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Microenvironment created by stromal cells is essential for a rapid expansion of erythroid cells in mouse fetal liver

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Summary

Mouse stromal cell lines (FLS lines), established from the livers of 13-day gestation mouse fetus, supported the proliferation and differentiation of the erythroid progenitor cells from mouse fetal livers and bone marrow in a semisolid medium in the presence of erythropoietin. A large erythroid colony of over 1000 benzidine-positive erythroid cells was developed from a single erythroid progenitor cell on the FLS cell layer after 4 days of culture. When in close contact with the layer, the erythroid progenitor cells divided rapidly with an average generation time of 9.6 h and mature erythroid cells, including enucleated erythrocytes, were produced. The present studies demonstrate that the microenvironment created by the stromal cells can support the rapid expansion of erythropoietic cell population in the fetal liver of mice.

Key words: erythropoietin, erythropoiesis, fetal liver, hematopoietic microenvironment, stromal cells.

Introduction

Hematopoietic stem cells and their progenitor cells can proliferate and differentiate in the microenvironment of bone marrow, spleen and fetal liver (Metcalf and Moore, 1971; Till and McCulloch, 1961). Understanding of the regulation of hematopoiesis requires a knowledge of the required microenvironment *in vitro*. By long-term bone marrow cultures (Dexter *et al.* 1977; Gordon *et al.* 1987; Zipori *et al.* 1985), it was demonstrated that bone marrow stromal cells support the proliferation and differentiation of hematopoietic stem cells *in vitro*. Several stromal cell lines have been successfully established from bone marrow cultures (Tsai *et al.* 1986; Kodama *et al.* 1986; Hunt *et al.* 1987; Whitlock *et al.* 1987; Itoh *et al.* 1989) and used in the study of the cellular interactions that are important in the regulation of hematopoietic stem cell proliferation and differentiation.

Spleens in adult mice and livers in fetal mice are other possible microenvironments for hematopoietic stem cells. No detailed analysis has been reported on the microenvironment of these organs for hematopoiesis, however. Recently we established mouse stromal cell lines (MSS lines) from the spleens of newborn mice, which support the proliferation and differentiation of the erythroid progenitor cells in the presence of erythropoietin (epo) (Yanai *et al.* 1989). Large erythroid colonies were developed from the fetal liver erythroid progenitor cells on the MSS cell layers after 6 days of incubation. These layers also supported the maturation of the erythroid cells. Thus, the established spleen stromal cells may create the *in vitro* microenvironment adequate for erythropoiesis in mouse spleen. This was the first clear demonstration of the *in vitro* reconstruction of erythropoietic microenvironment.

Fetal liver is a major erythropoietic organ in the mouse. The microenvironment produced by fetal liver stromal cells may be essential for the growth and differentiation of erythropoiesis. Thus, in this work, we established stromal cell lines (FLS lines) from fetal livers and used them for *in vitro* reconstruction of the erythropoietic microenvironment of fetal liver.

Materials and methods

Establishment of fetal liver stromal (FLS) cell lines

Stromal cell lines (9 FLS lines) were established from primary cultures of fetal liver cells from 13-day embryos of C57BL/6J mice. The livers of 13-day embryos were removed aseptically and prepared for culture by flushing with RITC 80–7 medium (Yamane *et al.* 1981; Kyokuto Pharmaceutical Co. Ltd., Tokyo, Japan) using 26 1/2-gauge needle. The cells were washed with same medium, then cultured on 35 mm plastic dishes (Falcon 3001, Becton Dickinson, Oxnard, CA) with RITC 80–7 containing 2% fetal bovine serum (FBS, Gibco New Zealand Ltd), 10 $\mu g\,ml^{-1}$ transferrin (Sigma, St. Louis, MO), 1 $\mu g\,ml^{-1}$ insulin (Shimizu Seiyaku Co. Ltd., Tokyo), 10 $ng\,ml^{-1}$ epidermal growth factor (recombinant EGF,

Ajinomoto Co. Ltd., Tokyo), and 2.5 ng ml^{-1} ethanolamine. The medium was changed on the 1st and 5th days. After 8 days in cultivation, primary cultures were subcultured by 25 μg ml^{-1} pronase (Behringer Mannheim, W. Germany) at room temperature for 5 min. Only adhered cells were subcultured, but contaminating macrophages did not detach from the substrate during such mild pronase treatment. After 8 weeks of successive culture and 8 passages, the cells were cloned by the colony formation during 3 to 4 weeks.

