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Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis

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Fas antigen is a cell-surface protein that mediates apoptosis. It is expressed in various tissues including the thymus and has structural homology with a number of cell-surface receptors, including tumour necrosis factor receptor and nerve growth factor receptor. Mice carrying the lymphoproliferation (lpr) mutation have defects in the Fas antigen gene. The lpr mice develop lymphadenopathy and suffer from a systemic lupus erythematosus-like autoimmune disease, indicating an important role for Fas antigen in the negative selection of autoreactive T cells in the thymus.

T-CELL precursors arise in the bone marrow and then mature in the thymus after interaction with the thymic microenvironment. During maturation, T cells recognizing self-antigens are destroyed by a process called apoptosis, whereas others are positively selected. The lymphoproliferation mutation (lpr) seems to interfere with T-cell maturation. The lpr is autosomal recessive and the phenotype includes formation of multiple autoantibodies and accumulation of large numbers of non-malignant CD4+CD8- T lymphocytes in lymph nodes and the spleen. Two independent spontaneous lpr mutations have been identified, lpr and lpr-+. The original lpr mutation occurred during the derivation of the MRL/MpJ strain and has been transferred onto a number of other inbred strain backgrounds, including C3H and C3HBL. The other mutation is lpr-+ in the CBA/K1Jms mouse strain. The clinical syndrome of lpr and lpr-+ mice is characterized by hypergammaglobulinemia, anti-DNA antibodies, rheumatoid factor, and circulating immune complexes as well as arthritis and glomerulonephritis, which resembles human systemic lupus erythematosus (SLE). Studies of lpr mice suggest that there is a defect in negative selection of self-reactive T lymphocytes in the thymus. Excessive numbers of self-reactive T lymphocytes released into peripheral organs seem to be responsible for the autoimmune disease in lpr mice.

Here we provide evidence that lpr encodes the structural gene for the mouse Fas antigen. A mouse monoclonal antibody has been prepared which has a cytotoxic activity on human cells expressing the Fas antigen. Cloning of Fas antigen complementary DNA from human and mouse cells indicates that the Fas antigen is a protein containing a single transmembrane domain with a calculated Mr of 35,000 (35 K) (refs 10, 12). Fas antigen from both species shows structural homology with a number of cell-surface receptors, including tumour necrosis factor (TNF) receptors and the low-affinity nerve growth factor receptor. Northern analysis indicates the Fas antigen messenger RNA is expressed in a limited number of tissues, including the thymus, liver, ovary and heart. When human Fas antigen is expressed in mouse cell lines, it can induce Fas antigen antibody-triggered cell death. Characterization of the process of cell death indicates that Fas antigen mediates apoptosis. Expression of Fas antigen in the thymus and its role in apoptosis provides an explanation for the phenotypes of lpr mice. These studies further suggest a role for the Fas antigen in the negative selection of autoreactive T cells in the thymus.

Expression of Fas mRNA in lpr mice

The mouse Fas antigen gene has been assigned to chromosome 19 by interspersive backcross analysis. When our linkage map of mouse chromosome 19 was aligned with a composite linkage map (GBASE, Jackson Laboratory) we found that the Fas antigen locus mapped near lpr.

