Osteoclast-like cells express receptor activity modifying protein 2: application of laser capture microdissection

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Abstract

Receptor activity modifying proteins (RAMPs) act as receptor modulators that determine the ligand specificity of receptors for the calcitonin (CT) family. The purpose of this study was to analyze the expression of RAMPs in osteoclast-like cells using the laser capture microdissection (LCM) technique. Mouse bone marrow and spleen cells were co-cultured on a film designed for LCM. After 10 days, 250 osteoclast-like cells were captured using the LCM system. Total RNA from these cells was used to synthesize cDNA and RT-PCR analysis was performed. Osteoclast-like cells expressed CT receptor (CTR), CT receptor-like receptor (CRLR) and RAMP2, but did not express RAMP1 or RAMP3. These results indicated (1) that a pure population of osteoclast-like cells can be prepared by LCM and gene expression of this population can be analyzed by RT-PCR and (2) that RT-PCR shows that osteoclast-like cells express RAMP2, CTR and CRLR, suggesting the potential for adrenomedullin binding to osteoclast-like cells. This is the first report that osteoclast-like cells express RAMP2.

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Introduction

The calcitonin (CT) family of peptides comprises five known members: CT, amylin (AMY), two CT gene-related peptides (CGRP1 and CGRP2) and adrenomedullin (ADM). Receptor activity modifying proteins (RAMPs) comprise a family of accessory proteins for G protein-coupled receptors, three of which act as receptor modulators that determine the ligand specificity of receptors for CT family members. CT receptor-like receptor (CRLR) has been shown to form a high affinity receptor for CGRP, when associated with RAMP1, or, when associated with RAMP2 or RAMP3, to specifically bind ADM (McLatchie et al. 1998, Sexton et al. 2001). RAMPs are type I transmembrane proteins that share ~30% amino acid identity and a common predicted topology, with short cytoplasmic C termini, one transmembrane domain and large extracellular N termini that are responsible for the specificity (McLatchie et al. 1998, Fraser et al. 1999). More recently, CT receptor (CTR) was demonstrated to form heterodimeric complexes with RAMP. CTR/RAMP1 and CTR/RAMP3 heterodimers exhibited the pharmacological profiles of receptors specific for AMY (Christopoulos et al. 1999, Muff et al. 1999, Sexton et al. 2001).

There is significant interest in analyzing gene expression of distinct cell populations. Heterogeneous populations of cells within tissues of various types possess correspondingly different patterns of gene expression, and these cell types must be separated from one another for accurate assessment of gene expression. Tong et al. (1994) has reported that a microisolation system using a micromanipulator tool was applied for mRNA phenotyping of a blood cell lineage. Laser capture microdissection (LCM) is a particularly useful tool for recovering small cell samples and even enables the collection of individual cells from tissue sections (Emmert-Buck et al. 1996). This method facilitates the separation of histologically distinct cells so that proteins, DNA or RNA from these cells can be analyzed in isolation from the surrounding cells (Bonner et al. 1997). Osteoclasts act centrally in the remodeling of bone in normal and diseased states. Nonetheless, because of their low numbers within bone, cell culture model systems have been increasingly used to investigate the biochemical functions of osteoclasts (Udagawa et al. 1989, Nakamura et al. 1998). However, because of their heterogeneity and adherence to the plate in such systems, there has been difficulty and controversy in analyzing these cell types. Thus, a more sensitive isolation method for osteoclasts is needed.
To address this problem, we used LCM techniques to isolate a pure population of osteoclast-like cells. We then analyzed RAMP gene expression in microdissected osteoclast-like cells using RT-PCR.

Materials and methods

An in vitro osteoclast model

Osteoclast differentiation in vitro was induced using the technique described by Udagawa et al. (1989). Both bone marrow cells and spleen cells were obtained from 10- to 14-week-old male C57BL/6 mice (Charles River, Sagamihara, Japan). The bone marrow cells were collected from tibiae and femora. Splenic tissue was cut with scissors and dispersed by pipetting, then the spleen cells were collected by centrifugation at 1000 r.p.m. for 5 min at 4 °C. Bone marrow cells were co-cultured with spleen cells (2 × 10^6 cells/ml for each cell type) on a film produced for use in LCM (Matsunami Glass Co., Osaka, Japan) for 10 days at 37°C in a humidified atmosphere of 5% CO_2. Cultures were fed with α-modified Eagle’s medium supplemented with penicillin and streptomycin, 10% fetal calf serum (Hyclone, Logan, UT, USA) and 10^{-8} M 1,25(OH)_2 D_3 (Calbiochem-Novabiochem Co., San Diego, CA, USA). Multinucleated osteoclast-like cells were then isolated using LCM. All the animal experimental procedures were approved by the Animal Care and Use Committee of Wakayama Medical University (Wakayama, Japan).

