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Expression of parathyroid hormone-related protein (PTHrP), osteoclast differentiation factor (ODF)/receptor activator of nuclear factor-κB ligand (RANKL) and osteoclastogenesis inhibitory factor (OCIF)/ osteoprotegerin (OPG) in ameloblastomas

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BACKGROUND: To clarify the roles of osteoclast regulatory factors in progression of odontogenic tumors, expression of parathyroid hormone-related protein (PTHrP), osteoclast differentiation factor (ODF)/receptor activator of nuclear factor-κB ligand (RANKL), and osteoclastogenesis inhibitory factor (OCIF)/osteoprotegerin (OPG) were analyzed in ameloblastomas as well as tooth germs.

METHODS: Tissue specimens of nine tooth germs and 36 benign and one malignant ameloblastomas were examined by reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry for the expression of PTHrP, ODF/RANKL, and OCIF/OPG.

RESULTS: Expression of PTHrP, ODF/RANKL, and OCIF/OPG mRNA was detected in all tooth germ and ameloblastoma samples. Immunohistochemical reactivity for PTHrP was recognized in both normal and neoplastic odontogenic epithelial cells. In ameloblastomas, PTHrP reactivity in peripheral columnar or cuboidal cells was stronger than that in central polyhedral cells, and keratinizing cells showed increased PTHrP reactivity. ODF/RANKL and OCIF/OPG were expressed predominantly in mesenchymal cells rather than in odontogenic epithelial cells in both tooth germs and ameloblastomas. Epithelial ODF/RANKL and OCIF/OPG expression was slightly lower in ameloblastomas than in tooth germs. Tumor cells in plexiform ameloblastomas showed slightly higher reactivity for PTHrP and ODF/RANKL than tumor cells in follicular ameloblastomas.

CONCLUSION: Expression of PTHrP, ODF/RANKL and OCIF/OPG in tooth germs and ameloblastomas suggests that these factors might locally regulate bone metabolism and dynamics in tooth development as well as in progression of ameloblastomas. These factors might also be involved in tumor cell differentiation and/or tumor tissue structuring in ameloblastomas.

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Odontogenic epithelium is responsible for tooth development under physiological conditions but can give rise to tumors or cysts in the jaws (1–3). Tumors arising from epithelium of the odontogenic apparatus or from its derivatives or remnants exhibit considerable histologic variation and are classified into several benign and malignant entities (2–5). Ameloblastoma is the most frequently encountered tumor arising from odontogenic epithelium and is characterized by a benign but locally invasive behavior with a high risk of recurrence (2, 4, 5). Histologically, ameloblastoma shows considerable variation, including follicular, plexiform, acanthomatous, granular cell, basal cell, and desmoplastic types (4). Malignant ameloblastoma is defined as a neoplasm in which the pattern of an ameloblastoma and cytological features of malignancy are shown by the primary growth in the jaws and/or by any metastatic growth (4). Recently, malignant ameloblastoma has been subclassified into metastasizing ameloblastoma and ameloblastic carcinoma on the basis of metastatic spread and cytological malignant features (3). These epithelial odontogenic tumors histologically resemble the epithelial odontogenic apparatus, such as enamel organ or dental lamina, in some respects; however, the detailed mechanisms of oncogenesis, cytodifferentiation, and tumor progression remain unknown.

