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Role of the bcl-2/bax pathway in hepatocyte apoptosis during acute rejection after rat liver transplantation

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Abstract It is well established that hepatocytes undergo apoptotic cell death in the course of rejection of liver grafts. The present study was designed to investigate the role of the bcl-2/bax pathway in liver allograft tissue. Orthotopic liver transplantation was performed in three groups of rats: group 1, a syngeneic combination (Lewis to Lewis), group 2, an allogeneic combination (ACI to Lewis), and group 3, an allogeneic combination (ACI to Lewis) treated with 15-deoxyspergualin. The number of apoptotic cells identified by the TUNEL method in the grafted liver reflected the severity of acute rejection. In group 1, both bcl-2 mRNA and bax mRNA were expressed in trace amounts. In group 2, bcl-2 mRNA was slightly expressed while the expression of bax mRNA rose steadily. In group 3, bcl-2 mRNA expression levels remained similar to group 1, while bax expression levels exceeded those in group 1, but were less than in group 2. Expression of bcl-2 mRNA was stationary in comparison with expression of bax mRNA. Significantly higher levels of bax mRNA were expressed from day 4 in group 2 than in group 1 (on postoperative days 4, 6, and 8, $P < 0.05$, group 2 vs group 1). We also investigated bax protein and results consistent with the mRNA analysis data were obtained. These findings suggest that apoptotic cell death in liver allograft rejection is regulated, at least in part, by bax.

Key words Liver transplantation · Apoptosis · TUNEL method · bax · bcl-2

Introduction

Recently our own group and other groups have shown that the incidence of apoptotic cell death in rat liver allografts increases steadily over time, but is not detected in syngeneic graft livers [10, 6]. This indicates that apoptosis is the mode of hepatocyte death in allografts during rejection after liver transplantation. The following molecules have been reported as regulators of the programmed cell death of hepatocytes: Fas ligand/Fas [15], TGF β 1/TGF β 1 receptor [13], TNF α/TNF α receptor [7], and IFN γ/IFN γ receptor [16]. Although the precise mechanism underlying apoptosis observed in allograft livers during rejection remains unclear, these molecules are probably involved.

In view of the well-known fact that cytokines, including TGF β 1, TNF α, and IFN γ, are produced during rejection reactions after liver transplantation [10, 21], it is reasonable to assume that these cytokines contribute to hepatocyte apoptosis in rejected allografts. Fas ligand/Fas is another potential pathway that may accelerate hepatocyte apoptosis during rejection reactions, judging from the fact that Fas ligand ist located on cytotoxic T cells, NK cells, and CD4+ cells and that these cells contribute to the alloimmune response [5]. In our prior study, a correlation between the Fas ligand mRNA level and the extent of apoptosis in rat liver allografts was actually confirmed.

All Fas and cytokine receptors described above are trigger molecules located on the cell membrane of tar-

get cells and may communicate programmed cell death activating signals to lower reaches of the chain reaction associated with apoptosis. The signal induced by these molecules probably interacts with regulators such as bcl-2/bax located in the cytosol or the nuclear membrane of the target cells. In order to determine the precise mechanism of hepatocyte apoptosis and to obtain clues for therapeutic options, that manipulate apoptotic regulators, it is important to identify the role of these intracellular regulators. In the present study, we focused on the role of the bcl-2/bax pathway in hepatocyte apoptosis observed during rejection of rat allografted livers.

Materials and methods

Animals

Male Lewis (RT-1l) and ACI (RT-1a) rats weighing 180–300 g maintained on a standard diet were used.

Liver transplantation

Orthotopic liver transplantation without rearterialization was performed using the cuff technique described by Kamada and Calne [9].

Experimental design

The experimental animals were divided into the following three groups:

1. Group 1, control isografts (Lewis to Lewis)
2. Group 2, untreated allografts (ACI to Lewis)
3. Group 3, 15-deoxyspergualin (DSG)-treated allografts (ACI to Lewis).

The ACI to Lewis combination is fully allogeneic and results in the acute rejection of liver grafts [20]. In our preliminary study, the mean survival time of Lewis recipients that received Lewis liver grafts ($n = 8$) or ACI liver ($n = 8$) grafts was more than 100 days and 11.12 ± 1.95 days, respectively. DSG (provided by Nippon Kayaku, Japan) was administered intraperitoneally from postoperative day (POD) 3 to 8 at a daily dose of 5 mg/kg body weigth. Our previous studies had demonstrated that DSG specifically inhibits lymphocyte clonal expansion at the onset of rejection and that this regimen provides potent rescue therapy [14, 18, 19]. Grafted liver samples were obtained on PODs 2, 4, 6, and 8 for histological examination and mRNA and protein assays. The number of liver samples on PODs 2, 4, 6, and 8 was five, in each of the three groups.

