Role of Bone Marrow Stromal Cells in the Growth of Human Multiple Myeloma

By Federico Caligaris-Cappio, Luciana Bergui, Maria Grazia Gregoretti, Gianluca Gaidano, Mirella Gaboli, Marina Schena, Alberta Zambonin Zallone, and Pier Carlo Marchisio

We have verified the hypothesis that multiple myeloma (MM) may be disseminated by circulating clonogenic cells that selectively home to the bone marrow (BM) to receive the signal(s) leading to proliferation, terminal differentiation, and production of the osteoclast activating factors. Long-term cultures of stromal cells have been developed from the BM of nine patients with MM. These cells were mostly fibroblast-like elements, interspersed with a proportion of scattered macrophages and rare osteoclasts. BM stromal cells were CDS+1, produced high levels of interleukin-6 (IL-6) and measurable amounts of IL-1p, and were used as feeder layers for autologous peripheral blood mononuclear cells (PBMC). After 3 weeks of cocultures, monoclonal B lymphocytes and plasma cells, derived from PBMC, developed and the number of osteoclasts significantly increased. Both populations grew tightly adherent to the stromal cell layer and their expansion was matched by a sharp increase of IL-6 and by the appearance of IL-3 in the culture supernatant. These data attribute to BM stromal cells a critical role in supporting the growth of B lymphocytes, plasma cells, and osteoclasts and the in vivo dissemination of MM.

MULTIPLE MYELOMA (MM) is a human B-cell neoplasm characterized by the bone marrow (BM) accumulation of plasma cells that secrete monoclonal immunoglobulins (Ig) and by multiple osteolytic lesions.1,2 The disease is widespread to the skeleton from the earliest recognizable stage, but very few plasma cells are seen in the peripheral blood (PB), where they can easily be appreciated only in the terminal phases of the disease.3 This observation has suggested that human MM, like murine plasmacytoma,1 may be disseminated by clonogenic cells circulating in the PB.1,2 Such a possibility is favored by different lines of evidence, including the presence of circulating B lymphocytes that express the relevant idio- type,4,5 the detection of a monoclonal Ig gene rearrangement within PB mononuclear cells (PBMC),6 and the identification of DNA-aneuploid cells in PB samples.7 Also, plasma cell colonies may be grown from PB7 and patients’ PBMC may be induced to plasma cell differentiation by the synergistic activity of interleukin-6 (IL-6) and IL-3.8

If MM clonogenic cells circulate, they must have the remarkable ability of selectively homing to the BM. In this environment they would receive the signal(s) leading to proliferation, terminal differentiation, and production of the osteoclast activating factors (OAF) that recruit osteoclasts and generate in situ osteolytic lesions.2,9 To verify this hypothesis, we focused our attention on the BM-inductive microenvironment that regulates the differentiation and proliferation of lymphohematopoietic precursors.10 Long-term cultures of stromal cells actively producing IL-6 were developed from the BM of MM patients and were used as feeder layers for autologous PBMC. In this experimental system, monoclonal B lymphocytes and plasma cells, derived from PBMC, as well as osteoclasts developed. Both populations grew tightly adherent to the stromal cell layer and their expansion was matched by a sharp increase of IL-6 and by the appearance of IL-3 in the culture supernatant. These data attribute to BM stromal cells a critical role in supporting the growth of B lymphocytes, plasma cells, and osteoclasts and the in vivo dissemination of MM.

PATIENTS

Nine patients with MM, three men and six women, age 48 to 72, and four patients with monoclonal gammopathy of unknown significance (MGUS), two men and two women, age 50 to 71, were studied. MM patients (Table 1) were classified according to Durie and Salmon.10 Five MM cases were studied at diagnosis, before the initiation of treatment; two who were unresponsive to therapy with Melphalan and prednisone, before starting aggressive chemotherapy; two who were partially responsive to therapy with Melphalan and prednisone, after five cycles of chemotherapy. The MGUS patients studied had a mean follow-up of 5 years. In each patient a BM aspirate and PB samples were concomitantly obtained after informed consent.

MATERIALS AND METHODS

Cells. BM nucleated cells were separated on methylcellulose, washed twice with phosphate-buffered saline (PBS), and cultured immediately. PBMC were separated on Ficoll-Hypaque and frozen in liquid nitrogen. Serum samples were stored at −80°C.