After the initial screening of 9 clones for the ability to support erythropoiesis, 3 clones (FLS3, FLS5, and FLS11) were used for the further experiments. Mouse fibroblast cell line, BALB3T3 A31 (Kakunaga and Crow, 1980), and mouse spleen stromal cell line, MSS 62 (Yanai *et al.* 1989), were used for comparisons. All cultivations were done at 37°C in a humidified atmosphere of 5 % CO_2 in air.

Preparation of hemopoietic progenitor cells and cocultivation of hemopoietic cells on stromal cell layers

Preparation of hemopoietic progenitor cells and cocultivation of hemopoietic cells on stromal cell layers were performed as described previously (Yanai *et al.* 1989). Hemopoietic progenitor cells were prepared from the liver of 13-day embryos of C57BL/6J mice (Mishina *et al.* 1986). Monolayers of FLS cell lines, MSS62 and BALB 3T3 were made in multiwell plates (Falcon 3047, Becton Dickinson, Oxnard, CA). Hemopoietic progenitor cells were cultured on these layers in 0.8 % methylcellulose semisolid culture media containing Iscove's modified Dulbecco medium (IMDM; Gibco), 30 % heat-inactivated FBS, 1 % bovine serum albumin, 100 mM 2-mercaptoethanol and various concentration of erythropoietin (epo) (recombinant human epo, generously supplied by Kirin-Amgen Co. Ltd). After cocultivation, erythroid colonies were scored by direct staining with benzidine.

Growth of the erythroid progenitor cells on the stromal cell layers

The growth of a single erythroid progenitor cell to form the large erythroid colony on the stromal cell layers was monitored under the microscope with a video camera and the number of cells were scored every 12 h for 4 days of culture.

Fractionation of erythroid progenitor cells from mouse fetal livers

Erythroid progenitor cells were fractionated by the method described previously (Mishina *et al.* 1986). Fetal liver cells were suspended in a solution containing 155 mM NH_4Cl, 10 mM $KHCO_3$, and 1 mM EDTA, and incubated to selectively destroy the differentiated erythroid cells before separation, thus enriching CFU-E (colony-forming unit-erythroid). Then, the cell suspension was layered on Percoll (Pharmacia) density gradient which was preformed by ultracentrifugation at 25 000 *g* for 15 min with 60 % Percoll and separated by centrifugation at 1200 *g* for 10 min. Fractions were taken from the gradient with an 18-gauge needle. The density of each fraction was monitored by density marker beads (Pharmacia).

Results

Establishment of FLS cell lines

FLS cell lines were established from primary cultures of livers of 13-day gestation fetuses of C57BL/6J mice (Fig. 1). These established FLS cell lines were maintained with RITC 80–7 supplemented with 2 % FBS, because they do not adapt to culture conditions with higher FBS concentrations.

