Interleukin-10 Inhibits Erythropoietin-Independent Growth of Erythroid Bursts in Patients With Polycythemia Vera

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In polycythemia vera (PV) erythroid colonies that grow in vitro in the absence of exogenous erythropoietin (EPO) arise from the abnormal clone that is responsible for overproduction of red blood cells. Although the mechanism of autonomous formation of burst-forming units-erythroid (BFU-E) is not fully understood, a spontaneous release of growth regulatory molecules by PV cells and/or by accessory cells is likely to be involved. Because of its cytokine synthesis inhibiting action, interleukin-10 (IL-10) could be a potentially useful molecule to modulate abnormal erythropoiesis in PV. We studied the effect of recombinant human IL-10 on the EPO-independent growth of erythroid bursts derived from peripheral blood mononuclear cells (PBMNCs) of patients with PV. IL-10 showed a profound, dose-dependent, and specific inhibitory effect on autonomous BFU-E formation. Ten nanograms per milliliter of IL-10 significantly suppressed spontaneous growth of erythroid colonies in methylcellulose in five of five PV patients tested with a mean inhibition by 81% (range, 72-94). To elucidate the possible mechanism of the inhibitory action of IL-10 we further studied the effect of anticytokine antibodies on autonomous BFU-E growth and the ability of exogenous cytokines to restore IL-10–induced suppression of erythroid colony growth. Among a panel of growth regulatory factors tested (granulocyte-macrophage colony-stimulating factor [GM-CSF], IL-3, granulocyte colony-stimulating factor, stem cell factor, and insulin-like growth factor-1) GM-CSF was the only molecule for which both an inhibition of spontaneous BFU-E formation by its respective antibody as well as a significant restimulation of erythroid colonies in IL-10-treated cultures by exogenous addition was found. Moreover, inhibition of GM-CSF production by IL-10 was shown in PV PBMNCs at the mRNA level. Our data indicate that autonomous BFU-E growth in PV can be profoundly inhibited by IL-10 and that this inhibitory effect seems to be at least in part secondary to suppression of endogenous GM-CSF production.

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POLYCYTHEMIA VERA (PV) is a clonal disease of the multipotential progenitor cell characterized by an increased red blood cell mass and varying numbers of granulocytes and platelets in the peripheral blood (PB). Whereas erythroid progenitors from normal individuals require addition of exogenous erythropoietin (EPO) to form erythroid colonies in vitro, a distinct population of erythroid progenitors from patients with PV can form hemoglobinized colonies in the absence of added EPO.[1] Because such spontaneous burst forming unit-erythroid (BFU-E) formation from PV cells can still be observed under serum-free culture conditions[2] or in the presence of anti-EPO and anti-EPO-receptor antibodies,[2,3] minute amounts of EPO in the culture system can be excluded as a possible cause of endogenous colony formation. Growth of BFU-E without addition of exogenous growth factors increases the possibility that PV cells secrete their own growth factors and/or are stimulated by growth factors released from accessory cells that might subsequently lead to hyperproliferation of erythroid cells in vitro and possibly in vivo.

Interleukin-10 (IL-10) is a 35-kD protein, originally identified by virtue of its ability to inhibit cytokine synthesis in T helper 1 clones.[4,5] It is primarily produced by mononuclear cells (MNCs)[6] and possesses a wide range of activities on a number of cell types including B cells,[7] T cells,[8] natural killer cells,[9] mast cells,[10] neutrophils,[11] eosinophils,[12] and monocytes.[13] The main feature of this cytokine is a suppressive effect on cytokine expression. Thus, IL-10 inhibits production of numerous cytokines in lipopolysaccharide or interferon-γ-activated monocytes, such as IL-1a, IL-1b, IL-6, IL-8, tumor necrosis factor-α, granulocyte- macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF).[13,14]

Because of its cytokine synthesis-inhibiting action, IL-10 could be a potentially useful molecule to modulate hematopoiesis in conditions in which the autocrine and/or paracrine secretion of cytokines plays a significant role. In fact, we have shown that IL-10 markedly suppresses the spontaneous formation of granulocyte-macrophage colonies (CFU-GM) from normal PBMNCs in semisolid cultures.[15] More importantly, we showed a profound inhibitory effect of IL-10 on the massively increased autonomous CFU-GM growth in patients with chronic myelomonocytic leukemia (CMML), suggesting that IL-10 was a potential therapeutic agent in this disease.[16] Here we investigated the effect of IL-10 on the spontaneous growth of erythroid colonies in patients with PV. We found that IL-10 profoundly inhibits EPO-independent BFU-E growth from such patients, at least in part through suppression of spontaneous release of GM-CSF.

MATERIALS AND METHODS

Patients. Five patients clinically diagnosed as having PV with the help of the PV Study Group guidelines[17] were used in this study. Clinical and laboratory data of these patients are shown in Table 1. All patients were being managed by phlebotomy at the time of study and none of them had been previously treated with cytostatic drugs or radioactive phosphorus.

