Ultrastructural study shows morphological features of apoptosis and para-apoptosis in megakaryocytes from patients with idiopathic thrombocytopenic purpura

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Running head: ultrastructural features of megakaryocytes in ITP

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Abstract

To investigate whether altered megakaryocyte morphology contributes to reduced platelet production in idiopathic thrombocytopenic purpura (ITP), ultrastructural analysis of megakaryocytes was performed in 11 ITP patients. Ultrastructural abnormalities compatible with (para-)apoptosis were present in 78 ± 14% of ITP megakaryocytes, which could be reversed by in vivo treatment with prednisone and intravenous immunoglobulin. Immunohistochemistry of bone marrow biopsies of ITP patients with extensive apoptosis showed an increased number of megakaryocytes with activated caspase-3 compared to normal (28 ± 4% versus 0%). No difference, however, was observed in the number of bone marrow colony-forming units-megakaryocyte (ITP: 118 ± 93 versus controls: 128 ± 101; p=0.7). To demonstrate that circulating antibodies might affect megakaryocytes, suspension cultures of CD34+ cells were performed with ITP or normal plasma. Morphology compatible with (para-)apoptosis could be induced in cultured megakaryocytes with ITP plasma (2/10 samples positive for antiplatelet autoantibodies). Finally, the plasma glycocalicin-index, a parameter of platelet and megakaryocyte destruction, was increased in ITP (57 ± 70 versus 0.7 ± 0.2; p=0.009) and correlated with the proportion of megakaryocytes showing (para-)apoptotic ultrastructure (p=0.02; r=0.7). In conclusion, the majority of ITP megakaryocytes show ultrastructural features of (para-)apoptosis, probably due to action of factors present in ITP plasma.
Introduction

Idiopathic or immune thrombocytopenic purpura (ITP) is an autoimmune disease characterized by isolated thrombocytopenia in an otherwise healthy person. The thrombocytopenia in ITP is caused by accelerated platelet destruction due to the action of antiplatelet IgG-autoantibodies that bind to antigens on the platelet cell membrane. The platelets are subsequently destroyed by tissue macrophages, predominantly in the spleen\(^1\). As a result of the accelerated destruction, platelet survival is usually greatly shortened and platelet production is thought to be compensatory increased\(^2^\). However, there is also evidence that platelet production can be impaired in ITP. This was demonstrated in platelet kinetic studies using radiolabeled platelets\(^4^\). The reduced platelet production rate might be mediated by the action of antiplatelet antibodies, which can bind to megakaryocytes in the bone marrow\(^7^\). Recent *in vitro* studies support this concept showing that human megakaryocyte colony formation and proplatelet formation is inhibited\(^10\) and that a reduced expansion of megakaryocytic progenitors can be observed especially in the presence of certain antiplatelet glycoprotein antibodies\(^11\). However, despite the evidence of a reduced platelet production in several ITP patients, numbers of megakaryocytes in the bone marrow are usually normal or increased\(^6\). This is compatible with the finding that plasma thrombopoietin (TPO) levels in ITP patients are not significantly different from normal controls, indicating that the total megakaryocytic mass has not been changed in ITP.

Investigating the relationship between thrombokinetic parameters and the glycocalcin-index (GCI), a parameter of platelet destruction\(^12\), we recently demonstrated that there is an inverse correlation between the platelet production rate and the GCI\(^13\). These results suggest that despite the normal number of megakaryocytes in the bone marrow an increased destruction of platelets and/or megakaryocytes might occur. These findings support the concept of
ineffective thrombopoiesis in the bone marrow. To investigate whether apoptosis or other forms of programmed cell death are responsible for this ineffective thrombopoiesis, we examined the ultrastructure of bone marrow megakaryocytes from ITP patients with electron microscopy. The results demonstrate that, independent of the refractoriness of ITP to therapy, in all patients a large majority of bone marrow megakaryocytes are extensively damaged showing ultrastructural abnormalities of apoptosis and para-apoptosis.
Materials and Methods

Patients

Eleven adult patients with ITP were investigated. The diagnosis required that an otherwise healthy person, after history, physical examination, complete blood count, and examination of the peripheral blood smear, had an isolated thrombocytopenia (platelets < 100 x 10^9/L) of undetermined etiology. Patients with associated systemic disease, such as human immunodeficiency virus infection or systemic lupus erythematosus, were excluded.

