Increased spontaneous *in vitro* apoptosis in double negative T cells of humans with a *fas/apo-1* mutation

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Abstract

We describe a 17 year old patient suffering from Canale-Smith syndrome (CSS) including chronic lymphadenopathy, splenomegaly, hypergammaglobulinemia and recurrent Coombs positive hemolytic crises. The parents are not consanguine, all other family members including two brothers are healthy. Peripheral blood mononuclear cells of the patient showed an increased rate of CD3 positive, CD4/CD8 double negative T-lymphocytes. *In vitro* assays showed these cells to have an increased rate of spontaneous apoptosis. Though expression of Fas/Apo-1 (CD95) and Fas-ligand (FasL) was detected on RNA- and protein level we found Fas/Apo-1 mediated apoptosis being significantly reduced. Sequencing of the *fas/apo-1* gene proved the patient RT and his father to carry a point mutation at position 804 located in exon 9 (death domain) leading to an amino acid substitution. For developing of CSS, a *fas/apo-1* mutation seems to be necessary but not sufficient. An additional independent mechanism must be involved in the pathogenesis of human *lpr*-phenotype.

Keywords: Canale-Smith syndrome; autoimmune lymphoproliferative syndrome; spontaneous *in vitro* apoptosis; *fas/apo-1* mutation

Abbreviations: ALPS, Autoimmune lymphoproliferative syndrome; CSS, Canale-Smith syndrome; DN, double negative; mAB, monoclonal antibody; FasL, Fas ligand; NHD, normal healthy donor(s); PBMC, peripheral blood mononuclear cells

Introduction

Apoptosis is known to play a role in a variety of physiological processes, e.g. in embryonic development and regulation of

the immune system. It can be programmed or triggered by different stimuli. Apoptotic cell death is mediated through the interaction of the Fas/Apo-1 receptor and its natural ligand (FasL) or through a variety of other mechanisms. This system is thought to be involved in the selection of autoreactive lymphocytes in the thymus and the periphery and in the elimination of T cells at the end of an immune response (Webb *et al*, 1990). The *lpr* and *gld* mouse strains, which develop lymphoproliferative disorders, have been found to carry mutations in the genes for *fas/apo-1* or *fasL*, respectively (Watanabe-Fukunaga *et al*, 1992; Takahashi *et al*, 1994). In carriers of these mutations, Fas-FasL interactions do not lead to apoptotic cell death.

In humans, a benign form of autoimmune lymphoproliferative syndrome (ALPS) in childhood was first described in 1967 and termed Canale-Smith syndrome (CSS). It is characterised by chronic lymphadenopathy, hepatosplenomegaly and autoimmune hemolytic anemia (Canale and Smith, 1967; Kellerer and Mutz, 1976; Malzberg *et al*, 1991). Moreover, CSS patients were found to have an increased DN T cell count (CD3⁺CD4[−]CD8[−]) in their PBMC (Sneller *et al*, 1992). Analysis of *fas/apo-1* and *fasL* genes revealed that CSS patients carry mutations in the *fas/apo-1* gene (Fisher *et al*, 1995; Rieux-Laucat *et al*, 1995; Drappa *et al*, 1996; Bettinardi *et al*, 1997; Sneller *et al*, 1997). Thus, CSS seems to be the human equivalent of the murine *lpr* phenotype. Table 1 summarises data on CSS patients known so far.

The case of CSS reported here is a 17-year-old boy who presented with the clinical picture of a severe general infection accompanied by pancytopenia. Despite thorough evaluation no infectious agent could be found. Bone marrow aspiration showed a dramatically reduced cell count without signs of malignancy. After recovery chronic lymphadenopathy, splenomegaly, autoimmune hemolytic anemia and hypergammaglobulinemia persisted. Further evaluation of the patient's history showed these symptoms to persist at least since his fourth year of life. DN T cells constantly exceeded 20%. Repeated bone marrow aspirations and lymph node biopsies showed hyperplasia of lymph node pulpa but no signs of malignancy. To confirm the diagnosis of CSS we sequenced the genes for *fas/apo-1* as well as *fasL* and found a point mutation in *fas/apo-1* exon 9 encoding the death domain of Fas/Apo-1. Moreover, functional tests revealed a defect in the Fas/Apo-1 mediated apoptosis pathway.

