Novel Fas (CD95/APO-1) mutations in infants with a lymphoproliferative disorder

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Abstract

Fas is an apoptosis-signaling receptor important for homeostasis of the immune system. In this study, Fas-mediated apoptosis and Fas mutations were analyzed in three Japanese children from two families with a lymphoproliferative disorder characterized by lymphadenopathy, hepatosplenomegaly, pancytopenia, hypergammaglobulinemia and an increase in TCRαβCD4–CD8– T cells. Apoptosis induced by anti-Fas mAb was defective in both activated T cells and B cells, and granulocytes from these patients. Truncated Fas receptor lacking the cytoplasmic death domain caused by a point mutation in the splice region of intron 7 were demonstrated in two siblings. A homozygous point mutation in the splice acceptor of intron 3 was found in the Fas gene of the third patient, which resulted in the skipping of exon 4 and complete loss of Fas expression. Corresponding to these mutations, soluble Fas concentrations were decreased and reciprocally soluble Fas ligands were increased in patients’ sera. Interestingly, co-stimulation by immobilized anti-Fas mAb in T cells from the two siblings was comparable to that seen in normal T cells. These results suggest that Fas-mediated apoptosis plays a pivotal role in immunological homeostasis in vivo, especially regarding clonal deletion of immune cells in humans.

Introduction

The Fas receptor (CD95/APO-1) is a 45 kDa type II integral membrane protein which induces apoptotic cell death in a variety of cells which express Fas antigen either directly through ligand binding or by cross-linking with agonistic anti-Fas or anti-APO-1 antibodies (1,2). Molecular analysis has shown that the Fas antigen belongs to the nerve growth factor receptor/tumor necrosis factor (TNF) receptor superfamiliy and its cytoplasmic domain has homology to the intracellular domain of the TNF type I receptor known to transduce a signal for cell death (3–6). The interaction of Fas receptor and its ligand (FasL) has been shown to play a central role in the regulation of apoptosis, especially in the clonal deletion of autoreactive T cells in the periphery and in activation-induced cell death of mature T cells (7–9). Furthermore, the Fas receptor–FasL system has been demonstrated to mediate apoptotic cell death of B cells (10–14), monocytes (14,15), granulocytes (15,16) and other non-lymphoid cells (17).

The physiological significance of this apoptosis-inducing system in vivo has been suggested by autoimmune and lymphoproliferative disorders in mice with lpr, lprcg or gld mutations as a consequence of the functional defects of the Fas receptor–FasL system (3,6,18–20). Mice with these mutations manifest lymphadenopathy, splenomegaly associated with non-malignant proliferation of TCRαβ+CD4−CD8− double-negative (DN) T cells and lupus-like illness. Fas mutations in humans have been also found in children showing similar manifestation associated with lymphoproliferation of DN T cells (21–26).

Fas receptor mainly mediates cell death, but a difference of susceptibility in leukocyte populations to Fas-mediated apoptosis was recognized (15). Furthermore, cross-linking of Fas receptor has been known to lead to enhanced proliferation of freshly isolated T cells in the presence of activation via the TCR (27,28) or induce proliferation of B cell leukemic cells...
Human IL-2 (Shionogi, Osaka) for 4 days in RPMI 1640 medium (San Diego, CA) in the presence of 100 U/ml recombinant Staphylococcus aureus Cowans I (SAC; Calbiochem-Behring). As described (15). T cell (E+), B cell (E–) enriched lymphocytes were isolated from dextran-treated blood by Hypaque gradient centrifugation from patients and controls (12,15). Briefly, cells were incubated with 1 µg/ml of anti-CD3 mAb for 30 min on ice. After washing with PBS, cells were incubated with 1 µg/ml of FITC-conjugated goat anti-mouse Ig (Southern Biotechnology Associates, Birmingham, AL) for 30 min.

