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Expression of bcl-2 homologue mRNAs in rat liver allograft: rejection-induced cell apoptosis is associated with upregulation of bax and bcl-xs expression

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Abstract Apoptosis is considered to play an important role in rejection of organ transplants, although the precise mechanism has not been elucidated. In this study, we screened for the expression of bcl-2 homologues (bcl-2, bax, bcl-xl, and bcl-xs) and Fas ligand (FasL) by RT–PCR method in grafts during acute rejection in rats following liver transplantation. Both bax and bcl-xs (inducers of apoptosis) mRNA levels increased steadily in the allografted group from postoperative day (POD) 2 to 8, while no remarkable changes of bcl-2 and bcl-xl expression (inhibitors of apoptosis) were recognized. Significant induction of FasL gene expression was observed in the allografted group on POD 4 and expression gradually decreased thereafter, although minimal FasL mRNA expression was seen in isografts. Our results indicated, for the first time, that rejection-induced cell apoptosis is closely associated with upregulation of bax and bcl-xs expression besides FasL, but not with down-regulation of bcl-xl.

Key words Liver transplantation · Apoptosis · bcl-2 · bax · bcl-x

Introduction

Acute rejection has been an obstacle to successful organ transplantation, despite the development of immunosuppressive agents. Recently, apoptosis, a distinctive mechanism of cell death, has been considered to play an important role in the rejection of organ transplants [6, 11]. Numerous positive and negative regulators of apoptosis have been identified. One of these regulators, the Fas/Fas ligand (FasL) pathway and/or perforine/granzyme pathway was recently reported to contribute, at the least, to apoptosis induction as part of the alloimmune response [19]. Meanwhile, it was also reported that Fas/FasL interactions are not essential mediators of T cell-induced allograft damage in experimental cardiac transplantation [12]. Thus, the role of the Fas/FasL pathway has not been elucidated. These studies, however, focused exclusively on the cell surface lytic pathway, without examining cytoplasmic events. bcl-2 [25] and its homologues [1–17] reside in the nuclear envelope, outer mitochondrial membrane, and endoplasmic reticulum and appear to control a downstream event in a pathway leading to apoptotic cell death. However, the relevance of homologues of the bcl-2 gene to rejection-induced cell apoptosis remains unknown. In this study, we have screened for the expression of bcl-2 homologues (bcl-2, bax, bcl-xl, and bcl-xs) and FasL by the RT–PCR method in grafts during acute rejection in rats following liver transplantation.

Table 1 Sequences of oligonucleotide primers used in RT-PCR. Each primer was designed based on published sequences with some modifications

Gene	Primer	Length (bp)	
bcl-2	Sense	5′ CACCCCTGGCATCTTCTCCTTC 3′	303
	Antisense	5′ CACAATCCTCCCCCAGTTCACC 3′	
bax	Sense	5′ CCAAGAAGCTGAGCGAGTGTCTC 3′	146
	Antisense	5′ AGTTGCCGTCTGCAAACATGTCA 3′	
bcl-x	Sense	5′ TTGGACAATGGACTGGTTGA 3′	765
	Antisense	5′ GTAGAGTGGATGGTCAGTG 3′	
FasL	Sense	5′ CAGAAGGAACTGGCAGAACTC 3′	282
	Antisense	5′ AGACTGACCCCGGAAGTATAC 3′	

Materials and methods

Animals

Inbred strains of male Lewis (RT-1) and ACI (RT-1) rats weighing 180–300 g maintained on a standard diet were used.

Transplantation

Orthotopic liver transplantation without rearterialization was performed using the cuff technique described previously by Kamada and Calne [9]. Briefly, the donor liver was harvested and retained at 4°C in 0.9% saline solution until transplantation (less than 1 h). In the recipient, the liver graft was implanted orthotopically after removal of the naive liver. Implantation surgery required less than 50 min, during which time the portal vein was clamped for 11–15 min.

Experimental design

The experimental animals were divided into three groups as follows:
Group 1, control isografts (Lewis-to-Lewis).
Group 2, untreated allografts (ACI-to-Lewis).
Group 3, 15-deoxyspergualin (DSG)-treated allografts (ACI-to-Lewis).

The ACI-to-Lewis combination is fully allogenic and results in acute rejection of liver grafts [27]. In our preliminary study, the mean survival time of group 1 was more than 100 days, while that of group 2 was 11.1 ± 1.95 days. DSG (provided by Nippon Kayaku, Tokyo, Japan) was administered intraperitoneally from postoperative day (POD) 3–8 at a daily dose of 5 mg/kg body weight. Recipients were killed periodically (POD 2, 3, 4, 6, and 8) in order to obtain grafted livers for histological and molecular examination.

