RAPID COMMUNICATION

Increased Basal and Induced Tyrosine Phosphorylation of the Insulin-Like Growth Factor I Receptor β Subunit in Circulating Mononuclear Cells of Patients With Polycythemia Vera

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We have previously shown that circulating progenitor cells in patients with polycythemia vera (PV) are hypersensitive to insulin-like growth factor I (IGF-I) with respect to erythroid burst formation in serum-free medium, and that this effect occurs through the IGF-I receptor. To investigate the molecular basis of this IGF-I hypersensitivity phenomenon, we examined tyrosine phosphorylation of the IGF-I receptor β subunit in peripheral blood mononuclear cells (PBMC) from eight PV patients and six normals. Cells were exposed to IGF-I at concentrations of 10⁻¹⁴ and 10⁻¹⁰ mol/L for 0, 1, 3, and 10 minutes, and then lysed. The IGF-I receptor β subunit was immunoprecipitated, and the protein was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotted with antiphosphotyrosine antibody (4G10). We found that, in the absence of exogenous IGF-I, there was a basal level of tyrosine phosphorylation of the IGF-I receptor β subunit, and it was substantially greater in PV than in normal. At 10⁻¹⁰ mol/L IGF-I in normals, no evidence of increased tyrosine phosphorylation was detected; however in PV, a pronounced increase in tyrosine phosphorylation was observed at both 10⁻¹⁴ and 10⁻¹⁰ mol/L IGF-I, and it occurred earlier and attained a higher level than in normal. In contrast, in PBMC from three patients with erythrocytosis, no significant increase above normal was seen in either basal or induced tyrosine phosphorylation of the IGF-I receptor β subunit. Thus, our findings show two distinctive features of the PV phenotype in PBMC: (1) an increased basal tyrosine phosphorylation of the IGF-I receptor β subunit; and (2) a hypersensitive and hyperresponsive receptor with respect to tyrosine phosphorylation. These features may influence the ability of the receptor to transmit a proliferative signal; thus, they may play a role in the pathogenesis of PV.

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POLYCYTHEMIA VERA (PV) is a chronic myeloproliferative disorder of as yet undetermined etiology characterized by a hyperplasia of all three major myeloid lineages. Because of the clonal nature of the disease, it is believed that dysregulation of a pluripotent stem cell leads to increased proliferation and expansion of the affected myeloid compartments, but with a particular emphasis on the erythroid lineage. PV is distinguished from secondary polycythemia in that the defect is intrinsic to the cells, and the relentless overproduction of red blood cells (RBCs) in PV occurs in the presence of normal O₂ saturation and with levels of serum erythropoietin (Epo), the key hormone of normal adult erythropoiesis, often depressed. Recently, work in our laboratory using an improved serum-free medium has shown that circulating erythroid progenitors (BFU-E) are not hypersensitive to Epo as previously believed, but exhibit a more than 100-fold increased sensitivity to insulin-like growth factor I (IGF-I). Furthermore, using an anti-IGF-I receptor antibody, we were able to abolish the IGF-I hypersensitivity, indicating that the effect was mediated via the IGF-I receptor.

The receptor for IGF-I is a member of the tyrosine kinase receptor family and is highly homologous to the insulin receptor. The IGF-I receptor is synthesized as a 180-kD precursor that is glycosylated, dimerized, and proteolytically processed to yield the mature αβ₂ heterotetramer consisting of two extracellular ligand-binding α chains disulfide linked to two β chains that span the membrane once and contain the intrinsic tyrosine kinase activity. Extracellular ligand binding to the receptor stimulates its intrinsic tyrosine-specific protein kinase activity, which leads to β subunit autophosphorylation. Autophosphorylation of the cytoplasmic domain of the β subunit leads to a dramatic increase in its kinase activity and its subsequent phosphorylation of intracellular substrates. It is thus believed to have an essential role in signal transduction.