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The integrity of bone metabolism is maintained by a delicate balance between bone resorption by osteoclasts and bone formation by osteoblasts, and is regulated by a wide variety of hormones, growth factors, and cytokines (6, 7). Parathyroid hormone-related protein (PTHrP) is an autocrine or paracrine factor responsible for humoral hypercalcemia of malignancy (HHM), an important paraneoplastic syndrome (8, 9). This peptide shares structural and functional homology with native parathyroid hormone (PTH) and causes increased osteoclastic bone resorption and increased renal tubular calcium reabsorption (10, 11). Production of PTHrP by neoplastic cells has been recognized in malignant tumors commonly associated with HHM, such as adult T cell lymphoma/leukemia (ATLL), pulmonary squamous cell carcinoma, and renal cell carcinoma (9, 10, 12) as well as in malignancies frequently associated with bone metastasis, such as breast and prostate carcinomas (13–15). Several cases of malignant ameloblastoma with hypercalcemia have been attributed to PTHrP (16, 17). Osteoclast differentiation factor (ODF)/receptor activator of nuclear factor-κB ligand (RANKL) is a cell membrane-bound and soluble molecule of the tumor necrosis factor (TNF) superfamily (6, 18). This molecule binds receptor activator of nuclear factor-κB (RANK) expressed on osteoclast precursors and stimulates osteoclast differentiation and activation through TNF receptor-associated factor (TRAF) adaptor molecules, c-*Jun* NH_2-terminal kinase (JNK), and nuclear factor-κB (NF-κB) (6, 19). Osteoclastogenesis inhibitory factor (OCIF)/osteoprotegerin (OPG) is a secreted molecule of the TNF receptor superfamily that functions as a decoy receptor for ODF/RANKL, preventing its interaction with RANK and consequently inhibiting osteoclastogenesis and osteoclast activation (20, 21). The balance between ODF/RANKL-RANK signaling and active OCIF/OPG levels regulates development and activation of osteoclasts and bone metabolism (6, 19). Alterations of these molecules have been proven to cause bone diseases resulting from imbalance between bone formation and resorption, such as osteoporosis and osteopetrosis (6, 7), inflammatory bone diseases, such as rheumatoid arthritis and marginal periodontitis (22, 23), and bone diseases of tumors, including primary bone tumors and metastatic malignancies (24–27).

Our previous studies confirmed matrix metalloproteinases and their inhibitors, adhesion molecules, and growth factors in tooth germs and epithelial odontogenic tumors, suggesting that these molecules play a role in regulating the progression of odontogenic tumors (29–33). Some osteolytic cytokines, such as interleukin (IL)-1α, IL-1β, IL-6, and TNF-α, have been investigated in ameloblastomas (34–36). In the present study, expression of PTHrP, ODF/RANKL, and OCIF/OPG was examined in ameloblastomas as well as in tooth germs using reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry to clarify the possible role of these bone resorption-related agents in odontogenic tumors.

Materials and methods

The study protocol was reviewed and approved by the Research Ethics Committee of Tohoku University Graduate School of Dentistry.

Tissue preparation

Specimens were surgically removed from 37 patients with ameloblastoma at the Department of Oral and Maxillofacial Surgery, Tohoku University Dental Hospital. The tumors were divided into several parts. The first part was fixed in 4% paraformaldehyde phosphate buffer for 1 to several days and was embedded in paraffin. The tissue blocks were sliced and stained with hematoxylin and eosin for histologic diagnosis according to the WHO histologic typing of odontogenic tumors (4). The tumors comprised of 36 ameloblastomas and one malignant ameloblastoma. The ameloblastomas were divided into 25 follicular and 11 plexiform types, including 12 acanthomatous and two granular cell subtypes. The malignant ameloblastoma was compatible with a metastasizing ameloblastoma according to the criteria of Eversole (3). The second part of each tumor was immediately frozen on dry ice and stored at −80°C until RT-PCR analysis. The third part of each tumor was embedded in Tissue-Tek OCT Compound (Sakura Finetechnical, Tokyo, Japan), quick-frozen in a mixture of acetone and dry ice, and stored at −80°C until immunohistochemical examination. Tooth germs of the mandibular third molars, enucleated from nine patients for orthodontic reasons at the stage of crown mineralization, were similarly prepared and compared with the ameloblastomas.

