Reduced incidence of thrombosis in mice with hereditary spherocytosis following neonatal treatment with normal hematopoietic cells

Nancy J. Wandersee, John C. Lee, Susan A. Deveau, and Jane E. Barker

Thrombosis is a life-threatening complication of hemolytic anemia in humans. Cardiac thrombi are present in all adult α-spectrin-deficient (sph/sph) mice with severe hereditary spherocytosis, providing a model for events preceding thrombosis. The current study evaluated (1) the timing of thrombosis initiation and (2) the effect of postnatal transplantation of normal cells on life span and thrombotic incidence in adult mice. Thrombi are detected histologically following necropsy in untreated sph/sph mice of various ages and are not observed until 6 weeks of age. Thrombotic incidence increases from 50% at 6 to 7 weeks of age to 100% at 9 weeks of age. As a potential therapy, nonablated sph/sph neonates were transfused with either genetically marked normal peripheral blood (PB), bone marrow (BM), or both and assessed for donor cells and thrombosis. A single transfusion of PB, with or without BM, significantly increases the percentage of sph/sph mice that survive to weaning (4 weeks of age). Replacement in all sph/sph recipients is limited to red blood cells (RBCs). RBCs derived from donor PB are lost within 5 weeks. PB plus BM prolongs high-level donor PB cell production better than BM alone. Thrombotic incidence is significantly reduced in all sph/sph mice treated with PB, BM, or both. Hence, the presence of normal blood cells in the peripheral circulation of neonatal and adult sph/sph mice rescues the former and abrogates the development of thrombosis in the latter. (Blood. 2001;97:3972-3975)

© 2001 by The American Society of Hematology

Introduction

One of the more serious complications of heritable hemolytic anemias in humans is thrombosis. Thrombotic events affect approximately 20% of patients with sickle cell disease, 5% to 10% of patients with β-thalassemia, and a small number of patients with hereditary spherocytosis (HS). In adult mice with severe hemolytic anemia caused by deficiency of red blood cell (RBC) cytoskeletal components, cardiac thrombi are prevalent. These mutant mice have severe HS typified by misshapen RBCs that are destroyed within a single day of entry into the peripheral blood (PB). Reticulocytes compose 75% to 95% of the total circulating erythroid cells; mean cell volume (MCV) is increased, reflecting the larger, immature cells in the circulation. Splenic and liver, sources of newly generated RBCs in anemic mice, are grossly enlarged. Iron deposits accumulate in the kidney and liver. Mice with HS are much more severely affected than most humans with HS, many of whom are not diagnosed until an unrelated health crisis arises.

There are few models for thrombosis in experimental animals with hemolytic anemia. The mice with HS adequately fill that void. The incidence of coronary thrombosis is highest (85% to 100%) in HS mice with mutations in α-spectrin (Spnα1), and lowest (15% to 22%) in HS mice with β-spectrin and ankyrin mutations. These mice, except for the mutation they carry, share identical genetic backgrounds and environments, permitting comparative analyses. Adult Spnα1<sup>+/−</sup> / Spnα1<sup>−/−</sup> (hereafter, sph/sph) mice have the highest incidence (100%) of thrombosis. They provide a model for determining thrombus ontogeny, pathological consequences, precipitating agents, and efficacy of therapeutic interventions. Death of 30% of untreated sph/sph mice occurs before 3 weeks of age; the average life span of the survivors is 26.8 weeks. It is not clear whether the postnatal deaths are caused by thrombosis or other pathology. Here, the temporal development of thrombosis is established in sph/sph mice.

