ANNALS OF SURGERY
Vol. 228, No. 6, 763–770

Isolated Hypoxic Hepatic Perfusion With Tumor Necrosis Factor-Alpha, Melphalan, and Mitomycin C Using Balloon Catheter Techniques

A Pharmacokinetic Study in Pigs

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Objective

To validate the methodology of isolated hypoxic hepatic perfusion (IHHP) using balloon catheter techniques and to gain insight into the distribution of tumor necrosis factor-alpha (TNF), melphalan, and mitomycin C (MMC) through the regional and systemic blood compartments when applying these techniques.

Summary Background Data

There is no standard treatment for unresectable liver tumors. Clinical results of isolated limb perfusion with high-dose TNF and melphalan for the treatment of melanoma and sarcoma have been promising, and attempts have been made to extrapolate this success to the isolated liver perfusion setting. The magnitude and toxicity of the surgical procedure, however, have limited clinical applicability.

Methods

Pigs underwent IHHP with TNF, melphalan, and MMC using balloon catheters or served as controls, receiving equivalent dosages of these agents intravenously. After a 20-minute perfusion, a washout procedure was performed for 10 minutes, after which isolation was terminated. Throughout the procedure and afterward, blood samples were obtained from the hepatic and systemic blood compartments and concentrations of perfused agents were determined.

Results

During perfusion, locoregional plasma drug concentrations were 20- to 40-fold higher than systemic concentrations. Compared with systemic concentrations after intravenous administration, regional concentrations during IHHP were up to 10-fold higher. Regional MMC and melphalan levels steadily declined during perfusion, indicating rapid uptake by the liver tissue; minimal systemic concentrations indicated virtually no leakage to the systemic blood compartment. During isolation, concentrations of TNF in the perfusate declined only slightly, indicating limited uptake by the liver tissue; no leakage of TNF to the systemic circulation was observed. After termination of isolation, systemic TNF levels showed only a minor transient elevation, indicating that the washout procedure at the end of the perfusions was fully effective.

Conclusions

Complete isolation of the hepatic vascular bed can be accomplished when performing IHHP using this balloon catheter technique. Thus, as in extremities, an ideal leakage-free perfusion of the liver can now be performed, and repeated, without major surgery. The effective washout allows the addition of TNF in this setting.

Presented at the fifth meeting of the European Surgical Association, Milan, April 24–25, 1998.
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Accepted for publication July 13, 1998.

The importance of developing new treatment modalities for primary and secondary liver tumors is evident. Hepatocellular carcinoma is one of the most common malignant tumors in the world today; it develops in approximately 1 million persons each year.[1] Recent advances in early detection have improved the prognosis.[2] In general, however,

tumors are not resectable at the time of diagnosis, and the prognosis of hepatocellular carcinoma remains poor.[3] The liver is a major site of metastatic spread of primary colorectal cancer, and it is the sole site of initial tumor recurrence in as many as 30% of patients.[4] Patients with resectable liver metastases can be cured by surgery, with reported 5-year survival figures ranging from 16% to 45%.[5–7] However, in 75% of patients with colorectal cancer metastases confined to the liver, these metastases are considered unresectable. Because the main determinant for duration of survival in these numerous patients is local tumor progression, new treatment modalities and schedules must be aimed at achieving local tumor control.

For most chemotherapeutic agents, steep dose-response curves can be demonstrated. Therefore, high drug concentrations are important for eradicating both sensitive and resistant tumor cells.[4,8] Systemic chemotherapy results in relatively low response rates both for colorectal liver metastases and primary liver tumors and has minimal effects on survival.[9]

The main principle of isolated regional chemotherapy is to achieve higher regional drug concentrations and thus higher exposure of tumor tissue to the agents, resulting in increased response rates, while shielding the organism from the systemic toxicity because of the much lower concentrations in the systemic circulation. These principles are even more important for the potential application of tumor necrosis factor-alpha (TNF). The toxicity of this cytokine has made systemic application of adequate doses with antitumor effect impossible.[10,11] However, in a leakage-free isolated perfusion setting, it can be safely used with remarkable results. Response rates of >80% have been observed in the treatment of unresectable extremity soft-tissue sarcomas using isolated limb perfusion with TNF plus melphalan.[12,13]