Histological examination

Specimens of the grafted livers were fixed in 10% formalin and stained with hematoxylin and eosin for light microscopy. Apoptotic cell death in the hepatocytes was identified by established criteria described by other authors [12].

Table 1 Sequences of oligonucleotide primers used in RT-PCR

Gene	Primer	Length (bp) (or Number of bp)
bcl-2	5'CACCCCTGGCATCTTCTCCTTC (rat 366–387) 3 CACAATCCTCCCCCAGTTCACC (rat 669–648)	303
bax	5'CCAAGAAGCTGAGCGAGTGTCTC (rat 61–73) 3'AGTTGCCGTCTGCAAACATGTCA (rat 207–184)	146

In situ assay for DNA fragmentation

DNA fragmentation was detected by in situ nick-end labeling, the so-called TUNEL method [4, 8]. The amount of hepatocyte apoptosis in the graft was evaluated by an apoptotic index (AI), which was defined as the number of TUNEL-positive apoptotic hepatocytes per 1000 hepatocytes.

Quantification of mRNA and cDNA

The mRNAs of bcl-2 and bax were obtained as follows. Total RNA was isolated from 1 g of tissue by the standard method described previously [10]. The mRNA level of each sample was analyzed by reverse transcription and subsequent polymerase chain reaction (RT-PCR). Primers for RT-PCR are shown in Table 1. RT-PCR was performed using a TaKaRa RNA PCR kit (TaKaRa Biomedicals, Japan) according to the manufacturer's instructions with some modification; 500 ng of total RNA was mixed with RT master mix (final concentrations in 20 μl reaction volume: 5 mM $MgCl_2$, 100 mM TRIS-HCl pH 8.3, 50 mM KCl, 1 mM dNTP mixture, 20 U RNase inhibitor, 5 U reverse transcriptase, 2.5 μM random 9mers). Then incubation at 30 °C was performed for 30 min to prolong the random 9mers for adequate reaction with sample RNA. RT was performed by incubation at 42 °C for 60 min. The reaction was stopped by heating at 99 °C for 5 min, and the reaction mixture was immediately placed on ice. Takara *Taq* polymerase, $MgCl_2$, TRIS-HCl pH 8.3, KCl, specific forward primer, reverse primer, and distilled water were added (final concentration in 100 μl reaction volume: 2.5 mM $MgCl_2$, 100 mM TRIS-HCl pH 8.3, 50 mM KCl, Takara *Taq* polymerase 0.25 U, specific forward primer 0.2 μM, reverse primer 0.2 μM). PCR was performed by 30 cycles of PCR (94 °C for 75 s, 56 °C for 2 min, 72 °C for 3 min), and finally the samples were incubated at 70 °C for 10 min.

Hybridization

Southern blotting was performed using the well-established procedure [11]. After gel electrophoresis, the PCR products were transferred to a nylon membrane by vacuum blotting. Probes were labeled with fluorescein-11-dUTP (ECL3' oligolabeling and detection systems, Amersham Life Science, UK, RPN2130) and hybridized with the nylon membrane (Hybond TM – N +; positively charged nylon membrane, Amersham Life Science). Hybridization was performed using antifluorescein horseradish alkaline phosphatase. Fluorescein-labeled PCR products were detected by enhanced chemiluminescence (ECL, RPN3510). Lumigraphs were produced by exposing X-ray film (Kodak scientific imaging film)

to the membrane for 1–3 min. The intensity of the hybridized PCR products was quantified with a Quantity One computing densitometer (pdi, N. Y., USA).

Immunoblot assay for proteins

Immunoblot analysis for bax was carried out as follwos: crushed frozen liver tissue was lysed in lysis buffer containing 50 mM TRIS-HCl, 125 mM NaCl, 0.1% Nonidet P-40 (Nakarai, Japan), 5 mM EDTA, and 2 mM sodium fluoride for 30 min on ice and centrifuged for 5 min at 12000 rpm. Aliquots containing 25 μg of total protein were size-fractionated in the SDS-polyacrylamide 12% gradient gel and electroblotted on nylon membrane. Blots were washed in PBS and blocked in 5% skim milk in PBS for 2 h. Preblocked blots were reacted with rabbit anti-human bax (P-19) at 1:200 dilutions (Santa Cruz Biotechnology, Calif., USA) in PBS containing 0.05% Tween 20 (PBS-T) at 4 °C for 16 h and then incubated with a 1:3000 goat anti-rabbit IgG antibody (Zymed, Calif., USA) in PBS-T for 50 min. Immunoblots were detected by using an ECL western blotting detection system (Amersham International, UK).

