Enhanced Recovery from Thrombocytopenia and Neutropenia in Mice Constitutively Expressing a Placental Hematopoietic Cytokine

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Expression of the placental hormone, prolactin-like protein E (PLP-E), a potent cytokine that acts on multiple myeloid lineages, is normally restricted to pregnancy and certain hematopoietic disease states. We hypothesized that the restricted pattern of PLP-E expression is necessary to avoid hyperstimulation of myelopoiesis. To test this idea, we have produced PLP-E transgenic mice and analyzed their steady-state blood cell levels. We find that blood cell levels remain in the normal range, and thus the constitutive expression of a cytokine of pregnancy fails to overcome the tight control of hematopoietic set points for blood cell levels. In contrast, an effect of constitutive PLP-E expression is detected during the recovery from low blood platelet levels (acute thrombocytopenia) and from low granulocyte levels (acute neutropenia) but not from anemia. Mice producing high circulating concentrations of PLP-E recover more rapidly from both thrombocytopenia and neutropenia, as seen both by an earlier increase of progenitor numbers in the bone marrow and the earlier return to normal circulating blood cell levels. (Endocrinology 146: 64–70, 2005)

Blood platelets are the end product of the pathway of megakaryocyte proliferation and differentiation. A broad array of cytokines act on the steps leading from the initial cell committed to the megakaryocyte lineage, colony-forming unit megakaryocyte (CFU-MK), through the terminal differentiation of the mature megakaryocyte. Most of these cytokines are constitutively present and include thrombopoietin, IL-3, IL-6, and stem cell factor. Two other cytokines that act on megakaryocyte progenitors are produced specifically by the placenta in mice and thus are present during pregnancy in the female but otherwise are not present in healthy female or male mice. These two hormones, prolactin-like protein (PLP)-E and PLP-F, by themselves stimulate megakaryocyte differentiation but not CFU-MK proliferation. In contrast, in the presence of other cytokines, PLP-E and PLP-F contribute a synergistic stimulation of CFU-MK growth (1, 2).

The effects of PLP-E are not restricted to the megakaryocyte lineage. This hormone has been found to stimulate proliferation and differentiation of erythroid cell lines (3). In experiments using myeloid progenitors from human bone marrow, mouse PLP-E was shown to act synergistically with thrombopoietin to enhance growth of CFU-MK, burst-forming unit erythroid (BFU-E) progenitors, and the common myeloid progenitor, CFU-granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEMM) (4).

Recently, we have found that PLP-E and PLP-F act on all mouse myeloid progenitors, including CFU-granulocyte/macrophage (CFU-GM), BFU-E, and CFU-GEMM (our manuscript in preparation).

PLP-E and PLP-F are closely related: the genes encoding these two proteins are adjacent on mouse chromosome 13 (5, 6); the secreted proteins share 54% identity in amino acid sequence (5); these hormones share the same receptor on megakaryocytes; and they function as agonists (2). Despite these similarities, the patterns of PLP-E and PLP-F synthesis display some striking differences. Transcription of the PLP-E gene initiates several days earlier in gestation than for the PLP-F gene, and the two genes are active in distinct cell types: the polyploid trophoblast giant cells for PLP-E and the diploid spongiotrophoblasts for PLP-F (5). Furthermore, the PLP-E gene can also become active in bone marrow cells in response to severely low platelet levels (thrombocytopenia), whereas the PLP-F gene appears to remain silent under this condition (7).

The production of PLP-E in bone marrow cells during thrombocytopenia suggests a functional role for this hormone in the recovery of platelet levels from the disease state. Indeed, exogenous administration of PLP-E during thrombocytopenia hastens recovery (7). We therefore decided to explore whether mice constitutively expressing high levels of PLP-E would be protected from thrombocytopenia. Our finding that PLP-E acts on all myeloid progenitor populations (manuscript in preparation) suggests that the constitutive expression of this hormone may also hasten recovery from other blood cell diseases, in particular anemia and neutropenia. Furthermore, by expressing PLP-E endogenously, delivery of this cytokine to the bone marrow compartment could be achieved more readily and continuously compared with any injection route.

By producing such mice, we would also be in a position...
to examine whether the presence of high levels of PLP-E continuously in the adult would alter the hematopoietic set point for platelet levels or for other myeloid lineages. Alterations in blood cell levels would be consistent with the restricted expression of this hormone during pregnancy and hematopoietic disease, both of which represent physiological states of significant stress. Conceivably, restricting PLP-E gene expression in this way would enable this hormone to enhance survival in stress situations while preventing PLP-E from stimulating excessive hematopoiesis under normal conditions.

