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DEFECTIVE FUNCTION OF THE FAS APOPTOTIC PATHWAY IN TYPE 1 DIABETES MELLITUS CORRELATES WITH AGE AT ONSET

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The Fas death receptor triggers lymphocyte apoptosis through an *extrinsic* and an *intrinsic pathway* involving caspase-8 and -9 respectively. Inherited defects of Fas function are displayed by a proportion of patients with Type 1 diabetes mellitus (T1DM) especially those with a second autoimmunity (T1DM-p). This study assesses activation of both pathways in Fas-resistant (FasR) patients to localize the defect. 21/28 (75%) T1DM-p, 14/50 (38%) T1DM, and 7/150 (5%) controls were FasR. Analysis of the 35 FasR patients and 20 Fas-sensitive (FasS) controls showed that caspase-9 activity was lower in T1DM-p and T1DM than in controls, whereas caspase-8 activity was lower in T1DM-p than in T1DM and the controls. Single patient analysis showed that 16/35 patients displayed defective activity of one (FasR1), whereas 19 displayed normal activity of both caspases (FasR2) Ages at onset of diabetes mellitus in T1DM and the second autoimmune disease in T1DM-p were lower in FasR than in FasS patients. All FasR1 patients developed diabetes mellitus before the age of 9 years, whereas a later onset was displayed by 26% FasR2 and 53% FasS patients. These data show that defective Fas function may involve both the extrinsic and intrinsic pathway in T1DM and severity correlates with the precocity of the autoimmune attack and its tissue polyreactivity.

Fas is a death receptor whose triggering by FasL induces programmed cell death by activating a caspase cascade (1-5). In the immune response, Fas is highly effective in activated effector lymphocytes and is involved in switching off the immune response, limiting clonal expansion of lymphocytes and favoring peripheral tolerance. The caspase cascade is activated through two partly interconnected pathways. The *extrinsic pathway* involves caspase-8-mediated direct activation of the cascade, whereas the *intrinsic pathway* proceeds through mitochondrial release of cytochrome-*c* and activation of caspase-9. Both pathways converge in the activation of effector caspases, such as caspase-3, -6 and -7.

Key words: Fas, type 1 diabetes mellitus, caspase, apoptosis, T cell

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The system is under the control of several inhibitors belonging to the FLIP (Flice inhibitory protein), IAP (inhibitor of apoptosis), and bcl-2 families. In the autoimmune lymphoproliferative syndrome (ALPS), inherited deleterious mutations hitting Fas function cause heterogeneous autommunities and accumulation of polyclonal lymphocytes in the secondary lymphoid organs (6-7). The mutated gene is Fas in ALPS type-IA, FasL in ALPS type-IB, caspase-10 in ALPS type-IIA, and caspase-8 in ALPS type-IIB, whereas that in ALPS type-III is not known (3, 8). We have also shown that inherited defects of Fas function may also be involved in the development of common autoimmune diseases, i.e. multiple sclerosis (MS), type 1 diabetes mellitus (T1DM), chronic inflammatory demyelinating polyneuropathy (CIDP), and thyroid autoimmunities (TA) (9-14). Intriguingly, the Fas function defect seemed to correlate with aggressive diseases. In MS and CIDP, it was more frequent in patients with progressive course than in those with relapsing remitting course (11, 14). In Hashimoto thyroiditis (HT), it was more frequent in patients requiring early replacement therapy, which may be a sign of aggressive immunological attack, than in those not requiring such therapy; moreover, the highest defect was displayed by patients with the highest levels of autoantibodies (13).

In T1DM, the Fas function defect is displayed by about 20% of patients, compared with as many as 70% of those with a second concomitant autoimmune disease (polyreactive T1DM or T1DM-p) (12, 15-16). The observation that Fas function is also decreased in the healthy parents of Fas-resistant (FasR) patients suggests an inherited component similar to that causing ALPS. Since the Fas, FasL, and caspase-10 genes are not mutated in T1DM, the defect probably hits Fas function downstream from Fas (12).