Preparation of cells. After informed consent, PB was collected into sterile tubes containing EDTA. MNCs were isolated from PB of patients

Table 1. Clinical and Laboratory Data in Patients With Polycythemia Vera at Time of Study

Patient	Age/Sex	Duration (mo)	RBC (×10¹²/L)	Hct	WBC (×10⁹/L)	Platelets (×10³/μL)	Spleen	Therapy
HR	82/M	5	6.88	42.3	17.4	594	Enlarged	Phlebotomy
KJ	75/M	216	6.15	42.1	16.1	340	Enlarged	Phlebotomy
KA	31/M	1	5.63	43.5	9.9	496	Normal	Phlebotomy
JH	70/F	1	6.59	46.1	6.1	365	Normal	Phlebotomy
SA	54/M	9	7.65	45.9	39.4	380	Enlarged	Phlebotomy

Abbreviations: RBC, red blood cells; Hct, hematocrit; WBC, white blood cells.

by Ficoll-Hypaque density gradient centrifugation (density 1.077 g/mL, 400g for 40 minutes). The low-density cells were collected from the interface between density solution and plasma, washed twice, and resuspended in Iscove's modified Dulbecco's medium (GIBCO, Paisley, Scotland).

Reagents. Recombinant human IL-10 (rhIL-10; specific activity 1-2 × 10⁶ U/mg) was kindly provided by Schering-Plough Corp (Kenilworth, NJ) and rhGM-CSF and rhIL-3 by Sandoz (Basel, Switzerland). RhG-CSF was purchased from British Biotechnology (Oxan, UK) and rhEPO from Boehringer Mannheim (Vienna, Austria). Recombinant human stem cell factor (rhSCF) and recombinant human insulin-like growth factor-1 (rhIGF-1) were obtained from Pharma Biotechnologie Hannover (Hannover, Germany). Antibodies directed against GM-CSF, IL-3, G-CSF, and SCF were purchased from Genzyme (Cambridge, MA), anti-IGF-1 from Serotec Ltd (Oxford, UK), and anti-IL-10 from R&D Systems Europe Ltd (Abington, UK).

Colony assay. PBMNCs were cultured in 0.9% methylcellulose, 30% fetal calf serum (FCS; INLIFE, Wiener Neudorf, Austria), 10% bovine serum albumin (Behring, Marburg, Germany), α-thioglycerol (10⁻⁴ mol/L) and Iscove's modified Dulbecco's medium with or without the addition of cytokines or anticytokine antibodies. Cultures were plated in triplicate at 75 to 120 × 10³ MNC/mL. In some experiments a neutralizing antibody against IL-10 was preincubated with IL-10 for 2 hours at room temperature. Neutralizing antibodies against GM-CSF, G-CSF, IL-3, SCF, or IGF-1 were used as recommended by the manufacturer. Plates were incubated at 37°C, 5% CO₂, and full humidity. After a culture period of 14 days, cultures were examined under an inverted microscope. Aggregates with at least 50 hemoglobinized cells, easily recognizable by their red color, were counted as BFU-E–derived erythroid bursts. For cultivation of BFU-E from normal individuals, 2 U/mL EPO was added to culture dishes.

Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of GM-CSF transcripts. A total of 2 × 10⁷ PV PBMNCs were cultured in suspension both with and without IL-10 (10 ng/mL) for 48 hours. After incubation, cells were washed twice in diethylpyrocarbonate-treated water and 10⁷ cell aliquots were lysed by addition of 1.6 mL RNAzol B (Biotecx, Houston, TX). Total RNA was extracted as described.[18] The integrity of RNA was controlled by electrophoresis through formaldehyde agarose gels. High quality RNA was quantitated by measuring absorbance at 260 nm and 1 g total RNA was subjected to cDNA synthesis as recently described.[19]

For semiquantitative analysis of GM-CSF mRNA, an RT-PCR technique that allows measurements of relative transcript levels was applied.[20,21] The oligonucleotide primer sequences for amplification of GM-CSF were 5′-CTGCTGCTGAGATGAATGAAACAG-3′ and 5′-TGGACTGGCTCCCAGCAGTCAAAG-3′, which bracketed a GM-CSF fragment of 286 bp.[22] PCR amplification of ABL (Abelson) transcripts was used as a reference to assess variation of total RNA or cDNA between samples. The primer sequences for amplification of ABL were as follows: 5′-CAGCGGCCAGTAGCATCTGACTTTG-3′ and 5′-CCATTTTTGGTTTGGGCATCACACCATTCC-3′ resulting in the production of a PCR fragment of 228 bp.[19] The linear ranges of PCR amplifications of GM-CSF and ABL were established as a function of cycle number and cDNA concentration as described.[15,20,21] Reaction conditions included 3 L cDNA, 20 pmol of each primer, 1.5 mmol/L

MgCL2, 200 mol/L of each dNTP, 2.5 U Ampli Taq DNA Polymerase (Perkin Elmer Cetus, Norwalk, CT), and (³²P) dCTP (150,000 cpm) in a 50-L reaction volume. The thermal cycling conditions were denaturation at 94°C (1 minute), annealing at 60°C (1 minute), and extension at 72°C (2 minutes), preceded by an initial denaturation step at 94°C for 5 minutes, and followed by a terminal extension of 10 minutes at 72°C. The number of PCR cycles for amplification of GM-CSF and ABL transcripts was 32 and 25 cycles, respectively. Reaction products were subjected to 6% polyacrylamide gels (Novex, San Diego, CA), and dried gels were exposed to Kodak XAR-5 films (Eastman Kodak, Rochester, NY) at −70°C for 12 hours.

