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RETICULO-FIBROBLASTOID STROMAL CELL PROGENITORS \(CFU-RF\) IN MURINE BONE MARROW

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Experimental **Hematology**

Reticulo-Fibroblastoid Stromal Cell Progenitors (CFU-RF) in Murine Bone Marrow

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Abstract. The hemopoietic inductive microenvironment (HIM) of the bone marrow is responsible for secretion of growth factors that regulate hemopoiesis. It is composed of an extracellular matrix and a complex variety of cell types with a range of functions related to blood cell development. In order to understand how such a complex system operates, it will first be necessary to determine the role(s) of the integral parts. Several of the stromal cell types have been identified morphologically in various culture systems, and some of their functions have been elucidated. We have identified a new stromal cell type in mouse bone marrow that appears similar if not identical to its human counterpart. When bone marrow cells are placed in methylcellulose/plasma clot culture with phytohemagglutinin-stimulated human leukocyte-conditioned medium in the presence of bovine calf serum (BCS), mercaptoethanol, and hydrocortisone, extensive branching colonies develop within 14 days. These "reticulo-fibroblastoid" (RF) colonies arise from a putative reticulo-fibroblastoid colony-forming unit (CFU-RF) stem cell, and many become adipocytic by day 14; the addition of fresh medium, methylcellulose, and BCS on day 7 inhibits this change. The batch of human citrated plasma used in the culture system and the type and source of stimulating factor all influence the number of RF colonies that develop as well as the percent of colonies that become adipocytic. Whether this adipogenesis represents functional maturity or terminal differentiation is not yet known. Information gained on the role of these RF cells in normal and impaired hemopoiesis should contribute to the elucidation of the complicated interactive role of the microenvironment in the support and modulation of hemopoiesis.

Key words: Stromal cell — Mouse — Bone marrow — Adipocyte — PHA-LCM

The regulation of mammalian hemopoiesis in vivo is dependent on a functional hemopoietic microenvironment (HIM), as described by Wolf and Trentin [1]. The HIM is composed of an extracellular matrix (ECM) and a variety of stromal cell types that are responsible for the support of hemopoietic growth and development [2]. In the adult mammal, hemopoiesis is restricted to the bone marrow cavity [3]. Long-term bone marrow culture (LTBMC), as described by Dexter et al. [4], has demonstrated that a HIM suitable for hemopoiesis can be set up in vitro. Adherent (stromal) cells grow and develop; upon transplantation to ectopic sites in animals they can support hemopoiesis [5], indicating a cellular basis for hemopoietic control. The role of stromal cells in control of hemopoiesis is not yet clearly understood, but efforts are being directed toward the identification and culture of the various cell types [6–9]. It will be necessary to first elucidate the role(s) of individual cell types, then to determine how they manage to orchestrate blood–cell development in a coordinated manner [10, 11].

The distinct subtypes of marrow stromal cells may have a dual role in hemopoiesis: first, by providing cell adhesion molecules [12] that permit homing of stem cells, and second, by producing and retaining growth factors. The growth factors are locally retained by binding to proteoglycans and sulfated glycosaminoglycans (GAGs) that are also produced by stromal cells [13, 14]. Monoclonal antibodies against a homing receptor (CD-44) can completely prevent the emergence of myeloid cells in LTBMC [12], suggesting that binding through adhesion molecules is an absolute requirement for stem cell growth and maintenance [6, 13–15]. Growth factors produced by stromal cells bind to heparan sulfate chains of transmembrane proteoglycans, and when hemopoietic stem cells attach, stimulation is initiated. Soluble granulocyte–macrophage colony-stimulating factor (GM-CSF) added to dishes coated with heparan sulfate will be bound to the proteoglycan molecules [16]; GM-CSF can also be eluted from cultured stromal cell layers [17], indicating that growth factors are produced by stromal cells but are retained on the cell surface instead of being released.

In 1986, Lim et al. [11] characterized a population of marrow stromal cells and called it "reticulo-fibroblastoid" (RF) cells, according to the appearance of the colonies that developed in tissue culture. The ECM consists of a structural matrix of reticular fibers that are apparently secreted by reticular cells in the bone marrow [18]. Many stromal cells seem to be fibroblastic in nature, arising from the progenitor fibroblast colony-forming unit (CFU-F) [8, 19, 20], and seem to be the in vitro equivalent of reticular cells [15]. However, Lim et al. [11] have established that RF cells are a distinct cell type and are not fibroblasts according to immunophenotypic markers such as production of collagen type IV (which is not produced by fibroblasts). The growth and appearance of the reticulo-fibroblastoid colony-forming unit (CFU-RF) colonies are also somewhat different from those of CFU-F colonies in tissue culture.

