Direct stimulation of osteoclastogenesis by MIP-1α: evidence obtained from studies using RAW264 cell clone highly responsive to RANKL

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Abstract

Macrophage inflammatory protein-1α (MIP-1α) is a member of the CC chemokines. We have previously reported the use of a whole bone marrow culture system to show that MIP-1α stimulates the formation of osteoclast-like multinucleated cells. Here we use rat bone marrow cells deprived of stromal cells, and clones obtained from murine macrophage-like cell line RAW264 to show that MIP-1α acts directly on cells in osteoclast lineage. We obtained several types of RAW264 cell clones, one of these clones, designated as RAW264 cell D clone (D clone), showed an extremely high response to receptor activator of NFκB ligand (RANKL) and tumor necrosis factor-α (TNF-α), while the other clone, RAW264 cell N clone (N clone), demonstrated no response to RANKL or TNF-α. Although both clones expressed receptor activator NFκB (RANK) before being stimulated for differentiation, only the D clone expressed cathepsin K when cells were stimulated to differentiate to osteoclasts. MIP-1α stimulated the formation of mononuclear preosteoclast-like cells from rat bone marrow cells deprived of stromal cells. MIP-1α also stimulated formation of osteoclast-like multinucleated cells from the D clone, when these cells were stimulated with RANKL and TNF-α. These findings provide strong evidence to show that MIP-1α acts directly on cells in the osteoclast lineage to stimulate osteoclastogenesis. Furthermore, pretreatment of RAW264 cell D clone with MIP-1α significantly induced adhesion properties of these cells to primary osteoblasts, suggesting a crucial role for MIP-1α in the regulation of the interaction between osteoclast precursors and osteoblasts in osteoclastogenesis.


Introduction

Chemokines, a family of the small cytokines, are known to be chemotactic factors against various types of white blood cells. Each chemokine has four conserved cystein residues and they can be classified into four types: C chemokines, CC chemokines, CXC chemokines and CXXXC chemokines; the classification depends on the number of amino acids between the first and the second cystein residue. Macrophage inflammatory protein-1α (MIP-1α) (Nakao et al. 1990), a member of the CC chemokines, has chemotactic activity against monocytes, lymphocytes, dendritic cells, eosinophils, and natural killer cells (Cook et al. 1995, Lukacs et al. 1996, Shimada et al. 1998, McDyer et al. 1999, Inngjerdingen et al. 2001). This cytokine was also shown to be involved in the regulation of growth and differentiation of hematopoietic cells (Shiozaki et al. 1992, Broxmeyer et al. 1993). Neote et al. (1993) identified MIP-1α receptor as a G-protein-coupled receptor with seven transmembrane domains.

Osteoclasts are multinucleated giant cells which resorb bone. These cells are hematopoietic in origin and mononuclear precursors of osteoclasts fuse to each other to form multinucleated osteoclasts. We have previously used in situ hybridization to demonstrate the expression of MIP-1α in human normal bone tissues (Kukita et al. 1997). We have also used a rat bone marrow culture system to show that MIP-1α induces the formation of osteoclast-like multinucleated cells (Kukita et al. 1997). Choi et al. (2000) and Han et al. (2001) reported that MIP-1α is involved in osteoclastogenesis associated with multiple myeloma. However, it remains unclear whether MIP-1α acts

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directly on cells in the osteoclast lineage or acts indirectly via bone marrow stromal cells.

RAW264 cells are known to have characteristics of murine macrophages with the ability to differentiate into osteoclast-like cells by receptor activator of NFκB ligand (RANKL) stimulation (Hsu et al. 1999). The use of cell lines of osteoclast precursors provides numerous advantages for studies on the mechanism of osteoclast differentiation at a molecular level. Here we isolated novel clones with a highly efficient ability to form osteoclasts from RAW264 cells and examined their ability to respond to MIP-1α. We further examined how MIP-1α modulated the binding properties of osteoclast precursors to osteoblasts. This study focused on clarifying the direct action of MIP-1α on cells in the osteoclast lineage.

