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Early down-regulation of Bcl-x_L expression during megakaryocytic differentiation of thrombopoietin-induced CD34⁺ bone marrow cells in essential thrombocythemia

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A B S T R A C T

Background and Objectives. Essential thrombocythemia (ET) is a chronic myeloproliferative disorder with abnormal megakaryocyte/platelet production. Recent studies have found that Bcl-x_L, as a member of the bcl-2 family of proteins that inhibit apoptosis, is essential in megakaryocytic differentiation. In this study the expression of Bcl-x_L was evaluated during megakaryocytic differentiation in ET patients.

Design and Methods. To study the role of Bcl-x_L in megakaryocyte differentiation, we evaluated the effect of small interfering RNA (siRNA) on the expression of Bcl-x_L. CD34⁺ cells from patients with ET, chronic myeloid leukemia (CML), polycythemia vera (PV) and normal individuals were cultured in serum-free medium supplemented with thrombopoietin (TPO). Immunocytochemical staining and flow cytometric analysis were used to evaluate the Bcl-x_L expression during megakaryocytic differentiation of CD34⁺ cells.

Results. When exposed to si-Bcl-x_L, the percentage of K562 cells induced into megakaryocytes in 72 hours was lower than the corresponding percentage of control cells. CD41a⁺ cells from the three groups of patients and the control group were cultured. At day 10, the percentage of Bcl-x_L⁻ cells in CD41a⁺ cells from ET patients was 61.0±28.1%, which was significantly higher than that from patients with CML (2.5±20.9%), PV (33.6±10.0%) or control subjects (15.1±13.0%).]

Interpretation and Conclusions. These results demonstrate that Bcl-x_L is down-regulated early during *in vitro* differentiation of megakaryocytes from ET patients; this might reflect an early entry of megakaryocytes into a degenerating mature stage.

Key words: essential thrombocythemia, megakaryocyte, Bcl-x_L, apoptosis.

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Essential thrombocythemia (ET) is a chronic myeloproliferative disorder characterized by a persistent increase in circulating platelet count and excessive proliferation of megakaryocytes in bone marrow.¹ The overproduction of megakaryocytes and platelets occurs in the absence of a recognizable physiologic stimulus, since the serum level of thrombopoietin (TPO) in ET is normal or slightly higher than normal,² and is not significantly different from that in reactive thrombocytosis.³ Furthermore, no mutation in either the *TPO* gene or its receptor (*c-mpl*) has been identified in patients with ET.^{4,5} However, spontaneous megakaryocyte colony formation has been repeatedly observed in a majority of ET patients.⁶⁻⁸ Further studies found that mature megakaryocytes derived from cultured CD34⁺ cells display apoptotic features,^{9,10} perhaps as a reflection of cellular

senescence. However, because pro-platelet formation represents the terminal phase in the megakaryocytes' life cycle, parallel programmed cell death has been suggested at several levels.¹¹ It remains unknown whether apoptosis and proplatelet formation of mature megakaryocytes in patients with ET are dysregulated.

Bcl-x_L, a member of the bcl-2 family of proteins that inhibit apoptosis, is expressed in human hematopoietic cells and may regulate their differentiation.¹²⁻¹⁵ Recent studies showed that Bcl-x_L is up-regulated during megakaryocytic differentiation but is absent from senescent megakaryocytes.^{16,17} Both inactivation and overexpression of Bcl-x_L could decrease platelet formation.^{18,19} Therefore, Bcl-x_L should play an important role in megakaryocyte differentiation and apoptosis as well as in platelet formation. To explore the characteristics of megakary-

ocyte differentiation of ET patients, we studied the role of Bcl-x_L in the differentiation of megakaryocytes using small interfering RNA (siRNA) and examined the expression of Bcl-x_L in megakaryocytes derived from CD34⁺ bone marrow cells of ET patients by immunocytochemical staining and flow cytometric analysis.

Design and Methods

Patients

Eleven patients with ET, 7 patients with polycythemia vera (PV), 9 patients with chronic myeloid leukemia (CML) and 8 normal individuals were studied. The diagnosis of ET was based on the following criteria: platelet count $\geq 600,000/\mu\text{L}$; hematocrit $<40\%$, or normal red blood cell (RBC) mass (males $<36 \text{ mL/kg}$, females $<32 \text{ mL/kg}$); stainable iron in marrow or normal serum ferritin or normal RBC mean corpuscular volume; absence of Philadelphia chromosome or *bcr/abl* gene rearrangement; none or less than 1/3 of the marrow biopsy showing collagen fibrosis with neither marked splenomegaly nor a leukoerythroblastic reaction; no cytogenetic or morphologic evidence of a myelodysplastic syndrome; and no cause for reactive thrombocytosis.

The diagnoses of PV and CML were made according to Goldman²⁰ and Spivak.²¹ All CML patients and 4 PV patients had high platelet counts. The clinical and laboratory characteristics of the subjects are shown in Table 1.

