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Immunohistochemical detection of uPA, uPAR, PAI-1, and maspin in ameloblastic tumors

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BACKGROUND: To evaluate the roles of extracellular matrix (ECM)-degrading serine proteinase in progression of odontogenic tumors, expression of urokinase-type plasminogen activator (uPA), uPA receptor (uPAR), plasminogen activator inhibitor-1 (PAI-1), and maspin was analyzed in ameloblastic tumors as well as in tooth germs.

METHODS: Tissue specimens of 10 tooth germs, 45 ameloblastomas, and 5 malignant ameloblastic tumors were examined immunohistochemically with the use of antibodies against uPA, uPAR, PAI-1, and maspin.

RESULTS: Immunohistochemical reactivity for uPA, uPAR, PAI-1, and maspin was detected in normal and neoplastic odontogenic tissues: uPA was recognized predominantly in mesenchymal cells, uPAR was evident in epithelial cells, PAI-1 was found in both epithelial and mesenchymal cells, and maspin was expressed only in epithelial cells. The levels of uPA and uPAR immunoreactivity in ameloblastic tumors were slightly higher than the levels in tooth germs, while PAI-1 reactivity in ameloblastomas tended to be lower than that in tooth germs. The level of maspin immunoreactivity in ameloblastomas was significantly higher than that in tooth germs, and ameloblastic carcinoma showed decreased maspin reactivity.

CONCLUSION: Expression of uPA, uPAR, PAI-1, and maspin in tooth germs and ameloblastic tumors suggests that interactions among these molecules contribute to ECM degradation and cell migration during tooth development and tumor progression. Altered expression of the serine proteinase and its associated molecules in ameloblastic tumors may be involved in oncogenesis of odontogenic epithelium.

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Introduction

Tumors arising from epithelium of the odontogenic apparatus or from its derivatives or remnants exhibit considerable histological variation and are classified into several benign and malignant entities (1, 2). 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 (1, 2). Histologically, ameloblastoma shows considerable variation, including follicular, plexiform, acanthomatous, granular cell, basal cell, and desmoplastic variants (2). Malignant counterparts of ameloblastoma are classified into metastasizing ameloblastoma and ameloblastic carcinoma on the basis of metastatic spread and cytological malignant features (2). Recent studies have identified genetic and molecular alterations in these epithelial odontogenic tumors (3, 4); however, the detailed mechanisms of oncogenesis, cytodifferentiation, and tumor progression remain unknown.

Extracellular matrix (ECM) degradation that occurs during developmental processes, tissue repair, inflammatory diseases, and tumor progression requires the action of a number of proteolytic enzymes (5). Among these proteinases, matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (uPA) catalyze degradation of ECM proteins at physiological pH and play important roles in the tissue remodeling processes (5, 6). uPA is a serine proteinase that converts the extracellular zymogen plasminogen into the active proteinase plasmin, which can degrade most ECM molecules and also activate the precursor forms of MMP-3, -9, -12, and -13 that lead further ECM degradation (6, 7). uPA receptor (uPAR) is a cell membrane-anchored uPA-binding protein that concentrates plasminogen activation activity at the cell surfaces (5, 6). The plasminogen activation activity is controlled by two main types of endogenous inhibitors, plasminogen activator inhibitor (PAI)-1 and -2 (6, 8). The balance of the uPA system components, including uPA, uPAR, and PAIs, ultimately determines the extent of ECM degradation in physiological and pathological conditions (6, 8). Maspin was identified as a novel serine proteinase inhibitor sharing structural homology with other serine proteinase inhibitors, such as PAI-1 and -2

(9). Despite the reported activity as a potent inhibitor of uPA, the inhibitory function of maspin is not completely established (10, 11). Recent studies have revealed that the uPA system and maspin are involved in progression and prognosis of human tumors (6, 8–10, 12, 13).

