No Stimulative Effect of Adipocytes on Hematopoiesis in Long-Term Human Bone Marrow Cultures

By Ivo Touw and Bob Löwenberg

Long-term cultures of human bone marrow were established for 5–13 wk to study the role of adipocytes in sustaining hematopoiesis. At weekly intervals, the numbers of nucleated cells and granulocyte-macrophage progenitor cells (GM-CFU) in culture were estimated in relation to the numbers of fat-containing cells present in the adherent stroma layer. In these quantifications, the numbers of GM-CFU trapped in the adherent cell layer were considered separately. It was found that the presence of adipocytes did not correlate with more active hematopoiesis. Fat cells appeared at late stages when successful cultures were being exhausted or early in cultures with poor activity. These observations suggest that human marrow continuous hematopoiesis in vitro, unlike hematopoiesis in the analogous murine bone marrow cultures, does not depend on the presence of adipocytes.

THE MAINTENANCE of hematopoiesis in long-term Dexter-type cultures of murine and human bone marrow requires the establishment of an adherent feeder layer derived from marrow stromal cells. The development of this layer is influenced by culture conditions. In the human system, the addition of fetal calf serum (FCS) to the cultures results in the formation of a confluent layer of fibroblast-like cells that does not properly support hematopoiesis. On the other hand, the addition of horse serum (HS) or a mixture of FCS and HS permits the development of a pluriform feeder layer of fibroblast-like cells, macrophages, and adipocytes. In the presence of this composite feeder layer, hematopoiesis can be maintained for many weeks. The exact roles of the different cellular components of the layer have not been determined. For mouse bone marrow, there is evidence suggesting that fat cells have a positive role in supporting GM-CFU proliferation; however, the effects of these stromal elements on human bone marrow have not been elucidated.

Long-term hematopoiesis by human marrow in vitro has generally been inferior to that of the mouse bone marrow cultures. The initial findings of Moore et al. and Hocking and Golde indicated that adherent layers in human Dexter cultures, unlike the murine system, contained only few or no adipocytes. One explanation for the relatively poor maintenance of GM-CFU in human bone marrow cultures is the lack of fat cells in the human cultures.

In this article, we describe experiments designed to clarify the relationship between developing fat cells in the stroma layer and the numbers of GM-CFU in culture. Our results indicate that the appearance of adipocytes is not of benefit in GM-CFU maintenance in long-term cultures of human bone marrow.

MATERIALS AND METHODS

Bone Marrow Specimens

Fresh bone marrow cells were obtained from posterior iliac spine aspirations from patients undergoing a diagnostic marrow puncture. Only marrow samples from patients who were proved to be hematologically normal were used in this study. Aspirates were collected in Hank’s balanced salt solution (HBSS) containing preservative-free heparin. Excess red blood cells were removed by sedimentation (20 min) of the marrow cells at unit gravity in 0.1% methylcellulose.

Establishing Cultures

Nucleated cells (2 × 10^6) were suspended in 8 ml of alpha medium containing 20% HS (Flow, Detroit, Mich.), 10^-4 M hydrocortisone acetate, and antibiotics (will be later referred to as “medium”), and seeded in 25 sq cm tissue culture flasks (Costar, Cambridge, Mass.). Cultures were maintained at 33°C in a humidified atmosphere of 7.5% CO_2 in air. Weekly feeding was done by replacement of 4 ml spent medium containing suspension cells by 4 ml of fresh medium. The removed nonadherent cells were counted and assayed. In other experiments, the adherent cell layer was allowed to develop for 2-4 wk before a second inoculum of autologous bone marrow was added. According to Schaefer et al., 1-2 × 10^7 viable cells, cryopreserved for this purpose on the day of aspiration, were inoculated in culture.

In each experiment, the complete adherent layers of 2-4 flasks were evaluated weekly for the presence of adipocytes by examination of the cultures using an inverted microscope. Fat cells were enumerated as numbers per flask.

Adherent cell layers were stained for neutral fat with Oil Red O after the medium had been removed from the culture flasks. In a couple of experiments, numbers of GM-CFU in the adherent layers were estimated. This was done following collection of these cells with a cell scraper (Costar) after removal of suspension cells. The adherent cells were suspended as single cells by vigorous pipetting through a Pasteur pipette.
GM-CFU Assay

Granulocyte-macrophage colonies were grown in a semisolid agar system with human placental conditioned medium (HPCM) as a source of colony-stimulating activity (CSA). Cells were cultured in plastic tissue culture Petri dishes (Costar: 35 mm) in a 0.2-ml agar overlay (0.25%) on top of a 1-ml basal layer (0.5% agar) containing the HPCM. The culture medium consisted of Dulbecco's modified Eagle's minimum essential medium, HS (6.7%), FCS (6.7%), and trypticase soy broth (6.7%) and was supplemented with dialyzed bovine serum albumin (0.75%), egg lecithin (3 x 10^{-7} M), NaSeO₄ (10^{-7} M), iron-saturated human transferrin (7.7 x 10^{-6} M), and beta-mercaptoethanol (10^{-4} M) as modified from Guilbert and Iscove. The dishes were incubated at 37°C in a humidified atmosphere of 7.5% CO₂ in air for 12 days. Colonies of more than 50 cells were scored by use of an inverted microscope.