The approach we took was to create transgenic mice in which transcription of the PLP-E cDNA is driven by the mouse metallothionein I (MTI) gene promoter to achieve expression at moderately high levels in a broad distribution of tissues. Importantly, we had shown that the PLP-E receptor is present on megakaryocytes of nonpregnant female and male mice (7), so myeloid cells should be capable of responding to circulating PLP-E in transgenic mice. We have generated several transgenic lines and have analyzed blood cell levels under normal conditions and after induction of blood cell disease.

Materials and Methods

PLP-E transgenic mice

An expression construct containing the MTI promoter (kindly provided by Kelly Mayo) followed by the full-length PLP-E cDNA and the SV40 splice/polyadenylation signals was generated. The DNA fragment containing the MTI promoter, PLP-E cDNA including its leader sequence, and SV40 splice and polyadenylation signals was released from the vector by restriction enzyme digestion, purified by GenElute Minus EtBr Spin Columns (Sigma Chemical Co., St. Louis, MO) after gel electrophoresis, and injected into fertilized mouse eggs. Embryos were transferred into the oviduct of pseudopregnant foster mothers later that day. Tail DNA from the F0 progeny were examined by PCR for integration of the transgene into mouse genomic DNA, and Western blotting of serum was used to assay for the accumulation of PLP-E protein in the circulation. Transgenic mice and control mice were maintained on 14-hr light, 10-hr dark cycles with food and water freely available. All procedures were conducted with the approval of the Northwestern University Animal Care and Use Committee.

DNA extraction, PCR, and Western blotting

Genomic DNA was extracted from mouse tails using a standard protocol. In brief, mouse tail tissues were digested with proteinase K at 55 C overnight. Samples were then extracted with phenol:chloroform (1:1) followed by two extractions with chloroform alone. Genomic DNA was precipitated in 300 m m

Bone marrow cell cultures

Femurs from 16-wk-old transgenic mice and wild-type CD-1 control mice were flushed with ice-cold modified Dulbecco’s medium (IMDM) and 10% fetal bovine serum (Life Technologies, Rockville, MD), and collected material was passed through 21-gauge and then 25-gauge needles to separate cells. After 1 h in IMDM at 37 C, nonadherent cells were collected by centrifugation and washed with PBS and resuspended with IMDM and 1% Nutridoma (Roche Molecular Biochemicals, Indianapolis, IN). These cells were then cultured in MegaCult C medium (Stem Cells Technology, Vancouver, Canada) for CFU-MK colony-forming assays or CollagenCult medium for CFU-GEMM, CFU-GM, and BFU-E colony-forming assays. Initial densities were 5 x 105 nucleated bone marrow cells per well in two-well chamber slides.

For colony formation and differentiation assays, cultures were maintained for 7–10 d for CFU-MK, 10–14 d for CFU-GEMM and CFU-GM, and 8 d for BFU-E. Slides were dried and stained for colony detection and counting. Acetylcholinesterase activity was used as surface marker for MK cells, and MK colonies were defined as clusters of at least three cells staining positive for acetylcholinesterase. To score CFU-GEMM, CFU-GM, and BFU-E colonies, slides were stained with May-Grünwald and Giemsa, and colonies were defined based on their morphological characteristics.

Results

PLP-E transgenic mice

The MTI promoter linked to the PLP-E cDNA was microinjected into fertilized eggs to generate transgenic mice. Preliminary transient transfection studies had demonstrated that this construct results in the synthesis and secretion of mature and functional PLP-E protein (data not shown). Neonates resulting from these eggs were screened by PCR of genomic DNA for integration of the transgene, and Western blotting of serum was used to identify the presence of PLP-E in the circulation. Four PLP-E transgenic lines were established: TG6, TG7, TG14, and TG21. TG6 and TG7 display moderate levels of circulating PLP-E, whereas TG14 and TG21 have high levels of expression of the transgene (Fig. 1).

