

A Naturally Occurring Soluble Isoform of Murine Fas Generated by Alternative Splicing

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Summary

We report a soluble isoform of mouse Fas, which is generated by alternative splicing of Fas mRNA to a newly identified exon located between exons 2 and 3 of the previously published Fas sequence. This splicing event creates a novel Fas transcript, Fas β , with the potential to encode a truncated form of the extracellular domain, termed Fas B. In vitro, P815 mastocytoma cells transfected with Fas B become resistant to Fas ligand-induced apoptosis, and the resistance is mediated by a secreted product of the transfected cells. In vivo, Fas β mRNA expression is correlated inversely with apoptosis among subsets of intrahepatic T lymphocytes, a cell population in which activation-induced T cell apoptosis occurs. We propose that Fas B is a new cytokine that acts physiologically to limit apoptosis induced by Fas ligand.

Soluble isoforms of cell surface receptors regulate receptor function in a number of biological systems. Such soluble receptors most commonly retain ligand-binding activity and compete for ligand, thus inhibiting receptor function (1–3). Soluble receptor isoforms may be generated by proteolytic cleavage of the transmembrane form or by alternative mRNA splicing to generate distinct molecular species. In this report, we describe a soluble inhibitory isoform of the CD95/Fas molecule, which is generated by alternative splicing and is differentially expressed in T cell subsets undergoing apoptosis in vivo.

The Fas antigen (CD95) is important in activation-induced cell death of T lymphocytes (4, 5) and is widely expressed in the immune system and in nonlymphoid tissues (6, 7). The susceptibility of cells to apoptosis induced by Fas ligation is regulated not only through the density of Fas on the cell surface, but also by other mechanisms that are poorly understood (8–10). We have cloned a variant Fas transcript that encodes a truncated, soluble isoform that we term Fas B. Expression of Fas B inhibits apoptosis induced by Fas–Fas ligand (FasL)¹ interaction, and the differential expression of the two Fas isoforms in vivo is consistent with a role in controlling apoptosis in a subset of activated T cells in the liver.

Materials and Methods

Reverse Transcription (RT) PCR, Cloning, and Sequencing. Murine Fas message was amplified by PCR from total thymus cDNA

using primers F1 (CGGGGATCCACCATGCTGTGGATCTGGGCTG) and RL1 (GCGGAATTCGATATCACTCCAGACATTGTCC). Reactions contained 2.0 mM MgCl₂ and were run for 30 cycles of 1 min at 94°C, 1 min at 58°C, and 1.5 min at 72°C. The two resulting products were separated by agarose gel electrophoresis, cloned into pBluescript, and sequenced by the Taq-Dye-deoxy termination method. λ -phage genomic clones of *fas* were isolated as described (11), using a probe encoding the extracellular domain published for murine Fas (115 to 558 bp) (6). The intronic regions surrounding exon 2A were sequenced from three of these clones using the exon 2A-specific primers 2AFOR (CGGGCGGCCGCGGACCAGTCTGATTCTGGCCACG) and 2AR (GCGGCGGCCGCTGCCTGCAAGATGTGCAGG).

Antibodies and Flow Cytometry. The following antibodies were used: H57-597-Cy Chrome (anti-TCR C β ; Pharmingen, San Diego, CA) (12); RA3-3A1-FITC (TIB-146, anti-B220; kind gift from Kim Bottomly, Yale University, New Haven, CT) (13); H129-19-FITC (anti-CD4; Gibco BRL, Gaithersburg, MD) (14); 53-6.7-Red 613 (anti-CD8 α ; Gibco BRL) (15); and Jo-2-PE (anti-Fas; Pharmingen) (16). FACS[®] staining was performed as described (17). Data were acquired with a FACScan[®] flow cytometer, using FACScan[®] research software, and analyzed using Lysys 1.7 (instrument and software from Becton Dickinson & Co., Mountain View, CA).

