Acceleration of the Hemoglobin Switch in Cultures of Neonate Erythroid Precursors by Adult Cells

By W. Vainchenker, U. Testa, A. Dubart, V. Beuzard, J. Breton-Gorius, and J. Rosa

Erythroid colonies from cord blood in which both late (CFU-E) and early (BFU-E) erythroid precursors are present, were grown by the plasma clot technique. Hemoglobin (Hb) synthesis was studied and compared in fresh reticulocytes, 7-day-old colonies, and 14-day-old colonies. In the 8 cases studied, the proportion of HbA synthesis progressively increased from circulating reticulocytes to 7-day-old colonies and finally in 14-day-old colonies. This result brings evidence that Hb switch is programmed at least at the level of early erythroid precursors. In order to modify the cellular environment of the culture and to examine their influence on globin genes expression, neonate and adult irradiated light density blood cells were added. Irradiated cells from adults, in contrast to those from neonates, were able to increase HbA synthesis in colonies derived from early erythroid progenitors. Under optimal conditions of culture (i.e., a high concentration of neonate plated cells), adult cells elicited a constant increase (22%) in the proportion of HbA synthesis. A linear relationship between this increase of HbA synthesis and the number of added cells was observed. In contrast, the plating efficiency was not significantly modified; however, the size of the erythroid bursts was increased upon the addition of adult irradiated cells. Under suboptimal conditions of culture (i.e., a low concentration of plated cells), adult irradiated cells markedly increased the plating efficiency, the size of the colonies, and HbA synthesis (85%). In contrast to the dramatic effects of irradiated adult cells, the cord blood irradiated cells had a very slight effect on HbA synthesis, the plating efficiency, and the size of the colonies derived from cord blood precursors. All these results suggest that only adult cells are able to amplify the Hb switch in the newborn at the level of BFU-E. Two possible mechanisms can be hypothesized: the adult irradiated cells may either modify the differentiation of the BFU-E or recruit precursors that have a higher capacity to express HbA in their progeny.

After the 32nd wk of gestation, there is a progressive switch from fetal (F) to adult (A) hemoglobin (Hb) in human. This switch ends a few months after birth. After the Hb switch period, most of the cells contain only HbA, however, a small proportion of adult erythroid precursors (<7%) synthesize HbF in addition to HbA. These developmental changes are not limited to Hb, but also involve other erythroid characteristics, such as the level of carbonic anhydrase, membrane antigens, and isoenzyme patterns. The control mechanisms governing these changes remain poorly understood. Two different models of the Hb switch at the cellular level have been suggested. First, Hb switch may occur at the level of pluripotent stem cells, which during fetal life switch over exclusively to HbA production; accordingly, a minority of stem cells throughout the adult life produce erythroid cells programmed for a small but variable amount of HbF. Second, globin gene expression may be regulated during the process of differentiation of erythroid precursors; in this case, Hb switch is due to a different pattern of erythroid differentiation in fetal and adult life.

The recent demonstrations that the expression of β and γ genes in adults is linked to the early phases of erythroid differentiation, affords some arguments in favor of the latter hypothesis. Furthermore, the cloning in culture of erythroid precursors has shown the heterogeneity of subcolonies derived from a single precursor in their ability to express HbA and HbF. This culture method also permits the investigation of the possible role of regulatory substances or cells involved in the hemoglobin switch.

It has been previously reported by several investigators that fetal and neonatal erythroid precursors are programmed for the expression in vitro of HbF and HbA, which correspond to their ontogenic stage. In the present study, we have investigated the role of irradiated mononuclear cells from adult blood or cord blood on the formation of erythroid colonies derived from cord blood precursors and their capability to produce HbA and HbF. The results show that addition in culture of adult irradiated cells, in contrast to neonatal irradiated cells, is able to modify Hb switch toward the adult condition.