![Fig. 1. Circulating PLP-E in transgenic mice. Serum samples were collected from wild-type and transgenic mice. Equal amounts of protein were fractionated by SDS-PAGE, transferred to a membrane, and probed with anti-PLP-E antiserum. Numbers on the left indicate the position of molecular weight markers. Serum from d10.5 pregnant mice (d10) was used as a positive control, and serum from wild-type CD-1 mice (wt) was used as a negative control. The four transgenic lines 6, 7, 14, and 21 show increasing levels of PLP-E accumulation.](https://academic.oup.com/endo/article-abstract/146/1/64/2499904)
Hematology of PLP-E transgenic mice

To determine whether the constitutive presence of PLP-E results in the chronic alteration of blood cell concentrations, blood samples of transgenic mice and nontransgenic littermates were assayed from wk 3 to wk 15 of age. No difference was seen at any time point between these groups in terms of platelet, erythrocyte, or total white blood cell levels (Fig. 2). Thus, continuous exposure to PLP-E beyond gestation does not reset steady-state blood cell levels.

In addition to monitoring blood cells in the circulation, myeloid progenitor populations in the bone marrow of transgenic and nontransgenic controls were also compared. Primary bone marrow cells were cultured in medium containing IL-3 at 10 ng/ml to stimulate proliferation and colony formation. After 7–14 d of incubation, the numbers of CFU-MK, CFU-GM, BFU-E, and CFU-GEMM colonies were counted. At least two mice from each transgenic line were tested (Fig. 3). Again, no differences were found.

Recovery from thrombocytopenia

We had previously noted that the PLP-E gene becomes active in the bone marrow a few days after the onset of thrombocytopenia and that injection of PLP-E into thrombocytopenic mice hastens the recovery to normal platelet levels (7). If PLP-E is indeed a limiting factor in the recovery period, these findings suggest that mice constitutively expressing PLP-E would recover faster than wild-type mice. Thrombocytopenia was therefore induced in transgenic and nontransgenic control mice by single injections of neuraminidase, and blood was collected daily for platelet counts (Fig. 4A). For wild-type mice, complete recovery of platelets requires 4–5 d. The recovery period for the two transgenic lines expressing moderate levels of PLP-E, TG6, and TG7, was indistinguishable from that for the wild-type mice. In contrast, the recovery period was significantly faster in the two transgenic lines, TG14 and TG21, that produce high concentrations of PLP-E. Platelet levels are significantly higher in these transgenic mice by the second day of the recovery period and return to normal by d 3. To verify that the effect of this treatment regime was specific for the platelet compartment, erythrocyte and white blood cell levels were also monitored and were found not to change (data not shown).

The more rapid recovery of platelet levels in TG14 and TG21 mice predicts that the high PLP-E-expressing transgenic mice would also show a rapid elevation in bone marrow myeloid progenitor numbers. Primary bone marrow cultures were prepared each day after neuraminidase injection from TG21 mice and from wild-type mice. Myeloid progenitor numbers were then determined in colony-forming assays (Fig. 4, B and C). As predicted, levels of the platelet progenitor CFU-MK, as well as the multipotential myeloid progenitor CFU-GEMM, increase more rapidly in TG21 mice. CFU-GEMM numbers increase first, by the first day of recovery, followed by an increase in CFU-MK numbers. These transgenic mice and the control mice were identical in terms of the levels of the myeloid progenitors, BFU-E and CFU-GM, that do not lead to platelet formation (data not shown).

Recovery from anemia

Because PLP-E cooperates with other cytokines to stimulate BFU-E growth, we next explored whether constitutive expression of PLP-E can stimulate recovery from acute anemia. Erythrocyte levels in the transgenic mice and in wild-type control mice were rapidly decreased by a single injection of phenylhydrazine. Blood samples were collected and examined every 24 h over the course of the next 9 d. No change in the rate of recovery is seen comparing PLP-E-expressing transgenic with wild-type mice (Fig. 5A). Furthermore, primary bone marrow cell cultures generated from the transgenic and wild-type mice challenged with phenylhydrazine show no difference in CFU-GEMM or BFU-E numbers (Fig. 5B and C).
Recovery from neutropenia

Finally, we tested the ability of PLP-E to bring about a more rapid recovery from neutropenia. Again, transgenic and wild-type control mice were injected, this time with monoclonal antibody Rb6-8C5. Neutrophil levels declined quickly after injection, and recovery in the wild-type mice required approximately 5 d (Fig. 6A). In the two high-expressing transgenic strains, neutrophil levels rose more rapidly and were significantly increased relative to control mice in the TG14 strain and in both the TG14 and TG21 strains. Consistent with this rapid recovery in the transgenics, bone marrow levels of the progenitor population CFU-GEMM and GFU-GM were elevated in Rb6-8C5 antibody-injected TG21 transgenics on d 2 and 3, respectively, compared with injected wild-type mice (Fig. 6, B and C).

