Haploinsufficiency rather than the effect of an excessive production of soluble CD95 (CD95∆TM) is the basis for ALPS Ia in a family with duplicated 3’ splice site AG in CD95 intron 5 on one allele

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Abstract

Autoimmune lymphoproliferative syndrome type Ia (ALPS Ia) is caused by mutations in the CD95/Apo-1/Fas (TNFRSF6) gene, which lead to a defective CD95 ligand (CD95L)-induced apoptosis. Soluble CD95 (sCD95) has been suggested to play an important role in the pathogenesis of diverse autoimmune and malignant diseases by antagonizing CD95L. Here we evaluate a family with four in five of its members harboring an ex-6 – 3C->G mutation that affects the splice cis regulatory region (cctaag/ex-6 ->cctagg/ex-6) of the CD95 gene. The mutation causes skipping of exon-6, which encodes the transmembrane region of CD95, and thereby leads to an excessive production of sCD95 in all four affected individuals. The mutation is associated with a low penetrance of disease phenotype and caused mild and transient ALPS in one male patient whereas all other family members are completely healthy. In all family members with the mutation we found that the cell surface expression of CD95 was low and the activated T cells were resistant to CD95-induced apoptosis. Unexpectedly, excessive production or addition of sCD95 had no effect on the CD95-induced apoptosis in diverse cells. In contrast, increasing the surface expression of CD95 was able to correct the defect in apoptosis. Thus we conclude that the ALPS in the patient was caused by haploinsufficiency of membrane CD95 expression. Our data challenges the hypothesis that sCD95 causes autoimmunity.
Introduction

CD95 (Fas, Apo1, TNFRSF6) is a transmembrane receptor belonging to the tumor necrosis factor superfamily \(^1\). Upon CD95 ligand (CD95L) stimulation, CD95 can induce programmed cell death (also referred to as apoptosis). Apoptosis is crucial for homeostasis of the immune system\(^2\)-\(^4\). It can downregulate an immune response after the elimination of antigens; it is also involved in removing autoreactive or futile cells and in extinguishing malignant lymphatic cells or their genetically altered precursors \(^5\);\(^6\).

Recently, defects in genes regulating apoptosis have been discovered causing the clinical picture of Autoimmune Lymphoproliferative Syndrome, ALPS\(^7\);\(^9\), or an ALPS-like clinical manifestation\(^10\). ALPS (also called Canale-Smith syndrome\(^6\);\(^10\)-\(^12\)) is characterized by recurrent or more often chronic, benign, sometime massive lymphoadenopathy; splenomegaly of early onset; autoimmune phenomena such as thrombocytopenia or hemolytic anemia; and less frequently, malignant lymphoma \(^5\);\(^6\). In addition, CD4/CD8 double negative, \(\alpha/\beta\) receptor-positive T cells are often present. Further immunophenotypic characteristics have been detailed previously \(^13\). However, even within one family, members that carry the same genetic defect can have diverse clinical phenotypes, including mild, reversible symptoms if any, severe autoimmune phenomena, and/or different malignancies \(^5\). Furthermore, many patients come up with improvement of their symptoms when they grow older. According to the OMIM nomenclature, several types of ALPS exist \(^7\);\(^9\);\(^12\);\(^14\)-\(^20\): ALPS Ia, the most common form, is associated with mutations in the CD95 gene, in ALPS Ib the CD95L gene is affected, whereas mutations in the caspase-10 gene lead to ALPS II, and cases without defined mutations are termed ALPS III. Other authors refer to the latter form as ALPS-like disease (ALD)\(^10\). ALPS induced by somatic mutations has also been described \(^21\).

The majority of ALPS Ia cases is due to mutations in the death domains of the CD95 gene. The penetrance of its phenotypes is high because the mutant proteins exert dominant-negative effects on function of the wild-type CD95 protein. In contrast, there are forms of ALPS Ia with low-penetrance, which have been shown to be due to
haploinsufficiency. In this case, CD95 gene mutations prevent the affected alleles from expressing a normal amount of functional CD95 proteins.  

Pro- and anti-apoptotic stimuli must be balanced carefully in the immune system in order to achieve effective homeostasis without causing “collateral tissue damage”. Alternative splicing plays a critical role in regulation of apoptosis under many circumstances. It has previously been strongly suggested that sCD95, a translational product of alternatively spliced isoforms of CD95 mRNA that lacks exon-6, can block apoptosis through CD95. CD95 exon-6 encodes the transmembrane domain and is therefore needed for cellular expression of the protein. This soluble form of CD95 has been postulated to bind and thus to neutralize CD95L. At the CD95 pre-mRNA level, the inclusion of exon-6 is upregulated by the binding of TIA-1 or TIAR to the U rich region in intron-5 directly downstream of the exon-5 (fig.1). This process facilitates the 5′ splice-site recognition and the selection of the splice partner at the 3′ end of the intron-5. We here describe for the first time four members of a family, who carry a heterozygous double “ag” in intron-5 directly upstream of exon 6. This mutation alters the 3′ splice-recognition site of the intron and therefore causes a defect in splice regulation, leading to excessive production and secretion of sCD95. Unexpectedly, such excessive sCD95 serum levels can be compatible with excellent health.
**Materials and methods**

Informed consent for blood drawing, functional tests, and genetic analysis was obtained from the patient and his relatives.

