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Regulation of IL-6 and IL-8 production by reciprocal cell-to-cell interactions between tumor cells and stromal fibroblasts through IL-1α in ameloblastoma

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ABSTRACT

Ameloblastoma is an odontogenic benign tumor that occurs in the jawbone, which invades bone and reoccurs locally. This tumor is treated by wide surgical excision and causes various problems, including changes in facial countenance and mastication disorders. Ameloblastomas have abundant tumor stroma, including fibroblasts and immune cells. Although cell-to-cell interactions are considered to be involved in the pathogenesis of many diseases, intercellular communications in ameloblastoma have not been fully investigated. In this study, we examined interactions between tumor cells and stromal fibroblasts via soluble factors in ameloblastoma.

We used a human ameloblastoma cell line (AM-3 ameloblastoma cells), human fibroblasts (HFF-2 fibroblasts), and primary-cultured fibroblasts from human ameloblastoma tissues, and analyzed the effect of ameloblastoma-associated cell-to-cell communications on gene expression, cytokine secretion, cellular motility and proliferation. AM-3 ameloblastoma cells secreted higher levels of interleukin (IL)-1α than HFF-2 fibroblasts. Treatment with conditioned medium from AM-3 ameloblastoma cells upregulated gene expression and secretion of IL-6 and IL-8 of HFF-2 fibroblasts and primary-cultured fibroblast cells from ameloblastoma tissues. The AM3-stimulated production of IL-6 and IL-8 in fibroblasts was neutralized by pretreatment of AM-3 cells with anti-IL-1α antibody and IL-1 receptor antagonist. Reciprocally, cellular motility of AM-3 ameloblastoma cells was stimulated by HFF-2 fibroblasts in IL-6 and IL-8 dependent manner. In conclusion, ameloblastoma cells and stromal fibroblasts behave interactively via these cytokines to create a microenvironment that leads to the extension of ameloblastomas.

1. Introduction

The WHO classification of ameloblastoma is defined as a benign tumor with odontogenic epithelial cell growth. This tumor is classified into subtypes such as follicular, plexiform, and mixed types and is comprised of tumor parenchyma consisting of tumor cells and rich tumor stroma [1]. Despite the benign nature of ameloblastoma, it demonstrates invasion and resorption of bone and tooth roots. Radical surgeries such as marginal resection, segmental resection and total resection of the jaw with wide margins are required to treat this tumor. While these treatments prevent postoperative recurrence, they significantly impair the patient quality of life [2]. Conservative surgical therapies are often chosen as the first alternative therapy. However, these procedures have a high recurrence rate [3,4]. The appearance of osteoclasts on the bone surface around ameloblastomas suggest that ameloblastoma cells secrete various bioactive factors that induce osteoclastogenesis [5].

Abnormal cell-to-cell interactions between cells that are normally located in different compartments might modulate cellular behavior [6]. Such interactions might regulate the pathogenic processes in cancerous invasion, inflammation, and wound healing.

Abbreviations: IL, interleukin; IL-1Ra, IL-1 receptor antagonist; D-KSFM, Defined keratinocyte serum-free medium; CM, conditioned medium; FCS, fetal calf serum.

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Recently, new roles of stromal cells have been highlighted in many diseases. For example, fibroblasts surrounding malignant tumors were important for the growth and invasive characteristics of tumor cells [7,8]. Synovial fibroblasts secrete inflammatory cytokines and RANKL and induce osteoclastogenesis [9,10]. Furthermore, epithelial cells under inflammatory conditions secrete interleukin (IL)-1α that stimulates neighboring fibroblasts [11].

Ameloblastomas consist of odontogenic tissue and abundant tumor stroma, which includes various cells such as fibroblasts and macrophages. Taken together, these studies indicate that interactions between epithelial-derived ameloblastoma cells and surrounding mesenchymal fibroblasts might contribute to the pathogenesis of this disease. However, few cell lines of ameloblastomas have been derived, and there have been few reports regarding intercellular communication between ameloblastoma and fibroblasts.

In this study, we investigated cell-to-cell communications between ameloblastoma cells and stromal fibroblasts using AM-3, an ameloblastoma cell line recently established by our group [12].