Cell cultures. BM cells were resuspended in Dulbecco medium and 5% horse serum (HS), layered onto coverslips in 24-well culture plates at the concentration of 2 × 10⁶ cells/mL, and cultured at 37°C in 5% CO₂. Every 4 days half of the spent medium was replaced with an equal amount of fresh one. Myeloid and lymphoid cells either did not adhere or progressively detached and were thus removed. A confluent layer of adherent cells was observed after 5 to 7 weeks of culture of MM samples and allowed an experimental plan based on two different steps. First, adherent cells were characterized as for the morphology, phenotype, and cytokine production. Second, when no residual lymphocytes or plasma cells could be identified by staining coverslips with CD19, CD3, anti-γ, μ, α, κ, λ antibodies (Abs; see below) and no Ig could be found in the culture supernatant, autologous PBMC (2 × 10⁶/mL) were thawed, washed twice with PBS, and seeded over the stromal cell layer. The percentage of plasma cells among PBMC was scored on cytopsins on the basis of the morphology and the immunoreactivity with Abs to the monoclonal light chain; B cells were identified as CD19+ cells. The BM-PBMC cocultures were...
performed for 3 weeks at 37°C in 5% CO₂, supplementing the medium with 20% fetal calf serum (FCS) and replacing every 4 days half of the spent medium with an equal amount of fresh one. The 3-week period was arbitrarily selected to give the putative circulating precursors enough time to develop and become detectable.

After 3 weeks of coculture, coverslip-adherent cells were processed for the morphology and phenotype. The cells freely floating in the medium were harvested, cytocentrifuged, and likewise characterized. The cytokines present in the culture supernatant were measured. The following culture controls were performed: (1) BM cell cultures were prolonged for up to 14 weeks in presence of HS without adding autologous PBMC; (2) after the first 7 weeks, BM cell cultures were switched from HS to autologous serum (AS; 5% to 20%) or FCS (20%) without adding PBMC; and (3) FCS (20%) was substituted with FCS (10%) in BM-PBMC cocultures.

**Characterization of cultured cells.** The morphology of cells growing in culture was studied on May-Grünwald-Giemsa (MGG)-stained coverslips. Staining for tartrate-resistant acid phosphatase (TRAP) was performed according to Ly et al. The presence of Ig in culture supernatants was measured with enzyme-linked immunosorbent assay (ELISA).

The phenotype was investigated with immunofluorescence (IF). Polyclonal antihuman γ, μ, α, κ, λ Abs directly conjugated with tetraethylrhodamine-isothiocyanate (TRITC; Dakopatts, Glostrup, Denmark, Cat. N. R 151, R-152, R-153, R-154, R-155) and monoclonal Abs (MoAbs) of the CD3 (Leu 4; Becton Dickinson, Mountain View, CA, Cat. N. 7340), CD14 (MO2; Coulter, Hialeah, FL, Cat. N. 6602141) and CD19 (B4; Coulter, Cat. N. 6602683) clusters were used to characterize lymphoid and monocyte cell populations. CD25 (Becton Dickinson, Cat. N. 7640), which detects the 55-Kd IL-2 receptor, HLA-DR (Becton Dickinson, Cat. N. 6602683) and CD19 (B4; Coulter, Cat. N. 6602141) clusters were used to characterize lymphoid and monocyte cell populations.

**Cytokine production.** IL-1β and IL-6 levels were measured with ELISA (Genzyme, Boston, MA) following the manufacturer's instructions. IL-3 levels were measured with a biologic assay using the megakaryoblastic M-07 cell line, whose proliferation is IL-3 or granulocyte-macrophage colony-stimulating factor (GM-CSF) dependent. In the absence of IL-3 or GM-CSF, cells stop growing within 28 hours; after readidation of the growth factor, DNA synthesis is restored and the peak of proliferative activity is reached 24 hours thereafter. To perform the biologic assay, stock M-07 cells grown in 1 to 5 ng/mL IL-3 were thoroughly washed to remove growth factors and grown at the concentration of 1.5 × 10⁶/mL in Iscove modified Dulbecco's medium (IMDM; GIBCO Europe, Paisley, Scotland) with 5% FCS at 37°C and 5% CO₂. After 28 hours, 5 × 10⁶ cells were resuspended in IMDM medium + 5% FCS and seeded in 200-μL wells in presence or absence of IL-3 (Genzyme). After 24 hours of incubation 1 μCi of [3H]-TdR/well (specific activity: 5 Ci/mmol; Amersham) was added for additional 4 hours and the growth response of M-07 cells was used to design an IL-3 titration curve: the smallest amount of IL-3 that induced proliferation was 0.1 U/mL and the plateau was reached with 2 U/mL. Sera and culture supernatants were added to the cultures instead of IL-3 at 10% concentration, after 2 hours of preincubation at 37°C with 10 μg/mL anti-IL-3 neutralizing Ab (British Bio-technology Ltd, Cowley, Oxford, UK).