Results

Family analysis of CSS patient RT

History as well as physical examinations revealed patient RT being the only family member suffering from symptoms of CSS, particularly chronic lymphadenopathy, splenome-

galy and Coombs positive autoimmune hemolytic anaemia (Figure 1). In addition, episodes of pancytopenia observed

twice in patient RT have not occurred in any other family member.

Table 1 Clinical picture of published CSS (Canale and Smith, 1967; Kellerer and Mutz, 1976; Malzberg *et al*, 1991; Sneller *et al*, 1992, 1997; Fisher *et al*, 1995; Rieux-Laucat *et al*, 1995; Drappa *et al*, 1996; Bettinardi *et al*, 1997) cases compared with patient RT and his father. Numbers from the literature on each clinical symptom: The first represents the number of cases positive for a symptom, the second the number of cases on which information was available

	Literature n=26	Patient RT	Father of patient RT
Family history positive			
a) phenotype	1/26	—	—
b) genotype	7/20	+	+
Age of onset	1.5 mo.- 5 years	3 years	—
Lymphadenopathy	26/26	++	—
Hepatomegaly	18/23	+	—
Splenomegaly	26/26	+++	—
Splenectomy	5/9	hemi- splenect.	—
Fever	—	—	—
Hypergammaglobulinemia	17/20	++	—
Hemolytic anemia (Coombs pos.)	18/25	+	—
Thrombopenia	12/23	+	—
Neutropenia	10/23	+	—
Biopsies: lymph node, bone marrows	17/17	+	—
Occurrence of malignoma	2/20	—	—
<i>fas/apo-1</i> mutation sequenced	18	+	+
Increased DN peripheral T cell count	19/19	+	+
Decreased Fas/Apo-1 mediated apoptosis	12/12	+	+

Analysis of apoptosis

Figure 2 summarises the results of DNA content analyses determined by PI staining of nuclei. Spontaneous *in vitro* apoptosis in PBMC cultured for 24 h was markedly increased in patient RT ($24 \pm 7\%$) as compared to NHD ($3 \pm 2\%$, $P < 0.05$). The overall apoptotic cell count further increased at day 2–3 ($38 \pm 6\%$ in patient RT *versus* $18 \pm 11\%$ in NHD). Supplementation of R^{10} with IL-2 decreased the rate of apoptosis in PBMC from NHD ($7 \pm 5\%$), but not in cells from patient RT ($34 \pm 11\%$) (Figure 2a). In order to look for other potential 'survival factors' inhibiting the increased rate of apoptosis in cultured PBMC from patient RT the following growth factors, cytokines as well as cytokine antagonists and mitogens have been employed: autologous serum (10% with RPMI 1640), R^{20} , TGF β , IL-1 β , IL-3, IL-4, IL-5, IL-6, GM-CSF, M-CSF, IFN γ , TNF α , anti TNF α , IL-2, G-CSF, IGF1, Epo, IFN α , insulin, PHA and PMA plus CD28 mAb. However, no reagent significantly reduced the increased rate of spontaneous *in vitro* apoptosis of cultured PBMC from patient RT (data not shown).

In OKT-3 stimulated PBMC from NHD signalling through Fas/Apo-1 receptor with Fas mAb significantly enhanced *in vitro* apoptosis (38% without *versus* 63% with addition of Fas mAb). No such increase was observed in PBMC from patient RT (49 *versus* 53%). NHD derived PHA blasts showed a rapid induction of apoptosis after withdrawal of growth factors. These cells may be rescued by further supplementation with IL-2 or other γ_c chain cytokines like IL-4, IL-7 or IL-15, but

Figure 1 Pedigree tree of patient RT and status of the *fas/apo-1* gene. Rectangles represent male, circles female relatives. Individuals 1, 3, 5, 7 and 8 are healthy homozygous carriers of wild type *fas/apo-1*. Individuals four (father, hatched) and nine (patient RT, black) are heterozygous for the *fas/apo-1* mutation (see Table 2), with the father being healthy. Individuals two and six (grey) were not available for analyses

