Functional significance of the Fas molecule in naive lymphocytes

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Abstract

The Fas molecule mediates apoptotic signal in many cell types. Mouse mutations (Ipr, Ipr², gld), which impair the function of Fas, cause spontaneous autoimmune disease. We generated Fas-deficient (Fas⁻⁻) mice by homologous recombination. In embryonic stem cells Fas⁻⁻ mice developed Ipr-like disease, confirming that the abnormality of Fas is causal in the Ipr phenotype. We also made Fas⁻⁻ chimeric mice composed of a mixture of Fas⁺/+ and Fas⁻⁻ cells. The chimeric mice also showed the Ipr phenotype. In Fas⁻⁻ chimeric mice, the Fas-deficient population expanded progressively among mature T and B lymphocytes. The expansion of Fas-deficient lymphocytes occurred at the naive, pre-primed, lymphocyte stage. These results suggest that the Fas molecule functions not only after antigenic stimulation, as previously hypothesized, but also at the naive lymphocyte stage.

Introduction

Fas/Apo-1 (CD95) was originally discovered as a transmembrane protein which mediated an apoptotic signal (1-4). Ipr mice, which show lymphoproliferative disease characterized by massive enlargement of lymph nodes and hypergammaglobulinemia, express reduced amounts of Fas molecule due to the insertion of a transposable element into the Fas locus (5-9). Ipr⁰ mice and gld mice develop lymphoproliferative disease similar to Ipr mice, and have point mutations in the genes encoding for Fas and Fas ligand respectively (6,10-13).

Antigenic stimulation enhances the expression of Fas on lymphocytes. Several days after stimulation, lymphocytes become sensitive to Fas-mediated cell death (14-21). Activation-induced death of lymphocytes is impaired in Ipr mice, and it is hypothesized that the major role of Fas is to cause cell death of activated lymphocytes and appropriately terminate the immune response (22-27).

We generated mice totally lacking Fas by homologous recombination in embryonic stem (ES) cells. By injecting Fas⁻⁻ ES cells into Fas⁺/+ blastocysts, we also made Fas⁻⁻ chimeric mice, which were composed of Fas⁺/+ and Fas⁻⁻ cells, and examined comparatively the phenotype of Fas⁺/+ and Fas⁻⁻ lymphocytes in vivo.

Methods

Construction of targeting vectors

A mouse Fas cDNA clone (generously provided by Dr S. Nagata; 28) was used as a probe to isolate genomic DNA clones corresponding to the Fas locus from a mouse strain 129 library (Stratagene, La Jolla, CA). In the targeting vectors, pFKO-Neo and pFKO-Hyg, the entire Fas gene except for exon 1 was replaced with either a PGK-neo-poly(A) cassette or a PGK-hyg-poly(A) cassette (29) respectively. Targeting vectors contained 1.3 kb of homology 5’ and 10.6 kb 3’ of the drug-resistance marker. The PGK-ik-poly(A) cassette (30) was ligated into a restriction site in a vector polylinker at the 3’ end of the insert (Fig. 1A).
Generation of Fas\(^{+/−}\) and Fas\(^{−/−}\) ES clones

Transfection and screening of E14 ES cells were performed as described previously (29). E14 ES cells were transfected with targeting vector, pFKO-Neo, to make Fas\(^{−/−}\) ES clones. For screening of pFKO-Neo-targeted clones by PCR, a Fas-flanking primer, Fl (5′-CTTGGCTGGCAATCCCTACACT-3′), and a PGK-promoter-specific primer (5′-TGCTAAAGCCCATGCTCAGACTG-3′) were used. Fas\(^{−/−}\) ES cells were further transfected with targeting vector, pFKO-Hyg, to make Fas\(^{−/−}\) ES cell clones. For screening of pFKO-Hyg-targeted clones, primer Fl and poly(A)-fragment-specific primer (5′-GGTGGGGTGGGATAGATAATGC-3′) were used. PCR positive clones were analyzed by Southern blot hybridization to verify for homologous recombination (Fig. 1B).

