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ORIGINAL ARTICLE

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Expression of HSP 70 as a potential prognostic marker for acute rejection in human liver transplantation

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Abstract In experimental models, the synthesis of heat shock protein 70 (HSP 70) has been recognized as an intracellular response to ischemia and reperfusion, insults inherent to transplantation. In this study, the HSP response in early stages of human liver transplantation was investigated. HSP 70 mRNA expression was detected by means of reverse transcriptase (RT)-PCR in liver biopsies (n = 28) and in cells obtained from the organ perfusate (n = 14) following cold preservation. The expression of HSP 70 differed substantially between individuals. Retrospective analysis revealed a close correlation of the amount of HSP 70 mRNA in perfusate cells and biopsies with the onset of organ dysfunction due to early graft rejection. Patients with early graft rejection had a significantly lower amount of HSP 70 mRNA than patients without rejection. These results suggest a protective role of HSP 70 expression. Low levels of HSP 70 may, therefore, represent a prognostic marker for early graft rejection.

Key words Heat shock Protein 70, acute rejection, liver transplantation · Rejection, acute, heat shock protein 70 · Liver transplantation, heat shock protein 70, rejection, cold preservation; PCR

Introduction

Heat shock proteins are a group of highly conserved intracellular proteins produced by most organisms in response to hyperthermia or other environmental insults. Since these proteins assist in the proper folding and stabilization of other proteins, they have been designated as "molecular chaperones". In myocardial ischemia and after experimental liver transplantation in rats, induction of heat shock protein 70 (HSP 70) has been found to occur after ischemic periods and is strongly enhanced during reperfusion [16, 17]. Since high levels of HSP 70 mRNA in rat hearts preconditioned by repeated ischemia correlate with a reduction in subsequent tissue damage, it has been suggested that these protein chaperones have a protective character [3]. This view is supported by the finding that transgenic mice overexpressing HSP 70 show an increased resistance to ischemic myocardial damage [10]. HSP 70 has been reported to be induced in response to hepatic ischemia in rats [7]. Although the precise mechanism of HSP 70 action with regard to ischemia/reperfusion protection is still unclear, its beneficial effects may be based on protein refolding, especially during the reperfusion period.

It has been shown in numerous studies that cytokines are involved in the inflammatory syndrome that can result from liver transplantation. In particular, TNFα and IL-8 were found to be induced during liver transplantation. The degree of their synthesis correlates with clinical complications such as rejection or infection [11, 18]. Antibodies to TNFα have proved to be protective in hepatic reperfusion injury [2]. It is, therefore, conceivable that HSP 70, or other chaperones may constitute an intracellular counterpart to proinflammatory cytokines with the potential to limit an exaggerated inflammatory response. The induction of hepatic heat shock proteins has not been investigated in clinical liver transplantation. We investigated the expression of HSP 70 by semi-