Figure 2 (a) Spontaneous apoptotic cell counts in cultured PBMC. Cells from NHD and patient RT were incubated for 3 days with and without IL-2. At day 0 and day 3 apoptosis was quantified by cytofluorometry of propidium iodide stained, Triton-X 100 lysed cells. NHD, normal healthy donor (healthy homozygous carrier of wild-type Fas/Apo-1); patient RT, patient with Canale-Smith Syndrome (heterozygous carrier of a Fas/Apo-1 mutation). (b) Apoptotic cell counts in PHA blasts. PHA blasts were cultured in the presence of IL-2. Apoptosis was induced by either soluble Fas ligand (sFasL) or by the monoclonal anti-Fas antibody (Fas-mAb, clone CH11). After 24 h apoptosis was quantified by cytofluorometry of propidium iodide stained, Triton-X 100 lysed cells. NHD, normal healthy donor (healthy homozygous carrier of wild-type Fas/Apo-1); patient RT, patient with Canale-Smith Syndrome (heterozygous carrier of a Fas/Apo-1 mutation)

remain susceptible for induction of apoptosis *via* engagement of Fas/Apo-1. In NHD PHA blasts withdrawal of growth factors like suboptimal concentrations of IL-2 led to an apoptotic cell count of 18% after 24 h. Supplementation with IL-2 (1 U/ml) slightly reduced this rate to 14%. In contrast, addition of Fas mAb or sFasL in the presence of IL-2 resulted in an increased apoptotic cell count of 32% (Fas mAb) and 21% (sFasL) respectively (see Figure 2b).

After growth factor withdrawal PHA blasts of patient RT showed an apoptotic cell count of 14% which was reduced to 8% by supplementation with IL-2. Importantly, neither the addition of Fas mAb (9%) nor of sFasL (8%) led to an increase in the apoptotic cell counts (Figure 2b). Analysis of T cell subsets in freshly isolated PBMC by cytofluorometry showed an increased DN T cell count in patient RT as compared to NHD, concordant to the diagnosis of CSS.

Moreover, after 24 h *in vitro* culture surface receptor staining revealed the presence of DN T cells mainly in the

apoptotic cell population (Figure 3a). In addition, as shown in Figure 3b, cultured PBMC of patient RT were found to have increased spontaneous apoptotic cell counts in the subsets of CD4⁺/CD8⁻ and CD4⁻/CD8⁺ T cells as well even though this was less pronounced when compared with DN T cells. Similar results were observed analyzing DN T cells in cultured PBMC of the father of patient RT. As shown in Figure 3c, when compared with cells from NHD and from the patient RT's mother, these DN T cells showed increased spontaneous apoptotic cell counts on days 1–3. However, spontaneous *in vitro* apoptosis in cells from patient RT was still more pronounced when compared to the cells of his father.

Analyses of transcripts and protein expression

Semiquantification of mRNA levels showed an increase in *fas/apo-1* expression in PBMC of patient RT as compared to cells of NHD, whereas mRNA levels for *bcl-2*, *bcl-x* and *fasL* were equal (data not shown). An average of 40% ($\pm 9\%$) of CD3⁺ cells from 10 NHD expressed Fas/Apo-1 as indicated by immune fluorescence (data not shown). While his father showed a Fas/Apo-1 expression practically within the normal range (53%), patient RT (68% $\pm 9\%$, analyzed at four different time points) and his healthy mother (63%) both had values exceeding $\bar{x} + 2SD$ (see Figure 4). No significant differences could be observed in the quantities of intracellular anti-apoptotic Bcl-2 protein expression between patient RT, his parents and NHD (data not shown).

Sequencing of *fas/apo-1*

Table 2 shows *fas/apo-1* exon 9 sequence in part. Patient RT and his father were shown to carry a point mutation at position 804 resulting in an A 804 C exchange when compared with the published sequences (Behrmann *et al*, 1994; Rudert *et al*, 1995). This mutation causes a single amino acid exchange at position 268 of the Fas/Apo-1 molecule (Q 268 P). It is located within the death domain of the molecule which is important for signal transduction to the FADD molecules (Itoh *et al*, 1991). The mutation was absent in any other relative as well as in all NHD investigated (see Table 2). Sequencing data could be confirmed by restriction enzyme digestion with BsmF1. It proves patient RT and his father being heterozygous for the point mutation identified by sequencing (see Figure 5).