Previous studies have confirmed the expression of MMPs and their associated molecules in ameloblastic tumors, suggesting that these matrix-degrading proteinases contribute to the local invasiveness of odontogenic tumors (14–16). In the present study, immunohistochemical expression of uPA, uPAR, PAI-1, and maspin was examined in benign and malignant ameloblastic tumors as well as in tooth germs to evaluate the roles of the uPA system and maspin in the progression of epithelial odontogenic tumors.

Materials and methods

Tissue preparation

Specimens were surgically removed from 50 patients with epithelial odontogenic tumors at the Department of Oral and Maxillofacial Surgery, Tohoku University Dental Hospital, and affiliated hospitals. The specimens were fixed in 10% buffered formalin for one to several days and were embedded in paraffin. The tissue blocks were sliced into 3-μm-thick sections for routine histological and subsequent immunohistochemical examinations. Tissue sections were stained with hematoxylin and eosin for histological diagnosis according to the WHO histological classification of odontogenic tumors (2). The tumors comprised 45 ameloblastomas and five malignant ameloblastic tumors. Ameloblastomas were divided into 22 follicular and 23 plexiform types, including nine acanthomatous, six granular cell, three basal cell, and four desmoplastic subtypes. Malignant ameloblastic tumors were classified into two metastasizing ameloblastomas and three ameloblastic carcinomas. Specimens of 10 tooth germs of the mandibular third molars, enucleated for orthodontic reasons at the initial stage of crown mineralization, were similarly prepared and compared with the epithelial odontogenic tumors.

Immunohistochemistry

The serial sections were deparaffinized, immersed in methanol with 0.3% hydrogen peroxide, and heated in 0.01 M citrate buffer (pH 6.0; for uPA and PAI-1) or 1 mM ethylenediamine tetraacetic acid (EDTA) buffer (pH 9.0; for uPAR and maspin) for 10 min by autoclave (121°C, 2 atm). Then, the sections were incubated with primary antibodies at 4°C overnight. The applied antibodies were mouse anti-uPA monoclonal antibody (American Diagnostica, Stamford, CT, USA; subclass IgG1; 33 μg/ml), goat anti-uPAR polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 5 μg/ml), rabbit anti-PAI-1 polyclonal antibody (Santa Cruz Biotechnology; 5 μg/ml), and mouse anti-maspin monoclonal antibody (Lab Vision, Fremont, CA, USA; subclass IgG2a; supplied as culture supernatant and diluted at 1:15) (11, 17, 18). The sections were allowed to react with peroxidase-conjugated anti-mouse IgG (for uPA and maspin), anti-goat IgG (for uPAR), or anti-rabbit IgG (for PAI-1) polyclonal antibody (Histofine Simple Stain MAX-PO; Nichirei, Tokyo, Japan) for 45 min, and reaction products were visualized by immersing the sections in 0.03% diaminobenzidine solution containing 2 mM hydrogen peroxide for 1–5 min. Nuclei were lightly stained with Mayer's hematoxylin. For control studies of the antibodies, the serial sections were treated with phosphate-buffered saline, mouse anti-OPD4 (CD45RO) monoclonal antibody (Dako, Glostrup, Denmark; subclass IgG1), normal goat and rabbit IgG, and mouse anti-L26 (CD20) monoclonal antibody (Nichirei; subclass IgG2a) instead of the primary antibodies and were confirmed to be unstained.

Immunohistochemical reactivity for uPA, uPAR, PAI-1, and maspin was evaluated and classified into three groups: (−) negative in epithelial or neoplastic cells, (+) slightly to moderately positive in epithelial or neoplastic cells, and (++) strongly positive in epithelial or neoplastic cells. The statistical significance of differences in the percentages of cases with different reactivity levels was analyzed by the Mann–Whitney *U*-test for differences between two groups or the Kruskal–Wallis test for differences among three or more groups. Values of $P < 0.05$ were considered to indicate statistical significance.