To date, we have shown that bone marrow (BM) hematopoietic cells transplanted from sph/sph mice to normal recipients are sufficient for thrombogenesis and that inversion of phosphatidyl serine to the outer leaflet of mutant RBCs is not a direct cause of thrombosis. Previous investigations showed a selective advantage for implantation of normal RBCs in anemic mice that received no conditioning regimen. These results also suggested an influence of normal PB on thrombogenesis in the highly susceptible sph/sph stock that could be assessed by monitoring mice with varying levels of donor cells. Three groups of nonablated mice were injected: the first received normal BM plus a transfusion of normal PB; the second and third received only normal BM or normal PB, respectively. None of the recipients was permanently implanted with any myeloid-derived elements other than RBCs. The data indicate that RBC transfusion increases the survival of newborn sph/sph mice to weaning and prolongs high-level implantation of normal BM cells. In addition, the results suggest that thrombosis is triggered by early events that can be blocked by exposure to normal PB cells soon after birth.

Materials and methods

Mice and treatments

Mice heterozygous for the sph mutation are maintained congeneric on both the WB/Re (WB) and C57BL/6J (B6) backgrounds. F1 hybrid (WB6F1) sph/sph and their normal sph/+ and +/+ (hereafter, +/+) littermates were generated by mating WB+sph/+ with B6-sph/+ mice.

From the Jackson Laboratory, Bar Harbor, ME.

Supported by National Institutes of Health (NIH) core grant CA34196 and NIH NRSA F32 DK09482 (N.J.W.); and NIH grant R01 HL29305 (J.E.B.).

Reprints: Jane E. Barker, The Jackson Laboratory, 600 Main St, Bar Harbor, ME 04609; e-mail: jeb@jax.org.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked “advertisement” in accordance with 18 U.S.C. section 1734.

© 2001 by The American Society of Hematology
For the transfusion/transplantation experiments, female congenic B6.CAST-+/+, Gpi1a/Gpi1a (hereafter, +/++) adult mice were used as BM and PB donors. Recipient WBB6F1-+/+ and WBB6F1-sph/sph mice were Gpi1a/Gpi1a. Glucose phosphate isomerase 1 (GPI1) is expressed in all cell types. Mice were housed and cared for according to American Association for the Accreditation of Laboratory Animal Care specifications.

Donor BM extraction, washing, and adjustment of cell concentration were performed as described previously.11 PB isolation for transfusion was as described.11 Recipient 1- to 5-day-old pups were injected on 1 to 3 successive days via the superficial temporal vein with donor cells in 0.1 mL phosphate buffered saline (PBS).11,20 Nonablated neonates were injected with BM, PB, or both. BM dose was 2.1 to 6.6 × 10⁶ cells delivered as a single injection or 9.5 to 11.9 × 10⁶ cells in 3 injections. PB was given in a single dose of 5 × 10¹³ total cells.

Measurements of blood parameters
Recipient blood was removed from the retro-orbital sinus in microhematocrit tubes at 5 weeks after transfusion/transplantation and at monthly intervals thereafter. RBC count, hematocrit, hemoglobin, MCV, and mean corpuscular hemoglobin concentration were evaluated by standard methods.12

GPI1 phenotype
RBCs retrieved from the packed pellet and white blood cells (WBCs) retrieved from the buffy coat were assessed for GPI1 phenotype as previously described.21 The concentration of each GPI1 isoform was quantified on a Molecular Dynamics (Sunnyvale, CA) densitometer. The sensitivity of the assay is 5%.

Histopathology
For assays of thrombotic incidence over time, untreated mutant and control mice were necropsied between 2 and 12 weeks of age. Treated mice were euthanized either when they became moribund or at 1 year of age or less. Heart, brain, liver, kidney, and spleen were collected from mice perfused either when they became moribund or at 1 year of age or less.

Thrombi in heart sections were distinguished from postmortem blood clots by their characteristic fibrous appearance. Prussian blue stain was used to detect nonhemoglobin iron (hemosiderin). Tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Histopathological surveys of renal and hepatic hemosiderosis were performed as described previously.21 The concentration of each GPI1 isoform was quantified on a Molecular Dynamics (Sunnyvale, CA) densitometer. The sensitivity of the assay is 5%.

Statistics
All statistical analyses were done with the unpaired nonparametric t test by means of the statistical package in Microsoft Excel (Seattle, WA).