Histological examination

The specimens of the grafted livers were fixed in 10% formalin, embedded in paraffin wax, and stained by hematoxylin and eosin.

RNA preparation

Total RNA was extracted by the guanidium thiocyanate–phenol-chloroform extraction method [11]. The quantity and purity of the nucleic acid preparation were estimated by measuring the ratio of the optical density of each sample at 260 and 280 nm.

RT-PCR

The levels of mRNAs were analyzed by RT and subsequent PCR. RT–PCR was performed using a RNA PCR kit version 2.1 (Takara, Kyoto, Japan) according to the manufacturer's instructions with the following modifications: 500 ng of total RNA was mixed with the RT master mix [final concentration in 20 μl reaction volume containing 5 mM $MgCl_2$, 10 mM TRIS-HCl, pH 8.3, 50 mM KCl, 1 mM dNTP mixture, 20 U RNase inhibitor, 5 U avian myeloblastosis virus reverse transcriptase (AMV reverse transcriptase XL; Life Science, Tokyo, Japan), and 2.5 μM random 9 mers]. The mixture was incubated for 30 min at 30°C in order to extend the primer for effective annealing followed by RT for 1 h at 42°C. The reaction was stopped by heating for 5 min at 99°C and subsequently cooled on ice.

Oligonucleotide primers were designed for specific amplification of each mRNA, based on published sequences with some modifications [5, 22, 26]. Each oligonucleotide primer sequence is shown in Table 1. PCR was performed by addition of PCR master mix containing 2.5 mM $MgCl_2$, 10 mM TRIS-HCl, pH 8.3, 50 mM KCl, 25 pmol forward and reverse primers, and 0.25 U *Taq* polymerase (Takara Taq; Takara) in a final volume of 100 μl. PCR was carried out in a Gene Amp PCR system 9600 (Perkin Elmer Cetus, Norwalk, Conn., USA) with 30 cycles of denaturation (94°C, 75 s), annealing (56°C, 2 min), and extension (72°C, 3 min), followed by a final extension at 70°C for 10 min. PCR products were visualized in ethidium bromide-stained 1.2% agarose gels.

Results

Histological findings

Histological characteristics of acute rejection were not recognized in group 1 but were seen on POD 4–8 in group 2. In group 2, mononuclear cell infiltration became apparent in the portal tracts on POD 3–4, progressively spreading into the parenchyma on POD 6. Loss of hepatocytes was evident on POD 8. In group 3, not only were mononuclear cells infiltrating sinusoids decreased, but also necrotic hepatocytes were recognized less often than in group 2. Signs of acute rejection diminished from POD 4 onward in group 3. TUNEL signals were detected in periportal hepatocytes and in a few lymphoid cells, mainly in the allografted group (data not shown).

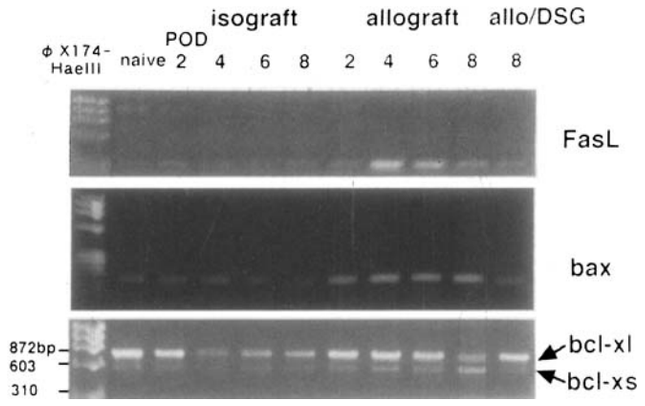

Fig. 1 RT–PCR analysis of bax, bcl-x, and FasL mRNA. Total RNA was prepared from naive liver, both syngenic and allogenic liver grafts on postoperative day *(POD)* 2, 4, 6, 8, and allogenic liver grafts treated with 15-deoxyspergualin *(allo/DSG)* on POD 8, and analyzed for FasL, bax, and bcl-x transcripts by RT–PCR. PCR products were visualized in ethidium bromide-stained 1.2% agarose gels. Both bax and bcl-xs transcripts were significantly upregulated in allogenic transplants, in addition to FasL

mRNA expression

The amounts of different mRNA species in the grafted liver were analyzed by RT–PCR. As shown in Fig. 1, agarose gel electrophoresis revealed a single amplified PCR product for both FasL and bax. Similar single bands were obtained for mRNAs of bcl-2 (data not shown in Fig. 1). bcl-x consisted of two forms porduced through alternative splicing (long and short), differing in size by only 189 bp. The two genes were amplified using the same primer pair and then visualized on the same ethidium bromide-stained gel. The mRNA expression data are summarized in Table 2.