RT-PCR

Total RNA was extracted from each frozen tissue using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. First-stranded complementary DNA (cDNA) was synthesized from 1 μg of RNA using Omniscript RT Kit (Qiagen) with oligo-(dT)15 primer (Roche Diagnostics, Mannheim, Germany) as outlined by the manufacturer. The cDNA samples were amplified using HotstarTaq Master Mix Kit (Qiagen) with specific primers in a DNA thermal cycler (Eppendorf, Hamburg, Germany). Primers used for detection of PTHrP, ODF/RANKL, and OCIF/OPG are listed in Table 1. A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as an internal control for the examination of human gene expression. PCR was performed in a total volume of 50 μl, containing 1 μl of the reverse transcriptase reaction mixture and 0.5 μM of each specific primer set. The procedure included 30 cycles at 94°C for 45 s, at 55°C for 45 s, and at 72°C for 60 s with an initial denaturation step at 95°C for 15 min and a final elongation step at 72°C for 10 min. The PCR products were electrophoresed on 2% agarose gel at 100 V for 40 min and visualized with ethidium bromide.

Immunohistochemistry

Serial cryostat 5-μm thick sections were obtained from each frozen block. The sections were fixed in cold acetone for 10 min and washed in cold phosphate-buffered saline (PBS). After treatment with normal rabbit serum for 30 min, the sections were incubated with primary monoclonal antibodies at 4°C overnight. The applied antibodies are listed in Table 1 (12, 28, 37). The standard streptavidin–biotin–peroxidase complex method was performed to bind the primary antibodies with the use of Histofine SAB-PO Kit (Nichirei, Tokyo, Japan). Reaction products were visualized by immersing the sections for 1–3 min in 0.03% diaminobenzidine

Table 1 Primers and antibodies

	Primer			*Antibody*			
	Sequence (5′–3′)	*Anneal (°C)*	*Product (bp)*	*Clonality*	*Source*	*Dilution*	*Reference*
PTHrP	Forward: GCGACGATTCTTCCTTCACC Reverse: AGAGTCTAACCAGGCAGAGC	55	285	Polyclonal (Goat lgG)	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1 : 100	12
ODF/RANKL	Forward: GCCAGTGGGAGATGTTAG Reverse: TTAGCTGCAAGTTTTCCC	55	486	Polyclonal (Goat IgG)	Santa Cruz Biotechnology	1 : 100	28
OCIF/OPG	Forward: GCTAACCTCACCTTCGAG Reverse: TGATTGGACCTGGTTACC	55	324	Polyclonal (Goat IgG)	Santa Cruz Biotechnology	1 : 100	37
GAPDH	Forward: GGAGTCAACGGATTTGGT Reverse: GTGATGGGATTTCCATTGAT	55	206				

solution containing 2 mM hydrogen peroxide. Nuclei were lightly counterstained with methylgreen. For control studies of the antibodies, the serial sections were treated with PBS and normal goat IgG instead of the primary antibodies and were confirmed to be unstained.

Evaluation of immunostaining and statistical analysis
Immunohistochemical reactivity for PTHrP, ODF/RANKL, and OCIF/OPG was evaluated and classified into three groups: (–) negative, (+) positive, and (++) strongly positive. The statistical significance of differences in the percentages of cases with different reactivity levels was analyzed by the Mann–Whitney's *U*-test for differences between two groups or the Kruskal–Wallis test for differences among three groups. *P*-values less than 0.05 were considered to indicate statistical significance.

Results

PTHrP, ODF/RANKL, and OCIF/OPG mRNA expression
RT-PCR analysis identified expression of mRNA transcripts for PTHrP, ODF/RANKL, and OCIF/OPG in all 36 ameloblastoma and one malignant ameloblastoma samples as well as nine tooth germ tissues (Fig. 1). The PCR products of PTHrP, ODF/RANKL, and OCIF/OPG were 285, 486, and 324 bp, respectively. There was no distinct difference in PTHrP, ODF/RANKL or OCIF/OPG mRNA expression among the different types of ameloblastomas or between the benign and malignant ameloblastomas.