RhsSuperCD95Ligand (super FasL; ALX-522-020-C005) has been purchased from Alexis Biochemicals, Lausen, Switzerland; recombinant human soluble CD95 (rh-sFas; Cat.-No.: 554336) from BD Biosciences, Pharmingen, Franklin Lakes, NJ, USA; IgM-CD95 agonistic antibody CH-11 clone from Clontech, Palo Alto, CA, USA; and human Apo-1/CD95 agonistic antibody (Cat.-No.: KHS9501) from Biosource, Camarillo, California, USA.

**Purification of genomic DNA**

For isolation of DNA, the QIAamp® kit (Qiagen, Hilden, Germany) was used according to the protocol provided. Briefly, 200 µL of whole blood were lysed by proteinase K, nucleic acids denatured by addition of ethanol (96%), absorbed to the silica membrane provided, washed, and eluted. The A260/280 ratio was 1.5-1.8, and the DNA yield was 4-6 µg in 200 µL of Tris, EDTA (TE) buffer.

**mRNA isolation and cDNA synthesis**

For the analysis of the CD95 mRNA from peripheral mononuclear cells (PBMC) and from EBV transformed B cells, the Dynabeads® mRNA direct kit (Dynal, Oslo, Norway) and the Superscript™ preamplification system for first strand cDNA synthesis (GibcoBRL, Eggenstein, Germany) were used according to the instruction manuals. Briefly, 4x10⁶ cells were lysed, homogenized, and incubated with oligo (dT)₂₅ beads. Using magnetic separation, washing steps included 2x LiDS and 3x standard washing buffer. After elution, 20µL of mRNA solution were incubated with oligo (dT)₁₂-₁₈ primer, polymerase chain reaction (PCR) buffer including MgCl₂, dNTP mix, Dithiothreitol (DTT), and superscript II reverse transcriptase for 50 min at 42°C.
PCR conditions

Thermo stable DNA polymerase, “Amplitaq Gold with Gene AmpR” and 10x buffer containing 15mM MgCl2 were purchased from Perkin Elmer (Weiterstadt, Germany). 10 µL of the buffer were mixed with 5 µL dNTPs (10mM of each), 5 µL of each primer (100 ng, tab.1, fig.1), 10 µL of genomic or cDNA solution, 0.5 µL polymerase (5U), and 64.5 µL ddH2O. The cycle program included 10 minutes at 94°C to denature the DNA, 35 cycles of 45 seconds at 94°C, 45 seconds at 60°C, and 90 seconds at 72°C and 5 minutes final extension at 72°C. The PCR products were partly cleaned and concentrated using Microcon-50 filter devices (Amicon, Witten, Germany). The retention was diluted with ddH2O to a total volume of approximately 30 µL, and the DNA content was determined by spectrometry (at 260 nm).

Real time TaqMan™ PCR (quantitative PCR)

Primers and probes (tab.1, fig.1) were designed using the primer express software provided by the manufacturer (Applied Biosystems PRISM™ 7700 sequence detection system, 7700 SDS, Foster City, CA, USA). The instruction procedures for the TaqMan EZ RT-PCR kit (same manufacturer) were optimized for Mn (OAC)2, primer (20µmol/l), and probe-FAM (10µmol/l) concentrations. For each experiment, 20ng mRNA was used and GAPDH-JOE (glyceraldehyd-3-phosphate dehydrogenase as a “housekeeping” gene) was co-amplified as an internal control. The results (ordinate, fig. 3B,C) are given as reciprocal values of cycle threshold (CT).

Cycle sequencing

The PE/ABI BigDye™ terminator-cycle-sequencing kit was purchased from Perkin Elmer (Weiterstadt, Germany) and was used to determine the sequence of the CD95 gene according to the provided protocol. Briefly, after mixing of 1 µL PCR product (100 ng DNA) with 1 µL primer (100ng), 2 µL terminator ready reaction mix (containing Mg2+, pyrophosphatase, and polymerase) and ddH2O to a final reaction volume of 10 µL, the following cycle conditions were applied: 25 x: 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes, 4°C forever (Trio Thermoblock, Biometra): The sequence was determined using a Sequagel 4.5% (National Diagnostics, Atlanta, GA) and a PE/ABI
377 automatic sequencer (Foster City, CA, USA). The sequences were aligned with both the standard sequence and the sequence of a healthy control using the ABI 377 sequence navigator software. Aberrant sequences were always confirmed on both DNA strands and in a second DNA sample to avoid PCR artifacts. On the genomic level, all exons of the CD95 gene including 20 or 30 bp of the adjacent introns and also the whole introns 5 and 6 were sequenced.

