Deregulated expression of \textit{A1}, \textit{Bcl-2}, \textit{Bcl-x\textsubscript{L}}, and \textit{Mcl-1} antiapoptotic proteins and \textit{Bid}, \textit{Bad}, and \textit{Bax} proapoptotic genes in polycythemia vera patients

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Apoptosis deregulation might have a role in the pathophysiology of polycythemia vera (PV). This study evaluated \textit{Bcl-2} molecule expression in CD34\textsuperscript{+} cells and leukocytes in 12 PV patients. Gene expression was investigated by real time PCR using SybrGreen Quantitect kit and protein expression was evaluated by western-blotting. JAK2 V617F mutation was detected according to Baxter et al. (2005). CD34\textsuperscript{+} cells from PV patients presented higher levels of \textit{A1} and \textit{Mcl-1} expression (median: 22.6 and 5.2, respectively) in comparison with controls (0.9 and 0.5, p = 0.004 and p = 0.020); while \textit{Bcl-2} and \textit{Bcl-x\textsubscript{L}} expression decreased in PV patients (0.18 and 1.19) compared with controls (1.39 and 2.01, p = 0.006 and p = 0.020). CD34\textsuperscript{+} cells in PV patients showed an elevated \textit{Bid} expression (14.4) in comparison with healthy subjects (1.0; p = 0.002). Patients’ leukocytes showed an \textit{A1} augmentation (7.41, p = 0.001) and a reduced expression of \textit{Bax} (0.19; p = 0.040) and \textit{Bad} (0.2; p = 0.030). There was no correlation between JAK2 V617F allele burden and molecular expression. PV patients showed alterations in \textit{Bcl-2} members’ expression, which may interfere with control of apoptotic machinery and contribute to disease pathogenesis.


A desregulação da apoptose parece participar da fisiopatologia da policitemia vera (PV). Este estudo avaliou a expressão das moléculas da família Bcl-2 em células hematopoéticas CD34\textsuperscript{+} e leucócitos de 12 pacientes com PV. Foram realizados: a quantificação da expressão gênica por PCR em tempo real utilizando kit Sybrgreen Quantitect, avaliação da expressão de proteínas por \textit{western-blot} e detecção da mutação JAK2 V617F segundo Baxter et al. (2005). Células CD34\textsuperscript{+} dos pacientes com PV apresentaram maior expressão de \textit{A1} e \textit{Mcl-1} (mediana: 22.6 e 5.2, respectivamente) em comparação com controles (0.9 e 0.5, p = 0.004 e p = 0.020) e expressão de \textit{Bcl-2} e \textit{Bcl-x\textsubscript{L}} diminuída nestes pacientes (0.18 e 1.19) em relação aos controles (1.39 e 2.01, p = 0.006 e p = 0.020). Células CD34\textsuperscript{+} dos pacientes com PV mostraram expressão elevada de \textit{Bid} (14.4) em comparação aos controles (1.0; p = 0.002). Leucócitos dos pacientes mostraram aumento de \textit{A1} (7.41, p = 0.001) e expressão reduzida do \textit{Bax} (0.19; p = 0.04) e \textit{Bad} (0.2; p = 0.030). Não houve correlação entre percentagem de alelos JAK2 V617F mutados e expressão molecular. Pacientes com PV apresentaram alterações na expressão de moléculas Bcl-2 que podem interferir no controle da apoptose e contribuir para a patogênese da doença.

INTRODUCTION

The 2001 WHO classification considered that the major myeloproliferative disorders (MPDs) are: chronic myelogenous leukemia (CML), polycythemia vera (PV), essential thrombocytopenia (ET), and idiopathic myelofibrosis (IMF). The discovery of a mutation in the Janus kinase led to an understanding of the pathogenesis of the classic MPDs. In 2008 the WHO classification revisions reorganized the MPDs into myeloproliferative neoplasms (MPNs) (Tefferi, Vardinam, 2008; Wadleigh, Tefferi, 2010).

Polycythemia vera is a clonal disorder arising in a multipotent hematopoietic progenitor cell, exhibiting accumulation of normal red and white cells, platelets, and their progenitors in the absence of a definable stimulus. The hallmark of PV is the trilineage hematopoietic cell hyperplasia, but erythrocytosis is its most remarkable clinical manifestation (Spivak, 2002). According to the 2008 WHO guidelines, the major PV diagnostic criteria are: hemoglobin levels > 18.5 g/dL in men, 16.5 g/dL in women or other evidence of increased red cell volume and presence of the Janus kinase 2 mutation (JAK2 V617F), or other functionally similar mutation such as JAK2 exon 12 mutation. The minor PV diagnostic criteria are: bone marrow biopsy showing hypercellularity with trilineage myeloproliferation; serum erythropoietin (EPO) level below the normal reference range; in vitro endogenous erythroid colony (EEC) formation. Diagnosis de PV requires meeting either both major criteria and one minor criterion or the first major criterion and two minor criteria (Tefferi, Vardinam, 2008; Wadleigh, Tefferi, 2010).

The JAK2 V617F somatic mutation has been detected in 95% of PV patients and in 40 to 60% of ET or IMF patients. It is responsible for the activation of downstream target genes of JAK/STAT pathways, which seem to be linked to cell apoptosis impairment. Furthermore, there are indications that there may be an association between the mutation status, PV pathogenesis, and prognosis. The JAK2 V617F homozygous mutation could promote the development of a polycythemic phenotype, since homozygosis for the JAK2 mutation occurs in roughly 30% of patients with PV (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005; Levine et al., 2007).