Immunohistochemical reactivity for PTHrP, ODF/RANKL, and OCIF/OPG
The results of immunohistochemistry for PTHrP, ODF/RANKL, and OCIF/OPG detection are summarized in Table 2. Immunohistochemical reactivity for PTHrP was detected in the cytoplasm of both normal and neoplastic odontogenic epithelial cells. PTHrP was expressed in the dental laminae in all but one tooth germ. Ameloblastomas showed PTHrP expression in 23 of 25 follicular cases and all 11 plexiform cases. PTHrP reactivity in peripheral columnar or cuboidal cells was stronger than that in central polyhedral cells (Fig. 2A). Keratinizing cells in acanthomatous ameloblastomas showed increased PTHrP reactivity (Fig. 2B), whereas granular cells in granular cell ameloblastomas showed decreased reactivity. The malignant (metastasizing) ameloblastoma showed a PTHrP expression pattern similar to that of benign ameloblastomas.

Immunohistochemical reactivity for ODF/RANKL and OCIF/OPG was detected in the cytoplasm of cellular components in both normal and neoplastic odontogenic tissues. In tooth germs, ODF/RANKL and OCIF/OPG were expressed in scattered epithelial cells of the dental laminae as well as in mesenchymal cells of the dental follicles and dental papillae. ODF/RANKL reactivity was more evident in endothelial cells than in fibroblasts, and OCIF/OPG was localized in fibroblasts but not in endothelial cells. Ameloblastomas showed ODF/RANKL and OCIF/OPG expression in stromal cells: ODF/RANKL reactivity was more evident in endothelial cells than in fibroblasts,

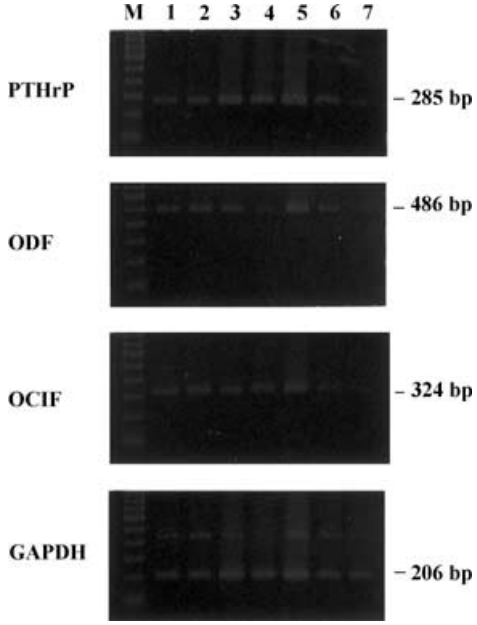

Figure 1 RT-PCR analysis of PTHrP, ODF/RANKL and OCIF/OPG mRNA expression in tooth germs and ameloblastomas. (M: molecular-weight standard, 1 and 2: tooth germs, 3–6: ameloblastomas, 7: malignant ameloblastoma) PTHrP, ODF/RANKL and OCIF/OPG mRNA expression was seen in all samples. GAPDH was run as a control to ascertain the integrity of mRNA/cDNA. The sizes of PTHrP, ODF/RANKL, OCIF/OPG, and GAPDH PCR products were 285, 486, 324 and 206 bp, respectively. The results of representative samples are shown.

Table 2 Immunoreactivity for PTHrP, ODF/RANKL and OCIF/OPG in tooth germs and ameloblastomas

