

## EXPRESSION OF Bcl-x IN ERYTHROID PRECURSORS FROM PATIENTS WITH POLYCYTHEMIA VERA

MAITE SILVA, PH.D., CARLOS RICHARD, M.D., ADALBERTO BENITO, PH.D., CRISTINA SANZ, PH.D., IGNACIO OLALLA, M.D., AND JOSÉ LUIS FERNÁNDEZ-LUNA, PH.D.

### ABSTRACT

**Background** Deregulating the expression of Bcl-x<sub>L</sub>, an inhibitor of apoptosis, in an erythropoietin-dependent erythroblast cell line averts apoptosis induced by the withdrawal of erythropoietin. Since in polycythemia vera an abnormal clone of erythroid progenitors is independent of erythropoietin, we investigated whether the endogenous expression of Bcl-x<sub>L</sub> was deregulated in these cells.

**Methods** Erythroid colonies from patients with polycythemia vera and normal subjects were cultured in the presence and absence of erythropoietin and assessed by immunocytochemical and flow-cytometric analysis with anti-Bcl-x antibodies that recognize the two species of Bcl-x (Bcl-x<sub>L</sub> and Bcl-x<sub>S</sub>). Reverse-transcriptase-polymerase-chain-reaction analysis was used to determine which one of the two species was responsible for anti-Bcl-x staining. Bone marrow mononuclear cells from 8 healthy bone marrow donors, 14 patients with polycythemia vera, 19 patients with other myeloproliferative syndromes, and 12 patients with secondary erythrocytosis were analyzed by flow cytometry with antibodies against Bcl-x and glycophorin A, an erythroid marker.

**Results** Erythroid cells from patients with polycythemia vera survived in vitro without erythropoietin, and this finding correlated with the expression of Bcl-x protein (Bcl-x<sub>L</sub> messenger RNA was the main species of Bcl-x found), even in mature erythroblasts that normally do not express Bcl-x. The mean ( $\pm$ SD) percentage of cells positive for both glycophorin A and Bcl-x in the 14 patients with polycythemia vera ( $21.8 \pm 3.6$  percent) was significantly higher than that in 8 normal donors ( $6.62 \pm 1.58$  percent), 12 patients with secondary erythrocytosis ( $6.87 \pm 1.95$  percent), 9 patients with essential thrombocythemia ( $3.81 \pm 0.97$  percent), and 10 patients with chronic myeloid leukemia ( $2.7 \pm 0.41$  percent).

**Conclusions** Deregulated expression of Bcl-x may contribute to the erythropoietin-independent survival of erythroid-lineage cells in polycythemia vera and thereby contribute to the pathogenesis of this disease. (N Engl J Med 1998;338:564-71.)

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**P**OLYCYTHEMIA VERA is a clonal hematopoietic disorder of stem cells characterized by erythrocytosis and, in most cases, granulocytosis and thrombocytosis. The overproduction of erythrocytes occurs in the absence of a recognizable physiologic stimulus, since serum levels of erythropoietin are normal or lower than normal.<sup>1,2</sup> In polycythemia vera there are normal erythropoietin-

dependent erythroblasts, but a fraction of the erythroid progenitors are independent of erythropoietin.<sup>3</sup> Normally, erythropoietin is essential for the survival and maturation of committed colony-forming unit-erythroid progenitors and early erythroblasts.<sup>4-6</sup>

We have shown that Bcl-x<sub>L</sub>, a member of the Bcl-2 family of proteins that inhibit apoptosis,<sup>7</sup> is expressed in human erythroleukemia cell lines and may regulate the survival of erythroid cells during differentiation.<sup>8,9</sup> We have also demonstrated that in the absence of erythropoietin, the erythropoietin-dependent murine erythroid progenitor cell line HCD-57 rapidly down-regulates the expression of both Bcl-x<sub>L</sub> and Bcl-2 and undergoes apoptosis.<sup>10</sup> Furthermore, the ectopic expression of Bcl-x<sub>L</sub> (by means of a retroviral vector) rescues erythropoietin-deprived HCD-57 cells from apoptosis, suggesting that erythropoietin inhibits apoptosis in erythroid progenitor cells through Bcl-x<sub>L</sub> and Bcl-2.<sup>10</sup>

In the present experiments, we analyzed the expression of Bcl-x in erythroid colonies derived from bone marrow or peripheral-blood cells of patients with polycythemia vera. We found that the erythropoietin-independent erythroid cells express high levels of Bcl-x when cultured in the absence of erythropoietin, that Bcl-x<sub>L</sub> was the predominant Bcl-x form as assessed by reverse-transcriptase-polymerase-chain-reaction (RT-PCR) analysis, and that the expression of Bcl-x protein in bone marrow erythroid cells is significantly higher in patients with untreated polycythemia vera than in those with other myeloproliferative disorders or secondary erythrocytosis.

### METHODS

#### Study Subjects

We studied 33 patients with myeloproliferative disorders (14 with polycythemia vera, 9 with essential thrombocythemia, and 10 with chronic myeloid leukemia), 12 patients with secondary erythrocytosis, and 8 normal subjects who were bone marrow donors.

