Gene Expression Profiling in Polycythemia Vera Using cDNA Microarray Technology

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ABSTRACT

Polycythemia vera (PV) is a myeloproliferative disorder characterized by an increased proliferation of all three myeloid lineages. The molecular pathogenesis of PV is unknown. Using cDNA microarrays comprising 6000 human genes, we studied the gene expression profile of granulocytes obtained from 11 PV patients compared with granulocytes obtained from healthy individuals. We found that 147 genes were up-regulated by ≥2.5 fold in the majority of PV patients. Eleven of these 147 genes were up-regulated in all PV patients studied and may represent a molecular signature for this disorder. An increase in the expression of several protease inhibitors with affinity for proteases that promote apoptosis in neutrophils (e.g., cystatin F, secretory leukocyte protease inhibitor), as well as the up-regulation of a number of antiapoptotic and survival factors was found (e.g., adrenomedullin, p38 mitogen-activated protein kinase). We speculate that the deregulation of these factors may inhibit normal apoptosis and promote cell survival in the granulocytes of patients with PV. These PV-specific expression changes are likely to be biologically important in the pathophysiology of this disorder.

INTRODUCTION

PV, idiopathic myelofibrosis, CML, and ET are classified as myeloproliferative disorders because they arise through the clonal expansion of a multipotent hematopoietic progenitor cell, with consequential excess production of one or more of the cells of the myeloid lineage (1). PV is characterized by an increased proliferation of all three myeloid lineages with subsequent overproduction of mature red cells, granulocytes, and platelets (1). X-inactivation studies have shown that red cells, neutrophils, monocytes, and platelets are clonal in PV, whereas T cells are polyclonal (2). Unlike CML, where the Philadelphia chromosome results in the BCR-ABL fusion gene, no specific chromosomal or gene abnormality has been reported in PV (2). The overproduction of erythrocytes in PV occurs in the absence of a recognizable pathological stimulus because serum levels of EPO are normal or lower than normal. Patients with PV have erythroid progenitors that develop in vitro without the addition of EPO (3). The presence of EPO-independent colonies in PV has lead to the suggestion that the primary defect in this disorder may be an abnormality in the EPO signaling pathway, possibly even involving the EPO receptor (2). Mutation of the EPO receptor has been reported in families with inherited erythrocytosis, but no abnormalities of the gene have been identified in PV, and the expression of the EPO receptor and its ability to bind EPO are also normal (2).

The erythroid progenitors of PV patients are hypersensitive to a variety of growth factors and cytokines, including stem cell factor, IL-3, granulocyte-macrophage colony-stimulating factor, and insulinderived growth factor (2). Despite these abnormal responses, the total number of receptors and binding affinities for each of these growth factors are normal. The observation that PV cells are hypersensitive to a large variety of growth factors may indicate that signal transduction pathways are altered in these cells (2). Interestingly, high levels of Bcl-x, an inhibitor of apoptosis, in erythroid precursors from patients with PV have been described by Silva et al. (4). The investigators speculated that this might explain the increased survival of PV cells without EPO. Most recently, increased expression of the cyclin-dependent kinase inhibitor INK4a/ARF locus has been reported in the erythroid cells of patients with PV (5). Other genes shown to be up-regulated in PV include CD44 and PRV-1, a novel member of the uPAR receptor superfamily (6). PRV-1 mRNA is highly expressed in the granulocytes from patients with PV but not detectable in normal control granulocytes and may be useful in establishing a diagnosis of PV (6).

Despite recent advances in the characterization of the malignant PV clone, the molecular abnormality(ies) associated with the development of this disorder remains unknown. As with an increasing number of other malignancies, including breast cancer and acute leukemia (7), it is most probable that the identification of genes differentially expressed in the myeloid cells of patients with PV will shed light on those gene/genes pathways important in its molecular pathogenesis. To investigate the molecular defects underlying PV, we have used cDNA microarray technology to determine the gene expression profile in granulocytes obtained from PV patients.