HPCM was essentially prepared by the method of Burgess et al. In brief a freshly obtained placenta was cut into small pieces (1 cu cm), which were subsequently rinsed in HBSS to remove the excess red blood cells from the tissue. The pieces of tissue were then mixed in a Waring blender, two times for 1 sec. One milliliter of the mixture was placed in T-75 culture flasks in 20 ml RPMI medium with 5% FCS and incubated at 37°C in a humidified atmosphere and 7.5% CO₂ for 7 days. The conditioned medium was harvested by centrifugation at 20,000 g for 30 min at 4°C and stored in aliquots of 10 ml at 70°C.

One batch of HPCM was used throughout the experiments in optimal concentration (20%), providing standard CSA levels.

RESULTS

The adherent layer was established during the first 2-4 wk of culture. This cell layer was organized as foci consisting of fibroblast-like cells, macrophages, and clusters of small spherical cells. Adipocytes appeared in most of the cultures, although at various times for different bone marrow specimens. They were absent in a minority of the cultures. An example of the morphology of the cultured adipocytes is shown in Fig. 1. These cells stained positive with Oil Red O.

Table 1 gives the results of three experiments in which cryopreserved marrow cells were inoculated in culture after the adherent layer had been formed. The recovery data of GM-CFU and nucleated cells are given without correction for the weekly losses as a consequence of the half-volume medium replacements. In experiment 1, reseeding was done at week 2 in the absence of adipocytes. GM-CFU numbers were maintained in these cultures for several weeks. In experiments 2 and 3, marrow cells were inoculated on the stroma at week 4, while adipocytes were apparent. In the latter two experiments, the adipocytes disappeared from the culture within 1 wk after the reinoculation.
Table 1. Numbers of Nonadherent Cells* and GM-CFU+ in Relation to the Numbers of Adipocytes in Stroma Preestablished Long-Term Cultures†

<table>
<thead>
<tr>
<th>Weeks in Culture After Remobilization</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nucleated Cells (x 10⁴) per Flask</td>
<td>GM-CFU per 10⁴ Cells</td>
<td>Numbers of Adipocytes per Flask</td>
</tr>
<tr>
<td>0</td>
<td>15.50</td>
<td>2790</td>
<td>18</td>
</tr>
<tr>
<td>1</td>
<td>6.00</td>
<td>3196</td>
<td>53</td>
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<tr>
<td>2</td>
<td>1.80</td>
<td>1620</td>
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<td>3</td>
<td>1.80</td>
<td>1547</td>
<td>85</td>
</tr>
<tr>
<td>4</td>
<td>0.77</td>
<td>870</td>
<td>113</td>
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<tr>
<td>5</td>
<td>0.34</td>
<td>401</td>
<td>118</td>
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<td>6</td>
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<td>47</td>
</tr>
<tr>
<td>8</td>
<td>0.16</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>9</td>
<td>1.10</td>
<td>6</td>
<td>6</td>
</tr>
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</table>

*No correction was made for the depletions due to the weekly replacement of half the culture medium volume.
†These continuous cultures were always set up in two stages: Autologous bone marrow cells were seeded onto a preformed adherent layer that had been initiated 2–4 wk earlier from marrow of the same donor.
‡Not present.
ND. Not determined.

Table 2. Numbers of Nonadherent Cells* and GM-CFU+ in Relation to the Numbers of Adipocytes in One-Stage Long-Term Cultures†

<table>
<thead>
<tr>
<th>Weeks in Culture</th>
<th>Exp. 4</th>
<th>Exp. 5</th>
<th>Exp. 6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nucleated Cells (x 10⁴) per Flask</td>
<td>GM-CFU per 10⁴ Cells</td>
<td>Numbers of Adipocytes per Flask</td>
</tr>
<tr>
<td>0</td>
<td>20</td>
<td>4,000</td>
<td>20</td>
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<tr>
<td>1</td>
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<td>4,033</td>
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<td>2</td>
<td>2.73</td>
<td>768</td>
<td>28</td>
</tr>
<tr>
<td>3</td>
<td>0.69</td>
<td>310</td>
<td>45</td>
</tr>
<tr>
<td>4</td>
<td>0.32</td>
<td>202</td>
<td>63</td>
</tr>
<tr>
<td>5</td>
<td>0.15</td>
<td>45</td>
<td>14</td>
</tr>
<tr>
<td>6</td>
<td>0.28</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*No correction was made for the depletions due to the weekly replacement of half the culture medium volume.
†In these experiments, stroma and hematopoiesis were established from a single marrow inoculum.
‡Not present.
This was a common observation in stroma preestablished cultures. In experiment 2, the cultures showed high numbers of GM-CFU per 10^5 cells through 7 wk of incubation in the continued absence of adipocytes, and fat cells appeared only when the cultures were exhausted. In experiment 3, the cultures showed an earlier decrease of GM-CFU per flask and low values per 10^3 nucleated cells, which coincided with the development of adipocytes at week 6. Nucleated cell numbers (i.e., nonadherent) paralleled the pattern of maintenance and decline of GM-CFU in these three experiments. These data indicate that the maintenance of GM-CFU did not depend on the presence of fat cells in the adherent layer.

