activity (42). Efficient in vivo transduction was again demonstrated, however, in this rhabdomyosarcoma tumor model we were confronted with dose limiting limb edema. Dose escalation to higher doses then used intravenously was not possible and tumor response could not be demonstrated. Favorable transduction efficacy remains an important prerequisite in cancer gene therapy, but the anti-tumor response can still not be predicted and depends also on tumor and vector specific factors.

Author	Year	Animal	Tumor	Vector	Toxicity	Transduction of Tumor	Response
Milas	1997	rat	FS	AV.LacZ	Limb function[1]	+[2]	NA
de Roos	2000	rat	STS+OS	AV.LacZ/Luc	-	+[3]	NA
de Wilt	2000	rat	STS+OS	AV.IL3β	Leukocytosis[4]	ND	PR & CR
van Etten	subm	rat	RMS	AV.Y28[5]	Limb edema[6]	+	SD & PD

Table 2 Gene therapy by Isolated Limb Perfusion (ILP) in tumor-bearing animals .

Abbreviations: FS, fibrosarcoma; STS, soft tissue sarcoma; OS, osteosarcoma; RMS, rhabdomyosarcoma; AV, adenovirus serotype 5; NA, not applicable; ND, not determined; PR, partial response; CR, complete tumor response; SD, stable disease; PD, progressive disease.

Notes:
1. Local toxicity of the perfused limb. Oedema and impaired limb function, resolved within 72 hours.
2. Transduction of tumors in 66-75% of animals. No quantification applicable.
3. β-galactosidase histochemistry showed homogeneous transfection of tumors after ILP, preferentially at the viable rim. Lucifase activity after ILP significantly higher compared to systemic treatment, and similar to intratumoral injection.
4. Transient dose-dependent leukocytosis accompanied with fever and elevated serum histamine levels after ILP.
5. Y28 is a single chain intracellular antibody to p21-*ras*.
6. Limb edema after ILP during 24 hours, no impaired limb function.

Other isolated perfusion models

Isolated Pulmonary Perfusion (IPP).

Only a few other isolated perfusion studies are reported up till now (Table 3). Isolated pulmonary perfusions with chemotherapeutic agents have already demonstrated promising results in pre-clinical models in experimental treatment of primary lung tumors or pulmonary metastases (43-47). Lee et al. demonstrated IPP liposome mediated gene transfer (48). The IPP was performed in rats and was in fact a 2-step procedure. First 10 minutes infusion of 5 ml saline solution carrying the liposomes via the pulmonary artery, followed by a stopflow period of 30 minutes for incubation with the same solution. The effluent was collected via the pulmonary vein. No heating of oxygenation was applied. After 48 hours selective expression in lung tissue of the perfused lung was observed. Moreover the duration of expression was longer than after systemic administration. Expression lasted for at least 21 days (end of experiment), while in the systemic group no expression could be detected at this time point. Brooks et al. investigated two IPP methods in a tumor bearing rodent model with herpes simplex virus (HSV) vectors (49). In a rat sarcoma lung metastases model IPP was performed with rapid arterial infusion of 0.5 ml perfusate followed by an indwelling, stopflow period of 20 minutes with venous outflow occlusion. The second method was a low, continuous flow of

100 µl/min during 10 minutes without venous outflow occlusion. This study demonstrated that higher transduction rates could be achieved by continuous flow: 5% transduced pneumocytes and macrophages versus 20%. But in the stopflow group the HSV-LacZ perfusion resulted in a significant tumor response, which was also observed in the saline only and the HSV-TNF group. Prolonged ischemia probably induced tumor growth inhibition in this tumor model. In the continuous IPP saline perfusion showed the same growth rate as the untreated control tumors. However, there was no difference in tumor response between HSV-LacZ and HSV-TNF. Both resulted in a significant decrease of tumor nodules. Studies in large animals have been carried out by the group of Parpala et al.: several models have been explored in pigs: IPP, isolated kidney perfusion (IKP) and isolated splenic perfusion (ISP) (50-52). Regarding the IPP they perfused a single lobe of the right lung for 60 minutes with a adenovirus vector and observed transgene expression in different cell types trough out the perfused lobe (52).

Isolated Kidney Perfusion (IKP).

The kidney is another organ that lends itself quite easily for isolated perfusion from an anatomical point of view. In cancer research studies isolated kidney perfusion (IKP) has been performed mainly with anti-tumor agents (53-55), but also for genetic disorders or other renal diseases IKP has been studied. Heikkila et al. performed AV-LacZ mediated IKP with recirculation in pigs during 120 minutes with a heated and oxygenated Krebs-Ringer solution (50). Gene tranfer into glomeruli (estimated 75% positive glomeruli) was demonstrated without significant toxicity. Whereas this and previous reports on single injection of this vector only results in scattered transduction pattern of endothelial cells in the renal medulla (56). These findings again demonstrate the importance of long and continuous exposure the viral vector. Stephan et al have also investigated IKP with an adenovirus construct (57). After a 15-minute IKP with venous outflow occlusion in rats they observed a dose dependent predominantly endothelial cell transduction in peritubular capillaries and arterioles. Upon IKP with the highest virus concentration focal tubular necrosis was observed.

Isolated Cardiac Perfusion (ICP).

Isolated cardiac perfusion is technically a demanding isolated organ perfusion and for complete isolation a heart-lung machine is essential. Cardiac adenovirus mediated gene therapy is mostly delivered via the coronary arteries by means of balloon catheter techniques (58). Up till now there is only one study in the literature describing the technique of an in vivo

ICP for gene transfer (59). This study was performed in pigs, which underwent sternotomy and cardiopulmonary bypass. During hyperkalemic cardioplegia adenoviruses encoding the human β-adrenergic receptor (AV-AR) or the LacZ gene (AV-LacZ) were injected in the aortic root and dwelled into the coronary arteries during 30 minutes. ICP with AV-AR with resulted in a 4 times higher expression in the myocardial of the left ventricle tissue compared to AV-LacZ. A systemic control group was not included in this study. No inflammatory response could be demonstrated in histological studies of the myocard at 1 week after ICP. It is known that the β-adrenergic receptor enhances cardiac performance, however since the ICP was carried out on healthy young pigs no data were available on myocardial function after treatment.

Isolated Splenic Perfusion (ISP)

Isolated splenic perfusion for gene transfer has been reported in literature only once as well (51). In this study infusion of adenovirus expressing β-gal in the splenic artery is described. No gene expression was detected after this infusion, but after ISP significant expression was mainly observed in macrophages, which located around capillaries and in the red pulp.

Several regional treatment methodologies have also been studied in other organs. Selective intra-arterial delivery of virus vectors has been investigated for brain neoplasma, lung tumors or liver metastases. Vesical instillations with adenoviruses for patients with bladder cancer are already reported as well. Since these routes of administration are not true isolated perfusions they are are not within the scope of this review and will not be discussed in further detail.

Author	Year	Organ	Animal	Vector	Perfusion Time(min)	Recircu-lation	Washout	Toxicity	Transduction/ Outcome
Davidson	2001	Heart	pig	AV	30[1]	-	+	-	Myocard
Heikkila	1996	Kidney	pig	AV	120	+	-	-	Glomeruli
Stephan	1997	Kidney	rat	AV	15[2]	-	-	+[3]	Endothelium
Lee	1998	Lung	rat	Liposome	40	-	+	-	Perfused Lung
Brooks	2001	Lung[4]	rat	HSV	10-20	-	-	-	Tumorresponse
Parpala	2001	Lung	pig	AV	60	+	-	-	Perfused Lung[5]
Parpala	2001	Spleen	pig	AV	60	+	-	-	Macrophages

Tabel 3 Gene therapy in other organ perfusion models.

Abbreviations: AV, adenovirus; HSV, herpes simplex virus.

Notes:
1. Infusion in coronary arteries during cardioplegia and cardiopulmonary bypass.
2. Infusion with vein outflow occlusion, follow by a stopflow period.
3. Focal necrosis at highest dose.
4. Fibrosarcoma lung metastases model perfused with HSV-TNF inducing a partial tumor response.
5. Transgene expression in alveolar epithelial cells, capillary endothelial cells, airway epithelial cells and alveolar macrophages of the perfused lung 7 days after perfusion.

Conclusions and future directions

Locoregional gene therapy by means of isolated perfusion (IP) of the target organ with vectors is a new, but promising strategy in gene therapy studies. It offers possibilities to selectively transduce cells in vivo with higher efficiency than systemic administration. Moreover, unwanted side effects by transduction of tissues in the rest of the body are minimized. As reported IP can be used in gene therapy protocols for inborn errors of metabolism but also in cancer directed gene therapy. Most studies discussed above stress the importance of high local concentration of the viral vectors at the target side during a time period that is long enough to effectively reach and transduce the cells. The IP methodology offers both conditions and thereby achieves encouraging transduction rates. Next to high transduction efficacy successful gene therapy depends on many factors. IP not only increases the number of transduced cells in vivo, but also delivers the transgene in a more homogeneous way. As shown by de Wilt et al. IT results in equal percentage of transduced tumor cells as compared to ILP, nevertheless a tumor response upon IT was absent where ILP showed impressive tumor shrinkage. Besides transduction rates, gene delivery at the accurate site is a prerequisite in gene therapy. With respect to future developments IP is a treatment strategy in gene therapy protocols, which needs further study especially in optimizing the vector type that

has to be delivered as well as the various transgenes. Lans et al. recently reported ex vivo Endothelial Monocyte Activating Polypeptide-II (EMAP-II) gene therapy followed by ILP with TNF in tumor bearing rats (60). Ongoing studies are performed at this moment to explore in vivo delivery of the EMAP gene into the tumor cells and tumor associated vasculature thereby inducing a more TNF sensitive tumor. Combination treatment of gene therapy with cytokines or chemotherapeutic agents may lead to a more targeted therapy.

Regarding liver directed gene therapy it is unlikely that in case of retroviral gene delivery partial hepatectomy will be performed in patients. Non- or less- invasive techniques have to be developed as an alternative to surgically induced liver regeneration. Portal vein ligation can be effective as demonstrated by Rozga et al. (22). In humans this can be performed by percutaneous catheter techniques with selective embolisation of portal branches. Patijn et al. demonstrated an alternative method by means of adenovirus pre-treatment of the liver (61). By expressing a modified form of urokinase type plasminogen activator, which is toxic to hepatocytes, hepatocellular proliferation was induced during an eight-day period. Within this period continuous infusion of retroviruses was performed achieving a hepatocyte transduction rate of 33%. Liver proliferation can also be stimulated by growth factors like keratinocyte growth factor or hepatocyte growth factor (62,63). When these growth factors are used in combination with retrovirus gene therapy an increase of transduced hepatocytes can be achieved as well.

In adenovirus based gene therapy we are investigating the immune response upon IHP. Recently an impaired neutralizing antibody production was observed after IHP in rats (24). Manipulation of adenoviruses to reduce antigenicity is now performed on a wide scale and this may in combination with local delivery be a future option for repeated delivery (64-67).

These encouraging results in the field of gene delivery by IP are a strong argument for further research in the development of in vivo vector delivery.

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IV

Clinical experience with isolated limb perfusion.

8

Efficacy of Repeat Isolated Limb Perfusions (ILP) with Tumor Necrosis Factor-α and Melphalan for multiple in transit metastases in patients who failed prior ILPs.

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Submitted.

Summary

Isolated limb perfusion (ILP) is an effective treatment modality for multiple in-transit melanoma metastases confined to the limb. Recurrences after ILP however, occur in approximately 50% of patients and are a challenge for further treatment. The efficacy of repeat ILPs to prolong local control in this patient category is evaluated in this report.

A prospective database in tertiary referral center is analyzed. Out of 100 TNF-based ILPs with TNF and Melphalan (TM-ILPs) in melanoma patients between March 1991 and July 2003, 25 repeat ILP procedures were performed in 21 patients who failed prior ILP treatment. All patients had bulky and/or numerous lesions and were treated with a mild hyperthermic TM-ILP using 2-4 mg TNF and 10-13 mg/L limb volume for the leg and arm respectively.

Complete response (CR) rate was 76%, Partial Response (PR) occurred in 20% and No Change (NC) was recorded in 4%. There was no difference in CR rate or local toxicity between first and repeat perfusions. Local recurrence occurred in 72%; median time to local progression was 14 months. Five-years survival rate was 47%, which compares favorably with known survival rates of stage III A/AB patients.

Patients who fail after previous ILP treatment respond very well to repeat perfusion and prolonged local control can thus be obtained, especially in stage IIIA patients with a long recurrence-free interval and in patients failing Melphalan ILPs. The subgroup of patients qualifying for repeat ILP represents a relatively favorable biological behavior of the melanoma.

Introduction

Recurrent melanoma of the limb remains a treatment challenge. Many options, varying from simple surgical excision, to intralesional injections with cytokines, to isolated limb perfusion (ILP), have been proposed. Depending on the size and the number of the lesions, each modality is more or less suitable [1]. ILP for melanoma has demonstrated to be very effective in patients with multiple metastases where surgical excision is impossible. Melphalan-based ILP is associated with CR rates of 40-50% and overall response rates of 75-80%. When Tumor Necrosis Factor-α (TNF) was introduced in the isolated limb perfusion model by Lejeune and Lienard in 1988 [2], CR rates of 80-90% were reported. The first report on the European multicenter experience with TNF-based ILP in melanoma patients revealed that a significant

increase in CR rates was observed in the 4 participating centers of 91% with TNF and Melphalan (TM)-ILPs compared to 52% CR rate for Melphalan-only-ILPs [3]. By this treatment, disease control in the limb can be obtained in patient categories with a poor prognosis quod vitam [4]. However, local progression in the limb after ILP with Melphalan has been reported to develop in 46-54% of patients, and is 55% in our own experience in 100 TM-ILPs with predominantly high numbers of metastases and/or bulky disease (Grünhagen D.J. et al.: unpublished data). For this patient category, treatment options are limited. Some patients however, qualify for re-treatment of the limb when they fail only locally without evidence of systemic metastases. Particularly for these patients repeat ILP might provide prolonged local control [5-7]. The use of TNF in the repeat ILP is hypothesized to improve response rates in patients who do not, or insufficiently, respond to Melphalan-only-ILPs, but definitive prove is still lacking [6].

In this study, we report on 25 TM-ILPs for locally recurrent melanoma in patients who have failed previous ILP treatment, both with and without TNF. Special attention is given to local and systemic progression after repeat ILP and on the locoregional toxicity of the repeat procedure.

Patients and Methods

Patients.

Out of 100 TNF-based ILPs in 87 patients performed in our institution between 1991 and July 2003, 25 ILPs in 21 patients were repeat ILPs because of failure of previous ILP treatment for extensive in transit melanoma metastases. Of these 25 ILPs, 12 were performed after failure of Melphalan-based ILPs performed in referring hospitals, one after a TM-ILP elsewhere, and 12 ILPs were second (N=10) or third (N=2) TNF-based perfusions performed in our own institution. Tumor stage was assessed according to the MD Anderson staging system: 7 stage IIIA patients (IIIA: only in-transit metastases, no lymph node metastases or distant metastases), 15 IIIAB patients (IIIAB: both in-transit metastases and lymph node metastases, but no distant metastases) and 3 stage IV patients (IV: in-transit metastases and distant metastases). At the time of a second perfusion in our institution, progression of tumor stage had occurred in 6 patients. Local control of the in-transit recurrences by surgery was impossible due to numerous lesions (>10 lesions in 21 ILPs) and/or size of the lesions (>40

mm in 12 ILPs). Patient and tumor characteristics of the 25 repeat ILPs in 21 patients are summarized in Table 1.

Patient and tumor characteristics of 21 patients undergoing 25 repeat TM-ILPs			
Sex	Female Male	15 6	
Age (years), mean+range		60	(25-83)
Stage	IIIA IIIAB IV	7 15 3	
Stage shift	IIIA → IIIAB IIIAB → IV IIIA → IV	4 1 1	
No. of tumors	<10 10-50 >50	4 9 12	
Size of largest lesion (mm)	<40 mm >40 mm	13 12	
Prior treatment (apart from ILP)	None XRT CT Immuno XRT + CT	20 1 2 1 1	

Table 1 Patient and tumor characteristics of 25 repeat ILPs in 21 patients.

Abbreviations:
ILP=Isolated Limb Perfusion, XRT=Radiotherapy, CT=Systemic chemotherapy, Immuno=Immunotherapy

Treatment.

All patients underwent an ILP of the lower extremity, either by iliac (N=15), femoral (N=6) or popliteal (N=4) approach. The method of ILP has been described by us in detail previously [8,9]. In short: isolation of the limb is achieved by clamping and canulation of the major artery and vein, connection to an oxygenated extracorporeal circuit, ligation of collateral vessels and application of a tourniquet proximal to the site of perfusion. Once tissue temperature has reached 38 ºC, recombinant TNF (Boehringer Ingelheim GmbH, Ingelheim/Rhein, Germany) is administered via the arterial line in a dose of 2-4 mg (mean 3.6 mg). Tissue temperatures are stabilized between 38-39.5 ºC and leakage monitoring is performed by using a precordial scintillation probe to detect leakage of radiolabelled albumen injected to the perfusion circuit [10]. After 30 minutes, Melphalan (L-PAM, Alkeran, Wellcome Ltd., London, UK) is added to the perfusate in a dose of 10 mg/L for leg- and 13 mg/L for arm perfusions. In 6 ILPs, performed between 1991 and 1994, interferon γ (IFN) was added to the schedule according to trial prescriptions consisting of the subcutaneous injection of 0,2 mg IFN on days –2 and –1

prior to the ILP and the injection of 0.2 mg IFN during the ILP procedure into the arterial line prior to the administration of TNF. Median dose of Melphalan was 100 mg (mean 92.6, range 40-140), and all 6 IFN-ILPs were performed with 0.2 mg IFN. At the end of the perfusion period, a washout procedure using 2-4 liters of a dextrane and/or electrolyte solution is performed. In patients undergoing an iliac perfusion routinely an iliac lymph node dissection is performed; an axillary lymph node dissection is performed in patients undergoing an axillary ILP. In patients with palpable nodal disease in the groin an ilio-inguinal lymph node dissection is performed in the same operative session as the ILP, the iliac lymph node dissection prior to the ILP and the inguinal lymph node dissection immediately after the ILP.

Evaluation of response and toxicity.

Acute local toxicity of the ILP procedure was classified according to Wieberdink et al. [11]: (I) no reaction, (II) slight erythema or edema, (III) considerable erythema or edema with some blistering, slightly disturbed motility permissible, (IV) extensive epidermolysis or obvious damage to the deep tissues, causing definite functional disturbance, and threatening or manifest compartmental syndrome, and (V) reaction that may necessitate amputation. Response evaluation was performed 4, 8, 12 weeks after ILP by clinical examination, hereafter at 3 months regular intervals for the first 2 years and subsequently at longer intervals. Response rates were reported according to WHO criteria [12], in which complete response (CR) is the complete disappearance of all lesions and no new areas of disease appearing within the field of ILP. Partial response (PR) is defined as a reduction of 50-99% of the total tumor size; no change (NC) is recorded if <50% of the total tumor size responds. Recurrence of tumor within the extremity after a CR, or progression of the lesions and the appearance of new lesions after a PR or after NC, is reported as local progression.

Statistical evaluation.

Estimates of survival and local/systemic progression were made using the Kaplan and Meier method and differences were evaluated using the logrank test. P-values <0.05 were considered to be significant.

Results

Of the 25 repeat ILPs performed in our institution, 19 (76%) resulted in a complete response. With an additional 5 ILPs with a PR, total response rate was 96%. One patient (pt no. 12) showed regression of the lesions of 20-25%, which is recorded as NC. There was no difference in CR rate between repeat ILPs after prior treatment elsewhere (83%) and repeat TM-ILPs in our institution (69%, p=0.36). In the 12 patients receiving multiple TM-ILPs in our institution, no difference in response rate was detected between first (70%) and second or third ILP (75%, p=0.58). Response rates for the different patient categories analyzed are reported in Table 2. Median time between first and repeat ILP was 25 months (range 3-76).

The median leakage of the repeat ILPs was 0% (range 0-13%), leading to absence of severe (grade IV) systemic toxicity (Table 3). Local toxicity was mild to moderate in all cases with Wieberdink grade I in 12%, grade II in 60% and grade III in 28% of the ILPs, and not a singe case of grade IV toxicity. No increase in local toxicity was observed in the patients receiving multiple TM-ILPs (Table 4).

Compared to our total database of TM-ILP for melanoma, consisting of 100 ILPs, CR rate of repeat perfusions did not differ from the CR rate of patients undergoing only one ILP (76% vs. 68%, p=0.61). Local progression occurred after 18 repeat ILPs (72%). The median time to local progression (TTLP) was 14 months for repeat perfusions vs. 16 months for the overall population and 18 months for single ILPs (p=0.40, Figure 1). Sixteen patients developed systemic metastases during the course of follow up; 3 of whom were already stage IV at time of repeat ILP. Time to systemic progression (TTSP) did not differ between patients receiving single (12 months) and multiple ILPs (15 months, p=0.27). In 3 patients (no. 1,2,5), repeat ILP (+ resection of a local recurrence on the lateral side of the proximal thigh in patient no. 1) contributed to sustained local control of disease in the leg. No systemic progression was observed in these patients during follow-up of 91, 129 and 72 months, respectively. The 21 patients who received multiple ILPs had a 5-years survival of 47% vs. 28% for patients receiving single treatment (p=0.05, Figure 2).

