Association Between Elevated Prolactin Levels and Circulating Erythroid Precursors in Dialyzed Patients (44503)

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Abstract. **The prolactin (PRL) receptor (R), a member of the cytokine hemopoietin receptor superfamily, has been shown to activate early differentiation steps along the erythroid pathway. In particular PRL, a product of bone marrow stroma, induces functional erythropoietin (EPO)-R on $CD34^+$ hemopoietic progenitors. In this study, expression of EPO-R mRNA and responsiveness to EPO were assessed on enriched hemopoietic progenitor cells (HPC) from seven hyperprolactinemic and three normoprolactinemic patients and two normal subjects. Expression of EPO-R mRNA by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was found in HPC of four out of seven hyperprolactinemic patients but not in normoprolactinemic patients or normal donors. Development of EPO-dependent Colony Forming Unit-Erythroid (CFU-E) colonies in semi-solid medium was observed only in hyperprolactinemic patients (six out of seven). A much higher number of CFU-E colonies was observed in the four patients with a positive EPO-R message. We conclude from these data that abnormally high levels of PRL may increase the number of EPO-responsive hemopoietic precursors *in vivo* as they do *in vitro*. Since hyperprolactinemia associates in these patients with depressed EPO production, it may be regarded as a compensatory mechanism for the reduced availability of the hemopoietic factor.**

[P.S.E.B.M. 2000, Vol 223:367–371]

Due to the paramount role of kidneys as a source of erythropoietin (EPO), anemia is very frequent in dialyzed patients. Its correction by administration of EPO (1, 2) reverts hyperprolactinemia (3) and the related sexual disfunctions often observed in these patients (4). Thus, hyperprolactinemia seems to be a consequence of EPO deficiency, although the final explanation for the rise of PRL is not clear. In this context, a clue may be provided by earlier literature pointing to a role for prolactin (PRL) in normal erythropoiesis (5–7). PRL receptors (R), which have recently been grouped into the family of the cytokine/hemopoietin receptors (8), are expressed on lymphoid and hemopoietic tissues (9–12). Engagement of PRL with its receptor on a very early population of hemopoietic progenitors promotes the expression of EPO-R and thus the responsiveness to the progression factor EPO (12). The PRL-R and severely truncated EPO-R support differentiation of erythroid progenitors (13), and the PRL-R rescues EPO-$R^{-/-}$ erythroid progenitors and replaces EPO-R in a synergistic interaction with c-kit (14). PRL produced by bone marrow stroma cells (BMSC) promotes local erythropoiesis (15). Finally, PRL has recently been shown to exert hematopoietic growth-promoting effects *in vivo* and partially counteract myelosuppression by azidothymidine (16).

Based on these premises, we have reasoned that hyperprolactinemia may serve to increase the number of EPO-responsive cells, thus compensating for the reduced availability of the hemopoietic factor. If so, hyperprolactinemia in dialyzed patients should be associated with an increased number of EPO-R positive (i.e., EPO-responsive) hemopoietic precursors. We have tested this hypothesis by studying

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Received June 9, 1999. [P.S.E.B.M. 2000, Vol 223]
Accepted November 18, 1999.

0037-9727/00/2234-0367$15.00/0

the EPO-R mRNA expression and the presence of committed Colony Forming Unit-Erythroid (CFU-E) precursors in enriched circulating hemopoietic progenitor cells (HPC) from hyperprolactinemic dialyzed patients.

Materials and Methods

Patients. Twenty-five patients (19 males and six females) not on treatment with EPO or bromocriptine were selected from a population of dialyzed patients, and their hemoglobin (Hb), EPO, and PRL serum levels were measured. Of these, only seven patients were found hyperprolactinemic and were enrolled in this study together with three normoprolactinemic patients and two normal subjects (Table I).

Cell Preparation. Blood samples were collected into preservative-free heparin according to institutional guidelines from the patients and normal donors. Peripheral blood lymphocytes (PBL) were obtained by Ficoll-Hypaque density gradient and depletion of adherent cells by 1-hr plastic adherence in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (Hyclone Laboratories, Inc., Logan UT). Enriched populations of HPC were isolated by negative selection with the immunorosetting technique from PBL treated with a mixture of anti-CD3, -CD2, -CD5, -CD20, -CD14, -CD56, -CD32, -CD16, -CD41, and -CD10 monoclonal antibodies (Serotech, Oxford, England), as previously described (17).