ES cells were microinjected into C57BL/6 (B6) blastocysts. Injected blastocysts were implanted into uteri of pseudo-pregnant ICR mice to generate chimeric mice.

Genotyping of mice

Tail DNA was analyzed by PCR amplification to determine the Fas locus. Neomycin-resistance-gene-specific primers (5′-ATTCGGCTATGACTGGGCACAACA-3′ and 5′-CAGGAACCATGAGTTGAGATAG-3′) were used to detect pFKO-Neo-targeted allele. Wild-type allele was detected by PCR amplification of a 0.2 kb fragment in the last exon of the Fas gene, using PCR primers (5′-GAAGGACCTTGGAAAATCAACC-3′ and 5′-TCTCAGCAACTGCAGAGAAAAT-3′).

Flow cytometry analysis

The following mAb conjugated with FITC, phycoerythrin (PE) or biotin were purchased from PharMingen (San Diego, CA) and used to stain cells: anti-CD4 (RM4-5), anti-CD8α (5-6.7), anti-CD44 (KM201) mAb were purchased from Southern Biotechnology Associates (Birmingham, AL). Red613-streptavidin (Gibco/BRL, Grand Island, NY) was used to reveal the biotin-conjugated mAb. Stained cells were analyzed on FACSscan flow cytometer with CELLQuest software (Becton Dickinson, Mountain View, CA). Viable lymphocytes were gated according to forward and side scatter and propidium iodide staining. In some analyses, to purify T cells, lymph node cell samples were incubated with rat anti-mouse B220 (RA3-6B2) mAb, washed and mixed with Dynabeads (Dynal) coated with sheep anti-rat IgG. Unbound cells were recovered by using a magnetic separator (Advanced Magnetics). After purification twice with Dynabeads, purity of the recovered cell population was established by flow cytometric analysis and was always >96% TCRαβ\(^{+}\) T cells.

ELISA

Blood samples were obtained from mouse tails and centrifuged to isolate sera. Microtitration plates (Immulone 4; Dynatech, San Diego, CA) were coated with goat antiserum to mouse IgM (Cappel, San Diego, CA), goat antiserum to mouse IgG (Cappel) or heat-denatured herring sperm DNA (10 μg/ml) at 37°C for 3 h. Plates were washed with washing buffer (PBS containing 0.05% Tween 20) and incubated with blocking buffer (PBS containing 1.0% bovine serum albumin and 0.05% Tween 20) at room temperature for 1 h. Serum samples diluted 200–3,125,000 times with blocking buffer were added to the plates and incubated at 4°C for 12 h. After washing, alkaline phosphatase (AP)-conjugated goat anti-mouse IgM antibody (Pierce, Chicago, IL) or AP-conjugated goat anti-mouse IgG antibody (Cappel) were added to the plates and incubated at room temperature for 2 h. After washing, p-nitrophenyl-phosphate (Sigma, St Louis, MO) in reaction buffer (0.1 M glycine, 1 mM MgCl\(_2\), 1 mM ZnCl\(_2\), pH 10.4) was added to the plates. Plates were incubated at room temperature for 1–12 h and color development was measured on a microtitration plate reader MR700 (Dynatech). Purified mouse IgM (MOPC-104E, PharMingen) or IgG (RPC5; Cappel) antibody was used as a standard to convert the OD values of data to the concentration (mg/ml).

Results

Germline-transmitted Fas\(^{−/−}\) mice develop the lpr phenotype

Using the targeting vector, pFKO-Neo, for homologous recombination, the Fas gene was deleted except for exon 1, which encodes half of the signal peptide (Fig. 1). This large deletion (>30 kb) was created to avoid any Fas-related residual function. Fas\(^{−/−}\) ES cell clones were obtained at a frequency of 1% from G418/gancyclovir-resistant pFKO-Neo-transfectant clones. Fas\(^{+/−}\) ES cell clones were injected into B6 blastocysts. Resulting chimeric mice were crossed to B6 mice. F1 offspring of agouti coat color were genotyped to select Fas\(^{−/−}\) mice. Fas\(^{+/−}\) mice were intercrossed, and Fas\(^{+/−}\), Fas\(^{+/+}\) and Fas\(^{−/−}\) F\(_2\) mice were obtained. Absence of the Fas molecule in Fas\(^{−/−}\) was confirmed by flow cytometric analysis (Fig. 2A).