For quantification of PCR products incorporated (³²P) dCTP was measured on autoradiograms by using the Bio Rad 670 Imaging Densitometer and the system's volume integration program (Bio Rad Gel DOC 1000 system, Molecular Analyst/PC software; BioRad, Richmond, CA). Potential differences of total cellular RNA/cDNA in PCR analyses were corrected by dividing GM-CSF values by the ABL value (mean value of six PCR analyses). The relative level of GM-CSF transcripts was measured in patient samples in three PCR analyses in duplicate using freshly synthesized cDNA.

Statistical analysis. The t-test was used to determine the significance of differences. A P value of $<.05$ was considered statistically significant.

RESULTS

Inhibitory effect of IL-10 on autonomous BFU-E growth in patients with PV. As it has been originally shown by Prchal and Axelrad,[1] erythroid colonies can grow in methylcellulose cultures containing PBMNCs from patients with PV in the absence of exogenous EPO. Treatment of PV cell cultures with IL-10 resulted in a profound and dose-dependent inhibition of autonomous BFU-E growth (Fig 1). The inhibitory effect of IL-10 became apparent at a concentration of 1 ng/mL and was even more pronounced at higher concentrations. This effect was specific, because a neutralizing anti-IL-10 antibody was able to prevent IL-10-induced suppression of BFU-E growth (Fig 1). The effect of 10 ng/mL IL-10 on autonomous BFU-E growth was investigated in PBMNCs from 5 patients with PV (Table 2). As shown in Table 2, autonomous formation of erythroid colonies greatly varied among different patients but was significantly inhibited by IL-10 in all of them. On average, 10 ng/mL of IL-10-inhibited EPO independent BFU-E growth from PV PBMNCs by 81% (range, 71% to 94%).

Effect of anticytokine antibodies on autonomous BFU-E growth from PV cells. To elucidate the possible mechanism of the inhibitory action of IL-10, we first tried to identify the factor responsible for autonomous BFU-E growth in PV by adding neutralizing antibodies against GM-CSF, IL-3, G-CSF, SCF, and IGF-1 to cell cultures from three patients with marked colony growth (Fig 2). Not unexpectedly the IGF-1 antibody significantly inhibited autonomous BFU-E growth in our FCS containing culture system (patient SA), because IGF-1 has been
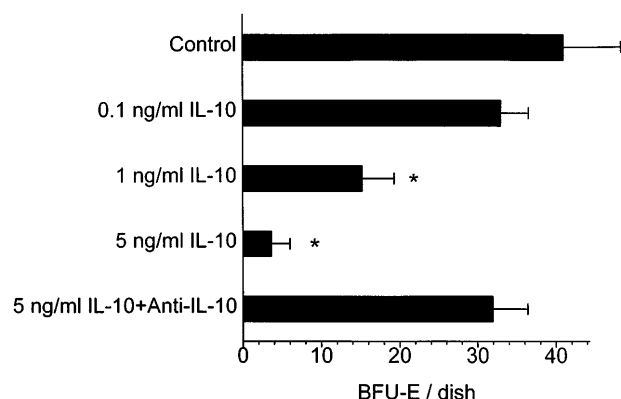
Fig 1. Dose-dependent inhibitory effect of IL-10 on autonomous BFU-E growth from PV cells and its abrogation by an anti-IL-10 antibody in patient HR. A total of 120 × 10³ PBMNC/mL were cultured in methylcellulose with medium alone, with increasing concentrations of IL-10, or with IL-10 plus a neutralizing antibody against IL-10. Colony growth was assessed after 14 days. Results represent the mean values ± standard deviation (SD) from triplicates. * Significant change from control with P value at least <.05.
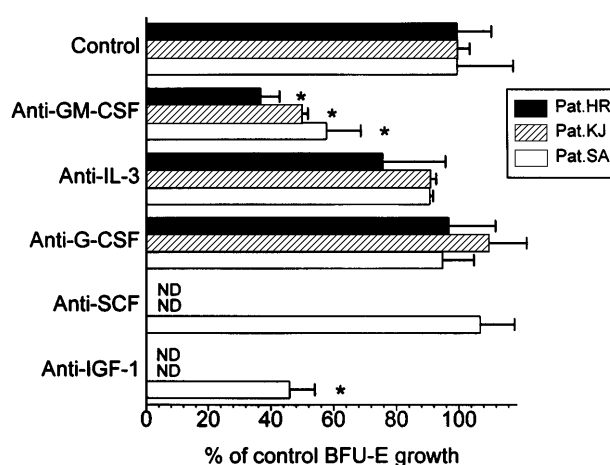

Fig 2. Effect of anticytokine antibodies on autonomous BFU-E growth from PV cells in three patients. PBMNCs were cultured in methylcellulose with medium alone or with antibodies against GM-CSF, IL-3, G-CSF, SCF, or IGF-1, respectively. Colony growth was assessed after 14 days. Results represent mean values ± SD from triplicates. * Significant change from control with P value at least <.05.

reported as the major EPO-like activity in FCS.[23] Among the other anticytokine antibodies tested only the anti-GM-CSF antibody significantly decreased spontaneous growth of erythroid colonies in all three patients tested, with a mean inhibition by 52% (range, 42% to 63%). This clearly suggested that GM-CSF and IGF-1 were involved in the spontaneous formation of erythroid colonies from PV PBMNCs in this culture system.

