Expression and role of mannose receptor/terminal high-mannose type oligosaccharide on osteoclast precursors during osteoclast formation

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Abstract

Osteoclasts are formed from hematopoietic precursors via cell–cell fusion. We have previously reported that mannose residues are expressed on the outer membranes of monocytes during osteoclast differentiation. In the present study, we have attempted to demonstrate the pattern of expression levels of terminal high-mannose type oligosaccharide and to show that the mannose receptor is expressed on osteoclast precursor cells. Osteoclasts were formed using three different systems, namely mouse bone marrow cell culture, co-culture of mouse spleen cells with stromal cells, and RAW264·7 cell cultures. During osteoclast differentiation, the expression of terminal high-mannose type oligosaccharide gradually increased and then peaked at the stage of fusion in all three systems. Expression of the mannose receptor gradually increased during osteoclast differentiation in bone marrow cells and the co-culture system. In contrast, that in RAW264·7 cells had already been detected in the absence of the soluble receptor activator of NF-κB ligand and did not change during osteoclast differentiation. To ascertain whether expression of high-mannose type oligosaccharide is involved in tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cell (MNC) formation, glycosidase inhibitors were used on RAW264·7 cell culture. Castanospermine, an inhibitor of glucosidase I, inhibited the TRAP-positive MNCs, and deoxymannojirimycin, an inhibitor of α-mannosidase I, increased the TRAP-positive MNC formation. These results indicate that the binding of terminal high-mannose and mannose receptor is important for the process of cellular fusion in osteoclast formation.


Introduction

Terminal high-mannose type oligosaccharide is very rare on the cell surface of mammalian cells, in spite of its being widely recognized that terminal high mannose in a lysosomal hydrolase is important for the recognition of GlcNAc phosphotransferase. Terminal high-mannose type oligosaccharide on the cell surface mediates cell–cell fusion reactions such as sperm–egg fusion (Primakoff et al. 1987, Blobel et al. 1990, 1992) and myoblast fusion (Kaufman et al. 1985, Rosenberg et al. 1985, Menko & Boettiger 1987, Rosen et al. 1992). The involvement of terminal high-mannose type oligosaccharide has also been reported in the events of viral infections such as the influenza virus (Anders et al. 1990) or human immunodeficiency virus (HIV) (Lifson et al. 1986, Matthews et al. 1987). For example, the mast cell function-associated antigen was found to bind terminal high-mannose residues in the influenza virus specifically (Binsack & Pecht 1997). Osteoclasts, the bone-resorbing cells, are multinucleated giant cells that develop from hematopoietic cells of the monocyte/macrophage lineage via cell–cell fusion (Udagawa et al. 1990). Using a mouse spleen cell co-cultured system, we have previously demonstrated that terminal high-mannose type oligosaccharide is expressed on the outer membranes of monocytes under pathophysiological conditions, and that it is also involved in osteoclast formation via cellular membrane fusion events (Kurachi et al. 1994).

The expression of terminal high-mannose type oligosaccharide proceeds as follows: (Glc)3(Man)9(GlcNAc)2 is first transferred from dolichol pyrophosphate to asparagine residues on proteins, and three glucoses are moved by glucosidases I and II to a terminal high-mannose type oligosaccharide, (Man)9(GlcNAc)2. The terminal high-mannose type glycan is transformed into complex type one via processing by mannosidases I and II (Oki et al. 1999).
The terminal high-mannose type oligosaccharide is believed to function in innate immunity by binding to the mannose receptor (MR). The MR is expressed on cells of the macrophage lineage (Pontow et al. 1992) and acts as a potential macrophage fusion-mediating receptor.

In the present study, we have demonstrated that both terminal high-mannose type oligosaccharide and MR were expressed on outer membranes of monocytes in three different osteoclast formation systems; mouse bone marrow cell culture, co-culture of mouse spleen cells with stromal cells, and RAW264·7 cell cultures. Moreover, we have also demonstrated the expression of terminal high-mannose type oligosaccharide promoted tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cell (MNC) formation in RAW264·7 cells using glycosidase inhibitors.