The diagnosis of polycythemia vera<sup>11</sup> required four of the following criteria: increased red-cell mass ( $\geq 32$  ml per kilogram of body weight in women and  $\geq 36$  ml per kilogram in men); splenomegaly; normal arterial oxygen saturation ( $\geq 91$  percent) in the presence of an increased red-cell mass; an increased platelet count

From the Servicio de Inmunología (M.S., A.B., C.S., J.L.F.-L.) and Servicio de Hematología (C.R., I.O.), Hospital Universitario Marqués de Valdecilla, Santander, Spain. Address reprint requests to Dr. Fernández-Luna at the Servicio de Inmunología, Hospital Universitario Marqués de Valdecilla, INSALUD, 39008 Santander, Spain.

TABLE 1. CHARACTERISTICS OF THE PATIENTS AND CONTROL SUBJECTS.\*

CHARACTERISTIC	PATIENTS WITH PV (N=14)	PATIENTS WITH SE (N=12)	PATIENTS WITH ET (N=9)	PATIENTS WITH CML (N=10)	CONTROL SUBJECTS (N=8)
Age (yr)					
Median	63	65.5	66	41	23
Range	39-80	36-77	45-75	11-68	14-56
Sex (M/F)	11/3	10/2	3/6	3/7	4/4
Hemoglobin (g/dl)					
Median	19.8	18.6	15.8	11.8	12.7
Range	18.5-24	17-20	11-18.6	10.8-12.9	11.5-15.1
Hematocrit (%)					
Median	59	55	46	35	42
Range	46-73	52-59	32-55	32-47	37-51
Leukocytes ( $\times 10^{-3}/\text{mm}^3$ )					
Median	14.7	7.6	13	98	5.7
Range	5.8-46	4.2-14.5	7.8-18.2	49-342	4.8-10.1
Platelets ( $\times 10^{-3}/\text{mm}^3$ )					
Median	654	208	860	490	302
Range	238-1747	161-515	617-1700	371-907	179-465
Red-cell mass (ml/kg)					
Median	45	36	24	ND	ND
Range	32-66	32-39	22-29		
Splenomegaly (no. of subjects)	8	0	5	10	0
Serum erythropoietin >30 U/ml (no. of subjects)	0	10	8	ND	ND
<b>Bone marrow studies</b>					
Bone marrow hyperplasia (no. of subjects)	12	1	8	10	—
Megakaryocytic hyperplasia (no. of subjects)	11	1	9	10	—
Erythroid precursors (%)†					
Median	4.7	4.6	5.3	5.1	—
Range	1-12	1-14	1-12	1-14	—
Bone marrow fibrosis $\leq 1/3$ (no. of subjects)‡	4	0	4	1	—
Months after diagnosis					
Median	33	—	17	30	—
Range	0-108		6-72	5-96	
Treatment (no. of subjects)					
Phlebotomy	10	5	—	—	—
Chemotherapy (hydroxyurea)	7	—	7	10	—

\*PV denotes polycythemia vera, SE secondary erythrocytosis, ET essential thrombocythemia, CML chronic myeloid leukemia, and ND not determined.

†Values are the percentages of bone marrow erythroid precursors identifiable by cytologic means (proerythroblasts and basophilic erythroblasts).

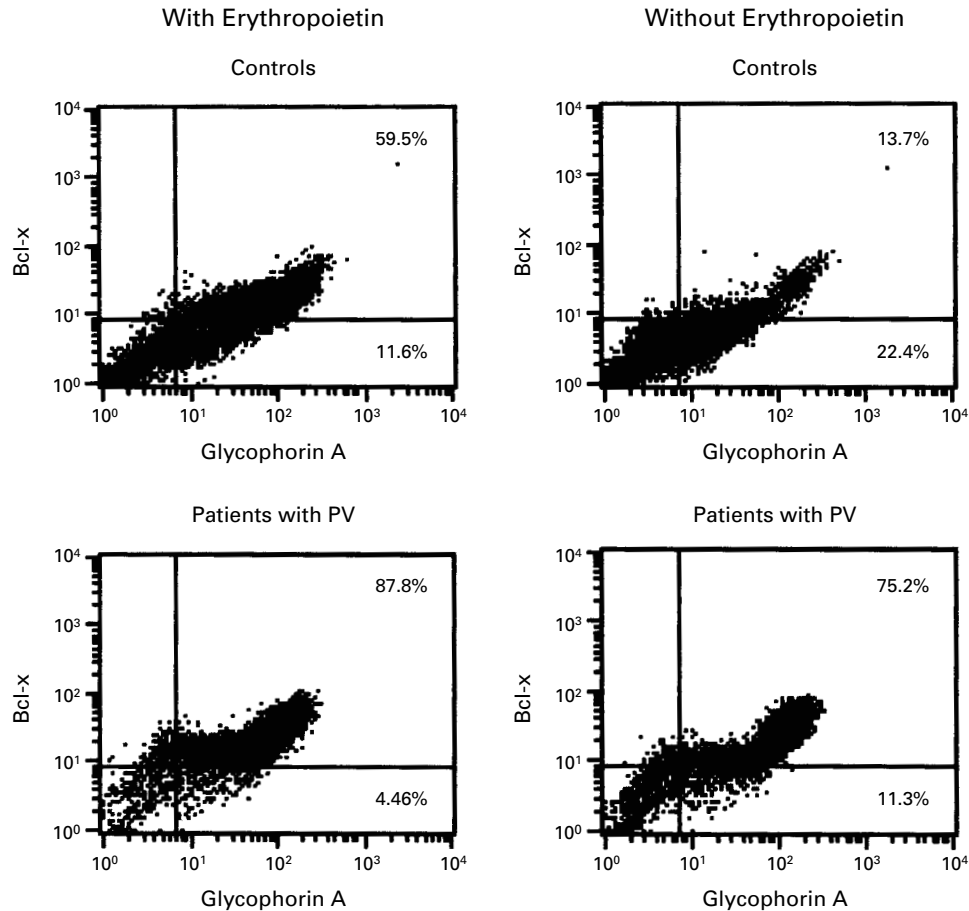
‡The numbers of subjects with bone marrow fibrosis of  $\leq 1/3$  of the cross-sectional area of the bone marrow-biopsy specimen are shown.