Notwithstanding the new knowledge about PV and the description of new prognostic and diagnostic markers, the etiologic and pathogenesis of this disease remains unclear. The mechanisms involved in PV molecular basis are still elusive. It seems that PV is a myelocumulative disorder rather than a myeloproliferative disorder (Spivak, 2002). In this context, we postulate that apoptosis deregulation may have a role in its pathophysiology.

Apoptosis could be triggered by mitochondrial (intrinsic) and death receptor (extrinsic) pathways. In the intrinsic pathway, cytochrome c is released upon stimulation by a variety of cell-death triggers such as ultra violet light, cellular stress, chemotherapy, virus infection, cytokine deprivation, and DNA damage. Cytochrome c release, in turn, leads to activation of Apaf-1 (apoptosis protease activating factor-1), caspase-9, and caspase-3 (Borner, 2005; Jin; El Deiry, 2005; Yan, Shi, 2005).

The extrinsic apoptotic pathway involves the interaction of death receptors such as Fas and caspase-8, which lead to caspase-3 activation and cell death by apoptosis (Fulda; Debatin, 2006). Cell apoptotic machinery is mainly regulated by Bcl-2 family members (Borner, 2005), probably acting through the control of mitochondrial membrane permeability. Bcl-2 members could be divided in pro- (Bid, Bik, Bim, Bmf, Bok, Puma, Noxa, Bak, Bad, and Bax) and anti- apoptotic (A1, Bcl-2, Bcl-xL, Mcl-1, and Bcl-w) molecules (Borner, 2005; Sharper, Arnoul, Youple, 2004).

The association between PV pathogenesis and apoptosis has been poorly investigated but its role in other myeloproliferative diseases was well established. The A1 protein seems to have an important role in the pathogenesis in several B-cell malignancies and acute leukemias (Simpson et al., 2006; Brien et al., 2007). The literature reports the association of the overexpression of the Mcl-1 antiapoptotic molecule with solid tumors, chronic lymphocytic leukemia, and chronic myeloid leukemia pathogenesis and with resistance to chemotherapy (Kitada, Reed, 2004). Economopoulos et al. (2008) described an increase in A1 and Mcl-1 mRNA levels in blastic cells of myelodysplastic syndrome patients. Del Poeta et al. (2008) reported an increase in the expression of Bcl-2, Bcl-xL, and Mcl-1 in acute myeloid leukemia. Zhang et al. (2004) showed that CD34+ cells from ET patients presented a high expression of Bcl-xL in the initial differentiation phase of megakaryocytes induced by thrombopoietin.

Zeuner et al. (2006) reported that PV patients had abnormal erythropoiesis, EPO independence or hypersensitivity, and c-Flip overexpression, which could have contributed to apoptosis resistance and erythropoiesis alteration in some patients with the disease. Garçon et al. (2006) observed that only a sustained level of Bcl-xL is capable of giving rise to Epo-independent erythroid colony formation suggesting that, in PV patients, JAK2 V617F may induce EEC via the STAT5/Bcl-xL pathway; the JAK2/STAT5/Bcl-xL pathway seems to be crucial to erythropoiesis and implicated in cell proliferation and
survival. Deregulated erythropoiesis in PV involves EPO hypersensitivity and apoptosis resistance of erythroid precursor cells associated with abnormally increased activation of RAS-ERK and phosphoinositide-3 kinase-AKT pathways (Laubach et al., 2009).

These literature observations are leading investigators to focus on studies to understand how cell death deregulation is relevant to the neoplasm’s pathophysiology and to develop more effective therapeutic strategies. Therefore, the objective of this study was to investigate the expression of genes and proteins of Bcl-2 family members in CD34+ hematopoietic stem cells and leukocytes in PV patients and healthy subjects. The potential correlation between gene and protein expression with JAK2 mutation allele burden and lymphocyte resistance to apoptosis is also analyzed.

SUBJECTS AND METHODS

Patients and controls

Twelve PV patients, not treated, were evaluated in this study and included only when PV diagnosis was confirmed according to the 2008 WHO criteria. Their demographic and clinical-laboratorial parameters are described in Table I. The patients’ mean age was 63.4 years old (range 50-84 years). Three males and nine females were included in the study.

Peripheral blood samples were provided by healthy subjects recruited at the Faculty of Pharmaceutical Sciences, University of São Paulo (USP) at Ribeirão Preto. Marrow donors were obtained at the Bone Marrow Transplantation Unit of the University Hospital, Faculty of Medicine of Ribeirão Preto-USP. The samples were collected from 19 healthy subjects, whose mean age was 38.4 years old (range 14-54 years), 12 males and 7 females. Leukocytes were obtained from 14 healthy individuals, 5 males and 9 females, whose mean age was 54.4 years (range 41-80 years). The local ethics committee approved the study and all samples were collected after the subjects signed the Informed Consent Form.

Peripheral blood mononuclear cell and leukocyte separation

Peripheral blood mononuclear cells (PBMC) were isolated by the Ficoll-Hypaque method (Histopaque®-1077, Sigma, St. Louis, Missouri, USA) according to instructions by the manufacturer and as described by Boyum (1976).

Peripheral blood leukocytes were obtained from patients and controls using Haes-Steril® reagent (Voluven®, Frasenius Kabi, Campinas, Brazil). Briefly, the peripheral blood is mixed to Haes reagent in a 3:1 proportion, the mixture is decanted for 120 minutes at room temperature and leukocytes are recovered from the supernatant by centrifugation at 1800xg for 15 minutes. The cells were resuspended in PBS-ACD buffer (1mL) for counting and viability determinations with 0.4% Trypan Blue (Sigma-Aldrich Chemie, Steinheim, Germany).

