Erythropoietic Stress, Macrocytosis, and Hemoglobin Switching in HbAA Sheep

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Hemoglobin switching and macrocytosis were studied in homozygous hemoglobin AA sheep. An abrupt initiation of erythropoietic stress, accompanied by a pulsed elevation of circulating erythropoietin levels, was induced by phlebotomy. Sequential blood samples were separated according to density on Stractan gradients to isolate cells newly entering the circulation from the marrow each day. Analysis of hemoglobin phenotype and cell volume distribution in these young reticulocytes revealed a distinct temporal separation in the appearance of hemoglobin C and increased cell volume. The appearance of macrocytes within 24 hr of erythropoietin elevation suggests that macrocytosis could be the result of the action of erythropoietin during the late stages of erythroid maturation. The 72-hr delay in the appearance of hemoglobin C indicates that commitment to a particular hemoglobin phenotype occurs at an early stage of differentiation and involves immature erythroid stem cells. The results of this study show that these consequences of erythropoietic stress are initiated at two different developmental stages, resulting in the production of macrocytosis and hemoglobin switching.

The cellular mechanism that regulates hemoglobin switching is of interest because of its potential importance in the amelioration of the effects of certain hemoglobinopathies. Recent success in the in vitro culture of erythroid cells has allowed the definition of some aspects of this regulation. The in vitro studies have helped to identify a maturational continuum of erythroid stem cells and have provided evidence that early erythroid progenitors (BFU-E) require high levels of erythropoietin (Epo) to differentiate and are capable of synthesizing fetal hemoglobin. On the other hand, late erythroid progenitors (CFU-E) require less Epo to differentiate and can only synthesize adult hemoglobin.

Early studies suggested a causal relationship between elevated erythropoietin levels and differentiation of BFU-E accompanied by synthesis of fetal hemoglobin. However, later studies raised the possibility that other factors, such as burst-enhancing factor, may also be involved in these processes.

It has not yet been possible to extrapolate these in vitro findings to a concept of in vivo differentiation of erythroid stem cells, and to determine whether an elevated Epo level is the sole physiologic regulator of hemoglobin switching. In contrast, the production of macrocytic red cells is clearly related to elevated Epo levels in vivo. This occurs in many species of animals and in humans, and is a consistently reliable indicator of ongoing erythropoietic stress.

The homozygote AA sheep (HbAA) has provided a reliable experimental system for the study of one hemoglobin switching process (HbA to HbC). Increased Epo levels have been convincingly shown to induce this switching in vivo as well as in vitro. We have thus chosen this experimental system to define the time course of hemoglobin switching and macrocyte production following abrupt erythropoietic stimulation. These observations were used to define the stage of erythroid differentiation at which commitment to hemoglobin phenotype and macrocytosis occurs in vivo. The use of a sensitive radioimmunoasay with highly purified Epo enabled us to accurately measure circulating Epo sequentially, a task previously difficult to accomplish. Our results show that in response to a pulsed elevation of Epo levels, macrocytic reticulocytes appeared in the circulation within 24 hr. In contrast, hemoglobin-C-containing reticulocytes appeared much later—72 hr after Epo levels were elevated. It is known that an erythroid stem cell in sheep drawn into hemoglobin synthesis requires 72 hr to complete its maturation. Therefore we conclude that once a cell has begun to synthesize hemoglobin, it cannot alter the type of hemoglobin produced. On the other hand, the appearance of macrocytes within 24 hr of elevation of Epo levels indicates that the erythroid cells retain additional sensitivity to Epo through a later maturational stage. Thus, macrocytosis is the result of Epo action on already differentiated erythroblasts, while hemoglobin switching is the result of the action of Epo and/or other factors on less differentiated erythroid stem cells.

Materials and Methods

The data presented were derived from two experiments on each of two homozygous AA sheep obtained from the NIH breeding colony.

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To produce a pulsed elevation of erythropoietin, 1800 ml of blood were withdrawn during a 12-hr period. Fifteen-milliliter blood samples were obtained on each subsequent day for 12 days and on every third day from days 13 to 22. After measurement of hematocrit by centrifugation and preparation of new methylene blue stained smears for reticulocyte counts, plasma was removed for subsequent measurement of Epo. Plasma Epo was determined by radioimmunoassay, using highly purified Epo (specific activity of 70,000 units/mg protein) as a label.