Results

Temporal development of thrombosis and hemosiderosis in untreated sph/sph mice
The high degree of organization, fibrin deposition, and endothelial growth noted previously in and surrounding thrombi11,12 suggested that thrombi formed at least several days, and perhaps even weeks, before necropsy of the animal. Although death is not always an immediate consequence of infarcts, thromboses must contribute to the subsequent morbidity.11,12 The temporal appearance of thrombi was investigated in untreated sph/sph mice since, as adults, all had cardiac thrombi at necropsy. Analysis of histological sections collected sequentially from 2 weeks of age indicated that thrombi are first observed at 6 weeks of age (Table 1). Our criteria for identifying thrombi are stringent, so thrombi may not be detected prior to 6 weeks. Thereafter, thrombotic incidence increased from 50% at 6 to 7 weeks of age to 100% by 9 to 10 weeks of age. As predicted from previous examination of adult +/+ mice, no thrombi were observed in the normal littermates.

Histopathological surveys of renal and hepatic hemosiderosis showed that iron deposition was not apparent in untreated sph/sph mice younger than 3.5 weeks of age (data not shown), despite onset of severe hemolytic anemia within hours of birth.22 Hemosiderosis in both organs increased with age, reaching levels typical of adult mice at 6 weeks. It is apparent from these studies that neither thrombosis nor severe hemosiderosis are responsible for the preweaning deaths of sph/sph mice but that thromboses occur in a narrow window after weaning.

Life span following transfusion/transplantation
Previous results with nonablated β-spectrin–deficient (ja/ja) neonates indicated that +/+ PB transfused alone or simultaneously with +/+ BM, but not +/+ BM alone, was therapeutic and dramatically increased life span from less than 1 week to 34.3 weeks of age.18 Thrombotic incidence, predicted to be 85% to 100% on the basis of concurrent studies in the α-spectrin–deficient mice, was actually 15%. Two questions arose from these experiments: Do normal RBCs rescue neonates and extend life in adults, and do normal RBCs decrease thrombotic incidence in the adult? The latter seemed unlikely since the implant in the ja/ja mice was lost before necropsy.23 These 2 questions were addressed in the sph/sph mice, 70% of which survived without intervention past weaning and had thrombi when necropsied as adults.

The posttransplantation survival to weaning and beyond was similar in ja/ja and sph/sph neonates.18 BM transplants alone did not rescue sph/sph mice from postnatal death (Table 2). The sph/sph mice responded well to BM transplantation when +/+ PB was included in the transfusate. The percentage of sph/sph pups that survived to weaning following neonatal injection of BM plus PB was significantly increased from that in untreated sph/sph pups. It was, in fact, similar to that in unaffected +/+ pups. All the sph/sph mice transfused with PB alone survived to weaning. In answer to the first question posed, a PB transfusion fostered survival beyond the critical neonatal period.

Long-term life span, on the other hand, was enhanced by all treatments (Table 3). Most PB recipients were necropsied while still healthy at an average age of 39.8 weeks. Average life span was, therefore, considerably longer than observed (26.8 weeks) in the untreated sph/sph mice that survived past weaning. In all of the BM transplantation experiments, the only donor cells found in the host PB were RBCs (Table 3). Very low levels of donor WBCs might have been present but undetected owing to sensitivity constraints of the GPI1 assays. Surprisingly, repopulation was more complete and longer lasting with BM plus PB than with BM injections alone.

With PB alone, all donor RBCs were lost by 5 weeks of age.

Thrombotic incidence following transfusion/transplantation
In answer to the second question, thrombotic incidence was considerably decreased in adult sph/sph mice regardless of the donor cells transfused neonatally (Table 3). Those sph/sph recipients of BM or of BM plus PB that retained donor RBCs at necropsy had no thrombi, whereas 55.5% of those without donor cells at
necropsy (3 of 10 BM-alone mice and 7 of 8 BM-plus-PB mice) had cardiac thrombosis. The incidence of thrombosis at necropsy (17 to 41 weeks of age) was 56% in the mice that received donor PB alone. Neither thrombosis nor donor cells were observed in any of the +/+ mice treated simultaneously.

