Identification and Characterization of Three Classes of Erythroid Progenitors in Human Fetal Liver


Erythroid progenitors from 18 fetal livers (7-15-wk gestational period) and 13 normal adult marrows were grown in methylcellulose cultures containing erythropoietin (Ep) and burst-enhancing factor(s) (BEF). Additional experiments were carried out on 1 fetal, 15 neonatal, and 26 normal adult blood specimens. Three classes of progenitors (primitive or mature erythroid burst-forming unit(s), P-BFU-E, M-BFU-E respectively; erythroid colony-forming unit(s), CFU-E) have been identified in fetal liver. Identification was based on their differential clonogenic characteristics (i.e., colony morphology and number, time/growth curve, Ep and BEF sensitivity, in vitro \(^{3}H\)-thymidine suicide index). The multistep differentiation of fetal erythroid progenitors is associated with: (1) gradual amplification of their pool size, (2) progressive decline of their BEF response, (3) gradual enhancement of their Ep sensitivity. Marked differences are observed, however, between the characteristics of corresponding fetal and adult erythroid progenitors. Thus: (1) Differentiation of adult precursors entails a gradual increase of their proliferative rate, while all fetal progenitors are characterized by maximal cycling activity. The doubling time of cells in fetal bursts is distinctly lower than in adult ones, while intermediate values are observed in cord blood bursts. These observations suggest that ontogenetic development is associated with a progressive decline of both (A) proliferative activity of early and intermediate erythroid progenitors, and (B) cell doubling time in erythropoietic differentiation. (2) Adherent cells apparently play a key role in BEF production in adult marrow cultures, but not in fetal liver ones. Finally, (3) the sensitivity to added Ep of fetal CFU-E and M-BFU-E is apparently more elevated than that of corresponding adult progenitors.

In both adult humans and mice, Eaves et al. \(^7\) identified a third class of erythroid progenitors, i.e., the "mature" BFU-E (M-BFU-E), which is apparently intermediate between "primitive" BFU-E (P-BFU-E) and CFU-E in respect to all the above parameters.

Erythroid progenitors generating colonies in semisolid cultures have been recently identified in human fetal liver. \(^9\) \(^11\) These studies include preliminary observations on their clonogenic properties. Our observations have been carried out on 18 fetal livers and 13 "normal" adult marrows, and further observations performed on 1 fetal, 15 neonatal, and 26 normal adult blood specimens. Three classes of fetal erythroid progenitors (P-BFU-E, M-BFU-E, CFU-E) have been thereby identified and characterized with respect to their clonogenic features (colony morphology and number, time/growth curve; in vitro \(^{3}H\)-thymidine suicide index, cell doubling time in bursts; BEF and Ep sensitivity). Their characteristics have been compared with those of corresponding progenitors in adult marrow. Similar observations have been comparatively carried out on fetal, neonatal, and adult blood BFU-E.

Differentiation of fetal erythroid progenitors entails a gradual elevation of their number and Ep sensitivity, versus a progressive decline of their BEF response. Marked differences, however, are observed between the characteristics of corresponding fetal and adult progenitors. Thus, (1) ontogenic development of the erythron is associated with a progressive decline of (A) proliferative activity of early erythroid progenitors and
(B) cell doubling time in erythropoietic differentiation. (2) Adherent cells apparently release BEF in adult marrow cultures, but not in fetal liver ones. Finally, (3) the sensitivity of fetal M-BFU-E and CFU-E to added Ep is apparently more elevated than that of corresponding adult precursors.

**MATERIALS AND METHODS**

**Fetal Livers, Adult Marrows, and Blood Samples**

Eighteen fetal livers, obtained from legal curettage abortions in the 7–15-wk gestational period, were rinsed and maintained in Iscove’s modified Dulbecco’s medium (IMDM) (GIBCO Co., Grand Island, N.Y.). Cells were plated within 6 hr after the abortion. Umbilical blood was obtained from 1 premature (sixth month of gestation) and 15 full-term healthy newborns. “Normal” bone marrow (13 specimens) and blood (26 samples) was obtained from 19–40-yr-old hematology-oncology patients without apparent marrow involvement, with seemingly normal erythropoiesis, and prior to initiation of treatment. Fully informed consent had been obtained in advance from the mothers and the adult volunteers.

