Suboptimal Erythropoietic Response to Hypoxemia in Idiopathic Pulmonary Fibrosis*

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Background and study objectives: Idiopathic pulmonary fibrosis (IPF) is a chronic inflammatory process characterized by severe derangement of gas exchange in the advanced stages of disease. However, erythrocytosis is infrequent in IPF. The aim of this study was to investigate the potential relation between the blunted erythropoietic response and the chronic inflammation.

Subjects: Nine patients (6 men and 3 women) with IPF and profound hypoxemia ($P_{O2} < 65 \text{ mm Hg}$) and 34 sex- and age-matched healthy volunteers participated in the study.

Methods: We evaluated the hematologic parameters, serum erythropoietin, tumor necrosis factor (TNF)-$\alpha$, interleukin (IL)-6, and IL-8 levels. We also studied the development of burst-forming unit-erythroid (BFU-E)–derived colonies in semisolid methylcellulose cultures in blood samples from all patients.

Results: Hemoglobin and serum erythropoietin levels were almost comparable between the two studied groups. On the contrary, serum TNF-$\alpha$, IL-6, and IL-8 values were significantly higher in patients with IPF ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively). IPF sera induced a significant growth inhibition of erythroid bursts arising from mononuclear cells of either patients or control subjects compared with heat-inactivated AB serum ($p < 0.05$ and $p < 0.01$, respectively). Moreover, there was an apparent increment in the number of BFU-E colonies when patients’ mononuclear cells were cultured in comparison with those of healthy subjects ($p < 0.05$).

Conclusions: Our findings suggest that in IPF there is an increased number of primitive erythroid progenitors, which fail to proliferate and differentiate in vivo, suggesting a kind of ineffective erythropoiesis. As a consequence, hemoglobin levels do not rise in proportion to the severity of hypoxemia. Cytokines released from alveolar macrophages seem to have not only local but also systemic effects, since the serum of these patients directly suppressed erythropoiesis; however, the suboptimal erythropoietic response to hypoxia cannot be entirely attributed to this suppression. It is possible that several other factors interfere, synergistically or additively. (CHEST 2003; 124:548–553)

Key words: chronic inflammation; cytokines; idiopathic pulmonary fibrosis; ineffective erythropoiesis

Abbreviations: BFU-E = burst-forming unit-erythroid; BSA = bovine serum albumin; IL = interleukin; INF = interferon; IPF = idiopathic pulmonary fibrosis; TNF = tumor necrosis factor

According to the prevailing views, idiopathic pulmonary fibrosis (IPF) is believed to arise from an uncontrolled chronic inflammatory process in the lower respiratory tract and alveoli of the lung. The process is initiated through activation of alveolar macrophages by immune complexes and undetermined antigen. This, in turn, causes release of various cytokines, such as tumor necrosis factor (TNF)-$\alpha$, interleukin (IL)-1, IL-6, IL-8, interferon (INF)-$\gamma$, followed by recruitment and migration of neutrophils, lymphocytes and monocytes, generation of chronic, persistent inflammation, lung injury and, ultimately, fibrosis. As a consequence, progression of IPF is associated with a severe derangement of gas exchange and profound hypoxemia; however, in disproportion with the severity of the latter, the expected erythropoietic response is usually absent.

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while several patients present anemia rather than compensatory polycythemia.\textsuperscript{1–6} The goal of this study was to explore the mechanisms that underlie the blunted erythropoietic response in patients with IPF.

**Materials and Methods**

**Subjects**

The study included nine patients (six men and three women) with severe IPF (median age, 65 years; range, 57 to 74 years). The diagnosis was suspected on the basis of clinical presentation, spirometry, and imaging data, and documented by lung biopsy. The selected patients fulfilled the following criteria: (1) stable hypoxemia ($P_aO_2 < 65$ mm Hg in room air) for at least 6 months prior to entering the study; (2) no history of infection over the last 2 months; (3) no renal or hepatic function derangement, no congestive heart failure (clinically), no diabetes mellitus, and no thyroid dysfunction; and (4) either nonsmokers or had stopped smoking for at least 12 months; (5) no evidence of other types of anemia, ie, megaloblastic, iron deficiency, and hemoglobinopathies. Secondary interstitial fibrosis was excluded by clinical and laboratory investigation. The control group included 34 sex- and age-matched nonsmoking, healthy volunteers (22 men and 12 women).

