Morphological and Ultrastructural Study of Megakaryocytopoiesis in Liquid Cultures of Hamster Spleen

By Chandra Choudhury, Irena Katsnelson, and Eugene Arnold

A long-term liquid culture system of hemopoietic tissue derived from adult hamster spleen has been described. These primary liquid cultures can maintain stem cell proliferation and differentiation for more than three months without secondary repopulation. A characteristic of the liquid cultures is the formation of clusters of hemopoietic cells around adherent stromal cells. Some islands were composed exclusively of megakaryocytes and adherent cells. Isolation of these clusters of differentiating megakaryocytes and their adherent cellular substrate permitted the analysis of the morphological and ultrastructural features of the interaction between cells of megakaryocytic lineage with the adherent stroma.

NUMEROUS studies have shown that an adequate hemopoietic microenvironment is necessary for hemopoietic stem cell differentiation. A liquid culture system that provides the best in vitro method to study this phenomenon has been described. Studies have shown that the rodent spleen provides an adequate microenvironment for hemopoietic stem cell differentiation. Other studies concerned specifically with megakaryocytopoiesis have reported that splenic stroma supports megakaryocyte colony formation in addition to erythroid and granulocytic colonies.

A long-term liquid culture system using adult hamster spleen has been described by Arnold et al., which maintains proliferation and differentiation of monocytes, myeloid, and megakaryocytic cells for more than four months. Typically, these liquid cultures produce supernatant cells and adherent cells analogous to the hemopoietic cell producing islets described by Dexter et al. We have used liquid cultures of adult hamster spleen to study the relation of megakaryocytes with adherent stromal cells.

MATERIALS AND METHODS

Cell Cultures

Female Syrian hamsters 6 to 8 weeks old of the LSH or F1D strain (Trenton Labs, Bar Harbor, Me), were used in these experiments. Spleen cell suspensions were prepared by teasing tissue placed in a Petri dish containing RPMI 1640 (GIBCO, Grand Island, NY). Ten million pooled spleen cells were inoculated into 25-cm² plastic flasks (Corning), which contained 5 mL RPMI 1640, supplemented with 20% horse serum (Flow Labs, McClean, Va) and gentamycin 10 mg/L of medium (Scherer Pharmaceutical Corp, Kenilworth, NJ). In some of the cultures, cloning coverslips (Belloco, Vineland, NJ) were placed for isolation of hemopoietic cell islets. Cultures were incubated at 37 °C in a fully humidified atmosphere of 5% CO₂ in air.

Feeding of the cultures was accomplished by removal of half of the growth medium (2.5 mL) along with suspended cells, followed by replacement with an equal volume of fresh medium. The cultures were fed once a week.

Cell Counts and Morphology

The cells that had been removed from the liquid cultures during feeding were counted in a hemocytometer. Cytospin preparations (Cytospin, Shandon Southern Instruments Inc. Sewickly, Pa) were made and stained by Wright’s stain for morphological assessment and for acetylcholinesterase (AchE) to identify megakaryocytes. Differential counts were done on 400 cells derived from eight liquid cultures.

The morphology of the adherent layer was surveyed using a Wild phase contrast inverted microscope. Islets identified as producing megakaryocytes were marked on the base of culture flasks, and further studies were done by staining in situ for AchE or examined by scanning or transmission electron microscopy.

Scanning and Transmission Electron Microscopy

Selected cell islets were fixed in situ in phosphate-buffered glutaraldehyde and removed for examination either by removal of the cloning coverslip or cutting out the region from the base of the culture container. Specimens were processed for transmission electron microscopy (TEM) by further fixation with 2.5% glutaraldehyde in 0.067 cacodylate buffer, followed by buffer washes in cacodylate buffer, with postfixation in 2% osmium tetinoxide in 0.067 mol/L cacodylate buffer. Following fixation, the tissue was dehydrated in graded alcohols and stained with 0.25% uranyl acetate in 75% alcohol. After embedding in Epon, sections of silver light interference were cut on an LKB ultramicrotome and picked up on copper grids and examined in an electron microscope.

For light microscopical studies, and for orientation, sections were cut 4 μ thick and stained with toluidine blue.

Processing for scanning EM was done as for TEM until the dehydration step. Following dehydration in graded acetones, the tissue was taken through critical-point drying and then coated with gold-palladium before examination by a scanning electron microscope.
<table>
<thead>
<tr>
<th>Weeks in Culture</th>
<th>Cells per Culture Supernatant ($\times 10^6$)</th>
<th>Differential Count (%)</th>
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<tr>
<td></td>
<td>Lymphs</td>
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<td>Input</td>
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Differential counts were done on 400 cells derived from eight liquid cultures.

RESULTS

**Supernatant Cells**

In a freshly prepared spleen cell suspension, 80% of the cells were lymphocytes (Table 1). After four to six weeks in culture, few lymphocytes were present. At this time, the cultures were predominantly composed of hemopoietic cells with 4% to 6% AchE-positive megakaryocytes (Table 1).

