Defective CD95/APO-1/Fas signal complex formation in the human autoimmune lymphoproliferative syndrome, type Ia

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ABSTRACT Heterozygous mutations in the CD95 (APO-1/Fas) receptor occur in most individuals with autoimmune lymphoproliferative syndrome (ALPS) and dominantly interfere with apoptosis by an unknown mechanism. We show that local or global alterations in the structure of the cytoplasmic death domain from nine independent ALPS CD95 death-domain mutations result in a failure to bind the FADD/MORT1 signaling protein. Despite heterozygosity for the abnormal allele, lymphocytes from ALPS patients showed markedly decreased FADD association and a loss of caspase recruitment and activation after CD95 crosslinking. These data suggest that intracytoplasmic CD95 mutations in ALPS impair apoptosis chiefly by disrupting death-domain interactions with the signaling protein FADD/MORT1.

Programmed cell death, or apoptosis, plays a critical role in regulating lymphocyte development and homeostasis. Apoptosis defects may contribute to abnormal lymphocyte accumulation, autoimmunity, and lymphoid malignancy (1). CD95/APO-1/Fas, a 45-kDa type I transmembrane glycoprotein in the tumor necrosis factor receptor superfamily (2) is a key molecule in lymphocyte apoptosis. CD95 functions as a homotrimer and contains a 68 amino acid cytoplasmic portion essential for apoptosis called the “death domain” (1, 3, 4). Engagement of CD95 triggers the recruitment of the cytosolic proteins FADD (or MORT1) and the inactive caspase-8 proenzyme (FLICE/MACH/Mch5) to form a complex with the death domain of CD95 (5, 6). Aggregation of procaspase-8 in the signaling complex initiates the autoprocessing and release of active caspase-8 into the cytoplasm where a proteolytic cascade leads to apoptosis (7, 8). Binding of FADD to CD95 is mediated by the interaction between death domains found in each molecule. The three-dimensional structure of the CD95 death domain has been solved and shown to have a characteristic fold of six antiparallel α-helices with a hydrophobic core (9). The lpr mutation has been shown to disrupt the α3 helix of the death domain and abrogate FADD binding (10).

Recently, we and others have described the human autoimmune lymphoproliferative syndrome (ALPS, also called the Canale–Smith Syndrome) that is characterized by defective lymphocyte apoptosis, lymphocyte accumulation, and humoral autoimmunity (11–17). The ALPS phenotype is associated with inherited mutations in the APT1 (CD95) gene (ALPS Type Ia) or the CD95 ligand gene (ALPS Type Ib) (11–18). In ALPS Type II, a similar, though more severe, clinical pheno-

type is presumed to be caused by an undefined inherited gene defect in the absence of mutations in the CD95 or CD95L genes (14). In inbred lpr and gld mice, essentially the same disease results from homozygous recessive mutations in the genes encoding CD95 and CD95 ligand, respectively (19–22). However, in ALPS Type Ia, individuals are most often heterozygous for mutant CD95 alleles. By an unknown mechanism, the coexpression of the CD95 protein encoded by the mutant allele interferes with the function of the wild-type (WT) CD95 protein, causing a severe defect in apoptosis (11). Rarely, homozygous “loss-of-function” mutations have been observed in ALPS Type Ia (12, 17). From the study of a large number of ALPS kindreds at National Institutes of Health, we have determined that the disease-producing mutations most often occur in the intracytoplasmic death domain of CD95 (11, 14). We therefore investigated the mechanism by which these mutations alter CD95 signaling.

MATERIALS AND METHODS

Patient Samples. All patients were studied at the National Institutes of Health under an approved protocol with informed consent. cDNA and genomic DNA samples were analyzed by PCR and sequencing as previously described (11). The genotypes and phenotypes of some patients have been reported previously (11, 14, 15, 23, 24).