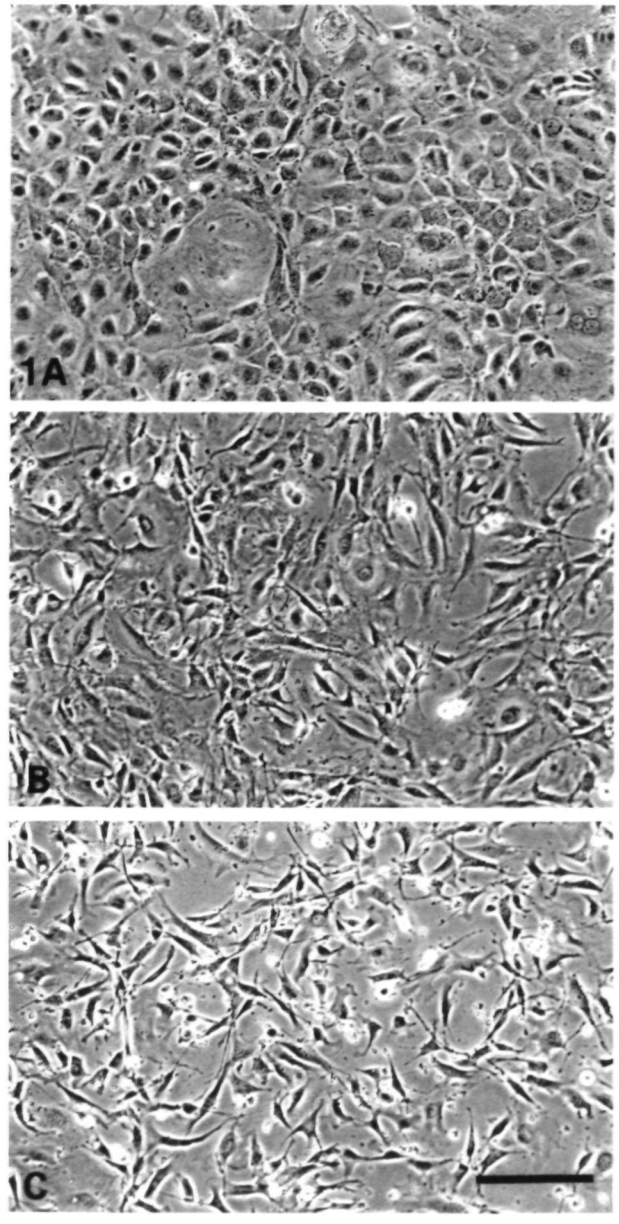

Fig. 1. Phase microscopic picture of three FLS cell lines. (A) FLS3 showed an epithelial cell-like morphology which appeared cobblestone monolayer at a confluent stage. (B) FLS5 showed a fibroblastic morphology. (C) FLS11 showed an endothelial cell-like morphology. Bar; 200 μm.

FLS cell lines support the proliferation and differentiation of erythroid progenitor cells

The ability of the FLS cell lines to support erythropoiesis was examined by culturing erythroid progenitor cells from mouse fetal liver on monolayers of FLS cell lines in a semisolid medium in the presence or absence of epo. The differentiated colonies were counted after benzidine-staining of hemoglobin-producing (benzidine-positive) cells. After 2 days of cultivation of fetal

