A bcl-2 Transgene Expressed in Hepatocytes Protects Mice from Fulminant Liver Destruction but Not from Rapid Death Induced by Anti-Fas Antibody Injection

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Summary

Stimulation of the Fas (APO-1, CD95) receptor, which is present on a variety of cells, usually triggers a process of programmed cell death. Systemic injection of anti-Fas antibody into mice leads to fulminant liver destruction resulting from massive hepatocyte apoptosis, and to rapid death. Hepatocytes bear Fas but do not express Bcl-2, a protein that plays, in a number of conditions, a protective role against apoptosis. We have generated mice whose liver expresses Bcl-2 as the result of a bcl-2 transgene placed under the control of the hepatocyte-specific α1-antitrypsin gene promoter, but is otherwise not distinguishable from that of normal mice. These mice display a marked to almost total resistance to liver damage induced by anti-Fas antibody injection. This protective effect of Bcl-2 occurs in the absence of significant variations, in the stimulated livers, in the level of expression of other proteins also involved in resistance or sensitivity to apoptosis, namely Bcl-x, Bax, Bad, Bak, and p53. Mice with protected livers, however, die almost as rapidly as normal mice, which indicates that acute lethality results from stimulation of Fas receptors present on other target organs or cells.

The Fas receptor (APO-1, CD95) is a membrane protein of the tumor necrosis factor–nerve growth factor receptor family, which, upon interaction with its ligand, Fas-L, another membrane protein, or upon triggering by anti-Fas antibodies, usually acts as an inducer of apoptosis (1, 2). This results from an incompletely understood chain of events that culminates in intracellular proteolysis involving the IL-1 converting enzyme (ICE)[1] and other proteases of similar specificity (3, 4). In a number of apoptotic conditions, expression or overexpression of the protein Bcl2 induces, in a variety of cells, some degree of protection against cell death. This has also been observed in vivo in transgenic mice overexpressing Bcl-2 in cells of the T or B lymphocytic lineages or in neurons (5–7). However, conflicting results have been published concerning the capacity of cells transfected with bcl-2 cDNA to resist Fas-mediated apoptosis (8–11).

Injection of anti-Fas antibodies into mice leads to a very rapid and massive destruction of hepatocytes and to death within a few hours (12). This results from the triggering of the Fas receptor present on the hepatocytes and not from another effect of the antigen–antibody reaction, since MRL

mice homozygous for the lpr^cg mutation are entirely resistant to anti-Fas injection; this mutation alters the intracytoplasmic domain of the Fas molecule, resulting in a lack of function (1). Since activated T lymphocytes express Fas-L on their membranes (13, 14), the high vulnerability of hepatocytes to stimulation of their Fas receptors may play an important role in liver damage of severe forms of immune-mediated hepatitis. In this report, we show that transgenic mice expressing Bcl-2 in their hepatocytes are protected against fulminant liver destruction resulting from anti-Fas antibody injection.

Materials and Methods

Construction of the Transgene and Generation of Transgenic Mice. A 2.9-kb DNA fragment containing an internal poliovirus sequence (ribosome landing pad) followed by a CAT gene and a SV40 poly (A) sequence was purified after PstI digestion of the plasmid pSVGHpolioCAT (15) and inserted into the PstI site of a pBluescript KS plasmid (Stratagene Inc., La Jolla, CA) to generate pRCAT. The pAATRCAT plasmid was generated by inserting the human α1-antitrypsin gene promoter (16), digested with XbaI and SacI into pRCAT previously digested with the same enzymes. The human bcl2 cDNA was obtained by digesting the phosphoglycerate kinase (PGK)–bcl-2 plasmid (7) with EcoRI and the resulting 0.9-kb fragment was, after blunting, inserted

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[1]Abbreviations used in this paper: ICE, IL-1–converting enzyme; PGK-1, phosphoglycerate kinase 1.

into the previously blunted SpeI site of pAATRCAT, to generate pAATbcl2RCAT. The transgene used for oocyte injections was excised by digesting pAATbcl2RCAT with BssHII, isolated on agarose gel and purified on NACS columns (GIBCO BRL, Gaithersburg, MD). Use of a transgene expressing a polycystronic mRNA with a reporter CAT sequence was intended as an attempt to follow more easily the expression level of the transgene with a CAT assay, but this approach was found less sensitive and not useful. Transgenic mice were generated as described (17) using (C57Bl/6×DBA2)F₁ eggs. Founder transgenic mice, identified by Southern blotting, were then mated with C57BL/6 mice. Three transgenic lines were obtained, with respectively 2, >10, and >30 copies of the transgene; only the second line, which corresponded to the highest expressor line as judged by Western blotting of liver extracts, was used for further experiments. Production of PGK-bcl2 transgenic mice has already been described (7); these last mice had also been bred on a C57BL/6 background. Mouse colonies were expanded by mating founder animals with C57BL/6 mice. All mice were purchased from IFFA CREDO (Lyon, France).