2. Materials and methods

2.1. Antibodies and reagents

Anti-human IL-1α, IL-1β, IL-6, and IL-8 antibodies, mouse IgG, and recombinant human IL-6 and IL-8 were purchased from R&D systems (Minneapolis, MN, USA). Recombinant IL-1 receptor antagonist (IL-1Ra) was purchased from Wako Pure Chemical (Osaka, Japan). Defined keratinocyte serum-free medium (D-KSFM) was purchased from Invitrogen Corp., (Carlsbad, CA, USA).

2.2. Tissue samples and cell culture

AM-3 ameloblastoma cells were maintained with D-KSFM as previously described [12]. HFF-2 fibroblasts were maintained with DMEM containing 10% fetal calf serum (FCS) [13]. Mix typed (follicular and plexiform) ameloblastoma tissue and cystic fluid aspirates were obtained from the left side of the mandible of a Japanese man who had provided written informed consent (Supplementary data 1). This study followed the Helsinki Declaration guidelines. The research protocol and consent form were approved by the institutional review board of Kagoshima University in advance. Preparation of primary-cultured fibroblasts from ameloblastoma tissues was performed as described previously [14]. In brief, diced ameloblastoma tissue was washed with DMEM three times and treated with collagenase solution (20 mg/mL) at 37 °C for 2 h with shaking and then plated into a tissue culture flask. Two days later, enriched fibroblasts were obtained by short period (up to 2 min) trypsin/EDTA treatment and maintained with DMEM containing 10% FCS.

2.3. Microscopic images

Microscopic images were obtained using ECLIPSE Ti-E (Nikon Corp., Tokyo, Japan) and PowerShot A640 (Canon Inc., Tokyo, Japan) as described previously [12].

2.4. DNA microarray

Total RNA were obtained from cells using Nucleospin® RNA XS (Machinery-Nagel AG, Oensingen, Switzerland) and analyzed by Hokkaido System Sciences (Sapporo, Japan) using 8 × 60 array of Agilent technologies (Santa Clara CA).mRNA expression data was normalized using the Quantile normalization method [15]. To prepare a gene list, we selected genes with a fold-change greater than 1.5 or less than 0.67 when compared with controls.

2.5. Preparation of AM-3-derived conditioned medium (CM)

A total of 5×10^5 of AM-3 ameloblastoma cells was cultured in a 100 mm-diameter dish with 10 mL of D-KSFM. After 5 days, the CM was collected.

2.6. Preparation of CM from HFF-2 fibroblasts or primary-cultured fibroblasts

Semiconfluent HFF-2 fibroblasts or primary-cultured fibroblasts from ameloblastoma tissue in a 100 mm-diameter dish were treated with D-KSFM (50% (v/v)), AM-3-derived CM (50% (v/v)) in the presence of anti-IL-1α antibody (1 μg/mL), IL-1Ra (100 ng/mL), or normal mouse control IgG (1 μg/mL). After 5 days, the CM was collected (Supplementary data 2).

2.7. Migration assay

To measure cellular motility, a modified Boyden-chamber (tissue culture treated, 6.5 mm in diameter, 10 μm thickness, 8 μm pores; Transwell [Corning, Inc., Cambridge, MA, USA]) was used as previously described [16]. The lower surface of the filters was coated with 10 μg/mL fibronectin for 2 h. Cells (2.5×10^4 cells) were suspended in D-KSFM and applied to the upper chamber. The same medium or CM was applied to the lower chamber. After 9 h, cells on the upper side of the filters were removed and cells that migrated to the lower side of the upper chamber were fixed with 4% paraformaldehyde in PBS and stained with propidium iodide (1 μg/mL).

