Hemoglobin Switching in Sheep: A Comparison of the Erythropoietin-Induced Switch to HbC and the Fetal to Adult Hemoglobin Switch

By Jane E. Barker, Joseph E. Pierce, and Arthur W. Nienhuis

Stimulation of sheep erythropoietic progenitor cells by erythropoietin (epo) has been studied with regard to its effect on the pattern of hemoglobin production. An analysis of hemoglobin (Hb) synthesis in BFU-E- and CFU-E-derived colonies from fetuses either homozygous for HbA (AA) (homozygous also for the β^c gene responsible for HbC production) or HbB (BB) (lacking the β^c gene) indicated the following. Colonies derived from precursor cells from 51- and 89-day fetuses exhibited small but detectable increments of HbB synthesis with prolonged incubation in vitro. This response was not dependent on the epo concentration. Erythropoietic precursor cells from a 124-day BB fetus were already committed to HbB synthesis, since HbF production was replaced by HbB on successive days in vitro as erythroid colonies matured; this switch was not affected by varying the epo concentration. In contrast, progenitor cells from a 124-day AA fetus responded to higher doses of epo by forming colonies in which more HbC was made at the expense of both HbF and HbA. Erythropoietic stress did not result in induction of HbF in vivo or in erythroid colonies derived from CFU-E in young adult BB sheep, whereas our prior studies had shown induction of HbC synthesis under analogous conditions in colonies derived from young adult AA sheep. We conclude that the epo-induced HbF (or HbA) to HbC switch and the fetal to adult hemoglobin switch are regulated by different mechanisms.

Many mammals synthesize primarily fetal hemoglobins during gestation, although more adult hemoglobin is produced as gestation progresses and a switch to predominantly adult hemoglobin occurs at birth. Both the mechanism(s) that suppresses the accumulation of adult hemoglobin in the fetus and the potential for re-expression of fetal hemoglobin in the adult merit investigation for the occurrence of fetal hemoglobin synthesis beyond the perinatal period would have obvious advantages for humans with sickle cell anemia or severe β-thalassemia.

Sheep synthesize fetal hemoglobin (HbF = α_2γ_2) almost exclusively from 30 to 125 days of gestation. Then adult hemoglobins HbA (α_2β_2) and/or HbB (α_2β^c), increase steadily until after birth (145 days gestation) when fetal hemoglobin synthesis ceases. The genes for β^A and β^B globin are alleles that may be found at the adult locus; linked to the β^A allele is the gene for β^C globin. This gene is not found in the genome of those animals homozygous for the β^B globin. HbC (α_2β^c) production can be induced in vitro by erythropoietin (epo) in cultures of fetal or adult erythroid stem cells, but its induction in vivo by epo injection or anemia only occurs near parturition and thereafter. The sheep therefore provides an opportunity to examine the cellular basis for fetal and adult hemoglobin synthesis in clonal erythroid cultures and also to characterize the epo-induced expression of the β^c globin gene.

Erythropoietic progenitor cells from mouse and man generate successive populations of erythroid colonies when challenged with epo in vitro. The erythroid precursors giving rise to the earliest appearing colonies have been designated CFU-E and those from which larger, later appearing colonies are derived are called BFU-E. We have recently described the characteristics of these progenitor cells in fetal sheep. In an attempt to characterize any differences in globin gene regulation at the cellular level during the epo-induced switch to HbC synthesis and during the normal augmentation of adult hemoglobin production near birth, we have compared the synthetic profiles of colonies derived from both CFU-E and BFU-E obtained from fetuses either homozygous for the β^A or β^B globin alleles. Finally, we have examined the effect of erythropoietic stress on hemoglobin synthesis in adult sheep lacking the β^C globin gene both in vivo and in clonal erythroid cultures.