SAMPLE COLLECTION AND CELL SEPARATION. Peripheral blood samples were obtained from 11 patients with PV, 2 patients with ET and 10 healthy volunteers. The diagnosis of PV and ET was established according to commonly accepted clinical and laboratory criteria (8). The study was approved by the ethics committee (C00.198), and informed consent was obtained. Granulocytes were isolated using Histopaque (Sigma-Aldrich, Dorset, United Kingdom) and pelleted after hypotonic lysis of erythrocytes and two washes in PBS (9). The purity of the granulocyte populations isolated from both PV patients and healthy volunteers was consistently high and was within the range 95–98% as assessed by standard morphology on Wright-Giemsa stained cytospin preparations.

RNA EXTRACTION AND POOLING. Total RNA from each sample was extracted using Trizol (Invitrogen-Life Technologies, Inc., Paisley, United Kingdom) following the protocol supplied by the manufacturer. Briefly, granulocyte pellets were resuspended in Trizol, incubated at room temperature for 2–3 min, and after addition of chloroform and separation of the phases, RNA was precipitated with isopropanol from the recovered aqueous phase. The RNA pellet was resuspended in diethyl pyrocarbonate-treated water and quantified by spectrophotometer measurement. An aliquot of each sample was conserved
for gel electrophoresis, inspection of RNA quality, and for real-time quantitative PCR analysis. Equal amounts of RNA extracted from the granulocyte fraction from 10 healthy volunteers were mixed to generate a pool of normal control RNA.

**RNA Labeling and Hybridization.** In each experiment, 1 PV patient was compared with the normal RNA pool; 25 μg of total RNA from the control pool were labeled with Cy3-dCTP and 25 μg of total RNA from each patient with Cy5-dCTP (Amersham Biosciences, Uppsala, Sweden). Labeling occurred via reverse transcription in the presence of 3 nmol of either Cy3-dCTP or Cy5-dCTP, 5 μg of oligo(dT), and 400 units of SuperScript II RT (Invitrogen-Life Technologies, Inc.). The labeled cDNAs were purified, mixed together, and after addition of 6 μg of poly(dA) (Sigma-Aldrich) and 6 μg of Cot-1 DNA (Invitrogen-Life Technologies, Inc.), hybridized competitively to a microarray slide (Sanger Institute, Hinxton, Cambridge, United Kingdom) containing 10,000 spots representative of 6000 known human genes.4 Hybridization occurred at 47°C overnight and then the slides were washed at room temperature once in 2× SSC for 5 min, twice in 0.1× SSC, 0.1% SDS for 30 min, and twice in 0.1× SSC for 5 min. All experiments were performed in duplicate according to the Sanger Institute’s own protocols.4

**Scanning and Analysis.** Slides were scanned immediately (ScanArray 4000; Perkin-Elmer/Packard BioScience, Boston, MA) with a 10-μm resolution and the generated TIFF images imported in QuantArray 3.0 (PE/Packard BioScience). After image and grid alignment and spot location, the Cy5 and Cy3 intensities were first normalized to the median value of the whole array and then the ratio for every spot was obtained. Analysis files were imported in GeneSpring 4.1 (Silicon Genetics, Redwood City, CA) and gene filtering and clustering performed.

**Real-Time Quantitative PCR.** Two μg of total RNA from each single patient and from each single healthy volunteer (included in the normal pool) were reverse transcribed using RETROscript kit (Ambion, Inc., Austin, TX). The generated first-strand cDNA was diluted and used as template for real-time quantitative PCR analysis (10, 11). This method is based on the use of two PCR primers and an additional fluorogenic probe in which the target sequence is located within the primer-generated amplicon. The probe has a fluorescent dye on the 5’-end (FAM) and a quencher on the 3’-end (TAMRA). During the extension phase of the PCR amplification, the 5’ exonuclease activity of

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4 Internet address: http://www.sanger.ac.uk/Projects/Microarrays/.