### TABLE I - Demographic features, hematological parameters, and JAK2 V617F allele burden from PV patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Skin Color</th>
<th>Hb (g/dL)</th>
<th>Ht (%)</th>
<th>WBC (/μL)</th>
<th>Plt (x10⁹/L)</th>
<th>JAK2 Allele Burden (%)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>F</td>
<td>B</td>
<td>19.1</td>
<td>57.9</td>
<td>8.08</td>
<td>207</td>
<td>Negative</td>
</tr>
<tr>
<td>2</td>
<td>66</td>
<td>F</td>
<td>W</td>
<td>20.1</td>
<td>60</td>
<td>5.20</td>
<td>247</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>80</td>
<td>F</td>
<td>W</td>
<td>15.9</td>
<td>44.5</td>
<td>8.80</td>
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<td>90</td>
</tr>
<tr>
<td>4</td>
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<td>25.0</td>
<td>65</td>
<td>5.10</td>
<td>169</td>
<td>13</td>
</tr>
<tr>
<td>5</td>
<td>59</td>
<td>M</td>
<td>W</td>
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<td>60.2</td>
<td>20.00</td>
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<td>W</td>
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<td>25</td>
</tr>
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<td>W</td>
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<td>7.47</td>
<td>276</td>
<td>52</td>
</tr>
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<td>8</td>
<td>61</td>
<td>F</td>
<td>B</td>
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<td>50</td>
</tr>
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<td>9</td>
<td>69</td>
<td>F</td>
<td>W</td>
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<td>57.3</td>
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<tr>
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<td>84</td>
<td>F</td>
<td>W</td>
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<td>53.9</td>
<td>21.30</td>
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<td>W</td>
<td>17.4</td>
<td>53.5</td>
<td>5.50</td>
<td>229</td>
<td>Negative</td>
</tr>
<tr>
<td>12</td>
<td>72</td>
<td>F</td>
<td>W</td>
<td>17.9</td>
<td>56</td>
<td>8.85</td>
<td>299</td>
<td>55</td>
</tr>
</tbody>
</table>

Abbreviations: PV, polycythemia vera; M, male; F, female; Hb, hemoglobin; Ht, hematocrit; WBC, white blood cell count; Plt, platelets; W, white; B, black.
CD34+ hematopoietic stem cell isolation

Bone marrow mononuclear cells were separated by Ficoll-Hypaque centrifugation (Histopaque®-1077, Sigma, St. Louis, MO, USA) and CD34+ hematopoietic stem cells (HSC) were obtained by positive selection using an immunomagnetic column according to the manufacturer’s protocol (MACS, Milteny Biotec, Bergisch Gladbach, Germany).

After selection, the purity of CD34+ HSC was verified by flow cytometry. CD34+ HSC population’s purity was more than 91.80% in bone marrow transplantation donors and 87.06% in PV patients.

SDS-PAGE and western-blot analysis

Peripheral leukocytes (2x10^6/cell/L) from 4 healthy individuals and 4 PV patients were chosen to represent and investigate the results obtained by gene expression. The samples were chosen because we have more leukocyte aliquots from them than the others patients and because according to gene expression results they present a gene expression close to the median value of the evaluated molecule, which were washed once in ice-cold PBS, lysed directly in SDS-sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 2.5% β-mercaptoethanol) and boiled for 5 min. Samples were resolved under reducing conditions for two hours at 80 volts in SDS-polyacrylamide gels. Separated proteins then were blotted onto polyvinylidene difluoride (PVDF) membranes (Pierce, Rockford, IL, USA) at 500 mA for two hours. Blots were blocked over night in TBST buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween) containing 0.1% sodium azide and 5% non fat dried milk and then probed during two hours or overnight with an appropriate dilution of primary antibody (Santa Cruz, Santa Cruz, California, USA), or overnight with an appropriate dilution of secondary antibody conjugated to horseradish peroxidase and detected by enhanced chemiluminescence (GE Amersham). A semi-quantitative assessment of protein expression was performed by band relative densities detection using the AlphaEase software (Alpha Inotech, San Leandro, California, USA). Protein expressions were normalized by β-actin (Sigma-Aldrich, St. Louis, Missouri, USA) or β-tubulin (Sigma-Aldrich) endogenous expression. The densitometry methodology was applied to quantify the proteins, which were expressed by integrated density value (IDV). The protein expression results are presented as supplementary data.

Total RNA extraction, cDNA synthesis and real time PCR

Leukocyte and CD34+ HSC total RNAs were obtained according to the Trizol® protocol. RNA concentration and purity was determined by measuring fluorescence at 260 nm and 280 nm. One microgram of RNA was used for cDNA synthesis by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), according to manufacturer instructions. Real time PCR assay was carried out in cDNA to determine proapoptotic (Bad, Bak, Bax, Bid, Bik, Bim, and Bok) and antiapoptotic gene expression (Bcl-2, Bcl-x, Bcl-w, Mcl-1, and A1) using SybrGreen PCR Master Mix Kit (Applied Biosystems), specific primers (Table II), and ABIPrism 7700 (Applied Byosytems). Beta-actin gene was used as housekeeping genes and the results were given as 2^-ΔΔCt. Beta-actin was chosen as a housekeeping gene because its expression is high in hematopoietic cells and it has a minimal expression variability between the samples evaluated in this study.

