Role of Tumor Necrosis Factor on Toxicity and Cytokine Production after Isolated Hepatic Perfusion


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ABSTRACT

Purpose: Isolated limb or liver perfusion with tumor necrosis factor (TNF) and melphalan results in regression of advanced cancers in the majority of treated patients. However, the contribution of TNF to the efficacy of isolation perfusion with melphalan has not been demonstrated conclusively in random assignment trials. Furthermore, TNF is an inflammatory cytokine and may be associated with significant systemic and regional toxicity. This study was conducted to characterize the toxicity and secondary cytokine production attributable to TNF by comparing these parameters in patients undergoing isolated hepatic perfusion (IHP) using melphalan with or without TNF.

Experimental Design: Thirty-two patients with unresectable colorectal cancer confined to the liver underwent a 60-min hyperthermic IHP using 1.5 mg/kg melphalan alone (n = 17) or with 1.0 mg of TNF (n = 15) with inflow via the gastroduodenal artery and outflow via an isolated segment of inferior vena cava. Complete vascular isolation was confirmed using the I-131 radiolabeled albumin-monitoring technique. Post-IHP parameters of hepatic and systemic toxicity and cytokine levels [TNF, interleukin (IL)-6 and IL-8] in perfusate and serum were measured.

Results: Levels of IL-6 and IL-8 in perfusate at the end of the 60-min IHP were significantly higher in TNF-treated patients (P ≤ 0.001). Peak systemic IL-6 and IL-8 levels post-IHP were also significantly higher in TNF-treated compared with non-TNF-treated patients (P < 0.0001) by 28- and 268-fold, respectively. The peak levels of these cytokines were associated with significantly lower systolic blood pressure and higher heart rate and mean pulmonary artery pressure in TNF-treated patients during the first 48 h post-IHP (P ≤ 0.03). Serum bilirubin levels were significantly higher (P = 0.017) and platelets lower (P = 0.03) in TNF-treated compared with non-TNF-treated patients. However, elevations in aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase were not significantly different between groups and returned toward baseline within 1 week after IHP.

Conclusions: Addition of TNF to melphalan during IHP results in significant differences in post-IHP production of IL-6 and IL-8 with associated changes in mean arterial blood pressure and greater regional toxicity, as reflected in higher levels of serum bilirubin. However, these measurable differences were transient and did not appear to be of major clinical consequence. Prior to its routine use, the benefit of TNF in isolation perfusion should be demonstrated in random assignment trials.

INTRODUCTION

TNF, a secreted M, 17,000 protein with a range of physiological activities, was originally identified as a circulating factor present in the sera of bacillus Calmette-Guérin-primed, endotoxin-treated mice that resulted in remarkable hemorrhagic necrosis of tumors when administered to tumor-bearing mice (1). After it became available in recombinant form, a number of clinical trials were performed with high expectations that it could produce similar results in patients with advanced cancer. However, it was shown that humans are exceedingly sensitive to the toxic effects of TNF, and at the maximum tolerated systemic doses, there was no meaningful antitumor activity (2). Furthermore, there was contemporaneous evidence that TNF was the primary endogenous mediator of acute inflammatory conditions such as endotoxic shock, and during this time, it appeared that inhibiting its effects might have broader clinical application than the protein itself (3, 4).

However, in 1992 Drs. Lienard and Lejeune reported their initial experience using TNF in ILP in combination with melphalan, IFN, and hyperthermia for in-transit extremity melanoma or high-grade unresectable extremity sarcoma (5). The complete response rate in 29 evaluable patients was 90% and rekindled considerable interest in the use of TNF with melphalan in isolation perfusion. Subsequent reports have confirmed complete response rates of >78% after ILP for patients with in-transit extremity melanoma, a limb salvage rate of 84% for patients with unresectable high-grade extremity sarcoma, and an overall response rate of 74% in patients with unresectable he-