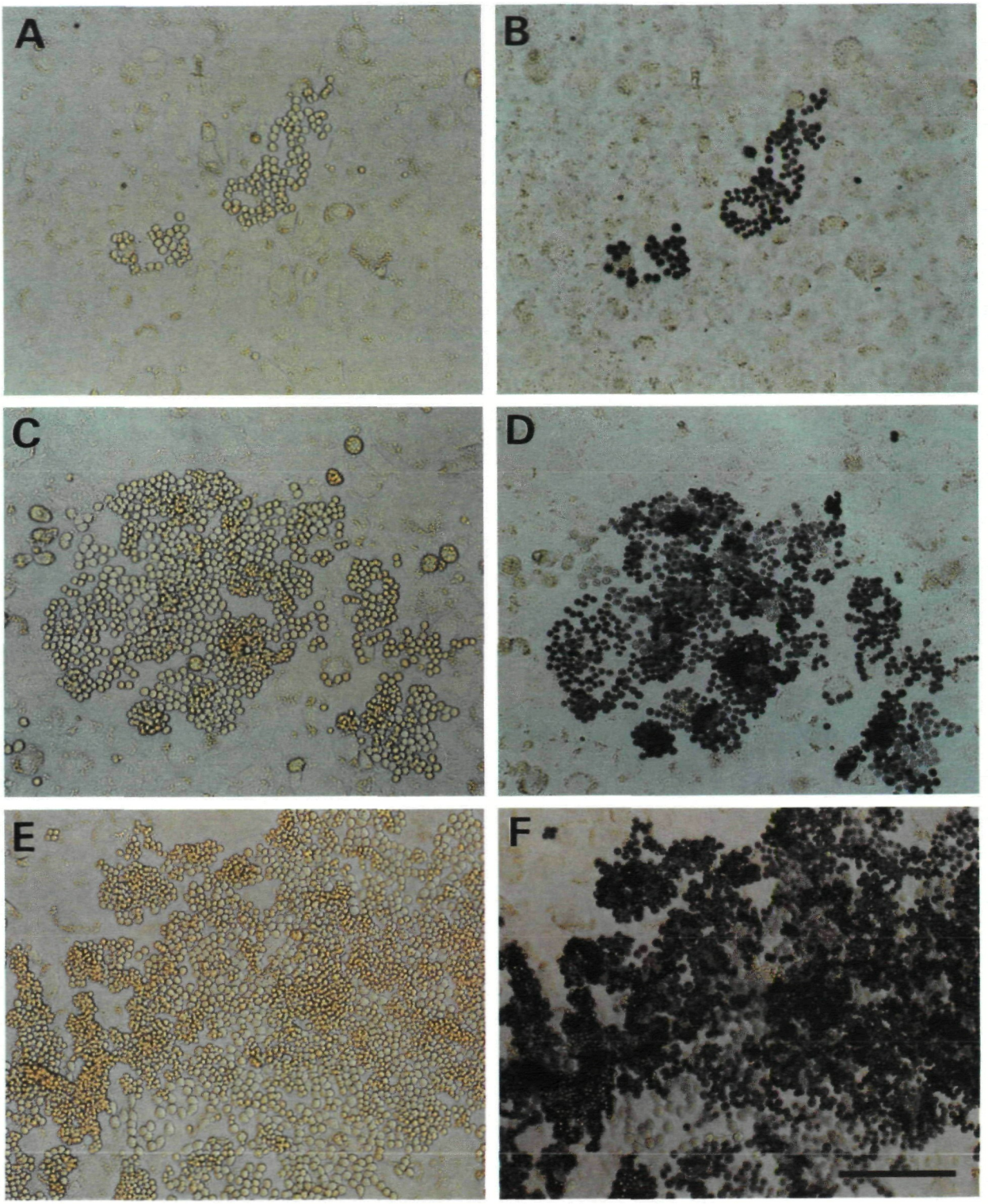

Fig. 2. Proliferation of hemoglobin-producing cells on the stromal cell layer. The time-dependent expansion of the erythroid colonies was monitored every day after seeding the erythroid progenitor cells on the FLS cell layer. Most of the cells in the colonies show reddish color showing high accumulation of hemoglobin and are positively stained with benzidine. A, C, and E show the colonies of 2, 3 and 4 days of culture, respectively. B, D, and F show the colonies after staining of 2, 3, and 4 days of culture. Bar; 200 μm.

Table 1. *Number of erythroid colonies developed on FLS cell lines*

Feeder cell	exp. 1	exp. 2	exp. 3
without feeder	0	0	0
BALB3T3A31	0	0	0
MSS62	63±9	40±0	93±4
FLS3	63±10	42±9	56±7
FLS5	75±10	62±3	93±12
FLS11	68±11	49±4	75±11

Fetal liver erythroid progenitor cells from 13-day gestation of mice were cultured on fetal liver stromal (FLS) cell lines in a semisolid medium in the presence of epo ($0.1\,U\,ml^{-1}$). After 4 days of incubation, cultures were directly stained with benzidine. Number of colonies were derived from 1×10^4 fetal liver cells in each experiment. All values are means±S.E.M. of the results of three wells. BALB3T3A31, mouse fibroblast cell line, and MSS62, mouse spleen stromal cell line (Yanai *et al.* 1989), were used as feeder cell lines for comparison.

liver cells in the presence of $0.1\,U\,ml^{-1}$ of epo in the semisolid medium, dark-blue benzidine-positive colonies derived from colony forming unit-erythroid (CFU-E) were formed, but they subsequently disappeared. Then, other types of colonies continued to proliferate on the stromal cell layers and developed to large randomly distributed ones after several days cultivation in the presence of $0.1\,U\,ml^{-1}$ epo as shown in Fig. 2.

