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TNF-α and heat-shock protein gene expression in ischemic-injured liver from fasted and non-fasted rats. Role of donor fasting in the prevention of reperfusion injury following liver transplantation

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Abstract We have previously shown that livers from long-term-fasted rats acquire tolerance to warm ischemic injury following transplantation, despite the fact that fasting depletes glycogen and ATP from the liver. The precise mechanism of the protective effect induced by donor fasting, however, is still a matter of controversy. In this experiment we determined heat-shock protein (GRP78) mRNA expression in livers during long-term fasting and TNF-α mRNA expression in transplanted livers exposed to warm ischemia. We also measured the concentration of TNF-α by ELISA in the ascitic fluid of fed and fasted rats injected intraperitoneally with zymosan to investigate why livers from fasted rats tolerate ischemic injury better. There seemed to be a positive correlation between GRP78 mRNA expression and survival. TNF-α secretion into the ascitic fluid of fasted rats was markedly suppressed, and fasting donor animals induced cytoprotective substances, such as GRP78, in the liver. These factors may contribute to the tolerance to ischemic injury produced by donor fasting.

Key words Liver transplantation · Fasting · Heat shock protein · GRP78 · TNF-α

Introduction

Livers from fasted animals have previously been thought to be more vulnerable to ischemic injury than livers from fed donors [1–4], but we have demonstrated that, as well as the liver, the heart and pancreas of long-term-fasted rats acquire tolerance to warm and cold ischemic injury following transplantation [5–7]. We have hypothesized that the sinusoidal lining cells, particularly Kupffer cells, plays an important role in the mechanism of the protective effect on the liver graft induced by prolonged donor fasting. Kupffer cell activation following ischemia/reperfusion can lead to the production of a large number of potentially cytotoxic substances such as TNF-α, oxygen free radicals, proteases, and arachidonic acid metabolites that could adversely affect hepatocytes or induce an inflammatory response and cause neutrophil infiltration.

Heat-shock protein (HSP) is present in prokaryotic and eukaryotic cells and increases in response to such stresses as heating [8–10], ischemia [11], hypoglycemia [8, 9], alcoholic intoxication [12], immune response [8, 9], and cell development [11, 13]. HSP plays an important role in the intracellular transport of proteins (chaperone function), protection of protein structure, and the switching of certain receptors, and the heat shock response seems to be a way by which cells protect themselves against different forms of stress.

In this study, we determined the expression of glucose-regulated protein 78 (GRP78), mRNA, a member of the 70-kDa HSP family, induced in the liver by long-term fasting, and TNF-α mRNA in transplanted liver grafts exposed to warm ischemia. We also examined the TNF-α response in rats following zymosan administration to investigate whether long-term fasting suppresses macrophage function.

Materials and methods

Animals

Male, Brown Norway (BN) rats weighing 180–250 g were obtained from Seiwa Experimental Laboratory (Fukuoka, Japan). BN rats were either fed or fasted (drinking water only) for up to 168 h. Their liver was then harvested, subjected to warm ischemia, and transplanted orthotopically into normally fed rats.

Orthotopic liver transplantation

The method of hepatectomy and orthotopic liver transplantation was based on the cuff technique described by Kamada and Calne [14]. The liver graft was stored in UW solution for 60 min at 37 °C. The outcome of liver transplantation was judged on the basis of survival for at least 7 days. Preliminarily, the effluent was collected after 45 min of warm ischemia and the concentrations of glutamic-pyruvic transaminase (GPT) and lactate dehydrogenase (LDH) were measured.

Examination of TNF-α and GRP78

Rats were serially sacrificed during fasting, and the liver was quickly removed and stored in liquid nitrogen until homogenized in guanidinium thiocyanate solution in a homogenizer. Livers were also biopsied 2 h after transplantation to measure TNF-α mRNA, and the intensity of mRNA expression in fasted liver grafts and non-fasted liver grafts was compared. RNA was extracted by the AGPC (acid guanidinium-thiocyanate-phenol-chloroform) method [15], and a reverse transcription polymerase chain reaction (RT-PCR) was performed using a TaKaRa RNA Kit (Takara Biochemicals, Japan). The following primers were designed: *TNF-α* upstream 5′CCACGCCGTAGCAAACCACCAAG 3′, downstream 5′GATCCACTCAGGCATCGACATTCG 3′, *GRP78* upstream 5′TCGGATCCGG(ACGT)GGAA(ACGT)GA(AG)GA (TC)TT(TC)GA(TC) 3′; downstream 5′GCGAATTC(GA)AA (ACGT)GT(ACGT)AC(TC)TC(AGT)AT(TC)TG3′