We recently evaluated Fas-induced activation of caspase-8 and -9, used as markers of the extrinsic and intrinsic pathway respectively, in FasR patients with TA to locate the defect (13). In HT, Fas resistance correlated with decreased activity of both caspases and thus appeared to be due to an early defect in the Fas pathway, whereas in Graves' disease activation of both caspases was not defective, which suggested a late defect located downstream from both caspases. It may thus be supposed that the biochemical and the genetic defect are different in these diseases.

The aim of this study is to dissect the extrinsic and intrinsic pathways of Fas signalling in T cells from FasR patients with T1DM and T1DM-p to provide a clearer picture of the Fas function defect and determine its correlations with the course of the disease.

MATERIALS AND METHODS

Patients

Seventy-eight pediatric and young adult patients with T1DM (median age 12.9, range 4-32) were recruited from the "Maggiore della Carità" Hospital of Novara (n=21), the "Regina Margherita" Children Hospital of Turin (n=49), and the "Brotzu" Hospital of Cagliari (n=8). Fifty displayed T1DM. The other 28 displayed T1DM-p; 21 T1DM-p patients displayed HT, 4 celiac disease, and 3 MS. Clinical characteristics are summarized in Table I and II.

All patients with HT displayed serum anti-thyroglobulin or anti-thyroperoxidase antibodies (Ab), and ultrasonographic features of thyroiditis (Sostre grading 2-3) (17). Twelve were treated with L-thyroxine because of low FT4 levels (free thyroxine) (< 0.80 ng/dl; normal range 0.80-1.90) or high TSH levels (>8 UI/ml; normal range 0.4-4), whereas nine displayed normal FT4 and TSH levels.

All patients with celiac disease were on a gluten free diet.

Patients with Down or Turner's syndrome were excluded.

Age-matched controls were children free from autoimmune or allergic diseases observed in the general practitioner outpatient clinic. Their Fas function was similar to that displayed by adult healthy donors (11, 14).

Informed consent was obtained from all patients or their parents. The study was approved by the local ethical committee.

Functional analysis of Fas

Fas-induced cell death was evaluated as previously reported (9) on T cell lines obtained by activating peripheral blood mononuclear cells (PBMC) with PHA at days 0 (1 mg/l) and 15 (0.1 mg/l) and cultured in RPMI 1640 medium + 10% fetal calf serum (FCS) + IL-2 (2 U/ml) (Biogen, Geneva, Switzerland). Fas function was assessed 6 days after the second stimulation (21 days of culture). Cells were incubated with control medium or anti-Fas monoclonal antibody (MAb) (CH11, IgM isotype) (1 mg/l) (UBI, Lake Placid, NY) in the presence of IL-2 (1 U/ml) to minimize spontaneous cell death. Cell

Table I. *Ages at onset of diabetes and the second autoimmune disease in patients with or without defective Fas function.*

Patient group	Age at onset					
	T1DM			Second autoimmune disease		
	Fas function		P† value	Fas function		P value
	FasS‡	FasR§		FasS	FasR	
T1DM	8.7* (7-13) n=36	5 (3.3-6) n=14	<0.01	-	-	-
T1DM-p	9.3 (7.5-12) n=7	6.9 (2-9) n=21	ns	12.9 (11-15)	8.6 (6-12)	<0.05
HT-T1DM-p	10 (8-11) n=6	7 (5-8) n=15	ns	13 (10-13.8)	8 (7-9.4)	<0.01

**: results are expressed as median age at onset in years; interquartile ranges are shown in the brackets*
†: statistical analysis was performed with the Mann-Whitney test
‡: Fas-sensitive
§: Fas-resistant

Table II. *Demographic and clinical parameters of the patients with and without defective Fas function.*

