THE EFFECT OF BISPHOSPHONATE ON THE OSTEOCLAST-LIKE CELL FORMATION IN A MOUSE BONE MARROW CULTURE

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Abstract.

Bisphosphonates are reported to have an inhibitory effect on bone resorption in vivo and in vitro. The present study examined the effect of bisphosphonate on the formation of osteoclast-like cells in vitro. When mouse bone marrow cells were cultured for 8 days with 10^{-8} M 1\alpha, 25-dihydroxyvitamin D_3 (1\alpha, 25(OH)_2 D_3) numerous clusters of mononuclear and multinucleated cells formed, which stained positive for tartrate-resistant acid phosphatase (TRAP-positive). 1\alpha, 25(OH)_2 D_3 is known to stimulate osteoclast-like cell formation in a mouse bone marrow culture. Adding 1-hydroxyethylidene-1, 1-bisphosphonate (HEBP) inhibited the increased formation of osteoclast-like cells stimulated by this stimulant. A time-course experimental model showed that the number of osteoclast-like cells decreased slightly when drugs were given early in the culture period and decreased markedly when the drugs were given later or continuously in the culture period. These findings suggested that bisphosphonate had an effect on mature stage and significantly inhibit bone destruction by inhibit osteoclast-like cells formation. The amount of PGE_2 production stimulated by 1\alpha, 25(OH)_2 D_3 was dose dependently higher with HEBP and 3-amino-1-hydroxypyropylidene-1, 1-bisphosphonate (APD). Showing that PGE_2 production is high at the end of culture when the cells are going to undergo apoptosis. This showed in part, the known bone-resorbing activity of these agents.

Introduction

The bisphosphonates are a new class of drugs that have been developed in the past two decades for use in various diseases of bone, tooth, and calcium metabolism. The pharmacological action of bisphosphonates is attributed to their inhibition of bone resorption. Clinical application in medicine has focused in patients with increased bone destruction such as osteoporosis, Paget's disease, hypercalcemia of malignancy and tumoral bone destruction. The bisphosphonate present a most interesting new develop
ment in the field of the treatment of bone disease.

Research on the effects of bisphosphonates in vivo showed degradation of both bone and cartilage and thus arrests the bone remodeling. Bisphosphonates also block bone resorption induced by various means in organ culture. Using a precipitation and dissolution of calcium phosphate in vitro and in vivo, it was found that bisphosphonate inhibits the bone resorption. However, this system only allowed examination of net effects on bone resorption, as the results were based on changes in the release of $^{45}$Ca. The primary mechanism that reduced bone resorption by this drug has not determined yet.

A mouse bone marrow culture system has been developed to study the formation of osteoclast-like cells from their progenitor. To clarify the inhibitory effect of bisphosphonates in the formation of osteoclasts, this present study investigated the effect of HEBP in a mouse bone marrow culture in which multipotential, mouse bone marrow cells proliferate into osteoclast-like cells. Understanding the inhibition process of bisphosphonate could lead to a better future of this drug and bone remodeling itself.

Materials and Methods

Animals and Hormones

Seven to 9-week-old male mice (ddy strain) were obtained from Nippon Bio Supply Center (Tokyo, Japan). $1\alpha$, $25(\text{OH})_2\text{D}_3$ was a generous gift from Teijin Pharmaceutical Co. Japan. All other chemicals and reagents use in this study are of analytic grade.

Bone Marrow Culture

The mice were killed by cervical dislocation and their femurs and tibiae were aseptically removed and dissected free of adhering tissues. The bone ends were cut off with scissors and the marrow cavity was flushed with 1 ml of $\alpha$-minimal essential medium ($\alpha$-MEM, GIBCO Lab., NY, USA) which was slowly injected into one end of the bone using a sterile 25-gauge needle. The marrow cells were collected into tubes, washed twice with $\alpha$-MEM, and cultured in wells containing 0.5 ml a-MEM (1.5 x $10^6$ cells/ml) and 10% fetal calf serum (Filtron, PTY Ltd., Victoria, Australia). The cells were held in 24-well plates (Corning Medical, Midfield, USA). Cultures were fed every other day by replacing 0.4 ml of the old media with fresh media.