First, expression of FasL was assessed. FasL mRNA was barely detectable in both normal livers and isografts. On the contrary, remarkable expression of FasL mRNA was detected in allografts on POD 4 then progressively decreased. In group 3, mRNA expression was suppressed on POD 8 compared to that of group 2.

Then, mRNAs of bcl-2 homologues were investigated. As shown in Table 2, a remarkable induction of bax (inducer of apoptosis) gene expression occurred on POD 4 in group 2 as rejection progressed, while bax expression was recognized in group 1. Thus, in group 3, bax mRNA expression was decreased on POD 8 compared to that of group 2. Low levels of bcl-2 (inhibitor of apoptosis) were detected in groups 1 and 3. bcl-2 was barely detectable in group 2. We also investigated expression of both bcl-xs (inducer of apoptosis) and bcl-xl (inhibitor of apoptosis) in each group. In the naive liver, bcl-xl was constitutively expressed, while bcl-xs was weakly expressed. In group 2, the expression of bcl-xs mRNA increased steadily from POD 2 to 8, while gradually decreasing over time in group 1. The bcl-xl gene expression of group 2 was slightly higher than that of group 1. Although the expression of bcl-xs in group 2 was usually weaker than that of bcl-xl in group 1, it was noteworthy that bcl-xs mRNA expression was stronger than that of bcl-xl on POD 8 in group 2. In group 3, the mRNA expression of bcl-xs was weaker than that of group 2 on POD 8.

Discussion

Rejection-induced apoptosis has been reported in organ transplantation [6, 11], but the role of apoptosis-related genes has not been elucidated. Members of the bcl-2 family regulate apoptosis cell death, and reside in the nuclear envelope, outer mitochondrial membrane, and endoplasmic reticulum [10] unlike the Fas/FasL, which is a cell surface protein that induces apoptosis [22]. Our data demonstrate, for the first time, expression of not only FasL but also bcl-2 homologues in liver allograft rejection.

The Fas antigen is a cell surface protein with a relative molecular weight of 45 kDa and is a member of the tumor necrosis factor/nerve growth factor receptor family. A high level of Fas mRNA expression has been detected in various tissues, such as the thymus, liver, lung, heart, and ovary [29]. Fas is activated, triggering apoptosis in susceptible tissues, following interaction with its

Table 2 mRNA expression of bcl-2 homologues and FasL. The degree of each mRNA expression is graded from – (none) to 3+ (strong). (*Group 1* Syngenic combination, *Group 2* allogenic combination without any immunosuppressive therapy, *Group 3* allogenic combination treated with 15-deoxyspergualin, *POD* postoperative day)

Gene	Group 1				Group 2				Group 3
	POD 2	POD 4	POD 6	POD 8	POD 2	POD 4	POD 6	POD 8	POD 8
bcl-2	1+	1+	1+	1+	±	±	±	±	1+
bax	1+	1+	1+	1+	2+	3+	3+	3+	2+
bcl-xl	2+	2+	2+	2+	3+	3+	3+	2+	3+
bcl-xs	1+	1+	1+	–	1+	2+	2+	3+	2+
FasL	1+	1+	1+	1+	1+	3+	2+	2+	1+

natural ligand, FasL, a 37-kDa type II protein [21]. Cytotoxic T cells can deploy FasL as a death effector molecule in their strategies for killing target cells [8, 14]. In our study, FasL mRNA was barely detectable in isografts, while the expression of FasL mRNA was regulated in allografts and this expression peaked on POD 4. It is difficult to identify the origin of mRNA in the grafted liver, but we can speculate that upregulation of FasL reflects the infiltration of mononuclear cells into the allografted liver. In fact, mononuclear cell infiltration in the portal tracts, regardless of venous endothelialitis and bile duct damage has been observed on POD 3 and 4 in allografted livers [16]. Moreover, FasL mRNA has not been detected in normal rodent and human livers [3, 4] but, rather, within hepatocytes in inflamed livers [4]. On the other hand, FasL can function in either paracrine or autocrine cell death pathways. Although not constitutively expressed in mature T cells, FasL is induced after activation of mature T cells by various stimuli [7, 28]. It is noteworthy that activated lymphocytes undergo death by apoptosis at the end of an immune response. The Fas/FasL system serves an important function in apoptosis of both lymphocytes and neutrophils [13, 20]. Numerous infiltrating lymphocytes are recognized in acute rejection. These lymphocytes are destined to die, due either to the absence of a survival signal or the activation of a killing signal. Thus, upregulation of FasL in allografted liver may reflect infiltration of mononuclear cells and its apoptosis.