Anti-Fas Antibody Injections and Histologic Examination. 5–8-wk-old nontransgenic littermates or transgenic mice bred on a C57BL/6 background were injected intravenously with 10 μg of an affinity-purified hamster monoclonal antibody against mouse Fas antigen(Jo2) (12) diluted in 100 μl of a 0.9-g/liter NaCl solution. Mice were killed at various times or autopsied immediately after death, and fragments of tissues were fixed in vivo with 4% paraformaldehyde in PBS, embedded in paraffin, and 5 μm sections were stained with 5 μm hematoxylin and eosin. For electron microscopy, organs were fixed by in vivo perfusion with 2% glutaraldehyde, 1% sucrose in PBS.

Northern and Western Blot Analysis. Total liver mRNA was extracted by using columns (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. RNAs (10 μg) were fractionated on 1% agarose–formaldehyde gel before blotting. Nitrocellulose blots of total RNA were hybridized with the following cRNA probes corresponding to the complete coding sequences: mouse fas (18), human bak, bcl-x, and bax (kind gifts of J.-C. Martinou, Glaxo IMB, Geneva, Switzerland). For Western blotting, proteins were extracted from minced liver fragments for 15 min at 4°C with RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing aprotinin and PMSF. After centrifugation at 15,000 g, protein in the supernatants was quantitated by the Bradford method and 100 μg protein per lane was run in 10% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, which were then blocked with 5% nonfat milk in TBS-T (TBS [20 mM Tris HCl, 500 mM NaCl, pH 7.5] and Tween 0.1%), and the washed membranes were incubated for 1 h at 20°C with one of the following: an anti–human Bcl-2 mouse monoclonal antibody (BCL2 100, kindly provided by D.Y. Mason, John Radcliffe Hospital, Oxford, UK) at a 1:200 dilution, a hamster anti–mouse Bcl2 monoclonal antibody (3F11; PharMingen, San Diego, CA) at a 1:1,000 dilution, a mouse anti-p53 monoclonal antibody (DO1; Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a 100 ng/ml concentration, a rabbit anti-mouse Bax (1696-4) (19), Bcl-x (1695-5) (20), a Bad (C-20; Santa Cruz Biotechnology Inc.), or a hamster anti-Fas antibody (Jo2) (12), all used at a concentration between 200 and 500 ng/ml). Rabbit anti–mouse ICE antibodies (R10311; a kind gift of D.G. Miller, Merck Research Laboratories, Rahway, NJ) were used at a 1:1500 dilution. Peroxidase-conjugated F(ab)₂ goat anti–hamster IgG (Jackson ImmunoResearch Labs., Inc., West Grove, PA), goat anti–mouse IgG, or anti–rabbit IgG (Santa Cruz

Biotech. Inc.) was used for 1 h as secondary antibody at 400–800 ng/ml. Membranes were washed with TBS-T, incubated in enhanced chemiluminescent (ECL) detection reagents (Amersham International, Amersham Bucks, UK) for 1 min at room temperature, and exposed to X-Omat film (Eastman Kodak Co., Rochester, NY).