Results

Immunohistochemical reactivity for uPA, uPAR, PAI-1, and maspin in tooth germs and ameloblastic tumors is summarized in Table 1. Expression of uPA was detected in the cytoplasm of cellular components in normal and neoplastic odontogenic tissues (Fig. 1). In tooth germs, weak reactivity was found in inner enamel epithelium (Fig. 1A). Many fibroblastic and endothelial cells in dental papillae and dental follicles were also reactive. All but two ameloblastomas and all metastasizing ameloblastomas showed scattered reactivity for uPA in peripheral columnar or cuboidal neoplastic cells (Fig. 1B,C). Keratinizing cells in acanthomatous ameloblastomas and granular cells in granular cell ameloblastomas exhibited no expression of uPA. Basal cell and desmoplastic ameloblastomas showed scattered uPA reactivity in neoplastic cells. In ameloblastic carcinomas, uPA reactivity was found in many neoplastic cells. In the stroma of these ameloblastic tumors, many fibroblasts and endothelial cells were also reactive with uPA.

Expression of uPAR was detected in the cell membrane and cytoplasm of cellular components in normal and neoplastic odontogenic tissues (Fig. 2). In tooth germs, uPAR reactivity was found in inner enamel epithelium (Fig. 2A). Some endothelial cells in dental papillae and dental follicles were also reactive. Ameloblastomas showed uPAR reactivity in peripheral columnar or cuboidal cells (Fig. 2B). Some central polyhedral neoplastic cells in a few ameloblastoma cases were reactive with uPAR. Keratinizing cells in acanthomatous ameloblastomas and granular cells in granular cell

Table 1 Immunohistochemical reactivity for uPA, uPAR, PAI-1, and maspin in tooth germs and ameloblastic tumors

		uPA			*uPAR*			*PAI-1*			*Maspin*		
	n	–	+	++	–	+	++	–	+	++	–	+	++
Tooth germ	10	0 (0)	10 (100)	0 (0)	0 (0)	8 (80)	2 (20)	0 (0)	2 (20)	8 (80)	0 (0)	6 (60)	4 (40)
Ameloblastoma	45	2 (4)	32 (72)	11 (24)	0 (0)	30 (67)	15 (33)	3 (7)	22 (49)	20 (44)	0 (0)	7 (16)	38 (84)
Follicular type	22	2 (9)	14 (64)	6 (27)	0 (0)	14 (64)	8 (36)	2 (9)	8 (36)	12 (55)	0 (0)	4 (18)	18 (82)
Plexiform type	23	0 (0)	18 (72)	5 (28)	0 (0)	16 (70)	7 (30)	1 (4)	14 (61)	18 (35)	0 (0)	3 (13)	20 (84)
Acanthomatous subtype	9	1 (11)	4 (44)	4 (44)	0 (0)	7 (78)	2 (22)	2 (22)	3 (33)	4 (44)	0 (0)	2 (22)	7 (78)
Granular subtype	6	1 (17)	5 (83)	0 (0)	0 (0)	5 (83)	1 (17)	1 (17)	4 (66)	1 (17)	0 (0)	1 (17)	5 (83)
Basal cell subtype	3	0 (0)	2 (67)	1 (33)	0 (0)	2 (67)	1 (33)	0 (0)	2 (67)	1 (33)	0 (0)	0 (0)	3 (100)
Desmoplastic subtype	4	0 (0)	4 (100)	0 (0)	0 (0)	4 (100)	0 (0)	0 (0)	4 (100)	0 (0)	0 (0)	2 (50)	2 (50)
Non-cellular variation	23	0 (0)	17 (74)	6 (26)	0 (0)	12 (52)	11 (48)	0 (0)	9 (39)	14 (61)	0 (0)	2 (9)	21 (91)
Malignant ameloblastic	5	0 (0)	3 (60)	2 (40)	0 (0)	2 (40)	3 (60)	0 (0)	2 (40)	3 (60)	0 (0)	3 (60)	2 (40)
Metastasizing ameloblastoma tumor	2	0 (0)	1 (50)	1 (50)	0 (0)	1 (50)	1 (50)	0 (0)	1 (50)	1 (50)	0 (0)	0 (0)	2 (100)
Ameloblastic carcinoma	3	0 (0)	2 (67)	1 (33)	0 (0)	1 (33)	2 (67)	0 (0)	1 (33)	2 (67)	0 (0)	3 (100)	0 (0)