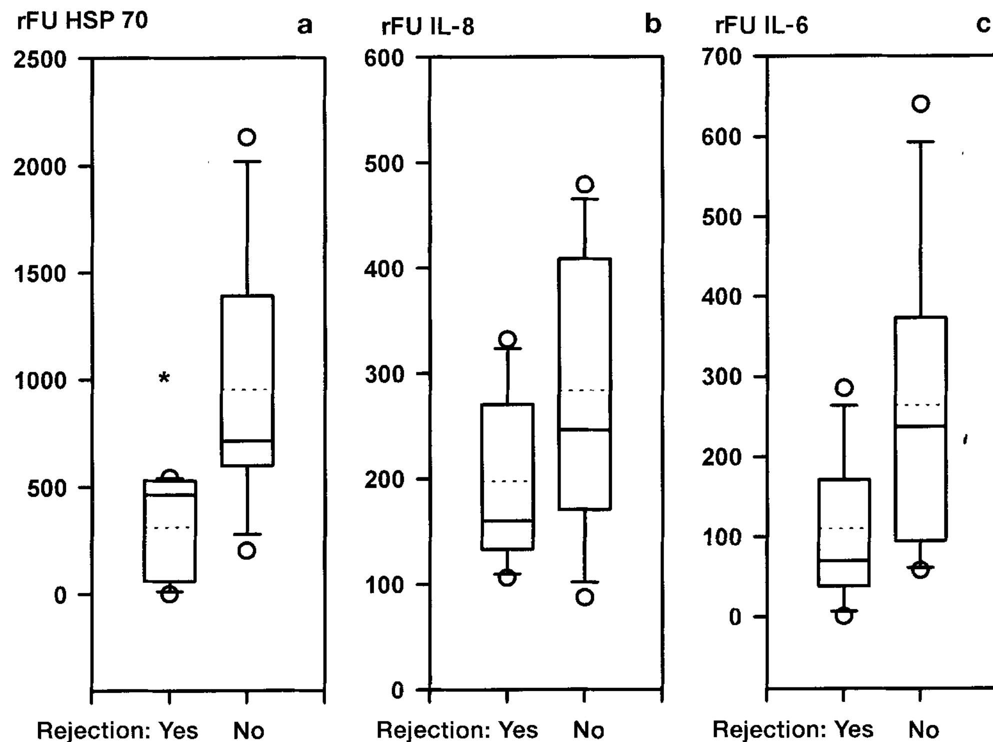

Fig. 1 a–c HSP 70, IL-8, and IL-6 mRNA levels in the cells obtained from the cold perfusate of 14 patients, 7 with and 7 without subsequent allograft rejection. HSP 70, IL-8, and IL-6 are expressed as relative fluorescence units (rFU), normalized on the β-actin mRNA level of the individual sample. The *box* shows the 25th–75th percentiles, the *line range* represents the 10th–90th percentiles. *Open circles* represent single values out of the 10th or 90th percentile. $^{*}P < 0.05$ (two-tailed Student's t test)

quantitative RT-PCR in liver biopsies and in cells obtained from the storage solution, both taken at the end of the cold preservation period before transplantation. These measurements were compared with cytokine expression and clinical parameters.

Material and methods

Patients

Liver biopsies of 28 patients and liver perfusates of 14 patients undergoing liver transplantation at the local transplantation center were included in this study. The study protocol was approved by the ethics committee of the university.

Cells from the cold perfusate

The first 10 ml of the cold storage solution that was flushed out of the hepatic veins during back table preparation of the donor liver (at a median time of 8 h after organ explantation and cold storage) was pelleted by centrifugation (5 min at 700 g). The cell pellet was washed once with sterile PBS and stored at –80 °C. Between 2 and 20* 10^6 cells were obtained and used for RNA isolation.

Liver biopsies

Liver biopsies were obtained by fine needle biopsy at the end of the cold preservation period and about 1 h after reperfusion (0 biopsy). Biopsies were stained with hematoxylin and eosin and evaluated histologically. The biopsies for RNA isolation were immediately frozen in liquid nitrogen.

Diagnosis of acute rejection

Acute rejection was diagnosed by clinical parameters, proven by histological assessment of liver biopsies, and graded from A1 to A3 according to the Hannover criteria [8]. Biopsies were obtained in cases of clinical suspicion of rejection or graft dysfunction.

RNA isolation and RT-PCR

Cell pellets or biopsies were thawed in guanidine thiocyanate buffer (GTC). Biopsies were homogenized and RNA was isolated using a commercially available extraction kit (RNeasy Qiagen, Düsseldorf, Germany). The amount of RNA was quantified by measuring the optical density at 260 nm. One microgram of total RNA was subjected to reverse transcription (Superscript, BRL, Eggenstein, Germany) with 25 pmol of oligo-dT-primer (Perkin Elmer, Weilerstadt, Germany) following the manufacturer's instructions. Semiquantitative PCR was achieved by using serial dilutions of cDNA and a minimum of cycles to obtain a PCR product so that the amount of the product reflected the quantity of the cDNA and mRNA, respectively, present in the preparation. The PCR product was analyzed after separation by 1.5 % agarose gel electrophoresis and ethidium bromide staining. The amount produced was quantified using a Fluor-Imager (Molecular Dynamics, Krefeld, Germany). Each cytokine was measured twice in two different cDNA dilutions. Very low amounts of the PCR product were controlled by increasing the amount of cDNA and the number of cycles. All PCR products measured were normalized to the amount of β-actin in each sample. β-Actin was also determined in the linear range of the reaction mea-