The most important primers for PCR and sequencing are given in tab. 1 and fig. 1.

*Flow cytometry determination of CD95 expression*

PBMCs were prepared using lymphocyte separation medium (Gibco BRL, Eggenstein, Germany) and isolated by standard gradient centrifugation techniques followed by hypotonic lysis of remaining erythrocytes. After activation with 10 \( \mu \text{g/ml} \) phytohemagglutinin (PHA, Sigma L9132) for 24 hrs and different time intervals (1-5 days) of activation with 1 \( \mu \text{g/ml} \) IL2 (Sigma I-2644), the cells were incubated for 30 min on ice with 5 \( \mu \text{g/ml} \) mouse IgM (Becton Dickinson, Heidelberg, Germany) isotype control or 5 \( \mu \text{g/ml} \) anti-CD95 mAb CH-11. Antibody binding was detected with FITC-conjugated goat anti-mouse IgG antibody (Becton Dickinson, Heidelberg, Germany). 10,000 lymphocytes were measured by flow cytometry and analyzed after gating on vital cells using a FACSCalibur (Becton Dickinson).

*Apoptosis measurement by Flow-cytometry*

PBMCs were incubated in PHA and later activated with IL2 for 5 days as described above. Apoptosis was induced by incubation of the cells with anti-CD95 mAb or soluble super CD95L for 16 hrs and then determined with the annexin V-FITC kit (PN IM3546, Immunotech, Marseille, France). This assay indicates an early phase of apoptosis by FITC-annexin binding to phosphatidyl serine, which turns to the outer cell membrane surface (green fluorescence), whereas cell death can be measured by propidium iodide, which stains the DNA of cells that lost its membrane integrity (red fluorescence). As agonistic antibodies the CH-11 IgM clone was used for diagnosis and when effectiveness in inducing apoptosis was essential whereas the Apo-1 antibody (ALEXIS Biochemicals Products) was used for graded responses.
**Determination of the sCD95 secretion**

PBMCs were incubated with PHA and activated with IL2 as described above for 0, 2 or 5 days. The medium containing IL2 was changed 48 hrs before measurement and the suspension was adjusted to 10^6 cells/ml. Aliquots of 50 µL of the cell supernatant were tested for sCD95 in appropriate dilutions using the MBL sCD95 (S) ELISA kit (No.5251, Medical and Biol. Laborat., Nagoya, Japan) through standard sandwich ELISA techniques. Measurements of CD95 expression, secretion of sCD95, apoptosis, and determination of the alternatively spliced forms of CD95 mRNA were also performed on optimally proliferating EBV-transformed B-cells without additional stimulation.

**Incubation of Jurkat cells in serum or cells supernatant containing sCD95**

Cells were incubated in 70% serum or supernatant and 30% standard medium before super CD95L was added. Apoptosis was measured 20 hrs later.

**Plasmids and transfection**

AU1-tagged CD95-encoding plasmid (pCI-backbone) has been used before. AU1 tagged CD95-del6 (CD95\(\Delta TM\)) plasmid for sCD95 production was made by applying site directied mutagenesis to the original CD95 plasmid using the Stratagene QuikChange XL Kit (Cat.-No.: 200516, La Jolla, CA, USA) according to the instructions of the manufacturer. In short, forward and reverse primers were designed comprising 12 bp of the 3’exon-5 and 12 bp of the 5’exon-7 to bridge exon-6. Methylated original CD95 plasmid derived from bacteria was used as the template and both primers were added for an 18-cycle PCR, the remaining methylated template was digested with the Dpn I provided, and the PCR product was used to transform XL 10-Gold competent bacteria. Bacterial colonies growing under appropriate selection were selected for further processing and correct plasmids were purified using the Marligen Biosciences Maxiprep system (Cat.-No.: 11452-026, Ijamsville, MD, USA). The correctness of CD95-del6 was verified by sequencing. Optimally proliferating Jurkat (ATCC, Rockville, MD, USA) or the mouse thymoma BW cells were suspended at 10^7 cells/ml, and used 0.4 ml per transfection in 4 mm cuvettes.
together with plasmids in the indicated amounts. The cells were electroporated using a BTX machine (260 V, 1050 µF, 720 Ohm; Pittsburgh, PA, USA), and resuspended in 6 ml-fresh medium in 6-well plates and cultured for approx. 20 hrs. EBV-transformed B-cells were transfected using the Amaxa biosystem (Cologne, Germany) according to the B-cell transfection protocols provided by the manufacturer.

