Hemoglobin Switching During Murine Embryonic Development: Evidence for Two Populations of Embryonic Erythropoietic Progenitor Cells

By Peter M.C. Wong, Siu-Wah Chung, Susan M. Reicheld, and David H.K. Chui

Explants of normal mouse embryonic tissues and disaggregated embryonic single cells were cultured in vitro to study the erythropoietic progenitor cells present during embryonic development. The results indicate that there are two populations of erythropoietic progenitor cells committed to different hemoglobin synthetic programs. These progenitor cells are present at an early gestational stage prior to the formation of the fetal hepatic primordium. One population of progenitors can be stimulated by erythropoietin alone to form usually small erythroid colonies after culture for six days in vitro. These erythroblasts primarily synthesize embryonic hemoglobin but produce some adult hemoglobin as well. The other population of progenitors requires stimulation by both erythropoietin and adult spleen cell-conditioned medium, and usually forms large erythroid colonies after culture for six days in vitro. These erythroblasts produce only adult hemoglobins.

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Erythropoiesis during normal murine embryogenesis provides an excellent model system to study the developmental cell biology of hemoglobin switching. The circulating primitive erythrocytes, derived from yolk sac blood islands, proliferate and mature in a relatively synchronized manner. These primitive erythrocytes synthesize embryonic hemoglobin as well as some adult hemoglobins. On day 9 of gestation, there are hematopoietic progenitor cells in the yolk sac and the embryonic circulation that, in culture, give rise to erythroblasts synthesizing adult hemoglobins. From day 11 on, the fetal hepatic erythroblasts as well as the erythropoietic progenitor cells in the fetal livers are committed to adult hemoglobin synthesis. In this study we have cultured embryonic tissues and disaggregated embryonic single cells on day 8 of gestation to document clearly the presence of different populations of erythropoietic progenitor cells during embryonic development.

The results indicate that on day 8 of gestation prior to the formation of the fetal hepatic primordium, there are two populations of erythropoietic progenitor cells. The first detectable population of progenitor cells, when cultured in vitro with erythropoietin alone for six days, usually give rise to small erythroid colonies containing erythroblasts that synthesize primarily embryonic hemoglobin but produce some adult hemoglobin as well. Addition of adult spleen cell-conditioned medium has no demonstrable effect upon the growth of these erythroid colonies. Shortly after, another population of progenitor cells were detected that, when cultured in vitro with both erythropoietin and adult spleen cell-conditioned medium for six days, usually produce large erythroid colonies synthesizing only adult hemoglobins.

Materials and Methods

Tissues and Cells. Normal adult FL/4Re inbred mice obtained from the Jackson Laboratory, Bar Harbor, Me., were mated, and the morning on which the vaginal plug was found was designated as day 0 of gestation. At approximately six-hour intervals on day 8 of gestation, embryos with their intact extraembryonic membranes were carefully dissected and washed free of maternal tissues and blood cells. They were placed in methylenecellose culture medium and cut into small fragments (0.5 mm) before culture. On days 9 and 10 of gestation, only the yolk sacs were dissected free and cut into similarly small fragments for culture. For disaggregated embryonic single cells, normal Balb/c mice obtained from the Cumberland View Farms (Clinton, Tenn.) were mated as was described. Around noon on day 8 of gestation, embryos with their extraembryonic membranes were dissected free, washed, and digested with collagenase (Sigma Chemical Co., St. Louis) at a final concentration of 0.1% in phosphate-buffered saline without calcium and magnesium but with 20% fetal calf serum (Flow Laboratories, McLean, Va.) for three hours at 37°C with intermittent agitation. At the end of the incubation, the digestion mixture was allowed to settle for five minutes, and the supernatant containing disaggregated embryonic single cells was harvested. Each day-8 conceptus would yield approximately 1 to 5 x 10³ cells.

