J Oral Pathol Med (2005) 34: 565–72

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Detection of mitochondria-mediated apoptosis signaling molecules in ameloblastomas

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BACKGROUND: To investigate the roles of the apoptosis signaling pathway mediated by mitochondria in oncogenesis and cytodifferentiation of odontogenic tumors, expression of pathway signaling molecules was analyzed in ameloblastomas as well as in tooth germs.
METHODS: Tissue specimens of 12 tooth germs, 41 benign ameloblastomas, and five malignant ameloblastomas were examined by reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry to determine the expression of cytochrome *c*, apoptotic protease-activating factor-1 (APAF-1), caspase-9, and apoptosis-inducing factor (AIF).
RESULTS: The mRNA expression of APAF-1, caspase-9, and AIF was detected in all samples of normal and neoplastic odontogenic tissues. Immunohistochemical reactivity for cytochrome *c*, APAF-1, caspase-9, and AIF was detected in both normal and neoplastic odontogenic tissues. Expression of cytochrome *c* and AIF was evident in odontogenic epithelial cells neighboring the basement membrane, and APAF-1 and caspase-9 were detected in most odontogenic epithelial cells. Immunoreactivity for cytochrome *c* in tooth germs was slightly weaker than that in benign and malignant ameloblastomas. Keratinizing cells in acanthomatous ameloblastomas and granular cells in granular cell ameloblastomas showed a decrease or loss of immunoreactivity for these mitochondria-mediated apoptosis signaling molecules. Expression of AIF was obviously low in ameloblastic carcinomas.
CONCLUSION: Expression of cytochrome *c*, APAF-1, caspase-9, and AIF in tooth germs and ameloblastomas suggests that the mitochondria-mediated apoptotic pathway has a role in apoptotic cell death of normal and neoplastic odontogenic epithelium. Expression of these mitochondrial apoptosis signaling molecules might be involved in oncogenesis, cytodifferentiation, and malignant transformation of odontogenic epithelium.
J Oral Pathol Med (2005) **34**: 565–72

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Accepted for publication May 24, 2005

Keywords: ameloblastoma; apoptosis; mitochondria; signal transduction

Introduction

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 (1–4). 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, 4). Histologically, ameloblastoma shows considerable variation, including follicular, plexiform, acanthomatous, granular cell, basal cell, and desmoplastic types (1). Malignant ameloblastoma is defined as a neoplasm in which the pattern of an ameloblastoma and cytologic features of malignancy are shown by the primary growth in the jaws and/or by any metastatic growth (1). Recently, malignant ameloblastoma has been subclassified into metastasizing ameloblastoma and ameloblastic carcinoma on the basis of metastatic spread and cytologic malignant features (3). Several recent studies have detected genetic and cytogenetic alterations in these epithelial odontogenic tumors (5, 6); however, the detailed mechanisms of oncogenesis, cytodifferentiation, and tumor progression remain unknown.

There are two alternative pathways that initiate apoptosis: one is mediated by death receptors on the cell surface, and the other is mediated by mitochondria (7, 8). Proapoptotic stimuli, including DNA damage and oxidative stress, require a mitochondria-dependent step that involves permeabilization of the outer mitochondrial membrane and release of mitochondrial proteins normally located in the intermembrane space (8–10). Cytochrome *c* is released into the cytoplasm, where the liberated cytochrome *c* initiates formation of an apoptosome along with apoptotic protease-activating factor-1 (APAF-1) in the presence of adenosine nucleotides (9, 11). The apoptosome recruits and activates caspase-9, an initiator of apoptosis (12). In contrast, apoptosis-inducing factor (AIF) translocates from mitochondria via the cytoplasm to the nucleus,

where it interacts with DNA and causes nuclear condensation and DNA fragmentation (10). Thus, the mitochondrial apoptotic pathway involves both caspase-dependent and caspase-independent processes in apoptotic cell death, and pathologic roles of the signaling pathway have recently been identified in several human tumors (13–17).

Our previous studies confirmed apoptotic cells and apoptosis-related factors, such as bcl-2 family proteins, inhibitor of apoptosis protein (IAP) family proteins, p53 and its associates, tumor necrosis factor (TNF)α, TNF-related apoptosis-inducing ligand (TRAIL), and their receptors, Fas, Fas ligand, and heat shock proteins, in tooth germs and ameloblastomas, suggesting that apoptotic cell death has an important role in oncogenesis or cytodifferentiation of odontogenic epithelium (18–23). We also showed that the apoptosis signaling pathway mediated by death receptors is low effective on biologic properties of odontogenic epithelial components (23). In the present study, the expression of cytochrome *c*, APAF-1, caspase-9, and AIF in benign and malignant ameloblastomas as well as in tooth germs was examined by reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry to investigate the expression and tissue distribution of these mitochondrial apoptosis signaling molecules in epithelial odontogenic tumors.

Materials and methods

Tissue preparation

Specimens were surgically removed from 46 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 1 to several days and were embedded in paraffin. The tissue blocks were sliced into 3-μm-thick sections for routine histologic and subsequent immunohistochemical examinations. Tissue sections were stained with hematoxylin and eosin for histologic diagnosis according to the WHO histologic typing of odontogenic tumors (1). The tumors comprised 41 ameloblastomas and five malignant ameloblastomas. Ameloblastomas were divided into 23 follicular and 18 plexiform types, including 11 acanthomatous, six granular cell, three basal cell, and four desmoplastic subtypes. Malignant ameloblastomas were classified into two metastasizing ameloblastomas and three ameloblastic carcinomas according to the criteria of Eversole (3). For RT-PCR analysis, tumor tissues were immediately frozen on dry ice and stored at −80°C. Specimens of 12 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.