An estimation of the accumulated production of GM-CFU in experiments 1 and 2 according to Gregory and Eaves and based on the assumption that no significant numbers of progenitors are retained by or released from the adherent layer at any time of culture, would give values of 270%-320% of the inoculated GM-CFU numbers. To verify the validity of this estimation, we measured the numbers of GM-CFU trapped in the adherent layer of successful cultures. In two different experiments, it was found that the numbers of adherent GM-CFU per flask were 20 and 35 at the time of reinoculation, 274 and 15 at week 3, 24 and 90 at week 5. These values represented, respectively, 0.005% and 0.005%, 16.6% and 4%, 6.5% and 15.8% of total GM-CFU numbers (i.e., adherent plus nonadherent) in culture.

Table 2 summarizes the results of three experiments in which the cultures were established in one phase, i.e., without additional seeding after stroma development. In these cultures, GM-CFU numbers always declined rapidly. In experiment 4, adipocytes emerged as early as week 3. In experiment 5, fat cells started to overgrow the culture from week 2 onwards. In both experiments, GM-CFU values per 10^3 cells remained low during total culture time. In comparison, somewhat higher GM-CFU numbers per 10^5 cells were recovered from culture in experiment 6 in which adipocytes were not seen throughout the observation time. Thus, in both reseeded and nonreseeded cultures, no positive effect of fat cells on GM-CFU maintenance was evident.

DISCUSSION

In murine long-term bone marrow cultures, the addition of 10^{-7}M hydrocortisone sodium hemisuccinate to deficient lots of horse serum (not giving rise to adipocyte formation in the feeder layer) led to the differentiation of fat cells from preadipocytes and an improved maintenance of GM-CFU. Thus, it was conceivable that hematopoiesis in the human cultures had the same dependency on the presence of adipocytes. Hydrocortisone-dependent fat cell formation was also observed in human bone marrow cultures. It was further demonstrated that the ability of human stromal cells to support maintenance of GM-CFU and nucleated cells in the long-term cultures was enhanced by hydrocortisone, although it remained uncertain whether this effect was mediated through the formation of adipocytes.

There are reported experimental data, as well, that indicate a negative effect of fat cells on GM-CFU survival in vitro. Blackburn and Goldman demonstrated that bone marrow fibroblast conditioned medium increased the survival of GM-CFU from human bone marrow in short-term (1 wk) liquid cultures. However, a medium conditioned by fibroblasts and fat cells was less active. Our series of experiments in which we investigated the relationship between the presence of adipocytes and the long-term maintenance of GM-CFU provide further evidence that fat cells do not play a significant role in the support of hematopoietic cell proliferation in human bone marrow cultures when numbers of GM-CFU and nucleated cells are taken as end-points. Fat cells grew at early stages in cultures with poor GM-CFU recoveries or emerged during final exhaustion of initially successful cultures.

The data in Table 1 were obtained in our standard experimental design in which autologous bone marrow is reseeded after formation of the stroma layer in culture. Contrary to the findings of Gartner and Kaplan, we have always observed a better maintenance of GM-CFU numbers in this design as compared with the “nonseeded” cultures, i.e., without a preformed adherent layer (Table 2).

We also measured numbers of GM-CFU attached to the adherent layer at different times in culture. Their numbers varied between 0% and ±16% of total GM-CFU. Thus, adherent GM-CFU represent a numerically small fraction of total GM-CFU in culture. One could question whether these low values were the result of inhibition of colony formation in the GM-CFU assay caused by the presence of stromal cells. This may be excluded since no inhibition was evident in coculture experiments of fresh bone marrow cells, and cells from adherent layers added up to 60% of the amount present in one culture flask (data not shown).

The question can be raised of whether adipocytes have an inhibitory influence on hematopoietic maintenance in human Dexter cultures, since their presence was associated with poor GM-CFU recoveries in some
of our experiments. On the basis of the data presented, the possibility of a suppression of GM-CFU numbers in culture by fat cells appears not very likely. In the unsuccessful cultures, i.e., experiment 3 (Table 1) and experiments 4 and 5 (Table 2), low GM-CFU values per $10^3$ cells were found before any fat cells were detectable.

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REFERENCES


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