( $\geq 400,000$  per cubic millimeter) or white-cell count ( $\geq 12,000$  per cubic millimeter), or both; and bone marrow hypercellularity with megakaryocytic hyperplasia and the absence of iron stores or low erythropoietin levels ( $< 30$  U/ml) in the presence of an increased red-cell mass. The diagnosis of essential thrombocythemia<sup>12</sup> was made when a patient met all the following criteria: platelet count greater than 600,000 per cubic millimeter; absence of an identifiable cause of thrombocytosis; normal red-cell mass; absence of clinically significant fibrosis of the bone marrow; and absence of the Philadelphia chromosome and the presence of bone marrow hypercellularity with marked megakaryocytic hyperplasia. The diagnosis of chronic myeloid leukemia was based on the presence of leukocytosis with a leftward shift, splenomegaly, a low leukocyte alkaline phosphatase score, and the Philadelphia chromosome.<sup>13</sup> The cases of secondary erythrocytosis included one case of sleep apnea, eight cases of chronic obstructive pulmonary disease, two cases associated with tumors (hypernephroma and uterine fibromyoma), and one case of polycystic renal disease.

The study was approved by the institutional review board of the Hospital Universitario Marqués de Valdecilla, Santander, Spain.

### Laboratory Findings and Treatments

The clinical and laboratory characteristics of the study subjects are summarized in Table 1. The studies were done at diagnosis in 4 of the patients with polycythemia vera, and a median of 20 months after diagnosis (range, 3 to 108) in 10 patients. These 10 patients were being treated with phlebotomy, and 7 of them had received cytoreductive chemotherapy with hydroxyurea for a median of 12 months (range, 6 to 45). In these 10 patients, the hemoglobin levels, hematocrit, and leukocyte counts were normal at the time of the study, whereas in 2 of the other 4 patients the platelet counts remained increased (729,000 and 899,000 per cubic millimeter). The studies were done at diagnosis in two patients with essential thrombocythemia, and after a median of 17 months (range, 6 to 72) in seven patients. These seven patients were receiving cytoreductive chemotherapy with hydroxyurea at the time of the study, and four of the nine had platelet counts below 600,000 per cubic millimeter. The 10 patients with chronic myeloid leukemia were studied a median of 30 months after diagnosis (range, 5 to 96). All of them were receiving cytoreductive chemotherapy with hydroxyurea, and 8 of 10 had white-cell counts



**Figure 1.** Expression of Bcl-x in Erythroid Cells from Patients with Polycythemia Vera (PV) and Control Subjects. Erythroid progenitors incubated with or without erythropoietin were labeled with anti-glycophorin A and anti-Bcl-x monoclonal antibodies. The values above and below the horizontal lines indicate the percentage of cells positive for glycophorin A and Bcl-x and the percentage positive for glycophorin A and negative for Bcl-x, respectively. The quadrants were drawn up on the basis of the results for isotype-matched negative controls. All dot plots are from a representative experiment run in triplicate.

of more than 20,000 per cubic millimeter. Nine of the patients with secondary erythrocytosis were studied at the time of diagnosis, and three were studied after phlebotomy treatment.