	PTHrP			*ODF/RANKL*				*OCIF/OPG*		
	(−)	*(+)*	*(++)*	*(−)*	*(+)*	*(++)*		*(−)*	*(+)*	*(++)*
Tooth germ ($n=9$)										
Dental lamina	1	1	3	0	3	2		0	2	3
	(20)	(20)	(60)	(0)	(60)	(40)		(0)	(40)	(60)
Dental follicle/papilla	9	0	0	0	2	7		0	3	6
	(100)	(0)	(0)	(0)	(22)	(78)	*	(0)	(33)	(67)
Ameloblastoma ($n=36$)										
Tumor cells	2	20	14	7	29	0		4	25	7
	(5)	(56)	(39)	(19)	(81)	(0)		(11)	(70)	(19)
Stromal cells	36	0	0	0	5	31		0	5	31
	(100)	(0)	(0)	(0)	(14)	(86)		(0)	(14)	(86)
Follicular type ($n=25$)										
Tumor cells	2	15	8	7	18	0		4	16	5
	(8)	(60)	(32)	(28)	(72)	(0)		(16)	(64)	(20)
Stromal cells	25	0	0	0	4	21		0	4	21
	(100)	(0)	(0)	(0)	(16)	(84)		(0)	(16)	(84)
Plexiform type ($n=11$)										
Tumor cells	0	5	6	0	11		0	0	9	2
	(0)	(45)	(55)	(0)	(100)	(0)		(0)	(82)	(18)
Stromal cells	11	0	0	0	1	10		0	1	10
	(100)	(0)	(0)	(0)	(9)	(91)		(0)	(9)	(91)
Acanthomatous subtype ($n=12$)										
Tumor cells	0	5	7	1	11	0		0	8	4
	(0)	(42)	(58)	(8)	(92)	(0)		(0)	(67)	(33)
Stromal cells	12	0	0	0	4	8		0	1	11
	(100)	(0)	(0)	(0)	(33)	(67)		(0)	(8)	(92)
Granular subtype ($n=2$)										
Tumor cells	0	2	0	1	1	0		0	2	0
	(0)	(100)	(0)	(50)	(50)	(0)		(0)	(100)	(0)
Stromal cells	2	0	0	0	0	2		0	0	2
	(100)	(0)	(0)	(0)	(0)	(100)		(0)	(0)	(100)
Malignant ameloblastoma ($n=1$)										
Tumor cells	0	0	1	0	1	0		0	0	1
	(0)	(0)	(100)	(0)	(100)	(0)		(0)	(0)	(100)
Stromal cells	1	0	0	0	0	1		0	0	1
	(100)	(0)	(0)	(0)	(0)	(100)		(0)	(0)	(100)

Immunohistochemical reactivity: (−) negative, (+) positive, (++) intensely positive.
Statistical significance: $^{*}P<0.05$.
Values in parentheses are in per cent.

and OCIF/OPG was localized in fibroblasts but not in endothelial cells, similar to tooth germs (Fig. 3). Some tumor cells exhibited ODF/RANKL reactivity in 27 of 36 ameloblastomas and OCIF/OPG reactivity in 32 of 36 ameloblastomas, and the reactivity in tumor cells was weaker than that in stromal cells. Keratinizing cells in acanthomatous ameloblastomas and granular cells in granular cell ameloblastomas were not reactive with ODF/RANKL or OCIF/OPG antibodies. ODF/RANKL expression in tumor cells of ameloblastomas was significantly lower than that in the dental laminae of tooth germs ($P<0.05$). The malignant (metastasizing) ameloblastoma showed ODF/RANKL and OCIF/OPG expression patterns similar to those of benign ameloblastomas.

Discussion

Originally described as the factor responsible for HHM, PTHrP is also produced by many normal cell types during fetal development and adult life, and physiological roles of PTHrP include regulation of calcium transport, cartilage development, smooth muscle relaxation, and keratinocyte differentiation (38–40). Expression of PTHrP has been confirmed during tooth development, and PTHrP gene knockout mice have shown disturbed tooth development, suggesting that this agent is involved in the morphogenesis and eruption of teeth (41–43). ODF/RANKL and OCIF/OPG are extensively characterized and are capable of regulating proliferation, differentiation, fusion, activation, and apoptosis of osteoclasts (6, 19, 21). ODF/RANKL gene knockout mice have demonstrated abnormalities of lymphocyte development and lymph node organogenesis, and expression of ODF/RANKL and OCIF/OPG has also been detected in extraskeletal and non-hematopoietic tissues throughout development, suggesting the possibility of several other functions (44–46). Recent studies have revealed that various dental cells express ODF/RANKL and OCIF/OPG, and these molecules are considered to play a role in

