Fas and *Fas Ligand* Gene Mutations in Hashimoto's Thyroiditis

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SUMMARY: To clarify whether *Fas* and *Fas ligand* (FasL) mutations are involved in the pathogenesis of Hashimoto's thyroiditis (HT), we examined the open reading frame of *Fas* and *FasL* in 21 cases. Mutations of *Fas* and *FasL* genes were detected in 8 (38.1%) and 1 (4.8%) of 21 cases, respectively. All but one of the *Fas* mutations were frameshift mutations, which affect the cytoplasmic region (death domain) known to be involved in apoptotic signal transduction and thus could be loss-of-function mutations. *FasL* mutation in one case was a 46-bp deletion from nucleotide 349 to 394, which corresponded to exon 2. Lack of exon 2 results in a frameshift, which generates a stop codon at residue 128. This mutant encodes the protein that contains only a part of the intracellular domain, thus the abnormal protein might not be expressed on the cell surface. The cells with *Fas* mutations were confined to the mantle zone and the germinal center, as determined by microdissection methods. These findings suggest that the cells with *Fas* mutations might accumulate in those areas and might be involved in the pathogenesis of Hashimoto's thyroiditis. (*Lab Invest 2002, 82:1611–1616*).

*F*as (Apo-1/CD95) is a 45-kDa membrane protein belonging to the TNF receptor family and mediates programmed cell death (apoptosis) upon trimerization induced by cross-linking by Fas ligand (FasL) (Nagata and Golstein, 1995; Suda et al, 1993). FasL is a type II membrane protein that belongs to the TNF family (Suda et al, 1993). The Fas/FasL system regulates a number of physiologic and pathologic processes of cell death (apoptosis) including immune responses. For example, activated T cells expressing both Fas and FasL undergo suicide, kill each other, or kill the Fas-expressing activated B cells, resulting in down-regulation of the immune reaction. Failure of apoptosis, or programmed cell death, is considered to contribute to the development of autoimmune diseases through impaired elimination of autoreactive T or B cells. FasL is one of the major effector molecules of cytotoxic lymphocytes (CTL). Therefore, it is possible that CTL-mediated tissue damage takes place through abnormal activation of FasL- or Fas-mediated apoptosis.

Hashimoto's thyroiditis (HT) is a well-known organ-specific autoimmune disease and results in hypofunction in severe cases. Apoptosis of thyrocytes is occasionally observed in histologic sections of normal thyroid tissues and is accelerated during the pathologic processes, with destructive changes in the thyroid gland, thus resulting in clinical hypothyroidism (Kotani et al, 1995; Mitsiades et al, 1998; Okayasu et al, 1995; Tanimoto et al, 1995). Fas expression and death signaling in thyrocytes have been demonstrated in HT (Giordano et al, 1997; Hammond et al, 1997; Mitsiades et al, 1998). Therefore, one hypothesis is that the thyrocytes expressing Fas are targets for CTL expressing FasL. Recent studies suggests that thyrocytes may kill themselves by constitutive expression of FasL and Fas, which are induced by IL-1β or IFN-γ secreted by infiltrating immune cells (De Maria and Testi, 1998; Giordano et al, 1997). Alternatively, the thyrocytes expressing FasL in combination with other molecules known to be induced by inflammatory cytokines that can increase cell-to-cell contact (intercellular adhesion molecule, lymphocyte function-associated antigen, MHC II) may act as a defense mechanism and lead to specific killing of the invading immune cell, thus providing an immune-privileged status to the thyroid (Dayan et al, 1997). Therefore, a defect of FasL may allow invasion of the immune cells in the thyroid.

Pathoepidemiologic study showed that thyroid lymphoma arise in the activated lymphoid cells infiltrating in the HT (Aozasa et al, 1986; Volpe, 1975). Recently we have reported that mutations of the *Fas* gene were detected in 65.4% of thyroid lymphoma but occasionally in HT, although the number of samples examined was relatively small (Takakuwa et al, 2001). These findings raise a possibility that lymphoid cells with *Fas* mutations accumulate in the HT, providing a basis for development of thyroid lymphoma.

In this study, we extensively examined the *Fas* and *FasL* mutations in HT to clarify whether *Fas/FasL* mutations are involved in the pathogenesis of HT.

Mutations of *Fas* and *FasL* genes were detected in 8 (38.1%) and 1 (4.8%) of 21 cases, respectively. Subsequently, we specified the location of cells with *Fas* mutations, with the use of a microdissection method.