Patients	Fas function	no.*	M/F†	Duration of T1DM‡	Age§	HbA1c‖	Second autoimmunity			Family history of autoimmune disease
							Thyroid	Celiac disease	Multiple sclerosis	
T1DM	FasR¶	14	8/6	6	11	7.7	-	-	-	5
	FasS¶	36	20/16	4	13.4	8.1	-	-	-	12
T1DM-p	FasR	21	4/17	4	15	7.8	15	4	2	8
	FasS	7	2/5	4.5	15	7.6	6	-	1	3

**: number of subjects; †: male/female ratio; ‡: duration of T1DM when analysed (median in years); §: age when analysed (median in years); ‖: percentage of glycated hemoglobin when analysed*
¶: Fas-sensitive (S) and Fas-resistant (R)

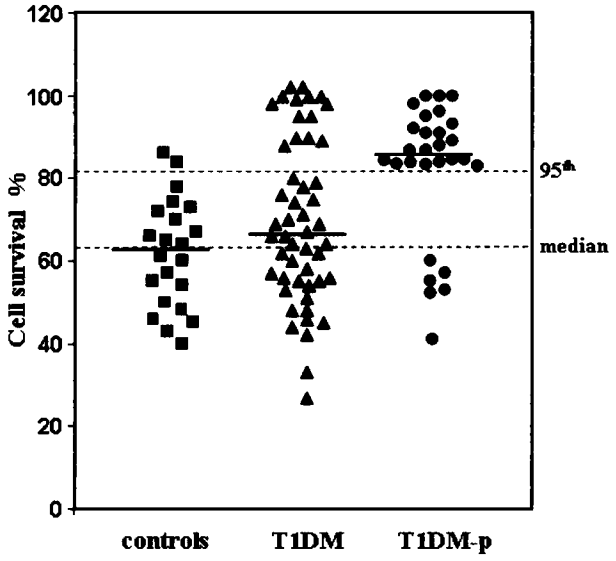

Fig. 1. *Fas-induced cell death in T cells from healthy controls, T1DM patients and T1DM-p patients. Activated T-cells were treated with anti-Fas MAb and survival was assessed after 18 hr. Results are expressed as specific cell survival %. The first lane shows the results from 22 age matched controls run in parallel with the patient samples; two or more were run in each experiment as positive controls. The highest dotted line indicates the upper limit of the normal range calculated as the 95th percentile of data obtained from 150 normal controls, the lowest dotted line their median. The full lines indicate the median value for each group. Statistical analysis using the Fisher's exact text: T1DM vs controls p<0.001, T1DM-p vs controls p<0.001, T1DM-p vs T1DM* p<0.001.

survival was evaluated after 18 hours by counting live cells in each well by the trypan blue exclusion test and by flow cytometry of cells excluding propidium iodide and unstained by annexin V-FITC (Becton Dickinson, San Josè, CA). The two methods gave overlapping results. Assays were performed in duplicate and analyzed by a blind observer. Cells from two normal donors were included in each experiment as positive controls. Results were expressed as specific cell survival %, calculated as follows: (total live cell count in the assay well/total live cell count in the control well) X100. Patients with a cell survival ≥82% (the 95th percentile of data obtained from 150 normal donors) were defined as FasR. Those with a survival <82% were defined as Fas-sensitive (FasS).

Caspase enzyme activity

Fas-induced activation of caspase-8, and -9 was evaluated on T cell lines obtained by activating PBMC from all FasR patients with PHA at days 0 (1 mg/l) and 8 (0.1 mg/l) and culturing cells with 10 U/ml IL-2 (13). Four days after the second stimulation (day 12), T cells were treated or not with the anti-Fas MAb (CH11) on ice for 30 min, then moved to 37°C for 3 h, centrifuged and lysed (MBL, Watertown, MA). Protein concentration was measured with the Biorad Protein Assay (Bio-Rad, Hercules, CA) and the same amounts were used to evaluate caspase activity by fluorimetry (MBL, Watertown, MA). Two or more control lysates from FasS normal donors were always run in parallel. Results were expressed as relative caspase activity (activity of Fas-stimulated cells/ activity of unstimulated cells).