**Materials and Methods**

**Materials**

α-Minimum essential medium (α-MEM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). 1α,25-Dihydroxyvitamin D₃ (1α,25-(OH)₂D₃) was purchased from Wako (Tokyo, Japan). Human soluble receptor activator of NF-κB ligand (sRANKL) was purchased from Peprotech, Inc. (Rocky Hill, NJ, USA). Naphthol AS-BI phosphoric acid sodium salt, fast red ITR, prostaglandin E₂ (PGE₂), mannan, castanospermine (CAS), 1-deoxymannojirimycin (DMJ), swainsonine (SW), and FITC-conjugated goat anti-rabbit IgG and Ficoll (type 400) were purchased from Sigma Chemical Co. (St Louis, MO, USA). FITC-labeled pradimicin was kindly provided by T Furumai (Toyama Prefectural University Biotechnology Research Center). Anti-alveolar macrophage MR antibody was raised in rabbits against synthetic peptide (RQFLYIYNEDHKRC) as previously described (Kawashima et al. 1997).

**Bone marrow cell culture**

All animals were treated according to the Tokyo Medical and Dental University Institutional Animal Care and Use Committee. Seven-week-old male ddY mice were purchased from Saitama Laboratory Animal Center (Saitama, Japan). Murine bone marrow cells were prepared by the previously described modified method of Takahashi et al. (1993). Spleen cells were prepared by the modified method previously described by Kurachi et al. (1993). Spleen cells were purified using a density gradient between Ficoll and 60% urografin, and red corpuscles were broken with 0·83% NH₄Cl. These cells were deposited into tubes and washed once with α-MEM containing 10% (v/v) FBS and then resuspended at a density of 5 × 10⁵ cells/ml in the same medium. A 1 ml aliquot of cell suspension was seeded onto TMS-14 stromal cells that had been cultured for 24 h (37 °C, 5% CO₂) in 24-well tissue culture plates (1 × 10³ cell/well). The cells were incubated in the presence of 1α,25-(OH)₂D₃ (10⁻⁸ M) with or without mannan (1 mg/ml). The cultures were maintained for 7 days with a change of medium every 3 days.

**Co-culture of spleen cells and stromal cells**

The mouse stromal cell line TMS-14 was isolated and cloned from bone marrow (Kurachi et al. 1993). Murine spleen cells were prepared by the modified method previously described by Kurachi et al. (1993). Spleen cells were purified using a density gradient between Ficoll and 60% urografin, and red corpuscles were broken with 0·83% NH₄Cl. These cells were deposited into tubes and washed once with α-MEM containing 10% (v/v) FBS and then resuspended at a density of 5 × 10⁵ cells/ml in the same medium. A 1 ml aliquot of cell suspension was seeded onto TMS-14 stromal cells that had been cultured for 24 h (37 °C, 5% CO₂) in 24-well tissue culture plates (1 × 10³ cell/well). The cells were incubated in the presence of 1α,25-(OH)₂D₃ (10⁻⁸ M) with or without mannan (1 mg/ml). The cultures were maintained for 7 days with a change of medium every 3 days.

**RAW264·7 cell culture**

RAW264·7 cells, a murine macrophage cell line, were prepared at a density of 4 × 10⁵ cells/ml in α-MEM containing 10% (v/v) FBS, and then 10 μl was seeded onto the spot in the middle of the well in 24-well tissue culture plates and incubated for 30 min. After cell adherence, 0·6 ml of the same medium containing sRANKL (50 ng/ml) and with or without mannan (1 mg/ml), CAS (100 μl/ml), DMJ (200 μl/ml), and SW (30 μl/ml) was added, and the cultures were incubated for 4 days.

**TRAP staining**

After 7 days of spleen and bone marrow culture and after 4 days of RAW264·7 cell culture, cells were washed with phosphate-buffered saline (PBS) and fixed with ethanol/acetone (1:1) for 1 min. The cultures were then dried and stained for TRAP by incubating them in 0·1 M sodium acetate buffer (pH 5·0) containing naphthol AS-BI phosphoric acid sodium salt and fast red ITR salt in the presence of 10 mM sodium tartrate as previously described (Burstone 1958). TRAP-positive cells with three or more nuclei were counted as MNCs. TRAP-positive MNCs formed in our culture system were considered to be osteoclast-like MNCs in the present study.

**Fluorescent image analysis with an interactive laser cytometer**

**FITC-labeled pradimicin** RAW264·7 cells were cultured on days 1 and 4, and tissue culture plates were washed twice with PBS containing 1 mM CaCl₂. The
cells were next incubated with 10 µg/ml FITC-labeled pradimicin in the same PBS for 20 min. After washing with the same buffer five times, a fluorescent image analysis was performed using the interactive laser cytometer, ACAS570 (Meridian Instruments, Okemos, MI, USA).