Materials and Methods

Materials

Rats were obtained from SEAC Yoshitomi (Fukuoka, Japan). Original RAW264 cells were obtained from American Type Culture Collection (Rockville, MD, USA), and RAW264-7 cells were provided by Dr Muta, Kyushu University School of Medicine. Culture vessels were from Becton Dickinson Labware (Lincoln Park, NJ, USA) and tartrate-resistant acid phosphatase (TRAP) staining kit was from Sigma-Aldrich. Soluble RANKL and MIP-1α were obtained from Peprotec Science (London, UK). Tumor necrosis factor α (TNF-α) was purchased from Roche. Reverse transcriptase and Taq-DNA polymerase were obtained from Takara (Kyoto, Japan).

Animal treatment

Rats were maintained in accordance with the Guide for the Care and Use of Laboratory Animals at Kyushu University.

Bone marrow cultures for forming mononuclear preosteoclast-like cells

Bone marrow cultures were obtained from the tibia and femur of 4-week-old male Sprague–Dawley rats. Bone marrow stromal cells were depleted by the use of a Sephadex G10 column as described previously (Kukita et al. 1993b). These cells were cultured in 24-multiwell plates (1 × 10⁶ cells/well) for 4 days in α-modified Eagle’s medium (α-MEM) containing 15% fetal calf serum (FCS) and 10⁻⁸ M 1α,25-dihydroxycholecalciferol (1α,25(OH)₂D₃) and 10% (v/v) heat-treated ROS17/2.8-cell-conditioned medium (Kukita et al. 1993a).

Culture conditions for forming osteoclast-like multinucleated cells from RAW264 cells

RAW264 cells were cultured in α-MEM containing 10% FCS (3·5 × 10⁴ cells in 500 µl/well for 24-multiwell culture plates or 6·8 × 10³ cells in 150 µl/well for 96-multinwell plates) for 3 days in the presence of various concentrations of soluble RANKL and TNF-α with various concentrations of MIP-1α.

Primary culture of osteoblasts and adhesion experiments

Primary osteoblasts were obtained from calvaria of 2-day-old Sprague–Dawley rats and cultured as described previously (Kukita et al. 1993b). Cells were cultured in α-MEM containing 10% FCS. The cell suspension of the D clone was seeded on the confluent culture of primary osteoblasts. After 3–6 h incubation at 37 °C, cultures were washed with PBS to remove non-adherent cells and were then stained for TRAP.

Cloning of RAW264 cells

Before isolating RAW264 cell clones, the culture condition was determined by optimizing the concentrations of cytokines to form a large number of osteoclast-like cells from the original RAW264 cells. Combinations of 20–100 ng/ml RANKL and 1 ng/ml TNF-α provided the most suitable conditions for osteoclastogenesis. The limiting dilution of RAW264 cell suspension was established by use of a standard protocol followed by culture for 10–14 days to form colonies. The ability to induce osteoclastogenesis was assessed for each clone by checking the ability to form osteoclast-like cells. Cells from each clone were cultured in the presence of 20 ng/ml RANKL and 1 ng/ml TNF-α for 3 days, followed by staining for TRAP. Cloning of the cells was repeated twice for each clone.

Reverse transcriptase (RT-PCR)

Total RNA was extracted using a commercial kit (ISOGEN Nippongene, Toyama, Japan). First-stranded cDNA was synthesized from 1 µg total RNA and was subjected to PCR using a RT-PCR kit (Takara, Japan) and PCR system (Gene Amp model 9700). PCR primers for murine RANK were TTAAGCCAGTGCTTCA CGGG (upstream primer) and ACGTAGACCAGAT GATGTCGC (downstream primer), which produced PCR products of 497 bp. PCR primers for murine GAPDH were ACCACAGTCCATGCCATCAG ATGATCAG (upstream primer) and TCCACCACCTGTGCTGTA (downstream primer), which gave PCR products of 452 bp. PCR primers for murine PGD were ACCACAGTCCATGCCATCAG (upstream primer) and TCCACCACCTGTGCTGTA (downstream primer), which gave PCR products of 452 bp. In PCR, annealing was performed at 55 °C for 30 s and primer extension was done at 72 °C for 1 min. After 30 cycles of the reaction, PCR products were separated on a 2% agarose gel and stained with ethidium bromide. Northern blotting analysis was performed according to a standard protocol. Briefly, total RNA was applied to 12% agarose–formaldehyde gel (10 µg/lane) and electrophoresis was performed in 20 mM 3-(N-morpholino)
Isolation of several types of RAW264 cell clones with diﬀerent potentials for osteoclastogenesis