Caspase expression was evaluated by SDS-PAGE and immunoblotting performed on 100 μg of cell lysates. Blots were blocked in TBS (10 mmol/l TrisHCl pH 7,9; 150 mmol/l NaCl) containing 5% non-fat milk and 0.1% Tween 20 and incubated with the anti-caspase-8 or -9 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and horse-radish-peroxidase-conjugated secondary antibodies, detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL). Bands were quantified with a Gel Doc EQ system (Bio-Rad)

Statistical analysis

Data were analysed with the non-parametric Mann-Whitney *U* test or Fisher's test, as reported. All p values are 2-tailed. Abnormal functions were those >95th percentile (Fas function) or <5th percentile (caspase activity) of data obtained from controls.

RESULTS

Fas-induced cell death was assessed in T cells from 50 patients with T1DM and 28 with T1DM-p to detect those displaying defective Fas function. It was found that 14/50 (38%) T1DM patients and 21/28 (75%) T1DM-p patients were FasR, and 36 T1DM patients (62%) and 7 T1DM-p (25%) patients were FasS (Fig. 1). The Fisher's test showed that the frequency of FasR subjects was significantly higher in both T1DM and T1DM-p than in 150 controls (7/150, 5%) (p<0.001), and in T1DM-p than in T1DM (p<0.001). A similar proportion of FasR subjects was detected in the HT subgroup (HT-T1DM-p) (15/21, 71%) and in those displaying a second non-thyroid autoimmune disease (6/7, 86%).

The Mann-Whitney test showed that age at onset of diabetes mellitus was significantly lower in FasR than in FasS patients in the T1DM group (p<0.01),