Light microscopy of the bone marrow megakaryocytes

In all patients bone marrow was aspirated from the sternum or posterior superior iliac spine and stained according to Giemsa. Normal bone marrow was obtained from non-hematological patients undergoing cardiac surgery after informed consent. The number of megakaryocytes was rated as normal [1 megakaryocyte per 1 to 3 low power fields (16x objective)], increased (>2 megakaryocytes per low power field), or decreased (1 megakaryocyte per 5 to 10 low power fields)⁶. Megakaryocyte morphology was studied with a 100x objective.

Electron microscopy of bone marrow megakaryocytes

Fresh bone marrow cells were washed in RPMI 1640 (Bio Whittaker Europe, Verviers, Belgium), pelleted and subsequently fixed in 2% glutaraldehyde, in 0.1 M phosphate buffer for 24 hours at 4°C. Cells were dehydrated, osmicated and embedded in Epon 812 according to routine procedures. Semi thin sections (1-0.5 μm) were inspected light microscopically to select megakaryocytes. To examine the ultrastructure in detail and to identify the developmental stage of the morphologically recognizable megakaryocytes (stage I, II, or III megakaryocyte) electron microscopic (Philips 201, Eindhoven, The Netherlands) analysis was
performed. The major criteria for classifying megakaryocytes into different stages are the quality and quantity of cytoplasm and the size, lobulation, and chromatin pattern of the nucleus. The immature or stage I megakaryocyte (megakaryoblast) is characterized by a large round, indented or bilobed nucleus, prominent nucleoli and cytoplasm containing scattered mitochondria, abundant free ribosomes, variable amounts of rough endoplasmic reticulum (RER), a small Golgi complex, a few α-granules and rudiments of the demarcation membrane system (DMS). The maturing or stage II megakaryocyte (promegakaryocyte) contains a lobulated nucleus with gradually condensed chromatin. Comparing to stage I the Golgi complex enlarges, the RER increases, the DMS penetrates the entire cytoplasm and the number of granules increases. Stage III or mature megakaryocytes are very large cells (40-56 µm) in which the nucleus is pushed to one pole of the cell, nucleoli are absent and granular cytoplasm is abundant. The well-developed DMS divides the cytoplasm into platelet fields. The Golgi complex and RER are greatly reduced.

A total of 30 megakaryocytes per sample were examined.

Ultrastructural characteristics of apoptosis and para-apoptosis

The ultrastructure of megakaryocytes was examined for the presence of apoptotic and para-apoptotic cell death.

Ultrastructurally apoptosis is characterized by margination of condensed chromatin, nuclear fragmentation and the formation of apoptotic bodies.

Para-apoptosis or non-classical apoptosis is a specific morphological type of non-necrotic cell death and is characterized by cytoplasmic vacuolization, condensed chromatin (but not early margination of the chromatin) and swollen mitochondria. Characteristics of apoptosis like surface blebbing and the formation of apoptotic bodies do not appear.
**Immunohistochemistry**

To identify apoptosis in megakaryocytes, immunohistochemical staining was performed with an antibody aimed at detecting activated caspase-3 as previously reported. For immunohistochemical staining, serial 3 µm-thick sections were cut from paraffin embedded bone marrow biopsies. After deparaffinization in xylene, antigen retrieval was performed using microwave heating at 700 W for 10 minutes in EDTA buffer. Following blocking of endogenous peroxidase with 3% hydrogen peroxide for 30 min, the primary antibody was applied for 1 h at room temperature. To identify activated caspase-3, immunostaining was used with a rabbit polyclonal antibody (1:100, New England Biolabs, Beverly MA, USA). Subsequently, the slides were incubated for 30 minutes with appropriate secondary and tertiary antibodies with streptavidin-conjugated peroxidase (DAKO, Glostrup, Denmark). Peroxidase activity was visualised with diaminobenzidine. Slides were counterstained with haematoxylin. As a positive control, a sample of colorectal carcinoma was included. As negative controls, slides were immunostained in the absence of the primary antibody. Evaluation of extension of staining was performed by light microscopy. Slides were evaluated by at least two independent investigators. If the evaluations did not agree they were re-evaluated under a multi-headed microscope. Samples were scored as negative (i.e. absence of detectable cytoplasmic staining) or positive. A total of 30 megakaryocytes per sample were examined.

