Controlled Environment Culture of Bone Marrow Explants from Human Myeloma

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SUMMARY

Bone marrow biopsy specimens from patients with myeloma were cultured in either 1 of 2 thin-film culture systems, a controlled environment steady state system or a rocker tube configuration of the system, for periods up to 42 days. Both functional and morphological characteristics of the myeloma cells were well-maintained in these systems. Cytocentrifuge preparations of the culture media disclosed hematopoietic cells that included from 5% to almost 100% plasma cells. Histological examination of the cultured specimens disclosed infiltration of the marrow with myeloma cells. Myeloma proteins were released at a steady rate throughout the period of culture after the 1st 4 days. Bone-resorbing activity was demonstrated in the culture media in 7 of 9 myeloma culture media and was well maintained, particularly during the 1st week of culture. This activity was associated with severe osteolytic lesions in the donor patients and marked infiltration of the cultured specimen by myeloma cells. The potential use of these organ culture systems for the further definitive identification of the factor responsible for bone destruction in myeloma is discussed.

INTRODUCTION

Bone destruction is one of the most common manifestations of multiple myeloma. Recently, it has been shown that the supernatant of the media of cultures of marrow aspirates obtained from patients with multiple myeloma contains an osteoclast-stimulating factor that produces bone lesions in vitro similar to those found clinically in myeloma (11). The bone marrow cells in these experiments were cultured in either an in vitro diffusion chamber or in Falcon plastic tissue culture tubes. Definitive identification of the nature of the myeloma bone-resorbing factor or factors has been limited by the small volumes of media obtained from individual cell cultures and by the requirement for serum supplementation of the medium. Isolation and characterization of this factor are of great importance for understanding the pathophysiology of bone destruction in myeloma and for designing a rational therapeutic approach to combat it. We wish to describe here a culture technique that could achieve this goal. Using a controlled environment rocker culture system (7-9), we have found that bone explants containing bone marrow cells obtained from patients with multiple myeloma could be maintained in culture for up to 6 weeks (longest period of observation). The morphology and function of the original cultured myeloma cells were well maintained and the volumes of spent culture media were greater than those obtained from other cell culture techniques.

MATERIALS AND METHODS

After obtaining informed consent, biopsy specimens of bone were obtained from the posterior iliac crest of patients with myeloma or other neoplastic diseases with a Westermann Jenson needle. These biopsy specimens ranged from 0.3 to 0.6 cm long. The bone explants were cultured in 2 thin-film culture systems (7-9); (a) an automated unit capable of being programmed for proportional media exchange on cultures (this unit will be referred to as the SS" culture system) and (b) a modified Leighton tube equipped with a 10-ml capacity side arm that permitted the culture of a small piece of tissue with maintenance of proper fluid tissue ratio (this unit will be referred to as the RT culture system).

For the RT system, the specimens were quickly and aseptically placed on the flat surface of the modified Leighton tubes. Ten ml of culture media were pipetted into the reservoir of the tubes, which were then closed by silicone rubber stoppers that contained glass tubing ports for gas exchange. The culture tubes were then attached to the tube rocking device and were individually gassed in an incubator at 37°. The tube platforms were automatically rocked with a traverse of 45° for 30 sec of each 3-min cycle. Thus, the film of culture fluid over the explant was exchanged 20 times/hr with media from the reservoir. About a 0.5-ml aliquot of the culture media was aspirated daily. pH measurements of the fluid served as a monitored function and thus indicated time for media renewal in the auxiliary reservoir.

In experiments where specimens were cultured in the SS system, a bone explant was placed in a roller tube that contained 1.5 ml of culture media. The culture tube was connected to the feed and drain manifolds of the tray prior to installation in the roller incubator. In this system about 0.4 ml of the culture media was automatically drained and substituted by an equal volume of fresh media based on a predetermined repetitive cycle of 1 to 6 hr. The time schedule of drainage and feeding was fixed according to the pH

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The abbreviations used are: SS, steady-state; RT, rocker tube; PG, prostaglandin.
change in any particular culture, and more frequent exchange was instituted if the pH dropped by more than 0.1 unit.