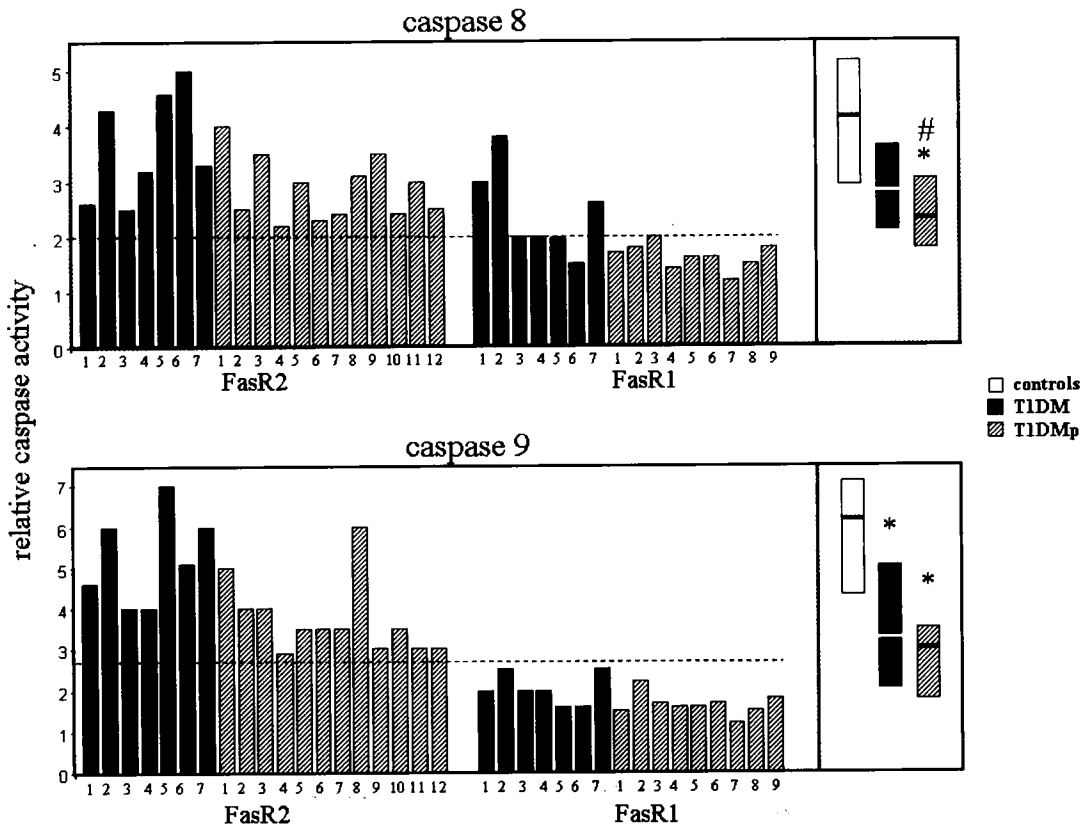

Fig. 2. *Fas-induced activation of caspase-8 and -9 in T cells from patients with T1DM or T1DM-p detected by fluorimetry. Results are expressed as relative caspase activity % (see Materials and Methods). The left panel shows results from individual patients, the right panel the overall data from each patient group: interquartile ranges (boxes) and medians (horizontal lines). Black bars: T1DM; striped bars: T1DM-p; white bars: FasS controls. FasR2 subjects are those with normal activation of both caspases, FasR1 those with defective activation of one or both caspases. The dotted horizontal lines indicate the 5th percentile of the activity displayed by normal controls. The symbols * and # mark comparisons that are significantly different from controls and T1DM respectively (Mann-Whitney test, see text for detailed p).*

but not in the T1DM-p group (Table I), though here the age at onset of the second autoimmune disease was significantly lower in FasR patients ($p<0.05$); this difference was also detected in the HT-T1DM-p subgroup ($p<0.01$) (Table I).

Duration of T1DM, age distribution, glycemic control, and family history for autoimmune diseases were not significantly different in any comparison (Table II). Females were prevalent in the T1DM-p group (M:F=6:22).

Function of the extrinsic and intrinsic Fas pathways was assessed by evaluating Fas-induced activation of caspase-8 and -9 in T cells from the 35 FasR patients (14 T1DM and 21 T1DM-p) and 20 age-matched FasS controls by fluorimetric enzyme assays. T1DM-p patients displayed significantly lower activation of both caspases than controls (caspase-8: $p<0.001$, caspase-9 $p<0.0001$, Mann-Whitney test), and lower activation of caspase-8 than T1DM patients ($p<0.05$, Mann-Whitney test) (Fig. 2). Moreover, T1DM patients showed lower activation of caspase-9, but not caspase-8, than controls ($p<0.01$, Mann-Whitney test). Single patient analysis showed that 16/35 (46%) of the FasR patients (7 T1DM and 9 T1DM-p) displayed a severe activation defect of one or both caspases (i.e activity <5th percentile of the controls) (pattern FasR1); in 13 (4 T1DM and 9 T1DM-p), activation of both caspases was defective, whereas in 3 (all T1DM) only activation of caspase-9 was defective. The remaining 19 patients displayed normal function of both caspases (pattern FasR2).