Results

Isolation of several types of RAW264 cell clones with diﬀerent potentials for osteoclastogenesis

In cultures of RAW264 cells, signiﬁcant heterogeneity of the cell population was noticed when these cells were cultured for the formation of osteoclast-like cells. In these cultures, colonies of TRAP-positive cells were frequently observed (data not shown). This observation strongly suggested the presence of several clones with diﬀerent potentials for osteoclastogenesis in the conditions stimulated by the diﬀerentiation factors. Therefore, we began separating clones with diﬀerent potentials for osteoclastogenesis from the original RAW264 cells by cloning these cells using a standard limiting dilution technique. We examined 36 clones with regard to their ability to form osteoclast-like cells. These clones were grouped into four categories with respect to their potential for osteoclastogenesis, as shown in Table 1. The ﬁrst type of clone (I) eﬃciently diﬀerentiated into multinucleated osteoclast-like cells with numerous nuclei. The second type of clone (II) diﬀerentiated into osteoclast-like cells with few nuclei (3–5 nuclei per multinucleated cell (MNC)). The third type of clone (III) diﬀerentiated into mononuclear TRAP-positive cells, however, these cells never diﬀerentiated into multinucleated cells. In contrast to the above three types of clones, the fourth type of clone (IV) diﬀerentiated into neither mononuclear TRAP-positive cells nor multinucleated osteoclast-like cells. We designated one representative clone from the ﬁrst type as RAW264 cell D (diﬀerentiation) clone (D clone) and one representative clone from the fourth type as RAW264 cell N (non-diﬀerentiation) clone (N clone). Figure 1 shows the microscopic observation of the D clone when stimulated by various concentrations of RANKL in the presence of 1 ng/ml TNF-α. Although the original RAW264 cells required 100 ng/ml RANKL to form TRAP-positive MNCs, the D clone was eﬃciently diﬀerentiated into TRAP-positive MNCs at a lower concentration (20 ng/ml) of RANKL. Figure 2 shows a comparison of the diﬀerentiation eﬃciencies among the D clone, the N clone, original RAW264 cells and RAW264·7 cells. The D clone showed the highest sensitivity to RANKL, forming numerous TRAP-positive MNCs, even when these cells were cultured in 96-multiwell plates. Although RAW264·7 cells formed TRAP-negative MNCs in response to RANKL, the D clone did not form TRAP-negative MNCs (data not shown). Figure 3 demonstrates the response of the D clone to TNF-α in the presence of 20 ng/ml RANKL. TNF-α markedly stimulated osteoclast-like cell formation from the D clone. To clarify if the D clone expressed characteristics of osteoclast precursors, we examined the eﬀect of calcitonin on the formation of TRAP-positive MNCs. As shown in

Table 1 Demonstration of four types of RAW264 cell clones

<table>
<thead>
<tr>
<th>Differentiation phenotype</th>
<th>TRAP-positive mononuclear cells</th>
<th>TRAP-positive MNCs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Staining intensity</td>
<td>Percent in total mononuclear cells</td>
</tr>
<tr>
<td>I</td>
<td>+++</td>
<td>100</td>
</tr>
<tr>
<td>II</td>
<td>+</td>
<td>10–50</td>
</tr>
<tr>
<td>III</td>
<td>++</td>
<td>20–50</td>
</tr>
<tr>
<td>IV</td>
<td>–</td>
<td>0</td>
</tr>
</tbody>
</table>

