Lipid Accumulation and Production of Colony-Stimulating Activity by the 266AD Cell Line Derived From Mouse Bone Marrow

By David L. Hines

The availability of cloned lines of bone marrow stromal cells could facilitate the analysis of their role in hemopoietic cell development. The 266AD cell line was isolated from a colony of lipid-accumulating bone marrow cells growing in a collagen gel. 266AD cells have subsequently been maintained by passage in tissue culture plastic flasks about every 10 days for greater than 10 mo. Subconfluent cultures of cells are fibroblast-appearing, but in confluent cell sheets, prominent foci of lipid-containing cells develop in both uncloned and four separate cloned cell lines. Supernatants from confluent cultures containing lipid-laden cells contain granulocyte-macrophage colony-stimulating activity (GM-CSA) for normal bone marrow cells and can induce differentiation of Abelson virus transformed murine monocytoic leukemia cells. 266AD cells were originally isolated in the presence of hydrocortisone, but hydrocortisone is not necessary for lipogenesis to occur.

Growth of bone marrow cells in a collagen gel matrix provided a way to isolate stromal cells, and the 266AD cell line provides a means to examine the relationships between stromal cell lipogenesis and regulation of granulopoiesis.

MATERIALS AND METHODS

Mice and Cells

BALB/c mice, specific pathogen free, obtained from Charles River Breeding Labs (Portage, Mich.) were brother-sister mated in our facility and used between 6 and 20 wk of age. Mice were killed by cervical dislocation. Femoral bone marrow cells for culture and as a source of GM-CFC were obtained by flushing the marrow cavity with RPMI 1640 medium (Grand Island Biological Company, Grand Island, N.Y., GIBCO) using a 26-gauge needle.

Culture of Bone Marrow Cells in Collagen Gels

Rat tail collagen (type I) solutions and polymerized collagen gels were prepared according to the method of Yang et al. Briefly, ethanol-sterilized rat tail tendons were dissolved in acetic acid (0.034N) to a initial concentration of 5 mg/ml protein. The stock collagen solution was stored at 4°C until use. Gels of collagen were prepared by raising the ph and ionic strength of the stock collagen solution to that of growth medium and then warming the isotonic collagen medium to 37°C to permit polymerization of the collagen fibers. Isotonic collagen medium was prepared by mixing at 4°C in this sequence 1.0 ml 10X concentrated RPMI 1640 medium (containing 10X sodium bicarbonate), 0.5 ml 0.34M NaOH, 1.8 ml horse serum, 1.2 ml fetal calf serum, 0.12 ml 10⁻³M hydrocortisone, and

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0.23 ml 2.5 x 10⁻³ M mercaptoethanol with 7.8 ml of stock collagen solution. Then, bone marrow cells were mixed with the isotonic collagen medium and 3 ml added to 60-mm tissue culture dishes, which were immediately incubated for 20 min at 37°C to allow collagen fiber polymerization. The collagen gel, containing 2.4 x 10⁴ bone marrow cells/ml, was covered with 4.0 ml of complete liquid growth medium (RPMI 1640 supplemented with 15% horse serum (GIBCO), 10% fetal calf serum (K.C. Biologicals, Lenexa, k.s.), 10⁻² M hydrocortisone sodium succinate (Sigma, St. Louis, Mo.), 50 µM 2-mercaptoethanol, and 5 µg/ml gentamycin) and incubated at 37°C in 5% CO₂. The cultures were fed at 4-day intervals by replacement of 3.0 ml of the liquid medium.

Selected colonies of cells were recovered from the gels with the aid of a 4-mm agar punch (Shandon Southern, SAE-3025). A plug of collagen gel containing cells was removed and placed in 0.5 ml of collagenase (Sigma, type II, 150 µg/ml in serum-free RPMI 1640) and digested for 20 min at 37°C with constant shaking. The freed cells were pelleted, resuspended in 0.6 ml of complete growth medium, plated in a 16-mm well of a Costar 24-well tissue culture plate, and incubated at 37°C in 5% CO₂ in air. Attached cells were routinely harvested for transfer by a 2-mm incubation in 0.05% trypsin, 0.02% EDTA (GIBCO). The capacity of conditioned medium and ES to induce the differentiation of the Abelson virus transformed, cloned, promonocyctic leukemia cell lines AC5 and AC8 was tested in a semisolid agar culture system similar to that described by Metcalf for WEHI-3B cells. Culture conditions were as described above for the assay of GM-CSA, except the leukemic cells were cultured at 10⁴/ml. After 7-day incubation at 37°C, all aggregates of 50 or more cells were scored as colonies using a dissection microscope. Colonies were scored as undifferentiated if they consisted of a tight aggregate with no outlying cells. Differentiated colonies were composed either of a loosely aggregated group of cells or a central aggregate of cells with a peripheral halo of dispersed cells. The validity of this scoring method was confirmed by morphological analysis of the cells from individual colonies. Undifferentiated colonies were composed of greater than 90% undifferentiated blast cells. Differentiated colonies contained from 20% to 100% maturing macrophages.