**Erythropoietin**

Urinary Ep from a red cell aplasia patient was purified by means of a 3-step chromatography method (final specific activity, >50,000 IU/mg of protein). This partially purified Ep is virtually free of activity stimulating or inhibiting, respectively, myelomacrophage or erythroid colony formation (in particular, the Limulus assay demonstrated 0.01 ng of endotoxin/1U of Ep). In some experiments, step III sheep plasma Ep (~5 IU/mg of protein, Connaught Medical Research Laboratories, Toronto) was utilized (the endotoxin titer, after its removal according to Zuckermann et al., was 0.1 ng/1U of Ep). Step III and low-titer (i.e., >50 <150 IU/mg) urinary Ep induced a virtually equivalent erythroid colony formation. These preparations were utilized in all experiments reported here, unless otherwise stated.

**BEF (Leukocyte-Conditioned Medium, LCM)**

Standard LCM was prepared essentially as described. Briefer, 106 leukocytes/ml were immobilized in 0.5% (v/v) agar with 10% fetal calf serum (FCS) (v/v) in IMDM. This semisolid agar base was overlaid with IMDM containing 10% FCS. The supernatant was harvested after 7 days of incubation at 37°C in a humidified atmosphere with 5% CO2.

**Cell Preparation and Culture**

A monocellular suspension of fetal liver in IMDM was obtained essentially as described. Light-density mononucleated cells from fetal, neonatal, or adult blood were obtained as reported. Cell separation from marrow aspirates was carried out as described by Eaves and Eaves, with an additional step involving passage of the cell-rich plasma, obtained from the centrifuged marrow, through progressively smaller needles (down to no. 25). Adherent cells were removed from adult marrow or fetal liver as reported. Each 1-ml plate contained the following components in IMDM: 0.8% methylcellulose (0.8%, final concentration), α-thioglycerol (10^{-4} M), FCS (25% or 40%), nucleated cells (0.5–1 × 10^6).

All assays were performed in duplicate. The plates, incubated in a humidified 5% CO2 in air atmosphere at 37°C, were removed from the incubator and examined, either once or up to 3 different times. All erythroid colonies were recognized in situ at the basis of their orange-red color.

** Colony Identification**

In adult marrow cultures, the earliest recognizable colonies are single aggregates of approximately 8–150 erythroblasts (time/growth curve described below), which are scored as CFU-E-derived clusters. Larger, late-hemoglobinizing colonies (containing approximately 4–6 × 10^2 up to 3–4 × 10^3 cells), almost always arranged in clusters or subcolonies (each composed from approximately 2 × 10^2 up to 2 × 10^3 cells), are scored as bursts derived from M- or P-BFU-E, essentially on the basis of criteria established by Eaves and Eaves. Thus, bursts of M- versus P-type, respectively, are colonies of smaller or larger size (i.e., composed of 2–12 versus 16–30 clusters), show earlier versus later hemoglobinization, and more versus less elevated Ep sensitivity (see below).

In fetal liver cultures, sequential waves of erythroid colonies are similarly expressed. The early-hemoglobinizing CFU-E clusters (containing approximately 20–150 cells) are virtually identical with adult marrow CFU-E clusters. Larger, late-hemoglobinizing colonies (containing approximately 4–6 × 10^3 up to 4–5 × 10^4 cells) are scored as bursts deriving from M- or P-BFU-E. The former, as compared to the latter ones, are characterized by: (A) a smaller size, (B) earlier hemoglobinization, and (C) more elevated Ep sensitivity. M- and P-bursts from fetal liver show virtually the same morphology described above for corresponding adult marrow colonies. The differential time of hemoglobinization and Ep sensitivity of fetal M- versus P-bursts is described below.