Receptor tyrosine kinases are important in mediating the effects of extracellular growth factors on cell proliferation and differentiation. Consistent with this view, an abnormal increase in the activity of receptor tyrosine kinases has been associated with unregulated growth. Studies indicate that an increased tyrosine kinase activity—attributed either to an increase in the number of tyrosine kinase receptors or to their constitutive activation—elicits increased signaling. Since we had previously observed hypersensitivity to IGF-I in PV, and as an increased tyrosine kinase activity had been shown to be associated with unregulated proliferation, we compared the kinase activities of the IGF-I receptor in cells from patients with PV with those from normal individuals. Here, we report that in PV tyrosine phosphorylation of the IGF-I receptor β subunit is increased in the absence of added ligand and its response to ligand is substantially enhanced. We suggest that these changes may provide a molecular mechanism for the hypersensitivity to IGF-I in this myeloproliferative disorder.

MATERIALS AND METHODS

Patients. Patients clinically diagnosed as having PV with the help of the PV Study Group guidelines were used in this study. All patients were being managed by phlebotomy at the time of study.

one PV patient had previously been treated with hydroxyurea. Patients with erythrocytosis were negative with respect to the criteria for PV, but had elevated hematocrits/RBC mass and either a decreased plasma volume, or an elevated Epo level.

**Cell preparations.** After informed consent, peripheral blood (PB) was obtained by venipuncture from healthy volunteer donors and patients, and was immediately placed in a polypropylene tube containing 10 U/mL preservative-free sodium heparin (no. 820 5077MF, GIBCO, Grand Island, NY). The heparinized blood was layered onto 15 mL of Ficoll-Hypaque (Pharmacia, Montreal, Quebec, Canada) and the light-density mononuclear cells (PBMNC) were collected after centrifugation at 450g for 30 minutes at room temperature. Cell suspensions were washed three times (450g, 10 minutes at room temperature) in a minimal essential medium (aMEM) containing 0.1% fatty-acid-free and globulin-free bovine serum albumin (FAF-BSA), and the cells were counted.

**Stimulation of cells with ligand.** After three washes in aMEM + 0.1% FAF-BSA (450g, 10 minutes at room temperature), PBMNC were resuspended in basal serum-free medium at a concentration of 5 x 10^5 cells/mL for 30 minutes at room temperature. The cells were then washed in aMEM + 0.1% FAF-BSA and resuspended at 5 x 10^5 cells/mL in aMEM + 0.1% FAF-BSA without or with IGF-I at a final concentration of 10^{-8} or 10^{-10} mol/L. PBMNC were incubated with and without ligand for 0, 1, 3, 10, or 30 minutes at 37°C. The cells were then washed in cold aMEM + 0.1% FAF-BSA, microfuged (Millicel, Hiettich, Diamond, Mississauga, Ontario, Canada) at 15,000 rpm for 5 seconds, and immediately lysed in sample buffer (0.5 mol/L Tris pH 6.8, 4% sodium dodecyl sulfate [SDS], 2% beta-mercaptoethanol, 5% glycerol, 0.1% Bromphenol blue) for immunoblotting with antiphosphotyrosine (UBI, Lake Placid, NY) or anti-IGF-I receptor antibody (UBI). Next day, after the appropriate washes (PBS containing 0.1% Triton X-100 [Sigma, St Louis, MO], 1% bovine hemoglobin, 0.1% SDS, and 0.5% sodium deoxycholate) for immunoprecipitation in the presence of protease inhibitors (stock in dimethyl sulfoxide containing 270 nmoL mL TLCK, 565 nmoL mL TPCK, 370 nmoL mL phenylmethylsulfonyl fluoride [PMSF], 280 nmoL mL E-64, 100 nmoL mL leupeptin, and 30 nmoL mL pepstatin) used at 1:1,000 stock concentration and 2 mmol/L sodium vanadate.

