Augmented Capillary Leak during Isolated Hepatic Perfusion (IHP) Occurs via Tumor Necrosis Factor-independent Mechanisms

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ABSTRACT
Isolated organ perfusion of the liver or extremity with tumor necrosis factor (TNF) and melphalan results in regression of bulky tumors in the majority of patients. The efficacy of TNF in this setting is not known, although data suggest that it may exert antitumor effects primarily on tumor-associated neovasculature. We studied the effects of TNF on capillary leak in liver and tumor tissue during isolated hepatic perfusion (IHP) with melphalan. Twenty-seven patients with unresectable cancer confined to the liver underwent a 60-min hyperthermic IHP using 1.5 mg/kg melphalan alone (n = 7) or with 1.0 mg of TNF (n = 20). Complete vascular isolation was confirmed in all patients using an intraoperative leak monitoring I-131 radiolabeled albumin technique. Samples of tumor and liver were collected just prior to and immediately after IHP. There was no difference in I-131 radiolabeled cpm/g of tissue (cpm) in liver versus tumor at baseline (P2 = 0.44). After IHP, I-131 albumin cpm were higher in tumor versus liver (10,999 ± 1,976 versus 3,821 ± 780, respectively; P2 < 0.005). However, I-131 albumin cpm in tumor were not effected by TNF (11,636 ± 2,518 versus 9,180 ± 2,674 without TNF; P2 = 0.59). TNF did not affect melphalan concentrations in tumor (1,883 ± 540 ng/g versus 1,854 ± 861 ng/g without TNF; P2 = 0.9). Capillary leak, as reflected by diffusion of I-131 radiolabeled albumin into the interstitial space, is comparable in liver and tumor before IHP but is significantly higher in tumor after IHP. The increased diffusion in the capillary tumor bed must occur through TNF-independent mechanisms such as intrinsic features of tumor neovasculature, hyperthermia, or other unrecognized perfusion-related factors. These data indicate that TNF must continue to be critically evaluated in clinical trials before it is routinely used with melphalan in isolated organ perfusion.

INTRODUCTION
Isolated organ perfusion with TNF2 and melphalan has resulted in remarkably high response rates in patients with intransit melanoma or unresectable high-grade sarcoma of the extremity or unresectable cancers confined to the liver (1–4). In 1992, Liénard et al. (1) published their initial results of ILP with TNF, melphalan, and IFN-γ for patients with intransit melanoma or extremity sarcoma. They reported an 89% complete response rate and an overall response rate of 100% (1). These results were interpreted to be superior to those obtained in previous reports of ILP using melphalan alone (2, 5), and based on these data, a number of centers initiated ILP clinical trials using TNF and melphalan administered via ILP to critically evaluate the potential therapeutic efficacy of this regimen. Subsequent series have reported complete response rates between 75 and 90% for patients with intransit melanoma and a limb salvage rate of over 80% in patients with unresectable high-grade extremity sarcoma (6–8). A Phase II trial of IHP using TNF and melphalan resulted in an overall response rate of 74% in patients with unresectable hepatic malignancies, including those with numerous metastases, bulky sites of disease, or patients who had failed extensive prior treatment (9).

When TNF is used with melphalan, there is a characteristic tumor response distinct to that observed following ILP with melphalan alone. There is often rapid necrosis of tumors with eschar formation of bulky cutaneous lesions and abrupt cystic changes in deeper lesions on computed tomography or contrast-enhanced magnetic resonance imaging. There are considerable experimental data (10–12) and clinical observations (13, 14) that indicate that the primary antitumor effects of TNF administered via isolated organ perfusion are on the tumor-associated neovasculature. In experimental models, TNF administration systemically results in rapid necrosis of tumors, and on histological evaluation, there is primarily endothelial cell injury with intravascular erythrostasis and fibrin deposition (10). Tumors resistant to direct TNF cytotoxic effects in vitro may be sensitive to TNF effects in vivo, particularly when >1 cm in diameter, a size at which the tumor neovasculature has been established (15). In ILP with TNF-based regimens, there is complete obliteration of the tumor-associated neovasculature after treatment (14).