** 3H-Thymidine Suicide Index**

The in vitro percent killing of erythroid progenitors following incubation with 3H-thymidine has been performed as previously indicated. Thus, pooled nucleated cells (2 × 10^6/ml) from each sample were placed in 4 tubes containing IMDM. Additionally, tubes II and III contained 20 and 100 µCi of 3H-TdR (methyl-3H-thymidine, 20 Ci/m mole) and a total 200 µg of cold TdR. All tubes were incubated at 37°C in a water bath for 20 min, thereafter, the tube contents were diluted with 12 ml of IMDM at 4°C, which contained 2% FCS and 200 µg/ml of unlabeled TdR. The cells were centrifuged, suspended in medium containing 40 µg of cold TdR/ml, and centrifuged. There was no significant difference in death of cells in tubes II and III, and in tubes I and IV. The cells from each tube were plated (5 × 10^4 or 10^5 nucleated cells/plating for assay of precursors in the presence of unlabeled TdR and other nucleosides. The percentage of precursors killed by 3H-TdR was calculated to be equal to 1 − T/C (T, sum of colonies in plates from tubes II, III; C, sum of colonies in plates from tubes I, IV).

**Doubling Time of Cells in Fetal, Neonatal, and Adult M-Bursts**

The doubling time of erythroid cells in M-bursts has been evaluated by counting twice the cell number in each colony. The first counting, by direct visual inspection under the microscope, was performed simultaneously on various colonies containing ~20~50 cells scattered among ~2–8 closely related clusters. Each colony was...
identified in situ and the plates further incubated. The second counting was carried out at different culture times on previously identified colonies: each one was picked up as described, and its cell number counted by means of a standard hemocytometer. Particularly, the first counting in fetal, neonatal, and adult cultures was performed 2 days after the peak of CFU-E growth (see Fig. 1). Subsequent countings were performed every 2 days, until the time of peak growth (Fig. 1). In this time period, a significant linear regression was observed when plotting time/log cell number values for M-bursts from fetal, neonatal, and adult cultures (unpublished results). This indicates that the cell doubling time remains fairly constant in the erythropoietic differentiation pathway comprised after the initial 4-6 mitoses and prior to terminal differentiation. On this basis, all cell doubling values were plotted together (Fig. 2), irrespective of the second cell doubling time. Control experiments insured that: (A) the volume of IMDM employed for burst pick up was constant (1 µl) and (B) cells were not lost before counting via surface adherence or lysis. The time of cell doubling was calculated as 
\[ \text{hr} = \frac{\log n_f - \log n_i}{\log 2} \] (hr: time between second and first counting; \( n_i, n_f \): number of cells at the time of pick up or visual counting, respectively).

**RESULTS**

**Time/Growth Curves of Fetal and Adult Erythroid Colonies**

As shown in Fig. 1, analysis of well hemoglobinized (i.e., at least in part orange-red) colonies in adult marrow cultures shows sequential emergence of CFU-E clusters, M-bursts, and finally P-BFU-E bursts. Peak colony expression is monitored, respectively, on days 8, 14, and 18. Partial overlapping of the 3 time/growth curves is observed. These results fully confirm those previously reported. Analysis of fetal liver cultures (Fig. 1) shows similarly sequential hemoglobinization of CFU-E clusters, M-bursts, and P-bursts: peak

*Marrow CFU-E clusters are indicated in Fig. 1 peak on day 8 and are relatively large (i.e., comprise ~20–150 cells); smaller CFU-E clusters, not shown on Fig. 1, contain ~8–30 cells, peak on day 4–5, and then persist up to day 8. Fetal CFU-E clusters, comparable to the larger adult CFU-E colonies (~20–150 cells), peak on day 5 (Fig. 1); smaller CFU-E clusters (~8–20 cells) are not observed in fetal cultures.