The media used in these experiments were McCoy's 5A media (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% horse sera, except for 3 experiments where serum-free media were used. The media were equilibrated with 5% CO₂ in air. For this purpose, a special water-jacketed tonometer held at 37°was used as described elsewhere (7), with periodic addition of 5% sodium bicarbonate solution to achieve a final pH of 7.4. The osmolality of the media was adjusted to 290 ± 4 mOsmole by sodium chloride solution.

In addition to daily pH monitoring, the following tests were performed on the culture media: (a) microscopic examination of cytocentrifuge slide preparations stained with Wright Giemsa stain with each media change; (b) in a selected number of experiments, measurement of human immunoglobulin (IgG, IgA, and IgM) concentrations by the double antibody techniques was performed on the spent media. Highly purified human IgG, IgA, and IgM were labeled with ¹³¹I by the chloramine T method (4). The sensitivity limit of the method is 2 ng/ml.³ (c) Identification of human immunoglobulins by the immunodiffusion technique (12) was performed on concentrated media obtained from two 2-week-old cultures; (d) the supernatant fluid harvested from the bone marrow culture media was assayed for bone-resorbing activity using the bioassay described in detail previously (13, 14, 17). Female rats were given injections of 0.4 mCi of ⁴⁵CaCl₂ (25 Ci/g Ca ICN) on the 18th day of gestation. The following day the rats were killed and the mineralized shafts of the ulnae and radii of each fetus were dissected out and incubated in 5% CO₂ in air at 37° in 0.5 ml of modified Biggers-Gwatkin-Judah medium (13, 14) (Grand Island Biological Co.) to permit exchange of the loosely complexed ⁴⁵Ca with the culture medium. The bones were then cultured in test or control media at 37° for 48 hr in an atmosphere of 5% CO₂ in air. The test media were prepared by adding an equal volume of Biggers-Gwatkin-Judah medium to the supernatant of the myeloma bone culture media, and the control was the same dilution of unused but similar culture media. Bone-resorbing activity was calculated from the percentage of total ⁴⁵Ca released into the medium during the test period and expressed as a ratio of the treated to the corresponding control samples. From each treatment group 4 assays were performed, and means and S.E.'s were calculated using Student's t test. Bone-resorbing activity was considered significant when the treated to control ratio was greater than 1.0 (p < 0.05).

At the conclusion of each culture, the myeloma bone explant specimen was fixed in Zenker's fixative with formalin, decalcified with acetic acid, and embedded in paraffin. Sections were stained with hematoxylin and eosin for microscopic examination. Electron microscope examination was carried out on 1 of the multiple myeloma bone specimens that had been in culture for 21 days.

³ The purified immunoglobulins, the specific rabbit anti-human IgG, IgA, and IgM sera and the goat anti-rabbit y-globulin sera used in these experiments were supplied by Dr. N. Tanigaki of the Department of Immunochmistry at Roswell Park Memorial Institute.

RESULTS

Ph Measurements. The pH measurement was a simple and sensitive indicator of the integrity of the cultures. In specimens cultured in the RT, there was a frequent tendency for the pH to drop by about 0.2 pH units/day from the base line during the 1st 2 to 3 days. Thereafter the pH of the media fluctuated within a narrow range of ±0.1 units as long as the exchange of the media was not delayed beyond 3 to 4 days. Similarly, the change of pH of the spent media in the SS system was used to determine the rate of automatic partial media feed exchange. A more frequent feed exchange was needed if the pH dropped by more than 0.1 units from the base line. Thus, it was possible to maintain the pH of the media at a relatively constant level.

Anoxia due to airway obstruction was often reflected by an elevation in pH, while bacterial contamination was reflected, in most instances, by increased acidity of the culture media. In these experiments, it was found that monitoring the pH appeared to be more sensitive and much simpler than measurement of glucose utilization or lactate production for assessment of the functions and the needs of the cultures.