Immunohistochemical reactivity: (–) negative in epithelial or neoplastic cells, (+) slightly to moderately positive in epithelial or neoplastic cells, and (++) strongly positive in epithelial or neoplastic cells. Values in parentheses denote percentage values. Statistical significance: $^*P < 0.05$.

ameloblastomas exhibited no expression of uPAR. Basal cell ameloblastomas showed uPAR reactivity in most neoplastic cells, and uPAR reactivity in desmoplastic ameloblastomas was detected in neoplastic cells near the basement membrane. Metastasizing ameloblastomas showed uPAR reactivity in peripheral columnar or cuboidal cells and some central polyhedral cells, and ameloblastic carcinomas showed uPAR reactivity in most neoplastic cells (Fig. 2C). In these ameloblastic tumors, some stromal endothelial cells were also reactive with uPAR.

Immunoreactivity for PAI-1 was detected in the cytoplasm of cellular components in normal and neoplastic odontogenic tissues (Fig. 3). Tooth germs showed strong PAI-1 reactivity in inner enamel epithelium and weak reactivity in other epithelial components (Fig. 3A). Many fibroblastic cells and some endothelial cells in dental papillae and dental follicles were also reactive. In all but three ameloblastomas and all metastasizing ameloblastomas, PAI-1 reactivity was found in many peripheral columnar or cuboidal cells and some central polyhedral cells (Fig. 3B). Acanthomatous ameloblastomas exhibited decreased reactivity for PAI-1 in keratinizing cells, and granular cells in granular cell ameloblastomas were not reactive with PAI-1. Basal cell and desmoplastic ameloblastomas and ameloblastic carcinomas showed PAI-1 reactivity in most neoplastic cells (Fig. 3C). In the stroma of these ameloblastic tumors, many fibroblasts and some endothelial cells were also reactive with PAI-1.

Expression of maspin was detected in the cytoplasm of normal and neoplastic odontogenic epithelial cells (Fig. 4). Tooth germs showed strong maspin reactivity in inner enamel epithelium and weak reactivity in other epithelial components (Fig. 4A). Ameloblastomas and metastasizing ameloblastomas showed strong maspin reactivity in peripheral columnar or cuboidal cells and weak reactivity in central polyhedral cells (Fig. 4B). Acanthomatous ameloblastomas exhibited decreased reactivity for maspin in keratinizing cells, and granular cells in granular cell ameloblastomas were reactive with maspin. In basal cell and desmoplastic ameloblastomas, maspin reactivity was found in most neoplastic cells. Ameloblastic carcinomas showed weak reactivity for maspin in some neoplastic cells (Fig. 4C). The level of immunohistochemical reactivity for maspin in ameloblastomas was significantly higher than that in tooth germs ($P < 0.05$, Table 1).

Discussion

Extracellular matrix provides an essential framework upon which cells grow, migrate, and differentiate, and its essential that ECM undergoes remodeling regulated by many kinds of proteinases during developmental processes (5). Mice targeted disruption of the genes for *uPA*, *uPAR*, and *PAI-1* develop to term, grow to adulthood, and are fertile, but show abnormal fibrinolysis and impaired leukocyte recruitment in inflammation, while mice deficient in *plasminogen*, whose product is controlled by the uPA system components, develop severe thrombosis, resulting in widespread organ damage, a low body weight, and a short life span (6). *Maspin* knockout mice are interfered with the formation of the visceral endoderm and cause lethality at the peri-implantation stage (19). Thus, the serine proteinase and its associated molecules affect a variety of developmental processes. uPA mRNA has been detected in developing teeth of normal mice at multiple sites of developmental stages, and targeted epithelial expression of *uPA* contributes to defective enamel formation (20, 21). In the present study, immunohistochemical reactivity for uPA, uPAR, PAI-1, and maspin was found in human tooth germs, suggesting that the proteinase activity play a role in physiological ECM degradation and odontogenic cell migration during tooth development.