Materials and Methods

Cell Cultures

Sheep fetuses of known gestational age homozygous for either HbA (AA) or HbB (BB) were obtained as described. Marrow cells were flushed from the long bones and treated in a similar manner to fetal liver, except that the step involving segregation of hepatocytes by gravity sedimentation was omitted. Cells were cultured and either fixed for histology or exposed to 3H-leucine for 24 hr and the globin synthetic pattern determined by carboxymethylcellulose chromatography in 8 M urea. Bone marrow cells from young adult animals were recovered as previously described. After Ficoll-paque centrifugation to enrich in nucleated cells, the cells were washed twice in minimal essential Eagles medium containing 10 mM Hepes, pH 7.4, and 2% fetal calf serum (MEM) and resuspended at a cell concentration of 10^7 cells/ml. Determination of the pattern of globin synthesis in the cells

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on explantation was as previously described. Aliquots of the cell suspension were cultured at various epo concentrations for up to 8 days in vitro. The epo used was obtained from Connaught Medical Research Labs, Toronto (Step III, 3–5 U/mg protein).

**Erythropoietic Stress in Adult Sheep**

An animal homozygous for the $\beta^a$ globin gene was anesthetized and phlebotomized to decrease the hematocrit and treated as outlined in Fig. 1. A hematocrit of 19% was achieved by removing 200–400 ml of blood and returning the plasma plus 100–150 ml of normal saline to the animal. This procedure was repeated four times with interval monitoring of the hematocrit. At the end of the phlebotomy, 100 U epo/kg was injected (total dose, 1200 U). The final hematocrit was 19% and fluctuated between 19% and 22% during the next 6 days. The reticulocyte count rose from zero initially to 3.6% on the fifth day. A bone marrow sample was obtained before and at various intervals after initiation of the erythropoietic stress using techniques that have already been described in detail. A second animal was studied in a similar fashion except that the hematocrit was reduced only to 25%.

The hemoglobins in selected culture lysates (as indicated in the results) were initially purified by gel filtration prior to ion-exchange chromatography. The sample, after addition of carrier and labeled hemoglobins, was diluted in 50 mM Tris-HCl, pH 7.5, and potassium cyanide, 1 mg/ml, and then passed over a 1.5 × 50 cm column of Sephadex G-75 previously equilibrated in the same buffer at 4°C. The fractions containing hemoglobin were pooled, dialyzed for 18–24 hr against deionized water, and lyophilized. The purified hemoglobin was dissolved in 1.5 ml of water and the globins recovered by acid-acetone extraction prior to ion-exchange chromatography.

**Messenger RNA Analysis**

Total RNA was prepared from bone marrow or fetal liver nucleated cells or the colonies released from plasma clot by trypsinization. Approximately $2 \times 10^6$ cells or the colonies contained in 40 ml of plasma clot culture were dissolved in 4 ml of 6 M guanidine-HCl–0.2 M potassium acetate, pH 5.0, as previously described. The total RNA was purified by cesium chloride buoyant density centrifugation. Separate aliquots of the total cellular RNA were assayed for their content of $\gamma$ or $\beta^a$ cDNA prepared from the recombinant plasmids pS$\gamma$56 or pS$\beta^a$2 exactly as previously described. These plasmids that contain the sheep globin genes derived from complimentary DNA have been approved for use at the P2-EKI level of containment under the NIH guidelines for Recombinant DNA Research as specified in a Memorandum of Understanding and Agreement approved by the NIH Biohazards Committee.

**RESULTS**

The Fetal to Adult Hemoglobin Switch in Clonal Erythroid Cultures

The potential for switching from fetal to adult hemoglobin synthesis in culture was studied in colonies derived from erythroid progenitor cells obtained from an 89-day BB fetus. Analyzed were the pattern of hemoglobin synthesis in erythroid colonies derived from bone marrow (Fig. 2 A and B) or fetal liver (Fig. 2 C, D, and E). In both bone marrow and fetal liver erythroblasts, the $\beta^a$ globin synthesis was less than 1% as measured by immediate incubation of the explanted cells in $^3$H-leucine (Fig. 2 A and C, respectively). At 1 U epo/ml, an estimated 12% of the total non-$\alpha$ globin synthesis in the colonies of bone marrow cultures and 4% in the colonies of fetal liver cultures, was $\beta^a$ between 4 and 5 days in vitro. Burst colonies arising in the fetal liver cultures synthesized an estimated 8% $\beta^a$ globin between 6 and 7 days in vitro. No effect of varying the epo concentration was observed in this experiment; compared were 0.1 and 1.0 U/ml. Analogous results were obtained in the cultures of fetal liver cells from a 51-day BB fetus (data not shown).

