Tumor Necrosis Factor in Isolated Hepatic Perfusion: credits, debits and future directions

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Tumor Necrosis Factor in geïsoleerde lever perfusie:
huidige balans en toekomstige ontwikkelingen

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CONTENTS

To my father

CHAPTER 1

General introduction and outline of thesis.

Irresectable hepatic malignancy

Patients with irresectable hepatic malignancies remain an intriguing clinical problem. Hepatocellular carcinoma (HCC) is the most common primary hepatic malignancy and approximately one million individuals will develop this tumor per year. The incidence of these tumors varies widely worldwide, being most common in the Far East [1]. Recent advances in the early detection of these tumors have improved the prognosis and long-term survival has been reported in patients with small, encapsulated malignancy [2-4]. Nevertheless, the overall prognosis of HCC remains poor and usually expressed in months rather than years [5, 6]

Metastatic disease from colorectal cancer is the most common hepatic malignancy in the Western countries. Most frequently, the liver is the site of dissemination with many other sites in the body (lung, brain, bone). On the other hand, in as many as 30 % of patients the liver is the sole site of initial cancer recurrence [7]. If left untreated the mean survival rate in these patients is approximately 6 to 9 months. In contrast, 5-year survival rates up to 35 % have been reported for patients amendable to resection [8-11]. Unfortunately in the majority (75 %) of the patients that have been diagnosed with colorectal cancer metastases confined only to the liver, these metastases are considered unresectable. These patients are eligible for other therapies.

Systemic chemotherapy

The effect of systemic chemotherapy on hepatic metastases depends on the primary site of metastatic disease and the dose of agents used. Since certain tumors (e.g. breast carcinoma) are responsive to chemotherapy, even when hepatic metastases develop, systemic chemotherapy may be the appropriate treatment. For patients with advanced colorectal carcinoma systemic chemotherapy with 5-fluorouracil (5-FU) based protocols has been the standard therapy. These therapies produce an average response rate of 20 to 30 % with median survival times of 6.5 to 13.5 months, if 5-FU plus folic acid (FA) at conventional doses is used for systemic (iv) therapy [12, 13]. Generally, with systemic combination therapy for hepatic metastases it is not likely to obtain response rates higher than 30 % with only minimal effect on survival and in most

cases this is possible only with considerable systemic toxicity [14]. Most patients eventually die due to intrahepatic progression and/or development and progression of extrahepatic disease. In order to gain improved control of intrahepatic disease and to reduce systemic toxicity of the applied therapy, locoregional therapies have been developed.

Locoregional chemotherapy

Common causes for failure of systemic chemotherapy are low concentrations at the tumor site and dose limiting systemic toxicity. The rationale behind regional administration of chemotherapeutic agents is based on the concept of achieving high local concentrations while minimizing systemic drug exposure and thus reducing dose-limiting side effects. The importance of achieving high local drug levels is based on the steep dose-response curves that have been demonstrated for both sensitive as well as resistant cancer cells. For the latter even extremely high levels are required in order to destroy them adequately [15, 16].

Although the best approach for regional infusion of the liver is still unknown, hepatic artery infusion (HAI) is the single most widely applied form. The rationale for the use of HAI is based on the fact that hepatic tumors obtain most of their blood supply from the hepatic artery. [17, 18]. Furthermore, on first passage through the liver, a significant amount of the anticancer drug could be extracted with subsequent reduced systemic drug levels with reduced systemic toxicity. This has indeed been firmly demonstrated in HAI with FUDR [19]. However, with high local drug levels, there is a greater risk of regional damage to normal hepatic tissue, as well as cancer cells [20]. Only a few completed randomized studies have been reported in patients with unresectable colorectal metastases confined to the liver. Although response rates of 50 to 55 % of HAI using either 5-FU, FUDR or fluoropyrimidines with or without other drug have been demonstrated, the median survival time of 11 to 14 months in most of these studies did not exceed the 11 months of 5-FU iv therapy that showed response rates of 11 to 20 % [21].

Therefore, with HAI, compared to systemic chemotherapy, improved response rates are achieved but convincing evidence of improved survival is lacking. Despite high extraction ratios in HAI, systemic exposure and toxicity cannot be fully eliminated and has been reported the dose-limiting factor. In order to further

increase (maximize) locoregional drug concentrations in the liver and at the same time completely shielding the patient from systemic toxicity isolated hepatic perfusion has been developed.

Isolated Hepatic Perfusion

In isolated hepatic perfusion (IHP) the vascular bed of the liver is completely isolated and perfused with a recirculating circuit. In short, an extracorporeal veno-venous bypass (VVB) circuit (pump aided) is created to shunt mesenteric, renal, and lower extremity blood around the liver to the heart. Next, inflow catheters are placed in the portal vein and/or hepatic artery, and an outflow catheter in the infrahepatic inferior caval vein. These catheters are connected to a heart-lung-machine, and the vascular isolation is completed by clamping the suprahepatic inferior caval vein and the suprarenal inferior caval vein. The liver is then perfused with a normo- or hyperthermic (> 38 °C) perfusate consisting of a mixture of saline and erythrocytes, to which drugs can be added. After the perfusion, the liver is washed thoroughly with a mixture of saline and Macrodex, decannulated, and the vascular continuity restored.

Due to the complete vascular isolation extremely high local drug concentrations can be achieved while on the same minimizing systemic exposure and thus toxicity. IHP is a means to further improve selectivity of administration of antitumor agents to the liver as compared with HAI. IHP with 5-FU in rats and pigs resulted in significantly higher 5-FU concentrations in liver tissue of animals in the higher dose groups [22]. When mitomycin C (MMC) was administered by IHP a 400% higher dose could be safely administered and resulted in a five times higher tumor tissue concentration as compared with HAI [23]. These data suggest that five times the HAI dose can be administered with IHP in order to achieve similar systemic drug levels. Therefore, IHP is a method to maximize selective administration of antitumor agents to the liver while maintaining very low systemic drug concentrations. As is true in HAI, it is clear from experimental data that in the IHP setting hepatic rather than systemic toxicity is dose limiting [22, 23].

Successful clinical experience with IHP is limited but promising. Thus far, several studies have been published with various chemotherapeutical or biological agents e.g. Nitrogen Mustard, 5-FU, MMC, and melphalan. Reported hepatic

toxicity is mild and transient. Furthermore, data thus far have made it clear that IHP can bring about 3 - 5 years of disease-free survival [24, 25]. More recently, tumor necrosis factor α (TNF) has come into focus as a result of the successes achieved in isolated limb perfusions (ILP) with this cytokine in combination with melphalan. Response rates greater than 80% have been observed in the treatment of irresectable extremity soft tissue sarcomas by ILP with TNF plus melphalan. This has recently led, in Europe, to the approval and registration of TNF for the treatment of locally advanced extremity soft tissue sarcomas by ILP with high dose TNF in combination with melphalan [26, 27].

Tumor Necrosis Factor α

TNF molecule

The structure of the human TNF protein is a homotrimeric complex of 52 kD that is biologically active [28]. TNF is produced by many cells but mainly by activated monocytes/macrophages [29-31]. Its expression and regulation is affected by a variety of other cytokines, as interferon-γ (IFN), interleukines (IL-1, IL-2, IL-12), GM-CSF, PAF as well as TNF itself [31]. TNF has pleiotropic effects that may depend on its concentration. It has been shown to have vasculotoxic effects at high concentrations while at low concentrations it may promote DNA synthesis and angiogenesis [32, 33]. High concentrations of TNF have antitumor activity in certain murine tumor models [34].

The effects of TNF are exerted by binding to two types of receptor, with molecular weights of 55 kD (TNF-R1) and 75 kD (TNF-R2) respectively, which are present on nearly all mammalian cells [29, 35, 36]. Apart from these two distinct types of receptors, also soluble forms consisting of the extracellular domain of the receptors have been described [37, 38]. The number of receptors on the cell does not predict the magnitude of response to TNF but upregulation (IFN) and downregulation (IL-1) of TNF receptors have been reported [39].

Clinical experience with TNF

The introduction of TNF in clinical trials commenced with systemic administration. Phase I/II trials in cancer patients demonstrated that dose limiting toxicity was already observed at dose levels too low to mediate anticancer effects. [40-42]. Locoregional administration of TNF seemed the only option for

successful application of TNF. Intralesional and intraperitoneal administration were reported to increase response rates somewhat but proved no essential improvement of the effective use of TNF [43-45]. This all changed when TNF based isolated limb perfusions (ILP) in melanoma and in soft tissue sarcoma patients were reported to yield impressive response rates [26, 27, 46]. This led to the exploration of its use in the isolated organ perfusion setting. It was attempted in lungs, kidneys as well as in livers [47-51].

TNF in combination with chemotherapeutics

TNF may potentate the effects of chemotherapy in various ways. The tumor associated vasculature (TAV) responds to TNF with rounding of the endothelial cells resulting in increased gaps, allowing easy passage of soluble materials and even cells [52, 53]. Moreover, i.v. injection of TNF in human melanoma xenograft-bearing mice resulted in significant reduction of the interstitial fluid pressure of the tumors [54]. This phenomenon could increase localization of cytotoxic drugs in the tumor interstitium and thus explain improved tumor response. Secondly, experimental and clinical results demonstrating massive destruction of the endothelial cells, as has been shown *in vitro* and on angiograms in patients after ILP, suggest that the TAV is the primary target for TNF and therefore that destruction of endothelial lining might be responsible for the antitumor response [46, 55, 56]. This process is accompanied by inflammatory responses and seemed to be dependent on infiltrating leucocytes [57]. Coagulative and hemorrhagic necrosis and destruction of the endothelial lining was also seen when TNF was used as a single agent in ILP, however without significant effect on tumor growth in rats. This indicates hat direct effects of TNF are most likely playing a minor role in its antitumor capacity [58, 59].

Outline of the thesis

A large animal model for IHP was developed in pigs in which the technique could be mastered and the toxicity of the combination TNF and melphalan evaluated. For this purpose, a modification of the original IHP technique was developed. The results of IHP in this model with the combination TNF and melphalan or melphalan alone are described in ***chapter 2***.
Following these experiments a phase I study of IHP with TNF and melphalan was started in nine patients with colorectal metastases confined to the liver. In ***chapter 3***, clinical and pharmacological results are presented of this phase I dose-escalation study.
It could be speculated that intrahepatic administration of TNF induces significant hepatotoxicity, as Kupffer cells are known to release various cytokines in response to TNF exposure. Therefore, we were interested in the effects of IHP with TNF on hepatic function, secondary cytokine production and hepatic acute phase response (APR). In ***chapter 4*** the APR in patients during and after IHP with TNF and melphalan or melphalan alone was evaluated regarding the levels and time dependency of TNF, IL-6, and the acute phase proteins C-reactive protein (CRP), α1-acidglycoprotein, α1-antitrypsin, and transferrin. It could be speculated that a similar TNF induced APR could be shown in patients with systemic leakage of TNF during isolated limb perfusion with TNF and melphalan. In ***chapter 5***, we investigated the APR pattern during and after ILP in patients with TNF leakage (> 10 %) and compared these results with patients who underwent an uncomplicated ILP.
TNF mediates its multiple effects by binding to specific high-affinity cell surface receptors. Two distinct TNF receptors (TNFR-p55 or type I and TNFR-p75 or type II) have been identified. These receptors do not only exist as cell surface membrane proteins but also as soluble proteins. Evidence indicates that these soluble TNF receptors (sTNFRs) are derived by proteolytic cleavage from the cell surface from a variety of cells. Since sTNFRs are known to influence the bioavailability of TNF (or modulate the effects of TNF), and since sTNFRs are known to be induced by TNF itself, we hypothesized that IHP with TNF and Melphalan induces *in-vivo* formation of sTNFRs. In ***chapter 6*** we present the effect of the addition of rhTNF to IHP with melphalan on sTNFR-levels in patients with irresectable colorectal metastases confined tot the liver.

Experimental as well as clinical ILP with TNF and melphalan have demonstrated that the tumor-associated vasculature (TAV) is the selective target for TNF. The effects of high dose TNF on the TAV lead to an increased permeability and a significant decrease of the interstitial pressure in the tumor. Both effects lead to a better penetration of cytotoxic drugs into the tumor tissue. Indeed our group demonstrated a 4 – 6 fold increase of intratumoral melphalan concentration when TNF was added to the perfusate in ILP with melphalan in rats. We were interested in the question whether the same holds true for TNF used in IHP. Therefore, we examined the intratumoral melphalan concentrations after IHP with TNF and melphalan in three different hepatic tumors in rats. The results of these experiments are outlined in ***chapter 7***.

In ***chapter 8***, the experimental and clinical results of IHP reviewed from the literature.

Finally, in ***chapter 9***, the conclusions are summarized.

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CHAPTER 2

Isolated Hepatic Perfusion in the pig with Tumor Necrosis Factor-α with and without melphalan.

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ABSTRACT

Isolated limb perfusion with tumor necrosis factor α (TNF) and melphalan is well tolerated and highly effective in irresectable sarcoma and melanoma. No data are available on isolated hepatic perfusion (IHP) with these drugs for irresectable hepatic malignancies. This study was undertaken to assess the feasibility of such an approach by analyzing hepatic and systemic toxicity of IHP with TNF with and without melphalan in pigs. Ten healthy pigs underwent IHP. After vascular isolation of the liver, inflow catheters were placed in the hepatic artery and the portal vein, and an outflow catheter was placed in the infrahepatic inferior caval vein. An extracorporeal veno-venous bypass was used to shunt blood from the lower body and intestines to the heart. The liver was perfused for 60 min with (1) 50 μg kg^{-1} TNF (n=5), (2) 50 μg kg^{-1} TNF plus 1 mg kg^{-1} melphalan (n=3) or (3) no drugs (n=2). The liver was washed with macrodex before restoring vascular continuity. All but one pigs survived the procedure well. A stable perfusion was achieved in all animals with median perfusate TNF levels of 5.1 ± 0.78 x 10^6 pg mL^{-1} (± s.e.m.). Systemic leakage of TNF from the perfusate was consistently < 0.02%. Following IHP, a transient elevation of systemic TNF levels was observed in groups 1 and 2 with a median peak-level of 23 ± 3 x 10^3 pg mL^{-1} at 10 min after washout, which normalized within 6 h. No significant systemic toxicity was observed. Mild transient hepatic toxicity was seen to a similar extent in all animals, including controls.

IHP with TNF with(out) melphalan in pigs is technically feasible, results in minimal systemic drug exposure and causes minor transient disturbances of hepatic biochemistry and histology.

INTRODUCTION

The liver is the commonest site of dissemination in patients with colorectal cancer [1-3]. Five-year survival rates up to 35% have been reported for patients amenable for partial hepatic resection [4-8]. Unfortunately, the vast majority of colorectal metastases confined to the liver are considered to be unresectable [9-11]. In addition, systemic chemotherapy has so far failed to provide satisfactory results in these cases [12, 13]. Therefore, it is mandatory to develop novel strategies in order to obtain tumor control in the liver.

The concept of locoregional administration of chemotherapy is aimed at achieving high local concentrations while minimizing systemic drug levels in an attempt to reduce dose-limiting side effects. This might enhance antitumor efficacy as steep dose-response curves have been described for most chemotherapeutic agents [14, 15]. Several techniques have been developed for regional therapy of hepatic malignancies, of which hepatic artery infusion (HAI) has become most widely used [16-19]. Although HAI has been shown to improve short-term tumor response rates over systemic chemotherapy, it only slightly affects survival, while significant dose-limiting toxicity has been encountered [13, 17-19]. Alternatively, isolated hepatic perfusion (IHP), including total vascular isolation of the liver, has been reported to significantly increase intrahepatic drug concentrations when compared with HAI, while maintaining sufficiently low systemic drug levels [20-27]. However, large animal studies have revealed systemic leakage of the perfused antitumor agent owing to incomplete vascular isolation in up to 20% of animals [22, 25, 27]. Although incidental clinical reports on IHP have confirmed its potential use in humans, it is clear that optimization of the IHP methodology is needed [20, 21, 26, 28]. In addition, a drug(s) that would provide optimal antitumor activity in the IHP setting is (are) at present unknown.

High dose tumor necrosis factor α (TNF) has been shown to be highly tumorocidal both *in vitro* and *in vivo* [29-31]. Many phase I and II studies have demonstrated that systemic administration of TNF in man results in considerable dose-limiting toxicity at dose levels at which no antitumor activity is observed [32-34]. On the other hand, isolated limb perfusion (ILP) with high-dose TNF in combination with the alkylating agent melphalan has recently been documented to be extremely effective in patients with

irresectable soft tissue sarcomas and in patients with stage III melanoma [35-37]. Although the exact mechanism of antitumor action by TNF is unknown, endothelial injury of the tumor associated vascular bed (TAV) has been suggested to play a pivotal role in inducing tumor necrosis [38, 39]. Thus, TNF may be effective against any histological tumor variant, provided the tumor has a well developed vascular bed.

It is not known whether intrahepatic administration of TNF via IHP is feasible with a satisfactory degree of safety. It is possible that TNF might induce significant hepatotoxicity, as Kupffer cells are known to release various cytokines in response to TNF exposure [40, 41]. The present study in healthy pigs was performed to determine the effects of IHP with TNF, with and without melphalan with emphasis on hepatic, as well as systemic toxicity. For this purpose, a modification of the previously reported IHP-techniques was developed and tested.

MATERIALS AND METHODS

Isolated hepatic perfusion

Ten healthy pigs weighing 25 - 33 kg (median 30 kg) were used. All animals received human care in compliance with the guidelines on animal welfare of the Erasmus University, Rotterdam. General anesthesia was induced and maintained with pavulon and fentanyl. Before surgery, all pigs received 0.1 mL kg^{-1} bodyweight depomycine consisting of 200.000 IU mL^{-1} of procainepenicillin and 200 mg mL^{-1} of dihydrostreptomycin. In all animals an arterial line was introduced into the right carotid artery; a tunneled double-lumen central venous catheter and Swan-Ganz catheter were placed in the right external and internal jugular veins respectively. In addition, the left external jugular vein was dissected in preparation for the veno-venous bypass shunt (see below). Via a midline abdominal incision, the liver was mobilized by transecting all ligaments, and the supra- and infrahepatic inferior vena cava (IVC) were dissected and encircled. All phrenic veins entering the suprahepatic IVC were ligated. The hepatoduodenal ligament was meticulously dissected preserving the portal vein (PV), celiac trunk, hepatic artery (HA) and the common bile duct. Branches of the PV and HA, particularly those arterial branches running towards duodenum and stomach, were ligated as needed to

obtain complete vascular isolation of the liver. The right common iliac vein was dissected free. After heparinization with 2 mg kg^{-1} heparin, a veno-venous bypass circuit (VVB) was established using an inverted Y-shaped cannula, to shunt mesenteric, renal and lower extremity blood around the liver back to the heart. For this purpose, a 20F cannula was introduced into the right common iliac vein, passed into the infrarenal IVC, and the free end was connected with one of the two lower limbs of the inverted Y. Next, the left jugular vein was cannulated (20F) and connected to the upper limb of the inverted Y. To complete the VVB, the distal PV was clamped, cannulated (20 French) and connected with the remaining lower limb. Directly before opening the VVB, a clamp was placed on the infrahepatic suprarenal IVC, proximal from the cannula tip. The VVB flow was aided by a centrifugal pump (Medtronic, Biomedics, USA) in a manner identical to the technique currently used during liver transplantation procedures [42]. The liver perfusion circuit was established by introducing a 20 French arterial cannula into the hepatic side of the PV. A 24 French venous outflow catheter was placed into the suprarenal, infrahepatic IVC via a longitudinal phlebotomy (including the pericaval hepatic tissue) and passed into the retrohepatic IVC. These two catheters were connected to the extracorporeal circuit (see below) and, after clamping of the suprahepatic IVC and the HA, portal liver perfusion was allowed immediately in an attempt to minimize anoxic liver damage (first anoxia time). Finally, the HA was cannulated with an 8F catheter, which was subsequently connected thus completing the isolated hepatic perfusion circuit. The extracorporeal perfusion circuit consisted of a double head roller pump, VPCML membrane oxygenator with integrated heatexchanger and reservoir, and arterial bloodfilters, analogous to the extracorporeal circuit used during cardiopulmonary bypass procedures. The circuit was primed with 500 mL of colloid solution (Haemacel) and 500 mL porcine blood. In addition, sodium hydrocarbonate 8.4 % was added to the priming solution (15 - 20 mL). Portal and arterial flow-rates and pressures, together with the oxygen saturation levels in the perfusate, were recorded as indicated by the heart-lung machine. The flow-rates in the VVB shunt were also documented. In addition, the portal flow-rates were measured before and immediately after IHP using a 8 mm 35B548 flow-probe (Transonic Systems, Inc., Ithaca, NY, USA) connected to a Transonic T206X flowmeter (A.B. Medical B.V., Roermond, NL). Once stable IHP was established, as judged by the reservoir level, absence of

systemic leakage from the IHP circuit was confirmed by injection of 1 cm^3 of a 1:10 dilution of fluorescein into the arterial circuit, followed by illumination with a UV (Woods) lamp. The perfusate was heated to 40 ^oC using a cooler/heater device and was kept at ≥ 39 ^oC throughout the drug perfusion period. After 60 minutes of perfusion the liver was washed with macrodex (>1500 mL) until the fluid from the hepatic veins was clear. In order to restore physiological hepatic perfusion, the HA was decannulated and repaired with Prolene 7-0 whereafter the HA and IVC clamps (second anoxia time) were released. Next, the IVC and PV were decannulated and sutured (Prolene 5-0). The VVB was further dismantled by decannulating and ligating the left internal jugular vein and right common iliac vein. Heparin was reversed by injection of protamine. Pigs were sacrificed 4 to 6 weeks after IHP.

Drugs

Recombinant human tumor necrosis factor-α (rhTNF) (0.2 mg per ampoule) was a kind gift from Boehringer Ingelheim, Germany. The cytostatic drug melphalan (Alkeran) was obtained as a sterile powder (100 mg) that was dissolved aseptically using solvent and diluent by Burroughs Wellcome (London, UK).

Treatment schedule

In five pigs, a 60 min hyperthermic IHP was performed with rhTNF (50 μg kg^{-1}) alone, while three pigs were treated by IHP with rhTNF (50 μg kg^{-1}) and melphalan (1 mg kg^{-1}). TNF was administered as a bolus in the arterial line of the perfusion circuit; melphalan was given directly following the rhTNF bolus. In 2 control pigs no drugs were added (sham group).

Sampling schedule

Perfusate was sampled at t = 0 (i.e., upon drug administration), 15, 30, 45 and 60 min. Systemic blood samples were collected at the day before IHP, during IHP at t = 0, 15, 30, 45 and 60 min, and after perfusion at t = 1, 10, 30, 60, 120 and 480 min, day 1, 3, and 7, and weekly thereafter. Blood samples were centrifuged at 5000 rpm for 5 minutes. Supernatants were stored at -70 ^oC until analysis. Biliary samples (approximately 5-10 mL) were taken by direct puncture of the gall bladder before IHP, immediately after IHP and upon closure of the abdomen.

TNF assay

TNF was measured by a sandwich-type ELISA using two monoclonal antibodies (Department of Immune Reagents, Central Laboratory of Blood Transfusion, Amsterdam, The Netherlands) raised against rhTNF (courtesy of Dr. A. Creasey, Chiron Corp., Emeryville, CA, USA). One mAb (mAb CLB-TNFα-7) was used for coating at a concentration of 2 μg mL^{-1}. The second mAb (mAb CLB-TNFα-5) was biotinylated and used in combination with streptavidin poly-horseradish peroxidase conjugate to detect bound TNF. Stimulated human mononuclear cell supernatant was used as a standard for comparison with purified rhTNF. Results were expressed as pg mL^{-1} by reference to this standard [43].

Histology

Multiple liver biopsies were taken before and directly after IHP and upon sacrifice at 4 to 6 weeks post-operatively. The tissue samples were fixed in formaldehyde and embedded in paraffin. Five-micrometer sections were stained with haematoxylin and eosin (HE). In addition, samples were taken from all animals in preparation for electron-microscopy (EM).

Statistics

Comparisons within and between groups were made by analysis of variance for repeated measurements (ANOVA) or by the t-test where appropriate. Correlations between maximum or minimum levels of parameters were calculated as Spearmann's rank correlations. The significance level was taken as a probability (two-sided) of < 0.05.

RESULTS

Operation

The duration of the operation ranged from 4 to 7 h (median 6 h). In all animals a stable perfusion was attained with no apparent leakage as demonstrated by the fluorescein dye injection. Further technical details are summarized in table 1. As indicated by the oxygen saturation levels in the perfusate, adequate tissue perfusion was attained in all cases. In addition, the measured flow-rates in the PV did not differ significantly before and after IHP in all groups. Median blood loss was 500 mL (range 300 - 1500 mL), including blood lost in the perfusion circuit. All pigs survived the operation. One animal in the TNF-alone group died on the first post-operative day. At necropsy clear, serosanguinous fluid was demonstrated in the abdomen without evidence of portal hypertension/thrombosis or surgical hemorrhage. One pig in the TNF/melphalan group underwent relaparotomy for hernia cicatricalis 2 weeks after IHP; one pig of the TNF alone group developed pneumonia with elevated leukocyte counts at 4 weeks after perfusion. At the time of necropsy all remaining animals were in good general condition, with weights ranging from 30 to 40 kg. In fact, 4 weeks after IHP, all surviving animals had gained weight. Weight gains did not differ significantly between groups. Macroscopic post-mortem examination did not reveal any intra-abdominal or intra-thoracic abnormalities.

		Control (n=2)	TNF (n=5)	TNF/Melphalan (n=3)
Anoxic period (min)				
	1 st	0 ± 0	5 ± 8	0 ± 0
	2 nd	6 ± 1.4	13 ± 3	13 ± 3
Flow rate VVB (mL min-1)		1125 ± 176	1053 ± 50	1117 ± 29
Perfusion pressure (mm Hg)				
	HA	125 ± 35	110 ± 46	178 ± 54
	PV	33 ± 4	38 ± 6	43 ± 6
Perfusion flow rate (mL/min)				
	HA	225 ± 14	237 ± 121	178 ± 21
	PV	470 ± 42	350 ± 71	407 ± 55
Perfusate O_2 - saturation (%)		77.7 ± 0	73 ± 5	72 ± 1

Table 1. Technical data. Technical perfusion data as indicated by pump and H-L machine. Data are presented as means ± s.e.m. First anoxic period is defined as time between clamping and portal perfusion; second anoxic period as time between initiation of washout and arterial recirculation.

Hepatic enzymes

In all animals, IHP resulted in significant elevations of aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), lactate dehydrogenase (LDH) and alkaline phosphatase levels, with peak values occurring at day 1 postoperatively (figure 1). Transaminase levels returned to normal within the first 7 to 10 postoperative days, wile alkaline phosphatase and LDH remained slightly elevated throughout the follow-up period. Total bilirubin values remained within the normal range, as did the serum values of urea, γ-GT and creatinin (data not shown). There were no significant differences in peak values or kinetics between the three groups. In all groups, serum albumin levels decreased to a nadir of approximately 22 g L^{-1} on the first postoperative day and returned to normal values within the following 7 to 14 days. Haemoglobin and haematocrit remained normal throughout the follow-up period (data not shown). In contrast, platelet counts decreased slightly, but not significantly, during the first postoperative day, and normalized within 3 to 7 days.

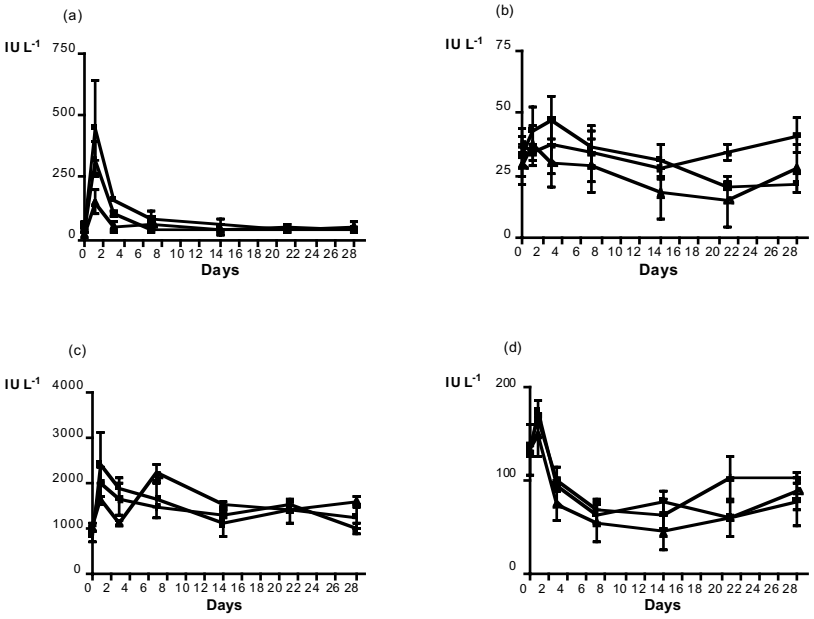

Figure 1. Course of liver biochemistry parameters as a function of time (days) following hyperthermic isolated hepatic perfusion (IHP) in pigs on day 0. (a) ASAT, aspartate aminotransferase; (b) ALAT, alanine aminotransferase; (c) LDH, lactate dehydrogenase; (d) alkaline phosphatase. -▲ -, control; - ■ -, TNF; -✳- , TNF + melphalan.