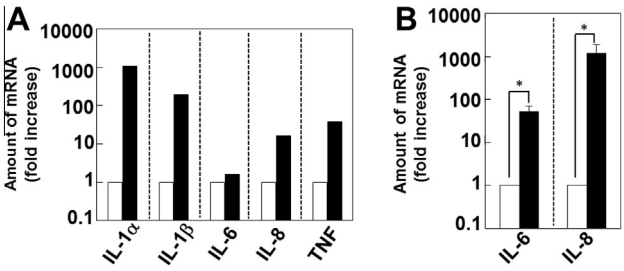

Fig. 1. Cytokine production in AM-3 ameloblastoma cells and HFF-2 fibroblasts stimulated with AM-3-derived CM. (A) Microarray analysis data of cytokine gene expression between AM-3 ameloblastoma cells and HFF-2 fibroblasts. White bars (controls) = HFF-2 fibroblasts; black bars = AM-3 ameloblastoma cells. (B) Fibroblasts stimulated by AM-3-derived CM secreted IL-6 and IL-8. Gene expression of cytokines from human fibroblasts treated with AM-3-derived CM. Results are means ± SE from at least three independent experiments. *$P < 0.05$. White bars (mock) = treated with serum-free medium (D-KSFM); black bars = treated with AM-3-derived CM.

Table 1
Concentration of cytokines in cystic fluid and cell-derived CM (pg/mL).

	IL-1α	IL-1β	IL-16	IL-18	TNF
Cystic fluid aspirate	218.91	688.47	13602.96	9220.11	139.84
AM-3 CM	61.02	11.44	596.20	619.97	57.77
HFF-2 CM	3.72	N.D.	2527.73	106.92	15.65
Primary fibroblasts CM	N.D.[a]	N.D.	1686.64	79.59	N.D.

[a] Not detectable.

2.8. Proliferation assay

AM-3 ameloblastoma cells (1×10^5 cells) were cultured in a 60 mm-diameter dish with D-KSFM in the presence of IL-6 (100 ng/mL) or IL-8 (100 ng/mL). Cells were counted at days 3 and 5.

2.9. Real-time RT-PCR

Total RNA was extracted from cells as described previously [14], and reverse-transcribed using a Transcriptor FirstStrand cDNA Kit (Roche Diagnostics, Indianapolis, IN, USA). Gene expression of cytokine mRNA was estimated by real-time RT-PCR using LightCycler TaqMan Master, LightCycler 1.5, and LightCycler software version 3.5 (Roche Diagnostics) according to the manufacturer's instructions. Expression of each mRNA was normalized using β-actin as a loading control. All primers used in this study were described previously [14].

2.10. ELISA

Concentrations of IL-1α, IL-1β, IL-6, IL-8, and TNF were analyzed by chemiluminescence based-Q-Plex® Human Cytokine array (Quansys Biosciences, Logan, UT, USA) in accordance with the manufacturer's instructions. Digital images were acquired using FluorChem® FC2 imaging system (Proteinsimple, Santa Clara, CA, USA) and analyzed with Quansys Q-View 2.5.2 software (Quansys Biosciences).

2.11. Statistical analyses

Statistical analyses were carried out using the nonparametric Wilcoxon rank sum test using StatMateIII for Macintosh® (ATMS, Tokyo, Japan).

3. Results

3.1. DNA microarray and multiplex ELISA

To identify cell-to-cell communication between AM-3 ameloblastoma cells and HFF-2 fibroblasts, we compared the gene expression between these cell lines in a mono-cell culture. The gene list was assigned KEGG annotations [17,18] by analysis of Genecodis [19–21] to identify molecular pathways. Besides typical differences in germ line marker expression were observed, genes in "cytokine–cytokine receptor interactions" were significantly elevated in AM-3 ameloblastoma cells (Supplementary data 3). Of these, IL-1α was expressed significantly in AM-3 ameloblastoma