We observed progressive enlargement of lymph nodes and spleens in Fas\(^{−/−}\) mice (Fig. 3). Atypical CD4\(^{+}\)CD8\(^{−}\) TCR\(^{+}\) lymphocytes appeared in enlarged peripheral lymphoid organs and peripheral blood of Fas\(^{−/−}\) mice (Fig. 2B and data not shown) Fas\(^{−/−}\) mice produced higher titers of total IgM, total IgG, anti-DNA IgM and anti-DNA IgG antibody than Fas\(^{+/+}\) or Fas\(^{+/−}\) littermates (Fig. 2C). Collectively, germline-transmitted Fas\(^{−/−}\) mice showed the same lymphoproliferative disease as lpr mice, confirming that a defect in Fas is the cause of the lpr phenotype.

Fas\(^{+/−}\) chimeric mice show the lpr phenotype

Fas\(^{+/−}\) ES cells were further transfected with the targeting vector, pFKO-Hyg, and selected to make Fas\(^{+/−}\) ES cell clones. Fas\(^+/−\) ES cells and parental ES cells (Fas\(^{+/+}\)) were separately injected into B6 blastocystos (Fas\(^{+/+}\)) to make chimeric mice (designated as Fas\(^{+/−}\) chimeric mice and control chimeric mice respectively) In Fas\(^{+/−}\) chimeric mice, the ES-derived population is Fas\(^{+/−}\) and the blastocyst-derived population is Fas\(^{+/+}\). In control chimeric mice, both the ES-derived and blastocyst-derived population are Fas\(^{+/+}\). Among the lymphocytes of chimeric mice, we identified ES-derived lymphocytes by an allotypic marker Ly-9.1, which is expressed on the cell surface of 129 (Ly-9.1) but not B6 (Ly-9.2) lymphocytes (29). As expected, both Ly-9.1\(^{+}\) (ES-derived) and Ly-9.1\(^{−}\) (control chimeric) mice showed lpr-like symptoms.
**Fig. 1.** Target disruption of the Fas gene (A) Schematic depiction of the mouse Fas gene, targeting vectors and mutant allele of the Fas gene. Hatched boxes indicate 3'- and 5'-homologous regions included in the targeting vectors. Closed boxes indicate exons. Locations of the initiation codon (ATG) and the termination codon (TGA) of the Fas gene are shown. Abbreviations for restriction sites were as follows. B, BglII, Bm, BamHI; E, EcoRI. The restriction map of EcoRI is not complete. (B) Southern blot analysis showing homologous recombination. Genomic DNA samples from parental ES cells, a Fas+ ES clone and a Fas− ES clone were digested with BglII. Location of the probe was as shown in (A). The 4.0 kb band indicates wild-type and the 6.0 kb band indicates the mutant allele as shown in (A).

**Fig. 2.** (A) Two-color flow cytometric analysis showing the absence of Fas molecule in germline-transmitted Fas− mouse. Thymocytes from a Fas− mouse and a Fas+ mouse were stained with anti-TCRαβ-FITC and anti-Fas-PE. (B) Appearance of atypical lymphocytes in germline-transmitted Fas− mouse. Lymph node cells from a 5-month-old Fas− mouse and a Fas+/mouse were stained with either anti-TCRαβ-PE and anti-B220-biotin or anti-CD4-PE and anti-CD8a-biotin, followed by Red613-streptavidin. Two-color flow cytometric profiles of TCRα/β-B220 (top) and CD4/CD8 (bottom) are shown. Percentages for quadrants are given. (C) Hypergammaglobulinemia and production of anti-DNA antibody in Fas− mice. Serum levels of total IgM, anti-ssDNA IgM, total IgG, and anti-ssDNA IgG antibody of Fas− mice (n = 4), Fas+/ mice (n = 5) and Fas− mice (n = 7) were measured by ELISA. Serum samples were obtained from 4-month-old mice. A serum from one Fas− mouse was used as a standard serum in assay of anti-DNA IgM and IgG antibody. The concentration of anti-DNA IgM and IgG antibody in each sample was expressed as a relative value. The average values and SD (error bars) are shown.

