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Regulated production of a pleiotropic cytokine—platelet-derived growth factor—by differentiating erythroid cells *in vitro* and *in vivo*

(erythropoiesis/erythropoietin)

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ABSTRACT **Erythroid progenitor growth *in vitro* is stimulated by exogenous platelet-derived growth factor (PDGF). We now report that both normal and transformed erythroid progenitor cells produce authentic PDGF *in vitro* and *in vivo*. Importantly, this production is highly regulated during erythropoiesis. Addition of soluble lysates from Rauscher murine erythroleukemia cells—an erythropoietin-responsive model progenitor cell line—to quiescent BALB/c 3T3 fibroblasts resulted in a mitogenic response identical to that observed with the addition of authentic recombinant PDGF. Polyclonal and monoclonal anti-PDGF antibodies immunoabsorbed 50–100% of this activity. Induction of Rauscher cell differentiation *in vitro* with dimethyl sulfoxide or erythropoietin for 48–72 hr markedly upregulated PDGF production by 17- to 18-fold and 14- to 38-fold, respectively. Importantly, stimulation of normal erythropoiesis *in vivo* in mice treated either with phenylhydrazine or with erythropoietin increased PDGF levels in the spleen by 11- to 48-fold and 20- to 34-fold, respectively. These results strongly suggest a role for erythroid cell-derived PDGF in normal erythropoiesis and provide documentation of the regulated production of a pleiotropic cytokine by erythroid cells.**

The growth and differentiation of erythroid progenitors *in vitro* are enhanced markedly by the addition of platelet-derived growth factor (PDGF) (1, 2). The mechanism through which PDGF exerts this effect is unknown. Stimulation of erythroid cells by PDGF may occur by direct activation of the progenitors themselves or may be mediated through another cell type via a so-called paracrine mechanism. Several types of cells, including some transformed cell lines with erythroid characteristics, produce PDGF-like activity or can be induced to do so (3, 4). In the case of the transformed erythroid cells, it had been speculated originally that the production of PDGF was consequential to their transformation rather than characteristic of their erythroid phenotype.

Previously, we demonstrated that both erythropoietin (Epo)-responsive Rauscher murine erythroleukemia cells and the splenic erythroid cells of phenylhydrazine (PHZ)-treated mice produce a PDGF-like activity that stimulates erythropoiesis *in vitro* (5). We now show that these "PDGF-like molecules" are authentic PDGF. Moreover, our results demonstrate that the production of erythroid cell-derived PDGF is highly regulated during erythropoiesis *in vivo* and erythroid differentiation *in vitro*.

MATERIALS AND METHODS

Cells. The continuous Rauscher murine erythroleukemia cell line (6, 7) (clone R404) and murine BALB/c 3T3 fibroblasts (8) (generous gift of C. D. Stiles, Dana–Farber Cancer Institute, Boston) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; GIBCO). In some experiments (see Fig. 4) Rauscher cells were induced with dimethyl sulfoxide (DMSO) or recombinant human Epo (Elanex Pharmaceuticals, Bothell, WA) for 48 or 72 hr. BALB/c 3T3 fibroblasts were made quiescent by incubating 90% confluent cultures in DMEM/0.5% FBS for 48 hr.

Control splenocytes, the majority of which are B and T lymphocytes, were obtained from $B_6C_3F_1$ mice. To induce splenic erythropoiesis, $B_6C_3F_1$ mice were injected subcutaneously either with recombinant human Epo (2000 units/kg) in phosphate-buffered saline (PBS)/1% bovine serum albumin on 4 consecutive days or with PHZ (60 mg/kg; Sigma) in PBS on 2 consecutive days (9–11). The mice were sacrificed on day 5 and their spleens were removed aseptically. The spleens of Epo-treated mice and PHZ-treated mice were processed separately. Splenic erythropoiesis was confirmed by microscopic examination of Wright's stained preparations.

Preparation of Cell Supernatants. The spleens were cut in half, and the pieces of tissue were dispersed between the ground glass ends of sterile microscope slides using gentle pressure and a circular motion. To prepare a single cell suspension, the dispersed tissue was resuspended in serum-free alpha MEM and triturated through an 18G needle and then through a 22G needle. The cells were washed three times by centrifugation in serum-free alpha MEM. Adherent cells were removed by incubation in serum-free alpha MEM in tissue culture dishes for 90 min at 37°C. The nonadherent cells were collected and washed in PBS. Rauscher cells were washed three times in PBS. Soluble lysates were prepared by homogenizing cells in a Dounce homogenizer (ball A, 70 strokes) in distilled H_2O followed by centrifugation at 27,000 × *g* for 30 min. Protein was quantified by the Bio-Rad protein assay (Bio-Rad). The supernatants were subjected to size-exclusion chromatography through Bio-Gel A-1.5m (Bio-Rad) in PBS/1% bovine serum albumin. This separated the PDGF activity from hemoglobin, which preliminary experiments had shown to be toxic to quiescent BALB/c 3T3 fibroblasts.