**Clinical and Laboratory Features of the Patients**

After obtaining informed consent, the following parameters were determined for each patient: complete blood counts and red cell indexes by a Technicon H2 automated analyzer (Bayer Diagnostics; Tarrytown, NY) and red cell morphology by light microscopy. Resting arterial blood gases and $pH$ were determined on sitting blood samples drawn on heparin from the radial artery using a 288 Blood Gas System (Ciba Corning Diagnostics; Tarrytown, NY) and red cell indexes by a Technicon H2 automatic analyzer (Bayer Corporation; Norwood, MA). Hemoglobin analysis was performed by hemolysate electrophoresis on cellulose acetate strips at alkaline pH. Serum vitamin $B_12$, folate, iron, and ferritin levels were quantified on an Assyn System autoanalyzer (Abbott Laboratories; Abbott Park, IL), while serum erythropoietin levels were determined by an enzyme-linked immunosorbent assay (Quintikine IVD Erythropoietin ELISA; R&D Systems; Minneapolis, MN). Serum TNF-$\alpha$, IL-$6$, and IL-$8$ concentrations were also measured by an enzyme-amplified sensitivity immunoassay (Easia; Biosource Europe S.A.; Nivelles, Belgium). All blood samples were collected between 9 am and 10 am.

**Effect of Sera From Patients With IPF on the Development of Erythroid Cultures**

The erythropoietic response in patients with IPF was studied in cultures of peripheral blood mononuclear cells by determining the influence of various experimental conditions on the number of developing burst-forming unit-erythroid (BFU-E) colonies. Cultures from normal persons matched for blood group, sex, and age were used as controls. Each cell culture was studied under the following conditions: (1) patient’s cells plus patient’s serum; (2) patient’s cells plus heat-inactivated AB serum; (3) control cells plus patient’s serum; and (4) control cells plus heat-inactivated AB serum. Growth of BFU-E colonies took place on semisolid methylcellulose according to a previously reported method.\textsuperscript{7} Peripheral blood was subjected to density-gradient ($1.077$) centrifugation on a cushion of Ficoll-Hypaque (Gibco BRL; Paisley, Scotland). Mononuclear cells collected from the interface were washed with phosphate-buffered saline solution (Gibco BRL) and bovine serum albumin (BSA) (Sigma; St. Louis, MO), BSA 1%. Cells were then resuspended in Iscove’s modified Dulbecco’s medium (Gibco BRL) with 5% fetal bovine serum (heat inactivated) and incubated for 1 h at $37^\circ$C on Falcon tissue culture plates to remove most of the adherent cells. Nonadherent mononuclear cells were counted on the Technicon H2 analyzer and diluted as necessary for plating. Four $\times 10^5$ nonadherent, light-density, mononuclear cells were plated on 35-mm Petri dishes. The methylcellulose culture medium consisted of Iscove’s modified Dulbecco’s medium supplemented with $42\%$ methylcellulose $1.1\%$ (MethoCult H4100; Stem Cell Technologies; Vancouver, Canada), $20\%$ fetal bovine serum, $1\%$ BSA, $1\%$ 2-mercaptoethanol (Sigma Aldrich; Munich, Germany), $1\%$ penicillin-streptomycin (Gibco BRL), $1\%$ L-glutamine (Gibco BRL), and $2\ U/mL$ erythropoietin (Human Recombinant EPO; Boehringer; Mannheim, Germany).

In each dish, the medium was further supplemented with either $15\%$ of heat-inactivated human AB serum (Pel-Freeze Clinical System LLC; Pel-Freez Biologicals; Rogers, AR) or $15\%$ of serum from the studied patients. The final volume of culture medium plated in each 35-mm Petri dish was $1.1\ mL$. All plates were prepared in duplicate and were examined after incubation for 14 days. Erythroid bursts in the dishes were estimated directly on an inverted light microscope. Identification of bursts was performed on the basis of standard morphologic features (size, color, time of maturation, and cluster composition).\textsuperscript{8}

**Statistics**

Because the values of most measured parameters were not normally distributed, nonparametric tests were used for the statistical evaluation. Differences for hemoglobin values, erythropoietin, and cytokine levels between control subjects and patients were assessed with the Wilcoxon rank sum (Mann-Whitney) test. The Wilcoxon matched-pairs signed-ranks test was applied for the analysis of culture results. For all tests, significance was assumed at $p < 0.05$.