Effect of exogenous growth factors on IL-10-induced suppression of autonomous BFU-E growth from PV cells. The fact that IL-10 has been shown to inhibit cytokine synthesis in different cell types of the mononuclear cell fraction[4,13] led us to hypothesize that inhibition of autonomous BFU-E growth by IL-10 was secondary to IL-10-induced suppression of endogenous release of growth regulatory molecules. If this was the case one would expect that exogenous addition of particular cytokines could at least in part reverse growth inhibition by IL-10. In contrast, restoration of colony growth by exogenous growth factors would not be observed if IL-10 had a direct cytotoxic effect on PV cells. In fact, exogenous GM-CSF and IL-3 significantly restimulated erythroid colony formation in IL-10 treated cultures (Fig 3). All other cytokines were not effective in correcting IL-10-induced growth inhibition. Also

Table 2. Inhibitory Effect of IL-10 on Autonomous BFU-E Growth in PV Patients

| Patient | MNC/Dish × 10³ | BFU-E ± SD/Dish | | Percent of Inhibition | P |
		Control	+IL-10		
HR	100	32.0 ± 3.5	9.3 ± 1.2	71	<.001
KJ	100	43.0 ± 1.7	10.3 ± 1.5	76	<.001
KA	100	5.0 ± 1.0	0.3 ± 0.6	94	<.005
JP	116	15.8 ± 4.2	3.0 ± 0.0	81	<.05
SA	75	107.3 ± 7.6	19.3 ± 2.5	82	<.001

PBMNCs from PV patients were cultured as described in the Materials and Methods with or without 10 ng/mL IL-10. Colony growth was assessed after 14 days. Results represent mean values ± SD from triplicates.

IGF-1 alone at concentrations up to 100 ng/mL was not able to restore autonomous BFU-E growth in IL-10 suppressed cultures, nor did it significantly potentiate the effects of GM-CSF when added in combination (Table 3). This observation and the suppressive effect of the anti-IGF-1 antibody in the culture system suggest that IGF-1 is important for EPO-independent erythroid colony formation in PV patients but is not involved in growth inhibition by IL-10.

Inhibitory effect of IL-10 on GM-CSF expression in PV PBMNCs. The demonstration of both the antiproliferative action of the anti-GM-CSF antibody on the autonomous BFU-E growth and the restoration of IL-10-induced growth suppression
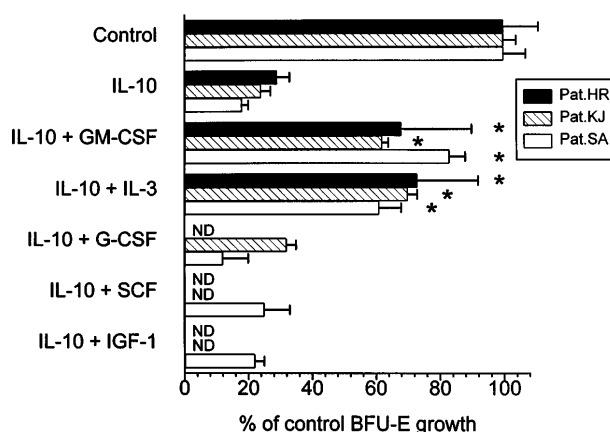

Fig 3. Effect of exogenous growth factors on IL-10-induced suppression of BFU-E growth from PV cells. PBMNCs were cultured in methylcellulose with 10 ng/mL IL-10 in the presence or absence of exogenous GM-CSF (100 U/mL), IL-3 (10 U/mL), G-CSF (100 U/mL), SCF (10 ng/mL), and IGF-1 (10 ng/mL), respectively. Colony growth was assessed after 14 days. Results represent mean values ± SD from triplicates. * Significant change from IL-10 treated cultures with P value at least <.05.