MATERIALS AND METHODS

Cultures

The mononuclear cells from the cord blood of 11 newborns were isolated by centrifugation on a cushion of Ficoll metrizoate (density: 1.077 g/ml; Lymphoprep, Nyegaard, Oslo) at room temperature. The cells were washed 3 times in cold α-medium (Eurobio, Paris, France), and the plasma clot cultures were established in 35-mm Falcon Petri dishes containing 6 x 10⁵ nucleated cells in 1 ml. Slight modifications were used as previously described: α-medium replaced the NCTC 109 medium; human AB serum was used instead of fetal calf serum; and CaCl₂ was added at a final concentration of 2.5 mM. The cultures were cultivated in a humidified incubator at 37°C in a mixture of 5% CO₂ and 95% air.
concentration of 0.026 mg/ml. Beef embryo extract was omitted. An erythropoietin step III preparation (Connaught Research Laboratories, Toronto, Canada), was added at zero time at a concentration of 1.5 U/ml. The cultures were then incubated for 7 or 14 days at 37°C in a fully humidified atmosphere with 3% CO2 in air. Each sample was grown at least in triplicate.

Light Microscopic Staining Procedure

On the 7th or 14th day, the cultures were directly stained in Petri dishes. The erythroblastic bursts were identified by revelation of the pseudoperoxidase activity of hemoglobin. Quantitation of the number of colonies was done under an inverted microscope at a 40x magnification.

Hemoglobin Study

Hb synthesis was evaluated in reticulocytes from samples of cord blood used for culture; it was also studied in 7- and 14-day-old colonies. Reticulocytes were incubated as outlined earlier. At day 6 or 13, 100 μCi of ³H-leucine (50 Ci/mMil, Commissariat à l’Energie Atomique, Saclay, France) was added to 0.3 ml of NCTC 109 medium (leucine-free, Eurobio, Paris, France) to one culture dish that had been incubated to produce erythroid colonies. Incubation was terminated 24 hr later by digestion of the clot with pronase (1 mg/ml, Calbiochem, San Diego, Calif.) for 10 min at room temperature; such treatment allowed the recovery of all the cells from one dish. Red blood cells were added as a carrier when cultures were grown at low concentration of cells in order to increase the size of the pellet. The cells were extensively washed with Hanks’ medium and then with sodium chloride (0.15 M); they were then frozen at -80°C until use.

Table 1. Comparison Between the Plating Efficiency and the Hemoglobin Synthesis at the 14th Day of Culture for Cord Blood BFU-E

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of BFU-E/Dish</th>
<th>With Adult Irradiated Cells</th>
<th>Without Adult Irradiated Cells</th>
<th>Ratio</th>
<th>With Adult Irradiated Cells</th>
<th>Without Adult Irradiated Cells</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>310</td>
<td>260</td>
<td>1.1</td>
<td>1.1</td>
<td>8</td>
<td>0.57</td>
<td>1.48</td>
</tr>
<tr>
<td>2</td>
<td>340</td>
<td>280</td>
<td>1.13</td>
<td>1.33</td>
<td>8</td>
<td>0.55</td>
<td>1.55</td>
</tr>
<tr>
<td>3</td>
<td>448</td>
<td>245</td>
<td>1.05</td>
<td>1.05</td>
<td>6</td>
<td>0.46</td>
<td>1.33</td>
</tr>
<tr>
<td>4</td>
<td>440</td>
<td>310</td>
<td>1.07</td>
<td>1.07</td>
<td>6</td>
<td>0.58</td>
<td>1.13</td>
</tr>
<tr>
<td>5</td>
<td>390</td>
<td>388</td>
<td>1.01</td>
<td>1.01</td>
<td>8</td>
<td>0.51</td>
<td>1.57</td>
</tr>
<tr>
<td>Mean value</td>
<td>385</td>
<td>361</td>
<td>1.06</td>
<td>1.06</td>
<td>8</td>
<td>0.53</td>
<td>1.51</td>
</tr>
</tbody>
</table>

6 x 10⁵ mononuclear cord blood cells were grown in the presence of 1.5 U/ml of erythropoietin. The radioactivity incorporated into the hemoglobin was determined after purification of hemoglobin from other proteins by affinity chromatography on Sepharose haptoglobin.

The proportion of radioactivity incorporated into the globin chains was evaluated by chromatography on carboxymethyl cellulose in urea, slightly modified after purification of hemoglobin by affinity chromatography on Sepharose haptoglobin.