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Fig. 1. Hierarchical clustering of 147 genes up-regulated in the granulocytes of PV patients. Each column represents a single gene on the microarray and each row a separate patient/control granulocyte sample. PV1 to PV11 are patients with PV; ET1 and ET2 are patients with essential thrombocythemia; N1 and N2 are normal healthy individuals.
the Taq DNA polymerase cleaves the probe releasing the fluorescent dye from the quencher. At each cycle of the PCR process, the increase of the fluorescence was monitored by an ABI Prism 5700 Sequence Detection System (Perkin-Elmer/Applied Biosystems; Refs. 10, 11). Using Primer Express 2.0 software (PE/Applied Biosystems), PCR primers for each gene were designed in adjacent exons and the fluorogenic probe spanning the junction between them. Fluorogenic probes were custom synthesized by PE/Applied Biosystems; PCR primers were synthesized by MWG Biotech (Ebersberg, Germany). The expression level of the β2-microglobulin gene was used to normalize for differences in input cDNA. For each gene, the sequences of the PCR forward primer, PCR reverse primer, and fluorogenic probe, respectively, are as follows: GYG, 5′-CAATCACGTTGACATCTTGGCT-3′, 5′-AAAATTGTTCTCAGTAGGTCTCT-3′, and 6FAM-5′-CCACATCAAAACTACCTTGCTCA-3′-TAMRA; CMAP, 5′-TCCTGATGACTGTGACTTCTCCAACAA-3′, 5′-AGGGCACAGCAGAGCCTCC-3′, and 6FAM-5′-CAACACACCTTGAGCACTGAC-3′-TAMRA; GNG10, 5′-AGGTGACTGATGGTCCCACCAA-3′, 5′-GGGTCATGAGGCTCCGCAGTTG-3′, and 6FAM-5′-CAGCAGTCAGGCCACCTATTCACCTCTTCT-3′-TAMRA. For the SGK, the BTG2 and the β2-microglobulin gene, we used commercially available predeveloped TaqMan assays (PE/Applied Biosystems). PCR reactions occurred in a volume of 25 μl in presence of 7.5 pmol of each primer, 2.5 pmol of fluorogenic probe, and 12.5 μl of TaqMan 2× Universal PCR mastermix. Five μl of diluted cDNA solution were used as template in each reaction. The thermal cycling program was: a first step at 50°C for 2 min, then 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 s and 60°C for 1 min.

Statistical Analysis. The results of the real-time quantitative PCR are expressed as difference in the expression levels between the gene of interest and the reference gene (β2-microglobulin) in each sample. Statistically significant differences between patients and control, were investigated using the Mann-Whitney U test. Analysis was performed using the StatView 5.0 software package (SAS).

RESULTS

Gene expression analysis of granulocytes obtained from PV patients compared with granulocytes obtained from healthy individuals showed that 147 genes were up-regulated by ≥2.5-fold in the majority of PV patients (defined as in at least 6 of 11 patients). The profile of these up-regulated genes was visualized in a gene tree (or dendrogram) that clusters together genes with similar expression patterns. Moreover, the tree clusters in families with the patient microarray expression profiles for these genes, and it shows that the patients with ET (a closely related myeloid disorder) clustered together with two individual normal controls, whereas the patients with PV were grouped in distinct families (Fig. 1). The gene tree revealed the presence of clusters of PV-specific gene expression changes. Among the 147 genes identified, a subset of 33 genes was up-regulated by a minimum of 3.5-fold in the majority of PV patients (Table 1). Eleven genes were up-regulated by ≥2.5 fold in all 11 PV patients included in the study: GYG, CMAP, SLPI, ADM, SFRS1, FCKERG, S100 CAAF1, IP30, PYGL, GNG10, and ANX3. Twenty genes were down-regulated (ratio < 0.7) in the granulocytes of the majority of the PV patients. These include, for example, SGK, BTG2, immune activation gene, nuclear factor 90, IL-7 receptor, and ribosomal phosphoprotein P0.

We studied the expression of the 147 genes up-regulated by ≥2.5-fold in the majority of PV patients in an ongoing study of 11 cases with myelodysplastic syndromes; interestingly, none of the 147 genes were up-regulated by ≥2.5 fold in the majority of the cases with myelodysplasia.