Antibodies and flow cytometry

The murine anti-human mAb against Fas (CD95) antigen were CH11 (IgM; MBL, Nagoya, Japan), ZB4 (IgG1; MBL), M38 (IgG1; kindly provided by Dr M. R. Alderson, Immunex) and 4B4-3A (IgG2a; kind gift of Dr S. Nagata, Osaka University). Expression of Fas antigen on T cells, B cells and granulocytes was evaluated by indirect immunofluorescent methods previously described (16). Other manifestations hematoma hydrops fetalis brain tumor

Table 1. Clinical and laboratory features of patients

<table>
<thead>
<tr>
<th></th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of onset</td>
<td>8 months</td>
<td>30 months</td>
<td>antenatal</td>
</tr>
<tr>
<td>Gender</td>
<td>F</td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>Liver (cm)b</td>
<td>5</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Spleen (cm)b</td>
<td>7</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Skin rash</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Hemolytic anemia</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Neutropenia</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>IgG (mg/dl)</td>
<td>3446</td>
<td>3355</td>
<td>4460</td>
</tr>
<tr>
<td>IgA (mg/dl)</td>
<td>97</td>
<td>231</td>
<td>147</td>
</tr>
<tr>
<td>IgM (mg/dl)</td>
<td>224</td>
<td>71</td>
<td>344</td>
</tr>
<tr>
<td>Lymphocyte phenotype (%)c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3+</td>
<td>81.4</td>
<td>75.3</td>
<td>47.1</td>
</tr>
<tr>
<td>CD4+</td>
<td>40.6</td>
<td>32.0</td>
<td>39.6</td>
</tr>
<tr>
<td>CD8+</td>
<td>24.8</td>
<td>32.2</td>
<td>6.3</td>
</tr>
<tr>
<td>CD16+</td>
<td>3.1</td>
<td>9.8</td>
<td>4.3</td>
</tr>
<tr>
<td>CD20+</td>
<td>10.4</td>
<td>9.7</td>
<td>44.4</td>
</tr>
<tr>
<td>TCRy+</td>
<td>7.3</td>
<td>5.1</td>
<td>0.7</td>
</tr>
<tr>
<td>TCRy+CD4–CD8–</td>
<td>8.1 (602)</td>
<td>5.6 (800)</td>
<td>6.4 (652)</td>
</tr>
<tr>
<td>Serum sFas (ng/mlf)</td>
<td>0.74</td>
<td>0.80</td>
<td>&lt;0.15</td>
</tr>
<tr>
<td>Serum sFasL (ng/mlf)</td>
<td>0.76</td>
<td>0.92</td>
<td>147.0</td>
</tr>
<tr>
<td>Other manifestations</td>
<td></td>
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</tr>
<tr>
<td>aLaboratory data were obtained at age 17 months.</td>
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<tr>
<td>bDepth (cm) below the costal margin.</td>
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<td></td>
</tr>
<tr>
<td>cData was obtained at October 1996.</td>
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<td></td>
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<tr>
<td>dAbsolute number of cells/mm³. The normal range for this cell population is &lt;50/mm³.</td>
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<td></td>
<td></td>
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<tr>
<td>eThe mean ± SD of serum sFas concentration in controls (n = 15) was 1.74 ± 0.46 ng/ml.</td>
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<td></td>
</tr>
<tr>
<td>fSerum sFasL concentration in controls (n = 8) was &lt;0.1 ng/ml.</td>
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</table>

Gibco, Grand Island, NY) containing 10% FCS, 5 × 10⁻⁵ M 2-mercaptopetoethanol, 200 U/ml penicillin G, 10 µg/ml gentamicin, and 25 mM HEPES. After 4 days culture medium was supplemented with 50 U/ml of IL-2 for 14–21 days. Epstein–Barr virus (EBV)-immortalized cells were obtained by infecting B cells with the EBV strain B95-8. For T cell proliferation assays, isolated E+ populations from patients and controls (1 × 10⁵/well) were stimulated for 4 days at 37°C in a 96-well flat-plate (Corning Glass Works, Corning, NY) pretreated with 0.5 µg/ml anti-CD3 mAb (OKT3) alone or together with different concentrations of anti-Fas mAb (CH11) in 0.05 M Tris–HCl (pH 9.5) for 2 h at room temperature. DNA synthesis was estimated by [³H]thymidine incorporation (0.2 µCi/well, 6.7 Ci/mM; New England Nuclear, Boston, MA) during the last 12 h of culture.