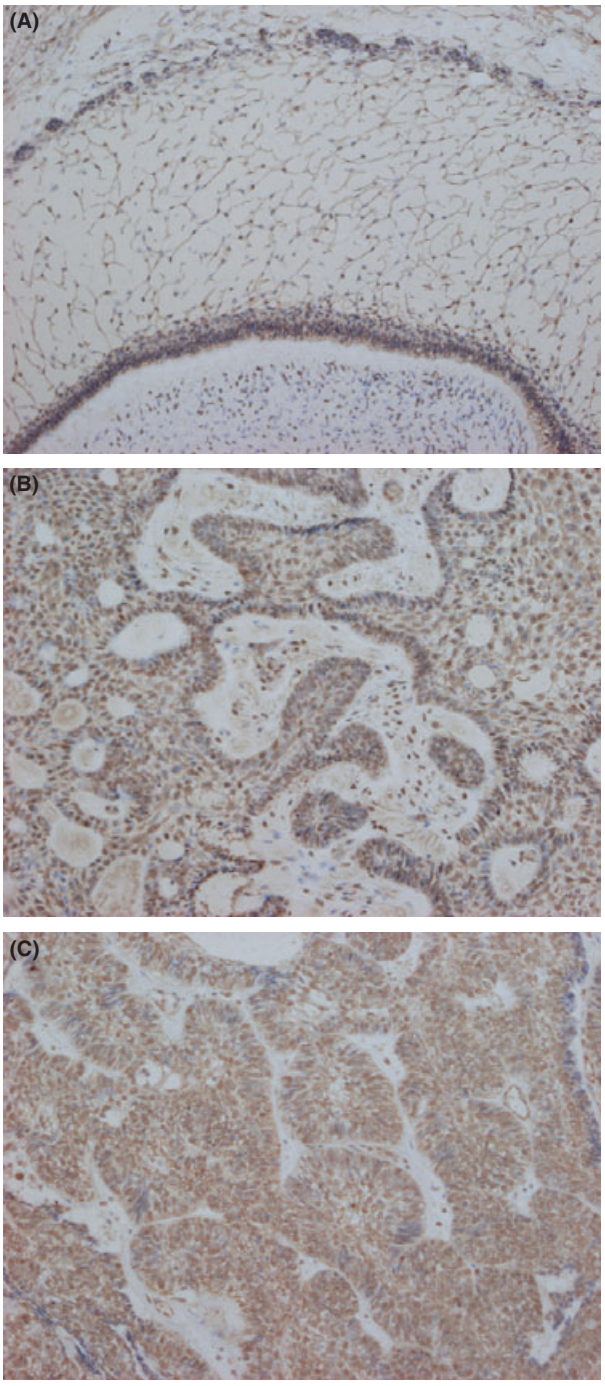

Figure 1 Immunohistochemical reactivity for uPA. (A) Tooth germ showing weak reactivity in inner enamel epithelium. Many mesenchymal cells in dental papilla and dental follicle are also positive (×70). (B) Plexiform ameloblastoma showing reactivity in some peripheral cuboidal neoplastic cells. Many stromal cells are also positive (×90). (C) Metastasizing ameloblastoma showing reactivity in many peripheral columnar neoplastic cells. Many stromal cells are also positive (×95). uPA = urokinase-type plasminogen activator.