Response Rates				
	Overall N=100	**Repeat** N=25	**Prior M-ILP** N=13	**TM-ILP repeats** N=12
CR	69/100=69%	19/25=76%	10/12=83%	9/13=69%
PR	26/100=26%	5/25=20%	2/12=17%	3/13=23%
NC	5/100=5%	1/25=4%		1/13=8%
Overall	94%	96%	100%	92%

Table 2 Response rates for the different patient categories.
Abbreviations:
M-ILP=Melphalan-only Isolated Limb Perfusion, TM-ILP=Isolated Limb Perfusion with TNF and Melphalan, CR=Complete Response, PR=Partial Response, NC=No Change

Local toxicity					
Wieberdink	**Grade I**	**Grade II**	**Grade III**	**Grade IV**	**Grade V**
	12%	60%	28%		
Systemic toxicity					
	Grade 0	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**
Neurologic*	96%	4%			
Liver*	100%	0%			
Renal*	100%	0%			
Haematologic*	96%	4%			
	<38ºC	38-39ºC	39-40ºC	>40ºC, <24 hrs	>40ºC, >24 hrs
Temp	36%	24%	32%	8%	
	Absent	Present			
Shock[&]	100%	0%			

Table 3 Local and systemic toxicity after TM-ILP.
Abbreviations:
Hrs=hours, Temp= temperature.
Notes:
*= WHO criteria
[&]= support of vasopressors needed

Pt no	Prior else-where	interval	1st TM-ILP			interval	2nd TM-ILP			interval	3rdTM-ILP		
		months	level	resp	tox	months	level	resp	tox	months	level	resp	tox
1	yes	14	Fem	CR	2	25	Iliac	PR	2				
2	yes*	29	Poplit	CR	2								
3	no		Iliac	CR	3	11	Iliac	CR	2	26	Iliac	CR	2
4	no		Poplit	PR	2	19	Iliac	CR	3				
5	yes*	42	Fem	CR	2	18	Poplit	CR	2	42	Fem	CR	2
6	yes&	76	Fem	CR	3								
7	no		Iliac	CR	4	9	Fem	CR	3				
8	no		Iliac	CR	2	58	Poplit	PR	2				
9	yes	47	Iliac	CR	3								
10	yes	40	Iliac	CR	1								
11	no		Iliac	CR	1	9	Iliac	CR	2				
12	yes$	28	Iliac	NC	2								
13	yes	4	Poplit	CR	2								
14	yes#	28	Iliac	CR	1								
15	no		Iliac	CR	2	30	Iliac	CR	2				
16	no		Fem	PR	2	13	Iliac	PR	2				
17	yes	36	Iliac	CR	3								
18	yes	17	Iliac	PR	3								
19	no		Iliac	PR	3	15	Iliac	CR	3				
20	yes	5	Fem	CR	2								
21	yes!	3	Iliac	PR	1								

Table 4 Patient characteristics, responses and toxicity after repeat ILP in 21 patients.

Abbreviations:
TM-ILP=Isolated Limb Perfusion with TNF and Melphalan, Fem=Femoral, Poplit=Popliteal, Iliac=Iliacal, CR=Complete Response, PR=Partial Response, NC=No Change, resp=response, tox=toxicity.

Notes:
*= previous ILP: M-ILP double schedule
&= adjuvant ILP
$= prior TM-ILP
#= 2 previous ILPs: 1st adjuvant, 2nd cisplatin
!= 3 previous ILPs

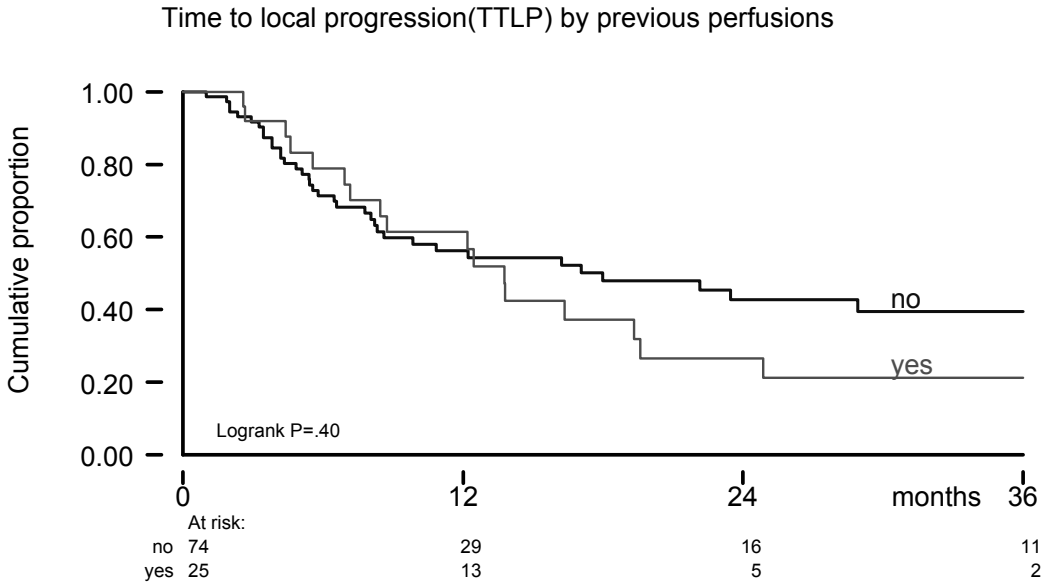

Figure 1 Time to local progression (TTLP), repeat (=Yes) vs. single (=No) ILPs
X-axis: time (months)
Y-axis: cumulative percentage

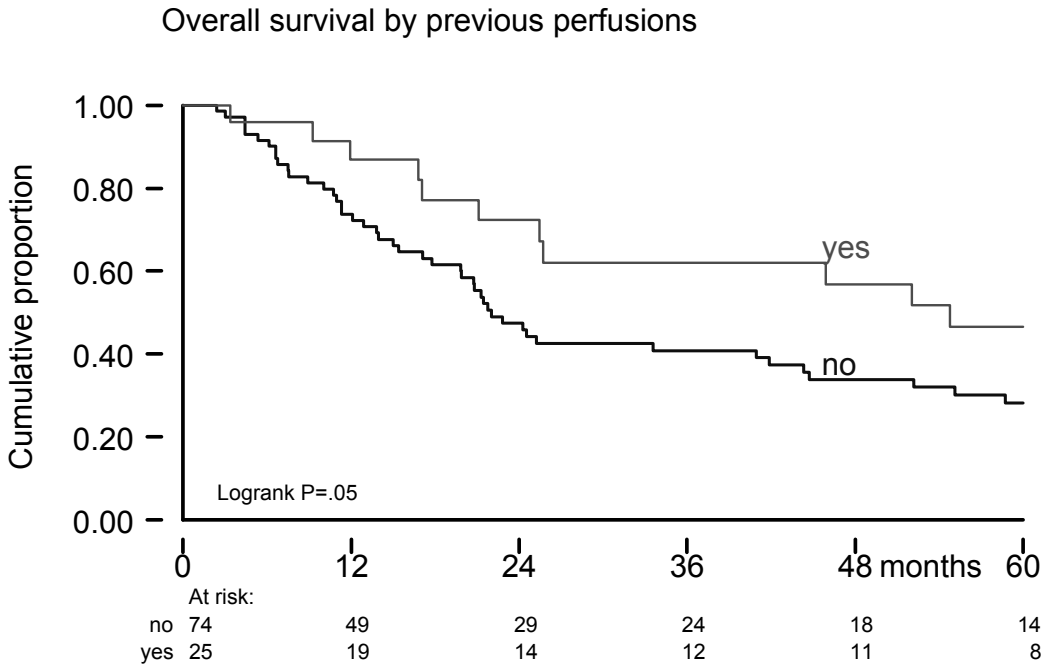

Figure 2 Overall survival (OS), repeat (= Yes) vs. single (=No) ILPs
X-axis: time (months)
Y-axis: cumulative percentage

Discussion

These results show that in a selected patient population of patients with local progression after previous ILP treatment, repeat TM-ILP is associated with excellent response rates (96%). The complete response rate after TM-ILP was 76%, which compares favorably with the CR rate of single ILPs in our institution. This is in line with the scarce literature on the efficacy of repeat ILPs [5-7] and indicative of the fact that a rather favorable group of patients, mostly patients with stage IIIA disease only and with a good response to the first ILP, are considered for repeat ILPs when they recur with in-transit metastases. The 5-years survival rate of 47% reported in this study compares favorably with recently published survival rates of patients with locally advanced melanoma (23-47% [4]) and is again an indication of relatively mild biologic behavior of the melanoma in this patient population.

The mechanism of recurrence after previous ILP treatment remains speculative. It is a common finding that in patients after a CR to the first perfusion, the new recurrent in-transit metastases arise at different locations than the original lesions that went into a CR after the initial ILP. Hypothetically this indicates that larger, well-vascularized lesions are completely eradicated but that dormant, (relatively) non-vascularized lesions are not eradicated by an ILP, because of inadequate drug exposure [13].

Patient selection for repeat ILP treatment is determined by the time to local progression after initial ILP and the absence of systemic metastases, unless the bulkiness of the disease is directly limb-threatening. Rapid disease progression after ILP is an indicator for aggressive disease and is therefore a relative contraindication for repeat ILP treatment, as the expected response rates are low [14]. The median interval between initial ILP treatment and repeat TM-ILP of 25 months, as indicated in table 4, is markedly longer than the median time to local progression both in this study after repeat ILP (14 months) and in our total database of 100 ILPs (16 months). In three patients (pt no. 13, 20, 21) repeat TM-ILP was performed 3-5 months after failure of ILP with other chemotherapeutics in order to obtain sufficient response, which resulted in 1 PR and 2 CRs. This shows that failure to respond to a M-ILP can be overcome by adding TNF, which in tumor models has been shown to enhance the uptake of Melphalan by the tumor [15, 16]. This is in line with the observation that Melphalan-only ILPs in STS patients is very poor, whereas TM-ILPs are associated with high response rates [3]. For our melanoma population we can conclude that, apart from these three patients with rapid failures after a M-ILP, the melanoma of patients qualifying for repeat treatment is

relatively indolent. This observation is sustained by the significant difference in overall survival between repeat- and single ILPs.

Whether to consider a patient for repeat ILP should be influenced by the stage of disease at first ILP. We found in our experience with 100 TM-ILPs that curves of TTLP and TTSP are virtually overlapping in stage IIIAB disease, whereas in stage IIIA patients, median TTSP was 55 months at a median TTLP of 18 months. This difference makes IIIA patients with local progression much better candidates for repeat ILP than IIIAB patients (Grünhagen D.J. et al.: unpublished data).

We found toxicity after repeat TM-ILPs to be mild to moderate in all performed procedures. Compared to the first ILP, repeat TM-ILP did not increase local toxicity, in contrast to a previous report using Melphalan alone [5]. In this report however more intensive schedules were used for the repeat ILP (higher tissue temperatures, ILPs at short intervals [5]. Other, smaller studies, do not report on increased local or systemic toxicity [6, 7]. On the basis of these data, we state that the use of mild hyperthermic TM-ILP for repeat perfusion is a safe procedure. Median leakage to the systemic compartment was zero percent in these 25 repeat ILPs, just like in our overall experience with TM-ILP in over 350 patients. TM-ILP is a safe procedure, provided adequate leakage monitoring is assured, and postoperative fluid management is generous to deal adequately with the TNF-mediated transient drop in the peripheral vascular resistance after ILP [17,18]. Moreover, the safety of the procedure is such that in our opinion there should be no age limit. Especially in limb threatening disease, limb salvage is of prime importance and this treatment should not be withheld from the elderly as we have demonstrated in our extensive experience in patients over 75 years old [19].

In conclusion, repeat TM-ILP is a valuable treatment option in patients with local progression after previous ILP treatment. In patients with bulky or numerous lesions that cannot be managed with surgery, repeat TM-ILP provides excellent disease control. Especially patients with recurrences after ILP for stage IIIA disease and patients with a long interval between initial and repeat ILP, who form a subcategory of melanoma patients with relatively favorable characteristics, profit from this treatment. Moreover patients with no response or rapid progression after a Melphalan-only ILP can still respond with a CR to a TM-ILP.

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Fifty TNF-based isolated limb perfusions for limb salvage in patients older than 75 years with limb threatening soft tissue sarcomas and other extremity tumors.

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Summary

Isolated limb perfusion (ILP) with TNF and Melphalan results in response rates greater than 80% and limb salvage rates above 70% in limb threatening soft tissue sarcoma (STS). Also in the management of multiple and bulky in-transit melanoma metastases the use of TNF improves complete response rates. Because of fear for TNF associated toxicity, ILP with TNF is not offered to old patients in some cancer centers, whilst especially in older patients an amputation may end their independence and completely limit their mobility. Thus every attempt to avoid amputation must be considered in these patients. We evaluated our experience in patients older than 75 years treated by TNF-based ILP.

Out of a total of 306 TNF-based ILPs between 1991 and 2001, 50 ILPs were performed for limb salvage in 43 patients older than 75 years (range 75-91). In 27 patients for STS or other sarcomas, in 2 for large squamous cell carcinomas and in 14 patients for multiple or bulky in-transit melanoma metastases.

In the STS group an overall response rate of 76% and a limb salvage rate of 76% was achieved at a median follow-up of 18.0 months. In the melanoma patients a 100 % response rate and a 93% limb salvage rate was observed. Local toxicity was mild to moderate (Wieberdink grade I-III) in 96% of the perfusions. The 3 postoperative deaths that have been observed in the total series of 306 TNF-based ILPs in Rotterdam (< 1%) have all 3 occurred in patients > 75 years old, due to cardiovascular events in 2 high-risk patients and due to sepsis in 1 patient with an enormous infected necrotic ulcerating tumor. These deaths all occurred after leakage free ILP's and were considered not to be associated to TNF.

Old patients should not be withheld a TNF-based ILP for limb salvage as also in these patients the procedure is safe and highly effective.

Introduction

Isolated limb perfusion (ILP) is a technique of cancer treatment which delivers high doses of cytostatic drugs to a tumor-bearing extremity that is isolated from the systemic circulation and connected to a heart-lung machine (1). By ILP regional concentrations of chemotherapeutic agents 15-20 times higher than those reached after systemic administration can be achieved without systemic toxicity (2). In the management of locally advanced soft tissue sarcoma

(STS) limb salvage is a major challenge. In patients with multiple and or bulky in-transit melanoma metastases obtaining local control is a major challenge. In contrast to the inefficacy of ILP with Melphalan alone in the management of irresectable extremity STS, with response rates between 10 and 35% only (3,4), the introduction of tumor necrosis factor-alpha (TNF) in combination with Melphalan in ILP has been reported in a European multicenter trial to result in response rates greater than 80% and limb salvage rates above 70% in STS (5,6). These results led to the approval of TNF in Europe (7). Also in the management of in-transit metastases TNF seems to improve CR rates (8), especially in patients with bulky tumors and in patients that have failed a previous ILP with Melphalan alone, as has been demonstrated by the reports from the Surgery Branch of the NCI in the USA (9,10).

The incidence of STS increases rapidly above the age of 50. About 18 % of the patient population is above the age of 70 (4). The incidence of malignant melanoma has more than doubled over the last 20 years (12). For both men and women rates increased relatively more in the older age groups. As a consequence of these epidemiological features we are confronted with a large group of elderly patients who might need a limb saving procedure such as ILP. Especially these patients will be severely limited in their mobility and loose independence when amputation of a limb has to be performed. Therefore especially in the elderly every attempt to avoid amputation must be considered and a TNF-based ILP must be considered in spite of the age of the patients. The perception that TNF-based ILP is a risky endeavor has been demonstrated to be false provided leakage is monitored and well controlled (13). But even in the event of significant leakage we have demonstrated that with correct fluid management and postoperative care patients do not develop major or life threatening complications (14). Therefore we do not think it is reasonable to consider age a limiting factor for patients to undergo a TNF-based ILP. Here we present our single Rotterdam institution experience in a large series of patients aged above 75 years with irresectable STS or in-transit melanoma metastases of an extremity. These patients were all treated by ILP with high dose TNF and Melphalan.

Patients and methods

ILP methodology

The procedure of the current TNF-based ILP has been described by us previously (1,2). In brief, isolation of the blood circuit of a limb is achieved by clamping and canulation of the major

artery and vein, connection to an oxygenated extracorporeal circuit, ligation of collateral vessels and application of a tourniquet. Once isolation is secured, drugs can be injected into the perfusion circuit. Drugs: Recombinant human Tumor Necrosis Factor-α (TNF, 4.9-5.8 x 10^7 U/mg) was administered at doses of 2-4 mg in the perfusion circuit; recombinant human Interferon-gamma (IFN: 0.2 mg or 1.5 x 10^6 U/ampoule) was administered in the ILPs performed in period 1991-1993 on days –2 and –1 subcutaneously and on day 0 into the perfusion circuit. Both TNF and IFN-gamma were obtained from Boehringer Ingelheim GmbH, Ingelheim/Rhein, Germany. Melphalan (L-phenyl-alaninemustard = L-Pam, Alkeran^R) was obtained as a sterile powder (100 mg) that was dissolved aseptically using solvent and diluent provided by Burroughs Wellcome (London, England). Because of its efficacy and low regional toxicity profile Melphalan is the standard drug, most commonly used a dose of 10 mg/L perfused tissue for a leg and at 13mg/L for an arm. The perfusate is heated to 39-40 ^{0}C to achieve mild hyperthermia tissue temperatures in the limbs to be kept stable at 39 ^{0}C. Radiolabeled albumen is injected into the extra corporeal circuit so leakage into the systemic circulation can be detected with a precordial scintillation probe (8). Leakage monitoring is mandatory especially now that high doses of TNF are used in the treatment of soft tissue sarcomas and melanomas. After 1.5 hours of perfusion the limb is rinsed with an electrolyte solution, cannulas are removed, and the vessels are repaired. Classification of postoperative limb toxicity is done according to Wieberdink *et al* (9): (I) No reaction; (II) Slight erythema and/or edema; (III) Considerable erythema and/or edema with some blistering, slightly disturbed motility permissible; (IV) Extensive epidermolysis and/or obvious damage to the deep tissues, causing definite functional disturbances; threatening or manifest compartmental syndrome; (V) Reaction which may necessitate amputation.

Patients

At the Erasmus University Medical Center – Daniel den Hoed Cancer Center a total of 306 TNF-based ILPs were performed in STS patients (224 ILPs) and melanoma patients (82 ILPs) between 1991 - 2001. In 43 patients (14 male and 29 female) older than 75 years 50 TNF-based ILPs were done. Twelve patients also received 0.2 mg of IFN-gamma subcutaneously on the two days prior to the ILP as well as 0.2 mg IFN-gamma in the perfusion circuit during ILP. All ILP and follow up data in our institution are recorded prospectively in a database. Patient, ILP characteristics, treatment results, and follow-up data are listed in Table 1 for STS or other non-melanoma limb threatening tumors and in Table 2 for melanoma. The mean age of this selected group of patients was 79 (range 75-91) years. Twenty-nine patients with

irresectable STS or other limb threatening tumors of the leg (n=19) or arm (n=10) underwent 34 ILP's (Table 1). Nineteen were treated for primary tumors and ten for recurrences. In this group the median size of single tumors (n=20) was 10 cm (range 6-38) and for multifocal tumors (n=9) 5 cm (range 1-20). Two patients with Stuart Treves syndrome have undergone 3 ILP's each. One patient underwent two perfusions. Fourteen patients with in-transit melanoma metastases (all lower extremity) underwent 16 ILP's (Table 2). Two patients underwent 2 ILP's.

Results

Sarcomas and non-melanoma limb threatening tumors (Table 1)

Response rates in the STS group: all patients but 2 (post operative deaths) were evaluated for response by MRI and histopathologic evaluation of tumor resection specimen of after response to the ILP: Complete responses were defined as clinical MRI-proven CRs, which were not operated on a second time and clinical MRI-proven PRs that after resection were demonstrated to be 100% necrotic and classified as histopathologic complete responses; Partial responses were defined as tumors that underwent shrinkage of greater than 50%, or in case of less than 50% clinical shrinkage revealed a > 50% necrosis upon resection. The overall response rate (CR+PR) was 76%, consisting of 38% CR, 38% PR. No change (NC) was defined as < 50% tumor shrinkage and < 50% necrosis, was seen in 18% of the patients. None of the patients had PD of the tumor within 3 months of the ILP.

In the soft tissue patient population 31 % of patients had multiple sarcomas and were not candidates for resections after ILP. In the patients with a single sarcoma resection was made possible by the induction treatment with ILP and performed in 16/20 patients at a median of 2.5 months after ILP. ILP and surgical treatment resulted in limb salvage in 22/29 patients (76%) after a median follow-up of 18 months. Amputation was performed in 7/29 patients. In 3 patients repeated ILP was performed after a median period of 20 months (range 13-24) after prior ILP.