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In this paper we report that murine marrow cells also contain a colony-forming cell that gives rise to stromal cell colonies similar in appearance to human RF cells, including formation of adipocytes and production of collagen type IV. This murine model for RF stromal cells will be of importance in the investigation of several features of stromal cells, such as cell membrane markers and their functional role(s). In particular, we want to discover what controls their activity and whether they produce, secrete, or bind growth factors to their membranes that would enable them to support hemopoiesis in whole or in part [15, 21]. Could RF cells be stimulated to enhance recovery of hemopoiesis after radiation damage?

Materials and methods

Animals. The animals used were 6- to 8-week-old female mice (CD-1; Charles River Laboratories, Montreal, Canada) weighing about 20 g. They were housed in groups of five in polycarbonate cages for 2 weeks after arrival to equilibrate them to the new environment (22°C, 55% humidity, and 12-h light/dark cycle). Purina mouse chow and acidified (pH 2.4) tap water were supplied ad libitum.

Preparation of bone marrow cell suspension. The mice were sacrificed by cervical dislocation and the femurs removed aseptically. The femurs were thoroughly cleaned of muscle tissue using sterile gauze pads in a vertical laminar flow unit, the proximal ends were cut off with surgical scissors, and the shafts were flushed with 5 ml Iscove's modified Dulbecco's medium (IMDM) by inserting a 23-gauge hypodermic needle into the distal end. The solution was aspirated twice through the needle, and the resulting single-cell suspension was centrifuged (1200 rpm, 10 min). Cells were resuspended in IMDM containing 10% bovine calf serum (BCS).

CFU-RF culture technique. The bone marrow cells were cultured in 35-mm plastic petri suspension dishes (Miles Scientific) using a methylcellulose (MC)/plasma clot system modified after Lim et al. [11]. The cell suspension was mixed thoroughly with the culture material (4 ml for triplicate cultures) consisting of 0.8%–0.9% MC (Sigma) supplemented with IMDM (GIBCO), 20% human citrated plasma (Canadian Red Cross, Ottawa, Canada), 10% BCS (Flow), 5×10^{-5} M 2-mercaptoethanol (2-ME; BDH) prepared freshly, 10^{-6} M hydrocortisone sodium succinate (HC; Sigma), and 10 μl 0.1 M $CaCl_2$ (added last to promote clotting). The colony-stimulating factor (CSF) used was phytohemagglutinin (PHA)-stimulated human leukocyte or T-cell-conditioned medium (PHA-LCM or PHA-TCM), pokeweed mitogen (PWM)-stimulated murine spleen-conditioned medium (PWM-SCM), or WEHI-3-cell (B-cell lymphoma cell line)-conditioned medium (WEHI-CM).

Bone marrow cells were plated in duplicate or triplicate at $2–5 \times 10^5$ per dish and incubated for 14 days in a humidified atmosphere at 37°C and 5% CO_2. The RF colonies were counted using an inverted binocular microscope, and the results were recorded as number of RF colonies per 10^6 bone marrow cells. Some cultures were fed on day 7 by overlaying the culture with fresh MC and IMDM containing 20% BCS and 2% Fungizone (GIBCO); incubation was then continued for the remaining 7 days until day 14.

The appearance of adipocytes in the RF colonies was confirmed by oil red O incorporation in lipid vesicles. MC RF cultures were overlaid with oil red O for 24 h to allow penetration of the dye through the MC and into the cells.

To characterize the murine RF colony cells, the presence of type IV collagen was used to distinguish them from fibroblasts. The human RF cells cultured by Lim et al. [11] were identified in this manner. RF colonies (only pure, branching, nonadipocyte) were picked on day 14 and placed in liquid culture (IMDM, 30% BCS) for 2–3 weeks. The adherent cells were then trypsinized, transferred to coverslips in 35-mm petri dishes, and incubated at 37°C with 5% CO_2 for 3–4 days (to allow collagen IV to be produced in sufficient quantity to be detected). After fixation, rabbit anti-mouse type IV collagen was introduced, followed by a fluorescent second antibody, goat anti-rabbit fluorescein isothiocyanate (FITC; Jackson Technology); the slide was dried and examined for positive cells using a Zeiss fluorescence microscope. As a negative control, the primary antibody (for collagen type IV) was deleted.