Polyclonal anti-alveolar macrophage MR antibody
RAW264·7 cells, spleen cells and bone marrow cells were cultured at the times indicated in the text, and then washed twice with PBS, fixed with 2% formaldehyde in PBS for 10 min, and rinsed twice with PBS. After blocking with PBS containing 1% FBS for 5 min, 10 µg/ml anti-alveolar macrophage MR antibody was applied for 60 min at room temperature. After washing the cells three times with PBS, FITC-conjugated goat anti-rabbit IgG was applied at a 1:40 dilution for 60 min at room temperature. Fluorescence imaging analysis was performed using the interactive laser cytometer ACAS570. The cells from two wells of a 24-well tissue culture plate (25 cells/well, 50 cells total) were assayed. The fluorescent intensity/cell area was calculated for each cell, and the percentage of the cells showing fluorescent intensity was determined.

Statistical analysis
Statistical significance was tested using ANOVA followed by Tukey’s test or Student’s t-test.

Results

Inhibitory effect of mannose-rich mannan on TRAP-positive MNC formation
In a previous paper (Kurachi et al. 1994), we demonstrated that terminal high-mannose type oligosaccharide has been shown to be expressed in hematopoietic cells during osteoclast differentiation. In order to characterize the counterpart of terminal high-mannose type oligosaccharide, we examined the effects of mannose-rich mannan as an antagonist of MR on TRAP-positive MNC formation. The addition of PGE2 (10⁻⁶ M) as well as 1α,25-(OH)₂D₃ (10⁻⁸ M) induced formation of TRAP-positive MNCs in bone marrow culture for 7 days. Treatment of bone marrow culture with mannan markedly inhibited MNC formation. Cultures of (a) bone marrow cells treated with PGE₂ (10⁻⁶ M) or (b) spleen cells on TMS-14 cells with 1α,25-(OH)₂D₃ (10⁻⁸ M) were maintained for 7 days with or without mannan (1 mg/ml). Cultures of (c) RAW264·7 cells treated with sRANKL (50 ng/ml) were maintained for 4 days with or without mannan (1 mg/ml). TRAP-positive cells with three or more nuclei were counted as MNCs. In each experiment, the number of TRAP-positive MNCs in the absence of mannan was defined as 100%. The number of MNCs without mannan was 42·0 with bone marrow, 249·5 with spleen and 45·7 with RAW264·7. Values are means ± S.E. of the results of four replicated experiments. *P<0·05, **P<0·01.

Expression macrophage MR on osteoclast progenitor cells
The MR is believed to function in innate immunity by recognizing unopsonized micro-organisms bearing terminal mannos, fructose, N-acetylglucosamine or glucose residues (Pontow et al. 1992). We therefore investigated the expression pattern of MR during osteoclast differentiation in three different culture systems. The expression of MR on osteoclast progenitors was quantified by indirect immunostaining and fluorescent imaging analysis. In bone marrow culture or spleen co-culture, the expression of MR was not observed in the absence of PGE₂ or 1α,25-(OH)₂D₃ or on days 1–3 in the presence of either one of the stimulants. The expression of MR on spleen cells in co-culture with TMS-14 as well as mononuclear cells in bone marrow culture was observed on days 4–7 (Fig. 3). In contrast, the MR in RAW264·7 cells was constitutively expressed during culture and expression was not changed by sRANKL treatment (Fig. 4). To evaluate the change in the MR expression during osteoclast differentiation, we analyzed osteoclasts using the interactive laser cytometer, ACAS570. The expression of MR during osteoclast formation was time-dependent in the case of both bone marrow culture and spleen co-culture (Fig. 5).

Expression of terminal high-mannose type oligosaccharide on RAW264·7 cells
In our previous report, we demonstrated that the number of osteoclast progenitors expressing terminal high-mannose type oligosaccharide on their plasma membrane...
increased during the latter term of co-culture of spleen cells and TMS-14. In order to confirm this phenomenon, we examined the expression patterns of terminal high-mannose type oligosaccharide on the RAW264·7 cells by using pradimicin derivatives, which specifically bind terminal high-mannose residues. During the 4-day cultivation, FITC-labeled pradimicin was observed to bind osteoclast progenitors and immature TRAP-positive MNCs (those containing more than three nuclei and small-sized cells) at the fusion stage (Fig. 6).