Retroviral transduction of Jurkat cells.

CD95del6 (Fas∆TM) was cloned into a bicistronic oncoretroviral transfervector (MFG-S-IRES-eGFP) \(^{35,36}\), which enables eGFP co-expression under a single promoter. For control transductions a monocistronic MFGS-eGFP vector was used. 293T cells were co-transfected with 10µg transfervector, 6.5µg gag pol packaging plasmid and 3µg VSV-G envelope plasmid (pMDG) in 10cm dish using PEI. Supernatant containing virus particles was harvested. Optimally proliferating Jurkat cells were suspended at 0.4x10^6 cells/ml, and 0.5ml cell suspension and 0.5ml virus supernatant were mixed in a 24 well plate. 8µg/ml polybrene was added and the plate was centrifuged at 2500rpm for 20min. 96h after transduction the efficiency was analyzed by flow cytometry and found to be more than 90%. sCD95 (sFas) expression of transduced Jurkat cells was analyzed by ELISA as described.

Co-incubation assay of Jurkat and CD95L-L5178Y cells, and calculation of % cell loss

CD95L (FasL) expressing cells were a kind gift from Dr. Sang-Mo Kang at UCSF. These cells are a transfected mouse thymoma line expressing uncleavable human FasL on their surface and are referred to as CD95L-L5178Y cells \(^{37}\). L5178Y wild type and L5178Y CD95L (FasL) overexpressing cells were co-incubated with Jurkat cells that either express sCD95 (sFAS) and GFP or GFP alone. For co-incubation assays all cells were suspended at 0.25x10^6 cells/ml. 0.25ml of Jurkat cell suspension was mixed with 0.75ml L5178Y cell suspension. GFP expressing cells were counted for 30s using flow cytometry at time points 0h and 22h. Cell loss induced by CD95L (FasL) expressing L5178Y cells was calculated as follows: One minus (number of vital GFP+ cells after 22hr incubation with FasL expressing cells divided by number of GFP+ cells found after
parallel co-incubation with L5178Y wildtype cells (not expressing FasL)), times 100%.
The same type of calculation was used in all experiments where % cell loss is given.

**Case report**

The family history of the patient is unremarkable. He presented with epistaxis, thrombocytopenia (min. 10,000/µl), splenomegaly, and cervical and axillary lymphoadenopathy at age 8 years. His mother reported that the splenomegaly had already been observed shortly after birth. From age 8 to 18, the patient had thrombocytopenia that was accompanied with petechia and hematomas of the skin once or twice a year. Lymph node biopsies were taken at age 10 and 12, both showing nonspecific, chronic inflammatory lymphatic hyperplasia. The patient also presented with recurrent neutropenia (250-600/µl). Anti-platelet autoantibodies were strongly positive and anti-neutrophil autoantibodies were weakly positive. Repeated cytological and histological examinations of the patient’s bone marrow revealed an enhanced granulocytopoiesis and normal megakaryocytes. All other findings and all routine immunological diagnostics were unremarkable. The thrombopenia and neutropenia responded well to steroids, but autoantibodies did not.

At age 12, 80% of the patient’s spleen was removed by surgery. However, it grew back to a moderately enlarged size within three years. Since age 18, the patient who is 24 years old now, has only had minor lymph node swellings and no further problems. Platelet and leukocyte counts have always been normal ever since. The CD4, 8 -double negative T cells have been normal lately.

All other family members including the father are completely healthy and have never had any similar symptoms. The father and one daughter participate in sports and other physical strains along with their profession.
Results

The clinical phenotype of our patient (see case report) was typical for ALPS. We found that as compared with normal controls, the CD95–induced apoptosis was clearly reduced in the peripheral blood lymphocytes from the patient, from all family members with the respective mutation (figs. 1D, 2A, tab.2) and in EBV- B cells (fig.2 B) from the patient. Since the majority of ALPS patients carry a mutation in the CD95 gene, we sequenced the patient genomic and cDNA for CD95 gene. We found a C->G point mutation at position –3 of intron-5 in one allele, which led to an alteration of the splice cis regulatory region (cctaag/G->cctaag/G, fig.1, C). In accordance with this finding, sequencing of cDNA that derived from the activated PBMCs and EBV-transformed B-cells revealed three splicing variants in the region of exons 5, 6, and 7, as diagrammed in fig.1. Allele specific, quantitative results were obtained by sequencing 50 individual cDNA clones from the patient. The SNP836 (pos. 74 in exon 7, fig. 1) clarified the maternal or paternal origin, tab.3: i) In the largest portion of patient cDNA, exon 6 was skipped (fig.1B, tab. 3, 2nd column); ii) a small portion was normally spliced and contained exon 6, (fig.1A, tab. 3, 3rd column); iii) a few cDNA clones were found to contain exon 6, but had a small two-bp insertion of AG in positions –1 and –2 upstream of this exon (fig.1A, tab.3, 4th column). This clearly demonstrated that the additional “ag”, which was created by the mutation, was weakly active as a splice site and led to an insertion of the normally intron-splicing consensus sequence “ag” into the CD95 RNA transcript. Also, we completely sequenced the CD95 cDNA and genomic DNA, including all its exons with extending 20-50 bp of adjacent introns from the patient. No mutation was detected on the second allele. The ALPS affected patient and one healthy sister shared the same second CD95 allele from their mother whereas the other healthy sister inherited the mother’s other allele (fig.1 D). Therefore, the maternal allele does not contribute to the patient’s ALPS, at least not critically.