Cell line and culture

K562 cell lines were routinely maintained in RPMI 1640 medium (GIBCO, Grand Island, NY, USA) containing 10% fetal calf serum, 100 units/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin at 37° in a humidified atmosphere containing 5% CO₂. The cells were seeded at an initial concentration of 2×10^5 cells/mL in DF12 medium (GIBCO) containing 1% fetal calf serum and grown in the presence of 50nM phorbol 12,13 dibutyrate (PDBu) (Sigma, USA). To assess megakaryocytic differentiation, cell surface expression of CD61 was measured by flow cytometry every 24 hours for 72 hours.

siRNA synthesis and transfection

siRNA was synthesized using the procedure described by Wilda²² (<http://www.mpibpc.gwdg.de/abteilung/100/105/siRNAuserguide.pdf>). The target sequence homologous to nt 48–69 of the Bcl-x_L mRNA sequence (GenBank accession number Z23115) was chosen (the target sequence: aagagaatcactaaccagaga, sense strand siRNA: gagaaucacuaaccagagatt, antisense strand siRNA: ucucugguagugauucuctt). The siRNA were pre-

Table 1. Characteristics of the patients and control subjects.

Characteristics	Patients with ET (n=11)	Patients with CML (n=9)	Patients with PV (n=7)	Normal subjects (n=8)
Age (years)				
Median	49.0	42	65	27
Range	20-61	17-67	49-74	16-53
Sex (M/F)				
	4/7	2/7	4/3	3/5
Hemoglobin (g/L)				
Median	138	111	194	122
Range	105-166	74-131	150-234	110-157
Leukocytes ($\times 10^9/\text{L}$)				
Median	7.0	22.9	7.39	6.7
Range	3.3-18.3	18.3-213.0	3.09-22.4	4.81-11.23
Platelets ($\times 10^9/\text{L}$)				
Median	744	900	363	297
Range	624-2430	597-2022	134-1025	200-349
Splenomegaly				
	4/11	5/9	3/7	0
BM studies				
BM hyperplasia	8/11	9/9	6/7	–
Megakaryocytic hyperplasia	10/11	9/9	4/7	–
Bone marrow fibrosis $\leq 1/3$	5/11	3/9	3/9	–
Therapy				
Aspirin	6/11	2/9	0	–
Hydroxyurea	8/11	9/9	5/7	–
α -interferon	4/11	1/9	0	–

Characteristics of the patients and control subjects*. *ET denotes essential thrombocythemia; CML, chronic myeloid leukemia; PV, polycythemia vera.

pared using the Silencer™ siRNA Construction Kit (Ambion, Austin, TX, USA) according to the instruction manual. To control the specificity of knockdown experiments, another siRNA duplex with one base pair mutated was also synthesized as a control. The siRNA duplexes were transfected according to the recommended procedure by using the Oligofectamine Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The cells were seeded at an initial concentration of 2×10^5 cells/mL in serum-free medium (Stemcell Technologies; USA) and 50nM PDBu (Sigma, USA) was added at the same time as transfection for differentiation induction. Megakaryocytic differentiation and apoptosis was assessed by the expression of CD61 every 24 hours for 72 hours after transfection.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from siRNA-treated cells and

control group cells (1×10^6) by using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, USA) every 24 hours for 72 hours. The reverse transcription reactions were done using Superscript First-Strand Synthesis System (Invitrogen Life Technologies) following the manufacturer's procedure. The complementary DNA generated was amplified with primers specific for human Bcl-x_L (5'-ggagctggtggttgactttc-3'; 5'-caaggtctaggtggtcatt-3'), and GAPDH (primer: 5'-accactcctccacctttg-3', 5'-gctgtagccaaattcggtg-3') was detected as an internal control. The PCR consisted of denaturation at 94°C for 45 seconds, annealing at 54.5°C for 30 seconds, and extension at 72°C for 30 seconds. After 35 cycles of amplification, the PCR products (562bp for Bcl-x_L and 96bp for GAPDH) were run on a 2% agarose gel, and stained with ethidium bromide.

Purification of bone marrow CD34⁺ cells

Ten to fifteen milliliters of bone marrow were obtained from patients and healthy donors after informed consent. Mononuclear cells were isolated from bone marrow using Ficoll-Hypaque (density, 1.077) (Union Stem Cell & Gene Engineering Company; China) density centrifugation. After two cycles of plastic adherence for 60 min, the cells were washed and suspended in phosphate-buffered saline (PBS, pH 7.4) containing 0.1% bovine serum albumin (BSA). The CD34⁺ cell fraction was positively isolated using anti-CD34 monoclonal antibody conjugated beads (Miltenyi Biotech; Germany) by the MiniMACS system (Miltenyi Biotech). The purity of the selected population was verified by flow cytometry using an anti-human CD34 antibody conjugated with fluorescein isothiocyanate (FITC; Becton Dickinson USA). Purity was consistently more than 95%. The numbers of isolated mononuclear cells and CD34⁺ cells were counted and the percentage of CD34⁺ cells in mononuclear cells was calculated.

Isolated CD34⁺ cells cultures

The CD34⁺ cells were cultured at a density of 5.0×10^4 cells/mL in serum-free medium (Stemcell Technologies; USA) in 24-well plates. Cultures were stimulated with only human recombinant TPO (100 ng/mL) kindly provided by Kirin Pharmaceutical Division (Japan). Cells cultured for up to 10 days were harvested for immunocytochemical staining and flow-cytometric analysis.

Immunocytochemical staining

Cells cultured for 10 days in serum-free culture with 100ng/mL TPO were cytocentrifuged onto microscope slides (1×10^5 cells slide), fixed in cold acetone, and stained with mouse anti-human Bcl-x_L monoclonal antibody (SouthernBiotech USA). Bcl-x_L was detected using a LSAB (Labeled Strept-Avidin-Biotin) kit (Zymed Laboratories, Inc. USA), with diaminobenzidine as a chromogen.