†Although results presented in Fig. 1 refer to 11–15-wk fetal livers, a virtually identical pattern has been observed for the 7–10-wk specimens.
growth is monitored at earlier times than for corresponding adult colonies (i.e., on days 5, 9, and 13-15, versus 8, 14, and 18, respectively). The time/growth curve of cord blood P- and M-bursts is intermediate between those of corresponding fetal and adult colonies (Fig. 1). The curve of adult blood P- and M-bursts is virtually identical to that of corresponding adult marrow colonies (results not shown).

Cell Cycling of Fetal, Neonatal, and Adult Progenitors

Table 1 indicates that the percentage of P-BFU-E and M-BFU-E in DNA synthesis (as evaluated by means of the in vitro \(^3\)H-thymidine suicide index) is more elevated in fetal liver than adult marrow. Late erythroid progenitors (i.e., CFU-E) show a high suicide index in both fetal liver and adult marrow. Interestingly, M-BFU-E in adult blood are never in DNA synthesis, whereas a significant proportion of fetal and neonatal M-BFU-E are killed by \(^3\)H-TdR in vitro (Table 1).

Doubling Time of Cells in Fetal, Neonatal, and Adult M-Bursts (Fig. 2)

The cell doubling time in M-bursts remains fairly constant in the erythropoietic differentiation phases comprised between after the first 4-6 mitoses and prior to terminal differentiation. Interestingly, the values observed in M-bursts from 2 fetal livers are significantly \((p < 0.01)\) lower than those from 1 cord blood sample. The latter ones are in turn clearly less elevated than those from 2 adult marrow \((p < 0.01)\).

Ep Dose–Response Curve

The Ep dose–response curves for erythroid progenitors in fetal liver and adult marrow liver are presented in Fig. 3 A and B. In marrow, the differentiation stage of progenitors is inversely related to their Ep sensitiv-

Table 1. Fetal, Neonatal, and Adult Erythroid Progenitors: \(^3\)H-TdR In Vitro Killing Index (Mean ± SEM Percent Values)*

<table>
<thead>
<tr>
<th></th>
<th>P-BFU-E</th>
<th>M-BFU-E</th>
<th>CFU-E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal liver</td>
<td>50 ± 3.0 (\dagger)</td>
<td>57 ± 2.5 (\dagger)</td>
<td>61 ± 4.0</td>
</tr>
<tr>
<td>Adult marrow</td>
<td>10 ± 0.6</td>
<td>35 ± 1.5</td>
<td>56 ± 4.9</td>
</tr>
<tr>
<td>Fetal blood</td>
<td>29</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>(sixth month)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neonatal blood</td>
<td>NE</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Adult blood</td>
<td>NE</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

NE, not evaluated.

*These studies were performed on 3 fetal livers, 3 adult marrows, 1 fetal, 2 neonatal, and 2 adult blood samples.

\(\dagger\) \(p < 0.01\) when compared with the corresponding adult marrow group.
THREE CLASSES OF ERTHROID PROGENITORS

Plateau growth is observed for CFU-E clusters starting from 0.5 IU/plate and for M- and P-BFU-E colonies from, respectively, 2.5 and 5 IU/plate. Differentiation of fetal liver precursors is similarly associated with a progressive rise of their Ep sensitivity, i.e., P-, M-bursts and CFU-E colonies show maximal growth starting from, respectively, 2.5, 0.5, and 0.25 IU Ep/dish. Fetal progenitors are thus apparently more sensitive to added Ep than corresponding marrow precursors. This is particularly relevant for M-BFU-E and CFU-E, while it is of minor significance for P-BFU-E, which are least responsive to Ep. Statistical analysis of CFU-E and M-BFU-E dose-response curves of fetal liver versus adult marrow confirmed the difference of their sensitivity. It is emphasized that both fetal and adult cultures were carried out under standardized, strictly controlled conditions, which in particular included the same Ep preparations. This standardization is necessary to allow a reliable comparative analysis. Indeed, the Ep dose-response curve for CFU-E with a more purified Ep preparation is moderately different (Fig. 3B) from that observed in standard cultures (Fig. 3A).

BEF Dose-Response Curves

It is generally conceded that FCS contains BEF. Indeed, only selected FCS batches support optimal BFU-E growth. The other ones must be supplemented with exogenous BEF to allow adequate burst formation.