Preparation of conditioned media. PHA-LCM was prepared from human peripheral blood leukocytes, separated from whole blood by Hypaque–Ficoll. The leukocytes were suspended in IMDM with 20% fetal calf serum (FCS) and 1% PHA (DIFCO) and incubated for 5 days at 37°C. Following centrifugation, the supernatant was removed, filter-sterilized using a 0.22-μm Millex GV filter unit, and stored at 4°C until tested for optimal stimulating concentration. Large batches of PHA-LCM were stored in small aliquots at −20°C.

PHA-TCM was prepared in essentially the same manner, but T cells were selectively segregated from the other leukocyte subpopulation by E-rosetting before incubation with 1% PHA for 5 days.

PWM-SCM was prepared from a single-cell suspension of mouse spleen cells in IMDM with 10% FCS, 5×10^{-5} M 2-ME, and 0.05 ml of a 1:10 dilution of PWM (GIBCO). The cells were incubated at 37°C in 5% CO_2 for 7 days, then centrifuged to obtain the supernatant, which was filter sterilized and stored in the same manner as the PHA-LCM.

WEHI-CM was prepared by incubating WEHI-3 cells in IMDM with 20% FCS for several days (1×10^5 cells/ml) until the culture became quite yellow. The supernatant was then obtained and handled in the same manner as the PHA-LCM.

Results

Morphological appearance of RF colonies

The RF colonies in the MC/plasma clot cultures appeared and were counted within the first week ("starts"); the final counting of the colonies was performed on day 14 of culture because only those colonies that continued to grow after day 7 developed into extensive multi-branched colonies (Fig. 1A). There was also variability in type and size, and occasionally a large burst-like colony formed. Less than 25% of all colonies were adipocytic on day 7, but by day 14 many of the colonies (up to 80% in some experiments) contained adipocytes (Fig. 1B) as identified by oil red O staining. Such colonies were of the typical reticular form shown in Figure 1A, but a few appeared to form more of a diffuse network (Fig. 1C). On a few occasions, a "mixed" colony, a granulocyte–macrophage colony-forming unit (CFU-GM) colony growing in conjunction with a large RF colony was also observed on day 14 (Fig. 1D). In some cases, it was possible to identify the underlying RF cells as adipocytes, but it was often difficult to do so when the granulocyte–macrophage (GM) colony was very large, obscuring much of the RF colony. Very rarely, an isolated CFU-GM colony was observed (depending on the type or batch of CSF), apparently not associated with an RF colony. All these colonies exhibited a similar morphology to those developing from human bone marrow [11]. Figure 1E–G illustrates that the cells comprising the RF colonies (when transferred to and cultured in liquid medium) produced collagen type IV (Fig. 1F and G), which fibroblasts do not produce; Figure 1E shows that cells do not stain when the primary antibody is missing.

Stimulating factors for CFU-RF

The source and concentration of CSF played a marked role in determining the proportion of pure RF colonies, adipocyte colonies, and mixed colonies. In Figure 2, a comparison of