Discussion

Here we report the case of a CSS/ALPS patient showing typical clinical findings of this rare disease like chronic lymphadenopathy, splenomegaly and autoimmune hemolytic anaemia. Cellular analysis revealed an increased count of DN T cells in PBMC and a mutation located in exon 9 ('death domain') of the *fas/apo-1* gene. Functional tests using Fas mAb as well as sFasL showed a defect in Fas/Apo-1 mediated apoptosis despite a significantly increased expression of the Fas/Apo-1 surface molecule on the T cells of the patient. Although several healthy carriers of *fas/apo-1* mutations have been reported in the literature (Sneller *et al*, 1992, 1997; Fisher *et al*, 1995; Rieux-Laucat *et al*, 1995; Drappa *et al*, 1996;

Bettinardi *et al*, 1997) there is no published data on spontaneous *in vitro* apoptosis so far. Spontaneous *in vitro* apoptosis of the patient's PBMC was markedly increased in comparison to NHD. This increase mainly affects DN T cells, but was also, though to a far lesser extent, observed in the

single positive T cell subsets. In contrast to NHD, apoptosis in the patient's cells could not be prevented by addition of IL-2 or other cytokines and growth factors like TGF β , IL-1 β , IL-3, IL-4, IL-5, IL-6, GM-CSF, M-CSF, IFN γ , TNF α , anti TNF α , IL-2, G-CSF, IGF1, Epo, IFN α and insulin. Our data are in part

Figure 3 (a) CD3, CD4, and CD8 surface expression of freshly isolated and cultured T cells. Cells were stained with monoclonal antibodies against CD3 (PE conjugated), CD4 and CD8 (FITC conjugated). Apoptotic lymphocytes were discriminated from viable lymphocytes by gating in the FS/SS log histogram. Single positive T cells (CD3⁺ CD4⁺ CD8⁻ or CD3⁺ CD4⁻ CD8⁺) are displayed in grey, double negative T cells (CD3⁺ CD4⁻ CD8⁻) are displayed in black, and CD3⁻ lymphocytes are displayed in white. Single positive T cells CD3⁺ CD4⁺ or CD3⁺ CD8⁺ were identified in a parallel experiment (data not shown) and are indicated in boxes in the PE/FITC histograms (upper and lower panel left). As shown in the FS/SS log histogram (lower panel right) most of the double negative T cells from patient RT are apoptotic (black dots) after 24 h of culture. NHD, normal healthy donor, CSS, patient RT (Canale-Smith syndrome). (b) Apoptosis of freshly isolated and cultured single positive and double negative T cells. Cells were stained with monoclonal antibodies against CD3 (PE conjugated), CD4 and CD8 (FITC conjugated) and analyzed by cytofluorimetry. Apoptotic lymphocytes were discriminated from viable lymphocytes by gating in the FS/SS log histogram. NHD, normal healthy donor, patient RT, patient with Canale-Smith syndrome. (c) Apoptosis of freshly isolated and cultured double negative T cells. Cells were stained with monoclonal antibodies against CD3 (PE conjugated), CD4 and CD8 (FITC conjugated) and analyzed by cytofluorimetry. Apoptotic lymphocytes were discriminated from viable lymphocytes by gating in the FS/SS log histogram. NHD 1/2, normal healthy donor 1/2 (healthy homozygous carrier of wild-type Fas/Apo-1); patient RT, patient with Canale-Smith Syndrome (heterozygous carrier of a Fas/Apo-1 mutation); father, father of patient RT (healthy heterozygous carrier of a Fas/Apo-1 mutation); mother, mother of patient RT (healthy homozygous carrier of wild-type Fas/Apo-1)