Hemopoietic Clonal Assay. Hemopoietic clonal assays were performed as described elsewhere in detail (18), by culturing in triplicate 1×10^5 HPC/ml/dish in Iscove's modified Dulbecco's Medium (Gibco) containing 0.9% methylcellulose, 30% FCS, 10% deionized bovine serum albumin fraction V, 10^{-4} *M* β-mercaptoethanol (Sigma, St. Louis, MO), in the presence of EPO (2 U/ml) (Eritrogen, Boehringer Mannheim GmbH, Germany) alone to detect the presence of EPO-R positive CFU-E, or EPO plus Interleukin (IL)-3 (50 ng/ml) and Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) (20 ng/ml) (kindly provided by Dr. S. Clark, Genetics Institute, Cambridge, MA) to detect the presence of EPO-R negative early erythroid progenitors Burst Forming Unit-Erythroid (BFU-E). Colonies were scored under an inverted microscope on Day 7 (CFU-E) and Day 14 (BFU-E).

Semiquantitative Reverse Transcriptase (RT)-PCR. Levels of EPO-R mRNA were assessed by a semiquantitative RT-PCR (19). Briefly, total RNA was extracted from EPO-R positive TF-1 erythroleukemia cell line (20) and HPC using the single-step RNAzol method (Cinna/Biotecx, Houston, TX). RNA (2 μg) was reverse-transcribed, using reverse transcriptase (SUPERSCRIPT II, Life Technologies, Inc., Gaithersburg, MD) and oligo (dT) primers according to the manufacturer's protocol. Each sample was subjected to an initial amplification using human β-actin–specific PCR primers as described by us previously (21). Based on the amount of amplified β-actin PCR product, an equal amount of reverse-transcribed product was amplified using the EPO-R primer pairs: 5′-GCACCGAGTGTG-TGCTGAGCAA-3′ (sense) and 5′-GGTCAGCAGCACCAGGATGAC-3′ (antisense) (22). PCR was performed in a reaction mixture containing 5 μl of cDNA, 200 μl of each dNTP, 0.4 μ*M* of each upstream- and downstream-specific primers, 1.5 m*M* $MgCl_2$, 2.5 U of Taq DNA polymerase (Life Technologies) and 1 μCi of [^{32}P]dCTP (3000 Ci/mmol; DuPont-New England Nuclear, Boston, MA) in reaction buffer supplied by the manufacturer. Thirty cycles were used: 2 min at 94°C for denaturation, 30 sec at 60°C for annealing, and 30 sec at 72°C for extension. The predicted size of EPO-R-PCR product was 196 base pairs. Samples were analyzed by electrophoresis through a 6% acrylamide Tris borate-EDTA gel, followed by autoradiography and quantitation by Molecular Imager and Molecular Analyst software analysis (Biorad, Hercules, CA). Diagnostic restriction enzyme digestion of the PCR amplimers with Alu I was used to confirm the specificity of the primers used for the EPO-R targeted.

Statistical Analysis. The Student *t* test was used for statistical analysis. Significance was defined as $P < 0.05$.

Table I. Association Between Hb, PRL, and EPO Serum Levels and *In Vitro* Erythroid Colony Formation

Subject	Sex	Hb (g%)	PRL[a] (ng/ml)	EPO[b] (mU/ml)	CFU-E	BFU-E
#1	M	9.5	54	20.2	19 ± 4[c]	46 ± 11
#2	F	9.1	41	25.7	13 ± 2	40 ± 2
#3	F	9.1	50	16.5	75 ± 6	16 ± 6
#4	M	9	31	15.3	30 ± 7	8 ± 2
#5	M	11.4	21	15.8	6 ± 3	10 ± 5
#6	M	11.9	22	nd	2 ± 1	7 ± 2
#7	M	8.3	21	nd	0	12 ± 1
#8	M	12.5	9.6	nd	0	6 ± 2
#9	M	8.9	10	37.3	0	17 ± 33
#10	F	7.5	11	43.9	0	20 ± 4
healthy	F	14	12	44.4	0	35 ± 9
healthy	F	14.2	9	65.3	0	33 ± 2