Cell labeling and flow cytometric analysis

Preparations of 2×10^5 mononuclear cells isolated from bone marrow were analyzed by flow cytometry Bcl-x_L expression. Cells fixed in 1% paraformaldehyde phosphate-buffered saline for 20 minutes on ice and permeabilized with 0.1% saponin (Sigma; USA) for 1 hour at 20°C were washed and incubated with FITC-conjugated mouse anti-human Bcl-x_L (Southern-Biotech; USA) for 30 minutes at 4°C. The cultured CD34⁺ cells were phenotyped for PE-CD41a and FITC-Bcl-x_L at day 10 by flow cytometry using a double-staining method. Collected cells were incubated with PE-conjugated mouse anti-human CD41a (Becton-Dickinson; USA) for 20 minutes followed by Bcl-x_L labeling with the same method as above. The labeled cells were resuspended after washing and analyzed within two hours using a FACScan flow cytometer (Becton-Dickinson; USA). Transfected K562 cells and control group cells were collected every 24 hours for 72 hours, and the level of apoptosis was assessed using combined FITC-annexin V and propidium iodide (PI) staining (Becton-Dickinson; USA) following the manufacturer's specifications. Binding of FITC-annexin V and PI was visualized on a FACScan flow cytometer (Becton-Dickinson; USA).

Statistical analysis

Student's t test was used to compare data from the ET patients with those of the other groups. The level of statistical significance was set at 0.05.

Results

Reduction of CD61⁺ cells by siRNA during differentiation induction of K562 cells

To investigate the function of Bcl-x_L in megakaryocytic lineage cells, we examined the change of CD61⁺ cells during K562 cell differentiation when exposed to si-Bcl-x_L. When K562 cells were cultured with 50nM PDBu, the percentage of CD61⁺ cells rapidly increased from $0.75 \pm 0.12\%$ to $67.4 \pm 1.7\%$ in 24 hours and maintained a high level of positivity for 72 hours. After exposure to si-Bcl-x_L, the percentage of CD61⁺ cells increased only slightly from $0.40 \pm 0.87\%$ to $8.6 \pm 0.31\%$ in 24 hours, and to 35.3% in 72 hours (Figure 1).

Meanwhile, we determined Bcl-x_L mRNA and protein expression during differentiation induction of K562 cells transfected by si-Bcl-x_L. After transfection, the expression of Bcl-x_L mRNA was assayed by RT-PCR every 24 hours for 72 hours. The Bcl-x_L transcription of transfected K562 cells was significantly less than that of the control group at all times (Figure 2A). To examine whether the Bcl-x_L protein was silenced correspondingly, its expression was measured by flow cytometry at the same time points after transfection and found to be sig-

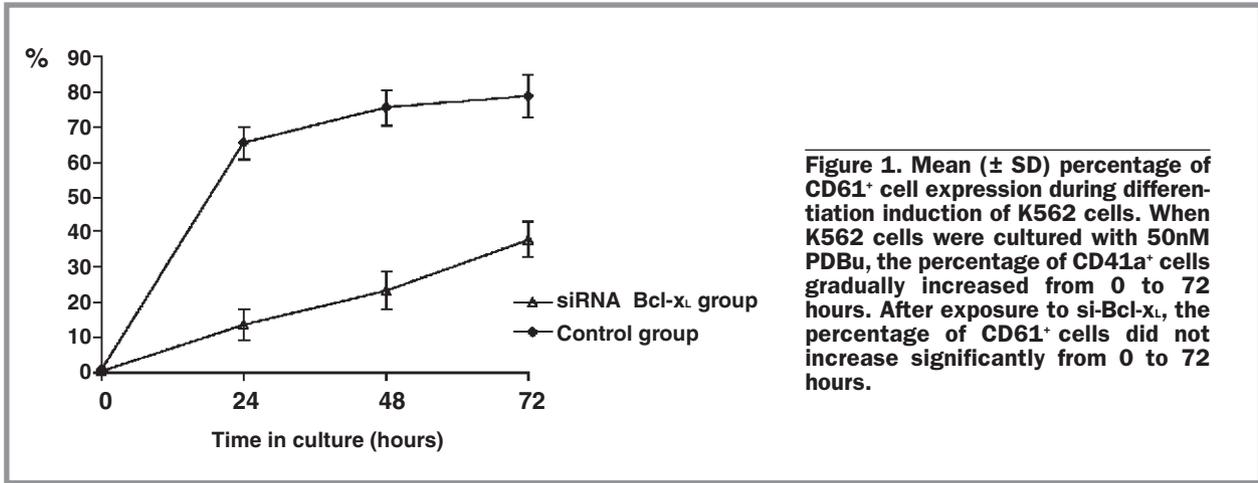


Figure 1. Mean (\pm SD) percentage of CD61⁺ cell expression during differentiation induction of K562 cells. When K562 cells were cultured with 50nM PDBu, the percentage of CD41a⁺ cells gradually increased from 0 to 72 hours. After exposure to si-Bcl-x_L, the percentage of CD61⁺ cells did not increase significantly from 0 to 72 hours.

