Disturbed apoptosis of T-cells in patients with active idiopathic thrombocytopenic purpura

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Summary

Idiopathic thrombocytopenic purpura (ITP) is an organ specific autoimmune disorder in which T-lymphocyte abnormalities have pathogenetic importance. In a DNA microarray screen of CD3+ T-lymphocytes from ITP patients and healthy controls we found an altered expression of genes associated with apoptosis, e.g. A20, caspase-8 and Bax. This together with our previous findings of increased gene expression of Fas, interferon-g and IL-2 receptor beta (IL2RB) indicated an altered activation induced cell death (AICD) of T-cells in ITP. Using a proliferation assay we found that CD3+ lymphocytes from ITP patients were significantly more resistant to dexamethasone induced suppression compared to normal lymphocytes. We also found that cultured CD3+ lymphocytes from ITP patients in remission were more susceptible to apoptosis both in the presence and absence of dexamethasone compared to cells from patient with active ITP and healthy controls, as indicated by increased staining of Annexin V binding. Our findings suggest that apoptotic resistance of activated T-lymphocytes in patients with active ITP may lead to defective clearance of autoreactive T-lymphocytes through AICD, which might cause a continued immune destruction of platelets. Conversely, a loss of resistance to AICD in ITP patients in remission might be an important mechanism for the achievement of remission.

Keywords

Immune thrombocytopenia, T-cells, apoptosis, microarray, autoimmunity

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Introduction

Chronic idiopathic thrombocytopenic purpura is an organ-specific autoimmune disorder, in which the platelets are prematurely destroyed. The thrombocytopenia is mainly attributed to the production of platelet-specific autoantibodies with accelerated phagocytosis of opsonized platelets in the reticuloendothelial system. Most likely, other mechanisms contribute to the platelet destruction, e.g. complement mediated lysis (1), ineffective thrombopoiesis (2) and cell-mediated cytotoxicity (3). It has become evident that T-lymphocytes play an important role in the pathogenesis of ITP. Several studies have reported a Th0/Th1 polarization of the immune response in ITP (4–8), whereas others have yielded inconsistent (9) or opposing results (10). Also, the presence of activated platelet-specific autoreactive T-cells that recognize and respond to autologous antigens and drive the production of platelet autoantibodies by B-lymphocytes has been reported (11, 12). Most recently, we studied the gene expression in T-lymphocytes from ITP patients and our results supported the concept of a platelet-specific T-cell mediated cytotoxicity in patients with active ITP (3).

Apoptosis is critical for the normal development and homeostasis of the immune system. There is emerging evidence that failure of apoptosis to eliminate potentially pathogenic, immature autoreactive T lymphocytes through negative selection in the thymus, may be involved in the pathogenesis of autoimmune diseases (13). However, autoreactive clones are found in healthy individuals but do not elicit an immune response. This is either due to low affinity of the T-cell receptor to the antigen, or to anergy i.e. tolerance, defined as the inability of a clone to respond by cytokine secretion, proliferation and differentiation (14, 15). Since this is not a foolproof system, several control steps exist and mature autoreactive T-cell clones have also been shown to be deleted peripherally through activation-induced cell death

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Table 1: Patient characteristics and the different assays in which the patients have been included

Patient	Gender	Age (year)	Plc ($x10^9$/L)	Medication	Category	Included in:		
						Gene Expression profiling	Apoptosis	Proliferation
1*	M	59	11	Prednisone 5 mg q.d.	Active	Yes	No	No
2*	F	53	45	Prednisone 5 mg q.d.	Active	Yes	Yes	Yes
3	F	28	50	None	Active	Yes	No	Yes
4*	F	44	33	Prednisone 10 mg q.d.	Active	Yes	No	Yes
5*	M	38	24	None	Active	Yes	Yes	Yes
6	M	37	35	None	Active	No	Yes	Yes
7*	F	26	20	None	Active	No	No	Yes
8*	F	22	23	None	Active	No	Yes	Yes
9	F	68	47	None	Active	No	No	Yes
10*	M	37	177	None	Remission	Yes	Yes	Yes
11*	M	56	299	None	Remission	Yes	No	Yes
12*	F	49	382	None	Remission	Yes	No	No
13	F	28	173	None	Remission	Yes	No	No
14	F	69	324	Prednisone 7.5 mg q.d.	Remission	Yes	No	No
15	M	49	163	None	Remission	No	No	Yes
16*	F	25	165	None	Remission	No	No	Yes
17*	F	75	265	Prednisone 2.5 mg q.d.	Remission	No	Yes	Yes
18	M	30	212	None	Remission	No	Yes	No
19*	M	70	150	None	Remission	No	Yes	No
20	F	30	237	None	Remission	No	Yes	No
21*	M	70	159	Prednisone 10 mg q.d.	Remission	No	Yes	No
22*	M	57	454	None	Remission	No	No	Yes