To determine whether the lpr mutation affects Fas antigen mRNA expression, northern analysis of wild-type and mutant lpr tissues was done using a mouse Fas antigen cDNA probe. Total cellular RNAs were isolated from two different strains carrying the lpr mutation (MRL lpr/lpr and C3H lpr/lpr) and their parental wild-type controls (MRL +/+ and C3H +/+). In agreement with previous observations, a 2.1 kilobase (kb) Fas antigen mRNA is detected in the liver and thymus of mice wild type at lpr (Fig. la). Almost no Fas mRNA is observed in homozygous lpr mice. Reprobing the same blot with human elongation factor-1a (EF-1a) cDNA reveals a band of 2.2 kb in all RNA preparations (Fig. 1b). To confirm the absence of Fas mRNA in lpr mice, single-stranded cDNAs were synthesized from thymus RNA of wild-type and lpr mice and amplified by the polymerase chain reaction (PCR), using Fas antigen oligonucleotide primers. PCR amplification of RNA from wild-type mice gave a band of the expected size (420 bp), whereas no such band was amplified from lpr mice (Fig. 1c).
Fig. 1. Little expression of Fas antigen mRNA in mice homozygous for the lpr mutation. Northern blot analysis of total cellular RNA from various mouse strains hybridized with mouse Fas antigen cDNA. (a) Human Fas cDNA probed with mouse Fas antigen cDNA. Total cellular RNA (9 μg) from the liver (lanes 1, 2, 6, 7) and thymus (lanes 4, 8, 9) of MRL/MpJ was hybridized with the mouse Fas cDNA clone. (b) Total cellular RNA (9 μg) from mouse L929 cells was also analysed (lanes 5, 10). Positions of 18S and 28S ribosomal RNAs are indicated. (c) The Fas antigen mRNA detected by reverse PCR. Single-stranded cDNA was synthesized using total cellular RNAs from the thymus of MRL/MpJ (lanes 1, 7), of C(3)H/EiJ (lanes 2, 4), and of C3H/HeJ (lanes 8, 10). Positions of the Fas antigen mRNA are indicated. METHODS: Total cellular RNAs, prepared by the guanidine isothiocyanate/acid phenol method, were denatured at 65°C for 5 min in 2.2 M formaldehyde, and 50% deionized formamide, and electrophoresed through 1.5% agarose gels containing 22.5 M formaldehyde. RNA was transferred to a nitrocellulose filter and hybridized with 32P-labelled probe DNA. The probe DNAs used were a 1.5-kb EcoRI fragment containing mouse Fas antigen cDNA, or a 1.8-kb BamHI fragment containing human Fas antigen cDNA. The reverse PCR was done as described. Random hexamer-primed single-stranded cDNA was synthesized in a final volume of 50 μl with 80 units avian myeloblastosis reverse transcriptase and 2 μg total RNA. An aliquot (5 μl) of the reaction mixture was diluted with 50 μl of the PCR buffer and the PCR reaction was done using 2.5 units Thermus aquaticus DNA polymerase (Taq polymerase). The conditions for the PCR were 1.5 min at 95°C, 1.5 min at 60°C, and 3 min at 72°C, 30 cycles. The primers spanned nucleotides 45-64 and nucleotides 448-467 in the coding sequence of mouse Fas antigen cDNA.

Rearrangement of the Fas antigen gene

To determine whether the absence of Fas antigen mRNA in lpr mice is associated with a rearrangement of the Fas antigen gene, Southern analysis of genomic DNAs from mutant lpr and wild-type mice was done using a full-length Fas antigen cDNA probe. Several bands were observed in EcoRI, BamHI, and PstI-digested genomic DNA from wild-type MRL and C3H mice (Fig. 2a, 8). Except for an extra 9-kb band observed in BamHI-digested DNA from C3H mice (Fig. 2b, lane 1), all bands were identical in size between the two strains. The genomic locus for the wild-type mouse Fas antigen gene has been isolated (R.W.-F. and S.N., unpublished observations). A comparison of the restriction map of the cloned genomic locus with DNA fragments observed in Southern analysis indicates that the Fas antigen gene is present in a single copy per haploid mouse genome and that some bands detected by Southern analysis represent closely migrating doublets. Southern analysis of DNA from MRL lpr/lpr mice showed extra bands of 8 kb or 9 kb in BamHI- or EcoRI-digested DNAs, respectively (Fig. 2a, lanes 3, 6). The 5.5-kb PstI fragment in wild-type DNA (Fig. 2a, lane 7) was replaced by a slightly larger fragment (Fig. 2a, lane 9). When DNAs from wild-type and mutant strains were mixed before restriction enzyme digestion, bands expected for both wild-type and mutant strains were observed (Fig. 2a, lanes 2, 5, 8), indicating that the extra bands seen in lpr DNA are not due to artefacts generated during restriction enzyme digestion. Southern analysis of DNA from C3H lpr/lpr mice gave bands identical to those in MRL lpr/lpr mice (Fig. 2b). These results indicate that the structural gene for the Fas antigen is rearranged in lpr mice.

To determine which region of the Fas antigen gene was rearranged, mouse Fas antigen cDNA was divided into four parts and each part was used as a probe for Southern analysis of wild-type and lpr DNAs. Rearrangement of the Fas antigen gene was detected only with the probe that contains nucleotides 115-580 of mouse Fas antigen cDNA. The 4-kb BamHI and 10-kb EcoRI DNA fragments seen in wild-type DNA were replaced by 8-kb and 9-kb fragments, respectively, in lpr DNA (Fig. 2c). Preliminary analysis of Fas antigen genomic DNA clones suggests that the 4-kb BamHI fragment detected in wild-type DNA contains exon 3 and that the rearrangement seen in lpr mice has occurred in intron 2 (R.W.-F. and S.N., unpublished observations).