Samples (1 μg each) were incubated in 20 μl 1 × reverse transcription buffer consisting of 5.0 m*M* $MgCl_2$, 1.0 m*M* dNTP mixture, 1.0 U/μl RNase inhibitor, 0.25 U/μl reverse transcriptase and 2.5 μ*M* random 9 mers. The reverse transcription reaction mixture was incubated as follows: 30 °C for 10 min, 42 °C for 30 min, 99 °C for 5 min, and 5 °C for 5 min. For PCR, the reaction mixture was brought to a final volume of 100 μl and the $MgCl_2$ concentration adjusted to 2.5 m*M*, and 2.5 U Taq DNA polymerase and primers added. PCR was carried out as follows: denaturing at 94 °C for 0.5 min, annealing at 55 °C for 1.5 min and extension at 72 °C for 1.0 min for 30 cycles. The cDNA obtained from the RT-PCR amplification procedure was subjected to electrophoresis on a 1.2% agarose gel, stained with ethidium bromide, and photographed using Polaroid film. The PCR signal intensities were quantitated by scanning the negatives with a scanner and analyzing the scans with Quantity One (PDI, USA) software.

TNF-α concentration in the ascitic fluid

Zymosan (5 mg/kg; Sigma Chemical Company, St. Louis, Mo.) was administered intraperitoneally to (nontransplant) rats that were either fed or fasted for 4 days. At various times after the injection, the rats were anesthetized with ether, and the peritoneal cavity was lavaged with 5 ml ice-cold saline. All peritoneal lavage fluid was collected and centrifuged, and the supernatant was stored at −70 °C for later determination of the TNF-α concentration using a Factor-Test-XTM Mouse TNF-α ELISA kit (Genzyme, USA).

Table 1 Enzyme levels in the effluent from warm ischemic rat livers. After 45 min of warm ischemia, rat livers were flushed with 5 ml cold saline and the concentrations of GPT and LDH were measured. The values are means with standard error, and *n* indicates the number of livers evaluated. Statistical comparison of the means was performed using the unpaired *t*-test.

	GPT (U/l)	LDH (U/l)
Fed	981 ± 112 ($n = 4$)	9430 ± 660 ($n = 4$)
Fasted	539 ± 78.2 ($n = 6$)	2620 ± 467 ($n = 6$)
P-value	0.001	0.0005

Statistical analysis

Statistical analysis was performed using the unpaired *t*-test and Fisher's test. A probability value < 0.05 was considered statistically significant.

Results

Enzyme levels in the effluent

The GPT and LDH contents of the effluent from livers after 45 min exposure to warm ischemia are shown in Table 1. The GPT concentrations in livers from fed and 4-day fasted rats were 981 ± 112 U/l and 539 ± 78.2 U/l, respectively, and the LDH concentrations were 9430 ± 660 U/l versus 2620 ± 467 U/l, respectively. The increased GPT and LDH levels in the effluent from the fed rats were considered to have been a result of plasma membrane injury occurring during the warm ischemia.

Survival and mRNA gene expression

The outcome after liver transplantation and GRP78 mRNA expression are shown in Fig. 1. All of eight recipient rats that had received a liver from a fed rat died within 24 h. However, the survival rate was increased to 50% when the liver had been obtained from a 2-day fasted rat, and when the donors had been fasted for 3 days, 89% (8 of 9) of the animals survived for 7 days. The expression of GRP78 mRNA was induced after 48 h of fasting, and was maintained thereafter. There seemed to be a positive correlation between GRP78 mRNA expression and survival. Livers from fasted rats showed a somewhat stronger expression of TNF-α mRNA 2 h after liver transplantation, than livers from fed rats (Fig. 2), but the difference between the two groups was not statistically significant.

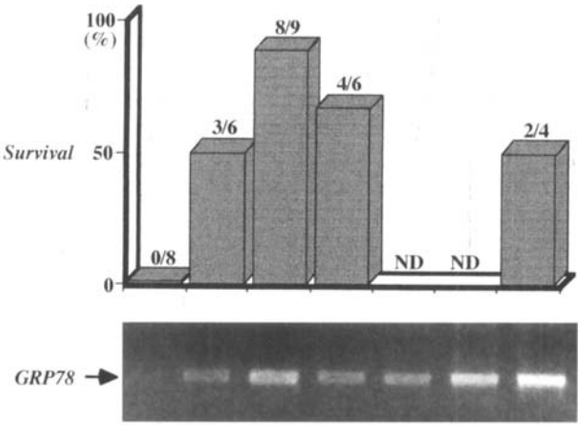

Fig. 1 Outcome after liver transplantation, and GRP78 mRNA expression. Long-term fasting induced GRP78 mRNA in rat livers, which acquired tolerance to preservation/reperfusion injury (*ND* not determined)