Despite considerable clinical evaluation, the exact contribution of TNF to the antitumor effects during hyperthermic isolated organ perfusion with melphalan are not known. Phase III random assignment trials are under way in Europe and the United States comparing these two treatment regimens administered via ILP for extremity melanoma. In a preliminary anal-

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2 The abbreviations used are: TNF, tumor necrosis factor; ILP, isolated limb perfusion; IHP, isolated hepatic perfusion; IVC, inferior vena cava; GDA, gastroduodenal artery.
yosis of a random assignment trial comparing these regimens in patients with stage IIIA or IIIB intransit melanoma, the complete response and overall response rate between groups were not significantly different, although patients with bulky disease or large lesions may have had a better response to TNF and melphalan compared with melphalan alone.3 Although TNF has potential effects on the tumor-associated neovasculature, when it is given alone via hyperthermic isolated organ perfusion of the limb or liver, there are no significant antitumor effects (16). In addition, leak of perfusate containing TNF can result in potentially significant systemic toxicity (5). Insight into the mechanisms of the effects of TNF in isolated organ perfusion will be important to use it most efficaciously in this setting. The present study was undertaken to determine the effects of TNF on endothelial cell permeability as measured by diffusion of radiolabeled I-131 albumin in normal and tumor tissue before and after hyperthermic IHP with melphalan.

PATIENTS AND METHODS

Patient Population. Between August 1996 and May 1997, 27 patients with primary or metastatic unresectable cancers confined to the liver were treated with a 60-min hyperthermic IHP using 1.5 mg/kg of melphalan with (n = 20) or without (n = 7) tumor necrosis factor. The treatment protocols were approved by Institutional Review Board and the Cancer Therapeutics Evaluation Program of the National Cancer Institute. Patients were treated on two consecutive Phase II protocols using identical IHP treatment parameters; the latter without TNF. All patients had multiple bilobar hepatic tumors. All patients who had tumors accessible for biopsy were included in the study. All patients had measurable, unresectable, biopsy-proven primary metastatic cancers confined to the liver. Patients underwent standard staging studies including computed tomography scan of the chest, abdomen, and pelvis and, as clinically indicated, brain magnetic resonance imaging or bone scan. Eligibility criteria included Eastern Cooperative Oncology Group performance status of 0 or 1, a serum bilirubin <2.0 mg/dl, a platelet count >150,000/ml, and a serum creatinine ≤1.5 mg/dl.

IHP. The treatment technique of IHP was performed as described previously (4). Briefly, via a laparotomy the liver is extensively mobilized by dividing the falciform ligament and the right and left triangular ligaments. The duodenum is extensively mobilized by dividing the falciform ligament and incorporating it into the veno-veno bypass circuit. The portal vein blood flow is shunted by inserting a cannula distally and incorporating it into the veno-veno bypass circuit. The GDA is ligated, the common hepatic artery is occluded, and a 3-4-mm GDA cannula is positioned at the orifice of the common hepatic artery. Finally, the suprahepatic IVC is cross-clamped just below the diaphragm and isolated hepatic perfusion is initiated.

The extracorporeal bypass circuit consists of a roller pump, membrane oxygenator, and heat exchanger. The perfusate consists of 700 ml of balanced salt solution primed with 300 ml of packed RBCs and 2000 units of heparin. Arterial and venous perfusate blood gases are obtained at regular intervals, and sodium bicarbonate was added to the perfusion circuit to maintain an arterial perfusate pH between 7.2 and 7.3. Hepatic parenchymal temperature probes are placed at various positions, and perfusate was temperature controlled using a Hemotherm cooler heated model #4 (Cincinnati SubZero Products, Cincinnati, Ohio). Flow rates are adjusted upward while monitoring for a stable reservoir volume and acceptable line pressures. Usually, stable perfusion parameters are achieved almost immediately, and there is rapid and uniform heating of the liver to target temperatures of 39.5-40°C. Melphalan at a dose of 1.5 mg/kg (Glaxo-Wellcome, Research Triangle Park, NC) and 1.0 mg TNF (Knoll Pharmaceuticals, Whippany, NJ) are added sequentially to the arterial inflow line of the perfusion circuit at time 0, and the perfusion continued for 60 min. At the conclusion of the perfusion, the liver is flushed through the arterial inflow cannula with 1500 ml of crystalloid, followed by 1500 ml of colloid, and the proximal portal vein is flushed with 1 liter of normal saline. After decannulation and repair of the IVC and portal venostomy, normal physiological blood flow is reestablished promptly to the liver.