(blastocyst-derived) thymocytes of control chimeric mice expressed the Fas molecule. On the other hand, Ly-9.1+ thymocytes of Fas− chimeric mice did not express the Fas molecule (Fig. 4A). We observed progressive lymphadenopathy and splenomegaly (Fig. 3), appearance of atypical lymphocytes (Fig. 4B), hypergammaglobulinemia and production of anti-DNA antibody (Fig. 4C) in Fas− chimeric mice, but not in control chimeric mice. In brief, Fas− chimeric mice also developed lpr syndrome.

Atypical CD4+CD8− TCR+ B220+ lymphocytes in Fas− chimeric mice were exclusively Ly-9.1+, indicating that they
Fig. 3. Massive enlargement of lymph nodes and spleens in Fas-deficient mice. A Fas$^{+/+}$ mouse (A), a 6-month-old Fas$^{-/-}$ mouse (B) and a 7-month-old Fas$^{+/-}$ chimeric mouse (C) are shown.

originated from Fas$^{+/-}$ ES cells. As observed in Ipr mice (31,32), enlarged peripheral lymphoid organs of Fas$^{+/-}$ chimeric mice contained conventional CD4$^+$, CD8$^+$ and IgM$^+$ lymphocytes in addition to the atypical lymphocytes (Fig. 4B).

Age-dependent expansion of Fas$^{+/-}$ lymphocytes in chimeric mice

We examined the age-dependent change of the ES-derived
**Fig. 4.** (A) Two-color flow cytometric analysis showing the absence of Fas molecule in ES-derived cells of Fas"' chimeric mice. Thymocytes from a Fas"' chimeric mouse and control chimeric mouse were stained with anti-Ly-9.1-FITC and anti-TCRap-PE. (B) Appearance of atypical lymphocytes in a Fas"' chimeric mouse. Lymph node cells from a 5-month-old Fas"' chimeric mouse and control chimeric mouse were stained with either anti-Ly-9.1-FITC, anti-TCRap-PE and anti-B220-biotin or anti-Ly-9.1-FITC, anti-CD4-PE and anti-CD8a-biotin, followed by Red613-streptavidin. Histogram plots of Ly-9.1 (top) and dot plots of TCRapB220 (middle) and CD4/CD8 (bottom) are shown. Cell populations gated on Ly-9.1* (as indicated in histogram plots) are shown in dot plots. Percentages for quadrants in Ly-9.1* cell populations are given. (C) Hypergammaglobulinemia and production of anti-DNA antibody in Fas"' chimeric mice. Serum levels of total IgM, anti-ssDNA IgM, total IgG and anti-ssDNA IgG antibody of control chimeric mice (n = 5) and Fas"' chimeric mice (n = 7) were measured by ELISA. Serum samples were obtained from 4-month-old mice. Serum from a Fas"' mouse was used as a standard in assay of anti-DNA IgM and IgG antibody. The concentration of anti-DNA IgM and IgG antibody in each sample was expressed as relative value to this standard serum. The average values and standard deviations (error bars) are shown.

**Fig. 5.** Progressive expansion of Fas"' population among peripheral blood T and B lymphocytes in chimeric mice. Tail blood samples were obtained from chimeric mice at 3, 8 and 12 weeks. To standardize the variation of original contribution of ES-derived population among mice and cell lineages, we calculated the relative ratio as (ratio at specific age)/(ratio at 3 weeks) for each chimeric mouse. As shown in Fig. 5, the Fas"' population progressively increased among T and B lymphocytes. Although the rate of increase was much greater in T cells than in B cells, these findings
indicate that the Fas molecule regulates the number of both T and B lymphocytes under normal situations, and that the absence of Fas molecules causes unregulated expansion of lymphocytes.