#### Cell Cultures

Erythroid colonies arise *in vitro* when progenitor cells from the bone marrow or peripheral blood of patients with polycythemia vera are cultured in the absence of erythropoietin, whereas normal erythroid progenitors undergo apoptosis in the absence of erythropoietin. These responses indicate the presence in patients with polycythemia vera of abnormal erythroid progenitors that do not require erythropoietin to proliferate and differentiate. The loss of cell viability in normal erythroid progenitors is accompanied by down-regulation of Bcl-x<sub>L</sub>, and in studies of a murine erythroid progenitor cell line, the ectopic expression of Bcl-x<sub>L</sub> blocked apoptosis induced by the withdrawal of erythropoietin.<sup>10</sup>

To study the participation of Bcl-x in the molecular mechanism of polycythemia vera, progenitor cells were obtained from peripheral blood of patients with the disease and control subjects after they had provided informed consent. Mononuclear cells were plated at a density of 250,000 cells per milliliter in a standard culture (Methocult, Stem Cell Technologies, Vancouver, B.C., Canada) containing 0.9 percent methylcellulose, 30 percent heat-inactivated

fetal-calf serum, 50 ng of stem-cell factor per milliliter, and 20 ng of interleukin-3 per milliliter. Cells were cultured in the presence or absence of 3 U of erythropoietin per milliliter and incubated until CFU-E was assessed on day 7. Erythroid cells were then incubated in liquid culture (Iscove's modified Dulbecco's medium, GIBCO-BRL, Grand Island, N.Y.) containing 30 percent fetal-calf serum with or without 3 U of erythropoietin per milliliter.

#### Immunocytochemical Staining

After 24 hours of incubation in liquid culture, erythroid cells were cytocentrifuged onto slides, fixed in 100 percent ethanol, and incubated with rabbit antihuman Bcl-x (this antibody recognizes both Bcl-x<sub>L</sub>, which inhibits apoptosis, and Bcl-x<sub>s</sub>, which induces apoptosis) (Transduction Laboratories, Lexington, Ky.) or antihuman Bcl-2 (Santa Cruz Laboratories, Santa Cruz, Calif.) as previously described.<sup>14</sup> Cells incubated with normal rabbit serum instead of primary antibodies were used as a negative control.

#### Flow-Cytometric Analysis

Cultured erythroid progenitors and mononuclear cells obtained from peripheral blood and bone marrow of controls (donors for bone marrow transplantation) and patients with polycythemia vera were analyzed for the expression of Bcl-x by flow

cytometry as described previously.<sup>14</sup> Antibodies used included anti-glycophorin A labeled with fluorescein isothiocyanate (Dako, Glostrup, Denmark) and mouse anti-Bcl-x (this antibody binds to both Bcl-x<sub>L</sub> and Bcl-x<sub>S</sub>) followed by biotin-conjugated goat antimouse IgG and phycoerythrin-labeled streptavidin.

**RT-PCR Analysis**

To assess the expression of messenger RNA, we used RT-PCR as previously described.<sup>9</sup> The complementary DNA generated was amplified with primers specific for human Bcl-x (5'CGGGC-ATTCAGTGACCTGAC3' and 5'TCAGGAACCAGCGGTTGAG3'). The PCR consisted of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. After 30 cycles of amplification, the expected PCR products (340 bp for Bcl-x<sub>L</sub> and 151 bp for Bcl-x<sub>S</sub>) were fractionated according to size onto a 2 percent agarose gel and stained with ethidium bromide.