Results and Discussion

By Western blotting (Fig. 1 *a*), normal mouse liver was found not to contain detectable Bcl-2 protein. To obtain mice with a strong hepatocyte expression of Bcl-2, we generated transgenic mice in which human bcl-2 cDNA was placed under the control of a α1-antitrypsin promoter, which we have found in various transgenic models to be a very effective hepatocyte-specific promoter (16, 21, and Kaya, G. and P. Vassalli, unpublished observations). Three independent mouse lines were obtained, and the line expressing the highest level of Bcl-2 on Western blots of liver extracts was selected (Fig. 1 *b*); no human Bcl-2 protein was detectable in the other organs tested (Fig. 1 *b*). Except for Bcl-2 expression, livers from these mice were indistinguishable from those of normal mice in gross appearance, histology, and expression of various proteins and mRNAs by Western and Northern blotting (not shown), including the Fas protein (Fig. 1 *c*). When transgenic and control mice were injected intravenously with 10 μg of the Jo2 monoclonal antibody, deaths occurred within a few hours in both types of mice, but gross appearance of the liver was strikingly different: massively hemorrhagic, shrunken, and diffluent in normal mice, and normal in transgenic mice. Pilot experiments showed that histologic lesions in general were inconspicuous in normal mice within the first 50–60 min after antibody injection, but appeared very rapidly afterwards. On this basis, comparison of the livers of transgenic and normal mice was performed on animals killed at various times before death, and mice examined at the time of death.

Histologic study of the livers of four normal mice killed 75 min after antibody injection showed extensive lesions characterized by massive hepatocyte destruction with cytoplasmic swelling of most surviving hepatocytes and frequent nuclear chromatin condensation, indicative of apoptosis, and by intraparenchymatous hemorrhages in the areas with the most extensive hepatocyte damage (Fig. 2, *a* and *c*). The livers of three transgenic mice killed 30 min later, i.e., 105 min after injection, showed, in contrast, no or discrete histologic changes (Fig. 2, *b* and *d*), with total preservation of the tissue architecture, no hemorrhages, and occasional swelling of some hepatocytes. To explore whether hepatocyte damage in normal mice is preceded or accompanied by changes in the levels of gene products that have been implicated in apoptotic death, changes that might be compensated by the expression of Bcl-2 in transgenic mice, Western and Northern blots were performed on liver extracts taken at various times after antibody injection. No significant variations were found in the levels of the Bcl-x protein (Fig. 1 *d*; a member of the Bcl-2 family expressed
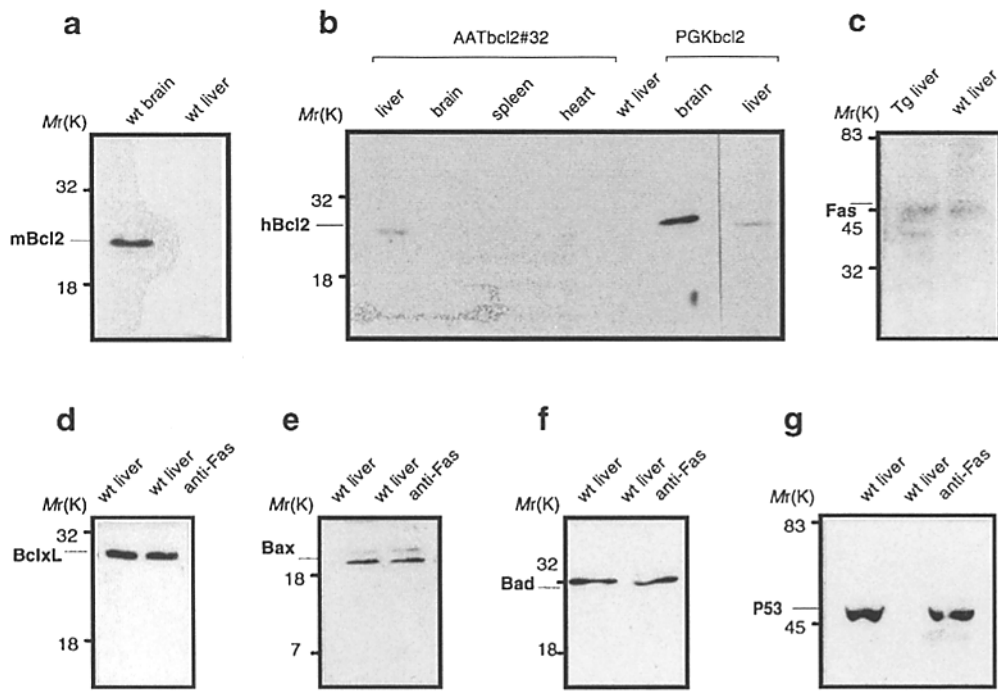
Figure 1. Western blot analyses. (a) Blots from lysates of brain and liver of a normal mouse, reacted with anti–mouse Bcl2 antibody. No Bcl2 is detected in the liver in contrast to the brain. (b) Blots from lysates of various organs of AAT-bcl2 and PGK-bcl2, or of normal mouse liver, reacted with anti–human Bcl2. In AAT-bcl2 mice, human Bcl2 is present only in the liver, in PGK-bcl2, it is present in the brain and liver (and other organs, not shown), and it is absent in normal mouse liver. (c) Blot from lysates of liver of a AAT-bcl2 transgenic mouse and its control littermate, reacted with anti–Fas antibody. (d–f) Blots from lysates of liver of normal mice, uninjected or 80 min after antibody injection, reacted with anti–Bcl-x, Bax, Bad, and p53 antibodies.