Discussion

We have succeeded in generating several transgenic lines of mice that constitutively express the placental hematopoietic hormone PLP-E. Expression levels of PLP-E vary among these lines, in effect providing a means of assessing dose-dependent biological effects. Based on polyacrylamide gel migration, the number, size, and relative amounts of glycoforms of PLP-E derived from the transgene and secreted by nonplacental tissues into the circulation are indistinguishable from the pattern of placental hormone in the circulation during pregnancy. Thus, the transgenic mice appear to be a valid model for testing the biological effects of PLP-E.

The production of these transgenic lines was intended to test the hypothesis that the restricted expression of PLP-E during pregnancy was crucial to eliminate the broad effects of this potent cytokine on the myeloid lineages outside of pregnancy. This hypothesis envisions pregnancy as a unique physiological state, including anemia and thrombocytopenia caused by blood volume expansion, and that as a result, pregnancy-specific regulatory factors have evolved to superimpose their activities on the homeostatic systems in place in the nonpregnant animal (13). The placenta provides the perfect endocrine gland for this pregnancy-specific restriction of cytokine expression, because that entire endocrine gland is discarded at the end of pregnancy and is not present in the developing newborn. In the transgenics, however, PLP-E expression would continue and myeloid development in the newborn might as a result be altered.

We analyzed the numbers of the various myeloid blood cell types longitudinally in the transgenic mice, but found no change in any of these numbers compared with control, nontransgenic siblings. Thus, the presence of PLP-E outside of pregnancy is insufficient to cause elevated platelet, erythrocyte, granulocyte, or macrophage levels. Two explanations seem most probable. First, hematopoiesis is controlled both by inducers and inhibitors of myeloid cell proliferation and differentiation (14); the presence of PLP-E may trigger a feedback mechanism that results in greater inhibitory activity, thereby maintaining the hematopoietic set points. Second, PLP-E by itself stimulates megakaryocyte differentiation but is dependent on the presence of other cytokines to enhance myeloid progenitor proliferation (1, 4). If the activity of other positive-acting cytokines is limiting, PLP-E may therefore not be able to bring about an expansion of cell numbers.

Although constitutive PLP-E does not result in altered hematopoiesis in the nonstressed state, we do observe an effect when platelet levels fall. Induction of a transient thrombocytopenia by injection of neuraminidase into nontransgenic mice is followed by a gradual recovery of platelet numbers over a 5-d period. In these mice, PLP-E expression

![Figure 3. Myeloid progenitor numbers in transgenic mice. Bone marrow was collected from transgenic lines and age-matched wild-type mice. Two mice were selected from each group, and measurements were made in triplicate. Bone marrow cells were grown in semisolid CollagenCult medium for CFU-GEMM (A), BFU-E (C), and CFU-GM (D); MegaCult C medium was used for CFU-MK (B) assays. CFU-MK colonies were identified by staining for acetylcholinesterase activity, and CFU-GEMM, CFU-GM, and BFU-E were stained by May-Grünwald and Giemsa solutions and scored based on their morphological properties. Statistical significance was determined by a one-way ANOVA and a post hoc Tukey’s test.](https://academic.oup.com/endo/article-abstract/146/1/64/2499904)
is observed in the bone marrow, but expression is detected only after approximately 3 d (7). The same recovery time course is seen in the transgenic lines that express moderate levels of PLP-E. In the two transgenic lines that have high levels of PLP-E, in contrast, platelet levels return to normal more rapidly. Consistently with this observation, bone marrow progenitor cells in the megakaryocyte lineage, first the multipotent myeloid progenitor CFU-GEMM and later the lineage-restricted progenitor CFU-MK, more rapidly increase in numbers in the transgenic high expressers. Importantly, these effects were specific, as no change is seen in the numbers of erythrocytes and their BFU-E progenitors, and no change is seen in total white blood cell numbers. Thus, constitutive expression of PLP-E at high levels eliminates the delay in production of this hormone in response to low platelet levels, and an important consequence is a more rapid recovery. These findings, coupled with the observation that the PLP-E gene is activated in bone marrow cells in response to thrombocytopenia (7), suggest that PLP-E may normally be a limiting factor in platelet production in this disease.