Identification of apoptotic cells

Activated T cells, activated B cells, EBV-transformed B cells or freshly isolated granulocytes were treated with different concentrations of anti-Fas mAb (CH11) for 12 h. Activation-induced cell death was estimated by reactivation of cultured T cells with anti-CD3 mAb (OKT3, 4 μg/ml) previously immobilized on a 96-well flat-plate. Apoptosis was evaluated by a flow cytometric method previously described (16).
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Table 2. PCR oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Region</th>
<th>Sequence</th>
<th>Position</th>
<th>Fragment size (bp)</th>
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<tbody>
<tr>
<td>A</td>
<td>5’-GGGAGCAGCTTTTCTC-3’</td>
<td>154-172</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5’-ACCATGCTTCTTCACACAC-3’</td>
<td>530-549</td>
<td>377</td>
</tr>
<tr>
<td>B</td>
<td>5’-GGAAGACAGAACAAGG-3’</td>
<td>461-480</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5’-GGAGAACAGAAGGACCACC-3’</td>
<td>735-753</td>
<td>275</td>
</tr>
<tr>
<td>C</td>
<td>5’-ACTTTCTGTCCTGCTGTC-3’</td>
<td>1018-1037</td>
<td>367</td>
</tr>
<tr>
<td></td>
<td>5’-AGAATGGAATCTGAAAGGCC-3’</td>
<td>946-965</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5’-CCCAAGTAAAAACACAGAG-3’</td>
<td>1298-1317</td>
<td>353</td>
</tr>
<tr>
<td>E</td>
<td>5’-TGCTTCTCATGACATTCATCAC-3’</td>
<td>219</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5’-AGTAACAAAAAGCCAAATCAC-3’</td>
<td>967-986</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>5’-ACTTTCTGTTCTGCTGTC-3’</td>
<td>1018-1037</td>
<td>367</td>
</tr>
</tbody>
</table>

The cDNA nucleotides correspond to Behrmann (40) and are available from GenBank (accession nos X81334–X81342).

Analysis of mutation in the Fas gene

Total RNA was extracted from an EBV-transformed B cell line or activated T cells from patients and controls by the acid guanidinium thiocyanate–phenol–chloroform method, and single-stranded cDNA was synthesized with reverse transcriptase (Takara, Tokyo, Japan). Primer sets of oligonucleotides (Funakoshi, Tokyo, Japan) were used in the PCR for amplifying the four different overlapping regions which included all nine exons of Fas mRNA (Table 2, regions A–D). PCR was performed on 5 µl of cDNA template in a total volume of 50 µl containing 2 µM of each primer, 200 µM dNTP, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2 and 1.25 U of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). Thirty-five cycles of amplification were performed in a GeneAmpTM PCR System 9600 R (Perkin-Elmer Applied Biosystems Division, Norwalk, CT) with denaturation at 95°C for 30 s, annealing at 54°C for 30 s and extension at 72°C for 30 s. An aliquot of each PCR product was separated by electrophoresis on a 3% agarose gel and visualized by staining with ethidium bromide. Each amplified product was directly subcloned into the pT7Blue T-Vector (Takara, Osaka, Japan) and transformed into JM109 high efficiency competent cells (Takara) with 42°C heat shock for 45 s. Recombinant colonies were subcloned and selected by blue–white screening on an indicator plate. The nucleotide sequence was determined by the dyeodeoxy terminator method with the DyeDeoxy Terminator Cycle Sequence Kit (Perkin-Elmer) according to the manufacturer’s recommendations with a DNA sequencer 371A (Perkin-Elmer). The cDNA nucleotides correspond to Behrmann (40) and are available from GenBank (accession nos X81334–X81342).

Measurement of soluble Fas (sFas) and soluble FasL (sFasL)

Serum sFas and sFasL concentrations were estimated by a commercial ELISA kit (MBL) according to the manufacturer’s recommendations. The sFas ELISA system detected only the soluble form of Fas antigen, which lacks the transmembrane portion, but not the soluble form of Fas, which lacks the intracytoplasmic death domain.