**Immunoprecipitation of IGF-I receptor.** Prepared cells were lysed in lysis buffer, protease inhibitors, and sodium vanadate at a final concentration of 5 x 10^5 cells/mL for 1 hour at 4°C. The lysate was then centrifuged twice at 3,000g for 10 minutes and again at 10,000g for 30 minutes. The supernatant was precleared overnight at 4°C with 100 lg of a prepared slurry of protein G-Sepharose (Sigma) plus dilution buffer (0.1% Triton X-100, 0.1% bovine hemoglobin in TSA [10 mmol/L Tris-HCl, pH 8.0, 140 mmol/L NaCl, 0.025% NaN_3]) and the supernatant was removed. Supernatants in 200-ml final volumes were placed in 1.5-ml microfuge tubes pre-coated with dilution buffer, 3 lg of anti-IGF-I receptor antibody directed against the beta subunit (αIR3) was added, and the contents of the tube were incubated for 1 hour at 4°C on an orbital shaker. Then, 25 lg of a prepared slurry of protein G-Sepharose was added to the mixture and the contents were incubated overnight at 4°C on an orbital shaker. The complexed Sepharose was then washed four times: twice in dilution buffer, once in TSA, and finally in 0.05 mol/L Tris Cl, pH 6.8. After the final wash, 50-lg sample buffer (10 mmol/L Tris-HCl pH 7.2, 1% SDS, 0.1 mmol/L beta-mercaptoethanol [β-ME], 10% glycerol, and 0.01% Bromphenol blue) was added to the complexed sepharose and the tubes were incubated at 100°C for 5 minutes.

**Immunoblotting with antiphosphotyrosine or anti-IGF-I receptor antibody.** To analyze tyrosine phosphorylation, cells prepared in sample buffer were centrifuged at 450g for 10 minutes. The supernatants were subjected to electrophoresis on an 8% SDS-gel. Proteins were electrophoretically transferred to supported nitrocellulose membrane. The membrane was blocked with non-specific proteins (Block 1, 5% w/vol powdered skim milk in phosphate-buffered saline [PBS]). After primary blocking, the membrane was incubated overnight with fresh Block 1 with primary monoclonal mouse antibody (αG10, UBI) at a dilution of 1 lg/mL, or polyclonal rabbit anti-IGF-IR (UBI). Next day, after the appropriate washes (PBS followed by Tris/NaCl [50 mmol/L Tris, 150 mmol/L NaCl, pH 7.5]), the nitrocellulose membrane was incubated with the secondary antibody, rabbit-antimouse horseradish peroxidase (HRP) conjugate (Sigma), or goat-antirabbit HRP conjugate (Sigma), or in the case of Fig 1, the rabbit-antimouse as secondary antibody, followed by the goat-antirabbit as tertiary antibody in Block 1 (5% w/vol powdered skim milk in Tris/NaCl). The blotted nitrocellulose membrane was treated with enhanced chemiluminescence substrates (ECL; Amersham, Arlington Heights, IL) and the membrane was used to expose x-ray film.

**Protein controls.** Protein loading was controlled by running duplicate gels and then either Western blotting with anti-IGF-I receptor antibody (outlined above), or by metabolically labeling cells with 35S-Met and 35S-Cys (Translabel; ICN, Montreal, Quebec, Canada) for Fig 1. In the latter case, prepared PBMNC were incubated at a concentration of 5 x 10^5 cells/mL in labeling medium (aMEM minus Met and Cys plus 25 mmol/L HEPES, pH 7.4, and 0.1% FAF-BSA) for 15 minutes at 37°C to deplete intracellular pools of Met and Cys. The cells were centrifuged and resuspended in labeling medium plus 0.2 mmol/L Translabel (ICN) to a concentration of 5 x 10^5 cells/mL. The cells were incubated at 37°C for 3 hours with gentle agitation. The prepared cells were used to immunoprecipitate the IGF-I receptor beta subunit (as described above).

**Densiometric analysis.** As LKB Ultrascan XL Laser Densitometer (LKB Biochem, Cambridge, UK) was used to scan immunoblots. Absorbance of the 95-kD band, ie, the IGF-I receptor beta subunit, was quantitated as the area under the peak for the band in arbitrary absorbance units. Table 1 contains representative data of a single PV patient compared with a normal individual. The highest absorbance value, whether normal or PV, was taken as 100%. All absorbance values were then normalized as percent of the maximum. The means of these percent values at each time point in six normal individuals and eight PV patients, together with their standard errors, were used to plot curves (Figs 2 and 3).