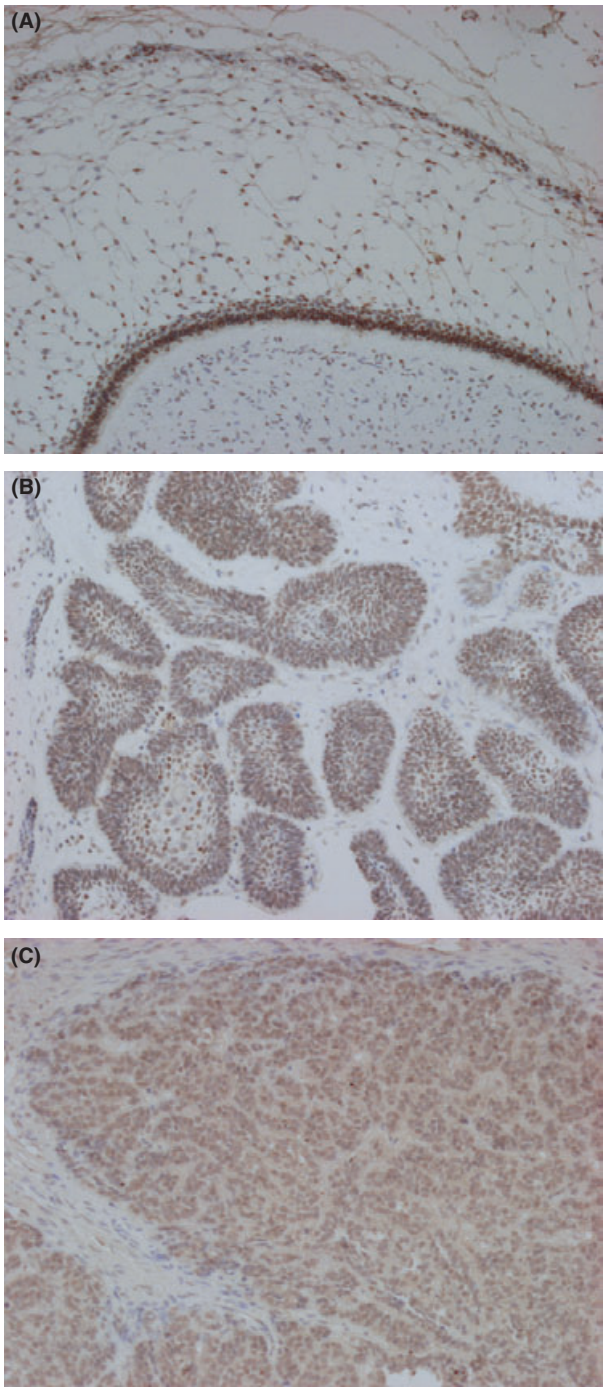

Figure 2 Immunohistochemical reactivity for uPAR. (A) Tooth germ showing reactivity in inner enamel epithelium. Some endothelial cells in dental papilla and dental follicle are also positive (×75). (B) Follicular ameloblastoma showing reactivity in peripheral columnar neoplastic cells. Some endothelial cells in the stroma are also positive (×95). (C) Ameloblastic carcinoma showing reactivity in most neoplastic cells. Some endothelial cells in the stroma are also positive (×95). uPAR = urokinase-type plasminogen activator receptor.

Extracellular matrix-degrading MMPs and uPA are universally expressed during tumor invasion and metastasis (5, 6). The levels of uPA, uPAR, and PAI-1 in human malignancies are significantly higher than in the corresponding normal tissues, and their levels are correlated with tumor angiogenesis and prognosis (6, 12, 13,

