Long-Term Maintenance of Multilineal Hemopoiesis in Collagen Gel Culture

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ABSTRACT. We examined the long-term maintenance of multilineal hemopoiesis in a collagen gel culture of mouse bone marrow cells. When cells were inoculated into the gel, stromal cells formed foci that were composed of sinusoidlike capillary structures, fibroblastic cells, adipocytes and macrophages. Many small hemopoietic foci similar to granulocyte-macrophage colonies (CFU-GM) appeared within a week and disappeared after two weeks. Several large hemopoietic foci appeared after two to three weeks of culture, without a second challenge of marrow cells. These large hemopoietic foci were composed mainly of myeloid cells. Megakaryocytes and mast cells were also observed. When erythropoietin (EPO) was added to the culture at the beginning, the erythroid focus appeared after 3 weeks and the number of megakaryocytes was greater than that in the culture without EPO. However, when EPO was added to the cultures after 6 or 12 weeks, erythroid cells appeared after 1 week and the number of megakaryocytes increased. This hemopoiesis lasted more than 6 months.

Bone marrow stromal cells form a microenvironment suitable for hemopoiesis. They secrete several types of stimulating factors (3, 8, 18) and sustain long-term proliferation and differentiation of hemopoietic cells in Dexter-type long-term bone marrow culture (1, 2, 5, 6, 14). Morphological investigations of this culture revealed that hemopoietic cells are located preferentially beneath the layer of fibroblastoid and endothelioid cells (1, 2, 6, 14), and adhere to macrophages (6, 14).

Stromal matrix also plays an important role in the proliferation and differentiation of hemopoietic cells (4, 19). Three-dimensional gels provide proven matrices for the examination of morphogenesis in vitro. Several cell types have shown three-dimensional organoid growth in collagen gel (10, 12, 16). In bone marrow culture, stromal cell colonies, unlike those in soft agar or methylcellulose, appeared together with granulocyte-macrophage colonies (CFU-GM) in collagen gel (7, 11). Lanotte (9) reported that cells of every lineage, including granulocytes, monocyte-macrophages, megakaryocytes, and cells of erythroid and lymphoid lineages, could be grown using collagen gel culture, provided that specific stimulators were added to the culture. However, until the present study, long-term maintenance of hemopoiesis in collagen gel culture had not yet been performed.

We recently reported that, in the collagen gel culture of bone marrow cells, capillary networks formed by endothelial cells occurred together with the proliferation of fibroblastic cells, adipocytes, and myeloid cells (13). Stromal cells in this culture appeared to be more differentiated than in Dexter-type culture, and the architecture of the stromal cells was similar to that seen in vivo. Further more, in collagen gel culture, it is easy to distinguish cell types, e.g., endothelial cells, fibroblastic cells, or adipocytes, from each other.

In this study, we report the long-term maintenance of granulopoiesis, erythropoiesis, megakaryopoiesis, and mast cells in collagen gel culture together with stromal cells.

MATERIALS AND METHODS

Cells. Bone marrow was obtained by flushing it from mice femora (C3H/He, 6–8 weeks old) with Fisher’s medium. The marrow was then carefully dispersed by passing it through a Pasteur pipet several times.

Collagen gel culture. Collagen gel was prepared as previously reported (14). Briefly, acidic collagen solution was mixed at 4°C with a reconstitution buffer and then with 10× Fisher’s medium (Gibco) to adjust osmotic pressure. The solution was then further mixed with an equal volume of marrow suspension. This mixture was plated onto 35 mm Petri dishes (0.4 ml per well; Corning, NY), and allowed to gel for 15 min at 37°C. Each dish contained approximately 2 × 10⁶ bone marrow cells. The dishes were then overlaid with 2 ml of Fisher’s medium containing 20% horse serum and 1 × 10⁻⁶ M hydrocortisone 21-hemisuccinate and were incubated in a humidified atmosphere of 5% CO₂ in air at 37°C. Half of the medium in the dishes was changed weekly. In some cultures, 2
U/ml of recombinant human erythropoietin (EPO) (Chugai Pharmaceutical, Tokyo) was added at the beginning; in others, it was added after 6 weeks or 12 weeks of culture.

Observations. Cultures were observed under phase contrast microscopy for up to 25 weeks. Every week, 2 gels were fixed with 2.5% glutaraldehyde for 2 hr and stained with toluidine blue. Two gels were cytochemically stained with acetylcholine esterase or alkaline phosphatase. The gels were then observed under light microscopy. Some gels were digested with 0.25% collagenase type I (Sigma, St. Louis), after which the cells were washed by centrifugation. Cytospin preparations of the cells were then stained with Giemsa solution and observed.

For electron microscopy, hemopoietic foci were cut off from truidine-blue-stained gels. They were postfixed with 1% OsO4 for 1 hr, dehydrated through a graded ethanol series and routinely embedded in Epon 812. Ultrathin sections, cut on a Reichert Ultracut E ultramicrotome, were stained with uranyl acetate and lead citrate and observed under a JEM 100CX electron microscope.

RESULTS

As reported in a previous study (14), fibroblastic cells began to migrate and proliferate one to two days after inoculation. Later, some of these fibroblastic cells were transformed into adipocytes. Capillary structures with lumina also appeared after one to two weeks. Four weeks after inoculation, capillaries and fibroblastic cells had formed networks among the adipocytes and hemo-

Fig. 1. a: Phase contrast micrograph of a myeloid hemopoietic focus. Round hemopoietic cells proliferate together with fibroblastic cells, sinusoidalike capillaries and adipocytes (4 weeks of culture). b: Large megakaryocytes together with small myeloid cells. (6 weeks of culture; toluidine blue stain). c: High-power view of a stromal cell focus. Adipocytes are in contact with a capillary network (12 weeks of culture; toluidine blue stain). d: Alkaline phosphatase-positive cells (stained black) are closely associated with myeloid cells (6 weeks of culture; alkaline phosphatase stain).
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hemopoietic cells.