Table 3. Effect of IGF-1 Alone or in Combination With GM-CSF on IL-10–Induced Suppression of Autonomous BFU-E Growth From PV Cells

Culture Conditions	BFU-E ± SD/Dish
Control (no cytokines)	96.0 ± 8.2
+ 10 ng/mL IL-10	12.7 ± 3.1
+ 10 ng/mL IL-10 + 1 ng/mL IGF-1	10.3 ± 0.6
+ 10 ng/mL IL-10 + 10 ng/mL IGF-1	14.0 ± 2.7
+ 10 ng/mL IL-10 + 100 ng/mL IGF-1	13.7 ± 1.5
+ 10 ng/mL IL-10 + 100 U/mL GM-CSF	83.7 ± 4.2
+ 10 ng/mL IL-10 + 100 U/mL GM-CSF + 10 ng/mL IGF-1	92.7 ± 24.6

A total of 10^5 PBMNC/dish from patient SA were cultured in methylcellulose in the presence or absence of exogenous cytokines as indicated in the Table. Colony growth was assessed after 14 days. Results represent mean values ± SD from triplicates.

by exogenous GM-CSF strongly suggested that the growth inhibitory effect of IL-10 in PV was at least in part caused by suppression of spontaneous GM-CSF production. Therefore, the effect of IL-10 on GM-CSF expression in unseparated PBMNCs from a patient with PV (HR) was studied by a semiquantitative PCR technique. We have previously shown that the PCR technique used by us allows at least semiquantitative measurements of GM-CSF transcript levels by establishing linear ranges of amplifications of GM-CSF as a function of cycle number and cDNA concentrations.[15] After culturing PV cells in the presence of IL-10 (10 ng/mL) for 48 hours, GM-CSF transcript levels were substantially lower than that of cells kept in suspension without IL-10 (Fig 4A). In comparison, ABL transcripts, which served as a control, remained unchanged during the time of culture (Fig 4B). Figure 4C shows corrected GM-CSF mRNA levels in PV PBMNCs at 48 hours. Comparison of corrected GM-CSF transcript levels between PBMNCs cultured with and PBMNCs cultured without IL-10 showed a mean decrease by 47%.

Effect of IL-10 on EPO-dependent BFU-E growth in normal individuals. To investigate the effect of IL-10 on the EPO-dependent in vitro growth of normal erythroid progenitors, PBMNCs from five healthy volunteers were cultured in the presence of EPO with or without 10 ng/mL IL-10 (Table 4). IL-10-induced suppression of EPO-dependent erythroid colony growth was seen in all five individuals, but this inhibition was less pronounced than in PV patients. The mean inhibition of normal EPO-dependent BFU-E growth by 10 ng/mL IL-10 was 45% ± 12% (standard deviation), which was significantly less than the inhibition of EPO-independent erythroid colony growth in PV patients (81% ± 12%, $P < .001$)

DISCUSSION

Elegant studies by Adamson et al[24] analyzing the isoenzyme pattern in different cell types of PV patients heterozygous for G6PD have shown that circulating red blood cells, granulocytes, and platelets expressed the same isoenzyme, suggesting that these cells were unicellular in origin and derived from an abnormal multipotential progenitor cell. Later, Prchal et al[25] could show that erythroid colonies from PV patients that formed in the absence of exogenous EPO contained the same G6PD isoenzyme type as that expressed by peripheral blood elements.
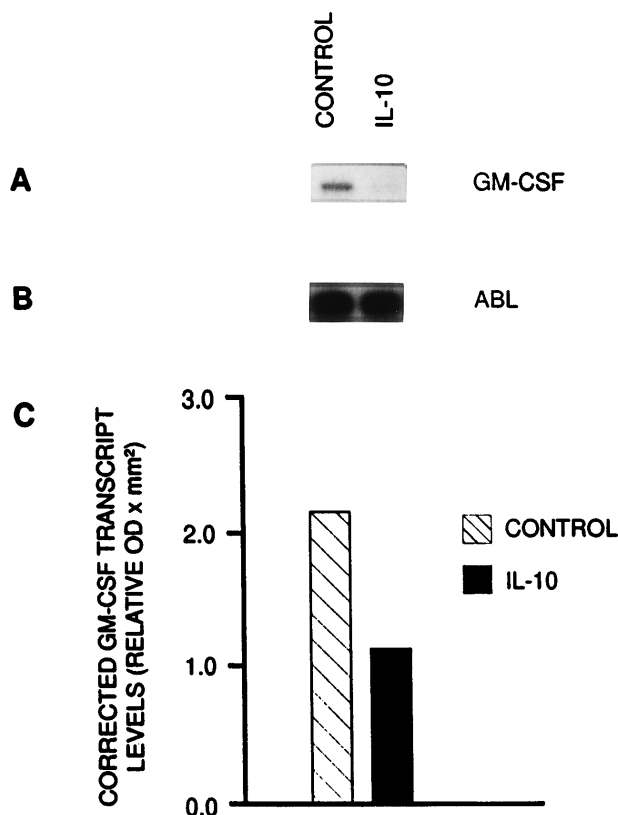

Fig 4. Semiquantitative RT-PCR analysis of GM-CSF transcript levels. (A) Autoradiogram showing incorporated radioactivity of amplification products obtained from PV PBMNCs cultured in suspension with or without IL-10 for 48 hours. (B) Autoradiogram showing ABL transcripts that served as a reference to correct for potential variations of RNA or cDNA samples. (C) Corrected GM-CSF transcript levels in cultured PV cells. Each sample was analyzed in three radioactive PCR analyses in duplicate using freshly synthesized cDNA. The quantity of ^{32}P incorporated into PCR product was determined by densitometric scanning of the autoradiograms. Results were corrected by dividing GM-CSF values by the mean values obtained from six ABL transcripts of that cDNA. The results are shown as mean values.