TNF levels

TNF levels in the perfusate of the pigs in the TNF alone group increased to a median of 5.0 x 10^6 pg mL^{-1} (range 4.9 - 6.3 x 10^6 pg mL^{-1}); compared with 5.2 x 10^6 pg mL^{-1} (range 5.1 - 6.6 x 10^6 pg mL^{-1}) in the TNF/melphalan group. These perfusate TNF levels remained virtually stable throughout the 1 h perfusion period. Perfusate TNF levels in the control group remained normal (i.e., < 5 pg mL^{-1}) throughout IHP (figure 2). At t=0 (i.e. at the beginning of the perfusion) all groups displayed normal systemic TNF levels. During IHP, systemic TNF levels in the control group increased to a median of 12 pg mL^{-1} (8.9 - 15 pg mL^{-1}) at t=60 min, compared with 76 pg mL^{-1} (41 - 120 pg mL^{-1}) in the TNF alone group, and 139 pg mL^{-1} (34 - 197 pg mL^{-1}) in the TNF/melphalan group. These figures indicate that, in both experimental groups, systemic leakage of TNF from the perfusate was less than 0.02% during the 60 min perfusion. However, following washout and decannulation at the end of the perfusion, systemic TNF levels increased significantly in the TNF alone group and the TNF/Melphalan group, with median peak levels of 3.2 x 10^3 pg mL^{-1} and 17 x 10^3 pg mL^{-1} respectively (figure 2). These peak levels occurred between 1 to 30 min (median 10 min) after washout, and returned to normal within 480 minutes after IHP. Again, there were no significant differences between the two experimental groups. Systemic post-perfusion TNF levels in the control group rose slightly, but not significantly, to a maximum value of 26 pg mL^{-1} at t=60 min after washout. None of the evaluated biliary samples contained detectable levels of TNF.

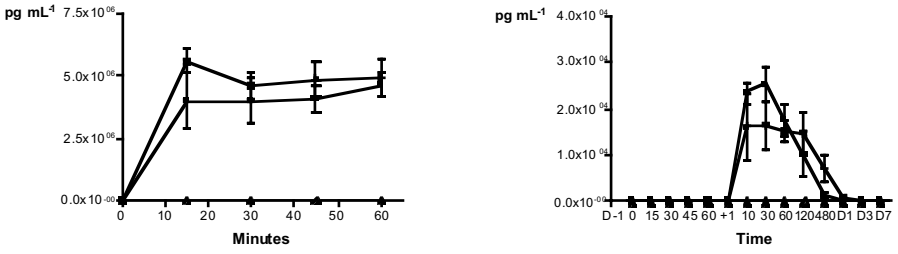

Figure 2. Perfusate (left panel) and systemic (right panel) TNF levels (pg mL^{-1}).as a function of time before, during and after IHP in pigs. - ■ -, TNF; - ✳ -, TNF + melphalan; - ▲ -, control

Histology

Compared with pre-perfusion histology, microscopic examination of HE stained sections taken directly after perfusion showed mild sinusoidal dilatation as well as septal edema with sporadic intraseptal polymorphonuclear cell (PMN) infiltration. These findings were documented in all animals, including controls. There was no apparent hepatocellular damage or parenchymal necrosis. At 4 to 6 weeks after IHP, all microscopical sections revealed normal pig liver histology (on both HE and EM) with the exception of sporadic PMN infiltrates in the liver parenchyma. The septal oedema and sporadic septal infiltration had disappeared in all specimens investigated. Again, these findings were similar in all three groups.

DISCUSSION

The data presented here demonstrate that, in the pig model used, hyperthermic isolated perfusion of the liver via both the HA and the PV is technically feasible and safe. Nevertheless the current IHP technique still involves a large operation, as illustrated by the median duration of 6 h and the one postoperative death. Additional modifications, including the use of balloon catheters are therefore being studied at present. Temporary exposure of normal porcine liver parenchyma to high dose rhTNF with and without melphalan, in combination with hyperthermia, is well accepted and results in mild, transient hepatotoxicity. This was illustrated by early elevation of liver enzyme levels, followed by spontaneous return to normal levels. On histological analysis immediate post-perfusion changes included sinusoidal dilatation and mild septal oedema, without any signs of hepatocellular injury. Sections taken 4 to 6 weeks after IHP revealed sporadic, periportal infiltrates in otherwise normal hepatic parenchyma. Most biochemical and histological alterations following IHP were similar in both control and experimental animals. This suggests that the mild hepatotoxicity phenomena observed were primarily caused by the IHP procedure itself, and that the addition of the drugs used, in particular rhTNF does not lead to additional hepatotoxicity. These findings are in agreement with those reported on IHP with hyperthermia and/or standard chemotherapeutics [25-27].

Complete vascular isolation of the liver during IHP is essential to avoid systemic exposure to high doses of antitumoral agents. Previous studies on IHP in large animals, using somewhat different methodologies, have mentioned technical difficulties resulting in incomplete vascular isolation and systemic leakage of drugs. Van de Velde et al reported leakage in 3 out of 15 pigs with IHP, whereas Sindelar et al encountered incomplete vascular isolation in 2 of 10 pigs resulting in severe systemic 5-FU toxicity and death [25, 27]. In these studies either a passive external or an internal venous shunt was employed to drain distal portal and lower body blood. In view of their findings, we modified the IHP technique in an attempt to minimize leakage. This modification involved the introduction of a separate, second active circuit which consisted of a pump-aided, extracorporeal veno-venous bypass shunt (VVB) connecting cannulas in the distal PV and infrarenal IVC with the external jugular vein. Besides simplifying the hepatic perfusion circuit in this manner (as opposed to internal venous shunts) the VVB has the additional advantage of more efficiently shunting blood from the lower body and intestines to the heart. As a result, the cardiac venous return increases, thereby augmenting haemodynamic stability throughout the procedure. In fact, we did not observe any haemodynamic instability during our experiments in pigs, generally considered to be haemodynamically sensitive. Moreover, we have been able to detect that there was no significant leakage from the liver perfusion circuit to the systemic circulation. This was achieved using either of two qualitative methods, i.e. observing fluorescent dye distribution or monitoring perfusate reservoir levels. This was confirmed in a quantitative manner by analyzing, during the vascular isolation period, systemic levels of TNF, which remained about 4 orders of magnitude lower than perfusate levels. In addition, all pigs survived the procedure and no animal demonstrated any of the known side effects of rhTNF during and after IHP [44, 45].

However, following IHP and washout, an additional rise in systemic TNF levels was seen upon restoration of vascular continuity. Although well below toxic concentrations of rhTNF in the pig, this phenomenon still has to be accounted for. It is possible that the washout procedure was not sufficiently effective in removing all remaining TNF from the perfusate. This may be particularly true in the non-tumor bearing pig liver, in which virtually no TNF uptake was observed during IHP, as judged by perfusate TNF levels (figure 2).

There is no consensus about the route of infusion (HA vs. PV vs. both). Normal hepatic parenchyma receives most of its blood supply from branches of the PV and to a much lesser extent from the HA. In contrast, the blood supply of hepatic metastases is reported to rely almost entirely on the HA [46]. Consequently, most regional approaches have been using the HA. More recently, however, attention has been drawn to the PV as very small liver tumors (< 5 mm), as well as the outer rim of larger hepatic metastases, are fed mainly by portal branches [46, 47]. In addition, most colorectal tumors are drained via the PV suggesting that spreading tumor cells will first proliferate in the portal system. Thus, by using the HA as well as the PV, drugs will reach both established and newly formed (micro) metastases. Taking this into consideration, we performed IHP via both the HA as well as the PV. However, since most normal hepatic parenchyme tissue is supplied primarily by the PV, it could be speculated that infusion via the PV might induce significant hepatotoxicity. Indeed, Boddie et al performed IHP solely via the PV and demonstrated significant hepatic damage [48]. In accordance with most other reports on IHP, we have not been able to confirm these findings [25, 26, 49].
At present, it is unknown which drug, or combination of drugs, would provide antitumoral efficacy in the IHP setting. TNF and melphalan were selected in this study based on its clinical success (100 % limb salvage and a 90 % overall response) in isolated limb perfusions for irresectable melanoma and sarcoma [35-37]. As at least part of the antitumor effect of TNF relies on the destruction of tumor associated vessels, irrespective of tumor histology, we reasoned that this combination might well be effective against colorectal hepatic metastases [38, 39, 50]. Indeed, Van der Schelling et al demonstrated that intratumoral administration of rhTNF under ultrasonographic guidance, was able to stabilize disease in eight patients with hepatic metastases at the cost of minimal systemic symptoms [51]. As demonstrated by Mavligit et al, HAI with rhTNF permits a more than sixfold dose increase of the maximum tolerated systemic (i.v.) dose before adverse systemic side effects are noted. In this setting, TNF was found to induce tumor regression in about 30% of patients with irresectable colorectal liver metastases [52]. Because of the synergy between melphalan and TNF as demonstrated in earlier reports, melphalan was chosen over 5-FU, the drug most frequently used in conventional regimens against colorectal (liver) metastases [35-37]. For reasons of comprehensiveness, we also performed IHP with TNF and 5-FU in

two pigs. No mortality was encountered, and (hepatic) response patterns were identical to the ones described above (data not shown).

On the other hand, Kahky et al have shown that intraportal administration of 100 μg kg^{-1} day^{-1} rhTNF results in 100% mortality in rats. Histological examination in their study revealed mild passive congestion of the liver combined with severe pulmonary edema. As systemic administration of the same dose of rhTNF did not result in death, it is unlikely that the high mortality after intraportal injection was caused solely by TNF [53]. TNF has been documented to induce the production of various cytokines (including IL-1, IL-6 and TNF) by macrophages (i.e. Kupffer cells) [40, 41]. As the vast majority of hepatic Kupffer cells are situated in the (peri) portal area, such a secondary cytokine release might explain the observed mortality. As far as we can judge, IHP with rhTNF and melphalan in the healthy pig does not lead to such dramatic cytokine-related side effects.

In conclusion, hyperthermic IHP with rhTNF and melphalan in pigs is technically feasible, resulting in minimal systemic leakage of drugs and mild hepatotoxicity. The addition of rhTNF and melphalan in the perfusate does not lead to additional hepatotoxic side effects. As pig liver physiology is similar to humans, IHP with rhTNF and melphalan should be considered for phase I evaluation in patients with irresectable hepatic malignancy.

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CHAPTER 3

Isolated Hepatic Perfusion with Tumor Necrosis Factor α and Melphalan in patients with colorectal hepatic metastases.

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ABSTRACT

We report our experience with isolated hepatic perfusion (IHP) with Tumor Necrosis Factor α (TNF) and melphalan in a phase I study in humans. An extracorporeal veno-venous bypass was used to shunt blood from the lower body and intestines to the heart. IHP was performed with inflow catheters in the hepatic artery and portal vein and an outflow catheter in the caval vein. The liver was perfused for 60 min with 1 mg kg^{-1} melphalan plus 0.4 mg TNF (n = 8) or 0.8 mg TNF (n = 1) with hyperthermia (> 41 °C). After the perfusion the liver was washed with macrodex before vascular continuity was restored. There was leakage in one patient (cumulative leakage 20%). There were three perioperative deaths (one possibly drug related). All patients demonstrated significant but transient hepatotoxicity. Survival ranged from 6 to 26 months (median 10.3 months). All patients demonstrated a tumor response (5/6 partial response, 1/6 stable disease) with a median duration of 18 weeks.

In contrast to our experimental program in pigs, many problems were encountered in the phase I study. By using both the hepatic artery and portal vein for IHP we encountered more toxicity than expected based on data from the pig program, resulting in fatal coagulative disturbances in one patient who received the second rhTNF dose. Furthermore, local control after one IHP with TNF and melphalan is only temporary.

INTRODUCTION

The liver is the most common site of dissemination in patients with colorectal cancer, and if left untreated the median survival in these patients is approximately 6-9 months [1]. In contrast, 5-year survival rates as high as 35% have been reported for patients amenable to partial hepatic resection [2]. Unfortunately, most colorectal metastases confined to the liver are not resectable. Therefore it is mandatory to develop novel strategies to obtain tumor control in the liver.

Several techniques have been developed for regional therapy of hepatic malignancies, of which hepatic artery infusion (HAI) is most widely used [3]. Although HAI has been shown to improve short-term tumor response rates compared to systemic chemotherapy, it hardly affects survival and significant dose-limiting toxicity has been encountered [3].

Alternatively, isolated hepatic perfusion (IHP), with total vascular isolation of the liver, significantly increased intrahepatic drug concentrations when compared with HAI while maintaining sufficiently low systemic drug levels [4-7]. With IHP hepatic rather than systemic toxicity may prove to be dose-limiting. Incidental clinical reports on IHP are promising, indicating the potential use of this technique in humans [4, 8-10]. It is clear that optimization of the IHP methodology is needed. In addition, it is presently unknown which drug(s) would provide optimal TNF activity in the IHP setting.

A promising drug with important *in vitro* and *in vivo* is tumor necrosis factor α (TNF), a cytokine produced mainly by activated macrophages [11]. In humans systemic administration of TNF in many phase I and II studies has resulted in considerable dose-limiting toxicity with dose levels at which no TNF activity was observed [12, 13]. Multicenter studies have now shown that high-dose TNF, in combination with the alkylating drug melphalan, can be used safely in isolated limb perfusion (ILP), where complete vascular isolation of the extremity involved ensures minimal systemic exposure to the drug [14-16]. Although the exact mechanism of antitumor action by TNF is unknown, endothelial injury of the tumor associated vascular bed (TAV) after ILP was ascribed to be essential in the genesis of tumor necrosis [17]. Thus TNF may be expected to prove effective against any histological tumor variant, provided the tumor has a well developed vascular bed [18].

It could be speculated that intrahepatic administration of TNF induces significant hepatotoxicity, as Kupffer cells are known to release various cytokines in response to TNF exposure [19]. On the other hand, based on the synergy between TNF and melphalan, considerable tumor responses could be anticipated. Following this step, we started a phase I clinical study in nine patients with colorectal metastases confined to the liver. For this purpose, a modification of the original IHP technique was developed and tested.

MATERIALS AND METHODS

Study Design

The study was designed as a dose escalation study to assess the toxicity and maximal tolerated dose (MTD) of recombinant human TNF (rhTNF) in combination with melphalan 1 mg kg^{-1} body weight in a hyperthermic, isolated hepatic perfusion (IHP). The study was performed in two centers: the Erasmus Medical Center Rotterdam - Daniel den Hoed Cancer Center and the Leiden University Medical Center. *Inclusion criteria* for IHP with TNF and melphalan were: (1) histological evidence of unresectable metastases of colorectal origin confined to the liver; (2) age between 18 and 70 years; (3) Karnofsky performance status of > 80%. *The exclusion criteria* (summary) included: (1) extrahepatic malignant disease; (2) > 50% hepatic tissue replacement by tumor; (3) liver cirrhosis; (4) signs of significant hepatic dysfunction (abnormal levels of aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) or alkaline phosphatase more than two times normal), and (5) ascites or portal hypertension.

From January to June 1995, nine patients underwent IHP with TNF and melphalan. All gave informed consent prior to treatment. The protocols were approved by the hospitals' ethics committees. There were six men and three women with a mean age of 59.8 years (range 49 - 65 years). The median replaced hepatic volume (RHV) was 20% (3.5 - 45%). Patient characteristics are outlined in table 1.

Operative procedure and leakage monitoring

The procedure of IHP has been described elsewhere [20]. Briefly, following systemic heparinization (200 U kg^{-1}), an extracorporeal veno-venous bypass (VVB) circuit (pump aided) was created to shunt mesenteric, renal, and lower extremity blood around the liver to the heart. Next, inflow catheters were placed in the portal vein and hepatic artery, and an outflow catheter in the infrahepatic inferior caval vein. These catheters were connected to a heart-lung-machine, and the vascular isolation was completed by clamping the suprahepatic inferior caval vein and the suprarenal inferior caval vein. The liver was then perfused with a hyperthermic (> 38 °C) perfusate consisting of a mixture of saline and erythrocytes. Once a stable counter-per-minute (cpm) baseline was obtained from scintillation probes placed over the perfusate reservoir and VVB, 200 μCi I^{131} - albumin was injected into the perfusate. Based on the systemic baseline count and the perfusion circuit volume, the percentage of leakage can be accurately calculated. If there was more than 1% leakage over 10 min, adjustments were made in the perfusion flow rates and cannula position in an attempt to identify the source of the leak prior to administering the rhTNFα and melphalan. The leak rate was monitored for the duration of the perfusion; and if the cumulative leak was more than 15%, the perfusion was halted and the perfusate flushed from the circuit. After the absence of leakage was confirmed, rhTNF (0.4 mg in eight patients, 0.8 mg in one.) was administered as a bolus in the arterial line of the perfusion circuit; melphalan (1 mg kg^{-1}) was given directly following the rhTNF bolus. After a 60 min perfusion, the liver was washed thoroughly with a mixture of saline and macrodex, decannulated, and the vascular continuity restored. Heparin was reversed with 1 mg kg^{-1} protamine sulphate (Novo-Nordisk AS, Rud, Norway) injection.

Postoperative Care

Postoperatively, the patients were monitored in the intensive care unit (ICU) for at least 48 h, primarily to evaluate for evidence of systemic toxicity due to rhTNF.

Routine laboratory tests were performed once a day for the first week, at days 10, 14, 21 and 28, and every 2 months thereafter. True-cut biopsies of the liver and tumor tissues were performed before and during operation, and 4 - 6 weeks after IHP. Tumor measurement was performed by computed tomography (CT) scan pre-operative, 2 and 4 weeks after IHP and every 2 months thereafter.

Drugs

RhTNF (0.2 mg per ampoule) was a kind gift from Boehringer Ingelheim, Germany. The cytostatic drug melphalan (Alkeran) came as a sterile powder (100 mg) that was dissolved aseptically using solvent and diluent obtained from Burroughs Wellcome (London, UK).

Sampling schedule

Blood samples were collected from a peripheral vein in siliconized 5 mL Vacutainer tubes (Becton Dickinson, Plymouth, UK) containing EDTA 10 nmol L^{-1}, soybean trypsin inhibitor 100 mg L^{-1}, and benzamidine 10 nmol L^{-1} (Sigma Chemicals, Detroit, USA) to prevent any *in vitro* activation. Samples were centrifuged immediately after collection, at 5000 rpm. for 5 minutes. Supernatant was stored at minus 70 ^{o}C until analysis. Perfusate was sampled at t = 0 (i.e., upon drug administration), 10, 20, 30, 40, 50, and 60 min. Systemic plasma samples were collected at the day before ILP, during ILP at t = 0, 30, and 60 min., and post-perfusion (after release of the VCI clamp) at t = 1, 5, 10, 20, 30, 60, 120, and 180 min., at 21:00 h, day 1, 3, 5, and 7, and weekly thereafter.

Assays

Tumor Necrosis Factor-α (TNF) levels were measured by a sandwich-type enzyme linked immunosorbent assay (ELISA) using two monoclonal antibodies (Dept. Immune Reagents, Central Laboratory of Bloodtransfusion, Amsterdam, Netherlands) raised against recombinant human TNF (courtesy of Dr. A. Creasey, Chiron Corp., Emeryville, CA, USA). One mAb (mAb CLB-

TNFα-7) was used for coating at a concentration of 2 μg mL^{-1}. The other mAb (mAb CLB-TNFα-5) was biotinylated and used in combination with streptavidin poly-horseradish peroxidase conjugate (CLB, Dept. Immune Reagent) to detect bound TNF. Stimulated human mononuclear cell supernatant was used as a standard for comparison with purified rhTNF. Results were expressed as pg mL-1 by reference to this standard [21].

Histology

Multiple liver biopsies were performed before and directly following IHP and 4 - 6 weeks after IHP. The tissue samples were prepared for hematoxylin and eosin (H-E) staining and electronmicroscopy (EM).

Toxicity

Toxicity and adverse events were assessed and recorded according to the World Health Organization (WHO) grading system (WHO Adverse Event Coding Thesaurus) [22].

Assessment of tumor response

Responses were defined by computed tomography. Radiologic findings were differentiated as follows (WHO response criteria): complete response (CR) as complete disappearance of all measurable tumor in the liver for more than 4 weeks, partial response (PR) as regression of tumor volume (the sum of the product of the perpendicular diameters of all measurable lesions) by > 50 % for more than 4 weeks, no change (NC) as regression < 50 % of the tumor volume or progression > 25 %, and progressive disease (PD) as progression > 25 % of the tumor volume. The primary efficacy endpoints in the study were best tumor response observed and duration of the best response, calculated from the date the best response was observed until the date of progression.

Statistics

Results are expressed as the mean ± standard error of the mean (SEM). Comparisons within groups were made by means of the Friedman nonparametric repeated measures test or by the Mann-Whitney test, where appropriate. The significance level was taken as a probability (two-sided) of < 0.05.

RESULTS

Operative Procedure

The median duration of the operation was 8 h (range 6 - 10h). Median blood loss was 5250 mL (range 4700 - 25000 mL), including blood lost in the perfusion circuit. A stable perfusion was attained in all patients. Mean flow-rates in the portal and arterial line were 480 mL (range 400 - 600) resp 293 mL (range 250 - 400). The mean veno-venous bypass flow-rate of the portal vein and infrahepatic IVC to the axillary vein during perfusion was 2425 mL (range 1750 - 3000). Systemic leakage was demonstrated in only one patient during the IHP procedure. In this patient this resulted in discontinuing the IHP after 43 min (cumulative leakage 20 %).

Three patients died during the perioperative period. One patient died from sepsis with multiple organ failure (MOF) due to biliary tract necrosis that was a result of common hepatic artery thrombosis following iatrogenic hepatic artery injury. In the second patient there was an unacceptably large blood loss prior to drug administration, possibly caused by preoperative abuse of aspirin (unknown to the physician), which caused severe coagulopathy leading to exsanguination. The third patient (given 0.8 mg rhTNF) was reoperated because of hypotension and shock about 25 min after a completely normal IHP procedure. Uncontrollable, diffuse intraperitoneal hemorrhage was demonstrated, probably caused by a coagulation disorder; it led to exsanguination. This adverse event was reported as probably drug related (rhTNF) and the phase I study was discontinued. The remaining six patients (five men, one woman) survived the operation and were evaluable for tumor response.

Toxicity

Toxicity within the first 30 days after IHP is summarized in table 1. A number of haemodynamic parameters were evaluated. In all patients slight fever, hypotension, pulmonary hypertension, and sinus tachycardia were demonstrated and was reported as drug related (figure 1). Most disturbances normalized within the first 3 days and did not appear to be of major clinical importance. Some degree of pulmonary hypertension was demonstrated in almost all patients, with two patients developing adult respiratory distress syndrome (ARDS). All patients demonstrated an anemia (nadir at day 3) and

thrombocytopenia (nadir 69.2 ± 14 x10^9 L^{-1} at day 3), returning to normal 10 days after IHP. Furthermore, all patients demonstrated initial significantly elevated serum levels of LDH, ASAT and ALAT, normalizing within the first 7 - 10 postoperative days (figure 2). No correlation could be demonstrated between IHP parameters nor TNF levels and the peak levels of LDH, ASAT, ALAT and bilirubin. Two patients had grade III and three grade IV hepatotoxicity. No significant renal toxicity could be demonstrated.

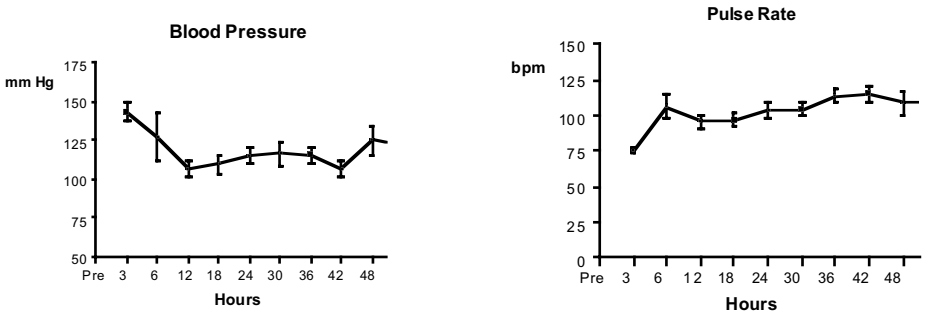

Figure 1. Pattern of blood pressure (left panel) and pulse rate (right panel) before and after IHP. On the y-axis, blood pressure is expressed as mm Hg and pulse rate as beats per minute (bpm). Time is expressed on the x-axis. Pre = day prior to IHP; 3, 6, 12, 24, 30, 36, 42, 48 = hours after IHP.

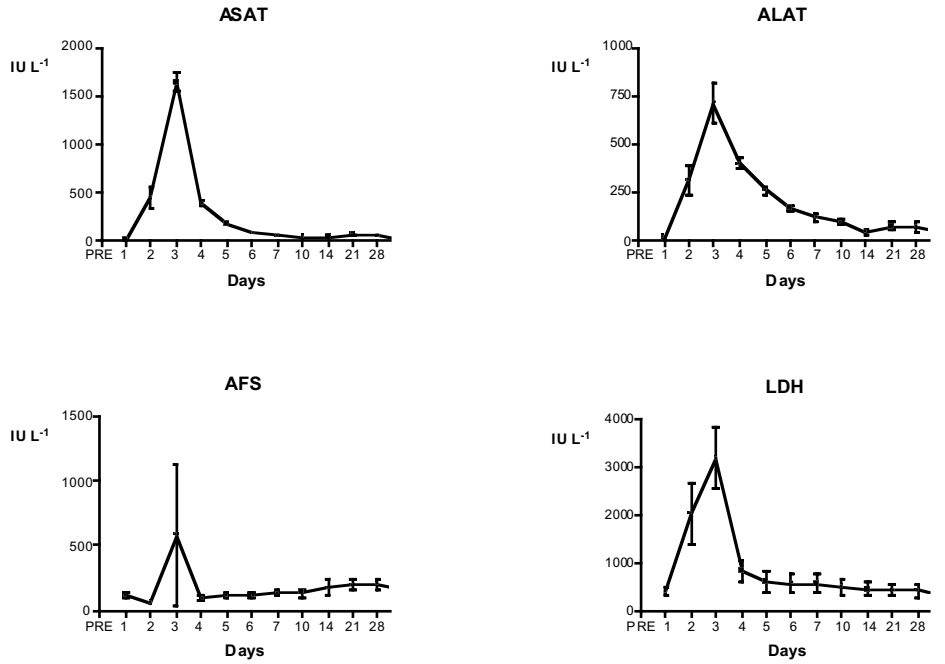

Figure 2. Course of ASAT, (aspartate aminotransferase); ALAT, (alanine aminotransferase); AFS, (alkaline phosphatase; and LDH, (lactate dehydrogenase) before and after IHP. Concentration is expressed on the y-axis in IU L^{-1}. Time is expressed on the x-axis. Pre = the day prior to IHP; 1, 2, 3, 4, 5, 6, 7, 10, 14 = days after IHP

Patient survival and Tumor response

Among the six evaluable patients the survival time ranged from 6 to 26 months. The median survival time was 10.3 months (mean 13.3 months). This best response was confirmed objectively in five patients by a true-cut biopsy and in the other patient by CT scan only. Among the six evaluable patients, five had partial responses and one had stable disease. In one patient, a complete response was observed at autopsy 26 days after IHP. The duration of best response ranged from 17.5 to 32.5 weeks (median 18 weeks). The first sites of progression were the lung (n = 2) and the brain (n = 1). In one other patient local recurrence of the primary tumor (rectal carcinoma) was observed. Two patients demonstrated local progression in the liver.

TNF levels

In the perfusate the initial TNF levels of 1.8 ± 0.5 pg mL^{-1} increased rapidly to 6.0 ± 2.0 x 10^4 pg mL^{-1} at 10 min, followed by a decrease to 2.8 ± 0.9 x 10^4 pg mL^{-1} at the end of IHP (figure 3). Systemic levels of TNF remained virtually unchanged during the IHP. After the washout, systemic TNF levels increased rapidly to a peak value of 169 ± 38 pg mL^{-1} at 1 min, and normalized within the next 2 h.