* indicates that the patient has been splenectomized

(AICD) (16). AICD is induced in T cells via different death pathways, of which Fas/FasL is the best characterized (17). Fas has also been shown to be upregulated in activated lymphocytes. Other candidate pathways for AICD include TNFα, TRAIL, IFN-γ, and IL-2 (18–21).

In our DNA microarray screen we found an altered expression of genes involved in apoptosis in ITP patients, which may promote resistance to apoptosis of potentially pathogenic T-lymphocytes and consequently, may allow for prolonged autoimmune platelet destruction. This concept was further explored in the present work. The results from assays for T-lymphocyte proliferation and apoptosis gave further support to this hypothesis, i.e. T-lymphocytes from patients with active ITP are resistant to apoptosis.

Material and methods

Patients

The patient characteristics are given in Table 1. Twenty-two patients with chronic ITP (12 females and 10 males, mean age 46.1 ± 3.8 years) were studied. All patients were diagnosed and treated at the Hematology Section of Sahlgrenska University Hospital. Thirteen patients (6 females and 7 males) were in remission, having a stable platelet count above 150 x 10^9/l and 9 patients (6 females and 3 males) had an active disease with a platelet count below 50 x 10^9/l. A control group consisted of 14 healthy volunteers (11 females and 3 males, mean age 40.3 ± 3.2 years). There was no significant difference in mean age between the ITP patients and healthy controls (Students' t-test). Informed consent was obtained from the patients and healthy controls and the study was approved by the local ethical committee.

Cell separation

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation. $CD14^+$ and $CD19^+$ cells were removed from PBMCs using magnetic microbeads (MACS; Miltenyi Biotec). The mean percentages of T-($CD3^+$) and NK-cells ($CD3^-/CD16^+/CD56^+$) in the cell preparations were 80 ± 2% and 13 ± 2%, respectively; the remaining 7% consisted of $CD14^+$ monocytes, $CD19^+$ B-cells and $CD3^+/CD16^+/CD56^+$ cells. These cell preparations were used for the proliferation and apoptosis assays. For the gene expression analysis the cell preparations were further purified by positive selection of $CD3^+$ T-cells, using magnetic microbeads (MACS; Miltenyi Biotec), as previously described (3).

Gene expression

Ten patients with chronic ITP were divided into two groups: active disease, i.e. platelet count (plc) <50 x 10^9/l (n = 5), and remission, plc >150 x 10^9/l (n = 5). Five healthy volunteers served as controls. T-cells ($CD3^+$/$CD14^-$) were isolated from peripheral blood as described above. Total cellular RNA was isolated from the cells and equal amounts from each individual were pooled together according to disease stage, i.e. "active ITP", "ITP in remission" and "controls". RNA isolations were performed using the Chomczynski method (22) followed by RNeasy clean up (Qiagen, Hilden, Germany) before reverse transcribed into cDNA. Preparation of biotin-labeled cRNA, hybridization to DNA microarrays (Human Genome U95A array version 2 (HU95Av2); Affymetrix, Santa Clara, CA) and detection of hybridized target cRNA was performed according to the Affymetrix Gene Chip Expression Analysis manual. Quality of the cDNA-synthesis and *in vitro* transcription was assessed by hybridization to Test2-arrays (Affymetrix). The Affymetrix GeneChip U95 array, version 2 (Affymetrix UK Ltd.) allows monitoring of the relative abundance of approximately 12,000 human mRNA transcripts from genes and ESTs. Scanned output files were visually inspected for hybridisation artifacts and then analyzed with MicroarraySuite 5.0 software (Affymetrix). The arrays were scaled to an average intensity of 500. Genes were clustered and selected using Genespring 5.0 (Silicon Genetics, Redwood City, CA).