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3 The abbreviations used are: TNF, tumor necrosis factor; ILP, isolated limb perfusion; IHP, isolated hepatic perfusion; IL, interleukin; GDA, gastroduodenal artery; VOD, veno-occlusive disease.
The patient was systemically heparinized with 200 units/kg, and after 5 min, a cannula was inserted into the saphenous vein and advanced into the IVC just below the renal veins. A second venous cannula was inserted into the axillary vein, and both were connected to a veno-veno bypass circuit. The IVC was occluded above the renal veins, and infrahepatic IVC blood flow was shunted to the axillary vein using a centrifugal pump. A short segment of infrahepatic IVC was isolated between vascular occluding clamps, and a 20–24 French cannula was inserted through a venotomy and positioned behind the retrohepatic IVC just beneath the hepatic veins. This cannula was connected to the venous outflow line of the extracorporeal bypass circuit. A cannula was positioned in the portal vein and connected to the veno-veno bypass circuit to shunt portal vein blood flow systemically. The GDA was ligated, the common hepatic artery was occluded, and a 3–4 mm GDA cannula was positioned at the orifice of the common hepatic artery. Finally, the suprahepatic IVC was cross-clamped just below the diaphragm, and IHP was initiated.

The extracorporeal bypass circuit consisted of a roller pump, membrane oxygenator, and heat exchanger. The perfusate consisted 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 were obtained at regular intervals, and arterial perfusate pH was maintained between 7.2 and 7.3 with sodium bicarbonate. Hepatic parenchymal temperature probes were placed, and perfusate was warmed using a Hemo-therm water heater model #4 (Cincinnati SubZero Products, Cincinnati, Ohio). Flow rates were adjusted upward while monitoring for a stable reservoir volume and acceptable line pressures. There is typically rapid and uniform heating of the liver to target temperatures of 39.5–40°C. Melphalan (1.5 mg/kg), with or without TNF (1.0 mg), was added sequentially to the arterial inflow line of the perfusion circuit at time 0, and IHP continued for 60 min. At the conclusion of IHP, the liver was flushed through the arterial inflow cannula with 1500 ml of crystalloid, followed by 1500 ml of colloid and through the portal vein with 1 liter of normal saline. After decannulation and repair of the IVC and portal venotomies, normal physiological blood flow was reestablished promptly to the liver.

Drugs. Melphalan was obtained from Glaxo-Wellcome (Research Triangle Park, NC), and recombinant human TNF-α was from Knoll Pharmaceuticals (Whippany, NJ).

Blood Sampling and Hemodynamic Monitoring. Perfusion was sampled at time 0, 15, 30, and 60 min during IHP. Blood samples were obtained at regular intervals beginning before and after IHP for clinical laboratory parameters; serum samples for cytokine determination were collected at the same intervals as perfusate samples during IHP and at regular intervals for 24 h after IHP. Hemodynamic, pulmonary, and other systemic end points were measured at intervals as clinically indicated but no less than every 8 h after IHP for up to 60 h.
Results for IL-6 and IL-8 are expressed as pg/ml; the lower limit of detection was 0.016 ng/ml.

**Cytokine Assays.** All blood and perfusate samples for cytokine determination were immediately refrigerated, and serum was separated and frozen until analyzed. TNF, IL-6, and IL-8 in perfusate and serum were measured by an ELISA using the Quantikine Immunoassay kits (R&D Systems, Inc.). In these assays, monoclonal antibodies raised against the cytokines were used according to procedures similar to those described earlier. Results for IL-6 and IL-8 are expressed as pg/ml; the lower limit of detection was 0.0312 ng/ml. Results for TNF are expressed as pg/ml; the lower detection limit for the assay was 0.016 ng/ml.

**Statistical Analysis.** Values are expressed as mean ± SE. For the regional toxicity variables, a Wilcoxon rank sum test was performed on the data collected 1 day before IHP to determine whether the two groups were the same at baseline. Aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and systemic vascular resistance were log 10 transformed, and serum bilirubin levels were square root transformed to reduce skewness in distributions. A repeated measured ANOVA was then performed on each variable, starting with time 0. To account for correlation between repeated measurements, we tested autoregressive, heterogeneous autoregressive, and Toeplitz covariance matrices for each variable and chose the best-fitting matrix according to Schwarz’s Information Criteria (16). For variables in which the interaction term was significant, Ps from the combined test of the treatment effects over time were reported. The assumption of normally distributed residuals was satisfied. \( P \leq 0.05 \) was considered to be statistically significant.