Erythroid Cell Cultures. Embryonic tissue fragments were cultured in methylenecellose culture medium at 37°C for six to eight days as described previously. Erythropoietin (Connaught Laboratories, Toronto; specific activity, 2 to 4 U/mg of protein) was added to these cultures, usually at 1 to 2 U/mL. Adult spleen cell-conditioned medium was prepared from C57BL/6J mice and was added to account for 20% of the erythroid cultures.

Cultures of disaggregated embryonic cells were done similarly except that the spleen cell-conditioned medium was prepared according to the method of Eaves et al. and was added to account for 1.25% of the culture. Erythropoietin was added to the cultures at a final concentration of 2 to 3 U/mL. A quantity of 1 to 3 x 10⁴ cells was plated in 0.7 mL of culture medium.

Hemoglobin Analysis. Hemolysates of the erythroblasts harvested from the cultures were analyzed by thin-layer isoelectric focusing and stained with benzidine dihydrochloride (Sigma) and scanned by a Beckman densitometer (Beckman Instruments, Inc., Burlington, Va.).

For immunocytochemical studies, individual in vitro erythroid colonies were carefully picked by means of a micropipette. They were deposited on glass slides, immediately air dried, and fixed in acetone/methanol. They were subsequently stained with rabbit IgG.
anti-HbA antibodies conjugated with fluorescein and rabbit anti-HbE antibodies conjugated with rhodamine. After washing, the slides were examined in a Zeiss microscope equipped for reflected light-fluorescence microscopy.

RESULTS

Cultures of embryonic tissue fragments. Early on day 8 of gestation, hemoglobinized cells were not observed in the embryonic tissues in situ. Cultures of small fragments of these early day 8 embryonic tissues and extraembryonic membranes in vitro for six to eight days with added erythropoietin produced clusters of well-hemoglobinized erythroblasts, each consisting of approximately 100 to 200 erythroid cells. All three embryonic hemoglobins (E1, x2y2; E11, a2y2; and E111, a2z2) were present in these cultures, but adult hemoglobins (x2beta2mnu and a2beta2min) were not detected by the isoelectric-focusing technique (Fig 1). The further addition of adult spleen cell-conditioned medium to these cultures did not alter the erythroid growths or the types of hemoglobins produced as determined by the isoelectric-focusing technique (Fig 1).

Cultures of day 8½ embryonic tissues and extraembryonic membranes with added erythropoietin produced more prominent erythroid growth. The hemoglobins produced were primarily embryonic hemoglobins, but some adult hemoglobins were also detected (Figs 1 and 2). Immunocytologic study revealed that virtually all the erythroblasts in the cultures contained both embryonic and adult hemoglobins (Figs 3A and 3B). When both erythropoietin and adult spleen cell-conditioned medium were added to the cultures of day 8½ embryonic tissues and extraembryonic membranes, there were some macroscopic erythroid bursts reminiscent of the bursts seen in cultures of days 9 to 12 embryonic peripheral blood cells in addition to the well-hemoglobinized erythroid cell clusters. Both embryonic and adult hemoglobins were produced in these cultures, and the proportion of adult hemoglobins synthesized was much augmented compared to the cultures with only erythropoietin added (Figs 1 and 2). Immunocytologic study revealed that, in addition to erythroblasts containing both embryonic and adult hemoglobins, there were also many erythroblasts containing only detectable adult hemoglobins (Figs 3C and 3D).

On days 9 and 10 of gestation, the yolk sacs with many hemoglobinized primitive nucleated erythroblasts present within the vasculature were dissected free of maternal tissues and the embryo proper. These yolk sacs were further cut into small pieces and cultured. Almost all of the hemoglobins detected in these cultures were adult hemoglobins (Fig 1). Moreover, the erythroid growth was much more prominent in the cultures to which both erythropoietin and adult spleen cell-conditioned medium had been added, as has been previously reported. That the cultures of days 9 or 10 yolk sac fragments produced adult hemoglobins and not embryonic hemoglobins indicates that the erythroid cell growth in the cultures after six to eight days in vitro represents the progeny of erythroid progenitor cells and not the terminal maturation of circulating primitive erythroblasts.