A point mutation in Fas antigen

To confirm that Fas antigen is the structural gene for lpr, a second lpr mutation, lpr/", was analysed. Northern analysis of RNAs from liver and thymus of CBA lpr/"/lpr/" mice indicates

Fig. 2. Southern analysis of the Fas antigen gene in various strains. DNA was prepared from the spleen of a wild-type C57BL/6 J, MRL/MpJ and C3H/HeJ mice. Genomic DNA (10 μg per lane) from wild-type (lanes 1, 4, 7) and lpr/lpr mutant mice (lanes 3, 6, 9) and their 1:1 mixture (lanes 2, 5, 8) was digested with BamHI (lanes 1-3), EcoRI (lanes 4-6) or PstI (lanes 7-9). Southern analysis was done using a full-length mouse Fas antigen cDNA (a, b) or coding sequences between nucleotides 115-580 (c). DNA fragments rearranged in lpr mice; open arrowheads, the corresponding DNA fragments in wild-type mice. Open circle, the polymorphic DNA fragment observed in BamHI-digested C57BL/6 J DNA.

METHODS: High M, chromosomal DNA, prepared from the spleen was digested with various restriction enzymes, electrophoresed on a 0.8% agarose gel, and transferred to a nylon membrane under alkaline conditions as described. Probe DNA fragments were prepared by PCR using mouse Fas antigen cDNA as a template, and labelled with 32P by the random primer labelling method (Boehringer). Hybridization was carried out under high stringency. Sizes of marker DNA are shown in kb on the right.
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that this mutant mouse expresses Fas antigen mRNA to the same extent as wild-type CBA +/- mice (Fig. 3a). After reverse transcription of CBA +/- RNA, the coding sequence of the Fas antigen cDNA was amplified by PCR and inserted into pBluescript. The PCR product has a sequence identical to that of mouse Fas antigen cDNA isolated from the mouse BAM3 cell line10 (derived from BALB/c mice), except at nucleotide positions 162, 163, 283, 284, 285 and 286. These differences probably represent polymorphisms between BALB/c and CBA mice. The sequence of three independent Fas antigen cDNA clones, obtained with RNA from CBA lpr+/+ lpr-/- mice, all show a transition of T to A at nucleotide 786, in addition to the polymorphic mutations mentioned above. This mutation causes the replacement of an asparagine for an isoleucine in the cytoplasmic region of the Fas antigen (Fig. 3b), which is highly conserved between the Fas antigen and TNF receptor type 1.

To establish whether this mutation abolishes the ability of the Fas antigen to transduce the apoptotic signal, and to overcome the lack of antibodies against the mouse Fas antigen, two chimaeric Fas antigen molecules were constructed from human and mouse CD3As. Both chimaeras carry the extracellular and transmembrane domains of the human Fas antigen cDNA. The cytoplasmic region of the chimaeric molecule was replaced by the respective domain from the wild-type or lpr-/- mouse, respectively. The chimaeric CD3As were expressed in mouse L929 cells, and the susceptibility of the transfectants to the Fas antibody-induced apoptosis was assayed11.

Transformants expressing the chimaera with the wild-type Fas antigen cytoplasmic tail were effectively killed by the Fas antibody (Fig. 4), indicating that the cytoplasmic region of the wild-type mouse Fas antigen can transduce the apoptotic signal into cells. Cells expressing the chimaera with the mutated mouse Fas antigen from lpr-/- mice were resistant to the cytolytic activity of the anti-human Fas antibody. These results indicate that the Fas antigen encoded by lpr-/- mutant mice is unable to transduce the apoptotic signal into cells, and that lpr encodes Fas antigen.

Discussion

We have shown that lpr mice carry transport defects in the Fas antigen which mediates apoptosis12. Bone marrow transplantation studies indicate that the lpr mutation causes an intrinsic T-lymphocyte abnormality responsible for lymphadenopathy and autoantibody production13. The Fas antigen has been expressed in the thymus14 and on activated or transformed T cells15. Thus it is possible that precursor T cells reacting with self-antigens express the Fas antigen during their thymic development and are killed by interaction with stroma cells in the thymus. No functional Fas antigen is expressed in lpr mice. Therefore, autoreactive lpr T cells can potentially contribute to abnormalities in the lymphadenopathy and autoimmune disease in lpr mice12,16.