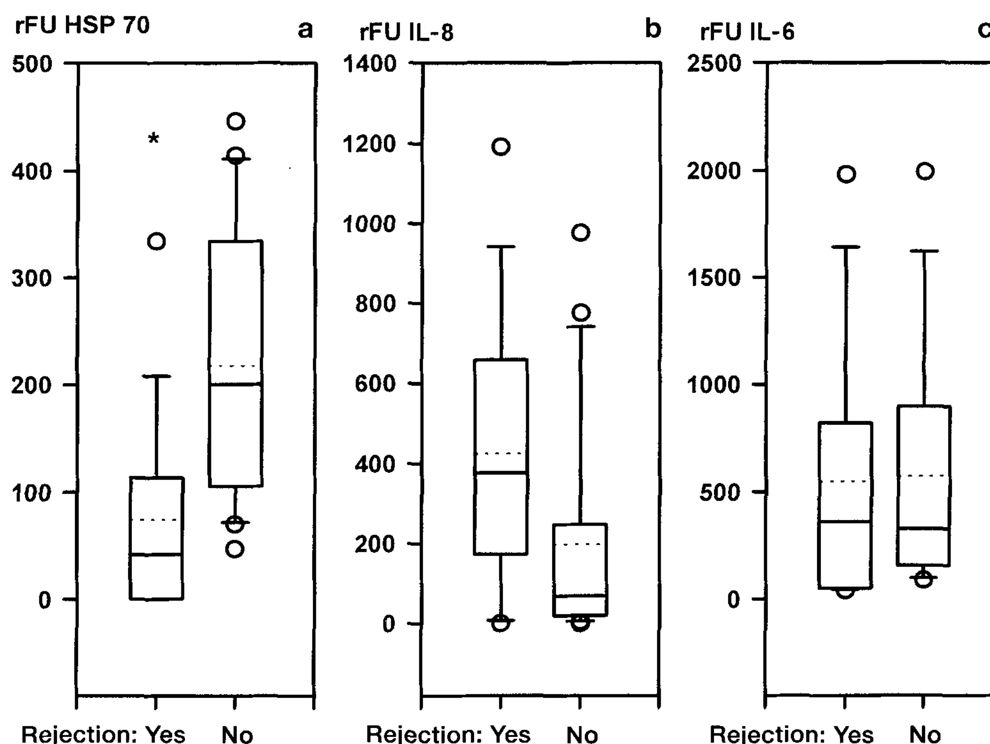

Fig. 2 a–c HSP 70, IL-8, and IL-6 mRNA levels in liver biopsies at the end of the cold preservation period from 16 patients without and 12 patients with subsequent acute allograft rejection. HSP 70, IL-8, and IL-6 are expressed as relative fluorescence units (rFU), normalized on the β-actin mRNA level of the individual biopsy. The *box* shows the 25th–75th percentiles, the *line range* represents the 10th–90th percentiles. *Open circles* represent single values out of the 10th or 90th percentile. $^{*}P < 0.05$ (two-tailed Student's t-test)

sured in two different cDNA concentrations. All values were expressed as relative units per β-actin.

The following primers were used: 5'TGAAGTCTGACGT GGACATC and 5'ACTCGTCATACTCCTGCTTG for β-Actin [8], 5'TGTGAAAGCAGCAAAGAGGC and 5'TGGGAGCGA ATGACAGAGGGT for IL-6 [21], 5'ACATACTCCAAA CCTTTCCAC and 5'TCTTCAAAAACTTCTCCACAA for IL-8 [15]; and 5'TGTGGCTTCCTTCGTTATTGG and 5'GCCAG CATCATTCACCACCAT for HSP 70 [4]. The expected size of the PCR products was 229 bp for the IL-6 primers, 245 bp for the β-actin primers, 168 bp for the IL-8 primers, and 342 bp for the HSP 70 primers. In the mononuclear cells of the perfusate as well as in the cold biopsy the mRNA of HSP 70 could be detected by means of RT-PCR after 35 and 40 PCR cycles, respectively. The mRNA of IL-6 and IL-8 could be detected in perfusate cells and biopsies in almost all patients after 35 PCR cycles.