Figure 4 Fas/Apo-1 surface expression of T cells. Freshly isolated PBMC were double-stained with monoclonal anti-CD3 (PE conjugated) and anti-Fas/Apo-1 (FITC conjugated) monoclonal antibodies. The percentage of Fas/Apo-1 positive T cells is indicated in each histogram. NHD, normal healthy donor (healthy homozygous carrier of wild-type Fas/Apo-1); patient RT, patient with Canale-Smith Syndrome (heterozygous carrier of a Fas/Apo-1 mutation); mother, mother of patient RT (healthy homozygous carrier of wild-type Fas/Apo-1)

Table 2 Sequence comparison of *fas/apo-1* exon 9

mRNA	792				804				816
<i>fas/apo-1</i> wild-type	AAU	GAC	AAU	GUC	CAA	GAC	ACA	GCA	GAA
Patient RT and father	***	***	***	***	***	***	***	***	***
	***	***	***	***	***	***	***	***	***
Fas/Apo-1 wild-type	Asn	Asp	Asn	Val	Gln	Asp	Thr	Ala	Glu
Patient RT and father	*	*	*	*	Pro	*	*	*	*
Protein	264				268				272

Sequences of the *fas/apo-1* mRNA were analyzed in Patient RT, healthy family members and normal healthy donor (NHD). Patient RT and his father are heterozygous for a point mutation at position 804 (A>C), which leads to an amino acid exchange at position 268 (Q>P) located in the death domain. The other family members and NHD show the wild-type sequence as published (Chomczynski and Sacchi, 1987).

consistent with the observations made in *lpr/lpr* mice. These animals also have a functional Fas/Apo-1 deficiency and a high proportion of DN T cells. Spontaneous *in vitro* apoptosis was found to be accelerated, too, and could not be inhibited by IL-2, IL-1, TGF β and TNF α (Van Houten and Budd, 1992). In contrast, stimulation of Protein Kinase C by PMA reverted the increased *in vitro* apoptosis in the animals, but not in the CSS patient.

The father of patient RT was also shown to be heterozygous for the *fas/apo-1* mutation. DN T cell counts as well as spontaneous *in vitro* apoptosis were increased as well. Despite these results he never showed any clinical findings of CSS/ALPS. Moreover, Fas/Apo-1 expression was found to be normal although in the upper normal range.

The mother of patient RT was clinically healthy, had functional *fas/apo-1* genes and a spontaneous *in vitro* apoptosis comparable to that of normal healthy donors. The only abnormality was an increased expression of the Fas/Apo-1 surface molecule on T cells.

Our data indicate a Fas/Apo-1 independent unrevocable pathway of apoptosis being responsible for the markedly increased spontaneous *in vitro* apoptosis of DN T cells from patient RT. This pathway may also be involved in the moderately accelerated *in vitro* apoptosis of CD4 and CD8 single positive T cells. The exact molecular mechanisms for these findings have to be defined by further experiments

addressing pathways to T cell apoptosis alternative to Fas/Apo-1. This mechanism may be active in cells lacking Fas/Apo-1 signals and thus may represent an emergency elimination pathway for T cells that have escaped Fas/Apo-1 mediated death control. The *in vivo* accumulation of atypical T cells in Fas/Apo-1 defective individuals indicates a higher potency of the Fas/Apo-1 mediated induction of apoptosis. Whether this increased spontaneous *in vitro* apoptosis seen in patient RT and his father is a general phenomenon of individuals heterozygous for a *fas/apo-1* mutation or with a defective Fas/Apo-1 apoptosis induction pathway remains unclear, because no data on spontaneous *in vitro* apoptosis in human Fas/Apo-1 deficiencies have been published so far. Moreover, the contribution of the mother of patient RT, or a putative environmental factor causing the development of ALPS/CSS remains to be determined. The increased expression of Fas/Apo-1 as the only peculiar finding shared by mother and son might be a candidate. Alternatively, as multiple transcriptional start sites have been found in the *fas/apo-1* promoter region, gene regulation may account to the pathogenesis of ALPS/CSS (Rudert *et al*, 1995). It seems reasonable that episodes of pancytopenia during infection as observed in our patient result from stimulation of *fas/apo-1* transcription as demonstrated for influenza infection by Wada and co-workers (Wada *et al*, 1995).