We found that in healthy donor lymphocytes, the ratio of wild-type (wt-) versus CD95-del6 was increased after lymphocyte activation as shown in fig.3. This confirms the previous observation that during lymphocyte activation, the splicing of complete wt-
CD95 mRNA is normally upregulated. However, we found that in the patient, the splicing variant CD95-del6 was upregulated above normal range during lymphocyte activation for both primary T and B cells, as well as in auto-proliferating EBV-B cell lines (fig.3A, B-cells). Our data suggests that the patient lymphocytes express an elevated amount of the alternatively spliced CD96-del6 mRNA during immune responses.

Next we investigated the impact of this variant splicing of CD95 on its cell surface expression. The full-length wt-CD95 mRNA is expressed as a membrane-bound, apoptosis-mediating receptor, which is capable of binding CD95L. We found that after activation, the membrane CD95-expression on the patient PBMC was decreased as compared to the control cells (fig.4 C, tab.2). This observation was recapitulated in EBV-cell lines derived from the patient versus five normal individuals by applying the same staining to these proliferating B cells, data not shown, and also in the other family members (fig.1D) affected by the ex-6 –3C->G mutation (fig.4 C, tab.2).

CD95-del6 mRNA splicing variants are translated to sCD95 proteins, which are secreted by lymphocytes. It has been postulated that the sCD95 can bind CD95L and thus antagonize CD95L-induced apoptosis. In accordance with the excessive production of CD95-del6 mRNA in the patient’s lymphocytes, an approximate of seven-fold increase in sCD95 was detected in the supernatant of the patient’s cells (fig.4 A, B), and in the serum of all affected family members (fig.4 D); as compared to that of the healthy control donors. The secretion of sCD95 was upregulated during activation of the patient lymphocytes (fig.4 B).

Next we evaluated the sCD95 at functional level. As mentioned above and shown in fig.2 (see also supplemental fig.1), the apoptosis of activated PBMCs from the patient and the other affected family members was obviously decreased as compared to control cells when these cells were stimulated by an agonistic anti-CD95 mAb CH-11 (or by super CD95L, data not shown), even though the induction of cell death was not abolished as seen in some ALPS patients previously described. A similar reduction of CD95-induced apoptotic response was also observed in patient EBV-B cells (fig.2 B). This
reduction could not be overcome by increasing the concentration of the mAb CH-11 to 25µg/ml (fig.2, B) or even 250µg/ml (data not shown). Despite the excess of agonist, the fraction of apoptotic cells did not exceed 25% (or 35%, respectively). These data indicate that the patient lymphocytes are defective in CD95-induced apoptosis.

Since excessive concentrations of the multi-valent anti-CD95 failed to enhance apoptosis of the patient lymphocytes, we hypothesized that the decrease in expression of membrane-bound CD95, but not the sCD95 produced by patient cells accounts for the defect of CD95-induced apoptosis. To test this hypothesis, we transfected patient EBV-B cells with a plasmid that expresses an epitope-tagged (AU1) wt-CD95. As shown in fig.5, cells supplemented with wt-CD95 (as indicated by AU1 expression) were more sensitive to anti-CD95-induced apoptosis compared to mock-transfected cells. We observed that the cells with high AU1 expression, indicating the greatest expression of CD95, were the most sensitive cells. They underwent apoptosis in response to as low as 5 ng/ml of anti-CD95 stimulation. By evaluating the dose-response, we found that cells expressing modestly increased CD95 responded well to 10-20ng/ml of anti-CD95 stimulation whereas mock-transfected patient cells were resistant to much higher-doses anti-CD95 stimulation (fig. 5). Thus, the level of membrane CD95 expression is a crucial determinant of the sensitivity of lymphocytes to CD95-induced apoptosis.