Results

Mutations of the Fas Gene

Mutations of the *Fas* gene were detected in 8 (38.1%) of 21 cases of HT (Table 1). All but one of the mutations were frameshift mutations and affected the death domain. *Fas* gene has a 6-(A) tract from nucleotides 1088 to 1094 of the *Fas* cDNA sequence (GeneBank accession no. M67454) (Itoh et al, 1991), and four cases of HT had 7-(A) tract as a result of a 1-bp insertion of (A) into nucleotide 1095. Mutations in these cases occurred in the death domain known to be involved in the apoptotic signal transduction and thus could be loss-of-function mutations.

Three cases showed a 25-bp deletion from nucleotides 846 to 870, which corresponded to exon 8. Lack of exon 8 results in a frameshift, which generates a stop codon at residue 205. As a result, the whole intracytoplasmic death domain is deleted (Cascino et al, 1996). Mutations in the acceptor splice site of *Fas* intron 7 or in the donor splice site of intron 8 might generate a splice variant of transcripts lacking exon 8. To examine this hypothesis, genomic DNA from the patients was amplified using primers flanking exon 8, cloned, and then sequenced. One case (Case 5) had mutations in the consensus sequence of the acceptor splice site of intron 7 and one (Cases 3) had the donor splice site of intron 8, although it is not certain whether these mutations cause exon skipping (data not shown). Mutations could not be detected in the other case (Case 4).

Mutation of FasL Gene

Mutation of the *FasL* gene was detected in 1 (4.8%) of 21 cases (Case 9). This case showed a 46-bp deletion from nucleotides 349 to 394 (Fig. 1) (Suda et al, 1993; Takahashi et al, 1994), which corresponded to exon 2. Lack of exon 2 results in a frameshift, which generates a stop codon at residue 128. This mutant encodes the
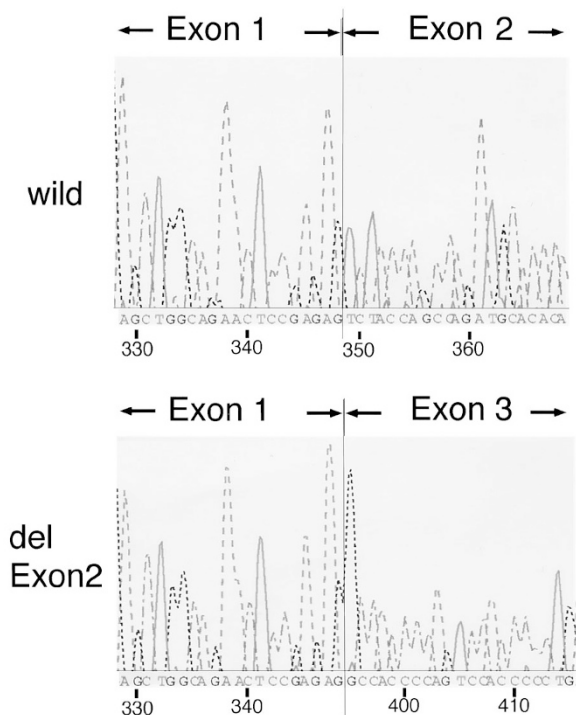

Figure 1.

Sequence of *FasL* gene cDNA derived from Case 9, showing a 46-bp deletion from nucleotides 349 to 394, which corresponds to exon 2.

protein that contains only a part of cytoplasmic domain, thus the abnormal protein might not be expressed on the cell surface. Frequency of mutations among clones was 40%. To examine whether mutations in the acceptor splice site of *FasL* intron 1 or in the donor splice site of intron 2 might generate splice variants of transcripts lacking exon 2, genomic DNA from Case 9 was amplified using primers flanking exon 2, cloned, and then sequenced. However, no mutations could be detected in that sequence (data not shown).