DNA Constructs and Apoptosis Assays. Plasmid cDNA constructs (WT CD95-pCI, mutant CD95-pCI, MC-159-pCI or pCI alone) and the H-2 L5-pSRα plasmid at a ratio of 3:1 (20 μg total DNA) were electroporated into 4–8 × 106 Jurkat T cells in 0.4 ml complete medium in an Electroporator 600 (BTX Corporation, San Diego) (10). After pulse discharge at settings 260 V, 1050 μF, and 720 Ω, the cells were immediately placed in 8 ml of complete medium and incubated for 16 hr at 37°C. Viable cells were isolated over a Ficoll gradient (Pharmacia). For the induction of programmed cell death, 5 × 104 cells in 200 μl were treated with 30 ng/ml of the anti-CD95 antibody, CH11 (Kamiya Biomedical, Thousand Oaks, CA) in triplicate wells for 7–10 hr; parallel control and annexin V, and transfected cells staining negative for annexin V and positive for H-2 Ld were enumerated over a constant time by flow cytometry. Nondetectable cells were excluded by using forward and side scatter parameters. A

Abbreviations: ALPS, autoimmune lymphoproliferative syndrome; HSQC, heteronuclear single-quantum coherence; WT, wild type; GST, glutathione S-transferase.

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Levels of GST-CD95 fusion protein bands were normalized by treatment, and exposed to BioMax MR (Kodak) film at glacial acetic acid, enhanced with Enlightening (DuPont) solution. *Assays performed on PHA-stimulated peripheral blood lymphocytes as described in Materials and Methods. The percent apoptosis induced in T cells from normal subjects under these assay conditions is shown in parentheses. All values reported are the results of the propidium iodide incorporation assay with the exception of the anti-CD95 assay on patient 30, which were done by the DNA content assay as described in ref. 23. ND, not determined.

<table>
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<th>Patient ID number</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>αβ T cells, CD4⁺/CD8⁺ (% &lt;1.0%)</th>
<th>Anti-CD3e apoptosis*, % (50–70%)</th>
<th>Anti-CD95 apoptosis*, % (70–90%)</th>
<th>NMR structure</th>
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§Assays performed on PHA-stimulated peripheral blood lymphocytes as described in Materials and Methods. The percent apoptosis induced in T cells from normal subjects under these assay conditions is shown in parentheses. All values reported are the results of the propidium iodide incorporation assay with the exception of the anti-CD95 assay on patient 30, which were done by the DNA content assay as described in ref. 23. ND, not determined.

†LY, lymphadenopathy; SM, splenomegaly; HSM, hepatosplenomegaly; AIHA, autoimmune hemolytic anemia; ITP, immune thrombocytopenic purpura; GN, glomerulonephritis, and NP, autoimmune neutropenia; direct antiglobulin—note that Coomb’s test was positive in all patients.

**NMR Spectroscopy.** The death domain of WT CD95 (residues 174–319) was cloned into the pET30b vector (Novagen) as described (9). Nonsense and missense mutations were made by using the Quik-Change mutagenesis kit according to manufacturer’s protocol (Stratagene) and confirmed by sequencing. The recombinant proteins were expressed in Escherichia coli and purified by affinity chromatography on a nickel-IDA column (Invitrogen) using a polyhistidine tag (<LHHHHHHHH> at the C terminus, 15N/H heteronuclear single-quantum coherence (HSQC) spectra were acquired at 30°C on Bruker (Billerica, MA) DDMX500 or AMX600 NMR spectrometers as described previously (9, 10).

