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Hemopoietic stem cells in murine embryonic yolk sac and peripheral blood

(embryonic stem cells/fetal liver/fetal transplantation/hemoglobin ontogeny)

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ABSTRACT **Disaggregated embryonic yolk sac cells and circulating peripheral blood cells were obtained from normal murine day 9 embryos, prior to the formation of the fetal liver. These cells were microinjected transplacentally into days 11–15 *W* mutant anemic fetuses, when the fetal liver was the major hemopoietic organ. In a small proportion of the recipient animals examined after birth, long-term repopulation by the embryonic donor hemopoietic cells was observed. The donor hemopoietic stem cells proliferated and differentiated in the hosts as evidenced by the presence of donor hemoglobins in the growing recipient host animals. Some mothers of the pups were also repopulated by the donor stem cells. These results provide direct evidence that, during early murine embryogenesis, there are functional hemopoietic stem cells which are capable of colonizing the adult hemopoietic organs and probably the fetal liver and spleen to initiate hemopoiesis in these tissues.**

Erythropoiesis during murine embryogenesis provides an excellent experimental model to study erythroid cell differentiation and hemopoietic stem cell migration. The circulating embryonic erythroblasts derived from the yolk sac synthesize primarily embryonic hemoglobins (1–3). Beginning on day 11 of gestation, small amounts of adult hemoglobins are also produced in the same population of maturing erythroblasts (4). Shortly after day 10 of gestation, the fetal liver is formed; it rapidly becomes the major erythropoietic organ until near birth, when it is supplanted by the spleen and bone marrow. Fetal hepatic erythroblasts produce adult hemoglobins (1–3, 5).

The issue of whether fetal hepatic erythropoiesis arises endogenously or is due to the colonization by circulating hemopoietic stem cells has been the subject of investigation by a number of laboratories (6–11). Definitive proof that yolk sac-derived stem cells seed the fetal liver requires an animal model which accepts *in utero* hemopoietic transplants. Adult mice with mutations at the *W* locus can be repopulated with normal stem cells without prior total body irradiation. It has also been shown that fetuses homozygous or heterozygous for various *W* alleles accept *in utero* transplants (12–15). In the present study, we have utilized *W* mutant fetuses to address the question of whether or not the yolk sac contains hemopoietic stem cells with long-term repopulating ability. Yolk sac cells or peripheral blood cells collected from normal donor day 9 embryos with β-globin genotype of Hbb^s/Hbb^d were microinjected transplacentally into recipient days 11–15 *W* mutant fetuses with β-globin genotype of Hbb^s/Hbb^s. Long-term repopulation of hemopoietic tissues by the donor hemopoietic stem cells, as evidenced by the presence of donor diffuse hemoglobins, was observed in a small number of recipient animals and their dams. These experimental results indicate that there are functional hemopoietic stem cells present in both yolk sac and circulating peripheral blood, and these stem cells are capable of long-term repopulation in adult and probably fetal animals.

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MATERIALS AND METHODS

Recipient Fetuses. Female C57BL/6J-$W^{39}/+$-Hbb^s/Hbb^s mice, and very occasionally female C57BL/6J-W^{39}/W^{41}-Hbb^s/Hbb^s mice, were mated to male C57BL/6J-W^{39}/W^{41}-Hbb^s/Hbb^s mice. The morning when the vaginal plug was found was designated as day 0 of gestation. The fetuses from such matings were C57BL/6J-Hbb^s/Hbb^s but differed in the alleles present at the *W* locus. Thus, they might be $W^{41}/+$, $W^{39}/+$, W^{41}/W^{41}, W^{39}/W^{41}, or W^{39}/W^{39}, identifiable by their characteristic coat color (16).

Adult Marrow Donor Cells. Normal adult female C57BL/6J-Hbb^d/Hbb^d mice were killed by cervical dislocation, and bone marrow cells were flushed from the femurs into phosphate-buffered saline (PBS) and later resuspended in Iscove's modified Dulbecco medium (IMDM). The marrow cells were disaggregated into a single-cell suspension by aspiration through a 26-gauge needle a few times. In some experiments, the disaggregated marrow cells were labeled with indium-111 oxine (17).

