Interleukin-1 Beta Rather Than Lymphotoxin as the Major Bone Resorbing Activity in Human Multiple Myeloma

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Human myeloma cells were purified from bone marrow aspirates from four patients having advanced myeloma, including one with common acute lymphoblastic leukemia antigen-positive myeloma. All of these myelomas had marked bone lytic lesions. From the culture supernatants of these purified myeloma cells, bone-resorbing activities were significantly revealed by $^{46}$Ca-release bone resorption assay, and IL-1 activities were also detected by IL-1 bioassay (mouse thymocyte comitogenic assay). Sandwich enzyme immunoassay for IL-1α or IL-1β revealed that IL-1β was responsible for IL-1 activity of these culture supernatants. Furthermore, the bone resorbing activities of these culture supernatants were completely neutralized by pre-treatment of anti-IL-1β, but not anti-IL-1α antibody. By Northern blot analysis, IL-1β mRNA was identified from these myeloma cells. Therefore, it is concluded that myeloma cells produce IL-1β, which acts as bone-resorbing activity in multiple myeloma.

In multiple myeloma, bone lytic lesion is a prominent feature, occurring often in advanced cases. In most of these, bone pain is the commonest presenting symptom. The mechanism of bone resorption in myeloma is still unclear. Mundy et al.1 reported that bone-resorbed surfaces in myeloma were associated with increased osteoclast accumulation adjacent to the myeloma cells.2,3 and they showed that myeloma cells produced an osteoclast stimulating factor (OAF) that could not be distinguished from OAFs produced by normal activated peripheral blood mononuclear cells. In the culture supernatants of normal activated peripheral blood mononuclear cells, several cytokines, such as interleukin-1 (IL-1), tumor necrosis factor (TNF), lymphotoxin (LT), and transforming growth factor β (TGFβ) are responsible for bone resorption in vitro.4,5 Recently, Garrett et al reported that cultured human myeloma cells produced LT, not IL-1, and the secreted LT may be responsible for the bone destruction in myeloma.6

In this paper, in contrast with Mundy et al., we found that IL-1 activity was detected in the culture supernatants of purified myeloma cells, and the bone resorbing activity produced by these myeloma cells was neutralized by anti-IL-1β antibody. We also found that these myeloma cells expressed IL-1β mRNA.

MATERIALS AND METHODS

Patients. Four patients with clinically advanced (stage III) multiple myeloma were studied either before treatment or at least 4 weeks after their last course of chemotherapy. All patients were well-characterized clinically and immunologically, and had marked punched out lytic lesions in the skeletal bones (Table 1). Special note was on myeloma cells from patient no. 4, which expressed common acute lymphoblastic leukemia antigen (CALLA). This patient had more marked multiple lytic bone lesions than the other three also with advanced myeloma. Informed consent was obtained from all patients according to institutional policy.

Myeloma cell culture. Myeloma cells were purified from bone marrow aspirates as described previously.7,8 In brief, bone marrow cells were centrifuged on Ficoll-Hypaque gradient, followed by Percoll (Pharmacia, Upplula, Sweden) discontinuous gradient ranging from 30% to 90% (vol/vol). The cells banding at the interface between 45% and 50% Percoll were collected and subjected to E-rosetting. Non-E-rosetting cells were treated with Lewis Mj antibody (Becton-Dickinson, Sunnyvale, CA) and rabbit complement (Hoechst Behring, Marburg, West Germany). The purified myeloma cells (>95%) were cultured at $1 \times 10^9$/mL in RPMI-1640 medium (Nissui, Tokyo) supplemented with 10% fetal calf serum (FCS, M. A. Bioproducts, Walkersville, MD) and $1 \times 10^{-7}$ mol/L 2-mercaptoethanol (2-ME) for 48 hours.

Bone resorption assay. Bone-resorbing activity was measured by a method described previously.12 In brief, two-day-old mice (ddy-ATD) were injected with $1 \mu$Ci of $^{45}$Ca CaCl$_2$ (Amersham, Buckinghamshire, UK). Two days later the parietal bones were dissected and each bone was incubated in Eagle’s minimal essential medium (Nissui) supplemented with 5% horse serum (GIBCO, Grand Island, NY). Twenty-four hours later, the medium was changed to a fresh one with or without the test substance. A culture was made to continue for the next 48 hours, then bone pieces and the culture supernatants were removed. The radioactivities in the bone pieces and the culture supernatants were measured in a β-scintillation counter (Tri-Carb 460C, Packard, IL). Bone resorbing activity was expressed as the percentage of $^{45}$Ca released or as the ratio of $^{45}$Ca released from the treated bones to the amount released from corresponding control bones. At least six bone pieces were used for each concentration of the tested substance. Statistical significance was evaluated by Student’s t-test.