Localization of Fas Gene Mutations

Three to five lesions from each histologic area (ie germinal centers, mantle zones, interfollicular areas,

Table 1. *Fas* Mutations in Hashimoto's Thyroiditis

No.	Age	Gender	Nucleotide change	Localization	Codon	Predicted effect	Frequencies of mutation-positive clones (%)
1	59	F	A416G	Exon 3	59	Silent	66.6
2	70	F	851 del AG	Exon 8	203	Frameshift (stop 208)	43.8
3	67	F	846−870 del (exon 8)	Exon 8	202−210	Frameshift (stop 204)	34.4
4	65	F	846−870 del (exon 8)	Exon 8	202−210	Frameshift (stop 204)	9.4
5	67	F	846−870 del (exon 8)	Exon 8	202−210	Frameshift (stop 204)	9.4
			1095 ins A	Exon 9	285	Frameshift (stop 303)	9.4
6	66	F	1095 ins A	Exon 9	285	Frameshift (stop 303)	6.3
7	52	F	1095 ins A	Exon 9	285	Frameshift (stop 303)	37.5
8	56	F	1095 ins A	Exon 9	285	Frameshift (stop 303)	9.4

F, female; del, deletion; ins, insertion.

and thyroid follicles) were selected for microdissection. *Fas* gene mutations were detected only in the mantle zones in two cases and in both the germinal centers and mantle zones in another two cases (Table 2). *Fas* mutations were never detected in the interfollicular areas and thyroid follicular epithelium.

Clinical and Histologic Findings

Serum thyroglobulin levels in HT patients with and without *Fas* mutations were 132.6 ± 253.1 ng/ml and 476.2 ± 571.0 ng/ml, respectively. Serum thyroid stimulating hormone and FT4 levels were within normal ranges in most of the cases. There were no significant differences in the serum levels of antithyroglobulin and antimicrosomal antibody between HT cases with and without *Fas* mutation. Germinal centers were hyperplastic in 3 of 8 *Fas*-mutated cases (Cases 2, 3, and 7) and in 1 of 13 cases without mutations. The degree of lymphocytic infiltration and fibrosis in the thyroid tissue was not significantly different between cases with and without *Fas* mutation.

Discussion

Fas-mediated apoptosis is a sophisticated mechanism for regulation of cell death and plays an important role in normal tissue development and homeostasis as well as in disease processes. A defect in apoptosis caused by abnormalities in the Fas/FasL system allows survival of autoreactive lymphocytes, which might result in systemic autoimmunity. Indeed patients who carry inherited defects in *Fas* and *FasL* genes exhibit a similar, albeit variable, pattern of phenotypes, including systemic lupus erythematosus (SLE), which have been collectively termed autoimmune lymphoproliferative syndrome (Drappa et al, 1996; Fisher et al, 1995; Nagata, 1998; Rieux-Laucat et al, 1995). An important role of Fas in SLE has been suggested from the observation that 60% of patients with SLE showed elevated levels of soluble Fas receptor in their serum (Cheng et al, 1994; Kovacs et al, 1997). Abundant soluble Fas receptor in the serum may protect lymphocytes from undergoing apoptosis, although this remains a subject of controversy (Goel et al, 1995; Knipping et al, 1995). Wu et al (1996) reported that a FasL defect is not a major factor contributing to the pathogenesis of SLE.

In the present study, mutations of the *Fas* gene were detected in 8 of 21 cases, suggesting that *Fas* gene mutations might play a role in the pathogenesis of HT. All but one of the *Fas* mutations were frameshift mutations, which affect the cytoplasmic region (death domain) known to be involved in the apoptotic signal transduction and thus could be loss-of-function mutations. In fact we have shown that mouse T-cell lymphoma cells transfected with frameshift mutations caused by insertion of a 1-bp (A) at nucleotide 1095 (as observed in Cases 5, 6, 7, and 8) were resistant to apoptosis induced by the anti-Fas antibody (Takakuwa et al, 2002). Deletion of exon 8 as a splicing variant of the *Fas* gene has been reported in the apoptosis-resistant clone, which was derived from human T-cell lymphoma cell line HUT78 (Cascino et al, 1996). The frameshift mutation found in Case 2 (851 del AG) might be a loss-of-function mutation because it generate a stop codon at residue 205. As a result, the whole intracytoplasmic death domain is deleted, which is equivalent to the deletion of exon 8. Only one case (Case 2) showed mutations of the *FasL* gene; this mutant gene encodes a protein that contain only a part of cytoplasmic domain, thus the abnormal protein might not be expressed on the cell surface. It is not clear whether the FasL mutation in this case contributed to the pathogenesis of HT.

Analysis of genomic DNA microdissected from the HT lesions showed that cells with *Fas* gene mutations were confined to the mantle zone and less frequently the germinal center. Thyrocytes and lymphoid cells outside the lymphoid follicles did not show *Fas* gene mutations. Although cytotoxic T cells and thyrocytes are reported to express FasL and kill Fas-expressing cells in HT (Giordano et al, 1997; Mitsiades et al, 1998), lymphocytes with *Fas* mutations may survive and accumulate in the tissues.