in normal liver, contrarily to Bcl-2), the Bax and Bad proteins (Fig. 1, e and f) or the bak mRNA (not shown), three genes whose increased expression has been shown to promote apoptosis (22–25). The level of the protein p53, surprisingly high in the liver, a nondividing tissue, was also unchanged after anti-Fas injection (Fig. 1 g).

10 normal and six transgenic mice were then injected with anti-Fas antibody and examined at the time of their death, which occurred in all cases, although slightly more rapidly with normal mice (2.1 ± 0.6 compared to 3.3 ± 1.2 h with transgenic mice; Fig. 3). Massive hemorrhagic destruction of the liver was observed in all control mice, whereas four transgenic mice showed no or minor hepatocyte alteration, consisting at most of some degree of cell swelling (Fig. 2 f). The two remaining transgenic mice had focal areas with more distinctive signs of cell damage and some apoptotic nuclei, but the liver structure was preserved without hemorrhages.

These observations raise two questions: (a) What are the mechanisms and the target tissue(s) of Fas-induced death, since what appears, in some cases at least, as a complete hepatic protection affords no protection against death? (b) What is the mechanism of the Bcl-2–induced hepatocyte protection?

In addition to hepatocytes and lymphoid cells in the thymus and peripheral lymphoid organs, the Fas receptor is expressed in several organs, in particular the heart, lung, kidney, and small intestine (26, 27). No histologic or ultrastructural (in the case of the myocardium) alterations that might explain the rapid death of animals were observed in these organs. To explore whether a transgenic expression of Bcl-2 expressed in many tissues besides the liver might afford a better level of protection against the lethal effect of