Embryonic Donor Cells. Normal female C57BL/6J-Hbb^s/Hbb^s mice were mated to normal male C57BL/6J-Hbb^d/Hbb^d mice. The hemoglobin phenotypes of all mating mice were confirmed by electrophoresis on cellulose acetate (18). The fetuses from such matings were C57BL/6J-Hbb^s/Hbb^d. On day 9 of gestation, the female mice were killed by cervical dislocation. The embryos with their membranes were removed and washed free of maternal tissues and blood cells in PBS, and the yolk sacs were dissected free. Circulating peripheral blood cells were obtained by allowing the embryos to bleed into PBS and were later resuspended in IMDM. The disaggregated yolk sac cells were obtained by incubating the yolk sacs for 3 hr in PBS with 10% fetal calf serum and 0.1% collagenase (Sigma) with frequent mixing as previously described (19). The disaggregated cells were then washed twice with PBS and finally resuspended in IMDM. All cell counts were done manually with a standard hemocytometer.

These embryonic donor Hbb^s/Hbb^d cells were injected into recipient Hbb^s/Hbb^s fetuses. The diffuse hemoglobins detected in the recipient animals could be derived only from donor embryonic cells, not from hemopoietic stem cells present in donor Hbb^s/Hbb^s maternal peripheral blood.

Microinjection of Cells into Recipient Fetuses. Females with the recipient fetuses were anesthetized with Avertin on days 11–15 of gestation. An incision was made on the abdomen and the uterine horns were exposed. Approximately 5×10^5 donor marrow cells or embryonic hemopoietic cells, suspended in 10 μl of IMDM, were injected through the uterine wall into each placenta via a micropipet 80–120 μm in diameter, in a manner similar to that described by Fleischman and Mintz (12). After the injections, the abdominal wound

was sutured and the pregnancies were allowed to go to term. The genotypes of the recipient newborn mice at the *W* locus were determined 2–4 weeks after birth by their distinctive coat colors.

Hematological Studies. Peripheral blood was obtained from the pregnant mice at operation, and subsequently from the dams and their offspring on a monthly basis. For quantitative determination of different globin chains, the hemolysates were run on Triton X-100/acetic acid/urea/polyacrylamide gel electrophoresis with cystamine (20). The gels were stained with Coomassie blue. The various globin bands were cut out and eluted with pyridine, and absorbance at 605 nm was determined by a spectrophotometer (21).

RESULTS

Fate of Microinjected Hemopoietic Cells. To demonstrate that hemopoietic cells injected transplacentally can enter the fetus, indium-111 labeled adult bone marrow cells were microinjected into the placentae of 32 (four pregnant mice) 12- to 13-day-old fetuses. Twelve hours after the injections, the animals were sacrificed. The fetuses were dissected, and radioactivity was determined.

Fig. 1 shows that 17 of the 32 fetuses displayed more than 20% of the injected radioactivity. In these 17, a mean of 21% of the total radioactivity occurred in the placentae, 28% in the fetal peripheral blood, 30% in the fetal yolk sac, 9% in the fetal liver, and 11% in the remaining parts of the fetus.

Hemopoietic Stem Cells in Adult Bone Marrow. One hundred fetuses in 21 pregnant mice were injected with adult bone marrow cells. Sixteen newborn mice survived to at least 1 month of age. Only two recipient newborn animals from two different litters showed evidence of donor cell repopulation of hemopoietic tissues (Fig. 2). One of these stemmed from a litter of 8 (injected on day 13) and the other from a litter of 7 (also injected on day 13). Three months later, one of these recipients (a male W^{39}/W^{41}) was totally repopulated, and the other (a female $W^{39}/+$) was partially repopulated. Donor hemoglobin levels in the latter recipient fluctuated between 8% and 34% over a period of 9 months. Moreover, 7 months after treatment, both $W^{39}/+$ dams had almost 100% diffuse hemoglobins of donor origin in their blood. These results indicate that adult bone marrow hemopoietic stem cells can seed fetal hemopoietic tissues as has been described (12–15).

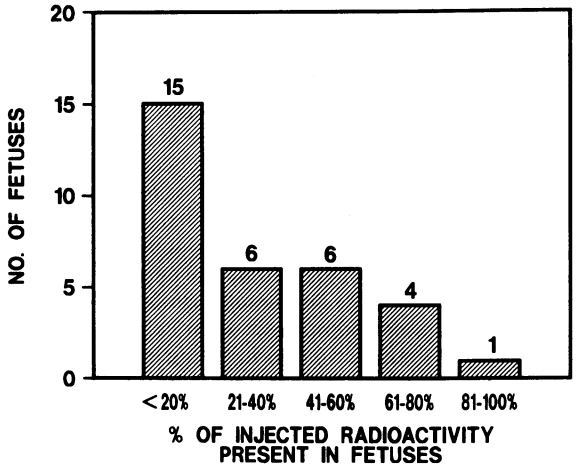

FIG. 1. Bone marrow cells from adult female C57BL/6J mice were labeled by indium-111 oxine by the method of Thakur *et al.* (17). The labeled cells were microinjected transplacentally into 32 recipient fetuses found in four pregnant mice of days 12 and 13 of gestation. Twelve hours later, the fetuses were dissected, and the radioactivity was determined.