Continuous Intraoperative Leak Monitoring with I-131 Radiolabeled Human Serum Albumin. A continuous intraoperative leak monitoring system as described previously for isolated limb and liver perfusion with TNF and melphalan was used in all patients. In addition, because the I-131 radiolabeled albumin was being used to assess capillary endothelial leak in the liver and tumor vascular beds just before and after IHP, tissue samples were obtained at consistent predetermined points. Once stable perfusion parameters were established, a gamma detection camera was positioned over the centrifugal pump housing, which served as a stable reservoir of systemic blood flow for the purposes of calculating leak rates. A 20 μCi dose of

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radiolabeled I-131 human serum albumin (Merck-Frosst, Quebec, Canada) was administered via a central vein, and a baseline level of radioactive cpm (cpm) was determined on a stripchart recorder. Subsequently, a 10-fold higher dose of I-131 radiolabeled albumin was then injected into the perfusion circuit. If any increase in cpm were detected on the stripchart recorder, this would indicate a leak from the perfusion circuit into the systemic circulation. On the basis of mean flow of systemic blood into the perfusion circuit. Small changes in reservoir volume are typically observed during the 60-mm IHP without. The perfusion parameters are shown in Table 2. The mean flow rate, central hepatic temperature, line pressure, and other parameters were comparable between groups. Using the radiolabeled I-131 albumin continuous leak monitoring system, there was no identifiable leak of perfusate into the systemic circulation in any patient. Conversely, the mean change in reservoir volume in each group was <100 ml, indicating no leak of systemic blood into the perfusion circuit. Small changes in reservoir volume are typically observed during the 60-min IHP and usually reflect small changes in passive filling or emptying of the hepatic vascular bed.

Fig. 1 illustrates the protocol for administration of radiolabeled I-131 albumin and biopsies of liver and tumor just before and immediately after IHP. After establishing a stable hepatic perfusion circuit, the systemic dose of I-131 albumin was administered through a central vein. Biopsies of liver and tumor were obtained 3–5 min after administration of the circuit dose of I-131 radiolabeled albumin. On the basis of mean flow rates of almost 850 ml/min and a circuit volume of 1 liter, this allowed the perfusion circuit containing I-131 albumin to recirculate through the hepatic vascular bed ~4 times. Melphalan, with or without TNF, was then added via the arterial line of the perfusion circuit. After 60 min, the circuit was flushed to remove melphalan and TNF from the intravascular space and also served to remove any intravascular I-131 radiolabeled albumin.

### Table 1 Patient demographics

<table>
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<tr>
<th></th>
<th>Total</th>
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<td>n</td>
<td>27</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>Male:female</td>
<td>18:9</td>
<td>13:7</td>
<td>5:2</td>
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<tr>
<td>Age</td>
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<td>Mean (range)</td>
<td>50 ± 2.2</td>
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<td>52.4 ± 3.5</td>
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<td>Range (yr)</td>
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<td>37–65</td>
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<td>Previous treatment (%)</td>
<td>13 (49%)</td>
<td>9 (45%)</td>
<td>4 (56%)</td>
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<tr>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Colon adenocarcinoma</td>
<td>17</td>
<td>13</td>
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<tr>
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<td>4</td>
<td>1</td>
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<td>0</td>
<td>2</td>
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<tr>
<td>Leiomyosarcoma</td>
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</table>

### Table 2 IHP parameters

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<th>Total</th>
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<tbody>
<tr>
<td>n</td>
<td>27</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>Melphalan dose</td>
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</tr>
<tr>
<td>Mean (mg)</td>
<td>117 ± 4.1</td>
<td>115 ± 4.3</td>
<td>124 ± 9.4</td>
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<tr>
<td>Range (mg)</td>
<td>75–160</td>
<td>75–144</td>
<td>95–160</td>
</tr>
<tr>
<td>Duration (min)</td>
<td>60</td>
<td>60</td>
<td>60</td>
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<tr>
<td>Flow rate</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Mean (mL/min)</td>
<td>853 ± 25</td>
<td>840 ± 21</td>
<td>889 ± 75</td>
</tr>
<tr>
<td>Range (mL/min)</td>
<td>600–1350</td>
<td>600–1050</td>
<td>720–1350</td>
</tr>
<tr>
<td>Mean central hepatic temperature (°C)</td>
<td>4.0 ± 0.06</td>
<td>40.1 ± 0.07</td>
<td>39.9 ± 0.07</td>
</tr>
<tr>
<td>Mean perfusion pressure (mm Hg)</td>
<td>176 ± 5.7</td>
<td>177 ± 6.3</td>
<td>171 ± 12.9</td>
</tr>
<tr>
<td>Perfusion pressure range (mm Hg)</td>
<td>105–240</td>
<td>110–240</td>
<td>105–223</td>
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<tr>
<td>Mean change reservoir volume (ml)</td>
<td>98 ± 14</td>
<td>110 ± 17</td>
<td>64 ± 23</td>
</tr>
<tr>
<td>% leak</td>
<td>0</td>
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**Statistics.** Data are presented as mean ± SE. Data were analyzed using Student’s t test, and significance of differences between data was determined by using the Bonferoni correction for multiple comparisons.