Irradiated Cells

Light density cells from cord blood and adult blood or bone marrow were isolated by Ficoll metrizoate gradient centrifugation as above. Viable cells were counted using the trypan blue exclusion test and were irradiated at 15 Gy, delivered by a ⁶⁰Co source at a dose rate of 1.50 Gy/min. These cells were usually added in a 1:1 ratio to the cord blood cells. In some experiments, increasing numbers of adult or neonatal irradiated cells were added in culture. The concentration and volumes of cells were suitably adjusted in order to maintain culture volume constant at 1 ml. As a control, in all cases, the irradiated cells were tested alone and were unable to give any type of erythroid colonies in presence of erythropoietin.

RESULTS

As previously reported, cord blood contains erythroid precursors that fully differentiate in various periods of time from 5 to 7 days to approximately 2 wk. The 14-day-old colonies derive from early erythroid precursors (BFU-E), the 7-day-old colonies are mainly derived from CFU-E, since they appear at day 4 or 5 of culture and are fully mature at day 7. At this date, these colonies only contain mature erythro-

Table 2. Comparison of the β/β + γ Ratio in Reticulocytes and in Culture of Erythroid Precursors in the Presence or Absence of Irradiated Cells

<table>
<thead>
<tr>
<th>Neonatal Blood</th>
<th>Reticulocytes</th>
<th>CFU-E</th>
<th>BFU-E + Adult Irradiated Cells</th>
<th>BFU-E + Cord Blood Irradiated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β/β + γ</td>
<td>β/β + γ</td>
<td>β/β + γ</td>
<td>β/β + γ</td>
</tr>
<tr>
<td>1</td>
<td>0.27</td>
<td>0.43</td>
<td>—</td>
<td>0.48</td>
</tr>
<tr>
<td>2</td>
<td>0.35</td>
<td>0.39</td>
<td>0.38</td>
<td>0.44</td>
</tr>
<tr>
<td>3</td>
<td>0.39</td>
<td>0.42</td>
<td>0.43</td>
<td>0.44</td>
</tr>
<tr>
<td>4</td>
<td>0.36</td>
<td>0.42</td>
<td>0.36</td>
<td>0.58</td>
</tr>
<tr>
<td>5</td>
<td>0.44</td>
<td>0.47</td>
<td>0.45</td>
<td>0.50</td>
</tr>
<tr>
<td>6</td>
<td>0.29</td>
<td>0.37</td>
<td>0.39</td>
<td>0.45</td>
</tr>
<tr>
<td>7</td>
<td>0.25</td>
<td></td>
<td>0.31</td>
<td>0.46</td>
</tr>
<tr>
<td>8</td>
<td>0.26</td>
<td>0.32</td>
<td>0.31</td>
<td>0.39</td>
</tr>
<tr>
<td>Mean value</td>
<td>0.33</td>
<td>0.40</td>
<td>0.38</td>
<td>0.45</td>
</tr>
</tbody>
</table>

In the two first cases, the adult irradiated cells were light density bone marrow cells, in contrast, in the six other cases, the cells had a blood origin.
blood irradiated cells. However, at day 7 of culture, some burst colonies may already be present.

The addition of adult or neonatal irradiated cells to 6 x 10^6 cord blood cells did not significantly modify the plating efficiency of the erythroid precursors. The number of BFU-E was, respectively 569/10^6 plated cells (SEM, 75) for cord blood cells alone, 588/10^6 plated cells (SEM, 71) for cord blood cells plus adult irradiated cells, and 546/10^6 plated cells (SEM, 72) for cord blood cells plus neonatal irradiated cells. However, the size of the burst colonies increased in the presence of adult irradiated cells. In 5 experiments, the total synthesis of hemoglobin at day 14 has been determined and compared in culture of samples grown in the presence or the absence of adult irradiated cells: almost a twofold increase (1.71) was observed when adult irradiated cells were added (Table 1).

The results of hemoglobin synthesis are shown in Table 2 and Fig. 1. In the first set of 8 experiments, a high concentration of cord blood cells were plated (6 x 10^5 cells/ml) in the presence or absence of irradiated cells (6 x 10^5 cells/ml). The results are as follows.