In three independent experiments, FLS3, 5 and 11 lines stimulated the formation of large erythroid colonies (Table 1). Formation of large colonies is dependent on the FLS cell layers and epo, since large colonies were not formed from fetal liver cells even in the presence of IL-3+epo or GM-CSF+epo without the cell layers and the large colony formation on the FLS cell layers was not stimulated with IL-3 or GM-CSF in the presence of epo (data not shown). On the FLS cell layers, benzidine-negative cells appeared after 4 days culture, but those colonies was not large, consisting of fewer than 50 cells. Mouse fibroblast cell line BALB 3T3, which we used for comparison, did not support erythropoiesis, although it has been shown to support granulocyte proliferation (Roberts *et al.* 1987). FLS cell lines could support the formation of as large erythroid colonies from the progenitor cells of adult bone marrow as do those from fetal liver cells.

The properties of the erythroid progenitor cells that form the large colonies on the stromal layers were examined by comparison of their epo sensitivity and cell density with those of the CFU-E-derived colonies. The dose-dependency of epo on the formation of both types of colonies from the erythroid progenitor cells of fetal liver is shown in Fig. 3. The large colonies on the FLS cell layers were formed at the same concentration of epo required for the formation of CFU-E-derived colonies. We obtained the same epo-dependency on the formation of both types of colonies from the progenitor cells of adult bone marrow (data not shown). Thus, large erythroid colonies on FLS cell layers were produced at epo concentration as low as $0.05\,U\,ml^{-1}$, enough for CFU-E, but not for BFU-E. To examine whether both types of colonies are generated from

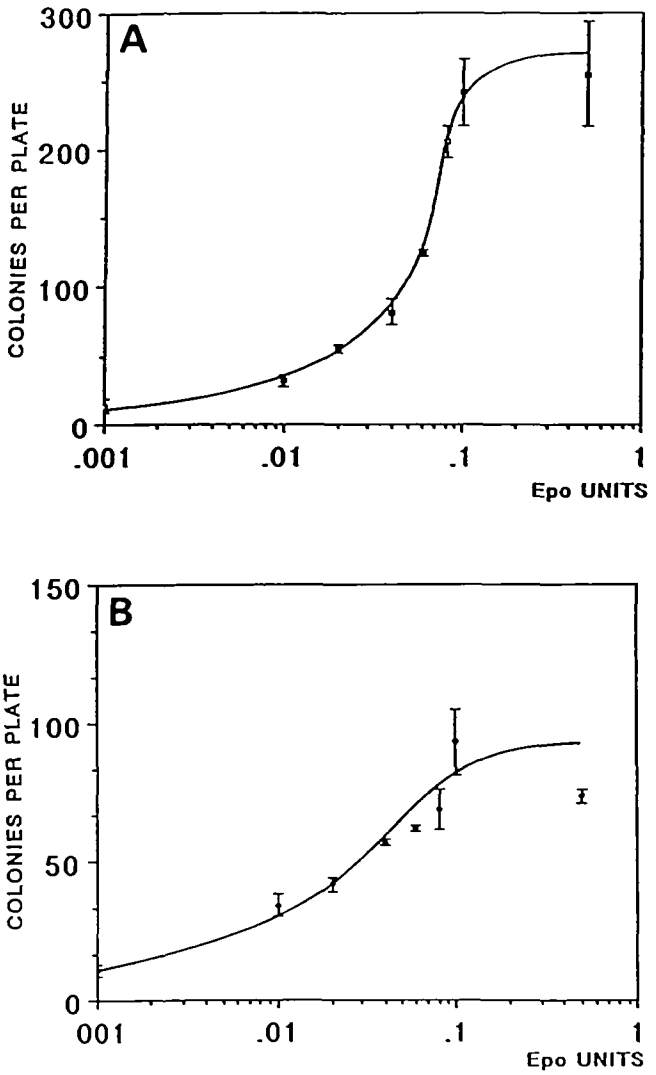

Fig. 3. Dose-dependency of epo on the formation of the large erythroid colonies and CFU-E-derived colonies. The erythroid progenitor cells from fetal livers were cultured on the monolayers of FLS cells in the presence of various concentrations of epo for 4 days. After direct benzidine staining, the large colonies (over 200 cells) were scored. All colonies were derived from 1×10^4 cells inoculated. The colony formation of CFU-E was done separately in the semisolid medium without the stromal layers in the presence of various concentration of epo. Colonies consisting of over 20 cells were scored as CFU-E- type colonies. (A) Formation of CFU-E type colonies; (B) formation of the large erythroid colonies.