**Effects of glycosidase inhibitors on TRAP-positive MNC formation**

To ascertain whether expression of terminal high-mannose type oligosaccharide is involved in TRAP-positive MNC formation, we used glycosidase inhibitors on RAW264·7 cell culture. CAS, an inhibitor of glucosidase I, inhibited the expression of high-mannose type oligosaccharide at the cell surface (Fig. 6c), and markedly inhibited the formation of TRAP-positive MNCs (Fig. 7). In contrast, the treatment of RAW264·7 cells with DMJ, an inhibitor of α-mannosidase I, increased to 2–9-fold the formation of TRAP-positive MNCs with enhancement of the expression of terminal high-mannose. SW, an inhibitor of α-mannosidase II, affected neither the formation of TRAP-positive MNCs nor the expression of terminal high-mannose type oligosaccharide (Fig. 7).

**Discussion**

Osteoclasts, the bone-resorbing cells, are multinucleated giant cells that develop from hematopoietic cells of the
monocyte/macrophage lineage via a cell–cell fusion process (Udagawa et al. 1990). Osteoclast differentiation factor (The American Society for Bone and Mineral Research 2000), also called osteoprotegerin ligand (Lacey et al. 1998), tumor necrosis factor-related activation-induced cytokine (Wong et al. 1997), and RANKL (Anderson et al. 1997), is known to be essential for osteoclast differentiation. It is a member of the TNF ligand family, and induces osteoclast differentiation from progenitor cells co-treated with macrophage colony-stimulating factor (M-CSF) (Lacey et al. 1998). Osteoblasts or stromal cells express RANKL and M-CSF on the surface of cell membranes. A co-culture system of spleen cells with osteoblasts or bone marrow stromal cells has been established to produce osteoclasts (Takahashi et al. 1988a, Udagawa et al. 1989). RAW264·7 cells express M-CSF continuously, and thus can be clearly differentiated from osteoclast-like cells in the presence of sRANKL alone (Hsu et al. 1999). The discovery of RANKL has enabled determination of the processes governing the proliferation of osteoclast progenitor cells, differentiation into osteoclasts, and activation and survival of osteoclasts; however, few details of the fusion mechanism of osteoclast progenitor cells are known.

The mechanisms that regulate cell–cell fusion events closely resemble those that regulate virus–cell fusion events. In viral infections such as influenza virus (Anders et al. 1990) or HIV (Lifson et al. 1986, Matthews et al. 1987) infection, the involvement of terminal high-mannose type oligosaccharide has been reported. Cell–cell

**Figure 4** Fluorescent imaging analysis of the expression of the macrophage MR on RAW264·7 cells. (Upper section) RAW264·7 cells were maintained for 4 days with or without sRANKL (50 ng/ml) and analysis of expression of MRs was performed in the absence (a) or the presence (b) of sRANKL. (Lower section, c) The fluorescence intensity was calculated for each mononucleated cell in the absence of sRANKL (open bars) and in the presence of sRANKL (solid bars) on days 1 and 4. Values are the means ± S.E. of 50 cells and similar results were obtained in each of the three experiments.

**Figure 5** Time-dependent change in the expression of MRs on bone marrow cells and spleen cells. (a) Bone marrow cells treated with PGE2 (10⁻⁶ M) or (b) spleen cells on TMS-14 cells treated with 1α,25-(OH)₂D₃ (10⁻⁸ M) were maintained, and analysis of the expression of MR performed on each day after 4 days of culture. The fluorescence intensity was calculated for each mononucleated cell. Each point represents the mean ± S.E. of about 50 cells and similar results were obtained in each of the two experiments. **P<0.01.

**Figure 6** Fluorescent imaging analysis of the expression of terminal high-mannose type oligosaccharide on RAW264·7 cells. RAW264·7 cells were maintained for 4 days with or without sRANKL (50 ng/ml), and analysis of expression of terminal high-mannose type oligosaccharide performed (a) in the absence of sRANKL, (b) in the presence of sRANKL, or (c) in the presence of sRANKL and 100 μg/ml of CAS, an inhibitor of glucosidase I.
fusion reactions such as sperm–egg fusion (Primakoff et al. 1987, Blobel et al. 1990, 1992), myoblast fusion (Kaufman et al. 1985, Rosenberg et al. 1985, Menko & Boettiger 1987, Rosen et al. 1992), monocyte fusion (Most et al. 1990), or preosteoclast fusion (Kurachi et al. 1993) are also protein mediated. From these observations (Kurachi et al. 1994), we have already reported that mannose residues are expressed on the outer membranes of monocytes during osteoclast differentiation using pradimicin derivatives, which are antiviral and antifungal both in vitro and in vivo, and recognize and bind specific sugars such as mannose residues (Oki et al. 1988, 1990).