In the wake of these promising clinical results, efforts have been made to translate this success to other regional perfusion settings. The liver is one of the most obvious targets, and successful isolated perfusions of the liver with cytostatic drugs, both in animal models and in humans, have been reported.[14–16] The applicability of TNF in the setting of leakage-free isolated liver perfusion has been reported by teams from the National Cancer Institute and by us.[17–19] Major drawbacks of clinical application, however, are the magnitude and associated toxicity of the surgical procedure and the complexity and costs of using a venous bypass system as well as a heart-lung machine operated by a team of perfusionists.[19] For this concept to become practical in a clinical setting, less invasive alternatives must be developed. Thus, if isolated liver perfusion is to become a treatment option applicable on a large scale, the extent, complications, and costs of the intervention must be acceptable, and the procedure should be repeatable and yield good response rates. Several groups have reported on regional arterial infusion of chemotherapeutic drugs in combination with complete or incomplete hepatic venous isolation; however, these procedures were combined with hemofiltration systems.[20–22]

New balloon catheter techniques allow relative vascular isolation of the abdomen and pelvis with only minimally invasive surgery.[23–25] Several authors have published articles on these methods and have used aortic hypoxic perfusion chemotherapy for unresectable tumors confined to the pelvis or abdomen.[26–28] Hypoxia causes dividing cells to halt their progression through the cell cycle by allowing them to progress to and then remain in the G_1-like susceptibility state.[29] Drugs whose cytotoxic action is particularly potentiated by hypoxia, such as mitomycin C (MMC) and melphalan, have been reported to be particularly effective when administered by aortic stop-flow infusion.[26,27,30]

With the use of a caval double-balloon catheter for complete venous isolation of the liver, in combination with an aortic occlusion catheter and a port catheter placed in the gastroduodenal artery, it has become a relatively simple procedure to perform an isolated liver perfusion. We tested the feasibility and quality of this approach and report here on this methodology and its validation by studying the distribution of TNF, melphalan, and MMC through the regional and systemic blood compartments in pigs.

MATERIALS AND METHODS

Animals

Dutch Yorkshire pigs weighing 30 to 40 kg were used. Animals were treated in compliance with the guidelines on animal welfare of the Erasmus University, Rotterdam. The study protocol was approved by the Ethics Committee on Experimental Studies in Animals of the Erasmus University, Rotterdam.

Drugs

Recombinant human (4.9 to 5.8 $\times$ 10^7 units/mg) was provided as a kind gift by Boehringer Ingelheim GmbH, Ingelheim/Rhein, Germany. Melphalan (L-pam, Alkeran, Wellcome Ltd., London, UK) was obtained as a sterile powder (100 mg) that was dissolved aseptically using solvent and diluent provided by Burroughs Wellcome (London, UK). MMC (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) was obtained as a sterile powder (40 mg) that was dissolved aseptically in 40 ml saline 0.9%.

Perfusion Set

Perfusion sets were kindly provided by PfM GmbH (Cologne, Germany) and consisted of a double-balloon catheter (12F, balloon capacity 25 ml, distance between balloons 4 cm) for venous isolation of the liver, an aortic occlusion balloon catheter (12F, balloon capacity 25 ml) for compensating the decrease in cardiac preload during the procedure, and a tubing set with a volume of 220 ml containing a

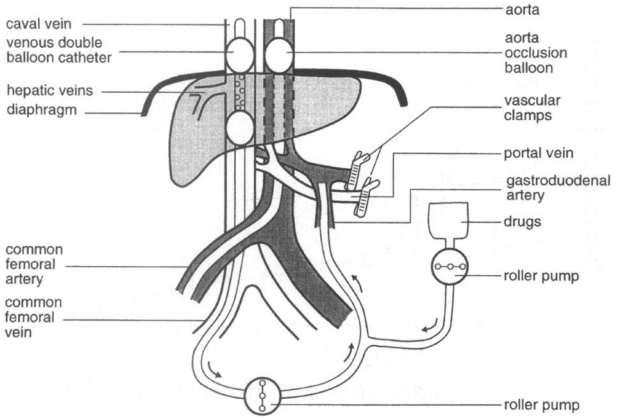

Figure 1. An isolated hypoxic hepatic perfusion with balloon catheter techniques.

bubble trap. In the perfusion circuit, flow was maintained by a roller pump, and the temperature of the perfusate was regulated by a heat exchanger (Cardioplegia heat exchanger, CSC14 Sorin, Biomedica, Italy). Drugs were infused through a sideline into the perfusion circuit.