**RESULTS**

Patient demographics are shown in Table 1. There was a similar mean age, a 2:1 male:female ratio, and a predominance of colon adenocarcinoma diagnoses in both groups. Twenty-seven patients were treated with a 60-min hyperthermic IHP using 1.5 mg/kg melphalan; 20 were treated with TNF, and 7 without. The perfusion parameters are shown in Table 2. The mean flow rate, central hepatic temperature, line pressure, and other parameters were comparable between groups. Using the radiolabeled I-131 albumin continuous leak monitoring system, there was no identifiable leak of perfusate into the systemic circulation in any patient. Conversely, the mean change in reservoir volume in each group was <100 ml, indicating no leak of systemic blood into the perfusion circuit. Small changes in reservoir volume are typically observed during the 60-min IHP and usually reflect small changes in passive filling or emptying of the hepatic vascular bed.
Establish IHP circuit

1. Biopsy liver and tumor
2. Add melphalan ± TNF

Fig. 1 Protocol for obtaining liver and tumor samples before and immediately after isolated hepatic perfusion.

I-131 Albumin (cpm/g)

Pre-IHP Post-IHP

Liver Tumor

Fig. 2 Mean I-131 albumin cpm in liver and tumor samples obtained immediately before and after IHP. Post-IHP I-131 albumin was significantly greater than post-IHP liver levels (*, P2 = 0.005) and pretreatment tumor levels (*, P2 = 0.006). There was no difference in I-131 albumin cpm in liver compared with tumor at baseline and a marginal increase in I-131 albumin cpm in post- compared with pre-IHP liver (P2 = 0.06). Bars, SE.

I-131 Albumin (cpm/g)

TNF No TNF

Fig. 3 I-131 albumin cpm in tumor samples post-IHP with or without TNF (P2 = 0.59). Bars, SE.

Methylalan (ng/gm)

TNF No TNF

Fig. 4 Methylalan concentrations (ng/gm) in tumor samples after IHP with n = 14 and without TNF (n = 5, P2 = 0.96). Bars, SE.

Therefore, all I-131 albumin detected in these samples is in the interstitial space.

Overall, there was no difference in I-131 albumin cpm in liver compared with tumor at baseline (P2 = 0.44; Fig. 2). There was a marginal increase in I-131 albumin cpm in post-compared to pre-IHP liver tissue (P2 = 0.06). I-131 albumin cpm were significantly higher in tumor after IHP compared with pre-IHP tumor (P2 = 0.006) and post-IHP liver (P2 = 0.005; Fig. 2). Interestingly, the I-131 albumin in tumor was not statistically different between patients treated with or without TNF (P2 = 0.59; Fig. 3). Furthermore, TNF did not affect methylalan tumor concentrations after IHP when compared with methylalan alone (P2 = 0.97; Fig. 4). These last data are based on sample analyses of 14 patients treated with TNF and 5 without. One other patient treated without TNF had a methylalan concentration in tumor that was >2-fold higher than any other value, almost 10-fold higher than the mean of either group and, therefore, was excluded from the statistical analysis. However, the outcome of the statistical analysis between the groups remained nonsignificant when the value was included in the data.

DISCUSSION

Because of the significant effects on tumor neovasculature that have been observed after hyperthermic isolated limb perfusion with TNF and methylalan (13, 14) and the experimental data suggesting that the primary antitumor effects of TNF may be exerted on the tumor neovasculature (11, 14), this study was performed to determine whether hyperthermic isolated hepatic perfusion may cause augmentation in tumor-associated microvascular permeability, thereby facilitating the selective delivery of chemotherapeutics to the tumor.