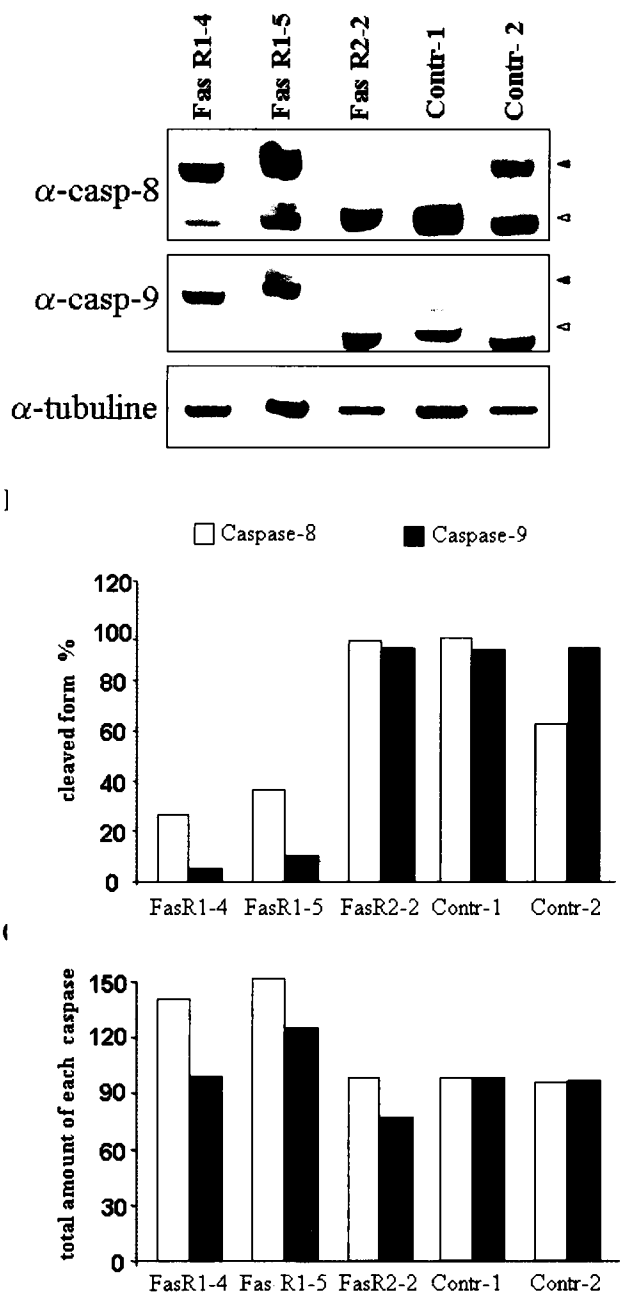

Fig. 3. *Western blot analysis of caspase-8 and -9 in T cells from 2 FasR1 and 1 FasR2 T1DM-p patients and 2 FasS controls. Cells were stimulated with anti-Fas MAb before lysis as in the caspase activity enzyme assay. Patients are patient 4 and 5 (FasR1) and patient 2 (FasR2) in Fig. 2. Panel (A): Western blots with antibodies to caspase-8, caspase-9, and tubulin. Black arrows indicate the uncleaved inactive caspases, white arrows the cleaved activated forms. Panel (B): Analysis of the proportion of the cleaved activated form of each caspase in relation to its total amount. Panel (C): Analysis of the total amount of each caspase (uncleaved plus cleaved forms). Data are relative to the mean values obtained in the controls (set as 100%) and are corrected on the amounts of tubulin for each subject.*

To determine whether these defects were due to decreased caspase expression, we performed a Western blot analysis of T cells treated as in the fluorimetric enzyme assays in 2 FasR1 and 1 FasR2 T1DM-p patients, and 2 FasS controls (Fig. 3A). Quantification of the activated (i.e. cleaved) and unactivated (i.e. uncleaved) forms of each caspase confirmed the Fas function patterns detected by fluorimetry in each subject (Fig. 3B). Moreover, quantification of the total amount of each caspase showed that the defects were not ascribable to defective expression since no substantial differences were found between FasR1, FasR2, and FasS subjects (Fig. 3C).

Since the maximum age at onset of diabetes mellitus in FasR1 patients was 9, we used this value as a cutoff to compare ages at onset of FasS, FasR2, and FasR1 patients. The results showed that in 16/16 (100%) FasR1, 14/19 (74%) FasR2, and 20/43 FasS (47%) patients the age at onset was less than 9 (FasR1 vs FasR2 $p<0.05$, FasR1 vs FasS $p<0.0001$, FasR2 vs FasS $p=0.053$, Fisher's test) (Fig. 4).

Lastly, we compared requirement of hormonal replacement therapy (L-thyroxin) in the 15 HT-T1DM-p patients. This was required by 6/6 (100%) FasR1 patients, but only 4/9 (44%) FasR2 patients and 1/6 (16%) FasS patients (FasR1 vs FasR2 $p<0.05$; FasR1 vs FasS $p<0.01$, Fisher's test). Age at onset, gender distribution, and duration of HT were similar in these subgroups (data not shown).