Pat. No.	Sex	Age (yrs)	Histology	Grd 1-3	Site	No. Tum	P/ R	Type ILP	Loc. Tox. 1-5	Clin. Resp	Hist. Resp. (% necr)	Final Out-come	Limb Salv.	Dead or Alive	F-up (mo)
1*	V	78	Angiosarcoma	2	Lo Arm	>100	R	Brach	2	CR	ND	CR			
	V	80	Angiosarcoma	2	Lo Arm	>100	RR	Axil	2	CR	ND	CR			
	V	82	Angiosarcoma	2	Lo Arm	>100	RR	Axil	3	PR	ND	PR	Y	D	47
2	V	75	Liposarcoma	1	Fo Arm	1	R	Brach	3	CR	ND	CR	Y	A	85+
3	V	82	Leiomyosarc	2	Up Arm	1	P	Brach	2	PR	>70	PR	Y	D	57
4	V	82	Mal Fibr Hist	3	Up Leg	1	P	Iliac	3	NC	ND	NC	Y	D	6
5	V	83	Liposarcoma	3	Up Leg	2	P	Iliac	2	PR	>90	PR	Y	A	55+
6*	V	81	Mal Fibr Hist	2	Lo Leg	4	R	Popl	2	NC	ND	NC			
	V	82	Mal Fibr Hist	2	Lo Leg	6	RR	Fem	2	CR	ND	CR	N	D	22
7	V	78	Pleiomorfsarc	3	Fos Popl	1	R	Iliac	2	NC	ND	NC	N	D	14
8†	M	91	Liposarcoma	3	Fos Popl	1	P	Iliac	2	ND	ND	ND	Y	D	0
9	V	87	Leiomyosarc	2	Foot	1	R	Popl	2	NC	ND	NC	Y	D	8
10	M	76	Leiomyosarc	3	Fo Arm	1	P	Brach	4	PR	90	PR	Y	A	43+
11	M	75	Clear cell tum	3	Up+Lo Leg	3	R	Iliac	3	CR	100	CR	Y	D	9
12	V	75	Mal Fibr Hist	2	Lo Foot	1	R	Fem	3	NC	>90	PR	Y	D	3
13	M	83	Extraskel Osteo	3	Up Leg	1	P	Iliac	3	NC	90	PR	Y	D	9
14†	M	76	Squamous cell	NA	Fo Arm	3	R	Axil	4	ND	ND	ND	Y	D	0
15	V	77	Leiomyosarc	3	Fos Popl	1	P	Popl	2	PR	90	PR	Y	A	39+
16*	V	77	Angiosarcoma	2	Fo Arm	30	P	Brach	3	CR	ND	CR			
	V	78	Angiosarcoma	2	Up+Lo Arm	20	R	Axil	2	CR	ND	CR			
	V	80	Angiosarcoma	2	Up+Lo Arm	>10	RR	Axil	1	CR	ND	CR	Y	A	46+
17	V	83	Mal Fibr Hist	3	Lo Leg	3	R	Popl	2	PR	<50	PR	N	D	26
18	V	76	Angiosarcoma	1	Fo Arm	21	P	Brach	3	CR	100	CR	Y	A	21+
19	M	83	Synoviosarc	2	Up Leg	1	R	Iliac	2	CR	ND	CR	N	A	28+
20	V	76	Schwannoma	3	Up Leg	1	P	Iliac	-	NC	<50	NC	Y	A	26+
21	M	79	Schwannoma	3	Fo Arm	1	P	Brach	2	PR	<10	PR	Y	A	10+
22	V	77	Kaposisarcoma	1	Lo Leg	30	P	Fem	1	CR	100	CR	N	A	18+
23	M	81	Mal Fibr Hist	3	Fo Arm	1	P	Brach	1	NC	<50	NC	Y	A	17+
24†	M	76	Undif Carc	2	Up Leg	1	P	Iliac	2	CR	100	CR	N	D	0
25	M	75	Liposarcoma	3	Fos Popl	1	P	Iliac	2	NC	>50	PR	N	A	11+
26	M	81	Epitheloid sarc	3	Lo Leg	1	P	Fem	1	PR	>90	PR	Y	A	8+
27	V	78	Angiosarcoma	1	Tot Arm	>20	P	Axil	2	CR	ND	CR	Y	A	6+
28	M	80	Squamous cell	NA	Hand	1	P	Brach	2	PR	ND	PR	Y	A	3+
29	M	86	Mal Fibr Hist	3	Lo leg	1	P	Fem	2	PR	ND	PR	Y	A	3+
		79.5	mean										22/29		26.0
		78	median										76%		18.0

Table 1 Characteristics of 29 patients with irresectable STS or other limb threatening tumors at the time of ILP (n=34) with TNF and Melphalan.

Abbreviations: Grd=Grade; Up Arm=upper arm; Lo Arm= lower arm; Up Leg= upper leg; Lo Leg= lower leg; Fos Popl= fossa poplitea; Iliac= Iliac ILP; Fem= femoral ILP; Popl= Popliteal ILP; Axil= Axilar ILP; Brach= brachial ILP; P= primary; R= recurrence; RR= re-recurrence; CR= complete response; PR= partial response; NC= no change; NA = not applicable; ND= not determined. Loc. Tox.=Local toxicity in Wieberdink grade; Clin. Resp= Clinical response; Limb Salv.=limb salvage; F-up=follow-up; mo=months.

Notes:
Overall Response rate is 76% (CR 38%, PR 38%).
* These have undergone multiple perfusions.
† Patients died postoperatively.

Patients with multiple or bulky melanoma in transit metastases (Table 2)

In the melanoma patients an overall response rate of 100% was achieved. Consisting of 75% CR and 25% PR. So all patients responded and no NCs or PDs were observed. This resulted in 93% limb salvage at a median follow-up of 16 months. The single amputation in this group (#4, Table 2) was non tumor related but due to severe arteriosclerosis more than 14 months after the

last ILP. Two patients underwent a second perfusion 19 and 25 months after the first ILP respectively. Both resulted in a CR after the repeated ILP. In the melanoma patients no postoperative deaths occurred.

Patient No.	**Sex**	**Age** (yrs)	**Site**	**No. Tum**	**Type ILP**	**Local Tox.** (Wieberdink) **1-5**	**Final Outcome**	**Dead or Alive**	**Follow-up** (months)
1*	V	78	Lo Leg	>20	Fem	2	CR		
	V	80	Tot Leg	>100	Iliac	2	CR	A	132+
2	V	76	Tot Leg	>100	Iliac	2	CR	D	10
3	V	75	Lo Leg	>10	Popl	3	CR	D	4
4*#	V	82	Lo Leg	>20	Popl	2	PR		
	V	83	Lo Leg	>100	Iliac	3	CR	D	44
5	V	77	Lo Leg	>10	Iliac	2	PR	D	11
6	V	76	Tot Leg	>50	Iliac	2	CR	D	45
7	V	75	Lo Leg	>50	Iliac	1	CR	D	21
8	V	76	Lo Leg	<10	Popl	2	CR	D	16
9	V	80	Tot Leg	>10	Iliac	2	CR	A	46+
10	V	78	Lo Leg	>10	Fem	2	CR	A	40+
11	V	79	Foot	>50	Fem	2	CR	A	17+
12	V	82	Lo Leg	>20	Fem	3	PR	A	3+
13	M	77	Lo Leg	>20	Iliac	3	PR	A	3+
14	V	91	Lo Leg	>100	Fem	2	CR	A	3+
Mean		78.7					CR 75%		27.0
Median		78					PR 25%		16

Table 2 Characteristics of 14 patients with in-transit melanoma metastases at time of ILP (n=16) with TNF and Melphalan.

Abbreviations: Up Leg= upper leg; Lo Leg= lower leg; Tot Leg =total leg; Iliac= Iliac ILP; Fem = femoral ILP; Popl = Popliteal ILP; CR= complete response; PR= partial response; NC= no change.

Notes:
Overall Response rate is 100% (CR 75%, PR 25%).
* Patients that have undergone multiple perfusions (n=2).
# This patient underwent an above knee amputation of the limb because of arteriosclerosis more than one year after the last perfusion.

Local toxicity

In 96% of the perfusions only mild to moderate (Wieberdink grade I-III) local toxicity was observed. In none of the patients an amputation had to be performed due to TNF-Melphalan related toxicity after ILP. Only in the patient with a large ulcerating undifferentiated carcinoma metastasis in the upper leg, that became completely necrotic after the ILP and subsequently got infected, a post-ILP amputation had to be undertaken as it was the source of (eventually fatal) sepsis.

Leakage Control and Systemic Toxicity

Leakage Control.

Median leakage rate was 0% (range 0-20%) in the 50 ILPs described in this series.

In the "sarcoma group" of 34 ILPs there were 26 ILPs with 0% leakage, 5 with < 7% leakage and 3 ILPs with significant leakage of 19, 20 and 20%

In the "melanoma group" of 16 ILPs there were 10 ILPs with 0% leakage and 3 with < 5% leakage. There were 3 ILPs with significant leakage of 10, 12 and 13 % .

None of these six patients with 10-20% leakage experienced grade 3-4 hypotension or any other form of grade 3-4 toxicity.

Systemic toxicity.

Most patients went through a slightly hyperdynamic postoperative period, that lasted usually 2-6 hours, with slightly increased heart rates and elevated temperature as a reaction to the circulating TNF immediately after the ILP. No major toxicity was observed in the immediate postoperative phase in any of the 43 old patients.

One patient with a history of multiple myocardial infarction and angina pectoris (# 19, Table 1) developed a postoperative myocardial infarction. The ILP was associated with a leakage rate of 3% and the postoperative course was associated with grade 2 hypotension.

Except in the patient (# 8, Table1) who developed a thrombosis of the mesenteric artery 3 days after the ILP and in the patient (# 24 Table 1), who developed a septic shock, because of infection of the massive necrosis of an large ulcerated tumor mass, 10 days after the ILP, no grade 2, 3 or 4 toxicity was observed with respect to liver function, renal function or shock like symptoms. Temporary mild fever (WHO grade II-III) occurred after 32 of the 50 perfusions.

Complications

In the total series of 306 TNF-based ILPs performed between 1991 and 2001 in the Daniel den Hoed Cancer Center 3 patients have died from postoperative complications. All 3 patients are in the above 75 years old patient category. Two patients with a high risk cardiovascular status and prior history died because of cardiovascular complications: The 91 year old patient # 8 in Table1, developed a thrombosis of the mesenteric artery 3 days after the ILP with fatal outcome. The 76 year old patient # 14 of Table 1, with a history of prior cerebrovascular accidents died from a massive CVA on day 3 after an uneventful and leakage free ILP. A cardiovascular complication also occurred in 83-year-old patient # 19 of Table 1. This patient had a history of multiple myocardial infarctions and angina pectoris developed a postoperative myocardial infarction after yet another well-controlled ILP with a leakage rate of 3% and no

significant postoperative hypotension. The third patient who died in the postoperative phase (# 24, Table 1) developed immediate and total necrosis of a very large ulcerating undifferentiated carcinomatosis metastasis in the upper leg. This necrotic mass got infected and the patient developed a septic shock. In spite of an amputation and optimal medical management the patient died of sepsis.

Discussion

In elderly people amputation of a limb has major impact on their independency and daily activities. However, exactly older patients with irresectable STS or recurrent melanoma of the extremities are often subjected to amputation, because the risk of an ILP is considered to be too high. Here we demonstrated that ILP is very efficient in achieving limb salvage and local tumor control in elderly patients, with acceptable morbidity and mortality. In the face of imminent amputation or unacceptable loss of control over locoregional tumor growth in melanoma patients we have not turned down any elderly patient above the age of 75 years for an TNF-based ILP. Thus we have accepted the considerable risk associated with this position. Here we report on the largest single institution experience on the efficacy and risks of TNF-based ILP in elderly patients. Out of 306 TNF-based ILPs, 50 ILPs were performed in this patient population with the goal to avoid amputation, which more than in any other age group determines the quality and independence of how these patients can continue their lives.

Until several years TNF was thought be inapplicable in a clinical setting because of its severe toxicity when administrated systemically. However, in an isolated perfusion setting it can be used in very safe way and is extremely efficient in inducing tumor responses when combined with cytostatic agents. We have demonstrated that this extremely potent synergy is based on the selective vasodestruction of the tumor associated vasculature (5,17) as well as on the greatly enhanced uptake of Melphalan or any other chemotherapeutic drug in the tumor when combined with TNF in the ILP setting (18,19)

Safe administration of TNF is guaranteed since we use continuous leakage monitoring and adequate hydration of patients. These components are both essential in the management of possible TNF related toxic side effects (10). The median leakage during ILP was < 0% (range 0-20). Even in cases with leakage this can be management very well. We described previously a series of patients with leakage from 12-65%. No fatal complications occurred and only a few shocks were observed which could be management without intubation and with little need for

vasopressor support (11). Also in case of leakage adequate hydration turned out to be the key in the treatment of TNF related toxicity.

Also in this group of older patients ILP can be performed safely without severe toxicity and morbidity. In this patient group, with a mean age of 79 year, the overall perioperative mortality was low and the local toxicity was in the vast majority of patients Wieberdink grade II or III. Vrouenraets et al. recently reported on regional toxicity after ILP with TNF and demonstrated that increasing age was not correlated with more severe toxicity (12). The median hospital stay in this patient population of elderly patients, after ILP is about 2 weeks. In the postoperative period most patients were completely mobilized and left the hospital without severe functional impairment.

The response rates and limb salvage percentage demonstrated in these is comparable to ILP studies reported in younger STS patients (1). In melanoma patients, especially the locally advanced bulky tumors may cause a lot of pain, hemorrhage or vascular compression that amputation of the affected limb sometimes has to be considered. ILP with TNF and Melphalan is an effective method to achieve local tumor control and thereby is of great anti-morbidity value. It is regarded as the treatment of choice in bulky cases of melanoma or failures after ILP with Melphalan alone. The 3 deaths reported in this series are the only 3 postoperative deaths that we have encountered in the 306 patients, treated by TNF-based ILP in Rotterdam between 1991 – 2001 and reflects the risks that have been accepted in this patient population. Two patients had extensive cardiovascular disease. The third patient died from sepsis, due to the infection of a totally necrotic massive tumor in the upper leg after ILP, which was confirmed to be a histopathological CR. The fact that this rapid tumor had penetrated the skin and was ulcerating should have kept us from performing the perfusion, since there was no way that infection of this necrotic mass could have been prevented. The fact that the complications occurred after leakage free ILPs further underlines the point that the complications were due to patient-specific risks and not to uncontrolled elements of the ILP.

This series of older patients with limb threatening sarcomas or multiple in-transit melanoma metastases demonstrates the safe and efficient use of TNF -based ILP. It results in high response rates and thereby high limb salvage rates and very good local tumor control rates in the melanoma patients. Old patients should be offered and not be withheld a TNF-based ILP for limb salvage.

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10

Isolated limb perfusion: an overview of current uses in the clinic and laboratory developments.

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Isolated Limb Perfusion (ILP)

ILP methodology

The technique of isolated limb perfusion was pioneered by Creech, Krementz and coworkers at Tulane University in New Orleans (1). Regional concentrations of chemotherapeutic agents 15-25 times higher than those reached after systemic administration can be achieved by ILP in the tumor bearing extremity without systemic side effects (2). Isolation of the blood circuit of a limb is achieved by clamping and canulation of the major artery and vein, connection to an oxygenated extracorporeal circuit, ligation of collateral vessels and application of a tourniquet. Once isolation is secured, drugs can be injected into the perfusion circuit. Because of its efficacy and low regional toxicity profile Melphalan (L-phenyl-alaninemustard) is the standard drug, most commonly used a dose of 10 mg/L perfused tissue for a leg and at 13mg/L for and arm (3). Tissue temperatures in the limbs are monitored and radiolabeled albumen or erythrocytes is injected into the extracorporeal circuit so leakage into the systemic circulation can be detected with a precordial scintillation probe (4). Leakage monitoring is mandatory especially now that high doses of tumor necrosis factor-alpha (TNF) are used in the treatment of soft tissue sarcomas. After 1-1.5 hours of perfusion the limb is rinsed with an electrolyte solution, cannulas are removed, and the vessels are repaired. Classification of acute tissue reactions after perfusion is done according to Wieberdink *et al* (5): (I) No reaction; (II) Slight erythema and/or edema; (III) Considerable erythema and/or edema with some blistering, slightly disturbed motility permissible; (IV) Extensive epidermolysis and/or obvious damage to the deep tissues, causing definite functional disturbances; threatening or manifest compartmental syndrome; (V) Reaction which may necessitate amputation.

ILP for in-transit melanoma metastases

In-transit metastases occur in 5-8% of melanoma patients. Treatment options are primarily dictated by the number and size of in transit metastases, and the great variety in aggressiveness that this form of metastatic melanoma may exhibit. Thus it may vary from simple excision(s), to evaporation of the lesions by carbon-dioxide laser, to isolated limb perfusion (6). Various attempts, such as hyperthermia and the use of TNF, to improve the efficacy of ILP for in-transit melanoma metastases have been undertaken over the years.

Hyperthermia

Prevention of vasoconstriction in cutis and subcutis by using a warm water mattress is advocated in the treatment of superficial in-transit melanoma metastases. *In vivo* drug uptake by in-transit metastases is two times higher at 39,5^0 C than at 37^0 C (7). Moreover, tumor cells per se are sensitive to heat and hyperthermia also improves uptake of drug by the tumor cells, especially at temperatures > 41^0 C (8,9). The term hyperthermia in the setting of ILP should be specified along lines as defined hereunder.

1. *normothermia (37-38^{0}C).* Normothermic ILP with Melphalan has been reported to result in a complete response (CR) rate of about 40% and a partial response (PR) rate of about 35%, and a median duration of local tumor control of about 6 months (10). Two perfusions at a 4 week interval improves the the CR-response rate (77%) but does not improve duration of local control (11).
2. *"mild" hyperthermia (39-40^{0}C).* Several reports have claimed improved response rates (12-16) but a comparative study did not show a significant benefit for mild hyperthermia over normothermia (17).
3. *"borderline true" hyperthermia (40-41^{0}C)*; seems to be associated with higher CR-rates, but may also be associated with increased regional toxicity (18-20).
4. *"true" hyperthermia (41-43^{0}C)*, yields high CR rates (21,22), but is in some reports associated with unacceptable regional toxicity and even amputations (23).

Isolated Limb Infusion (Percutaneous Hypoxic Isolated Limb Perfusion)

Isolated limb infusions with application of a tourniquet, developed by Thompson and coworkers in 1993, is essentially a low-flow, normothermic, hypoxic isolated limb perfusion with Melphalan, but has been called isolated limb "infusion" (ILI) to differentiate it from ILP. The ILI procedure is technically much less complex than ILP, as small caliber catheters are inserted percutaneously into the vessels of the limb via the common femoral artery and vein in the contralateral groin. The patient is anaesthesized and a pneumatic tourniquet is applied and Melphalan (5-10 mg/L limb tissue) and Actinomycin-D (50-100µg/L limb tissue) in 300-400 ml heparinised normal saline at 40^{0}C is infused rapidly through the arterial catheter using a standard intravenous fluid pump set. Using a syringe and a three-way tap the infusate is continuously circulated by aspiration from the venous catheter and re-injection into the arterial catheter, via a standard blood-warming coil immersed in a hot water bath at 41-42^{0}C. After 30 minutes the limb is flushed with saline, the tourniquet is deflated and the catheters are removed.

With a CR rate of 41% and PR in 44% in 207 cases the results of ILI seem similar to those obtained by classical ILP (24). This is doubtful as these results are obtained in patients without lesions all the way up to the groin. It should be realized that only lesions in the distal 2/3 of the legs are treated as an inflatable tourniquet is used positioned at about the 1/3-2/3 level of the upper leg. ILP achieves higher CR rates for distal lesions are higher than for proximal lesions close to the groin, which may be due to suboptimal perfusion conditions high up in the leg on the one hand and to more extensive disease or more aggressive disease in patients who have extensive disease throughout the leg all the way up to the groin. Two ILI procedures at a 6 weeks interval did not yield better results than a single perfusion (25). Yet ILI offers an effective, simple and cheep alternative to the technically (hart-lung machine) more complicated and more expensive isolated limb perfusion procedure. It is easily repeatable, requires litte operation theatre time, simple equipment, less personnel and is therefore more widely applicable than ILP.

Tumor Necrosis Factor alpha (TNF) in melanoma

The application of TNF in ILP was pioneered by Lejeune and Lienard in 1988 and has moved the field forward as it has resulted in very high response rates in all series reported thus far. TNF is a pleiotropic agent, with direct and indirect anti-tumor effects, and a mediator of septic shock (26). Systemic administration of TNF in cancer patients has been ineffective as dose-limiting toxicity is encountered at concentrations 10-50 times lower than needed for anti-tumor effects in murine tumor models. (27). Such a difference can be overcome in the setting of isolated limb perfusion. The results of ILP with TNF + Melphalan with or without IFN-gamma have been published since 1992: Liénard *et al* (28); and Lejeune *et al* (29); Hill *et al* (30); Vaglini *et al*, (31); Eggermont *et al*, (32); Fraker et al. (33), and Lienard et al (34). Interferon-gamma (IFN) was added in the schedule in most studies because of synergistic anti-tumor activity of IFN plus TNF (35). Its role has been evaluated in a randomized phase II trial, which showed only a marginal improvement of outcome for the combination with IFN (34). The question whether TNF significantly improves outcome in melanoma patients over ILP with Melphalan alone is currently being addressed in a randomized trial in the USA. Fraker and collegues at the Surgery Branch of the National Cancer Institute observed a significantly better response (CR 64%) with TNF+Melphalan in bulky ("sarcoma-like") melanoma in transit metastases than with Melphalan alone (CR 17%) (36). This is in line with our observations and the spectacular success of TNF+Melphalan in soft tissue sarcoma. Therefore we consider bulky "sarcoma-like" melanoma

metastases and failure to respond to or recurrences after an ILP with Melphalan alone, strong indications for the use of TNF in melanoma (37).