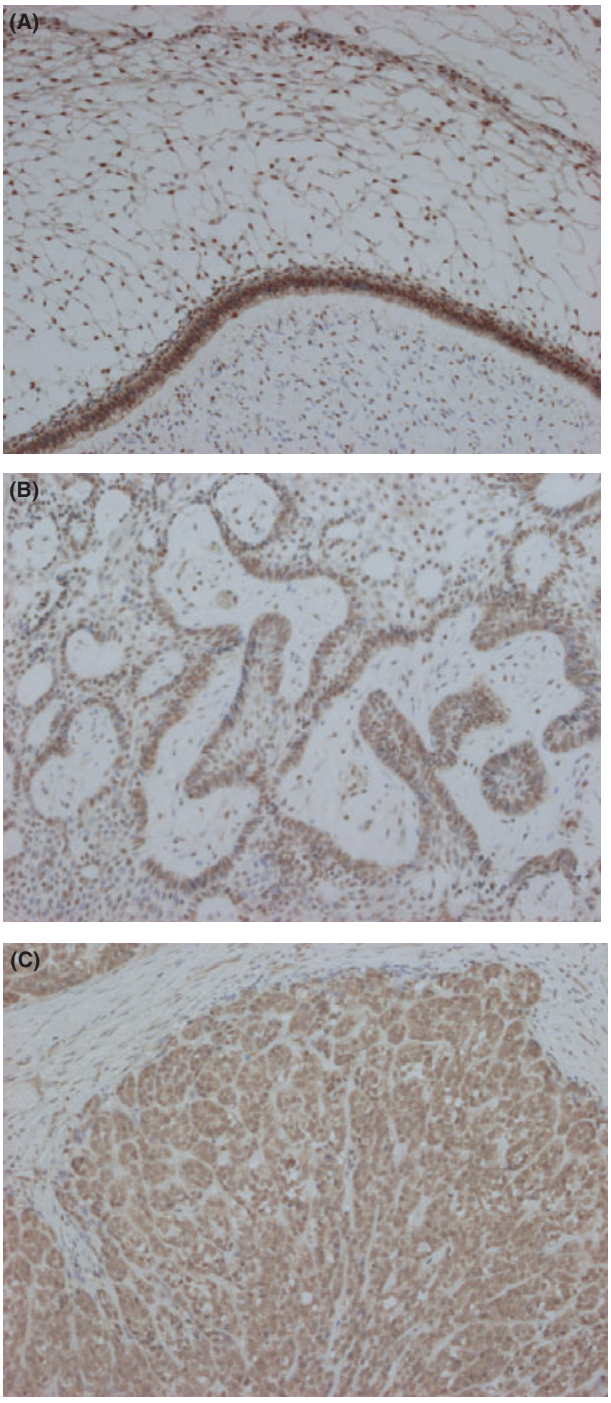

Figure 3 Immunohistochemical reactivity for PAI-1. (A) Tooth germ showing reactivity in inner enamel epithelium and weak reactivity in other epithelial components. Many mesenchymal cells in dental papilla and dental follicle are also positive (×75). (B) Plexiform ameloblastoma showing reactivity in many peripheral cuboidal cells and some central polyhedral cells. Many stromal cells are also positive (×95). (C) Ameloblastic carcinoma showing reactivity in most neoplastic cells. Many stromal cells are also positive (×95). PAI-1 = plasminogen activator inhibitor-1.