**RESULTS**

**Tyrosine phosphorylation of the IGF-I receptor in normal and PV.** It has been shown that in cells expressing the IGF-I receptor, stimulation with IGF-I rapidly induces autophosphorylation of the IGF-I receptor beta subunit on specific tyrosine residues that play an essential role in the cells' response to IGF-I. Using PBMNC isolated from normal individuals, we first showed that, in the presence of 10^{-8} mol/L IGF-I, autophosphorylation of the beta subunit occurred within 1 minute, with phosphorylation peaking between 3 and 10 minutes (data not shown), thus indicating that PBMNC have IGF-I receptors which respond to IGF-I in the expected way. Next, we compared tyrosine phosphorylation of the receptor beta subunit in PBMNC obtained from normal individuals and patients with PV. Figure 1 compares tyrosine phosphorylation of the IGF-I receptor beta subunit in PV with that of the normal in high concentration of ligand. It illustrates that at time zero, in the absence of added IGF-I, the amount of tyrosine phosphorylation in PV cells was substantially greater than in normal. With time, in the presence of 10^{-8} mol/L IGF-I, there was an increase in tyrosine phosphorylation in both PV and normal cells, but the beta subunit...
IGF-I RECEPTOR TYROSINE PHOSPHORYLATION IN PV

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**Fig 1.** Comparison of tyrosine phosphorylation in normal and PV cells at high IGF-I concentration for similar amounts of IGF-I receptor.

Time course of tyrosine phosphorylation after immunoprecipitation of the IGF-I receptor \( \beta \) subunit in the presence of \( 10^{-9} \text{ mol/L} \) IGF-I. PBMNC were metabolically labeled with \( \text{\textsuperscript{35}S-Met and \text{\textsuperscript{35}S-Cys (Translabel; ICN) and placed in serum-free culture. They were then exposed to } 10^{-8} \text{ mol/L IGF-I for 0, 1, 3, and 10 minutes. The cells were lysed, and the lysates were used to immunoprecipitate the IGF-I receptor } \beta \text{ subunit with a monoclonal anti-IGF-I receptor antibody (\( \alpha \text{IR3)}. Immunoprecipitates were resolved on an 8% gel by SDS-PAGE and the proteins were then transferred to nitrocellulose membrane and Western blotted with a monoclonal antiphosphotyrosine antibody (4G10). As a control for the amount of IGF-I receptor, a duplicate gel was dried and used to expose x-ray film.**

Phosphorylated earlier and to a much higher level in PV. In contrast, the amount of IGF-I receptor \( \beta \) subunit was similar in PV and normal, as shown by immunoprecipitation of the IGF-I receptor \( \beta \) subunit from metabolically labeled cells with an anti-IGF-I receptor antibody (\( \alpha \text{IR3) (Fig 1). Therefore, in PV cells the increased ability of the receptor to respond to IGF-I, as evidenced by increased tyrosine phosphorylation, appeared to be specific, and was not simply caused by an increase in the amount of receptor protein. **