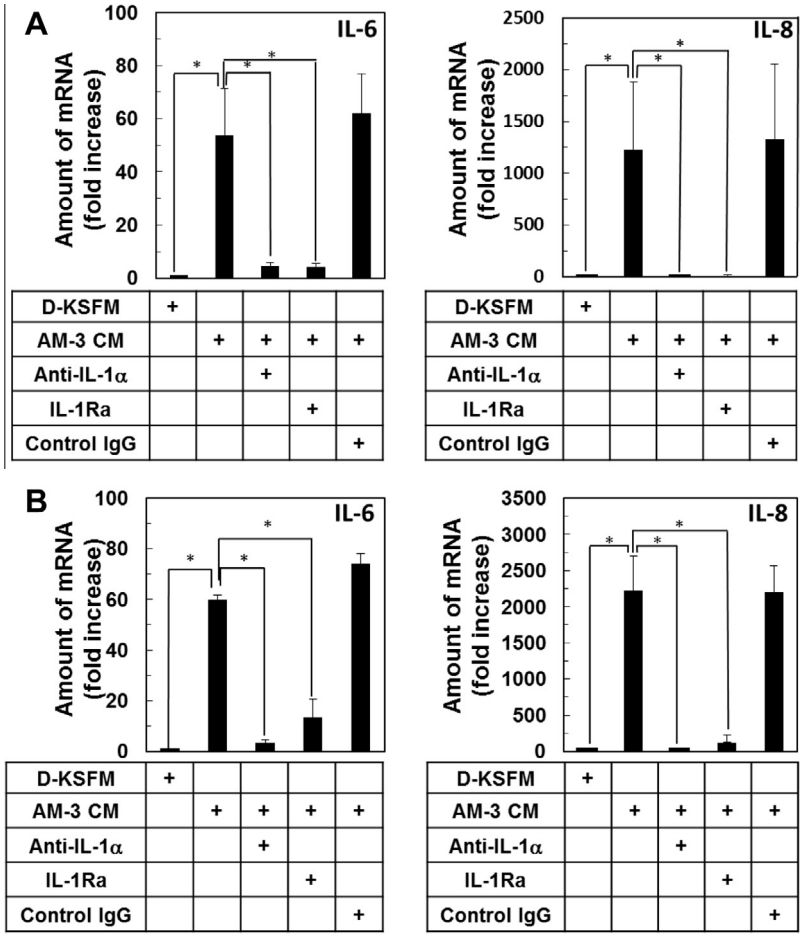

Fig. 2. Ameloblastoma-derived IL-1α regulates IL-6 and IL-8 gene expression in fibroblasts. (A) mRNA expression in HFF-2 human fibroblast cell lines. Induction of IL-6 (left panel) and IL-8 (right panel) in HFF-2 fibroblasts. HFF-2 fibroblasts were treated with D-KSFM (as control), AM-3-derived CM in the presence of anti-IL-1α antibody (1 μg/mL), IL-1Ra (100 ng/mL), or normal mouse control IgG (1 μg/mL) for 24 h and the amounts of mRNA of IL-6 and IL-8 were analyzed. (B) mRNA expression in human primary-cultured fibroblasts from ameloblastoma tissue. Induction of IL-6 (left panel) and IL-8 (right panel) in human primary fibroblasts from ameloblastoma tissue. Human primary-cultured fibroblasts were treated similarly as above. Results are means ± SE from at least three independent experiments. $^*P < 0.05$.

cells. IL-1β, IL-6, IL-8, and TNF were elevated slightly (Fig. 1A). These cytokines were also present at high levels in cystic fluid aspirate from human ameloblastoma. IL-1α was highly detected not only in cystic fluid aspirate but also in present in the CM of AM-3 ameloblastoma cells (Table 1).

To identify whether ameloblastoma cells affected the gene expression of mesenchymal cells, we investigated the gene expression of HFF-2 fibroblasts treated with AM-3-derived CM. The gene expression of IL-6 and IL-8 in HFF-2 fibroblasts was significantly upregulated in response to AM-3-derived CM (Fig. 1B). These findings suggested that AM-3-derived soluble factors could modulate the gene expression of HFF-2 fibroblasts.

3.2. IL-1α-dependent cell-to-cell communication between ameloblastoma cells and fibroblasts

Because IL-1α was highly secreted from AM-3 ameloblastoma cells, we added IL-1 antagonists to AM-3-derived CM to examine the effect of IL-1α on fibroblasts (Fig. 2). The gene expression of IL-6 and IL-8 in HFF-2 fibroblasts was elevated by the treatment of AM-3-derived CM, and was completely neutralized by the addition of anti-IL-1α antibody and IL-1Ra (Fig. 2A). Primary-cultured fibroblasts from ameloblastoma tissue responded similarly (Fig. 2B). Treatment with anti-IL-1β antibody did not neutralize the activity of AM-3-derived CM in contrast to anti-IL-1α antibody (data not shown).