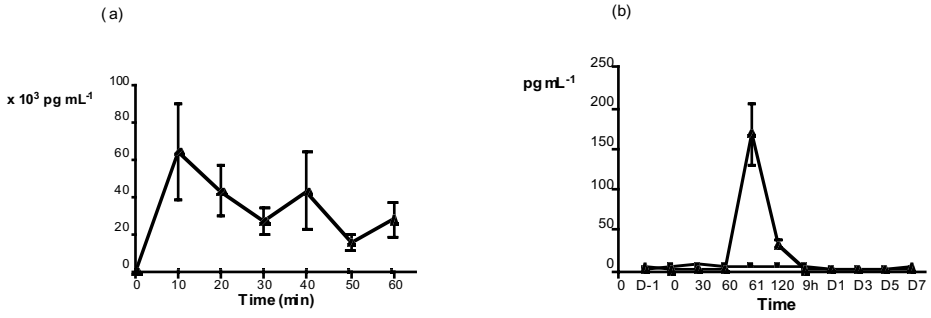

Figure 3. Course of TNFα levels before, during and after IHP. Left panel (a) shows perfusate levels, right panel (b) systemic levels. Time is expressed on the x-axis; left panel: minutes during perfusion; right panel: D-1 = the day prior to IHP; 0, 30, 60, 61, 120 = 0, 30, 60, 61, 120, 240 minutes after start of IHP (61 min represents the time just after vascular restoration; 9h = 9h after IHP; D1, D3, D5, D7 = first, third, fifth and seventh day after IHP.

Table 1. Patient characteristics.

Nr	Sex	Age (years)	HRV (%)	Primary Dukes'	Previous Chemo	TNF (mg)	Melphalan (mg)	Leak %	[TNF]p (pg mL-1)	[TNF]s (pg mL-1)	Pulm	BP	Renal	Hep1	Hep2	Throm	Leuco	Response (weeks)	Survival (months)
1	M	61	25-50	C	yes	0.4	84	0	99062	59	III-IV	-	-	III	-	III	-	18	20.5
2	M	63	40	B2	yes	0.4	66	0	66864	389	III	III	-	IV	IV	III	-	n.a.	n.a.
3	F	50	3.5	D	no	0.4	73	0	16635	27	III	-	-	-	-	-	-	26	7
4	M	61	15	D	yes	0.4	83	0	19807	785	III	-	-	-	IV	III	-	17.5	26
5	M	66	25-50	C2	no	0.4	85	0	105547	6.6	-	-	-	III	III	-	-	n.a.	6
6	M	52	40	C1	no	0.4	90	23	16675	312	-	-	-	IV	III	-	-	18	11
7	F	62	25-50	B2	no	0.4	75	0	41896	8.5	-	-	-	-	-	-	-	n.a.	n.a.
8	M	65	15	C	no	0.4	75	0	27509	115	-	-	-	IV	IV	-	-	32.5	9.5
9	F	63	10	C	no	0.8	69	0	unk	unk	(III)	(IV)	-	(IV)	(IV)	(III)	-	n.a.	n.a.

DISCUSSION

Several IHP methods were employed in the past [5, 8, 23] that initially encountered technical difficulties resulting in incomplete vascular isolation and systemic leakage of drugs. In view of these findings, we modified the IHP technique in an attempt to minimize leakage. The first modification consisted of the development of a pump-aided, extracorporeal veno-venous bypass shunt (VVB) as a second circuit during IHP. The VVB has the additional advantage of sufficiently shunting the blood from the lower body and intestines, resulting in better haemodynamic control during IHP. In fact, no haemodynamic instability was observed during the perfusions. During IHP, complete vascular isolation of the liver is essential to avoid systemic exposure to chemotherapeutic agents. Vascular isolation was complete in all but one patient. In this patient the IHP had to be stopped after 43 min because of progressive systemic leakage (cumulative leakage 20%). Despite the leakage, with higher systemic TNF levels during IHP this patient did not exhibit additional toxicity, demonstrated by clinical and biochemical parameters, compared to the other patients studied.

In contrast to our experimental animal studies in the pig, protracted (hepatic) toxicity was encountered in five out of nine patients. It might therefore be speculated that the effects of rhTNF on the porcine liver are different than those in the human liver. Furthermore, three patients died during the postoperative period. Two of these deaths demonstrated protracted bleeding with one of these possibly drug related, after which the study was discontinued. Unfortunately, we were not able to evaluate coagulation and fibrinolysis in this patient by analyzing TAT, PAP, PAI, and t-PA levels. In the six evaluable patients, measurement of these parameters indicated significant activation of both coagulation and fibrinolysis. Apart from a direct TNF effect the extent of the surgical procedure with subsequent bloodloss may have contributed.

All surviving patients developed some degree of pulmonary hypertension, with two patients developing ARDS. Kahky et al. demonstrated that intraportal administration of rhTNF 100 $\mu g\ kg^{-1}$ per day resulted in 100% mortality in rats. Histological examination demonstrated significant gastric and small intestinal mucosal injury, mild passive congestion of the liver, and severe pulmonary edema. Animals that had received rhTNFα systemically

followed a relatively benign course with only mild pulmonary edema and no renal or gastrointestinal injury [29]. Furthermore, Boddie et al showed significant hepatic damage after IHP via the portal vein only [30]. Most hepatic Kupffer cells are situated in the (peri) portal area, and TNF is known to induce production of various cytokines (including IL-1, IL-6, and TNF) by macrophages (i.e. Kupffer cells). Therefore, this could be an explanation for the toxicity we encountered, as we performed IHP via both hepatic artery and portal vein [19]. Indeed high IL-6 and IL-8 levels were seen in our patients; and they were significantly higher when compared with levels in the ILP setting [31, 32]. Paradoxically, we could not demonstrate a significant correlation between peak levels of TNF, IL-6 or IL-8 and clinical parameters such as fever or hypotension. Furthermore, cytokine levels in the two patients with ARDS were not different from those measured in the other patients. The investigated acute phase proteins demonstrated the same course as in the ILP setting, with CRP reaching peak levels 48 h after IHP, with no significant differences between patients [33].

The second modification of the IHP technique involved the use of both hepatic artery (HA) and portal vein (PV) as inflow routes. Normal hepatic parenchyma receives most of its blood supply from branches of the PV and to a much lesser extent from the HA. In contrast, the blood supply of hepatic metastases has been ascribed to rely almost entirely on the HA, although very small (< 5 mm) liver tumors and the outer rim of large hepatic metastases have been shown to be fed mainly by portal branches [27, 28]. Most colorectal tumors drain via the PV, suggesting that spreading tumor cells first proliferate in the portal system. Thus by using the HA and PV drugs reach both established and newly formed (micro) metastases. It could therefore be speculated that an improvement of response could be achieved. In our study, almost all surviving patients demonstrated a partial response or stable disease with only one of the non-surviving patients showing a complete response. In contrast, other groups demonstrated a higher complete response rate by performing IHP via the hepatic artery only. A possible explanation for this difference might be the mechanism by which TNF exerts its anti-tumor effect in isolated perfusion setting (ILP/IHP). TNF without melphalan or any other chemotherapeutic agent does not appear to have any significant antitumor activity when administered in IHP. Fraker et al demonstrated a response rate of only 20% in 17 patients treated in a study with IFN and escalating doses of

TNF administered in IHP [36]. Similar results have been demonstrated in ILP with TNF only. Therefore, as was concluded from the ILP experiments in rats, TNF needs a chemotherapeutic agent to in order to be effective. The antitumor effects of TNF in the Isolated Perfusion setting are based on synergism with a cytostatic drug [18, 37, 38]. Probably, TNF is suggested to be responsible for the disruption and subsequent leakage of the TAV whereas melphalan (or in theory any other chemotherapeutic drug) causes a non-specific necrosis of the tumor cells [39]. The effects of high dose TNF on the TAV lead to an increased permeability and a significant decrease of the interstitial pressure in the tumor. Both effects lead to a better penetration of cytotoxic drugs into the tumor tissue [40-42]. Indeed our group demonstrated a 4 – 6 fold increase of intratumoral melphalan concentration when TNF was added to the perfusate in ILP with melphalan [43]. Similarly, an increased uptake of doxorubicin in tumor tissue was shown after TNF based ILP [44]. Probably these findings are one of the most important mechanisms behind the successes demonstrated by ILP with TNF and melphalan. In contrast, Alexander et al showed an increased capillary leakage during IHP and an increased uptake of I^{131} albumin in tumor tissue compared to liver tissue. However, the addition of TNF did not affect melphalan concentrations in the tumor tissue compared to liver tissue [45]. Several reasons for the discrepancy are possible such as concentrations of TNF used, sampling method and duration of perfusion. Of more importance however, might be the type of tumor with associated difference in tumor vasculature since colorectal metastases are hypovascular and largely necrotic, whereas soft tissue sarcoma are usually hypervascular. The results of Van der Veen et al and De Wilt et al implicate that the better the vascularisation of a specific tumor, the more explicit the effects of TNF on the TAV and the better the overall response of the tumor to the treatment [43, 44]. This has been proven from experimental as well as clinical results. In the rat, best response rates after ILP with TNF and melphalan have been demonstrated by our group for the highly vascularised BN soft tissue sarcoma bearing rats whereas the less vascularised rat osteosarcoma (ROS) showed subsequent lower responses [46, 47]. Similar results have been shown by Van IJken et al in an IHP model in rats using the same tumors (BN, ROS) but now localized in the liver. In case a coloncarcinoma was used, only few responses had been shown [48]. In accordance with these results, best clinical responses have been demonstrated

after ILP with TNF and melphalan in patients with unresectable soft tissue sarcoma of the extremities [15, 16]. The same holds true for the clinical result of IHP. Therefore, the vascularisation of the tumor seems to be of utmost importance in the treatment with TNF, independent of its localization.

Two goals of IHP are an increase in response rate and a prolongation of duration of response. The technique of IHP as presented here is not repeatable. Furthermore, IHP is a technically demanding procedure. One way to overcome these two problems could be the development of the balloon catheter technique in combination with IHP (IHHP) [53]. With this technique, the procedure is simplified and can be repeated. Van IJken et al performed IHHP with TNF, melphalan and mytomicinC (MMC) in pigs. They 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. Because of the leakage free quality of this procedure in combination with the efficacy of the washout procedure, TNF may be used in this setting [53]. After the promising results in pigs, our group started a phase I-II study on IHHP with melphalan in patients with irresectable hepatic metastases of colorectal origin [54]. In our first six patients we have had no serious adverse events. Moreover, antitumor activity has been demonstrated at the dose level of 1 mg kg^{-1} melphalan. With further experience with this technique, TNF may be introduced in order to further increase antitumor activity.

Despite the very promising experiences with our IHP experiments in pigs, many problems were encountered during the phase I study. Contributing factors were the magnitude of the surgical procedure of IHP, and the overall blood loss (median 5500 mL). By using both the hepatic artery and portal vein in the IHP we encountered more toxicity than expected from the pig program, resulting in fatal coagulative disturbances in one patient who received the second rhTNF dose. Furthermore, local control after one IHP with TNF and melphalan is only temporary. Taking into account the current aims and indications for IHP it is clear that simplification and further optimization of the IHP technique is necessary to render it a smaller, preferably less invasive as well as repeatable technique. One possibility that may be explored is the employment of multiple balloon catheters to achieve vascular isolation.

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CHAPTER 4

Acute phase response patterns in Isolated Hepatic Perfusion with Tumor Necrosis Factor α and melphalan in patients with colorectal liver metastases.

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ABSTRACT

In this study, we have evaluated hepatotoxicity, secondary cytokine production and hepatic acute-phase response (APR) in patients who underwent isolated hepatic perfusion (IHP) with tumor necrosis factor α (TNF) and melphalan for irresectable colorectal liver metastases.

An extracorporeal veno-venous bypass was used to shunt blood from the lower body and intestines to the heart. Inflow catheters were placed in the hepatic artery and portal vein, and an outflow catheter in the infrahepatic, inferior caval vein. The liver was perfused for 60 min with 1.0 mg kg^{-1} melphalan and TNF (0.4 mg (n=8) or 0.8 mg (n=1)), IHP_{TM} group or 1 mg kg^{-1} melphalan (n=3), IHP_{M} group. The liver was washed with macrodex before restoring vascular continuity.

After the washout procedure, a TNF peak (169 ± 38 pg mL^{-1}) was demonstrated in the IHP_{TM} group only. Both groups demonstrated peak levels of IL-6 in perfusate as well as systemically. These were significantly higher in the IHP_{TM} group. Acute phase protein (APP) levels followed a similar pattern as has been demonstrated after major surgery, with no significant differences between both groups. The addition of TNF to the perfusate did not lead to a significant difference in APP levels as well as time course between groups.

IHP with TNF and melphalan is followed by a transient systemic peak of TNF directly after liver washout. Secondary IL-6 induction was seen in the present study after IHP with and without TNF, which was highest when TNF was added. This phenomenon cannot be extrapolated to APP induction, which appeared unaffected by the addition of TNF, presumably because the surgical procedure itself already causes maximal stimulation of APP production.

INTRODUCTION

The acute phase response (APR) is a general systemic reaction induced by injury or various kinds of inflammatory states such as burns, infection or surgical procedures. As part of this reaction, the liver responds to several mediators by the increased synthesis of a series of glycoproteins called acute phase proteins (APP). The function of the APPs is pleiotropic: they may both mediate and inhibit inflammation, scavenge free oxygen radicals, act as transport proteins for products of the inflammatory process, or have an active role in tissue repair and remodeling.

Both tumor necrosis factor α (TNF) and interleukin 6 (IL-6) have been shown to induce the APP synthesis of the liver, of which the latter seems to be the major inducer and mediator of this APP production [1-4]. TNF derives its name from the observation that it can cause hemorrhagic necrosis of tumors. However, TNF is also known to be an important mediator in shock. Systemic administration of small amounts of TNF leads to influenza-like symptoms; at higher dose levels a septic shock syndrome develops, characterized by pulmonary edema, adult respiratory distress syndrome (ARDS) and severe inflammatory response syndrome (SIRS). For this reason, many phase I and II trials studying the systemic administration of TNF have failed to reproduce the experimental successes because the severe, systemic toxic side effects of TNF limited the usable dose of TNF to a level at which no effective antitumor activity could be seen [5-7].

It has been shown recently that this concentration gap can be overcome by the application of cytostatic drugs in isolated perfusion systems. These allow safe increase of doses to > 20 x the systemic maximum tolerated dose (MTD) [8]. This also holds true for the application of TNF in such systems. It has become firmly established that TNF can be applied safely and successfully in isolated limb perfusion (ILP) both in melanoma patients and in patients with irresectable soft tissue sarcomas [9-11]. Furthermore, this remarkable antitumor effectivity of TNF in combination with melphalan, proved to be effective against a wide range of histologies [5].

The results of the ILP studies led to the development of isolated perfusion of other organs, such as isolated hepatic perfusion (IHP) [12, 13]. With this technique, the liver circulation is completely separated from the systemic circulation, thus allowing high drug levels within the liver while keeping systemic

exposure low. However, unlike limbs, the liver is a metabolically active organ that contains a large amount of tissue macrophages, the Kupffer cells. As Kupffer cells are known to release various cytokines in response to TNF, it could therefore be speculated that IHP with TNF might induce considerable (hepato)toxicity. Therefore, we were interested in the effects of IHP with TNF on secondary cytokine production and hepatic APR. To obtain further insight, we evaluated patients who underwent IHP with TNF and melphalan for colorectal metastases confined to the liver. The APR was evaluated regarding the levels and time dependency of TNF, IL-6, and the APP C-reactive protein (CRP), α1-acidglycoprotein, α1-antitrypsin, and transferrin.

PATIENTS AND METHODS

IHP patient groups

Twelve patients with colorectal metastatic liver disease gave informed consent for undergoing isolated hepatic perfusion (IHP) with TNF and melphalan (IHP_{TM} group, n = 9) or melphalan alone (IHP_M group, n = 3). There were 9 men and 3 women with a mean age of 59.8 years (range, 49 – 65). All gave informed consent before treatment in protocols approved by the hospitals' ethical committees. The study was carried out in accordance with the principles of the Declaration of Helsinki, as revised in Hong Kong 1989. Inclusion criteria for IHP with TNF and melphalan included histological evidence of irresectable metastases of colorectal origin confined to the liver, Karnovsky performance status of > 80%. Exclusion criteria included extra-hepatic malignant disease, > 50% hepatic tissue replacement by tumor, liver cirrhosis, signs of significant hepatic dysfunction (abnormal levels of ASAT, ALAT or Alkaline Phosphatase > 2 x norm), and ascites or portal hypertension.

IHP technique

The procedure of IHP has been described elsewhere [12]. Briefly, the vasculature of the liver was dissected free and isolated. After systemic heparinization (200 IU kg^{-1}), an extracorporeal veno-venous bypass (VVB) circuit (aided by a passive centrifugal pump) was created to shunt mesenteric, renal, and lower extremity blood around the liver to the heart. Next, inflow catheters were placed in the portal vein and hepatic artery, and an outflow catheter in the infrahepatic inferior caval

vein. These catheters were connected to a heart-lung machine, and the vascular isolation was completed by clamping the suprahepatic inferior caval vein and the suprarenal inferior caval vein. The liver was then perfused with a hyperthermic (> 38 °C) perfusate consisting of a mixture of saline and erythrocytes. Once a stable perfusion was attained, leakage from the perfusion circuit into the systemic circulation was measured by the addition of 200 μci I^{131} -albumin into the perfusate and the continuous monitoring of radioactivity scintillation probes placed over the perfusate reservoir and VVB. The leak rate was monitored for the duration of the perfusion and if the cumulative leak was greater than 15%, the perfusion would be halted and the perfusate flushed from the circuit. After the absence of leakage was confirmed, drugs (see treatment schedule) were administered as a bolus into the arterial line of the perfusion circuit. After a 60 min perfusion, the liver was washed thoroughly with a mixture of saline and macrodex, decannulated, and vascular continuity restored. Heparin was reversed with 1 mg kg^{-1} protamine sulphate (Novo-Nordisk AS, Rud, Norway) injection. Postoperatively, the patients were monitored at the ICU for at least 48 hours, primarily to evaluate for evidence of systemic toxicity due to rhTNF.

Drugs

Recombinant human TNF (0.2 mg per ampoule) was a kind gift from Boehringer Ingelheim GmbH, Ingelheim am Rhein, Germany. The cytostatic drug melphalan (Alkeran) was obtained as a sterile powder (100 mg) that was dissolved aseptically using solvent and diluent by Burroughs Wellcome, London, UK.

Treatment schedule

In nine patients (IHP_{TM} group), rhTNF (0.4 mg (n=8), 0.8 mg (n=1)) was administered as a bolus; melphalan (1 mg kg^{-1}) was given directly following the rhTNF bolus. In 3 patients (IHP_M group) only melphalan (1 mg kg^{-1}) was administered.

Sampling schedule

Blood samples were collected from a peripheral vein in siliconized 5 mL Vacutainer tubes (Becton Dickinson, Plymouth, UK) containing EDTA (10 nmol L^{-1}) and soybean trypsin inhibitor (100 mg L^{-1}), and benzamidine (10 nmol L^{-1}) (Sigma Chemicals, Detroit, Michigan). Samples were centrifuged immediately after collection, at 5000 rpm. for 5 minutes. Supernatants were stored at -70 ^{o}C

until analysis. Perfusate was sampled at t = 0 (i.e., upon drug administration), 10, 20, 30, 40, 50 and 60 min. Systemic plasma samples were collected the day before IHP, during IHP at t = 0, 30 and 60 min., and post-perfusion (after release of the inferior caval vein clamp) at t = 1, 5, 10, 20, 30, 60, 120 and 240 min., day 1, 3, and 7, and weekly thereafter, until 3 weeks post-operatively.

Assays

TNF levels were measured by a sandwich-type enzyme linked immunosorbent assay (ELISA) using two monoclonal antibodies (Dept. Immune Reagents, Central Laboratory of Blood transfusion, Amsterdam, Netherlands) raised against recombinant human TNF (courtesy of Dr. A. Creasey, Chiron Corp., Emeryville, CA, USA). One mAb (mAb CLB- TNFα-7) was used for coating at a concentration of 2 μg mL^{-1}. The other mAb (mAb CLB-TNFα-5) was biotinylated and used in combination with streptavidin poly-horseradish peroxidase conjugate (CLB, Dept. Immune Reagent) to detect bound TNF. Stimulated human mononuclear cell supernatant was used as a standard for comparison with purified recombinant human TNF. Results were expressed as pg mL^{-1} by reference to this standard [14]. The detection limit of this essay was 10 pg mL^{-1}.

IL-6 was measured by an ELISA modified from that described in detail before [15]. Briefly, purified monoclonal anti-human-IL-6 antibody (mAb CLB-IL-6-16) was used as a capture antibody, and biotinylated sheep antibodies in combination with streptavidin poly-horseradish peroxidase conjugate were used to detect bound IL-6. Results were expressed as pg mL^{-1} by reference to a standard consisting of recombinant human IL-6. Normal healthy control subjects values were < 10 pg mL^{-1} and the limit of detection was 5 pg mL^{-1}.

Acute-phase proteins

C-reactive protein (CRP), α1-antitrypsin (α1-AT), α1-acidglycoprotein (α1-AG) and transferrin (TRF) levels were measured by means of a nephelometric assay [16]. The antisera used were obtained from the Central Laboratory of the Blood Transfusion Service (CLB), Amsterdam, The Netherlands. Normal values (obtained from 100 blood donors) were: CRP < 5 mg L^{-1}, α1-AT 1.4 - 3.2 g L^{-1}, α1-AG 0.4 - 1.3 g L^{-1}, and TRF 2.3 - 4.3 g L^{-1}.

Statistics

Results are expressed as means ± standard error of the mean (SEM). Comparisons within groups were made by means of the Friedman Nonparametric Repeated Measures Test or by the Mann-Whitney Test, where appropriate. Correlations between maximum levels of parameters were calculated as Spearman's rank correlations. A two-side P-value < 0.05 was regarded as significant.

RESULTS

Operative procedure

The median duration of the operation was 8 h (range 6 - 10 h). In all patients a stable perfusion was attained. In one patient, progressive systemic leakage resulted in discontinuation of the IHP after 43 minutes (cumulative leakage 20%). In all other patients no leakage was demonstrated. Three patients in the IHP_{TM} group died in the immediate postoperative period as a result of surgical complications of the operation. As the purpose of this study was to describe APR in uncomplicated IHP, these patients were excluded for this study.

Survival

In the six evaluable patients the survival time ranged from 6 to 26 months. The median survival time was 10.3 months (mean 13.3 months).

Tumor response

The primary efficacy end points in the study were best tumor response observed [WHO response criteria: complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD)] and duration of the best response, calculated from the date the best response was observed until the date of progression. True-cut hepatic tissue samples and/or computerized tomography (CT) scan evaluations were used to describe the best tumor response. In the IHP_{TM} group 5 out of 6 patients demonstrated CR or PR and one patient demonstrated SD. Patients treated with melphalan alone demonstrated PR or SD. The duration of best response ranged from 17.5 to 32.5 weeks (median 18 weeks).

Toxicity

Toxicity and adverse events were assessed and recorded according to the WHO grading system (WHO Adverse Event Coding Thesaurus) [17].

General toxicity

In the IHP_{TM} group, all patients developed slight fever, hypotension, pulmonary toxicity, and sinus tachycardia that was reported as drug related. All patients demonstrated anemia and thrombocytopenia (nadirs at day 3), returning to normal after 10 days post IHP ($p>0.05$ between groups).

Hepatotoxicity

All patients demonstrated significant initial elevations in liver enzyme levels, normalizing within the first two postoperative weeks ($p>0.05$ between groups). In contrast, bilirubin and alkaline phosphatase levels increased more slowly after IHP but remained elevated over a longer period. Overall, two (IHP_{TM} group) patients demonstrated grade III and three (IHP_{TM} group) patients grade IV according to the WHO classification (figure 1).

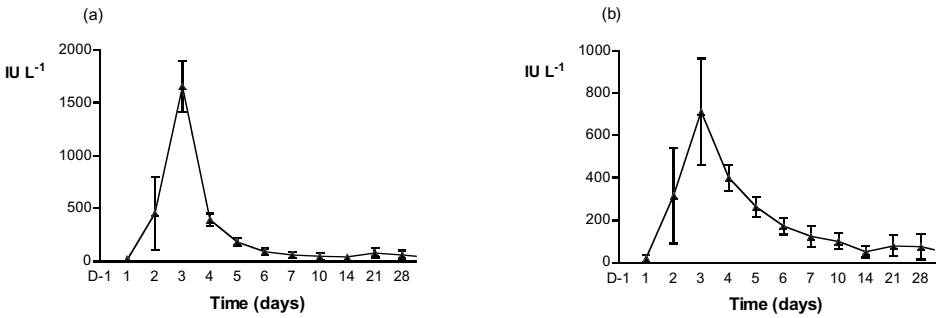

Figure 1. Course of liver enzyme levels as a function of time following IHP. Time is expressed in days on the x-axis, where D–1 represents the day prior to the operation. Left panel (a): ASAT, aspartate aminotransferase; right panel (b): ALAT, alanine aminotransferase. Note: since liver enzyme levels were virtually equal in both groups, the IHP_M group levels are not shown for reasons of clarity.

Cytokine levels

TNF levels

In the perfusate, TNF levels in the IHP_{TM} group were 1.8 ± 0.5 pg mL^{-1} at t = 0 min and increased rapidly to 6.0 ± 2.0 x 10^4 pg mL^{-1} at t = 10 min. Thereafter, TNF levels decreased to 2.8 ± 0.9 x 10^4 pg mL^{-1} at the end of IHP (t = 60 min). In the IHP_M group TNF levels did not demonstrate any significant changes. (figure 2)
Baseline serum TNF levels were similar in both groups, although these levels were higher than normal (4.3 ± 0.5 pg mL^{-1} and 4.5 ± 1 pg mL^{-1} in the IHP_{TM} and IHP_M group resp.). During IHP TNF levels did not change significantly, indicating that vascular isolation was effective. However, after washout, TNF levels increased rapidly to a peak value of 169 ± 38 pg mL^{-1} at t = 1 min in the IHP_{TM} group, and normalized within the next 3 hours. In the IHP_M group, TNF levels demonstrated a slight increase to 7.2 ± 5 pg mL^{-1} within the first 30 minutes after IHP. However, this elevation of TNF levels in the IHP_M group was not statistically significant (figure 2).

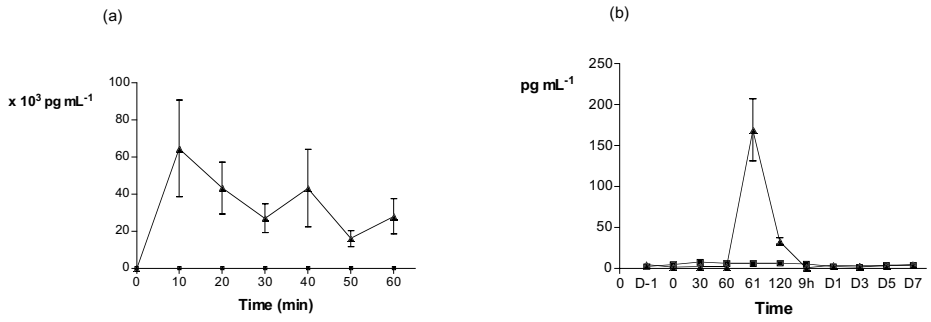

Figure 2. Course of tumor necrosis factor α (TNF) levels before, during and after IHP. Left panel (a) shows perfusate levels, right panel (b) shows systemic levels. Time is expressed on the x-axis; left panel: minutes during perfusion; right panel: D-1, the day prior to IHP; 0, 30, 60, 61, 120, 0, 30, 60, 61, 120 minutes after start of IHP (61 represents the time just after vascular restoration, not shown in following panels); 9h, 9 hours after IHP; D1, D3, D5, D7, first, third, fifth and seventh day after IHP. (▲--▲), represents the group of patients receiving TNF and melphalan; (■--■), the group of patients receiving melphalan alone.

Interleukin-6 levels

In the perfusate, baseline IL-6 levels were elevated in both the IHP_{TM} and IHP_M group (91 ± 45 pg mL^{-1} resp 94 ± 58 pg mL^{-1}). During IHP, IL-6 levels increased over time to 2.1 ± 0.7 x 10^3 pg mL^{-1} in the IHP_{TM} group ($p<0.01$) and to 227 ± 128 pg mL^{-1} ($p>0.05$) in the IHP_M group ($p<0.01$ between groups, figure 3).