RESULTS

Growth of Bone Marrow Cells in Collagen Gel

Three distinct colony morphologies were seen within 7 days after plating bone marrow cells in collagen gel: (1) compact oval colonies of spherical cells that resembled colonies produced by GM-CFC in soft agar gel in the presence of GM-CSA (although no exogenous source of GM-CSA was added to the collagen gel); (2) stellate colonies of apparent stromal cell origin growing attached to collagen fibers; and (3) stellate colonies with other hemopoietic cells closely associated with them. The stellate colonies were observed at a low frequency and resembled those previously described by Lanotte et al. and Metcalf. The individual cells within the stellate colonies often arranged themselves in linear fashion along collagen fibers, and by 12 days, lipid-accumulating cells were observed in almost 60% of the colonies. Omission of hydrocortisone from the collagen gel reduced the number of stellate colonies that contained lipid-accumulating cells without affecting the frequency of stellate colonies. Most stellate colonies were apparently multicellular in origin, arising from small cellular aggregates. When precautions were taken to culture only very well dispersed single cell suspensions, few stellate colonies were seen, grew slowly, and remained small.

After culture for 14 days, stellate colonies containing lipid-accumulating cells were removed from the collagen gel, and following collagenase digestion, the cells were cultured in plastic tissue culture dishes using complete growth media. Cells resembling fibroblasts, endothelial cells, cells containing lipid, and in some cases a significant number of macrophages, adhered to the plastic. The fibroblastic cells grew more readily than the others, while cells containing multiple lipid droplets appeared not to replicate. Cells from most colonies appeared to have a limited replicative capacity and could not be readily transferred following confluence of the cell sheet. Cells that transferred readily became more homogenous and fibroblast appearing.

The cells derived from one colony, 266AD, were observed at the second passage to begin to accumulate lipid as the culture reached confluence. The 266AD cells have since been subcultured approximately every 10 days for 10 mo by transfer of 1/25 of a 25 sq cm tissue culture flask to fresh media. The cells retain a fibroblast appearance when subconfluent, but upon reaching confluence, some cells begin to accumulate lipid, often in focally discrete areas of the culture. In early passages, the number of lipid-containing cells...
Table 1. Stimulation of GM-CFC Colony and Cluster Formation in Response to Various Sources of GM-CSA

<table>
<thead>
<tr>
<th>Source of GM-CSA</th>
<th>10% Colony*</th>
<th>10% Cluster</th>
<th>5% Colony</th>
<th>5% Cluster</th>
<th>2% Colony</th>
<th>2% Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>L cell CM</td>
<td>85 ± 7†</td>
<td>107 ± 4</td>
<td>73 ± 11</td>
<td>100 ± 18</td>
<td>0</td>
<td>27 ± 5</td>
</tr>
<tr>
<td>266AD CM with hydrocortisone</td>
<td>133 ± 10</td>
<td>162 ± 15</td>
<td>136 ± 13</td>
<td>158 ± 23</td>
<td>3 ± 1</td>
<td>77 ± 9</td>
</tr>
<tr>
<td>266AD CM without hydrocortisone</td>
<td>132 ± 18</td>
<td>150 ± 20</td>
<td>131 ± 19</td>
<td>156 ± 12</td>
<td>26 ± 7</td>
<td>135 ± 15</td>
</tr>
<tr>
<td>Endotoxin serum</td>
<td>Not tested</td>
<td>135 ± 18</td>
<td>161 ± 32</td>
<td>120 ± 18</td>
<td>132 ± 21</td>
<td></td>
</tr>
<tr>
<td>Pokeweed mitogen stimulated spleen cells</td>
<td>66 ± 9</td>
<td>157 ± 18</td>
<td>10 ± 3</td>
<td>164 ± 22</td>
<td>1 ± 1</td>
<td>11 ± 3</td>
</tr>
</tbody>
</table>

†Mean ± standard deviation.

*Colony equals more than 50 cells, cluster equals 10-50 cells.

increases until about 50% of the cells at confluence contain fat droplets. After prolonged passage, the percentage of fat-containing cells decreases. Observation of cell growth following transfer of confluent cultures suggests that fat-containing cells do not replicate.

Production of GM-CSA by 266AD Cells

Supernatants were harvested from confluent 10-day-old cultures of 266AD cells and examined for GM-CSA activity using fresh bone marrow cells. Conditioned medium from 266AD cells (266AD-CM) stimulated the production of a large number of GM-CFC colonies and clusters. The colonies were of both the granulocyte and macrophage type. No stimulation of erythroid, megakaryocyte, or lymphoid colony formation was observed in the 0.3% agar-gel system.