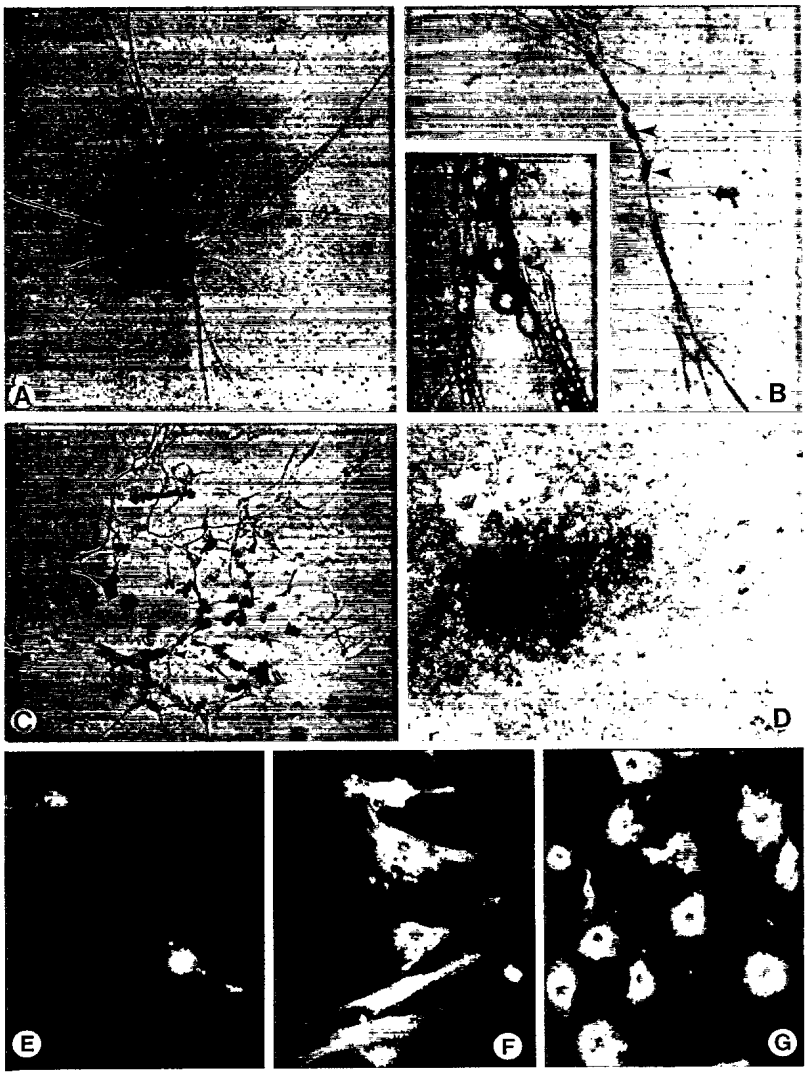

Fig. 1. Morphological appearance of RF colonies in MC/plasma clot culture derived from murine bone marrow (CD-1 mice) on day 14 (photomicrographs at 25× magnification). **(A)** Typical multi-branched RF colony. **(B)** RF colony with adipocyte transformation/differentiation. *Inset:* enlargement of adipocyte RF colony (100×). **(C)** Occasional variant type of RF colony (also adipocyte). **(D)** "Mixed" colony (rare); myeloid colony growing in conjunction with a very large RF colony. **(E–G)** Presence of collagen type IV on RF colony cells transferred to and grown in liquid culture. **(E)** Negative control without primary antibody. **(F** and **G)** Positively staining cells (50×).

the types and concentrations of four different types of CSF is seen. In this experiment, all cultures received fresh medium (MC, IMDM, and 20% BCS) on day 7, so adipocyte formation was negligible for all CSFs. There was a baseline RF colony formation in the absence of CSF. The addition of PHA-TCM (T-lymphocytes), PHA-LCM (leukocytes), or WEHI-CM (WEHI-3 B-lymphoma cell line, known to produce interleukin 3 [IL-3] and granulocyte–macrophage colony-stimulating factor [GM-CSF]) resulted in an increase in the number of RF colonies (day 14) compared to unstimulated cultures; a few CFU-GM colonies were sometimes observed and occasionally some "mixed" colonies (RF plus CFU-GM). PWM-SCM (spleen cells) did not increase RF colony number (colonies were also generally smaller), but it did markedly increase the number of CFU-GM; not unexpectedly, it also resulted in an increased number of RF-mixed colonies at lower concentrations. When these mixed colonies were removed and replated under the same conditions (results not shown), the resulting colonies were of all types: RF, GM, and mixed.

The most effective CSF, giving the largest colonies and the maximum number of RF colonies by day 14 (180 per 10^6 bone marrow cells plated), was derived from leukocytes stimulated with PHA; the optimal concentration was 10%. Therefore, PHA-LCM was chosen as the most appropriate stimulating factor for all subsequent experiments.

A comparison of five different batches of PHA-LCM prepared in our laboratory from human leukocytes (Canadian Red Cross) revealed differences in the capacity of each batch to stimulate growth of RF colonies in culture (duplicate cultures); however, all batches of PHA-LCM consistently increased the number of RF colonies. A concentration of 10% was found to be optimal.

