Discrimination of ameloblastomas from odontogenic keratocysts by cytokine levels and gelatinase species of the intracystic fluids

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Abstract
In the present study, we measured the levels of the cytokines and gelatinase species in the fluids of ameloblastomas and odontogenic keratocysts, and showed that ameloblastomas can be distinguished from odontogenic keratocysts by the use of these biochemical data. We found that interleukin (IL)-1α and IL-1β levels in the intracystic fluids of ameloblastomas were significantly lower than those in the fluids of odontogenic keratocysts, while IL-6 levels in the fluids of ameloblastomas were significantly higher than those in the fluids of odontogenic keratocysts. On the other hand, no significant differences in tumor necrosis factor (TNF)-α levels of the fluids were detected between ameloblastomas and odontogenic keratocysts. An immunohistochemical study revealed that the staining intensity of IL-1α, IL-1β and TNF-α in the tumor cells of ameloblastomas was significantly weaker than that in the epithelial cells of odontogenic keratocysts, while the staining intensity of IL-6 in the tumor cells was significantly stronger than that in the epithelial cells of odontogenic keratocysts. Gelatin zymography of the fluids showed that only a small amount of pro-MMP-9 was detected in ameloblastomas, while both pro-MMP-9 and the active form of MMP-9 were detected in 8 of 10 cases of odontogenic keratocysts. Thus, ameloblastomas can be distinguished from odontogenic keratocysts by measuring IL-1α and IL-6 levels, and gelatinase species in the fluids.

Key words: ameloblastoma; odontogenic keratocyst; cytokine; gelatinase


Ameloblastoma is a benign odontogenic tumor which consists of proliferating odontogenic epithelium in a sparsely cellular connective tissue stroma (1). On the other hand, odontogenic keratocyst is a common jaw cyst characterized by a lining of keratinized stratified squamous epithelium with a thin fibrous capsule (1). It is recognized that multilocularity and root resorption are the most common radiographic signs of ameloblastoma (2). It is often difficult to distinguish ameloblastoma from an odontogenic keratocyst because, in
most cases, the clinical findings that include radiographic views are similar to each other (3). In addition, it has been reported that the tumor is sometimes misdiagnosed even by a pathological examination, especially in the cases of mural ameloblastomas (3). It is, therefore, indispensable to develop diagnostic methods for the cystic lesions in jaws.

Inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) precede bone resorption by inducing the production of prostaglandin (4) and collagenase (5–9), and the formation of osteoclast-like cells (4, 10). It has been shown that these cytokines are expressed in odontogenic jaw cysts (11–14) and ameloblastomas (15). Therefore, it is speculated that these cytokines play a crucial role in the expansion of cystic lesions, including ameloblastomas.

Matrix metalloproteinases (MMPs) are involved in the degradation of extracellular matrix macromolecules in various pathological conditions. MMP-2 and MMP-9 are gelatinases of the MMP family, and degrade many types of collagens, such as native type IV, V and X collagens, and denatured fibrillar type I, II, and III collagens (16). It has been reported that these gelatinases are involved in normal tissue remodeling and pathological processes, such as arthritis, tumor metastasis, wound healing, angiogenesis, inflammation, embryonic development, and bone resorption (17–19). MMP-2 and MMP-9 are also detected in odontogenic jaw cyst wall extracts and cyst fluids (20–22). Therefore, it is likely that these gelatinases are involved in pathological processes in the expansion of the cystic lesions. Both MMP-2 and MMP-9 are secreted as inactive forms (pro-MMP-2 and pro-MMP-9) and need to be converted to the active forms in order to work. Recently, we have shown that the activity ratio of MMP-9 (pro-MMP-9 gelatinolytic activity/total MMP-9 gelatinolytic activity) depends on the type of odontogenic jaw cyst (22). In addition, IL-1α stimulates the production and activation of pro-MMP-9 in the epithelial cells of odontogenic jaw cysts (22).

Taken together, the above findings suggest that the differences in the cytokine levels and the gelatinase species of the intracystic fluids would help us to diagnose cystic lesions, although there are few reports about the cytokine levels and the gelatinase species of the intracystic fluids of ameloblastomas.