Mice carrying the generalized lymphoproliferative disease mutation (gld) show a clinical syndrome which is indistinguishable from that of lpr mice16,17. Like lpr, gld is an autosomal recessive but gld maps to mouse chromosome 17. Bone marrow transplantation18 indicates that these two mutations are expressed by different cell compartments and affect an interacting pair of molecules, suggesting that the structural gene for gld may encode the ligand for Fas antigen. The molecule affected by the gld mutation is thought to be expressed in bone marrow-derived cells19. That the gld responsible for T-cell depletion are derived from the bone marrow18,19,20 supports this.

Transgenic mice expressing a rearranged T-cell receptor for the H-Y male antigen and carrying the lpr mutation have been constructed12,21. Although male lpr mice carrying the transgene show an increased number of autoreactive T-cells in the spleen and an enhanced production of autoantibodies relative to female mice, clonal deletion of autoreactive T-cells was still observed. These results indicate that in addition to the Fas antigen, other molecules have a role in the clonal deletion of autoreactive T-cells, although it is not possible to rule out the weak expression of the Fas antigen in lpr mice. The involvement of defective B-cells in the autoimmune disease of lpr mice has also been suggested22,23,24. Because Fas antigen can be expressed in activated B lymphocytes24, B-cell producing autoantibodies could survive longer in lpr mice than normal mice. Such an extension of the functional lifespan of B lymphocytes could cause autoimmune disease in a similar way to that proposed for transgenic mice carrying an unregulated bel-2 gene25.

Transfer of bone marrow stem cells from lpr mice into irradiated wild-type mice causes a severe graft-versus-host-like disease (GVHD)23. But this is not seen when bone marrow stem cells from lpr-/- mice are transferred into irradiated wild-type residues at an aligned position are boxed by solid lines, sets of three residues regarded as favoured substitutions are boxed by dotted lines. The arrowhead indicates the position of the mutation in the Fas antigen of lpr-/- mice.

METHODS: Total RNA (2 μg) from the liver or thymus of CBA. +/- or CBA lpr+/+ lpr-/- mice was used as a template for single-stranded cDNA synthesis. PCR was carried out as described above. The PCR products (400 bp) were separated by gel electrophoresis and transferred to nitrocellulose. The specific primers were 5′-GAAATTCCGCTTGTCCCTTGCGCA (5′ primer) and 5′-GAGTGGCAGTGGATCCGATG (3′ primer), which contains the sequence in the 5′ or 3′ non-coding region of the mouse Fas antigen cDNA, respectively. PCR conditions were 1 min at 95°C, 15 min at 60°C, and 3 min at 72°C, 50 cycles. The amplified DNA fragment was digested with EcoRI and SalI, and inserted into pBluescript KS+ (Stratagene). The sequencing reaction was done by the dideoxy nucleotide chain-termination method using T7-DNA polymerase (Pharmacia) and [α-35S]dATP (Amersham). As primers for the sequencing reaction, a set of 20-base oligonucleotides specific for the coding region of the mouse Fas antigen gene was used.

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FIG. 3. Structure of the Fas antigen gene in mice homozygous for the lpr-/- mutation. A Northern analysis of Fas antigen mRNA in lpr-/- mice, CBA/K.lms +/+, and CBA/K.lms lpr+/+ lpr-/- mice total cellular RNA (5 μg) prepared from the liver (lanes 1, 2) or thymus (lanes 3, 4) of wild-type (lanes 1, 3) or mutant (lanes 2, 4) mice was analysed using mouse Fas antigen cDNA as probe. Total RNA from L929 cells (lane 5) was also analysed. Positions of 18S and 28S RNA are indicated. A, Point mutations in the cytoplasmic region of the Fas antigen gene of lpr-/- lpr-/- mice. The coding region of the Fas antigen gene expressed in the thymus and liver of wild-type and mutant mice was amplified by PCR and the nucleotide sequence determined after the addition of the Fas antigen cDNA sequence to the 5′-end. PCR conditions were 1 min at 95°C, 1 min at 60°C, and 3 min at 72°C, 50 cycles. The amplified DNA fragment was digested with EcoRI and SalI, and inserted into pBluescript KS+ (Stratagene). The sequencing reaction was done by the dideoxy nucleotide chain-termination method using T7-DNA polymerase (Pharmacia) and [α-35S]dATP (Amersham). As primers for the sequencing reaction, a set of 20-base oligonucleotides specific for the coding region of the mouse Fas antigen gene was used.