Apoptosis assay

T-cells ($CD3^+$/$CD19^-$/$CD14^-$) were isolated from peripheral blood as described above. The cell concentration was adjusted to 5 x 10^5 cells/ml and cultured in RPMI-1640 (Life Technologies, USA) medium supplemented with 5 % heat-inactivated human AB^+ serum. The cells were activated by adding IL-2 (final concentration (f.c.) 5 units/ml; Roche, Mannheim, Germany). The activated cells were then cultured for 72 hours in the presence or absence of dexamethasone (f.c. 5 x 10^{-7} M; Sigma-Aldrich, St. Louis, MO) and assayed for apoptosis using Annexin V/propidium iodine (PI) staining and flow cytometry, as previously described (23, 24). Briefly, cells were immuno-stained directly with PI, fluorescein isothiocyanate (FITC)-conjugated Annexin V, and Peridine-Chlorophyll-Cy5.5 (perCP)-conjugated MoAbs specific for CD^3. All MoAbs were from Becton Dickinson Bioscience (San Diego, CA). Flow cytometry was performed using a FACScan (Becton Dickinson, Mountain View, CA). 10 000 events were collected and the data were analysed using the WinMDI-software (j.trotter@scripps.edu). The fraction of CD3+ cells expressing Annexin V and PI was determined by setting a quadrant gate in the fluorescence dotplot for the respective cell population and expressed in percent.

Dexamethasone induced suppression of T-cell proliferation

$CD3^+$/$CD14^-$/$CD19^-$ PBMC were isolated as described above and 100 ml of the cell suspension (total 1 x 10^5 cells) in RPMI was added to each well of a 96-well U-bottomed tissue culture plates (Nunclon, Nalge Nunc Int., Denmark). Thereafter, IL-2 was added (f.c. 5 units/ml) and the cells cultured at 37°C and 5% CO_2, in the presence (f.c. 10^{-6} – 10^{-9} M) or absence of dexamethasone for 2 days. After 48 hours, 1.0 μCi [3H]-thymidine was added to each well and the cells were further cultured for 18 h. The cells were then harvested (Harvester 96, Tomtec, USA) onto filterpaper and incorporation of [^{3}H]-thymidine was determined in a scintillation counter (1450 Microbeta Plus, Wallac, Finland). The results are presented as % inhibition, defined as mean cpm for triplicate wells with dexamethasone divided by the mean cpm for the corresponding triplicate wells not holding dexamethasone.

Statistics

Unless otherwise stated, the mean values ± SEM are reported. Differences between groups were evaluated using analysis of variance (ANOVA for repeated measurements or one-way ANOVA).

Results

Microarray analysis

In the DNA microarray analysis we identified an altered expression of several genes that could be linked to apoptotic pathways, in T-lymphocytes from patients with ITP. The genes *BAX, A20, CALPASTATIN, CASPASE-8, NFKBIA, P50-NF-KAPPA B, TANK, CASPASE-1* and *TRADD* displayed an expression pattern supporting the hypothesis of an altered apoptotic response of T-cells in ITP (Fig. 1). Bcl-2 has been shown to inhibit apoptosis (25), and overexpression at the mRNA and protein level is found in lymphoproliferative disorders (26). However, Bcl-2 associated X protein (Bax), that heterodimerizes with Bcl-2, is proapoptotic (27). The ratio of Bcl-2 and Bax seems to determine whether a cell will remain viable or die through apoptosis (27). A20 has been implicated in both TNFa and Fas mediated apoptosis (28–31). P50-NF-Kappa B, NFKBIA, TRADD and TANK are all members of the NFKB signaling pathway which have been shown to participate in apoptosis (32, 33). Calpastatin has been shown to be an inhibitor of calpain and is the target of cas-