**Material and methods**

**Tissues and intracystic fluids**

Fourteen cystic ameloblastomas (10 men, 4 women; mean age 30.8±6.4 years) and 10 odontogenic keratocysts (6 men, 4 women; mean age 30.3±7.5 years) were selected at random from patients who underwent surgical treatment at the Kyushu University Dental Hospital after informed consent was obtained. The patients had no history of infection in the cystic lesions, and they were not treated with any antibiotics. No oro cystic communications were clinically detected before surgery. The intracystic fluids were taken just before surgery. Blood sera from the same patients were used as a control. The intracystic fluids and blood samples were centrifuged at 2500 g for 15 min at 4°C, and the supernatant and sera were stored at −80°C until further use (22). The tissue samples were fixed immediately in phosphate-buffered saline (PBS) (150 mM NaCl and 10 mM phosphate buffer, pH 7.4) containing 4% paraformaldehyde (Polysciences Inc., Warrington, PA, USA) for 24 h. Cases with Gorlin’s syndrome were excluded from the present investigation.

**Measurement of IL-1α, IL-1β, and TNF-α concentrations**

The concentrations of IL-1α, IL-1β, IL-6 and TNF-α in the intracystic fluids were measured by the enzyme-linked immunosorbent assay (ELISA) (Amersham International plc, Buckinghamshire, UK), according to the manufacturer’s instructions. A group of serially diluted standard samples of the recombinant human IL-1α, recombinant human IL-1β, recombinant human IL-6, and recombinant human TNF-α were used to generate the standard curves. The system could measure human IL-1α and human IL-1β in the range from 3.9 pg/ml to 250 pg/ml, human IL-6 in the range from 31 pg/ml to 300 pg/ml, and human TNF-α in the range from 15.6 pg/ml to 1000 pg/ml. Absorbance was measured at 450 nm using an ELISA reader (Bio-Rad Laboratories, Richmond, CA, USA).

**Immunohistochemistry**

An immunohistochemical procedure was performed on paraffin sections by the avidin-biotin-peroxidase complex (ABC) method using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). Briefly, the deparaffinized and rehydrated sections (4 μm in thickness) were treated with 0.3% hydrogen peroxide (H2O2) in 96% methanol for 30 min to block endogenous peroxidase activity. After washing in PBS, the sections were treated with normal serum for 20 min to eliminate any non-specific binding of conjugated secondary antibodies, and then incubated with anti-human IL-1α, IL-1β, IL-6 or TNF-α antibody overnight at 4°C. After rinsing with PBS, the sections were incubated with a second antibody for 1 h at room temperature, and then incubated with an ABC reagent for 45 min. As negative controls, the primary antibody was substituted with normal serum at the same dilution rate. The antibodies for IL-1α,
IL-1β, and TNF-α were purchased from Genzyme Corporation (Cambridge, MA, USA) and the antibody for IL-6 was purchased from R&D System, Inc. (Minneapolis, MN, USA). The staining intensity for antibodies was graded as follows: intense (++), moderate (+), mild (+), or almost negative (−).

**Gelatin zymography**

Gelatin zymography was performed in 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels impregnated with 2 mg/ml gelatin (22, 23). Briefly, the gelatin had been labeled with 2-methoxy-2,4-diphenyl-3(2H)-furanone, as described previously (22, 24). The supernatant of the intracystic fluid (0.2 ml) was electrophoresed under non-reducing conditions. After running gels, the gels were washed with 2.5% Triton-X 100 for over 1 h at room temperature, and then incubated in 200 mM NaCl, 5 mM CaCl₂, 30 mM Tris-HCl (pH 7.6), and 0.02% NaN₃ for 18 h at 37°C. The lysis of gelatin was visualized under long-wave UV light, and the images of the gels were captured with a computer system (17, 22). The gelatinolytic activities of the samples were calculated from the integrated density of each band. A group of serially diluted purified MMP-9 (Yagai Co, Yamagata, Japan) was run with the samples in each gel to make the standard curve. Finally, the gels were stained in 0.2% Coomassie Brilliant Blue R250 in 50% methanol and 10% acetic acid, and destained in 30% methanol and 10% acetic acid.

**Western immunoblotting**

Samples were run on 10% SDS-polyacrylamide gels, and transferred onto nitrocellulose paper in 20% methanol, 20 mM Tris, and 150 mM glycine (pH 8.8) at 100 mA for 18 h, as described previously (22). Non-specific binding was blocked by incubating the nitrocellulose paper with 5% bovine serum albumin in Tris-buffered saline Tween-20 (TBST) containing 150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 0.05% Tween-20, and 0.02% NaN₃ for 1 h. The nitrocellulose papers were incubated with monoclonal antiserum for MMP-2 and MMP-9 (each from Calbiochem, Cambridge, MA, USA) in TBST overnight at room temperature, and developed by a horseradish peroxidase ABC kit. The antibodies for MMP-2 and MMP-9 recognized both latent and active forms of MMP-2 and MMP-9.

**Statistical analysis**

Data are expressed as mean±SE. Statistical significance was assessed by Student’s t-test or the χ²-test, and P values <0.05 were considered significant.