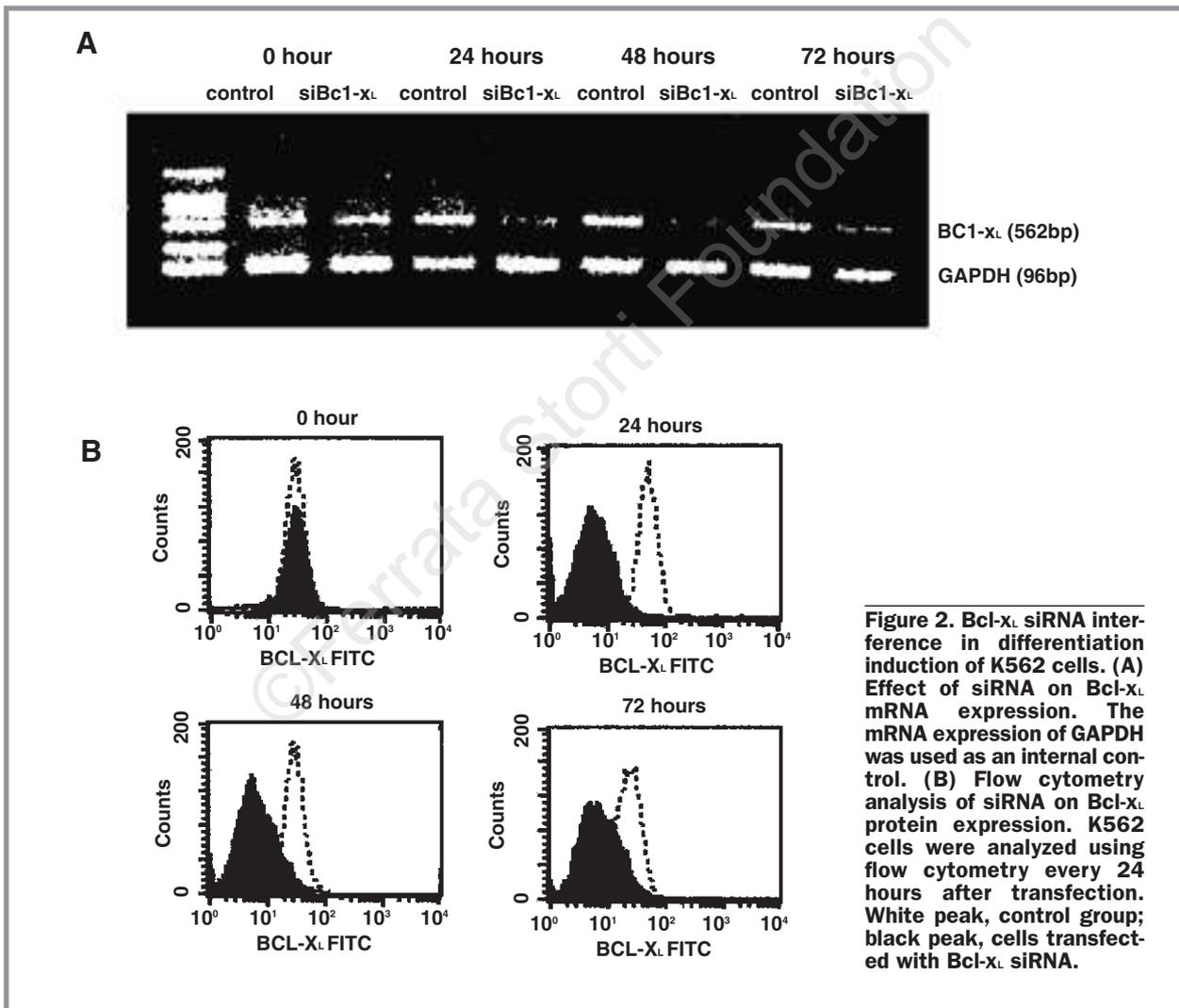


Figure 2. Bcl-x_L siRNA interference in differentiation induction of K562 cells. (A) Effect of siRNA on Bcl-x_L mRNA expression. The mRNA expression of GAPDH was used as an internal control. (B) Flow cytometry analysis of siRNA on Bcl-x_L protein expression. K562 cells were analyzed using flow cytometry every 24 hours after transfection. White peak, control group; black peak, cells transfected with Bcl-x_L siRNA.

nificantly reduced (Figure 2). Apoptosis of transfected cells was measured at the same time. The percentage of apoptosis in the control group was 1.3 \pm 0.1%, 15.5 \pm 1.2%, 20.2 \pm 2.3% and 22.1 \pm 3.4% at 0, 24, 48 and

72 hours, respectively; the corresponding percentages for the transfected cells were 1.2 \pm 0.2%, 19.3 \pm 1.4%, 25.5 \pm 2.5%, and 27.5 \pm 2.6%, respectively, being slightly higher than in the control group.