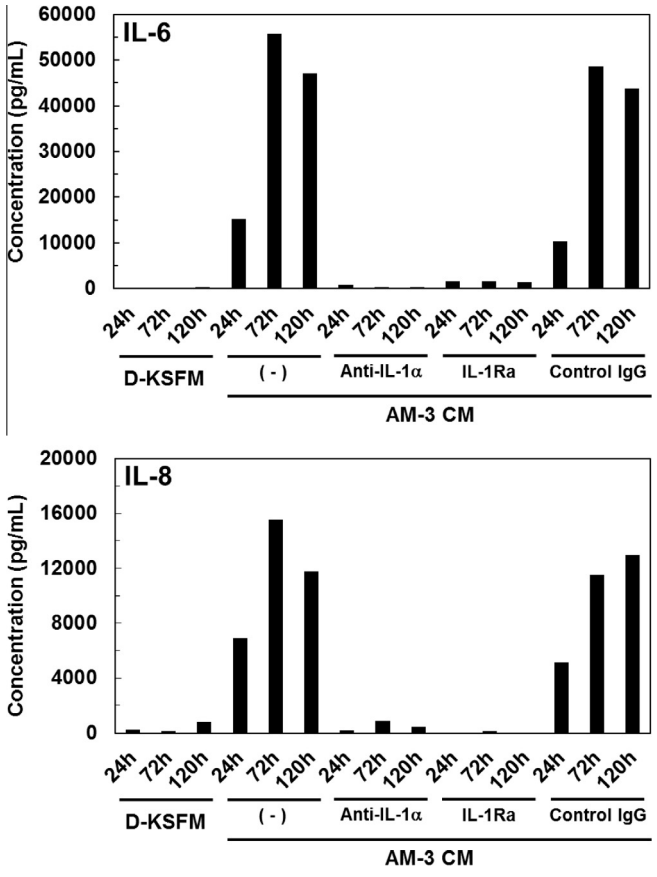

Fig. 3. Secretion of IL-6 and IL-8 from HFF-2 fibroblasts stimulated by AM-3-derived CM through IL-1α. After HFF-2 fibroblasts were treated with D-KSFM or AM-3-derived CM in the presence of IL-1α antibody (1 μg/mL), IL-1Ra (100 ng/mL), or normal mouse control IgG (1 μg/mL) for 24 h, 72 h, and 120 h, the concentration of IL-6 (upper panel) and IL-8 (lower panel) protein in CM was quantified by Q-Plex ELISA kit.

AM-3 cell-dependent secretion of IL-6 and IL-8 from HFF-2 fibroblasts was neutralized by IL-1 antagonists (Fig. 3). AM-3 cell-dependent secretion of IL-6 and IL-8 from primary-cultured fibroblasts was also similarly neutralized by IL-1 antagonists (data not shown).

3.3. IL-6 and IL-8-dependent cell-to-cell communication between HFF-2 fibroblasts and AM-3 ameloblastoma cells regulates cellular motility and proliferation

We examined the effect of IL-6 and IL-8 secreted by HFF-2 fibroblasts on the cellular motility of ameloblastoma cells. Cellular motility of AM-3 ameloblastoma cells stimulated by IL-6 or IL-8 was significantly increased compared with controls (Supplementary data 4). When AM-3 cells were treated with HFF-2 fibroblast-derived CM prepared in the presence of AM-3 derived CM, cellular motility of AM-3 ameloblastoma cells was significantly enhanced compared with controls. Furthermore, these effects were neutralized by incubation with anti-IL-6 antibody and anti-IL-8 antibody (Fig. 4A). When HFF-2-derived CM was produced by treatment with AM-3-derived CM and IL-1 antagonists, the cellular motility of AM-3 ameloblastoma cells was not elevated compared with controls (Fig. 4B). The cellular proliferation of AM-3 ameloblastoma cells treated with IL-6 or IL-8 was significantly increased compared with controls (Supplementary data 5). These results suggested that ameloblastoma and stromal cells could communicate via cytokines to modulate mutual cellular responses.

4. Discussion

It was reported that malignant tumor cells and stromal fibroblasts secrete soluble factors such as cytokines and growth factors into the microenvironment, which upregulate the growth and invasion of malignant tumor cells [22,23], and therefore it is believed a number of interactions occur between malignant tumor cells and fibroblasts [7]. Furthermore, in the case of rheumatoid arthritis, an autoimmune disease that destroys bone cartilage, synovial fibroblasts produced soluble factors such as IL-1α, IL-1β, IL-6, IL-8, and RANKL, which induced osteoclastogenesis and significant bone destruction [24–26].