PDGF Assays. PDGF activity was quantified by measuring [^{3}H]thymidine incorporation into newly synthesized DNA of quiescent fibroblasts (12). Briefly, quiescent fibroblasts were incubated in the presence of specified concentrations of cell lysates or recombinant human PDGF-BB homodimer (Boehringer Mannheim) and 1 μCi of [^{3}H]thymidine per ml (1 Ci = 37 GBq) for 24 hr at 37°C in DMEM/0.5% FBS. The medium was removed by aspiration. The cells were washed in PBS, treated with 5% trichloroacetic acid at 0°C for 5 min, and

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Abbreviations: PDGF, platelet-derived growth factor; Epo, erythropoietin; PHZ, phenylhydrazine; DMSO, dimethyl sulfoxide.

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washed three times in distilled water. The cells were dissolved in 0.25 ml of 200 mM NaOH/0.1% SDS for 5 min, and the solution was neutralized with 0.25 ml of 200 mM HCl. [^{3}H]Thymidine was measured by β scintillation spectrometry (Beckman LS 3801) using Insta-Gel XF liquid scintillation mixture (Packard).

Immunoadsorption of PDGF Activity. To determine whether the fibroblast growth-promoting activity found in the erythroid cell lysates was due to the presence of authentic PDGF, purified anti-human PDGF-AB antibodies (goat polyclonal IgG; Collaborative Research) and mouse monoclonal anti-PDGF-B-chain-(1–18) antipeptide antibody (Quality Biotech, Camden, NJ) were used in immunoadsorption assays. Cell supernatants were incubated for 2 hr at 37°C with specified concentrations of antibodies. Then 50 μl of IgGsorb (The Enzyme Center, Malden, MA) was added and incubation was continued for 30 min followed by centrifugation. The resulting supernatants were filter sterilized and assayed for residual PDGF activity. Control immunoadsorptions were performed with purified rabbit anti-mouse IgG (whole molecule).

RESULTS

Rauscher Murine Erythroleukemia Cells Produce PDGF Activity That Stimulates Fibroblast Growth. Our previous study demonstrated that uninduced Rauscher murine erythroleukemia cells and splenic erythroid cells produce PDGF-like molecules that stimulate the growth of human erythroid cells in serum-free culture (5). The focus of the present study was to determine whether these molecules are authentic PDGF and whether their expression is relevant to normal erythropoiesis. To determine whether these molecules are authentic PDGF, we tested their ability to act as a mitogen for BALB/c 3T3 fibroblasts—a cell line whose proliferative response to PDGF has been well-documented (8). Quiescent BALB/c 3T3 fibroblasts were treated with specified concentrations of uninduced Rauscher cell supernatants for 24 hr in the presence of [^{3}H]thymidine. After 24 hr, the proliferative state of the cells was determined by assaying the relative amount of [^{3}H]thymidine incorporated into DNA. Rauscher cell supernatants stimulated the proliferation of BALB/c 3T3 fibroblasts in a concentration-dependent manner (Fig. 1, solid squares) identical to the stimulation seen with recombinant human PDGF-BB homodimer (Fig. 1, solid circles). Similar results were obtained in several other experiments. This strongly suggested that the Rauscher cell-derived PDGF-like molecules may be authentic PDGF.

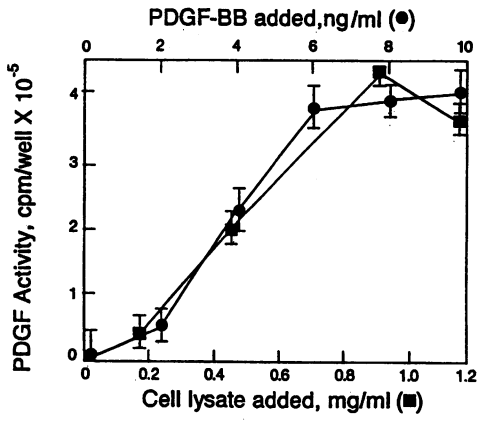

FIG. 1. Erythroid cell-derived PDGF activity stimulates DNA synthesis in quiescent BALB/c 3T3 fibroblasts. Note parallel slopes of Rauscher cell lysate (■) and recombinant PDGF-BB homodimer (●). Values are means ± SD.