The Ep dose-response curves for fetal erythroid progenitors has been evaluated here at either optimal (40%) or suboptimal (25%) concentrations of FCS. Suboptimal amounts dampen the growth of P- and M-BFU-E, (i.e., cause a shift to the right of their Ep dose-response curve; Fig. 3A versus 5, and data not shown). The shift is fully corrected by addition of LCM (i.e., BEF; Fig. 4, also unpublished results): thus, it is apparently caused by the decrease of BEF levels due to the lower FCS concentration. On the other hand, the Ep sensitivity of CFU-E is similar in both 40% or 25% FCS cultures (Fig. 3A versus 5, and data not shown). In adult marrow cultures, a decrease of BEF concentration similarly dampens growth of early and intermediate progenitors, but not of late ones.

These results suggest that BEF represents a key modulator of fetal BFU-E growth. This postulate is strongly supported by the LCM dose-response curve of fetal erythroid precursors (in presence of suboptimal amounts of FCS and hence of BEF) (Fig. 4). Indeed, LCM addition induces a dose-related elevation of the number of M-bursts and even more markedly of P-bursts, but does not modify or may even inhibit CFU-E growth. Similar results have been obtained in FCS-free cultures (results not presented here).

Adherent Cells Removal

Removal of adherent cells from adult marrow does not affect CFU-E growth, but dampens significantly that of M- and even more of P-BFU-E (Fig. 5B). These results confirm previous observations. Our studies were performed in cultures containing suboptimal amounts of BEF (i.e., 25% FCS); therefore, they imply that adherent cells cooperate to induce marrow BFU-E growth, presumably via release of BEF in culture. Furthermore, we observed that in marrow cultures the cell/colony regression is linear for CFU-E clusters, but not for bursts (results not presented here). Once again, this suggests cooperation of two cell populations for burst, but not CFU-E, colony growth.

In fetal liver cultures containing 25% FCS, removal

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‡Plateau values are defined as those above 90% of peak cloning efficiency.

§Statistical analysis involved: (1) probit transformation of mean values of fetal liver versus adult marrow dose-response curves; (2) comparison of the 2 straight lines thereby obtained for each progenitor (CFU-E, p < 0.05; M-BFU-E, p < 0.05; P-BFU-E, not significant).
of adherent cells does not significantly modify colony formation by early, intermediate, and late erythroid progenitors (Fig. 5A). Accordingly, linearity of the cell/colony regression is observed for both CFU-E colonies, M- and P-bursts (Fig. 6).

Addition of PGE, in adult marrow cultures induces enhanced growth of BFU-E. This phenomenon is dependent on adherent cells, in that it is fully abolished by prior removal of these elements. No modification of fetal burst formation has been observed following PGE, addition (results not presented here). Once again, this may be referred to lack of influence by adherent cells on fetal BFU-E growth.

**DISCUSSION**

**Identification of Fetal Liver Erythroid Progenitors**

These studies have been focused on identification and characterization of erythroid progenitors in fetal liver, as compared with those in adult marrow.

Although adult erythroid precursors are characterized by a continuous spectrum of differentiation stages, their subdivision in distinct classes facilitates analysis of their clonogenic features and regulatory mechanisms. The present results on adult marrow progenitors confirm previous observations by Eaves and Eaves. At least 3 classes of precursors (CFU-E, M-, and P-BFU-E) have been identified on the basis of: (1) their capacity to form different types of colonies undergoing sequential hemoglobinization; (2) their differential sensitivity to Ep and BEF; and (3) their different proliferative activity.*

Erythroid progenitors in fetal liver have been subdivided here in 3 classes (P- and M-BFU-E, CFU-E) by means of the same criteria. Indeed, both fetal liver and adult marrow cultures show sequential hemoglobinization of progressively larger colonies, which derive from gradually less differentiated progenitors. Furthermore, the morphology of corresponding fetal and adult colonies is virtually identical. Finally, differentiation of progenitors of both fetal and adult type entails a progressive increase of their Ep sensitivity, as well as a gradual decline of their BEF response.*