Figure 5 Restriction enzyme analysis of *fas/apo-1* exon 9 PCR products. Since the mutation (see Table 2) of *fas/apo-1* resulted in generation of a BsmFI site, which is absent in the wild-type gene, sensitivity of a *fas/apo-1* exon 9 PCR product for BsmFI reflects the mutated allele. Lane 1 shows the restriction pattern of patient RT (nine), lane 2 mother of patient RT (three), lane 3 father of patient RT (four), lane 4 grandfather of patient RT (one), lane 5 aunt of patient RT (5), lanes 6–7 brothers of patient RT (7–8). The numbers in brackets refer to the numbers in the pedigree tree (figure 1)

Table 3 Primer sequences and length of PCR products

Gene	Primer 5'-3'	Length /bp
GAPDH	GCAGGGGGGAGCCAAAGGG GGTACCTGCCAGCCCCAGCGTCAAAG	571
bcl-2	GGTGCCACCTGTGGTCCACCTG CCTCACTTGTGGCTCAGATAGG	459
β -Actin	ATGGATGATGATATCGGCGCG CTAGAAGCATTTCGGTGGACGATGGAGGGGCC	1126
<i>fas/apo-1</i> (expression)	TTATCGTCCAAAAGTGTTA TTCTGTTCTGCTGTGTCTTG	770
<i>fas/apo-1</i> (sequencing)	ACTTCGGAGGATTGCTCAAC GTTTAAGTCAAGACTCATTCCG	1067
<i>fas/apo-1</i> exon 9 (death domain)	GCATATACTCAGAACTGAATTTG GTTTAAGTCAAGACTCATTCCG	332
<i>fas</i> -ligand (expression)	AAAAAAGGAGCTGAGGAAAG GTGCCTGTAACAAGAATCATA	472
<i>fas</i> -ligand (sequencing)	GCCTGACTCACCAGCTGC TAATACTAAGAAACAATGTCCG	910

In conclusion we confirm published data that a Fas/Apo-1 misfunction is not sufficient for development of the clinical picture of CSS/ALPS in humans. In addition we present data of a high spontaneous *in vitro* apoptosis of CD4⁺CD8⁺ double negative T cells in humans with functional Fas deficiency.

Materials and Methods

Blood donors and isolation of PBMC

Peripheral blood was obtained from the CSS patient RT, four healthy relatives of first (parents, brothers), and two of second (grandfather and aunt) degree (see family tree in Figure 1) as well as from normal healthy volunteers (NHD). PBMC were isolated with Histoprep (BAG GmbH, Lich, Germany) using standard methods. Cells were maintained in R^{10/20} (RPMI 1640 supplemented with 10/20% heat inactivated foetal calf serum, L-glutamine, penicillin, and streptomycin (Gibco, Eggenstein, Germany) at 37°C in an atmosphere containing 5% CO₂.

Analysis of apoptosis

Spontaneous *in vitro* apoptosis was analysed in PBMC cultured without any stimulus or growth factor at 1×10^6 /ml in R¹⁰. Apoptosis inhibition studies included experiments, in which several growth factors and cytokines as well as cytokine antagonists and mitogens were added: autologous serum (10% with RPMI 1640), R²⁰, IGF1, Epo (10 U/ml each), IFN α (100 U/ml), insulin (4×10^{-5} U/ml), TGF β (1 ng/ml), IL-1 β , IL-3, IL-4, IL-5, IL-6, GM-CSF, M-CSF, IFN γ , TNF α (10 ng/ml each), anti TNF α (4 μ g/ml), G-CSF, IL-2 (1 U/ml each, Boehringer Mannheim, Germany), phytohemagglutinin (PHA, 1 μ g/ml, Sigma Chemical, Deisenhofen, Germany) and phorbol myristate acetate (PMA, 10 ng/ml) together with CD28 mAb.

To prepare cells for Fas/Apo-1 mediated apoptosis, PBMC were either stimulated via CD3 or PHA. PBMC stimulated with CD3 mAb (OKT-3, 1 μ g/ml, Ortho, Raritan, NJ, USA) were incubated for 7 days at 37°C. PHA blasts were generated by stimulation with 1 μ g/ml PHA and 10 μ g/ml IL-2 for 6 days followed by expansion with IL-2 for another two days.