The amount of GM-CSA activity produced by confluent 266AD cells was compared to the GM-CSA activity of conditioned medium from L cells (LC-CM), and endotoxin serum (ES) (Table 1). While 266AD cells were originally isolated with hydrocortisone in the medium to stimulate the rapid appearance of lipid-containing cells, lipid accumulation in confluent cultures is not now dependent on the presence of hydrocortisone. Cultures were initiated at 2.5 × 10⁵ cells/T-25 flask with and without 10⁻⁸M hydrocortisone and refed on days 5 and 9 after plating. On day 10, culture supernatants were harvested and tested for the presence of GM-CSA. Conditioned media from 266AD cells grown with or without hydrocortisone was more stimulatory than LC-CM or PWMSCM at a final concentration of 10%, 5%, and 2% (v/v). At 5% final concentration, 266AD-CM was as stimulatory as ES, but at 2%, 266AD-CM induced significantly fewer colonies than ES.

Clone 266AD Cells Retain Capacity for Lipogenesis and GM-CSA Production

Because of the probable multicellular origin of the collagen gel stellate colony from which the 266AD cells were isolated, they were cloned to determine if GM-CSA production and the capacity for lipid accumulation were conjoint phenotypic characteristics. Cloning was done at the fifteenth passage by dilution plating using 96-well Costar tissue culture cluster plates. Four cloned lines were obtained. Despite some differences in morphology, saturation density, and growth rate, confluent cultures of all 4 clones contained lipid-accumulating cells. Conditioned media from all 4 clones also contained GM-CSA activity when tested on normal bone marrow cells (Fig. 1). Although there are statistically significant differences

![Fig. 1. Comparative stimulation of GM-CFC, both colonies and clusters, by a 10% concentration of conditioned medium from four clones and uncloned 266AD cells. Conditioned medium was from cultures established 10 days previously at 2.5 × 10⁵ cells/T-25 flask. Values represent mean ± standard deviation of 4 replicate cultures.](http://www.bloodjournal.org)
in the amount of GM-CFC stimulation by CM from
the various clones, the biologic significance of these
differences and the relationships between the amount
of GM-CSA activity and parameters such as saturation
density, amount of lipogenesis, and cellular mor-
phology are not yet established.

266AD CM Induced Differentiation of Promonocytic
Leukemia Cells

In addition to inducing proliferation and differentia-
tion of normal GM-CFC, unpurified biologic sources
of GM-CSA may contain substances capable of induc-
ing in vitro differentiation of some human and mouse
myelomonocytic and promonocytic leukemia cell lines.
266AD CM was tested for its ability to influence the
growth and differentiation of two cloned Abelson virus
transformed promonocytic leukemia cell lines (AC5
and AC8). Endotoxin serum was used as a positive
control source of differentiating activity and LC-CM
as a negative control. Both ES and LC-CM have
GM-CSA activity for normal bone marrow cells, but
LC-CM is normally inactive in inducing differentia-
tion of leukemic cells. Both the AC5 and AC8 cell
lines exhibit some spontaneous differentiation in semi-
solid agar cultures, but differ in that ES significantly
increases the frequency of differentiation in AC8 cells
but does not affect the frequency of differentiation of
AC5 cells.

One-thousand AC8 or AC5 cells suspended in 1.0
ml of complete growth media containing 0.3% agar
and 10% 266AD-CM or LC-CM or 2% ES were plated
in 35-mm Petri dishes. After 7-day incubation, colo-
nyes were counted using a dissecting microscope, and
cellular differentiation within the colony scored based
on colony morphology. Similar to WEHI-3B cells,
colony morphology with AC8 and AC5 cells accu-
rately reflects the extent of differentiation of cells
within the colony and the replication capacity of the
cells within the colony (unpublished observations). As
indicated in Table 2, CM from 266AD cells grown in
either the presence or absence of hydrocortisone
increased significantly the percentage of differentiated
AC8 colonies, but had no effect on AC5 colonies. Direc
t addition of hydrocortisone alone to leukemic
cells at the maximal concentration that might be
present in this assay (10^{-8} M) had no effect.