A function of the Fas molecule on mature lymphocytes

To determine the differentiation stage at which the Fas<sup>−/−</sup> lymphocyte population expands, we analyzed the Ly-9<sup>+</sup>: Ly-9<sup>−</sup> ratio in T and B lymphocytes in thymi, lymph nodes, and bone marrow of 8-week-old chimeric mice, based on two-color flow cytometric analysis. The relative ratio was calculated as described in the text. CD4<sup>+</sup> or CD8<sup>+</sup> cells in lymph nodes and spleens were defined as mature T cells. IgD<sup>+</sup> cells were defined as mature B cells. The average values and SD (error bars) of Fas<sup>−/−</sup> chimeric mice (n = 5) and control chimeric mice (n = 5) are shown.

We calculated the relative Ly-9<sup>+</sup>: Ly-9<sup>−</sup> ratio for T cell lineage (ratio in a specific population of T cell lineage): (ratio in TCR<sup>hi</sup> thymocyte population) for each mouse. As shown in Fig. 6(A), the relative ratio in the TCR<sup>hi</sup> thymocyte population in Fas<sup>−/−</sup> chimeras was not different from that in control chimeras. On the other hand, in mature T cell populations in lymph nodes and spleens, the relative ratio in Fas<sup>−/−</sup> chimeras was higher than that in control chimeras.

We calculated the relative Ly-9<sup>+</sup>: Ly-9<sup>−</sup> ratio for B cell lineage (ratio in a specific population of B cell lineage): (ratio in B220<sup>+</sup> IgM<sup>+</sup> bone marrow cell population) for each mouse. As shown in Fig. 6(B), the relative ratio in B220<sup>+</sup> IgM<sup>+</sup> bone marrow cell population in Fas<sup>−/−</sup> chimeras was not different from that in control chimeras. On the other hand, in the populations of IgD<sup>+</sup> mature B cells in spleens and lymph nodes, the relative ratio in Fas<sup>−/−</sup> chimeras was higher than that in control chimeras.

More than 80% of lymphocytes were of conventional phenotype (CD4<sup>+</sup>, CD8<sup>+</sup> or IgM<sup>+</sup>) in 8-week-old Fas<sup>−/−</sup> chimeric mice, whose lymph node populations contained at least six times as many lymphocytes as control chimeric mice in the case that coat-color-chimerism was 70% or more. Therefore, Fas<sup>−/−</sup> lymphocytes increased not only relatively to Fas<sup>−</sup/+ lymphocytes but also in absolute number.

Taken together, the Fas<sup>−/−</sup> population abnormally increased in mature T and B lymphocytes in peripheral lymphoid organs, but not in thymus or bone marrow. These results suggest that Fas functions at the mature lymphocyte stage, although we cannot rule out that Fas has a function also at the immature stages.

Functional significance of the Fas molecule in naive lymphocytes

Because IgD<sup>+</sup> B cells are at the naive or pre-primed stage (33), the result described above that the Fas<sup>−/−</sup> population increased among IgD<sup>+</sup> B lymphocytes indicates an abnormal increase of Fas<sup>−/−</sup> naive B cells. Therefore, in normal situations, the Fas molecule functions to regulate the number of naive B cells.

We examined whether Fas has a regulatory role also in naive T cells. To avoid the complexity in flow cytometric
analysis due to the existence of atypical lymphocytes, we analyzed 5-week-old chimeric mice, in which atypical lymphocytes were <4% of total lymph node cells of Fas+ chimeric mouse.

High expression of CD62L (L-selectin) and low expression of CD44 (Pgp-1) are markers for naive lymphocytes (34–37). We calculated the relative Ly-9.1−:Ly-9.1+ ratio for lymph node T cell populations classified according to the level of expression of CD62L and CD44, by the same formula as described above. In both CD62Lhigh and CD62Llow as well as both CD44high and CD44low T cell populations, the relative ratio in Fas−/− chimeric mice was higher than that in control chimeric mice (Fig. 7) Thus, the unregulated increase of Fas−/− T cell occurred before the differentiation to memory cells, suggesting that Fas plays a regulatory role in naive T cells.