Statistics

For statistical evaluation, a two-tailed Student's t test was used. If a normality test failed, a Mann-Whitney rank-sum test was applied (evaluation of AST serum levels). A P value equal to or below 0.05 was considered statistically significant. Correlation coefficients were calculated by Pearson correlation.

Results

The semiquantitative analysis of the PCR products obtained from biopsies and from cellular material perfusates revealed marked interindividual differences with regard to the levels of mRNA expressed for HSP 70. When the individual HSP 70 expression in perfusate cells was compared to the patients' later clinical course with regard to the onset of an acute allograft rejection within the first 30 postoperative days, two groups could be distinguished. Patients undergoing acute early graft rejection within the first 30 days after transplantation showed significantly lower HSP 70 expression than patients without transplant rejection. Analogous to that, the expression of HSP 70 mRNA in donor liver biopsies taken after cold storage was low in specimens of patients with acute rejection and high in patients without rejection (Figs. 1a, 2a). No correlation, however, was observed between HSP 70, IL-6, or IL-8 expression during cold ischemia and complications such as local infection of the liver (CMV infection $n = 5$, hepatitis B or C $n = 1$, cholangitis $n = 2$) or systemic septic events ($n = 5$). In contrast to HSP 70, the levels of IL-8 mRNA in the perfusate cells and biopsies did not show any significant difference between the patients with and without allograft rejection, although there seemed to be a tendency towards higher IL-8 expression in donor livers with subsequent rejection (Figs. 1b, 2b).

The mRNA expression of IL-6 also showed obvious interindividual differences. Although the correlation between the clinical course and IL-6 mRNA expression was not statistically significant, there was a trend towards lower IL-6 mRNA levels in the perfusate of livers

Table 1 Clinical details of patients with and without acute rejection periods

Patients	Without rejection (uneventful course)	Acute rejection	*P*
Allograft rejection	–	A1: $n = 11$ A2: $n = 5$	–
Age of the recipient	50.7 ± 9.8	43.1 ± 10.4	NS
Cold ischemia time (h)	8.5 ± 2.3	8.8 ± 4.5	NS
Warm ischemia time (min)	52.0 ± 11.5	48.2 ± 17.6	NS
Number of erythrocyte concentrates given	9.8 ± 11.1	13.7 ± 18.3	NS
Sex of the recipient: male/female	8/10	10/5	NS
Histological changes in biopsies after reperfusion	No changes: $n = 2$ Mild reactions: $n = 11$ Moderate/severe reactions: $n = 2$	No changes: $n = 2$ Mild reactions: $n = 7$ Moderate/severe reactions: $n = 1$	–
Serum AST (U/dl) peak on days 1–3	728 ± 651	1195 ± 2145	NS
on day 5	34 ± 22	113 ± 206	< 0.05
on day 10	17 ± 15	38 ± 31	< 0.05
Serum LDH (U/dl) peak on days 1–3	1801 ± 1052	2101 ± 3555	NS
Serum bilirubin (mg/dl) on day 5	6.7 ± 5.6	9.2 ± 4.2	NS
on day 10	6.6 ± 7.5	13.6 ± 8.8	< 0.05

that were acutely rejected in the postoperative course (110 ± 100 relative units IL-6/β-actin with rejection vs 264 ± 205 relative units IL-6/β-actin without rejection; Fig. 1c). In the biopsies obtained at the end of cold storage, no significant differences in IL-6 expression were observed (Fig. 2c).