**Megakaryocyte progenitor cell assay**

Colony-forming units-megakaryocyte (CFU-Mk) were evaluated quantitatively using a commercially available kit (MegaCult-C; Stem Cell Technologies, Vancouver, Canada), according to manufacturer’s instructions. In short, 1 x 10^5 mononuclear bone marrow cells from ITP patients and normal controls were seeded per double chamber culture slide in
serum-free medium containing thrombopoietin (TPO; 50 ng/mL), interleukin (IL)-3 (10 ng/mL), IL-6 (10 ng/mL) and collagen (1.1 mg/mL). Cultures were incubated for 12 days, followed by dehydration, fixation and immunocytochemical staining on slides. Megakaryocyte colonies were detected using anti-glycoprotein (GP)IIb/IIIa antibody (CD41) and the alkaline phosphatase detection system (APAAP). Cultures were scored for the presence of pure megakaryocytic colonies consisting of at least five nucleated cells expressing GPIIb/IIIa without any negatively stained cells (mixed and non-Mk colonies). Colonies were divided into megakaryocyte progenitors with low-proliferative capacity, when colonies contained 5-20 megakaryocytes, and progenitors with high-proliferative capacity, when CFU-Mk contained more than 20 megakaryocytes per colony.²⁰

Isolation of CD34⁺ cells

Peripheral blood cells were obtained from leucapheresis samples of a patient undergoing autologous peripheral blood stem cell mobilization after informed consent. Cells were collected by apheresis during the regeneration phase after high-dose cyclophosphamide in the presence of granulocyte colony-stimulating factor (G-CSF) in patients with multiple myeloma. CD34⁺ cells were isolated by positive selection using Isolex-300 (Baxter, Deerfield, IL, USA) magnetic cell sorting system according to manufacturer’s instructions. At the end of the procedure, CD34⁺ cell purity was reanalysed by flow cytometry using anti-HPCA-2 and was greater than 90%.

Suspension cultures

Purified normal CD34⁺ cells were seeded at 2 x 10⁵ cells/ml in liquid serum free expansion medium (StemSpan™ SFEM; Stem Cell Technologies, Vancouver, Canada) plus growth factors (TPO 20 ng/mL and stem cell factor (SCF) 10 ng/mL) in 24-well plates (Costar,
Cambridge, MA, USA). Cultures were performed for 7 and 12 days in the presence of 10% plasma from ITP patients, 10% plasma from normal controls and without plasma. All cultures were performed at 37°C under a 5% CO₂ in air–humidified atmosphere. Viable cells were evaluated by trypan blue exclusion and cell counts were determined using a haemocytometer.

**Antiplatelet antibody detection**

A commercially available ELISA (PakAuto™, GTI, Brookfield, USA) was used to detect antibodies reactive with GPIIb/IIIa, GPIb/IX and GPIa/IIa in plasma of ITP patients according to manufacturer’s instructions. Test results showing absorbance values equal or greater than twice the value obtained for the mean of the negative controls were regarded as positive.

**Plasma glycocalicin**

Plasma glycocalicin (GC) concentrations were measured by EIA (Takara Shuzo Co, Ltd., Ohtsu, Japan). Citrate-anticoagulated blood was processed within 2 hours after blood collection. Since GC levels are dependent on the platelet count, the GC-index (GCI) was calculated by using the following formula: \[ \text{GCI} = \frac{\text{GC} (\mu g/mL) \times (250 \times 10^9/L)}{\text{individual platelet count} (10^9/L)} \] \(^{12,21}\). Normal value of the GCI is 0.7 ± 0.2.

**Statistical analysis**

Data are reported as mean ± SD. Differences between groups were calculated using the Mann-Whitney U-test. Correlations were calculated using Spearman’s rank correlation test. P-values below 0.05 were considered significant.
Results

Patients

Eight female and 3 male patients with ITP were studied. Mean age was 34 ± 19 years. The mean platelet count at the time of study was 21 ± 30 x 10^9/L. Eight patients (73%) had platelet counts < 15 x 10^9/L (Table 1). During the time of study no patients were on treatment. Four patients had undergone a splenectomy before they were studied.