Next we incubated Jurkat cells in cell-culture supernatant from patient EBV-B cells or serum from the family members carrying the mutation and added agonistic antibody or CD95L. The concentrations of sCD95 in these fluids are given in fig.4. We also incubated PKH26 labeled Jurkat cells with patient EBV-B cells to test if sCD95 is effective in the microenvironment. We found no inhibition of apoptosis of Jurkat cells in any of these experiments (Supplemental Fig. II). Furthermore, we observed that overexpression of sCD95 in Jurkat cells by transfection had no protective effect whether the cells were exposed to high or to low levels of agonistic antibodies (Supplemental Fig. III).
The mouse thymoma BW cells have been successfully used to demonstrate dominant negative effects of mutations in the CD95 death domain \(^8\). Therefore, these cells were transfected with either wt-CD95 or CD95-del6 or both (and with empty plasmid as a control, fig.6). As shown in fig.6 C, the expression of wt-CD95 was necessary to render BW cells sensitive to apoptosis induced by specific anti-human CD95. However, the presence of human sCD95 did not interfere with the induction of apoptosis. Thus, in this co-expression model we found no inhibitory effect of sCD95.

To further explore the potential functions of sCD95 in apoptosis, we incubated Jurkat cells with different, at certain occasions extremely high, concentrations (10-1000ng/ml) of recombinant human (rh) sCD95, and induced apoptosis of the cultured cells by super CD95L (fig.7 A). We only observed modest inhibition of apoptosis at the highest concentration of rh sCD95, and yet the inhibition was easily reversible by a moderate increase in CD95L concentration. We obtained a similar result using activated peripheral T-cells from healthy donors (fig.7 B). In another experiment we induced apoptosis by effector cells expressing CD95L on their surface to evaluate if cell to cell contact was more sensitive to sCD95 than to apoptosis induced by soluble CD95L (fig.7 C, D). However, again only modest inhibition of apoptosis at the highest concentration of rh sCD95 was detected (fig.7 C). This minor inhibition is statistically significant if all experiments are combined (fig.7 A, B, C). Nevertheless, these data do not reveal a potent blocking effect for sCD95.

Finally, it remained possible that sCD95 is effective in inhibiting apoptosis in a cell to cell contact microenvironment if secreted by target cells. Therefore, we transduced Jurkat cells with an MFG-S-sFas(sCD95)-IRES-GFP retroviral vector (or MFG-S-GFP as a control) and checked the permanent and high sCD95 secretion one week later (fig.7 D, E). It was clearly higher than the sCD95 production by patient cells (fig.4). The sCD95 secreting Jurkat target cells were identified by GFP and incubated with CD95L expressing effector cells or control cells not expressing CD95L. We found no difference in the loss of GFP positive target cells whether or not these cells secreted sCD95 (fig.7 D).
Discussion

The most frequent form of ALPS is type Ia, which is caused by a variety of different mutations in the CD95 gene. In the majority of cases, the mutations occur in the region encoding the intracellular death domain, and the mutant proteins exert a dominant negative effect on CD95 signals. This can be explained as follows: Transmembrane wild-type CD95 associates with the mutated form (2:1 or 1:2) via the pre-ligand assembly domain to form trimers (or bigger complexes) that are incapable of appropriately recruiting the FADD/MORT1 adaptor protein upon CD95L occupancy of the extracellular domain of CD95. Thus, the majority of the receptor trimers are defective in intracellular signaling, even if 50% of the total CD95 protein in the cell is normal. In contrast, type Ia ALPS mostly caused by extracellular mutations in CD95, that have no dominant negative effect, is a result of haploinsufficiency of CD95 expression. In this case, the disease phenotype is inherited in a dominant mode with low penetrance.

We found that the lymphocytes from our patient were resistant to in vitro CD95-induced apoptosis (fig. 2) and that their level of cellular CD95 expression was decreased (fig. 4) whereas their secretion of sCD95 was elevated. However, all other family members that carry the same splice mutation seemed to be healthy albeit their high serum levels of sCD95 (fig. 4). We examined if the functional defect for CD95-induced apoptosis was caused by decreased CD95-expression, by excessive sCD95 production, or both.

Our data show that a moderate enhancement of wt-CD95 expression in patient cells by transfection could easily restore their sensitivity to CD95-induced apoptosis even in the presence of excessive sCD95 production (fig.5). However, attempts to antagonize the sCD95 by an excess of antibody did not result in any significant change in sensitivity to apoptosis (fig. 2B). The incubation of Jurkat cells in cultures containing sCD95 from the mutant-affected family members (serum and cell supernatant) did not inhibit apoptosis, even when induced by very low amounts of CD95L. In addition, expression of sCD95 in Jurkat cells by transfection failed to reduce their sensitivity to CD95-induced
apoptosis. Thus, our data suggests that sCD95 is not potent in neutralizing CD95L for apoptosis induction.

Results using BW cells 8,22 (fig.6), rh-sCD95, and target cells expressing sCD95 (fig.7) further strengthen our conclusion that the soluble receptor is not a very potent regulator of cell death under any condition tested. We therefore conclude that the regulatory and pathogenic function of sCD95 may have been overestimated 32,34,40-43.