Transfection. Fas B cDNA was obtained by RT-PCR using primers F1 and X3REV (GCGGCGGCCGCGATGGGGCAGGTTGGTG), digested with XbaI and SacI, then cloned into the same sites of the pSR α -72(N.1) eukaryotic expression vector. The pSR α -72(N.1) vector drives expression of the inserted sequence under control of the SV40 early promoter and the HTLV I 5' enhancer. The integrity of the insert was confirmed by sequencing as discussed earlier, and the vector DNA was electroporated into P815 cells as described (18). From >700 neomycin-resistant clones, 200 were tested by PCR for presence of the cDNA insert. Of several dozen positive clones, four were

¹ Abbreviations used in this paper: Etn, early transposable element; FasL, Fas ligand; IHL, intrahepatic T lymphocyte; RT, reverse transcription.

chosen for further study. These clones expressed similar levels of Fas A, but different levels of Fas B, as tested by RT-PCR.

A FasL expression vector was made using the pSR α -72(N.1) vector and the FasL PCR product as described earlier for Fas B using primers FASLIGF1 (GCCTCTAGAGCCACCATGCAG-CAGCCCATG) and FASLIGR4 (GCGGAGCTCTTTTAAAGCTTATACAAGC) (125 to 965 bp in Takahashi et al. [19]). This vector was transfected into NIH-3T3 cells by using calcium phosphate precipitation as described (18). Parallel to this transfection, NIH-3T3 cells were also transfected with the empty pSR α -72(N.1) vector, and all transfected 3T3 cells were selected in 0.5 mg/ml G418-supplemented medium. G418-resistant clones were tested for FasL expression by their ability to differentially kill wild-type but not *lpr* thymocytes. One clone was selected for expansion and use in further studies. All transfected cells were expanded and maintained in 0.5 mg/ml G418-supplemented medium until the passage before their use in experiments.

Isolation of Intrahepatic Lymphocytes. Intrahepatic T lymphocytes (IHLs) were isolated from livers of C57Bl/6 mice by collagenase IV digestion and metrizamide flotation as described (20).

RNAse Protection Analysis. Uniformly labeled riboprobes for RNase protection were synthesized as described (11) at two different specific activities. A γ -actin-specific probe, which protected a 105-bp fragment from the 3' untranslated region of the message, was synthesized using 800 μ M cold rCTP and 25 μ M UTP, of which 5 μ M was supplied as α -[³²P]UTP. A Fas-specific probe, which protected a 121-bp fragment from Fas α and a 272-bp band from Fas β , was synthesized using 9 μ M each of rCTP and UTP, of which 5 μ M was supplied as α -[³²P]rCTP and α -[³²P]UTP. Protected fragments were separated by electrophoresis through a 5% polyacrylamide/8 M urea gel, and the dried gel was exposed to a phosphor-imaging screen for 4–6 d. Under the conditions used, the protected fragments frequently ran as doublet bands. In these cases, both bands were included in quantitation. The specific signal from each protected Fas band was divided by 1% of the specific signal from γ -actin in that sample to determine Fas message intensity in “actin units.”

Results

Cloning and Sequence of Fas B. In an RT-PCR analysis of mouse Fas expression in thymocyte subsets, an amplified product of the expected size was consistently accompanied by a larger fragment that resulted from an alternative transcript of the Fas gene. The alternative transcript (which we term Fas β) arose from a 158-bp insertion between the second and third exons of the reported Fas sequence. A λ -phage mouse genomic DNA library was screened with a plasmid containing the extracellular domain of the Fas sequence, and three clones were isolated in which the novel sequence could be detected by PCR. The sequence of this insertion, which we term exon 2A, and its surrounding intronic splice signals are shown in Fig. 1 A. The incorporation of exon 2A caused a frame shift in exon 3 of the gene, leading to a stop codon 27 bp into exon 3. The translated product of this message, Fas B, would be a truncated Fas protein containing a single cysteine-rich subdomain followed by a highly charged COOH-terminal tail. The mature Fas B polypeptide has a predicted molecular mass of 11 kD, with two potential N-linked glycosylation sites as well as O-linked sites.