IL-1 bioassay. Mouse thymocytes were obtained from 4- to 6-week-old C3H/He mice (Clea Japan Inc., Osaka), and cultured in plastic flasks for two hours at 37°C in RPMI-1640 medium supplemented with 10% FCS to remove adherent cells. Nonadherent thymocytes ($1 \times 10^6$ cells/mL) were seeded into the wells of microculture plates (Corning, Corning, NY) in 0.2 mL of RPMI-1640 medium.
medium supplemented with 10% FCS and 1 μg/mL PHA-P (Difco, Detroit). Then 0.05 mL of sample fluid to be assayed was added and the plates were incubated for 72 hours at 37°C. DNA synthesis was measured by adding 1 μCi of H thymidine (3H-TdR, 5 Ci/mmol, Amersham) nine hours before harvesting. One unit per mL of IL-1 activity was defined as the reciprocal of the dilution causing 50% of the maximal response.13

Quantitation of IL-1α or IL-1β by sandwich enzyme immunoassay. The enzyme immunoassays for IL-1α and IL-1β were performed with some modifications.14 Polyclonal rabbit antibodies and monoclonal mouse antibodies specific to human IL-1α and IL-1β were made using recombinant IL-1α and IL-1β as immunogens, respectively, as described previously.14 The immunoplate coated with monoclonal antibody was further treated with blocking solution. Samples were added to each well and incubated at 4°C overnight. The plates were washed and polyclonal rabbit antibodies specific to IL-1α or IL-1β were added to each well. After an incubation at 37°C for two hours, the plates were washed, horse radish peroxidase-conjugated goat anti-rabbit immunoglobulin antibodies were added to each well, and the plates were incubated at room temperature for two hours. After washing, substrate (0-phenylenediamine) was added to each well. After an incubation at room temperature for ten minutes, 2N H2SO4 was added and absorbance at 492 nm was measured. The lowest detectable levels of IL-1α and IL-1β were 10 pg/mL and 100 pg/mL, respectively.

Bioassay for LT/TNF activity. L.P3 cells (2 × 106), a subclone of L929 cells, were plated to the micromonolayers, and 25 μL of a twofold dilution of the culture supernatants of purified myeloma cells and 25 μL of 4 μg/mL actinomycin D (Makor Chemicals Ltd, Jerusalem) were added as described previously.15 After incubation for 24 hours at 37°C, the cells were fixed with glutaraldehyde and stained with Giemsa. The number of morphologically intact cells in a given area was counted under a microscope. One unit per milliliter of LT/TNFβ activity was defined as the concentration required to give a 0.5 survival ratio when the initial number of target cells was 355/μL and the culture volume was 100 μL.

IL-1 mRNA expression. Total RNA was isolated from myeloma cells after 24-hour culture by a guanidine isothiocyanate method with cesium chloride modification.14 RNA (10 μg) was subjected to electrophoresis through 1% agarose gel containing 6% formaldehyde. After electrophoresis, RNA was transferred to NYTRAN filter (Schleicher & Schuell, Keene, NH). Filters were dried, baked at 80°C for two hours in vacuo, prehydrized at 42°C for 24 hours in prehybridization buffer containing 50% formamide, 5 × SSC, 1 × Denhardt's solution, 10% dextran sulfate, 50 mM sodium phosphate buffer (pH 6.5), 1% sodium dodecyl sulfate (SDS), 0.1 mg/mL denatured salmon sperm DNA, and 0.05 mg/mL tRNA, and hybridized at 42°C for 48 hours in the same buffer containing a heat-denatured plasmid DNA encoding human IL-1β17 radiolabeled with [α-32P]dCTP (3,000 Ci/mmol, Amer sham) by a random primer method. Filters were washed twice in 2 × SSC and 1% SDS at 25°C for 30 minutes and twice in 0.2 × SSC and 1% SDS at 60°C for 30 minutes, and were exposed at −70°C to Kodak XAR film.

RESULTS

Myeloma cells freshly isolated from patients with advanced stage myeloma, having marked bone lytic lesions, were investigated for the production of bone resorbing activities. According to our devised separation procedure,14 myeloma cells were highly purified to more than 95% and contaminated with a very small number of myeloid and erythroid cells. These myeloma cell–rich fractions were cultured for 48 hours and the resulting culture supernatants were harvested for bone resorbing activities and IL-1 activity. As shown in Table 1, all tested myeloma cells produced significant bone resorbing activities (1.56 to 2.22 of treated control ratios). IL-1 activities were also detected by IL-1 bioassay (mouse thymocyte comitogenic assay), although levels of bone resorbing activities did not directly correlate with those of IL-1 biologic activities. These IL-1 biologic activities (thymocyte comitogenic activities) in the culture supernatants were completely neutralized by anti-IL-1β, but not anti-IL-1α antibody14 (data not shown). In sandwich enzyme-linked immunosorbent assay (ELISA), furthermore, it was confirmed that IL-1 biologic activities were derived from IL-1β and not IL-1α molecules (Table 1). However, it is not unusual that the amount of IL-1 measured by ELISA method exceeds or is dissociated from the amount of IL-1 biologic activity by IL-1 bioassay, for antibody to IL-1β used in this paper reacts to the precursor form of IL-1β,14 and the precursor is inactive in IL-1 bioassay.14 On the other hand, no significant LT/TNFβ activities for bone resorption assay were detected in those four patients; in our bone resorption assay, recombinant human LT/TNFβ at the final concentration of 10 U/mL had almost no significant effect on 40Ca-release.20