Serum IL-6 levels at the start of the IHP procedure were elevated in both groups and rose slightly during IHP ($p>0.05$ between groups). However, after the washout procedure IL-6 levels increased significantly, reaching the highest levels, 1 hour after washout, in the IHP_{TM} group ($p<0.01$ between groups). Respective maximum IL-6 levels were 9.8 ± 0.8 x 10^3 pg mL^{-1} and 2.4 ± 1 x 10^3 pg mL^{-1} in the IHP_{TM} and IHP_M group. Thereafter levels dropped, and returned to pre-operative levels within the first post-operative day (figure 3).

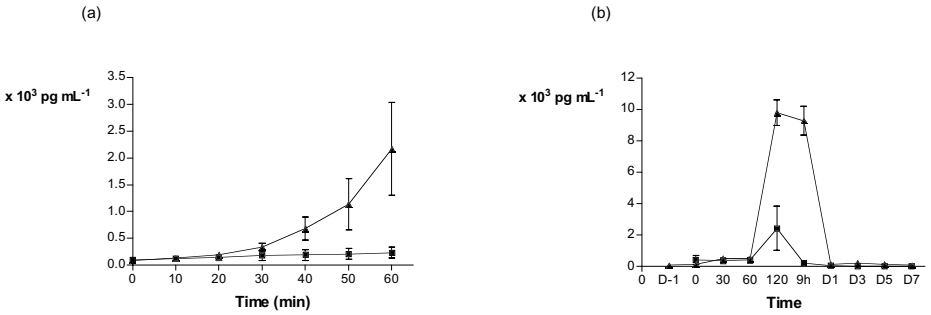

Figure 3. Course of IL-6 levels before, during and after IHP. Left panel (a) shows perfusate levels, right panel (b) shows systemic levels. Time is expressed on the x-axis; (a): minutes during perfusion; (b): D-1, the day prior to IHP; 0, 30, 60, 120, 0, 30, 60, 120 minutes after start of IHP; 9h, 9 hours after IHP; D1, D3, D5, D7, first, third, fifth and seventh day after IHP. (▲--▲), represents the group of patients receiving TNF and melphalan; (■--■), the group of patients receiving melphalan alone.

Acute-phase protein response

C-reactive protein levels started to rise 9 hours after washout and maximum levels (143 ± 3 mg L^{-1} and 129 ± 4 mg L^{-1} in the IHP_{TM} and IHP_M group respectively) were reached at the fifth postoperative day. Thereafter, levels dropped and normalized slowly within the next two weeks ($p>0.05$ between groups, figure 4). From the start of the IHP procedure α1-antitrypsin and α1-acidglycoprotein levels

first decreased in both groups. At 60 min after washout, levels increased gradually ($p<0.01$), and they were still elevated at day 10 ($p>0.05$ between groups, figure 4) The "negative" acute phase protein transferrin levels in both groups decreased until 30 - 60 min after washout, where after levels increased to normal values within the first postoperative days ($p>0.05$ between groups, figure 4)

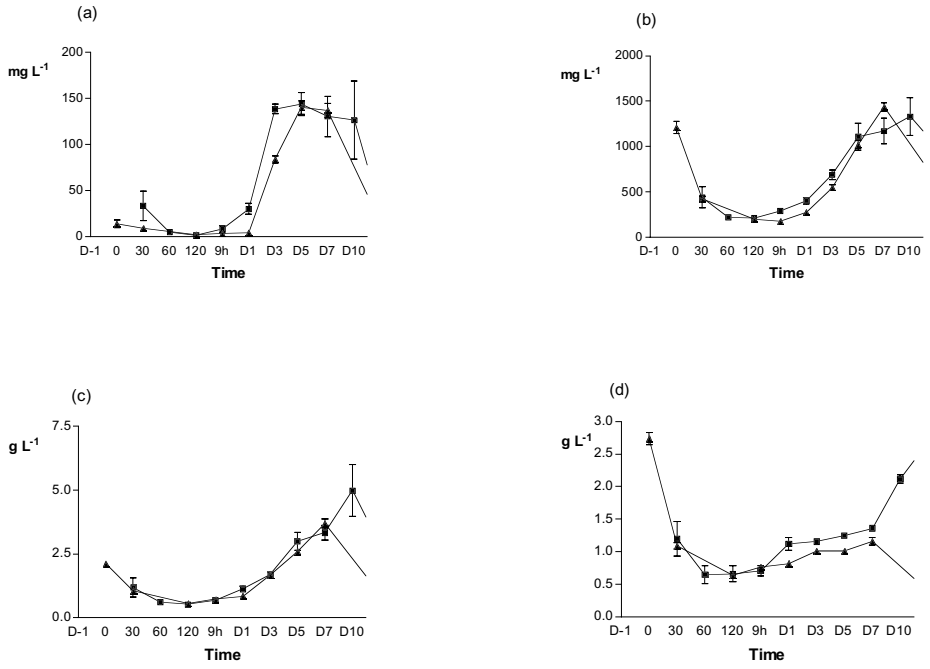

Figure 4. Course of (a) C-reactive protein (CRP), (b) α1-antitrypsin (a1-AT), (c) α1-acidglycoprotein (a1-AG) and (d) Transferrin (TRF) levels before, during and after IHP. Time is expressed on the x-axis as follows: D-1, the day prior to IHP; 0, 30, 60, 120, 0, 30, 60, 120 minutes after start of IHP; 9h, 9 hours after IHP; D1, D3, D5, D7, first, third, fifth and seventh day after IHP. (▲--▲), represents the group of patients receiving TNF and melphalan; (■--■), the group of patients receiving melphalan alone.

DISCUSSION

In this study, we describe the effects of IHP with TNF and melphalan, or melphalan alone, on secondary cytokine and APP production. After the washout procedure, IHP was followed by a TNF peak in the IHP_{TM} group only, normalizing within 2h. TNF levels in the melphalan alone group remained virtually unchanged. In both groups, peak levels of IL-6 were observed 1h after the washout, although levels were significantly higher in those patients with TNF added to the perfusate. IL-6 levels normalized at day 1 post-operatively. The APP production, followed a pattern similar to that demonstrated after various kinds of surgery, including major hepatic surgery, with no differences between both groups.

Despite its promising *in vitro* antitumor effects, the *in vivo* administration of TNF has been demonstrated to be accompanied with dose limiting systemic toxicity at dose levels at which no antitumor effect could be seen. Furthermore, phase I and II studies demonstrated specific organ toxicity with increasing dosages of TNF [6, 7]. We were particularly interested in the potential hepatotoxicity caused by IHP with TNF and melphalan. All our patients displayed moderate transient hepatotoxicity as demonstrated by transient elevated hepatic enzymes (figure 1). Since similar elevations were also observed in the IHP_M group, we believe that the addition of TNF did not lead to additional hepatotoxicity. These findings are in accordance with the results of our experimental IHP program in pigs [12]. Therefore, more likely, we think that the hepatotoxicity encountered in our patients is the result, at least in part, of the IHP procedure itself.

To avoid systemic exposure to chemotherapeutic agents or cytokines, the main goal of the isolation perfusion technique is the complete vascular isolation of the limb or organ. In our study vascular isolation of the liver was complete in all patients but one. In this patient progressive systemic leakage (cumulative leakage: 20%) led to premature termination of the IHP procedure after 43 min. However, despite this leakage, this patient did not demonstrate additional toxicity as demonstrated by clinical as well as biochemical parameters, compared with the other patients studied. The same observation has been demonstrated by Pinsky and co-workers, who showed that the outcome of patients with septic shock did not correlate with peak levels but with the persistence of both TNF and IL-6 levels [18]. In our eight patients without leakage, systemic TNF levels did not change significantly during IHP, indicating that vascular isolation was complete.

After the washout procedure, however, systemic TNF levels in the IHP_{TM} group peaked rapidly and normalized within the next two to three hours (figure 2). A possible explanation for this TNF peak in the IHP_{TM} group could be the release of remnant TNF in the liver after the washout procedure, a phenomenon also described in isolated limb perfusions (ILP) with TNF and melphalan [16, 19, 20]. However, peak TNF levels in our study were much lower than those levels in ILP, indicating a more efficient washout procedure [19, 21]. Furthermore, endogenous TNF production may have attributed to this peak, since surgery, extra corporeal circulation circuits, and intravascular plastic catheters are known to induce TNF [22, 23]. However, the latter explanation may be of less importance since only mild elevation of systemic TNF levels could be demonstrated in the IHP_{M} group (figure 2).

TNF is also know as a proinflammatory cytokine being able to stimulate the production of several interleukines like IL-6. In this study, perfusate IL-6 levels increased significantly in all patients during IHP, with highest levels in the IHP_{TM} group (figure 3). The same pattern has been demonstrated in the perfusate of ILP circuits in patients with melanoma stage III/IV or irresectable sarcoma of the limbs, although perfusate IL-6 levels in our patients (IHP_{TM} group) were much higher at a lower TNF dose [20]. Macrophages are known to produce various cytokines, e.g. IL-6, in response to TNF. As the liver contains the largest amount of fixed tissue macrophages, Kupffer cells, this induction of IL-6 production by the Kupffer cells could be an explanation not only for the difference in IL-6 levels between our study groups, but also between IHP and ILP. Furthermore, all patients demonstrated elevated systemic IL-6 levels at the start of the actual perfusion, which further increased, with perfusing time. Several studies have demonstrated increased IL-6 levels after various surgical procedures, including hepatic surgery [24-29]. Cruickshank *et al*. described elevated IL-6 levels in patients undergoing elective surgery of varying severity. Levels of IL-6 were shown to increase with the extent of surgery [29]. Several other studies confirmed this finding but peak IL-6 levels were usually less than 500 pg mL^{-1} [24-29]. In this context, it should be borne in mind that, at the beginning of the perfusion, patients already had undergone a major surgical procedure, with subsequent increased IL-6 concentrations resembling levels described in other surgical procedures (figure 3, t = 0 min). The slow initial increase, during IHP was followed by a further steep increase after IHP to a peak at 1 hour after the washout procedure (figure 3). Although this peak occurred in both groups, it was significantly higher in those patients perfused with TNF and

melphalan. Since both groups underwent the same surgical procedure with melphalan, the significant difference between groups can only be explained by the addition of TNF to the perfusate of the IHP_{TM} group. The same observation was reported by Thom *et al.* In their study serum IL-6 levels in patients with ILP with TNF, interferon-γ, and melphalan were significantly higher compared to ILP with melphalan alone, with a trend toward higher IL-6 levels with increased exposure to TNF [20]. Taking into account the aforementioned Kupffer cell response to TNF, both the TNF peak after washout in the IHP_{TM} group as well as the hepatic IL-6 production could be an explanation for the difference in systemic peak IL-6 levels.

An important function of the liver is the production of so called APPs in response to various stimuli. In all our patients a clear APR could be observed. Levels of all proteins first decreased, probably caused by haemodilution in combination with extravasation after the washout procedure. Thereafter the negative APPs normalized, whereas the positive APPs started to increase and remained elevated for at least two weeks with the exception of CRP which normalized within this period (figure 4). Furthermore, there were no significant differences in APP levels between both groups. Several *in vitro* and *in vivo* studies demonstrated that IL-6 is secreted in the early stages of the APR, and is the main mediator of the hepatic production of APPs like α1-AG, α1-AT and CRP [1, 2]. To emphasize the central role of IL-6, *in vivo* studies have revealed clear correlations between IL-6 and several APP levels [3, 30-33]. Since in our study significant differences in systemic IL-6 levels were demonstrated, we speculated that this might lead to a different APP production profile between groups. In contrast, in our study, there were no significant differences in APP profiles or peak levels between groups. Moreover, we could not demonstrate a significant correlation between peak IL-6 and CRP levels. Instead, the APP pattern, including peak levels, as described here is similar to patterns demonstrated after elective surgery of various extent, including ILP [16, 31, 32]. A possible explanation for the identical profile in both groups could be that the IHP, as a major surgical procedure (and identical in both groups), already caused a maximal induction of the APPs by the liver. Consequently, the addition of TNF to the perfusate, with subsequent short-lived systemic TNF peak and resulting IL-6 peak, could not further influence the APR. Thus, the conditions under which TNF induced IL-6 production occurs may be essential to the extent in which the hepatic APR is induced [34].

In conclusion, IHP with TNF and melphalan is followed by a transient systemic peak of TNF directly following liver washout. Secondary IL-6 induction was seen

in the present study after IHP with and without TNF, which was highest when TNF was added. This phenomenon cannot be extrapolated to APP induction, which appeared unaffected by the addition of TNF, presumably because the surgical procedure itself already causes maximal stimulation of APP production.

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CHAPTER 5

Systemic toxicity and cytokine/acute phase protein levels in patients after isolated limb perfusion with tumor necrosis factor-alpha (TNFα) complicated by high leakage.

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ABSTRACT

Since the introduction of high dose tumor necrosis factor α (TNF) in the setting of isolated limb perfusion (ILP) in the clinic prevention of leakage to the body of the patient is monitored with great precision for fear of TNF-mediated toxicity. The fact that we observed remarkably little toxicity in patients with and without leakage prompted us to determine patterns of cytokines and acute phase proteins (APPs) in patients with high leakage and in patients without any leakage.

TNF, IL-6, IL-8, CRP and $sPLA_2$ were measured at several time-points during and after (till 7 days) ILP in 10 patients with a leakage to the systemic circulation varying in percentage from 12 to 65%. As a control the same measurements, both in peripheral blood and in perfusate, were done in 9 patients without systemic leakage.

In patients with systemic leakage levels of TNF increased during ILP reaching values up to 277 ng mL^{-1} . IL-6 and IL-8 peaked 3 hours after ILP with values significantly higher compared with patients without systemic leakage. CRP and $sPLA_2$ peaked at day 1 in both patient groups. $sPLA_2$ with significant higher levels and CRP, in contrast, with lower levels in the leakage-patients.

High leakage of TNFα to the systemic circulation caused by a complicated ILP led to a ten- to more than hundredfold increased levels of TNF, IL-6 and IL-8 in comparison to patients without leakage. The increase of the APPs was limited. Even when high leakage occurs, this procedure should not lead to fatal complications. The most prominent clinical toxicity was hypotension (grade III in 4 patients), which was easily corrected. No pulmonary or renal toxicity was observed in any patient. It is our experience that even in the rare event of significant leakage during a TNF-based ILP postoperative toxicity is usually mild and can be easily managed by use of fluid and in some case vasopressors.

INTRODUCTION

The technique of regional isolated limb perfusion (ILP) utilizing an extracorporeal circuit was pioneered by Creech and coworkers [1]. The advantage of this treatment modality is that high dose of cytostatic drug can be delivered to the tumor bearing extremity without producing systemic side effects. ILP permits regional cytostatic concentrations 15 to 20 times higher than those reached after systemic administration [2]. The standard drug in this setting is melphalan (L-phenyl-alaninemustard). In patients with multiple melanoma in-transit metastases an ILP with melphalan results in a complete remission in about 50% of the patients [3]. Addition of Tumor Necrosis Factor α (TNF) to melphalan has proven most effective in terms of response rate, yielding a 80-90% complete response rate, and an overall response of about 100% [4, 5]. In locally advanced soft tissue sarcomas the use of TNF in combination with melphalan has proven remarkably effective in rendering irresectable tumors resectable and thereby preventing amputations [4, 5]. The efficacy of TNF against the drug-resistant soft tissue sarcomas has led to the approval of TNF by the EMEA (European Medicine Evaluation Agency) for its use in combination with melphalan [6].

In the ILP system, TNF is administered in a 10-fold higher dose compared with the maximum tolerable dose (MTD) in systemic administration. The MTD of TNF in single dose intravenous (i.v.) or intramuscular (i.m.) administration is limited by toxicity at 400 μg m^{-2} [7, 8]. Toxicity consists of fever, hypotension, chills and transient leucopenia. Hardly any tumor response has been reported after systemic administration of TNF [8-10].

Despite careful precautions systemic leakage of more than 10% appeared in 10 patients during the last 8 years in our hospital. These patients had a remarkably mild clinical course. To get more insight in the cause of this discrepancy between the high systemic concentration of TNF and the mild clinical symptoms, we studied cytokine levels and the acute phase response.

PATIENTS AND METHODS

Patients

From the 212 patients who underwent an ILP with TNF and melphalan in the past 8 years in our hospital, 10 patients were selected because of very high systemic levels of TNF, caused by leakage from the perfusate. These patients were treated because of an irresectable sarcoma (n=6) or melanoma with multiple in transit metastases (n=4). Demographic and treatment characteristics are summarized in table 1. As a control group we studied 9 comparable patients undergoing ILP without systemic leakage. These patients were all sarcoma-patients, who underwent a 90-minutes long ILP with 3 to 4 mg TNF. Mean age was 48 years (range, 21 to 77).

Patient no.	age	sex	Diagnosis	Arm/ leg	Duration of perfusion	dose (mgTNF)	% leak	max systTNF (ng mL^{-1})
1	55	F	Sarcoma	leg	90 min	2	23%	169
2	52	F	Melanoma	leg	90 min	4	20%	178
3	66	M	sarcoma	arm	90 min	3	12%	30
4	61	F	sarcoma	arm	30 min	3	65%	277
5	71	F	melanoma	leg	90 min	2	24%	112
6	56	M	sarcoma	leg	90 min	2	15%	77
7	65	M	melanoma	leg	90 min	2	32%	108
8	64	F	melanoma	leg	90 min	4	13%	104
9	83	M	sarcoma	leg	75 min	4	19%	174
10	55	F	sarcoma	leg	90 min	4	16%	90

Table 1. Patient characteristics

Treatment schedule

The procedure of ILP has been described previously [5]. Briefly, ILP consisted of a 90 minutes long perfusion with 3 to 4 mg of recombinant-human-TNF (Boehringer Ingelheim, Germany) and 10 to 13 mg L^{-1} perfusion tissue of melphalan (Alkeran) (Burroughs Wellcome, London, UK) at mild hyperthermia (39 to 40°C). Composition of the perfusate was as follows: priming volume of 700 to 850 mL consisted of 400 to 500 mL blood (50% red blood cells, 50% plasma), 200 to 400 ml 5% dextran-40 in glucose 5% (Isodex, Pharmacia, Uppsala, Sweden), 10 to 30 ml 8.4% sodium bicarbonate and 0.5 mL 2500 to 5000 IU heparin. TNF was injected as a bolus into the arterial line provided limb tissue temperature was > 38°C. Melphalan was administered 30 minutes later at limb temperatures between 39-40°C. At the end of perfusion, the limb was washed with at least 2 liters of 6% dextran-70 (Macrodex, Pharmacia, Uppsala, Sweden). During and after ILP vital signs of the patients, including body temperature, heart rate, blood pressure and fluid balance were recorded. Toxicity was registered according to the World Health Organization criteria [11].

Leakage monitoring

During ILP, there was a dynamic balance between two pressure compartments, the systemic vasculature and the isolated circuit, which could be influenced by adjusting the systemic blood pressure and/or the extracorporeal flow rate. Throughout the perfusion period any potential leakage of the drugs was monitored using a radioactive tracer. A small calibration dose of human serum albumin radiolabeled with iodine 131 or technetium 99m was injected into the systemic circulation and a 10-fold higher dose of the same isotope into the isolated extremity. Continuous monitoring was performed with a precordial scintillation probe. Systemic leakage was expressed quantitatively as a percentage such that 100% leakage represented a homogeneous distribution of the isotope in the body.

Blood sampling procedure

Venous blood samples were collected at several time-points, i.e., the day before ILP, just before administration of TNF in the perfusate, halfway perfusion, just before release of the tourniquet (after completion of the washout procedure at the end of the perfusion). Then after ILP, 5, 10, 30 minutes, 3 and 7 hours after release of the tourniquet and once a day until 7 days after ILP. Samples from the

perfusate were obtained at time-points: 0 (just before administration of TNF) and 10, 30, 60 and 90 minutes after administration of TNF. Blood samples were immediately centrifuged; plasma was collected and stored at -70°C until tested.

Assays for cytokine and acute phase protein analysis

Cytokine and acute phase protein levels were measured using enzyme-linked immunosorbent assay (ELISA). Used antibodies were obtained from the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands). For measuring TNF, as described previously, flat-bottomed micro titer plates (Nunc, Kamstrup, Denmark) were coated overnight with purified monoclonal antibody (mAb) against TNF (CLB-TNF/7) [12]. After washing, serial dilutions of TNF-containing samples were added. Bound TNF was detected by biotinylated sheep anti-TNF. The detection limit of the assay was 5 pg mL^{-1} . Healthy controls were at less than 5 pg mL^{-1} .

The IL-6 specific ELISA has been described previously [13]. A coat of CLB-IL-6/16 was applied overnight and bound IL-6 was detected by biotinylated affinity-purified polyclonal sheep anti-IL-6. Lower detection limit was 1 pg mL^{-1} and normal healthy control subjects were at less than 10 pg mL^{-1}.

For IL-8 a coat of CLB-IL-8/1 MAb was applied overnight and bound IL-8 was detected by biotinylated affinity-purified polyclonal sheep anti-IL-8. The lower detection limit of this assay was 8 pg mL^{-1}. Normal values were at less than 20 pg mL^{-1} [14].

C-reactive protein (CRP) levels were measured by a sandwich ELISA using polyclonal rabbit anti-human CRP Abs as catching Abs and biotinylated mAb anti-CRP (CLB anti-CRP-2) as a detecting Ab. Results were referred to a standard (Behringwerke AG, Marburg, Germany) and expressed in mg L^{-1}. The detection limit was 10 ng L^{-1} [15].

The ELISA used for measuring secretory phospholipase A_2 ($sPLA_2$) has been described before [16]. Two different mAbs against human $sPLA_2$ were used as coating and catching antibodies respectively. The lower limit of detection was 0.1 ng mL^{-1}. Normal healthy volunteers were at less than 5 ng mL^{-1}.

Statistics

Median values are expressed with range. Comparison between the cytokine and acute phase protein levels in the 2 groups (with and without leakage) were made by the Mann-Whitney test. Values of $p \leq 0.05$ were considered to be statistically significant.

RESULTS

Systemic toxicity

Ten patients with a systemic leakage percentage of more than 10% were entered in this study. Leakage varied from 12 to 65% (mean 24%, table 1). Because of expected toxicity all patients were well monitored at our intensive care unit post-operatively. Systemic toxicity is summarized in table 2.

During ILP blood pressure and pulse rate remained stable with adequate fluid management. The body-temperature did not increase above 38°C. After ILP, all patients received indomethacin to suppress flu-like symptoms. Eight out of 10 patients developed fever grade II (range, 38 to 40°C) within a few hours after ILP (mean maximal temperature 38.9°C). In the patients without detectable leakage the mean maximal temperature was 38.1°C. In 4 leakage-patients the heart rate increased to more than 110 beats per minute (range, 120 to 132). Four patients had a hypotension, which was not quickly restored to normal values by fluid administration alone, requiring additional treatment with dopamine (3 to 6 $\mu g\ kg^{-1}\ min^{-1}$) during 2 to 3 days. From start of surgery a mean of 8 liters was administered to the leakage-patients over the first 16 hours versus 5 liters for non-leakage patients. Leucopenia and thrombocytopenia was absent or mild. Grade IV leucopenia and thrombocytopenia in one patient was induced by leakage of melphalan. No transfusion was required. Patients without leakage did not develop hematological toxicity. All leakage-patients had a hyperbilirubinemia after 2 days and the transaminases increased (grade I-II). In the no leakage group only 1 patient had a mild hyperbilirubinemia. No pulmonary or renal toxicity was observed in any patient.

Toxicity		WHO grade		
LEAKAGE	Grade 0-I	Grade II	Grade III	Grade IV
Fever	2	8	0	0
Hypotension	3	3	4	0
Leukocytes [a]	8	0	1	1
Platelets [b]	6	3	0	1
Bilirubin [c]	3	3	4	0
OT/PT [d]	7	3	0	0
Nausea	8	2	0	0
NON-LEAKAGE	Grade 0-I	Grade II	Grade III	Grade IV
Fever	6	3	0	0
Hypotension	9	0	0	0
Leukocytes	9	0	0	0
Platelets	9	0	0	0
Bilirubin	9	0	0	0
OT/PT	9	0	0	0
Nausea	8	1	0	0

[a] gr. III: 0-1.9 x 10^9 L-1iter; gr.IV: < 1.0 x 10^9 L-1iter

[b] gr. II: 50-74 x 10^9 L-1iter; gr.IV: < 25 x 10^9 L-1iter

[c] gr. II: 2.6-5 x N; gr. III: 5.1-10 x N (N = upper limit of normal value)

[d] gr. II: 2.6-5 x N

Table 2. Systemic toxicity following ILP in the 10 patients with leakage to the systemic circulation compared to 9 patients without leakage.

Plasma cytokine and acute phase protein levels

In figure 1, median values with range are represented for the cytokines TNF and IL-6 and the acute phase proteins (APPs) CRP and $sPLA_2$. Median peak levels of all measured cytokines and APPs in both patient groups, depicted in table 3, were significantly different (p-values in table 3). Because we know the curves of IL-8 and $sPLA_2$ from previous published experiments we restricted the determinations to the pre-operative and the maximum level time points [17, 18].

In the leakage group very high circulating concentrations of TNF are found *during* perfusion (at – 45 minutes) in contrast to the non-detectable TNF levels in patients without leakage. Plateau circulating concentrations are measured at the end of the perfusion lasting up to 30 minutes after ILP. The small amount of TNF that remains in the limb after the washout procedure does not increase the colossal systemic levels any further in these patients in contrast to what is observed in patients without leakage. There we observed a brief peak of systemic levels more than hundredfold less than in leakage patients. Moreover the peak occurs typically at 5-10 minutes after ILP and represents the TNF that was left behind in the limb after the washout. TNF-levels decreased already after 30 minutes, because rapid clearance of this cytokine with a short half-life time of 17 minutes is operational. Thus, in leakage-patients very high TNF-concentrations are present for about 4 hours, whereas a very short peak of 20-30 minutes of "moderate" increased TNF-levels is present in leakage free patients. There was a strict correlation between the degree of leakage estimated by isotope monitoring, the (adjusted) dose of TNF and the measured maximum systemic levels of TNF, depicted in table 1.

IL-6 increased already during perfusion in leakage-patients, immediately induced by TNF. In the non-leak patients IL-6 increased 5 to 10 minutes after ILP, i.e., after TNF from the washed out limb appeared in the systemic circulation. Maximum values of IL-6 were reached 3 hours after ILP. IL-8 showed the same pattern as IL-6 (data not shown). In the control group, values of IL-6 and IL-8 were 10-60 times lower than in the leakage-patients.

The acute phase protein CRP was increased from 3 hours after ILP until over 7 days after ILP. Peak levels occurred at day 1. The CRP curve in patients without leakage was comparable, but the peak-value was higher. Levels of $sPLA_2$ were very different for each patient. However, the pattern was consistent, with the start of increase at 3 hours after ILP and the peak at day 1. Levels were still increased after 7 days. Levels in the non-leakage patients were factor 6 lower.

Levels of cytokines and acute phase proteins in perfusate

No significant difference was observed in perfusate-levels of cytokines and acute phase proteins in patients who underwent ILP with systemic leakage and without leakage. From the ILP-patients with systemic leakage, only 5 series of perfusate samples were available. Curves are presented in figure 2. TNF levels remained stable around 7.5 µg mL^{-1}. IL-6 increased after 10 minutes of perfusion, to 4.3 ng mL^{-1} at the end of perfusion. IL-8 increased from 65 pg mL^{-1} to 1600 pg mL^{-1} during perfusion. CRP did not change during perfusion, with values hardly detectable or at less than the detection limit. In 3 out of 5 patients $sPLA_2$ increased during perfusion; the median value at the end of perfusion was 14 ng mL^{-1}, range 8.5 to 266 ng mL^{-1}.