DISCUSSION

This work follows a previous report showing that a minority of T1DM and a majority of T1DM-p patients display defective Fas function, and suggesting that the defect has a genetic component (12). It confirms that finding in a larger group of subjects and assesses Fas function in terms of apoptotic response and triggering of caspase-8 and -9 activation. Moreover, our results detected three patterns of Fas function: FasS patients displayed normal apoptotic response and caspase activation,

FasR1 defective apoptotic response and caspase activation, and FasR2 defective apoptotic response but normal caspase activation.

The Fas function defect influences the disease course

In previous studies on MS, TA, and CIDP, we showed that Fas function was mainly decreased in patients displaying signs of aggressive disease (11-14). This work reports three lines of data showing that this is also true in patients with type1 diabetes mellitus. Firstly, FasR patients are significantly more frequent in the T1DM-p group. This suggests that defects of Fas function build up an autoimmune-prone background favoring development of multiple autoimmune reactions. Secondly, defective Fas function correlates with early age at onset. Ages at onset of diabetes mellitus in the T1DM group, and the second autoimmune disease in the T1DM-p group were lower in FasR patients. Moreover, an early onset was displayed by 100% FasR1, 74% FasR2, and 47% FasS. These findings are in line with those of a multicentre study, in which patients affected by T1DM and celiac disease (with or without HT) displayed lower age at onset of T1DM than those with diabetes alone (18). Thirdly, in the HT-T1DM-p subgroup, need for replacement therapy was progressively more frequent in FasS (16%), FasR2 (44%), and FasR1 (100%) subjects. Since HT duration was similar in all groups, it can be assumed that development of hypothyroidism is a sign of aggressive immunological attack of the gland that causes rapid and extensive tissue damage.

A further factor influencing T1DM-p development was gender, since females were significantly prevalent in this group. This is in line with the notion that females are predisposed to development of HT which is the most frequent second autoimmune disease in T1DM-p patients. However, previous works showed the gender did not influence Fas function (9-14). Moreover, HT *per se* cannot account for the striking frequency of Fas-Resistance of T1DM-p patients since patients with HT alone display a much lower frequency (12-13).

Fas function differences were also not ascribable to age, disease duration or glycemic control differences, since age does not influence Fas function (9, 14); adult T1DM patients display a similar frequency of FasR subjects as children (data not shown), and glycemic control was similar in the different patient groups. Moreover, analysis of HLA genotypes in a subset of patients ruled out a correlation with Fas function (data not shown). However, analysis of a larger cohort of patients is needed to assess the cooperative role of HLA genotypes and the defect of Fas function in development of T1DM and T1DM-p.

Molecular heterogeneity of the Fas function defect

Fas signaling starts from aggregation of Fas, the adaptor molecule FADD, and caspase-8 forming the Death Inducing Signaling Complex (DISC) which triggers caspase-8 activation by initiating both the intrinsic and the extrinsic pathway (1-6). The extrinsic pathway proceeds through activation of a caspase cascade comprising caspase-6, -7, and -3. The intrinsic pathway proceeds though cleavage of cytosolic *bid*, whose cleaved form induces release of cytochrome-*c* from mitochondria and aggregation of the apoptosome formed by Apaf-1, cytochrome-*c*, ATP, and caspase-9, which is thus activated and activates caspase-3. This process is under the control of several inhibitors, such as FLIP, which inhibits DISC formation, members of the bcl-2 family, that block mitochondrial release of cytochrome-*c*, and members of the IAP family, such as IAP-1, IAP-2, and XIAP, that directly inhibit the activity of several caspases. Theoretically, the defective Fas signaling displayed by our patients may be caused by either decreased function of signal transducers or increased function of inhibitors.

