Large Volume Hepatic Freezing: Association with Significant Release of the Cytokines Interleukin-6 and Tumor Necrosis Factor α in a Rat Model

Joachim K. Seifert, M.D.,1,2 Malcolm P. France, B.V.Sc., Ph.D.,3 Jing Zhao, M. Med. Sci.,1 Elaine J. Bolton, Ph.D.,1 Ian Finlay, B.M., B.S.,1 Theodor Junginger, M.D.,2 David L. Morris, M.D., Ph.D.1

1Department of Surgery, University of New South Wales, St. George Hospital, Kogarah, Sydney, New South Wales 2217, Australia
2Klinik für Allgemein- und Abdominalchirurgie, Johannes Gutenberg-Universität, Langenbeckstrasse 1, 55101 Mainz, Germany
3Department of Veterinary Anatomy and Pathology, University of Sydney, Sydney, New South Wales 2006, Australia

Published Online: September 26, 2002

Abstract. Although cryotherapy of liver tumors is generally considered a safe procedure, a syndrome of coagulopathy and fatal multiorgan failure has been observed in some patients and is called the cryoshock phenomenon. Our aim was to establish an animal model of this phenomenon and examine the effects of the basic parameters of freezing or cryotherapy on it. A group of 75 female Sprague-Dawley rats were allocated randomly to five groups: (1) sham laparotomy (n = 15); (2) small (25% liver volume) single freeze (n = 15); (3) small (25% liver volume) double freeze (n = 15); (4) large (50% liver volume) single freeze (n = 15); (5) large (50% liver volume) double freeze (n = 15). Blood samples were collected at different postoperative times, and organs were harvested for histopathology. There was a significant release of tumor necrosis factor-α (TNFα) and interleukin 6 (IL-6) following hepatic freezing, which was greatest in group 5. Postoperative serum cytokine levels were significantly associated with hepatocellular injury, as measured by postoperative serum aspartate transaminase (AST) concentrations. Severe hemoglobinuria and renal injury, as demonstrated by the serum creatinine level and the glomerular neutrophil count, were observed and were greatest in group 5. Hepatic cryosurgery is associated with release of IL-6 and TNFα and renal injury in a rat model. It is likely that the cryoshock phenomenon is another form of the systemic inflammatory response syndrome. Based on the results of this study, it is possibly mediated by cytokines released from the frozen liver tissue. We therefore caution against cryotherapy of large tumor volumes.

Cryotherapy gained importance as a focal therapy for nonresectable primary and secondary liver tumors during the last decade [1-9]. Mortality following cryotherapy is rare, and complications are usually minor [1], although a syndrome of multiorgan failure, severe coagulopathy, and disseminated intravascular coagulation (DIC), similar to septic shock but without evidence of systemic sepsis has been described [5] and is referred to as the cryoshock phenomenon [6]. This syndrome was responsible for the death of two patients following cryoablation of large central liver lesions in the Pittsburgh series [5]. Severe DIC necessitating repeated infusions of fresh-frozen plasma (FFP), cryoprecipitate, platelets, and tranexamic acid was also observed by Guenther et al. in two patients [10]. In a recent survey of the morbidity associated with cryotherapy the cryoshock phenomenon was observed in 21 of 2173 patients following hepatic cryotherapy, with 6 of these 21 patients dying during the postoperative period [11].

We have shown previously that hepatocellular injury, measured by serum aspartate aminotransferase (AST) levels on the first postoperative day, is associated with the duration of freezing and is greater following double freeze-thaw cycles [12]. We have also demonstrated a drop in the platelet count after hepatic cryotherapy, which was associated with the degree of hepatocellular injury [13, 14]. Because the clinical features of cryoshock are similar to those of the systemic inflammatory response syndrome (SIRS) observed with severe sepsis, we hypothesized that the same mediators could be involved. The cytokines tumor necrosis factor-α (TNFα) and interleukin-6 (IL-6) in particular seem to be key mediators of this syndrome [15-17]. The liver is a major source of these cytokines, which probably originate from Kupffer cells [18-21]. Elevated serum levels of TNFα and IL-6 have been demonstrated following liver resection [21-25], hepatic ischemia-reperfusion [26, 27], and gut-derived portal venous endotoxemia [21]. The aim of this study was to assess serum concentrations of the cytokines TNFα and IL-6 following hepatic cryotherapy of various liver volumes using single or double freeze-thaw cycles and to relate these concentrations to the postoperative platelet count and serum AST and creatinine concentrations as well as to postoperative histopathologic renal and lung tissue changes in a rat model.

Materials and Methods

The experimental protocol was approved by the University of New South Wales Animal Care and Ethics Committee.

Animals

A group of 75 female 12- to 16-week-old Sprague-Dawley rats (Animal Resources Centre, Perth, Australia), weighing 206 to
338 g, were used. The animals were kept under controlled temperature (22°C), humidity, and lighting (12-hour light-dark cycles) and were allowed water and standard laboratory food ad libitum.

Surgical Procedure
Anesthesia was induced by inhalational halothane 1% to 2% in oxygen via a mask. The animals were kept on a heating pad and allowed to breathe spontaneously.