Light microscopy of bone marrow megakaryocytes

Eight patients (73%) had an increased number and 3 patients a normal number of megakaryocytes in the bone marrow. In smears made from bone marrow aspirates for clinical use no abnormalities were found in megakaryocyte morphology.

Electron microscopy and immunohistochemistry of bone marrow megakaryocytes

ITP patients versus normal controls

Electron microscopic study of bone marrow megakaryocytes of ITP patients and healthy controls demonstrated that the percentage of stage III (mature) megakaryocytes was significantly decreased in ITP patients (n=11) (48 ± 21) compared to normal controls (n=4) (72 ± 11; p=0.05). The percentage of stage I (immature) megakaryocytes in ITP patients was not significantly different from controls (12 ± 6 vs 11 ± 7; p=0.8), while stage II (maturing) megakaryocytes were significantly increased in ITP patients compared to controls (40 ± 19 vs 17 ± 5; p=0.02).

Ultrastructural examination of megakaryocytes in all ITP patients showed extensive damage in 37 ± 37% of stage I, 65 ± 37% of stage II and 84 ± 16% of stage III megakaryocytes (Figure 1). Of all stages together 78 ± 14% were morphologically abnormal.
The morphological alterations consisted primarily of cytoplasmic vacuolization due to mitochondrial swelling and distended demarcation membrane system (DMS), and chromatin condensation within the nucleus, without margination of the chromatin, all ultrastructural features of para-apoptosis. Megakaryocytes showing these abnormalities all had an intact, mostly thickened, peripheral zone, which did not seem to contain any functional cellular material (i.e. organelles or DMS). Continuity between the extracellular space and the DMS was only very rarely observed. The cytoplasm of these damaged cells lacked platelet territories. Practically all abnormal but none of the normal ITP megakaryocytes, were surrounded by neutrophils or macrophages, some being in a state of phagocytosis (Figure 3B).

The majority of ultrastructural alterations in damaged ITP megakaryocytes consisted of characteristics of para-apoptosis. Morphological features of apoptosis were observed in 4/11 ITP patients and only in stage III megakaryocytes (Figure 2B). Of stage III megakaryocytes 62 ± 31% showed ultrastructural features of apoptosis, 31 ± 31% of para-apoptosis, while 7 ± 8% were morphologically normal. In these 4 patients all stage I and the majority of stage II megakaryocytes were morphologically intact (15-20% of stage II megakaryocytes showing features of para-apoptosis).

In more than 90% of the abnormal megakaryocytes all ultrastructural characteristics of apoptosis and para-apoptosis were found. The amount and type of alterations in the megakaryocytes were not correlated with the platelet count.

To confirm the process of apoptosis immunohistochemical staining for activated caspase-3 was performed on bone marrow biopsies from ITP patients (n=2; nrs 6 and 10, see Table 1) whose megakaryocytes showed ultrastructurally prominent morphological features of apoptosis. Twentyfive and 30% of the megakaryocytes of these ITP patients were positive for activated caspase-3, while megakaryocytes of bone marrow biopsies from normal controls (n=4) were negative (Figure 4).
**ITP patients before and after treatment**

In two patients the effects of prednisone or high-dose immunoglobulin could be analysed. In the first patient the bone marrow aspiration was repeated 2 weeks after start of prednisone treatment. In this period the platelet count had significantly increased to $120 \times 10^9/L$. Ultrastructural examination of this bone marrow sample demonstrated a distinct reduction in the number of megakaryocytes showing programmed cell death (in stage I/II from 100 to 55%, in stage III megakaryocytes from 100 to 61%). The percentage of stage III megakaryocytes increased from 20 to 26%. In the second patient bone marrow examination was repeated after high-dose intravenous immunoglobulin therapy, which resulted in an increase in platelet count from 20 to $160 \times 10^9/L$. The percentage of stage III megakaryocytes increased from 20 to 43%, which coincided with a diminished number of damaged megakaryocytes (in stage I/II from 80 to 35%, in stage III megakaryocytes from 50 to 23%).