Allograft rejection was diagnosed by histological evaluation of biopsies obtained upon clinical suspicion of a graft rejection [9]. Patients with or without graft rejection could not be differentiated as far as duration of cold or warm ischemia or sex and age of the donor or recipient was concerned (Table 1). The mean maximum AST serum level during the first 3 days after transplantation was higher in the patients who developed an acute rejection than in those without rejection; however, this difference was not statistically significant ($P = 0.259$, Table 1). Moreover, there was no significant correlation between the expression of HSP 70 during cold ischemia and the peak AST level ($r = 0.13$). In addition, no correlation between maximum AST or LDH serum levels within the first 3 days after transplantation and the duration of either cold preservation (AST/LDH vs cold ischemia $r = 0.275/-0.049$) or warm ischemia (AST/LDH vs warm ischemia $r = 0.29/0.318$) was determined. Furthermore, the histologically observed signs indicating preservation injury in the biopsy obtained after reperfusion were not reflected in corresponding increases in serum AST or LDH. Bile flow or bile acid concentrations could not be routinely considered for assessment of liver function after transplantation since a T drain was inserted in only 50% of the patients. These findings illustrate the difficulties in quantifying preservation and reperfusion injury.

Death of the organ donors was predominantly caused by intracerebral bleeding, stroke, or brain trauma, and only one donor died after a status asthmaticus. There was no obvious relationship between the cause of death of the donor and the onset of rejection in the recipient. Therefore, the available clinical data are not sufficient to relate the extent of HSP 70 expression after cold storage to donor history.

The patients suffering from acute allograft rejection also showed reduced liver function, as determined by significantly higher serum AST and bilirubin levels on days 5 and 10 after transplantation (Table 1). The histological evaluation of the liver biopsies obtained approximately 1 h after reperfusion (0 biopsies) did not show any differences between the two groups as far as histologically detectable cell damage is concerned. Mostly minor or no ischemic damage to the liver tissues was diagnosed in both groups (Table 1). The differences in HSP 70 expression during cold ischemia were, therefore, not reflected in histological changes. The mean cold preservation time was 8.6 ± 3.6 h (SD) and did not correlate with the expression of HSP 70.

Discussion

A number of investigators have previously reported that HSP 70 increases as a result of ischemia and subsequent reperfusion in experimental models [12]. In order to determine whether this also holds true in human hepatic ischemia during liver transplantation, we analyzed mRNA for HSP 70 in cellular material of the effluent and in biopsies of donor livers after cold preservation. The severity of preservation injury has been found to

be associated with a higher risk of subsequent acute allograft rejections. This has been shown by correlating serum levels of liver enzymes (e.g. ALT) early after transplantation with the rate of graft rejections [6]. In the present study, no significant correlation was found between maximum AST serum levels after transplantation and acute rejection episodes. RT-PCR analysis of cellular effluents and biopsies obtained after cold preservation of human donor livers revealed that HSP 70 mRNA is expressed in transplants and is detectable subsequent to cold preservation before transplantation and reperfusion of the organ. Moreover, retrospectively it was found that high levels of HSP 70 mRNA correlate with a lower incidence of acute rejection periods later on. Therefore, elevated levels of HSP 70 during cold preservation may protect the liver from ischemia and reperfusion damage. Evidence to support this suspected protective effect of HSP 70 was found by Currie et al., who reported that HSP 70 is expressed preferentially at the border of the ischemic area in myocardial infarction, and who concluded that it thereby limits the extent of the infarct [3]. Ischemic preconditioning is known to provide protection against a subsequent myocardial infarction and is accompanied by an increase in HSP 70 expression [16]. It has also been shown that anaerobic metabolism strongly induces HSP 70 expression in ischemic rat hearts [12]. Finally, overexpression of HSP 70 in transgenic mice has been found to reduce the susceptibility for ischemic injury [10]. Thus, HSP 70 expression appears to represent an endogenous protective mechanism against ischemic injury.