The concentrations of HEBP ranged from $10^{-7}$-$10^{-4}$ M. The $1\alpha$, $25(\text{OH})_2\text{D}_3$ (10$^{-8}$ M) was used as bone-resorbing stimulators in this experiments. These chemicals were added simultaneously to the media when it was refreshed every other day. The drugs are added as follows: continuously in the first group (group A), during the first 4 days of culture in the second group (group B), and during the last 4 days of culture in the last group (group C). All cultures were maintained at 37 $^\circ$C in a humidified atmosphere of 5% CO$_2$ in air. After 8 days, cells adherents to the wall surface were rinsed once with ethanol-acetone (50:50 vol/vol) for 1 minute. Culture plates were dried at room temperature for 10 minutes and the cells were stained for tartrate-resistant acid phosphatase (TRAP), using the method described below.

Histochrometry for TRAP

TRAP was used as a marker for osteoclast (6-8). Staining for TRAP was performed according to the modified method of Burstone (9). In brief, the fixed cells were incubated for 20 minutes at room temperature in acetate buffer (0.1 M sodium acetate, pH 5.0). The buffer contained naphthol AS-MX phosphate (Sigma Chemical Co., St. Louis, Mo., USA) as a substrate, 10 mM sodium tartrate, and red violet LB salt (Sigma) as a stain for the reaction product (10). TRAP positive cells appeared as dark red cells under light microscopy.
Prostaglandin E₂ (PGE₂) Assay

PGE₂ was assayed in the supernatants from some cultures using commercially available ¹²⁵I radioimmunoassay (Du Pont Canada Inc., NEN Research Products, Ontario). Culture supernatant (100 ml) was taken for assay immediately before feeding and stored at -20 °C until the assay.

Statistical analysis

All values are expressed as the mean ± SD Statistical significance is determined by Student's t-test. A P values less than 0.05 is considered statistically significant.

Results

Treatment of culture with the bone resorbing stimulant 1α, 25(OH)₂ D₃ (10⁻⁸ M) alone for 8 days caused the appearances of TRAP-positive Multinucleated cells (MNC) (Fig. 1-2). Adding HEBP continuously for 8 days during the culture period (group A) or for the last 4 days of culture (group C) inhibited the total number of TRAP-positive MNC stimulated by 1α, 25(OH)₂ D₃ in a dose dependent manner (Fig. 1). In contrast, there was no inhibitory effect on the 1α, 25(OH)₂ D₃ stimulated TRAP-positive MNC by adding HEBP at 5x10⁻⁶ M for the first 4 days of culture period (group B). Increasing the concentration of HEBP caused inhibition of TRAP-positive MNC but there was no significantly different in group B compared with group A or C.

Counting the large osteoclast alone by adding HEBP on the early period of culture (group B) caused no inhibitory effect (Fig. 2). However, adding HEBP continuously for 8 days or for the last 4 days of culture (group A and C) inhibited the large osteoclast number of TRAP-positive MNC significantly in a dose dependent manner, stimulated by 1α, 25(OH)₂ D₃.

1α, 25(OH)₂ D₃ (10⁻⁸ M) stimulated the production of PGE₂ released into the culture media (Fig. 3). Adding HEBP and ADP continuously in the culture media increased PGE₂ production dose dependently.

Discussion

1α, 25(OH)₂ D₃ was used in this present study to enhance the recruitment of osteoclast-like cells. This compound is known to stimulate osteoclast-like cell formation in a mouse bone marrow culture with specific mechanisms of action. The addition of 1α, 25(OH)₂ D₃ to the present culture increased the formation of TRAP-positive stimulated by this stimulant.