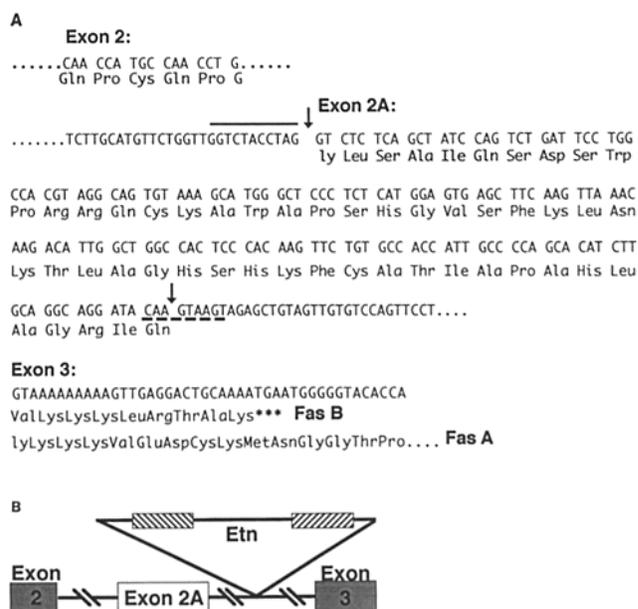


Figure 1. (A) Genomic sequence of Fas exon 2A and flanking sequences. The 158-bp nucleotide sequence of exon 2A and its flanking sequences in genomic DNA are shown. The interpolation of exon 2A sequence results in a frameshift and premature stop codon 27 bp into exon 3. The Fas β RNA therefore encodes a soluble protein, Fas B, corresponding to a single subdomain of the extracellular domain of CD95/Fas. (B) Diagram of exon 2A and surrounding region. The relative positions of exon 2A and the Etn insertion in *lpr* in the genomic Fas sequence are shown. The positions were determined by RT-PCR using cDNA from *lpr* and wild-type mice.

Mutant *lpr* mice lack functional Fas expression because of the insertion of a retroviral early transposable element (Etn) into this same region of the Fas gene (21, 22). To determine the relationship of exon 2A to the Etn insertion in *lpr*, we performed RT-PCR for Fas using cDNA from C57Bl/6 mice or congenic B6-*lpr* thymocytes. Identical products were obtained from either template from reactions with a forward primer in exon 1 and a reverse primer in exon 2A. When reactions contained a forward primer in exon 2A and a reverse primer in exon 3 or at the 3' end of the coding region, however, we observed amplicons of the expected size only from the C57Bl/6 template. No product was seen from the B6-*lpr* template. Similar results were obtained for MRL.MP-+/+ and MRL.MP-*lpr/lpr* template. Because Fas RNA was spliced efficiently to exon 2A in both wild-type and *lpr* mice, but not from exon 2A to exon 3 in *lpr* mice, we conclude that the exon 2A sequence is 5' of the Etn insertion in *lpr* (Fig. 1 B).

Function In Vitro. Such a molecule might be expected to act as a soluble competitor for the FasL, thereby inhibiting FasL-induced apoptosis. To test this hypothesis, P815 cells, which express endogenous transmembrane Fas (Fas A), but not Fas B, were transfected with a Fas B-encoding cDNA using the eukaryotic expression vector pSR α -72(N.1). Four clones were selected for study. Clones 1B7 and 1D1 had low level expression of Fas B, as determined by competitive PCR, whereas clones 1D7 and 1E11 had