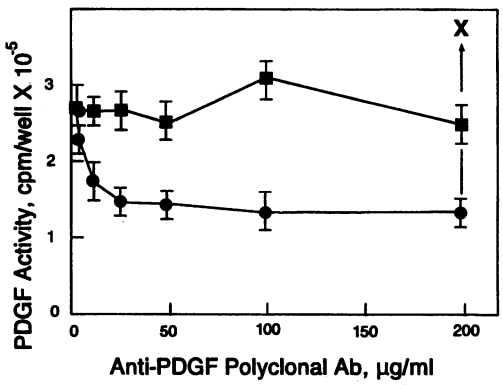

FIG. 2. Polyclonal antibodies to authentic human PDGF-AB heterodimer recognize Rauscher cell PDGF. ●, Polyclonal antibodies to human PDGF-AB; ■, control IgG. Values are means ± SD. Addition of excess PDGF-BB reverses the antibody effect (arrow X).

The PDGF Activity Produced by Rauscher Cells Is Removed by Anti-PDGF Antibodies. We obtained further evidence that the stimulation of DNA synthesis in BALB/c 3T3 fibroblasts by Rauscher cell supernatants was attributable to authentic PDGF by removing the mitogenic activity with anti-PDGF antibodies. Two antibody preparations were used to immunoadsorb the activity. Polyclonal anti-human PDGF-AB antibodies (Fig. 2) consistently removed 50–60% of the mitogenic activity from the supernatant. This antibody-dependent reduction in mitogenic activity was not due to toxicity of the antibody preparation. We showed this by adding excess recombinant PDGF-BB to replicate wells containing the highest concentration of antibody used. Excess PDGF-BB resulted in a marked increase in DNA synthesis (3.6×10^5 cpm), completely reversing the antibody-dependent reduction (Fig. 2, arrow X). The lack of complete immunoadsorption may be due to a structural difference between murine PDGF and human PDGF-BB to which the antibodies are directed. Monoclonal anti-PDGF-B-(1–18) antibody (Fig. 3) removed 100% of the mitogenic activity. This effect was also completely reversed by adding excess PDGF-BB, demonstrating the lack of antibody toxicity. In contrast, the addition of control nonimmune IgG (Figs. 2 and 3, solid squares) had no effect on the PDGF activity of the cell lysate. Virtually identical results were obtained in two additional experiments. These results, taken together, indicate that Rauscher murine erythroleukemia cells produce authentic PDGF.

PDGF Production by Rauscher Cells Is Increased Markedly During Erythroid Differentiation *in Vitro*. The observations that (*i*) PDGF stimulates erythropoiesis (1, 2) and that (*ii*)

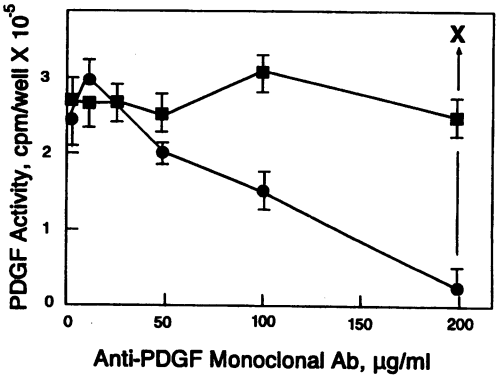

FIG. 3. Monoclonal anti-PDGF-B-(1–18) antibody immunoadsorbs 100% of erythroid cell PDGF. ●, Monoclonal antibody; ■, control IgG. Values are means ± SD. Addition of excess PDGF-BB reverses the antibody effect (arrow X).

erythroid cells produce authentic PDGF (present study) suggested a positive feedback mechanism—i.e., that erythroid cells may amplify their own growth by production of PDGF. This prompted us to ask whether the production of erythroid cell-derived PDGF is regulated. We found that PDGF production is upregulated markedly by inducers of erythroid differentiation.

Rauscher cells differentiate either in response to the natural inducer of erythropoiesis, Epo, or in response to several chemical inducers such as DMSO (6, 7, 13, 14). We have shown previously that Epo and DMSO trigger erythroid differentiation in these cells by dissimilar mechanisms (7, 13–15), including different signal transduction pathways to the protooncogene c-*myb* (14). Therefore, we investigated the effect of DMSO and Epo in order to determine whether increased PDGF production is a general feature of erythropoiesis.