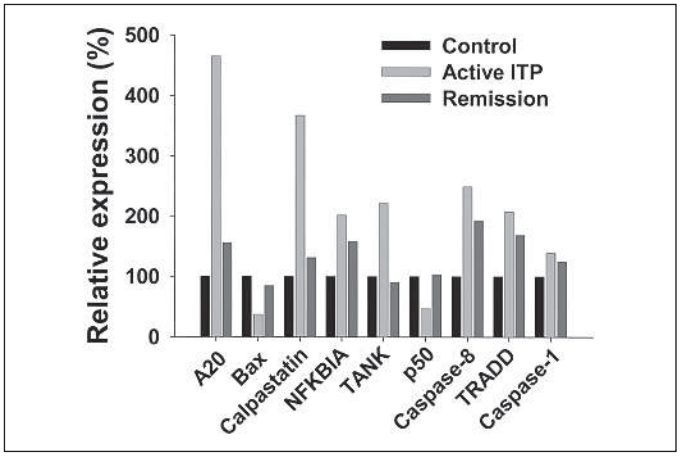

Figure 1: Gene expression of T-cells in ITP and healthy controls. Expression of genes associated with apoptotic pathways in CD3+ lymphocytes from patients with active ITP, ITP in remission and healthy controls. Data is derived from 5 individuals pooled in each group. The controls are set as 100 % and the values in the other groups are normalized to their respective control.

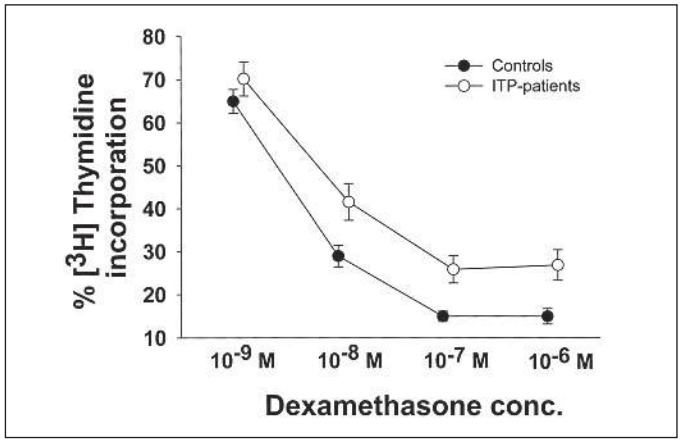

Figure 2: Proliferation assay of T-cells from ITP patients and healthy controls. Dexamethasone suppression of IL-2 induced proliferation (mean ± SEM) of CD3+ lymphocytes from ITP patients (n = 14) and healthy controls (n = 9). The proliferation was measured by [^{3}H]-thymidine incorporation and the results were normalized to the baseline value, i.e. cultures not exposed to dexamethasone. The ITP patients displayed a significantly higher [^{3}H]-thymidine incorporation compared to the controls (p = 0.02; ANOVA for repeated measurements). No difference was seen between patients with active ITP (n = 8) and patients with ITP in remission (n = 6).