To investigate the validity of the array data, the expression levels of seven differentially expressed genes were measured using the TaqMan 5′ nucleolar fluorogenic quantitative PCR assay. Relative expression levels initially determined with the cDNA microarrays were correlated with the expression levels determined using quantitative PCR for each of the patient samples. We chose to investigate GYG, CMAP, SLPI, IL-4 receptor, and ADM, all of which were consistently up-regulated in PV; we also chose SGK and BTG2, which were down-regulated in PV. The correlation between the two assays for all seven genes validated was high, indicating a high level of agreement between the two assays for the identification of dysregulated genes (Fig. 2). Statistical analysis performed on results obtained with real-time quantitative PCR in the group of PV patients and controls showed that the two groups are significantly different. Using the Mann-Whitney U test, a p ≤ 0.002 was obtained for all of the genes selected for validation.

DISCUSSION

We have used global expression profiling to characterize gene expression in the granulocytes of PV patients and to compare it to that of granulocytes from healthy controls. The gene expression patterns of granulocytes from PV patients were clearly distinct from those obtained from the granulocytes of healthy controls. We have determined that the abnormal pattern of gene expression observed in PV is remarkably consistent across the 11 patients studied. Gene expression analysis of granulocytes obtained from PV patients compared with granulocytes obtained from healthy individuals showed that 147 genes were up-regulated by ≥2.5-fold in the majority of PV patients. This study has identified many genes that are differentially expressed in PV, several of which comprise antiapoptotic factors or genes that promote cell survival.

We have shown the up-regulation of several protease inhibitors,

Table 1 List of the genes up-regulated by ≥3.5-fold in the majority of PV patients

<table>
<thead>
<tr>
<th>Cellular function</th>
<th>Genes</th>
<th>Product</th>
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<tr>
<td>Cell adhesion</td>
<td>CD44</td>
<td>CD44 antigen</td>
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<td></td>
<td>CD48</td>
<td>CD48 antigen</td>
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<td>Cell growth and</td>
<td>PBR</td>
<td>Benzozaepine receptor, peripheral type</td>
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<td>maninestence</td>
<td>SNX1</td>
<td>Sorting nexin 3</td>
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<td>ANX3</td>
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<td>DEFA1</td>
<td>Defensin 1</td>
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<td>SFRS1</td>
<td>SFRS protein kinase 1</td>
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<td></td>
<td>IP30</td>
<td>IP30</td>
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<tr>
<td>Signal transduction and transcription factors</td>
<td>MAPK</td>
<td>p38 MAPK</td>
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<td>CAAF1</td>
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<td>MAPK-interacting serine/threonine kinase 1</td>
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<td>6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3</td>
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<td>Apoptosis</td>
<td>IL-4R</td>
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<td>CMAP</td>
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<td>E1</td>
<td>EL, monocyte/neutrophil</td>
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<td>NAP</td>
<td>Neuronal apoptosis inhibitory protein</td>
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including SLPI, (12), CMAP (13), and neutrophil EI (14) in the majority of patients with PV included in this study. These protease inhibitors are known to have a high affinity for proteases that promote apoptosis in neutrophils (15). For example, the protein encoded by SLPI has a high affinity for three enzymes: trypsin, chymotrypsin, and elastase, all of which are known to induce a dramatic stimulation of apoptosis in neutrophils (15). Similarly, EI has a high affinity for the neutrophil serine protease elastase (14, 15). It is possible that the up-regulation of protease inhibitors with affinity for proteases that promote apoptosis in neutrophils may contribute to the increased cell survival observed in PV neutrophils.

Interestingly, the p38 MAPK (16) is up-regulated in the majority of PV patients included in this study. The MAPK pathway regulates growth and survival of many cell types, and its constitutive activation has been implicated in the pathogenesis of a number of cancers (16). Inappropriate MAPK activation may play a role in the leukemic transformation of myeloid cells (16), and there is much evidence to suggest that p33 MAPK plays a role in regulating antiproliferative and inflammatory responses (17). It has been shown, for example, that p33 MAPK contributes to the survival of human neutrophils and that the early reduction in p33 MAPK activity during both spontaneous and Fas-induced apoptosis (18) constitutes an important regulatory step in the initiation of neutrophil apoptosis. The exposure of human neutrophils to the p33 MAPK inhibitor, SB203580, has been shown to cause an increase in cell apoptosis. Thus, the pharmacological inhibition of p33 MAPK activity promotes apoptosis in neutrophils. It is possible that p33 MAPK may represent a valuable drug target in PV. The up-regulation of p33 MAPK observed in the granulocytes of patients with PV may contribute to the increased cell survival observed in PV.