Failure of Prophylactic ILP in High Risk Melanoma

Retrospective studies suggested, as studies using historic control invariably seem to do, that a prophylactic ILP improved outcome in patients with high-risk primary melanoma (38). The only adequately sized trial addressing the question of the value of a prophylactic ILP with Melphalan in the management of high-risk primary melanoma of the extremity is the intergroup trial of the EORTC-WHO and NAPG (North American Perfusion Group) in 832 evaluable randomized patients. At a median follow up of > 6 years a definitive analysis showed that ILP had only a regional effect (reduction of in-transit metastasis from 6% to 3%). A reduction of regional lymph node metastases was noted without any effect on the appearance of distant metastases or survival. Prophylactic ILP should no longer be performed. It is a harmful procedure with significant morbidity and costs and without any impact on survival (39).

In the symptomatic in-transit metastasis setting the benefit of an ILP after excision of metastases has not been demonstrated. Adjuvant isolated limb perfusion after excision of in transit metastases has not been demonstrated to have a significant impact on outcome of disease although improved disease free interval has been reported in a randomized phase III trial in this patient population (40).

TNF- based ILP for extremity soft tissue sarcoma

The Advent of TNF

ILP with Melphalan, Doxorubicin and a variety of other drugs was tried in the seventies and eighties with poor results and was largely abandoned (41-44). This situation changed dramatically with the advent of TNF. TNF-based ILP has been established as a highly effective new method of induction biochemotherapy in extremity soft tissue sarcomas with a 20-30% complete remission (CR) rate and about a 50% Partial Remission (PR) rate (45-52). On the basis of results in a multicenter program in Europe TNF was approved and registered in Europe for the sarcoma-indication in 1998 (51). The European TNF/ILP assessment group evaluated 246 patients with irresectable STS enrolled in 10 years in 4 studies. All cases were reviewed by an independent review committee and compared with conventionally treated patients (often by amputation) of a population based Scandinavian STS database. In short: there were 246 patient switch locally very advanced disease: Primary sarcomas in 55%, local recurrent sarcomas in

45%, multifocal primary or multiple local recurrences in 22 %. Overt concurrent metastatic disease in 15%. Tumors >10 cm in 46%. Grade III tumors in 66%. Previous radiotherapy (13%), chemotherapy (15%). Patients underwent 1 ILP (236pts) or 2 ILPs (12 pts) of 90 minutes at 39-40 ^{0} C with 2-4 mug TNF + Melphalan (10-13 mg/L limb volume). The first 62 pts also received IFNγ. A delayed marginal resection of the tumor remnant was usually (75%) done 2-4 months after ILP. Major responses were seen in 56.5 to 82.6 % of the patients after which usually resection of the sarcoma became possible.

Results

Limb salvage was achieved in 74%-87% in these 4 studies and in 71 % of the 196 patients who had been classified by the independent review committees as cases that normally could only have been managed by amputation (87%) or by functionally debilitating resection + radiotherapy (13%). Comparison with the survival curves based on a matched control study with cases from the Scandinavian Soft Tissue Sarcoma Databank showed that TNF had no negative effect on survival (p=0.96). It was concluded that the application of TNF in combination with Melphalan in the setting of isolated limb perfusion represents a new and successful option in the management of irresectable locally advanced extremity soft tissue sarcomas (51). Smaller single center studies with TNF+Melphalan have been reported recently by Lejeune (53) reporting a 17% CR and 64% PR rate in 22 STS patients treated for limb threatening STS tumors, achieving limb salvage in 77% of the patients. From Rotterdam results in 2 special patient populations have been reported: one on the efficacy of the procedure in patients with Stewert Treves Lymphangiosarcomas. In 16 perfusions a CR rate of 56% and a PR rate of 31% were observed, achieving a limb salvage rate of 80% in this rare and otherwise non-responsive condition (54). An important message is given by the report on the Rotterdam experience with 50 TNF-based ILPs in patients older than 75 years with limb threatening tumors. Results were very favorable in the 34 perfusions for limb threatening sarcomas, with a 38% CR and a 38% PR rate, achieving limb salvage in 76% of the patients as well as in 16 perfusions for bulky melanoma in-transit metastases resulting in a 75% CR and 25% PR rate. The procedure was proven safe in the elderly with the high reward of limb salvage which is of overriding importance in this age group as amputations lead to loss of independency in lives in the elderly (55)

Italian perfusion groups have obtained very similar results with the drug doxorubicin in combination with TNF. Interestingly similar response and limb salvage rates are achieved while

using lower doses of only 1 mg TNF instead of the usual doses of 2-4 mg used in combination with Melphalan (52). The perfusions were performed at much higher temperatures (40-41 degrees), which lead to higher locoregional toxicity. Grade IV locoregional toxicity was reported in 25% as opposed to only 5% in the large TNF+Melphalan series (46,49,51). We found that with Melphalan ILPs grade IV toxicity was clearly related to tissue temperatures of above 39 degrees when Melphalan was administered (56) Therefore we have only allowed for tissue temperatures to rise to 39 degrees after Melphalan has been added to the perfusion circuit the last 8 years and have hardly seen any cases with grade IV toxicity since, without a drop in response rates (49,53-55). Most likely therefore the higher regional toxicity in the Italian experience with doxorubicin is primarily related to the hyperthermia although doxorubicine may be responsible in part.

Apart from activity in some 20 different histological types of soft tissue sarcoma and activity in melanoma, the efficacy of TNF + Melphalan ILP has also been demonstrated in various skin tumors (57), bony sarcomas (58) and limb desmoid tumors (59).

Target is the Tumor Vasculature

The target of TNF is the tumor-vasculature. This common denominator in all these tumors makes the use of TNF very attractive and explains its efficacy in combination with chemotherapy across all these different histology's. The selective destructive effects of TNF-ILP on tumor-associated vessels have been illustrated in previous publications by means of pre- and post perfusion angiographies (46). Moreover in sarcoma patients magnetic resonance spectrometry studies we have clearly shown that the metabolic shut down of the tumor is virtually complete within 16 hours after the perfusion, confirming the likelihood of TNFα mediating its most important effects on the vasculature of the tumor (60). At the histopathological level we have also studies these intravascular effects such as thrombocyte aggregation, erythrostasis, endothelial and vascular destruction already in the early and late stages after ILP (61-62).

Developments in the Laboratory

To further insight in the mechanisms underlying the positive results obtained with ILP in humans we developed in rats extremity perfusion models using the BN175 non immunogenic fibrosarcoma in Brown Norways rats and the ROS-1 osteosarcoma in WAG rats. In both models we could demonstrate that the tumor cells were resistant to TNF in vitro and that ILP in vivo

with TNF alone had no major impact on tumor growth. In both models a strong synergistic anti-tumor effect leading to CRs in some 60-70 % was observed after ILP with TNF+Melphalan (63,64). TNF alone only caused some central necrosis and no regression of the tumor was observed as has been reported for the clinical setting as well. Histopathologically hemorrhagic necrosis was most prominent after ILP with both drugs. Early endothelial damage and platelet aggregation in the tumor vessels are observed after ILP with TNF + Melphalan and this is believed to lead to ischemic (coagulative) necrosis, which is in line with observations in patients. Our observations confirm that TNFα has its major effect on larger tumors, with well-developed vasculature in contrast to small tumors (diameter < 3 mm) with lack of developed capillary bed. TNF may exert its effect mainly through the neovasculature of the tumor, which is more abundant in large tumors. Moreover there are distinct similarities between tumor stroma generation and wound healing and observations by us that sites other than the tumor (recent wounds or skin overlying tumors only when invaded by tumor), which undergo angiogenesis, also become necrotic after ILP with TNF+ Melphalan, but not after ILP with Melphalan alone.

Prerequisites for an Effective ILP

We have demonstrated a number of crucial elements in our rat tumor models identifying the mechanisms for the strong synergy between TNF and cytostatic drugs in ILP and have identified the prerequisites for an effective ILP.

1) *Tumor vessel destruction:*

The vasculotoxic effects of the combination of TNF + Melphalan leading to haemorrhagic and anoxic coagulative necrosis as described above.

2) *Enhanced drug uptake by the tumor:*

We have recently demonstrated that the addition of high dose TNF to the perfusate results in a 4-6 fold increased uptake by the tumor of the cytostatic drug. For Melphalan and for Doxorubicin is was demonstrated that this uptake was tumor specific and that no increased uptake was noted in the normal tissues, thus emphasizing the relatively selective action of TNF on the tumor-associated vasculature (65) This increase in concentration was also observed with doxorubicin (66). Whether the acute drop in interstitial pressure in the tumor after exposure to TNF, as reported by Kristensen and coworkers (67), plays an essential role, remains speculative.

3) *Role of Leukocytes:*

We have shown that leukocytes play also an important role in the TNF-mediated antitumor effects. In rats that underwent total body irradiation and underwent an ILP at the time of

absolute leukopenia the antitumor effect of an ILP with TNF+Melphalan was very similar to the effects of a perfusion with Melphalan alone. In the leukopenic rat the TNF-effect was lost and the synergy between TNF and Melphalan was no longer observed (68)

4) *Dose range for TNF:*

It was shown that 10 micrograms of TNF (a fivefold reduction of the "standard dose of 50 microgram" was the threshold dose for activity of TNF in our rat tumor extremity perfusion model. At 2 microgram all TNF-effects were lost (69). This finding would suggest that also in the clinical setting dose reduction without loss of activity could be explored as been also suggested by the clinical results in the UK (30) and in Italy (52).

5) Duration of ILP:

As the pharmacokinetics of Melphalan demonstrate that almost all Melphalan uptake occurs over 20-30 minutes the minimal duration for an effective ILP should be 30 minutes. Shorter perfusion times are associated with a drop in CR and PR rates whereas longer than 30 minutes ILPs do not seem to further improve the results (69)

6) Mild Hyperthermia:

Temperatures of 38-39 degrees Centigrade were shown to be essential for obtaining a good anti-tumor response without damage to the normal tissues in the limb. True hyperthermia (42-43degrees) resulted in an increase of CRs but was associated with very sever damage to the normal tissues. All anti-tumor efficacy was lost when perfusions were performed at room temperature.(69)

7) Hypoxia:

It was shown that hypoxia can enhance the anti-tumor effects of an ILP with either TNF alone or Melphalan alone. Hypoxia did not further enhance the anti-tumor efficacy of an ILP with TNF+Melphalan as the synergy between these two agents "overrode" any minor enhancement mediated by hypoxia (69).

8) Interferon-gamma:

In spite of many reports of the synergy between IFN-gamma and TNF both in vitro as well as in vivo in murine tumor models the role of IFN-gamma not very strong in our rat models. We demonstrated that about a 10% increase in CR rate and an increase of about 20% in overall response rate was observed in our animal models (70), which resembles the situation in the clinic (34)

9) Idiosyncratic toxicity:

Interestingly unexpected interactions may lead to idiosyncratic reactions between TNF and certain cytostatic agents. Actinomycin D is commonly used in combination with Melphalan in

the clinical setting. When whether TNF would enhance the efficacy of Actinomycin D we discovered that it did in an idiosyncratic and nondiscriminative way. The combination was more effective against the tumor than TNF+Melphalan, but this advantage was annulled by the toxicity of TNF+Actinomycin D to the normal tissues, which was such that virtually all extremities in these animal models had to be amputated, bringing a strong warning to the clinic not to use TNF in combination with Actinomycin D (71)

10) Vasoactive drugs:

Various vasoactive drugs have been and are being studied in our laboratory models. Nitric Oxide (NO) is an important molecule in the maintenance of both vascular tone and the integrity of the vascular wall and is highly produced in experimental and human tumors. We postulated that its inhibition could lead to hypoxia and an enhancement of TNF early vascular effects in the tumor. In our ILP BN175 rat model we performed a response study with TNF in combination with the Arginine analogues L-NAME and LNA, which inhibit NO synthase. In rats treated with TNF combined with L-NAME/LNA important and immediate anti-tumor effects were observed in all rats and necrosis of the skin at the tumor site. These effects are normally only observed when hypoxia or Melphalan are added to TNF as described above. Typical TNF tumor response was observed, when NO synthase was inhibited during ILP. (72).

Another vasoactive drug is histamine that is currently being studied. Also in this case we see a clear synergy with Melphalan in our tumor models (manuscript under preparation) This finding shows the importance of agents that can change the (patho)physiology of tumorvasculature, and thereby can improve drug uptake in tumors. These findings underline the importance of investigating how to modulate tumor physiology and the potential that this approach has to improve efficacy of various standard agents.

ILP and Gene Therapy

Isolated limb perfusion is an interesting method for new treatment modalities such as adenoviral-vector mediated gene therapy. We have shown that ILP is the best and most selective method for effective homogeneous transvascular local gene-delivery by using adenoviral vectors. Experiments in our soft tissue sarcoma, osteosarcoma and rhabdomyosarcoma isolated limb perfusion models have clearly shown this by making use of luciferase-marker gene and LacZ gene methodology (73,74). Moreover we have shown that ILP with adenoviral-vector gene delivery of the cytokine IL-3 is the only method in these tumor models to achieve good tumor responses in contrast to the failure of other methods such as IV

administration, intra-arterial administration or intratumoral administration to do so (75). It demonstrates that ILP is a valuable method to treat advanced limb tumors and to develop new treatment modalities.

Conclusions

Isolated limb perfusion methodology provides us an excellent tool in the clinic to obtain local control and avoid amputations of limbs in patients with limb threatening tumors. This has been largely achieved by the success of the antivascular TNF-based biochemotherapy in this setting. TNF, for the first time, has brought us an effective treatment against large, bulky tumors.

Moreover Isolated limb perfusion is a albeit somewhat exotic, but very interesting research model to develop and study agents that modify the pathophysiology of large tumors that blocks effective penetration of cytotoxic drugs into the tumor.

We can now manipulate and study the tumor vascular bed in ways that will identify “new” drugs that can enhance the activity of “old” drugs. Moreover it has proven to be a model system that may also facilitate the development of vector-mediated gene therapy and other innovative approaches.

Much of these developments have been initiated by the application of TNF in this setting. TNF-based isolated limb perfusion is a very successful treatment option to achieve limb salvage in the management of advanced, multiple or drug resistant extremity tumors. TNF-based ILPs are now performed in thirty cancer centers in Europe with referral programs for limb salvage. TNF-based antivascular therapy of cancer is here to stay and its potential needs to be studied further (76). Other drugs will follow and we may well learn through this model how to use them systemically more effectively as well.

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V

Clinical isolated hepatic perfusion studies.

11

Isolated hypoxic hepatic perfusion (IHHP) with orthograde or retrograde flow in patients with irresectable liver metastases using percutaneous balloon catheter techniques. *A phase I-II study.*

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Summary

Isolated hepatic perfusion (IHP) for irresectable metastases confined to the liver has reported response rates of 50-75%. Magnitude, costs and non-repeatability of the procedure are its major drawbacks. We developed a less invasive, less costly and potentially repeatable balloon-catheter mediated isolated hypoxic hepatic perfusion (IHHP).

In this phase I-II study 18 consecutive patients with irresectable colorectal or ocular melanoma hepatic metastases were included. Two different perfusion methods were used, both with inflow via the hepatic artery, using Melphalan at a dose of 1 mg/kg. In the first eight patients the portal vein was occluded and outflow was via the hepatic veins into an intracaval double balloon catheter. This orthograde IHHP had on average a 56% leakage. In next 10 patients we performed a retrograde outflow-IHHP with a triple balloon blocking outflow into the caval vein and allowing outflow via the portal vein. The retrograde IHHP still had a 35% leakage on average.

Although local drug concentrations were high with retrograde IHHP, systemic toxicity was still moderate to severe. Partial responses were seen in 12 % and stable disease in 81% of patients. Median time to local progression was 4.8 months.

We have abandoned occlusion balloon methodology for IHHP as it failed to obtain leakage control. We are presently conducting a study utilizing a simplified surgical retrograde IHHP method, where leakage is fully controlled which translates into high response rates.

Introduction

Approximately 50-60 % of colorectal cancer patients will develop liver metastases during follow up. In nearly a quarter of these patients the liver is the only site of disease (1). If hepatic metastases of colorectal cancer are resectable, five-year survival rates are reported between 25-45% depending on several prognostic factors (2). Patients with irresectable hepatic metastases have a 0-2% five year survival rate. Therefore, aggressive, selective treatment of the liver seems justified since control of hepatic metastases translates into improved overall survival. There is no standard treatment for unresectable hepatic metastases confined to the liver, so novel treatment modalities have to be developed.

Although response rates with novel systemic chemotherapeutic agents such as oxaliplatin and irinotecan in combination with 5-FU are promising, overall survival remains poor (3-5). In order to improve responses and survival loco-regional chemotherapeutic regiments have been

developed such as hepatic artery infusion (HAI), chemoembolization and isolated hepatic perfusion (IHP). For most chemotherapeutic agents a steep dose response curve can be demonstrated and exposure of the liver metastases to higher drug concentrations by means of loco-regional treatment, might result in improved control of hepatic metastases. HAI exploits the first pass effect of the liver, resulting in high local, but low systemic drug exposure. Several repeated hepatic artery infusions regimens produced higher response rates, compared to systemic chemotherapy, with a 2 –year survival of 50-60% (6-11).

In animal studies Marinelli et al demonstrated that significantly higher intrahepatic concentrations can be reached by IHP compared to HAI (12,13). In a leakage free perfusion setting IHP shields the systemic compartment to drug exposure and in combination with a washout procedure it protects against systemic toxicity. Classic surgical IHP (SIHP) with Melphalan or mitomycine C has been studied in animal models and resulted in high response rates (14-16). Clinical studies using Melphalan with or without tumor necrosis factor-alpha (TNF) have shown promising results (17-24). The phase II trial performed by the NCI of SIHP with Melphalan and TNF demonstrated an overall response rate of 75% (18).

SIHP is a major, complex, expensive and time-consuming operation. These features in combination with potential toxicity are major drawbacks towards wide clinical application. Moreover, this procedure can only be performed once. In order to address these problems we developed a leakage free isolated hypoxic hepatic perfusion (IHHP) technique with balloon catheters in pigs (25). Using Melphalan and TNF it was demonstrated that an isolated perfusion with balloon catheters was feasible and showed minimal systemic leakage. On the basis of these favourable pharmacokinetic results a phase I-II study with Melphalan was developed for patients with irresectable liver metastases. In this report we present the results of the first 18 patients who underwent an IHHP using balloon catheter techniques with orthograde or retrograde flow through the liver.

Materials and methods

Patient selection criteria: In all patients a radical resection of the primary tumor was performed prior to entering the study protocol. The liver metastases were considered irresectable, on the basis of multiple lesions in multiple segments of the liver or location near vascular structures. Tumor involvement had to be less than 50% of the total liver volume, to prevent massive necrosis in case of a response. Absence of extrahepatic tumor growth was evaluated by CT scan of thorax and abdomen. All patients had a Karnofsky performance score of at least 90, liver

enzymes (ALAT, ASAT and AF) not higher than 5 times the normal values and bilirubin not higher than 2 times the normal values. Exclusion criteria included: age younger than 18 or older than 75, portal hypertension, significant central nervous system disease, significant cardiovascular, pulmonary or renal disease, uncontrolled infections, presence of organ grafts, and chemotherapy or radiation therapy within 4 weeks prior to the IHHP. Routinely an angiography was performed to exclude aberrant hepatic arteries or to visualize other anatomic anomalies. Patients with severe arteriosclerosis of the aortic-iliac-femoral vessels which made placement of balloon catheters impossible were also excluded. All IHHP's were performed at the Daniel den Hoed Cancer Center. The study protocol was approved by the Medical Ethical Committee of the Erasmus University Medical Centre and written informed consent was obtained from all patients.

Perfusion circuit: Perfusion sets (PfM, GmbH, Cologne, Germany) consisted of a double balloon catheter (12 F, balloon capacity 25 ml, distance between balloons 4-5 cm) for venous isolation of the liver. An aortic-occlusion balloon catheter (12 F, balloon capacity 25 ml) for compensating the decrease of cardiac preload during the procedure and a tubing set with a volume of 220 ml, containing a bubble trap. All IHHP's were performed with inflow via the hepatic artery. In the first 8 patients predominantly an open technique was used in order to cannulate the proper hepatic artery via the gastroduodenal artery with a 8F catheter (Figure 1). From patient 9 up to 18, we used a percutaneous 5F stopflow occlusion catheter (PfM, GmbH, Cologne, Germany) introduced pre-operatively via the groin using the Seldinger technique (Table 1 and Figure 2). Except for patient no. 12 and patient no. 15 who had a double hepatic artery. The balloon was positioned in the proper hepatic artery.
The first 8 patients were perfused with a double balloon catheter in the caval vein with occlusion of the portal vein and outflow via the side holes in the caval vein catheter (Figure 1). To improve leakage control a triple balloon occlusion catheter was developed (PfM, GmbH, Cologne, Germany) and the outflow was diverted to the portal vein, creating a retrograde flow. This triple balloon was used from patient 9-18. It occludes the retrohepatic caval vein in a section of 18 cm vs. 12 cm with the double balloon catheter (Figure 2). In the perfusion circuit flow was maintained by a roller pump and pressure was measured via a side-line.