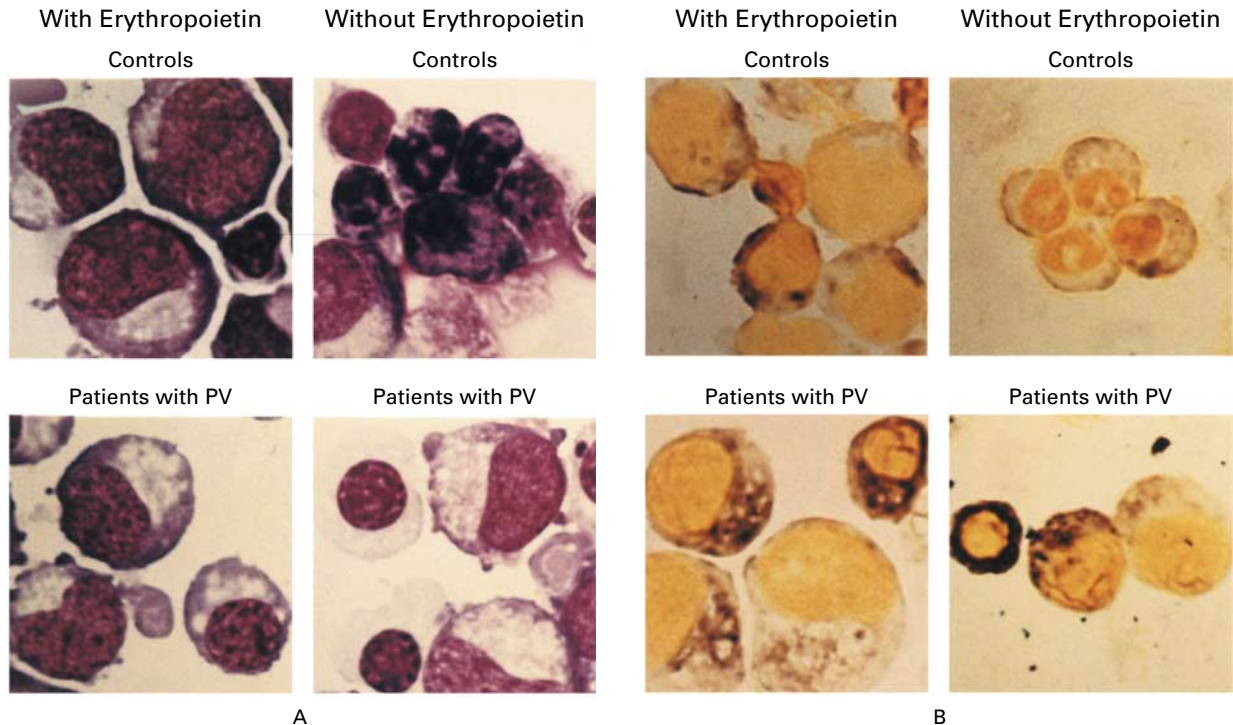
**RESULTS**

**Bcl-x Protein in Erythropoietin-Independent Colonies from Patients with Polycythemia Vera**

To assess the expression of Bcl-x in the erythroid colonies, we used flow-cytometric analysis of glycophorin A-positive cells that had been cultured in the presence of erythropoietin and in its absence. Glycophorin A is an erythroid-specific surface marker. A

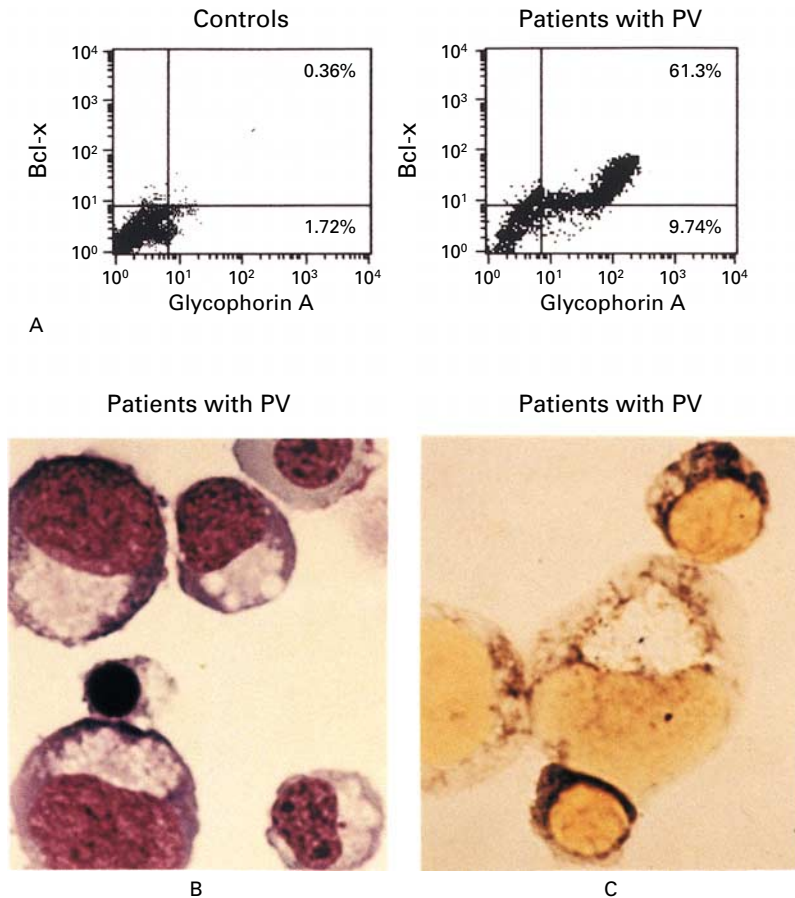
representative experiment is shown in Figure 1. After 24 hours of culture, the percentages of cells positive for glycophorin A and Bcl-x in the control cultures were 59.5 percent in the presence of erythropoietin and 13.7 percent in the absence of erythropoietin, whereas the percentage of cells positive for glycophorin A and negative for Bcl-x was 11.6 percent with erythropoietin and 22.4 percent without erythropoietin. By contrast, the presence or absence of erythropoietin had no significant effect on the expression of Bcl-x in the glycophorin A-positive population of cells from patients with polycythemia vera (values, 87.8 percent with erythropoietin and 75.2 percent without erythropoietin) (Fig. 1).

These results were confirmed by immunocytochemical analysis. Figure 2 shows a representative experiment. In the presence of erythropoietin, Bcl-x was expressed mainly in immature erythroid cells from both control subjects and patients with polycythemia vera. However, when erythroid colonies were cultured without erythropoietin for 24 hours, control erythroid progenitors underwent apoptosis as assessed morphologically (Fig. 2) and on the basis of the typical pattern of DNA fragmentation in apoptosis (not



**Figure 2.** Morphologic Analysis (Panel A) and Immunocytochemical Analysis (Panel B) of the Expression of Bcl-x in Erythroid Cells from Patients with Polycythemia Vera (PV) and Control Subjects.