Fig. 4. Inability of the Fab antigen encoded by lpr2 mice to mediate apoptosis. Mouse L929 cells transformed by and expressing a chimeric human-mouse Fab antigen containing the wild-type mouse Fab antigen (C), or the mutated mouse Fab antigen (C), or a hybrid polyclonal region were incubated at 37°C for 6 h with various concentrations (0.03-1000 mg ml⁻¹) of anti-human Fab antibody in the presence of 0.5 μg ml⁻¹ actinomycin D. After incubation, viable cells were stained with crystal violet, and dye uptake was quantified by measuring absorbance at 540 nm using an automated Micro-ELISA autoreader. The parental L929 cells (∗) were also treated with anti-human Fab antibody in the presence of actinomycin D. Assays were done in duplicate for two independent cell lines transformed by and expressing the chimeric Fab antigen, and the average values for each transformation were plotted.

Methods. A hybrid cDNA between mouse and human Fab antigen cDNAs was constructed by theTagName PCR method. The primary PCR was done with two sets of primers. A part of the human Fab antigen cDNA was amplified with a forward primer (primer A) containing human sequence from 696-715, and a reverse hybrid primer (primer B) containing the sequence complementary to mouse Fab antigen cDNA from 586-608, and to human Fab antigen cDNA from 725-743. A part of mouse Fab antigen cDNA was amplified using primer A and the cDNA from the lpr mouse as a template. The oligonucleotides complementary to primer B, and a 20-base oligonucleotide (primer C) complementary to the sequence from 964-1003 of the mouse Fab antigen cDNA were used as primers. The extension for the PCR were 1 min at 94°C, 2 min at 55°C and 2 min at 72°C, 15 cycles. Products were isolated by agarose gel electrophoresis, and treated with T4 DNA polymerase to flush the ends. The two DNA fragments obtained by the primary PCR were then mixed at 1:1, and the secondary PCR was done for 20 cycles as above using primers A and C. The product was digested with BamHI and KpnI, and the 330-bp DNA fragment was isolated. This fragment was ligated with the 700-bp XbaI-BamHI fragment containing the 5′ part of human Fab antigen cDNA and the 660-bp KpnI-XbaI fragment containing the 3′ part of mouse Fab antigen cDNA, and introduced into the mammalian expression vector pEF-BOS. All constructions were confirmed by DNA sequencing. The expression plasmid for the human-mouse chimeric Fab antigen was introduced into mouse L929 cells. After 9 days, individual G418-resistant colonies were isolated, and the primary plates of clones were transfected by and expressing the chimeric Fab antigen were identified by FACS analysis using mouse anti-human Fab monoclonal antibody.

Pillars of Immunology

These two observations can be explained as follows. Fab antigen an N-truncated form in RNA is not expressed in lpr cells. When lpr stem cells introduced in wild-type mice become mature T-cells, they recognize the Fab antigen expressed in the host cells as a foreign antigen or "non-self" and induce the GVHD syndrome. Mice carrying the lpr allele express the Fab antigen, although in a non-functional form. Therefore, mature T-cells derived from stem cells of lpr mice can recognize host cells expressing the normal Fab antigen as "self".

Our results expand the phenotype observed in double heterozygous lpr+/+, gid/+ and lpr+/-, gid/+ mice. Although the original lpr mutation fails to complement gid in double heterozygotes, lpr/+ can complement gid1 in lpr+/-, gid/+ double heterozygotes develop a disease similar to that observed in lpr or gid, gid mice, although it is less pronounced. These phenotypic differences can be explained by the nature of the Fab antigen lesions found in lpr and gid mice. In lpr mice, Fab antigen is not expressed and cannot bind ligand. In gid mice, a non-functional receptor is expressed that can still presumably bind ligand. In lpr+/-, gid/+ double heterozygotes, the non-functional receptor could compete for ligand, further lowering the effective concentration of ligand below that normally found in gid/+ mice. This reduced concentration of ligand could produce a phenotype similar to that seen in lpr/lpr or gid/gid mice.

Fas antigen is expressed in the thymus and in the liver, heart and ovary. Abnormalities of these tissues have not been described in lpr mice, although CD4+ CD8+ T-lymphocytes which accumulate in lpr mice seem to proliferate in the liver17. Because the liver can support extrathymic development of T-lymphocytes if the thymus is inactive or absent18, it is possible that cells expressing Fas antigen in the liver are precursor cells for T-lymphocytes.

We have provided evidence that the lpr locus encodes Fas antigen. This finding should help to elucidate the process regulating normal T-cell maturation in the thymus, and the mechanism underlying formation of autoimmune disease in mice. Finally, human patients displaying a similar phenotype as lpr mice have been identified19 and it will be interesting to examine the structure of the Fas antigen gene in these patients.

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