To examine the behavior of erythroid colonies derived from a fetus at a point in gestation just prior to the fetal to adult switch, bone marrow from an animal...
at 124 days of gestation was cultured in vitro. Between 2 and 4 days, only a small amount of $\beta^A$ globin synthesis was detected, but between 4 and 5 days, 30% of the total non-$\alpha$ globin synthesis was $\beta^A$ (Fig. 3). The effect of varying the epo concentration on $\beta^A$ globin synthesis is shown in Table 1. The fraction of $\beta^A$ globin synthesis was similar at the two concentrations tested during both labeling periods.

### Table 1. $^3$H-Thymidine Suicide of CFU-E

<table>
<thead>
<tr>
<th>Amount of Thymidine* ($\mu$Ci/ml)</th>
<th>Number of Colonies (± SEM)*/(per 0.1 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>522 ± 1.0</td>
</tr>
<tr>
<td>100</td>
<td>106 ± 2.4</td>
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<tr>
<td>200</td>
<td>111 ± 1.4</td>
</tr>
<tr>
<td>500</td>
<td>108 ± 11</td>
</tr>
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</table>

*Sixteen nanomoles of nonradioactive thymidine was added to the control culture; 100 $\mu$Ci of $^3$H-thymidine is equivalent to 3.5 n mole. Incubation in the presence of thymidine was for 30 min.

†Colonies in six 0.1-ml microwells were counted after 5 days in vitro. The epo concentration was 1.25 U/ml. The cells were plated at 10^5/ml.

### Induction of HbC Synthesis in Erythroid Colonies From the Perinatal Period

An experiment quite analogous to the last one described above except that the fetus was homozygous for the $\beta^A$ allele was performed in order to examine the induction of HbC synthesis. The bone marrow cultures were derived from an animal at 124 days gestation just prior to the onset of the HbF to HbA switch in vivo. The following observations were made (Fig. 4): (1) $\gamma$ synthesis declined and $\beta^A$ synthesis increased in erythroid colonies during the 6 days of in vitro observations; (2) $\beta^C$ synthesis was induced particularly at the higher epo concentration (1.0 U compared to 0.01 U/ml) at the expense of both $\gamma$ and $\beta^A$ synthesis; (3) $\beta^C$ synthesis was higher at 6 days than 4 days; and (4) there was no difference in the switching pattern observed in bone marrow versus fetal liver cultures.

### Attempts to Induce an HbB to HbF Switch

Bone marrow from a young animal (homozygous for HbB) was cultured under standard in vitro conditions at 1.0, 2.5, 5.0, 7.5, and 10 U epo/ml; globins were labeled between 4 and 5 days and analyzed by ion-exchange chromatography after preliminary purification of the hemoglobin by gel filtration. No evidence of $\gamma$ globin synthesis was detected. Cultures containing 5 U epo/ml were labeled for 5 successive 24-hr intervals up to 5 days, but again, no $\gamma$ globin synthesis was detected. No colonies formed in sheep bone marrow cultures after 5 days; specifically, no burst colonies were observed.

To examine further the potential for switching from adult to fetal hemoglobin synthesis in the sheep both in vivo and in vitro, we designed the experiment outlined in Fig. 1. During the course of this study, we had the opportunity to look for an HbB to HbF switch in colonies derived from an unstressed animal. Also, the pattern of hemoglobin synthesis was examined in colonies arising in cultured bone marrow obtained at various intervals up to 6 days after severe erythropoietic stress was imposed by bleeding the animal to a
hematocrit of 19% and injection of epo at a dose of 100 U/kg. Colonies were formed at several epo concentrations in vitro (Table 2). Illustrated in Fig. 5 is the globin synthetic pattern in colonies derived from stem cells obtained at 2 and 6 days after imposition of erythropoietic stress and grown in vitro at 0.5 or 2.5 U epo/ml, respectively. The labeling period was from 4 to 5 days. Less than 1% γ synthesis was observed during this interval (Fig. 5). Similarly, extraction of total RNA from the plasma clot cultures followed by