Follicular type ameloblastoma often shows a directional follicular or tuft-like growth, which implies the existence of chemoattraction from surrounding tissues. This type of ameloblastoma accompanies tumor stroma including fibroblasts and blood corpuscle cells and commonly invades neighboring bone [4]. Currently, there are very few immortalized ameloblastoma cell lines available, which makes the cellular biological analysis of ameloblastomas difficult.

It was reported that ameloblastoma cells expressing RANKL and TNF directly induced osteoclastogenesis [5,27]. However, the physiological role of cell-to-cell communication in the pathogenesis of ameloblastoma has not been studied intensively. We hypothesized that tumor cells and stromal fibroblasts might be activated reciprocally through cytokines to co-operatively produce a microenvironment that promotes the growth of ameloblastoma. In this study, we investigated the interactions between ameloblastoma cells and fibroblasts using AM-3 ameloblastoma cells and HFF-2 fibroblasts.

AM-3 ameloblastoma cells expressed mRNAs of IL-1α, IL-1β, IL-6, IL-8, and TNF at higher levels than HFF-2 fibroblasts. IL-1α is a cytokine associated with inflammatory reactions. Epithelial cells in an inflammatory environment secreted IL-1α that activated fibroblasts to express IL-1α, IL-1β, and IL-6 [11]. IL-1α was also reported to be expressed in ameloblastoma [28]. HFF-2 fibroblasts stimulated with AM-3-derived CM significantly enhanced the gene