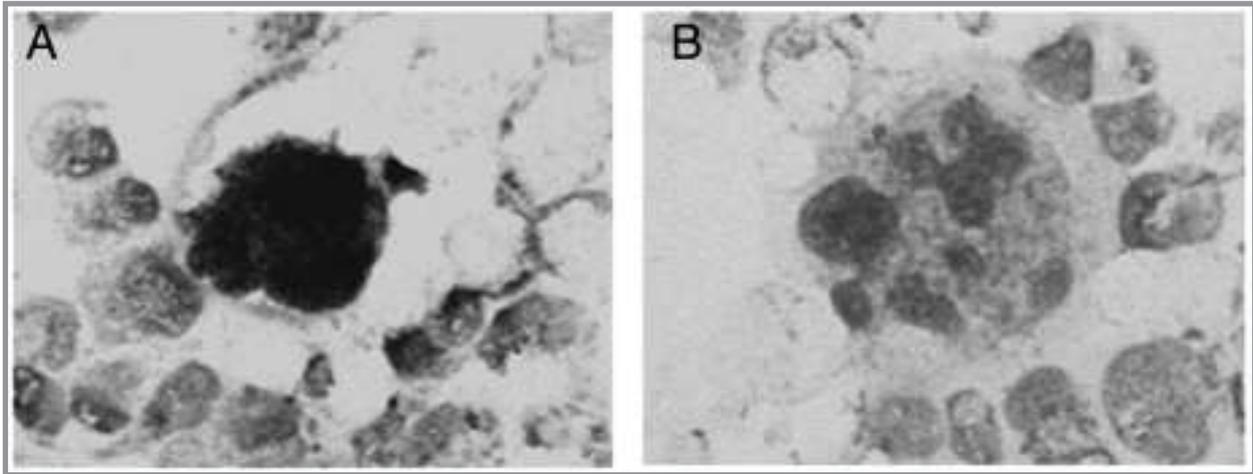


Figure 3. Immunocytochemical analysis of Bcl-x_L expression in megakaryocytes. At day 10 of culture in the presence of TPO, expression of Bcl-x_L was analyzed by an immunocytochemical procedure using a mouse antibody against human Bcl-x_L. A strong expression of Bcl-x_L in immature megakaryocytes (A) and decreased expression of Bcl-x_L in degenerating mature megakaryocyte (B) was shown. Original magnification $\times 200$.

Isolation and culture of CD34⁺ cells

The percentage of CD34⁺ cells sorted from bone marrow mononuclear cells of patients with ET, CML, PV and normal subject was $1.8 \pm 1.2\%$, $2.8 \pm 2.1\%$, 1.7 ± 1.8 and $1.5 \pm 1.2\%$, respectively. There was no significant difference between ET and the other groups. After 10 days of liquid culture, the number of cells from ET, CML, PV and the normal group increased by 6.9 ± 1.6 , 9.5 ± 2.6 , 6.7 ± 3.8 and 2.6 ± 1.1 fold, respectively.

Expression of Bcl-x_L in immature and mature megakaryocytes

Megakaryocyte apoptosis and platelet formation are presumably two concurrent and correlated events in healthy physiology. To explore the expression of Bcl-x_L in megakaryocytes in different phases, the CD34⁺ cells cultured in the presence of TPO for 10 days were stained for expression of Bcl-x_L using an immunocytochemical technique. A strong expression of Bcl-x_L was detected in immature megakaryocytes (Figure 3A). The expression of Bcl-x_L was obviously decreased in degenerating mature megakaryocytes (Figure 3B).

Expression of Bcl-x_L in marrow mononuclear cells and CD41a⁺ cells from CD34⁺ cells induced by TPO

The percentage of Bcl-x_L⁺ cells in mononuclear cells of bone marrow of patients with ET, CML, PV and normal subject was $45.6 \pm 26.7\%$, $62.3 \pm 22.8\%$, $53.1 \pm 19.3\%$ and $40.4 \pm 17.1\%$, respectively. The Bcl-x_L expression in patients with ET was similar to that in normal controls and lower than that in patients with PV or CML, but without the differences being statistically significant (Table 2). CD41a is a specific marker for megakaryocytes. We found that the percentage of CD41a⁺ cells after 10 days in serum-free culture with 100 ng/mL TPO was

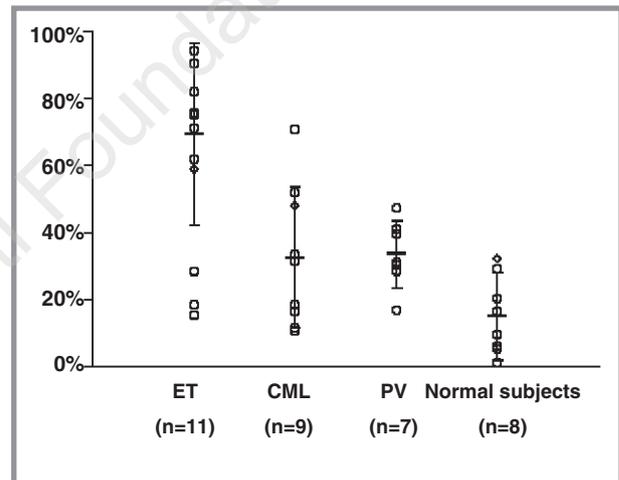


Figure 4. The percentage of Bcl-x_L negative cells in total CD41a⁺ positive cells. Mean (\pm SD) percentage of Bcl-x_L cells in total CD41a⁺ cells from induced CD34⁺ cells isolated from patients with ET, CML, PV and normal subjects in serum-free medium with TPO and cultured for 10 days. The results were obtained by flow cytometry.