Drugs: A dosage of 1 mg/kg Melphalan (L-Pam, Alkeran, Wellcome Ltd. London, UK) was used in all patients and infused through a side-line into the perfusion circuit.

Surgical procedure of the orthograde flow IHHP: A small right subcostal incision was performed, and cannulation of the gastroduodenal or hepatic artery was established. A cholecystectomy was routinely performed in the first 5 patients only. When percutaneous techniques were used pre-operatively, palpation confirmed the position of the balloon in the proper hepatic artery. Surgical exposure of the femoral artery and vein in the groin and cannulation of the artery with an aorta-occlusion catheter positioned under radiographic control just above the celiac axis. Patients were subsequently heparinized with 2mg/kg heparin. Hereafter, cannulation of the femoral vein was performed with the caval double balloon catheter positioned under radiographic control. The proximal balloon at the level of the diaphragm and the distal balloon just below the liver, which was confirmed by palpation. In between the 2 caval balloons 20ml contrast solution is rapidly injected to visualize the hepatic veins at their confluence into the retrohepatic caval vein. After clamping of the portal vein, connecting the hepatic artery catheter and the caval balloon catheter to the perfusion circuit primed with 220 ml Haemaccel (Behring Pharma, Amsterdam, The Netherlands) the orthograde isolated perfusion was performed. (Figure 1, Table 1 patients 1-8).

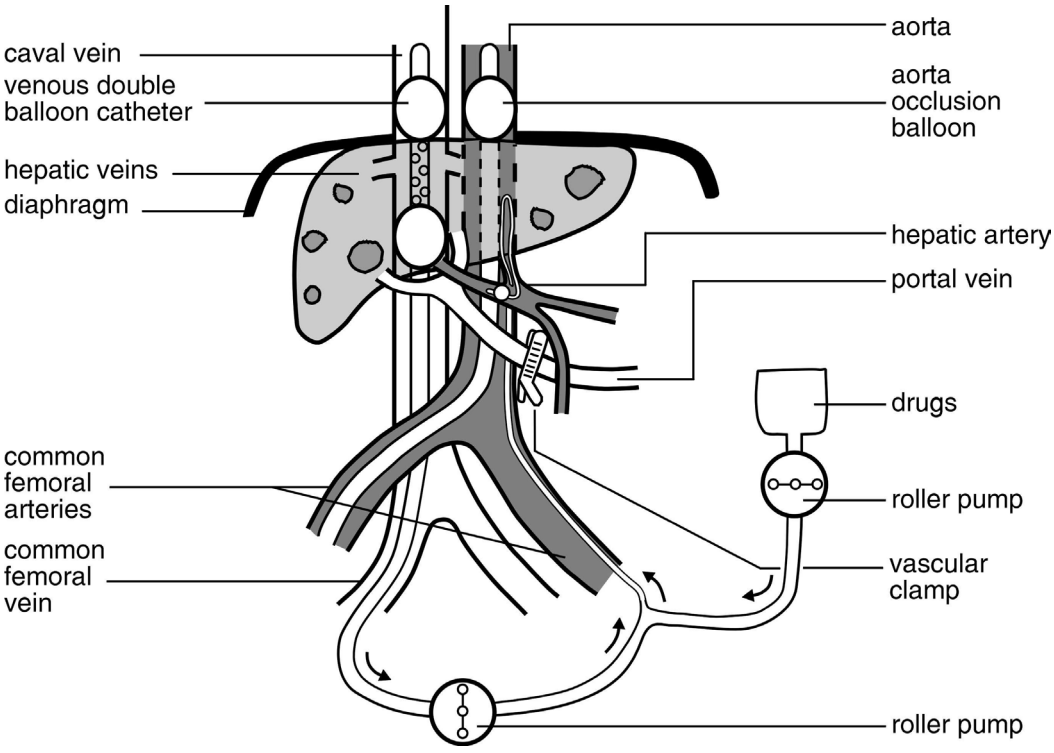

Fig. 1 Schematic representation of IHHP with a percutaneous catheter in the hepatic artery (inflow) and double balloon catheter in the caval vein (outflow). **Orthograde flow method**.

Surgical procedure of the retrograde flow IHHP: Prior to the operation the hepatic artery was cannulated via the groin as described above. Via the abdominal incision the portal vein was dissected. The femoral artery and vein were cannulated and the occlusion balloons were positioned both in the vena cava and the aorta. Hereafter, the portal vein was cannulated with 14 F catheter for outflow (Table 1 patients no. 9-18). Patients are subsequently heparinized with 2mg/kg heparin. Following connection to the perfusion circuit a retrograde perfusion was commenced through the portal veins. The retrograde perfusion set-up is depicted in Figure 2.

The perfusate was circulated by a constant flow (see Table 1). Stable perfusion was monitored by pressure measurement and the perfusate level in the bubble trap. Methylene blue was injected into the arterial catheter to check homogeneous distribution over both lobes of the liver. Hereafter, Melphalan was infused into the circuit and the perfusion was conducted for 20 minutes. After 20 minutes a washout procedure was performed by 1 litre of Haemaccel collecting the venous effluent. Total liver ischemia time never exceeded 60 minutes.

The isolation was terminated by deflating the caval balloon followed by the aortic balloon and releasing the ligature of the portal vein (orthograde IHHP) or decanulation and closing the venotomy of the portal vein (retrograde IHHP).

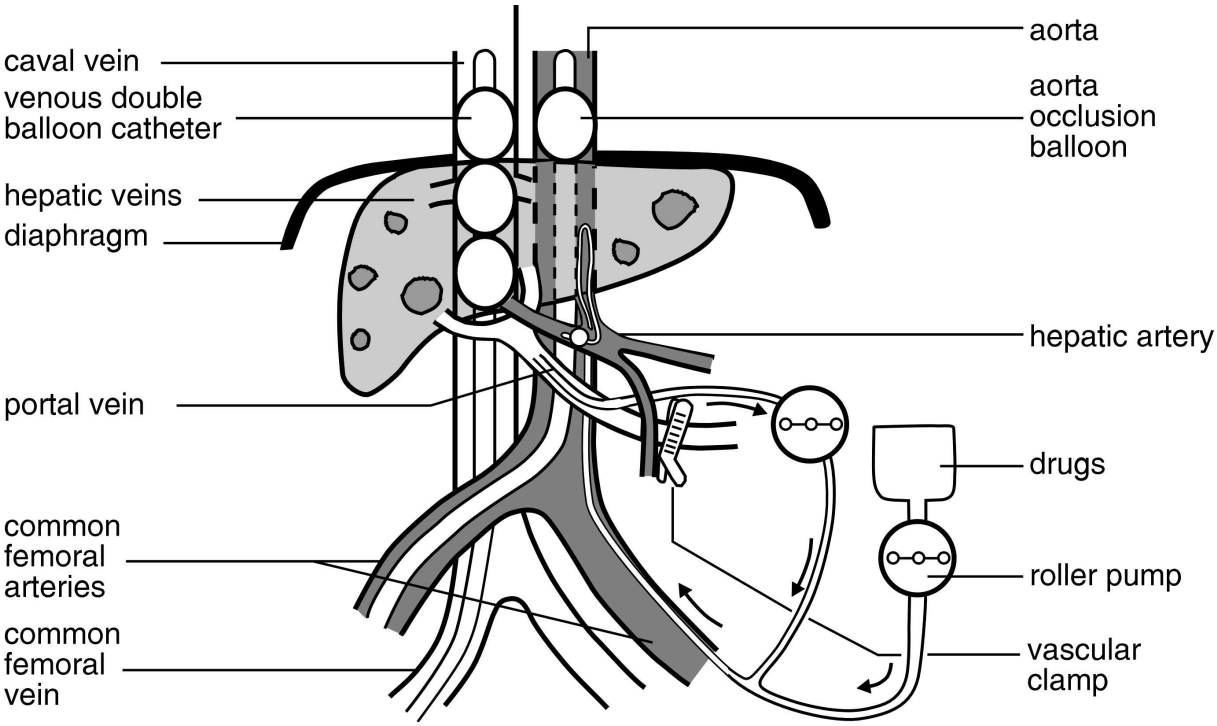

Fig. 2 Schematic representation of IHHP with a percutaneous catheter in the hepatic artery (inflow), a triple balloon occlusion catheter in the caval vein and an outflow catheter in the portal vein. **Retrograde flow method**.

Pat	Sex	Age	Tumor	Balloon	Outflow	Leakage	Toxicity				Response			TTLPD
Nr		(years)	type	V.Cava	(site)	(%)	Hematol	Renal	Hepatic	GI	CEA pre	CEA 6 w	CT	(mo)
1	M	67	Colorect	Double	Caval	50	3	0	3	0	236	56	SD	4
2	M	70	Colorect	Double	Caval	60	4	0	2	0	74	10	SD	3
3	F	67	Colorect	Double	Caval	20	4	0	3	0	211	171	SD	3
4	F	58	Colorect	Double	Caval	100	3	0	3	0	9980	2260	SD	3
5	M	69	Colorect	Double	Caval	50	3	0	2	0	13,7	5,9	SD	5
6	M	66	Colorect	Double	Caval	33	1	0	1	0	29,7	30,2	SD	13
7	M	68	Colorect	Double	Caval	40	4	0	3	0	45,9	27,6	PD	5
8	M	39	Colorect	Double	Caval	100	4	0	2	0	12,0	20,7	SD	3
9	F	68	Colorect	Triple	Portal	15	0	0	1	0	6,33	2,74	PR	5
10	M	71	Colorect	Triple	Portal	30	4	0	2	0	15,73	21,5	SD	7
11	F	64	Colorect	Triple	Portal	17	0	0	3	0	81,65	58,75	SD	3
12	F	69	Ocul.mel	Triple	Portal	5	0	0	4	0	NA	NA	NA	NA
13	M	65	Colorect	Triple	Portal	35	3	0	3	0	480	87,74	SD	3
14	M	51	Colorect	Triple	Portal	85	1	0	3	0	5,12	2.0	PR	5
15	F	64	Colorect	Triple	Portal	55	0	0	3	0	2330	NA	SD	4
16	M	71	Colorect	Triple	Portal	65	3	1	3	0	54	NA	abces	NA
17	F	52	Colorect	Triple	Portal	16	0	0	2	0	43,5	15,5	SD	4
18	M	59	Colorect	Triple	Portal	50	3	0	3	0	14,27	NA	SD	6
						Gr 4					Mortality	Response		
mean		63.2				Toxicity	5/18	0/18	1/18	0/18	1/18	SD	81%	4.8
med		66.5				(%)	27%	0%	5%	0%	5%	PR	12%	4.0

Table 1 Characteristics of 18 patients with irresectable liver metastases treated by IHHP with Melphalan.

Abbreviations: SD= stable disease, PD=progressive disease, PR=partial response, NA=not available, TTLPD= time till local progression of disease (at the liver); mo=months after IHHP; med=median.

Leakage monitoring: During IHHP potential leakage of drugs was monitored using a radioactive tracer. A small calibration dose of human serum albumin radiolabelled with ^{131}I was injected into the systemic circulation prior to the perfusion and 10-fold higher dose of the same isotope was injected into the isolated hepatic perfusion circuit. Continuous monitoring was performed with a precordial scintillation probe. Systemic leakage is expressed quantitatively as a percentage (100% leakage representing a homogeneous distribution of the isotope in the body (21).

Blood sampling: Before, during and after the perfusion blood samples were taken and collected to study pharmacokinetics of Melphalan and haematological, renal, hepatic and gastro-intestinal toxic side effects. Toxicity is graded according to the standardized WHO common toxicity criteria (26).

Measurement of Melphalan concentrations: Melphalan was measured in plasma by gas chromatography-mass spectrometry (GC-MS). P-[Bis(2-chloroethyl)amino]-phenylacetic acid methyl ester was used as an internal standard. Samples were extracted over 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 described earlier by Tjaden et al (27).

Assessment of tumor response: This was done by comparing pre-perfusion CT and MRI scans of the liver with scans made at 6-8 weeks after IHHP. The tumor marker carcinoembryonic antigen (CEA) was monitored (when indicated) preoperatively and 6-8 weeks post-perfusion, but was not used for response assessment. Clinical responses are assessed by standardized WHO criteria (26): Complete remissions (CR) of all measurable disease in the liver > 4 weeks; partial remissions (PR): regression of the tumor size by more than 50% for > 4 weeks; stable disease (SD): regression less than 50% of the tumor in the liver or progression less than 25% for > 4 weeks; progressive disease (PD) progression more than 25%

Results

Patient characteristics. In total 18 patients were included in the protocol. Seven women and eleven men with a mean age of 63.2 years (range 39-71). Seventeen patients with irresectable colorectal liver metastases, and one patient with ocular melanoma hepatic metastases.

Leakage control. In the first 8 IHHP's with a double balloon intracaval catheter a mean leakage of 56% (range 20-100) was measured. Repeated adaptations to the catheter design in terms of balloon size and interballoon distance were performed. This lead to a change of concept and the design of a triple balloon caval vein occlusion catheter with outflow via the portal cannula and a retrograde flow direction (patients 9-18). Now leakage decreased to an average of 35% (range 5-85).

Toxicity study (Table 1). Regional toxicity consisted mainly of a transient rise of liver enzymes during the first week after IHHP, WHO grade II-III in 83 % of the patients. No coagulopathy was observed. In 1 patient we were confronted with severe hepatic toxicity (grade IV). Unfortunately this patient died within 30 days of the operation (discussed in detail in the *Complications* section).
Because of the leakage of Melphalan during the perfusion most patients were treated with G-CSF (Neupogen, Amgen B.V., Breda, The Netherlands) trying to prevent severe leucopenia.

Systemic toxicity consisted mainly of leucopenia, WHO grade I-III in 44% and severe grade IV leucopenia in 27%, during 10-20 days after perfusion. In most patients with relatively less leakage no or only mild leucopenia was observed. No renal or gastro-intestinal toxicity was observed.

Melphalan pharmacokinetics. Figure 3 shows a drug-concentration vs. time curve in the isolated circuit and in the systemic circulation. It shows Melphalan concentrations during a retrograde IHHP (patient no. 9). Very high regional and negligibly low systemic Melphalan concentrations were observed. Post-operatively this patient only had mild hepatic toxicity and no signs of systemic toxicity. Area under the concentrations versus time curve (AUC) calculation showed a regional concentration advantage with AUC ratio regional/systemic of 28.2.

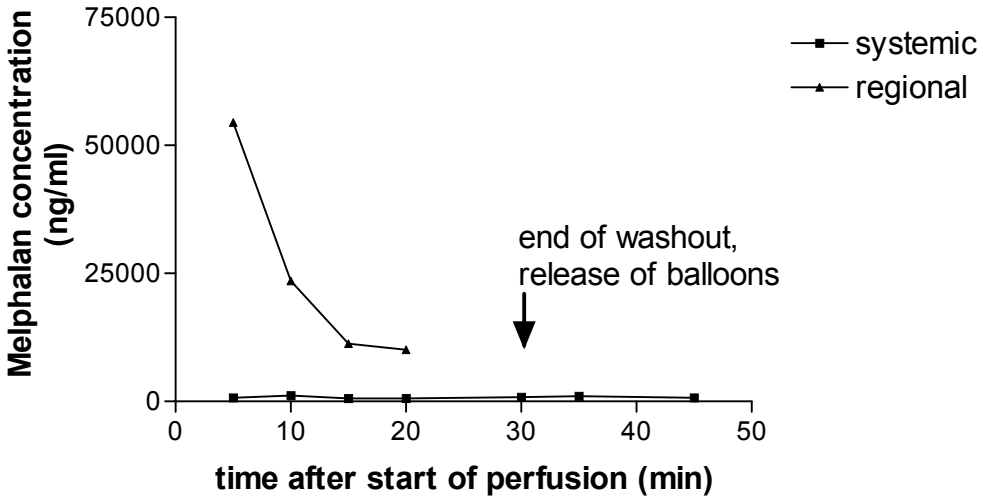

Fig. 3 Pharmacokinetics of Melphalan during IHHP.
IHHP no. 9 with 15% leakage during perfusion. Area under the concentration vs. time curve (AUC) calculated between t=5 and t=20 minutes. Regional 335500 (ng x min/ml) vs. systemic 11870. The AUC ratio regional/systemic is 28.2.

Complications. Patient no. 3 developed a paralysis of both legs a few days after the procedure. During the clinical observation period the symptoms decreased and after about 3 months the paralysis had disappeared completely. This temporary neurological feature was probably caused by peroperative ischemia of spinal marrow by occlusion of the Adamkiewicz artery. This *arteria radicularis magna* supplies part of the spinal marrow and was probably occluded by the aortic balloon catheter.

One patient developed liver abscesses 2 weeks after IHHP (no.20). He underwent the perfusion with 65% leakage and developed grade III haematological and hepatic toxicity. Then a period with fever occurred and CT scan demonstrated multiple abscesses in the liver. These abscesses were located at the former sites of the colorectal metastases. Moreover, an abscess was apparent at the blind end of the rectum as a result of the Hartmann procedure performed for his primary tumor several months before. This was the possible focus for the bacteraemia causing the infected necrotic masses in the liver. After a period of 2 months with multiple percutaneous draining periods of the liver abscesses and antibiotic treatment he finally developed a aspiration pneumonia and died because of respiratory failure.
One patient died within 30 days of the operation, resulting in a mortality during this phase I-II study of 5%. This patient (no 12.) presented with ocular melanoma metastases and had an uneventful IHHP with only 5% leakage. Post-operatively she developed severe dyspnoea and grade IV hepatic toxicity. Mechanical ventilation was indicated because of respiratory failure. Hepatic dysfunction increased rapidly and 8 days post-operatively the patient died. Autopsy showed pneumonia. There were no signs of pulmonary (thrombus or tumor) embolism. An ischemic liver was found with almost total necrosis of the melanoma metastases. Surprisingly almost 70% of liver was replaced by tumor. Although a CT-scan 4 weeks prior to perfusion showed an estimated tumor involvement of < 40%. We assume that the metastases must have grown very rapidly the last weeks before IHHP and the remnant of normal liver tissue was not enough to survive the hepatic toxicity due to the IHHP.

Tumor response and patient survival. Stable disease was demonstrated in 81% of evaluable patients (13 out of 16) after 6-8 weeks (Table 1). Patients no. 12 and 16 were not evaluable with respect to tumor response. In 12 % of patients a PR was seen (2/16). Two patients developed progressive disease after IHPP (12%). In both patients who had a PR, CEA levels decreased to normal (<5 μg/ml) levels. CEA levels decreased in at least 8 of the SD patients but none had reached normal levels. Progressive disease at the liver occurred with mean interval after IHHP of 4.8 months (range 3-13). Seven patients developed systemic metastases. Five of them developed pulmonary metastases in range from 3 to 7 months after IHHP. One patient had metastatic lesions in the sacral bone at the same time of liver metastases progresion at 4 four months after perfusion. In one patient peritoneal carcinomatosis was detected 5 months after IHHP. In one patient a local recurrence at the rectum was detected. Median patient survival was 11.1 months (range 0-32).

Discussion

In the last decade isolated liver perfusions are performed by a few centres world-wide and the anti-tumor effect showed promising response rates up to 75% and a potentially prolonged mean survival of 16-24 months (17,20). The major drawbacks of the technique are the magnitude, the costs and the non-repeatability of the surgical procedure. In the open procedure the whole liver has to be mobilised and all lumbar veins have to be ligated to guarantee a leakage free perfusion. Classical SIHP also uses a veno-venous bypass and a heart-lung machine which is a time-consuming procedure necessitating a specialized perfusion team. The mean SIHP operation time is 8.6 hours with a mean number of packed RBC's transfusion of 5.7 units. The main goal of our study was to develop a less invasive, less costly and potentially repeatable percutaneous technique, allowing safe perfusion in a much shorter time, which can be repeated and performed without a heartlung machine or veno-venous bypass. Moreover, IHHP makes use of hypoxia which renders tumor cells more sensitive to cytostatic agents in general and which enhances in particular the anti-tumor effects of drugs such as Melphalan (28,29). In pigs we previously demonstrated a leakage free IHHP technique with an open double balloon catheter in the caval vein (25). The same technique in patients resulted in major leakage in this study. This despite positioning the two balloons directly above and below the orifices of the hepatic veins and trying to occlude the lumbar veins. By replacing the open double balloon catheter by a triple balloon occlusion catheter, which should occlude the adrenal vein and all lumbar veins, but also the hepatic veins, more successful hepatic perfusion could be performed with inflow via the hepatic artery and outflow via the portal vein. The mean operating room time was reduced to 3 hours and mean transfusion is 1 RBC unit compared to SIHP.

The mean leakage decreased from 56% in the orthograde IHHP to 35% in the retrograde setting. We anticipated on a decrease in leakage along the learning curve, however unless increasing experience and technical modifications IHHP it is still not possible to perform it a leakage free in this current setting. We assume that diaphragmatic, lumbar, and adrenal veins are the main cause for the type of leakage we observed. Veins around the common bile duct in the hepatic ligament could also be a potential cause, but temporary ligation of the ligament during perfusion was performed routinely since we started with the retrograde perfusion and leakage remained. Leakage started directly after start of the perfusion and remained at a constant level during the procedure. Due to this persisting leakage problem and subsequent dose limiting systemic toxicity we were not able to escalate to higher Melphalan dosages.