Firstly, the average proportion of γ and β chains synthesized was constantly increased in erythroid colonies (β/β + γ ratio: 0.40 at day 7, 0.45 at day 14) in comparison to the in vivo situation (0.33). Furthermore, this increase was always higher at the 14th day of culture than at the 7th day.

Second, the addition of adult irradiated cells increased an increase in the β/β + γ ratio in 14-day-old colonies but not in 7-day-old colonies. The value of this ratio was 0.45 in BFU-E colonies grown without adult irradiated cells, and 0.55 in BFU-E colonies grown in the presence of adult irradiated cells. This 22% increase in the β/β + γ ratio could be obtained either with marrow cells (first 2 cases) or blood cells from adults. The differences observed with the addition of adult irradiated cells are highly significant, since purification of hemoglobin from other proteins and the chromatographic pattern (Fig. 2) permit an accuracy of about 1%-2% for the β/β + γ ratio.

Thirdly, the addition of irradiated cord blood cells in culture was unable to increase HbA synthesis in cord blood BFU-E colonies. In two circumstances, the irradiated cord blood cells were from the same sample and from a different neonate. No difference was observed in the proportion of HbA synthesis.

In order to further investigate the mechanisms that are involved in the effect of adult irradiated cells on globin gene expression in cord blood BFU-E colonies, three other experiments were designed.

In the first, increasing amounts (from 2 x 10^5 to 1.2 x 10^6) of adult irradiated cells were added to 6 x 10^6 cord blood cells as above (Fig. 3). Only a very slight modification of the plating efficiency was observed. In contrast, a progressive and linear increase was observed in the proportion of β chain synthesized. A plateau was not reached despite the addition of 1.2 x 10^6 irradiated adult blood cells (ratio 2:1).

In the second, an increasing number of irradiated blood cells from adults or neonates were added to a lower concentration of cord blood cells (3 x 10^5 cells/ml) in two different samples.

Under these conditions, adult irradiated cells dramatically increased the plating efficiency and particularly the number of large BFU-E colonies, defined as colonies involving more than 16 subcolonies. In addition, the β/β + γ ratio was enhanced from 0.3 to 0.55, revealing a 83% increase (Fig. 4). In this experiment, a plateau was not attained, although 3 x 10^6 adult irradiated cells were added (ratio 9.5:1). Cord blood irradiated cells only slightly modified the plating efficiency and the HbA expression (Fig. 5).
In a final experiment (not reported in Table 2), the behavior of adult or neonatal irradiated blood cells was compared for their action on the plating efficiency of the same sample of cord blood studied at low concentration of cells (1.6 x 10^5/ml). Adult irradiated cells increased the plating efficiency and particularly that of large BFU-E. Irradiated cord blood cells had a very slight effect (Fig. 6).

DISCUSSION

The pattern of Hb production during the Hb switch period in man has been studied in detail, but the control mechanisms governing the process at the cellular and molecular levels remained unknown. The experiments described in this article represent an attempt to further progress in the knowledge of the cellular mechanisms involved in this process.

The present study confirms previous results showing that erythroid progenitors from neonates or fetuses grown in culture contain a program of HbF and HbA expression, characteristic of their ontogenic stage. In addition, these data provide evidence that the Hb switch is programmed at the level of the early erythroid precursors, since HbA synthesis progressively increased from in vivo reticulocytes to 7-day-old erythroid colonies derived mostly from the late erythroid precursor, and finally to 14-day-old colonies derived from the early erythroid precursors. In 14-day-old colonies, the proportion of HbA synthesis is similar to that expected 2 or 3 wk after birth, a delay that corresponds to the differentiation time in vitro of the erythroid precursor. Another explanation of these results could be that the genetic program for globin genes expression can be more easily modulated during the process of differentiation of early than late erythroid precursors, as previously suggested by Rowley et al. In the adult, reactivation of fetal hemoglobin also occurred mainly in colonies derived from early precursors. After having determined the cellular level at which the hemoglobin switch occurs, we have tried to modify the expression of the program. Earlier studies indicated that the concent-
unable to modify globin chain synthesis at the 7th day of culture. It can be concluded therefore that adult cells are able to accelerate the Hb switch in culture, but only at the level of the early erythroid precursor present in the culture. This result is in agreement with the statement that the Hb switch is programmed at the level of BFU-E.