**RESULTS**

Patient demographics and tumor characteristics are shown in Table 1. There was a similar mean age, a roughly 2:1 male:female ratio, and the percentage of patients who had been previously treated was comparable between groups. In addition, the tumor burden, as reflected by number of liver metastases, size of largest lesion, percentage of hepatic replacement, and preoperative carcinoembryonic antigen level were similar between groups. The overall radiographic response rate was 72% and not different between those receiving and not receiving TNF (Table 1). Fifteen patients were treated with a 60-min hyperthermic IHP using 1.5 mg/kg melphalan; 17 patients also received 1 mg of TNF. IHP perfusion parameters are listed in Table 2. All parameters were comparable between the two groups except for flow rate that was higher, but not significantly (\( P = 0.06 \)), in the no-TNF group. The reason for this is not clear and not attributable to any bias or attempt to achieve higher flow rates in patients not receiving TNF. Because there was no difference in the pH of the perfusate between groups, we presume that the differences in flow rates were physiologically inconsequential and did not influence hepatic toxicity. Using a radiolabeled I-131 albumin continuous leak monitoring system, there was no identifiable leakage of perfusate into the circulation in any patient. Because of the unique vascular anatomy of the liver, it is our experience that complete vascular isolation with no leak of perfusate can be achieved routinely during IHP (8). This is further supported by the fact that the mean change in reservoir volume in each group was minimal, <100 ml, indicating no leak of systemic blood into the perfusion circuit. Small changes in reservoir volume that occur during IHP most likely reflect changes in passive filling or emptying of the hepatic vascular bed.

A number of hemodynamic parameters were compared between groups during the first 48 h after IHP. There were no significant differences in systemic vascular resistance, cardiac output, or temperature. However, patients who received TNF had a significantly higher mean heart rate, lower systolic blood pressure, and higher peak mean pulmonary artery blood pressure compared with melphalan alone (Fig. 1). However, these differences were gone by 48–60 h and did not appear to be of major clinical consequence. Hypotension was treated with fluid resuscitation, and no patient in the TNF group required circulatory support with cardiopressor agents.

In patients receiving TNF, initial mean perfusate levels determined 15 min after beginning IHP were 637 ± 333 \( \mu g/ml \) and decreased to 520 ± 185 \( \mu g/ml \) at the end of IHP, consistent with our findings reported previously (17). Per fusate IL-6 and IL-8 levels are shown in Fig. 2. Baseline levels of each cytokine were comparable between groups and significantly higher after IHP in the TNF group compared with melphalan.
alone. In patients receiving TNF, systemic levels were consistently measurable only at 1.5 h after IHP (7.5 ± 20 µg/ml; range, 1–83), whereas in those not receiving TNF there was no detectable systemic TNF in any sample at any time point after IHP. Systemic IL-6 and IL-8 levels are shown in Table 3. Time 0 represents the time immediately after IHP when native hepatic vascular blood flow had been reestablished. In patients receiving TNF, serum levels of IL-6 increased rapidly to a peak value of 23,543 ± 4,144 pg/ml, and IL-8 increased to 7,333 ± 1,113 pg/ml between 4 and 6 h after IHP and returned toward baseline within 24 h. The peak systemic levels of cytokines were significantly correlated (Spearman rank correlation coefficient, \( r = 0.68; P = 0.006 \)). The peak values were significantly greater than those observed with melphalan alone and occurred coincident with the maximum changes in systolic blood pressure.