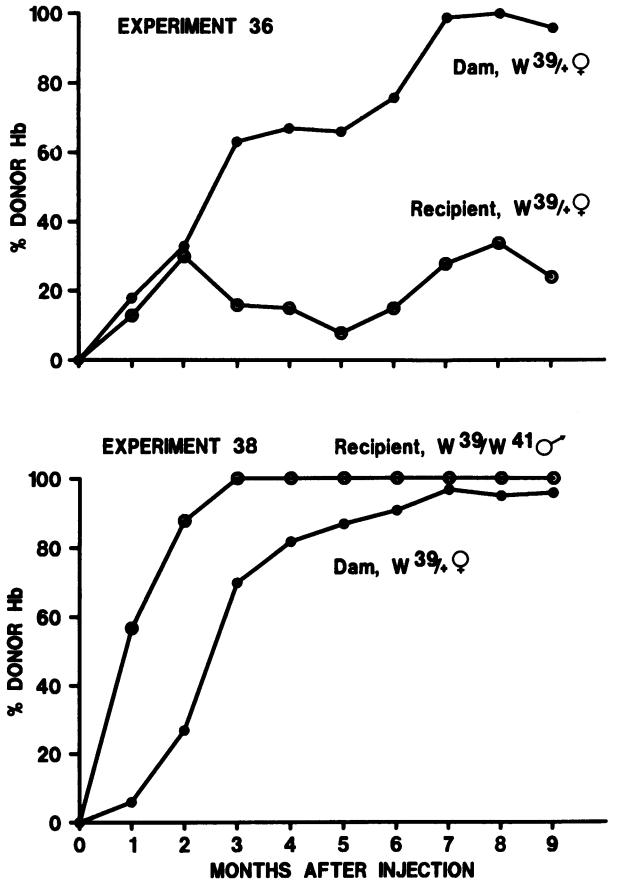

FIG. 2. Changes in hemoglobin phenotypes in two fetal recipients and their dams after microinjection with adult bone marrow cells. The hemoglobin genotype for the donors is Hbb^d/Hbb^d, while the hemoglobin genotype for the recipients is Hbb^s/Hbb^s. In experiment 36, eight fetuses were injected on day 13 of gestation. In experiment 38, seven fetuses were injected on day 13 of gestation.

Hemopoietic Stem Cells in Day 9 Yolk Sacs. One hundred and sixty-three fetuses in 26 pregnant mice were injected. Thirty-nine newborn mice survived to at least 1 month of age. Nine newborn recipients from five different litters had donor type hemoglobins in their blood. Two of these recipients were injected with donor yolk sac cells on day 12, five on day 14, and two on day 15 of gestation. When examined at 1 month of age, the levels of donor hemoglobins in these nine recipients varied from barely detectable in a male $W^{39}/+$ mouse which was injected on day 12, to 30% in another male $W^{39}/+$ mouse which was injected on day 14. Six recipient animals survived to 5 months of age, and the levels of donor hemoglobins in them varied between 21% in a male $W^{39}/+$ mouse to 98% in a female W^{39}/W^{41} mouse. Three recipient animals survived to be more than 2 years old, and the levels of donor hemoglobins in these animals were 70%, 94%, and 98%.

Nine dams also had donor hemoglobins in their blood. Eight dams were $W^{39}/+$, and their levels of donor hemoglobins varied between 8% and 48% when examined 3 months after their fetuses were injected. One dam was W^{39}/W^{41}, and had 32% donor hemoglobins at 1 month after injection and complete replacement with donor hemoglobins when examined 2 and 3 months after injection.