Immune detection of Fas receptor

Detection of Fas protein was estimated by Western blot analysis as previously described (31). Briefly, 20 mg of total protein of lysates from activated T cells from normal control and patients was size fractionated in the 5–20 % gradient SDS–PAGE gel (ATTO, Tokyo, Japan) under non-reducing conditions and electrophoresed on nitrocellulose membranes. Preblocked membranes were incubated with 10 µg of anti-Fas mAb (4B4-3A) and then incubated with 1:2000 diluted horseradish peroxidase-conjugated goat anti-mouse Ig antibodies (Zymed, San Francisco, CA). Finally, specific bands were detected by using ECL detection system (Amersham International, Amersham, UK).

Results

Defective Fas-mediated apoptosis on different immune cell populations from patients

Since the clinical symptoms and manifestations of our patients were quite similar to those of lpr/gld mice (3,6–9) and Fas knockout mice (32,33), we hypothesized that an abnormality in Fas-mediated apoptosis was responsible for their immunodeficient status. The significant increase of DN T cells within peripheral blood from patients (Table 1) supports this idea. Fas receptor expression on activated T cells from patient 1 was comparable to controls (Fig. IA and C), but Fas antigen was not detected on T cells from patient 2 (Fig. 1E) by indirect immunofluorescence analysis with M38 anti-Fas mAb. Similar Fas unreactivity was obtained with other antibodies against Fas antigen, CH11, 4B4-3A and ZB4. Although Fas was normally expressed on activated T cells from patients 1 and...
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2 (results not shown), Fas-induced apoptosis was defective in these patients even after treatment with high concentrations of anti-Fas mAb (Figs 1D and 2A). Activated T cells from patient 3, which did not express Fas antigen, were similarly resistant to anti-Fas mAb treatment (Fig. 1F). Similar patterns of Fas expression were found on SAC-activated B cells, EBV-transformed B cells and freshly isolated granulocytes from patients (results not shown). EBV-immortalized B cells from normal individuals were sensitive to treatment with anti-Fas mAb, while there was no induction of apoptosis in EBV-transformed B cells from patients (Fig. 2B). Fas-induced apoptosis was also defective in B cells cultured with SAC and IL-2 from patients (data not shown). Acceleration of Fas-mediated apoptosis was not seen in granulocytes from patients as well (Fig. 2C). Recently, the Fas–FasL system has been shown to play an important role in activation-induced T cell death (7–9). We compared apoptosis of cultured T cells induced by anti-CD3 mAb among patients and controls. Apoptosis of T cells from patients was weaker than those from controls (Fig. 3), but cross-linking with a higher concentration of anti-CD3 mAb induced comparable levels of apoptosis on T cells from patient 3.

Fas gene mutations in patients

The above results suggested loss of Fas-mediated function in cells from patients 1 and 2. Fas-mediated apoptosis was not induced even with normal cell surface expression of Fas. No differences were noted in the size and quantity of RT-PCR products amplified with the four primer sets obtained from...
patients and controls (results not shown). Nucleotide sequence analysis showed an insertion of four bases (GCAG) between exon 7 and 8 of Fas mRNA in half of the cloned PCR products using primer set C (Fig. 4A). The genomic DNA sequence of PCR products from the primer E showed a T to C point mutation at the splicing site of intron 7. This mutation predicted a frameshift and premature termination after 210 amino acids and a change of nine missense amino acids which resulted in a truncated Fas protein expressed on the cell surface but lacking the intracytoplasmic region including death domain. This mutation created a PstI digestion site. PstI treatment of genomic PCR products from primer E cleaved the 219 bp into 144 fragments of 75 bp in two siblings and their mother but not in the father, suggesting dominant inheritance of the mutation (Fig. 4B). Indeed, Fas-induced apoptosis was defective in T cells from their mother but not the father (Fig. 4C).

The complete absence of Fas receptor expression and consanguinity of parents for patient 3 could suggest a homozygous abnormality of the Fas gene. RT-PCR analysis demonstrated shorter PCR products from primer B (results not shown) and the skipping of exon 4 was found by direct sequencing of cDNA (Fig. 4A). This shorter mRNA of patient 3 was predicted to have a frameshift and premature termination after missense codons. This mutation was confirmed by sequencing of PCR products from primer F revealing an A to G point mutation in the acceptor of intron 3 (Fig. 4A). Homozygous inheritance of this mutation was evaluated by digesting genomic DNA with MaeI. PCR products from controls were digested to 134 and 173 bp fragments by MaeI (Fig. 5B, lane 1), but no digestion was observed in patient 3 (Fig. 5B, lane 6). Samples from both parents, the elder brother and paternal grandmother demonstrated heterogeneity for this mutation (Fig. 5B, lanes 2–5). Western blot analysis by anti-Fas mAb demonstrated small truncated Fas molecules (32 kDa) as well as normal Fas molecules (45 kDa) in activated T cells from patients 1 and 2, but no protein was detected in T cells from patient 3 (Fig. 6).