Schiaffonati et al. reported that HSP 70 is barely detectable in rat livers during the ischemic period but that it is strongly enhanced after reperfusion [14]. In contrast to this finding, we observed increased mRNA levels of HSP 70 already before reperfusion. A possible explanation for these discrepant findings could be the substantially longer ischemic periods during human liver transplantation (the mean cold preservation time in this study was > 8 h). On the other hand, the trauma or disease that led to the death of the organ donor may have resulted in induction of HSP 70. Hyperthermia and ischemic periods are very likely to occur in the donor before organ explantation. In mice, induction of HSP 70 could not be detected until 24 h after the injection of endotoxin [20]. This suggests a delayed response of HSP 70 to an inflammatory stimulus. Hence, when searching for a cause for elevated mRNA levels of HSP 70 in liver biopsies and perfusate cells at the end of the cold preservation period, one should not only consider a longlasting ischemia, but also possible host deterioration before organ explantation. The fact that the expression of HSP 70 does not correlate with the duration of the cold storage period may also support the possibility of HSP 70 induction before organ explantation. Since, in the present study, all patients died of cerebral injuries or diseases, one may conclude that events preceding organ explantation do not necessarily determine the extent of HSP 70 expression. In order to elucidate whether HSP 70 expression pre-exists in the donor organ or is induced during the cold preservation period, future studies should include biopsies taken prior to organ harvesting.

Other possible inflammatory mediators, such as IL-6 and IL-8, have been investigated in liver transplantation [9, 13]. IL-8 serum levels have been reported to be increased during acute rejection, bacterial infection, and CMV disease as early as 2 days before the appearance of clinical symptoms [18]. We did not observe a significant association of IL-8 expression and acute rejection, but merely a tendency towards higher IL-8 expression in donor organs with subsequent rejection. However, in this study, a very early phase of liver transplantation (i.e., the cold preservation period) was analyzed. High systemic and bile IL-6 levels after human liver transplantation have been found to correlate with the onset of acute graft rejection [19]. Yet, Bishop et al. reported that low levels of IL-6 mRNA occurred in liver biopsies during the rejection period compared to high levels in biopsies of nonrejected organs [1]. Since IL-6 has been said to possess potentially anti-inflammatory activity via the induction of the acute phase reaction [5], locally expressed IL-6 in the transplanted organ itself may be beneficial.

In conclusion, a variety of endogenous protective mechanisms may exist, including the stress protein HSP 70. To fully ascertain its prognostic value, a larger number of patients needs to be examined. In addition, the situation after reperfusion and during the rejection period should be analyzed in the same fashion to determine whether HSP 70 remains low during acute rejection periods and high in uneventful courses, which would support its role as a protective factor. However, before one begins manipulating organ donors in order to examine HSP induction in human liver transplantation, such procedures need to be extensively evaluated in experimental studies. The methodology outlined here may represent an approach to identifying donor organs at risk of acute allograft rejection and, thus, contribute to organ evaluation. More generally, the identification of factors involved in the local response of the donor organ by means of sensitive techniques such as RT-PCR early during transplantation may provide some insight into the mechanisms leading to organ dysfunction and early graft rejection.