[a] Normal values < 15 ng/ml in males, < 20 ng/ml in females
[b] Normal values 5–53 mU/ml
[c] Colony number/dish, mean ± SD

Results

The semiquantitative RT-PCR (Fig. 1, Panel A) revealed expression of the EPO-R mRNA in HPC from peripheral blood of four (Lanes 1–4) out of seven (Lanes 1–7) hyperprolactinemic patients but not in normoprolactinemic (Lanes 8–10) patients or normal donors (Lanes 11 and 12). Patients with positive EPO-R mRNA (#1, #2, #3, #4) have higher levels of PRL (44 ± 10, mean ± SD, $P < 0.01$) than the hyperprolactinemic pts (#5, #6, #7) with no message (21 ± 0.6, mean ± SD) (Table I).

Development of colonies from EPO-R–positive CFU-E in the presence of EPO alone was observed in six of the seven hyperprolactinemic patients, but not in normal donors. Interestingly, a much higher number of CFU-E colonies (range 13 ± 2–75 ± 6; mean ± SD) was observed in patients with a positive EPO-R message, compared with the negative ones (range 0–6 ± 3). The number of CFU-E colonies and PRL levels was positively correlated ($r = 0.73$, $P < 0.01$).

No difference in the number of BFU-E colonies, unresponsive to EPO alone, was observed between patients and normal donors (Table I).

Discussion

We show here that hyperprolactinemia in dialyzed patients is associated with the presence in blood of EPO-R positive, EPO-responsive erythroid precursors, which are present neither in patients with normal PRL levels nor in healthy subjects.

Enhanced erythropoiesis in pregnant women and direct erythropoietic effect *in vitro* of plasma from pregnant and lactating mice have been observed (5, 6). Furthermore, pituitary grafts under the kidney capsule, a model of *in vivo*