**Quantification of megakaryocyte progenitor cells**

In view of the observed abnormalities in immature megakaryocytes, we were interested whether this was also reflected at progenitor level, i.e. a reduced number of megakaryocyte progenitors (CKU-Mk). The total number of CKU-Mk per $10^5$ bone marrow cells in ITP was not significantly different from normal controls ($n=10$) ($118 \pm 93$ vs $128 \pm 101$; $p=0.7$). In addition, there was no significant difference in the number of progenitors with low- and high-proliferative capacity between ITP and normal control bone marrow ($p=0.7$ and 0.9, respectively).

**Megakaryocytes derived from CD34+ cells after incubation with normal and ITP plasma**
To investigate whether normal megakaryocytopoiesis can be influenced by factors in ITP plasma, normal CD34+ cells were incubated with 10% plasma from 3 ITP patients (nrs 1, 2 and 10; Table 1) and 3 normal controls and cultured for 7 and 12 days in serum-free medium containing TPO and SCF. After 7 days incubation with ITP or normal plasma, electron microscopy of the suspension cultures showed only immature megakaryocytes without abnormalities. After 12 days of culture similar cytoplasmic and nuclear alterations as observed in the ITP megakaryocytes were seen in 60 ± 14% of the megakaryocytes derived from incubation with ITP plasma (42 ± 23% with apoptotic and 18 ± 9% with para-apoptotic ultrastructure). Ninety percent of megakaryocytes incubated with normal plasma were morphologically normal and ultrastructural features of para-apoptosis were not observed.

**Antiplatelet antibodies**

Plasma from 2/10 patients tested positive for the presence of antiplatelet antibodies (Table 1). Plasma used for incubation studies was derived from patients nrs 1, 2 and 10. The plasma from patient nr 2 tested positive for antibodies reactive with GPIIb/IIIa, GPIb/IX and GPIa/IIa. In the plasma of the other 2 patients test results for antibodies were negative.

**Plasma GCI**

GCI in ITP patients (n=10) was significantly increased compared to normal controls (n=16) (57 ± 70 vs 0.7 ± 0.2; p=0.009) (Table 1). There was a significant correlation between the GCI and the proportion of ITP megakaryocytes with morphological features of (para-) apoptosis (p=0.02; r=0.7) (Figure 5).
Discussion

In the present study extensive ultrastructural alterations in megakaryocytes from ITP patients are described. Previous studies on megakaryocyte morphology and ultrastructure in ITP show conflicting results. Earlier light microscopic observations showed megakaryocytes with degenerative characteristics and greatly reduced platelet production, although the number of megakaryocytes was increased in ITP patients compared to normal\textsuperscript{22}. Ultrastructural studies of megakaryocytes in ITP showed results varying from moderate vacuolization, deficient formation of demarcation membrane system (DMS) and invaginations in the peripheral membrane\textsuperscript{22} to practically normal morphology of megakaryocytes\textsuperscript{24,25}. Stahl et al\textsuperscript{26} found that 50 to 75\% of the ultrastructurally examined megakaryocytes from 4 ITP patients had extensive damage, with marked dilatation of the DMS and a disrupted peripheral zone. ITP megakaryocytes with an intact peripheral zone did not show any morphological alterations. The present investigation, however, reveals ITP megakaryocytes with extensive abnormalities within an intact and often thickened peripheral zone. These cells are all surrounded by neutrophils and macrophages, suggesting an inflammatory response against these megakaryocytes.

The ultrastructural alterations observed in the present study are compatible with the morphological criteria for apoptosis and para-apoptosis. Para-apoptosis, first described by Asher et al\textsuperscript{16}, is characterized by condensed chromatin and cytoplasmic vacuolization due to swollen mitochondria and endoplasmic reticulum. In contrast to necrosis there is no cell membrane disruption. Recently, Sperandio et al defined a form of para-apoptosis (which they called paraptosis) with similar ultrastructure by cellular characteristics and response to apoptosis inhibitors\textsuperscript{18}. They found that this form of nonapoptotic cell death is TUNEL (terminal deoxynucleotidyl transferase-mediated nick end labeling)-negative and resistant to
caspase-inhibitors and Bcl-xL, and is driven by a catalytic mutant of caspase-9 that is APAF-1 independent.