MNC, multinucleated cell. – , no cells were stained for TRAP; +, cells were weakly stained for TRAP; ++, cells were moderately stained for TRAP; ++++, cells were strongly stained for TRAP.
Figure 4, calcitonin significantly suppressed the formation of osteoclast-like MNCs from the D clone. Northern blotting analysis showed that a significant expression of cathepsin K mRNA was detected in the D clone when stimulated with RANKL and TNF-\(\alpha\), while no expression of this mRNA was detected in the N clone treated with these cytokines (Fig. 5). We also confirmed that osteoclast-like MNCs formed from the D clone resorbed dentin (data not shown). We further examined the expression of RANK, the receptor for RANKL, in these clones before they were stimulated with RANKL and TNF-\(\alpha\). Semiquantitative RT-PCR analysis showed that both clones (D and N) expressed RANK at least by RNA (Fig. 6). These data suggest that the remarkable difference between the D clone and the N clone in their potentials for osteoclastogenesis is not a result of differences in RANK expression. These findings demonstrate the usefulness of the D clone for studies on the molecular mechanisms of osteoclastogenesis.

MIP-1\(\alpha\) stimulates formation of preosteoclasts from bone marrow cells deprived of stromal cells

We reported previously that MIP-1\(\alpha\) stimulates osteoclastogenesis in a rat whole bone marrow culture system containing bone marrow stromal cells. The direct action of MIP-1\(\alpha\) on cells in the osteoclast lineage was assessed by use of rat bone marrow cells deprived of stromal cells. MIP-1\(\alpha\) significantly stimulated formation of TRAP-positive preosteoclast-like mononuclear cells as shown in Fig. 7. Peak stimulation was obtained with 1 ng/ml

Figure 2 Comparison of the efficiency of osteoclast-like cell formation among various RAW264 cells. The dosage effect of RANKL on osteoclastogenesis was examined among different RAW cells. The D clone, N clone, RAW264 original cells and RAW264·7 cells were cultured in 96-multweli plates in the presence of 1 ng/ml TNF-\(\alpha\) with various concentrations of RANKL (10–80 ng/ml). In cultures of RAW264 original cells, TRAP-positive mononuclear cells were observed, however, few TRAP-positive MNCs were observed. Numerous TRAP-positive MNCs at greater than 4000 per well were formed from the D clone. No osteoclast-like cells were formed from the N clone. Low numbers of osteoclast-like MNCs were formed from RAW264·7 cells. *\(P<0.01\); **\(P<0.001\). Data represent a typical experiment for three independent experiments.
MIP-1α in the presence of 1α,25(OH)2D3. However, MIP-1α showed no stimulatory effect in the absence of 1α,25(OH)2D3. These results strongly suggest that MIP-1α acts directly on cells in the osteoclast lineage and stimulates the formation of preosteoclasts from bone marrow cells deprived of stromal cells.

**MIP-1α stimulates formation of osteoclast-like MNCs from RAW264 cell D clone**

To confirm that MIP-1α acts directly on cells in the osteoclast lineage, we examined the effect of MIP-1α on osteoclastogenesis using RAW264 cell D clone as shown in Fig. 8. MIP-1α stimulated formation of TRAP-positive MNCs from the D clone when cells were stimulated with RANKL and TNF-α, while no MIP-1α-induced osteoclastogenesis was observed in the N clone, even in the presence of RANKL and TNF-α. To establish the optimum concentration of MIP-1α for osteoclastogenesis from cells of the D clone, we used finer doses of MIP-1α to assess osteoclastogenic activity. As shown in Table 2,

![Figure 4](image1.png)

**Calcitonin Conc. (ng/ml)**

Figure 4  Suppressive effect of salmon calcitonin on the formation of osteoclast-like cells from the D clone. Cells were cultured in 96-well culture plates for forming osteoclast-like MNCs in the presence of 20 ng/ml RANKL and 1 ng/ml TNF-α in the presence of various concentrations of salmon calcitonin. Calcitonin significantly inhibited the formation of TRAP-positive MNCs in a dose-dependent manner. *P<0.05; **P<0.01. Data represent a typical experiment for three independent experiments.