LCM of samples

Before LCM, cells were fixed in ethanol for 1 min and stained for 3 min with filtered hematoxylin. They were then washed with sterilized water and air-dried for 10 min. LCM of cultured osteoclast-like cells was performed using the Application Solutions Laser Microdissection System (Leica Microsystems Co., Tokyo, Japan) according to the manufacturer’s instructions.

RNA isolation

Total RNA was extracted from 250 LCM-captured cells and 250 spleen cells. The spleen cells used for RNA extraction were from an aliquot of those prepared for the co-culture system. Total RNA extraction was performed using TRIzol LS Reagent (Invitrogen Life Technologies Co., Carlsbad, CA, USA) as described by the manufacturer. Briefly, 170 µl TRIzol reagent was added to a tube containing LCM cells and this was incubated for 5 min at room temperature. Forty microliters of chloroform were then added and the tube was incubated at room temperature for a further 15 min. The aqueous phase was transferred to a new tube and isopropyl alcohol was added followed by centrifugation at 12 000 g for 10 min. The RNA precipitate was washed with 70% ethanol and dissolved in 20 µl sterilized water.

RT-PCR

The SUPERSCRIPT One-Step RT-PCR with PLATINUM Taq (Invitrogen Life Technologies Co.) was used to synthesize cDNA and PCR was performed as described by the manufacturer. The nucleic acid sequences of primers used for RT-PCR are shown in Table 1. RT-PCR reactions were initially performed in a 25 µl reaction volume containing 1 µl of each primer (at 100 ng/µl) and 3 µl RNA as template. The reactions were run at 55°C for 30 min (cDNA synthesis) and 94°C for 2 min (pre-denaturation), followed by 45 cycles of 94°C for 30 s (denaturation), 53°C for 30 s (annealing) and 72°C for 30 s (extension), followed by 7 min at 72°C (final extension). To increase the detection capacity, we performed a second round of PCR. The second-round PCR reactions were carried out using Taq polymerase (Perkin-Elmer-Cetus, Norwalk, CT, USA) with 8 µl RT-PCR products as template (final 25 µl reaction mixture) under the following conditions: 35 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s. In the second-round PCR, CTR was amplified using 2nd sense and anti-sense primers (Table 1). The primers of CRLR, RAMP1, 2, 3, CTR and β-actin for the second-round PCR were the same primers as those used in the initial RT-PCR. The samples were electrophoresed in 3% agarose gels and stained with ethidium bromide.

Results and discussion

We have developed a rapid and precise method for the isolation of pure populations of osteoclast-like cells using LCM. Figure 1 illustrates two osteoclast-like cells before and after LCM. Two hundred and fifty cells with > three nuclei each were isolated. Total RNA was extracted and RT-PCR was used to analyze multiple gene expressions. Figure 2 shows the RT-PCR results for CTR, RAMP1, 2 and 3, CRLR and β-actin. The predicted sizes were clearly visualized by ethidium bromide staining. RT-PCR results showed that the ubiquitous gene, β-actin, was amplified from both spleen and osteoclast-like cells, whereas CTR mRNA was amplified from osteoclast-like cells alone. RAMP1 and RAMP3 mRNAs were amplified from spleen cells alone. RAMP2 and CRLR mRNAs were amplified from both types of cells.

In the present study, we were able to isolate a pure population of osteoclast-like cells and detect a series of gene expressions. Two hundred and fifty cells were...
used for RNA extraction and cDNA synthesis. Three microliters of the 20 µl cDNA solution was successful for each gene amplification. Approximately 40 cells were therefore used for RT-PCR analysis. Naot et al. (2001) have reported that osteoclastic cells such as primary osteoblasts and UMR 106-06 cells expressed all three types of RAMP analyzed using RT-PCR. A very high expression of mRNA for RAMP2 was detected in those cells, compared with those for RAMP1 and RAMP3. Previous studies showed that osteoblast but not osteoclast cells express ALP (Tong et al. 1994). To exclude the possibility of osteoblast contamination, we investigated ALP mRNA expression in the microdissected osteoclast-like cells. The result showed that no ALP mRNA was detectable (Fig. 3), which supported the idea that RAMP2 was amplified from osteoclast-like cells. Thus, LCM is a useful technique for isolation of small cell samples, and our strategy might be extended to other procedures, such as quantitative RT-PCR to measure mRNA levels in the osteoclast. The bone marrow macrophages are the precursors of osteoclasts; it will be interesting to compare gene expression between osteoclasts and bone marrow macrophages. Immunostaining of the Fc receptor, C5 receptor or vitamin D receptor will help to distinguish those cell types in our co-culture system. However, in order to perform RNA