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**Results**

**Intracystic concentration of cytokines**

IL-1α, IL-1β, IL-6, and TNF-α levels in the intracystic fluids of ameloblastomas and odontogenic keratocysts were measured by ELISA. Both IL-1α and IL-1β levels in the fluids of ameloblastomas [32.1±11.6 pg/ml (n=14) and 91.6±49.8 pg/ml (n=14), respectively] were significantly lower than those in the fluids of odontogenic keratocysts [378.4±33.1 pg/ml (n=10) and 653.7±176.3 pg/ml (n=10), respectively], while the IL-6 levels in the fluids of ameloblastomas [1045.9±163.7 pg/ml (n=14)] were significantly higher than those in the fluids of odontogenic keratocysts [31.8±13.5 pg/ml (n=10)]. On the other hand, no significant difference in the levels of TNF-α was detected in the fluids of ameloblastomas and odontogenic keratocysts (Fig. 1). The serum IL-1α levels were 0.01±0.02 pg/ml (n=24), and the serum IL-1β, IL-6 and TNF-α levels (each n=24) were below the sensitivity of the assay system.

**Immunostaining of cytokines**

Expression of IL-1α, IL-1β, IL-6 and TNF-α in the sections of ameloblastomas and odontogenic keratocysts was examined immunohistochemically.
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In the tumor cells of ameloblastomas, + (moderate) staining for IL-6 was detected in most all specimens (13 of 14 specimens), although + staining for IL-1α and IL-1β was detected in 4 and 6 of 14 specimens, respectively. No + or ++ (intense) staining for TNF-α was detected in ameloblastomas. The positive staining for IL-1α, IL-1β and TNF-α was detected in the epithelial cells as well as in the endothelial cells and fibroblasts in the subepithelial layers of odontogenic keratocysts. In the epithelial

**Fig. 2.** Immunohistochemical staining of cytokines in ameloblastoma and odontogenic keratocyst. Immunohistochemical staining with anti-IL-1α (**a and e**), anti-IL-1β (**b and f**), anti-IL-6 (**c and g**), and anti-TNF-α (**d and h**) antibodies in ameloblastoma (**a-d**) and odontogenic keratocyst (**e-h**) was shown. (Magnification ×200).
cells of odontogenic keratocysts, + ~ ++ staining for IL-1α and IL-1β was detected in all and in 9 of 10 specimens, respectively, and + staining for TNF-α was detected in the half specimens. Moderate (+) staining for IL-6, however, was detected only in 3 of 10 specimens. Statistical analysis revealed that the staining intensity of IL-1α, IL-1β and TNF-α in the tumor cells of ameloblastomas was significantly weaker than that in the epithelial cells of odontogenic keratocysts, but the staining intensity of IL-6 in the tumor cells of ameloblastomas was significantly stronger than that in the epithelial cells of odontogenic keratocysts (Table 1). In addition, the staining intensity of IL-1α, IL-1β and TNF-α in the endothelial cells of ameloblastomas was significantly weaker than that in odontogenic keratocysts and was significantly stronger than that in odontogenic keratocysts (Table 1).

### Table 1. Immunostaining of cytokines in ameloblastomas and odontogenic keratocysts

<table>
<thead>
<tr>
<th></th>
<th>IL-1α</th>
<th>IL-1β</th>
<th>IL-6</th>
<th>TNF-α</th>
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<tr>
<td><strong>Ameloblastomas (n=14)</strong></td>
<td></td>
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<tr>
<td>Tumor cells</td>
<td>++</td>
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<td>−</td>
<td>7</td>
<td>4</td>
<td>0</td>
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<tr>
<td>Endothelial cells</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>6</td>
<td>2</td>
<td>3</td>
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<td></td>
<td>±</td>
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<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>8</td>
<td>10</td>
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</tr>
<tr>
<td><strong>Odontogenic keratocysts (n=10)</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>++</td>
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<td>7</td>
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<td>+</td>
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<td>Endothelial cells</td>
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### Table 2. MMP-9 activities of the fluids of ameloblastomas and odontogenic keratocysts

<table>
<thead>
<tr>
<th></th>
<th>MMP-9 (mU/ml)</th>
<th>Activity ratio</th>
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<tr>
<td></td>
<td>92 kDa</td>
<td>83 kDa</td>
</tr>
<tr>
<td><strong>Ameloblastomas (n=14)</strong></td>
<td>73.1±35.7</td>
<td>0</td>
</tr>
<tr>
<td><strong>Odontogenic keratocysts (n=10)</strong></td>
<td>535.2±108.6</td>
<td>347.2±125.8</td>
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</table>