![Figure 5](image2.png)

**Cathepsin K / GAPDH**

Figure 5  Induction of cathepsin K mRNA in the D clone but not in the N clone after being stimulated with factors for osteoclastogenesis. Both the N clone and the D clone were stimulated with 20 ng/ml RANKL and 1 ng/ml TNF-α for 48 h; total RNAs were then prepared from these cells. Expression of cathepsin K was examined by use of Northern blotting analysis as described in Materials and Methods (10 μg total RNA per lane). Clear induction of cathepsin K mRNA expression was observed in the D clone but not in the N clone. Lane 1, unstimulated N clone; lane 2, unstimulated D clone; lane 3, the N clone stimulated by RANKL and TNF-α; lane 4, the D clone stimulated by RANKL and TNF-α; lane 5, the D clone stimulated by RANKL, TNF-α and MIP-1α (1 ng/ml). The membrane sheet was hybridized with DIG-labeled cathepsin K probe and the same membrane was reprobed with DIG-labeled GAPDH probe.

![Figure 6](image3.png)

**N- D- clone clone**

**RANK**

**GAPDH**

Figure 6  Expression of RANK mRNA in both the N clone and the D clone, before stimulation with factors for osteoclastogenesis. Cells were cultured in the absence of factors for osteoclastogenesis and total RNAs were prepared from these cells; RANK mRNA expression was then examined using RT-PCR analysis, as described in Materials and Methods. Both the N clone and the D clone expressed mRNA for RANK.

![Figure 7](image4.png)

**MIP-1α Conc. (ng/ml)**

Figure 7  Dosage effect of MIP-1α on the formation of mononuclear preosteoclast-like cells from rat bone marrow cells deprived of stromal cells. Rat bone marrow cells deprived of stromal cells were cultured in 24-well plates in the presence of various concentrations of MIP-1α as described in Materials and Methods. MIP-1α significantly stimulated the formation of preosteoclast-like mononuclear cells in the presence of 10−8 M 1α,25(OH)2D3 (right panel, D3 (+)) but not in the absence of 1α,25(OH)2D3 (left panel, D3 (−)). Peak stimulation was observed at 1 ng/ml MIP-1α. *P<0.01. Data represent a typical experiment for three independent experiments.

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maximal stimulation was observed at 1–2 ng/ml MIP-1α. We further examined whether the type II clone responded to MIP-1α. Osteoclastogenesis was also significantly stimulated by 2–6 ng/ml MIP-1α in the type II RAW264 clone (Table 3). These results provide strong evidence to show that MIP-1α acts directly on cells in the osteoclast lineage to stimulate osteoclastogenesis. We also confirmed the cathepsin K mRNA expression formed in osteoclast-like cells in the presence of MIP-1α, as shown in Fig. 5 (lane 5).

MIP-1α stimulates fusion among osteoclast-like MNCs formed from the D clone

MIP-1α markedly stimulated the formation of osteoclast-like MNCs (Fig. 8), however, higher concentrations of MIP-1α tended to suppress the number of MNCs formed in these cultures. This apparently suppressive effect of high doses of MIP-1α could be attributed to stimulation of fusion among osteoclast-like MNCs. Therefore, we examined this possibility by creating a histogram in which the relation between MIP-1α dose and the degree of multinucleation was assessed, as shown in Fig. 9. Higher concentrations of MIP-1α significantly stimulated formation of MNCs with a large number of nuclei of greater than 41 nuclei per osteoclast-like MNC. These findings strongly suggest that the apparent decrease in the number of osteoclast-like MNCs formed in the presence of 10 ng/ml of MIP-1α (Fig. 8) is a result of stimulation of fusion in osteoclast-like MNCs.

MIP-1α-induced adhesion of the D clone to primary osteoblasts

The adhesion of osteoclast precursors to osteoblasts is an important step in the regulation of osteoclastogenesis. Therefore, we further investigated whether MIP-1α modulates the adhesion properties of the D clone to primary osteoblasts. As shown in Fig. 10, the MIP-1α-treated D clone adhered efficiently to primary osteoblasts in comparison with untreated D clones. These results

Table 3 MIP-1α stimulation of osteoclastogenesis from cells of type II clone RAW264 cells