This study has demonstrated the up-regulation of several other antiproliferative factors in the granulocytes of patients with PV. These included ADM, a peptide that elevates intracellular cyclic AMP and is a mitogen, angiogenic factor, and apoptosis survival factor highly expressed in a wide range of human tumors and malignant cell lines (19). It has been proposed that ADM can function as an autocrine growth factor that could drive neoplastic proliferation (19). IL-4 receptor (20) is also up-regulated in the granulocytes of patients with PV. Responses to IL-4 are mediated by IL-4 receptor, and IL-4 has an antiproliferative effect on human neutrophils (20). We speculate that the increase in IL-4 receptor in PV may result in an amplification of the protection from apoptosis conferred by IL-4.

Cancer-related genes shown to be deregulated by this study include the proto-oncogene ETS2 (21). The ETS family of genes encode transcription factors and are implicated in hematological malignancy, including acute myeloid leukemia and chronic myelomonocytic leukemia (21). The proto-oncogene ETS2 is up-regulated in the majority of patients with PV in our study. Interestingly, up-regulation of ETS2 is associated with blast crises in CML.

G-CSF is not only a specific growth factor for the granulocyte colony-forming unit but also an activator for mature neutrophils (22). G-CSF enhances the survival of neutrophils and stimulates superoxide production and phagocytosis (22). A number of genes known to be induced by G-CSF treatment of human neutrophils (22) were also shown to be up-regulated in the granulocytes of PV patients and include CD44 antigen, CD48 antigen, and Fc fragment of IgE and high affinity I receptor. Similarly, several genes known to be induced in human neutrophils after activation by lipopolysaccharide (17) and including toll-like receptor 5, CD44 antigen, CD48 antigen, ETS2, and glycerol kinase were also found to be up-regulated in the granulocytes of patients with PV. Thus, there are similarities between the gene expression profile of granulocytes from PV patients with those of activated neutrophils from normal individuals.

In contrast to gene up-regulation, gene down-regulation is not a marked feature of the granulocytes of patients with PV. Only a few genes were down-regulated in the majority of patients. Genes down-regulated in the majority of PV patients in this study include the antiproliferative BTG2 gene, a p53 target that negatively regulates the cell cycle in response to DNA damage (23) and the transcriptional regulator nuclear factor 90.

Eleven genes were up-regulated by ≥2.5 fold in all 11 PV patients included in the study (GYG, CMAP, SLPI, ADM, SFRS1, FCER1G, CAAFI, IP3O, PYGL, GNG10, and ANX3). Analysis of the differential gene expression profiles of normal and PV granulocytes has identified a select set of genes that may define a molecular signature for PV. A robust set of molecular markers for PV would clearly be of great diagnostic use (24).

The molecular pathogenesis of PV is unknown. The array data presented here shows an increase in the expression of several protease inhibitors with affinity for proteases that promote apoptosis in neutrophils as well as the up-regulation of a number of antiproliferative and survival factors. We speculate that the deregulation of these factors may inhibit normal apoptosis and promote cell survival in the granulocytes of patients with PV. These PV-specific expression changes are likely to be biologically important in the pathophysiology of this disorder.

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9. Boyum, A. Isolation of mononuclear cells and granulocytes from human blood: Isolation of mononuclear cells by one centrifugation and of granulocytes by combination using a high affinity I receptor. Similarly, several genes known to be induced in human neutrophils after activation by lipopolysaccharide (17) and including toll-like receptor 5, CD44 antigen, CD48 antigen, ETS2, and glycerol kinase were also found to be up-regulated in the granulocytes of patients with PV. Thus, there are similarities between the gene expression profile of granulocytes from PV patients with those of activated neutrophils from normal individuals.

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