Surgical Procedure

Figure 1 illustrates the methodology of an isolated hypoxic hepatic perfusion (IHHP) with balloon catheters. It shows the position of the occlusion catheters, the inflow catheter in the gastroduodenal artery, and the other components of the perfusion circuit. The IHHP procedure was carried out as follows. General anesthesia was induced in pigs by standard methods and maintained with pancuronium and fentanyl. An arterial line was introduced into a carotid artery and a double-lumen central venous catheter was placed in an external jugular vein on either side. After a midline laparotomy was performed, the gastroduodenal or common hepatic artery was cannulated with the arterial infusion catheter (7F). Pigs were subsequently heparinized with 2 mg/kg heparin. After formal surgical exposure of the femoral vessels, the aortic occlusion catheter was introduced into the common femoral artery. Subsequently the double-balloon catheter was introduced into the common femoral vein and retrogradely moved up into the caval vein, positioning the balloons above and below the hepatic veins. To compensate for the decrease in cardiac preload, the aorta was clamped or the aortic occlusion balloon was inflated. Caval balloons were then inflated and temporary isolation of the hepatic vascular bed was obtained by clamping the hepatic artery and subsequently the portal vein. After connecting the double-balloon catheter and arterial cannula to the perfusion system primed with 220 ml Haemaccel (Behring Pharma, Amsterdam, The Netherlands), the perfusate was circulated by means of a roller pump with a constant flow of 200 ml/minute. The temperature of the perfusate was kept at 38.5°C by means of a heat exchanger. Stable perfusion was assessed by monitoring the blood level in the bubble trap.

Drugs were rapidly administered as a bolus infusion into the perfusate. After 20 minutes of perfusion, the remaining agents were washed out of the hepatic vascular bed by perfusing the isolated circuit with Haemaccel for 10 minutes and collecting the venous effluent. Isolation was then terminated by releasing the temporary clamps on the portal vein and the hepatic artery, and the caval balloons and the aortic balloon were deflated. The total time of clamping the portal vein did not exceed 35 to 40 minutes.

Control animals were given an equivalent amount of drugs as a bolus injection through a central venous line placed in the external jugular vein. Samples were obtained from a carotid arterial line.

Blood Sampling

Baseline blood samples were obtained before administration of the drugs into the regional circuit. Throughout and after the procedure, blood samples were simultaneously drawn from the hepatic blood compartment through a sampling port on the caval double-balloon catheter and from the systemic blood compartment through the central venous catheter at 5, 10, 15, 20 (before washout started), 30 (before termination of isolation), 35, 55, and 85 minutes after infusion of the agents. Blood samples of animals that received the drugs as an intravenous bolus infusion were drawn at 5, 10, 15, 20, 25, 35, 55, 85, 150, and 180 minutes after administration. Blood samples were collected in glass tubes containing EDTA and immediately stored in the dark on ice. Samples were centrifuged at 2600 r.p.m. for 6 minutes at 4°C, and the obtained plasma was stored at −70°C until analysis.

Treatment Schedule

Five pigs underwent IHHP and five pigs received the drugs as an intravenous bolus infusion. Of the group that underwent IHHP, two pigs were given MMC (0.25 mg/kg) and melphalan (0.25 mg/kg) alone, and three pigs were given the same amount of MMC and melphalan in combination with TNF (0.02 mg/kg). Of the pigs that received an intravenous bolus, two were given MMC (0.25 mg/kg) and melphalan (0.25 mg/kg) alone, and three were given the same amount of MMC and melphalan in combination with TNF (0.02 mg/kg).

Bioanalysis

Plasma TNF concentrations were determined using ELISA for rhTNF as described by Engelberts et al.[31] In short, a 96-well Immuno-Maxisorp plate was coated with murine antihuman TNF mAb 61E71. A standard titration curve was obtained by making serial dilutions of a known sample of human rhTNF in normal porcine serum. The

plates were incubated with a polyclonal rabbit antihuman TNF antiserum, followed by addition of an enzyme-labeled antirabbit reagent and enzyme reaction. The detection limit for human TNF was 20 pg/ml.