Human citrated plasma for clot formation

The quantity, size, and proportion of RF colonies depended to a certain extent on the sample of bone marrow cells obtained (cells from femurs of four mice were pooled), but more definitely on the combination, source, and batch of stimulating factor (LCM), human citrated plasma, and BCS. In Figure 3, three different batches of platelet-rich human plasma (R, L, and J, from the Red Cross Blood Donor Clinic, Ottawa, Canada) were tested with two different stimulating

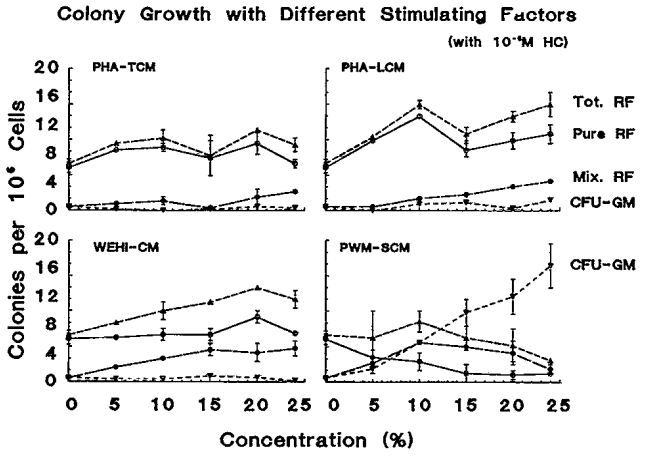

Fig. 2. Comparison of stimulating effect on RF colony growth by various conditioned media (CSF) derived from different cell types: PHA-stimulated human T-cells (*PHA-TCM*) or lymphocytes (*PHA-LCM*); WEHI-3 cell line (*WEHI-CM*); and pokeweed mitogen-stimulated murine spleen cells (*PWM-SCM*). Duplicate cultures; fresh medium added on day 7. Colony type: –▲–, total RF; –○–, RF (pure); –●–, RF (mixed); and –▽–, CFU-GM.

factors (LCM-BL and LCM-406) for the ability to support growth and development of RF colonies. The colonies were examined on days 7 and 14. There was no apparent difference among the plasma batches with respect to the total number of RF colonies grown by day 14 (Fig. 3A) with either of the two LCMs tested.

The colonies counted on day 14 were in most cases already present on day 7, but they had grown larger and many had differentiated into adipocytes (Fig. 3B). There were very few, if any, adipocyte colonies to be found on day 7. The percentage of adipocytes was markedly greater on day 14 than on day 7 in all cases. Plasma R was the most effective plasma for induction of adipocyte transformation with either LCM; with LCM-BL on day 7 about 20% of the colonies were adipocytic, but on day 14 about 80% had become adipocytic. The other plasmas were much less effective in inducing adipogenesis, and LCM-406 was less active than LCM-BL.

The transformation of the colonies into adipocytes by day 14 could be inhibited by refeeding the cultures on day 7, that is, by adding fresh MC, medium (IMDM), and serum (BCS) (Fig. 3C). The effect was similar for all plasmas and LCMs tested, but it was most striking for the combination of plasma R and LCM-BL. However, although adipocyte formation with plasma J was not as strong as with the other plasmas by day 14, refeeding completely prevented adipocytes from forming.

Dependence of RF colony numbers on cell concentration

There was a linear increase in number of CFU-RF colonies per plate with increasing number of bone marrow cells plated (Fig. 4), from 1×10^5 to 8×10^5 cells per plate. The size and type of colonies was fairly consistent in all cultures. The cell concentration used in the experiments reported in this paper ranged from 2×10^5 to 5×10^5 per plate, within the region of linearity for RF colony formation.