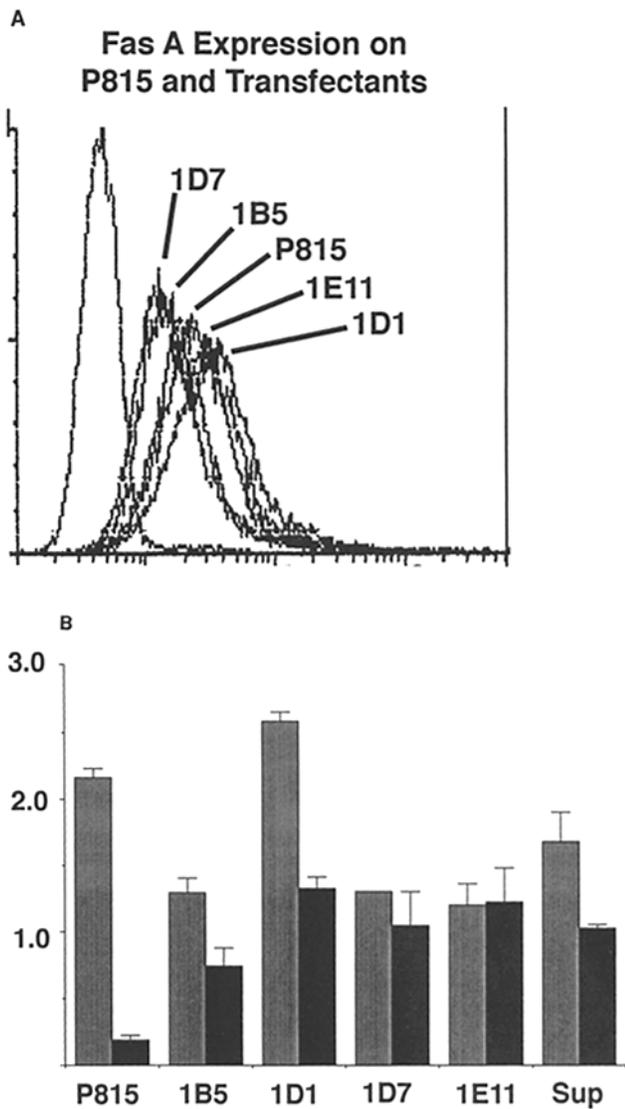


Figure 2. (A) Surface Fas A expression on P815 cells and transfectants. The expression of endogenous Fas on parental P815 cells and Fas B-transfected clones was examined by FACS[®] with anti-Fas-PE. Transfectants continued to express Fas on the membrane. The order of the labels reflects the level of expression. Note that clone 1E11, shown in B to be completely protected, expressed the second highest level of Fas. (B) Protection from Fas-mediated death by Fas B expression. Parental P815 or Fas β transfectants were cocultured overnight with FasL or empty-vector-transfected 3T3 cells, and the surviving P815 cells counted. Four Fas β transfectants—clones 1B5, 1D1, 1D7, and 1E11—were relatively protected from apoptosis by FasL. Values are mean \pm SD of the viable cells recovered from overnight cultures in millions. Gray bars represent viable cells recovered from control 3T3 monolayers and black bars represent cells from FasL-transfected 3T3 monolayers. The effect was due to soluble Fas B protein, because supernatant from 1E11 cells protected wild-type P815 cells from FasL-induced apoptosis. A representative experiment of five is shown. Differences in cell yield from control monolayers were not consistent from experiment to experiment and most likely represent differences in the rate of growth within each culture. The protective effect of Fas B transfection was consistent in all experiments, however.

\sim 10-fold higher expression (data not shown). All clones expressed Fas A on the cell surface, some more and some less than wild-type P815 (Fig. 2 A). Fas B was therefore not simply interfering with Fas A expression.

To test the biological activity of excess Fas B, these cells were cultured overnight on monolayers of 3T3 fibroblasts that had been transfected with FasL (Fig. 2 B). After overnight (18 h) coculture, viable P815 cells were counted by nigrosin exclusion. Less than 10% of the wild-type P815 cells survived the treatment with FasL-positive 3T3 cells, whereas 20–50% of clones 1B5 and 1D1, expressing Fas B at low levels, survived. Among the high expressing clones 1D7 and 1E11, 75–100% of the cells survived FasL exposure, indicating that Fas B expression can block Fas-mediated