Cultures of disaggregated embryonic cells. Day 8½ embryonic tissues and extraembryonic membranes were digested with collagenase in the presence of 20% fetal calf serum, and single-cell suspensions were obtained. These
single cells could form erythroid colonies in vitro under the appropriate culture conditions (Table 1). Erythroid colonies were not observed in cultures to which erythropoietin and adult spleen cell conditioned-medium were not added. Erythropoietin alone stimulated the growth of many small erythroid colonies when examined after six days in culture. Adult spleen cell conditioned-medium alone produced a few small as well as large erythroid colonies. However, in cultures with both erythropoietin and adult spleen cell conditioned medium added, there were a significant number of large erythroid colonies in addition to numerous small erythroid colonies (Table 1).

Twelve small erythroid colonies were picked from cultures in which only erythropoietin was added. These were examined by the immunocytochemical technique, and all were found to consist of erythroblasts containing both embryonic and adult hemoglobinins (Table 2). On the other hand, in cultures with both erythropoietin and adult spleen cell conditioned-medium added, seven erythroid colonies, four small and three large or mixed colonies, were found to be made up

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Table 1. Erythroid Colonies in Cultures of Disaggregated Embryonic Single Cells on Day 8½ of Gestation

<table>
<thead>
<tr>
<th>Erythropoietin  (2-3 U/mL)</th>
<th>Adult Spleen Cell Conditioned-Medium (125%)</th>
<th>Small Erythroid, &lt;300 Hemoglobinized Erythroblasts</th>
<th>Large Erythroid, More Than 300 Hemoglobinized Erythroblasts</th>
<th>Mixed Erythroid, More Than 200 Cells Containing Hemoglobinized Erythroblasts and Other Cell Types Including Granulocytes and Macrophages</th>
<th>Nonerythroid, More Than 100 Cells Without Detectable Hemoglobinized Erythroblasts</th>
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Balb/c embryonic tissues and extraembryonic membranes on day 8½ of gestation were digested with collagenase in the presence of 20% fetal calf serum to obtain disaggregated embryonic single cells. The average egg cylindrical lengths of these embryos vary between 1.0 and 1.4 mm. A quantity of 2 to 4 x 10⁶ cells/mL were plated in methylcellulose and cultured for six days. Colonies were enumerated under an inverted microscope without staining. Data are expressed as mean numbers of colonies per 5 x 10⁶ nucleated cells plated per experiment. For each experiment, two to four methylcellulose cultures were done.
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Table 2. Immunocytotoxicological Studies of Individual Erythroid Colonies Picked From Cultures of Disaggregated Embryonic Single Cells on Day 8 of Gestation

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Erythropoietin (2-3 U/mL)</th>
<th>Adult Spleen Cell-Conditioned Medium (1.25%)</th>
<th>Erythroid Colonies, No.</th>
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Disaggregated single cells of Balb/c embryonic tissues and extraembryonic membranes on day 8 of gestation were cultured in methylcellulose for six days. The average egg cylindrical lengths of the embryos used in experiments A and B were 0.9 and 1.2 mm, respectively. Individual erythroid colonies were carefully picked with a micropipette under an inverted microscope, deposited on slides, air dried, fixed, and double stained with monospecific anti-HbA antibodies conjugated with fluorescein and anti-HbE antibodies conjugated with rhodamine. Abbreviations: EA, erythroid colonies made up of erythroblasts containing both embryonic and adult hemoglobin as detected by the immunocytotoxicological technique; A and EA, erythroid colonies made up by many erythroblasts containing only demonstrable HbA and some other erythroblasts containing both embryonic and adult hemoglobins; A, erythroid colonies made up of erythroblasts containing only demonstrable HbA. See text for details.

of cells containing both embryonic and adult hemoglobins (Table 2 and Figs 4A and 4B). Seven other erythroid colonies, one small and six large or mixed colonies, were made up of cells containing only detectable adult hemoglobins (Table 2 and Figs 4C and 4D). There were two other large or mixed erythroid colonies that were made up of erythroblasts containing both embryonic and adult hemoglobins as well as erythroblasts containing adult hemoglobins only (Table 2).