The different ability of Fas triggering to activate caspase-8 and caspase-9 in FasR patients suggests that dissimilar molecular alterations are involved in different subjects. Patients with defects of both caspases (n=13) may carry an early defect of Fas signaling hitting both pathways, as suggested by a report showing decreased activation of caspase-8 and increased recruitment of FLIP into the DISC in T1DM patients (16). In those with normal activation of both caspases (n=19), the defect could be due to alterations downstream from both caspases, as suggested by a report showing decreased expression of caspase-3 in T1DM patients (15). Alternatively, this pattern may be due to mild defects hitting both pathways, as suggested by the finding that function

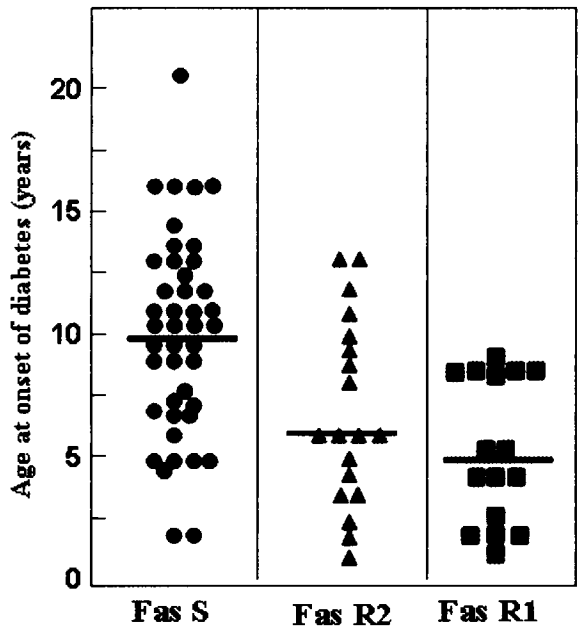

Fig. 4. *Ages at onset of Fas Sensitive patients (FasS) and Fas Resistant patients with normal (FasR2) or decreased caspase activity (FasR1). The full lines indicate the median value for each group. Statistical analysis using the Fisher's exact text: FasR1 vs FasR2 $p<0.05$, FasR1 vs FasS $p<0.0001$, FasR2 vs FasS $p=0.053$*

of both caspases was borderline (i.e. in the 5th-25th percentile range of normal controls) in 7/19 (37%) of these patients versus 3/20 (15%) of controls. Finally, the 3 patients with a defect of caspase-9 only may carry a defect specifically affecting the intrinsic pathway, as we have already proposed for subjects showing normal apoptotic response to Fas, but decreased response to apoptotic stimuli acting on mitochondria (9-10).

In our patients, we did not detect decreased expression of caspase-8 or -9 (Fig. 3), nor increased expression of FLIP, bcl-2, IAP-1, IAP-2, and XIAP by Western blotting (data not shown). However, these analyses cannot rule out mild variations of expression with a pathogenic role nor qualitative alterations affecting their function but not their expression.

In conclusion, these data suggest that, in addition to favoring development of T1DM, defective Fas signaling identifies a subgroup of patients with aggressive autoimmune attack characterized by low age at onset and increased risk of a second autoimmune disease. It is intriguing that defective apoptosis, decreased expression of caspase-8 and -3, and increased expression of c-FLIP have also been described in NOD mouse T cells (19-20).

Apoptosis defects may favor development of autoimmunity by altering the immune response switching-off system and increasing the risk of cross-reactions with self antigens by "molecular mimicry" when the non-self antigens have been cleared. The same mechanism may also favor an expansion of the autoimmune response following the initial autoimmune damage ("epitope spreading") (21-23), which may account for the early onset and polyreactivity.

However, other mechanisms may also be involved. For instance, β-cell apoptosis has been suggested to trigger T1DM development since initiation of anti-islet immune response in NOD mice coincides with a post-natal wave of β-cell apoptosis during organ remodeling at about 15 days of age. A failure to clear apoptotic cells might result in secondary necrosis and stimulation of inflammatory reactions that favor recognition of self-antigens by the immune system (24-25). In this view, defective Fas function may favor this process by slowing down cell apoptosis and/or inducing exposure of abnormal amounts of apoptosis-related antigens.

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