Melphalan in plasma was measured by gas chromatography/mass spectrometry. 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 derivatives were separated on a methyl phenyl siloxane GC capillary column and measured selectively by single ion monitoring mass spectrometry in the positive EI mode. The detection limit for melphalan is 10 pg/ml. Details of this assay were described by us.[32]

Concentrations of MMC were analyzed by high-performance liquid chromatography with ultraviolet detection. Porfiromycin was used as an internal standard. Samples were extracted over XAD-2 columns. After elution with methanol and evaporation, the dry residue was diluted in the mobile phase, which consisted of methanol and water (40‰). The sample was injected and separated over a C18 column and detected at 362 nm. The detection limit for MMC is 10 pg/ml. Details of the assay are described elsewhere.[33]

Calculation of Area Under the Curve

Areas under the plasma concentration *versus* time curve (AUCs) were calculated applying the trapezoid rule using GraphPad Prism software. AUCs were calculated from 5 to 20 minutes after the start of perfusion or from 5 to 20 minutes after administration of the intravenous bolus.

RESULTS

All pigs survived the procedures. Data were obtained from five subsequent procedures. Apart from transient tachycardia in a minority of pigs, stable hemodynamics were observed throughout the procedures. After deflating the balloons, a short period of hypotension was observed, easily managed by fluid challenge. The addition of TNF to MMC and melphalan did not alter the plasma concentration *versus* time profile of MMC and melphalan during and after the procedure (data not shown). Therefore, data from pigs perfused with melphalan and MMC alone and from pigs perfused with melphalan and MMC plus TNF were pooled.

Plasma Concentrations

Figure 2 shows the mean systemic plasma concentrations of MMC, melphalan, and TNF after administration of these agents as an intravenous bolus. A typical one-phase exponential decay curve was observed for TNF and MMC with an elimination half-life of 31 minutes for TNF and 7.2 minutes for MMC. Two-phase exponential decay was observed for melphalan with a λ_1 of 2.8 and a λ_2 of 161 minutes.

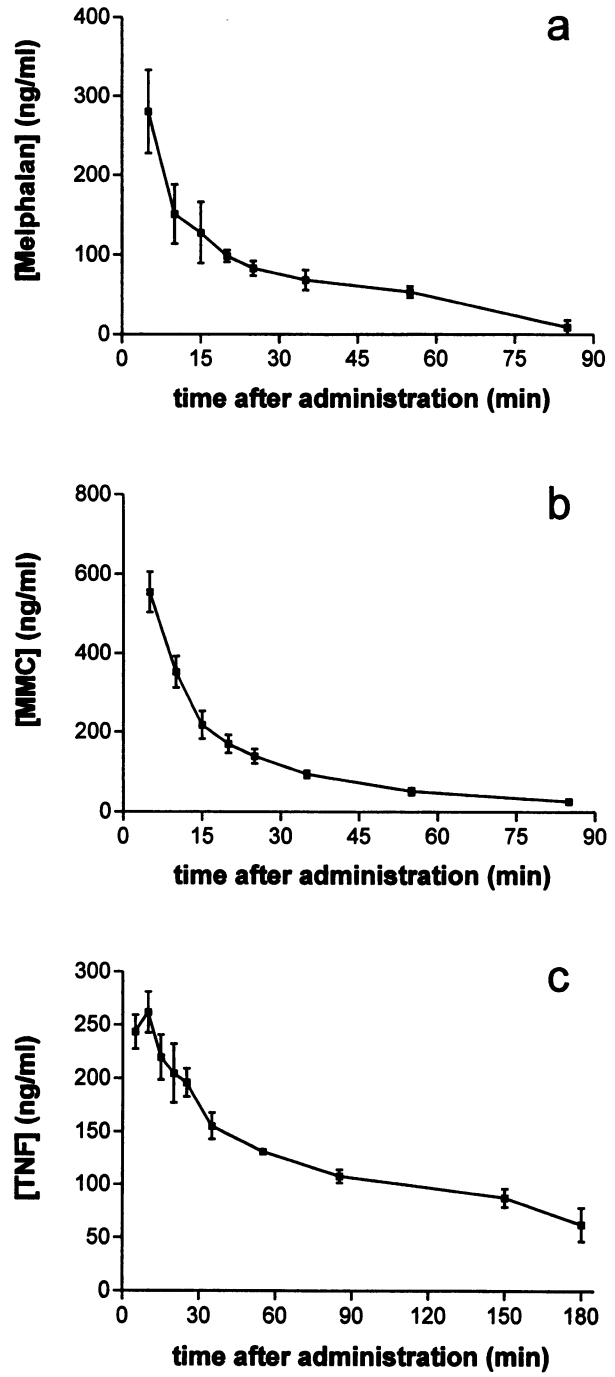

Figure 2. Plasma concentration curves after intravenous bolus injections of melphalan (0.25 mg/kg) (A), mitomycin C (0.25 mg/kg) (B), and tumor necrosis factor-alpha (0.02 mg/kg) (C). For mitomycin C and melphalan, mean plasma concentrations of five pigs ± SEM are shown. For tumor necrosis factor-alpha, mean plasma concentrations of three pigs ± SEM are shown.