**Adherent Cells**

Adherent cells appeared on the base of the culture flask within seven to ten days of culture. These cells were comprised of two main types. One was small stellate cells with short cytoplasmic processes as described by Steinman et al. This cell type was not associated with hemopoietic cell clusters. The other type was extremely large flattened cells with multiple long processes. Along these long processes clusters of hemopoietic cells were attached. These cells were similar to the large adherent stromal cells described by Dexter and co-workers.

**Megakaryocytopoiesis**

Two kinds of cell clusters or islets were observed, which eventually were proved, by specific staining, to contain large numbers of megakaryocytes. In one (Fig 1), multiple cell types were present along the processes of the adherent cell, including megakaryocytes. In these islets, there were several contiguous adherent cells forming the base of the cluster. The second variety of cell islet also had associated with it at least one giant cell consistent, by light microscopy, with a megakaryocyte. In this variety of cell islet, a single adherent cell formed the stromal base. In 34 cell islets identified by the above criteria, selected and stained for AchE, more than 95% of the cells were found to be positive, including a number of small cells that were unrecognizable by light microscopy as being megakaryocytes (Fig 2). All of the 34 cell islets tested contained at least some AchE-positive cells.

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**Fig 1.** Cell islands from hamster spleen cell culture showed megakaryocytes (arrow) and cells of other lineage attached to cytoplasmic processes of an adherent cell (phase contrast). (Original magnification ×150; current magnification ×127.)

**Fig 2.** The presence of large recognizable megakaryocytes and small unrecognizable megakaryocytes were confirmed by staining for acetylcholinesterase in situ. (Original magnification ×250; current magnification ×199.)
Selected megakaryocyte cell islets were examined by scanning electron microscopy (Fig 3). The adherent stromal cell was anchored to the surface by a process. Attached to the adherent cell were megakaryocytes. Thin filamentous folds were seen traversing the smooth surface of the megakaryocytes. In ultrathin sections examined by TEM, the earliest stages of the demarcation membrane system (DMS) could be seen as a concentric arrangement of channels in a binucleate megakaryocyte (Fig 4). In larger, more mature megakaryocytes, extensive DMS formation was evident (Fig 5). Some areas of the megakaryocyte cytoplasm with extensive channel formation by the DMS also had dense granules present, similar in appearance to organelles seen in blood platelets (Fig 6). Occasionally, cytoplasmic particles, constricting and breaking off from the body of the megakaryocyte were observed, usually in an area where the cisternae of the DMS were very prominent (Fig 7). Segregation of a small circumferential area of the megakaryocyte cytoplasm by the DMS channel was also observed (Fig 7).

DISCUSSION

The presence of a microenvironment that is conducive for hemopoietic stem cell differentiation is essential. The necessity of an adequate microenvironment has been confirmed in vitro using the two hereditary murine anemias. In cocultures from the W/W^v mouse, which involves a stem cell defect, and the SL/SL^d...
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Fig 7. Cytoplasmic fragments were observed breaking off from the body of the megakaryocyte, usually in an area where the cisternae of the DMS were prominent. An area of the megakaryocyte cytoplasm circumscribed by DMS channel formation was also seen. (Original magnification x 12,500; current magnification x 11,063.)

Mouse, which has a defective microenvironment, has achieved an in vitro correction of the defective hemopoiesis in liquid cultures.

The prolonged hemopoiesis maintained in long-term liquid cultures of adult hamster spleen, without repopulation, indicates the presence of an adequate microenvironment. This microenvironment permits the proliferation and differentiation of hemopoietic cells of all lineages, including a significant number of megakaryocytes. The importance of reasonably consistent levels of megakaryocytes in the supernatants of these cultures lies in the fact that differentiated megakaryocytes have not been demonstrated for extensive periods in other in vitro systems. This suggests that the regulatory factors present in the hemopoietic microenvironment in vivo, which are necessary for stem cell differentiation into megakaryocytic cell lineage, were also present in the in vitro liquid cultures.

Megakaryocyte maturation occurs with nuclear endoreduplication and extensive DMS formation in the cytoplasm. During megakaryocytopoiesis, initiation of DMS occurs after completion of DNA synthesis. Platelet genesis ensues from the cytoplasm of mature megakaryocytes. During feeding, the liquid cultures were depleted of half of the supernatant together with suspended cells and replaced with fresh media. Release of cells from the adherent layer replenished the supernatant, indicative of a reservoir of immature cells on the adherent layer. Light microscopic examination and ultrastructural studies demonstrated the presence of megakaryocytes in all stages of maturation on the adherent layer of the cultures. The intimate association of megakaryocytes with adherent stromal cells suggests that the adherent cells are probably important in the regulation of megakaryocytopoiesis.

Previous reports and the observations described here indicate that an initial event in platelet genesis is fragmentation of cytoplasmic particles from the megakaryocyte. In all probability these particles later give rise to mature platelets. The formation of mature platelets may be dependent on humoral factors. Since mature platelets were not seen in the liquid cultures, it may be inferred that the regulatory role of stromal cells is primarily on the earlier events in megakaryocyte differentiation. This in vitro culture system provides a useful model to investigate the role of cell–cell interactions on hemopoietic progenitor cell differentiation.

Acknowledgment

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References


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