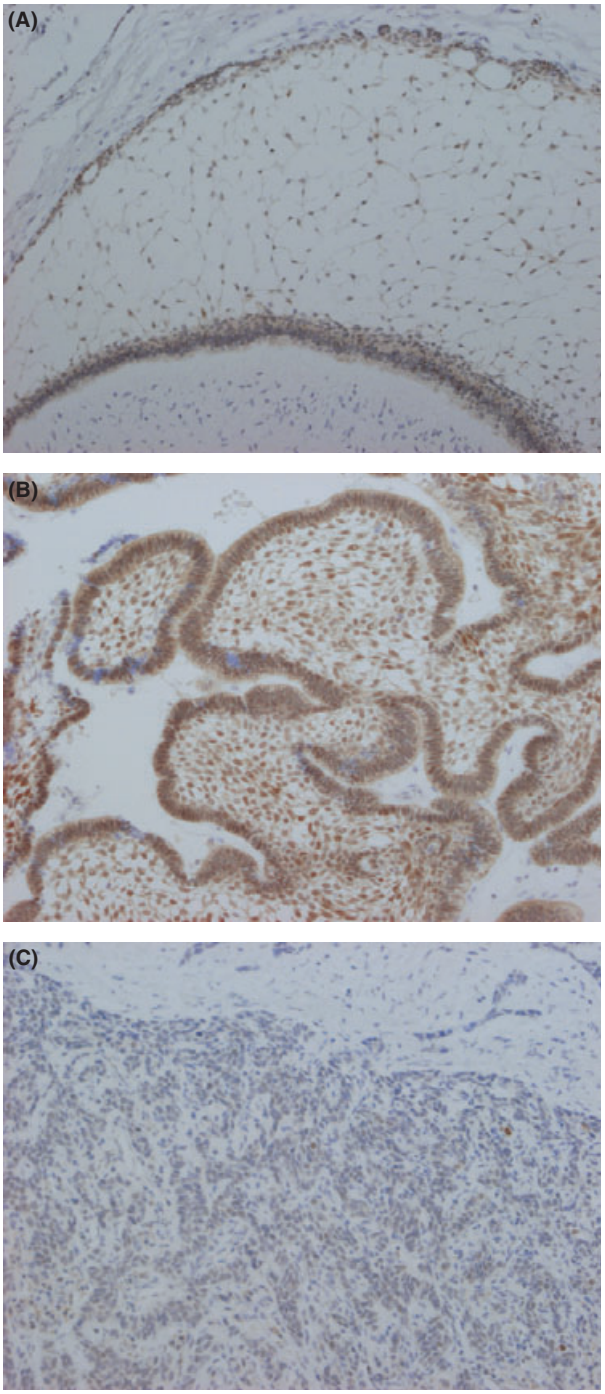

Figure 4 Immunohistochemical reactivity for maspin. (A) Tooth germ showing strong reactivity in inner enamel epithelium and weak reactivity in other epithelial components (×x80). (B) Follicular ameloblastoma showing strong reactivity in peripheral columnar cells and weak reactivity in central polyhedral cells (×95). (C) Ameloblastic carcinoma showing weak reactivity in some neoplastic cells (×95).

22). A previous study has revealed that ameloblastoma shows high fibrinolytic activity when compared with odontogenic cysts, suggesting that ameloblastoma tissue has a great capacity to induce locally activated plasmin (23). In the present study, immunohistochemical reactivity for uPA, uPAR, and PAI-1 was detected in ameloblastic tumors: uPA was expressed predominantly in stromal cells, uPAR expression was evident in neoplastic cells, and PAI-1 was found in both neoplastic

and stromal cells. These features suggest that interactions among the uPA system components contribute to progression of the epithelial odontogenic tumors by regulating the extent of ECM degradation. In this study, the levels of immunoreactivity for uPA and uPAR in benign and malignant ameloblastic tumors tended to be higher than that in tooth germs, while the level of PAI-1 immunoreactivity in ameloblastomas was slightly lower than that in tooth germs. These features suggest that altered expression of the uPA system components might be involved in oncogenesis of odontogenic epithelium. Signal transduction of hepatocyte growth factor (HGF), a pleiotropic cell growth effector, mediates the activation of the uPA proteolysis system, and extracellular matrix metalloproteinase inducer (EMMPRIN) is known to stimulate not only MMPs but also the uPA system, thus promoting tumor cell progression (24, 25). Our previous studies have confirmed expression of HGF and its receptor c-Met and EMMPRIN in ameloblastic tumors (16, 26), and the uPA system-dependent ECM degradation is considered to be affected by HGF signaling and EMMPRIN synthesis in the epithelial odontogenic tumors.

Maspin gene was initially isolated from normal mammary epithelium, and its expression was down-regulated in mammary carcinoma cells, supporting the role of *maspin* gene as a tumor suppressor gene (9). Reduced maspin expression associated with increased levels of malignancy has been recognized in other human malignant tumors (10, 13, 27). In the present study, immunohistochemical reactivity for maspin was detected in neoplastic cells of ameloblastic tumors, suggesting that this potent proteinase inhibitor plays a role in tumor cell progression of the epithelial odontogenic tumors. The level of maspin immunoreactivity in ameloblastomas was significantly higher than that in tooth germs, and ameloblastic carcinomas showed decreased reactivity for maspin. These features suggest that aberrant maspin expression might be involved in oncogenesis and malignant potential of odontogenic epithelium. In this study, distribution and levels of maspin immunoreactivity exhibited considerably distinct patterns from PAI-1 reactivity in tooth germs and ameloblastic tumors. These features suggest that the serine proteinase inhibitor family members PAI-1 and maspin might differentially function in both developing and neoplastic odontogenic tissues.

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