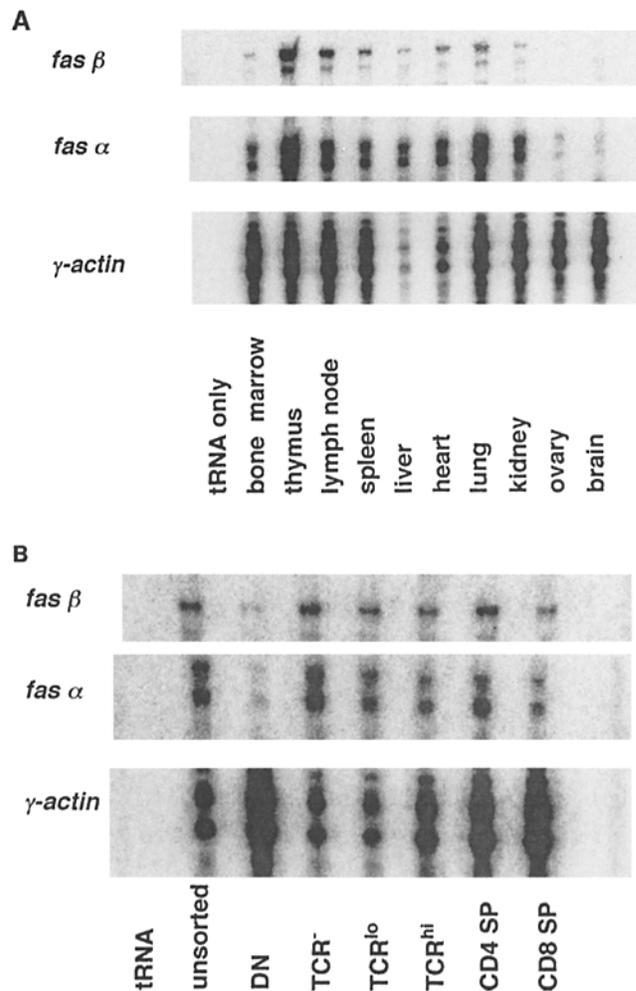


Figure 3. (A) Expression of Fas B-encoding RNA in vivo. RNase protection was used to examine Fas β versus Fas α in 10 μ g of RNA from various tissues. The relative expression of Fas β was lower in nonlymphoid than in lymphoid tissues. A representative experiment of five is shown. Similar results were obtained from poly-A⁺ RNA. (B) Expression of Fas B-encoding RNA during thymic development. Thymocytes from C57Bl/6 mice were sorted by FACS[®] according to CD4 and CD8 expression or by TCR density. RNase protection was used to examine expression of Fas β versus Fas α in samples of RNA from subsets of thymocytes. 4.0×10^6 cells were used for each sample. A representative experiment of three is shown.