The data presented here show that comparable amounts of
radiolabeled I-131 albumin are present in liver and tumor tissue just prior to treatment with isolated hepatic perfusion. However, after a 60-min hyperthermic hepatic perfusion using melphalan with or without TNF, there is a significant and selective increase in interstitial I-131 albumin in tumor compared with both pre-treatment tumor levels and posttreatment liver tissue. The pre-treatment I-131 albumin cpm in liver and tumor also reflect some I-131 albumin contained in the intravascular space in the tissue biopsies obtained. However, posttreatment values reflect true interstitial amounts of I-131 albumin because the circuit is flushed with 3 liters of crystalloid and colloid solution, which effectively removes the I-131-containing perfusate from the intravascular space. The 27 patients analyzed in this report underwent an identical hepatic perfusion treatment protocol. This cohort represents consecutively treated patients who had tumor that could be easily biopsied before and after treatment. All but two patients had metastatic cancers to the liver, and two-thirds of those were colorectal tumors. All identifiable perfusion-related parameters that may have influenced microvascular permeability were comparable between the 20 patients treated with TNF and the 7 who received no TNF. Remarkably, using the maximum safe tolerated dose of TNF when combined with 1.5 mg/kg melphalan, there was no significant difference in interstitial I-131 albumin in patients treated with TNF compared with those who received no TNF. This suggests that other unrecognized perfusion-related factors must be accounting for the augmented capillary leak in the tumor bed during isolated perfusion. The number of possibilities are considerable and would include the artificial flow dynamics of the extracorporeal bypass circuit, local pH changes potentially related to nonphysiological flow rates, or local production of cytokines or other factors that may render the tumor-associated neovascularure selectively sensitive to hyperthermia or melphalan. Interestingly, the overall initial response rates between the groups are similar.4

The comparable levels of melphalan in liver tissue after IHP in patients treated with or without TNF may be related to the increased diffusability of I-131 albumin. The larger macromolecule, I-131 albumin, has a molecular weight of 66,000, whereas melphalan is a considerably smaller molecule with a molecular weight of 305. Therefore, its delivery into the interstitial space may not be as dependent on changes in endothelial permeability as I-131 albumin. Although mean melphalan concentrations were similar in patients treated with TNF compared with those who were not, because there was only a single time point available for analysis, there are limited conclusions that can be drawn from these data. The tissue concentrations of melphalan over time or the possible cellular or molecular effects of melphalan with or without TNF have not been addressed in this study.

A large number of institutions are evaluating the use of TNF in isolated limb perfusion for intransit melanoma or unresectable high grade extremity sarcoma, and a smaller number of centers are evaluating its use in IHP for unresectable cancers confined to the liver. In both limb and liver perfusion TNF is associated with considerable regional and systemic toxicities (9, 17, 18) and has not been conclusively demonstrated to improve response rates.5 On the basis of presently available data, it appears that TNF causes a very characteristic pattern of tumor regression manifested by rapid tumor necrosis with eschar formation of cutaneous lesions, significant angiographic and radiographic changes of large extremity sarcomas, and a more rapid time course of response compared with patients treated with melphalan alone (2, 14, 19). Taken together, these points highlight the fact that the use of TNF in isolated perfusion must be critically evaluated in appropriately designed clinical trials to determine efficacy and mechanisms of its antitumor effects, optimal dosing schedules, and appropriate patient selection.

We have recently reported results of a Phase II trial of IHP with melphalan and TNF for patients with unresectable cancers confined to the liver (9). The 60-min perfusion interval has been used based upon previously conducted studies, and there are no data indicating that a longer or shorter interval would be superior. The overall response rate in 33 evaluable patients was 74% including patients with large or numerous lesions as well as those who had undergone extensive previous treatment. Seventy-five percent of all patients experienced transient grade 3 or 4 (National Cancer Institute common toxicity criteria) hepatic toxicity, and one patient developed fatal hepatic veno-occlusive disease. Complete vascular isolation was achieved during IHP on the basis of the results of continuous intraoperative leak monitoring and the fact that systemic levels of TNF could not be detected, except for low transient levels in two patients who had a correctable small (<4%) leak of perfusate during treatment. However, there are significant hemodynamic changes associated with this procedure, and circulating levels of interleukin 6 and interleukin 8 between 2 and 12 h after the procedure are as high as those reported in patients with life-threatening septic shock (20–22). These secondary cytokines are presumably released by the liver in response to the perfusion. In patients undergoing isolated limb perfusion with TNF and melphalan, systemic toxicity is associated with the presence of a perfusate leak during treatment, and leak rates of 5% or greater occur in ~15% of patients (23, 24). The question as to whether the routine use of TNF is justified in isolated organ perfusion has not yet been determined.

In summary, the data presented in this report show that hyperthermic IHP results in a selective and significant increase in capillary endothelial permeability in tumor-associated neovascularure. This has important implications for the use of isolated organ perfusion with various therapeutic agents. Although the exact mechanisms for this increase in microvascular permeability are not known, it does not appear that TNF contributes significantly to this effect.

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2362 IHP and Capillary Leak


Augmented capillary leak during isolated hepatic perfusion (IHP) occurs via tumor necrosis factor-independent mechanisms.


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