We had previously shown that circulating progenitor cells in patients with PV are hypersensitive to IGF-I with respect to erythroid burst formation in serum-free medium. In normals, no bursts developed at IGF-I concentrations below \( 10^{-9} \text{ mol/L} \), whereas in PV burst formation occurred at IGF-I concentrations below \( 10^{-11} \text{ mol/L} \). To determine whether the IGF-I receptor is hypersensitive to IGF-I with respect to tyrosine phosphorylation, PV and normal cells were exposed to IGF-I at concentrations of \( 10^{-8} \text{ mol/L} \) and \( 10^{-10} \text{ mol/L} \), and then lysed. Proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotted with antiphosphotyrosine antibody. To quantitate tyrosine phosphorylation of the IGF-I receptor in response to ligand, densitometric analysis was performed. Figure 2 shows data on Western blots from eight PV patients and six normals, normalized as percent of maximum \( \pm \) SEM. The densitometric data demonstrate an increased tyrosine phosphorylation in PV over normal cells in the absence of added IGF-I (.01 \( > \) \( P > .001 \)) and in the presence of low concentration of ligand, \( 10^{-10} \text{ mol/L IGF-I}, \) at 1 and 3 minutes (.02 \( > \) \( P > .01 \) and .001 \( > \) \( P \), respectively). Tyrosine phosphorylation in PV appeared to peak at 3 minutes and then decrease; however, in the normal, the apparent increase in tyrosine phosphorylation at 10 minutes did not reach the level of statistical significance (\( P > .05 \)). In the presence of high phosphorylation earlier and to a much higher level in PV. In contrast, the amount of IGF-I receptor \( \beta \) subunit was similar in PV and normal, as shown by immunoprecipitation of the IGF-I receptor \( \beta \) subunit from metabolically labeled cells with an anti-IGF-I receptor antibody (\( \alpha \text{IR3) (Fig 1). Therefore, in PV cells the increased ability of the receptor to respond to IGF-I, as evidenced by increased tyrosine phosphorylation, appeared to be specific, and was not simply caused by an increase in the amount of receptor protein. **

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**Table 1. Densitometric Analysis of the 95-kD Band in Normal and PV**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>N Absorbance (arbitrary units)</th>
<th>N Percent maximum absorbance</th>
<th>PV Absorbance (arbitrary units)</th>
<th>PV Percent maximum absorbance</th>
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<td>10</td>
<td>0.452</td>
<td>47.0</td>
<td>0.500</td>
<td>52.0</td>
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</table>

**A** Absorbance (arbitrary units) and Percent maximum absorbance for normal and PV cells. IGF-I receptor was immunoprecipitated from metabolically labeled cells with a monoclonal anti-IGF-I receptor antibody (\( \alpha \text{IR3) (Fig 1). Immunoprecipitates were resolved on 8% SDS-PAGE and the proteins were then transferred to nitrocellulose membrane and Western blotted with a monoclonal antiphosphotyrosine antibody (4G10). As a control for the amount of IGF-I receptor, a duplicate gel was dried and used to expose x-ray film.

**B** Absorbance (arbitrary units) and Percent maximum absorbance for normal and PV cells. IGF-I receptor was immunoprecipitated from metabolically labeled cells with a polyclonal anti-IGF-I receptor antibody (UBI). The highest absorbance value, whether normal or PV, was taken as 100.0% and percent maximum for each time point was calculated.
Fig 2. Comparison of tyrosine phosphorylation of the 95-kD band from PV and normal cells at $10^{-10}$ mol/L and $10^{-9}$ mol/L IGF-I. Western blots derived from eight PV patients and six normal individuals were used for quantitation of tyrosine phosphorylation of the 95-kD band by densitometric analysis. The data are presented as percent maximum ± SEM. (A) Tyrosine phosphorylation of the 95-kD band in response to the low concentration of IGF-I in cells from patients with PV (□) and normal individuals (●). (B) Tyrosine phosphorylation of the 95-kD band in response to the high concentration of IGF-I in cells from patients with PV (□) and normal individuals (●).

concentration of ligand, $10^{-4}$ mol/L IGF-I in normal, tyrosine phosphorylation was significantly increased at 10 minutes over time 0 ($P < .001$); in PV, tyrosine phosphorylation was significantly increased at 3 minutes (.01 > $P > .001$). Together these data indicate that not only is the IGF-I receptor in PV hyperresponsive to IGF-I as compared with the normal in that, at a given concentration of ligand, the magnitude and/or rate of the response are substantially greater, but it is also hypersensitive in that it responds to ligand concentrations 100-fold less than required to activate the receptor in normal cells (Fig 2). This mirrors the increased sensitivity to IGF-I previously observed in PV with respect to erythroid burst formation in vitro.