Cells were stained with May-Grunwald-Giemsa solution (Panel A) and analyzed immunocytochemically for the expression of Bcl-x (Panel B) (×1000). There are apoptotic cells in the control preparation without erythropoietin, and the expression of Bcl-x increases with the maturation of the erythroid cells in patients with polycythemia vera in the absence of erythropoietin. No signal was detected when cells were incubated with normal rabbit serum instead of rabbit anti-Bcl-x. All samples are from a representative experiment run in triplicate.



**Figure 3.** Expression of Bcl-x in Erythroid Cells from Patients with Polycythemia Vera (PV) and Control Subjects Cultured in the Absence of Erythropoietin.

Erythroid cells were analyzed by flow cytometry with anti-glycophorin A and anti-Bcl-x (Panel A), stained with May-Grunwald-Giemsa solution for morphologic analysis (Panel B), and analyzed immunocytochemically for the expression of Bcl-x (Panel C) ( $\times 1000$ ). There are no erythroid colonies in the normal controls under these culture conditions. The values above and below the horizontal lines in Panel A indicate the percentage of cells positive for both glycophorin A and Bcl-x and the percentage positive for glycophorin A and negative for Bcl-x, respectively. All results are from a representative experiment run in triplicate.

shown). These changes were accompanied by reduced expression of Bcl-x (Fig. 2). In contrast, in patients with polycythemia vera, erythroid progenitors cultured without erythropoietin for 24 hours did not show any evidence of apoptosis (Fig. 2), and the expression of Bcl-x was maintained in the immature erythroblasts and was even increased in the more mature cells (Fig. 2). Interestingly, the percentage of mature erythroblasts was greater when polycythemia vera erythroid progenitors were cultured without erythropoietin than when they were cultured with erythropoietin (mean [ $\pm$ SD],  $54 \pm 6$  percent vs.  $29 \pm 8$  percent), suggesting that the absence of erythropoietin facilitates the maturation of erythroid progenitor cells in patients with polycythemia vera.

To test further whether the expression of Bcl-x was

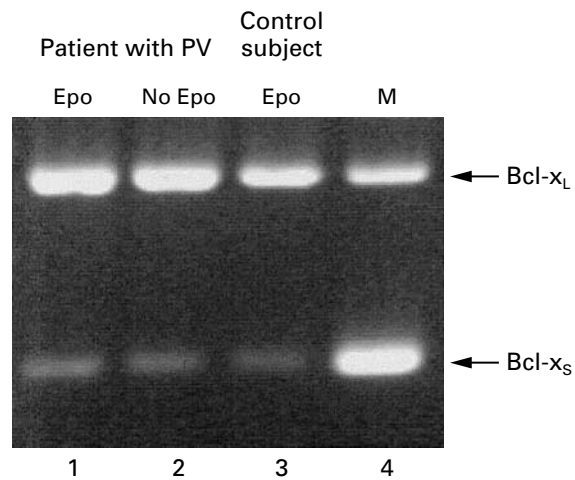
independent of erythropoietin in erythroid progenitors from patients with polycythemia vera, mononuclear cells from patients with polycythemia vera and normal subjects were plated in parallel in methylcellulose cultures in the absence of erythropoietin. On day 7, the colonies were transferred to a liquid culture with or without erythropoietin and incubated for an additional 24 hours. Control erythroid progenitors exhibited no growth without the addition of erythropoietin, and no colonies were found in the methylcellulose cultures. In contrast, erythroid colonies arose from progenitor cells from patients with polycythemia vera cultured in the absence of erythropoietin (Fig. 3B). A representative flow-cytometric analysis of this erythropoietin-independent erythroid population showed that the majority of glycophorin A-positive

cells expressed Bcl-x (61.3 percent, as compared with 9.7 percent that were negative for Bcl-x) (Fig. 3A). This result was confirmed by immunocytochemical staining, which showed increased expression of Bcl-x in the more mature erythroid cells (Fig. 3C).

There are two species of human Bcl-x, Bcl-x<sub>L</sub> and Bcl-x<sub>S</sub>.<sup>7</sup> Since the anti-Bcl-x antibodies that we used cannot distinguish between the two species, we used RT-PCR to determine whether Bcl-x<sub>L</sub> or Bcl-x<sub>S</sub> was responsible for anti-Bcl-x staining in erythroid cells. A representative experiment is shown in Figure 4. In all the normal subjects and the patients with polycythemia vera, Bcl-x<sub>L</sub> was the predominant form of Bcl-x in colony-forming unit-erythroid progenitors.