Higher local Melphalan concentrations seem to be a prerequisite for improving tumor responses. Vahrmeijer et al. reported a correlation between high dose Melphalan SIHP (3 mg/kg) for colorectal liver metastases and patient survival (20).

Leakage free perfusion is of major importance for the potential application of tumor necrosis factor-alpha (TNF), a cytokine with significant anti-tumor effects at high concentrations. The adequate concentration for TNF to induce its synergistic anti-tumor effect is to high to administer it intravenously. The use of TNF has led to excellent clinical responses observed after isolated limb perfusion (ILP) with Melphalan and TNF for irresectable soft tissue sarcomas and melanomas (30,31). In ILP TNF proved to be very effective and safe since these perfusions are performed with minimal systemic leakage of 0-10% (30,31). Whether TNF contributes to therapeutic efficacy in IHP remains unclear. We recently demonstrated in a pre-clinical rat liver metastases model that increased intratumoral Melphalan uptake is strongly correlated with the microvessel density of the tumor (32). Only hypervascularized tumors showed improved Melphalan uptake in tumor tissue and synergistic anti-tumor effects after IHP with Melphalan and TNF. Since colon carcinoma metastases are hypovascular, IHP with Melphalan alone might be probable just as effective as combined with TNF. This was demonstrated in our colorectal liver metastases model, which showed no increased intratumoral Melphalan concentrations and lack of therapeutic efficacy compared to IHP with Melphalan alone. Results from the NCI showed same duration of response after SIHP for colorectal metastases with or without TNF (18,33,34). But in patients with highly vascularized ocular melanoma metastases addition of TNF in SIHP yielded to prolonged duration of response compared to SIHP with Melphalan alone: 14 months vs. 6 months (35). These clinical results seem to confirm the hypothesis about the indication for the utility of TNF in IHP. Since the minimal invasive IHHP methodology we report here is not without leakage the addition of TNF in this setting seems impossible.

Savier et al. recently reported a phase I study with 4 patients repeatedly treated by ten courses of Melphalan based SIHP and percutaneous IHP (36). At percutaneous IHP the hepatic artery was used for inflow of the perfusate and an open double caval balloon catheter was used for outflow. The portal vein was occluded by a percutaneous balloon catheter to complete isolation. This group was also confronted with major leakage starting as soon as the perfusion commenced. This was postoperatively measured by systemic Melphalan levels. Severe (grade III-IV) systemic toxicity (haematological) was observed postperfusion in this study. Due to lower Melphalan doses varying from 15 to 45 mg used no severe hepatic toxicity was observed. Others have used percutaneous IHP techniques combined with extracorporal

chorcoal hemoperfusion filters for the venous effluent, predomininantly in patients with hepatocellular carcinoma (37-39). This technique is completely different compared to the balloon catheter technique described in our study, but results look promising although toxicity is also significant. Systemic toxicity might not only be related to the surgical technique or catheter, but influenced by the used drug as well. Our study with eighteen consecutive patients in this phase I-II study showed advantages compared to SIHP regarding magnitude and operating time, although morbidity and mortality are still significant. Despite technical modifications and increasing experience leakage is still observed and regional and systemic toxicity remained.

With respect to regional toxicity we emphasise that at least 50 % of functional liver tissue should be present. Especially in fast growing melanoma metastases liver imaging should be performed shortly prior to the operation as well as laboratory investigation. The only fatal complication occurred after a technically uncomplicated IHHP with less leakage, but was due to hepatic failure based on massive liver replacement by fast growing metastases

Systemic toxicity is directly correlated with leakage of cytostatic agents during perfusion and an effective washout procedure. Vahrmeijer et al. could escalate up to 3 mg/kg Melphalan safely provided leakage was minimal (20). In our series leakage less than 20% prevented leucopenia almost completely. In a leakage free perfusion setting hepatic toxicity will be the dose limiting factor.

In conclusion balloon catheter mediated IHHP has failed since no good leakage control was achieved by neither the orthograde nor the retrograde method. We therefore have abandoned this program. Instead we have used the experience to develop a surgically much simplified method to perform a retrograde IHHP with fully controlled leakage and thus improved local drug concentrations, improved washout at the end of the perfusion, and much improved toxicity and response rates. Lastly we want to emphasize that systemic or locoregional maintenance therapy after IHHP also has to be considered in order to control the liver metastases. Continuing locoregional treatment by HAI after an IHP procedure is technically feasible and appears to prolong the duration of the response and survival (18).

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12

Isolated hepatic perfusion: experimental evidence and clinical utility.

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Introduction

The optimal treatment for patients with primary or metastatic malignancies confined to the liver is surgical resection with curative intent. Isolated liver metastases occur frequently in colorectal and neuroendocrine tumors, but other malignancies such as gastrointestinal cancers, sarcomas, ocular melanoma and even breast cancer, occasionally develop isolated liver metastases as well (1). The liver is the only site of initial colorectal cancer recurrence in as many as 30 % of patients (2,3). If left untreated, the mean survival rate in these patients is poor and will rarely surpass one year. In contrast, 5-year survival rates of up to 40 % have been reported for patients who underwent a radical resection, the standard treatment for isolated liver metastases (1,3). Similar results with long-term survivors have been reported for endocrine and non-colorectal, non-endocrine liver metastases (1,3).

Many patients with tumors confined to the liver are not amenable for surgical resections because of tumor size, number of tumors, tumor location, involvement of vascular structures and/or significantly compromised liver function. Several treatment options are available in this situation, systemic chemotherapy being the most widely used. In case of colorectal liver metastases, standard treatment with the combination of 5-FU and leucovorin provides a median survival of 10-14 months (1,4). Recently developed drugs such as oxaliplatin or irinotecan seem to improve survival rates up to 20 months or more in multiple sequential combinations of these agents (5).

Most patients eventually die due to intrahepatic progression and/or development and progression of extrahepatic disease. In order to achieve a better control of intrahepatic disease and to reduce systemic toxicity of the applied therapy, locoregional therapies have been developed. These therapies include hepatic arterial embolization (6), intratumoral injections of ethanol (7), acetic acid, biological agents (8,9), stereotactic or intraarterial radiotherapy, intralesional laser therapy, cryotherapy, radiofrequency ablation (10) and regional infusion or perfusion of chemotherapeutic drugs (11). The best approach for regional infusion of chemotherapeutics in the liver is unknown (12). Hepatic artery infusion (HAI), hepatic artery ligation with hepatic artery and portal vein infusion, or portal vein infusion, have all been attempted (4,13). Of these modalities, HAI is the most widely applied form. A number of studies have been conducted comparing systemic chemotherapy with HAI and a modest but significant improvement of survival was demonstrated by HAI in a meta-analysis (4). Isolated hepatic perfusion with chemotherapeutic drugs is another locoregional therapeutic option, which allows maximal locoregional drug concentrations, and at the same time protects the

patient from systemic toxicity.

Isolated Hepatic Perfusion

Isolated hepatic perfusion (IHP) was first performed more than 40 years ago by Ausman (14), but experience with the technique has been limited to a few centers worldwide. The main principle for an IHP is to achieve high local drug concentrations and thus high exposure of tumor tissue to these agents. Local hepatic toxicity will be the dose-limiting factor using this treatment modality, but systemic toxicity will be minimal provided leakage from drugs to the systemic circulation is prevented. Since for most chemotherapeutics steep dose-response curves have been demonstrated, this should theoretically result in greater tumor responses. Other theoretical advantages of IHP are the possibility to use perfusion drugs that do not have a high first pass hepatic extraction rate. Perfusion drugs with high systemic toxicity such as tumor necrosis factor (TNF) might be used because the liver can be washed out after a perfusion and thus prevents systemic toxicity. Hyperthermic and/or hypoxic conditions can be achieved during IHP, which might induce synergistic effects in combination with chemotherapeutic drugs (15).

Different ways of performing an IHP are developed using experimental animal models and in several institutions IHP is currently used in controlled trials for patients with inoperable primary or metastatic liver disease.

Animal studies

Large Animals.

The first experimental IHP studies have been performed in the 60's in large animal models such as pigs and dogs, since the liver anatomy and vasculature in these animals resemble those in humans (14,16). When the technique proved to be feasible, hyperthermia was introduced by Skibba in a dog IHP model and demonstrated to be possible with preservation of good hepatic function (17). A few years later, IHP studies performed in pigs with increasing doses of 5-FU with or without hyperthermia demonstrated temporary hepatic enzyme disturbances, but without systemic toxicity in all animals. Levels of 5-FU tolerated by the liver were significantly higher than maximum levels achieved by routine systemic, intra-arterial or intraperitoneal administration (18). In another reliable and technically feasible IHP model in pigs developed by Van de Velde et al. increasing doses of 5-FU were used and demonstrated that at least four times the conventional dose of this drug can be safely administered (19).

As a result of the dramatic responses observed after the use of tumor necrosis factor (TNF) in the isolated limb perfusion setting in melanoma (20) and in large soft tissue sarcomas in combination with or without IFN-gamma (21,22,23) the utility of this cytokine was studied also in the IHP setting.

We investigated in pigs the possibility to utilize the highly toxic cytokine tumor necrosis factor (TNF) in the classic surgical IHP procedure (24). For an agent like TNF to be used successfully a leakage free IHP is a prerequisite and therefore leakage monitoring is essential. We demonstrated that IHP with Melphalan with or without TNF in pigs resulted in very high and stable concentrations of Melphalan and TNF in the perfusate, without measurable systemic TNF concentrations during the perfusion, which proofs a leakage free system (24). Hepatotoxicity was observed in all control animals after IHP, but the transient rise in hepatic enzymes was normalized within one week. Histological sections of the liver demonstrated mild septal edema with polymorph nuclear cell infiltration, without apparent hepatocellular damage or parenchyma necrosis. The addition of high dose TNF and Melphalan did not seem to cause additional hepatotoxic side effects, so most of the changes observed, were interpreted as the result of the IHP procedure itself. Similar results have been reported by others who also showed that washout of the liver with a protein solution reduces systemic TNF levels as well (25).

From other experiments in large animals it became clear that IHP with hyperthermia is technically feasible and safe. Since transplantable tumor cell lines do not exist in large animals, evaluation of anti tumor effects of IHP is studied in tumor bearing IHP models in rodents, but the obvious technical difficulty of performing hepatic perfusions in these animals has limited the number of available data.

Rodent models.

De Brauw et al developed the first IHP model in hepatic tumor bearing rats (26). They demonstrated that a 200-400 % higher dose of mitomycin C (MMC) or Melphalan could be safely administered during IHP and resulted in a 4-5 times higher tumor tissue concentration compared with HAI (27-30). A well-tolerated dose of mitomycin C could induce complete tumor remissions in IHPs, which could not be achieved in tumor bearing rats using HAI (31). Others demonstrated a significantly decreased tumor growth using 5-FU in IHP, but there was also some decreased tumor growth in the control group suggesting an anti tumor effect of the hyperthermic IHP alone (32). In a rabbit hepatic metastases model, hyperthermia preferentially increased vascular permeability in tumors compared with liver tissue in a dose-dependent fashion, thus providing a mechanism for inducing anti tumor effects using IHP

(33).

IHP studies performed in our institution in hepatic sarcoma bearing rats with TNF and Melphalan demonstrated a dramatic increase in regional concentrations of perfused agents (34). IHP with carrier solution only resulted in a significantly decreased growth rate of liver tumors compared to the growth rate of tumors in non-perfused rats. Perfusion with Melphalan alone resulted in minimal antitumor effects and perfusion with TNF only had no effect on tumor growth. When TNF was used in combination with Melphalan, a dramatic anti-tumor effect was observed similar as has been demonstrated in isolated limb perfusions (35-37). These results could not be extrapolated to a colon carcinoma hepatic metastases model in the rat. The difference in response rates between the two tumor models seem to correlate with the hyper – and hypovascular properties of the tumors (38).

Nakamoto et al demonstrated in a similar model that anti-tumor activity of a less toxic TNF mutant (TNF-SAM2) was similar to standard TNF (39). In a recently published study TNF was combined with 5-FU and no additive or synergistic effect was demonstrated with the combination of these drugs (40).

New perfusion methods to improve IHP were recently studied by Rothbarth et al (41). They analyzed Melphalan concentrations in a retrograde perfusion system with occlusion of the hepatic veins and performing hepatic inflow via the hepatic artery and outflow via the portal vein. It was demonstrated that retrograde IHP with continuous infusion of Melphalan in the hepatic artery provides high Melphalan concentrations in the tumor with reduced Melphalan concentration in the liver tissue compared with an orthograde hepatic perfusion. (41)

Gene therapy is another treatment modality that has recently been evaluated in IHP models. In the development of gene therapy protocols it has often been shown that data from in vitro experiments do not predict anti-tumor effects in vivo. To expose viral vectors mainly to the organ of interest and minimize systemic exposure, the IHP setting seems an elegant administration option. A transendothelial route is important in targeting unresectable liver metastases because there is often a wide spread disease trough out the whole liver. Other groups and we already demonstrated effective administration of adenoviral and herpes simplex vectors in regional limb perfusion models (42,43). However, we recently were not able to demonstrated significant transfection or tumor responses after IHP, HAI, intratumoral or systemic treatment (44). Repeated HAI could induce transfection and responses in this study using an adenoviral vector. Thus gene therapy might be a promising option, but needs further experimental studies to establish the optimal route of administration for further clinical trials.

IHP in rodents has confirmed the theoretical advantages of IHP in terms of achieving higher tumor tissue concentrations of the used drugs with subsequent significant better response rates as compared to HAI. With the combination of TNF and Melphalan in IHP, similar synergistic response rates have been demonstrated in highly vascularized tumors as in the isolated limb perfusion setting.

IHP Technique

IHP is an extensive surgical procedure in which the liver and its vasculature are completely isolated from the systemic circulation. The technique involves briefly a bilateral subcostal incision and the liver is mobilized from its retroperitoneal and diaphragmatic attachments and a prophylactic cholecystectomy is performed. Tributaries to the vena cava such as the adrenal, lumbar and diaphragmatic veins are dissected and ligated. The vena cava is isolated and clamped above and below the liver to prevent venous leakage and the isolated segment of the inferior vena cava is cannulated to collect the hepatic venous outflow. A veno-venous bypass is established to maintain venous return from the subhepatic inferior vena cava and the portal venous system. Cannulas are introduced in the saphenous vein and the superior mesenteric vein and connected to a pump that returns blood to the systemic circulation via the axillary vein. The portal vein, proper hepatic artery and gastroduodenal artery are dissected and the hepatic artery and portal vein are cannulated for inflow of the perfusate usually via the gastroduodenal artery and a venotomy of the portal vein. Temperature probes are placed in the liver to measure adequate tissue hyperthermia. Leakage can be monitored using different radioactive labeled agents that are introduced in the perfusion circuit. The perfusate is circulated for 60 minutes using an oxygenator and a roller pump. After the isolated perfusion a washout procedure is performed with crystalloid or colloid solutions. When the cannulas are removed, the liver vascularization is restored.

The best mode of perfusion is not known, but determination of the most optimal route of infusion involves issues of vascular variations, technical aspects of cannulation, as well as tumor blood supply. Normal hepatic parenchyma receives most of its blood supply from branches of the portal vein, but hepatic metastases rely almost entirely on the hepatic artery for its blood supply (45,46). Regardless the route of administration, drugs are delivered to the sinusoid where it is extracted by the hepatocyte and the route of regional administration might not be important for hepatic uptake of drugs. Although most perfusion centers started performing IHP with a dual infusion system, inflow of chemotherapeutic drugs is now mainly via the hepatic artery only, which makes the procedure less invasive.

Clinical experience

A limited number of studies has described IHP inpatients using chemotherapeutics and/or hyperthermia only (Table 1). Hyperthermia enhances cytotoxic effect of several chemotherapeutic drugs, by increasing cell membrane permeability and altering drug transport and cell metabolism (15). Skibba et al. used hyperthermia as the only antitumor modality in a 4 hour IHP and demonstrated a high response rate, but with significant mortality (47). Several chemotherapeutic agents have been used in IHP, of which 5-FU, cisplatinum, MMC are the most widely used (48,49). Chemotherapy-related hepatotoxicity turned out to be significant and the finding of severe veno-occlusive disease was observed when high doses of MMC were applied in clinical phase I/II studies (50-52). In IHP experiments in rats was demonstrated that Melphalan was not only more effective than MMC, but also resulted in lower hepatotoxic side effects. This resulted in the use of Melphalan in phase I/II dose-findings studies (51). Results after several Melphalan based IHP studies indicate that median survival after one IHP treatment is at least comparable to the results obtained from the most effective (multiple treatments) HAI schedules (15,53).

The dramatic increase in response rate reported after addition of TNF in combination with Melphalan to the isolated circuit in ILP (20-23) has stimulated to investigate the possible application of TNF in IHP in patients. TNF acts mainly by damaging the tumor-associated neovasculature and as a result increased intratumoral drug concentrations have been reported in both ILP and IHP animal models (34-36). TNF as a single agent does not appear to have any significant anti-tumor activity when administered in IHP (54). In patients with unresectable hepatic metastases of ocular melanoma the combination of Melphalan and TNF yielded a 62% response rate, which was similar to IHP with Melphalan only. But more interesting, overall median duration of response was significantly longer in those treated with the combination of Melphalan and TNF (14 vs. 6 months, respectively) (55).

IHP with TNF and Melphalan in patients with unresectable hepatic metastases of different origin resulted in a similar overall response rate in a study from our institution and from various reports of the group of the National Cancer Institute (56-60). However, Alexander et al demonstrated that TNF did not improve intratumoral uptake of Melphalan in colorectal metastases (59). We have demonstrated in animal models that TNF significantly enhances drug uptake in highly vascularized tumors, both in the isolated limb perfusion models (35.36), as well as in the IHP models for liver metastases (38). Our observations would correspond quite well with the lack of improvement of drug uptake in colorectal metastases in patients as these metastases are notoriously hypovascular. Together these results suggest strongly that

indeed also in the clinic the effect of TNF and chemotherapeutics is dependent on the vascularity of the tumor, which is recently confirmed in animals. For well vascularized tumors the addition of TNF seem to improve responses or duration of response, but in hypovascularized tumors such as colorectal metastases this effect remains to be defined. In a Swedish study group using both Melphalan and TNF in IHP similar results were seen with only partial responses in patients with hepatic metastases from melanoma or leiomyosarcoma. Patients with liver metastases from colorectal origin showed no response (61). In order to prolong duration of response after IHP, Bartlett et al reported the results of IHP with TNF and Melphalan followed by HAI with FUDR and leucovorin in patients with unresectable colorectal hepatic metastases. Response rates were similar, but median duration of response and survival was improved using the maintenance therapy. This combined strategy could be a manner to improve the present results of IHP (62).

Toxicity

Morbidity is significant after IHP and varies between centers and used perfusion agents. A transient rise of liver enzymes and bilirubin is normal, subsides usually after one week and seems to be mainly related to the surgical procedure itself. Surgical complications and systemic side effects have been described such as postoperative symptoms such as malaise and nausea or symptoms that reflect high leakage during the procedure such as myelodepression and hair loss. Mortality is reported between 0-33% and was due to veno-occlusive disease, TNF-related coagulation disorders and hepatic failure (63). Lans et al showed that the production of secondary mediators in the liver after IHP with TNF and Melphalan might result in subsequent transient hemodynamic alterations not observed with Melphalan alone (64). Most of these side effects can be minimized by a complete isolation and a thorough washout in order to keep systemic levels of perfusion agents during and after IHP as low as possible.

Percutaneous IHP

A new method to improve the current IHP technique is the development of a percutaneous balloon catheter technique, which makes the surgical procedure must easier and faster and in theory would allow for repeated perfusions. Several groups have developed different modifications (65-67) but the technique usually involves an inflow catheter inserted into the proper hepatic artery advanced from the groin. Balloon catheters inserted at the site of the femoral artery or vein and positioned at the upper side just above the hepatic vein and at the

lower side just above the renal vein. A roller pump circulates venous outflow from the liver into the hepatic artery. The portal vein is clamp to avoid leakage. A balloon catheter in the aorta is positioned above the diaphragm to compensate for the blocked venous return. The balloons are inflated and the bloodstream is blocked and perfusion commenced under hypoxic conditions to create an Isolated Hypoxic Hepatic Perfusion (IHHP). We performed IHHP with TNF, Melphalan and MMC in pigs and demonstrated that a leakage free isolated IHHP can be performed with a small surgical procedure and is well tolerated in pigs. Regional drug levels were 20 to 40 times higher than after systemic drug injection (68). After these promising results in pigs, our group started a phase I-II study on IHHP with Melphalan in patients with unresectable hepatic metastases of colorectal origin (66). In our first patients we have had no serious adverse events, but responses were marginal presumably due to substantial leakage, which was present and exceeded 30% in most patients. In these patients a double balloon catheter, which had been shown to allow for a leakage-free IHHP in pigs, was used. This experience in patients lead to a modification of the perfusion concept by using a triple balloon catheter that would completely block the inferior caval vene over a trajectory that spanned the immediate suprahepatic, the whole retrohepatic and the immediate infra-hepatic part of the inferior caval vein. In this setting also the hepatic veins are blocked and thus Inflow was via the hepatic artery but outflow of the perfusate was retrograde via the portal vein. Using this modifications we assumed that venous leakage though tributaries from the inferior vena cava would be stopped and leakage minimal. In 10 patients perfused with this modified technique we demonstrated that the technique of retrograde venous outflow via the portal vein was feasible, but again systemic leakage was a dose-limiting problem. We concluded that with the current balloon catheter techniques no complete isolation of the liver could be established which makes the use of agents such as TNF impossible. Using hypoxia and Melphalan we could not demonstrate responses as were reported with oxygenated leakage-free IIHP (66). Using a percutaneous IHP in its current form must be combined with other treatment modalities such as systemic or HAI to become clinically significant. Another means of using the percutaneous technique is to repeat it during several courses. This has recently been reported by Savier et al., who used a complete percutaneous technique, which they performed repetitively in 4 patients (65). Our disappointing experience with the various forms of occlusion catheters has brought us now to perform a smaller surgical procedure maintaining the method of retrograde outflow through the portal vein: the surgical IHHP. This procedure takes 2-2.5 hours, includes a 30 minutes isolated hypoxic perfusion period and an overall hypoxia of the liver of maximally 1 hour. The procedure is leakage-free and is

consistently associated with clinical responses, including CRs. After 7 patients it is too early to express this in percentages.