$25.9 \pm 10.3\%$ for ET, $31.5 \pm 9.8\%$ for CML, $34.6 \pm 22.4\%$ for PV and $35.0 \pm 17.1\%$ for control subjects. The percentage of CD41a⁺ cells was lower in ET patients than in the other groups, but the differences were not statistically significant. The percentage of Bcl-x_L⁻ cells among CD41a⁺ cells of patients with ET was $61.0 \pm 28.1\%$, which was significantly higher than that of patients with CML ($32.5 \pm 20.9\%$), PV ($33.6 \pm 10.0\%$) and particularly the control subjects ($15.1 \pm 13.0\%$) (Figure 4). The percentage of Bcl-x_L⁻ cells in CD41a⁺ cells was $77.0 \pm 16.3\%$ in 3 ET patients without prior treatment, $62.2 \pm 28.5\%$ in 6 ET patients receiving aspirin, $75.5 \pm 13.6\%$ in 8 ET patients receiving hydroxyurea and $56.1 \pm 35.7\%$ in 3 ET

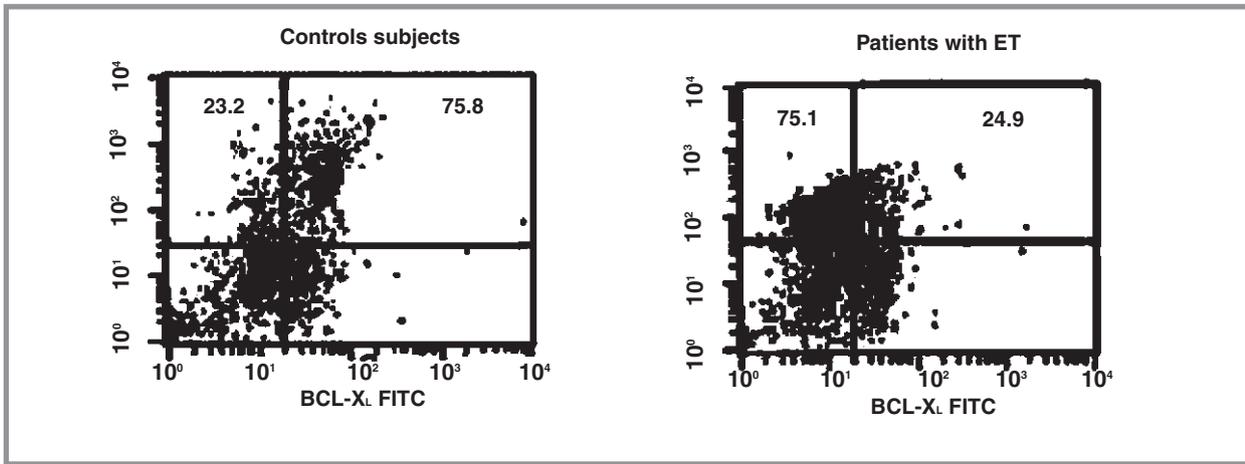


Figure 5. Analysis of Bcl-x_L expression in CD41a⁺ cells. Isolated CD34⁺ cells from patients with ET and control subjects were cultured in serum-free medium with TPO. On the 10th day, cells were double labeled with mouse anti-human Bcl-x_L and anti-human CD41a. In each graph, the percentage of Bcl-x_L⁺ cells (right) and of Bcl-x_L⁻ cells (left) in total CD41a⁺ cells are shown. The quadrants were drawn up on the basis of the results for isotype-matched negative controls. Both dot plots are from a representative experiment run in triplicate.

patients receiving α -interferon. Figure 5 shows representative flow cytometric data.

Discussion

At present the pathological mechanisms underlying ET are not well established. Exploring the molecular alterations should improve our understanding of ET. We evaluated the expression of Bcl-x_L during megakaryocytic differentiation of cells from ET patients.

Recent studies showed that apoptosis occurred predominantly in mature megakaryocytes rather than in immature megakaryoblasts. The kinetics of platelet release into culture supernatants correspond to the onset of apoptosis in these cells.^{9,23} Thus, platelet formation may be the consequence of apoptotic activation of megakaryocytes. Bcl-x_L, BAD and BAX are members of proapoptotic bcl-2 family of proteins. Sanz *et al.*²⁴ found that expression of Bcl-x_L decreased in hematopoietic progenitor cells induced to undergo

apoptosis while the expression of BAD and BAX remained unchanged. The expression of bcl-2 family members was further studied in megakaryocytes at various stages of differentiation. Sanz *et al.* reported that Bcl-x_L expression increased during the course of differentiation in megakaryocytes derived from cord blood CD34⁺ cells induced by TPO and in cells of the UT7 megakaryoblastic line induced by phorbol 12-myristate 13-acetate (PMA), while the expression of BAD and BAX remained unchanged at all culture times in a UT7 cell line similarly induced.¹⁷ According to Sanz, senescent megakaryocytes do not express Bcl-x_L, perhaps because most of the Bcl-x_L protein has already been released from the platelets leaving the senescent megakaryocytes with no detectable Bcl-x_L protein. Other investigators also found up-regulation of Bcl-x_L expression during megakaryocyte differentiation of K562 cells induced by PMA.¹⁶ It was shown that platelet counts dropped by 85% in mice after inactivation of Bcl-x_L. Upon cell culture *in vitro*, the rate of apoptosis in the mutant cells was significantly increased, suggesting

Table 2. The expression Bcl-x_L in CD41a⁺ cells after 10 days of culture.