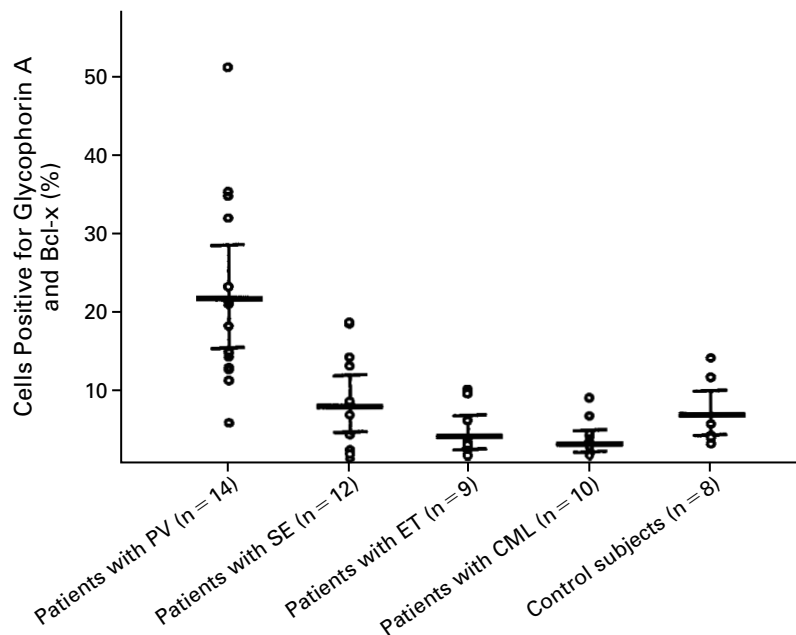
**Expression of Bcl-x by Glycophorin A-Positive Cells in Bone Marrow**

Given that cells in the erythropoietin-independent stages of erythroid differentiation express Bcl-x in patients with polycythemia vera, we hypothesized that the number of glycophorin A-positive cells in the bone marrow that express Bcl-x would be higher in patients with polycythemia vera than in normal subjects or patients with other myeloproliferative disorders or secondary erythrocytosis. We tested this



**Figure 4.** Expression of Bcl-x Messenger RNA in Erythroid Colonies from a Patient with Polycythemia Vera (PV) and a Control Subject.

Total RNA was subjected to reverse-transcriptase-polymerase-chain-reaction analysis with oligonucleotide primers that amplify both Bcl-x<sub>L</sub> and Bcl-x<sub>S</sub>. The simultaneous amplification of two plasmids containing Bcl-x<sub>L</sub> or Bcl-x<sub>S</sub> complementary DNA (1:1 molar ratio) was used as a positive control (M). Epo denotes erythropoietin.



**Figure 5.** Mean (±SD) Percentage of Glycophorin A-Positive Cells Expressing Bcl-x in Bone Marrow of Patients with Polycythemia Vera (PV), Secondary Erythrocytosis (SE), Essential Thrombocythemia (ET), or Chronic Myeloid Leukemia (CML) and Control Subjects.

The results were obtained by flow cytometry.

idea by determining the percentage of mononuclear cells that were positive for both glycophorin A and Bcl-x in bone marrow from 8 bone marrow donors, 14 patients with polycythemia vera, 12 with secondary erythrocytosis, 9 with essential thrombocythemia, and 10 with chronic myelogenous leukemia. The percentage of erythroid cells and the ratio between immature cells (proerythroblasts and basophilic erythroblasts) and mature cells (polychromatophilic and orthochromatic erythroblasts) were similar in the control subjects and the patients. However, the percentage of cells that were positive for both glycophorin A and Bcl-x was significantly higher ( $P < 0.001$  by one-way analysis of variance) in the patients with polycythemia vera ( $21.8 \pm 3.6$  percent) than in the patients with secondary erythrocytosis ( $6.87 \pm 1.95$  percent), the patients with essential thrombocythemia ( $3.81 \pm 0.97$  percent), the patients with chronic myelogenous leukemia ( $2.7 \pm 0.41$  percent), and the normal controls ( $6.62 \pm 1.58$  percent) (Fig. 5). Interestingly, the four patients with polycythemia vera who were studied at the time of diagnosis had the highest percentages of cells that were positive for both glycophorin A and Bcl-x (range, 33.1 to 52.6 percent).

#### DISCUSSION

Erythroid progenitor cells from normal subjects develop into colonies in culture only when erythropoietin is added.<sup>15</sup> In contrast, erythroid progenitors from bone marrow or peripheral blood of patients with polycythemia vera give rise to colonies in the absence of erythropoietin.<sup>16,17</sup> The erythropoietin-independent formation of erythroid colonies is a hallmark of polycythemia vera and can be used to distinguish polycythemia vera from secondary polycythemia.<sup>18</sup> Erythropoietin is needed for normal erythroid maturation,<sup>15,19</sup> but the mechanism by which it controls this process is unknown. It is possible that erythropoietin maintains cellular viability during the differentiation of erythroid progenitor cells. We have recently found that erythropoietin inhibits apoptosis of murine HCD-57 erythroid progenitor cells by promoting the expression of Bcl-x<sub>L</sub> and Bcl-2, two inhibitors of apoptosis.<sup>10</sup> In the present study we showed that Bcl-x is mainly expressed in the erythropoietin-dependent stages of normal erythroid differentiation. Mature erythroblasts express little or no Bcl-x, whereas immature erythroblasts (proerythroblasts and basophilic erythroblasts) are positive for Bcl-x. However, in patients with polycythemia vera, erythroid cells at all stages of differentiation express Bcl-x; indeed, the more mature erythroblasts express the highest level of Bcl-x. In vitro, polycythemia vera progenitors undergo erythroid differentiation in the presence of erythropoietin, but in the absence of erythropoietin, the differentiation process is accelerated. A similar phe-