Statistical analysis

Results are expressed as means ± SEM, and the unpaired Student's *t*-test was used to calculate P values. A P value of < 0.05 was considered indicative of statistical significance.

Results

Histological findings

Histological examination of the grafts confirmed the presence of acute rejection in the allograft groups and the absence of characteristic features of rejection in the control isograft group. In group 2, no evidence of rejection was observed on POD 2. Mononuclear cell infiltration in the portal tracts with or without venous endothelitis and bile duct damage was observed on POD 4. Extensive cell infiltration in the portal tracts and parenchyma was observed on POD 6, and marked mononuclear cell infiltration in the portal tracts and parenchyma with confluent dropout of hepatocytes was evident on POD 8. In group 3, the sinusoids contained various numbers of mononuclear cells, but always fewer than in group 2. On POD 8, there were some scattered individual necrotic hepatocytes, but no zonal necrosis was evident. Thus, although mild rejection remained during the observation period, a significant therapeutic effect of DSG was observed.

In situ assay for DNA fragmentation

TUNEL signals were detected in periportal hepatocytes and in a few lymphoid cells (Fig. 1). Bile duct epithelial cells producing TUNEL signals were rarely recognized. Figure 2 shows the respective AI of the hepatocytes in-

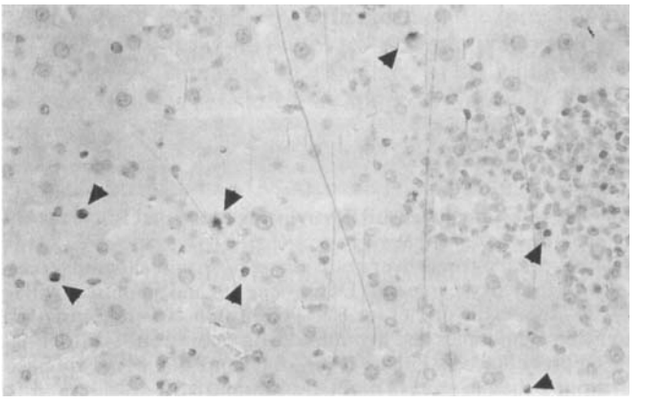

Fig. 1 The histological findings demonstrate apoptosis in an ACI to Lewis untreated liver allograft during rejection 6 days after transplantation. TUNEL signals *(arrowheads)* were detected mainly in the periportal hepatocytes and in a few lymphoid cells. Magnification x · 50

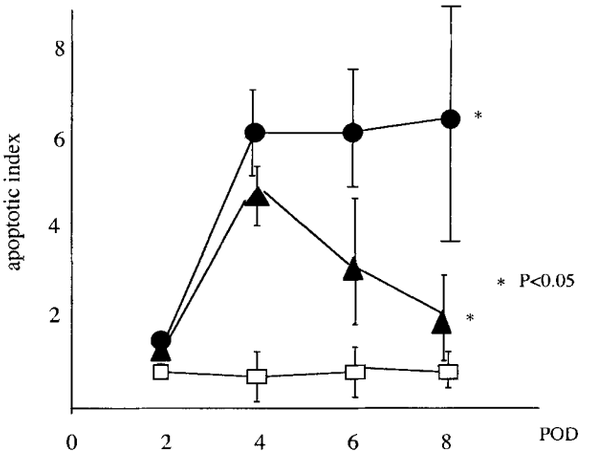

Fig. 2 Apoptotic index (AI) of the grafted liver in the three groups. AI is the number of apoptotic cells detected by the TUNEL method per 1000 hepatocytes. The AI remained low in group 1 (□) throughout the observation period. The AI rose until postoperative day (POD) 4 in groups 2 (●) and 3 (▲). The index did not change significantly from POD 4 to 8 in group 2. In contrast, the AI declined as the acute rejection abated on PODs 6 and 8 in group 3. On POD 8, the AI was significantly higher in group 2 than in either group 1 or group 3 ($P < 0.05$, $n = 5$)

the three groups. The AI rose significantly on PODs 4, 6, and 8 in group 2, while the index remained significantly constant in group 1 ($P < 0.05$, group 2 vs group 1). In group 3, the AI rose on POD 4, but declined as the acute rejection abated on PODs 6 and 8. On POD 8, there was a significant difference in the AI between group 2 and 3 ($P < 0.05$).