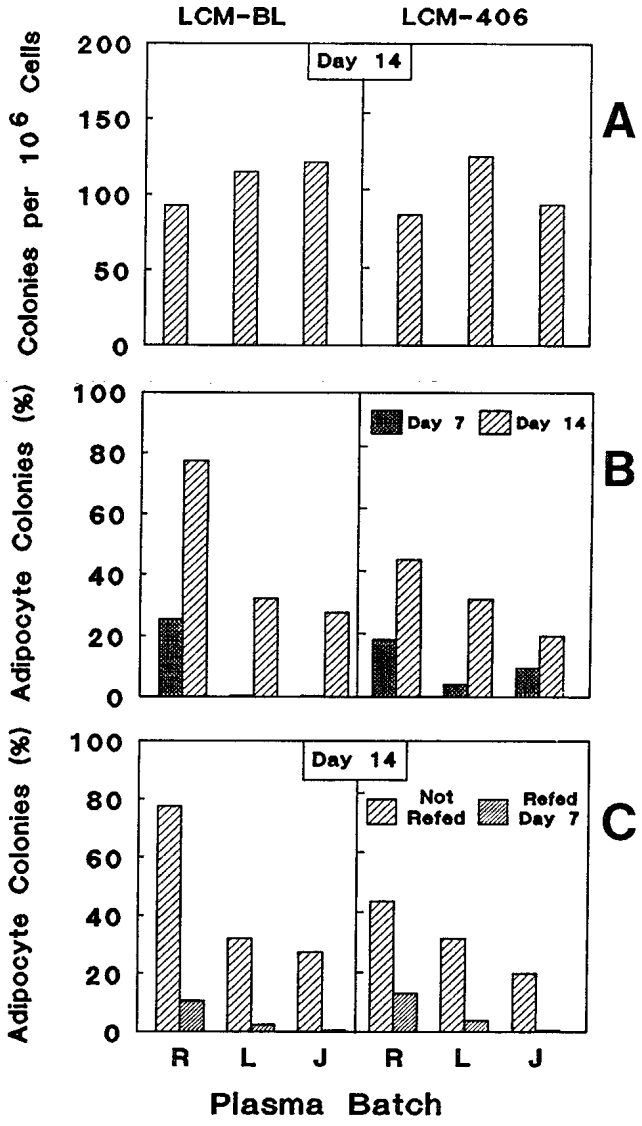

Fig. 3. Growth and development of RF colonies: influence of plasma, LCM, time, and addition of fresh medium. Triplicate cultures. (**A**) Three different batches of human citrated plasma did not differ in their ability to affect formation, nor did either batch of LCM. (**B**) Transformation of RF colonies into adipocyte colonies was influenced by both plasma batch and LCM, mostly between days 7 (*shaded*) and 14 (*striped*). (**C**) Adipocyte transformation (*striped column*) was strongly inhibited by refeeding cultures with fresh complete medium on day 7 (*shaded column*) for all plasma and LCM batches tested.

Discussion

We have demonstrated that there is a precursor of the RF type of stromal cell in murine bone marrow. Its growth requires MC, clotted human plasma, and PHA-LCM as stimulator. The characteristics of murine RF cells match those of the human RF cells described by Lim et al. [11], based on morphological appearance and production of type IV collagen. Myeloid colonies were rarely seen under the culture conditions that were used. However, after 7 days in culture we observed the sudden appearance of large myeloid colonies developing in intimate association with RF colonies. This observation suggests that some RF precursors were associ-

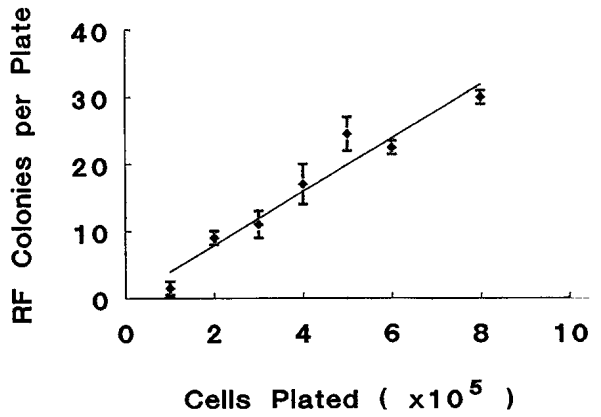

Fig. 4. RF colony number increased linearly with increasing number of bone marrow cells plated, from 1 × 10^5 to 8 × 10^5. Each cell concentration was performed in triplicate.

ated with (perhaps attached to) some myeloid stem cells, and that a well-developed RF was capable of providing growth factors to sustain growth and differentiation of hemopoietic stem cells. It is likely that the growth factor(s) needed for hemopoietic growth was produced locally by the RF colonies and either kept on their membranes or at relatively effective levels in the microenvironment provided by each RF colony. It has been shown that growth factor can be compartmentalized at the membrane levels by sulfated GAGs [22] with variable efficacy [23]. Thus, it is possible that RF colonies can provide an in vitro model for the study of the interaction between microenvironment and hemopoietic stem cell localization and development. In human bone marrow, hydrocortisone also caused a marked increase in the number of RF colonies, but it did not seem to exert such an effect on murine CFU-RF.