Thus, the so-called endogenous colonies arose from the abnormal clone that was responsible for the overproduction of red blood cells in PV. When exogenous EPO was added, increasing numbers of erythroid colonies were formed containing cells that expressed the other G6PD isoenzymes, indicating the existence

Table 4. Effect of IL-10 on EPO-Dependent BFU-E Growth in Normal Individuals

Patient No.	BFU-E ± SD/Dish		Percent of Inhibition
	Control	+IL-10	
1	13.0 ± 5.0	8.3 ± 2.1	36
2	33.0 ± 2.7	13.7 ± 10.1	58
3	36.0 ± 4.4	23.7 ± 5.0	34
4	30.3 ± 2.1	19.0 ± 3.0	37
5	53.7 ± 3.5	22.7 ± 2.5	58

A total of 10^5 PBMNC/dish from 5 healthy volunteers were cultured in the presence of 2 U/mL EPO as described in the Materials and Methods with or without 10 ng/mL IL-10. Colony growth was assessed after 14 days. Results represent mean values ± SD from triplicates.

of both malignant and nonmalignant populations of hematopoietic progenitor cells in PV marrow. Here we show a profound inhibitory effect of IL-10 on endogenous erythroid colony formation in vitro in patients with PV. A significant inhibition of spontaneous erythroid colony formation by IL-10 was observed in all five PV patients investigated. The effect of IL-10 was dose-dependent and specific, because a neutralizing anti-IL-10 antibody was able to prevent IL-10-induced suppression of BFU-E growth.

The mechanism of spontaneous erythroid colony formation in vitro and the factors that lead to in vivo expansion of clonal cells in patients with PV remain to be fully understood. Spontaneous erythroid colony formation in the presence of anti-EPO antibodies (3) and in serum-free culture conditions (2) exclude minute amounts of EPO in the culture system as a potential stimulus for endogenous colony formation. The marked reduction of spontaneous erythroid colony formation by cells from PV patients by depletion of monocytes and its restoration by readdition of adherent cells or adherent cell supernatant[26,27] strongly suggests monocyte-derived molecules as stimulatory factors for erythroid colony formation in PV. Several cytokines including GM-CSF,[28] IL-3,[28] SCF,[29] and IGF-1[30] have been shown to enhance EPO independent BFU-E growth if added to semisolid cultures containing PV MNCs. Moreover a hypersensitivity of PV BFU-E to each of these growth factors compared with normal erythroid progenitors has been shown,[28-30] which may be a principal factor in the profound marrow hyperplasia and increased blood counts of patients with PV. Recently, a defect in phosphatase activity in PV cells has been proposed as the molecular basis for the enhanced response of PV BFU-E to growth regulatory molecules. Thus, increased basal and induced tyrosine phosphorylation of the IGF-1 receptor β subunit in circulating MNCs in such patients has been reported.[31] Moreover, a diminished enhancement of erythroid colony formation in the presence of orthovanadate,[32] an inhibitor of protein tyrosine phosphatases, in PV cells has been shown.

There is substantial evidence that the inhibitory effect of IL-10 on autonomous BFU-E growth in PV is indirect. Spontaneous formation of erythroid colonies from PV cells has been shown to be decreased after removal of adherent cells.[26,27] In unseparated PV PBMNCs, we found, in agreement with observation by others,[33] that autonomous BFU-E growth was markedly reduced by addition of an anti-GM-CSF antibody, suggesting spontaneous release of GM-CSF as an important mechanism of EPO-independent erythroid colony formation. Not unexpectedly, an anti-IGF-1 antibody also inhibited autonomous BFU-E growth in our culture system because IGF-1 has been shown to be the major EPO-like activity in FCS.[23] The antiproliferative action of anti-GM-CSF antibody on EPO-independent BFU-E formation in PV and the fact that IL-10 has been shown to inhibit cytokine synthesis, including GM-CSF in human monocytes,[13] led us to hypothesize that inhibition of BFU-E growth by IL-10 was mainly caused by IL-10-induced suppression of endogenous GM-CSF release. Our observation that exogenous GM-CSF was able to, at least in part, reverse growth inhibition by IL-10 strongly supports this concept. In fact, analysis of the effect of IL-10 on GM-CSF expression in MNCs from a patient with PV clearly showed that the IL-10-induced inhibition of erythroid colony growth correlated with a substantial decrease in GM-CSF transcripts in PV PBMNCs. Exogenous IL-3 also restimulated autonomous BFU-E formation in IL-10-treated PV cell cultures, but an anti-IL-3 antibody had no antiproliferative effect on spontaneous growth of erythroid progenitors. These data suggest that in addition to GM-CSF-responsive PV BFU-E, there seems to exist an IL-3-responsive population, which however, cannot be sufficiently stimulated by the endogenously released growth factors in unstimulated cultures.