nomenon occurred with HCD-57 erythroid progenitor cells that were transduced with a Bcl-x<sub>L</sub> retrovirus vector.<sup>10</sup> Taken together, these studies suggest that erythropoietin does not induce erythroid differentiation, but rather promotes differentiation by providing survival signals.

Our finding that erythroid progenitors from patients with polycythemia vera do not undergo apoptosis in vitro in the absence of erythropoietin was made with cells obtained from patients who had been treated with hydroxyurea or studied at the time of diagnosis. In both cases the erythroid colonies that arose in culture were indistinguishable in terms of the expression of Bcl-x and erythroid maturation (data not shown); however, the number of colonies was consistently lower in the treated patients, most likely because of the inhibitory effect of hydroxyurea on the cell cycle.<sup>20</sup> Our results suggest that the deregulation of Bcl-x in erythroid progenitors may be responsible for the accumulation of erythroid cells in patients with polycythemia vera, since the predominant form of Bcl-x messenger RNA in these cells is Bcl-x<sub>L</sub>, which is a repressor of apoptosis. We also found little or no expression of Bcl-2 in erythroid progenitors from patients with polycythemia vera cultured without erythropoietin (data not shown). However, the staining pattern of Bcl-2 in erythroid cells is very weak, making it difficult to rule out the possibility that Bcl-2 contributes to the accumulation of erythroid cells in patients with polycythemia vera.

The diagnosis of polycythemia vera is based mainly on criteria used by the Polycythemia Vera Study Group<sup>21</sup> in order to obtain a uniform population of patients for therapeutic evaluations. However, some patients have a myeloproliferative disorder resembling polycythemia vera but do not fulfill all the criteria. The erythropoietin-independent growth of erythroid colonies in semisolid culture may be a useful diagnostic tool to distinguish polycythemia vera from other myeloproliferative disorders and secondary erythrocytosis; however, the erythroid progenitors of some patients with essential thrombocythemia have similar in vitro behavior.<sup>22</sup> It is clear that a better understanding of the molecular alterations could improve our ability to diagnose polycythemia vera.

We found that the mean number of Bcl-x-positive cells in the population carrying the erythroid marker glycophorin A was significantly higher in the bone marrow of patients with polycythemia vera than in the bone marrow of normal subjects and patients with essential thrombocythemia, chronic myelogenous leukemia, or secondary erythrocytosis. However, the percentage of Bcl-x-positive cells in 7 of 14 patients with polycythemia vera was similar to that found in patients with secondary erythrocytosis. Six of these seven patients were treated with hydroxyurea. Since erythroid cells from patients with polycythemia vera who are treated with hydroxyurea give

rise to fewer colonies in semisolid culture than cells from normal subjects, it is likely that the cytoreductive effect of hydroxyurea accounts for the relatively low number of cells positive for both glycophorin A and Bcl-x in the bone marrow of treated patients. By contrast, the four patients studied at the time of diagnosis had the highest percentages of Bcl-x-positive erythroid cells. These preliminary data suggest that the number of cells positive for both glycophorin A and Bcl-x could be helpful in confirming the diagnosis of polycythemia vera.

In conclusion, we propose that the erythropoietin-independent expression of Bcl-x may cause the accumulation of erythroid cells in polycythemia vera. A constitutively activated or hypersensitive erythropoietin receptor might be involved, but mutations of the erythropoietin-receptor gene are detected in only some cases of hereditary polycythemia.<sup>23,24</sup> A recent study proposed that the increased level of insulin-like growth factor-binding protein 1 in patients with polycythemia vera may account for the increased sensitivity of erythroid progenitors to insulin-like growth factor I and the consequent overproduction of erythroid cells.<sup>25,26</sup> In line with this proposal, insulin-like growth factor I has been shown to suppress apoptosis in erythroid progenitors and myeloid cells.<sup>27,28</sup> The role of insulin-like growth factor I in the erythropoietin-independent expression of the apoptosis-inhibitory protein Bcl-x will need to be addressed in future studies.

Supported by a grant (FISS-94/1415) from Fondo de Investigaciones Sanitarias, a grant (SAF-96/0274) from Plan Nacional de Investigación y Desarrollo (to Dr. Fernández-Luna), and a postdoctoral fellowship from the Fundación Marqués de Valdecilla (to Dr. Benito).

*We are indebted to Dr. Craig Thompson for generously supplying anti-Bcl-x monoclonal antibody and to Drs. Francesco Lo Coco, Mario Cazzola, and Gabriel Núñez for critical review of the manuscript.*

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