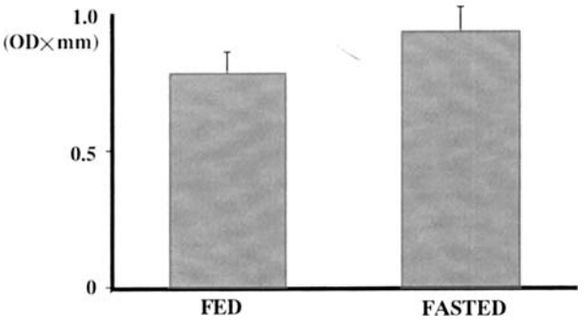

Fig. 2 TNF-α mRNA expression in liver grafts 2 h after transplantation. Livers from fasted rats showed slightly stronger expression than livers from fed rats, but the difference was not significant

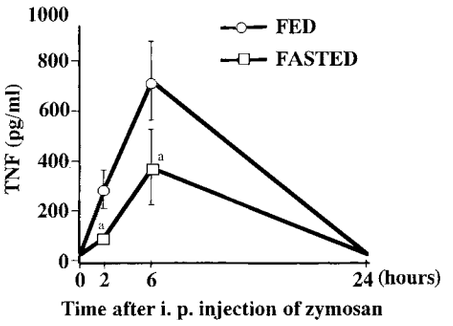

Fig. 3 TNF-α production during acute inflammation. Acute inflammation was induced by an i.p. injection of 5 mg/kg zymosan. TNF-α production was suppressed in the fasted rats as compared with to the fed rats ($P < 0.05$) (*open circles* fed, *open squares* fasted)

TNF-α assay

The TNF-α concentrations in the ascitic fluid from the fasted rats treated with zymosan were lower than in the ascitic fluid from fed rats, as shown in Fig. 3. In rats fed a standard diet, the TNF-α concentrations were 286 ± 75.6 pg/ml 2 h after and 719 ± 157 pg/ml 6 h after i.p. injection of zymosan. The TNF-α concentrations in the ascitic fluid of 4-day fasted rats were 94.5 ± 10.8 pg/ml 2 h after and 374 ± 151 pg/ml 6 h after injection. Thus, TNF-α secretion in the fasted rats was significantly suppressed compared with the fed rats ($P < 0.05$).

Discussion

Our studies clearly show that the survival of liver grafts from fasted animals is far better than the survival of livers from fed animals, and this is presumably due to induction of HSP expression and suppression of Kupffer cell function in the liver graft.

We have previously shown that the livers of rats fasted for 4 days weigh 42% less than normal livers, and that hepatic glycogen and ATP contents are 5% and 36%, respectively, less than their contents in normal livers from fed animals [5]. In view of the well-established fact that the higher the energy status of a liver graft, the better the functional recovery, our experimental findings were unexpected, and we have no plausible explanation at this moment why such nutritionally poor livers showed better survival after preservation and transplantation. Are biochemical substances such as ATP and glycogen in the liver graft before transplantation not very important to obtaining a well functioning liver?

One reason why nutritionally poor livers showed better survival after transplantation may be that the above-described biochemically disadvantageous events were masked by other more advantageous factors such as cytoprotective substances induced in the livers. It is hypothesized that long-term fasting alters cellular metabolism and induces several different cytoprotective substances such as glycine [16], alanine [17], ketone bodies and HSP in the cells.

Recent studies have investigated possible mechanisms by which cells may be protected from further injury or recover from injury already sustained [18]. HSP, in particular, is thought to be an important cytoprotective protein in the reactivation of protein denatured by ischemia. HSP has been demonstrated to be induced in cells when they are exposed to hyperthermia [8–10] or to a variety of metabolic stresses such as anoxia [11], fasting [8, 9] and metabolic inhibitors [19], among others. HSP acts as a molecular chaperone, binding to unfolded proteins and refolding them via an ATP-dependent mechanism, providing cellular protection [20–22].

Thus, HSP may contribute to the correct protein folding during preservation and after reperfusion, and be crucial for cellular survival after ischemia.

Another reason why nutritionally poor livers showed better survival after transplantation may be that preservation/reperfusion injury may not be directly related to the energy status of the parenchymal cells, but principally to the function of nonparenchymal cells such as tissue macrophages (Kupffer cells). This hypothesis is based on our findings that the hepatocyte membrane was better preserved in the fasted livers (low GPT and LDH levels) and that TNF-α production was more suppressed in fasted rats. Paradimitriou and Bruggen have shown that prolonged malnutrition (fasting) decreases phagocytosis and diminishes macrophage division in the liver [23]. Fusaoka et al. have demonstrated that livers from fasted rats require less oxygen during reperfusion [24]. These findings support our hypothesis and suggest that inhibition of Kupffer cell function plays an important role in the suppression of preservation/reperfusion injury.

In conclusion, fasting donor animals for a long period induces cytoprotective substances in the liver and markedly suppresses TNF-α production. These factors may contribute to the tolerance to ischemic injury produced by donor fasting.

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