Studies of Immunoglobulin Released into the Media. Human IgG and IgA were measured by radioimmunoassay in the spent media of 2 myeloma bone cultures. IgG predominated in the media of the bone culture obtained from a patient with κ myeloma, with concentrations maintained between 12 and 18 μg/day after the 4th day of culture, while IgA predominated in the media of the culture from a κα myeloma (Chart 1). The immunoglobulins released into the media of these 2 cultures were also characterized by immunodiffusion. Aliquots obtained on Day 14 of culture were concentrated 30-fold by passage through Amicon filter PM30 and were then tested by immunodiffusion. The results confirmed the preponderance of κ light chain and α heavy chain in the media of κα myeloma culture. IgM, IgG, and IgA levels were also measured by radioimmunoassay in the media of a bone culture obtained from a patient with κμ myeloma whose disease was characterized by osteolytic bone lesions, increased numbers of plasma cells in marrow aspirates, and monoclonal μκ on serum immunoelectrophoresis. The concentrations of IgM in the media of this culture were greater (15 to 20 μg/day) than those of IgG and

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IgA. As found with normal bone cultures, the initial level of the immunoglobulins was much higher than subsequent measurements. However, after this initial drop, there was a steady release throughout the period of observation (up to 42 days) provided the culture conditions remained satisfactory. The initial high concentration of immunoglobulins most probably reflects contamination of the media with the small amount of serum present in the bone culture inoculum.

**Morphological Observations.** Microscopic examination of stained cytocentrifuge slide preparations of cells that shed into the media of specimens cultured in the RT system throughout the culture period disclosed the presence of hematopoietic cells, including plasma cells. The percentage of plasma cells ranged from less than 5% to about 80% and reflected the degree of infiltration of the original bone marrow aspirate by myeloma cells. Although it was not possible to quantitate the number of cells in the cytocentrifuge preparations precisely, the number was higher in the 1st 2 to 3 media exchanges (around 1 week of culture), after which the number of cells declined progressively so that by the 3rd week of culture only a few cells were seen in each cytocentrifuge preparation.

Hematopoietic cells that were shed from bone specimens cultured in the SS system tended to be retained in the culture tubes, and only a few cells were seen on the cytocentrifuge slide preparations of the spent media. On the other hand, examination of media obtained directly from the culture tube continued to disclose the presence of hematopoietic cells, including plasma cells, throughout the period of culture (Fig. 1). Occasional cells in mitosis were seen up to 21 days of culture. The percentage of plasma cells in these preparations ranged from 30% to more than 90%.

Microscopic examination of histological sections of the myeloma bone explant at the conclusion of the culture (usually 21 days) disclosed a good preservation of the bone marrow architecture and a variable but easily recognizable infiltration with plasma cells (Fig. 2).

Electron microscope examination of thin sections of a 21-day-old culture specimen obtained from a patient with multiple myeloma showed both fibroblasts and plasma cells. The latter were frequently found in clusters associated with fibroblasts. The plasma cells contained prominent and often eccentric nuclei and heterogeneous nucleoli. The cells contained large Golgi regions, usually close to the nucleus. Mitochondria, lipid droplets, and small lysosomes were observed in the cell cytoplasm. The main portion of the cytoplasm was filled with rough endoplasmic reticulum that contained a homogeneous electron-dense matrix thought to represent sequestrated proteinaceous products (Fig. 3).

**Bone-resorbing Activity.** The supernatant fluid of the media of 7 of 9 myeloma bone cultures harvested during the 1st week of therapy contained significant bone-resorbing activity (Table 1). Cultures of 6 patients with marked bone-resorbing activity had greater than 20% myeloma cells in the cytocentrifuge specimens. All of these patients had multiple lytic bone lesions. One patient with no radiological bone lesions had less bone-resorbing activity and fewer myeloma cells in the culture medium, and 2 patients with less than 5% myeloma cells in the medium had less severe bone lesions and did not secrete detectable bone-resorbing activity into the culture medium.

