Reduction 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 potentially life-threatening complication of hereditary 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)
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.21 PB isolation for transfusion was as described.31 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).12 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 sinuses in microhemocrit 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. 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.31 Two of these 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

| Table 1. Temporal development of cardiac thrombosis in sph/sph mice |
|------------------|---|---|---|---|---|---|---|
| Age              | < 4 wk | 4-5 wk | 5-6 wk | 6-7 wk | 7-9 wk | 9-10 wk | 11-12 wk |
| No. mice*        | 12 | 8 | 7 | 6 | 8 | 7 |
| Cardiac thrombi; † % | 0 | 0 | 0 | 50 | 75 | 100 |

*There were equal numbers of males and virgin females—or in columns with odd numbers of animals, as close to these values as possible—at 6 to 12 weeks of age.

†Mice with thrombi are those that have a large thrombus in either the mitral valve or left atrium of the heart.
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>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>
<td>0</td>
</tr>
<tr>
<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>
<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>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>
<td>0</td>
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<tr>
<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>
<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 thromboses 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 × 10¹² +/+ +/+ total PB cells.

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.

Pathophysiologicals 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. All could exacerbate damage to the endothelial vasculature. 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 ja/ja and sph/sph mice is reminiscent of the physiologic anemia of pregnancy, where the proliferative response is to increased blood volume. Reticulocyte...
percentages decrease to normal levels in treated ja/ja mice and hematopoietic organs subside to 1.5- to 10-fold enlargement, as noted for the sph/sph mice as well. Results suggest that the mice no longer respond to the demand for additional cells. Alternatively, it is possible 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

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References

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