Both inducers increase Rauscher cell PDGF production markedly (Fig. 4). Uninduced cells exhibited endogenous PDGF activity. The value obtained in this experiment (8500 cpm per 50 μg of protein per ml) was consistent with that seen previously for uninduced cells (see Fig. 1). Induction with DMSO (0.7%) for 48 and 72 hr increased PDGF activity by 17- and 18-fold, respectively. Induction of the cells with Epo (50 units/ml) increased Rauscher cell PDGF activity by 14- and 38-fold, respectively. These effects were highly reproducible. Such marked increases in PDGF production during erythropoiesis *in vitro* caused by these two very different inducers strongly suggested that upregulation of erythroid cell-derived PDGF may be a normal part of the erythropoiesis program.

The Production of PDGF Accompanies Epo-Induced Erythropoiesis *in Vivo*. We next demonstrated that production of erythroid cell-derived PDGF is regulated during normal erythropoiesis *in vivo* (Table 1). We did this in two ways. First, we measured the PDGF content of splenocytes from PHZ-treated mice. PHZ causes a brisk hemolytic anemia that results in a marked increase in circulating Epo and the appearance of enhanced erythropoiesis in the murine spleen (9–11). Mice were injected subcutaneously with PHZ (60 mg/kg) on two consecutive days. PHZ caused the average hematocrit of the mice to fall from 45% to 20% and the appearance of large numbers of erythroid precursors in the spleens (>50% of total cells) as evidenced by microscopic examination of Wright's stained samples. On day 5, spleen cell supernatants were prepared and assayed for PDGF activity (see *Materials and Methods*). As shown in Table 1, only a small amount of PDGF activity was detected in the spleens of normal (untreated) animals. As expected, the spleens of these animals contained only rare erythroid cells by microscopic examination. However, the erythroid spleens of PHZ-treated mice contained

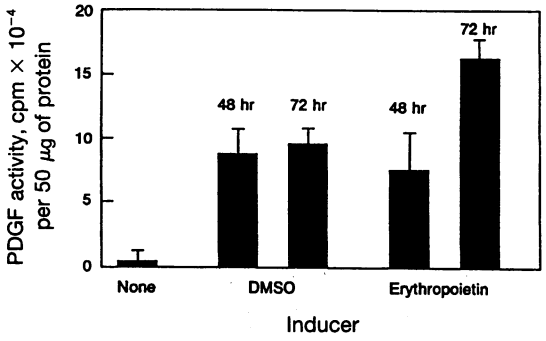

FIG. 4. Inducers of erythropoiesis markedly upregulate PDGF production by Rauscher cells *in vitro*. DMSO, 0.7%; Epo, 50 units/ml. Values are means ± SD. Note that in the absence of inducer (None), Rauscher cells expressed PDGF activity constitutively (8500 cpm per 50 μg per ml; see Fig. 1, 38,500 cpm per 0.18 mg per ml). DMSO and Epo upregulated PDGF expression 17- to 18-fold and 14- to 38-fold, respectively.

Table 1. Regulated production of PDGF during erythropoiesis *in vivo*

Spleen	No.	PDGF activity* cpm per μg of protein	PDGF activity* cpm per spleen ($\times 10^{-5}$)	Fold increase† Per μg of protein	Fold increase† Per spleen
Normal	6	21 ± 3	1.4 ± 0.2	—	—
PHZ treated	4	230 ± 41	67 ± 12	11	48
Epo treated	2	410 ± 60	48 ± 7	20	34

*Determined by [^{3}H]thymidine assay. Values are means ± SD.
†Mean cpm for PHZ- or Epo-treated animals/mean cpm for normal (untreated) animals.

greatly increased PDGF levels, 11- to 48-fold that of untreated mice.

Second, we demonstrated that Epo treatment of mice greatly increases the production of erythroid cell-derived PDGF in their erythropoietic spleens. Mice were injected subcutaneously with Epo (2000 units/kg) on 4 consecutive days, which raised the average hematocrit from 45% to 55%. On day 5, the spleens were harvested. Microscopic examination of Wright's stained samples confirmed the presence of active erythropoiesis. As shown in Table 1, the spleens of Epo-treated mice contained 20- to 34-fold more PDGF activity than did normal spleens of untreated animals. These results demonstrate that an upregulation in production of erythroid cell-derived PDGF accompanies erythropoiesis *in vivo* and is stimulated by Epo.