Fig. 3 illustrates the changes in peripheral blood globin chain phenotypes of a $W^{39}/+$ dam and one of her offspring, a W^{39}/W^{41} male. The pregnant $W^{39}/+$ female was found to have eight fetuses on day 14 of gestation. These fetuses were injected with day 9 disaggregated donor yolk sac cells. The dam had barely detectable donor β-globin chains 1 month after injection. By 5 months after injection, the level of donor

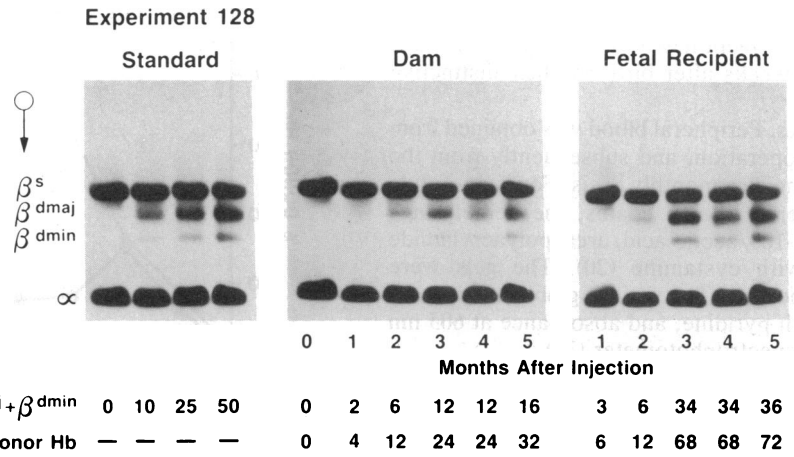

FIG. 3. Polyacrylamide gel electrophoresis of the globins obtained from a dam (hemoglobin genotype Hbb^s/Hbb^s) and a fetal recipient (hemoglobin genotype Hbb^s/Hbb^s) after microinjection transplacentally with donor day 9 disaggregated yolk sac cells (hemoglobin genotype Hbb^s/Hbb^d). (*Left*) (Standard) Series of control hemolysate mixtures consisting of single hemoglobin with 0%, 10%, 25%, and 50% diffuse hemoglobins. Eight fetuses were microinjected on day 14 of gestation, five of which were subsequently delivered. (*Center*) (Dam) Hemoglobin phenotype of the dam. Since the hemoglobin genotype of the donor cells was Hbb^s/Hbb^d, the percent of donor hemoglobin is twice the amounts of diffuse hemoglobins observed. (*Right*) (Fetal Recipient) Hemoglobin phenotype of the only newborn with donor hemoglobins, a W^{39}/W^{41} male mouse.

β-globin chains in the dam rose to 32%. Of the five animals that were delivered by this female, only one, a W^{39}/W^{41} male, was found to have donor β-globin chains. In this male, the level of donor β-globin chains was 6% at 1 month of age, and it rose to 72% at 5 months of age.

Hemopoietic Stem Cells in Day 9 Embryonic Peripheral Blood. One hundred and sixty-two fetuses in 24 pregnant mice were injected with peripheral blood cells obtained from day 9 embryos. Of these, 23 newborn mice survived to at least 1 month of age. Two female W^{39}/W^{41} recipients from one litter injected on day 15 of gestation had increasing amounts of donor hemoglobins in their blood, up to 58% in one and 96% in the other when examined at 3 months of age. The dam of this litter, a $W^{39}/+$ female, also was found to have 24% donor hemoglobins in her blood 3 months after injection.

In addition, four other dams were also found to have donor hemoglobins in their blood. A $W^{39}/+$ dam had 36% donor hemoglobins at 9 months after injection, while another $W^{39}/+$ dam had 41% donor hemoglobins at 3 months after injection. The third $W^{39}/+$ dam had 12% donor hemoglobins at 1 month after injection, but the donor hemoglobin became almost nondetectable 2 and 3 months after injection. The fourth dam was a female W^{39}/W^{41} mouse, and had 58%, 60%, and 96% donor hemoglobin in her blood when tested 1, 2, and 3 months after injection, respectively.

DISCUSSION

During murine embryogenesis, the first site of red blood cell formation is the yolk sac blood islands at around day 8 of gestation. The yolk sac-derived erythroblasts proliferate and mature intravascularly in a relatively synchronous manner. They synthesize primarily embryonic hemoglobins but produce some adult hemoglobins as well (1–4). Shortly after day 10, the fetal liver rapidly becomes the major organ of erythropoiesis (6). The fetal hepatic erythroblasts produce adult hemoglobins (1–3, 5).