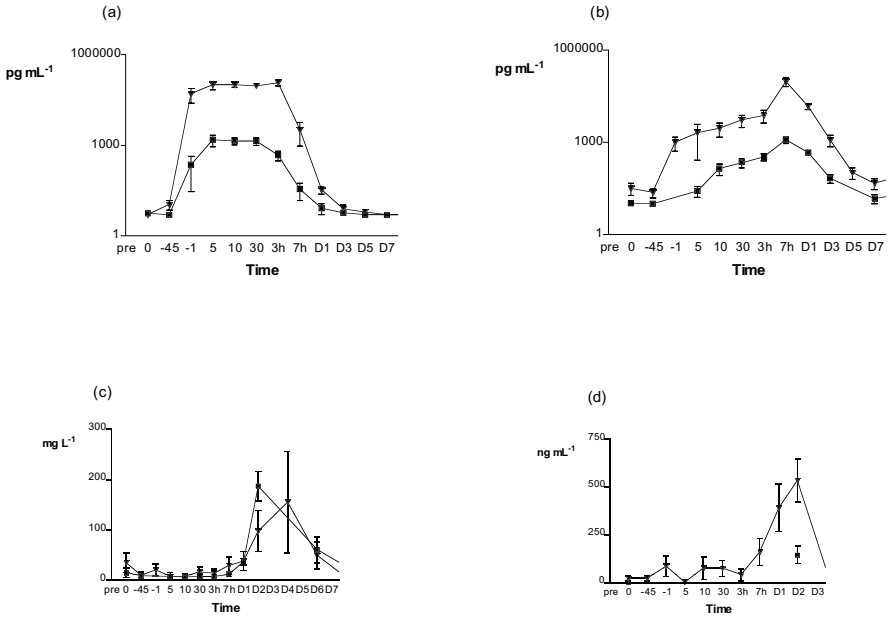

Figure 1. Median (with range) levels of TNF (a), IL-6 (b), CRP (c) and $sPLA_2$ (d) in plasma from 10 patients who underwent an ILP complicated by high leakage (> 12%) to the systemic blood circulation (▼). For TNF (a), IL-6 (b) and CRP (c) the curves of the non-leakage group (■) are also depicted, for sPLA2 only two control levels (pre and day 1) are shown. Time-points were as follows: pre, pre-operative; 0', just before perfusion; -45', half-way perfusion; -1', just before release of the tourniquet; 5' (10', 30'), 5 (10, 30) minutes after release of the tourniquet; 3h and 7h, 3 and 7 hours after ILP; d1 to d7: number of days after ILP

Cytokine/acute phase protein	time-point	Leakage-patients median (range)	no leakage median (range)	p-value
TNFα ng mL^{-1}	10 min after ILP	108 (26-277)	1.4 (0.3-3.4)	p<0.001
IL-6 ng mL^{-1}	3 hours after ILP	49 (13-257)	0.8 (0.3-3.3)	p<0.001
IL-8 ng mL^{-1}	3 hours after ILP	14 (1.3-49)	0.2 (0.01-1.7)	p<0.001
CRP mg L^{-1}	day 1	76 (34-419)	166 (93-350)	p<0.01
$SPLA_2$ ng mL^{-1}	day 1	568 (123-986)	84 (20-390)	p<0.01

Table 3. Median and range of peak-levels of cytokines and acute phase proteins in 10 patients who underwent ILP with >10% leakage compared with 9 patients without leakage in the systemic circulation

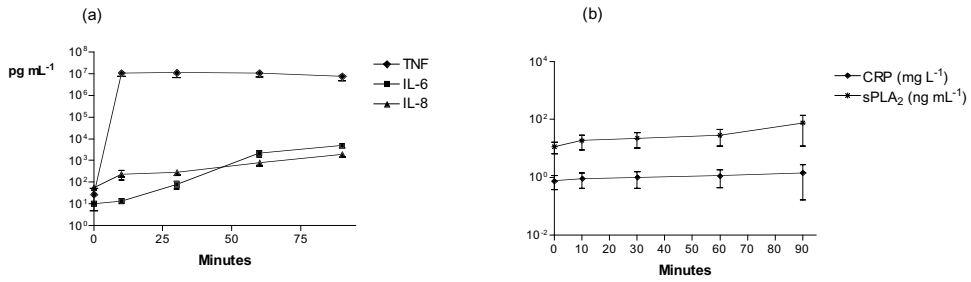

Figure 2. Median (with range) of cytokine (a) and acute phase protein (b) profile in perfusate from 5 patients who underwent an ILP complicated by high leakage (> 12%) to the systemic blood circulation. Time-points: 0', just before administration of TNF in the perfusate; 10' (30', 60'), 10 (30,60) minutes after start of perfusion; 90', end of perfusion, just before release of the tourniquet.

DISCUSSION

The aim of our study was to quantify cytokine levels and acute phase response in patients who underwent an ILP with high dose TNF complicated by high leakage compared with the same variables in patients without leakage, and correlate findings with clinical toxicity. In this study, we measured TNF plasma-levels up to 277 ng mL^{-1}. Nevertheless, most of the patients needed only extra intravenous fluid administration. Systemic levels of this magnitude have been described before in the same setting and once after a 30-min intravenous infusion of recombinant TNF [19-21].

In our patients the necessity for dopamine administration was not related to the highest levels of TNF in the systemic circulation. This finding is in accordance with previous studies in which no correlation between maximum TNF concentrations in the peripheral blood of an individual and the side effects could be found, which indicates that patients vary in their sensitivity to TNF [19, 22].

Systemic toxicity seems to be determined by the duration of exposure to high levels of TNF. Our data demonstrate this clearly with levels of 1000 to more than 100.000 pg mL^{-1} for 4 hours in high leakage patients, and only "moderate" levels (~ 1000 pg mL^{-1} for 20 minutes) in non-leakage patients. In non-leakage patients, 20 minutes of "moderate" TNF-levels were not enough to cause hypotension. This is in accordance with the findings reported previously in this journal by Vrouenraets et al., who described minimal toxicity after leakage-controlled ILP in 20 patients [23]. The IL-6 curves demonstrate the effect of prolonged exposure to high TNF levels even more pungently. Even at 24 hours after ILP, IL-6 levels are still higher in the leakage-patients than the peak IL-6 levels observed in ILP-patients without leakage. IL-6 levels remained elevated for at least 3 whole days.

In the 10 patients with high leakage, 4 hours of exposure to very high levels of TNF resulted in 3 patients with grade II and 4 patients with grade III hypotension. Four patients required dopamine support temporally with good response. In phase I-II studies on the systemic administration of TNF dose-limiting toxicity was observed at TNF-concentrations similar to those observed in our 10 patients described here. For instance Schaadt et al. reported dose-limiting hypotension in 32% of the patients after administration of 650 μg m^{-2} intravenously resulting in a systemic TNF peak concentration of approximately 270 ng mL^{-1}. Moreover grade II hepatotoxicity was observed in 80% of the patients. This is quite different from the relative lack of toxicity observed in our 10 ILP-patients who had similar

systemic TNF peak concentrations. In other studies hypotension was dose limiting at lower doses, with serum TNF levels of 10 ng mL^{-1} [7, 8].

In comparison with septic shock the duration of exposure to elevated levels of TNF in the leakage-patients is relatively short. The prolonged exposure in septic shock, despite of concentrations many times lower than the short peak levels after ILP, results in the typically unresponsiveness of the hypotension to fluid challenge in the septic shock patients [24-27]. Adequate diuresis plays a key-role to keep the period of high circulating TNF-levels as short as possible to prevent a septic shock like state in perfusion patients. That patients are well hydrated at the time of exposure to TNF, and that their blood pressure is optimally maintained by fluid challenge, and only if necessary also the use of dopamine, prevents septic shock like situation. This explains why these patients have little toxicity in view of the very high circulating TNF-levels. It is a fundamental difference with often poorly hydrated patients with metastatic cancer who received intravenous TNF in phase I-II studies in the past. Moreover, septic patients are infected and have significant levels of endotoxin, which has been shown to be synergistic with TNF for toxicity (in rats) [28, 29]. In addition, Feelders et al. have shown in patients who underwent ILP, cortisol is already increased prior to the TNF-peak as a result of surgery and anesthesia [30]. The cortisol response may have a down-regulatory effect on TNF.

The increased TNF-levels in the patients with systemic leakage were followed by significant higher levels of IL-6 and IL-8. This is in accordance with previous studies [17, 21, 31]. In our study, we also determined CRP and $sPLA_2$ as parameters of the acute phase response. $sPLA_2$ levels were more increased in the leakage-patients; CRP, in contrast, had significantly lower levels in these patients at the time points of maximum values. Lower levels of CRP than expected were also observed in patients who underwent an isolated hepatic perfusion (IHP) [32]. That CRP had even lower levels in leakage-patients could be ascribed to a higher expenditure of CRP in the neutralization of the effects of exposure to higher levels of TNFα [33-35].

In conclusion, ILP complicated by high leakage to the systemic circulation resulted in high systemic levels of TNF up to 277 ng mL^{-1}. IL-6 and IL-8 followed with significantly higher levels compared with values measured in patients without leakage. The pattern of the acute phase proteins CRP and $sPLA_2$ resembled each other, except that CRP levels had significantly lower maximum levels in leakage-patients compared with patients without leakage. Overall the

patients with high systemic leakage had a marked mild clinical course. It is our experience that even in patients with very high leakage life-threatening reactions have not occurred and that temporary hypotension can be easily dealt with by fluid challenge and sometimes by temporary vasopressor support. Our observations support the need for further study regarding potential use of TNF systemically.

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CHAPTER 6

Soluble Tumor Necrosis Factor Receptor patterns in Isolated Hepatic Perfusion with Tumor Necrosis Factor-α (TNFα) + Melphalan or melphalan alone in patients with colorectal liver metastases.

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ABSTRACT

In this study we have evaluated soluble tumor necrosis factor α receptor (sTNFR) p55 and p75 levels in patients who underwent Isolated Hepatic Perfusion (IHP) with tumor necrosis factor α (TNF) and melphalan for irresectable colorectal liver metastases.

An extracorporeal veno-venous bypass was used to shunt blood from the lower body and intestines to the heart. Inflow catheters were placed in the hepatic artery and portal vein, and an outflow catheter in the infrahepatic inferior caval vein. The liver was perfused for 60 min with TNF (0.4 mg (n=8), 0.8 mg (n=1)) plus 1 mg kg^{-1} melphalan (IHP_{TM} group) or 1 mg kg^{-1} melphalan alone (IHP_M group, n = 3). The liver was washed with macrodex (washout) before restoring vascular continuity. After the washout procedure, a TNF peak (169 ± 38 pg mL^{-1}) was demonstrated in the IHP_{TM} group only. In both groups levels of both sTNFR increased after the IHP and were still higher than pre-operative values after two weeks. In the patients with TNF added, sTNFR-p55 levels were higher than sTNFR-p75 levels, whereas patients in the melphalan alone group demonstrated the reverse. Furthermore, sTNFR-p55 concentrations were significantly higher in the IHP_{TM} group, and remained so during the following 2 weeks. In contrast, there were no significant differences in sTNFR-p75 levels between groups.

INTRODUCTION

Tumor Necrosis Factor-α (TNF) is a 17 kD trimeric polypeptide cytokine, produced mainly by activated mononuclear phagocytes. It was originally recognized for its cytotoxic effect on tumor cells *in vitro* and for its capacity to cause necrosis of solid tumors *in vivo*. TNF has diverse biological activities, of which this antitumor effect has clinical desirability and great therapeutic potential. However, TNF cannot be administered systemically in cancer patients at effective doses because of prohibitive toxicity as was experienced in phase I-II studies [1, 2]. In fact, it is estimated that only 1/20 to 1/50 of doses with consistent antitumor effectivity in murine tumor models can be administered in humans [3]. This concentration gap is unlikely to be overcome in any mode of systemic administration. Thusfar only isolated perfusion systems have been shown to increase cytostatic drug concentrations more than 20-fold the maximum tolerated dose (MTD) when given systemically [4]. It has become firmly established that TNF can indeed be successfully applied in isolated limb perfusion (ILP) in melanoma patients as well as in patients with very large tumors such as irresectable soft tissue sarcomas [5-7]. ILP with TNF and melphalan resulted in a 30% complete response (CR) and a 50% partial response rate (PR) in the latter patient category and resulted in the approval of TNF by the EMEA (European Medicine Evaluation Agency) for its clinical use in the setting of ILP [8]. Furthermore, this remarkable antitumor effectivity of TNF in combination with melphalan proved to be effective against a wide range of histologies.

TNF mediates its multiple effects on cell function by binding to specific high-affinity cell surface receptors. Two distinct species of TNF receptors, one of 55 kD (TNFR-p55 or type I) and one 75 kD (TNFR-p75 or type II) have been identified and purified [9, 10]. These receptors do not only exist as cell surface membrane proteins but also as soluble proteins. Evidence indicates that these soluble TNF receptors (sTNFRs) are derived by proteolytic cleavage from the extracellular domain of the corresponding cell surface from a variety of cells, mostly neutrophils, activated T-cells and monocytes. The formation of sTNFRs *in vitro* is triggered by certain immune-stimulating agents and is enhanced by a number of cytokines, including TNF itself [11, 12]. Elevated levels of sTNFRs have been described in patients with cancer, HIV infection, sepsis and malaria [13-15]. The exact function of sTNFRs is not known yet.

The loss of cell surface TNF receptors may lead to a temporary decrease in sensitivity of the cell to TNF. Furthermore, by binding to the TNF molecule, soluble receptors could antagonize TNF function. On the other hand, the same sTNFRs are proposed to augment TNF activity by the formation of so called 'slow-release' complexes, depending of the relative concentrations of sTNFRs and TNF [16].
Recently, the technique of isolated perfusion of the liver has been developed for the treatment of patients with irresectable hepatic malignancy. With this technique, the liver circulation is completely separated from the systemic circulation, thus allowing high drug levels within the liver whilst keeping systemic exposure low [17]. Since sTNFRs are known to influence the bioavailability of TNF (or modulate the effects of TNF), and since sTNFRs are known to be induced by TNF itself, we hypothesized that Isolated Hepatic Perfusion (IHP) with TNF and melphalan induces *in-vivo* formation of sTNFRs. In the present study we therefore evaluated the effect of IHP with recombinant human TNF (rhTNF) and melphalan on sTNFR-levels in patients with irresectable colorectal metastases confined tot the liver.

PATIENTS AND METHODS

Patients

Twelve patients with colorectal metastatic liver disease gave informed consent for undergoing Isolated Hepatic Perfusion (IHP) with TNF and melphalan (IHP_{TM} group, n = 9) or melphalan alone (IHP_{M} group, n = 3). There were 9 men and 3 women with a mean age of 59.8 years (range, 49 - 65). Inclusion criteria for IHP with TNF and melphalan included: histological evidence of (irresectable) metastases of colorectal origin confined to the liver, Karnofsky performance status of > 80%. Exclusion criteria included: extra-hepatic malignant disease, > 50% hepatic tissue replacement by tumor, liver cirrhosis, signs of significant hepatic dysfunction (abnormal levels of ASAT, ALAT or Alkaline Phosphatase > 2 x Norm), and ascites or portal hypertension. The protocol was approved by the hospitals' ethical committees. The Study was carried out in accordance with the principles of the Declaration of Helsinki, as revised in Hong Kong in 1989.

Isolated Hepatic Perfusion Technique

The procedure of IHP has been described elsewhere [17]. Briefly, following systemic heparinization (200 U kg^{-1}), an extracorporeal veno-venous bypass (VVB) circuit (pump aided) was created to shunt mesenteric, renal, and lower extremity blood around the liver to the heart. Next, inflow catheters were placed in the portal vein and hepatic artery, and an outflow catheter in the infrahepatic inferior caval vein. These catheters were connected to a heart-lung-machine, and the vascular isolation was completed by clamping the suprahepatic inferior caval vein and the suprarenal inferior caval vein. The liver was then perfused with a hyperthermic (> 38 °C) perfusate consisting of a mixture of saline and erythrocytes. Once a stable perfusion was attained, leakage from the perfusion circuit into the systemic circulation was measured by the addition of 200 µci I^{131} -albumin into the perfusate and the continuous monitoring of radioactivity scintillation probes placed over the perfusate reservoir and VVB. The leak rate was monitored for the duration of the perfusion and if the cumulative leak was greater than 15%, the perfusion would be halted and the perfusate flushed from the circuit. After the absence of leakage was confirmed, rhTNF (0.4 mg in eight patients, 0.8 mg in one) was added as a bolus in the arterial line of the perfusion system (IHP_{TM} group); melphalan (1 mg kg^{-1}) was given directly following the TNF bolus. In 3 patients (IHP_M group), serving as a control group with regards to this study, only melphalan (1 mg kg^{-1}) was administered. After a 60 min perfusion, the liver was washed thoroughly with a mixture of saline and macrodex, decannulated, and vascular continuity restored. Heparin was reversed with 1 mg kg^{-1} protamine sulphate (Novo-Nordisk AS, Rud, Norway) injection. Postoperatively, the patients were monitored in the ICU for at least 48 hours, primarily to evaluate for evidence of systemic toxicity due to rhTNF.

Drugs

Recombinant human TNF (0.2 mg per ampoule) was a gift from Boehringer Ingelheim GmbH, Ingelheim am Rhein, Germany. The cytostatic drug melphalan (Alkeran) came as a sterile powder (100 mg) that was dissolved aseptically using solvent and diluent obtained from Burroughs Wellcome, London, UK.

Sampling schedule

Blood samples were collected from a peripheral vein in siliconized 5 ml Vacutainer tubes (Becton Dickinson, Plymouth, UK) containing EDTA 10 nmol L^{-1}, soybean trypsin inhibitor 100 mg L^{-1}, and benzamidine 10 nmol L^{-1} (Sigma Chemicals, Detroit, USA) to prevent any *in vitro* activation. Samples were centrifuged immediately after collection, at 5000 rpm. for 5 minutes. Supernatant was stored at minus 70 ^{o}C until analysis. Perfusate was sampled at t = 0 (i.e., upon drug administration), 10, 20, 30, 40, 50, and 60 min. Systemic plasma samples were collected at the day before IHP, during ILP at t = 0, 30, and 60 min., and post-perfusion (after release of the VCI clamp) at t = 1, 5, 10, 20, 30, 60, 120, and 180 min., at 21:00 h, day 1, 3, 5, and 7, and weekly thereafter.

Assays

Tumor Necrosis Factor-α (TNF) levels were measured by a sandwich-type enzyme linked immunosorbent assay (ELISA) using two monoclonal antibodies (Dept. Immune Reagents, Central Laboratory of Blood Transfusion, Amsterdam, Netherlands) raised against recombinant human TNF (courtesy of Dr. A. Creasey, Chiron Corp., Emeryville, CA, USA). One mAb (mAb CLB-TNFα-7) was used for coating at a concentration of 2 μg mL^{-1}. The other mAb (mAb CLB-TNFα-5) was biotinylated and used in combination with streptavidin poly-horseradish peroxidase conjugate (CLB, Dept. Immune Reagent) to detect bound TNF. Stimulated human mononuclear cell supernatant was used as a standard for comparison with purified recombinant human TNF. Results were expressed as pg mL^{-1} by reference to this standard [18].

Soluble TNFα receptor p55 (sTNF-R55) and soluble TNFα receptor p75 (sTNF-R75) levels were measured by an ELISA as described previously [12]. Briefly, polyclonal anti-sTNF-R55 (anti-sTNF-R75) antibody was used as a capture antibody, and biotinylated, polyclonal rabbit antibodies in combination with streptavidin conjugate were used to detect bound sTNF-R55 (sTNF-R75). Results were expressed as ng mL^{-1} by reference to a standard consisting of human sTNF-R55 (sTNF-R75). The ELISA was shown to be not affected by TNF concentrations as high as 1 μg mL^{-1}. Normal values are < 2 ng mL^{-1} for both sTNFRs.

Statistics

Results are expressed as means ± standard error of the mean (SEM). Comparison within groups was made by means of the Friedman Nonparametric Repeated Measures Test or by the Mann-Whitney Test, where appropriate. Correlations between maximum levels of parameters were calculated as Spearmann's rank correlations. The significance level was taken as a probability (two-sided) of < 0.05.

RESULTS

Operative procedure

The median operation time was 8 hours (range, 6 to 10). In all patients a stable perfusion was attained. In one patient progressive systemic leakage resulted in discontinuation of the IHP after 43 minutes (cumulative leakage: 20%). In all other patients no leakage could be demonstrated. Three patients in the IHP_{TM} group died peroperatively (n = 1) or in the direct postoperative period (n = 2) due to complications of the operation. As the purpose of this study is the description of the sTNFR levels in uncomplicated IHP, these patients were excluded for this study.

Tumor Response and Survival

The primary efficacy endpoints in this study were the best tumor response observed (WHO response criteria: complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD)) and duration of the best response, calculated from the date the best response was observed until the date of progression. True-cut hepatic tissue samples and/or CT-scan-evaluations were used to describe the best tumor response. In the IHP_{TM} group five out of six patients demonstrated CR or PR, one patient SD. Patients treated with melphalan alone demonstrated PR or SD. The duration of best response ranged from 17.5 to 32.5 weeks (median 18 weeks). In the evaluable patients the survival time ranged from 6 to 26 months. The median survival time was 10.3 months (mean: 13.3).

TNF levels

In the *perfusate*, TNF levels in the IHP_{TM} group were 1.8 ± 0.5 pg mL^{-1} at t = 0 min and increased rapidly to 6.0 ± 2.0 x 10^4 pg mL^{-1} at t = 10 min. Thereafter, TNF levels decreased to 2.8 ± 0.9 x 10^4 pg mL^{-1} at the end of IHP (t = 60 min). In the IHP_M group TNF levels did not demonstrate any significant changes. (figure 1, left panel)

Baseline *serum* TNF levels were similar in both groups, although these levels were higher than normal (4.3 ± 0.5 pg mL^{-1} and 4.5 ± 1 pg mL^{-1} in the IHP_{TM} and IHP_M group resp.). During IHP TNF levels did not change significantly, indicating that vascular isolation was effective. However, after washout, TNF levels increased rapidly to a peak value of 169 ± 38 pg mL^{-1} at t = 1 min in the IHP_{TM} group, and normalized within the next 3 hours. In the IHP_M group, TNF levels demonstrated a slight increase to 7.2 ± 5 pg mL^{-1} within the first 30 minutes after IHP. However, this elevation of TNF levels in the IHP_M group was not statistically significant (figure 1, right panel)

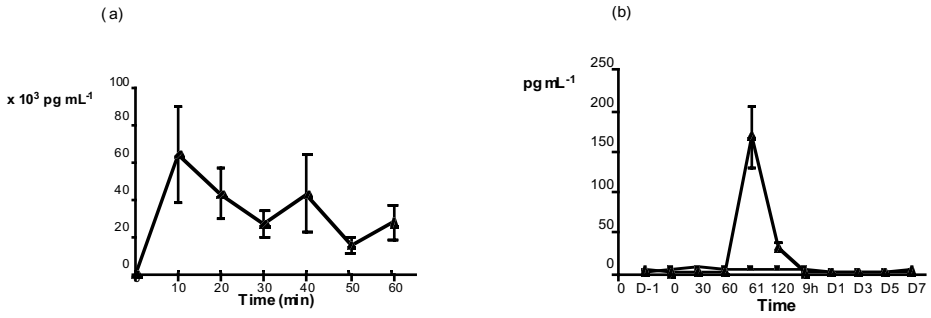

Figure 1. Course of TNF levels before, during and after IHP. Left hand panels show perfusate levels, right panels show systemic levels. Time is expressed on the x-axis; left panel: minutes during perfusion; right panel: D-1 = the day prior to IHP; 0, 30, 60, 61, 120 = 0, 30, 60, 61, 120, 240 minutes after start of IHP (61m represents the time just after vascular restoration, not shown in following panels); 9h= 9h after IHP; D1, D3, D5, D7 = first, third, fifth and seventh day after IHP. (▲--▲), represents the group of patients receiving TNF and melphalan; (■--■), the group of patients receiving melphalan alone.

Soluble TNF receptor levels

sTNFR-p55 levels

In the *perfusate*, baseline sTNFR-p55 levels were mildly elevated in both the IHP_{TM} and IHP_{M} group (5.4 ± 0.7 ng mL^{-1} resp 2.66 ± 0.62 ng mL^{-1}). During IHP, sTNFR-p55 levels increased over time to 9.9 ± 0.7 ng mL^{-1} in the IHP_{TM} group ($p<0.01$) and to 4.57 ± 1 ng mL^{-1} (n.s.) in the IHP_{M} group. The difference between groups was significant ($p<0.01$) (figure 2, left panel).

Serum sTNFR-p55 levels before and during the IHP did not change significantly in both groups. After the washout procedure, the sTNFR-p55 levels rose significantly in all patients, reaching the highest levels in the IHP_{TM} group ($p<0.01$ between groups). Maximum sTNFR-p55 levels were 12.7 ± 2.7 ng mL^{-1} and 3.0 ± 0.6 ng mL^{-1} in the IHP_{TM} and IHP_{M} group respectively. Furthermore, after 2 weeks, sTNFR-p55 levels were still elevated in the IHP_{TM} group (9.1 ± 2.9 ng mL^{-1}), whereas levels in the IHP_{M} group had normalized (figure 2, right panel).

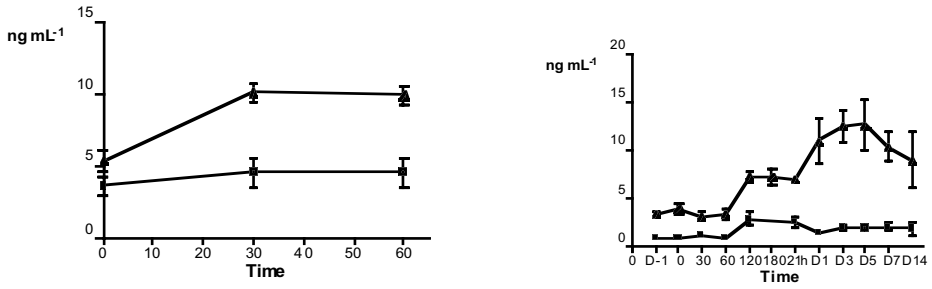

Figure 2. Course of soluble TNF receptor p55 (sTNFRp55) levels before, during and after IHP. Left panel show perfusate levels, right panel show systemic levels. Concentration is expressed on the y-axis in ng mL^{-1}. Time is expressed on the x-axis; left panel: minutes during perfusion; right panel: D-1 = the day prior to IHP; 0, 30, end, 120, 180 = 0, 30, 60, 120, 180 minutes after start of IHP; 21h = 9 hours after IHP; D1, D3, D5, D7, D14 = first, third, fifth, seventh and fourteenth day after IHP. (▲--▲), represents the group of patients receiving TNF and melphalan; (■--■), the group of patients receiving melphalan alone.

sTNFR-p75 levels

Perfusate baseline sTNFR-p75 levels were also slightly elevated in both the IHP_{TM} and IHP_M group (1.4 ± 0.3 ng mL^{-1} and 1.96 ± 0.26 ng mL^{-1}, respectively). During IHP, sTNFR-p75 levels rose in both groups, to 4.1 ± 0.9 ng mL^{-1} in the IHP_{TM} group ($p<0.05$) and to 2.52 ± 0.08 ng mL^{-1} in the IHP_M group (n.s.). (figure 3, left panel).

Serum sTNFR-p75 levels during the IHP did not change significantly in both groups. After the washout procedure, sTNFR-p75 levels rose significantly in all patients. In contrast with sTNFR-p55 levels there was no significant difference between groups. Maximum sTNFR-p75 levels were 6.9 ± 2.0 ng mL^{-1} and 6.3 ± 3.0 ng mL^{-1} in the IHP_{TM} resp IHP_M group. Although not as high as sTNFR-p55 levels, sTNFR-p75 levels remained elevated in the both the IHP_{TM} group (3.6 ± 0.7 ng mL^{-1}) and IHP_M group (3.9 ± 0.8 ng mL^{-1}) after 2 weeks (n.s. between groups). (figure 3, right panel)

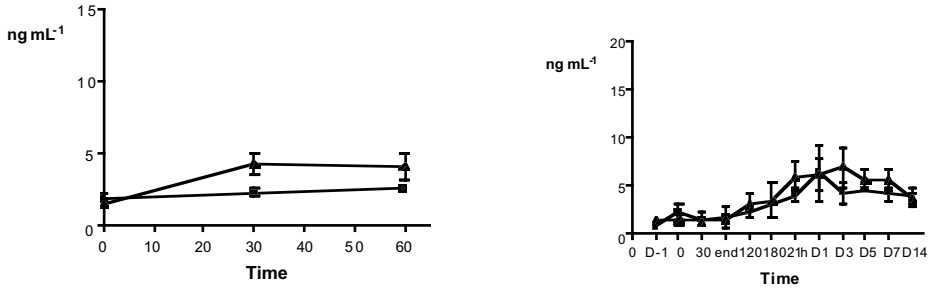

Figure 3. Course of soluble TNF receptor p75 (sTNFRp75) levels before, during and after IHP. Left panel show perfusate levels, right panel show systemic levels. Concentration is expressed on the y-axis in ng mL^{-1}. Time is expressed on the x-axis; left panel: minutes during perfusion; right panel: D-1 = the day prior to IHP; 0, 30, end, 120, 180 = 0, 30, 60, 120, 180 minutes after start of IHP; 21h = 9 hours after IHP; D1, D3, D5, D7, D14 = first, third, fifth, seventh and fourteenth day after IHP. (▲--▲), represents the group of patients receiving TNF and melphalan; (■--■), the group of patients receiving melphalan alone.