different progenitors, the erythroid progenitor cells of fetal livers were fractionated using Percoll gradient centrifugation. The CFU-E-derived and the large erythroid colonies on the stromal layers were formed from the progenitors that migrated in the same density (Fig. 4). These results show that both types of colonies are generated from erythroid progenitor cells with the same epo sensitivity and cell density. From the examination of the morphological character of the erythroid cells of the colonies by May–Gruenwald–

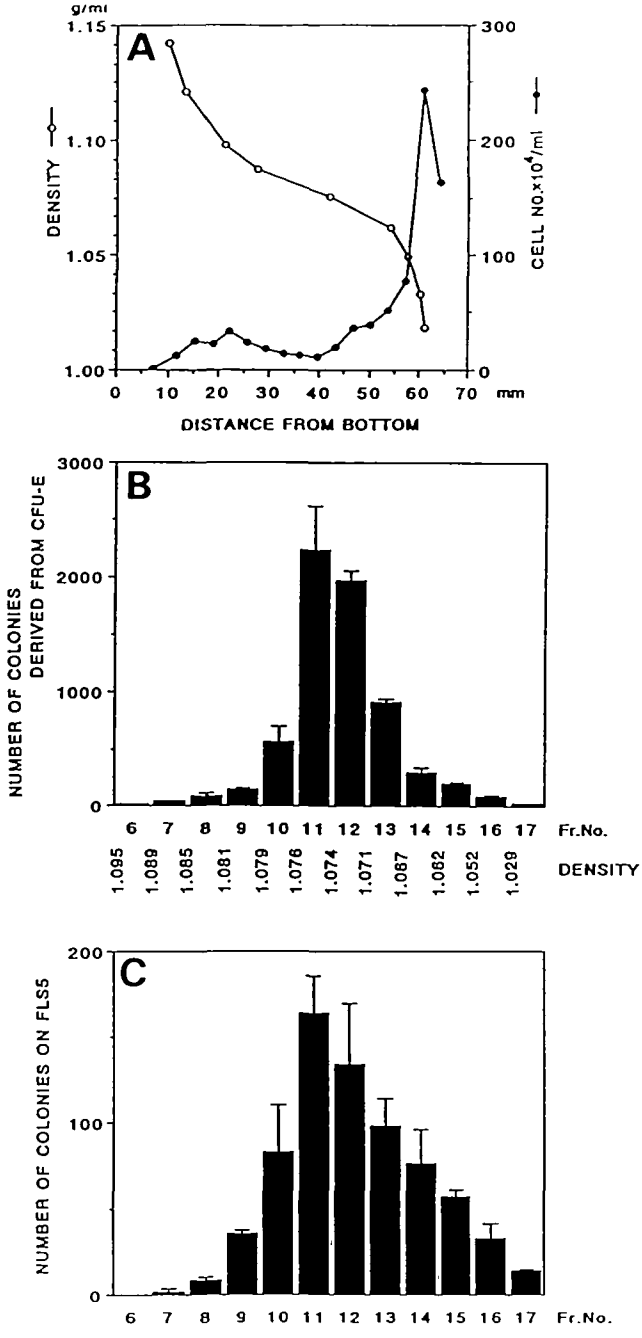

Fig. 4. Distribution of the erythroid progenitor cells forming the large erythroid colonies and CFU-E in the Percoll gradient. (A) The erythroid progenitor cells of 13-day fetal livers were enriched by lysing treatment and put on the preformed Percoll gradient and fractionated by the centrifugation at 1200*g* for 10 min. The formation of the large erythroid colonies and the CFU-E of the fractionated cells was performed as described in Table 1. (B) CFU-E type colonies were formed from 1×10^4 fractionated cells. (C) Large erythroid colonies were formed from 1×10^4 fractionated cells. All values are means±S.E.M. of the results of three separate cuiltures.