Study	*Year*	*N*	*Drugs*	*Art/ Dual*	*Mortality*	*Responses*	*Median survival*
Ausman	1961	5	Nitr Mus	?	?	?	2 LT surv
Aigner	1984	32	5-FU	Dual	20%	?	8 months
Skibba	1986	8	None	?	25%	RR 83%	-
Schwemmle	1987	32 14 4	5-FU MMC Cisplatin	Dual	?	CR 22 %, PR 68%	14 months
Hafstrom	1994	29	Melph/Cispl	Dual	14%	PR 20%	7 months
Marinelli	1996	9	MMC	Dual	11%	CR11% , PR 11%	17 months
De Vries	1998	8 1	Melph + TNF TNF	Dual	33%	PR 83%	10 months
Alexander	1998	34	Melph + TNF	Art	3%	CR 3%, PR 77%	11 months
Oldhafer	1998	6 6	Melph + TNF MMC	Art	0%	CR 17%, PR 33% CR 0%, PR 33%	9 months
Hafstrom	1998	11	Melph + TNF	Dual	18%	PR 27%	16 months
Vahrmeijer	2000	24	Melphalan	Dual	4%	RR 29%	19 months
Alexander	2000	11 11	Melph + TNF Melphalan	Art Art	3% 0%	CR 18%, PR 36% CR 0%, PR 64%	11 months 11 months
Bartlett	2001	19 32	Melph + HAI-FUDR Melph + TNF	Art Art	0% 3%	RR 74% RR 77%	27 months 11 months

Table 1 Overview of literature concerning clinical IHP.

Abbreviations: N, number of patients included in the study; art, arterial inflow site of IHP; dual, arterial and portal inflow site of IHP; Nitr Mus, nitrogen mustard; 5-FU, 5-fluorouracil; MMC, mitomycine-C; Melph, Melphalan; TNF, tumor necrosis factor-alpha; HAI-FUDR, hepatic artery infusion with FUDR; RR, response rate, CR, complete response; PR, partial response; LT surv, long-term survivors.

Future directions

Clinical experience with IHP is limited and is still in its developmental stage. It's efficacy needs to be established in a large phase II experience and only when a response rate of over 70% is achieve I(H)HP would need to be evaluated in a randomized trial to establish its role as compared to systemic chemotherapy and/or HAI. Improvement can be expected using more effective or less toxic drugs. The combination of IHP with maintenance HAI or systemic chemotherapy is tested and looks promising. Results from preclinical research of the past have demonstrated that tumor growth requires an adequate blood supply. In order to obtain a sufficient vasculature, tumor cells stimulate the formation of new vessels (angiogenesis). Subsequently, this dependency of tumors on angiogenesis provides the possibility to limit tumor outgrowth by specific inhibition of new vessel formation (69,70). It is conceivable to combine IHP with adjuvant anti-angiogenic treatment strategies, in order to reduce the recurrence rate, which is the major determinant of the survival rate after IHP. Other modalities such as gene therapy might be used in the IHP setting to increase viral vector uptake to the organ of interest and to minimize exposure systemically. This would minimize the costs and reducing systemic toxicity, herewith morbidity and even mortality of gene therapy. Further simplification and refinement of the technique using percutaneous balloon catheters is expected and may make the technique more applicable. However our own experience has been quite negative with these occlusion catheters since substantial leakage was the common denominator during this period of trials. New endovascular techniques, the growing experience and instrumental improvements in laparoscopic surgery could make a minimal invasive approach feasible. The main problem of percutaneous IHP at this moment is to obtain a leakage free system. The major advantage of the percutaneous technique (if demonstrated effective) would, in contrast to the "open" surgical IHP, a repeatable procedure.

Conclusions

Many patients with tumors confined to the liver are not amenable for surgical resection and an increasing number of these patients are treated by local ablation methods. Isolated hepatic perfusion is another treatment option especially suitable for patients with multiple or bulky primary or metastatic tumors and can mediate clinical regression of advanced liver metastases. Experience with IHP is still limited to a few centers in the world because of the technical difficulties, surgery-related morbidity and unproven efficacy. IHP remains an

experimental modality restricted to specialized units dedicated to treat this difficult group of patients. Experimental animal IHP models have lead to explore new ways of improving efficacy, reducing technical difficulties and minimizing regional and systemic toxicity. Future research should be directed to the identification of suitable biological or chemotherapeutic agents, defining clinical indications, and development of technical modifications to make it more generally applicable.

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VI

13

Summary and Conclusions.

Samenvatting en Conclusies.

Summary

In **Chapter 1** an introduction to the several studies is given. The aims of the thesis are described here.

Chapter 2 addresses the efficacy of TNF in isolated hepatic perfusion (IHP). In an isolated limb perfusion (ILP) model in rats we previously observed synergistic anti-tumor effects of tumor necrosis factor-alpha (TNF) and Melphalan on BN-175 soft tissue sarcoma extremity tumors. The aim of the present study was to investigate if similar synergy in anti-tumor effect could be achieved by treating experimental BN-175 soft tissue sarcoma liver tumors by IHP with these agents. We demonstrated in our IHP model with TNF and Melphalan a dramatic increase in regional concentrations of perfused agents with virtually no concomittant systemic leakage. IHP with only carrier solution resulted in a diminished growth rate of BN-175 liver tumors compared to the growth rate of tumors in non-perfused rats. Perfusing with Melphalan alone resulted in minimal anti-tumor effects. When TNF was added to Melphalan a dramatic anti-tumor effect was observed. Thus, TNF induced synergy with Melphalan is tumor-site independent. Strikingly, potentiation of tumor response in IHP occurred at relatively low concentrations of TNF compared to concentrations eliciting synergy with Melphalan in ILP.

The study performed in **Chapter 3** is based on the favourable results of the a forementioned study of Chapter 2. Previous studies led to the hypothesis that the use of TNF in isolated limb perfusion causes specific destruction of tumor endothelial cells and thereby induces an increased permeability of tumor vasculature. However, whether TNF contributes to the therapeutic efficacy in IHP still remains unclear. In a rat liver metastases model we studied three different tumors: colon carcinoma CC531, ROS-1 osteosarcoma and BN-175 soft tissue sarcoma which exhibit different degrees of vascularization. IHP was performed with Melphalan with or without addition of TNF. IHP with Melphalan alone resulted in all tumor types in a decreased growth rate. In the BN-175 tumor addition of TNF again resulted in a strong synergistic effect. In the majority of the BN-175 tumor bearing rats a complete response was achieved. Secondly, the response rate in BN-175 tumor bearing rats when TNF was co-administrated with Melphalan, was strongly correlated with drug accumulation in tumor tissue, as only in these rats a 5-fold increased Melphalan concentration was observed. Thirdly, immunohistochemical analysis of microvascular density of the tumor showed a significantly higher MVD for BN-175 tumor compared to CC531 and ROS-1. These results indicate a direct relationship between vascularity of the tumor and TNF-mediated effects.

Assessment of the tumor vasculature of liver metastases would be a way of establishing an indication for the utility of TNF in this setting.

In **Chapter 4** gene therapeutic strategies are investigated in our experimental colon carcinoma liver metastases model. In order to target the ras-oncogene we studied the transduction efficacy and anti-tumor activity of an adenoviral vector expressing a intracellular, neutralizing single chain antibody to p21-ras (Y28). Different routes of administration were evaluated to determine which regimen resulted in the best transfection levels and tumor responses: intravenous injection (IV), intratumoral injection (IT), isolated liver perfusion (IHP), or hepatic artery infusion (HAI). Intravenous injection did not result in any measurable transfection. Intratumoral injection resulted only in the transfection of tumor cells along the needle track. IHP as well as single HAI achieved low transfection levels of tumor tissue. Expression of Y28 was demonstrated in tumors after IT injection, HAI and IHP. Whereas, repeated HAI's clearly achieved expression in and around tumor associated vessels. Only five times repeated HAI's with Y28 resulted in significant tumor responses.

Immunological effects of adenoviral gene therapy in IHP are studied in **Chapter 5**. IHP is a methodology that offers possibilities to deliver high concentrations of viral vectors locally without systemic exposure and washout possibilities for non-bound viruses. By means of IHP a very high transduction efficacy is achieved in vivo without significant toxicity using adenoviral vector in fully immunocompetent rats. Moreover a remarkable decreased immune response to the adenoviral vector after IHP was observed. Significantly impaired neutralizing antibody formation and decreased leukocytes proliferation was demonstrated. These findings are a strong argument for further development of gene therapy in pre-clinical isolated perfusion settings.

The study described in **Chapter 6** explores the anti-tumor activity of the previously mentioned adenoviral construct encoding an intracellular single-chain antibody (scFv) against p21*ras* (Y28). In order to determine the influence of the ras status on the efficacy of the scFv we used a wild type rat rhabdomyosarcoma and its ras–oncogene transfectant, for in vitro studies. In vivo we used the ILP delivery method to study anti-tumor activity on established limb tumors. In vitro studies demonstrated an inhibition of growth caused by the Y28 construct. No significant difference between transfected and wild type cell lines could be demonstrated. Upon ILP homogeneous transduction in the tumor with on estimate 5 % transduction of tumor cells was observed. Perfusion with the Y28 construct however, did not result in any additional anti-tumor activity compared to controls.

Chapter 7 reviews the current literature on gene therapy in *in vivo* isolated perfusion models. Locoregional administration of the genetic construct by means of isolated perfusion (IP) of the target organ or extremity is a method that may increase in vivo efficacy. Vascular isolation and perfusion minimizes systemic exposure and thereby reduces unwanted side effects. Several models of IP like hepatic perfusion and limb perfusion, but also isolated kidney, lung and spleen perfusion are discussed. IP delivers vectors highly selectively, with a long exposure time and high concentrations at the target side. This results in higher transduction rates and thereby may improve therapeutic effects.

A clinical ILP study was performed in patients with recurrent melanoma of the limb in **Chapter 8**. Many treatment options are possible, mainly depending on tumor site, the size and the number of the lesions. Repeat biochemotherapeutic ILP with Melphalan and TNF (TM-ILP) is a valuable treatment option in patients with local progression after previous ILP treatment. Of the 25 repeat ILPs performed in our institution, 19 (76%) had a complete response (CR). With an additional 5 ILPs with a PR, total response rate was 96%. Compared to our total database of TM-ILP for melanoma, consisting of 100 ILPs, CR rate of repeat perfusions did not differ from the CR rate of patients undergoing only one ILP. In patients with bulky or numerous lesions that cannot be managed with surgery, and in patients without evidence of systemic disease, repeat ILP provides excellent disease control.

We described our experience in patients older than 75 years treated by TNF-based ILP in **Chapter 9**. The incidence of soft tissue sarcoma (STS) increases rapidly above the age of 50. About 18 % of the patient population is above the age of 70. Also in melanoma the incidence increases in older age groups. Because of fear for TNF associated toxicity, ILP with TNF is not offered to old patients in some cancer centers, whilst especially in older patients an amputation may end their independence and completely limit their mobility. Thus every attempt to avoid amputation must be considered in these patients. In elderly STS patients ILP and surgical treatment resulted in limb salvage in 22/29 patients (76%). In the elderly melanoma patients (n=14) an overall response rate of 100% was achieved. This resulted in a 93% limb salvage. We demonstrated that ILP is very efficient in achieving limb salvage and local tumor control in elderly patients, with acceptable morbidity and mortality. The fact that the 3 fatal complications occurred after leakage free ILPs further underlines the point that the complications were due to patient-specific risks and not to uncontrolled elements of the ILP.

Chapter 10 is review of the results ILP methodology performed in the clinical and experimental setting in different cancer centers worldwide. Several perfusions strategies are discussed such as the isolated limb infusion, the impact of hyperthermia, and the addition of

TNF. The role of prophylactic ILP is evaluated, as well as the importance of targeting the tumor vasculature. Laboratory studies performed to get better insight in the mechanism of TNF based ILP are described. Future directions based on current experimental ILP developments like gene therapy and new vaso-active drugs are discussed.

The results of a clinical isolated hepatic perfusion (IHP) study are described in **Chapter 11**. IHP for irresectable metastases confined to the liver has reported response rates of 50-75%. Magnitude, costs and non-repeatability of the procedure are its major drawbacks. We developed a less invasive, less costly and potentially repeatable balloon-catheter mediated isolated hypoxic hepatic perfusion (IHHP). In a phase I-II study 18 consecutive patients with irresectable colorectal or ocular melanoma hepatic metastases were included. Two different perfusion methods were used: IHHP with orthograde or retrograde flow, both with inflow via the hepatic artery and using Melphalan at a dose of 1 mg/kg. IHHP using balloon catheters resulted in high local drug concentrations but failed to obtain leakage control by neither an orthograde nor a retrograde flow method. Due to this persisting leakage problem and subsequent dose limiting systemic toxicity we were not able to escalate to higher Melphalan dosages.

In **Chapter 12** an overview is given on the worldwide experience of isolated liver perfusion in the treatment liver tumors. Animal studies, IHP techniques, clinical results, toxicity and new developments like percutaneous techniques are reviewed.

Conclusions

Based on the studies performed in this thesis the following conclusions can be drawn:

- Addition of TNF to Melphalan in an isolated hepatic perfusion may induce synergestic anti-tumor effects compared to Melphalan alone.
- Intra-tumoral Melphalan uptake can be enhanced by TNF and is correlated with the microvessel density of the tumor.
- A high intra-tumoral Melphalan concentration improves tumor regression.
- Isolated hepatic perfusion in patients using balloon catheter techniques is less invasive than classical IHP but a leakage free perfusion can not be achieved.
- Colorectal liver metastases are difficult to transduce transvascularly by recombinant adenoviruses. Repeated locoregional administration seems to be essential in achieving a tumor response in vivo.
- IHP with recombinant adenoviruses results in an impaired neutralizing antibody formation compared to systemic treatment.
- Adenoviral gene therapy mediated anti-tumor activity *in vitro* does not always predict *in vivo* activity.
- ILP with recombinant adenoviruses in a rat sarcoma model results in transduction of tumor cells especially at the viable rim and around tumor associated vessels.
- Amputation of limb should be avoided especially in elderly patients: TNF based ILP is safe and effective in elderly people with limb threatening sarcoma and melanoma.
- In patients with recurrent melanoma repeated TNF based ILPs remain highly effective after failure of previous ILP.

Samenvatting

In **Hoofdstuk 1** wordt er een introductie aangaande de diverse studies gegeven. Het doel van het proefschrift wordt eveneens beschreven.

Hoofdstuk 2 onderzoekt de bruikbaarheid en effectiviteit van TNF in de geïsoleerde lever perfusie (IHP). In een geïsoleerd ledemaat perfusie (ILP) model in ratten hebben we reeds eerder een synergistisch anti-tumor effect van TNF en Melphalan op BN-175 weke delen tumor gezien. Het doel van de huidige studie was om te onderzoeken of dit synergistisch anti-tumor effect ook kon worden behaald bij BN-175 lever tumor in een IHP model met deze agentia. In ons IHP model met TNF en Melphalan een zeer sterke stijging van regionale concentraties zonder evidente gelijktijdige systemische lekkage. IHP met alleen perfusie vloeistof resulteerde in een licht vertraagde groeisnelheid vergeleken met onbehandelde tumoren. Perfusie met Melphalan alleen resulteerde eveneens in een minimaal anti-tumor effect. Wanneer TNF werd toegevoegd aan Melphalan resulteerde dit in een sterke tumor respons. Dus TNF gemedieerde synergie met Melphalan is onafhankelijke van de plaats van de tumor. Bovendien trad dit effect op bij relatief lage dosering TNF gezien in vergelijking met de benodigde dosis in de ILP.

De studie uitgevoerd in **Hoofdstuk 3** is gebaseerd op de veelbelovende resultaten uit het hierboven beschreven Hoofdstuk 2. Eerdere studies hebben geleid tot de hypothese dat het gebruik van TNF in de ILP specifieke destructie veroorzaakt van tumor endotheel cellen en daarbij een toegenomen vaatpermeabiliteit induceert. Echter of TNF ook van therapeutisch waarde is in de IHP is nog steeds niet geheel duidelijk. In een rat lever metastases model hebben we drie verschillende tumoren onderzocht: colon carcinoom CC531, ROS-1 osteosarcoma en BN-175 weke delen sarcoom. Elk van deze drie tumoren heeft een andere vascularisatiegraad. IHP werd uitgevoerd met Melphalan met of zonder TNF. IHP met Melphalan alleen resulteerde in alle genoemde tumor types in een afname van de groeisnelheid. Toevoeging van TNF resulteerde bij de BN-175 tumor opnieuw in een sterk synergistisch effect. In de meeste van de BN-175 tumoren werd een complete respons bereikt. Ten tweede was de tumor respons zoals gezien in de BN-175 tumoren sterk gecorreleerd aan de Melphalan opname in het tumor weefsel. Slechts in deze tumoren werd een 5-voudige verhoogde Melphalan concentratie in de tumor gemeten. Ten derde toonde bij immuno-histochemische analyse van de microvaatdichtheid (MVD) een significant hogere MVD van de BN-175 tumor vergeleken met de CC531 en de ROS-1. Deze resultaten wijzen op een directe relatie tussen tumor vascularisatie en TNF gemedieerde effecten. Pre-operatief vaststellen van de tumor vascularisatie graad van lever

metastasen zou een methode zijn om de indicatie voor het gebruik van TNF in de IHP wel of niet te stellen.

In **Hoofdstuk 4** worden gentherapie strategieën in ons experimenteel colon carcinoom lever metastasen model onderzocht. In deze studie hebben we gebruik gemaakt van een adenovirus vector welke een intracellulair antilichaam (Y28) tegen p21-ras herbergt. Dit ras-oncogen is vaak gemuteerd in colon carcinomen. Transfectie (infectie van een cel met dit virus) efficientie en anti-tumor werking werden bestudeerd. Verschillende toedieningswegen zijn geëvalueerd om te bepalen welke het beste transfectie resultaat behaalde evenals een mogelijke tumor respons: intraveneuze injectie (IV), intra-tumorale injectie (IT), geïsoleerde lever perfusie (IHP) en arteria hepatica infusie (HAI). Intraveneuze injectie gaf geen meetbare transfectie van de tumor. IT toonde slechts transfectie langs het traject van de naald. Zowel IHP als HAI gaven meer homogene, doch lage transfectie percentages in tumor weefsel. Y28 expressie kon worden aangetoond in tumoren na IT, HAI en IHP. HAI biedt de mogelijkheid voor herhaalde locoregionale toedieningen. Slechts een 5 x herhaalde HAI met Y28 virus resulteerde in een tumor respons: in alle onderzochte ratten werd een tumor groei remming gezien, en in 3 van de 8 was er een complete tumor respons.

Immunologische effecten van adenovirus gentherapie door middel van IHP werden bestudeerd in **Hoofdstuk 5**. IHP is een methode welke de mogelijkheid biedt tot het bereiken van hoge virale vector concentraties lokaal in het doelgebied zonder systemische blootstelling en met de mogelijkheid van een spoel procedure om aan het eind van de perfusie niet gebonden virussen uit te spoelen. Door middel van IHP met een adenovirale vector werd een hoge transductie efficiëntie van het leverweefsel bereikt zonder significante toxiciteit. Bovendien bleek er een sterk verminderde antilichaam formatie tegen de virussen en was er ook een verminderde leucocyten aanmaak. Deze bevindingen zijn een sterk argument om door te gaan met het ontwikkelen van gentherapie protocollen in pre-klinische geïsoleerde perfusie modellen.