	CD41a ⁺ cells (%)				CD41a ⁺ /Bcl-x _L cells (%)			The percentage of Bcl-x _L cells total CD41a ⁺ cells (%)			p
	No.	Median	Mean	Range	Median	Mean	Range	Median	Mean	Range	
ET	11	25.1	25.9	12.0-49.7	14.2	15.6	5.1-37.4	71.1	61.0	15.4-94.3	
CML	9	32.8	31.5	19.0-44.6	12.7	10.0	2.0-17.0	31.7	32.5	10.6-71.0	0.021
PV	7	34.9	34.6	7.0-66.2	9.9	11.9	2.4-21.9	31.3	33.6	17.1-47.3	0.025
Normal subjects	8	38.0	35.0	8.1-55.8	4.4	5.0	0.01-9.6	13.0	15.1	1.2-32.4	<0.001

ET denotes essential thrombocythemia; CML, chronic myeloid leukemia; PV, polycythemia vera. The difference between ET and the other three groups (p<0.05), as derived by Student's t test.

that Bcl-x_L deletion causes a defect in megakaryocyte maturation that prevents platelet release.¹⁸ One study of transgenic mice over expressing Bcl-x_L gene in megakaryocytes indicates that dysregulated, high-level expression of Bcl-x_L impairs the ability of the cells to fragment into platelets.¹⁹

In the present study, we found that although cell apoptosis increased after RNA interference, the magnitude of the increase in the percentage of apoptosis of transfected cells was smaller than the decrease in the percentage of CD61⁺ cells, suggesting that decreased expression of Bcl-x_L mRNA and protein could lead to a reduction of CD61⁺ K562 cells during differentiation induction when exposed to si-Bcl-x_L. We also observed strong expression of Bcl-x_L in immature megakaryocytes and an obviously decreased expression in degenerating mature megakaryocytes. These results suggest that increased Bcl-x_L expression might be essential to megakaryocyte maturation. The down-regulation of Bcl-x_L in mature megakaryocytes may be crucial to platelet formation.

Osada *et al.*²⁵ reported that mature megakaryocytes lose their ability to respond to TPO. Unlike immature megakaryocytes, whose viability depends on the presence of TPO, late-stage mature megakaryocytes undergo apoptosis even if maintained with a sufficient amount of TPO and fresh culture medium. This suggests that mature megakaryocytes undergo *spontaneous* apoptosis.¹¹ Down-regulation of the anti-apoptotic protein Bcl-x_L in late-stage megakaryocytes presumably triggers this apoptosis of mature megakaryocytes, resulting in the formation of platelets.

In the present study, we found that the percentage of CD34⁺ cells in bone marrow of ET patients was not significantly different from that in normal subjects. The expression of anti-apoptotic Bcl-x_L protein in bone marrow mononuclear cells of patients with ET was similar to that of normal subjects but lower than that of patients with CML or PV. After 10 days of culture, the percentage of Bcl-x_L⁻ cells in total CD41a⁺ cells of ET patients was much higher than that of other groups. Because Bcl-x_L⁻ megakaryocytes possibly represent late-stage mature megakaryocytes,¹⁷ it is reasonable to

believe that the accelerated maturation of megakaryocytes derived from CD34⁺ cells isolated from patients with ET. More late-stage mature megakaryocytes fragmenting into platelets might explain why the percentage of CD41a⁺ cells is slightly lower from ET patients than from normal controls after 10 days of culture. It has been shown that progenitor cells in ET are hypersensitive to TPO,²⁶ leading to a more rapid differentiation of CD34⁺ cells into megakaryocytes. However, mutations of the gene for the *c-mpl* receptor, the receptor for thrombopoietin, were not detected⁵ and the number and function of platelet thrombopoietin receptors are markedly decreased in patients with essential thrombocythemia.^{27,28} Hydroxyurea, aspirin or α -interferon might affect apoptosis of hematopoietic cells. Our study population included only three patients with ET without prior treatment, and the number of patients receiving each of the three medications was also very small. Furthermore, some patients used combinations of more than two of the medications, making it difficult to define whether therapy affects the result.

In conclusion, we demonstrate for the first time that in ET the anti-apoptotic Bcl-x_L protein is down-regulated early during megakaryocytic culture *in vitro* in serum-free medium with TPO. Our data suggest that dysregulation of Bcl-x_L expression may be responsible, at least in part, for the overproduction of megakaryocytes and platelets.

LZ did the experiments including cell cultures, FACS manipulation, data collection and analysis, and prepared the manuscript. HZ and AS purified the bone marrow CD34⁺ cells and established the cell cultures. SL and BL did the immunocytochemical staining of megakaryocytes. FY, YF and LZ participated in the design of siRNA synthesis and transfection. RY and ZCH contributed to the concept and design of the study, revised it, gave final approval, obtained funding and provided administrative support. All authors were involved in the revision of the manuscript and have approved the final version of the manuscript. The authors are listed in an order based on the contributions they made to the experiments. The authors reported no potential conflicts of interest.

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References

1. Tefferi A, Murphy S. Current opinion in essential thrombocythemia: pathogenesis, diagnosis, and management. *Blood Reviews* 2001;15:121-31.
2. Espanol I, Hernandez A, Cortes M, Mateo J, Pujol-Moix N. Patients with thrombocytosis have normal or slightly elevated thrombopoietin levels. *Haematologica* 1999;84:312-6.
3. Wang JC, Chen C, Novitsky AD, Lichter SM, Ahmed F, Friedberg NM. Blood thrombopoietin levels in clonal thrombocytosis and reactive thrombocytosis. *Am J Med* 1998;104:451-5.
4. Harrison CN, Gale RE, Wiestner AC, Skoda RC, Linch DC. The activating splice mutation in intron 3 of the thrombopoietin gene is not found in patients with non-familial essential thrombocythemia. *Br J Haematol* 1998;102:1341-3.
5. Kiladjian JJ, Elkassar N, Hetet G, Briere J, Grandchamp B, Gardin C. Study of the thrombopoietin receptor in essential thrombocythemia. *Leukemia* 1997; 11: 1821-6.
6. Han ZC, Briere J, Abgrall JF, Sensebe L, Nedellec G, Parent D, et al. Spontaneous colony formation of megakaryocyte progenitors (CFU-MK) in primary thrombocythaemia. *Acta Haematologica* 1987; 78:51-3.
7. Han ZC, Briere J, Abgrall JF, Sensebe L, Parent D, Guern G. Characteristics of megakaryocyte colony formation in normal individuals and in primary thrombocythemia: studies using an optimal cloning system. *Exp Hematol* 1989;17:46-52.
8. Han ZC, Bellucci S, Tenza D, Caen JP. Negative regulation of human megakaryocytopoiesis by human platelet factor 4 and