Specificity of increased tyrosine phosphorylation of the IGF-I receptor in PV. To test the specificity of the observed increase in tyrosine phosphorylation, we examined three patients with erythrocytosis who had elevated hematocrits/RBC mass, but did not fulfill the criteria for PV. As Fig 3 illustrates, there appeared to be no significant difference ($P > .05$ for all points) with respect to tyrosine phosphorylation of the 95-kD band, either in the absence of IGF-I, or in the presence of IGF-I at low or high concentrations. These results were confirmed when tested against a larger pool of normals (data not shown). Thus, the phenomenon of a receptor that had increased basal tyrosine phosphorylation, and was hypersensitive and hyperresponsive in the presence of ligand, appears to be specific to PV.

DISCUSSION
We have found that in patients with the myeloproliferative disorder PV, first, the IGF-I receptor β subunit of circulating mononuclear cells, in the basal unstimulated state, exhibited an increased level of tyrosine phosphorylation over that of normal cells. This was not caused by an increase in the amount of receptor protein in PV (Fig 1). Second, the induction of tyrosine phosphorylation by IGF-I in PV occurred at approximately 100-fold lower ligand concentration than in normal, ie, it was hypersensitive to ligand. It also peaked earlier and reached higher intensity, ie, it was hyperresponsive to ligand. These findings are consistent with, and extend and provide a molecular mechanism for our previous observation that in PV, erythroid progenitor cells among circulat-
ing mononuclear cells are hypersensitive to IGF-I with respect to erythroid burst formation in vitro. IGF-I is known to play a role in erythropoiesis in vitro and in vivo and in adults whose Epo-driven erythropoiesis is impaired.

Whether the enhanced basal tyrosine phosphorylation observed in cultures of PBMCN from PV patients is caused by a constitutively activated receptor tyrosine kinase, or represents a hypersensitive response to minute amounts of ligand, which may be released by the PBMCN during the experiment, is unknown. Furthermore, the observed increase in tyrosine phosphorylation probably reflects an increase in biologic activity of the IGF-I receptor in PV cells, but this remains to be formally established. Experiments designed to resolve these questions are currently in progress.

Either of two hypotheses could account for our findings. The first hypothesis postulates that the PV phenotype results from the abnormal expansion of an otherwise normal subpopulation of cells that possesses an enhanced basal tyrosine kinase activity and an enhanced sensitivity and response to IGF-I. The normal fetal IGF-I receptor, which has an increased tyrosine kinase activity, would fit such a model. However, this hypothesis leaves unanswered the question of what is responsible for the self-renewal and selective amplification of this particular cell population in PV. The second hypothesis postulates that the PV phenotype results from a mutation in the IGF-I receptor, or in its signaling pathway, which renders the proliferative signal constitutively active, hypersensitive, and hyperresponsive to IGF-I. Where such a defect would lie is open to discussion. An altered IGF-I receptor with the β subunit having a relative molecular weight of 105 kD has been found in leukemic cells. But from Fig 1 above, as well as from data not shown, we found no evidence of an abnormality in the IGF-I receptor β subunit that would be manifest as a change in its relative molecular weight. A lesion in the α subunit that affects the tyrosine kinase activity of the receptor, or a discrete lesion in the β subunit that does not alter its relative molecular weight is of course possible, and would not be detected by the methods used here. If the lesion does not lie within the IGF-I receptor, but lies within its signal transduction pathway or its regulatory proteins, then it must somehow be closely associated with the receptor, because the defect appears to have a direct effect on its kinase activity, as evidenced by increased autophosphorylation in PV cells. Among the primary candidates for such a defect would be those elements involved directly in IGF-I receptor activation and/or desensitization.

ACKNOWLEDGMENT

We extend our thanks to the patients and other individuals who kindly volunteered to take part in this study, and to Drs Dominick Amato and Irving Rother who referred these patients to us and who were helpful throughout the course of the study. We also appreciate the useful comments of Dr Alan Bernstein on the manuscript, and thank Dr Steven Jacobs for his kind gift of the αIR3 monoclonal antibody.

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