De studie beschreven in **Hoofdstuk 6** verkent opnieuw de anti-tumor effect van het reeds genoemde adenovirus construct tegen p21-ras oncogen (Y28). Dit maal hebben we gebruik gemaakt van een ratten rhabdomyosarcoma welke het ras-oncogen in verschillende sterkte tot expressie brengt: een “wild-type” rhabdomyoasrcoma en een voor ras-oncogen genetisch gemanipuleerde vorm. Deze verschillende tumor cel lijnen werden in vitro getest op hun gevoeligheid voor het Y28 virus. Er werd een duidelijke groei remming van door Y28 gezien in vitro, maar was geen significant verschil tussen de tumor cel lijnen. In vivo werd het anti-tumor effect bestudeerd in het ILP model met een solide tumor ter plaatse van de achterpoot van de rat. De transfectie efficiëntie studie toonde ongeveer 5% getransfecteerde tumor cellen. Echter dit

resulteerde bij Y28 ILP niet in een additionele tumor response ten opzichte van de controle groepen.

Hoofdstuk 7 geeft een overzicht van de huidige aanwezige literatuur over gentherapie in *in vivo* geïsoleerde perfusie (IP) modellen. Locoregionale toediening van het genetisch construct door middel van IP van het doel orgaan of extremiteit is een methode welke de efficiëntie kan doen toenemen. Vasculaire isolatie en perfusie minimaliseert de systemische blootstelling en vermindert daardoor ongewenste neveneffecten. Verschillende IP modellen passeren de revue van lever en ledemaat perfusie tot milt, long en nier perfusie. Door de sterk selectieve toediening, een lange blootstelling en hoge concentraties resulteert dit in hogere transductie graad en daardoor mogelijk ook tot een verbetering van therapeutische effecten.

Een klinische ILP studie uitgevoerd in patiënten met recidief melanomen van een extremiteit is beschreven in **Hoofdstuk 8**. Voor recidief melanomen zijn meerdere behandelopties voorhanden, voornamelijk afhankelijk van plaats van de tumor, de grootte en het aantal laesies. Herhaalde biochemotherapeutische ILP met Melphalan en TNF (TM-ILP) zou een waardevolle optie kunnen zijn voor patiënten met lokale progressie na eerdere ILP behandeling. In de Daniel den Hoed Kliniek hebben we 25 herhaalde ILP uitgevoerd met daarbij in 76% (19/25) een complete respons (CR). In 5 ILPs werd een partiele respons bereikt, een totale respons van dus 96%. Vergeleken met onze complete database TM-ILPs voor melanomen (100 ILPs) was het CR percentage hetzelfde als voor patiënten met slechts een ILP. Voor patiënten met grote of talrijke laesies die niet middels chirurgie behandeld kunnen worden, waarbij er geen systemische ziekte is waarvoor systemische chemotherapie beter is, biedt herhaalde ILP zeer goede locale controle van de ziekte.

Onze ervaringen opgedaan in patiënten met weke delen tumoren (WDT) en melanomen ouder dan 75 jaar welke een TM-ILP hebben ondergaan zijn beschreven in **Hoofdstuk 9**. De incidentie van WDT neemt snel toe in patiënten ouder dan 50. Ongeveer 18% van patiënten populatie is ouder dan 70 jaar. Ook in melanomen stijgt de incidentie in de oudere patiënten groepen. Als gevolg van angst voor TNF geassocieerde bijwerkingen worden in sommige ziekenhuizen TNF ILPs niet aan de oudere patiënten aangeboden. Terwijl juist in oudere patiënten een amputatie van een ledemaat een einde kan betekenen van hun onafhankelijkheid en mobiliteit. Dus juist alles moet worden ondernomen om een amputatie te voorkomen. In oudere WDT patiënten behandeld met TM-ILP en aanvullende chirurgie kon in 76% (22/29) het ledemaat gespaard worden. In de oudere melanoompatiënten (n=14) een totaal respons percentage van 100% werd behaald. Dit resulteerde in ledemaat sparing in 93%. Hierbij hebben we dus aangetoond dat ILP is zeer efficiënt in oudere patiënten, met daarbij acceptabele morbiditeit en mortaliteit. Het feit

dat in het geval van de 3 besproken fatale complicaties er een lekvrije perfusie aan vooraf was gegaan onderstreept het feit dat de complicaties het gevolg waren van patiënt specifieke risico's en niet als gevolg van ongecontroleerde elementen van de TNF-ILP.

Hoofdstuk 10 is een overzichtsartikel dat de resultaten bespreekt van de ILP methode zoals uitgevoerd in zowel de klinisch als de pre-klinische experimentele setting. Verschillende perfusie condities worden besproken zoals de geïsoleerde ledemaat infusie, het effect van hyperthermie en de toevoeging van TNF. De rol van een profylactische ILP wordt geëvalueerd, evenals het belang van het doelgericht behandelen van het tumorvaatbed. Verder worden laboratorium studies beschreven welke zijn uitgevoerd om de werking van TNF in de ILP beter te begrijpen. Toekomstige ontwikkelingen gebaseerd op recente experimentele ontwikkelingen zoals gentherapie en nieuwe vaso-actieve stoffen worden besproken.

De resultaten beschreven in **Hoofdstuk 11** betreffen in klinische geïsoleerde lever perfusie (IHP) studie. IHP voor irresectabele lever metastasen heeft gerapporteerde respons percentages van 50-75%. Een aantal nadelen aan de procedure zijn de grootte en kosten van de ingreep en tevens het feit dat behandeling niet herhaald kan worden. We hebben een minder ingrijpende, goedkopere en potentieel herhaalbare balloncatheter gemedieerde geïsoleerde hypoxische lever perfusie (IHHP) ontwikkeld. In een fase I-II studie werden 18 opeenvolgende patiënten met irresectabele colorectale of melanoom lever metastasen geïncludeerd. Twee verschillende perfusie methoden werden gebruikt: IHHP met orthograde of retrograde stroomrichting door de lever, beide met de instroom via de arteria hepatica en gebruikmakend van Melphalan (dosis 1 mg/kg). IHHP met behulp van balloncatheters resulteerde in hoge locale Melphalan concentraties. Het lukte helaas niet om de perfusie lekkage vrij te krijgen, zowel bij de orthograde als retrograde stroomrichting methode. Als gevolg van dit persisterend lekkage probleem en de daarbij optredende toxiciteit konden we niet doorgaan met hogere Melphalan doseringen.

In **Hoofdstuk 12** wordt een uitgebreid overzicht gegeven over de wereldwijde ervaringen van de IHP in de behandeling van lever tumoren. Pre-klinische diermodellen, diverse IHP technieken, klinische resultaten, toxiciteit en nieuwe ontwikkelingen zoals percutane methoden worden geëvalueerd.

Conclusies

Gebaseerd op de studies zoals uitgevoerd in dit proefschrift kunnen de volgende conclusies worden getrokken:

- Toevoeging van TNF aan Melphalan in een geïsoleerde lever perfusie kan een synergistisch anti-tumor effect induceren in vergelijking tot perfusie met Melphalan alleen.
- Intra-tumorale Melphalan opname kan versterkt worden door TNF en is gecorreleerd aan de microvaatdichtheid van de tumor.
- Een hoge intra-tumorale Melphalan concentratie bevordert tumor regressie.
- Geïsoleerde lever perfusie in patiënten met behulp van balloncatheter technieken is minder invasief dan de klassieke IHP, maar een lekkage vrije perfusie kan niet worden bereikt.
- Colorectale levermetastasen zijn moeilijk te transfecteren door adenovirussen. Een herhaalde locoregionale toediening lijkt essentieel om een tumor respons te bereiken.
- IHP met recombinante adenovirussen resulteert in een verminderde antilichaam productie in vergelijking tot systemische behandeling.
- Gentherapie met adenovirussen resulteren in anti-tumor activiteit *in vitro*, voorspelt niet altijd een tumor respons *in vivo*.
- ILP met adenovirussen in een sarcoom model in de rat resulteert in transductie van tumor cellen met name in de vitale rand van de tumor en rond tumor geassocieerde bloedvaten.
- Juist in oudere patiënten dient een amputatie te worden voorkomen: TNF gebaseerde ILP is veilig en effectief in oudere patiënten met ledemaat bedreigende sarcomen en melanomen.
- In patiënten met recidief melanomen blijven herhaalde TNF gebaseerde extremiteit perfusies zeer effectief, zelfs na eerdere ILP behandeling.

Appendices

Color figures

Chapter 4 (page 65)

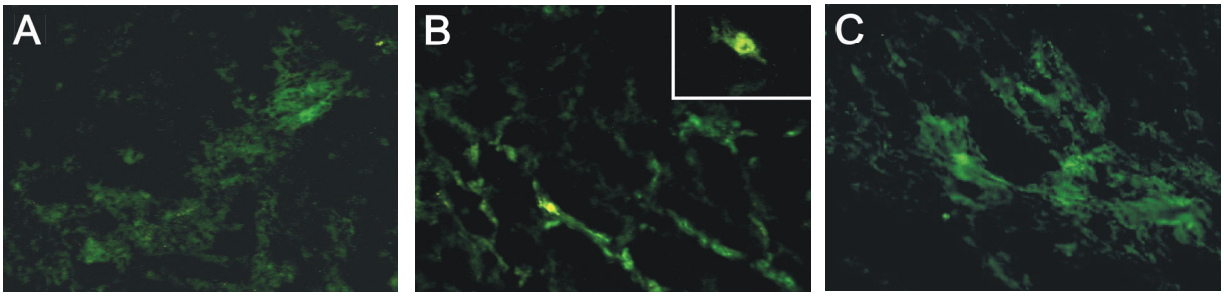

Fig. 2 Y28 fluorescence immunohistochemistry on cryosections of tumours collected 24 hours after treatment *in vivo* with AV.1.0CMV.Y28 (Y28). **A**: tumour after IT, transfection around the needle track. **B:** foci of Y28 expression in tumour after IHP. **C:** expression in tumour after HAI.
Original magnification: A, B and C: 16 x, insert 40 x. No staining was found in case of treatment with AV1.0CMV.

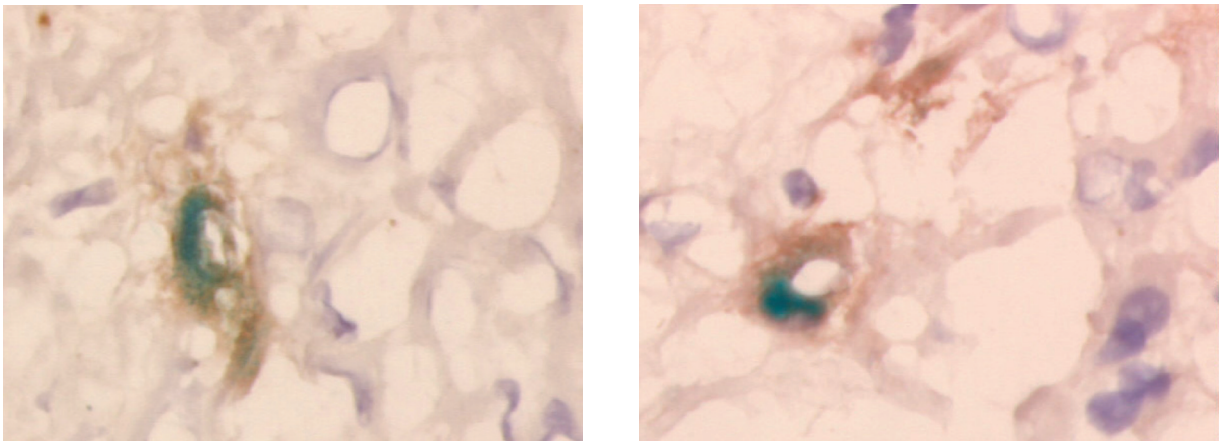

Fig. 3 Two examples of X-gal stained and RECA stained cryosections of tumours of different animals after HAI treatment with AV.1.0CMV.LacZ (left and right). Tumour vessel (brown) and perivascular orientation of transfected cells (dark blue) are clearly visible. Original magnification: 40 x. No staining was found in case of treatment with AV1.0CMV.

Chapter 5 (page 77)

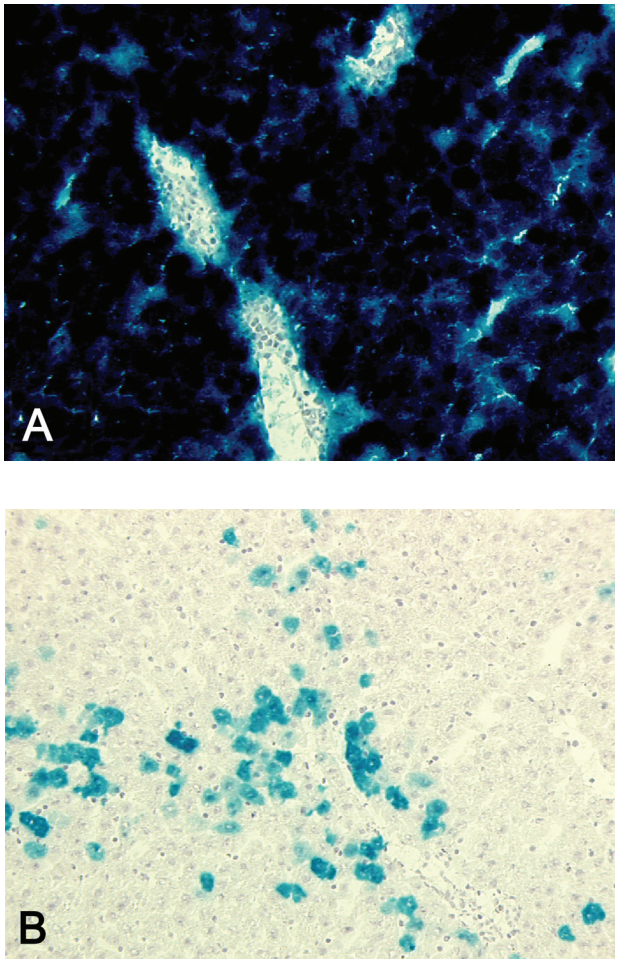

Fig. 1

X-gal staining of liver tissue after treatment with AV1.0CMV.LacZ.
A After IHP, estimated transduction of 80-90 % **B** After IV injection, estimated transduction of about 5 %.

Chapter 6 (page 92)

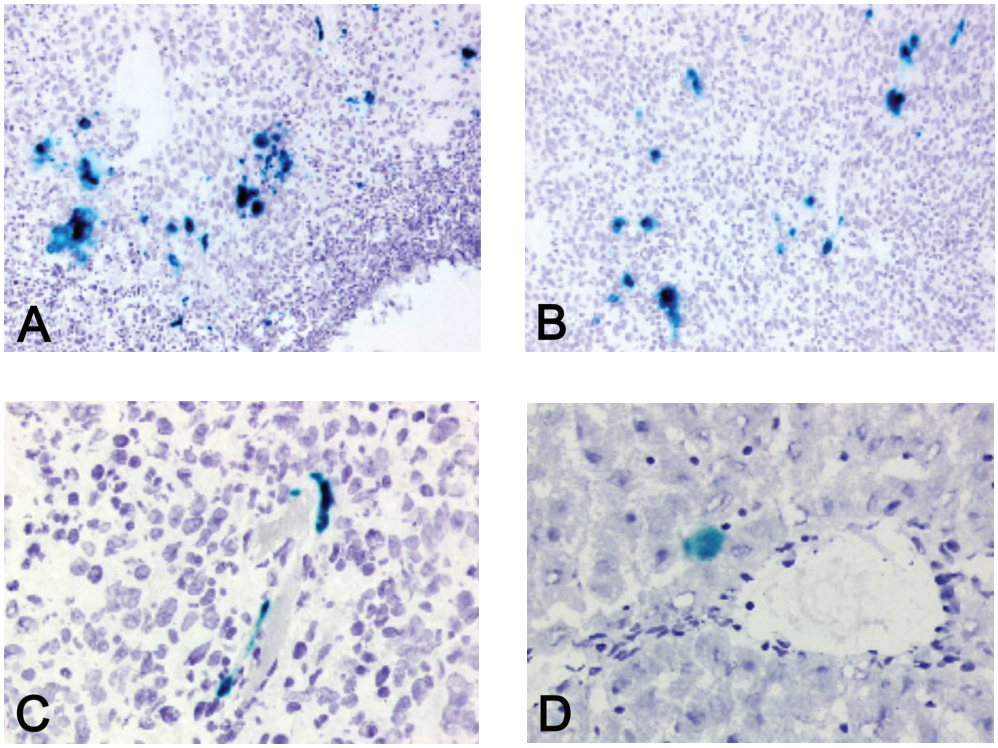

Fig. 3 Light microscopy of X-gal stained cryosections of tumor and liver 24 hours after in vivo treatment by ILP with 2.5 x 10^{11} vp AV1.0.CMV.LacZ. Showing: **A** R2T24 tumor, viable rim area **B** R2T24 tumor, central area, **C**: R2T24 tumor, detail: perivascular transduction , **D**: liver. Original magnification: A, B and C: 160 x, D: 400x.

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- M.G.A. van IJken, B. van Etten, J.H.W. de Wilt S.T. van Tiel, T.L.M. ten Hagen and A.M.M. Eggermont: TNFα augments anti-tumor efficacy in isolated hepatic perfusion with melphalan in a rat sarcoma model. *J Immunotherapy 2000;23:449-4.*
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Curriculum Vitae

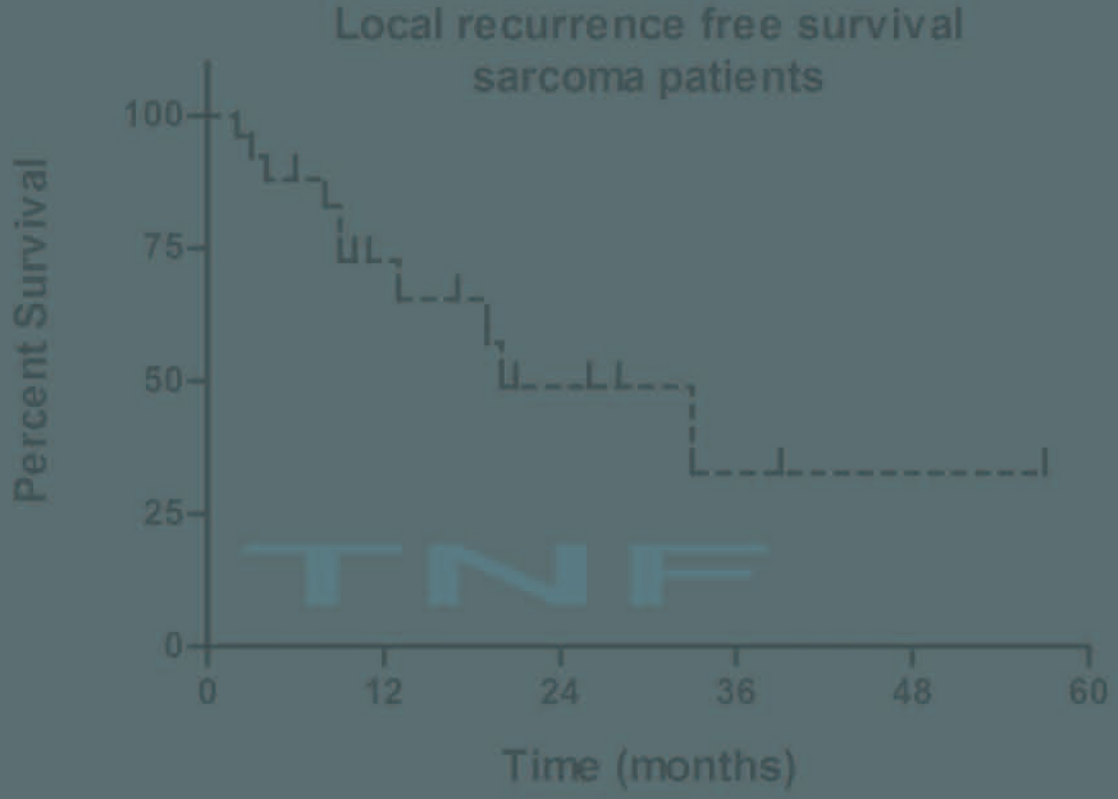
Boudewijn van Etten werd geboren op 28 augustus 1971 te Breda. In 1990 haalde hij het V.W.O. diploma aan de Nassau Scholengemeenschap te Breda. Hetzelfde jaar begon hij aan zijn studie geneeskunde aan de Erasmus Universiteit te Rotterdam. Tijdens de studie doorliep hij klinische stages in Darwin, Australië aan de afdeling Anesthesiologie van het Royal Darwin Hospital en bij de afdeling Thoraxchirurgie van het Royal Prince Alfred Hospital te Sydney, Australië. Afstudeeronderzoek werd verricht aan de Coronary Care Unit van het Thoraxcentrum, Academisch Ziekenhuis Rotterdam-Dijkzigt.

In 1997 behaalde hij het Artsexamen en werd er begonnen als arts-assistent chirurgie in de Daniël den Hoed Kliniek te Rotterdam.

Vanaf maart 1998 werkte hij als arts-onderzoeker op het Laboratorium Experimentele Chirurgische Oncologie te Rotterdam onder leiding van Dr. T.L.M. ten Hagen en Prof.dr. A.M.M. Eggermont, alwaar gedurende enkele jaren de basis werd gelegd voor dit proefschrift. In 2001 was hij gedurende een half jaar werkzaam als AGNIO Chirurgie aan de afdeling Algemene Heelkunde van het AZR-Dijkzigt.

Sinds maart 2002 is hij in opleiding tot algemeen chirurg en werkt momenteel aan de afdeling Chirurgie van het Reinier de Graaf Gasthuis te Delft.

Hum



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