JAK2/V617F mutation detection and allele burden calculation

The JAK2 V617F mutation was detected by real time PCR according to Baxter et al. (2005) and the results were presented as negative or allele burden of JAK2 positive mutation.

Apoptosis resistance assay

Mononuclear cells from patients and controls were cultured with 2μg/mL of phytohemagglutinin (Sigma, St. Louis, MO, USA) in complete RPMI 1640 medium (Sigma) supplemented with 10% bovine fetal serum, 10mM HEPES, 2mM L-glutamine, 100units/mL of penicillin and 100μg/mL streptomycin, at 37 °C in a 5% CO₂ during three days.

Then, the mononuclear cells were treated with apoptosis inducers such as: actinomycin (ACT, 1 and 5 μM, Sigma-Aldrich), cycloheximide (CHX, 25 and 50 μM, Sigma-Aldrich), cytarabine (ARA-C, 25μM, Pfizer®, Milan, Italy), etoposide (VP-16, 25μM, Bristol-Meyers-Squibb®, Mayaquez, Porto Rico), teniposide (VM-26, 25 μM, Bristol-Meyers-Squibb®), staurosporine (STS, 1 and 5 μM, Sigma-Aldrich) for twelve hours.

After incubation, mononuclear cells were recovered from the culture medium by centrifugation (240x g,
Deregulated expression of A1, Bcl-2, Bcl-xL, and Mcl-1 antiapoptotic proteins and Bid, Bad, and Bax proapoptotic genes

TABLE II - Primers sequences, amplicon size and their specific annealing temperature for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>5’→3’ sequence</th>
<th>Amplicon (bp)</th>
<th>AT (ºC)</th>
</tr>
</thead>
</table>
| Bax  | F: CCC TTT TGC TTC AGG GTT TC  
R: TCT TCT TCC AGA TGG TGA GTG | 500 | 56 |
| Bad  | F: CCG AGT GAG CAG GAA GAC TC  
R: GGT AGG AGC TGT GGC GAC T | 205 | 60 |
| Bak  | F: TTT TCC GCA GCT ACG TTT TT  
R: TGG TGG CAA TCT TGG TGA AGT | 249 | 60 |
| Bid  | F: GCT TCC AGT GTA GAC GGA GC  
R: GTG CAG ATT CAT GTG TGG ATG | 203 | 62 |
| Bik  | F: TCT GCA ATT GTC ACC GGT TA  
R: TTG AGC ACA CCT GCT CCT C | 193 | 60 |
| Bok  | F: TTT TCC GCA GCT ACG TTT TT  
R: TGG TGG CAA TCT TGG TGA AGT | 249 | 59 |
| Bim<sub>el</sub> | F: TCT GCA ATT GTC ACC GGT TA  
R: AAG ATG AAA AGC GGG GCT CT | 196 | 59 |
| Bcl-2 | F: ACG AGT GGG ATG GGG GAG ATG TG  
R: GGC GTA GCG GCG GGA GAA GTC | 204 | 67 |
| Bcl-w | F: AGT TCG AGA CCC GCT TCC  
R: CCC GTC CCC GTA TAG AGC | 308 | 60 |
| Bcl-x<sub>L</sub> | F: CTG AAT CGG AGA TGG AGA CC  
R: TGG GAT GTC AGG TCA CGT AA | 211 | 60 |
| A1   | F: GGC TGG CTC AGG ACT ATC  
R: CCA GTT AAT GAT GCC GTC | 227 | 50 |
| Mcl-1 | F: AGA AAG CTG CAT CGA ACC AT  
R: CC AGC TCC TAC TCC AGC AAC | 183 | 56 |
| Noxa | F: AGC TGG AAG TCG AGT GTG CT  
R: ACG TGC ACC TCC TGA GAA AA | 167 | 54 |
| Puma | F: GGA GCA GCA CCT GGA GTC  
R: TA CTG TGC GTT GAG GTC GTC | 156 | 60 |
| β-actin | F: GCC CTG AGG CAC TCT TCC A  
R: CCA GGG CAG TGA TCT CCT TCT | 192 | 58 |

Abbreviations: AT: annealing temperature; bp: base pair; F: forward; R: reverse.

10 minutes, 4 ºC), washed once with annexin buffer (10 mM HEPES pH 7.4; 150 mM NaCl; 5 mM KCl; 1 mM MgCl₂; 1.8 mM CaCl₂) and incubated for 20 minutes with 100 μL of Annexin-V FITC. Subsequently, ten thousand cells were acquired with 40 μL of a propidium iodide solution (50 μg/mL) and the data were analyzed by the DIVA software in a FACSCanto Flow Cytometer (Becton & Dickinson, San Jose, CA, USA). The results were expressed as percentage of apoptotic cells labeled with Annexin-V-FITC.

Statistical analysis

The Mann Whitney U-test was used to determine statistical differences in gene expression and protein expression between controls and PV patients. The Spearman’s correlation test was carried out to detect the associations of JAK2 V617F allele burden and gene expression, JAK2 V617F allele burden and percentage of apoptotic mononuclear cells, and gene expression and percentage of apoptotic mononuclear cells. The results were considered statistically significant when p values were < 0.05.