During perfusion, concentrations of MMC and melphalan were markedly greater in the isolated hepatic vascular compartment than in the systemic compartment (Fig. 3). After 5 minutes of perfusion, mean MMC peak levels were 38-fold

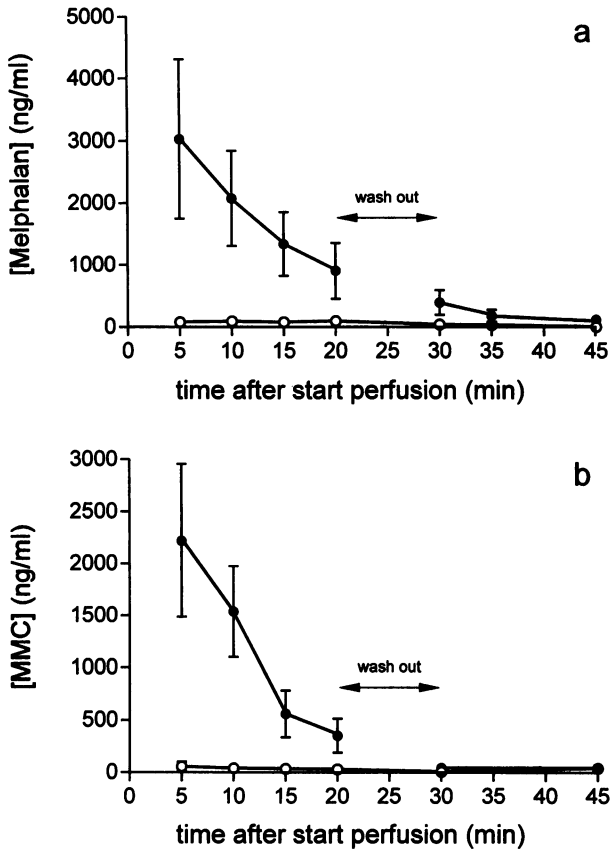

Figure 3. Regional (black circles) and systemic (white circles) plasma concentration curves throughout and after 20 minutes of isolated hypoxic hepatic perfusion with melphalan (0.25 mg/kg) (A) and mitomycin C (0.25 mg/kg) (B). Perfusion was followed by a 10-minute washout. Mean plasma concentrations of five pigs ± SEM are shown.

higher than systemic peak MMC plasma concentrations (2222 ng/ml *vs.* 59 ng/ml). Mean melphalan peak levels at 5 minutes were 33-fold higher than mean systemic plasma concentrations (3028 ng/ml *vs.* 93 ng/ml). During the 20 minutes of perfusion, MMC and melphalan plasma levels in the isolated hepatic vascular compartment steadily declined. However, systemic levels of melphalan and MMC remained at <93 ng/ml and 59 ng/ml, respectively, throughout the procedure, indicating no significant leakage from the isolated circuit.

The mean plasma concentration of TNF at 5 minutes was 840 ng/ml; it remained virtually stable throughout perfusion, not exhibiting the regional concentration decline observed for MMC and melphalan (Fig. 4). Systemic TNF concentrations remained at <22 ng/ml throughout the 20 minutes of perfusion. As a result of the washout procedure, regional TNF concentrations declined from 648 ng/ml to 99 ng/ml. Five minutes after termination of isolation, systemic TNF levels showed only a small rise to 50 ng/ml.