<table>
<thead>
<tr>
<th>MIP-1α concentration (ng/ml)</th>
<th>No. of TRAP-positive MNCs per culture (mean ± S.E.M.)</th>
<th>Level of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9.25 ± 1.18</td>
<td>*P&lt;0.05</td>
</tr>
<tr>
<td>0.25</td>
<td>13.25 ± 1.18</td>
<td>*P&lt;0.05</td>
</tr>
<tr>
<td>0.5</td>
<td>17.25 ± 3.92</td>
<td>*P&lt;0.05</td>
</tr>
<tr>
<td>1</td>
<td>17.25 ± 5.18</td>
<td>*P&lt;0.05</td>
</tr>
<tr>
<td>2</td>
<td>22.75 ± 4.38</td>
<td>*P&lt;0.05</td>
</tr>
<tr>
<td>4</td>
<td>23.5 ± 3.66</td>
<td>*P&lt;0.05</td>
</tr>
<tr>
<td>6</td>
<td>21.25 ± 3.61</td>
<td>*P&lt;0.05</td>
</tr>
<tr>
<td>8</td>
<td>14.25 ± 2.78</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>14.0 ± 2.54</td>
<td></td>
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</table>

Cells of type II clone were cultured in 96-multwell plates (4.5 × 10⁴ cells/ml, 150 µl/well) to form osteoclast-like MNCs in the presence of 20 ng/ml RANKL and 1 ng/ml TNF-α. Various concentrations of MIP-1α were added. MIP-1α significantly stimulated the formation of TRAP-positive MNCs at concentrations of 2–6 ng/ml. *P<0.05. Data represent a typical experiment for three independent experiments.
suggest that MIP-1α induces expression of adhesion molecules on the cell surface of the D clone. Previously, we reported that lymphocyte function-associated antigen-1 (LFA1) is expressed on mononuclear precursors of rat osteoclasts and that this molecule participates in the regulation of osteoclastogenesis (Harada et al. 1998). Therefore, expression of LFA1α and β on the surface of the D clones was examined by use of specific antibodies. Both molecules were expressed on the cell surface of the D clone, however, the level of the expression was not altered when these cells were treated with MIP-1α (data not shown).

**Discussion**

The macrophage-like cell line RAW264 is useful for studying osteoclastogenesis. The D clone used in the present study, is able to differentiate into osteoclast-like MNCs at a very high rate even at low concentrations of RANKL, while the N clone has no ability to differentiate into osteoclast-like cells. Although these two types of clones were derived from the same origin, sensitivity to RANKL differed between these clones. We also obtained other types of clones that had intermediate potentials for osteoclastogenesis. One type of clone differentiated into TRAP-positive MNCs, however, their number of nuclei was limited to 3–5 per cell. Another type of clone differentiated into mononuclear TRAP-positive preosteoclast-like cells, but they never fused to each other to form MNCs. These four types of RAW264 cell clones are suggested to be suitable for molecular analysis of osteoclastogenesis involving signal transduction studies. They will be of value for studies of the molecular mechanisms of osteoclast differentiation, to isolate and maintain stable RAW264 cell clones with different responses against differentiation stimuli. RT-PCR analysis and Northern blotting analysis showed that both the D clone and the N clone expressed significant levels of RANK, the receptor for RANKL, but that only the D clone expressed cathepsin K after being stimulated for osteoclastogenesis. Anderson et al. (1997) stated that RANK mRNA levels do not accurately predict RANK protein expression on the cell surface. It is true that there is a limitation of using RANK mRNA to predict RANKL responsiveness and the potentiation of the MIP-1α signal; further analysis is required in respect of the RANK protein expression on the cell surface of RAW264 cell clones. However, the current data may suggest that the N clone has some defects in the signaling pathway, downstream of RANK, that leads to the expression of molecules required for osteoclastogenesis. The D clone, N clone and other

![Image of Figure 9](https://example.com/figure9.png)

**Figure 9** Dosage effect of MIP-1α on the nuclear number of formed osteoclast-like MNCs. Cells of the D clone were cultured in 96-multiwell plates for forming osteoclast-like MNCs in the presence of 20 ng/ml RANKL and 1 ng/ml TNF-α with various concentrations of MIP-1α. After staining for TRAP, 16 fields (12.6% of the total area of each well) were randomly selected from each well and the numbers of osteoclast-like MNCs with different ranges of nuclear number were separately counted. The Figure shows the number of MNCs in each nuclear number range. At higher concentrations of MIP-1α, the numbers of MNCs with numerous nuclei (greater than 41 nuclei per MNC) was markedly increased. *P < 0.05, **P < 0.01. Data represent a typical experiment for three independent experiments.
clones obtained in this study would provide powerful tools for molecular biological analysis of osteoclast differentiation.