Apoptosis was induced by addition of a monoclonal Fas antibody (Fas mAb, clone CH11, IgM 1 μ g/ml; Dianova-Immunotech, Marseilles, France), or soluble Fas ligand (sFasL, 100 ng/ml; Alexis Deutschland, Grünberg, Germany) added for 24 h.

For quantification of apoptosis, 4×10^5 cells were lysed in buffered hypotonic Triton-X 100 solution containing propidium iodide (PI) for DNA staining (PI-T: 0.1% sodium citrate and 0.1% Triton-X 100, 50 ng/ml PI (Sigma Chemical, Deisenhofen, Germany)). After 6 h incubation in the dark at 4°C, fluorescence was determined using a Coulter cytofluorometer (Coulter, Hialeah, USA) as described elsewhere (Nicoletti *et al*, 1991). Since T cells executing apoptotic cell death change size as well as shape apoptotic cells were discriminated in immunofluorescence assays in the forward scatter versus log side scatter histograms. This allowed us to measure expression of surface molecules of apoptotic and viable cells separately.

PCR

Genomic DNA was prepared from all family members using the GITC/2P method as described by Ciulla *et al* (1988) followed by selective PCR for *fas/apo-1* exon 9. In case of the patient direct sequencing was performed and the fragment was additionally cloned in *E. coli* using the pGEM-T vector system.

RT-PCR experiments were done on the basis of total RNA purified by the Trizol technique according to the manufacturer's instruction (Gibco, Eggenstein, Germany) (Chomczynski and Sacchi, 1987). cDNA synthesis was done using M-MuLV reverse transcriptase and random hexamer oligonucleotides (Stratagene, Heidelberg, Germany). PCR was performed in a final volume of 10 μ l with 120 μ M dNTPs each, 3 mM MgCl₂, 0.5 μ M each primer and 0.8 U Taq using a hot air rapid cycler (Idaho Technologies, Birmfeld, Switzerland). Primers were designed according to published sequences (Behrmann *et al*, 1994; Rudert *et al*, 1995). Primers used for analysis of transcription quantities of *fas/apo-1* and *fasL* and for sequencing purposes are shown in Table 3.

Sequence analysis of *fas/apo-1*

cDNA (complete coding region) and genomic DNA (exon 9) derived PCR products were sequenced directly. To check for heterozygosity of genomic DNA a 332 bp fragment of the Fas/Apo-1 death domain (exon 9) was additionally cloned in *E. coli* using the pGEM-T vector system (Promega, Heidelberg, Germany). Sequence reactions were performed with dye terminators, samples were loaded on a prewarmed 5% sequencing gel, run on an automated DNA sequencer type 373,

and analyzed using Prism Software (ABI, Weiterstadt, Germany). Since sequencing of the *fas/apo-1* mRNA revealed generation of a new restriction enzyme recognition site in the mutated allele cleaved by BsmFI (GGGAC(N)₁₀^ we confirmed our sequencing results by restriction enzyme digest (New England Biolabs Beverly MA, USA). Therefore exon 9 PCR products (330 bp) were digested with BsmFI according to the manufacturer's instruction and analyzed on 1% agarose gels. BsmFI only cuts the mutated gene (mutated: GGGAC wt: TGGAC) resulting in generation of two small fragments with 114 bp and 216 bp, respectively.

Cytofluorometry

PBMC were incubated in 96 well plates (1.2×10^5 cells/well) with 5 μ l of fluorescence conjugated antibodies to human cell surface receptors CD3-PE, CD4-FITC, and CD8-FITC (Immunotech, Marseilles, France). After 30 min on ice cells were fixed by Q-prep (Coulter, Hialeah, USA) and analysed using an EPICS XL cytofluorometer (Coulter, Hialeah, USA). All immunofluorescence data are presented as mean fluorescence intensities.

Statistical analysis

For statistical analyses nonparametric Mann-Whitney tests were performed with the InStat program 2.01.

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