- beta thromboglobulin: comparative analysis in bone marrow cultures from normal individuals and patients with essential thrombocythaemia and immune thrombocytopenic purpura. *Br J Haematol* 1990;74:395-401.
9. Zauli G, Vitale M, Falcieri E, Gibellini D, Bassini A, Celeghini C, et al. In vitro senescence and apoptotic cell death of human megakaryocytes. *Blood* 1997;90:2234-43.
 10. Ryu KH, Chun S, Carbonnier S, Im SA, Kim HL, Shin MH, et al. Apoptosis and megakaryocytic differentiation during ex vivo expansion of human cord blood CD34⁺ cells using thrombopoietin. *Br J Haematol* 2001;113:470-8.
 11. Li J, Kuter DJ. The end is just the beginning: megakaryocyte apoptosis and platelet release. *Int J Hematol* 2001;74:365-74.
 12. Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, et al. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* 1993;74:597-608.
 13. Benito A, Grillot D, Nunez G, Fernandez-Luna JL. Regulation and function of Bcl-2 during differentiation-induced cell death in HL-60 promyelocytic cells. *Am J Pathol* 1995;146:481-90.
 14. Benito A, Silva M, Grillot D, Nunez G, Fernandez-Luna JL. Apoptosis induced by erythroid differentiation of human leukemia cell lines is inhibited by Bcl-XL. *Blood* 1996;87:3837-43.
 15. Silva M, Richard C, Benito A, Sanz C, Olalla I, Fernandez-Luna JL. Expression of Bcl-x in erythroid precursors from patients with polycythemia vera. *N Engl J Med* 1998;338:564-71.
 16. Terui Y, Furukawa Y, Kikuchi J, Iwase S, Hatake K, Miura Y. Bcl-x is a regulatory factor of apoptosis and differentiation in megakaryocytic lineage cells. *Experimental Hematology* 1998;26:236-44.
 17. Sanz C, Benet I, Richard C, Badia B, Andreu EJ, Prosper F, et al. Antiapoptotic protein Bcl-x_L is up-regulated during megakaryocytic differentiation of CD34⁺ progenitors but is absent from senescent megakaryocytes. *Exp Hematol* 2001;29:728-35.
 18. Wagner KU, Claudio E, Rucker EB 3rd, Riedlinger G, Broussard C, Schwartzberg PL, et al. Conditional deletion of the Bcl-x gene from erythroid cells results in hemolytic anemia and profound splenomegaly. *Development* 2000;127:4949-58.
 19. Kaluzhny Y, Yu G, Sun S, Toselli PA, Nieswandt B, Jackson CW, et al. BclxL overexpression in megakaryocytes leads to impaired platelet fragmentation. *Blood* 2002;100:1670-8.
 20. Spivak JL. Polycythemia vera: myths, mechanisms, and management. *Blood* 2002;100:4272-90.
 21. Goldman JM, Melo JV. Chronic myeloid leukemia: advances in biology and new approaches to treatment. *N Engl J Med* 2003;349:1451-64.
 22. Wilda M, Fuchs U, Wossmann W, Borkhardt A. Killing of leukemic cells with a BCR/ABL fusion gene by RNA interference (RNAi). *Oncogene* 2002;21:5716-24.
 23. Falcieri E, Bassini A, Pierpaoli S, Luchetti F, Zama L, Vitale M, et al. Ultrastructural characterization of maturation, platelet release, and senescence of human cultured megakaryocytes. *Anatom Rec* 2000;258:90-9.
 24. Sanz C, Benito A, Inohara N, Ekhterae D, Nunez G, Fernandez-Luna JL. Specific and rapid induction of the proapoptotic protein Hrk after growth factor withdrawal in hematopoietic progenitor cells. *Blood* 2000;95:2742-7.
 25. Osada M, Komeno T, Todokoro K, Takizawa M, Kojima H, Suzukawa K, et al. Immature megakaryocytes undergo apoptosis in the absence of thrombopoietin. *Exp Hematol* 1999;27:131-8.
 26. Axelrad AA, Eskinazi D, Correa PN, Amato D. Hypersensitivity of circulating progenitor cells to megakaryocyte growth and development factor (PEG-rHu MGDF) in essential thrombocythemia. *Blood* 2000;96:3310-21.
 27. Li J, Xia Y, Kuter DJ. The platelet thrombopoietin receptor number and function are markedly decreased in patients with essential thrombocythaemia. *Br J Haematol* 2000;111:943-53.
 28. Horikawa Y, Matsumura I, Hashimoto K, Shiraga M, Kosugi S, Tadokoro S, et al. Markedly reduced expression of platelet c-mpl receptor in essential thrombocythemia. *Blood* 1997;90:4031-8.