The sph/sph recipients of BM or BM plus PB that thrombosed prior to necropsy had no characteristic temporal pattern of donor RBC loss during postnatal life that distinguished them from mice without thrombi (data not shown). For example, all were devoid of donor cells by 16 weeks of age as were 4 of the mice with no thrombi. Eight of the 10 mice with thrombi and 2 of those without thrombi were moribund at necropsy. Mice injected with PB alone were also variable. Mice necropsied when they became moribund at 17, 26, and 34 weeks of age all had heart thrombi. The other 6 mice in this group, 2 of which developed thrombi, remained healthy until necropsy at 37 to 41 weeks of age. A unifying rationale for the difference in response within each group is not obvious and requires more extensive analysis beyond the scope of the current paper. It is clear that, in the sph/sph adult, the continued presence of donor RBCs prevents thrombosis and that donor RBC exposure postnatally reduces the incidence of thrombosis.

**Therapeutic effects of transfusion/transplantation on host pathophysiology**

**BM alone.** Anemia was not corrected but splenomegaly and cardiomegaly were decreased in 3 mice with fewer than 30% donor cells at necropsy (Table 4). Hepatomegaly was not significantly reduced (Table 4). Renal and hepatic hemosiderosis was reduced in 3 mice, only 1 of which still had donor cells at necropsy (data not shown).

**BM plus PB.** There was no significant improvement of the anemia in sph/sph mice with donor RBCs (data not shown). Splenomegaly and cardiomegaly were reduced in mutant recipients, with donor RBC levels ranging from 10% to 100% at necropsy (Table 4). Hepatomegaly was not significantly decreased in 3 mice with fewer than 30% donor RBCs at necropsy (data not shown).

**PB alone.** None of the parameters improved in PB mice prior to or at necropsy (Table 4).

**Discussion**

There are 4 major conclusions from this research. First, thrombosis in the α-spectrin-deficient prototype of hemolytic anemia occurs just after puberty and, therefore, is not responsible for the preweaning deaths of 30% of the pups. Second, thromboses can be inhibited by treatments that introduce normal hematopoietic cells into the PB of these mice prior to the onset of thrombosis. Third, maintenance of transplanted normal cells prevents thrombosis throughout life, but short-term exposure early in life is sufficient to reduce the risk of thrombosis from 100% to 56% or less in the adult. Fourth, organomegaly and anemia are not major contributors to the thrombotic incidence since they still occur in mice that do not thrombose. The data strongly support the hypothesis that events predisposing to thrombosis occur early in life.

Two events occur postnatally in the sph/sph mice. First, the mice become anemic within hours of birth. This immediately precedes a period of rapid growth and an increased demand for erythroid cells. Second, the mice develop hyperbilirubinemia owing to the destruction of their short-lived RBCs. Hemosiderosis is a later event (3.5 weeks of age) that, since it precedes the first appearance of thrombi, could be a factor contributing to thrombotic events. It is clear from the present results that normal PB cells need be present only for the first 4 to 5 weeks to prevent thrombosis in the critical window of 6 to 10 weeks of age. Necropsy of these mice well after the loss of normal cells suggests that the donor cells do not merely delay thrombosis but prevent it.

Pathophysiology that contribute to hemostatic changes and could predispose to thrombosis include hyperbilirubinemia, hemosiderosis, and the increasing demand for erythroid cells since the neonate doubles in size daily. These pathologies may be preceded or accompanied by changes in blood viscosity, in serum levels of free iron and homocysteine, or in the “stickiness” of aberrant RBCs. Normal RBCs could reverse some of the adverse hemostatic changes or provide a hemostatic response missing in the untreated mutant mice.