**Analysis of CD95/FADD Binding.** The cytoplasmic domains (residues 174–319) of WT CD95 and mutant CD95 from patients 3, 5, 6, 17, and 26 were amplified by PCR using patient CD95 cDNAs as templates (see above) and cloned in-frame with the WT CD95 pGEX 4T-1 construct (Pharmacia). For patients 29, 30, 31, and 33, the CD95 cDNAs as templates (see above) and cloned in-frame with the WT CD95 pGEX 4T-1 (Pharmacia). For patients 29, 30, 31, and 33, the CD95 cDNAs as templates (see above) and cloned in-frame with the WT CD95 pGEX 4T-1 construct was used as a template for site-directed mutagenesis (Quik-Change, Stratagene). pGEX 4T-1 (Pharmacia). For patients 29, 30, 31, and 33, the CD95 cDNAs as templates (see above) and cloned in-frame with the WT CD95 pGEX 4T-1 construct was used as a template for site-directed mutagenesis (Quik-Change, Stratagene). pG- STag CD95-lpr was a gift of Vishva Dixit. The pGEX 4T-1 constructs were transfected with 1.0 μg of the indicated constructs with 0.1 μg FADD (as indicated in the figure) by using Superfect (Qiagen, Chatsworth, CA). The cells were lysed on ice with 1 ml E1A lysis buffer [50 mM Hepes, pH 7.6/250 mM NaCl/0.1% NP40/5 mM EDTA/1× Complete proteinase inhibitor mix (Boehringer Mannheim)] for 30 min. Lysates were spun for 10 min at 14,000 × g at 4°C, and the supernatants were immunoprecipitated for 1 hr with 5 μg/ml mouse APO-1 (anti-human CD95, Kamiya) at room temperature. Twenty microliters per sample of a 1:1 slurry of protein A-Sepharose beads (Pharmacia) was added for an additional hour at room temperature with agitation, after which the beads were washed twice with E1A buffer, twice with high-salt E1A buffer (1 M NaCl), and twice more with E1A buffer. The precipitates were fractionated on 12% SDS/PAGE gels and transferred to a nitrocellulose membrane. CD95-FADD co-immunoprecipitations were probed with anti-human FADD mAb (Transduction Laboratories, Lexington, KY) followed by HRP-conjugated rabbit anti mouse Ig (Bio-Rad) and developed with Pierce super-signal chemiluminescent substrate.

**Death-Inducing Signal Complex (DISC) Analysis and Caspase Assays.** Epstein–Barr virus-transformed B cells (1 × 10⁷) from the indicated patients were stimulated with the anti-CD95 antibody APO-1 (IgG3) at a concentration of 1 μg/ml at 37°C for 10 min (7). The cells were lysed in 500 μl DISC buffer (30 mM Tris-HCl, pH 7.5/150 mM NaCl/1% Triton X-100 (Sigma)/10% glycerol/5 mM iodoacetamide/1× Boehringer Mannheim Complete proteinase inhibitors/2 μM of the caspase inhibitor zVAD-fmk) for 15 min on ice. The samples were centrifuged at 14,000 × g for 15 min. Supernatants were added to either 25 μl of protein A-Sepharose suspension (50%) (Pharmacia) or anti-mouse IgG-conjugated magnetic beads (Dynal, Great Neck, NY) and incubated for at least 2 hr at 4°C with rotation. The beads were then washed five times with lysis buffer, resuspended in reducing SDS sample buffer, and boiled for 5 min. As a negative control, the same number of cells was lysed without anti-APO-1 stimulation, and anti-APO-1 at 1 μg/ml was added to the lysates. The samples were separated by 12% SDS/PAGE, transferred to nitrocellulose (Bio-Rad) and blotted with a mixture of anti-CD95 antisera diluted 1:100 (C18 and N20, Santa Cruz Biotechnology) followed by horseradish peroxidase (HRP)-conjugated donkey anti-rabbit Ig (Amersham); anti-FADD mAb (Transduction Laboratories) at 1 μg/ml followed by HRP-conjugated anti-mouse IgG1, or anti-FLICE mAb C 15 (39), followed by HRP-conjugated anti-mouse IgG2b. After washing with PBS/0.05% Tween-20, the blots were developed with Pierce Super Signal or Super Ultra chemiluminescence reagents. Caspase
RESULTS

We studied nine unrelated patients referred to the National Institutes of Health who exhibited the characteristic clinical features of ALPS Type Ia: defective lymphocyte apoptosis, the accumulation of CD4+ CD8+, CD3+ αβ T lymphocytes, and heterozygous mutations in APT1 exon 9 encoding the death domain of the CD95 protein (Table 1 and Fig. 1A) (11, 14, 23, 24). We found that lymphocytes from all nine patients had defective apoptosis after stimulation of the T cell receptor or CD95 (Table 1). To confirm that the CD95 mutations were indeed the cause of defective apoptosis, we tested these mutant alleles in a transient transfection assay in Jurkat cells and found that all dominantly interfered with apoptosis triggered by Fas crosslinking (Fig. 1B and C). These mutations were dispersed along the length of the death domain (Fig. 1A). When we mapped these amino acid changes on the three-dimensional structure of the CD95 death domain, illustrated as a bundle of six antiparallel α-helices (8), no clear pattern emerged (Figs. 1A and 2A).

