Bone Marrow Matrix Modulation of HL-60 Phenotype

By Sharon D. Luikart, James L. Sackrison, and Charles A. Maniglia

The initiation and maintenance of cellular differentiation for a variety of cell types has been shown to be influenced by the microenvironment. To investigate the influence of bone marrow stroma on leukemic cell differentiation, HL-60 human promyelocytic leukemia cells were grown in the presence of Triton-treated extracellular matrix derived from normal human bone marrow stromal cells. This bone marrow matrix microenvironment had a dramatic impact on the phenotypic expression of this malignant line. HL-60 cellular proliferation, morphology, nonspecific esterase activity, formation of Fc rosettes, and sensitivity to induction by 12-O-tetradecanoyl-phorbol-13-acetate (TPA) were all influenced by the presence of matrix molecules. In contrast, stromal cell-conditioned media did not alter HL-60 phenotype. Thus, HL-60 cells appear to retain responsiveness to a human bone marrow stromal cell-derived matrix despite their ability to grow autonomously. Studies of the interaction of leukemic cells and marrow stroma in vitro may provide important information concerning the regulation of leukemic cell behavior.

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suspension cultures in uncoated flasks, the nonadherent HL-60 cells on stroma appeared to be in close association with the bone marrow matrix, but spontaneously detached with removal of the media.

Assessment of differentiation. After cells were on substrata for 72 hours, suspension and attached cells were combined, and morphological maturation was assessed using Wright-Giemsa staining, nonspecific acid esterase staining, and NBT reduction. Induction of Fc rosettes was measured by modification of the method of Bianco et al. Maturation was also assessed in the presence of 1 nmol/L 12-0-tetradecanoylphorbol-13-acetate (TPA; Chemicals for Cancer Research, Inc, Eden Prairie, MN) and various concentrations of retinoic acid (Sigma).

RESULTS

Since uncontrolled proliferation is a characteristic of leukemic pathophysiology, the effects of bone marrow stroma on the rate of proliferation of HL-60 cells in culture were measured (Fig 1). Bone marrow-derived matrix was capable of modifying HL-60 cell proliferation. Thus, after six days in culture, cell numbers decreased by 54% in matrix-coated flasks compared with uncoated flasks. In contrast, fibronectin, the control substrate, had no significant effect on the rate of proliferation.

Concomitant with the dramatic inhibition of neoplastic cell proliferation, bone marrow stroma also induced morphological changes in HL-60 cells. As can be seen in Fig 2, Wright-Giemsa staining of cells grown in stromal cultures exhibits a reduced nuclear:cytoplasmic ratio, irregular cytoplasmic borders, and cytoplasmic vacuoles. Cells grown on fibronectin were similar in morphology to those on plastic, although vacuolization was only rarely observed. Nonspecific acid esterase slides, which were coded and read by independent observers, showed 86% of cells on stroma were positive, while cells on plastic or fibronectin were essentially negative (Table 1). Another parameter of leukocyte maturation is the induction of Fc rosettes. As can be seen in Table 1, cultivation of cells on matrix enhanced induction of Fc rosettes, whereas control substrata did not. However, with 1,000 µg fibronectin, which exceeds the weight of our stroma (10 µg/cm²), we did observe that 50% of cells formed rosettes. None of the HL-60 cells on the various substrata demonstrated significant nitroblue tetrathiazolium (NBT) positivity.

In order to assess the specificity of the bone marrow stroma, HL-60 cells were also grown on matrix derived from human foreskin fibroblasts. HL-60 cells did not adhere to this matrix and, as shown in Table 1, did not display esterase activity. Although there was some vacuolization of the cytoplasm, the cytoplasmic size and borders appeared less mature than cells on bone marrow stroma (data not shown). However, this matrix did enhance rosette formation, as shown in Table 1.

There have been reports of soluble factors that are capable of influencing HL-60 cell behavior. However, as can be seen in Table 1, media conditioned by bone marrow stroma cells did not alter the morphology, esterase production, or rosette formation of HL-60 cells in plastic culture flasks. Thus, free diffusion of the component of the matrix that is regulating HL-60 phenotype did not take place under our culture conditions. However, nonadherent cells in cocultures of HL-60 and stromal cells displayed the morphology, esterase production, and rosette formation seen with HL-60

Table 1. Percentage of HL-60 Cells Expressing Nonspecific Esterase Activity or Fc Rosette Induction

<table>
<thead>
<tr>
<th>Substrata</th>
<th>Esterase*</th>
<th>Fc Rosettes*</th>
</tr>
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<tbody>
<tr>
<td>Plastic</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Stroma</td>
<td>86</td>
<td>40</td>
</tr>
<tr>
<td>Fibronectin (4 µg/cm²)</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Foreskin fibroblast matrix</td>
<td>4</td>
<td>34</td>
</tr>
<tr>
<td>Stromal cell-conditioned media</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>HL-60/stromal cell coculture media</td>
<td>25</td>
<td>29</td>
</tr>
</tbody>
</table>

*Cells were collected 72 hours after seeding and were assayed. Each value is the average of a minimum of 200 cells scored by two independent observers with <5% variation.
cultures on matrix alone (data not shown). Furthermore, the cell-free media from cocultures was able to alter the phenotype of other HL-60 cells (Table 1). This suggests that the matrix-exposed leukemic cells produced and/or released active factors into the media. Since conditioned media from dimethyl sulfoxide-treated or 1,25-dihydroxyvitamin D$_3$-treated HL-60 cells has been recently reported to induce monocytic differentiation in fresh HL-60 cells, an autonomously-produced HL-60 differentiation factor has been proposed.