Our results strongly suggest that the transient ALPS of the patient in this family is caused by haploinsufficiency of membrane CD95, and that the increased sCD95 is of minor importance, if it has any effect at all, in causing the cellular resistance to CD95-induced apoptosis. The assumption of haploinsufficiency is supported by the observation of the decreased expression of wt-CD95 in patient cells; the reconstitution of normal CD95 mediated apoptosis by transfection of wt-CD95; the lack of significant interference on the wt-CD95 from the mutated allele; and the low penetrance in the family. As mentioned above, this low penetrance has previously been observed in other ALPS families with haploinsufficiency 7,14,22,39. In accordance with these reports, we propose that a sufficient expression of full-length CD95 protein on lymphocyte membrane is crucial for keeping normal apoptosis responses to CD95 stimulation.

An important role for sCD95 has been hypothesized in malignant and autoimmune diseases 32,34,40-43. However, in these reports, high concentrations were often used in the analysis of in vitro and in vivo functions of sCD95, and we confirm some effect of such high concentrations (fig.7). The impact of physiological concentrations as measured by other investigators and us remains unclear. The biochemical mechanism by which sCD95 could antagonize the ability of CD95L in triggering membrane-CD95 receptor has not been established. Efficient ligand (CD95L) binding requires oligomerization of the receptor (sCD95) and it has not been shown that such proper oligomerization or conformation of the shed receptor is achieved.

Even though the general role of sCD95 remains inconclusive at the moment, our data do not support the hypothesis of its great importance in the pathogenesis of autoimmune diseases, and alternative assumptions should be discussed. Sensitizing leukocytes for CD95-apoptosis includes, among other regulatory steps, upregulation of CD95 transcription and regulation of pre-mRNA splicing. The physiological role of this
splice regulation may simply be to ensure that there is no premature sensitivity to CD95 mediated apoptosis. High levels of membrane CD95 at an early phase of activation can be prevented without necessarily interfering the CD95 function, and this can be achieved by just excluding the exon 6 and thereby membrane integration domain from the CD95 molecule. It is also likely that CD95 transcription and splicing are regulated by different signals. If this is the case, expression high levels of CD95 will depend on the simultaneous presence of more than one proapoptotic signal.

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SUPPLEMENTAL MATERIAL IS AVAILABLE ONLINE AT THE TIME OF FINAL PUBLICATION ONLY.
Figure legends

Fig. 1. Positions of primers and probes in the CD95 genomic and cDNA, and overview of important sequences in the ALPS patient. A: cDNA including exon-6. It has an AG insert in a few strands that is linked to a C in the polymorphic pos. 74 (pos. 74 downstream of the 5’ end of exon-7, same as pos. 836 bp downstream of the first translated ATG). Most normally spliced cDNA (including exon-6, but not the extra AG) has a T in pos. 74. Primers CF1, CR1 were used for cloning, CF2, CR2 for RT-PCR and TF1, P1,TR1 for TaqMan PCR to quantify the normally spliced form. B: Splice forms that lack exon-6 and hence the transmembrane domain. They mostly have a C in pos. 74. These forms were quantified by TaqMan PCR using TF2, P2, TR2. C: Genomic sequence with a mutation C->G in pos. –3 upstream of exon 6 on one allele and with the C/T polymorphism in exon 7. The primers used for genomic sequencing (GF1, GF2, GR1, GR2), the TIA-1 binding site and the transmembrane domain are marked. D: Pedigree and SNP haplotyping. Analysis of intronic SNPs upstream of exon-5 (a,b) and downstream of exon-7 (c), the C/T SNP expressed in exon-7 (d) 836 bp downstream of the first translated ATG (see pos. 74 A, B, C, also used in tab. 2), and the mutation ex-6 – 3C->G (c) that is associated with excessive sFas production, decreased mFas expression, and apoptosis (figs. 2 and 4).

Fig. 2. Apoptosis of lymphocytes from the patient and the other family members. A: PBMCs were activated for 5 days (2 days PHA, 3 days IL2) and incubated in the absence and presence of agonistic mAb CH-11 (2.5 µg/ml) for another 24 hrs. Ordinate: % PI and annexin positive cells; induced minus spontaneous apoptosis is given. B: Proliferating EBV-transformed B-cells from the patient were incubated with mAb CH-11 at different concentrations. Five experiments with 2.5 and 25 µg/ml mAb CH-11 each. Circles: Cells from a healthy donor, diamonds: cells from the patient. Induced minus spontaneous apoptosis is given. Ordinate as in A.