<table>
<thead>
<tr>
<th>Patient</th>
<th>% of myeloma cells in marrow aspirates</th>
<th>Skeletal radiology</th>
<th>Wt of cultured bone explant (mg)</th>
<th>% of myeloma cells in cytocentrifuge preparation of media</th>
<th>48-hr Ca release (treated/control ratio)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>86</td>
<td>Multiple lytic lesions</td>
<td>69.5</td>
<td>80</td>
<td>3.14 ± 0.45*</td>
</tr>
<tr>
<td>2</td>
<td>52</td>
<td>Multiple lytic lesions</td>
<td>40</td>
<td>40</td>
<td>2.9 ± 0.38*</td>
</tr>
<tr>
<td>3</td>
<td>36</td>
<td>Multiple lytic lesions</td>
<td>28</td>
<td>25</td>
<td>2.05 ± 0.56*</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>Multiple lytic lesions</td>
<td>56</td>
<td>30</td>
<td>2.5 ± 0.5*</td>
</tr>
<tr>
<td>5</td>
<td>51</td>
<td>Multiple lytic lesions</td>
<td>42</td>
<td>30</td>
<td>2.79 ± 0.48*</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>Multiple lytic lesions</td>
<td>35</td>
<td>20</td>
<td>2.29 ± 0.19*</td>
</tr>
<tr>
<td>7</td>
<td>1.5</td>
<td>Normal</td>
<td>30</td>
<td>&lt;5</td>
<td>1.35 ± 0.3*</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>Few lytic lesions</td>
<td>27</td>
<td>&lt;5</td>
<td>1.12 ± 0.6*</td>
</tr>
<tr>
<td>9</td>
<td>1.6</td>
<td>Osteoporosis</td>
<td>50</td>
<td>None</td>
<td>0.95 ± 0.09</td>
</tr>
</tbody>
</table>

* Values are means and S.E.'s for 4 bone cultures.

b Treated to control ratio greater than 1.0 (p < 0.05).
Culture media of two 48-hr normal bone cultures, 2 bone cultures from patients with lymphosarcoma, and 1 with Waldenstrom’s macroglobulinemia contained no bone-resorbing activity. Seven of 12 acute leukemic bone marrow culture media contained bone-resorbing activity, although the activity was less marked than that seen in the media of most of the myeloma cultures. Only 1 of the acute leukemia cultures had a treated to control ratio greater than 2.0. The mean and S.E. of bone-resorbing activity in the media of the 12 leukemic bone marrow cultures were 1.38 ± 0.09 (p < 0.05).

Bone explants from patients with myeloma were cultured in the SS system without the supplementation of the media with sera. In 1 culture from a patient with myeloma, 55 ml of serum-free media were obtained during 5 days of culture. Potent bone-resorbing activity was present in the media taken during each 24-hr period, and the treated/ control ratio of the spent media from the 5th day of culture was 1.98 ± 0.28 (mean and S.E. for 4 bones). The cytocentrifuge specimen at the end of culture showed clumps of morphologically intact myeloma cells. Similar results in serum-free media were obtained from a pool of 2 bone cultures from 2 other patients with myeloma. After 24 hr of culture, bone-resorbing activity was present in the spent medium (1.98 ± 0.28). After the 4th day of culture, the treated to control ratio was 1.35 ± 0.14 (mean and S.E. for 4 bones).

DISCUSSION

Primary cultures of normal and neoplastic mammalian tissue should aim at achieving maintenance of the morphological characteristics and functions of the cultured cells in vitro. This goal requires the stabilization of optimal nutritional and physical requirements of the cultured cells (5). The controlled environment thin-film rocker culture systems (7-9) offer an approach to this problem. The basic unit has a functional configuration that ensures proper gaseous diffusion coupled with an automatic media feed and/or large enough media volume to maintain proper concentration of nutrients. Provisions are made for frequent monitoring of the culture media for factors that are basic for assessment of the state of the culture, such as chemical, cytological, and pH changes. The demonstration of a steady pH-dependent release of immunoglobulins into the media of normal bone marrow explants in the thin-film culture systems (2) led to this study of human myeloma bone cultures.