DISCUSSION

In this study, we describe the effects of Isolated Hepatic Perfusion (IHP) with TNF and melphalan, or melphalan alone, on the patterns of TNF and its p55 and p75 soluble receptor levels in patients with unresectable colorectal malignancy confined to the liver. After the washout procedure, the rapid and transient appearance of TNF could be demonstrated in the serum of patients in the IHP_{TM} group, which was absent in the IHP_{M} group. In all patients, levels of both sTNFRs increased significantly after IHP demonstrating a different pattern. In the patients with TNF added, sTNFR-p55 levels were higher than sTNFR-p75 levels, whereas patients in the melphalan alone group demonstrated the reverse. Furthermore, sTNFR-p55 concentrations were significantly higher in the IHP_{TM} group, and remained so during the following 2 weeks. In contrast, there were no significant differences in sTNFR-p75 levels between groups.

In order to avoid systemic exposure to chemotherapeutic agents or cytokines, the main goal of the isolation perfusion technique is complete vascular isolation of the limb or organ. In our study vascular isolation of the liver was complete in all patients but one (IHP_{TM}-group). In this patient progressive systemic leakage (cumulative leakage: 20%) led to premature termination of the IHP procedure after 43 min. However, despite this leakage, this patient did not demonstrate additional toxicity as demonstrated by clinical as well as biochemical parameters, compared to the other patients studied [19]. Furthermore, this patient did not demonstrate any significant differences in sTNFRs levels. In our eight patients without discernable leakage, systemic TNF levels did not change significantly during IHP, indicating that vascular isolation was complete. After the washout procedure, however, systemic TNF levels in the IHP_{TM} group peaked rapidly and normalized within the next two to three hours (figure 1, right panel). A possible explanation for this TNF peak in the IHP_{TM} group could be the release of remnant TNF in the liver after the washout procedure, a phenomenon also described in Isolated Limb Perfusions (ILP) with TNF and melphalan [20, 21]. Furthermore, endogenous TNF production may have attributed to this peak, since surgery, extra corporeal circulation circuits, and intravascular plastic catheters are known to induce TNF [22, 23]. However, the latter explanation may be of less

importance since only mild elevation of systemic TNF levels could be demonstrated in the IHP_M group (figure 1).

Our finding of elevated pre-operative sTNFR levels is in contrast with the normal levels described in Isolated Limb Perfusion [21, 24]. Several other studies however reported elevated sTNFR levels in patients with various malignancies, with highest levels in disseminated disease and lower to normal levels in patients with more localized cancer [16, 25]. This could well be the explanation of the virtually normal preoperative sTNFRs levels in patients undergoing Isolated Limb Perfusion (ILP) for irresectable sarcomas of the extremities (localized cancer) compared to the patients described in this study [21, 24].

After IHP, levels of both sTNFRs increased significantly. Elevated sTNFR levels have also been demonstrated in patients with chronic renal failure or dialysis, and a positive correlation with plasma creatinine was demonstrated in patients with different degrees of chronic renal failure [26-29]. Indeed, recent studies indicate that soluble TNF receptor levels are influenced by renal function. Furthermore, a central role of the kidney in the clearance of TNF and TNF - sTNFR complexes has been demonstrated by Bemelmans et al. in mice [30]. Therefore, renal function has to be monitored, in order to evaluate sTNFR levels. Since one of the systemic side effects of TNF is (acute) renal failure/renal impairment, it could be suggested that the elevation in sTNFR levels after the IHP procedure are the result of a decrease in renal clearance. None of the patients presented here demonstrated renal failure as indicated by serum creatinine and urea levels (data not shown). Therefore, in our study, the increased sTNFR levels before, during and after IHP cannot be explained by a decrease in renal clearance.

Our finding of sTNFRs induction after IHP with TNF and melphalan confirms previous studies by others on the systemic administration of TNF. Lantz et al showed a rapid and transient release of TNF binding protein (TNF-BP, sTNFR) in five patients with various malignancies [11]. Furthermore, an inductive role of TNF on the release of sTNFRs has been suggested by Jansen et al, who demonstrated that blocking TNF with neutralizing antibodies, strongly inhibited sTNFR induction in chimpanzees [31]. More recently, elevated sTNFR levels have been demonstrated after ILP with TNF and melphalan, with higher sTNFR concentrations in those patients with leakage of TNF from the circuit during the perfusion [32]. Since elevated sTNFR

levels were also demonstrated in our patients without TNF added, there must be an inductive role for the IHP procedure (with melphalan) itself. Indeed, several reports describe elevated sTNFR levels after major surgical procedures, including liver surgery [33, 34]. Schroder et al demonstrated prolonged elevated sTNFR concentrations after gastrectomy and liver resection, whereby levels after liver surgery remained elevated for a longer (up to one week) period [34]. Similar results have been described in ILP with TNF and melphalan for irresectable sarcomas of the limb, with higher sTNFR levels in those patients with leakage of TNF.

Several hypotheses about the *in vivo* function of sTNFRs have been postulated with evidence of both antagonistic and carrier (buffer) effects. There are indications that the soluble forms of TNF receptors are derived by proteolytic cleavage from the extracellular domain of the corresponding cell surface form (shedding) [35]. The result of this cleavage of sTNFRs from the cell surface might result in a down regulation of the number of cell surface receptors, with a subsequent decreased responsiveness of the cell to circulating TNF [36]. Since both sTNFRs are still capable of binding TNF, they can compete for TNF with the cell surface form. *In vitro* the shedding of the sTNFR-p75 is triggered with TNF-mimetic antibodies. The shedding of the sTNFR-p75, leading to the above mentioned down regulation of this receptor on the cell-surface, can reduce the cell-associated TNFR-p75 passing of TNF to the p55 receptor, and thus acting as a desensitization mechanism [16]. This mechanism has been confirmed *in vivo*, and could explain the observation that in most studies, sTNFR-p75 levels are higher than p55 levels. In accordance with these reports are the sTNFR patterns and maximal concentrations demonstrated here in IHP with melphalan alone. In contrast, patients with TNF added to the perfusate showed a reverse pattern with higher sTNFR-p55 concentrations. Possibly, in IHP with TNF and melphalan different shedding mechanisms are responsible for the induction of sTNFR-p55 and –p75, which might lead to excessive shedding of the TNFR-p55 instead of p75. The reason for this is unclear, but other factors than TNF, e.g. interleukin-6 (IL-6) might be involved in the induction of sTNFR-p55 as has been demonstrated by Tilg et al [37]. This alternative (p55 mediated) desensitization mechanism has also been demonstrated by Leist et al who showed that extensive for induction of hepatocyte apoptosis and liver failure [38]. Arguing that the increased sTNFR-p55 levels indicate a down regulation of the membrane bound

receptor, this process might make the hepatocyte less prone to the induction of TNF mediated apoptosis. In this light, an interesting question would be whether the down regulation of membrane bound TNF receptors, with subsequent (possible) protection against TNF, may have implications for the antitumor effects of TNF treatment. Further research is mandatory in order to evaluate this putative sTNFR-mediated antitumor effect of TNF.

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CHAPTER 7

Degree of tumor vascularity predicts drug accumulation and tumor response upon Tumor Necrosis Factor based isolated hepatic perfusion.

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ABSTRACT

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 to 90 % in patients with in transit melanoma and unresectable sarcoma of the extremities. 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. Moreover in our pre-clinical ILP model we observed drastic alterations in tumor microvasculature integrity. These findings led to the hypothesis that TNF causes specific destruction of tumor endothelial cells and thereby induces an increased permeability of tumor vasculature. Isolated hepatic perfusion (IHP) with melphalan with or without TNF is currently performed in clinical trials in patients with hepatic metastases. 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 vascularisation. 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 α (TNF) is a cytokine with an interesting potential in the treatment of cancer [1]. However when administered systemically it is accompanied with severe toxicity, but when TNF is used locoregionally in combination with chemotherapy without systemic exposure it has very potent antitumor effects. Clinical trials of isolated limb perfusion (ILP) with recombinant human TNF (rhTNF) and melphalan resulted in high complete response rates of 75-90 % in patients with in-transit melanoma and unresectable sarcoma of the extremities [2-7]. This is in contrast to ILP with melphalan alone which is relatively effective against small in transit melanoma metastases but achieves very poor results against large tumors such as soft tissue sarcomas [8-10].

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 [11]. 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 [12]. In patients, angiographic studies performed pre and post TNF perfusion showed selective destruction of tumor associated vasculature and histologic studies demonstrated hemorrhagic necrosis of the tumor [2, 6]. 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 favorable 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 antitumor effect of TNF is strongly 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.

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 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 α (TNF, 4.9-5.8 x 10^7 units mg^{-1}) 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. 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 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^{-1} 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.

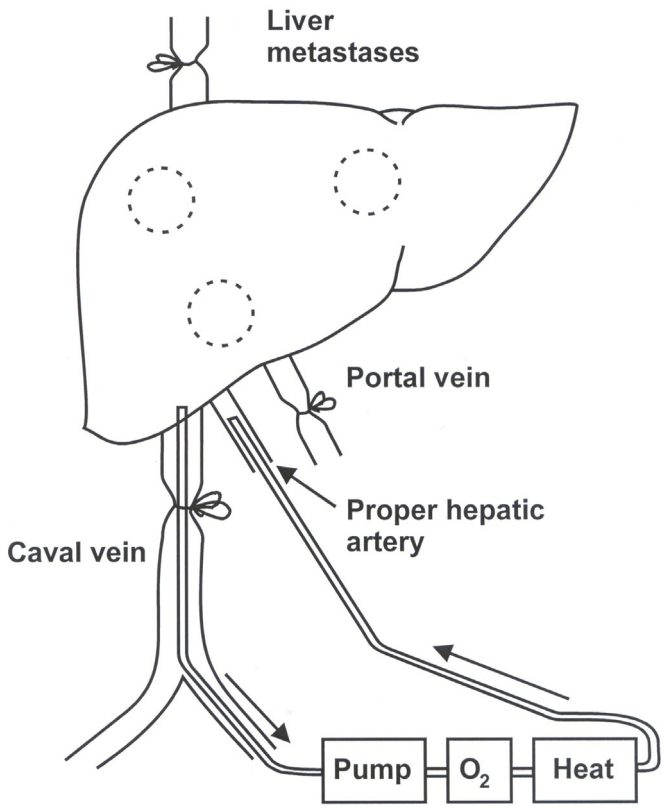

Figure 1. Schematic representation of an isolated hepatic perfusion (IHP).

In vivo Antitumor 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. CC531 bearing rats were treated with 50 mg melphalan (n=6), 20 µg TNF (n=6), or a combination of 50 mg melphalan and 20 µg TNF (n=6). ROS-1 bearing rats were perfused with 50 mg melphalan (n=6), 20 µg TNF (n=8), or a combination of 50 mg melphalan and 20

µg TNF (n=6). In the BN-175 bearing rats perfusions were carried out with 200 mg melphalan (n=6), 20 µg TNF (n=6), or a combination of 200 mg 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}), 1% streptomycin (5000 IU mL^{-1}) 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^{-1} . 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 antitumor efficacy of IHP with melphalan with or without TNF was evaluated for the CC531, ROS-1 and BN-175 tumor. 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 and untreated control 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 in 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.

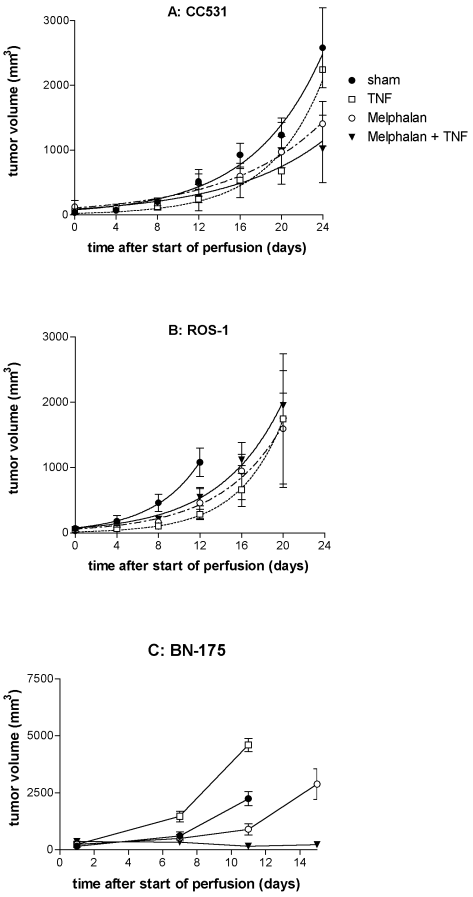

Figure 2. Growth curves of in vivo tumors after isolated hepatic perfusion: (A) CC531, (B) ROS-1, (C) BN-175. Each group contained at least six animals. Mean values (± SEM) are shown.

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^{-1}. So *in vitro* tumor cells were exposed to a range of TNF concentrations varying from 0 to 10 µg mL^{-1}. 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^{-1} was observed.

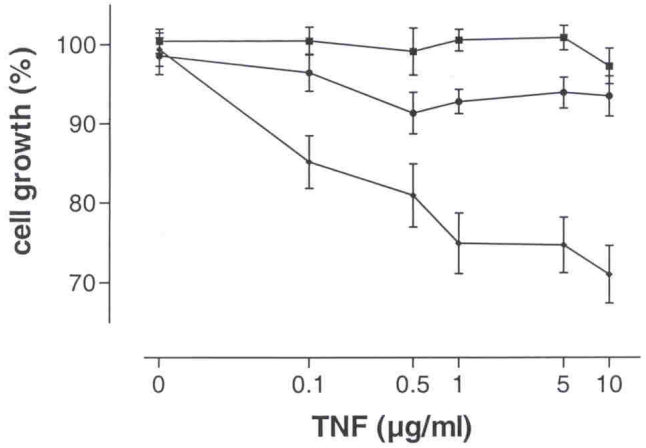

Figure 3. In vitro growth curves of tumor cells upon exposure to TNF. CC531 (●), ROS-1 (♦), BN-175 (■). Six independent assays were performed in duplicate for each point on the line. Mean values (± SEM) are shown.

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 , which is in correspondence with the tumor response observed.

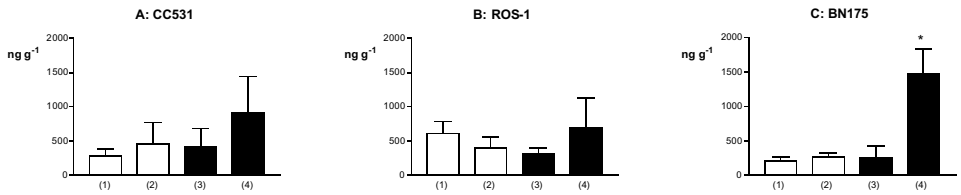

Figure 4. Melphalan concentrations in liver and tumor tissue after IHP with melphalan with or without TNF. Six IHPs were performed per tumor type (a) CC531, (b) ROS-1, (c) BN-175. Y-axis: melphalan concentration in ng g^{-1}. X-axis: (1) liver tissue, IHP with melphalan only, (2) liver tissue, IHP with melphalan + TNF, (3) tumor tissue, IHP with melphalan only, (4) tumor tissue, IHP with melphalan + TNF. Mean values (±SD) are shown. (★ = $p<0.05$ versus tumor melphalan concentration after IHP with 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 direct relationship between vascularity of the tumor and TNF-mediated effects.

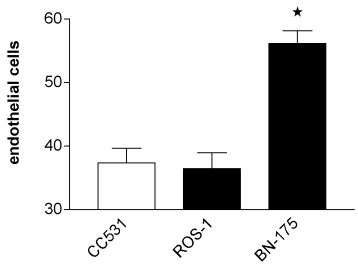

Figure 5. Microvessel count of CC531, ROS-1 and BN-175 tumors. Mean values (±SEM) are shown. (★ = p<0.001 versus CC531 and versus ROS-1)

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 antitumor 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 [11, 12]. 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. So TNF has specific tumor vascular mediating capacity in this perfusion model, which results in enhanced tumor responses in highly vascularised tumors. As a result of our findings in ILP and now also in IHP we know that TNF is able to augment the accumulation of melphalan. We are of the opinion that this observation is essential in understanding and explaining the impressive responses observed.

Changes in vascular permeability in patients who underwent IHP with TNF were 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 vascularised BN-175 tumor. The results of Alexander et al. reported on intratumoral 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 vascularised. 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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CHAPTER 8

Tumor Necrosis Factor and Isolated Hepatic Perfusion from preclinical tumor models to clinical studies:

credits, debits and future perspectives

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INTRODUCTION

Tumor Necrosis Factor α

The American surgeon Coley was the first to observe the spontaneous regression of cancers in patients with concurrent bacterial infection [1]. These regressions were acute and a hallmark was rapid hemorrhagic necrosis with bouts of fever. Many decades later a factor causing hemorrhagic necrosis in experimental tumors was discovered and called tumor necrosis factor α (TNF) [2, 3].

TNF is a homotrimeric complex of 52 kD which is produced by many cell types but mainly by activated monocytes/macrophages [4, 5]. Its expression and regulation is affected by a variety of other cytokines, as interferon-γ (IFNγ), interleukines (IL-1, IL-2, IL-12), GM-CSF, platelet aggregating factor (PAF) as well as TNF itself [5]. TNF is directly cytostatic or cytotoxic to only a few cancer cell lines [6]. On other cell types TNF shows a growth inhibitory or even a growth stimulatory effect [5, 7]. The effects of TNF are exerted by binding to two types of receptor with molecular weights of 55 kD (TNF-R1) and 75 kD (TNF-R2) respectively, which are present on nearly all mammalian cells [4, 8]. The number of receptors on the cell does not predict the magnitude of response to TNF, but up-regulation (IFNγ) and down-regulation (IL-1) of TNF receptors have been reported [7]. TNF has pleiotropic and concentration dependent effects. At high concentrations, TNF has been shown to have vasculotoxic effects, while at lower concentrations promotion of angiogenesis and DNA synthesis may be demonstrated [9, 10].

In vitro, synergism between TNF and a number of cytotoxic agents may be present [11]. Our group investigated several cell lines on susceptibility of TNF and certain cytotoxic drugs (e.g. the alkylating agent melphalan or the topoisomerase-II inhibitor doxorubicin). No direct cytotoxic effects of TNF, nor synergism with melphalan or doxorubicin were observed *in vitro* but only additive antitumor effects were noted [12-14]. Cytotoxic effects of TNF can be enhanced by a number of other biological response modifiers like IFNγ or IL-1, by hyperthermia and by irradiation [11, 15-17]. The mechanisms by which TNF exerts its cytotoxic effects are not yet fully understood. The number of receptors on the tumor cell is probably of less importance than the role of oxygen free radicals in TNF cytotoxicity, and activation of lysosomal enzymes [5].

Many animal studies have demonstrated antitumor effects of TNF *in vivo*, leading to hemorrhagic necrosis in tumors [2, 11]. Systemic application of TNF in

humans however, proved to be deleterious to patients and in phase I-II studies severe toxicity was reported. A variety of side effects were noted, hypotension being the dose limiting factor [18-20]. The maximal tolerated dose (MTD) varied between 200 and 400 μg m^{-2}, which was only 1/50 of the effective dose in murine tumor models [21].

So only low doses could be explored by systemic administration and due to these low concentrations of TNF negligible response rates were achieved in phase II studies [22-24]. Addition of IFNγ or IL-2 did not enhance antitumor efficacy but further increased toxicity [25, 26]. Because of severe toxicity after systemic use of TNF in clinical trials, other routes (locoregional) of administration were explored to achieve high local concentration of TNF in tumor tissue. Intratumoral injection revealed only slightly better responses than intravenous injection with similar side effects [27-29]. Hepatic artery infusion and intraperitoneal administration of TNF revealed only modest results [30, 31].

The major breakthrough for TNF was its introduction in isolated limb perfusion (ILP) as a treatment for patients with irresectable soft tissue sarcoma or in transit metastases of melanoma [32-36].

The impressive response rates achieved with this treatment modality have resulted in the approval of the drug in Europe in 1998 for the treatment of irresectable extremity soft tissue sarcomas and has renewed interest in the application of TNF in other isolated organ perfusion settings such as isolated perfusion of lung, kidney, and liver [37-42].

Antitumor effects of TNF

Experimental as well as clinical ILP with TNF and melphalan have demonstrated that the tumor-associated vasculature (TAV) is the selective target for TNF. The effects of TNF on this vascular bed are concentration dependent: at high concentrations mostly vasculotoxic effects have been shown, whereas at lower concentrations it may promote DNA synthesis and angiogenesis [9]. Renard et al described the effects of TNF on the TAV as early endothelium activation, up regulation of adhesion molecules and invasion of polymorphonuclear cells, all leading to coagulative necrosis with or without hemorrhagic necrosis [43, 44]. However, examination of the melanomas and sarcomas of patients treated by ILP with TNF, melphalan and IFNγ did not demonstrate differences in expression of adhesion molecules as ICAM-1, E-selectin (ECAM-1), VCAM-1 or PECAM-1 in tumors compared with normal tissue [45]. More recently, detachment and

apoptosis of the integrin αvβ3 positive endothelial cells was demonstrated in melanoma metastases of patients treated by ILP with TNF, melphalan and IFNγ, again aiming at the importance of the selective disruption of the TAV [46]. NMR further indicated this and angiography studies which clearly showed the disappearance of only TAV after TNF based ILP [47-49].

The antitumor effects of TNF in the isolated perfusion setting are based on synergism with a cytostatic drug [7, 12, 13, 19]. Probably, TNF is suggested to be responsible for the disruption and subsequent leakage of the TAV whereas melphalan (or in theory any other chemotherapeutic drug) causes a non-specific necrosis of the tumor cells [50, 51].

The TNF induced endothelial damage was demonstrated to occur as early as three hours after the onset of TNF based ILP [44]. On the other hand, also a delayed type of TAV hyperpermeability may be present which would explain the fact that complete tumor regression frequently requires longer periods after TNF based ILP [52]. The effects of high dose TNF on the TAV lead to an increased permeability and a significant decrease of the interstitial pressure in the tumor. Both effects lead to a better penetration of cytotoxic drugs into the tumor tissue [53-55]. Indeed our group demonstrated a 4 to 6 fold increase of intratumoral melphalan concentration when TNF was added to the perfusate in ILP with melphalan [56]. Similarly, an increased uptake of doxorubicin in tumor tissue was shown after TNF based ILP [14]. Probably these findings are one of the most important mechanisms behind the successes demonstrated by ILP with TNF and melphalan [57].

Alexander et al showed an increased capillary leakage during IHP and an increased uptake of I^{-131} albumin in tumor tissue compared to liver tissue. However, the addition of TNF did not affect melphalan concentrations in the tumor tissue compared to liver tissue [58]. Several reasons for the discrepancy are possible such as concentrations of TNF used, sampling method and duration of perfusion. Of more importance however, might be the type of tumor with associated difference in tumorvasculature since colorectal metastases are hypovascular and largely necrotic, whereas soft tissue sarcoma are usually hypervascular. The results from our laboratory implicate that the better the vascularisation of a specific tumor, the more explicit the effects of TNF on the TAV and the better the overall response of the tumor to the treatment [14, 56]. This has been proven from experimental as well as clinical results. In the rat, best response rates after ILP with TNF and melphalan have been demonstrated by our

group for the highly vascularised BN soft tissue sarcoma bearing rats whereas the less vascularised rat osteosarcoma (ROS) showed subsequent lower responses [12, 13, 57]. Similar results have been shown by Van IJken et al in an IHP model in rats using the same tumors (BN, ROS) but now localized in the liver. In case a coloncarcinoma was used, only few responses had been shown [59]. In accordance with these results, best clinical responses have been demonstrated after ILP with TNF and melphalan in patients with unresectable soft tissue sarcoma of the extremities [33, 34]. Therefore, the vascularisation of the tumor seems to be of utmost importance in the treatment with TNF, independent of its localization (this thesis chapter 7).

Primary and secondary malignant hepatic disease

Patients with unresectable primary and secondary (metastatic) hepatic malignancy remain a challenging clinical problem. Of the primary hepatic malignancies, Hepato Cellular Carcinoma (HCC) is the most frequent, although the incidence of these tumors varies widely worldwide, being most common in the Far East [60]. Recent advances in the early detection of these tumors have improved the prognosis and long term survival has been reported in patients with small, encapsulated malignancy [61-63]. Nevertheless, the overall prognosis of HCC remains poor and usually expressed in months rather than years [64].

In the Western countries the most common hepatic malignancy is metastatic disease from colorectal cancer. Most frequently, the liver is the site of dissemination with many other sites in the body (lung, brain, bone). On the other hand the liver is the sole site of initial cancer recurrence in as many as 30 % of patients [65]. If left untreated the mean survival rate in these patients is approximately 6 to 9 months. In contrast, 5-year survival rates up to 35 % have been reported for patients amendable to resection. Unfortunately in the majority (75%) of the patients that have been diagnosed with colorectal cancer metastases confined only to the liver, these metastases are considered unresectable. These patients are eligible for other therapies.

Systemic chemotherapy

The effect of systemic chemotherapy on hepatic metastases depends on the primary site of metastatic disease and the dose of agents used. Since certain tumors (e.g. breast carcinoma) are responsive to chemotherapy, even when hepatic metastases develop, systemic chemotherapy may be the appropriate

treatment. The standard therapy for patients with advanced colorectal carcinoma has been systemic chemotherapy with 5-fluorouracil (5-FU) based protocols. These therapies produce an average response rate of 20 to 30 % with median survival times of 6.5 to 13.5 months, if 5-FU plus folonic acid (FA) at conventional doses is used for systemic (iv) therapy [66-68].
In general, with systemic combination therapy it is not likely to obtain response rates higher than 30 % with only a minimal effect on survival and in most cases this is possible at the cost of considerable systemic toxicity, especially with the use of TNF. 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.

Locoregional therapy

The main principle for locoregional therapy is the achievement of higher local drug concentrations and thus higher exposure of tumor tissue to the agents, resulting in increased response rates, while shielding the patient from systemic toxicity because of much lower concentrations in the systemic circulation. Since for most chemotherapeutic steep dose-response curves have been demonstrated, this should result in a greater tumor response. On the other hand, there is a greater risk of regional damage to normal cells in the tissues surrounding the tumors. The most direct mode of locoregional therapy is the injection of drug into the tumor. TNF has also been used in this treatment modality with only slightly better responses than iv injection with similar side effects at the same MTD [27-29].
In case of intra-arterial chemotherapy to the liver (hepatic artery infusion, HAI), depending on the type of drug used, there should be the additional factor of extraction of a significant amount of the anticancer agents on the first passage through the organ and thus reduced outflow into the systemic circulation with a distinctly reduced risk of systemic toxicity.
However, the best approach for regional infusion of the liver is still unknown. Hepatic artery infusion (HAI), hepatic artery ligation with hepatic artery and portal vein infusion, or portal vein infusion have all been attempted [69-71]. Of these modalities, HAI is the single most widely applied form. Only a few completed randomized studies have been reported in patients with unresectable colorectal metastases confined to the liver. Although response rates of 50 to 55 % of HAI using either 5-FU, FUDR, fluoropyrimidines with or without other drug

have been demonstrated, the median survival time of 11 to 14 months in most of these studies did not exceed the 11 months of 5-FU iv therapy that showed response rates of 11 to 20 % [67].

TNF has also been used in HAI in an attempt to improve response rates with lower systemic toxicity. Mavligit et al treated 22 patients with HAI with recombinant human TNF (rhTNF) and showed that the MTD was 150 µg m^{-2}, which is six times the MTD of rhTNF that could be given systemically on the same schedule. The dose-limiting toxicity, hypophosphatemia and associated myocardial dysfunction, was severe but transient. However, only modest responses were demonstrated [30]. In summary, with HAI improved response rates are achieved but convincing evidence of improved survival is lacking. The application of TNF in HAI is possible but still limited by systemic toxicity.

For most chemotherapeutic agents steep dose-response curves can be demonstrated. Therefore, high drug concentrations are important for both sensitive and resistant tumor cells. For resistant cells extremely high exposure is needed for adequate cell kill. With HAI higher locoregional drug concentrations have been demonstrated with subsequent better response rates. However, despite high extraction ratios in HAI, systemic exposure and toxicity cannot be fully eliminated and has been reported the dose limiting factor. In order to maximize locoregional drug concentrations and at the same time completely shielding the patient from systemic toxicity isolated perfusion techniques have been developed.