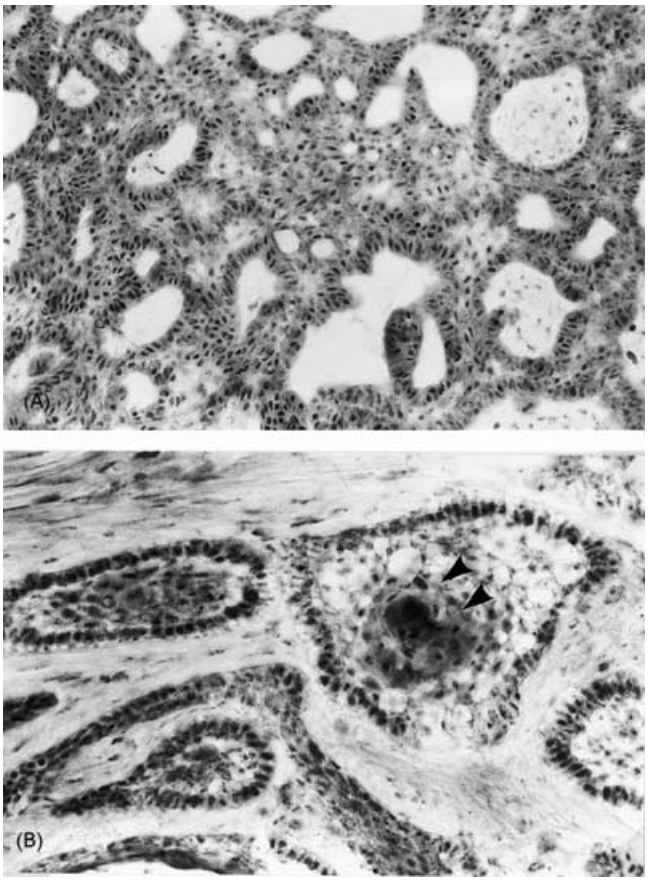

Figure 2 Representative immunohistochemical reactivity for PTHrP in ameloblastomas. (A) Plexiform ameloblastoma showing stronger reactivity in peripheral columnar cells than in central polyhedral cells (×140). (B) Acanthomatous ameloblastoma showing increased reactivity in keratinizing cells (arrowheads) (×200).