Areas Under the Curve

Table 1 summarizes the regional *versus* systemic ratios for TNF, MMC, and melphalan. AUCs were calculated

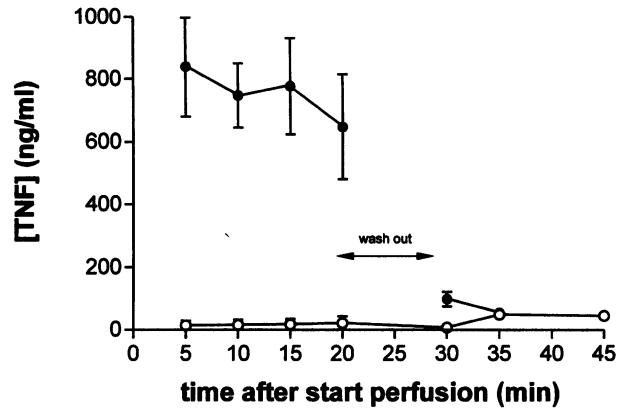

Figure 4. Regional (black circles) and systemic (white circles) plasma concentration curves throughout and after 20 minutes of isolated hypoxic hepatic perfusion with tumor necrosis factor-alpha (0.02 mg/kg). Perfusion was followed by a 10-minute washout. Mean plasma concentrations of three pigs ± SEM are shown.

using values of drug concentrations from 5 to 20 minutes. Much higher AUCs were found during IHHP for the isolated hepatic vascular compartment than for the systemic compartment, or as compared with plasma levels after administration of an intravenous bolus. Regional drug concentration *versus* time advantages of 20- to 40-fold were observed during IHHP (melphalan, 20.4; MMC, 27.9; TNF, 43.3). When regional AUCs were compared with systemic AUCs after systemic bolus administration of equivalent doses, regional advantages varied from 3.2 to 11.5 (TNF, 3.2; MMC, 3.6; melphalan, 11.5).

DISCUSSION

We have demonstrated in this series of experiments in pigs the feasibility and safety of a leakage-free IHHP using balloon catheter methodology. Regionally, tissue exposure

Table 1. AREA UNDER THE CONCENTRATION *VERSUS* TIME CURVE AND RATIOS DURING ISOLATED HYPOXIC HEPATIC PERFUSION IN PIGS

AUC (ng × min/ml)	Melphalan	MMC	TNF
Regional*	26860	16940	11340
Systemic†	1319	608	262
Intravenous‡	2339	4657	3528
AUC ratios			
Regional/systemic	20.4	27.9	43.3
Regional/intravenous	11.5	3.6	3.2

* Regional is regional plasma concentration in isolated hepatic vascular blood-compartment during IHHP.
† Systemic is systemic plasma concentration during IHHP.
‡ Intravenous is systemic drug concentrations after intravenous bolus administration.
AUC was calculated between t = 5 and t = 20 minutes after start of perfusion.

to perfused drugs was 20 to 40 times greater than systemic exposure. Because of the leakage-free quality of the method, these ratios are just as good as those observed with classic surgical isolated liver perfusions.[17,18,34] Regional MMC and melphalan levels declined rapidly during perfusion, indicating a fast uptake by the liver tissue; concomitant minimal systemic concentrations indicated virtually no leakage to the systemic blood compartment. This holds true not only for the parent drugs, but also for possible toxic metabolites and degradation products.[32] During isolation, concentrations of TNF in the perfusate declined only slightly, indicating limited uptake by the liver tissue; no leakage of TNF was observed. After termination of isolation, systemic TNF levels showed only a minor transient elevation, indicating that the washout procedure at the end of the perfusion was fully effective.

One could argue that regional/intravenous AUC ratios (see Table 1) become less favorable if they are calculated over the time period from 0 to 180 minutes instead of over the time period of isolation. For instance, calculated regional/intravenous ratios for melphalan, MMC, and TNF would become 7, 13, and 1, respectively. However, the high concentrations of TNF that are maintained during isolation have a different effect[35]: the high concentrations have been shown to have vascular toxicity but are necessary to obtain an optimal effect,[36] thus promoting enhanced uptake of MMC and melphalan. It is important that high concentrations are achieved for a short period, because in previous studies by us in patients with isolated limb perfusion, immediate TNF-induced vascular leakage was observed.[37]

Isolated hepatic perfusion has been developed as a treatment modality that maximizes drug concentrations in the target organ while shielding the organism from systemic toxicity.[14–16] It is a means to improve the selectivity of administration of antitumor agents to the liver, as compared with hepatic artery infusion (HAI). When MMC was administered by IHP, a fourfold higher dose could be safely administered; this resulted in a tumor tissue concentration five times greater than that of HAI.[38] Similar results were obtained with melphalan.[39] The relative pharmacologic advantage of IHHP for MMC in our experiments may seem relatively small when compared with HAI with MMC. However, in IHHP the dose of MMC can be increased and the exposure time can be prolonged, whereas these parameters cannot be further exploited with HAI. It is clear from experimental data that in this setting liver toxicity rather than systemic toxicity is dose-limiting.[34,38] In earlier experiments, we performed classic isolated hepatic perfusion on pigs with melphalan and TNF for 90 minutes. In these experiments a transient elevation of liver enzyme levels reflected acute but overall moderate toxicity; no late toxicity was observed.[17]