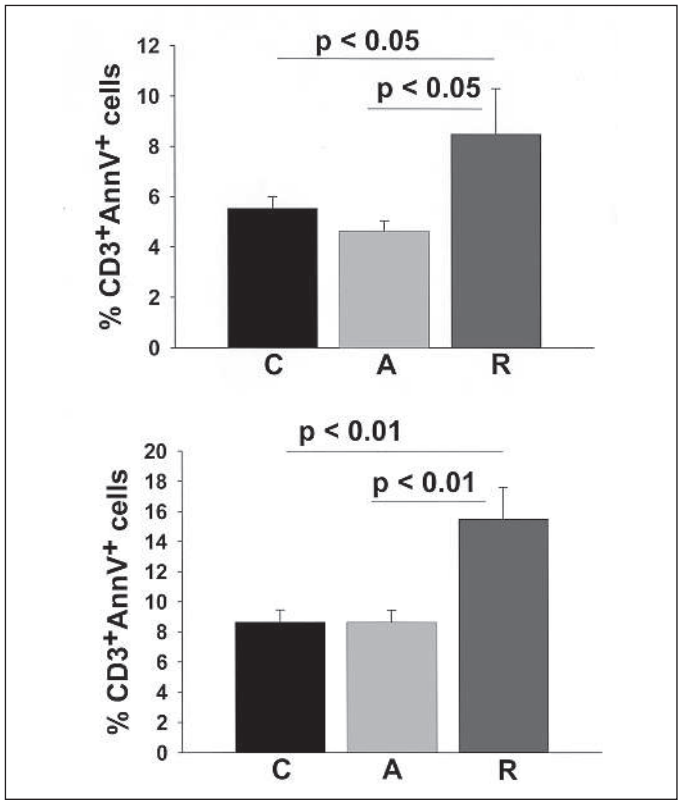

Figure 3: T-cell apoptosis in ITP patients and healthy controls. Apoptosis was measured as the percentage of CD3+AnnexinV+ cells by flow cytometry, in patients with active ITP (A; n = 5), ITP in remission (R; n = 6) and healthy controls (C; n = 9). The cells were activated by IL-2 and cultured in the absence (A) or presence (B) of dexamethasone. Values are presented as mean ± SEM. Differences between groups were evaluated using ANOVA.

pase-1 (34). Cleavage of calpastatin results in apoptosis. Caspase-1 has also been shown to cleave TRADD which interacts with TNF receptor 1 and mediates TNF mediated apoptosis (31). Caspase-8 (FLICE/MACH) is an important member of the intracellular apoptotic proteolytic cascade following a death signal (35, 36) and lack of caspase-8 may cause lymphoproliferative disorder and immunodeficiency (37). Microarray data is available at the NCBI's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/; series no. GSE574 and sample nos. GSM8814-GSM8819).

IL-2 induced T-cell proliferation assay under the influence of dexamethasone

Any disruption in the interactions between the Fas and IL-2 pathways will interfere with AICD and support the expansion of self-reactive T-lymphocytes, which are normally targeted for elimination (38, 39). Since IL-2 is crucial for T-cell sensitization to AICD, we analyzed the influence of exogenous IL-2 on T-lymphocyte proliferation, i.e. [^{3}H]-thymidine incorporation, and the effect of dexamethasone suppression. The IL-2 induced proliferation of cultured T-lymphocytes from ITP patients, both those with an active disease and those in remission, was suppressed to a lesser extent by dexamethasone compared to healthy controls ($p < 0.05$; ANOVA) (Fig. 2). In the absence of dexamethasone, there was no statistical significant difference between active ITP, ITP in remission and controls, regarding the IL-2 induced mean [^{3}H]-Thymidine incorporation (809 ± 208, 818 ± 160 and 725 ± 116 cpm, respectively).

Dexamethasone-induced apoptosis

To test our hypothesis of altered apoptosis of T-cells in patients with ITP in remission compared to active ITP patients, we analyzed Annexin V staining as a marker for apoptosis on cultured T-lymphocytes, by flow cytometry. Cultured T-lymphocytes from ITP patients in remission displayed an increased apoptosis both in the presence and absence of dexamethasone, a potent inducer of apoptosis in T-cells (40), compared to patients with active ITP and healthy controls ($p < 0.05$; ANOVA) (Fig. 3).

Discussion

We found altered gene expression of *BAX, A20, CALPASTATIN, CASPASE-8, NFKBIA, P50-NF-KAPPA B, TANK, CASPASE-1* and *TRADD* in CD3$^+$ T-lymphocytes between patients with active ITP, ITP in remission and healthy controls (Fig. 1). These molecules are known to be involved in apoptosis through different pathways. Thus, our DNA microarray data indicated alterations of apoptotic pathways in T-lymphocytes from ITP patients.

This finding corroborates previous studies. Yoshimura et al. found a higher level of soluble Fas and FasL in patients with chronic ITP (41). Shenoy et al. proposed that altered Fas-pathway signalling probably is involved in the aetiology of autoimmunity in haematological disorders; they found T-cells resistant to Fas-mediated cell death, which could support the expansion of self-reactive clones (42).