In the present study, we have shown that terminal high-mannose type oligosaccharide is expressed on the surface of RAW264·7 cells as well as spleen cells in the process of osteoclast differentiation. To investigate the involvement of terminal high-mannose type oligosaccharide in osteoclast formation, we used glycosidase inhibitors of glucosidase I or mannosidase I. Treatment of RAW264·7 cells with a glucosidase inhibitor resulted in a marked decrease in the number of mannose residues on the cell surface, while the formation of TRAP-positive MNCs was attenuated (Fig. 7). Furthermore, the increased expression of the terminal high-mannose type oligosaccharide by the inhibition of mannosidase I caused a dramatic increase in the formation of TRAP-positive MNCs (Fig. 7). Thus, the activities of both glucosidase I and mannosidase I are important in the cell–cell fusion process.

We then focused on the MR, which is a supposed counterpart of mannose residues. The MR, expressed on some macrophages, epithelial, and endothelial cells (Takahashi et al. 1998, Linehan et al. 1999), is the prototype member of a family of multi-lectin receptors that recognize carbohydrates such as terminal mannose, fucose, N-acetylgalcosamine, or glucose residues on the cell walls of infectious organisms (Stahl & Ezekowitz 1998). The MR is believed to mediate both endocytosis of glycoproteins and phagocytosis of micro-organisms or glucose residues (Astarie-Dequeker et al. 1999, DeFife et al. 1999, Lansink et al. 1999). MR-mediated fusion occurs via a filamentous action-dependent pathway (DeFife et al. 1999). The MR is expressed on cells of the macrophage lineage, is not detectable on blood monocytes, and has its expression modulated by inflammatory mediators (Stein & Ezekowitz 1992). The MR is also associated with a signal transduction pathway leading to cytokine production (Stahl et al. 1998). The expression of the MR on mononuclear cells in both bone marrow culture and spleen cells co-cultured with stromal cells gradually increased in the presence of bone-resorbing factors (Fig. 5), while those on RAW264·7 cells were constant and independent of the addition of sRANKL (Fig. 4). These differences in the expression of MR among the three systems may affect the time required for osteoclast formation by RAW264·7 cells compared with that by bone marrow and spleen cells. These results also suggest that MR expression is regulated by M-CSF, because it is recognized that RAW264·7 cells release M-CSF. Moreover, several lines of evidence have shown that M-CSF induces cell fusion of osteoclasts, resulting in...
multinucleation of the cells (Amano et al. 1998, Jimi et al. 1999a). We therefore attempted to examine the effect of either M-CSF or anti-M-CSF neutralizing antibody on MR expression in these systems. However, expression levels of MR were not affected by the addition of either M-CSF or anti-M-CSF antibody, suggesting that the mechanism of M-CSF for cell fusion of osteoclasts is independent of the expression of both MR and terminal high-mannose type oligosaccharide (data not shown).

In a recent study, Abe et al. (1999) demonstrated that antisense oligonucleotides of meltrin α involved in egg–sperm fusion inhibited by 70% the formation of multinucleated osteoclast-like cells expressing TRAP in co-cultures of bone marrow cells. Moreover, anti-fusion regulatory protein-1/CD98 antibody has been shown to induce stimulated multinucleated giant cell and osteoclast formation (Higuchi et al. 1998). These are recognized as protein-related cellular fusion events. Several lines of evidence have shown that M-CSF, interleukin-1, RANKL and vitronectin receptors are involved in the fusion process in osteoclast differentiation (Amano et al. 1998, Nakamura 1998, Jimi et al. 1999a,b). However, there are no reports that the expression of either meltrin α or CD98 is regulated by several cytokines and receptors. In our experiments, we used 1α,25-(OH)2D3, PGE2, M-CSF and RANKL as stimulators of osteoclast differentiation, but the expression of both MR and terminal high-mannose type oligosaccharide was not affected directly. Taken together, the binding between the MR, and terminal high-mannose type oligosaccharide on the cell surface of osteoclast progenitor cells appears to be one of the processes of cell–cell adhesion, after which the cells are fused by protein-related fusion machinery systems.

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