Clinical experience with isolated hepatic perfusion is limited. Aigner[40] treated patients using classic surgical isolated hepatic perfusion chemotherapy, reporting 5-year survival rates of >10%. However, classic procedures have been associated with significant mortality and morbidity rates of >10%.[16,41,42]

The development of balloon catheter-mediated IHHP represents a significant improvement over the classic procedure in four ways. First, it limits the scale of the procedure and thus reduces the associated complications and high costs. IHHP, using described techniques, takes about 2 hours; the classic procedure takes an average of >6 hours,[17] which in patients was found to be associated with a high morbidity rate.[18,19] Moreover, IHHP is less costly in terms of personnel (no perfusionists required) and material (no heart-lung machine or portocaval venous bypass). Second, IHHP is a technique that in principle allows for repeated isolated perfusions when the arterial infusion catheter is left in place after the first perfusion and connected to a subcutaneous port. This is of particular importance because multiple treatments are needed to keep the disease in the liver under control. Third, IHHP makes use of hypoxia, which renders tumor cells more sensitive to cytostatic agents in general and enhances in particular the antitumor effects of drugs such as MMC and melphalan.[29,30]

Finally, by limiting the extent of the surgical intervention, while still achieving full isolation and thus allowing complete washout of perfused agents after perfusion, IHHP in theory makes possible the introduction of drugs such as TNF. This is important because the combination of TNF and melphalan has been shown to exert highly synergistic antitumor effects against otherwise resistant tumors such as soft-tissue sarcomas and melanoma in the setting of isolated limb perfusion. Therefore, IHHP facilitates the translation of the successful experience with TNF in isolated limb perfusion into its application in isolated hepatic perfusion.

The pharmacokinetic profiles of TNF, MMC, and melphalan in balloon catheter-mediated isolated pelvic and abdominal perfusions have been virtually identical in our experience in pigs[43] and in humans (manuscript in preparation). Therefore, we are confident that a similar correlation will exist between the pharmacokinetic profiles of these agents during IHHP in pigs and those that will be obtained in patients in a phase I–II study we have recently started in our clinic.

A crucial point in cancer therapy is to use the right drug in the right patient. A promising substance with important *in vitro* and *in vivo* antitumor effects is TNF.[18,44] Because of its general toxicity, however, TNF cannot be given in adequate doses intravenously.[10,11] When administered at adequate concentrations, such as in isolated limb perfusions, it is effective in humans.[12,13,45,46] High doses of TNF can induce tumor necrosis with acute softening of the tumor brought about by selective destruction of the tumor's microvasculature, causing acute hemorrhagic necrosis of the tumor.[12,35,47,48] TNF can be used in high doses in the extremity perfusion setting because leakage to the systemic circulation is usually limited to 0% to 10%, and in theory similar doses could be used in IHHP because a complete washout of agents can be achieved. Hepatic toxicity, how-

ever, is expected to be the dose-limiting factor. This was indicated by the experience of Fraker and Alexander[18,49] at the National Cancer Institute with classic isolated hepatic perfusions: responses were achieved in about 75% of the patients, which corresponds with our limited clinical experience with classic isolated hepatic perfusion with TNF and melphalan.[19]

In conclusion, we have demonstrated that IHHP has favorable pharmacokinetic characteristics for both TNF and cytostatic agents that equal those that can be achieved by the major procedure of a classic surgical isolated liver perfusion. Moreover, the balloon catheter procedure can be repeated, which is of eminent importance in prolonging control of metastatic disease in the liver. IHHP may provide a new chance to reintroduce TNF into the clinical setting in an attempt to translate its success in combination with chemotherapy in isolated limb perfusions into the treatment of the much more common problem of managing unresectable malignant disease to the liver.

Acknowledgments

The authors thank Boehringer Ingelheim GmbH for the generous supply of rhTNF, and PfM GmbH for providing the balloon catheter sets.

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