In order to further investigate the action of irradiated cells on Hb expression, cord blood BFU-E were grown under different culture conditions.

Fig. 3. Effect of increasing amounts of adult irradiated blood cells on the plating efficiency of BFU-E and the hemoglobins synthesized studied by the $\beta/\beta + \gamma$ ratio in a culture of $6 \times 10^5$ cord blood cells at day 14. The upper diagram shows an almost linear relationship between the increase in the $\beta/\beta + \gamma$ ratio and the number of adult irradiated cells, while the plating efficiency was not significantly modified (lower diagram).

Fig. 4. Effect of increasing amounts of adult irradiated blood cells on the plating efficiency of BFU-E and the hemoglobin synthesis (studied by the $\beta/\beta + \gamma$ ratio) in a culture of $3 \times 10^5$ and blood cells at day 14. The upper diagram shows a marked increase in the $\beta/\beta + \gamma$ ratio with increasing amounts of irradiated adult cells. This augmentation in the Hb synthesis is associated with an increase in the plating efficiency of BFU-E, particularly of the large ones defined as BFU-E with more than 16 subcolonies (lower diagram).
Fig. 5. Effect of increasing amounts of neonatal irradiated blood cells on the plating efficiency and the hemoglobin synthesis studied by the $\beta/\beta + \gamma$ ratio in culture of $3 \times 10^5$ cord blood cells at day 14. Irradiated cord blood cells did not significantly modify the $\beta/\beta + \gamma$ ratio (upper diagram) or the plating efficiency.

At low concentrations of plated cells, adult irradiated cells dramatically increased both the plating efficiency and the proportion of HbA synthesis (83% increase). This experiment may suggest that a factor mainly produced by adult irradiated cells recruits early precursors and permits their differentiation. This activity would be similar to a burst-promoting activity. In these conditions, adult irradiated cells produce a higher quantity of burst-promoting activity than neonatal ones.

At high concentrations of plated cells, adult irradiated cells still increased HbA synthesis but without modifying the plating efficiency. A direct relationship was found between the increase in HbA synthesis and the number of added cells. A plateau was not seen in the stimulation of HbA synthesis.

This experiment is consistent with another mechanism that might accelerate the Hb switch in vitro. This action may be linked to a modification of BFU-E differentiation in vitro. In fact, the addition of adult irradiated cells increased the size of BFU-E, even if this parameter is difficult to measure accurately. The amount of Hb synthesis at day 14 of culture was higher in BFU-E grown in the presence than in the absence of adult irradiated cells for a similar number of colonies. This phenomenon may be related either to a higher number of mitoses between BFU-E and mature erythroblasts or to a higher synthesis of hemoglobin by individual erythroblasts; this remains to be investigated.

Recent results afforded evidence that the degree of maturity of erythroid cells also influences the expression of globin genes. More particularly, it was shown that the process of erythroid maturation determines a decrease of $\gamma$ gene expression and thus an increase of $\beta$ chain synthesis in culture of adult and neonatal erythroid bursts. In culture of erythroid progenitors from cord blood, the proportion of $\beta$ chain synthesis was higher in the bursts composed by mature erythroblasts than in those composed by immature erythroblasts. The difference in the proportion of $\beta$ chain synthesis in these two populations of erythroid bursts was similar to that we have observed in the present study. An additional explanation of our findings could be that adult irradiated cells accelerate the hemoglo-
bin switching because they increase the degree of erythroid maturation of erythroblasts contained in BFU-E colonies. However, cytologic analysis of the cultures performed in different conditions (with and without irradiated cells) has not shown a significant difference in the degree of maturation of erythroid cells present in BFU-E colonies. In fact, in both conditions at the 14th day of culture, more than 80% of the BFU-E colonies were composed by mature and well hemoglobinized erythroblasts.

In any case, these results underline the role of cellular factors in the changes occurring during Hb switch, at least in vitro. In agreement with this result, it has recently been demonstrated that the in vivo transplantation of fetal liver cells in an adult sheep leads to the synthesis of adult hemoglobin by the progeny of fetal cells.29

Further studies in vitro and purification of factors regulating erythropoiesis will permit a better understanding of the regulation of globin gene expression during ontogenesis and differentiation.

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