RESULTS

Gene expression of Bcl-2 family members in hematopoietic stem cells and peripheral leukocytes

CD34<sup>+</sup> HSC from PV patients presented higher levels of A1 and Mcl-1 expression (median: 22.6 and 5.2, respectively) in comparison with controls (median: 0.9 and 0.5, p=0.002 and p=0.008) (Figure 1; Supplementary Table S1). Bcl-2, Bcl-x<sub>L</sub>, Bax and Bad gene expressions...
were decreased in PV patients CD34+ HSC (0.18, 1.19, 6.76, 0.24) compared with controls (1.39, 2.01, 10.5 and 4.42; \( p = 0.006, p = 0.020, p = 0.010 \) and \( p = 0.010 \)) (Figures 1 and 2). CD34+ HSC of PV patients showed increased Bid expression (14.4) in relation to normal individuals (1.0; \( p = 0.002 \)) (Figure 2; Supplementary Table S1).

In contrast to controls (median \( A1 = 1.02, Bax = 1.25 \) and \( Bad = 1.18 \)), patient leukocytes showed an augmented \( A1 \) gene expression (7.41, \( p = 0.001 \)) (Figure 1) and a reduced expression of \( Bax \) (0.19; \( p = 0.020 \)) and \( Bad \) (0.2; \( p = 0.030 \)) (Figure 2). No significant differences between controls and PV patients were verified regarding expression of genes \( Bcl-w \) (Figure 1), \( Bik, Bim_{EL}, Bok, Noxa, \) and \( Puma \) (data not shown).

**FIGURE 1** - Antiapoptotic genes expressed in CD34+ HSC and leukocytes from PV patients and controls. Beta-actin gene was used as endogenous control. The genes relative expression were detected by real time PCR and results were given as \( 2^{-\Delta\Delta Ct} \). The horizontal lines represent the median of gene expression in each group analyzed. \( A1 \) and \( Mcl-1 \) are overexpressed (median: 22.6 and 5.2, respectively) in CD34+ HSC from PV patients in comparison with controls (median: 0.9 and 0.5, \( p = 0.002 \) and \( p = 0.008 \)). \( Bcl-2 \) and \( Bcl-x_L \) mRNA levels were decreased in PV patients’ CD34+ HSC (0.18 and 1.19) in relation to controls (1.39 and 2.01; \( p = 0.006 \) and \( p = 0.020 \)). Patients’ leukocytes also showed an \( A1 \) gene expression augmentation (7.41, \( p = 0.001 \)). There were no statistically significant differences in mRNA levels of \( Bcl-2, Bcl-x_L \) and \( Mcl-1 \) in patient leukocytes. No significant differences in \( Bcl-w \) expression between controls and PV patient CD34+ HSC and leukocytes were detected (\( p > 0.05 \)).
Deregulated expression of A1, Bcl-2, Bcl-xL, and Mcl-1 antiapoptotic proteins and Bid, Bad, and Bax proapoptotic genes

**Bad, Bim-EL, Bcl-xL, and A1 protein expression in peripheral leukocytes**

**FIGURE 2** - mRNA levels of *Bid, Bax* and *Bad* proapoptotic molecules in CD34+ HSC and leukocytes from PV patients and controls. Beta-actin gene was used as endogenous control. Gene relative expressions were detected by real time PCR and results were given as $2^{-\Delta\Delta C_t}$. The horizontal lines represent the median of gene expression in each group analyzed. PV patients’ CD34+ HSC showed *Bid* elevated expression (median=14.4, $p=0.002$) in relation to normal individuals. The *Bax* (median= 6.76, $p=0.010$) and *Bad* (0.24, $p=0.010$) expression were reduced in patients’ CD34+ HSC. The *Bad* (0.2; $p=0.030$) and *Bax* (0.19; $p=0.020$) levels were also decreased in PV patient leukocytes compared with controls.

We chose samples with gene expression values similar to median of *Bad, Bim_{EL}, Bcl-xL* and *A1*. Evaluation of protein expressions was performed to confirm gene expression data. The results showed high *Bcl-xL* and *A1* expression and low levels of *Bad* protein in PV patients in comparison to controls. *Bim_{EL}* expression was performed just because it is known that this molecule is susceptible to post-transcriptional regulation mechanisms. *Bim_{EL}* proteic
levels were also reduced in patients, PV leukocytes having only 35% of Bim-EL normal values. Bad protein expression was significantly lower in PV patients in relation to controls, which were 60% higher. PV patients had triple Bcl-xL expression compared with control leukocytes. Hence, Bcl-xL and Bim-EL protein expression are different; they were opposite from that observed in PV gene expression profiles, whereas A1 and Bad data supported the mRNA level results.

**JAK2 V617F mutation allele did not correlate with gene expression of Bcl-2 family members**

No correlation was detected between JAK2 V617F allele burden and antiapoptotic protein (A1, Bcl-2, Bcl-w, Bcl-xL, and Mcl-1) or proapoptotic gene expression (Bad, Bak, Bax, Bid, Bik, Bim-EL, and Bok) in CD34+ HSC or leukocytes (Supplementary Table S2).

**Mononuclear cells from PV patients were resistant to apoptosis**

Concerning assessment of mononuclear cell apoptosis, PV patients cells were more resistant to ACT-D 1 µM (median = 29.95; p=0.015), CHX 25 µM and 50 µM (median = 5.53 and 19.80; p=0.002 and 0.001), STS 1 µM (median = 6.59; p<0.001), ARA-C 25 µM (median = 2.76; p<0.001), VM26 25 µM (median = 22.80; p<0.003) and VP16 25 µM (median = 12.25; p<0.001) than controls (median = 39.70) (Figure 3).