The majority of lymphocytes in the germinal center and mantle zone are of B-cell origin. Our previous study showed that the lymphomas arising from HT are of B-cell origin and showed frequent *Fas* mutations (Takakuwa et al, 2001). These findings suggest that *Fas* gene mutations occur among the B cells in the lymphoid follicles, which might provide a basis for development of lymphoma. Recent studies showed that expression of functional Fas on B cells is essential for the regulation of autoantibody production (Fukuyama et al, 1998; Shinohara et al, 1997). Therefore, unless the autoreactive or activated B cells are killed through the interaction of Fas expressed on the B-cell surface and FasL expressed on the activated T cells, these activated B cells might continue to pro-

Table 2. Localization of Cells with *Fas* Gene Mutations

| Case | Age/gender | Number of mutations per examined area | | | |
		Germinal center	Mantle zone	Interfollicular area	Thyroid follicle
5	67/F	0/192 (4)	5/176 (5)	0/144 (3)	0/144 (3)
6	66/F	0/104 (3)	1/128 (4)	0/128 (4)	0/128 (4)
7	52/F	2/272 (5)	5/144 (4)	0/160 (4)	0/148 (4)
8	56/F	2/220 (4)	4/86 (3)	0/160 (4)	0/128 (4)

The number of areas examined are shown in parentheses.

duce autoantibodies. Taken together, B lymphocytes with *Fas* mutations in the lymphoid follicles of HT become resistant to the apoptosis induced by the Fas/FasL system and continue to produce thyroid autoantibodies, which destroy the thyroid follicular epithelium.

It is also possible that cells other than B cells in the lymphoid follicles carry the *Fas* mutations. Autoreactive T cells might remain alive in thyroid tissue as a result of *Fas* mutations and provide helper signals necessary for B-cell proliferation and differentiation. These activated B cells might overproduce the autoantibodies. Future studies will examine which subsets carry the *Fas* mutation and whether the presence of cells with *Fas* mutations is involved in the pathogenesis of HT.

In conclusion, the present results suggest that mutations of *Fas* occur in lymphoid cells but not thyrocytes of HT. *FasL* mutations are infrequent both in thyrocytes and lymphocytes. These findings indicate that further study on lymphoid follicles formed in the thyroid gland of HT may provide insight on the disease mechanism of HT.

Patients and Methods

Cases

Thyroid specimens were collected from 21 patients with HT. They were admitted to Kuma Hospital (Kobe City, Japan) with varying degrees of goiter from 1995 to 2001. All but two cases were women, with age at admission ranging from 45 to 70 (median 66) years. All patients underwent surgery including total or partial thyroidectomy or open biopsy, and the histologic specimens were fixed in 10% formalin and routinely processed for paraffin embedding. Histologic sections cut at 4-μm thickness were stained with hematoxylin and eosin and were reviewed for histologic diagnosis. Samples in all cases were snap-frozen with optimal cutting temperature compound at $-150°$ C and stored at $-80°$ C until use. Criteria for the diagnosis of HT included increased consistency of the thyroid gland,

occasional hypothyroidism, a high level of thyroid stimulating hormone, low ^{123}I uptake, and the presence of antimicrosomal and/or antithyroglobulin antibodies in the serum. Histologic findings of HT included lymphocytic infiltration, usually forming lymphoid follicles with germinal centers, varying degrees of fibrosis, and oxyphilic change or squamous metaplasia of epithelial cells of the thyroid follicles. The presence of these clinical and histological parameters was compared between cases with and without *Fas* mutations.

Isolation of Total RNA, RT-PCR, and Detection of Mutations

Fresh-frozen samples from HT were homogenized, and total RNA was extracted in the presence of TRIzol reagent (GIBCO BRL, Grand Island, New York). Five micrograms of total RNA was reverse-transcribed by random hexamer priming, and 1 μg was subjected to PCR as a template. The open reading frames of *Fas* and *FasL* were amplified using the oligonucleotide primers Fas-F, -R and FasL-F, -R, respectively (Table 3). Thermocycling conditions for each reactions were 35 cycles of denaturation at 94° C for 15 seconds, annealing at the various temperatures shown in Table 3 for 15 seconds, and extension at 72° C for 1 minute, followed by a final 7-minute extension at 72° C. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Studio City, California), and cloned in pCR 2.1-TOPO according to the indications of the manufacturer (Invitrogen, San Diego, California). Sequencing was performed using the DNA sequencing kit (Applied Biosystems, Foster City, California) and analyzed by the Genetic Analyzer (ABI PRISM 310'; Applied Biosystems). Twelve to 16 clones derived from two different PCR reactions were sequenced. When common mutations were found in two PCR, we regarded them as definite mutations. Frequency of clones with *Fas* mutations among clones sequenced was defined as frequency of mutation-positive clones.