$sFas$ and $sFasL$ concentration in sera reflects the Fas mutation

We evaluated serum levels of sFas by ELISA which detects sFas containing the death domain. As expected from the results of sequence analysis, serum concentrations of sFas were decreased in patients 1 and 2, and undetectable in patients 3 (Table 1). The mother of the two siblings and the heterozygous members in the family of patient 3 revealed decreased concentrations of sFas (0.34–0.68 ng/ml) compared with controls (1.74 ± 0.46 ng/ml). On the other hand, the $sFasL$ concentrations in sera of patients were apparently...
Fig. 6. Immunoblot analysis of the Fas receptor. Detergent lysates of activated T cells from normal control (lane 1), patient 1 (lane 2) and patient 3 (lane 3) were electrophoresed in the 5–20 % SDS–PAGE gel, and specific Fas protein was immunodectected by anti-Fas mAb.

Fig. 7. Role of the truncated Fas receptor in Fas-mediated co-stimulation of T cells. Isolated E1 populations from patients, their families and controls (1 × 10^5/well) were stimulated for 4 days at 37°C in 96-well plates previously immobilized with anti-CD3 mAb (OKT3) and indicated concentration of anti-Fas mAb (CH11) diluted in 0.05 M Tris–HCl (pH 9.5) for 2 h at room temperature. DNA synthesis was examined by [3H]thymidine incorporation during the last 12 h. Data expressed are the mean ± SD obtained from triplicate experiments.

increased, especially an extremely high concentration of sFasL was noticed in the complete Fas-deficient patient 3.

Role of the truncated Fas receptor in Fas-mediated co-stimulation of T cells

Fas receptor is known to be involved in co-stimulation of freshly isolated T cells, as well as inducing apoptosis (25, 28). Similar to a previous report (28), immobilized CH11 anti-Fas mAb enhanced the anti-CD3 mAb-stimulated proliferation of freshly isolated normal T cells. Freshly isolated T cells from patients 1 and 2 showed a comparable augmentation of proliferation when co-stimulated with anti-Fas mAb (Fig. 7). This augmentation of T cell proliferation by anti-Fas mAb was not seen in patient 3 and augmentation of proliferative responses of T cells from the heterozygous carrier patient 3 were less than that in normal T cells. When CH11 was used in soluble form or soluble M38 antibody, neutralizing anti-Fas mAb were added, augmentation of proliferation was not seen (data not shown).

Discussion

The Japanese patients we analyzed showed novel Fas mutations which predicted the truncation of the intracytoplasmic domain of the Fas receptor in two siblings and the loss of Fas antigen expression by skipping of exon 4 in Fas mRNA of patient 3. Although Fas mutations were previously reported in humans in 18 cases from 14 families, which were distributed through exon 3 to exon 9 (21–26), sites of mutations seen in our two siblings were different from these reported mutations. However, similar to the mutation in our patients 1 and 2, aberrant splicing due to mutation in the 3′ splice site of intron 6 was noticed in a previously reported case (21) and exon skipping similar to patient 3 was also demonstrated in exon 3 of a previously reported case (21). Although the mutations of the Fas gene in humans and in lpr mice were usually seen in only one allele, our patient 3 has a homozygous mutation of the Fas gene. Similar to our case, rare homozygous mutations of two families, one patient has the completely homozygous mutation and another sibling case having combined different mutations inherited from their parents, were also noticed (24, 26). Although the expression of the Fas receptor on the cell surface markedly differed among the two siblings and another patient, the principal abnormality in these patients was a loss of function in Fas-mediated apoptosis. Signal transduction through Fas needs trimerization of Fas on the surface and close association of the death domain in the cytoplasm; thus, the truncated Fas receptor derived from the mutant allele in the siblings may interfere with trimerization of the normal death domain. This may prevent association of second messenger molecules with the death domain complex, which has been shown to have IL-1-converting enzyme- or CPP32-like protease activity (34). In patient 3, absent surface expression of the Fas receptor precludes binding of FasL, leading to severe clinical symptoms with prenatal onset, like one case reported as complete Fas deficiency (24).