**RESULTS**

**Characterization of MM BM stromal cells.** BM adherent cells were mostly CD54 (ICAM-1)⁺ fibroblast-like cells (Fig 1a) interspersed with a proportion of 10% to 15% scattered macrophages; no endothelial cells were found on the basis of von Willebrand factor immunoreactivity. Few large multinucleated cells (Fig 1b) were scattered within the culture; these cells were positively stained with the TRAP reaction, organized cytoskeletal F-actin in podosomes located at the periphery (Fig 1a, at arrow) and displayed receptors for calcitonin (Fig 1c) thus fulfilling the major criteria for identifying osteoclasts. Few calcitonin-positive, podosome-bearing mononuclear cells were also found interspersed with larger cells. The stromal cell features and relative proportions were unmodified by prolonging the cultures for up to 14 weeks; also, the addition of AS (5% to 20%) and the substitution of HS with FCS after the first 7 weeks of culture did not lead to the appearance of lymphoid cells or increase the number of osteoclasts.

In the supernatant of 7-week BM stromal cell cultures high levels of IL-6 and low levels of IL-1β were found, while IL-3 was undetectable (Table 2).

**Characterization of BM-PBMC cocultures.** The proportion of plasma cells among PBMC was 0.03% to 0.5% and the percentage of CD19⁺ B cells was 3% to 6%. After 3 weeks of coculture the morphology showed that two series

<p>| Table 1. Main Features of MM Patients Studied |
|------------------|------------------|------------------|------------------|------------------|</p>
<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>Ig isotype</th>
<th>Stage</th>
<th>% PC (BM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>61</td>
<td>M</td>
<td>Ga</td>
<td>I A</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>70</td>
<td>F</td>
<td>Gc</td>
<td>I A</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>F</td>
<td>Gc</td>
<td>II A</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>F</td>
<td>Ga</td>
<td>II A</td>
<td>23</td>
</tr>
<tr>
<td>5</td>
<td>48</td>
<td>F</td>
<td>Gc</td>
<td>III A</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>49</td>
<td>M</td>
<td>Ga</td>
<td>III A</td>
<td>19</td>
</tr>
<tr>
<td>7</td>
<td>57</td>
<td>M</td>
<td>Ga</td>
<td>IIIB</td>
<td>40</td>
</tr>
<tr>
<td>8</td>
<td>70</td>
<td>F</td>
<td>Ga</td>
<td>IIIB</td>
<td>48</td>
</tr>
<tr>
<td>9</td>
<td>49</td>
<td>F</td>
<td>Gc</td>
<td>IIIB</td>
<td>45</td>
</tr>
</tbody>
</table>

Abbreviations: PC, plasmacells; BM, bone marrow.

*According to Durie and Salmon.¹⁴
of events had taken place: (1) variable proportions of lymphoid cells and plasma cells (Fig 2) were interspersed with and tightly adherent to the CD54+ stromal cell layer and (2) the number of stromal cell-bound giant multinucleated cells had significantly increased (Fig 3). These findings were observed in all nine samples studied, irrespective of the Ig isotype or the stage of the disease. The lymphoid cells and plasma cells expressed in each case the same light chain produced by BM malignant plasma cells (Fig 2b). The number of monoclonal B lymphocytes ranged between 5 and 30 and the number of plasma cells between 2 and 25 in each high power microscopic field. In two selected experiments performed in the absence of AS, the levels of Ig measured in the culture supernatant (Table 2) proved an active secretion of monoclonal Ig. In three of nine cases, sporadic lymphocytes and plasma cells expressing the other light chain were also observed; the mean ratio between monoclonal versus nonmonoclonal cell populations was around 30:1. The cell growth was unaffected by the presence of FCS versus AS. Slight differences in the morphology of developing cells were observed with different culture protocols: with FCS, plasma cells were the prevalent monoclonal B cell population, while with AS, lymphocytes outnumbered the proportion of plasma cells. B lymphocytes

Table 2. IL-1β, IL-3, and IL-6 Levels in Culture Supernatants and Patients’ Sera

<table>
<thead>
<tr>
<th>Case</th>
<th>IL-6 (ng/mL)</th>
<th>IL-1β (pg/mL)</th>
<th>IL-3 (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9†</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(A) Sera from the patients whose stromal cell cultures were studied for the production of ILs; (B) BM stromal cell culture supernatants (7 weeks); (C) supernatants from 3-week-old cocultures between PBMC and autologous BM stromal cells. The data are the mean of experiments performed in triplicate (IL-6 and IL-1β) or in quintuplicate (IL-3).