Unlike FasL on the cell surface, members of the bcl-2 family reside in the nuclear envelope, outer mitochondrial membrane, and endoplasmic reticulum [10], where they regulate apoptotic cell death. bcl-2 blocks cell death following a variety of stimuli, while bax, a homologue of bcl-2, accelerates apoptotic death [17]. bcl-x is also a bcl-2-related gene that can function as a bcl-2-independent regulator of programmed cell death [1]. Alternative splicing results in two distinct bcl-x mRNAs, bcl-xl and bcl-xs. Despite having only 63 fewer amino acids, the product of bcl-xs has an effect completely opposite to that of bcl-xl. bcl-xs mRNA encodes a protein that inhibits the ability of bcl-2 to enhance the survival of growth factor-deprived cells, while bcl-xl inhibits cell death upon growth factor withdrawal when stably transfected into an interleukin-3-dependent cell line [1]. In our study, both bcl-x and bax mRNAs were detected in a naive liver but bcl-2 was not. Furthermore, mRNA expression of both bax and bcl-xs, an inducer of apoptosis, were upregulated in allografted liver, but not in isografted liver. Thus, upregulation of apoptosis-stimulating bcl-2 homologues was recognized in acute rejection. On the contrary, the bcl-xl gene expression in allografts was stronger than in isografts. This may suggest that rejection-induced apoptosis is not related to down-regulation of the apoptosis suppressor, bcl-xl, but closely related to upregulation of apoptosis inducers such as bax and bcl-xs. On POD 8, the bcl-xl mRNA expression in allografts was slightly suppressed compared to that on POD 6. The precise interaction between bcl-xl and bcl-xs has not been elucidated; up-regulation of bcl-xs may reflect suppression of bcl-xl.

In contrast to the remarkable expression of FasL in the early stages of acute rejection, movement of functional bcl-2 homologues was delayed. Both bax and bcl-xs (inducers of apoptosis) mRNA levels increased steadily in allografts from POD 2 to 8, while no remarkable changes in these mRNA levels were observed in isografted livers. This may suggest that upregulation of bax and bcl-xs mRNAs may reflect a mechanism not involving Fas/FasL. We can hypothesize two possible scenarios in which bcl-2 homologues play key roles in acute rejection. One possible explanation is that bcl-2 homologues regulate hepatocyte death in the allografted liver. We have already confirmed that the extent of hepatic apoptosis might reflect the magnitude of acute rejection using TUNEL analysis (data not shown). Both bcl-x mRNAs are present in adult rat liver and in mouse hepatocytes [18], and bax immunoreactivity is present in mouse hepatocytes, whereas bcl-2 is expressed in bile ductules and the small bile duct epithelium, but not by normal hepatocytes [2]. In addition, hepatocytes constitute most of the grafted liver volume and, thus, bcl-xs and bax mRNAs may both be derived from hepatocytes undergoing apoptotic cell death. The second scenario involves bcl-2 homologues mediating lymphocyte apoptotic cell death [20], such that upregulation of both bcl-xs and bax mRNAs may reflect this lymphocyte apoptosis, at least in part.

For its specific inhibition of lymphocyte clonal expansion at the onset of rejection, DSG serves as an immunosuppressant in the event of rescue therapy [15, 23, 24]. In this study, mRNA expressions of bax, bcl-xs, and FasL were suppressed in the DSG-treated as compared to the allografted group. Our results are consistent with the former study. The inhibition of clonal expansion caused by DSG may result in diminishing the number of cytotoxic T lymphocytes expressing FasL and/or the suppression of apoptosis induced by bcl-2 homologues. Further precise analysis focused on each bcl-2 homologue in combination with other immunosuppressants will elucidate the mechanism of rejection-induced apoptosis.

In conclusion, we have shown, for the first time, that upregulation of not only FasL but also bax and bcl-xs mRNA expression correlates with rejection-induced cell apoptosis in rat liver transplantation.

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