**Differential Characteristics of Fetal Versus Adult Erythroid Progenitors**

The growth curve of fetal CFU-E clusters, M- and P-bursts precedes that of corresponding adult colonies

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*Studies by means of velocity sedimentation at unit gravity showed that P-BFU-E, M-BFU-E, and CFU-E from both adult marrow and fetal liver (our preliminary results) are characterized by different cell elution profiles.
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from peripheral blood or marrow. Furthermore, cord blood bursts show a growth pattern intermediate between that of fetal and adult ones. Indeed, the doubling time of cells is lower in fetal than adult M-bursts, while intermediate values are observed in corresponding neonatal colonies.

Parallel studies have shown that early, intermediate, and late fetal erythroid progenitors are characterized by elevated values of the \(^{3}\text{H}-\text{dTR}\) in vitro killing index. It is generally conceded that this parameter allows a reliable measurement of the percentage of cells in DNA synthesis, and hence in cycling activity. It is postulated, therefore, that both P-, M-BFU-E, and CFU-E in early fetal life are characterized by an elevated proliferative activity, i.e., \(~50\%\) are in DNA synthesis, and presumably all are cycling. Furthermore, since in mice P-BFU-E are very closely related to stem cells, it may be suggested by analogy that human fetal stem cells are virtually all in cycle too.

The elevated proliferative activity of fetal P- and M-BFU-E is in sharp contrast with that of correspondingly low or intermediate levels of the \(^{3}\text{H}-\text{dTR}\) killing index. Thus, differentiation of adult erythroid progenitors entails a gradual increase of their proliferative activity up to maximal levels, while fetal progenitors are always in cycling, irrespective of their differentiation stage. Similarly, virtually all adult blood BFU-E are out-of-cycle, whereas a significant proportion of fetal or neonatal blood BFU-E are in DNA synthesis.

In conclusion, ontogenic maturation of the erythron is associated with: (1) progressive decline of the cycling activity of M- and even more of P-BFU-E (as well as, presumably, of stem cells) and (2) a gradual increase of the doubling time of erythropoietic elements. Similarly, fibroblasts undergoing prolonged proliferation in culture show a gradual decline of their proliferative activity.\(^9\)

A further difference between fetal versus adult erythroid progenitors relates to their interaction with adherent cells. In this regard, it has been suggested that, in adult marrow cultures, BEF is released by the adherent elements.\(^8\) Their removal does not significantly modify CFU-E growth, but dampens proliferation of M-BFU-E and even more of P-BFU-E,\(^8\) as confirmed here. On the other hand, although BEF is necessary for fetal BFU-E growth, removal of fetal liver adherent cells does not modify burst formation in cultures with suboptimal BEF levels (with 25% FCS, see above). This suggests that BEF is not released by these adherent elements. Furthermore, the linearity of cell/colony regression for fetal BFU-E indicates that BEF activity is not released either by nonadherent hepatic cells. In this regard, BEF is closely related to the myelomacrophage colony-stimulating factor,\(^14\) and both are produced in large amounts by the placenta (and our unpublished data). It may be suggested, therefore, that BEF, released by the placenta, is conveyed to fetal liver via blood, until liver-marrow migration of stem and progenitor cells renders essential BEF production in marrow microenvironment.

Finally, human fetal CFU-E and M-BFU-E are more sensitive to added Ep than corresponding marrow progenitors. A similar phenomenon has been observed for murine CFU-E.\(^{13}\) The significance of these observations is uncertain, in view of the fact that fetal liver cells release Ep\(^2\) (as originally suggested for hepatic macrophages\(^23\)). The in vitro produced Ep might possibly add to the hormone plated in the dish. Furthermore, different FCS batches may contain different levels of Ep activity: Ep-rich batches may thus induce formation of “endogenous” fetal CFU-E colonies in the absence of added Ep.\(^9\)
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Identification and characterization of three classes of erythroid progenitors in human fetal liver

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