Whether fetal hepatic erythropoiesis arises endogenously or is due to the colonization by circulating hemopoietic stem cells has not yet been settled by incontrovertible experimental results. Using the spleen colony assay in lethally irradiated adult recipient mice, Moore and Metcalf reported (7) that hemopoietic stem cells originating initially in the yolk sacs were responsible for colonization of other fetal and adult hemopoietic organs. However, the interpretation of these experimental data is controversial (22). Moreover, other investigators failed to detect significant numbers of yolk sac-derived hemopoietic stem cells as assessed by the spleen colony assay (8, 10, 11). Weissman *et al.* (11) transplanted days 8–10 yolk sac cells into days 8–9 host embryos differing in cell surface antigens. By the indirect immunocytochemical technique, donor cells were shown to be present in recipient host animals at low levels, but chimerism could not be confirmed by isoenzyme analysis.

We have previously shown that, beginning on day 9, there are hemopoietic progenitor cells in the yolk sac and in the embryonic circulation which can give rise *in vitro* to large mixed erythroid colonies synthesizing adult hemoglobins (23, 24). These results suggest that there are multipotential hemopoietic progenitor cells in the embryonic yolk sac and peripheral blood and support the hypothesis that circulating hemopoietic progenitor cells seed the fetal liver to initiate erythropoiesis in that organ.

In the present study, we have investigated the origin of fetal hepatic erythropoiesis by an *in vivo* experimental approach. Disaggregated yolk sac cells or peripheral blood cells from normal day 9 embryos with a β-globin genotype of Hbb^s/Hbb^d were injected into days 11–15 mutant W^{39} or W^{41} fetuses with a β-globin genotype of Hbb^s/Hbb^s. Adult homozygous or compound heterozygous *W* mutant mice have macrocytic anemia, white hair, and germ cell depletion. Normal hemopoietic stem cells can repopulate these *W* mutant adult and fetal mice without lethal irradiation (12–15, 25–28). The presence of donor hemoglobins in the recipient peripheral blood is accompanied by the appearance of donor granulocytes, mast cells, and immunoglobins, indicating repopulation by donor hemopoietic stem cells (25–28).

The results of the experiments injecting indium-111-labeled bone marrow cells into fetuses demonstrate that our transplacental injection technique resulted in the delivery of a significant number of hemopoietic cells into fetuses *in utero* in approximately half of the cases. Normal adult marrow cells were injected into 100 fetuses *in utero*. Of the 16 mice born that survived to at least 1 month of age, 2 were found to have been repopulated by donor marrow hemopoietic stem cells. In a similar set of experiments performed previously, 56 fetuses were injected, 27 mice were born and survived, and 6 of these were found to have been repopulated (13). The

causes for the lower success rate of repopulation in the present experiments are unclear.

Using the experimental model of injecting genetically marked embryonic hemopoietic cells into days 11–15 recipient fetuses, we tested the hypothesis that there are hemopoietic stem cells in yolk sac and peripheral blood of day 9 fetuses. The results of the present investigation reveal that 9 recipient fetuses were repopulated by day 9 yolk sac cells, and 2 recipient fetuses were repopulated by day 9 peripheral blood cells. In addition, a total of 14 dams were also repopulated by these same embryonic cells. The presence of diffuse hemoglobins of the donor type in the growing recipient *W* mutant animals clearly indicates that donor hemopoietic stem cells have seeded, proliferated, and differentiated in the recipient hemopoietic tissues. These data demonstrate that there are functional hemopoietic stem cells in day 9 disaggregated yolk sac cells and peripheral blood cells and that these stem cells are capable of long-term repopulation. It has previously been reported that fetal liver provides a receptive microenvironment for the proliferation and differentiation of circulating hemopoietic stem cells (9). Taken together, the present experimental observations provide direct *in vivo* evidence that adult erythropoiesis results from colonization by circulating embryonic hemopoietic stem cells and, by inference, that fetal hematopoiesis is also supported by migratory stem cells.

The origin of these embryonic hemopoietic stem cells is not yet clearly defined. The donor embryonic yolk sac cells and peripheral blood cells used in the current study were obtained from embryos of day 9 of gestation. In C57BL/6J mice, immature hematopoietic precursor cells can be recognized within the fetal liver only beginning on days 10 1/2 and 11 of gestation (6). In the present investigation, we have examined histologically the serial sections of a few day 9 donor embryos, and we have confirmed that no recognizable hemopoietic precursor cells are present in the area presumably of the rudimentary fetal liver (data not shown). Therefore, the donor embryonic cells used in the present study could not be of fetal liver origin. The presence of these hemopoietic stem cells in day 9 disaggregated yolk sac cells suggests that these stem cells are likely derived from embryonic yolk sac blood islands. Additional experimentation is needed to determine definitively the origin of these embryonic hemopoietic stem cells.

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