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Age of Fetus</th>
<th>3H-TdR</th>
<th>Colonies</th>
<th>Burst†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>% Kill</td>
<td>% HBc</td>
</tr>
<tr>
<td>1</td>
<td>60 days</td>
<td>−</td>
<td>256.0 ± 0.4</td>
<td>38.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>186.8 ± 0.3</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>65 days</td>
<td>−</td>
<td>64.8 ± 1.4</td>
<td>68.1</td>
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<td></td>
<td>+</td>
<td>20.7 ± 1.6</td>
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</tr>
<tr>
<td>3</td>
<td>60 days</td>
<td>−</td>
<td>117.0 ± 2.6</td>
<td>59.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>48.8 ± 0.2</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>60 days</td>
<td>−</td>
<td>390.2 ± 14.7</td>
<td>47.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>206.8 ± 11.2</td>
<td>54.4 ± 0.4</td>
</tr>
</tbody>
</table>

|            | −            | +      | % Kill | % HBc | % Kill | % HBc |
| 6 65 days  |              | 64.8 ± 1.4 | 68.1 | 50   | 7.8 ± 0.3 | 61.5 | − |
| 6 60 days  |              | 117.0 ± 2.6 | 59.3 | 8    | 6.2 ± 0.4 | 54.1 | 19 |
| 6 60 days  |              | 390.2 ± 14.7 | 47.1 | 11.3 ± 0.2 | − | 20 |
| 6 60 days  |              | 206.8 ± 11.2 | 54.4 ± 0.4 | 5.4 ± 0.4 | 52.2 | 19 |

Table 2. Effect of 3H-Thymidine Suicide of CFU-E and BFU-E on HbC Synthesis

*Colonies were scored at 5 days, and the labeling period was between 4 and 5 days in vitro. The epo concentration was 1–2.5 U/ml, and 10^6 cells/ml were plated.
†Bursts were scored at 8 days, and the labeling period was between 7 and 8 days in vitro. The epo concentration was 1–2.5 U/ml, and 10^6 cells/ml were plated.
‡Colonies in 4–6 0.1-ml wells were counted (± SEM).
hybridization analysis against pure $\gamma$ and $\beta^b$ complementary DNA derived from recombinant plasmids, failed to detect any $\gamma$ mRNA (Fig. 6). A second experiment of similar design also failed to provide any evidence for an HbB to Hbf switch either in vivo or in vitro. The data from these two experiments are summarized in Table 2.

**DISCUSSION**

The studies described here as well as previously published data allow us to compare the pattern of fetal and adult hemoglobin production to the induction of HbC synthesis in clonal erythroid cultures from the fetal, perinatal, and adult developmental periods of the sheep. In cultures derived from erythropoietic precursor cells of fetuses homozygous for the $\beta^b$ globin allele (and therefore lacking the $\beta^c$ globin gene), the predominant hemoglobin produced was Hbf. As the erythroid colonies matured, there was a slight tendency toward enhanced adult hemoglobin synthesis, which never exceeded 12%. Our data in this regard are similar to the observations of Rowley et al. and Stamatoyannopoulos et al. in cultures of human fetal liver. Darbre et al. have also observed a similar small enhancement of HbB synthesis in cultures of sheep fetal erythroid cells obtained late in gestation but did not observe any HbB synthesis in cultures derived from cells of fetuses of less than 100 days gestation. In contrast to this limited and epo-independent ability to produce adult hemoglobin synthesis in cultures from fetuses homozygous for the $\beta^b$ allele, induction of HbC synthesis may be readily accomplished in clonal erythroid cultures derived from fetuses homozygous for the $\beta^a$ allele. HbC synthesis increases with increasing epo concentration between 4 and 5 days in vitro but is maximal during the maturation of burst colonies between 7 and 8 days in vitro.

The pattern of hemoglobin synthesis in clonal erythroid cultures from the perinatal period provides an interesting comparison between animals homozygous for the $\beta^a$ or $\beta^b$ allele. An augmentation of adult hemoglobin synthesis occurs in fetuses in vivo beginning at 125 days of gestation. Erythrocyte progenitor cells from an animal of 124 days of age and homozygous for the $\beta^a$ allele, produced a small amount of $\beta^b$ globin between 2 and 4 days in vitro but a much larger quantity between 4 and 5 days; this increase was not dependent on the epo concentration (Table 1). These data suggest that the erythrocyte precursor cells whose colonies matured between 4 and 5 days in vitro were already committed to form erythroblasts in which substantial amounts of adult hemoglobin synthesis occurred. The immediately preceding cohort of progenitor cells, namely those whose colonies matured between 2 and 4 days in vitro, were committed to form erythroblasts making only a small amount of adult hemoglobin. Hoffman et al. have observed a similar augmentation of adult hemoglobin production in human erythroid bursts derived from umbilical cord blood; the colonies made 34% HbA compared to 27% HbA synthesized by reticulocytes at the time of explantation. Similar data were interpreted by Stamatoyannopoulos and coworkers to indicate that human erythroid stem cells from umbilical cord blood are precommitted in vivo to form erythroblasts making substantial amounts of HbA when their progeny colonies are observed in vitro.