### Table 3

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>0 h after IHP</th>
<th>4–6 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Serum IL-6 (pg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With TNF</td>
<td>312 ± 43</td>
<td>23,543 ± 4,144</td>
<td>478 ± 95</td>
</tr>
<tr>
<td>Range</td>
<td>60–670</td>
<td>4,661–60,033</td>
<td>15–1,312</td>
</tr>
<tr>
<td>No TNF</td>
<td>287 ± 31</td>
<td>841 ± 335</td>
<td>264 ± 77</td>
</tr>
<tr>
<td>Range</td>
<td>44–451</td>
<td>96–4,763</td>
<td>31–1,035</td>
</tr>
<tr>
<td><strong>B. Serum IL-8 (pg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With TNF</td>
<td>250 ± 73</td>
<td>7,333 ± 1,113</td>
<td>53 ± 14</td>
</tr>
<tr>
<td>Range</td>
<td>48–1,190</td>
<td>672–12,492</td>
<td>9–221</td>
</tr>
<tr>
<td>No TNF</td>
<td>65 ± 13</td>
<td>274 ± 42</td>
<td>52 ± 7</td>
</tr>
<tr>
<td>Range</td>
<td>23–195</td>
<td>71–580</td>
<td>22–112</td>
</tr>
</tbody>
</table>

*Time starting just after IHP when native blood flow to liver had been established.

All patients had significant elevations in aspartate aminotransferase and alanine aminotransferase that returned toward baseline within the first week after IHP, as reported previously for patients undergoing IHP with melphalan and TNF (8). However, after IHP there is a transient marked elevation in serum bilirubin in patients receiving TNF that was not observed in
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**DISCUSSION**

The data presented in this study show that in patients undergoing IHP with virtually all major treatment variables equal and without any measurable leak of perfusate into the systemic circulation, the addition of TNF to melphalan in the perfusate is associated with significant regional and systemic toxicity compared with those receiving melphalan alone. Interestingly, the significantly lower mean systolic blood pressure in patients treated with TNF was coincident with significantly higher peak levels of IL-6 and IL-8 occurring 3–4 h after IHP. Other studies have reported systemic toxicities associated with the use of TNF in ILP or IHP that were attributable largely, if not exclusively, to systemic exposure of TNF secondary to perfusate leak during treatment (10, 13, 14, 18). Thom et al. (10) observed that patients experiencing a perfusate leak of >1% during ILP with TNF and melphalan had significantly greater systemic toxicity associated with higher circulating levels of IL-6 and IL-8. Activation of the fibrinolytic system, as reflected by significant increases in circulating tissue plasminogen activator activity, has been observed after systemic TNF leak in ILP patients (18). Similarly, increases in circulating levels of the soluble TNF receptor are observed after systemic TNF leak during ILP in amounts proportional to the serum TNF up to levels of 1.5 ng/ml (11). TNF-induced hypotension appears to occur when the capacity of released soluble TNF receptors to neutralize circulating TNF levels is exceeded. Under conditions in which serum TNF levels are considerably >1.5 ng/ml after ILP, refractory hypotension with associated hematological, hepatic, and pulmonary toxicities can occur (19, 20). Lidner et al. (13) reported considerable toxicity when performing IHP with melphalan and 30–200 μg of TNF, which resulted in a 22% mortality that was most likely attributable to systemic exposure of TNF and melphalan. These previous data highlight the importance of controlling perfusate leak of TNF during isolation perfusion. The data in this study are consistent with a previous report of 6 patients undergoing IHP using melphalan with or without TNF (21). In that study, data from 3 patients who received 0.4 mg of TNF and 1 mg/kg of melphalan and 3 patients who received melphalan alone showed that systemic TNF levels peaked soon after IHP in the former group and were not detectable in the latter. In addition, perfusate levels of IL-6 increased during IHP only in those receiving TNF, and systemic levels were significantly higher between 2 and 9 h after IHP compared with those who received melphalan alone. The current study demonstrates that with complete vascular isolation of the liver and virtually all other treatment parameters equal, the production of secondary mediators in the liver after IHP with TNF and melphalan may result in subsequent transient hemodynamic alterations not observed with melphalan alone.