In addition to compartmentalized growth factor production, Izaguirre et al. [18], Aye and Izaguirre [24] and Aye [25] have shown that human RF cells release into the supernatant a factor that enhances erythroid burst-forming unit (BFU-E) formation, in the presence of erythropoietin, without stimulating CFU-GM colony formation. Such growth stimulation has also been shown for other stromal cells and other hemopoietic cell lines [25–27], although LTBMCs do not seem to release any detectable CSF [14]; the CSF remains bound to membrane components so that stem cells must come into intimate contact in order to be stimulated [15, 16, 23]. Retention of growth factors in the ECM seems to be necessary for local regulation (modulation) of the numerous stimulation factor concentrations [23, 28]. The RF cells with their long branching arms may play an important role in setting up local environments that differ in the myeloid cell lineage that can be supported. Spooncer et al. [22] have indicated that different sulfated GAGs are distributed in consistent, distinctive patterns in LTBMC and thus may play a role in setting up differing microenvironments in the ECM. Also, growth factors differ in binding affinity to the different GAGs [23], so selective compartmentalization of growth factors may therefore be important for hematopoietic regulation and establishment of different microenvironments. Recently, the genetic defect accounting for the Sl mutation (a microenvironmental defect in hemopoiesis) has been identified [29, 30]. It is due to the lack of a specific growth factor, kit-ligand (KL), coded by the Sl locus. KL is an integral transmembrane protein, perhaps produced by RF cells, that is the ligand for *c-kit* (product of the W locus). Thus, several conditions must be met locally in order that hemopoietic stem cells can grow and differentiate to form mature functional cells, and the RF colony assay may be used to study such functions.

When progenitor cells are not in contact with the stroma, they become vulnerable to terminal differentiation [7, 12, 15] even though soluble stimulatory molecules are present. Damage to this functional environment (e.g., by irradiation) prevents hemopoiesis from continuing until repairs have been effected, and a defective stroma appears to be the cause of the genetically-based anemia in *Sl/Sl^d* mice [8, 31].

In our cultures, once the colonies became adipocytic, proliferation appeared to slow or cease, and the size of colony was maintained. However, most adipocyte colonies remain viable, with no evidence of degeneration or cell death. In liquid cultures that were set up using nonadipocyte RF colonies, the adherent cells continued to grow (with no evidence of adipocyte formation). The addition of fresh medium and serum on day 7 strongly inhibited adipocyte colony development; thus, refeeding may have prevented terminal differentiation of RF cells. Myeloid colonies were seen growing in conjunction with RF–adipocyte colonies, so adipogenesis certainly appears to be compatible with, if not a prerequisite for, hemopoietic stimulation ability.

The development of adipocytes within colonies may be related to terminal differentiation of the RF stromal cells and/or to specialization and onset of production of certain growth factors. Several workers have reported production of stimulation factors in preadipocyte [27, 32] and adipocyte cells [32–34] of the marrow stroma, and Dexter et al. [4] found that in the absence of giant fat cells (with radiating foci and clusters of granulocytes) there was no spleen colony-forming unit (CFU-S) maintenance. Knospe et al. [35] have found evidence that preadipocyte and endothelial phenotypes seem to be necessary for stromal support of trilineal hemopoiesis. Also, foci of hemopoiesis were frequently associated with adipose tissue on cellulose ester membranes (CEM) implanted ip. Although fat cells are virtually absent from active marrow, in LTBMCs their presence is used as the benchmark for hemopoietically productive cultures [15]. Thus, there still seems to be some disagreement as to whether adipogenesis is the signal to start or to cease production of stimulating factors and how this would affect differentiation.

An emerging model of hemopoiesis integrates three essential elements in a single unit—the stem cells, the stromal cells, and the extracellular matrix—with a tight spatial control of growth factor activities [7, 10, 26, 31, 36]. This architectural arrangement of hemopoiesis is likely to be of major significance in the formation of blood cells. The murine stromal colony assay may be a useful in vitro model to test this functional unit: for example, to establish the type of stem cell attached to the stromal cell, the type of proteoglycan and GAG produced by stromal cells, the growth factors retained within the functional unit and the factors released to the supernatant, and the response of the unit to chemicals, radiation, and various physiological stimulators. Thus, the CFU-RF assay may serve as an experimental model for studying hemopoiesis, its damage, and recovery.

Acknowledgments

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