DISCUSSION

For several years it has been recognized that exogenous PDGF stimulates the proliferation and differentiation of erythroid cells in human bone marrow cultures (1, 2). However, the physiological relevance of this observation was unknown. In a previous study, we used serum-free bone marrow cultures to assay for erythroid growth-promoting activity produced by transformed and normal erythroid cells (5). We showed that both transformed Rauscher murine erythroleukemia cells—a model Epo-responsive progenitor cell line—and nontransformed erythroid splenocytes from PHZ-treated mice produce molecules that stimulate human erythroid progenitor growth *in vitro* in a manner similar to that of PDGF. Because these molecules could be detected on Western blots, immunohistochemically, and by immunoprecipitation with anti-PDGF antibodies, we designated them "PDGF-like molecules" (5). In the present study, we have used the classic BALB/c 3T3 cell proliferation assay to detect PDGF activity (8). The results of these assays, taken together with immunoprecipitation experiments, provide strong evidence that erythroid progenitor cells produce authentic PDGF. This work provides documentation of the regulated production of a pleiotropic cytokine by erythroid progenitor cells.†

It has been well-documented that other hematopoietic cells produce cytokines that can modulate hematopoiesis. For example, T cells, in response to an antigen, produce interleukin 2 (IL-2) (18), IL-3 (19), IL-4 (20), transforming growth factor β (21), interferon γ (22), and granulocyte–macrophage colony-stimulating factor (GM-CSF) (23). Cells of the mononuclear/phagocyte lineage produce multiple cytokines including PDGF (24), fibroblast growth factors (25), IL-1 (26), GM-CSF (27), and interferon α (28). Megakaryocytic cell lines have also been reported to express several cytokine genes (29). In addition, both B cells (30–32) and NK cells (33–35) produce hematopoietic growth factors. It appears that the production

†There is evidence that early hematopoietic progenitors may produce Epo (16). Also, a mitogenic activity, apparently not due to PDGF, has been detected in lysates of peripheral blood mononuclear cells from polycythemia vera patients (17).

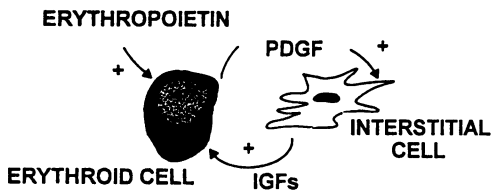

FIG. 5. Proposed model for paracrine effect of erythroid cell-derived PDGF *in vivo*.

of cytokines by hematopoietic cells is central to hematopoiesis, serving to amplify blood cell growth and differentiation.

Our experiments *in vitro* demonstrate a profound increase in PDGF production during erythroid differentiation induced by either the natural inducer, Epo, or by a chemical one, DMSO. Thus, PDGF production is regulated during erythropoiesis and is not simply constitutive. This implies that erythroid cell-derived PDGF may serve to amplify erythroid cell growth as part of Epo's growth-promoting action.

There are no reports of the detection of PDGF receptors on erythroid progenitor cells, and the work of Delwiche *et al.* (2) indicates that the erythropoiesis-stimulating action of PDGF is mediated via an adherent, intermediate cell. Considering this in light of our previous and present studies, we propose that erythroid cell-derived PDGF acts as a paracrine effector in stimulating erythroid cell growth (Fig. 5). We hypothesize that, under the regulatory influence of Epo, erythroid cell-derived PDGF acts on one or more types of interstitial cells to trigger the production of additional cytokines and especially the insulin-like growth factors IGF-I and IGF-II. Such PDGF regulation of IGF production has been documented in numerous relevant cell types, including vascular smooth muscle (36, 37), monocytes (38), bone cells (39), and fibroblasts (40). This possibility gains greater significance in view of studies that have demonstrated a growth-promoting effect of the IGFs on erythroid progenitors *in vitro* (41–49). Also, recent evidence suggests a role for the somatomedins in patients with primary polycythemia (50–52). Thus, we speculate that the following sequence constitutes an Epo–PDGF–IGF paracrine loop: Epo→erythroid progenitor→PDGF→interstitial cells→IGF-I and -II→erythroid progenitor.‡

Our results demonstrate the production of a pleiotropic cytokine—PDGF—by differentiating erythroid cells and suggest a role for it in enhancing erythroid growth. They also raise the possibility of other effects of erythroid cell-derived PDGF, including (*i*) an action on normal or malignant cells, including nonhematopoietic cells, within or outside the bone marrow, especially during Epo therapy and (*ii*) a role in the pathology observed in chronic conditions of an expanded erythropoietic compartment such as sickle cell disease and thalassemia. Moreover, they suggest that erythroid cells be considered as possible sources of other cytokines.

‡Also of note in this regard is the demonstration that PDGF stimulates the growth of primitive hematopoietic cells (pre-CFC multi) by inducing IL-1 production in a subset of macrophages (53).

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