Table 3. Oligonucleotide Primers Used for PCR Reactions

	Primer sequence	Ta (° C)	PCR products (bp)
cDNA primers			
Fas-F	5′-CACTTCGGAGGATTGCTCAACA-3′	54	1,163 (nt. 170–1336)
Fas-R	5′-TATGTTGGCTCTTCAGCGCTA-3′		
FasL-F	5′-TGACTCACCAGCTGCCATGCAG-3′	54	886 (nt. 170–1336)
FasL-R	5′-GGAAAGAATCCCAAAGTGCTTCTC-3′		
Genomic primers			
8-F	5′-ATTAAGGAAAAATTAGAAGTTCACATT-3′	52	217
8-R	5′-ATCCCATAATATGTCACTGAAA-3′		
9-F1	5′-GAAAGTTCAACTGCTTCGTAATTGG-3′	50	102
9-R	5′-GCAAGAGTACAAAGATTGG-3′		
9-F2	5′-CTTCGTAATTGGCATCAACTTCAT-3′	50	89
2-F	5′-TTTATTTGACGATTCTGCCTCTTTT-3′	50	186
2-R	5′-TAACATAGTTCTGTGCTGAGGAT-3′		

Ta, annealing temperature; nt, nucleotides.

Detection of Mutations in the Exon/Intron Boundary of Fas *Exon 8 and* FasL *Exon 2*

Cases 3, 4, and 5 had deletions in exon 8 of the *Fas* gene, and Case 9 had a deletion in exon 2 of the *FasL* gene. Genomic DNA extracted from the frozen tissues of these cases were subjected to PCR with the oligonucleotide primers, 8F and 8R, and 2F and 2R (Table 3), which flank *Fas* exon 8 and *FasL* exon 2, respectively. PCR products were cloned in pCR 2.1-TOPO (Invitrogen). Twenty-four clones were sequenced.

Laser Capture Microdissection and DNA Extraction

Thyroid tissues in patients with HT contain lymphocytic infiltration, usually forming lymphoid follicles with germinal centers. To specify which kind of histologic lesions have *Fas* mutations, microdissection was performed on the paraffin-embedded samples of the selected cases (Cases 5, 6, 7, and 8) with an insertion (A) at nucleotide 1095 of the *Fas* gene. Lymphoid cells in the germinal centers, mantle zones, interfollicular areas, and thyroid follicular epithelium, all in the HT lesions, were microdissected with the use of a PixCell laser capture microscope (Arcturus Engineering, Santa Clara, California) according to previously described methods with some modifications (Bonner et al, 1997; Emmert et al, 1996). Briefly, histologic sections were dehydrated and then the histologic fields of interest were selected, overlaid with a thermoplastic film mounted on a transparent cap, and captured by film through laser energy. The dissected pieces were allowed to adhere to the transparent cap and were collected in 0.5-ml Eppendorf tubes. The procured cells were subsequently resuspended in 20 to 50 μl of extraction buffer containing 10 mM Tris (pH 8.0), 2 mM EDTA, 0.2% Tween 20, and 200 μg/ml proteinase K and were incubated overnight at 37° C. The mixture was heated at 100° C for 10 minutes to inactivate the proteinase K, and a 3% to 5% of solution was used for a template for each PCR.

Detection of a 1-bp (A) Insertion at Nucleotide 1095 of Fas *Exon 9*

Genomic DNA extracted by microdissection methods from cases 5, 6, 7, and 8 were subjected to PCR with the oligonucleotide primers, 9F1 and 9R (Table 3), which flank the 6-(A) tract from nucleotides 1088 to 1094 of the *Fas* cDNA sequence of *Fas* exon 9. PCR products were cloned in pCR 2.1-TOPO (Invitrogen). Thirty-two to 96 bacterial colonies were subjected to PCR as a template using the oligonucleotide primers, 9F2 and 9R. To detect the difference of 1 bp as a result of insertion, reaction products (3 μl) were denatured and separated on 8% polyacrylamide gels containing 8 M urea. The gels were analyzed with FMBIO-II (Takara, Kusatsu, Japan).

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