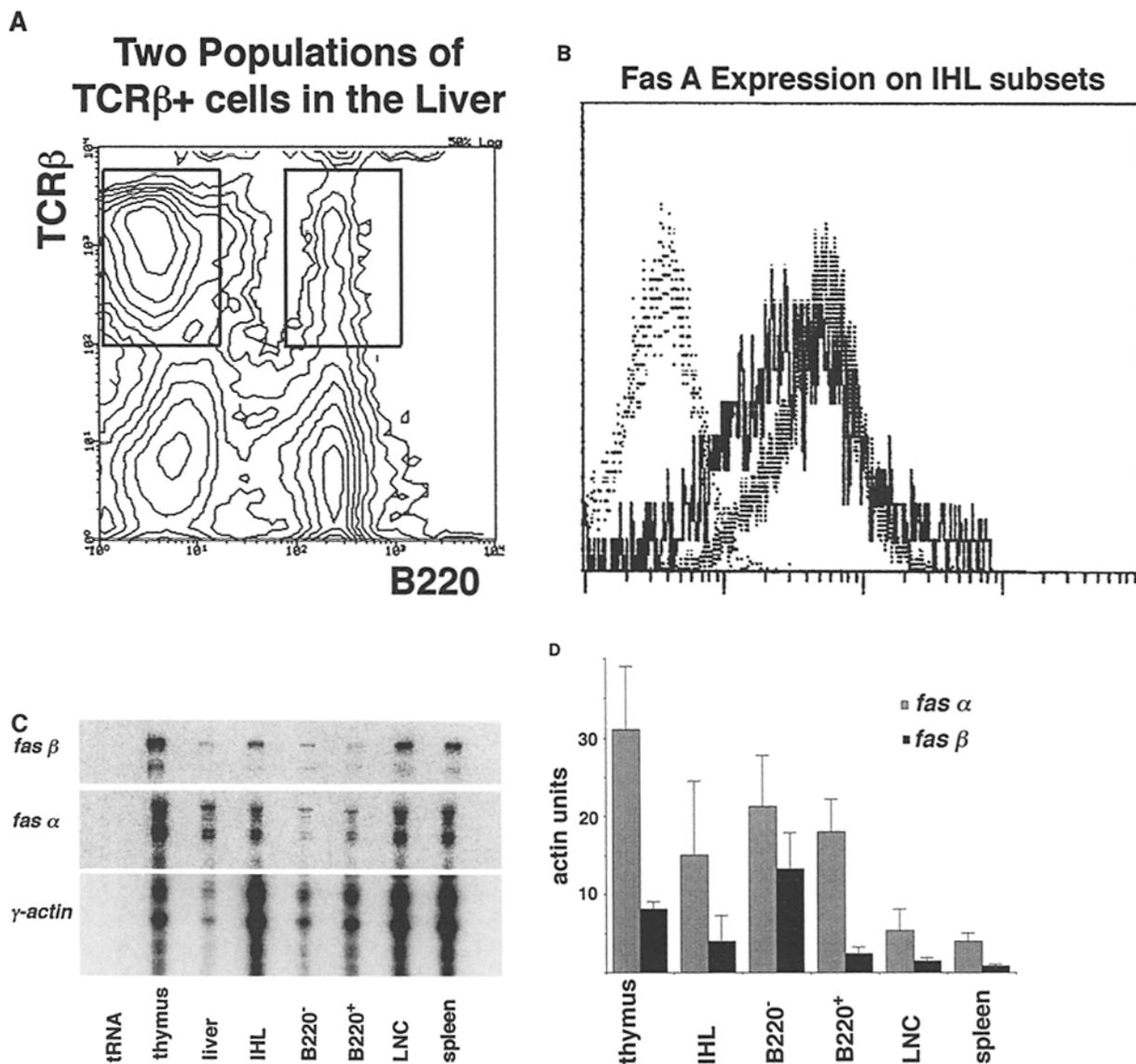


Figure 4. (A and B) Surface Fas expression in IHL subsets. IHLs were isolated from C57Bl/6 mice as described (20) and stained with anti-TCR β , anti-B220, and anti-Fas and examined by FACS[®] in three colors. The gates shown for TCR β and B220 expression in Fig. 4 A were used to generate the Fas expression histograms shown in Fig. 4 B. The solid line represents the B220⁺ subset, and the dashed line represents the B220⁻ subset. Unstained cells (dotted line) are shown for comparison. (C) Expression of Fas B–encoding RNA in IHL subsets. IHLs were sorted into two populations of TCR β ⁺ cells based on B220 expression as shown in Fig. 4 A. RNase protection was used to examine expression of Fas β versus Fas α in RNA from 0.5×10^6 B220⁻ IHLs and 1.5×10^6 B220⁺ IHLs. This expression was compared with that of 4×10^6 thymocytes, total IHLs, LNCs, and splenocytes. In addition, Fas expression in 5 μ g of liver RNA was tested. A representative experiment of three is shown. (D) Quantitation of Fas β expression in IHL subsets. Intensity of expression of Fas relative to γ -actin in subsets of IHLs and controls is shown. Values are mean expression in three experiments; error bars show standard deviation. Because hepatocytes primarily express β -actin rather than γ -actin, quantitation of the total liver RNA sample is not shown. Quantitation was performed using a phosphorimager as described in Materials and Methods.

ated apoptosis. The protection is caused by a soluble material, since supernatant from clone 1E11 protected parental P815 cells from apoptosis. We conclude that Fas B is secreted by the transfected cells as a soluble protein that inhibits apoptosis induced by Fas–FasL interaction.

Expression In Vivo. The messages encoding Fas A and Fas B were differentially expressed in different cell populations. The expressions of Fas α and Fas β in RNA from various tissues were analyzed by RNase protection (Fig. 3,

A and B). Fas β was more abundant in lymphoid tissues; 11–20% of the Fas message in thymus, LN, spleen, and bone marrow, but <5% of the total Fas message from liver, kidney, heart, and lung was in the Fas β form. Two T cell populations, thymocytes and IHLs, were selected for detailed analysis. Thymocyte subsets were tested because thymocytes are Fas positive (16, 23), highly susceptible to apoptosis (24–26), and differentially susceptible to Fas-mediated apoptosis (27–29). Thymocytes were sorted by FACS[®] into sub-

sets at different developmental stages based on TCR expression or CD4 and CD8 phenotype. Fas mRNA expression was very low in CD4⁻CD8⁻ progenitor thymocytes, high in CD4⁺CD8⁺ immature cells, and lower on mature CD4⁺CD8⁻ and CD8⁺CD4⁻ cells, following the pattern of Fas expression on the membrane (29, 30). The Fas α /Fas β ratio, however, did not differ among these subsets (Fig. 3 B). We conclude that the recently reported exquisite sensitivity of CD4⁺CD8⁺ thymocytes to apoptosis induced by Fas ligation (27–29) is not caused by differential low expression of Fas B in these cells.