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References

1. Bishop GA, Rokahr KL, Napoli J, McCaughan GW (1993) Intragraft cytokine mRNA levels in human liver allograft rejection analyzed by reverse transcription and semiquantitative polymerase chain reaction amplification. Transpl Immunol 1: 253–261
2. Colletti LM, Remick DG, Burtch GD, Kunkel SL, Strieter RM, Campbell DA Jr (1990) Role of tumor necrosis factor-alpha in the pathophysiologic alterations after hepatic ischemia/reperfusion injury in the rat. J Clin Invest 85: 1936–1943
3. Currie RW, Tanguay RM, Kingma JG Jr (1993) Heat-shock response and limitation of tissue necrosis during occlusion/reperfusion in rabbit hearts Circulation 87: 963–971
4. Dworniczak B, Mirault ME (1987) Structure and expression of a human gene coding for a 71 kD heat shock "cognate" protein. Nucleic Acids Res 15: 5181–5197
5. Geiger T, Andus T, Klapproth J, Hirano T, Kishimoto T, Heinrich PC (1988) Induction of rat acute-phase proteins by interleukin 6 in vivo. Eur J Immunol 18: 717–721
6. Howard TK, Klintmalm GB, Cofer JB, Husberg BS, Goldstein RM, Gonwa TA (1990) The influence of preservation injury on rejection in the hepatic transplant recipient. Transplantation 49: 103–107
7. Hutter MM, Sievers RE, Barbosa V, Wolfe CL (1994) Heat-shock protein induction in rat hearts. A direct correlation between the amount of heat-shock protein induced and the degree of myocardial protection. Circulation 89: 355–360
8. Kemnitz J (1991) The histopathological classification of liver allograft rejection. In: Engemann R, Hamelmann H (eds) Experimental and clinical liver transplantation. Elsevier, Amsterdam, New York, Oxford, pp 9–14
9. Le Moine O, Marchant A, Durand F, Ickx B, Pradier O, Belghiti J, Abramowicz D, Gelin M, Goldman M, Deviere J (1994) Systemic release of interleukin-10 during orthotopic liver transplantation. Hepatology 20: 889–892
10. Marber MS, Mestril R, Chi SH, Sayen MR, Yellon DM, Dillmann WH (1995) Overexpression of the rat inducible 70-kD heat stress protein in a transgenic mouse increases the resistance of the heart to ischemic injury. J Clin Invest 95: 1446–1456
11. McCurry KR, Campbell DA Jr, Scales WE, Warren JS, Remick DG (1993) Tumor necrosis factor, interleukin 6, and the acute phase response following hepatic ischemia/reperfusion. J Surg Res 55: 49–54
12. Myrmel T, McCully JD, Malikin L, Krukenkamp IB, Levitsky S (1994) Heat-shock protein 70 mRNA is induced by anaerobic metabolism in rat hearts. Circulation 90: 299–305
13. Ohzato H, Monden M, Yoshizaki K, Gotoh M, Kanai T, Umeshita K, Tono T, Nishimoto N, Kishimoto T, Mori T (1993) Serum interleukin-6 levels as an indicator of acute rejection after liver transplantation in cynomologous monkeys. Surg Today 23: 521–527
14. Schiaffonati L, Cairo G, Tacchini L, Pappalardo C, Gatti S, Piazzini Albani A, Bernelli Zazzera A (1993) Protein synthesis and gene expression in transplanted and postischemic livers. Transplantation 55: 977–982
15. Schmid J, Weissman C (1987) Induction of mRNA for a serine protease and a beta-thromboglobulin like protein in mitogen stimulated human leukocytes. J Immunol 139: 250–257
16. Sun JZ, Tang XL, Knowlton AA, Park SW, Qiu Y, Bolli R (1995) Late preconditioning against myocardial stunning. An endogenous protective mechanism that confers resistance to postischemic dysfunction 24 h after brief ischemia in conscious pigs. J Clin Invest 95: 388–403
17. Tacchini L, Pogliaghi G, Radice L, Anzon E, Bernelli Zazzera A (1995) Differential activation of heat-shock and oxidation-specific stress genes in chemically induced oxidative stress. Biochem J 309: 453–459
18. Tilg H, Ceska M, Vogel W, Herold M, Margreiter R, Huber C (1992) Interleukin-8 serum concentrations after liver transplantation. Transplantation 53: 800–803
19. Umeshita K, Monden M, Tono T, Hasuike Y, Kanai T, Gotoh M, Mori T, Seu P, Shaked A, Busuttil RW (1992) Interleukin-6 in bile as a marker of allograft rejection following liver transplantation in man. Transplant Proc 24: 1462–1464
20. Zhang YH, Takahashi K, Jiang GZ, Zhang XM, Kawai M, Fukada M, Yokochi T (1994) In vivo production of heat shock protein in mouse peritoneal macrophages by administration of lipopolysaccharide. Infect Immun 62: 4140–4144
21. Zilberstein A, Ruggieri R, Korn JH, Revel M (1986) Structure and expression of cDNA and genes for human interferon-beta-2, a distinct species inducible by growth-stimulatory cytokines. EMBO J 5: 2529–2537