With agents such as dimethyl sulfoxide, HL-60 differentiation is an irreversible process with cessation of proliferation once commitment has occurred. Thus, we wanted to determine whether matrix-induced phenotypic changes were reversible on the removal of the matrix. HL-60 cells were grown for three to six days on matrix or plastic substrate. Cells were then removed and resuspended in fresh media in 25-cm$^2$ plastic tissue culture flasks. After 24 hours of incubation, the cell number and adhesion were not significantly different in cultures that were initially grown on matrix or uncoated plastic (data not shown). The morphological changes that occurred during exposure to stroma gradually resolved after several passes on uncoated plastic flasks. Thus, these phenotypic changes are dependent on the continued presence of matrix molecules.

A variety of agents can induce HL-60 cells to exhibit a mature phenotype. To determine the effect of matrix on HL-60 cell sensitivity to inducers, cells were seeded on plastic or matrix in the presence of 1 nmol/L TPA, an inducer of monocytic differentiation. After 72 or 96 hours, differentiation was assessed. As seen in Table 2, the adhesive response revealed synergistic activity of TPA and matrix. The TPA and matrix cultures also demonstrated greater morphologic maturation with more dramatic cytoplasmic changes and more intense esterase staining than cells on matrix alone. In contrast, this dose of TPA had no effect on cells in uncoated plastic culture flasks. However, the effects of low dose (50, 100, or 200 nmol/L) retinoic acid, an inducer of myeloid maturation, were not enhanced by the presence of matrix.

**DISCUSSION**

A number of observations support the importance of the microenvironment in the regulation of hematopoietic cell maturation. Transplanted marrow fibroblasts are able to generate their own specific hematopoietic environment, with those from red marrow forming hematopoietic ossicles and those from yellow marrow forming fatty ossicles. The molecules of the microenvironment may possibly exert their effects on cell phenotype via interaction with the cytoskeleton. For example, it has been suggested that protein synthesis may be affected by changes in arrangement and organization of the polyribosomes that are associated with cytoplasmic microtubules.

The influence of the microenvironment on neoplastic cells has been reported for a variety of malignant cell types. In hematological malignancy, long-term cultures infected with Friend murine leukemia virus produce immature cells that produce leukemia in recipient mice. However, these cells retain their dependency on the marrow-adherent layer for in vitro growth. The HL-60 cell line grows in suspension culture and does not require growth factors for long-term maintenance, but our results demonstrate that expression of a mature phenotype by HL-60 cells can be influenced by the presence of the marrow stroma. Similarly, Steinberg et al reported an increase in NBT-positive colonies when HL-60 cells were grown in coculture with Dexter cultures of human bone marrow compared to cultures without stromal cells. These investigators suggested that cell-cell contact mediated the effect. In addition, Schölzel and Löwenberg reported expression of mature leukocyte surface antigens by freshly-isolated myeloblasts grown in coculture with adherent cells from a normal marrow. In that study morphologic maturational was incomplete, and leukemic cell replication was stimulated compared with cultures without stroma. However, our results were dependent on intact stromal cells.

The marked differences in HL-60 cells grown on marrow stroma imply these myeloid leukemia cells, despite their autonomous growth in vitro, retain responsiveness to the environment. Other examples of myeloid leukemia cells that no longer require growth factors but are sensitive to physiological inducers of differentiation have been reported. Other malignant cells have loss of additional controls leading to blocks in differentiation, but even these cells may differentiate in the presence of nonphysiological agents that use other maturation pathways. This uncoupling of growth and maturation regulation has been suggested as a cause of leukemia.

The change in sensitivity of HL-60 cells to the monocytic differentiation inducer, TPA, in the presence of matrix may be quite important in considering future in vitro screens for therapeutic efficacy. Similarly, Gospodarowicz et al found that corneal epithelial cells maintained on collagen were sensitive to epidermal growth factor, but those grown on plastic were not. We plan to investigate further the effect of bone marrow matrix on the sensitivity of myeloid leukemic cells to a variety of potential differentiation agents.

The precise role of the various components of the complex hematopoietic microenvironment in regulation remains to be determined. In long-term cultures in which adherent stromal cells support hematopoiesis, growth-regulating proteins are not found in large quantities in the media. In the experiments reported here, stromal cell-conditioned media did not alter HL-60 phenotype. Thus, the importance of cell interactions and local control over hematopoietic cell development as opposed to diffusible molecules has been recognized. Whether or not the phenotypic changes seen here are due to a local concentration of regulatory proteins or other compo-

### Table 2. Percentage of HL-60 Cells Adherent to Substrata

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Drug</th>
<th>Adhesions*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plastic</td>
<td>None</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>1 nmol/L TPA</td>
<td>5</td>
</tr>
<tr>
<td>Stroma</td>
<td>None</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>1 nmol/L TPA</td>
<td>50</td>
</tr>
</tbody>
</table>

*Cells were counted 96 hours after seeding. Each value is the mean of duplicate flasks with -5% deviation between flasks, representing one of four experiments with similar results.
ments of extracellular matrix (collagen, laminin, or proteoglycan) is under current investigation in our laboratory. Gordon et al. recently reported that GM-CSF binds to glycosaminoglycan components of the bone marrow matrix leading to selective compartmentalization. However, studies of the effects of recombinant human GM-CSF and G-CSF on HL-60 cells demonstrated no morphological maturation, although membrane antigen expression was altered. Our results also suggest that various characteristics may be influenced by different components, since rosette formation is much less specific than the other markers we have used. Investigations of the phenotype of HL-60 cells grown on defined matrices and on partially degraded marrow stroma are currently underway.

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