These data are suggestive that on day 8½ of gestation there is a population of erythropoietic progenitor cells that give rise usually to small erythroid colonies after culture in vitro for six days with erythropoietin. The erythroblasts derived from these progenitor cells synthesize primarily embryonic hemoglobins, but produce some adult hemoglobins as well, similar to the yolk sac-derived primitive erythroblasts in vivo. In addition, there is another population of erythropoietic progenitor cells that needs to be stimulated by both adult spleen cell-conditioned medium and erythropoietin to form usually large erythroid colonies after culture for six days in vitro. These erythroblasts produce only adult hemoglobins, similar to the fetal liver-derived erythroblasts in vivo.

DISCUSSION

It has been proposed that there are two cell lineages in mammalian embryonic erythropoiesis committed to different hemoglobin synthetic programs. The results of the present study support such a concept. Moreover, these studies show that the erythropoietic progenitor cells, capable of giving rise to progeny synthesizing primarily embryonic hemoglobins as well as progenitor cells committed to adult hemoglobin production, are detected on day 8 of gestation, prior to the appearance of the fetal hepatic primordium. A model of normal murine embryonic erythropoiesis is proposed and illustrated in Fig 5.

Fig 4. Immunocytologic studies of erythroblasts harvested from individual colonies in cultures of Balb/c disaggregated single-cell suspension of day-8 embryonic cells. Both erythropoietin and adult spleen cell-conditioned medium were added to these cultures. Original magnification x 380; current magnification x 304. (A and B) Small erythroid colony estimated to consist of less than 300 erythroblasts. (A) Immunofluorescence of rhodamine-conjugated anti-HbE. (B) Immunofluorescence of fluorescein-conjugated anti-HbA. Note that all the cells contain both HbE and HbA. (C and D) Large erythroid burst colony. (C) Immunofluorescence of rhodamine-conjugated anti-HbE. (D) Immunofluorescence of fluorescein-conjugated anti-HbA. Note that all the cells contain only detectable HbA.
The first population of erythropoietic progenitor cells is found early on day 8 of gestation. More recently, we were able to detect similar erythropoietic progenitor cells by culturing disaggregated embryonic cells of day 7 of gestation. These progenitor cells usually form small erythroid colonies after culture for six days in vitro with erythropoietin alone. Adult spleen cell–conditioned medium does not have a demonstrable effect upon them. The erythroblasts in the erythroid colonies derived from these progenitor cells obtained from early day 8 embryos produced embryonic hemoglobins demonstrable by isoelectric focusing (Fig 1). It is conceivable that a very minute amount of adult hemoglobins are also present in these erythroblasts but not detectable by the comparatively less-sensitive isoelectric-focusing technique. By mid to late day 8 of gestation, these progenitor cells give rise to erythroid colonies that can be shown clearly to synthesize mostly embryonic hemoglobins but some adult hemoglobins as well, as shown by the isoelectric-focusing technique (Figs 1 and 2) and immunocytochemistry (Figs 3A, 3B, 4A, and 4B). By day 9, these erythroid progenitor cells are not found either in the yolk sac or in the embryonic circulation.6,8

Later, on day 8 of gestation, another population of erythropoietic progenitor cells appears whose progeny are committed to adult hemoglobin synthesis (Fig 4C and 4D). These progenitor cells are responsive to stimulation by the adult spleen cell–conditioned medium and erythropoietin (Figs 1 and 2). It is likely that the factor(s) present in the adult spleen cell–conditioned medium stimulates cellular proliferation and differentiation of erythropoietic progenitor cells already committed to adult hemoglobin synthesis. This hypothesis is supported by the following observations. First, the adult spleen cell–conditioned medium does not alter the types of hemoglobins produced in cultures of early day 8 embryonic tissues (Fig 1). Second, in the cultures of late day 8 embryonic tissues with both erythropoietin and adult spleen cell–conditioned medium added, there were in addition to the small erythroid colonies large erythroid bursts that were rarely seen in the cultures with only erythropoietin added (Table 1). Last, most of these large erythroid colonies that were found in cultures to which both adult spleen cell–conditioned medium and erythropoietin were added consisted of erythroblasts containing only adult hemoglobins demonstrable by immunocytochemistry (Fig 4C and 4D). Thus, the emergence of progenitor cells committed to adult hemoglobin synthesis is accompanied also by the appearance of receptors for the putative mitogenic factor(s) present in the adult spleen cell–conditioned medium. More definitive studies are required to rule out the other possibility that the adult spleen cell–conditioned medium has a direct effect upon the hemoglobin synthetic program of murine embryonic erythropoietic progenitor cells.