Fig. 3. RT-PCR products of CD95 and CD95-del6 mRNA. A: RT-PCR before (-) and after (+) activation (5 days as in fig. 2) of PBMCs (T-cells) and of EBV transformed B-
cells (B-cells, n=5). P, patient; C, healthy donor; primers: CF2, CR2. B: Real time TaqMan RT-PCR of the splice product containing exon-6 (closed line) and of the alternatively spliced mRNA without exon-6 (dashed line), both from PBMCs. Primers and probes: TF1, P1, TR1; TF2, P2, TR2; abscissa: days of activation; ordinate: reciprocal cycle threshold. The same experiment was performed with EBV transformed B-cells (same result as on day 5, n=3, not shown).

Fig. 4. Secretion and serum levels of sCD95, and CD95 expression. Cell supernatant from proliferating EBV-transformed B-cells (A, n=5) and from PBMC (B, n=2) that were activated with PHA (2 days) and IL2 for 3 days (5 days of total incubation), was collected to determine sCD95 secretion; p: patient, c: healthy donor. C, CD95 expression by PBMCs after five days of activation, representative result, see also tab.3. Right two curves: m, mother and c, healthy control donor; curves in the middle: d, the four affected family members; left curves: second antibody alone in three affected family members and c. The same result was seen in EBV-transformed B-cells (n=5), data not shown. D, Serum levels of sCD95 were measured in the patient, the other family members affected by the mutation (father, sister 1 and 2), in the healthy mother (n = 2 each), and in six healthy control donors.

Fig. 5. Functional correction of EBV-transformed B-cells from the ALPS patient by transfection (Amaxa system) with 20 µg wt-CD95 AU1-tagged plasmid (dark line). As negative control, patient cells were transfected with empty plasmid (gray line). The region M1 is set to contain 0.2% of negative control cells. Apoptosis was induced by incubation with the agonistic mAb Apo1 for 20 hrs. AU1-staining is shown; one representative of two transfection experiments is given.

Fig. 6. Production of human sCD95 did not interfere with anti-CD95 induced apoptosis in mouse BW cells. A: Expression of human CD95 (Fas) after transfection of mouse BW cells with 20µg of plasmids as indicated. B: Secretion of human sCD95 (sFas) into the cell supernatant by BW cells 2 days after transfection. C: Loss of transfected BW mouse cells by mAb Apo1 (200ng/ml, gray columns; 500ng/ml, dark
columns) and Protein A or by Protein A alone (white columns; the average of 2 experiments is given).

**Fig. 7. Insufficient inhibition of super CD95L-induced apoptosis by rh-sCD95.** A, Jurkat cells and B, normal activated peripheral T-cells were incubated with rh-sCD95 (rh-sFas). Abscissa: Concentration of rh-sCD95 (rh-sFas), ng/ml; ordinate: % cell loss; diamonds 1 ng/ml, squares 5 ng/ml, circles 25 ng/ml super CD95L (FasL) added to cell medium; n = 2; incubation time 36 hr. C, effect of rh-sCD95 (rh-sFas) on apoptosis of Jurkat target cells induced by CD95L (FasL) expressing effector cells; effector to target ratio: 3:1; abscissa and ordinate same as in A and B; n = 6; incubation time 22 hr; Note: compared with the respective values at 0, 10 or 100 ng/ml the moderate inhibition of apoptosis by 1000 ng/ml rh-sCD95 (rh-sFas) is significant if all experiments (A, B, C) are included into the calculation (in each series cell loss is lowest when 1000 ng/ml rh-sCD95 (rh-sFas) were added), p<0.016, Sign-test. D, Apoptosis of transduced, permanently sCD95 (sFas) secreting Jurkat target cells after incubation with CD95L (FasL) expressing effector cells; effector to target ratio: 3:1; ordinate same as in A; incubation time 22 hr, n = 6; vectors used: MFG-S-GFP, left column; MFG-S-sFas(sCD95)-IRES-GFP, right column; cells used one week after transduction; E, respective amounts of CD95 (sFas) produced by the transduced cells in 48 hr.

**Tab. 1. Sequences of primers and probes used.** Positions are marked in fig. 1. Approx. 40 more primers were used for complete genomic sequencing (not shown).

**Tab. 2. Phenotype of the ex-6 –3C->G mutation.** a Mean channel fluorescence, arbitrary units; membrane-CD95, n = 8 measurement on affected family members; b n = 8; c annexin and PI positive activated peripheral T-cells in percent (induced minus spontaneous apoptosis is given), see materials and methods; p< 0.01 for a,b,c, signed rank test.
Tab. 3. Different expression of the paternal and maternal CD95 allele of the patient in activated PBMCs. The number and percentage of CD95 cDNA clones with the respective sequence are given.
Reference List


Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Fig. 7
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Tab.1
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<td>clinical phenotype</td>
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Tab.2

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Tab.3
Haploinsufficiency rather than the effect of an excessive production of soluble CD95 (CD95 δTM) is the basis for ALPS Ia in a family with duplicated 3’ splice site AG in CD95 intron 5 on one allele

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