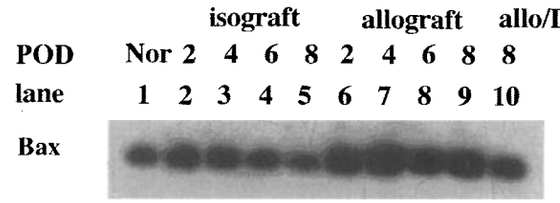

Fig. 3 Expression of bax mRNA in liver grafts. Total RNA was obtained from normal native liver *(lane 1)*, syngeneic liver grafts *(lanes 2–5)*, allogeneic liver grafts *(lanes 6–9)*, and allografts treated with 15-deoxyspergualin (DSG) *(lane 10)* and amplified by RT-PCR with specific primer for bax. PCR products were transferred to nylon membranes and hybridized with fluorescein-11-dUTP-labeled probe for bax. Higher levels of bax mRNA were expressed in group 2 than in group 1 on PODs 4, 6, and 8

Quantification of mRNA by RT-PCR and Southern blotting

Expression of bcl-2 mRNA in group 1 amounted to only trace amounts. In group 2, hardly any was expressed, while in group 3, expression remained relatively constant at the same levels as in group 1. Expression of bax mRNA in group 1 was maintained at a constant level, the same as in normal liver. In contrast, expression in group 2 rose significantly from POD 4 to 8 (on PODs 4, 6, and 8, $P < 0.05$, group 2 vs group 1; Figs. 3, 4). In group 3, bax expression on POD 8 exceeded expression in group 1, but was less than in group 2.

Immunoblot assay for proteins

Western blotting was performed to investigate bax protein in groups 1 and 2. The typical immunoblot profile of bax protein is shown in Fig. 5. The amount of bax protein was markedly larger in group 2 on PODs 6 and 8 than in group 1.

Discussion

The present study was performed to investigate the mechanism of hepatocyte apoptosis in allografted livers during rejection by analyzing the alteration in expression of the apoptosis-associated genes bcl-2 and bax and their products. Initially, we demonstrated by the TUNEL method that the number of apoptotic hepatocytes parallels the pathological severity of acute rejection of the liver. The AI rose steadily until POD 4 and remained level on PODs 6 and 8 in untreated allogeneic combinations, while the characteristic histological findings of acute hepatic allograft rejection were observed on POD 4 and acute rejection progressed in the graft of untreated allogeneic combinations on POD 6. Thus, it was revealed that hepatocyte apoptosis similarly correlated with a mechanism of cell death in liver allograft rejection.

Although the mechanisms responsible for hepatocyte apoptosis are unclear, cytokines, including TNF α, IFN γ and TGF β 1, produced in the grafted liver may contribute to it. TNF α kills cultured rat hepatocytes by increasing apoptosis, and this effect is potentiated by the addition of IFN γ [16]. TGF β 1 induces apoptosis in hepatocytes both in vitro and in vivo [13]. Consistent with these findings, it has been reported that the mRNA of TGF β 1 [10] and of IFN γ [3] increases during the process of rejection of allografted rat liver. Fas ligand/Fas is another pathway that may accelerate hepatocyte apoptosis during rejection reactions, judging from the fact that the Fas ligand is located on cytotoxic cells, NK cells, and CD4+ cells, and that these cells contribute to the alloimmune response [5]. In our prior study, expression of Fas ligand in allografted liver was actually shown to increase gradually by POD 8 (data not shown). Thus, several molecules may regulate apoptosis in allografted liver. However,