Isolated Hepatic Perfusion

The isolated perfusion concept is easy: due to the complete vascular isolation high local drug concentrations can be achieved whilst minimizing systemic exposure and thus toxicity. In case of the liver isolated hepatic perfusion (IHP) has been developed. In IHP the vascular bed of the liver is completely isolated and perfused with a recirculating circuit. IHP is a means to further improve selectivity of administration of antitumor agents to the liver as compared with HAI. IHP with 5-FU in rats and pigs resulted in significantly higher 5-FU concentrations in liver tissue of animals in the higher dose groups. When mitomycin C was administered by IHP a 400% higher dose could be safely administered and resulted in a five times higher tumor tissue concentration as compared with HAI [72, 73]. These data suggest that, to achieve similar systemic drug levels, 5 times the HAI dose can be administered with IHP. Therefore, IHP is a method to improve selective administration of antitumor agents to the liver

while maintaining very low systemic drug concentrations. As is true in HAI, it is clear from experimental data that in the IHP setting hepatic rather than systemic toxicity is dose limiting. Another advantage of the complete vascular isolation of the liver is the possibility to apply hyperthermia. Hyperthermia has been demonstrated to synergize with chemotherapeutic and biological agents. Furthermore, it has direct independent antitumor effects as well [74-76].
Successful clinical experience with IHP is limited but promising. Thus far, several studies have been published with various chemotherapeutical or biological agents e.g. Nitrogen Mustard, 5-FU, mitomycin C, and melphalan. Reported hepatic toxicity is surprisingly mild and transient. Furthermore, data thus far have made clear that only IHP can bring about 3 – 5 years of disease-free survival [77-79]. More recently, tumor necrosis factor α (TNF) has come into focus as a result of the successes achieved in isolated limb perfusions (ILP) with this cytokine in combination with melphalan.

IHP Technique

Since the liver has a dual blood supply both the hepatic artery (HA) and portal vein (PV) allow access for IHP. However, the best mode of infusion is not known. Determination of the most optimal route of infusion involves issues of vascular variations, technical ease of cannulation, as well as tumor blood supply. There is no consensus about the route of infusion (HA vs. PV vs. both). Normal hepatic parenchyma receives most of its blood supply from branches of the portal vein (PV) and to a much lesser extent from the hepatic artery (HA). In contrast, the blood supply of hepatic metastases was ascribed to rely almost entirely on the HA [70, 80, 81]. Consequently, most regional approaches have been using the HA. More recently, however, attention has been drawn to the PV since very small liver tumors (< 5 mm), as well as the outer rim of larger hepatic metastases, are fed mainly by portal branches [81, 82]. In addition, most colorectal tumors drain via the PV suggesting that spreading tumor cells will first proliferate in the portal system. Furthermore, the presence of dye in metastatic foci within the liver could be demonstrated when the color agent had been injected through the portal system, convincing evidence that blood carrying from the antitumor drug would reach the same site. Thus, by using the HA as well as the PV, drugs may reach both established and newly formed (micro) metastases. However, since primarily the PV supplies most normal hepatic parenchyma tissue, it could be speculated that infusion via the PV might induce significant hepatotoxicity. Indeed, Boddie

et al performed IHP via the PV only and demonstrated significant hepatic damage [83]. Furthermore, Kahky et al demonstrated an increased mortality in rats in case TNF was delivered via the PV [84]. In contrast, several IHP studies in pigs, including ours (this thesis, chapter 2) using both vessels could not confirm these findings [41, 85, 86].

Several studies have demonstrated that liver uptake of various drugs after portal vein infusion is not significantly different from liver uptake after hepatic artery infusion [80]. Regardless the route of administration, the drug is delivered to the sinusoid, where it is extracted by the hepatocyte. Therefore, hepatic uptake of drugs is not dependent on the route of regional administration. In contrast, the blood supply of most hepatic metastases is predominantly arterial .

Uptake of drugs by the tumor is dependent both on perfusion of the tumor and cellular uptake of drug. Most anatomical studies of the blood supply of colorectal hepatic malignancies are based on bolus injection of dye or radiolabeled compounds. In a clinical randomized study comparing chronic hepatic artery or portal vein infusion of FudR for colorectal metastases, the measured uptake of drug by the tumors correctly predicted the clinical response: 50% of the HAI group responded, whereas no patient in the portal vein group responded [87]. Therefore, with respect to flow through a tumor, there is a pre for the HA (high flow through tumor) vs. the PV (low flow through tumor).

Apart from these theoretical considerations there are also some practical ones. First, the HA is small and local anatomy varies widely whereas the PV is a larger vessel with limited anatomical variation. Second, the hepatic venous drainage is through multiple small veins and meticulous dissection of all these veins is necessary in order to obtain the desired complete isolation. Although difficult, the same techniques apply as used in orthotropic liver transplantation. This also includes the use of a veno-venous bypass in order to shunt blood from the intestines, kidneys and limbs back to the heart without the risk of intestinal congestion and subsequent bacterial translocation.

Animal studies

Several groups have been experimenting with IHP and several models have been developed in rodents, dogs and pigs. The first experimental IHP studies have been performed in large animal models (pigs and dogs). Since the anatomy of the liver and its vasculature in these animals resemble those in humans they used to develop techniques of IHP and study the pharmacokinetics of several

chemotherapeutics including TNF. Skibba showed that hyperthermic (42°C) IHP (dual system), without drugs, in dogs with survival (20 out of 26) and with preservation of good hepatic function was possible [88]. Sindelar et al performed IHP in pigs with increasing doses of 5-FU with or without hyperthermia (41°C) and demonstrated temporary hepatic enzyme disturbances without systemic drug toxicity in all animals. Levels of 5-FU tolerated by the liver in the IHP setting (500 mg kg^{-1}) were more than 1000-fold greater than maximum levels achieved by routine systemic, intra-arterial or intraperitoneal administration [85]. Van de Velde et al performed IHP in pigs with increasing doses of 5-FU (20, 40 and 80 mg kg^{-1}) and demonstrated that at least four times the conventional dose of this drug can be safely administered [86].

Regarding TNF: In addition to its systemic side effects, TNF is also known to be cytotoxic for hepatocytes [89-91]. Experience with the use of TNF in IHP in large animals is scarce. We performed leakage free IHP with melphalan and/or rhTNF in pigs demonstrating stable perfusate TNF with a cumulative systemic leakage of rhTNF during IHP of 0.02 %. (this thesis, chapter 2) As to hepatotoxicity, after IHP a significant but transient rise in hepatic enzymes (ASAT, ALAT, LDH, Alk.Phosphatase) was observed in all pigs, including the controls, which normalized within one week. Histologic sections of the liver showed mild sinusoidal dilatation as well as septal edema with sporadic intraseptal polymorphonuclear cell infiltration, without apparent hepatocellular damage or parenchymal necrosis. Since the addition of high dose rhTNF and melphalan to the perfusate did not lead to additional hepatotoxic side effects, most of the changes shown are the result of the IHP procedure itself. Similar results have been reported by Lang who furthermore showed that washout of the liver with a protein solution reduces systemic rhTNF levels as well as associated lethal cardiocirculatory and hepatotoxic side effects [92]. Although it is unknown what the effects of rhTNF on the cytokine release in pigs are, Lang et al demonstrated that in IHP the addition of rhTNF to the perfusate in pigs led to a statistically significant release of porcine TNF [92]. In accordance with this study, Pogrebniak et al showed that i.v. infusion of 80 μg kg^{-1} BW led to a 100 % mortality due to cardiocirculatory and pulmonary depression with 24 h in experiments in pigs [92, 93].

From experiments in large animals (pigs and dogs) it is clear that IHP with hyperthermia is technically feasible and safe, and results in transient hepatotoxicity. Much higher dosages of chemotherapeutics can be applied since

with complete vascular isolation systemic exposure and toxicity are and have been proven to be minimal. With respect to the application of TNF it is important that isolation is complete and the washout procedure is carried out with protein solution instead of crystalloids in order to minimize systemic exposure to this cytokine. Up to date no transplantable tumor cell lines exist in large animals. Therefore, these models are not suitable for the evaluation of antitumor effects of IHP. Fortunately tumor models do exist and have been described in rodents (rats and rabbits) but the obvious technically difficulty of performing hepatic perfusions in these animals has limited the number. Therefore, contrasting with the situation for ILP, from animal studies there are limited data available with respect to IHP for metastases.

Rodent models

In an IHP model in hepatic tumor bearing rats, Marinelli et al and De Brauw et al demonstrated that when mitomycin C (MMC) resp. melphalan was administered by IHP a 400 % resp. 200 % higher dose could be safely administered and resulted in a 5 resp. 4 times higher tumor tissue concentration compared with HAI. Furthermore, they showed that IHP in a rat coloncancer CC531 liver tumor model was superior to HAI and allows the administration of a well-tolerated dose of mitomycin C being high enough to induce marked DNA synthesis inhibition and even complete tumor remission [72, 73, 94, 95]. Radnell et al demonstrated that the addition of 5-FU (70 mg kg^{-1}) to the perfusate significantly retarded tumor growth evaluated 10 days after IHP compared to rats perfused without 5-FU. There was however also some degree of tumor growth retardation in the group without 5-FU demonstrating an antitumor effect of the IHP with hyperthermia also [96]. The influence of hyperthermia on the permeability of tumor neovasculature has been shown by Gnant et al in a rabbit hepatic metastases model (VX-2/New Zealand White). They demonstrated that hyperthermia (41°C) preferentially increased vascular permeability in tumors compared with liver tissue in a dose-dependent fashion, thus providing a mechanism for antitumor effects of IHP [97].

We performed IHP with TNF and melphalan in rats bearing BN-175 (soft tissue sarcoma) hepatic tumors and demonstrated a dramatic increase in regional concentrations of perfused agents. IHP with only carrier solution resulted in a significantly diminished growth rate of BN 175 liver tumors compared with the growth rate of tumors in nonperfused rats. Perfusion with melphalan alone

resulted in minimal antitumor effects. Perfusion with only TNF had no effect on tumor growth. When TNF was added to melphalan, a dramatic antitumor effect was observed. Thus, as in the isolated limb perfusion setting, the antitumor effect is augmented when TNF is added to IHP with melphalan to treat BN 175 soft-tissue sarcoma tumor-bearing rats. In contrast, these results could NOT be extrapolated to the coloncarcinoma (CC531) hepatic metastases model in the rat. The difference in response rates between BN-175 and CC531 tumors correlated with the hyper – and hypovascular properties of the respective tumors (this thesis, chapter 7).
In summary, the experimental IHP in rodents demonstrate that the much higher drug concentrations used in IHP result in higher tumor tissue concentrations of the drugs used with subsequent significant better response rates as compared to HAI. In case the combination TNF and melphalan is used in IHP, similar response rates have been demonstrated as in ILP. The response rates are, however, dependent of the vascularity of the tumor: best responses in highly vascularised tumors.

Clinical experience

Isolated hepatic perfusion (IHP) was first clinically applied almost 40 years ago, and over the subsequent 20 years a limited number of studies have reported results indicating its feasibility but also its morbidity and even treatment related mortality (10% - 25%). Furthermore, it was not clear what drugs had to be to use since in IHP higher concentrations can be used than in regional or systemic setting and hepatic rather than systemic toxicity will be the dose limiting factor.
In 1961 Ausman was one of the first to report the results of IHP in patients. Of the five patients treated with IHP with Nitrogen Mustard there were 2 “long term” survivors. Furthermore he showed that IHP was accompanied by acceptable toxicity [98]. After this report a few other reports have followed, describing IHP in patients with chemotherapeutics and/or hyperthermia alone (Table 1). The influence of hyperthermia has been demonstrated by Skibba who performed a 4 hour IHP with hyperthermia (42 °C) alone in 8 patients. Two patients died after surgery but of the surviving six patients five were reported as responders [99]. Several chemotherapeutic agents have been used in IHP, of which 5-FU, cisplatinum, MMC are the most widely used. The fifty patients treated by Schwemmle et al with IHP with 5-FU, MMC or cisplatinum had a median survival of 14 months and showed a 22 % complete and 68 % partial response

rate [100]. Aigner et al. treated 34 patients with IHP with MMC and/or 5-FU and described an impressive 34 % CR. Unfortunately, in all patients IHP was followed by 5 courses of HAI with MMC and 5-FU, such that the impact from the IHP can not be clearly delineated [77]. Despite the stimulating results of the IHP studies, results have to be taken with care since there was no standardized protocol for measurement of responses. In most studies a drop in CEA levels was used, in others the appearance of central necrosis on CT scans.

As Aigner, MMC was also used in IHP by Oldhafer et al, who reported veno-occlusive disease (VOD) in 2 out of 6 patients treated by IHP with MMC [101]. The finding of severe VOD has also been described by Marinelli et al. In their clinical phase I/II study with MMC 30 mg m^{-2} administered as a bolus in the isolated circuit, two of nine patients had a complete remission, with a median survival of 17 months. Four patients developed VOD of the liver, and as a result one patient died [102]. The same group showed that in IHP experiments in rats the melphalan MTD of 12 mg kg^{-1} was even more effective than MMC and did not cause hepatotoxic side effects. In the following phase I/II dose-findings study with melphalan in IHP in patients with colorectal metastases confined to the liver, the MTD in humans was approximately 3.0 mg kg^{-1}. As in the rats, systemic toxicity was dose-limiting. The median survival of the whole group was 19 months, which is comparable to results after IHP with MMC. However, when patients were treated with higher doses of at least 1.8 mg kg^{-1} (dose median), median survival after IHP was 30 months [102-104]. Their results indicate that median survival after one IHP treatment is at least comparable to the results obtained from the most effective (multiple treatments) HAI schedules. After these promising results they started a phase II study of melphalan in IHP with a fixed dose of 200 mg melphalan, which is still ongoing [102, 105].

Since the addition of TNF to the isolated circuit in ILP with melphalan has resulted in a dramatic increase in response rate, trials were started to investigate the possible application of TNF in IHP. TNF without melphalan or any other chemotherapeutic agent does not appear to have any significant antitumor activity when administered in IHP. Fraker et al demonstrated a response rate of only 20% in 17 patients treated in a study with IFNγ and escalating doses of TNF administered in IHP [40]. Similar results have been demonstrated in ILP with TNF only. Therefore, as was concluded from the ILP experiments in rats, TNF needs a chemotherapeutic agent to in order to be effective. Alexander et al treated 22 patients with unresectable hepatic metastases of ocular melanoma with IHP: in

11 patients melphalan was added, in the other 11 melphalan and TNF. There was one treatment related mortality. Overall response rate was 62 % (2 CR, 11 PR). More interesting, overall median duration of response was 9 months (range, 5 – 50) and was significantly longer in those treated with TNF (14 vs. 6 months, respectively). Overall median survival was 11 months [106]. Therefore, TNF appears to significantly prolong the duration of response at least in ocular melanoma metastases. Their experience has been extended with other tumor types. More recently, the same group presented the results of IHP with 1 mg TNF and 1.5 mg kg^{-1} melphalan in 50 patients with unresectable hepatic metastases (37 colorectal, 8 ocular melanoma, 5 other). The overall response rate in the 48 surviving patients was 75 % (2 % CR, 73 % PR) and the median time to recurrence was 6 months with a range of 2 to 50 months [107]. Lindner et al reported results of 11 patients treated by IHP with 0.5 mg kg-1 melphalan and 30 to 200 µg TNF. Six patients underwent re-operation due to post-operative bleeding. Two patients died of coagulopathy or multiple organ failure within the first post-operative month. Three of six patients with hepatic metastases from malignant melanoma or leiomyosarcoma showed a partial response whereas none of the patients with liver metastases from colorectal origin showed any response. The mean survival time in their study was 20 months [108]. In Germany, the group of Oldhafer treated 12 patients by IHP, of whom 6 received MMC alone and 6 the combination TNF (200 to 300 µg) with melphalan (80 to 140 mg). Although there were 3 deaths after treatment, there were no deaths in the patients treated with TNF and melphalan. Of the 10 evaluable patients, four patients showed a partial response, three no change and the others progression. We used the combination of 1 mg kg^{-1} melphalan with 400 µg (n=8) or 800 µg (n=1) TNF in patients with irresectable colorectal metastases confined to the liver. Of the three post-operative deaths, there was probably one TNF related (uncontrollable coagulopathy). Five of the evaluable patients demonstrated a PR and in one patient the IHP had no effect on the tumor load [109].
Therefore, concluding from clinical experience, a total of 68 patients with irresectable metastases confined to the liver have been treated by IHP with TNF and melphalan. There were 2 CR (2%) and 45 PR (66%) with an overall response rate of 69 %. The duration of response varied widely from 2 to 50 months.

Toxicity

From our IHP experiments in rats we learned that the MTD of TNF used in the

perfusate is rat strain dependent: the Brown-Norway (BN) rats tolerated higher TNF concentrations than the WAG/Rij rats used in the CC531 experiments. Therefore, it could be speculated that the MTD in the WAG/Rij is too low in order to be effective. A possibility to further increase the MTD for TNF in our CC531 model could be the application of TNF mutants with better chemotherapeutic indices. Nakamoto et al performed IHP with the TNF mutant TNF-SAM2 in a in a hepatic metastases (syngeneic coloncarcinoma cell line) rat model. Their group was the first to develop this TNF mutant, the biological activities of which have been shown to be more beneficial for antitumor therapy than those of TNF. The chemotherapeutic index of TNF-SAM2 was at least 5 times higher than that of TNF. In addition, the detrimental toxic side effects were milder than TNF [110, 111]. Despite these results, they also described a rat strain specific sensitivity to TNF-SAM2 added in the perfusate as demonstrated by us for TNF. However, in the sensitive rats, they demonstrated that dexametasone (4 mg) administered subcutaneously or low molecular weight lipopolysaccharide (LPS) (50 mg/rat) administered intradermally could increase the tolerable dose of TNF significantly to concentrations by which antitumor effects have been observed in ILP [112].

Apart from strain dependent factors, it has been demonstrated that the intraportal application of rhTNF results in a significantly higher morbidity and mortality rate that after intravenous application of this cytokine. This could be shown by Kahky et al who found 100 % mortality in rats after intraportal perfusion of rhTNF 100 $\mu g\ kg^{-1}$ BW compared to no mortality after infusion of the same dose of rhTNF into the caval vein [84] Consistently, Tracey et al described that a dosage of 3600 $\mu g\ kg^{-1}$ BW resulted in 100 % mortality after i.v. application of rhTNF in rats and a mean lethal dosage of 700 $\mu g\ kg^{-1}$ BW rhTNF when given as a 5 min bolus via the tail vein [113]. The data from these studies suggest that intraportal application of TNF leads to a much stronger activation of macrophages and Kupffer cells followed by cytokine release (IL-1, IL-6 and TNF) which act synergistically to TNF (this thesis, chapter 4). We used PV and/or HA as a mode of inflow in IHP in the rats but could not demonstrate a difference in morbidity or mortality dependent on route of inflow, besides the rat strain dependent sensitivity (this thesis, chapter 7) [59]. Concluding, TNF can be used in IHP in rats and the TNF based toxicity is rat strain dependent. Furthermore, the route of infusion might be of importance. With respect to TNF induced toxicity, the application of TNF mutants, like TNF-SAM2, or treatment with dexamethasone or LPS can reduce

symptoms.
In virtually all patients, temporary elevated hepatic enzymes have been demonstrated after IHP. These elevations seem more procedure-related than drug-related and normalize within 2 weeks. In our group we could not demonstrate a significant difference between hepatic enzyme level patterns of patients treated with melphalan and patient treated with melphalan and TNF [114].
When administered systemically, TNF leads to severe side effects mimicking a septic shock like syndrome with severe hypotension, central nervous system dysfunction, as well as thromboembolic and cardiopulmonary phenomena. Despite the isolation of the liver, more toxicity than expected has been described after IHP with the combination of TNF and melphalan. Linder et al described considerable toxicity and performed a reoperation in six patients for bleeding and there were two deaths [108]. Most likely the encountered toxicity was attributable to systemic exposure to TNF and melphalan. Three of our patients died after IHP, two of surgical complications, one probably TNF related [114]. Oldhafer et al demonstrated severe hypotension and capillary leak in one patient [109]. Lans et al showed that with complete vascular isolation and virtually all other variables equal, the production of secondary mediators in the liver after IHP with TNF and melphalan may result in subsequent transient haemodynamic alterations not observed with melphalan alone [115]. Therefore, most of these side effects can be minimized by a complete isolation and a thorough washout with colloidal fluids in order to keep systemic TNF levels during and after IHP as low as possible.
Because of the toxicity of TNF a total isolation of the liver is of utmost importance in order to minimize systemic toxicity. Since the liver is not an inert organ like the limbs, hepatic toxicity will be dose-limiting. Indeed Fraker et al demonstrated that the MTD of TNF in IHP is 1.5 mg, which is far less than the dose used in ILP, again stressing that local (hepatic) instead of systemic toxicity is dose limiting [40]. However, this does not implicate that the dose used in IHP is thus less effective since De Wilt has demonstrated that lower doses of TNF in ILP can be as affective as the high doses normally used [57].
The systemic toxicity of TNF is related to the systemic concentrations during and after IHP. As stated, with a meticulous technique, systemic levels during IHP can be nihil [79, 116]. On the other hand, as has been described in chapter 5 even if leakage occurs, the effects might be limited. We demonstrated that in complicated ILP, high leakage of TNF to the systemic circulation led to a ten- to more than

hundredfold increased levels of TNF, IL-6 and IL-8 in comparison to patients without leakage. The increase of the APPs was limited. Even when high leakage occurs, this procedure should not lead to fatal complications. The most prominent clinical toxicity was hypotension (grade III in 4 patients) which was easily corrected and no pulmonary or renal toxicity was observed in any patient. It is our experience that even in the rare event of significant leakage during a TNF-based ILP postoperative toxicity is usually mild and can be easily managed by use of fluid and in some case vasopressors [117].

After IHP, all studies describe a brief TNF peak almost immediately after the restoration of the hepatic vessels. Most probably, the peak consists of remnant TNF in the liver after washout that flushes out of the liver after release of clamps. However, this peak varies between groups. In order to minimize the peak concentration a thorough washout procedure has to be accomplished. Furthermore, as has been demonstrated by Lang et al, with the use of colloids, toxicity will be further minimized [92].

In chapter 4 we describe elevated levels of IL-6 and IL-8 after IHP in all patients. Significantly higher IL-6 and IL-8 levels were shown in those patients with TNF added to the melphalan in the perfusate [116]. In contrast, we could not demonstrate significant differences in acute phase response (APR) in those patients receiving melphalan and TNF compared with those receiving melphalan only. Presumably, the IHP procedure itself already causes a maximal stimulation of APR. Similar, Lans et al demonstrated that the addition of TNF to melphalan in IHP resulted in significant higher systemic levels of both cytokines after washout with associated changes in mean arterial blood pressure. Contrasting with our results they also demonstrated greater regional toxicity, as reflected in higher levels of serum bilirubin levels. However, these measurable differences were transient and did not lead appear to be major clinical consequence [115].

Similarly to elevated IL-6 and IL-8 levels, we also demonstrated a significant difference in soluble TNF receptor (sTNFR) level with higher levels in those patients treated with melphalan and TNF (chapter 6). These receptors do not only exist as cell surface membrane proteins but also as soluble proteins. Evidence indicates that these soluble TNF receptors (sTNFRs) are derived by proteolytic cleavage from the extracellular domain of the corresponding cell surface from a variety of cells, mostly neutrophils, activated T-cells and monocytes. The formation of sTNFRs *in vitro* is triggered by certain immune-stimulating agents and is enhanced by a number of cytokines, including TNF itself [118, 119]. The

exact function of sTNFRs is not known yet. The loss of cell surface TNF receptors may lead to a temporary decrease in sensitivity of the cell to TNF. Furthermore, by binding to the TNF molecule, soluble receptors could antagonize TNF function. On the other hand, the same sTNFRs are proposed to augment TNF activity by the formation of so called 'slow-release' complexes, depending of the relative concentrations of sTNFRs and TNF [120]. TNF induced hypotension appears to occur when the capacity of released sTNFRs to neutralize circulating TNF levels is exceeded. Under conditions in which serum TNF levels are considerably greater than 1.5 ng mL^{-1} after ILP, refractory hypotension with associated hematological, hepatic and pulmonary toxicities can occur [121, 122].
In conclusion, IHP with TNF and melphalan leads to transient hepatotoxicity, as reflected by elevated hepatic enzyme levels. Moreover, most of this local toxicity seems to be procedure related. Nevertheless, since the liver is not an inert organ, local MTD of TNF has been demonstrated to be much lower than the MTD in ILP. Systemic toxicity is dependent of systemic drug levels during and after IHP and can be minimized by a meticulous isolation technique and a thorough washout preferably with colloids. Toxicity (clinical, secondary mediators) encountered in this way will be mild and transient, even when TNF is used.

Future directions

The main disadvantages with the IHP concept in its present form are the hepatotoxicity of the drug used (TNF), the magnitude of the procedure, and the non-repeatability of the procedure. In an attempt to further decrease the toxicity of the drug used, in particular TNF, less toxic TNF mutants have been developed, aiming at enhancement of cytotoxicity whilst reducing systemic side effects. Nakamoto et al described their experience with TNF-SAM2, a TNF mutant with a chemotherapeutic index at least 5 times higher than the index of TNF. The MTD of TNF-SAM2 administered systemically to a patient was at least twice that of TNF. In addition, the detrimental side effects such as hypotension were milder than with TNF [111]. In sarcoma bearing rats, De Wilt et al demonstrated that ILP with TNF-SAM2 has similar antitumor activity in combination with melphalan or doxorubicin as rhTNF [123]. In the event of unexpected massive leakage during ILP it is obvious that the reduced toxicity of TNF-SAM2 can make ILP a safer procedure. More important, this may lead to the application of TNF-SAM2 in IHP, as has been described by Nakamoto et al in rats [111].
Systemic tumor progression is the site of initial failure in the majority of patients

treated with IHP, which is a major drawback of being a locoregional treatment. Wu et al investigated the possibility that IHP may result in significant embolization of tumor cells as a potential cause of subsequent systemic tumor progression. However, using PCR with primers for tyrosinase or carcinoembryonic antigen with a sensitivity of detecting 1 –10 cells mL^{-1} of blood, they were unable to detect any circulating tumor cells systemically or in the perfusate samples in patients with ocular melanoma or colorectal cancer during or immediately after IHP [124].

Next to an upgrade of response rates (70 % in IHP with TNF and melphalan) is the prolongation of the duration of response. Bartlett et al reported the results of IHP with TNF and melphalan or IHP with melphalan only followed by HAI with FUDR and leucovorin in patients with unresectable colorectal hepatic metastases. Twenty-four of 31 patients (77%) had a PR after IHP alone and 14 of 19 (74%) after IHP followed by HAI. Interestingly, median duration of response was 8.5 months after IHP alone and 14.5 months after IHP and HAI. Median survival was 16 and 27 months, respectively. In their study, HAI appeared to prolong the duration of response after IHP, and therefore this combined strategy could be a manner to improve the present results of IHP [125].

Conclusions

In its present form, IHP is a technically demanding procedure and is not repeatable. One way to overcome these two problems could be the development of the balloon catheter technique in combination with IHP (IHHP). With this technique, the procedure is simplified and can be repeated. To apply IHHP, an inflow catheter is inserted into the proper hepatic artery via the right or left femoral artery whilst a single or double balloon catheter is inserted at the site of the femoral artery or vein respectively. Under X-ray the position of the double balloon catheter is fixed at the upper side just above the hepatic vein and at the lower side just above the renal vein and the position of the single balloon catheter in the aorta is above the upper side of the double balloon catheter. The balloons are inflated and the bloodstream is blocked. In this way, the lower part of the body under the single balloon enforces the hypoxic condition because the blood supply is stopped due to blockade of the aorta. Van IJken et al performed IHHP with TNF, melphalan and MMC in pigs. They 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 [126]. Because of the leakage free quality of this procedure in combination with the efficacy of the washout procedure, TNF may be used in this setting. After the promising results in pigs, our group started a phase I-II study on IHHP with melphalan in patients with irresectable hepatic metastases of colorectal origin [127]. In this study the technique has been further simplified by replacing the operative placement of the hepatic artery catheter by a percutaneously inserted and angiographically controlled positioned balloon infusion catheter in the common hepatic artery. Percutaneously insertable occlusion catheters for the aorta and caval vein are under development and would be the next step towards a fully percutaneously managed IHHP. In our first six patients we have had no serious adverse events. Moreover, antitumor activity has been demonstrated at the dose level of 1 mg kg^{-1} melphalan. With further experience with this technique, TNF may be introduced in order to further increase antitumor activity.