The inhibitory effect of IL-10 on hematopoietic colony formation is not specific for PV BFU-E. However the growth suppression of IL-10 on EPO-independent erythroid colony growth in PV seems to be more pronounced than that on EPO-dependent normal BFU-E. In contrast to a mean growth inhibition by 81% on autonomous BFU-E formation from PV PBMNCs, IL-10 decreased the number of EPO-stimulated BFU-E by approximately 45% in PBMNC cultures from normal individuals. Moreover, the inhibitory action of IL-10 is not restricted to the erythroid lineage, because we have also shown IL-10-induced inhibition of spontaneous formation of myeloid colonies in normal individuals[15] and patients with CMML[16] through suppression of endogenous GM-CSF release. Thus, the inhibition of GM-CSF synthesis by IL-10 makes it useful in indirectly modulating hematopoiesis in a variety of conditions.

Despite its well-defined effects in vitro, the in vivo role of GM-CSF in normal and abnormal hematopoiesis is much less clear. Mice deficient in GM-CSF (GM^-/-) through homologous recombination in embryonal stem cells show no alterations in hematopoiesis[34] suggesting substantial overlap of function with other colony-stimulating factors. On the other hand, animals transplanted with cells containing the GM-CSF cDNA developed a fatal syndrome resembling myeloproliferation, which however, was non-neoplastic in nature.[35] In PV patients it is not known if GM-CSF, as in the in vitro system, plays a significant role in the expansion of clonal cells. If this is the case, therapy with IL-10, a cytokine that has been well tolerated in first clinical trials,[36,37] could be considered as a strategy to suppress the excessive production of red blood cells in PV.

REFERENCES

1. Prchal JF, Axelrad AA: Bone marrow responses in polycythaemia vera. N Engl J Med 290:1382, 1974

2. Fisher MJ, Prchal JF, Prchal JT, D'Andrea AD: Anti-erythropoietin (EPO) receptor monoclonal antibodies distinguish EPO-dependent and EPO-independent erythroid progenitors in polycythemia vera. Blood 84:1982, 1994

3. Kralovics R, Indrak K, Stopka T, Berman BW, Prchal JF, Prchal JT: Two new EPO receptor mutations: Truncated EPO receptors are most frequently associated with primary familial and congenital polycythemias. Blood 90:2057, 1997

4. Fiorentino DF, Bond MW, Mosmann TR: Two types of mouse T helper cell: IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J Exp Med 170:2081, 1989

5. Veiira P, de Waal-Malefyt R, Dang MW, Johnson KE, Kastelein R, Fiorentino DF, de Vries JE, Roncarolo MG, Mosmann TR, Moore KW: Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: Homology to Epstein-Barr virus open reading frame BCRFI. Proc Natl Acad Sci USA 88:1172, 1991

6. Spits H, de Waal-Malefyt R: Functional characterization of IL-10. Int Arch Allergy Immunol 99:8, 1992

7. Rousset F, Garcia E, Defrance T, Péronne C, Vezzio N, Hsu DH, Kastelstein R, Moore KW, Banchereau J: Human and viral IL-10 are

potent growth and differentiation factors for activated human B lymphocytes. Proc Natl Acad Sci USA 89:1890, 1992

8. Ralph P, Nakoinz I, Sampson-Johannes A, Fong S, Lowe D, Min HY, Lin L: IL-10, T lymphocyte inhibitor of human blood cell production of IL-1 and tumor necrosis factor. J Immunol 148:808, 1992

9. Hsu DH, Moore KW, Spits H: Differential effects of interleukin-4 and -10 on interleukin-2-induced interferon-gamma synthesis and lymphokine-activated killer activity. Int Immunol 4:563, 1992

10. Thompson-Snipes LA, Dhar V, Bond MW, Mosmann TR, Moore KW, Rennick DM: Interleukin 10: A novel stimulatory factor for mast cells and their progenitors. J Exp Med 173:507, 1991

11. Cassatella MA, Meda L, Bonora S, Ceska M, Constantin G: Interleukin 10 (IL-10) inhibits the release of proinflammatory cytokines from human polymorphonuclear leukocytes. Evidence for an autocrine role of tumor necrosis factor and IL-1 beta mediating the production of IL-8 triggered by lipopolysaccharide. J Exp Med 178:2207, 1993

12. Takanaski S, Nonaka R, Xing Z, O'Byrne P, Dolovich J, Jordana M: Interleukin 10 inhibits lipopolysaccharide-induced survival and cytokine production by human peripheral blood eosinophils. J Exp Med 180:711, 1994

13. De Waal-Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE: Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: An autoregulatory role of IL-10 produced by monocytes. J Exp Med 174:1209, 1991

14. Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A: IL-10 inhibits cytokine production by activated macrophages. J Immunol 147:3815, 1991

15. Oehler L, Foedinger M, Koeller M, Kollars M, Reiter E, Bohle B, Skoupy S, Fritsch G, Lechner K, Geissler K: Interleukin-10 inhibits spontaneous colony-forming unit-granulocyte-macrophage growth from human peripheral blood mononuclear cells by suppression of endogenous granulocyte-macrophage colony-stimulating factor release. Blood 89:1147, 1997

16. Geissler K, Oehler L, Foedinger M, Virgolini I, Leimer M, Kabrna E, Kollars M, Skoupy S, Bohle B, Rogy M, Lechner K: Interleukin-10 inhibits growth and granulocyte/macrophage colony-stimulating factor production in chronic myelomonocytic leukemia cells. J Exp Med 184:1377, 1996