To understand the structural effects of these mutants, we compared the NMR HSQC spectra of the mutated ALPS death domains to the WT HSQC spectrum (Fig. 2B, yellow = WT and red = ALPS mutant) (8). Some mutations, such as T225P, R234P, Q257X, and I294S, produced many differences in the 15N/1H amide chemical signals compared with WT, represented by the noncoincidence of the crosspeaks from WT and mutant CD95 proteins (Fig. 2B, R257X, and I294S, and Table 1). The collapse of 1H chemical shift dispersion for these mutants indicates unfolding of the death domain into random coils. This unfolding was caused either by destabilization of the hydrophobic core as in the I294S mutation or by deletion of portions of the death domain as for Q257X. In the other mutants, R234Q, A241D, and D244V, the signals in the HSQC spectra were mostly coincident with the WT, indicating no major structural perturbations (Fig. 2B, A241D and D244V). These mutations are located in the α2 and α3 helices and could directly affect the association of FADD or the oligomerization of CD95 (9, 10). Thus, all of these mutant CD95 proteins, except that of Pt. 30, which was aggregated and not suitable for analysis, were either misfolded or had local perturbations in the α2 or α3 helices (10).

To test the effect of these mutations on FADD binding, we examined the ability of immobilized GST-CD95 intracytoplasmic domain fusion proteins to bind FADD in vitro. In contrast to the WT GST-CD95 protein, we found that all nine mutations, as well as the human version of the lprcg mutation, failed to bind FADD (Fig. 3A). When overexpressed in 293T cells under conditions where the WT full length CD95 protein binds FADD, each of these mutant full length CD95 molecules also failed to bind FADD in vivo (Fig. 3B). These experiments provided examples of both local and global changes in the CD95 death-domain structure that severely disrupt FADD binding.

To determine whether defective FADD association could account for the apoptosis defects in lymphocytes from the ALPS patients, we stimulated and immunoprecipitated CD95 from ALPS patient EBV-transformed B cell lines.

Under these conditions, FADD and procaspase-8 associate with the CD95 death domain only after stimulation of the receptor (7, 8). In comparison to normal cells (Fig. 4A, lane 2), we found that FADD and caspase-8 recruitment was dramatically decreased in cells from ALPS patients (Fig. 4A, lanes 4, 6, 8–10), with densitometric analysis yielding less than 25% of the normal levels. Control blots showed that cytosolic extracts from the ALPS lymphocytes contained equivalent levels of CD95, FADD, and caspase-8 compared with normal lymphocytes (Fig. 4A and data not shown).

Finally, we assessed activation of the apoptosis-inducing mechanism by carrying out a sensitive fluorometric assay for caspase activity on ALPS patients’ lymphocytes after CD95 stimulation (25). Correlating with the decreased formation of CD95 signaling complexes, we found greatly reduced activation of downstream caspases in the ALPS patient cells, measured by the DEVD-AMC substrate, compared with control cells (Fig. 4B).
patients are given in Table 1. The representative ALPS patients as indicated; full data for all nine HSQC spectra of bacterially synthesized death domains from four treating the location of the amino acid changes that were analyzed (9).