Giemsa staining, the majority were identified as erythroblasts, but some were enucleated mature erythrocytes. Many cytoplasmic buddings were observed among these erythroblasts in the process of enucleation. Thus, the enucleation process of erythroid cells may be facilitated on FLS cell layers.

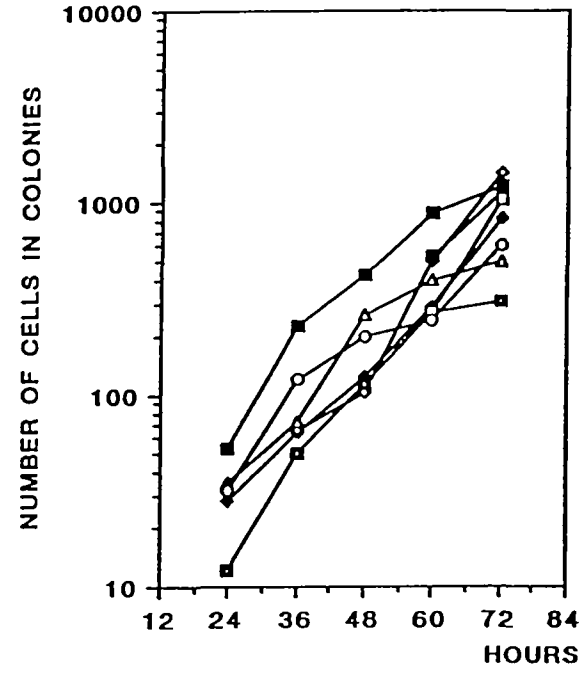

Fig. 5. Clonal growth of the erythroid cells on the stromal cell layers. The erythroid progenitor cells were enriched by lysing treatment of 13-day fetal livers. The enriched erythroid progenitors were cultured on the stromal cell layers. The proliferation of each erythroid progenitor cell was followed by the microscope attached to the video TV camera every 12 h. The patterns were documented in the video film and after several days of culture, number of cells in the single expanding colonies were counted. Each line in the figure shows the cumulative increase in cell number in a single colony. The generation time of each colony forming erythroid progenitor cells is between 6 and 10 h approximately 9.6 h on average.

Kinetics of production of erythroid colonies

The erythroid cells grew on top of the FLS cell layers with some cells dispersed or detached from the layer. The growth was so rapid that colonies consisted of more than 500 and sometimes over 1000 cells after 4 days of culture. Their orange color revealed an accumulation of hemoglobin and most cells in the colonies were stained with benzidine. To show the rapid proliferation and differentiation of single erythroid progenitor cells, we followed the formation of colonies from several independent single cells on FLS cell layers by observing the colonies every 12 h using a microscope attached to a video TV set. Cell numbers of the individual continuously growing colonies were counted as shown in Fig. 5. The result clearly indicated that individual colonies were derived from single cells and 500 to 1000 hemoglobin-positive cells were generated from a single erythroid progenitor cell on the FLS cell layer after 4 to 5 days. The generation time of the colony-forming erythroid cells was from 6 to 10 h (average=9.6 h). The colonies were stained everyday, and most of cells in a

single colony were benzidine-positive, starting from the small colonies consisting of 20–30 cells to the large colonies of over 1000 cells (Fig. 2). Thus, the erythroid cells producing hemoglobins could divide more than 10 times on the FLS cell layers in the presence of epo.

Discussion

The stromal cells of hematopoietic organs are thought to create a microenvironment adequate for the proliferation and differentiation of hematopoietic stem cells and their progenitor cells. The survival, proliferation and differentiation of the hematopoietic stem cells and their progenitor cells in an *in vitro* culture were earlier demonstrated on layers of stromal cells established from bone marrow (Tsai *et al.* 1986; Kodama *et al.* 1986; Hunt *et al.* 1987; Whitlock *et al.* 1987; Itoh *et al.* 1989). It has been proposed that stromal regulation of hematopoiesis occurs by cell–cell contact (Kodama *et al.* 1986; Itoh *et al.* 1989), by extracellular matrices (Zipori *et al.* 1985; Bentley and Foidart, 1980; Zuckerman and Wicha, 1983; Campbell *et al.* 1987), or through the secretion of hematopoietic growth factors from the stromas (Tsai *et al.* 1986; Hunt *et al.* 1987; Lipton and Nathan, 1983).