**Results**

Hemoglobin and erythropoietin values were higher in patients with IPF compared to normal control subjects; however, this difference did not reach statistical significance, despite the severity of hypoxemia observed in the patient group (mean $P_aO_2$, $56$ mm Hg; range, $42$ to $65$ mm Hg). On the contrary, the serum TNF-$\alpha$, IL-$6$, and IL-$8$ values showed statistically significant differences from the control subjects (Table 1, Fig 1).

The number of BFU-E colonies in cultures arising from mononuclear cells of either patients with IPF or control subjects grown in presence of heat-inactivated AB serum was clearly higher than that of the cultures grown in presence of IPF sera ($p < 0.05$ and $p < 0.01$, respectively; Table 2, Fig 2). In addition, the number of BFU-E colonies obtained from mononuclear cells of patients with IPF in the presence of either IPF sera or AB heat-inactivated serum was clearly higher than that of the control subjects ($p < 0.05$; Table 2, Fig 2).
In spite of the severe hypoxemia of all patients with IPF reported in this study (with $P_{O_2}$ values $< 65$ mm Hg), their hemoglobin levels were not different than those of matched, healthy control subjects. This observation of blunted erythropoietic response is in keeping with other earlier reports, and contrasts with the secondary polycythemia that is the standard feature of hypoxemic patients, and therefore warrants further exploration. Moreover, erythropoietin levels were almost comparable in the two groups, but are considered as relatively low in spite of pronounced hypoxemia of our patients.

Identification of the mechanisms underlying the anemia associated with chronic inflammatory diseases has attracted a major scientific interest. A variety of cytokines, such as IL-1, INF-γ, and TNF-α, all secreted by activated macrophages, exert a significant

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control Subjects (n = 34)</th>
<th>Patients With IPF (n = 9)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin, g/dL</td>
<td>13.55 (12.5–15.8)</td>
<td>14.6 (12.2–18.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Erythropoietin, mIU/mL</td>
<td>6.2 (1–14.9)</td>
<td>6.9 (3.1–16.9)</td>
<td>NS</td>
</tr>
<tr>
<td>TNF-α, pg/mL</td>
<td>6 (0–20)</td>
<td>7 (5–70)</td>
<td>0.034 (&lt; 0.05)</td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>3.5 (0–6.5)</td>
<td>8 (3–40)</td>
<td>0.0021 (&lt; 0.01)</td>
</tr>
<tr>
<td>IL-8, pg/mL</td>
<td>7 (2.5–16)</td>
<td>18 (10–45)</td>
<td>0.0004 (&lt; 0.001)</td>
</tr>
</tbody>
</table>

*Data are presented as median (range). NS = not significant.

**Figure 1.** Comparison of serum erythropoietin (EPO), TNF-α, IL-6, and IL-8 levels between the two studied groups. Results are presented as one-way scatterplots combined with box-and-whisker plots. Erythropoietin levels were almost comparable between control subjects and patients with IPF. Serum TNF-α, IL-6, and IL-8 values were significantly higher in patients with IPF (p < 0.05, p < 0.01, and p < 0.001, respectively).
inhibition on the growth of erythroid cultures, implying that these agents may play an important causative role (TNF-α⁹–¹² and INF-γ¹⁰,¹³–¹⁵). However, other authors¹⁰,¹⁵,¹⁶ suggest that these factors attenuate the erythropoietic response by enhancing the apoptotic mechanism through induction of the Fas protein, while others¹¹,¹³ support an inhibitory effect mediated through the activity of accessory cells. It has been documented that chronic administration of TNF in experimental animals induces ineffective erythropoiesis accompanied by erythroid hyperplasia.¹⁷ Furthermore, IL-1 is considered to play a significant role in the pathogenesis of the anemia of chronic disease, either by depressing hypoxia-stimulated erythropoietin production¹² or by inhibiting the stimulatory influence of erythropoietin on mature erythroid progenitors.¹⁸,¹⁹

To explore the relevance of these mechanisms in IPF, we compared the levels of several of the above-mentioned cytokines in a representative number of patients with IPF selected on the basis of long-standing severe hypoxemia (PO₂ < 65 mm Hg) to the levels of fully matched control subjects; we also studied the erythropoietic response of the above-mentioned patients and control subjects in vitro by defining the growth of erythroid cultures deriving from their mononuclear cells under the influence of either “normal,” heat-inactivated AB serum or serum from patients with IPF. The use of methylcellulose cultures supplemented with 2 U/mL

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**Table 2—Erythroid Bursts Produced by Mononuclear Cells When Either Patient’s Serum or Heat-Inactivated AB Serum Was Added to the Culture Medium**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Patient No.</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Patient’s cells plus normal AB serum</td>
<td>210</td>
</tr>
<tr>
<td>Patient’s cells plus patient’s serum</td>
<td>186</td>
</tr>
<tr>
<td>Control cells plus normal AB serum</td>
<td>72</td>
</tr>
<tr>
<td>Control cells plus patient’s serum</td>
<td>57</td>
</tr>
</tbody>
</table>

*Data are presented as No. of BFU-E colonies (14th day).