In our study about 80% of mature (stage III) megakaryocytes, in which most of the platelet production occurs, showed ultrastructural features of apoptosis and para-apoptosis. Significantly less stage I megakaryocytes showed morphological alterations, indicating that immature megakaryocytes are less affected by (para-)apoptotic cell death. This is further supported by the finding that the total number of CFU-Mk in our ITP patients was not significantly different from normal. Previous studies on the number of CFU-Mk in chronic ITP showed conflicting results, depending on the assay that was used, the stage (acute or chronic) of the disease and the refactoriness of the ITP to therapy. Several authors found a significant increase in number of CFU-Mk in chronic ITP patients. Others reported a reduced number of CFU-Mk in chronic ITP. The present group of ITP patients was heterogeneous, consisting of patients responding to prednisone and splenectomy and patients with recurrent disease after splenectomy.

Morphological changes compatible with (para-)apoptosis similar to those found in megakaryocytes from ITP patients could be induced in normal CD34+ cells that were cultured to megakaryocytes in the presence of ITP plasma. The data from these incubation studies are consistent with earlier phase-contrast microscopic studies of ITP megakaryocytes in which cytoplasmic vacuolization, loss of granularity and lack of platelet release were observed. In these studies the morphological alterations in the megakaryocytes could be reproduced in vivo by infusing healthy controls with ITP plasma. The results suggest that the defect in ITP is not only at the level of platelets but also at the level of megakaryocytes. This is further supported by the finding that during successful treatment with prednisone the number of damaged megakaryocytes decreases. Suprisingly, this was also the case in an ITP patient treated with intravenous immunoglobulin, indicating that this treatment not only protects
peripheral blood platelets from destruction by macrophages but might also protect megakaryocytes against destruction by bone marrow macrophages. A possible explanation for this finding could be a reduced concentration of antiplatelet antibodies, since recent observations in a rat model of immune thrombocytopenia showed that intravenous immunoglobulin administration can lead to increased clearance of an antiplatelet antibody\textsuperscript{32}. Since antiplatelet antibodies bind to antigens present on the surface of megakaryocytes\textsuperscript{8} it is likely that this interaction plays an important role in initiating the cascade of programmed cell death. However, in only 20\% of ITP patients plasma antiplatelet antibodies were detected. This may in part be due to the limited sensitivity of the ELISA test for detecting antiplatelet autoantibodies. Previous studies, however, have also demonstrated a low frequency of free circulating autoantibodies\textsuperscript{33}, suggesting that alternative mechanisms for initiating cell death are of importance, including the release of cytokines\textsuperscript{34}.

The presence of extensive ultrastructural abnormalities in megakaryocytes indicates that ITP is essentially different from other autoimmune cytopenias such as autoimmune hemolytic anemia (AIHA). In contrast to AIHA, which presents in practically all cases with clear erythroid hyperplasia\textsuperscript{35,36}, bone marrow examination in ITP shows normal numbers of megakaryocytes in a large number of patients\textsuperscript{37}, suggesting that ITP megakaryocytes might be affected contrary to erythroid precursors in AIHA. Only a small proportion of AIHA patients has reticulocytopenia, which is associated with an increased apoptosis of erythroid progenitors in the bone marrow\textsuperscript{38}.

In a recent study\textsuperscript{6} megakaryocyte levels were normal in 65\% of 141 ITP patients and increased in only 33\%. Twenty-four percent had an increased platelet production rate (PPR, i.e. the number of platelets entering the circulation), and only 8\% had an increased PPR in conjunction with an increased number of megakaryocytes. Moreover, there are many reports about a reduced PPR in a subgroup (30-50\%) of ITP patients\textsuperscript{4,5,6,39,40}. Experiments in which
thrombokinetic studies were repeated after splenectomy and prednisone treatment showed that both forms of treatment can induce a distinct increase in PPR\textsuperscript{41}, indicating that the release of platelets to the circulation (i.e. PPR) in active ITP is relatively depressed, perhaps due to the action of autoantibodies directed against megakaryocytes.