Our findings of altered expression of apoptotic genes together with our previous findings of increased gene expression of Fas, interferon-γ and IL-2 receptor beta (IL2RB) (3), in-

dicated an altered AICD response in T-lymphocytes from ITP patients.

Since activation and proliferation is a prerequisite for AICD, we established an assay for the analysis of T-lymphocyte proliferation. We cultured the T-lymphocytes, from patients with active ITP, ITP in remission and healthy controls, in the presence of IL-2 and dexamethasone. We found that T-lymphocytes from both active ITP patients and ITP patients in remission displayed a lower dexamethasone suppression of the IL-2 induced proliferation compared to T-cells from healthy controls, as measured by [3H]-thymidine incorporation, indicating a higher degree of proliferation/activation (Fig. 2). However, another possibility for this observation is that T-cells from healthy controls are more sensitive to dexamethasone induced apoptosis, leaving fewer cells able to proliferate. This latter possibility was explored in another set of experiments; IL-2 stimulated T-cells were cultured in the presence or absence of dexamethasone and apoptosis was evaluated by Annexin V staining. It was found that T-cells from ITP patients in remission displayed a higher percentage of apoptosis compared to T-cells from controls and active patients, both in the presence and absence of dexamethasone; no difference was found between the controls and active ITP patients (Fig. 3). Taken together, these experiments show that T-cells from ITP patients, cultured in the presence of IL-2 and dexamethasone, have a higher degree of proliferation/activation, and rules out an increased percentage of apoptosis in the T-cells from controls, as an explanation for the results seen in the proliferation assay. Furthermore, this increased T-cell proliferation/activation could possibly be explained by our previous finding of increased gene expression of IL2RB (CD122) in T-cells from both active ITP patients and in ITP in remission (3).

The normal reaction of activated T-cells is to die through AICD. Indeed, we observed an increased apoptosis in T-cells from ITP patients in remission compared to healthy controls. However, T-cells from active ITP patients had a lower percentage of apoptosis compared to T-cells from ITP in remission, even though T-cells from both groups had a higher degree of activation/proliferation. This suggests that T-cells from patients with active ITP are resistant to AICD and that correction of the AICD response might induce remission. Of the genes involved in apoptosis that showed altered expression in our microarray experiment, Bax is the most studied. Bax has been shown to be proapoptotic (27) and to heterodimerize with Bcl-2, which in most instances acts as an inhibitor of apoptosis (25). The ratio of Bcl-2 and Bax has been shown to determine whether a cell will remain viable or die through apoptosis (27). Thus, Bax is a candidate for our findings of resistance to AICD of T-cells in patients with active ITP. However, many proteins of the apoptotic machinery act in concert, making it impossible to conclude from these studies which pathway is responsible for the AICD resistance observed in active ITP.

The phenomenon of resistance to apoptosis and AICD has also been shown for T-cells from other autoimmune diseases and experimental models of autoimmunity, e.g. multiple sclerosis (MS) (43), systemic lupus erythematosus (SLE) (44), rheumatoid arthritis (RA) (45) and experimental allergic encephalomyelitis (EAE) (46). Thus, it appears reasonable to assume that a disturbed AICD is a common pathogenic mechanism in autoimmunity. Serially testing apoptotic markers in patients with autoimmunity who are first active and then in remission, would give further support for this hypothesis.

In conclusion, our findings suggests that apoptotic resistance of T-lymphocytes in patients with active ITP may lead to defective clearance of potentially pathogenic autoreactive T-lymphocytes through AICD and consequently, may allow continuing autoimmune platelet destruction. i.e. platelet antibody production and cell-mediated cytotoxicity. Furthermore, the normalization in sensitivity of T-lymphocytes to AICD in ITP patients might be an important mechanism for the achievement of remission. Also, besides the well-known effects of glucocorticoids on phagocytosis and antibody production by B-lymphocytes, the deletion of autoreactive T-cells by apoptosis is another and intriguing mechanism, that might explain the beneficial effect of glucocorticoids in ITP that merits further studies.

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