On the contrary, hemopoietic cells formed many clusters, similar to granulocyte-macrophage colonies, one week after inoculation; the majority of these degraded after 2 weeks of incubation. However, several small hemopoietic foci then developed to form a large focus (more than 1 mm in diameter at 3 or 4 weeks). In these hemopoietic foci, hemopoietic cells were intermingled with capillaries, fibroblastic cells and adipocytes (Figs. 1a, 3a). Dozens of large megakaryocytes, which were acetylcholine esterase positive, were observed together with myeloid cells (Figs. 1b, 3b). In the stromal cell foci without hemopoietic cells, the association of sinusoid-like capillaries and adipocytes was observed frequently (Fig. 1c). Alkaline phosphatase (Al-p) histochemistry showed a close association between Al-p positive fibroblastic cells and hemopoietic cells in almost all of the myeloid foci (Fig. 1d). Capillaries were negative for Al-p. Mast cells with metachromatic granules were observed both inside and outside the hemopoietic foci (Figs. 2a, 3c); these mast cells increased in the late phase of the culture. Some hemopoietic foci degraded at different times during the culture period, but new foci appeared and hemopoiesis lasted more than 6 months.

In cultures to which EPO had been added at the beginning of culture, orange-colored erythroid clusters appeared after around 3 weeks (Figs. 2b, 3d). Some of these erythroid clusters grew in the gel, but migrated preferentially on the surface of the gel. They often associated with macrophage to form erythroblastic islands.

![Fig. 2. a: Mast cells with abundant metachromatic granules (4 months of culture; toluidine blue stain). b: Large hemopoietic foci at 6 weeks of culture. Orange-colored clusters are composed of erythroid cells. c: Erythroid cells in capillary lumina (8 weeks of culture; toluidine blue stain). d: Cytospin preparation of cells at 12 weeks of culture. Several myeloid and erythroid cells are seen around a large megakaryocyte (Giemsa stain).]
(Fig. 3d). In this EPO-containing culture, the number of erythroid cells was greater than the number of myeloid cells, and the number of megakaryocytes was greater than that in the culture without EPO. In cultures to which EPO was added at 6 or 12 weeks, the orange-colored erythroid clusters appeared at an sooner stage than in the cultures containing EPO from the beginning; also the number of megakaryocytes was increased. Occasionally, a large number of erythroid cells were observed in the capillary, as shown in Fig. 2c. Myeloid cells and megakaryocytes were also observed in the capillary. Figure 2d shows the cytospin preparation of collagenase-digested culture at 16 weeks of culture. Large mature megakaryocytes were observed together with myeloid and erythroid cells. This hemopoiesis also lasted more than 6 months.

**DISCUSSION**

In this study, we found that megakaryocytes, myeloid cells, erythroid cells, and mast cells actively proliferated for a long period. On the other hand, in conventional Dexter-type long-term culture of bone marrow, predominantly myeloid cells proliferated with stromal cells (1, 2, 5, 6, 14). When EPO was added to Dexter-type culture, erythropoiesis also occurred with myeloid cells (1, 15), but megakaryocytes were not consistently observed. Williams et al. (20) reported that megakaryocytes were observed in the stromal cell layer that was produced by femur bone marrow cells which were not
broken up. However, megakaryocyte production persisted for only up to 6 weeks in their culture. They postulated that the relatively short period of megakaryopoiesis might be due to the loss of some general function in the adherent cell populations.

Campbell and Wicha (4) reported that the extracellular matrix (ECM) from bone marrow stimulated stromal cell adhesion and the proliferation and differentiation of hemopoietic progenitor cells. They further suggested that ECM stimulated stromal cells to produce soluble factors. In our study, multilineal hemopoiesis was maintained for more than 6 months without a second challenge of marrow cells. This indicates that collagen gel provides a suitable environment similar to that of ECM for the proliferation and differentiation of bone marrow stromal cells, i.e., differentiation such as the formation of sinusoid-like capillaries. These stromal cells provide a suitable “niche” for hemopoietic cells and will also excrete more hemopoietic stimulators.

The addition of EPO induced erythropoiesis and an increase in the number of megakaryocytes. Oddos et al. (15) reported the appearance of erythropoiesis when EPO was introduced together with normal murine serum or with frequent changes of medium in Dexter-type culture, but they did not report megakaryocytepoiesis. The appearance of erythroid cells occurred faster when EPO was added 6 or 12 weeks after culture began than when EPO was added at the beginning, probably because of the already well-developed stromal cells which stimulated erythropoiesis. The increase in megakaryocyte numbers was due to the stimulating effect of EPO on megakaryopoiesis (17).

Morphological studies in Dexter-type cultures have demonstrated interactions between hemopoietic cells and stromal cells (1, 2, 5, 6, 14). However, in Dexter-type cultures, it is very difficult to distinguish fibroblastic and endothelial cells because these cells are flat and endothelial cells are difficult to distinguish fibroblastic cells, as shown in Fig. 3a. These features make it very easy to examine the interaction of hemopoietic cells and stromal cells.

As mentioned above, collagen gel culture provides more suitable conditions for hemopoiesis than does Dexter-type culture. Furthermore, collagen gel culture provides a better tool for studying hemopoietic cell-stromal cell interactions and migration of hemopoietic cells into sinusoids.

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