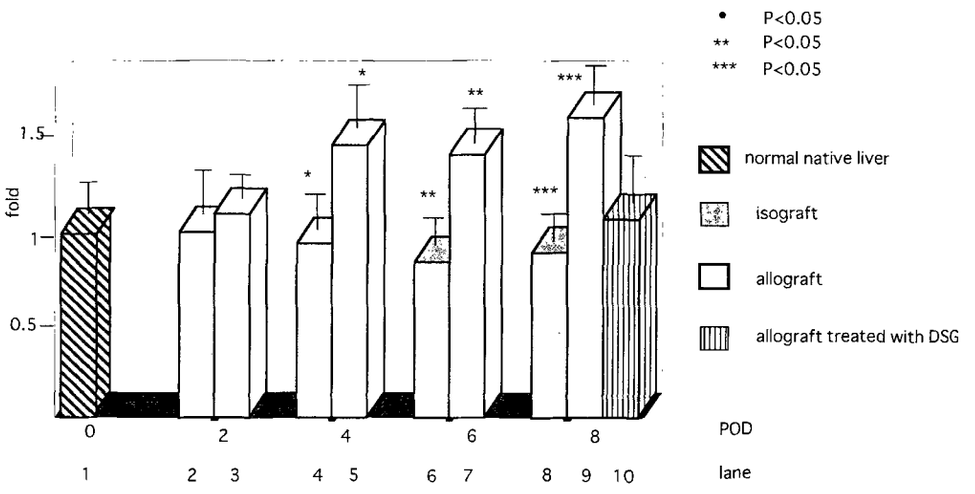

Fig. 4 Quantification of bax mRNA in liver grafts. The intensity of hybridized PCR products was quantified with a Quantity One computing densitometer. The bax mRNA level of normal liver was defined as 1. The columns show expression of bax mRNA of normal native liver *(lane 1)*, syngeneic liver grafts *(lanes 2–5)*, allogeneic liver grafts *(lanes 6–9)*, and allografts treated with DSG *(lane 10)*. Higher levels of bax mRNA were expressed in group 2 than in group 1 on PODs 4, 6, and 8 (on PODs 4, 6, and 8, $P < 0.05$, group 2 vs group 1, $n = 5$). In the DSG-treated group, the bax mRNA level had risen 1.1 – fold on POD 8

Fig. 5 Typical profile of immunoblot analysis for bax protein. The liver tissue bax protein was obtained from syngeneic liver grafts *(lanes 1–8)* and allogeneic liver grafts *(lanes 9–16)*. Western blotting was performed. The amount of bax protein in allografts was greater than in isografts

all of the molecules described above, Fas and cytokine receptors, are just trigger molecules located on target cell membranes. Identification of the precise mechanism of hepatocyte apoptosis will require analysis of the regulators located intracellularly, including bcl-2 and bax.

Bcl-2, the protein encoded by the bcl-2 (B cell lymphoma/leukemia 2) protooncogene, contributes to the prolongation of cell survival by blocking apoptosis, and bax, the protein encoded by the bax (bcl-2-associated X protein) gene, induces apoptosis to target cell in opposition to bcl-2. In trace amounts, bcl-2 was identified in allo- and isografted liver tissue, but no difference in expression of bcl-2 was detected between the isograft group and the allograft group. In this study, the tissues examined contained hepatocytes, bile ducts, blood vessels, and blood cells. It is known that bcl-2 is expressed in bile ductules and small bile ducts, but not in hepatocytes or the epithelium of large bile ducts, based on the results of an immunohistochemical study of the liver [2]. Expression of bcl-2 in grafted liver probably reflects its expression in bile ductules and the epithelium of small bile ducts. Conversely, it is thought that apoptosis of hepatocytes during acute liver rejection is induced because of a lack of bcl-2 in the hepatocytes. Bergese and colleagues [1] have reported apoptosis in murine cardiac grafts. According to that report, there were not many apoptotic myocytes in the rejected cardiac graft, and they hypothesized that expression of bcl-2 in myocytes contributes to their survival by blocking apoptosis. It may be that the lack of bcl-2 in hepatocytes diminishes resistance to apoptosis in rat liver during rejection.

In the DSG-treated group (group 3), the AI decreased after POD 6. DSG exerts an immunosuppressive effect by blocking lymphocyte clonal expansion [14, 18, 19]. Although, the mechanism of immunosuppression by DSG has not been elucidated, in this study DSG was found to have a role in suppressing acute rejection in allogeneic combinations and to contributed to prolonging survival. In view of the fact that apoptosis of liver tissue during acute rejection was induced in part by apoptosis-associated gene products on CTL, Fas ligand and several cytokines may decrease in the DSG-treated group. We have already confirmed less expression of Fas ligand mRNA on POD 8 in allografted livers treated with DSG than in untreated allografted livers (data not shown). Expression of bax mRNA on POD 8 in group 3 was less than in group 2, but greater than in group 1. Bax may be affected by stimulation of the Fas ligand/Fas pathway or other pathways associated with CTL.

The continued higher level of bax known to induce apoptosis to target cells, on PODs 4, 6, and 8 after liver transplantation in allogeneic combinations, suggests that bax in the graft contributes to hepatocyte apoptosis. Krajewski and colleagues [17] immunohistochemically demonstrated the presence of bax in hepatocytes. Thus, it is possible that the bax that was detected regulates hepatocyte apoptosis. Parallel changes in bax expression and AI suggest that bax regulates apoptosis in cooperation with regulators in higher reaches, for example, Fas ligand and cytokines. In conclusion, the results of present study show that bax protein plays an important role in the hepatocyte apoptosis observed in allografted liver tissue.

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