The data presented in this study show that myeloma cells in cultured bone explants maintain the morphological characteristics of the original neoplastic cells, the ability to produce myeloma proteins for up to 6 weeks, and the ability to release bone-resorbing activity for at least 1 week. These results compare favorably with previous experience using cells obtained from marrow aspirates grown in Falcon plastic tissue culture tubes, where the morphological characteristics of the cells are not maintained for prolonged periods and bone-resorbing activity is not present in the supernatant fluid after the 1st day of culture (B. G. Durie and G. R. Mundy, unpublished observations). The results in the present study are similar to those described using the in vitro diffusion chamber technique (11) except for 2 differences: (a) much larger volumes of active serum-free media were obtained in the controlled environment method and (b) the bone-resorbing activity produced at each time point was much greater.

Recently, it has been found that cell cultures derived from bone marrow aspirates of patients with myeloma (11) and cultured in tissue tubes or using the in vitro diffusion chamber technique secrete a bone-resorbing factor into the supernatant fluid that stimulates osteoclasts to resorb bone. Similar bone-resorbing activity has been demonstrated in the supernatant fluid of mouse myeloma cell cultures (G. R. Mundy and D. N. Buell, unpublished observations) and lymphoid cell lines derived from patients with a number of hematological neoplasms including myeloma (10). The bone-resorbing factor(s) produced by these cultured cells was assessed by a number of biological and chemical criteria including dose-response curves, behavior on gel chromatography, heat lability, and ultrafiltration and was found to resemble the osteoclast-activating factor produced by phytohemagglutinin-activated peripheral blood leukocytes (6). Other stimulators of osteoclast activity such as PG’s, parathyroid hormone, and active vitamin D metabolites were excluded by immunoassay (parathyroid hormone and PG’s) and extraction in lipid solvents (for PG’s and vitamin D metabolites).

Although the bone-resorbing factor produced by these myeloma cell cultures resembled osteoclast-activating factor secreted by normal activated peripheral blood leukocytes by a number of biological and chemical characteristics, both the authors (10, 11) and the accompanying editorials (1, 16) were careful to point out that these data did not prove that the osteoclast-stimulating factor in myeloma was osteoclast-activating factor or that, in the in vivo situation, osteoclast-activating factor was the only factor involved. Although no role could be found for PG’s in the cell culture systems, PG’s may still have a role either in vivo or in bone explant models. Normal fetal rat long bone cultures have been shown recently to produce PG’s of the E series when cultured with complement-sufficient sera (15). The production of PG’s is inhibited by heat in activation of the sera or by treatment with indomethacin. When C6-deficient sera are used, no PG’s are produced, but when C6 is added, PG’s are again released into the medium. We have preliminary evidence that PG’s are released by the myeloma bone cultures (3), but the concentrations do not correlate well with the bone-resorbing activity found in the supernatants. We also have preliminary evidence that macro-molecular bone-resorbing activity not ascribable to PG’s is present in the supernatants (G. R. Mundy and J. O. Shapiro, unpublished observations).

Further characterization and the definitive identification of the factor responsible for the stimulation of osteoclast activity in myeloma is extremely important for a rational therapeutic approach to this disorder (1). Attempts to define this factor further should be facilitated using the culture system we have described, because, unlike other systems for human myeloma cell cultures, the myeloma cells can be maintained in a healthy condition both morphologically and functionally for sufficient time to permit the collection of
sufficient quantities of serum-free media to make this goal possible.

ACKNOWLEDGMENTS

We are indebted to James Shapiro, Holly Simmons, and Nicholas Christoff for excellent technical assistance.

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Fig. 1. A cytocentrifuge preparation of media of 12-day-old multiple myeloma culture in the 55 system showing a number of myeloma cells. Wright Giemsa, × 970.
Fig. 2. A histological section of a myeloma bone explant cultured for 3 weeks in the RT system. Wright Giemsa. × 970.

Fig. 3. An electron micrograph of a myeloma cell from a bone explant cultured for 3 weeks in the RT system. × 7250.
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