In contrast to thymocytes, there is a strong case that Fas is important in the apoptosis of mature T cells and that this apoptosis occurs in the liver (4, 31). The two major subsets of intrahepatic T cells defined by B220 expression expressed Fas on the cell membrane at similar density (Fig. 4, A and B). These cell subsets had similar overall Fas mRNA levels but very different ratios of Fas α to Fas β . The B220⁺CD4⁻CD8⁻ subset, in which ~20% of the cells are in the process of apoptosis (20), expressed a high level of Fas α with a low level of Fas β . In contrast, the B220⁻ subset, composed of both CD4 SP and CD8 SP cells (20), expressed high levels of both Fas transcripts (Fig. 4, C and D). Because the two populations express a similar amount of Fas A on the cell surface, their difference in Fas β expression probably accounts for their difference in apoptosis.

Discussion

While superantigen-induced deletion of mature CD4⁺ T cells results in apoptosis in the lymph nodes (32), peptide-driven deletion of CD8⁺ T cells leads to accumulation and apoptosis at a specific anatomical site: the liver (31). Activated T cells express an increased level of Fas on the cell membrane and are susceptible to Fas-induced apoptosis (5, 8, 10). We propose that the liver is the site of activated T cell apoptosis because it is a site in which the T cells are exposed to FasL. This implies that all Fas-positive T cells among the IHL are vulnerable to FasL-induced apoptosis unless specifically protected. The CD4⁺ and CD8⁺ T cells in the liver appear to be protected by their elevated expression of Fas B. The function of these cells is unknown; their resistance to apoptosis suggests that unlike the B220⁺CD4⁻CD8⁻ cells, they are not proceeding along the peripheral deletion pathway. It is possible that, instead, they are the effectors of deletion.

A very different splice variant of the human Fas gene has been cloned by using PCR from T cells of a patient with systemic lupus erythematosus (33). This cDNA resulted from deletion of the transmembrane region, leaving a sequence with the potential to encode a soluble molecule consisting of the extracellular domain directly linked to the signaling domain (sFas). The structure of sFas is highly unusual for a soluble receptor isoform. Whereas a number of other transmembrane receptors (for example, IL-7 and G-CSF) generate soluble isoforms by splicing out of the transmembrane region, such deletions cause frameshifts or stop codons, as we describe here for Fas B (3, 34). An ELISA with anti-Fas antibody showed that a form of soluble Fas was present in the sera of some lupus patients, but whether this is in fact transmembrane-deficient sFas, a human equivalent of murine Fas B, or simply Fas A shed from the surface of dying cells remains to be determined. The expression pattern of transmembrane-deficient sFas in normal human T cells has not yet been defined. It is possible that both humans and mice regulate Fas-mediated apoptosis through soluble isoforms but generate these in completely different ways. It is also possible that sFas and Fas B exist in both species but perform different functions. A recent study of mouse Fas protein expression by immunoprecipitation showed three molecular species, which may correspond to Fas A, Fas B, and sFas (35).

The abnormal Fas mRNA species generated in mice with the *lpr* mutation have the potential to encode truncated forms of Fas similar to Fas B (22). The insertion of the Etn transposon in this mutation leads to frequent splicing of RNA to a site just 5' of the LTR; this generates truncated messages that use the Etn poly A addition site and can be translated (22). These aberrant transcripts could include exon 2A and generate a protein very similar to Fas B. In preliminary experiments by RNase protection, we found a high proportion of Fas transcripts with exon 2A in *lpr* thymus RNA (unpublished data). Because *lpr* thymocytes were recently shown to express a very low level of Fas protein (35), the presence of Fas B-like isoforms may contribute to the *lpr* phenotype by blocking signaling through the few Fas molecules that are expressed. This also raises the question whether Fas B-like molecules are overexpressed in other mouse strains with spontaneous autoimmunity, where they may act to subvert normal apoptosis of potentially self-reactive T cells.

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