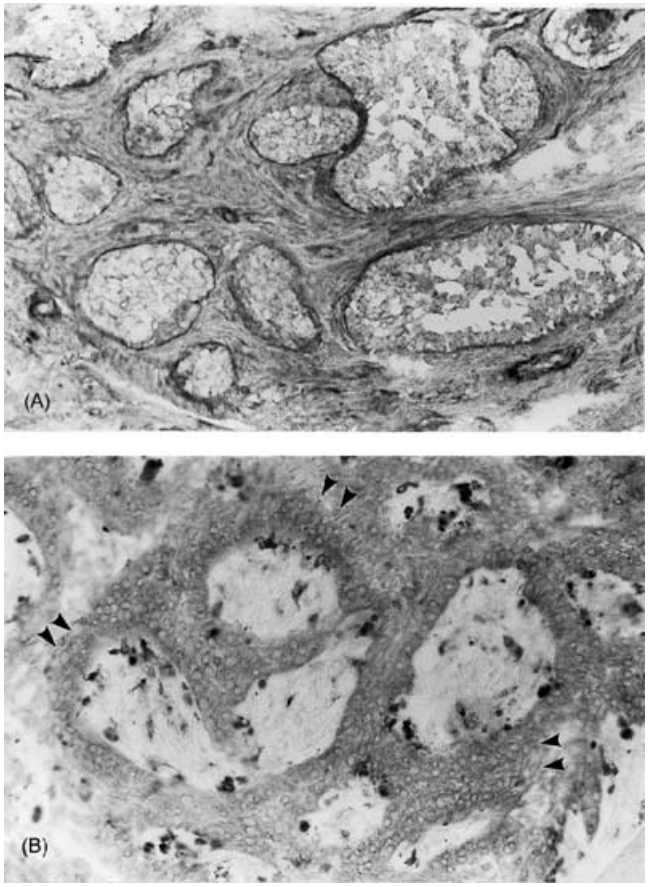

Figure 3 Representative immunohistochemical reactivity for ODF/RANKL and OCIF/OPG in ameloblastomas. (A) Follicular ameloblastoma showing ODF/RANKL reactivity in stromal endothelial cells and some fibroblasts. Tumor cells are not reactive (×160). (B) Plexiform ameloblastoma showing OCIF/OPG reactivity in stromal fibroblasts. Some tumor cells are weakly reactive (arrowheads) (×140).

regulation of osteoclastogenesis and bone resorption during tooth development and eruption (37, 47). In the present study, during the crown mineralization stage of tooth germs, expression of PTHrP was detected in epithelial components, while ODF/RANKL and OCIF/OPG were recognized in epithelial and mesenchymal components. These features suggest that these molecules are associated with regulation of bone metabolism and dynamics during tooth development (37, 41–43, 47).

Humoral hypercalcemia of malignancy (HHM) is caused most commonly by the humoral action of PTHrP, which is released by tumor cells (9, 10, 12). Malignant ameloblastoma is occasionally accompanied by hypercalcemia with or without metastasis, and several cases have been ascribed to tumor-derived PTHrP (16, 17, 48, 49). Tumor-derived PTHrP is also known to participate in bone metastasis and to establish osteolytic bone lesions in metastatic sites, even in the absence of hypercalcemia or increased plasma PTHrP concentration (13–15). Li et al. (50) have shown that PTHrP is found in a variety of odontogenic cysts, especially in odontogenic keratocysts, and suggest that PTHrP modulates cyst expansion and bone resorption. In the present study, expression of PTHrP was detected in tumor cells of benign and malignant ameloblastomas, in patients who had normal serum calcium and phosphorus levels. These features suggest that tumor-produced PTHrP might have a role in local bone destruction associated with progression of ameloblastomas. PTHrP is proven to participate in keratinocyte growth and differentiation during fetal development (38, 39). In our study, acanthomatous ameloblastomas showed slightly stronger expression of PTHrP, especially in keratinizing cells, than other ameloblastomas, suggesting that PTHrP might be associated with tumor cell differentiation in ameloblastomas.

Expression of osteoclastogenesis regulators, ODF/RANKL and OCIF/OPG, has been investigated in various primary and metastatic bone tumors, and abnormalities of the ODF/RANKL-OCIF/OPG system have been implicated in the pathogenesis of these bone lesions (24–28). In the present study, benign and malignant ameloblastomas expressed ODF/RANKL and OCIF/OPG predominantly in stromal cells rather than tumor cells. This expression pattern suggests that these molecules might have a role in regulation of local bone metabolism via parenchymal–stromal interactions in ameloblastomas. Immunohistochemical reactivity for ODF/RANKL and OCIF/OPG in epithelial components

was slightly lower in ameloblastomas than in tooth germs, indicating decreased regulation capacity of bone metabolism because of neoplastic transformation of odontogenic epithelium. In addition, tumor cells showed slightly higher expression of ODF/RANKL as well as PTHrP in plexiform ameloblastomas than in follicular ameloblastomas, suggesting that these molecules are involved in tissue structuring of ameloblastomas. Current evidence indicates that tumor-derived PTHrP promotes ODF/RANKL formation and inhibits OCIF/OPG production (24, 51). In our study, PTHrP, ODF/RANKL, and OCIF/OPG were identified in various components of benign and malignant ameloblastomas at both mRNA and protein levels, and were considered to function as local regulating factors for bone resorption and tumor progression in these epithelial odontogenic tumors.

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