IL-6 and IL-8 are inflammatory cytokines with known effects on the cardiovascular system (22–25); whether they are the direct cause of the relatively greater degree of hypotension after IHP in this study is not known. Of note, the differences in blood pressure between those who did or did not receive TNF were clearly transient and did not appear to be a major clinical consequence. No patient treated with TNF required cardiopressor support because of hypotension. Elevated plasma levels of IL-6 have been reported in patients with sepsis and shown to correlate with heart rate, hypotension, thrombocytopenia, and death (22, 23, 26). The mean peak systemic IL-6 level in patients treated with TNF and IHP was >23,000 pg/ml (maximum, >60,000 pg/ml), which is higher than the reported mean levels of patients in septic shock of >10,000 pg/ml (22, 23). Similarly, elevated plasma IL-8 levels have been reported in patients with sepsis and shown to correlate with hypotension and death (24, 27). The mean peak systemic levels in TNF-treated IHP patients (>7,000 pg/ml) is 2-fold greater than plasma levels in patients with lethal septic shock. The correlation between systemic IL-6 and IL-8 levels in this study have also been observed in septic patients (27). The fact that the high circulating levels of IL-6 and IL-8 observed in this study were associated with only transient hemodynamic changes is most

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**Fig. 3** Mean platelet count and serum bilirubin levels in patients after IHP with (n = 15) or without (n = 17) TNF. Platelet levels were significantly lower in patients treated with TNF on days 4 through 7 (P < 0.05), and serum bilirubin levels averaged over days 1 through 7 were significantly higher (P = 0.017) in patients receiving TNF compared with those that did not. There were no differences in baseline values between groups. Bars, SE.
likely attributable to the limited and controlled exposure to TNF during IHP compared with septic individuals with uncontrolled bacteremia and ongoing cytokine production. Our data indicate that hepatic synthesis and release of proinflammatory cytokines may be a major source of endogenous mediators during sepsis and that TNF can stimulate significant hepatic proinflammatory cytokine production clinically.

Although there are limited clinical data, during IHP for in-transit melanoma of the extremity, the use of TNF alone is associated with minimal regional toxicity (28). However, because of the metabolic nature of the liver, the use of TNF alone in IHP has significant toxic effects. In a previous Phase I study of escalating dose TNF administered with 0.2 mg IFN-γ via IHP, dose-limiting coagulopathy was encountered at 2.0 mg of TNF and did not result in any meaningful antitumor activity (12). A subsequent Phase I study of alternating dose escalations of TNF and melphalan was conducted (15), and dose-limiting melphalan toxicity was observed at 2.0 mg/kg (renal and liver) and TNF toxicity at 1.5 mg (coagulopathy). The maximum safe-tolerated dose of 1.0 mg of TNF when used with melphalan in IHP determined in our clinical trials was considerably less than the 3–4-mg dose used in ILP at various institutions and highlights the different tissue tolerances of the protein. In a previous Phase II trial of IHP using TNF and melphalan, significant transient elevations in hepatic transaminases and bilirubin levels were observed (8). Hepatic failure from VOD is a known complication of high-dose chemotherapy with a grave prognosis, manifested initially by progressive elevation in serum bilirubin, followed by ascites and progressive liver failure (29). Although serum bilirubins were significantly higher in patients treated with TNF in this series, there were no other clinical or laboratory indications of hepatic VOD in any patient treated in this cohort. Liver biopsies were not routinely obtained; therefore, the pathological changes responsible for the elevations in bilirubin are not known. However, because of the transient nature of the hyperbilirubinemia, it was presumed to be related more to cholestasis than VOD.

In summary, the data in this study provide additional insights of the pathophysiology of TNF toxicity during isolation perfusion and suggest that the liver may be a significant source of proinflammatory cytokine production secondary to TNF. As in animal models of endotoxin or TNF-induced shock (30, 31), strategies to neutralize the IL-6 or IL-8 may ameliorate the proinflammatory cytokine production secondary to TNF. As more is learned about the pathophysiology of TNF toxicity during isolation perfusion, future studies should take into account the transient nature of the hyperbilirubinemia, when planning the use of TNF in IHP.

REFERENCES