The animals were randomly allocated to five treatment groups of 15 animals each: (1) sham laparotomy; (2) laparotomy and cryosurgery of 25% of the liver with a single freeze-thaw cycle; (3) laparotomy and cryosurgery of 25% of the liver with a double freeze-thaw cycle; (4) laparotomy and cryosurgery of 50% of the liver with a single freeze-thaw cycle; and (5) laparotomy and cryosurgery of 50% of the liver with a double freeze-thaw cycle.

Preoperatively, a blood sample of 0.5 ml was collected from the tail vein. For analgesia a subcutaneous injection of buprenorphine (0.3 mg/kg body weight) (Temgesic; Reckitt & Colman PL, Berkshire, UK) was given preoperatively and then every 8 hours postoperatively. Cryosurgery was performed by direct application of a 3 mm cryosurgical probe connected to a liquid nitrogen-based cryotherapy system (LCS 3000 Cryosystem; Spembly, Hampshire, UK) to the liver surface (Fig. 1). The underlying bowel was actively warmed with an infrared lamp. Free access to food and water was allowed immediately after operation.

At 4, 8, and 24 hours postoperatively, with five animals in each group, respectively, the animals were anesthetized again, and a 3 ml blood sample was collected by cardiac puncture as a terminal procedure. Animals were killed immediately by cervical dislocation. Frozen and unfrozen liver, kidneys, and lungs were collected for histopathology. Urine was collected by puncturing the bladder.

Samples
A 200 μl aliquot of each blood sample was collected in an EDTA tube and analyzed for a full blood count immediately using a commercial cell counter (Abbott Cell-Dyn 3500 System, Sydney, New South Wales, Australia). The remaining blood was collected in endotoxin-free tubes; the serum was separated and stored at −70°C before analysis.

Cytokine Assays and Biochemical Measurements
Serum concentrations of TNFα and IL-6 were determined with commercially available enzyme-linked immunosorbent assay (ELISA) kits (Cytoscreen, Biosource International, Camarillo, CA, USA). For TNFα the lower limit of detection was 4 pg/ml, and the coefficients of variation were 2.6% to 2.7% (intraassay precision) and 3.5% to 4.3% (interassay precision) for the kits used. For TNFα the lower limit of detection was 31 pg/ml, and the coefficients of variation were 4.5% to 6.3% (intraassay precision) and 8.3% to 9.3% (interassay precision) for the kits used. Serum AST, creatinine, and hemoglobin were determined by standard laboratory techniques in the postoperative serum samples only.

Urine Analysis
In some animals with macroscopically blood-stained urine, the urine was tested for the presence of heme (Ecur-Test strips; Roche Diagnostics, Sydney, NSW, Australia). If positive, urine was centrifuged through a filter (Centriprep-30 microconcentrator; Amicon, Millipore, Sydney NSW, Australia) with a 30,000 MW cutoff at 3200 rounds per minute for 15 minutes to differentiate myoglobin and hemoglobin. In addition, native polyacrylamide gel electrophoresis (PAGE) (Bio-Rad, Sydney, NSW, Australia) of diluted urine and serum samples with myoglobin and hemoglobin standards was performed. The concentration of hemoglobin in the urine was assessed with spectrophotometry at 546 nm (Abbott Cell-Dyn 3500 System).

Histopathology
Frozen and unfrozen liver, kidney, and lung specimens were fixed in 10% phosphate-buffered formalin solution. They were then embedded in paraffin, sectioned, and stained with standard hematoxylin and eosin. The stained sections were reviewed by an experienced animal histopathologist.

In kidney sections, the presence of proteinuria was assessed histologically by examining tubular lumens in 10 fields of renal cortex along the length of a longitudinal section of kidney under ×200 magnification. The number of fields containing one or more tubules with hyaline casts, red-orange (hemoglobin-like) crystals, or finely granular protein in the lumen was then recorded, assigning a score of 0 to 10 for each rat. The number of neutrophils (identified by their relatively small size, their dark distinctly lobed nuclei, and their pale to moderately eosinophilic cytoplasm) was counted in 30 randomly selected glomeruli examined under ×400 magnification. To avoid observer bias, sections were "blind"-labeled so the histopathologist was unaware of the treatment any rat had received. In addition, glomerular neutrophil counts were obtained and the slides were then examined a second time for independent assessment of tubular protein.

Statistical Analysis
The data are presented as means (± SEM). Because data sets were too small to assume normal distribution, nonparametric tests were used. The Kruskal-Wallis H-test was used to test for overall significant differences between the five treatment groups. If it was significant at a particular time point, the Mann-Whitney U-test was used to check for significant differences between individual groups. Pearson's correlation was used to assess associations between variables. Statistical analysis was performed with SPSS for Windows, version 7.0 (SPSS-GmbH, Munich, Germany).

Results
Five animals died before the end of the required follow-up. Each of the five animals had undergone cryotherapy of 50% of the liver volume, and four of the five had had double freeze-thaw cycles. No animal after freezing 25% of the liver volume or sham laparotomy died before the end of follow-up (Table 1). Four animals had preoperatively elevated serum TNFα levels (> 20 pg/ml) and were excluded from the analysis, as an unidentified infection as a cause for TNF elevation could not be excluded. Table 1 summa-