However, there has been no report of established stromal cells that support erythropoiesis. In the previous work, we demonstrated clearly that the established mouse spleen stromal (MSS) cell lines selectively support formation of the large erythroid colonies only in the presence of epo. In this work, we have tried to reconstruct the erythropoietic microenvironment *in vitro* with the established mouse fetal liver stromal cell (FLS) lines to explain the phase of rapid expansion of the erythropoietic cell population observed in mouse fetal liver during 12 to 16 days of gestation (Wolf and Trentin, 1967).

On the FLS cell layers, we detected the large colonies composed of 500 to 1000 benzidine-positive erythroid cells. They were produced only on the FLS cell layers from the erythroid progenitor cells from fetal livers and from adult bone marrows in the presence of epo. The erythroid cells producing hemoglobins could divide more than 10 times on the FLS cell layers in the presence of epo. A large expansion of a single erythroid progenitor cell observed in our stromal cell lines is striking since such large colonies were not observed in the *in vitro* erythroid colony formation in the usual semisolid assay. Although expansion of late erythroid progenitor cells occurs at the stage of BFU-E (burst-forming unit-erythroid) in the semisolid medium without feeder cells after 7 days of culture (Gregory and Eaves, 1977), the colonies are smaller and their cell population is not homogeneous. The large colony-forming cells from fetal liver and adult bone marrow observed in the present study require the same optimum epo concentration (as low as 0.05 U ml^{-1}), enough for CFU-E, but not for BFU-E. They are not separated from CFU-E by fractionation of the erythroid progenitor cells of fetal livers. BFU-E can be separated from the CFU-E by density gradient experiments (Heath *et al.* 1976; Mishina *et al.* 1986), requires a higher epo-concentration than CFU-E and cannot be detected in the fetal liver erythroid cells in the semisolid culture (Gregory and Eaves, 1977; Iscove *et al.* 1974). In fact, large colonies were not formed from fetal liver cells in the IL-3+epo or GM-CSF+epo without FLS cell layers. Thus, the progenitors that form the large erythroid colonies appear not be BFU-E, but may be at a stage close to or identical with the CFU-E.

The *in vitro* microenvironment created by the fetal liver stromal cell layers is essential for the large expansion of the colonies from erythroid progenitor cells. An *in vitro* microenvironment that supports more than 10 cycles of cell division of the erythroid progenitor cells can explain the phase of rapid expansion of the erythropoietic cell population observed in the fetal liver (Wolf and Trentin, 1967). The *in vitro* microenvironment seems to be created by direct contact and/or short-range communication between the erythroid progenitor cells and the stromal cell layers since large erythroid colonies were not formed by separation of the progenitor cells from the stromal cells by a diffusion chamber or a nucleopore filter and the large erythroid colonies were formed only in the presence of semisolid support (data not shown). Supplementing the conditioned medium of stromal cells did not show any effects on the colony formation (data not shown). Thus, IL-3, GM-CSF or other diffusive factors that stimulate the erythroid colony formation in the presence of epo may not be produced from the FLS cells. In this microenvironment, many mature erythrocytes and erythroblasts showing cytoplasmic budding were produced as also observed on the MSS cell layers (Yanai *et al.* 1989). Thus, FLS cells may support the maturation of enucleation of the erythroblasts *in vitro*. Morphological and biological studies on the MSS cell lines suggest these cell lines may be derived from endothelial cells (Yanai *et al.* in preparation) and most of FLS cell lines examined in this study seemed to be epithelial-like cells in their morphology. Although their morphological features are different, they show similar selective ability to support erythropoiesis *in vitro*. It will be interesting to examine at the cellular and molecular levels how the clonally established stromal cell creates the microenvironment sufficient for the proliferation and differentiation of erythroid cells.

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