We have previously reported that MIP-1α participates in osteoclast formation in rat whole bone marrow cultures (Kukita et al. 1992, 1997). We have also reported that MIP-1α is expressed in human osteoblasts and that this chemokine induces osteoclast formation on dentin slices. However it has not yet been clear whether MIP-1α acts directly on cells in the osteoclast lineage or not. The current study demonstrates that MIP-1α acted directly on cells in the osteoclast lineage and induced differentiation in culture systems in which other types of cells were not involved. One of the culture systems used was the stromal cell-deprived bone marrow culture system for forming mononuclear preosteoclast-like cells. The other culture system used was the pure culture system in which only a particular RAW264 cell clone was involved. As MIP-1α showed stimulation of osteoclastogenesis in both culture systems, it is concluded that MIP-1α directly acts on cells in the osteoclast lineage to augment formation of osteoclast-like cells. Han et al. (2001) recently reported that CC-chemokine receptor (CCR1) and CCR5 are involved in MIP-1α-induced osteoclastogenesis in culture systems of bone marrow cells from patients with multiple myeloma. In our culture systems for osteoclastogenesis induced by MIP-1α, these chemokine receptors are supposed to play an important role in the stimulation of osteoclastogenesis. Our culture system using the D clone would provide a suitable system for investigating signal transduction mediated by MIP-1α in osteoclastogenesis. In osteoclast differentiation induced by RANKL, signal transduction through TNF receptor-associated factor 6 (TRAF6) is important (Kobayashi et al. 2001). As stimulation of osteoclastogenesis by MIP-1α was only observed when cells were treated with RANKL, it is supposed that MIP-1α could modulate the TRAF6 signaling pathway triggered by RANKL. These studies are underway in our laboratories.

Adhesion of osteoclast progenitors to osteoblasts has been recognized as an essential step for osteoclastogenesis (Takahashi et al. 1988, Tanaka et al. 1998b). Here it has been demonstrated that MIP-1α dramatically enhances adhesion of the D clone to the monolayer of primary osteoblasts. The differentiated D clone expressed significant levels of LFA-1α and β, however, the level of the expression of these molecules was not altered by the addition of MIP-1α (data not shown). We can not rule out the possibility that MIP-1α acts as the upregulator of osteoclastogenesis in bone marrow. In our culture systems for osteoclastogenesis, it is supposed that MIP-1α directly acts on cells in the osteoclast lineage to augment formation of osteoclast-like cells. Han et al. (2001) recently reported that CC-chemokine receptor (CCR1) and CCR5 are involved in MIP-1α-induced osteoclastogenesis in culture systems of bone marrow cells from patients with multiple myeloma. In our culture systems for osteoclastogenesis induced by MIP-1α, these chemokine receptors are supposed to play an important role in the stimulation of osteoclastogenesis. Our culture system using the D clone would provide a suitable system for investigating signal transduction mediated by MIP-1α in osteoclastogenesis. In osteoclast differentiation induced by RANKL, signal transduction through TNF receptor-associated factor 6 (TRAF6) is important (Kobayashi et al. 2001). As stimulation of osteoclastogenesis by MIP-1α was only observed when cells were treated with RANKL, it is supposed that MIP-1α could modulate the TRAF6 signaling pathway triggered by RANKL. These studies are underway in our laboratories.

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The use of a combination of RAW264 cell clones with different potentials for osteoclastogenesis could provide a powerful tool for clarifying the genes and their products that are essential for each stage of the osteoclast differentiation. This work is now underway in our laboratories.

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