To isolate the very young reticulocytes entering the circulation each day, the washed red cells were layered onto discontinuous gradients of Stractan. Four layers with densities of 1.065, 1.074, 1.083, and 1.092 were used. The gradients were centrifuged on a Beckman SW 27.1 rotor at 15°C for 30 min at 20,000 rpm. After centrifugation, four populations of cells were removed with a Pasteur pipet and were washed three times in buffered saline containing potassium and glucose (BSKG*). Samples from each subpopulation were stained with new methylene blue for reticulocyte counts. Samples were taken for cell counts in each fraction and were fixed in 0.25% glutaraldehyde in Isoton cell counting medium (Coulter Electronics, Hialeah, Fla.) for measurement of cell volume distribution. Cell volume distributions were determined using a Coulter type of electronic sizing instrument equipped with a 48-μm diameter aperture. The pulse-height analysis, data acquisition, and data analysis were performed by an on-line computer (PDP 8/I, Digital Equipment Corporation).

Hemolysates were prepared from samples of each red cell subpopulation and from samples of whole blood and were analyzed by electrophoresis on cellulose acetate in 130 mM Tris-borate buffer pH 9.1. The proportion of HbC was determined by scanning Ponceau-S-stained electrophoretograms using a Joyce Loebl densitometer.

**RESULTS**

The schedule of acute bleeding resulted in an abrupt drop in the hematocrit followed by a gradual recovery during the next 3 wk as shown in Fig. 1. The hematocrit rose approximately one percentage unit each day. This sudden drop in hematocrit produced a sharp, transient increase in circulating Epo in all the experiments. A representative example is shown in Fig. 1. Epo levels reached the maximal point of increase within 24 hr and subsided rapidly after the third day following bleeding. The accompanying production of reticulocytes was modest, as is usual for sheep. The percentage of reticulocytes in the whole blood was initially low, we obtained a dramatic increase of these youngest cells, as evidenced by intense staining with new methylene blue (Fig. 2). The percentages of reticulocytes contained in the top fractions throughout the experiment are shown graphically in Fig. 3.

A modest increase in the mean cell volume of the least dense population, compared to that of normal mature cells before bleeding, was initially evident. This increase became substantial by the second day after bleeding, and macrocytes continued to appear in this population through the 11th day. The variation in mean cell volume of this newly circulating cell population over time is also shown in Fig. 3. The entire cell volume distribution for that same population on selected days are diagrammed in Fig. 4. It can be seen that the volume distribution for the whole population shifted to higher values during erythropoietic stress and reverted toward normal as the stress was relieved. On days 3 and 4 after bleeding, the mean cell volume of the newly entering reticulocyte population was more than twice the normal volume of the least dense cells before bleeding.

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*BSKG: 7.808 g NaCl, 0.373 g KCl, 2.302 g NaH2PO4·7H2O, 0.194 g NaH2PO4·H2O, 2.0 g glucose, made up to 1 liter with distilled water. The pH and osmolality were adjusted to 7.4 and 290–295 mosmole/kg, respectively, if necessary.
In all four experiments, hemoglobin C was first detected in the top fraction on day 4, 2 days after the appearance of macrocytosis. It reached a peak of up to 50% of the total hemoglobin on day 6 and rapidly fell to undetectable levels by the 11th day (Fig. 3). In Fig. 5 we have combined the response of mean cell volume and HbC of the newly circulating cells after elevation of Epo levels. This shows the distinct separation between the 2 responses, with an increase in mean cell volume occurring at 24 hr and HbC first appearing at 72 hr after elevation of Epo levels.

**DISCUSSION**

By removing a large quantity of blood within a short period of time, we produced a pulsed increase of Epo. This well defined physiologic response was used as a temporal reference point for sequential studies of responses in hemoglobin synthesis and cell volume. All previous studies of hemoglobin switching in sheep have been performed using repeated phlebotomy over a prolonged period of time or repeated injections of phenylhydrazine. Responses occurred over a long period of time, and some sequential events were obscured by the lack of a well defined initiation of erythropoietic stress. Moreover, circulating Epo levels were not monitored during these studies, and thus, the erythropoietic stimulus was not well defined. The use...
of Stractan gradients provided us with a highly enriched population of cells newly entering the circulation from the bone marrow each day. The isolation of these cells from the much larger numbers of random aged cells thereby allowed us to observe the precise sequence of macrocyte production and HbC synthesis. In most of the earlier studies, measurements were performed on whole blood, precluding this type of observation.