DISCUSSION

The adherent cell layer necessary to maintain active
hemopoiesis in the Dexter long-term bone marrow
liquid culture system is actually a complex multilayer
of cells and consists of several cell types. Determina-
tion of the role of the various cell types in maintaining
hemopoiesis could be facilitated by the availability of
functionally active cloned cell lines. In an attempt to
develop such cell lines, initial efforts were focused on
isolating lipid-accumulating cells, since accurate mor-
phological identification of viable cells was possible
and their association with granulopoiesis provided an
indication of one possible functional role. The three-
dimensional collagen fiber matrix is an excellent sub-
strate for growth of anchorage-dependent normal cells
and has been shown to support in vitro hemopoiesis and
growth of bone marrow stromal cells. The collagen gel
allowed selective removal of stellate-shaped bone mar-
row stromal colonies containing lipid-accumulating
cells. Subsequent culture led to the derivation of the
266AD cell line. When subconfluent, the 266AD cells
are fibroblastic in appearance, but upon reaching
confluence, numerous cells accumulate lipid. Superna-
tant medium from confluent cultures contains a high
concentration of GM-CSA and induces the formation
of macrophage, granulocyte, and mixed macrophage-
granulocyte colonies from normal bone marrow cells.

GM-CSF, the purified active component of crude
substances containing GM-CSA, is required for the clonal
growth in vitro of colonies derived from GM-
CFC and has been implicated in the in vivo regulation
of granulopoiesis. In the Dexter culture system, granu-
lopoiesis typically occurs in close proximity to adherent
cells undergoing active lipid synthesis. Although the

Table 2. Stimulation of Growth and Differentiation of 10^3 Abelson Virus Transformed Promonocytic Leukemia Cells

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>AC8 Cells</th>
<th>AC5 Cells</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Total No. Colonies</td>
<td>Percent Colonies</td>
</tr>
<tr>
<td>None</td>
<td>162 ± 20*</td>
<td>23%</td>
</tr>
<tr>
<td>Endotoxin serum</td>
<td>146 ± 14</td>
<td>82%</td>
</tr>
<tr>
<td>266AD CM with</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hydrocortisone</td>
<td>241 ± 24</td>
<td>93%</td>
</tr>
<tr>
<td>266AD CM without</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hydrocortisone</td>
<td>162 ± 33</td>
<td>48%</td>
</tr>
<tr>
<td>L cell CM</td>
<td>114 ± 14</td>
<td>7%</td>
</tr>
</tbody>
</table>

*Mean ± standard deviation of four replicates.
mechanism of stimulating granulopoiesis in the Dexter culture system is not understood, the production of GM-CSA by a cell line with properties similar to those of the cells associated with granulopoiesis in Dexter cultures suggests GM-CSA may be involved. Initial efforts to detect GM-CSA in the supernatants of Dexter cultures were unsuccessful, and addition of purified GM-CSF or antibody to GM-CSF also failed to affect granulopoiesis.\(^\text{16-19}\) However, recent evidence suggests GM-CSA is produced by the adherent layer cells, but in hemopoietically active cultures, is rapidly consumed by differentiating myeloid cells.\(^\text{19}\) The properties of the 266AD cells suggest that lipid-accumulating cells may be a major source of GM-CSA in the Dexter culture system.

In addition to containing GM-CSA, 266AD CM is able to induce the differentiation of Abelson virus transformed murine promonocytic leukemic cells in vitro. Purification of murine GM-CSF has shown it to be a heterogeneous population of glycoproteins that have distinct biologic activities.\(^\text{10,20}\) In addition to stimulating the in vitro proliferation and differentiation of normal granulocytic and macrophage cells, some GM-CSF species can also induce differentiation of mouse myeloid or myelomonocytic leukemia cells. Different sources of GM-CSA can have varying concentrations of the known GM-CSF species.\(^\text{19}\) Whether the induction of differentiation of leukemic cells is due to the existence of a molecular form (differentiation factor, DF) distinct from all other CSF species is unclear.\(^\text{11}\) However, 266AD CM could serve as a source for purification of both GM-CSF and DF activity.

The derivation of the H-1 cell line from the adherent layer of a 14-wk-old Dexter culture was recently described by Harigaya et al.\(^\text{21,22}\) Like the 266AD cell line, the H-1 cells are fibroblastic appearing, accumulate lipid, and secrete GM-CSF. In contrast to the approach used to isolate the 266AD cells, the H-1 cells were isolated by the sequential passage of an adherent cell layer from a Dexter culture rich in lipid-containing cells. The independent isolation of these two similar cell lines suggests that in the mouse, the marrow fibroblasts or reticular cells that accumulate lipid have an extensive replicative capacity.

There is presently no direct evidence that the 266AD cells are transformed or malignant. Growth in vitro is anchorage dependent, for they will not grow in 0.3% agar. Tumors have not developed during 5 mo observation of syngeneic BALB/c following subcutaneous inoculation of \(5 \times 10^6\) cells, and despite extensive testing, no evidence has been obtained that the cells are producing infectious murine ecotropic, xenotropic, or amphotropic retroviruses (D.L. Hines, unpublished observations).

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Lipid accumulation and production of colony-stimulating activity by the 266AD cell line derived from mouse bone marrow

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