**FIG. 4.** Recovery from thrombocytopenia. Neuraminidase was injected at d 0 into groups of transgenic and wild-type mice (n = 3 for each group) to induce thrombocytopenia, and blood samples were collected daily for cell counting. A, Platelet levels in two transgenic strains with high PLP-E expression (TG14 and TG21) are significantly above the controls on d 2 (P < 0.001) and d 3 (P < 0.01). By d 3, platelet levels in TG14 and TG21 mice have returned to normal, whereas wild-type mice and the two transgenic mouse lines with low PLP-E expression recover from thrombocytopenia by d 4–5 after injection. Bone marrows were also collected from TG21 and wild-type mice, and equal cell numbers (5 × 10⁵) were plated per well in MegaCult for CFU-MK (B) or in CollagenCult for CFU-GEMM (C) colony-forming assays. CFU-MK numbers are elevated in the transgenic strain on d 2 (P < 0.01), and CFU-GEMM numbers are higher in the TG21 mice on d 1 and 2 (P < 0.01). Statistical significance (marked by asterisks) was determined by a one-way ANOVA and a post hoc Tukey's test.

**FIG. 5.** Recovery from anemia. A, Phenylhydrazine was injected on d 0 into transgenic (TG14 and TG21) and wild-type control mice (n = 4 for each group). Blood samples were collected daily for cell counting. No statistically significant difference in erythrocyte levels between transgenic and wild-type mice is seen at any time point. Blood marrows were also collected from transgenic strain TG21 and wild-type mice at each time point. Equal cell numbers (5 × 10⁵) were plated per well in CollagenCult for BFU-E (B) or CFU-GEMM (C) colony-forming assays. No significant differences are detected between transgenics and controls.
Because PLP-E acts synergistically with other cytokines to enhance progenitor growth, several cytokines may be rate limiting for recovery. Indeed, other cytokines have also been found to enhance recovery from thrombocytopenia in mice (15–18). Although the result with the high-expressing lines supports a role for endogenous PLP-E expression in the bone marrow, the finding that the two transgenic lines expressing comparatively moderate levels of PLP-E do not recover more rapidly from acute thrombocytopenia suggests that pharmacological levels of PLP-E may be needed to stimulate recovery. Alternatively, it may be that circulating levels of PLP-E in these transgenic mice are not indicative of bone marrow levels of expression of the transgene, or that the distribution of PLP-E-expressing cells for the transgene and the endogenous gene differ in the bone marrow.

In contrast to the results in recovery from thrombocytopenia, and despite the effects of PLP-E in cell culture on BFU-E growth (our manuscript in preparation), no difference is seen between high-expressing PLP-E transgenics and wild-type mice in the kinetics of recovery of circulating red blood cell levels from phenylhydrazine-induced anemia. The simplest explanation is that some factor other than PLP-E is rate limiting for recovery and that high levels of PLP-E are not able to substitute for the limiting activity that is required for restoration of normal erythrocyte concentrations. Notably, neither of the myeloid progenitors, CFU-GEMM and BFU-E, increases in number in the transgenics compared with wild-type mice under conditions of anemia, even though this hormone does stimulate CFU-GEMM growth during thrombocytopenia. As shown previously (1, 2), the effect of PLP-E on progenitor cell proliferation requires the presence of one or more other cytokines, consistent with the interpretation that another cytokine is limiting during recovery from anemia. It is possible that PLP-E would have a positive effect on the rate of recovery from anemia when present in combination with erythropoietin and other cytokines, as has been seen for erythropoiesis in the mouse in response to other combinatorial treatments (19–22).

Similar to the recovery from thrombocytopenia (and distinct from the recovery from anemia), the return to normal neutrophil levels after induction of neutropenia occurs more rapidly in PLP-E transgenics than in wild-type mice, as has been seen with other cytokine treatments (23–26). Preceding and accompanying the faster rise in circulating neutrophils are significantly greater increases in the transgenic bone marrow populations of CFU-GEMM and CFU-GM. Thus, PLP-E can contribute a rate-limiting activity at the level of progenitor expansion during the recovery period in this disease model. In contrast to the observed expression of PLP-E in the bone marrow in response to low blood platelet levels, we have not been able to detect PLP-E mRNA in the bone marrow under conditions of neutropenia. Thus, PLP-E may be able to play a pharmacologically significant role in the recovery from neutropenia and a physiologically significant role in the recovery from thrombocytopenia.

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