Gelatinolytic activity

Gelatin zymography showed that 92-kDa gelatinase was present in the fluids of odontogenic keratocysts. In addition, 83-kDa gelatinase was detected in 8 of 10 cases of odontogenic keratocysts. On the other hand, only a small amount of 92-kDa gelatinase, but no 83-kDa gelatinase, was detected in the fluids of ameloblastomas and serum (Fig. 3A). In immunoblotting, 92-kDa and 83-kDa gelatinases were reacted with anti-MMP-9 antibody, which suggested that both gelatinases were pro-MMP-9 and the active form of MMP-9, respectively (Fig. 3B). No immunoreactivity against anti-MMP-2 antibody was detected in the fluids of odontogenic keratocysts or ameloblastomas (data not shown). Both total MMP-9 activities (92-kDa and 83-kDa MMP-9 gelatinolytic activities) and the activity ratio (83-kDa MMP-9 activities/total MMP-9 activities) of odontogenic keratocyst fluids were significantly higher than those of ameloblastoma fluids (Table 2).

### Discussion

We have demonstrated that there are significant differences in the levels of IL-1α, IL-1β and IL-6 between the fluids of ameloblastomas and odontogenic keratocysts, although the levels of TNF-α in the intracystic fluids were not significantly different between...
them. The levels of these cytokines in the fluids were significantly higher than those of sera, and the protein concentration of the fluids of ameloblastomas (21.0±6.9 mg/ml) and odontogenic keratocysts (24.0±13.1 mg/ml) was significantly lower than that of sera (57.4±1.7 mg/ml). Therefore, the intracystic fluids of ameloblastomas and odontogenic keratocysts are different from those of the sera. Our immunohistochemical study supported the differences in the levels of the cytokines in the fluids. IL-1α and IL-1β were expressed strongly in the epithelial cells, endothelial cells, and fibroblasts of odontogenic keratocysts, as reported previously (25), while weaker or no expression of IL-1α and IL-1β was detected in the tumor cells of ameloblastomas. On the other hand, IL-6 was expressed in the tumor cells of ameloblastomas more strongly than in the epithelial cells of odontogenic keratocysts. Recently, it was reported that IL-1α and IL-1β were expressed strongly in the epithelial cells, endothelial cells, and fibroblasts of odontogenic keratocysts, as reported previously (25), while weaker or no expression of IL-1α and IL-1β was detected in the tumor cells of ameloblastomas. On the other hand, IL-6 was expressed in the tumor cells of ameloblastomas while faint staining for IL-1α and IL-1β was detected in the tumor cells of ameloblastomas. On the other hand, IL-6 was expressed in the tumor cells of ameloblastomas while faint staining for IL-1α and IL-1β was detected in the tumor cells of ameloblastomas.

Gelatinases/type IV collagenases are thought to be involved in the cyst expansion in the jaws (20, 21). An interesting finding in the present study was that only a small amount of pro-MMP-9 was present in the fluids of ameloblastomas, although both pro-MMP-9 and the active form of MMP-9 were present in the fluids of odontogenic keratocysts in most cases, as shown previously (20–22). Since a larger amount of pro-MMP-9 is present in the fluids of dentigerous cysts (1362.3±402.9 mU/ml) and radicular cysts (1057.1±231.1 mU/ml) than in the fluids of ameloblastomas (22), ameloblastoma can be distinguished from three major types of odontogenic jaw cyst by measuring gelatinolytic species of the fluids.

It has been shown that IL-1α precedes bone resorption in odontogenic jaw cysts (14, 25). In addition, we showed that IL-1α stimulated both pro-MMP-9 secretion and its activation in the epithelial cells of odontogenic jaw cysts (22). Therefore, IL-1α should play a crucial role in the odontogenic jaw cyst expansion. On the other hand, the precise pathological functions of IL-6 in odontogenic jaw cysts are not well understood. Since the intensity of IL-6 expression in the sections of ameloblastomas was stronger than that of IL-1α, the expansive mechanisms may be different between ameloblastomas and odontogenic keratocysts. It is sometimes difficult to distinguish an ameloblastoma from an odontogenic jaw cyst by clinical findings and a usual pathological examination, especially in the case of mural ameloblastoma (3). Only 200 μl and 0.2 μl supernatant of intracystic fluid sample is needed for measuring; respectively, the cytokine concentration and the gelatinase species when our assay systems are employed. Therefore, these assay systems can be used for relatively small cystic lesions, and the combination of the assay systems would support a pathological diagnosis of the cystic lesions.

References

Cytokine levels and gelatinase activity


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