The sFas molecule is known to be produced by alternative splicing of the exon encoding the transmembrane portion of Fas and is reported to be involved in the pathogenesis of autoimmunity by competing for binding of FasL to membrane-associated Fas receptors (36–37). As expected from mutations and cell surface Fas expression, sFas was not detected in the serum of patient 3 with complete Fas deficiency. As the ELISA method cannot detect sFas lacking the death domain, the level of sFas in patients 1 and 2 was decreased by half. The determination of sFas in sera may be a useful method for detection of Fas deficiency in patients and in healthy carriers with mutations since family members with the mutation showed decreased serum sFas. Furthermore, sFasL levels of sera from patients were significantly increased. The levels of sFasL concentration in patients 1 and 2 were comparable to those in patients with NK cell- or large granular lymphocyte-related malignancy (41) and another patient 3 revealed >100-fold higher sFasL concentration in serum. This extremely high level of sFasL in the
complete Fas-deficient patient may be due to accumulation in sera resulting from no binding sites of this TNF family cytokine that was produced in the activated state.

Although stimulation through the Fas receptor usually transduces death signals, ligation of the Fas receptor with immobilized anti-Fas mAb has been demonstrated to co-stimulate proliferation of freshly isolated T cells in the presence of activation via TCR (25,28), and similar stimulatory effects of Fas were seen in B lymphocytic leukemic cells (13) and normal diploid fibroblasts (29). Since the mechanism for stimulatory effects through Fas is unknown, we compared stimulatory effects of immobilized anti-Fas mAb on freshly isolated T cells from controls and patients 1 and 2, who have Fas mutation leading to expression of the truncated Fas and normal Fas on the same cell surface. Comparable co-stimulation of T cells from patients 1 and 2 was due not to neutralizing effects of anti-Fas mAb against FasL binding to Fas because only the immobilized (not the soluble) form of antibody showed co-stimulation of T cell proliferation. No co-stimulation of T cells in patient 3 confirmed that normal or truncated Fas receptor in patients 1 and 2 transduced the stimulatory signal for cell proliferation. One explanation for this result is that the intracytoplasmic portion, which includes the death domain, is not needed for the co-stimulatory signal of Fas. The role of these stimulatory functions through the Fas receptor in freshly isolated T lymphocytes is unknown in the physiological state. However, no co-stimulation through the Fas receptor in the complete Fas-deficient patient 3 in vitro may be involved in prolonged inflammation with more severe clinical manifestations in this patient, because these co-stimulatory signals through the Fas receptor were suggested to contribute to the differentiation of the pre-cytotoxic T cells into mature functional ones (27).

We showed that activation-induced death of T cells from patients treated with anti-CD3 mAb was diminished but was recovered with higher concentrations of anti-CD3 mAb. We could detect apoptotic cells in the lymph nodes and skin of patients 1 and 2 by TUNEL methods (our unpublished observations), and the presence of apoptotic death in vivo has also been shown in other human Fas-deficient patients (24). Furthermore, patients with Fas deficiency (including our cases) did not show fatal outcomes and survived (21–26,39). These observations suggest the presence of compensation mechanisms of apoptosis other than the Fas–FasL system in vivo, such as cytotoxic T lymphocytes using the perforin-granzyme-dependent pathway. Involvement of other factors regarding induction of apoptosis in vivo are unclear. Further studies on mechanisms mediating apoptosis may clarify the pathogenesis of many diseases including lymphoproliferative disorders associated with Fas deficiency.

Acknowledgements
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Abbreviations
DN T cells  CD4<sup>-</sup>CD8<sup>-</sup> double-negative T cells

EBV  Epstein–Barr virus
FasL  Fas ligand
PHA  phytohemagglutinin
sFas  soluble Fas
sFasL  soluble Fas ligand
SAC  Staphylococcus aureus Cowans I
TNF  tumor necrosis factor

References
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