Discussion
The genomic region encoding mature Fas protein was completely deleted by the targeting constructs which span at least 30 kb. Although we have not obtained contiguous DNA clones covering all the deleted region, the deletion may be >50 kb, based on the report that the mouse Fas gene consists of >70 kb (7). Not only germline-transmitted Fas−/− mice but also Fas−/− chimeric mice showed the ipr phenotype. These results are consistent with a report on allophenic chimeras developed from ipr/+ and +/+ embryos (38). On the other hand, bone marrow chimeras, irradiated +/+ mice implanted with ipr/+ bone marrow grafts, did not show the ipr phenotype but showed graft-versus-host-disease-like syndrome and atrophy of lymphoid organs (39–41). The difference between embryonic chimeras and bone marrow chimeras is that Fas−/− non-bone-marrow-derived cells exist in embryonic chimeras, but not in bone marrow chimeras. Therefore, the existence of Fas−/− non-bone-marrow-derived cells, probably stromal cells in lymph node and spleen, may be a requisite for lymphoproliferation of ipr mice. This explanation is supported by the report of Matsuzawa et al. (42) that ipr/+ bone marrow grafts caused enlargement of simultaneously implanted lymph nodes derived from ipr/+ mice but not those derived from +/+ mice.

From the data shown in Figs 6 or 7, we cannot rule out that Fas has a function on immature cells which manifests itself in more mature cells. However, an age-dependent increase of the discrepancy of Ly-9.1−:Ly-9.1+ ratio between the mature and immature populations supports the notion that Fas functions at the mature lymphocyte stage. In 2-week-old chimeric mice, there was no effect of the absence of Fas on the discrepancy in the Ly-9.1−:Ly-9.1+ ratio between mature and immature populations (data not shown). On the other hand, we observed a progressively increasing discrepancy in older Fas−/− chimeric mice (Figs 6 and 7, and data not shown). These results suggest that mature lymphocytes, probably self-renewing, may accumulate in peripheral lymphoid organs when they are Fas-deficient, although a possibility still remains that the effect of Fas on immature cells increases in an age-dependent manner.

There are many reports that Fas is involved in activation-induced lymphocyte cell death (19–26). It is hypothesized that the major role of Fas in lymphoid systems is to kill activated lymphocytes and terminate the immune response. The current idea is that the failure of Fas-mediated death of activated lymphocytes causes abnormal lymphoproliferation in lpr and gld mice (43). However, there is no evidence directly connecting the impairment of activation-induced cell death with lymphoproliferation and autoimmune disease of lpr mice. In our in vitro experiment, TCR-mediated stimulation caused cell death even in Fas−/− T cells, although the rate of death was lower than that in Fas+/+ T cells (unpublished observation). Thus, activation-induced cell death does not totally depend on Fas.

In the Fas−/− chimeric mice, the Fas−/− population expanded in the naive T and B lymphocytes, suggesting that the Fas molecule plays a regulatory role in lymphocytes even before they are stimulated with exogenous antigen. In agreement with this role, the Fas molecule is expressed on most of the mature T lymphocytes (most of them are naive T cells) and >20% of IgD+ B lymphocytes in normal mice (data not shown). Our results are supported by data from Sidman et al. (44), who reported that T lymphocytes bearing male-antigen-specific TCR participated in the lymphoproliferative process even in female lpr or gld mice. Their results imply that interactions with specific antigen are not necessary for abnormal proliferation of Fas-deficient lymphocytes. Ettinger et al. observed that autoreactive T lymphocytes existed mainly in the naive T cell fraction of ipr mice (45). We propose that, in addition to the failure of activation-induced cell death, the impairment of function of Fas in naive lymphocytes also contributes to the development of lymphoproliferation and autoimmune disease.

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Abbreviations

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<th>Description</th>
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<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
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<tr>
<td>B6</td>
<td>C57BL/6</td>
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<td>ES</td>
<td>embryonic stem</td>
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<td>PE</td>
<td>phycoerythrin</td>
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