In the experiments described here, we directly compared the induction of HbC synthesis in the clonal erythroid cultures from an animal at the point of switching from fetal to adult hemoglobin synthesis. In contrast to the data obtained with respect to HbB, induction of HbC synthesis was observed at the expense of Hbf and HbA production in erythroid colonies formed from stem cells during this critical switching period. An augmentation of HbC production in vitro was also obtained by increasing the epo concentration. Thus, a fundamental difference between the Hbf (or HbA) to HbC switch and the HbF to HbB switch is revealed by our analysis. The
former is epo-dependent and readily inducible in clonal erythroid cultures, while the latter cannot be currently manipulated in vitro.

Further support for this conclusion is found in our attempts to induce HbF synthesis in cultures of bone marrow from young adult animals. Previously published data have indicated that HbC synthesis can be readily induced in those animals in this developmental period who are homozygous for the β^A allele. Reports of substantial HbF production in cultures of adult human bone marrow and small amounts in cultures of lamb bone marrow, in addition to the observations that erythropoietic stress induces HbF synthesis in vivo in the baboon, prompted us to reexamine the role of erythropoietic stress as a potential inducer of HbF synthesis in the sheep. Despite acute but fairly prolonged and severe erythropoietic stress imposed by both anemia and epo injection, we were unable to find any evidence for significant γ globin synthesis or any γ mRNA production either in vivo or in erythroid colonies in vitro. These results differ from those reported by Darbre et al. for they observed up to 12% γ chain synthesis in cultures of sheep adult bone marrow cells. This discrepancy is unlikely to be due to our failure to accurately assess the potential for γ globin production, since our studies include biosynthetic measurements and mRNA analysis. However, the data of Darbre et al. are also convincing for they demonstrated labeled globin tryptic digest peptides from the γ globin peak isolated by ion-exchange chromatography. Perhaps under certain conditions a population of precursor cells giving rise to colonies making a small amount of HbF can be obtained from sheep bone marrow comparable to the reproducible observation of this phenomenon in cultures of human bone marrow and peripheral blood. Our failure to generate burst colonies in adult sheep bone marrow cultures may be one factor that minimizes the production of γ globin, since in human cultures, the highest levels of HbF synthesis are associated with colonies formed from the earliest progenitor cells. BFU-E-derived colonies are formed in cultures of sheep fetal liver possibly due to the presence of cells producing a burst-enhancing activity in this tissue. By using spleen cell conditioned media, we are attempting to obtain burst colonies in adult sheep bone marrow.

The erythroid colony culture system as currently constituted provides an opportunity to reproducibly induce and examine the mechanism of HbC synthesis in sheep in which the β^C gene is present. Our data indicate that a cycling precursor cell at or just before the CFU-E level of differentiation may be committed by exposure to a high concentration of epo to form erythroblasts making HbC. However, the earliest progenitor cells detectable in fetal liver or adult bone marrow are already committed in vivo to form erythroblasts making predominantly fetal or adult hemoglobin, respectively, even when these progenitors form erythroid colonies under identical conditions in vitro. Therefore, more refined methodologies are necessary to examine the fetal to adult (or adult to fetal) hemoglobin switches. Fortunately, two recent developments may offer a new experimental approach. Discovery of the role of burst-enhancing activity in erythroid colony formation and the attempts of Iscove and others to formulate defined culture media may permit more reproducible manipulation of stem cells during their development in vitro. Furthermore, use of long-term culture systems in which the most primitive erythroid precursor cells regenerate provides some hope that more purified erythroid progenitor cell populations may be obtained. Growth of these cells under totally defined conditions with experimental manipulation may provide direct insights into the fetal to adult switching mechanism.

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