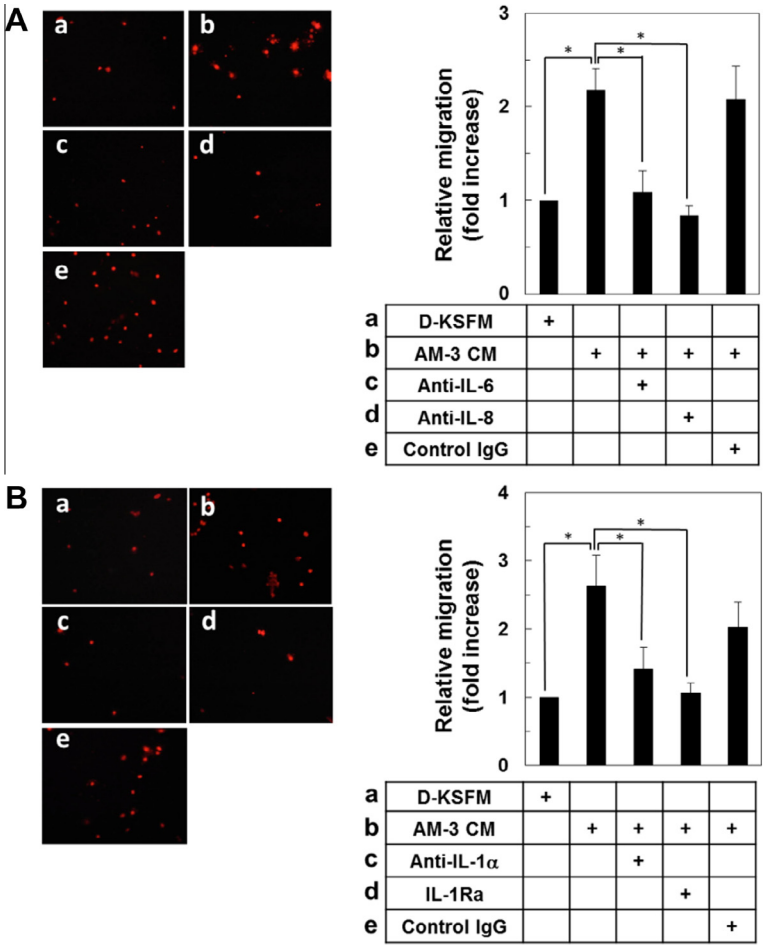

Fig. 4. Cellular motility of AM-3 ameloblastoma cells is regulated by fibroblast-derived IL-6 and IL-8. (A) AM-3 ameloblastoma cells migrated for 9 h in the presence of CM derived from HFF-2 fibroblasts treated with D-KSFM (a) or AM-3-derived CM (b–e) for 5 days. Anti-IL-6 antibody (1 μg/mL) (c), anti-IL-8 antibody (1 μg/mL) (d), or normal mouse control IgG (1 μg/mL) (e) were added to medium at the beginning of the migration assay. Left panels are representative microscopic images (magnification 10×) and relative cellular motility of AM-3 ameloblastoma cells is expressed as relative migration (right panel) (see supplementary data 2).(B) AM-3 ameloblastoma cells migrated for 9 h in the presence of CM derived from HFF-2 fibroblasts pretreated with D-KSFM (a) or AM-3-derived CM (b–e) for 5 days. Anti-IL-1α antibody (1 μg/mL) (c), IL-1Ra (100 ng/mL) (d), or normal mouse control IgG (1 μg/mL) (e) were added to the medium at the beginning of the CM preparation (see supplementary data 2). Left panels are representative microscopic images (magnification 10×) and relative cellular motility of AM-3 ameloblastoma cells is expressed as relative migration (right panel). Results are means ± SE from at least three independent experiments. $^{*}P < 0.05$.

and protein expression of IL-6 and IL-8. Furthermore, IL-1α, IL-6, and IL-8 enhanced osteoclastogenesis [24,29,30] and we previously showed that AM-3 ameloblastoma cells induced osteoclastogenesis [12]. Because anti-IL-1α antibody and IL-1Ra significantly suppressed the AM3-derived CM-dependent cellular responses of HFF-2 fibroblasts and primary-cultured fibroblasts isolated from ameloblastoma tissue (Figs. 2 and 3), IL-1α secreted from ameloblastoma cells might induce surrounding mesenchymal cells to produce IL-6 and IL-8. Although anti-IL-1β antibody was expected to act similarly to anti-IL-1α antibody, it was unable to suppress the AM3-derived CM-dependent cellular responses of HFF-2 fibroblasts and primary-cultured fibroblasts. This might be due to the relative low accumulation of IL-1β in AM-3-derived CM (Table 1). Reciprocally, IL-6 and IL-8 from fibroblasts promoted the cellular motility and proliferation of ameloblastoma cells (Supplementary data 5). It was also reported that IL-6 was associated with the proliferation and cellular motility of tumor cells [31]. IL-8 correlated with invasion metastasis for various malignant tumors [32–34]. We (this study) and other groups reported that several cytokines were retained in ameloblastoma cysts [35], suggesting cytokines are closely related to the pathogenesis of ameloblastoma.

Directional cell migration is necessary for many biological events, including proper embryogenesis and wound healing, as well as for diseases such as chronic inflammation and tumors [36]. Taken together, ameloblastoma might show directional follicular growth in response to chemoattractant gradients generated by IL-6 and IL-8 reciprocally released from surrounding tissues.

Recently, the neutralization of cytokines was applied to the therapy of inflammatory disease. For example, antibodies against IL-1 and IL-6 receptor were used to treat rheumatoid arthritis [26,37].

We have shown that several cytokines were overexpressed at higher levels in ameloblastoma compared with fibroblasts in a mono-cell culture. Treatment of fibroblasts with CM from ameloblastoma cells induced large amounts of IL-6 and IL-8 from fibroblasts, which were suppressed by IL-1α antagonists. In contrast, the cellular motility of AM-3 ameloblastoma cells was accelerated by CM from fibroblasts by an IL-6- and IL-8-dependent mechanism.

These findings suggested that ameloblastoma cells and surrounding stromal cells reciprocally could affect each other to create a microenvironment that enhanced the invasion and growth of ameloblastoma (Supplementary data 6). Neutralizing reagents against IL-1α, IL-6, and IL-8 might have high therapeutic potential for the local control of ameloblastoma, as well as for rheumatoid arthritis. We hope these treatments will be used as supportive therapy for minimal surgical operation to enhance the quality of life of ameloblastoma patients. Further animal and clinical studies

are needed to establish the practical usability of cytokine-antagonizing reagents for ameloblastoma.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.07.137.

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