**JAK2 mutation allele burden is not associated with the percentage of mononuclear cells apoptosis in PV patients**

No significant correlation between the percentage of JAK2 V617F allele burden and mononuclear cell apoptosis
induced by STS, CHX, ACT-D, ARA-C, VP16 and VM26 (data not shown) was found in PV patients.

Gene expression of Bcl-2 family members is linked to mononuclear cells’ resistance to apoptosis in PV patients

A negative correlation was detected between the percentage of PV cells apoptosis induced by ACT-D (1uM), CHX (50 uM) and ARA-C (25 uM) and A1 mRNA levels (r=-6527, p=0.044); r=-.527; p=0.033 and r=-.900; p=0.041, respectively); ACT (5 uM), STS (1 uM) and CHX (50 uM) and Bcl-2 (r=-.857; p=0.012; r=-.691; p=0.038 and r=-.9; p=0.040, respectively); ARA-C and Mcl-1 (r=-.728, p=0.015). Another interesting result was that Bad expression was positively correlated with cell percentage of apoptosis induced by VM26 (r=0.7857; p=0.024). Thus, low levels of Bad gene are related to a decrease in cell apoptosis percentage mediated by VM26.

These results indicate that death resistance to apoptotic inducers in PV patients is linked to A1, Mcl-1 and Bcl-2 antiapoptotic protein and Bad proapoptotic genes levels.

DISCUSSION

Several studies have addressed the clinical and laboratorial features in chronic MPDs these diseases, but the cellular and molecular mechanisms involved in the pathogenesis of MPD cells are still unclear.

Altered apoptosis regulation seems to contribute to the physiopathology of several diseases like neoplasia, autoimmune disorders, neurodegenerative disorders, myelodysplastic syndromes, and acute and chronic leukemia (Del Poeta et al., 2008; Economopoulou et al., 2008; Yan, Shi, 2005). The association of apoptosis-related proteins deregulation with PV pathogenesis, examined in the present study, has been scarcely explored elsewhere.

A differential profile of Bcl-2 family members’ genes expression was verified in PV patients in relation to controls. Data suggest disequilibrium in anti- and proapoptotic gene expressions related to the intrinsic pathway, which is represented by a significant increase in A1 and Mcl-1 expression in marrow stem cells and A1 in peripheral blood and by a reduction in mRNA levels of Bad and Bax molecules. In addition, the low expression of these proapoptotic molecules is related to drug-induced mononuclear cells resistance, such as topoisomerase inhibitors, cytarabine, and cycloheximide. Furthermore, our results indicate a potential augmented survival in CD34+ HSC and peripheral leukocytes, leading to promoted clonal expansion and perpetuation of myeloid lineage cells characteristic of the disease.

The literature shows that inhibition of apoptosis by A1 and Mcl-1 genes may occur through inactivation of the Bak protein. Conformational change of Bak and mitochondrial MOMP formation are prevented by the A1 and Mcl-1 molecules, blocking release of cytochrome C and other apoptogenic factors, not activating caspase-3 and suppressing apoptosis (Chipuk, Green, 2008; Thomadaki, Scorilas, 2006). It is possible to speculate that the patients included in the present study showed cell resistance to apoptosis through this mechanism, as stem cells or leukocytes showed elevation of A1 and Mcl-1 and decrease of Bad, Bim-, and Bax in comparison with controls.

Another finding is related only to the increase in the Bcl-xL protein (supplementary data), a potent antiapoptotic molecule, which may contribute to a phenotype with a higher survival and myeloaccumulation of myeloid cells in PV patients. The findings in this investigation also include decreased levels of Bcl-2 and increased Bid, a paradox, since these molecules promote apoptosis potentiating and not blocking. It is possible that these molecules do not play a relevant role in the physiopathology of PV.

In addition, since apoptosis unleashing is dependent on the interaction and equilibrium of several proteins of the Bcl-2 family (Chipuk; Green, 2008), the increase of A1, Mcl-1, and Bcl-xL antiapoptotic molecules and the decreased expression of Bax and Bad would be sufficient to prevent cellular death in PV.

A1 protein is abundantly expressed in bone marrow cells, spleen, and lung in several lineages originating in thymus, testicle, and intestine cancers (Thomadaki; Scorilas, 2006). It has an important role in high risk leukemia and other B cell malignancies. This molecule is a direct target in the transcription of NF-κB, which is involved in myeloid and lymphoid differentiation, in apoptosis resistance by different stimuli, like the activation of death receptors (Simpson et al., 2006; Brien et al., 2007).

Mcl-1 protein probably participates in the immune system development and hematopoiesis. In hematopoietic cells the protein is regulated by ERK, JAK/STAT, p38, MAPK, P13K/AKT signaling and phosphorylation of elf2α and E2F1 and may be phosphorylated by JNK, GSK-3α/β, a cyclin-dependent kinase (Inoue et al., 2008). It may be inactivated and/or degraded by a caspase mediated cleavage or other proteins (ubiquitin-proteasome pathway) (Wuilleume-Toumi et al., 2006). In chronic myeloid leukemia (Aichberger et al., 2005), expression of Mcl-1 protein is frequently associated with resistance to chemotherapy and disease progression. In multiple myeloma (MM), chemoresistance is also caused by apoptosis deregulated pathways, with over expression of Mcl-1 and
an insignificant expression of Bcl-2 (Wuillème-Toumi et al., 2005). Increase in Mcl-1 levels is correlated with disease severity, turning the molecule into a potential therapeutic target in MM.