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**Erythroid bursts in IPF**

![Graph showing erythroid bursts in IPF](image)

**Figure 2.** Columns represent the median number of erythroid bursts produced by 4 × 10⁵ mononuclear cells in nine separate experiments, when the following combinations were applied: patient’s cells plus patient’s serum; patient’s cells plus AB serum; control cells plus patient’s serum; and control cells plus AB serum. Intergroup comparisons are presented.
of erythropoietin ensured the maximal stimulation of erythropoiesis and excluded dependence of the growth of erythroid progenitors on the erythropoietin concentration in the employed sera.20,21

In fact, in all patients with IPF, the serum TNF-α, IL-6, and IL-8 values were significantly higher than those of the control subjects (p < 0.05, p < 0.01, and p < 0.001, respectively); this is in accordance with the findings of other investigators,22,23 and suggests that cytokines released from alveolar macrophages have not only local but also systemic effects. The significant scatter of the observed values may be related to the progressive nature of the disease.24

The culture studies left no doubt that the number of BFU-E colonies derived from the mononuclear cells of patients with IPF was significantly higher than that obtained from the control subjects, and that the number of BFU-E colonies developing in the presence of IPF sera was significantly lower than that observed in presence of sera from normal control subjects. The former finding is taken to reflect that the marrow of patients with IPF harbors a greater than normal number of erythroid progenitors, which fail to complete their proliferation and maturation in vivo because of interference of various inhibitory factors.

This view is corroborated by the marked inhibition of the BFU-E growth when the culture milieu contained serum from patients with IPF. The fact that most of the adherent cells (including monocytes and macrophages) had been removed suggests that the suppression was a direct humoral effect brought about by various factors contained in the IPF sera; however, the presence in the cultures of other accessory cells, such as lymphocytes, cannot be excluded, and their effects on erythropoiesis remain to be investigated.

Erythropoietin values of our patients varied within normal range in spite of their severe hypoxemia. This finding may reflect the inhibitory effects of inflammatory cytokines (TNF-α, IL-1) on hypoxia-induced erythropoietin production.12 The relatively low erythropoietin may contribute to the blunted erythropoietic response, since erythropoietin levels are known to determine the rate of erythroid differentiation through promotion of the survival of early erythroblasts by either increasing the expression of the Bcl-Xl protein25 or by down-regulating the Fas/FasL system.26 Cytokines or inflammatory factors may also influence the balance between erythropoietin and Fas/FasL by increasing the Fas sensitivity of immature erythroblasts and, hence, by causing further erythropoietic suppression.15,26 Moreover, IL-1 has been found to antagonize the effects of erythropoietin on late-stage erythroid progenitors.18 Other authors27 support the existence of an inverse correlation between RBC precursor mass and serum erythropoietin concentration.

On the basis of these findings, it appears that the marrow of patients with IPF contains an increased number of early erythroid progenitors, which fail to proliferate and differentiate normally, suggesting a kind of ineffective erythropoiesis. As a consequence, hemoglobin levels do not rise in proportion to the severity of hypoxemia, and the patients remain relatively “anemic.” The serum of these patients directly suppressed erythroid burst growth; it is possible that this inhibition would be more pronounced if the high erythropoietin levels contained in the culture medium did not abrogate the TNF-α- and IL-1-induced suppression of erythropoiesis.18,28 Similarly, the administration of high doses of erythropoietin may prove useful in the clinical setting, since it could circumvent the suppressive effects on erythropoiesis and thus correct hemoglobin level; however, such an approach would rather apply to a subset of carefully selected patients, especially those with frank anemia or with an unacceptably low hemoglobin value. For the rest, any expected benefit in terms of improving oxygen transport and tissue oxygenation should be weighted against potential hazards, for example the impeding of blood flow in small vessels. Besides, the suboptimal erythropoietic response to hypoxia observed in patients with IPF cannot be entirely attributed to suppression by inflammatory mediators; an extensive research toward the identification of other synergistic or additive influences is warranted before the full explanation of the phenomenon and a safe therapeutic intervention is possible.

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