Table 1 Oligonucleotide sequences used for PCR

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
<th>Size</th>
</tr>
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<tbody>
<tr>
<td>CTR</td>
<td>5’-GTCTTGCAACTTCTGGATGC-3’</td>
<td>255 bp</td>
</tr>
<tr>
<td></td>
<td>5’-AAGAAGAAGTGACCACCCAGAC-3’</td>
<td>255 bp</td>
</tr>
<tr>
<td></td>
<td>5’-GTCTTGCAACTTCTGGATGC-3’</td>
<td>104 bp</td>
</tr>
<tr>
<td></td>
<td>5’-GAAGATAGTACCCACTGAGGC-3’</td>
<td>(U18542*)</td>
</tr>
<tr>
<td>RAMP1</td>
<td>Sense, 5’-CACCTCTCTCTCATGTCACTG-3’</td>
<td>187 bp</td>
</tr>
<tr>
<td></td>
<td>Antisense, 5’-CAATCTGTCGCCACGCAGTCG-3’</td>
<td>(AJ250489)</td>
</tr>
<tr>
<td>RAMP2</td>
<td>Sense, 5’-GCAAGTATGGACATGTTTCCG-3’</td>
<td>217 bp</td>
</tr>
<tr>
<td></td>
<td>Antisense, 5’-GGAAGATAGTACCAGCGTAGGC-3’</td>
<td>(AJ250490)</td>
</tr>
<tr>
<td>RAMP3</td>
<td>Sense, 5’-GTGTGTCGTTGAGGTGTCACCGG-3’</td>
<td>189 bp</td>
</tr>
<tr>
<td></td>
<td>Antisense, 5’-GCCATGATTTGGTCTCCATG-3’</td>
<td>(AJ250491)</td>
</tr>
<tr>
<td>CRLR</td>
<td>Sense, 5’-TTGTTAGGATGATGGTCCAGG-3’</td>
<td>225 bp</td>
</tr>
<tr>
<td></td>
<td>Antisense, 5’-GTATGGCCACTGCCGTGA-3’</td>
<td>(AF146525)</td>
</tr>
<tr>
<td>ALP</td>
<td>Sense, 5’-ATCGGAACGTGTACCTGGATT-3’</td>
<td>152 bp</td>
</tr>
<tr>
<td></td>
<td>Antisense, 5’-ATCGAGTTCTTGGCTCCGTAC-3’</td>
<td>(NM007431)</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Sense, 5’-GTGGGGCGGTCTAGGCACCA-3’</td>
<td>246 bp</td>
</tr>
<tr>
<td></td>
<td>Antisense, 5’-GGTTGGCCTTAGGTTGTC-3’</td>
<td>(Flores-Delgado et al. 1998)</td>
</tr>
</tbody>
</table>

*Accession number of GeneBank.
ALP, alkaline phosphatase.

Figure 1 Osteoclast-like cells were isolated by using LCM. Two osteoclast-like cells (A) before and (B) after microdissection are shown. Stained by hematxylin; bar denotes 50 µm.
analysis after immunostaining, further efforts should be made to modify the conventional staining protocol to protect RNA from degradation.

Our findings that osteoclast-like cells expressed RAMP2 and CRLR as well as CTR provide the first evidence that osteoclasts express RAMP2. These results suggest that osteoclasts may have the ability to bind ADM through the CRLR/RAMP2 heterodimer. ADM is a 52 amino acid peptide first described in a human phaeochromocytoma but subsequently found to be present in many tissues, including the vascular system and bone tissue (Kitamura et al. 1993). Naot et al. (2001) has suggested that ADM is mitogenic to osteoblasts, raising the possibility that ADM is a local regulator of bone growth; however, the action of ADM or RAMP on the osteoclast is not clear. It has been reported that bone abnormalities were observed in both CTR +/− and AMY +/− mice, thereby ruling out the possibility that AMY uses CTR to inhibit osteoclastogenesis in vivo (Dacquin et al. 2004).

In summary, we have demonstrated that LCM is a useful solution for osteoclast research. We found that osteoclast-like cells expressed mRNAs for CTR, CRLR and RAMP2 but not RAMP1 or RAMP3; RAMP2 may therefore play an important role in osteoclast function. Further study is needed to elucidate the role of RAMP2 and its relationship to the CT family of receptors.

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References


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