Besides the results of reduced platelet production in platelet kinetic studies the present study gives additional evidence for reduced platelet production by injured megakaryocytes. In a recent study\textsuperscript{13} we observed a significant inverse correlation between the PPR and the glycocalcin-index (GCI) in ITP patients. The GCI is used as a parameter of platelet destruction. A low PPR is associated with an elevated GCI. These findings reflect an increased release of GPIb-complex in the circulation, as a result of shedding of the receptor complex from megakaryocytes and/or platelets destructed in the bone marrow. In the present data a significant correlation was observed between the percentage of megakaryocytes in programmed cell death and the GCI, underscoring the view that the low PPR in ITP might be due to an increase in (para-)apoptosis of platelet producing megakaryocytes.

In summary, the present study demonstrates that in ITP patients a large majority of morphologically recognizable megakaryocytes show ultrastructural alterations compatible with (para-)apoptosis, which can be mimicked by incubating normal differentiated CD34\textsuperscript{+} cells with ITP plasma.

**Acknowledgments**

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References


Legends

Figure 1. Percentage of damaged ITP megakaryocytes in different stages of differentiation. Significantly more stage III (mature) megakaryocytes than stage I (immature) megakaryocytes showed the characteristic ultrastructural features of (para-)apoptosis.

Figure 2. Ultrastructure of megakaryocytes in normal control and ITP.
(A) Mature megakaryocyte (stage III) of healthy donor, showing the characteristic ultrastructure. The inset shows a higher magnification of intact mitochondria (arrowheads) and a normal demarcation pattern. Magnification: 3.000x, inset: 18.000x.
(B) Mature megakaryocyte of an ITP patient showing apoptotic characteristics. The inset shows a detail of the nuclear fragmentation (arrow) and chromatin condensation to the margins of the nucleus (arrowheads). Note that the cells shown in B and C are taken at a higher magnification than those in A. The shrinkage of the cells and rounding up has reduced cellular volume considerably. Magnification: 7.000x, inset: 18.000x.
(C) Mature megakaryocyte of another ITP patient showing ultrastructural features of para-apoptosis. In the inset details are shown of cytoplasmic vacuolization, partly as a result of swollen mitochondria (arrowheads) dilatation of endoplasmic reticulum and dilatation of demarcation membrane system. The thick, enlarged peripheral margin (open arrow) in the cytoplasm lacks organelles. Magnification: 7.000x., inset: 18.000x.

Figure 3. Ultrastructure of normal and ITP megakaryocytes.
(A) Normal megakaryoblast (stage I megakaryocyte) showing a lobulated nucleus (N). In the cytoplasm characteristic demarcation membrane system (asterisks) and normal mitochondria (arrowheads) can be found. Magnification: 3.000x.
(B) Megakaryoblast of an ITP patient in the process of para-apoptosis, N indicates nucleus. The cell has an intact enlarged peripheral margin (large arrow). Magnification: 4.500x.
(C) The inset of B shows a higher magnification with mitochondria, some slightly swollen (open arrowheads) and others with completely collapsed cristae, that appear as empty vacuoles (asterisks). The enlarged peripheral margin in the cytoplasm is free of organelles (open arrow). Magnification: 15.000x.

Figure 4. Immunohistochemical detection of activated caspase-3 in ITP megakaryocytes.
(A) Normal bone marrow megakaryocytes are negative for activated caspase-3. Some neutrophil granulocytes are positive (arrows). In (B) and (C) megakaryocytes of 2 ITP patients with extensive ultrastructural features of apoptosis are shown. These megakaryocytes stain positive for activated caspase-3. Magnification: 400x.

Figure 5. Correlation between percentage of damaged megakaryocytes and glycocalcin-index (GCI). The GCI is significantly correlated with the proportion of megakaryocytes showing morphological features of (para-)apoptosis (p=0.02; r=0.7).
Table 1. Characteristics of ITP patients

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<td>Negative</td>
</tr>
</tbody>
</table>

F=female; M=male; GCI= glycocalicin-index; GP=glycoprotein; ND=not determined; Plt=platelets; y=years. Normal values: Platelet count: 150-350 x 10⁹/L; GCI: 0.7 ± 0.2.
Figure 1

% (para) apoptotic precursors

- Immature
- Maturing
- Mature

p = 0.0008
Fig. 4
Figure 5
Ultrastructural study shows morphological features of apoptosis and para-apoptosis in megakaryocytes from patients with idiopathic thrombocytopenic purpura

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