Table 1. Overview of literature concerning clinical IHP.

Study	Year	n	Drugs	Dose	art/port/ dual	Hyper-thermia	Duration	Mortality	Responses	
Ausman	1961	5	Nitrogen Mustard	0.2-0.4 mg/kg	?	no	?			
Aigner et al	1984	15	5-FU		?	yes	60 min	20%	unknown	median survival: 8 months
		19	5-FU (+ 5xHAI)	750-1250 mg		yes	60 min	0%	3 CR (16%), 15 PR+MR (79%)	median survival: 18 months
		15	5-FU (+ 5xHAI) MMC	750-1250 mg 15 - 50 mg		yes	60 min	0%	10 CR (26%), 4 PR+MR (26%)	idem
Skibba et al	1986	8	none	-	?	yes	60 min	2/8	5/6 response rate	
Schwemmle et al	1987	32	5-FU	300-1250 mg	?	yes	60 min		CR 41 %, PR 68%	median survival: 14 months
		14	MMC	5-50 mg		yes	60 min			
		4	Cisplatin	50 mg		yes	60 min			
Hafstrom et al	1994	29	melphalan cisplatin	0.5 mg/kg 0.2-0.7 mg/kg	dual	yes	60 min	14%	PR 20%	
Marinelli et al	1996	9	MMC	30 mg/m2	dual	no	60 min		1 CR, 1 PR (RR 28%)	
van de Velde et al	1996	24	melphalan	0.5-4 mg/kg	dual	yes	60 min	14%	1/17 CR, 4/17 PR (29%)	
de Vries et al	1997	9	TNF melphalan	0.4 mg, 0.8 mg (n=1) 1 mg/kg	dual	yes	60 min	33%	5 PR (%)	
Alexander et al	1998	34	TNF melphalan	1 mg 1 mg/kg	art	yes	60 min	3%	1 CR, 26 PR (75%)	
Oldhafer et al	1998	6	TNF melphalan	200-300 ug 60-140 mg	art	yes	60 min	0%	1 CR, 2 PR (50%)	
Lindner et al	1999	11	TNF Melphalan	30-40-50-100-200 ug 0.5 mg/kg	dual	39	60 min	18%	3 PR (33%)	6 reoperations (bleeding), 2 death caused by coagulopathy
Bartlett et al	2001	19	melphalan HAI with FUDR	0.2 mg/kg/day	art	yes	60 min monthly		14 PR (74%)	

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CHAPTER 9

Summary and Conclusions

Samenvatting en Conclusies

Summary

In *Chapter 1* a general introduction to this thesis is given. An overview is presented regarding the different treatment options (systemic and locoregional modalities) for patients with irresectable hepatic malignancy. An introduction to the isolated perfusion model in general and the isolated hepatic perfusion (IHP) in particular is described. A summary of the objectives of this thesis concludes this chapter.

Chapter 2 describes the results of experimental IHP in pigs. This study was undertaken to assess the feasibility of such an approach by analyzing hepatic and systemic toxicity of IHP with TNF with and without melphalan in pigs. Ten healthy pigs underwent IHP. After vascular isolation of the liver, inflow catheters were placed in the hepatic artery and the portal vein, and an outflow catheter was placed in the inferior vena cava. An extracorporeal veno-venous bypass was used to shunt blood from the lower body and intestines to the heart. The liver was perfused for 60 min with (1) 50 μg kg^{-1} TNF (n=5), (2) 50 μg kg^{-1} TNF plus 1 mg kg^{-1} melphalan (n=3) or no drugs (n=2). The liver was washed with macrodex before restoring vascular continuity. All but one pigs survived the procedure well. A stable perfusion was achieved in all animals with median perfusate TNF levels of 5.1 ± 0.78 x 10^6 pg mL^{-1} (± s.e.m.). Systemic leakage of TNF from the perfusate was consistently < 0.02%. Following IHP, a transient elevation of systemic TNF levels was observed in groups 1 and 2 with a median peak-level of 23 ± 3 x 10^3 pg mL^{-1} at 10 min after washout, which normalized within 6 h. No significant systemic toxicity was observed. Mild transient hepatic toxicity was seen to a similar extent in all animals, including controls. Therefore, IHP with TNF with(out) melphalan in pigs is technically feasible, results in minimal systemic drug exposure and causes minor transient disturbances of hepatic biochemistry and histology.

After the experimental hepatic perfusions with melphalan and TNF, nine patients with irresectable hepatic metastases of colorectal origin underwent this procedure. In *Chapter 3* the results of this phase I-II study are presented. The technique used was the same as described in *Chapter 2*. An extracorporeal veno-venous bypass was used to shunt blood from the lower body and intestines to the heart. IHP was performed with inflow catheters in the hepatic artery and portal vein and an outflow catheter in the caval vein. The liver was perfused for 60 min with 1 mg kg^{-1} melphalan plus 0.4 mg TNF (n = 8) or 0.8 mg TNF (n = 1) with hyperthermia

(> 41 °C). After the perfusion the liver was washed with macrodex before vascular continuity was restored. During the perfusion, one patient demonstrated leakage (cumulative leakage 20%) after which the perfusion was ended after 45 minutes. Three patients died in the perioperative period (one possibly drug related). All patients demonstrated significant but transient hepatotoxicity. Survival ranged from 6 to 26 months (median 10.3 months). All patients demonstrated a tumor response (5/6 partial response, 1/6 stable disease) with a median duration of 18 weeks. In contrast to our experimental program in pigs, many problems were encountered in the phase I study. By using both the hepatic artery and portal vein for IHP we encountered more toxicity than expected based on data from the pig program, resulting in fatal coagulative disturbances in one patient who received the second rhTNF dose. Furthermore, local control after one IHP with TNF and melphalan proved to be only temporary.

In *Chapter 4* the acute phase response during and after IHP with TNFα and melphalan is shown. In this study, we have evaluated hepatotoxicity, secondary cytokine production (interleukin-6, IL-6) and hepatic acute-phase response (APR) in nine patients, mentioned in *Chapter 3,* who underwent IHP with TNF and melphalan for irresectable colorectal liver metastases (IHP_{TM} group). Since we were interested in the effects of the addition of TNF to the perfusate, results were compared with those obtained from three patients who underwent IHP with melphalan only (ongoing Phase 2 study).
After the washout procedure, a TNF peak (169 ± 38 pg mL^{-1}) was demonstrated in the IHP_{TM} group only. Both groups demonstrated peak levels of IL-6 in perfusate as well as systemically. These were significantly higher in the IHP_{TM} group. Acute phase protein (APP) levels followed a similar pattern as has been demonstrated after major surgery, with no significant differences between both groups. The addition of TNF to the perfusate did not lead to a significant difference in APP levels as well as time course between groups. Therefor, IHP with TNF and melphalan is followed by a transient systemic peak of TNF directly after liver washout. Secondary IL-6 induction was seen in the present study after IHP with and without TNF, but IL-6 levels were highest when TNF was added. This phenomenon could not be extrapolated to APP induction, which appeared unaffected by the addition of TNF, presumably because the surgical procedure itself already causes maximal stimulation of APP production.

In *Chapter 5* patients with significant leakage of TNF from the perfusion circuit into the systemic compartment were investigated. Systemic toxicity in these patients was studied in detail after an ILP with leakage to the systemic circulation of 12-65%, and the clinical course of these patients was compared with patients with uncomplicated (no leakage) ILP. Additionally, cytokine and acute phase levels were measured in both groups. In four patients, grade III hypotension was the most prominent clinical toxicity which was corrected with fluid administration and if necessary dopamine. Hematological toxicity was mild and no renal or pulmonary toxicity was observed. TNF levels up to 277 ng mL^{-1} could be demonstrated in those patients with leakage. Also IL-6, IL-8 and maximum $sPLA_2$ levels were significantly higher in these patients. In contrast, CRP levels were lower, suggestive for a higher expenditure of CRP in the removal of injured cells. Concluding, ILP with high leakage of TNF to the systemic circulation is accompanied by manageable systemic toxicity and an elevated cytokine and acute phase protein response.

In *Chapter 6* the effects of the addition of TNF to the perfusate in patients (as described in *Chapter 4*) with irresectable hepatic colorectal metastases treated by IHP with melphalan, on the soluble TNFα receptor (sTNFR) p55 and p75 levels are investigated. After the wash-out procedure a TNFα peak was demonstrated in the IHP_{TM} group only. In patients with TNFα added to the perfusate, sTNFR-p55 levels were higher than sTNFR-p75 levels, whereas patients in the melphalan alone group demonstrated the reverse. Furthermore, sTNFR-p55 concentrations were significantly higher in the IHP_{TM} group and remained so during the following 2 weeks. In contrast, there were no significant differences in the sTNFR-p75 levels between groups.

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. Moreover in our pre-clinical ILP model we observed drastic alterations in tumor microvasculature integrity. These findings led to the hypothesis that TNF causes specific destruction of tumor endothelial cells and thereby induces an increased permeability of tumor vasculature. IHP with melphalan with or without TNF is currently performed in clinical trials in patients with hepatic metastases. However, whether TNF contributes to the therapeutic efficacy in IHP still remains unclear. In *Chapter 7*, we evaluated this efficacy in IHP in an *in vivo* hepatic metastases model in rats. We studied three different

tumors: colon carcinoma CC531, ROS-1 osteosarcoma and BN-175 soft tissue sarcoma, which exhibit different degrees of vascularisation. 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.

In *Chapter 8* the results of the presented studies are described in a general discussion and compared with the available literature.

Conclusions:

- Hyperthermic IHP with TNF and melphalan in pigs is technically feasible, results in minimal systemic leakage of drugs and causes mild transient hepatotoxicity.
- After IHP with TNF and melphalan in patients with irresectable hepatic metastases a temporary tumor response can be demonstrated.
- A transient peak of TNF levels follows IHP with TNF and melphalan in patients with irresectable hepatic metastases directly after liver wash-out.
- After IHP with melphalan with and without TNF, a secondary IL-6 induction was seen. Highest IL-6 levels were demonstrated when TNF was added to the perfusate.
- IHP with melphalan with and without TNF is followed by the induction of acute phase proteins. This acute phase response appears to be independent of the addition of TNF, presumably because the surgical procedure itself already causes maximal stimulation of acute phase protein production.

- During isolated limb perfusion with melphalan and TNF, high leakage of TNF to the systemic circulation results in a clear acute phase and cytokine response with manageable toxicity.
- In IHP with melphalan higher soluble TNF receptor p75 than p55 levels can be demonstrated, whereas in those patients with TNF added to the melphalan in the perfusate, a reverse pattern is seen.
- The addition of TNF to IHP with melphalan results in an increased intratumoral melphalan concentration which is dependent of the tumor vascularity.

Samenvatting

Hoofdstuk 1 vormt de algemene inleiding tot dit proefschrift. Er wordt een kort overzicht gegeven van de verschillende behandelingen voor patienten met niet verwijderbare kwaadaardige levertumoren (meestal uitzaaiingen van darmkanker) in het algemeen en de geisoleerde lever perfusie (isolated hepatic perfusion, IHP) in het bijzonder. Aangezien voor de meeste tumoren geldt dat hoe hoger de concentratie van het werkzame antitumor middel is, hoe sterker het antitumor effect zal zijn, is het van belang een zo hoog mogelijke dosis van het desbetreffende middel toe te dienen. Echter: hoe hoger de toegdiende dosis, hoe meer bijwerkingen de patient daarvan ondervindt. De oplossing van dit dilemma zou kunnen liggen in de IHP. De geisoleerde lever perfusie is een techniek die het mogelijk maakt dat alleen de lever (in het lichaam van de patient) wordt aangesloten aan een hart-long-machiene. De lever wordt dan van zuurstofrijk bloed voorzien via de hart-long machiene. Als men er zeker van is dat er geen lekkage vanuit de hart-long-machiene naar de patient optreedt, kan een zeer hoge dosis van het chemotherapeuticum worden toegediend aan het lever circuit, zonder dat daarbij de patient dus wordt blootgesteld aan het middel.
Tevens wordt een bondig overzicht gegeven van de geschiedenis van Tumor Necrosis Factor α (TNF) alsmede de klinische toepassing van TNF. Samenvattend blijkt het antitumor effect van TNF vooral te worden veroorzaakt door de specifieke destructie van tumorbloedvaten. De klinische toepasbaarheid van TNF wordt echter bemoeilijkt door de ernstige bijwerkingen die reeds optreden bij doseringen (indien toegdiend aan de bloedbaan) die te laag zijn om enig antitumor effect waar te nemen. Naast de effecten op de tumorbloedvaten speelt TNF ook een belangrijke rol bij allerlei systemische processen in het lichaam. Het hoofstuk besluit met een overzicht van de doelstellingen van dit proefschrift gegeven.
In *Hoofdstuk 2* worden de resultaten beschreven van experimentele IHP in het varken. Het doel van deze studie was het evalueren van de toepasbaarheid van deze techniek. Hierbij werd tevens gekeken naar algemene en lever specifieke bijwerkingen van het gebruik van TNF en melphalan. Tien gezonde varkens ondergingen IHP. Na het isoleren van alle vaten van en naar de lever werden zgn in-flow catheters geplaatst in de aanvoerende leverslagader (a. hepatica) en de aanvoerende ader van de ingewanden naar de lever (poortader, v. portae) en een out-flow catheter in de naar de lever gaande onderste holle ader. Deze catheters

werden vervolgens aangesloten op aan hart-long-machiene. Via een buiten het lichaam verlopend pompsysteem (zgn veno-veneuze bypass, VVB) werd het bloed afkomstig van de onderste lichaamshelft en de ingewanden, de lever omzeilend, naar het hart gepompt. Aldus werd de lever na het starten van de hartlongmachiene en de VVB als het ware buiten de lichaamscirculatie gehouden. Vervolgens werd de lever gedurende 60 minuten geperfundeerd (1) met 50 µg kg^{-1} TNF (n=5), (2) met 50 µg kg^{-1} TNF plus 1 mg kg^{-1} melphalan (n=3) of (3) zonder toevoegingen (n=2). Na de 60 minuten werd de lever gespoeld met macrodex (plasma), werden de catheters verwijderd, de bloedvaten hersteld en de lever aldus weer "toegevoegd" aan de lichaamscirculatie.
Met uitzondering van 1 varken overleefden alle varkens de operatie. Lekkage van TNF (en melphalan) naar het lichaam tijdens de IHP bedroeg < 0.02 %. Tien minuten na IHP vertoonden de gemeten TNF concentraties een piek waarde in de groepen (1) en (2), waarbij deze concentraties 6h na de IHP weer genormaliseerd waren. Geen van de varkens vertoonde systemische bijwerkingen. Wel werden bij alle varkens tijdelijke tekenen van geringe (reversibele) leverschade waargenomen. Concluderend is IHP in het varken technisch haalbaar, resulteerd in zeer geringe blootstelling aan de toegepaste middelen (in dit geval TNF en melphalan) en leidt tot tijdelijke en reversibele geringe leverschade
Na de experimentele IHP met TNF en melphalan in het varken werd gestart met een studie naar de toepasbaarheid van TNF in IHP met melphalan als behandeling van patienten met niet verwijderbare kwaadaardige tumoren (meestal uitzaaiingen van darmkanker) in de lever. In *Hoofdstuk 3* wordt op deze studie nader ingegaan. Negen patienten met niet verwijderbare uitzaaiingen van darmkanker in de lever werden behandeld met IHP met melphalan en TNF. De IHP techniek was dezelfde zoals beschreven in *Hoofdstuk 2*. De lever werd gedurende 60 min geperfundeerd met 1 mg kg^{-1} melphalan en 0.4 mg (n=8) of 0.8 mg (n=1) TNF waarbij het perfusaat werd verwarmd tot > 41 C. Na de IHP werd de lever, zoals beschreven, gespoeld met macrodex waarna de vascularisatie weer werd hersteld. Bij 1 patient werd de perfusie na 45 minuten voortijdig beeindigd in verband met oncontroleerbare lekkage (totaal 20%). Drie patienten overleden in de post-operatieve periode waarvan 1 patient mogelijk als gevolg van het toedienen van TNF. Alle patienten vertoonden tijdelijke maar significante tekenen van leverbeschadeging. De overleving van de patienten varieerde van 6 tot 26 maanden (mediaan 10.3 maanden). Bij alle evalueerbare patienten werd een tumor respons waargenomen: bij 5 patienten werden de tumoren kleiner (partiele

respons) en bij 1 patient vertoonde de levertumoren geen verdere groei (stabiele ziekte). De duur van deze tumor respons varieerde van 17.5 tot 32.5 weken (mediaan 18 weken). De bijwerkingen van de IHP met TNF en melphalan waren ernstiger dan verwacht. Mogelijk dat het gebruik van zowel de a hepatica alsook de v portae hierbij een rol heeft gespeeld. Daarnaast resulteert IHP met TNF en melphalan slechts in een tijdelijke tumor respons.

De acute fase reactie (APR) kan omschreven worden als een algemene, systemische reactie van het lichaam op trauma, ontstekingen, etc. Behalve koorts, kenmerkt deze APR zich door de productie van zogenaamde acute fase eiwitten (acute phase proteins, APPs) door de lever waardoor verhoogde concentraties van deze eiwitten zullen worden aangetroffen in het bloed. Meting van de spiegels van deze eiwitten, waaronder het C-reactieve proteine (CRP), maken het mogelijk de duur en omvang van de APR te beschrijven. De laatste jaren is bovendien duidelijk geworden dat cytokines (waaronder interleukin 6 (IL-6) maar ook TNF) een centrale rol spelen bij het op gang brengen van de APR. In *Hoofdstuk 4* wordt de APR beschreven na IHP met TNF en melphalan. Hierbij werd bij de patienten zoals beschreven in Hoofdstuk 3 de hepatotoxicititeit, de secundaire cytokine productie en de APR van de lever geevalueerd. Omdat TNF zelf ook een rol speelt bij de inductie van de APR werden de resultaten vergeleken met die zoals waargenomen bij 3 vergelijkbare patienten die behandeld werden met IHP met alleen melphalan (lopende studie). De techniek was zoals beschreven in Hoofdstuk 2 en 3. Na de spoelprocedure werd alleen in de met TNF en melphalan behandelde groep een piek in TNF concentraties waargenomen (gemiddeld 169 ± 38 pg mL^{-1}). In beide groepen kon een piek van IL-6 concentraties worden waargenomen maar deze maximale concentraties waren hoger in de groep waar TNF werd toegegvoegd. De APP concentraties vertoonden eenzelfde patroon gedurende onderzochte periode zoals ook gezien wordt na grote operaties, waarbij geen verschillen konden worden aangetoond tussen beide groepen. Concluderend werden in beide groepen verhoogde IL-6 concentraties (piekwaarden) aangetoond al waren deze hoger in de groep waar TNF werd toegevoegd aan melphalan. Het toevoegen van TNF had echter geen effect op de APP productie na IHP aangezien vergelijkbare waarden en een vergelijkbaar beloop werd gezien in beide groepen. Een verklaring hiervoor zou kunnen zijn dat de IHP, als chirurgische procedure, al tot een maximale stimulatie van de APR leidt.

De APR is ook het onderwerp van *Hoofdstuk 5*. Hier zijn patienten die een geisoleerde extremiteits perfusie (isolated limb perfusion, ILP) met TNF en melphalan ondergingen, gecompliceerd door significante lekkage van TNF naar de systemische circulatie, onderwerp van de studie. De lekkage percentages varieerden van 12 tot 65 %. De als gevolg hiervan opgetreden toxiciteit werd vergeleken met het klinisch beloop bij een groep patienten die een ongecompliceerde ILP ondergingen (dus zonder meetbare lekkage). Tevens werd het beloop van de concentraties van cytokines en acute phase eiwitten vergeleken. De meest prominente bijwerking was hypotensie (te lage bloeddruk), graad 3 bij 4 patienten. Deze hypotensie was echter vrij eenvoudig te corrigeren door extra vocht toe te dienen en zo nodig bloeddrukverhogende middelen (dopamine). Hematologische toxiciteit was mild en er waren geen nier of long gerelateerde bijwerkingen. Hoge TNF concentraties tot 277 ng mL^{-1} werden aangetoond in de patienten met lekkage. Ook de concentraties van de cytokines IL-6 en IL-8 waren in deze groep significant hoger dan in de patienten zonder lekkage. De maximale concentraties van het acute phase eiwit $sPLA_2$ waren eveneens hoger in de lekkage groep. De C-reactive protein (CRP), ook een acute phase eiwit, concentraties waren echter lager in de lekkage groep, waarschijnlijk wijzend op een hoger verbruik van CRP bij het opruimen van beschadigde cellen. Concluderend gaat ILP met TNF en melphalan, gecompliceerd door lekkage van TNF (en melphalan) naar de systemische circulatie, gepaard met behandelbare bijwerkingen en een verhoogde cytokine en acute fase respons.
De effecten van TNF vinden plaats nadat TNF bindt aan twee receptoren die zich in de celwand bevinden. Van beide receptoren komen ook zogenaamde oplosbare varianten voor (sTNFR-p55 en sTNFR-p75), bestaande uit een afgesplitst deel (dat deel van de receptor dat buiten de celwand uitsteekt) van de receptor. Als een cel door wat voor reden dan ook veel TNF receptoren afsplitsts zou deze dus ongevoeliger voor TNF kunnen worden. Daarnaast zijn de oplosbare receptoren in staat TNF te binden waarbij ze het TNF kunnen verhinderen zich aan een celgebonden receptor te binden. Aan de andere kant kunnen ze ook fungeren als een soort buffer waardoor het TNF molekuul beschermd vervoerd kan worden naar een ander deel van het lichaam om daar vervolgens werkzaam te kunnen zijn. Bovendien komen grote hoeveelheden oplosbare TNF receptoren voor bij verschillende, veelal chronische ziekten (rheumatoide arthritis, AIDS, TBC, etc). In *Hoofdstuk 6* worden de oplosbare TNF receptoren nader onderzocht in de groep patrienten die een IHP met TNF en melphalan ondergingen. De gegevens

werden weer vergeleken met de groep patienten waarbij alleen melphalan werd toegevoegd aan het perfusaat. Na de spoelprocedure werd zoals eerder beschreven een piek in TNF concentraties waargenomen in de IHPTM groep maar niet in de IHPM groep. Beide sTNFR concentraties waren verhoogd bij alle patienten maar er waren ook verschillen. Bij de groep met TNF toegevoegd aan de melphalan waren de p55 concentraties hoger dan de p75 terwijl juist het omgekeerde gezien werd in bij de IHPM groep. Daarnaast waren de p55 concentraties in de IHPTM groep hoger dan die in de IHPM groep waarbij de concentraties na 2 weken nog niet genormaliseerd waren. De p75 concentraties vertoonden echter geen significante verschillen tussen beide groepen.

Kort geleden hebben wij aangetoond dat toevoeging van TNF aan IHP met chemotherapie leidt tot een maximaal 6 voudige toename in intra-tumoral concentraties van het gebruikte chemotherapeuticum. Verder werden drastische veranderingen waargenomen in de tumor microvascularisatie in ons pre-klinische ILP model in de rat. Deze waarnemingen kunnen waarschijnlijk een verklaring vormen voor het antitumor effect van TNF in ILP. De vraag of deze mechanismen ook gelden in IHP met TNF en melphalan staat centraal in *Hoofdtsuk 7*. In dit hoofdstuk wordt experimentele IHP met TNF en melphalan in een *in vivo* levermetastasen model in de rat beschreven. Hierbij werden drie tumoren bestudeerd: CC531 colon carcinoom, ROS-1 osteosarcoom, en BN-175 weke delen sarcoom waarbij elk tumortype een specifieke mate van tumor vascularisatie vertoont. IHP werd verricht met melphalan waaraan al dan niet TNF werd toegevoegd. IHP met alleen melphalan resulteerde bij alle tumor modellen in een afgenomen groeisnelheid van de tumoren. Echter in het BN-175 tumor model resulteerde de toevoeging van TNF tot een zeer sterk synergistisch effect. Bij de meerderheid van de ratten met het BN-175 levermetastasen model leidde dit to een complete respons (geen tumor meer aantoonbaar). *In vitro* cytotoxiciteits studies (tumor cellen worden hierbij blootgesteld aan verschillende concentraties chemotherapeuticum) lieten geen gevoeligheid (CC531 en ROS-1) of alleen lichte gevoeligheid (BN-175) voor TNF zien, zodat een directe interactie van TNF met tumor cellen onwaarschijnlijk is. De mate van respons in de BN-175 groep indien TNF werd toegevoegd aan melphalan correleerde met de concentratie van melphalan in het tumor weefsel, aangezien alleen in deze groep ratten een 5-voudige toename van de melphalan concentratie in het tumor weefsel werd aangetoond. Daarnaast toonde immunohistochemische analyse van de microvasculaire dichtheid (MVD), oftewel de hoeveelheid bloedvaten in de

tumor, aan dat de MDV in de BN-175 tumoren significant hoger was in vergelijking met ROS-1 en CC531. Met andere woorden: de BN-175 tumoren zijn dus het vaatrijkste van de drie onderzochte tumoren. De resultaten van deze studie tonen een directe relatie tussen de mate van vascularisatie van een tumor en de door TNF gemedieerde effecten aan. Het bepalen van de mate van vascularisatie van een tumor kan een manier zijn om de effectiviteit van de toevoeging van TNF aan een IHP met chemotherapeutica te voorspellen.
Hoofdstuk 8 vormt de discussie van dit proefschrift waarin tevens een overzicht wordt gegeven van literatuur aangaande IHP.

Conclusies

- Hypertherme IHP met TNF en melphalan in het varken is een technisch mogelijke ingreep en leidt tot minimale systemische lekkage en geringe, tijdelijke leverbeschadeging.
- Hypertherme IHP met TNF en melphalan leidt bij patienten met niet verwijderbare uitzaaiingen in de lever tot een tijdelijke tumor respons.
- Na hypertherme IHP met TNF en melphalan bij patienten met niet verwijderbare uitzaaiingen in de lever wordt een tijdelijke TNF concentratie piek waargenomen vlak na de spoel procedure van de lever.
- Na IHP met TNF en/of melphalan werd een tijdelijke IL-6 concentratie piek waargenomen. Hierbij werden de hoogste concentraties gezien indien TNF was toegevoegd aan het perfusaat.
- IHP met TNF en/of melphalan leidt tot de productie van acute fase eiwitten. Deze acute fase reactie lijkt onafhankelijk te zijn van het toevoegen van TNF. Mogelijk dat de IHP als chirurgische procedure al tot een maximale stimulatie leidt.
- ILP met TNF en melphalan, gecompliceerd door hoge lekkage van TNF, leidt tot een evidente acute fase en cytokine respons met behandelbare bijwerkingen;
- Na IHP met melphalan leidt tot hogere sTNFR-p75 concentraties dan sTNF-p55 concentraties, terwijl na toevoeging van TNF aan het perfusaat een omgekeerd patroon wordt gezien.
- Het toevoegen van TNF aan melphalan tijdens IHP resulteert in een toegenomen intratumorale melphalan concentratie en is afhankelijk van de mate van vascularisatie van de tumor.

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Dankwoord

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Curriculum Vitae

Mark Rem de Vries werd geboren op 13 maart 1968 te Maastricht. In 1986 behaalde hij het atheneum-B diploma aan de Albert Schweitzer Scholengemeenschap te Geleen. Hetzelfde jaar begon hij aan de studie geneeskunde aan de Erasmus Universiteit te Rotterdam. In 1994 behaalde hij het artsexamen. In datzelfde jaar werkte hij als arts-onderzoeker aan de afdeling chirurgische oncologie van de Dr. Daniel den Hoed Kliniek te Rotterdam alwaar de basis werd gelegd voor dit proefschrift (onder leiding van prof.dr. A.M.M. Eggermont). De opleiding tot algemeen chirurg werd in april 1995 begonnen in het Sint Franciscus Gasthuis te Rotterdam (opleider: dr. J.C.J. Wereldsma). In april 1998 werd de opleiding vervolgd in het Academisch Ziekenhuis Dijkzigt te Rotterdam (opleider: prof.dr. H.A. Bruining/prof.dr. H.J. Bonjer). Van januari 1999 tot januari 2000 onderbrak hij de opleiding voor een jaar onderzoek in het kader van een KWF/NKB arts-assistenten beurs. Op 1 april 2002 voltooide hij de opleiding tot chirurg waarna hij begon met de chirurgische vervolgopleiding Traumatologie aan de afdeling Traumatologie van het Erasmus Medisch Centrum Rotterdam (opleider: prof.dr. A.B. van Vugt). Vanaf 1 april 2003 werkt hij als chirurg in de Reinier de Graaf Groep te Delft, een plek waar hij nog vele jaren hoopt te blijven.