17. Berlin NI: Diagnosis and classification of the polycythemias. Semin Hematol 12:339, 1975

18. Chomczynski P, Sacchi N: Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156, 1987

19. Maurer J, Janssen JW, Thiel E, van Denderen J, Ludwig WD, Aydemir Ü, Heinze B, Fonatsch C, Harbott J, Riehm H, Hoelzer D, Bartram CR: Detection of chimeric BCR-ABL genes in acute lymphoblastic leukaemia by the polymerase chain reaction. Lancet 337:1055, 1991

20. Russell ME, Adamd DH, Wyner LR, Yamashita Y, Halnon NJ, Karnovsky MJ: Early and persistent induction of monocyte chemoattractant protein in rat cardiac allografts. Proc Natl Acad Sci USA 90:6086, 1993

21. Khoury SJ, Gallon L, Chen W, Betres K, Russell ME, Hancock WW, Carpenter CB, Sayegh MH, Weiner HL: Mechanism of acquired thymic tolerance in experimental autoimmune encephalomyelitis: Thymic dendritic-enriched cells induce specific peripheral T cell unresponsiveness in vivo. J Exp Med 182:357, 1995

22. Miyatake S, Otsuka T, Yokota T, Lee F, Arai K: Structure of the chromosomal gene for granulocyte- macrophage colony stimulating factor: Comparison of the mouse and human genes. EMBO J 4:2561, 1985

23. Kurtz A, Hart W, Jelkmann W: Activity in fetal bovine serum that stimulates erythroid colony formation in fetal mouse livers is insulinlike growth factor I. J Clin Invest 76:1643, 1985

24. Adamson JW, Fialkow PJ, Murphy S, Prchal JF, Steinmann L: Polycythemia vera: Stem-cell and probable clonal origin of the disease. N Engl J Med 295:913, 1976

25. Prchal JF, Adamson JW, Murphy S, Steinmann L, Fialkow PJ: Polycythemia vera. The in vitro response of normal and abnormal stem cell lines to erythropoietin. J Clin Invest 61:1044, 1978

26. Nissen C, Hasler E, Moor K, Moser Y, Speck B: Polycythemia vera: Spontaneous growth of hemoglobinized colonies is mediated by adherent cells. Exp Hematol 14:549, 1986

27. Shabbad E, Cassal A, Froom P, Aghai E: Effect of adherent cells on the regulation of BFU-E in patients with myeloproliferative disease. Am J Hematol 33:225, 1990

28. Dai CH, Krantz SB, Dessypris EN, Means Jr RT, Horn ST, Gilbert HS: Polycythemia vera II. Hypersensitivity of bone marrow erythroid, granulocyte-macrophage, and megakaryocyte progenitor cells to interleukin-3 and granulocyte-macrophage colony-stimulating factor. Blood 80:891, 1992

29. Dai CH, Krantz SB, Green WF, Gilbert HS: Polycythaemia vera III. Burst-forming units-erythroid (BFU-E) response to stem cell factor and c-kit receptor expression. Br J Haematol 86:12, 1994

30. Correa PN, Eskinazi D, Axelrad AA: Circulating erythroid progenitors in polycythemia vera are hypersensitive to insulin-like growth factor-1 in vitro: Studies in an improved serum-free medium. Blood 83:99, 1994

31. Mirza AM, Correa PN, Axelrad AA: Increased basal and induced tyrosine phosphorylation of the insulin-like growth factor I receptor b subunit in circulating mononuclear cells of patients with polycythemia vera. Blood 86:877, 1995

32. Dai CH, Krantz SB, Sawyer ST: Polycythemia vera V. Enhanced proliferation and phosphorylation due to vanadate are diminished in polycythemia vera erythroid progenitor cells: A possible defect of phosphatase activity in polycythemia vera. Blood 89:3574, 1997

33. De Wolf JTM, Hendriks DW, Esselink MT, Halie MR, Vellenga E: The effects of IL-1 and IL-4 on the Epo-independent erythroid progenitor in polycythemia vera. Br J Haematol 88:242, 1994

34. Stanley E, Lieschke GJ, Grail D, Metcalf D, Hodgson G, Gall JA, Maher DW, Cebon J, Sinickas V, Dunn AR: Granulocyte/ macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology. Proc Natl Acad Sci USA 91:5592, 1994

35. Lang RA, Metcalf D, Cuthbertson RA, Lyons I, Stanley E, Kelso A, Kannourakis G, Williamson DJ, Klintworth GK, Gonda TJ, Dunn AR: Transgenic mice expressing a hemopoietic growth factor gene (GM-CSF) develop accumulations of macrophages, blindness, and a fatal syndrome of tissue damage. Cell 51:675, 1987

36. Van Deventer SJH, Elson CO, Fedorak RN for the Crohn's Disease Study Group: Multiple doses of intravenous interleukin 10 in steroid-refractory Crohn's disease. Gastroenterology 113:383, 1997

37. Geissler K: Current status of clinical development of interleukin-10. Curr Opin Hematol 3:203, 1996