The present finding showed that HEBP strongly inhibited the formation of TRAP-positive MNC more during the later stage (days 5-8) of the culture period than during the earlier stage (days 1-4) indicates that they have a stronger effect on the later stage of osteoclast-like cell formation. Since the differentiation of TRAP-positive precursor cells and TRAP-positive preosteoclasts is reported to occur between days 4 and 6 of this culture system, HEBP may either inhibit the differentiation of precursor cells into TRAP-positive preosteoclasts or the differentiation of TRAP-positive preosteoclasts into TRAP-positive MNC. Since HEBP significantly inhibited large or mature osteoclast-like cell, this drug appears to have stronger inhibitory effect on differentiation rather than proliferation of osteoclast precursor. This finding is in accord with the study by Vitte et al. Since osteoblasts mediate the bisphosphonate inhibition which influence the osteoclast differentiation.

Bisphosphonate act directly on mature osteoclast-like cell, suggested that this drug worked on osteoclast-like cell before the nuclear apoptosis. The inhibitory action of bisphosphonate in this study differs from the inhibitory action of sodium salicylate showed by Soekanto et al. These findings are in accord with the theoretical perspective that during bone remodeling, osteoclasts undergo apoptosis for physiological purpose.

The finding that the amount of PGE₂ stimulated by 1α, 25(OH)₂ D₃ production
Fig 1. Effects of HEBP on the formation of TRAP-positive MNCs induced by 1α25(OH)2D3 (10⁻⁸M). Mouse bone marrow mononuclear cells were cultured with the bone resorbing agents in the presence of HEBP at different concentrations.

Group A was administered HEBP continuously, Group B was administered HEBP only on the first 4 days of culture, Group C was administered HEBP only on the last 4 days of culture.

After 8 days, the number of total TRAP-positive MNCs was counted. Data are expressed as the means ± SD of eight cultures (* P < 0.05 versus bone-resorbing agents treated alone).

Fig 2. Effects of HEBP on the formation of TRAP-positive MNC induced by 1α25(OH)2D3 (10⁻⁸M). Mouse bone marrow mononuclear cells were cultured with the bone resorbing agents in the presence of HEBP at different concentrations.

Group A was administered HEBP continuously, Group B was administered HEBP only on the first 4 days of culture, Group C was administered HEBP only on the last 4 days of culture.

After 8 days, the number of large osteoclast from TRAP-positive MNCs was counted. Data are expressed as the means ± SD of eight cultures (* P < 0.05 versus bone-resorbing agents treated alone).

Fig 3. Increase in PGE₂ production in response to 1α25(OH)2D3 (10⁻⁸M). PGE₂ assayed at the end of the mouse bone marrow culture showed an increase PGE₂ production in response to 1α25(OH)2D3 (10⁻⁸M). PGE₂ production was increased dose dependently by adding 10⁻⁴-10⁻⁵ M of APD and HEBP continuously. Data are expressed as the means ± SD of six cultures (*P < 0.05 versus 1α25(OH)2D3 treated alone).
was dosed dependently higher with bisphosphonate (HEBP or ADP). The mouse bone marrow culture consists of a mixed population of cells, including osteoclast precursors and other bone marrow cells. Among these cells, bone derived stromal cells are known to have the capacity to support osteoclast differentiation. The addition of 1α, 25(OH)2D3 may induce the development of osteoclast by producing cytokines and other substrate(s) that regulate hematopoiesis, also apoptosis of osteoclast itself may increase the PGE2 production. Bisphosphonate proved to be very powerful inhibitors of bone resorption when tested in a variety condition in vitro. Although bisphosphonate as a class have been studied for more than 25 years, their mode of action as inhibitors of bone resorption is still unclear and is probably different for different members of the class. It is showed that PGE2 production is stimulated by 1α, 25(OH)2D3 may explain, in part, the known bone-resorbing activity of these agents.

The present studies showed an inhibitory effect of bisphosphonate on osteoclast-like cell differentiation in vitro. This inhibitory effect was more pronounced when the drugs were given during the later stage of the culture period (days 4-8), suggested that bisphosphonate had an effect on mature stage which was larger than normal osteoclast-like cells. Bisphosphonate had an effect on osteoclast-like cells before the osteoclast nuclear apoptosis. These finding are in accord with the theoretical perspective that during bone remodeling, osteoclasts undergo apoptosis for physiological purpose.

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