Because our caspase assays on CD95-stimulated patient lym-
trophs used the DEVD substrate, which is preferred by nonreceptor caspases related to and including caspase 3, our

30 reported examples of pathogenic human APT1 (CD95) mutations, all except two are heterozygous and dominant interfering, and nearly three-fourths occur in the death domain (11–17, 23, 24). The mechanism of receptor dysfunction is important because in outbred human populations, deleterious mutations are most likely to be found in the heterozygous state. In this report, we show that death-domain mutations associated with ALPS markedly decrease the ligand-induced binding of the FADD signaling protein. FADD signaling leads to a secondary antibody. Control experiments showed equivalent levels of CD95 in immunoprecipitates and FADD in cell lysates (not shown).

results suggest that the failure of caspase-8 recruitment prevents any downstream caspase activation (26). Our data support the idea that the FADD/caspase-8 signaling complex (7, 8), and not other potential CD95 signals (27–31), is essential for lymphocyte apoptosis after CD95 crosslinking.

Our results provide the first important insight into the apoptosis signaling defect in ALPS Type I but unveil a key unanswered question. The loss of FADD association in ALPS patient cells is much greater than expected in the heterozygous state. If the WT and mutant CD95 alleles are codominantly expressed, then half of the CD95 molecules would have intact death domains and should be capable of binding FADD after appropriate stimulation. The fact that we consistently observed a much greater than 50% decrement in FADD recruitment in the patients’ cells indicates that the mechanism of dominant interference is more complex than a simple failure of FADD binding to the mutant CD95 protein. Several possibilities can be envisioned. Competition for ligand between mutant and WT CD95 molecules is unlikely as the amounts of crosslinking antibody used in these studies are far in excess of equilibrium. Other possibilities are that the WT allele may be poorly expressed, leading to selective expression of the mutant allele, or the mutant allele could bind the WT in an intracellular compartment and prevent surface CD95 expression. These two conjectures are unlikely, as CD95 expression on lymphocytes from all the patients in this report is equivalent to normal lymphocytes (10, 13, and data not shown). In addition, transfection of each of these mutant CD95 molecules with or without WT Fas produces surface CD95 receptors expression comparable to WT by flow cytometry (L.Z, C.J., J.P., and M.J.L., unpublished observations). We favor the possibility that the mutant CD95 protein interferes with signaling by preventing the binding of FADD to WT CD95 protein. We hypothesize that the formation of the CD95 signaling complex may be a cooperative process requiring the preassembly of CD95 subunits. This preassembly is not ligand induced but caused by spontaneous association of the assorted
chains to form mixed WT and mutant heterotrimers. The fact that completely unfolded death domains can occur in association with dominantly interfering CD95 chains implies the interaction takes place outside the death domain. Preliminary biochemical data suggest that the extracellular domain may play an important part in CD95 preassembly. Such a mechanism could also play a role in dominant-interfering phenomena for other receptors in the TNFR superfamily that are structurally related.

The development of autoimmunity associated with genetic defects in CD95 may require immunoregulatory defects in addition to impaired apoptosis (32, 33). The lpr mutation on certain inbred mouse genetic backgrounds is associated with much less severe disease than on the MRL background, implying that cofactors in addition to the apoptosis defect are important (1, 2). Similarly, in ALPS, members of a kindred that harbor the same CD95 mutation may differ greatly in the severity of their clinical phenotype (24). Smith et al. have shown that transgenically expressing CrmA in T cells can block apoptosis but does not, in and of itself, cause autoimmune disease (32). Nonetheless, emerging evidence from our studies of large kindreds suggests that the CD95 death domain mutations are associated with a high penetrance of clinical autoimmune disease compared with defects in other parts of the receptor (23, 34). Also, all cases in which germline CD95 mutations are associated with the development of lymphoma involve death-domain alterations, although the number of patients is as yet too small to validate this observation statistically (S.E.S., J.D., J.M.P., and M.J.L., unpublished work). Therefore, it will be very important to determine the molecular mechanism by which CD95 receptor chains with abnormal death domains could prevent FADD recruitment to WT CD95 chains.

In closing, it is noteworthy that knockout and dominant-negative transgenic mice have shown that FADD plays an essential role in lymphocyte proliferation (35–37), yet we have observed no obvious defects in proliferative responses in ALPS lymphocytes (data not shown). Indeed, activated proliferating lymphocytes are a prominent feature of the lymphoid hyperplasia in this disease (38). This suggests that other CD95-independent functions of FADD are required for proliferation, and these pathways are unimpaired in ALPS.

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