anti-Fas antibody injection, we used mice expressing a human bcl-2 transgene placed under the control of the PGK-1 promoter, since the promoter of this ubiquitous protein should lead to the expression of the transgene in a variety of tissues. These PGK-bcl2 transgenic mice have been used, for instance, to detect the effect of the constitutive expression of Bcl-2 in the brain during fetal development (7). Western blot analysis of various organs from these mice indeed showed the presence of the human Bcl-2 protein (Fig. 1 b). In the liver, the level of this protein was comparable to that found with the AAT-bcl-2 transgenic line (Fig. 1). Two PGK-bcl2 transgenic mice of the same C57Bl/6 genetic background were injected with antibody and died, but only about 9 h later (Fig. 3). By gross appearance, the livers were not hemorrhagic, but by histology, apoptotic lesions were severe (Fig. 1 e) with, however, preservation of a number of hepatocyte trabeculae and no massive hemorrhagic infiltrates. More extensive studies were not performed with these mice, which are difficult to breed. This limited observation is nevertheless compatible with the possibility that constitutive overexpression of Bcl-2 elsewhere than in the liver exerts some protective effect against the most immediate mechanisms of death resulting from anti-Fas antibody injection. These mechanisms may be related to the impressive systemic effects observed within 10 min after antibody injection (prostration and progressive deep hypothermia). These effects represent more than the mere consequences of a widespread antigen–antibody reaction, since mice homozygous for the lpr[cg] mutation are resistant to anti-Fas antibody injection, although they express as much antigen on their membranes (26). The basis for these systemic effects has remained so far elusive: death by cardiovascular or respiratory failure ap-
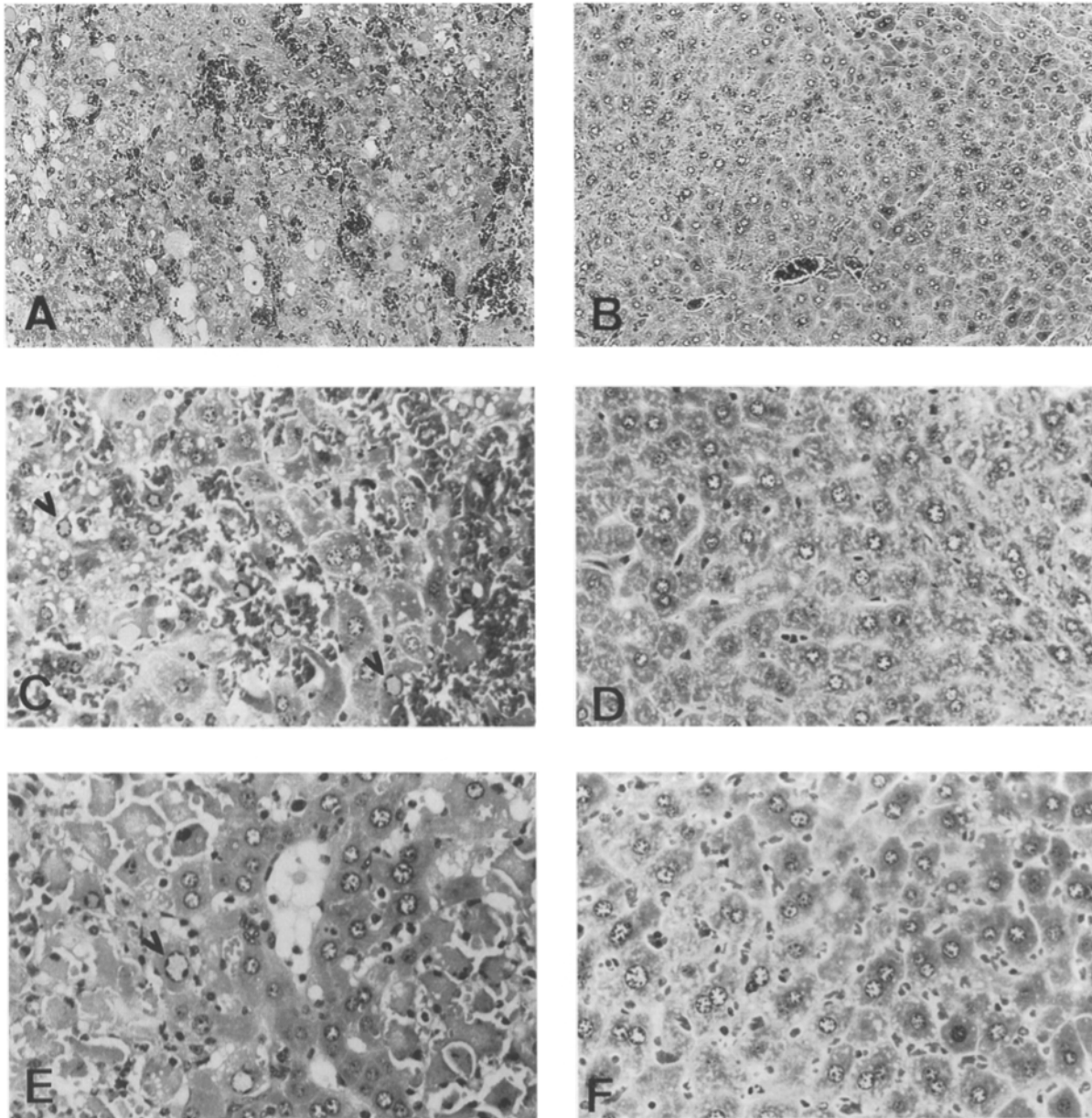
Figure 2. Histology of the livers. (*A* and *C*) Liver from an 8-wk-old nontransgenic littermate 75 min after injection of anti-Fas antibody. At low magnification (*A*, ×100), the architecture of the liver parenchyme is destroyed; the scattered dark areas represent hemorrhagic foci. At higher magnification (*C*, ×400), some very swollen hepatocytes are seen, as well as apoptotic nuclei characterized by peripheral chromatin condensation (*arrows*); there are numerous red blood cells. (*B* and *D*) Liver from an 8-wk-old transgenic AAT-bcl2 mouse 115 min after injection of anti-Fas antibody. At ×100 (*B*) and ×400 (*D*), liver appears normal and without hemorrhagic foci. (*E*) Liver from a transgenic mouse of the PGK-bcl2 line at the time of death (8.5 h after antibody injection; ×400). In the middle of the picture, hepatocytes appear normal, whereas laterally, most hepatocytes are at different stages of apoptosis (see *arrow* for an example of apoptotic nucleus). No significant intraparenchymatous hemorrhage is seen. (*F*) Liver from an 8-wk-old AAT-bcl2 mouse at the time of death (2.3 h after anti-Fas antibody injection; ×400). Hepatocytes appear almost normal although some are swollen; there is some degree of intercellular edema, but not hemorrhage.