Our results show a clear difference in time between the first appearance of macrocytosis and the new hemoglobin phenotype. These findings can be interpreted in the context of a model for erythroid differentiation proposed initially by Schofield and Lajtha (Fig. 6). This model considers differentiation to be a two-stage process. The maturation of morphologically recognizable pronormoblasts into reticulocytes takes place during the second stage, which in sheep, occupies the 60–72-hr period before the release of reticulocytes from the marrow into circulation. Thus, the appearance of macroticreticulocytes within 24 hr of Epo level elevation suggests that sensitivity to Epo for macrocyte production is present during this second stage of erythroid differentiation (pronormoblasts to reticulocytes), and that macrocytic reticulocytes are produced as a result of the action of increased Epo on erythroblasts at all stages of maturation. On the other hand, the considerably longer delay of 72 hr before the appearance of HbC-containing reticulocytes is consistent with previous conclusions that Hb switching requires the presence of stimulation on early erythroid precursors in the first stage of differentiation.

A more detailed analysis of the events during the first stage of differentiation is included in a model proposed by Papayannopoulou et al. based on data from human erythroid cultures. They proposed that erythropoietic stimulation draws erythroid stem cells at three levels of differentiation into maturation accompanied by hemoglobin synthesis. In this model, the cells drawn from the youngest population of stem cells synthesize fetal hemoglobin. Cells from the intermediate level synthesize both fetal and adult hemoglobin, while the cells that complete the normal course of differentiation synthesize only adult hemoglobin. Our observations of HbAA sheep can be interpreted with reference to a similar model of erythroid differentiation (Fig. 6). We suggest that in HbAA sheep, erythroid stem cells can be divided into three classes, depending on the range of capacity for commitment to the synthesis of a particular type of hemoglobin. The most immature stem cells can be stimulated to initiate synthesis of fetal hemoglobin, cells of intermediate maturity to synthesis of HbC, and the most mature cells to synthesis of HbA only.

Our studies suggest that erythropoietic stress in adult sheep is only capable of recruiting the intermediate erythroid stem cells to maturation and hemoglobin C synthesis. Other in vivo studies have also failed to demonstrate synthesis of fetal hemoglobin in response to severe anemic stress. Even transplantation of fetal hemoglobin-producing hematopoietic cells into adult sheep was followed by switching to adult hemoglobin synthesis. However, recent in vitro culture studies have demonstrated limited fetal hemoglobin synthesis by adult sheep cells. Thus, the in vivo regulatory factors that specify the commitment of the most immature erythroid stem cells to synthesis of fetal hemoglobin in sheep have not yet been identified, and we have indicated this uncertainty in the model illustrated in Fig. 6.

Increased erythropoietic stress by itself cannot induce fetal hemoglobin synthesis in vivo in humans either, as evidenced by the absence of substantial fetal hemoglobin synthesis in patients with certain chronic anemias or those recovering from severe transient...
anemias. It appears that other humoral factors or cellular interactions, as yet unidentified, may be involved in regulating fetal hemoglobin switching. The observations that fetal hemoglobin synthesis is frequently activated in the regenerating marrow of patients after bone marrow transplantation, in regenerating marrow of leukemic patients following chemotherapy, and in the regenerating marrow of children with acquired aplastic anemia and congenital hypoplastic anemia strongly suggest that cellular or humoral factors associated with regenerating hypocellular marrow, with little or no erythroid elements present, may play a decisive role in the recruitment of immature stem cells to synthesize hemoglobin. Once recruited, these cells synthesize fetal hemoglobin. Sequential studies, modeled after the one described here, on such groups of patients, may provide important information on the regulatory factors of fetal hemoglobin synthesis.

The present study has shown that erythropoietic stress exerts its influence on erythroid differentiation at two different developmental stages that lead to the production of macrocytes and hemoglobin switching. The results have also demonstrated that cell separation techniques can be used to explore sequential changes in the properties of circulating red cells produced as a result of abrupt variations in the microenvironment of differentiating erythroid stem cells.

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