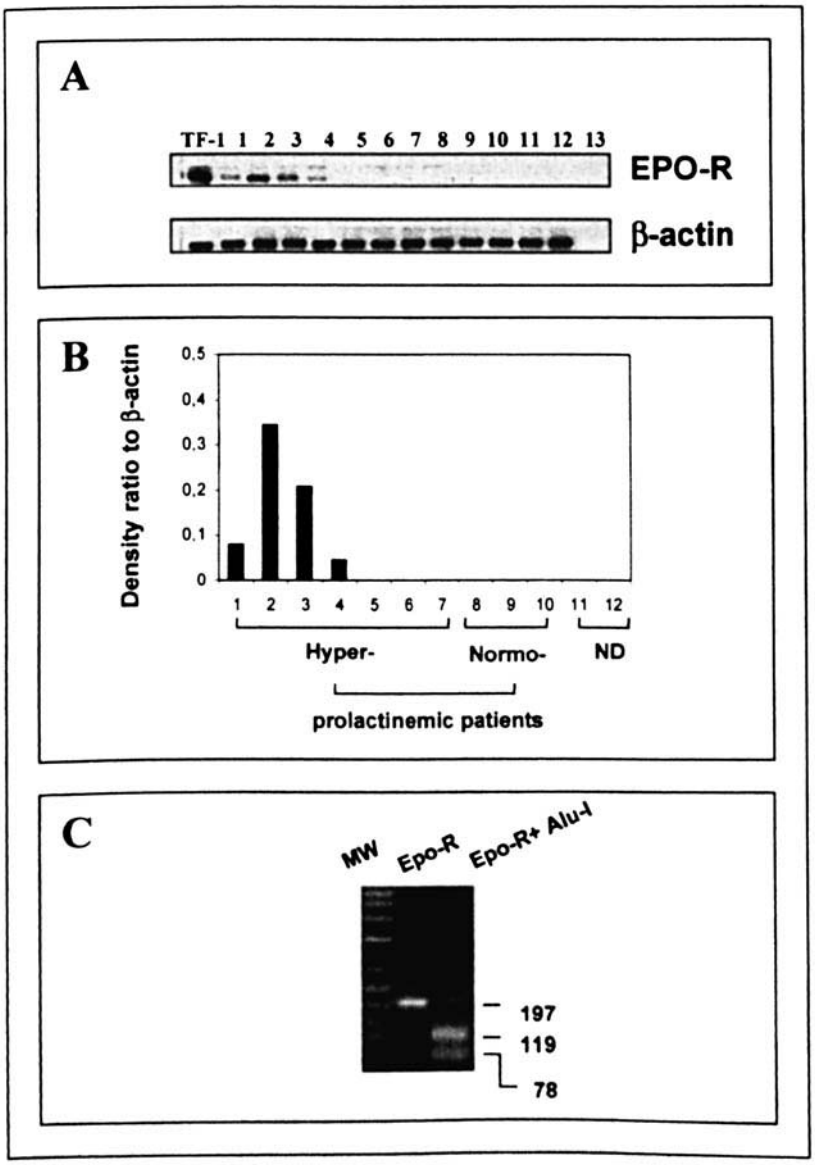

Figure 1. (Panel A) RT-PCR analysis of EPO-R mRNA expression by freshly isolated HPC from hyperprolactinemic patients (Lanes 1–7), normoprolactinemic patients (Lanes 8–10), and two normal donors (ND) (Lanes 11 & 12). Amplification was performed as described in Materials and Methods. The EPO-R–expressing TF-1 erythroleukemia cell line was used as the positive control. Lane 13 shows lack of amplification products in the absence of mRNA template. (Panel B) RT-PCR data were normalized for the expression of β-actin, and the ratio of EPO-R to β-actin was determined. (Panel C) Diagnostic restriction enzyme digestion of the PCR amplimers was used to confirm the specificity of the primers and amplification conditions. Amplicon sizes were compared with 1-kb DNA ladder (left).

hyperprolactinemia, favor the development of Friend murine virus-induced leukemias (FMLV) and switch their histotype from predominantly lymphoid to erythroid (7) and, conversely, regression of erythroblastic leukemia has been observed in a significant number of rats after hypophysectomy (23). These data suggest that a moderate increase of PRL above the physiological range can up-modulate the EPO-R on the HPC *in vivo* as it does *in vitro* (12). In our previous study, optimal increase of colony formation was observed with 25–50 ng/ml of PRL (12).

In the dialyzed hyperprolactinemic patients studied here, the presence of CFU-E cells in blood may represent either extra-bone marrow maturation or abnormal dismission from the bone marrow. We favor the latter hypothesis since maturation of BFU-E to CFU-E by PRL has been shown to require, at least *in vitro,* the presence of IL-3 and GM-CSF (12), which are undetectable in blood (24). High concentrations of PRL at the site of hemopoiesis may be provided by both locally circulating blood and stromal cells. There is in fact convincing evidence to show that the effects of PRL are not entirely attributable to pituitary release, and autocrine circuits have been shown in the immune system (25). In particular, we have recently shown that PRL is produced by human BMSC and contributes to differentiation of co-cultured BFU-E (15). In that study, basal production of PRL by BMSC was strongly increased by exogenous Platelet Activating Factor (PAF), suggesting that extrapituitary PRL, too, may be controlled by the blood levels of biological factors. We are currently addressing the modulatory effect of EPO on BMSC PRL synthesis.

Migration of HPC from bone marrow to the periphery is observed during acute bone marrow reconstitution, and it has been exploited for therapeutic purposes. Administration of IL-3 and GM-CSF is, in fact, used to increase the pool of blood HPC in protocols of blood transplant (26). In the case of dialyzed patients, enlargement of the pool of EPO-responsive (i.e., EPO-R positive) HPC may counteract the EPO deficiency, thus optimizing the erythroid maturation process. This final interpretation is in line with the reported normalization of PRL levels in hyperprolactinemic dialyzed patients after EPO-treatment (3) and with the present observation of lower levels of EPO in blood of hyperprolactinemic compared (#1–#5) with normoprolactinemic patients (#9, #10).

Although formal assessment of this hypothesis would require experimental demonstration of the effect of decreasing the levels of PRL on the hematological parameters, the present observation may unveil a so far neglected role of pituitary hormones on erythropoiesis.

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