The continued anemia of treated jufa and sph/sph mice is reminiscent of the physiologic anemia of pregnancy, where the proliferative response is to increased blood volume. Reticulocyte

---

**Table 2. Survival to weaning in injected and uninjected sph/sph mice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. pups surviving to wean/total no.</th>
<th>% surviving</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninjected sph/sph mice</td>
<td>91/130</td>
<td>70.0</td>
</tr>
<tr>
<td>BM</td>
<td>15/23</td>
<td>65.2</td>
</tr>
<tr>
<td>BM + PB</td>
<td>17/18*</td>
<td>94.4</td>
</tr>
<tr>
<td>PB</td>
<td>10/10*</td>
<td>100.0</td>
</tr>
</tbody>
</table>

BM indicates bone marrow, PB, peripheral blood.

*Significantly different from uninjected sph/sph mice, P < .02.

---

**Table 3. Donor red blood cell replacement and incidence of thrombosis**

<table>
<thead>
<tr>
<th>Donor cells</th>
<th>No. mice</th>
<th>Recipient genotype</th>
<th>No. BM cells injected × 10⁶</th>
<th>Age* at necropsy</th>
<th>Donor RBCs at 5 wk, %</th>
<th>Donor RBCs at 16 wk, %</th>
<th>Donor cells at necropsy, %</th>
<th>Mice with cardiac thrombi, %†</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM</td>
<td>10</td>
<td>sph/sph</td>
<td>4.8-6.0</td>
<td>30-42</td>
<td>20-80</td>
<td>0-20</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>sph/sph</td>
<td>4.8-6.0</td>
<td>38-42</td>
<td>5-80</td>
<td>30-65</td>
<td>13-26</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>+/+</td>
<td>4.8-6.0</td>
<td>48-50</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BM + PB</td>
<td>8</td>
<td>sph/sph</td>
<td>2.1-11.9*</td>
<td>23-29</td>
<td>0-50</td>
<td>0-30</td>
<td>0</td>
<td>87.5</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>sph/sph</td>
<td>2.1-11.9*</td>
<td>25-52</td>
<td>10-90</td>
<td>30-100</td>
<td>10-100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>+/+</td>
<td>2.1-11.9*</td>
<td>50-52</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PB</td>
<td>9</td>
<td>sph/sph</td>
<td>‡</td>
<td>17-41</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>56</td>
</tr>
</tbody>
</table>

RBC indicate red blood cell; for other abbreviations, see Table 2.

*Age in weeks.

†The percentage of mice with thrombosises needs clarification in a larger cohort of animals. This does not detract from the conclusions that the 11 mice with donor cells present at necropsy do not have thrombi.

‡Each mouse was injected with approximately 5 × 10¹⁵ +/+/* total PB cells.
The number of physiological responses from contention. Subsequent experiments set the window of response, describe cells that alter present postnatally that predispose to thrombosis. The current anemia does not seem to affect the incidence of thrombosis. They provide sufficient oxygen to tissues. In either case, the persistent possibility that aberrant host cells are still generated but so rapidly destroyed that, although they cannot be quantified by the observer, they provide sufficient oxygen to tissues. In either case, the persistent anemia does not seem to affect the incidence of thrombosis. The current experiments set the stage for analysis of factors present postnatally that predispose to thrombosis. The current experiments set the window of response, describe cells that alter thrombotic incidence when present neonatally, and eliminate a number of physiological responses from contention. Subsequent experiments can address the issue of whether sph/sph WBCs and platelets are required for thrombosis; when normal donor RBCs are most efficacious; whether alternative means of curing bilirubinemia are protective; whether iron overload is a contributing factor; and whether “stickiness” of the sph/sph RBCs is causative.

Acknowledgments
The authors thank Drs. David Serreze and Ji Chen Chen for critical review of the manuscript, and the Biomedical Imaging Service at The Jackson Laboratory for technical assistance.

References
17. Eppig JJ, Kozak LP, Eicher EA, Stevens LC. Ovarian teratomas in mice are derived from oocytes that have completed the first meiotic division. Nature. 1977;269:517-518.
Reduced incidence of thrombosis in mice with hereditary spherocytosis following neonatal treatment with normal hematopoietic cells

Nancy J. Wandersee, John C. Lee, Susan A. Deveau and Jane E. Barker