Mcl-1 protein neutralizes the proapoptotic function of Bim and prevents activation of death receptors (Wuillème-Toumi et al., 2006). Regulating the Mcl-1 expression is important to break the Bim/Mcl-1 complex and induce Bim activation in HMCL-human myeloma and Hek-293-human embryonic kidney cell lines. It has been shown recently that Bim, a direct activator of Bax/Bak, protects the mitochondrial membrane permeability dependent on the pair of proteins by loosing its activity (Chipuk, Green, 2008). Thus, increased levels of this protein interfere with mitochondrial membrane permeability and activation of apoptosis extrinsic pathway, both contributing to the resistance profile to apoptosis shown by PV patient cells.

The results in this study suggest that A1 and Mcl-1 contribute to apoptosis resistance by means of direct or indirect Bax inhibition, by blocking the activation of Bad, tBax or Bim molecules and/or compromising the activation equilibrium of other proapoptotic proteins such as Bok and Noxa, which inhibit cytochrome liberation and caspase 3 activation and suppress the apoptotic cascade.

Increased amounts of Bcl-xL protein in PV patients’ leukocytes confirm the data in the literature. Bcl-xL protein is increased in erythroid precursors and provokes cell accumulation in PV (Silva et al., 1998). Other reports consider the molecule as the most potent antiapoptotic agent, its high levels being partially responsible for cell apoptosis resistance in patients with chronic myeloid leukemia (CML). It seems that Bcl-xL instead of Bcl-2 mediates the most prominent antiapoptotic effect of Bcr-abl tyrosine kinase in CML patients. Amarante-Mendes et al. (1998) showed that treatment with antisense oligonucleotides targeted to Bcl-xL downregulates the expression of Bcl-x and increases Bcr-Abl positive cell susceptibility to apoptosis induced by staurosporine in CML.

The sensitive profile of PV mononuclear cells to several apoptogenic drugs (actinomycin D, staurosporine, cycloheximide, cytarabine, etoposide, and teniposide) was also evaluated in this research. Substances with diverse mechanisms of action were employed to induce cell death, aiming to analyze cell apoptotic machinery at a functional level and its relation to the expression profile of molecules of the Bcl-2 family members.

Our results suggest that PV patients’ mononuclear cells resistance to cell death is related to alterations in the gene expression profile of the molecules regulating apoptosis intrinsic pathway.

The percentage of JAK2 mutated alleles reflects the tumoral load in the disease and is linked to worse prognosis (Passamonti, Rumi, 2009; Finazzi, Barbui, 2008; Gangat et al., 2008; Vannucchi et al., 2007). Lack of correlation between the JAK2 (V617F) mutation and the genes in the study does not exclude the relation between the expression of these genes to the activity of JAK2-mutated tyrosine kinase or the presence of different mutations in JAK2 or other kinases. It is worth emphasizing that in the present study we only evaluated the JAK2 V617F mutation in the exon 14 in PV patients, we did not measure JAK2 activity or detect other mutations associated with PV pathogenesis, such as MPL or JAK2 mutation in the exon 12 (Vainchenker, W. et al., 2011).

Nussenzveig et al. (2007) showed that events related to the JAK2 V617F mutation may not give rise to PV, but identification of the genetic lesion is essential to the development of therapeutic strategies, since inhibitors of active, constitutive tyrosine kinase JAK2 are not sufficient to eradicate the disease. The authors correlated the frequency of the JAK2/V617F mutant allele with clonality and presence of endogenous erythroid colonies with allele homozygosis and demonstrated that PV is not only provoked by the mutation, which seems to be a secondary genetic event. However, the primary molecular lesion responsible for the clonal hemapoiesis was not defined.

JAK2 inhibitors have been explored as the most efficient therapeutic agents in MPDs but some patients do not respond to therapy and some are JAK2 negative. Thus, the importance of describing new therapeutic targets, as inserted in this context, for Bcl-2 family members.

Apoptosis resistance to stimuli by drugs such as actinomycin D, cycloheximide, cytarabine, teniposide, and staurosporine correlates with high levels of A1, Mcl-1 and low expression of Bad and Bax.

Actinomycin D acts inhibiting the synthesis of RNA and it seems to potentiate Fas-mediated apoptosis, prevent binding of NF-κB to DNA (Wang et al., 2007), decrease the expression of the extrinsic antiapoptotic pathway, c-Flip, and stimulate Puma and Noxa expression of p53 and APAF-1 pathway (Kalousek et al., 2007). ACT-D and cycloheximide induce apoptosis via the TRAIL protein or TNF-α receptor. Both drugs are able to reduce RIP expression, a kinase having a death domain involved in TNF-R1 signaling that mediates NF-κB activation, which, in turn, may be antiapoptotic. They also reduce XIAP expression, a member of the IAP family responsible for the regulation of the common apoptosis pathway by inactivating effector caspases (Fulda et al., 2000). ACT-D and STS require Bax and Bak to induce cytochrome c liberation to take the cell to its death. These data indicate.
Deregulated expression of A1, Bcl-2, Bcl-xL, and Mcl-1 antiapoptotic proteins and Bid, Bad, and Bax proapoptotic genes

that PV patients’ lymphocyte resistance to apoptosis induced by STS and ACT-D may be a consequence of low levels of Bax mRNA.

Cytarabine, a cytotoxic antineoplastic agent shows cell phase specificity, initially destroying cells that synthesize DNA and, under certain conditions, blocking progression of phase G1 to phase S. The mechanism of action is not completely known, but the drug seems to inhibit DNA polymerase during replication and damage DNA, thus activating the p53 pathway. Other antineoplastic drugs tested in this study were etoposide (Vepesid) and teniposide, both inhibitors of topoisomerase II; their main effects may be related to rupture of DNA double loop by enzyme interaction or production of free radicals; the drugs increase TRAIL receptor expression and stimulate p53 pathway by damaging DNA (Karpinish et al., 2002; Li et al., 2006).