pears to be ruled out by failure to detect significant alterations by electrocardiographic monitoring, determination of blood gases and lactate levels, and histologic and ultrastructural examinations of the heart and lung. Glycemia and blood electrolytes remained within control values, except for an increase in blood potassium level, as previously noted (12). Histologic study of all tissues known to express

Fas (27) and of the hypothalamic region did not reveal conspicuous alterations. Since injection of limited amounts of anti-Fas antibody may be considered a possible therapeutic approach in the case of well-differentiated, not surgically removable hepatomas, it will be necessary to elucidate the mechanisms of the rapid systemic and life-threatening effects of anti-Fas antibody injection.
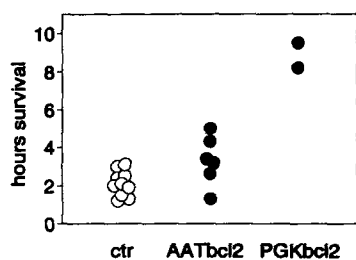
Figure 3. Times of death after anti-Fas injections. Times of death of controls (ctr) and transgenic mice of the AAT-bcl2 and PGK-bcl2 lines, respectively, after intravenous injection of 10 µg of anti-Fas antibody.

Triggering of the Fas receptors expressed on the hepatocytes induces the apoptotic destruction of these cells more rapidly than with any other cell types studied so far. This may be related to: (a) the intensity of the signal received; (b) shortage of protective mechanisms; or (c) a high basic level of effector mechanisms of cell death in this cell type. The effects of Fas receptor stimulation appear not to be merely related to the number of receptors involved, but also to the amount and variety of phosphatases constitutively expressed by the cells (28); protein tyrosine phosphorylation is a very rapid consequence of Fas triggering (29). In this regard, the Fas-binding phosphatase FAP-1 is not expressed in the liver (30), which is consistent with the extreme sensitivity of this organ to the effects of anti-Fas antibody. Possible differences in protein phosphorylation after antibody injections in normal and Bcl-2-protected livers have not been explored. As for a possible shortage of apoptotic-protective mechanisms in normal hepatocytes, it was striking to observe that Bcl-2 expression is not likely to act by compensating a decrease in Bclx or an increase in Bax, Bad, or Bak proteins, since, in contrast to what is observed with some apoptotic-inducing conditions with other cell types (31–33), no significant variations of these proteins or their mRNAs were found in Fas-stimulated livers. Thus, it may be that constitutive expression of Bcl-2 is more protective than that of Bclx in hepatocytes. There is indeed evidence that expression of Bcl-2 and Bclx may not be in all cells or conditions equally protective against apoptosis (34). Use of AAT-bclx transgenic mice might offer some information in this respect. Finally, it has been shown with other cell types that the effector mechanism of Fas-mediated apoptosis involves proteolytic events, in particular activation of the proteolytic enzyme ICE and possibly other cysteine proteases of the same family (3, 4). Comparable proteolytic events are likely to occur in Fas-stimulated hepatocytes. Since hepatocytes contain high amounts of ICE (not shown), it will be of interest to explore whether a proteolytic cascade involving ICE can be especially easily induced or activated in hepatocytes, thus explaining the high sensitivity of these cells (which also synthesize high levels of precursors of the coagulation proteolytic cascade) to Fas-mediated apoptosis.

Fas-mediated liver injury is likely to play a critical role in some forms of immune-mediated hepatitis, since it has become evident that activated T lymphocytes have the ability to kill Fas-bearing targets through their membrane expression of Fas-L (1). The present observations raise the possibility of new therapeutic approaches in life-threatening forms of these diseases, by the use of gene targeting of Bcl-2 into hepatocytes in vivo or into liver transplants in vitro, or by the temporary perfusion of specific antiproteolytic peptides that can penetrate within cells.

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