Next, we investigated whether IL-1β was responsible for the bone resorbing activities in myeloma cell–culture supernatants. We used neutralizing antibodies to recombinant human IL-1α and IL-1β.14 Myeloma cell culture supernatants were pretreated with rabbit antihuman IL-1α antibody or anti-human IL-1β antibody at the final X1,000 dilution of serum at 4°C overnight. Thus, treated fluids at the final X10 dilution were assayed for bone resorption activity. Bone resorbing activity of the culture supernatants could be completely neutralized by pretreatment with anti-IL-1β antibody, not anti-IL-1α antibody (Fig 1). Recombinant human IL-1β (rIL-1β) at the concentration of 10 pg/mL, which is
Culture Condition

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![Graph showing bone resorption assay](image)

Fig 1. IL-1β secreted from myeloma cells had a bone-resorbing activity. Culture supernatants from myeloma cells in patient no. 4 were pretreated with or without rabbit anti-human IL-1α or IL-1β antibody at 4°C overnight. Fluids were assayed at the final X10 dilution for bone-resorbing activity. PTH represents synthetic human parathyroid hormone (PTH)- (1-34) (6,000 U/mg). Columns and bars represent mean ± SE (N = 6), respectively.

Almost equivalent to the concentration of biologic IL-1 activity (1 U/mL = 50 pg/mL) in the X10 diluted culture supernatant of patient no. 4, induced ⁴⁵Ca release as much as the X10 diluted culture supernatant of patient no. 4. Bone resorbing activity induced by rIL-1β 10 pg/mL was also completely abrogated by pretreatment with anti-IL-1β antibody.

To confirm whether myeloma cells could synthesize IL-1β, we used the Northern blot technique to detect the expression of IL-1β mRNA. Total RNA was extracted from myeloma cells after 24-hour culture. As shown in Fig 2, IL-1β mRNAs (1.6 Kb) were detected in myeloma cells from patient no. 1 and patient no. 4 (Lanes 3 and 4, respectively), although the levels of IL-1β mRNA in these myeloma cells were much less than those of LPS-stimulated monocytes (Lane 2).

Therefore, these results show that myeloma cells from patients with advanced stage myeloma, who have marked bone lytic lesions, produce IL-1β. In addition, it is very likely that IL-1β secreted from these myeloma cells has some effects on bone resorption in vivo.

**DISCUSSION**

The data presented in this paper demonstrate that myeloma cells produce IL-1β and the secreted IL-1β may be responsible for bone resorption in myeloma. IL-1β mRNA was detected in myeloma cells, and the bone resorbing activity of the myeloma cell culture supernatants was neutralized by anti-IL-1β antibody. Our results are in sharp contrast with a recent report by Garrett et al., who reported that cultured myeloma cells produced LT/TNFβ, not IL-1, and that these myeloma cells expressed LT/TNFβ and TNFα mRNA, but not IL-1 mRNA. We also examined LT/TNFβ activity in four myeloma cell culture supernatants presented in this paper. In all these culture supernatants, LT/TNFβ activities exhibited 5 U/mL as shown in Table 1. Such levels of LT/TNFβ activity induced no significant ⁴⁵Ca-release in our bone resorption assay. Therefore, our data indicate that, in the culture supernatants of myeloma cells examined in this paper, LT/TNFβ was not responsible for bone resorption; instead, IL-1β could be one of the principal mediators for bone resorption, although other bone-resorbing factors and substances may be released in small amounts by these myeloma cells.

In addition to myeloma, adult T cell leukemia (ATL) is frequently associated with hypercalcemia and thus increased osteolysis. Recent reports showed that primary leukemic ATL cells produced IL-1β and expressed IL-1β mRNA and, in contrast, long-term ATL cells lines produced variable amounts of IL-1α, not IL-1β. However, osteoclastic bone resorption is considered to be mediated by several cytokines, including IL-1α, IL-1β, TNFα, LT/TNFβ, and TGFβ. It is likely that these cytokines synergistically or additively stimulate bone resorption in vivo.

Interestingly, considerable amounts of IL-1 biologic activ-
ility were observed in the myeloma cells of patient no. 4 (Table 1), compared with the other three myelomas tested in this report. Myeloma cells in patient no. 4 expressed CALLA and this patient had severe multiple lytic bone lesions, continuous moderate fever, and advanced renal failure. The increased amounts of IL-1β produced by myeloma cells in this case may account for these features; IL-1β stimulates bone resorption, and is an endogenous pyrogen. IL-1 is also capable of stimulating the proliferation of glomerular mesangial cells, and thus, prolonged cellular activation by IL-1 may lead to progressive glomerulosclerosis and severe renal failure. But, the exact mechanism associated with the striking features in CALLA-positive myeloma remains to be clarified.

This paper, thus, demonstrates that myeloma cells produce IL-1β, and this secreted IL-1β may be responsible for bone resorption in myeloma.

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