Many of the biologic activities ascribed to the adult spleen cell–conditioned medium have been found to be due to interleukin 3 (IL-3) present in the conditioned medium.14,15 It should be informative to determine whether purified IL-3 preparation can replace the adult spleen cell–conditioned medium in stimulating the proliferation and differentiation of murine embryonic erythropoietic progenitor cells committed to adult hemoglobin synthesis. We have previously shown that embryonic fluid obtained from the exocoelom of day 10 embryos could induce the erythroid colony growths in vitro from circulating embryonic hematopoietic progenitor cells, and these erythroblasts in vitro synthesize only adult hemoglobins.9 A recent report suggests that mouse yolk sacs can produce factors capable of inducing large erythroid colonies in vitro.10 The nature and the mechanism of action for these activities remain to be clearly defined.

In cultures of day 8 disaggregated embryonic cells with both erythropoietin and adult spleen cell–conditioned medium added, 14 out of a total of 16 individual erythroid colonies examined were found to consist of either erythroblasts containing only adult hemoglobins or erythroblasts containing both embryonic and adult hemoglobins, as shown by the immunocytochemical technique (Table 2). The specificity of the antibodies used has been previously reported.10 The sensitivity of the immunocytochemical technique has not been quantitatively determined. However, in a recent publication, the results obtained by the immunocytochemical studies were clearly corroborated by two different experimental approaches.15 In the same cultures, there were two large erythroid colonies, each of which was found to consist of erythroblasts containing only adult hemoglobins and erythroblasts containing both embryonic and adult hemoglobins. It is conceivable that each of these two erythroid colonies was derived from two different erythroid progenitor cells that happened to be in close proximity to each other when first plated. Alternatively, these observations may indicate the presence of some embryonic erythropoietic progenitor cells with bipotentiality with regard to their commitment to the hemoglobin synthetic programs.

Beginning on day 9 of gestation, the erythropoietic progenitor cells committed to adult hemoglobin synthesis are found in the yolk sac and in the embryonic circulation (Fig 1) as has been previously reported.2 From day 11 on, the fetal hepatic erythropoietic progenitor cells as well as the fetal hepatic erythroblasts are committed to adult hemoglobin synthesis also.1–3,10 It is therefore probable that the circulating progenitor cells are responsible for the seeding of the fetal
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Liver on days 10 and 11 of gestation to initiate fetal hepatic erythropoiesis, although direct and definitive experimental evidence is not yet available.

The first population of murine embryonic erythropoietic progenitor cells can be clearly detected early on day 8 of gestation. They respond to the stimulation of erythropoietin in culture and give rise usually to small erythroid colonies. Their erythroblasts produce both embryonic and adult hemoglobins. By day 9 of gestation and thereafter, these progenitor cells can no longer be detected. On the other hand, the second population of progenitor cells was detected beginning later on day 8 of gestation. Their proliferation and differentiation in vitro require stimulation by both erythropoietin and spleen cell-conditioned medium. Usually they give rise to large or mixed erythroid colonies, and their erythroblasts synthesize only adult hemoglobins. The interrelationship of these two populations of embryonic erythropoietic progenitor cells is presently unknown. They may arise independently, or the progenitor cells committed to adult hemoglobin synthesis may evolve directly from the progenitor cells committed primarily to embryonic hemoglobin synthesis but producing some adult hemoglobins as well.

Acknowledgment

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References

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