The percentages of cell death detected in PV patients suggest mononuclear cells resistance to most drugs and indicate that cells may show alterations in at least one of the regulatory mechanisms of cellular apoptosis. The decreased cell susceptibility to death may be associated with cell accumulation in PV, being partially responsible for the morbidity/mortality of patients with the disease.

In conclusion, high levels of A1 and Mcl-1 and a reduced expression of antiapoptotic Bcl-2 and Bcl-xL and proapoptotic Bad and Bax genes in CD34 HSC were detected in PV patients. In leukocytes, A1 expression was elevated and Bad and Bax mRNA levels were decreased. Thus, overexpression of the above antiapoptotic genes and a low mRNA level of Bax and Bad proapoptotic molecules may be associated with PV pathogenesis.

Additionally, a low expression of Bad and Bax proapoptotic genes also seems to be linked to PV lymphocyte resistance to apoptosis and contribute to the disease physiopathology.

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REFERENCES


Deregulated expression of A1, Bcl-2, Bcl-xL, and Mcl-1 antiapoptotic proteins and Bid, Bad, and Bax proapoptotic genes


SUPPLEMENTARY MATERIAL

TABLE S1 - Percentiles 25 and 75 of gene expression $2^{\Delta\Delta Ct}$ from Bcl-2 family members in hematopoietic stem cells and peripheral leukocytes in PV patients and controls

<table>
<thead>
<tr>
<th>Genes</th>
<th>CD34+ HSC</th>
<th>Peripheral Leukocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls P25 - P75</td>
<td>PV Patients P25 - P75</td>
</tr>
<tr>
<td>A1</td>
<td>0.3596-4.212</td>
<td>4.068-83.69</td>
</tr>
<tr>
<td>Bad</td>
<td>0.0003-9.596</td>
<td>0.005-7.556</td>
</tr>
<tr>
<td>Bax</td>
<td>0.007-9.659</td>
<td>0.0009-8.017</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>0.3801-2.539</td>
<td>0.0277-0.7124</td>
</tr>
<tr>
<td>Bcl-x^L</td>
<td>0.4549-1.704</td>
<td>0.1067-42.67</td>
</tr>
<tr>
<td>Bcl-x^L</td>
<td>1.607-3.491</td>
<td>0.5042-1.457</td>
</tr>
<tr>
<td>Bid</td>
<td>0.4190-3.681</td>
<td>4.015-26.11</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>0.3248-1.044</td>
<td>1.803-12.33</td>
</tr>
</tbody>
</table>

HSC: Hematopoietic Stem Cells; PV: Polycythemia Vera; P: Percentile.
### FIGURE S1

**A1, Bcl-xL, Bad, and Bim-EL** protein expression determined in peripheral leukocyte lysates from four PV patients and four controls. *A1, Bcl-xL, Bad,* and *Bim-EL* expression were assessed by immunoblot with specific antibody. Protein loading is controlled by beta-actin or tubulin. Results were shown by blotting figures and protein expression quantification detected by densitometry and expressed by the ratio of target protein integrated density values (IDV) and beta-actin or tubulin IDV. *Bcl-xL* and *A1* presented a high expression while *Bad* protein levels were decreased in PV patients in comparison with controls. *Bim-EL* protein levels are also reduced in PV patient leukocytes being only 35% of the values in normal individuals.

### TABLE S2

**Correlation between JAK2 V617F allele burden and genes expression of Bcl-2 family members in PV patients**

<table>
<thead>
<tr>
<th>Genes</th>
<th>CD34+ HSC r</th>
<th>p</th>
<th>Peripheral Leukocytes r</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A1</em></td>
<td>0.1763</td>
<td>p&gt;0.05</td>
<td>-0.03647</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td><em>Bad</em></td>
<td>-0.3465</td>
<td>p&gt;0.05</td>
<td>-0.09726</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td><em>Bak</em></td>
<td>-0.3404</td>
<td>p&gt;0.05</td>
<td>-0.08113</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td><em>Bax</em></td>
<td>-0.2979</td>
<td>p&gt;0.05</td>
<td>-0.03647</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td><em>Bcl-2</em></td>
<td>-0.6565</td>
<td>p&gt;0.05</td>
<td>0.1216</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td><em>Bcl-w</em></td>
<td>-0.1398</td>
<td>p&gt;0.05</td>
<td>0.3536</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td><em>Bcl-xL</em></td>
<td>-0.2675</td>
<td>p&gt;0.05</td>
<td>0.01824</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td><em>Bid</em></td>
<td>-0.2614</td>
<td>p&gt;0.05</td>
<td>-0.2349</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td><em>Bik</em></td>
<td>-0.1094</td>
<td>p&gt;0.05</td>
<td>-0.04863</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td><em>Bim-EL</em></td>
<td>-0.2675</td>
<td>p&gt;0.05</td>
<td>-0.1702</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td><em>Bok</em></td>
<td>-0.2371</td>
<td>p&gt;0.05</td>
<td>-0.1885</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td><em>Mcl-1</em></td>
<td>0.1216</td>
<td>p&gt;0.05</td>
<td>0.3161</td>
<td>p&gt;0.05</td>
</tr>
</tbody>
</table>

HSC: Hematopoietic stem cell.