Abbreviations: U, undetectable; ND, not done.
*Numbers referred to patients detailed in Table 1.
†The levels of monoclonal IgG were measured in the culture supernatants with ELISA: in (B) they were undetectable, in (C) they increased to 9.0 mg/dL (case 7) and 12.7 mg/dL (case 9).
and plasma cells were always admixed with the stromal cell layer and were not found in the culture supernatant, where instead, T cells were floating together with variable (10% to 30%) percentages of macrophages. A proportion of 15% to 40% T cells reacted with CD25 and HLA-DR Abs, thus proving their state of activation. The number of TRAP$^+$ giant multinucleated cells expressing the calcitonin receptor and exhibiting the cytoskeleton organization of osteoclasts was significantly increased in all samples (Fig 3). IL-3 became measurable and IL-6 levels were increased in the supernatant of 3-week-old cocultures, while IL-1β levels were unchanged (Table 2).

Control experiments performed with cocultures of BM cells and PBMC of four patients with MGUS showed no obvious enrichment in plasma cells and osteoclasts. Also, the growth and survival of stromal cells was greatly reduced and IL-6 was undetectable.

**DISCUSSION**

In this work we have developed long-term cultures of stromal cells from the BM of MM patients. The cell populations growing in culture include fibroblasts, macrophages, and bona fide osteoclasts, are CD54$^+$, and produce high levels of IL-6. The addition of PBMC to autologous BM stromal cells supported the growth and final differentiation of malignant B cells of peripheral origin and further promoted the growth of osteoclasts. B cells and osteoclasts were tightly adherent to the stromal cell layer and their expansion was matched by an increased production of IL-6 and IL-3. These events have not been observed in MGUS.

The significance of this work is threefold. First, it demonstrates that clonogenic cells circulate in the PB of MM patients and display their potential when brought in close contact with IL-6--producing BM stromal cells. Malignant plasma cells may express adhesion structures on their surface, like H-CAM (CD44), N-CAM (CD56), and sometimes also the ICAM-1, CD56$^+$ ligand LFA-1. It is thus not unreasonable to suspect that in vivo the physical interaction between stromal cells and malignant B lineage cells may contribute to compartmentalization malignant plasma cells within the BM. In vitro experiments with anti-LFA-1, anti-CD54, and anti-CD56 Abs have to be performed to evaluate the operative role of these molecules in the clonogenic/stromal cell interactions. Further, BM stromal cells from MM actively produce IL-6, while normal BM stromal cells as well as MGUS BM stromal cells do not; normal BM stromal cells produce IL-6 only after activation with inflammatory mediators. Our findings would indicate that MM stromal cells are in a state of activation, thereby differing from both normal and MGUS BM stromal cells. IL-6 has a potent MM growth promoting activity and its production by BM stromal cells lends further credit to the possibility of a paracrine regulation of MM cell growth.

The second point is the feasibility of cultivating in vitro human osteoclasts from the BM of MM patients. The giant cells that we have grown in vitro satisfy the morphologic, cytochemical, and phenotypic criteria of osteoclasts, including the peculiar cytoskeleton organization and the presence of calcitonin receptors. Experiments with coverslips replaced by bone slices are currently underway to define these cells also in functional terms. The number of osteoclasts significantly increased when PBMC were added to the stromal cell layer, while it was unmodified by the mere addition of FCS or AS. This increase may be due to a recruitment of circulating osteoclast precursors or to the addition of cytokine-producing cells facilitating the in situ proliferation of osteoclasts or favoring the differentiation of
osteoclast precursors growing within the BM stromal cell layer.

Finally, our findings point out the network of cells and cytokines that may operate in the dissemination of the disease. The role of some cytokines in the growth of MM B-lineage cells and osteoclasts has already been stressed and led us to concentrate upon IL-1β, IL-3, and IL-6. Besides IL-6, IL-3 also may be involved in the proliferation of MM cells and IL-1β is able to accelerate the growth of MM through IL-6. Further, IL-1β, IL-3, and IL-6 are all active in this culture system. We have observed that BM stromal cells produce IL-1β and high amounts of IL-6 (Table 2); when PBMC are added to autologous stromal cell cultures, IL-6 levels are increased and measurable amounts of IL-3 appear in the culture supernatants. These findings suggest a role for activated T cells of PB origin that might release IL-3 and contribute to the production of IL-6.

In conclusion, our findings attribute to BM stromal cells a critical role in favouring the growth and dissemination of MM. Conceivably, the culture system used in these experiments may be used to identify MM circulating clonogenic cells, to investigate the kinetics of development of B-lineage cells, and to characterize the paracrine milieu operating in the disease, thereby defining the immunoregulatory circuits amenable to therapeutic manipulation.

REFERENCES


'Role of bone marrow stromal cells in the growth of human multiple myeloma

F Caligaris-Cappio, L Bergui, MG Gregoretti, G Gaidano, M Gaboli, M Schena, AZ Zallone and PC Marchisio