RT-PCR

Total RNA was extracted from frozen tissue samples of 12 tooth germs, 32 ameloblastomas, and one malignant ameloblastoma, using an 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 an Omniscript RT Kit (Qiagen) with oligo-$(dT)_{15}$ primer (Roche Diagnostics, Mannheim, Germany) as outlined by the manufacturer. The cDNA samples were amplified using a HotstarTaq Master Mix Kit (Qiagen) with specific primers in a DNA thermal cycler (Eppendorf, Hamburg, Germany). The primers used in this study are listed in Table 1. A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as an internal control to examine human gene expression. PCR was performed in a total volume of 50 μl, containing 1 μg of template cDNA and 0.5 μM of each specific primer set. The procedure for amplification included 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and elongation at 72°C for 60 s with heat starting at 95°C for 15 min and final elongation at 72°C for 10 min. The PCR products were electrophoresed on 2% agarose gel at 100 V for 30 min and visualized with ethidium bromide.

Immunohistochemistry

Tissue sections of 10 tooth germs, 39 ameloblastomas, and five malignant ameloblastomas were deparaffinized and immersed in methanol with 0.3% hydrogen peroxide. The sections were heated in 0.01 M citrate buffer (pH 6.0; for cytochrome *c* and APAF-1) or 1 mM of ethylenediaminetetraacetic acid (EDTA) buffer (pH 8.0; for caspase-9 and AIF) for 10 min by autoclave (121°C, 2 atm) and were incubated with primary antibodies at 4°C overnight. The applied antibodies are listed in Table 1. After washed with phosphate-buffered saline (PBS), the sections were allowed to react with peroxidase-conjugated antimouse immunoglobulin G (IgG; for cytochrome *c* and caspase-9) or antirabbit IgG (for APAF-1 and AIF) polyclonal antibody (Histofine Simple Stain MAX-PO; Nichirei, Tokyo, Japan) for 45 min. After washed with PBS, reaction products were visualized by immersing the sections in 0.03% diaminobenzidine solution containing 2 mM hydrogen peroxide for 1–3 min. Nuclei were lightly counterstained with methylgreen. For control studies of the antibodies, the serial sections were treated with PBS, mouse antichromogranin A monoclonal antibody (Dako, Glostrup, Denmark; subclass IgG_{2b}), mouse antidesmin monoclonal antibody (Nichirei; subclass IgG_1), and normal rabbit IgG instead of the primary antibodies and were confirmed to be unstained in the odontogenic tissues. Immunohistochemical reactivity for mitochondria-mediated apoptosis signaling molecules was evaluated and classified into three groups: (−) negative, (+) scatteredly positive, and (++) diffusely positive.

Results

mRNA expression

The RT-PCR analysis identified expression of mRNA transcripts for mitochondria-mediated apoptosis signaling molecules in all 32 ameloblastomas and one metastasizing ameloblastoma as well as in the 12 tooth germ tissues (Fig. 1). The PCR products of APAF-1,

Table 1 Primers and antibodies

	Primer		*Antibody*			
	Sequence (5′–3′)	*Product (bp)*	*Clonality*	*Source*	*Dilution*	*Pretreatment*
Cytochrome *c*			Monoclonal (mouse IgG_{2b})	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:20	Autoclave (citrate)
APAF-1	Forward: CCATATGATCGAGGACATCCAG Reverse: GAAATTAAAGCAACCAGGCATCT	1107	Polyclonal (rabbit IgG)	Lab Vision, Tremont, CA, USA	1:20	Autoclave (citrate)
Caspase-9	Forward: CTTTTCCCTGGATTGGATTAAAG Reverse: ATCTGGTGAAAATCTGCAGTGAT	750	Monoclonal (mouse IgG_1)	Lab Vision	1:20	Autoclave (EDTA)
AIF	Forward: GGATCCTGGGGCCAGGGTACTGAT Reverse: CTCGGGGAAGAGTTGAATCACTTC	550	Polyclonal (rabbit IgG)	Oncogene, San Diego, CA, USA	1:30	Autoclave (EDTA)
GAPDH	Forward: GGAGTCAACGGATTTGGT Reverse: GTGATGGGATTTCCATTGAT	206				

APAF-1, apoptotic protease-activating factor-1; AIF, apoptosis-inducing factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EDTA, ethylenediaminetetraacetic acid.

Figure 1 Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of mRNA transcripts for mitochondria-mediated apoptosis signaling molecules in tooth germs and epithelial odontogenic tumors (M: molecular weight standard; 1, 2: tooth germs; 3–6: ameloblastomas; 7: malignant ameloblastoma). mRNA expression of apoptotic protease-activating factor-1 (APAF-1), caspase-9, and apoptosis-inducing factor (AIF) was seen in all samples. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was run as a control to ascertain the integrity of mRNA/cDNA.

caspase-9, and AIF were 1107, 750, and 550 bp, respectively. There was no distinct difference in the mRNA expression of mitochondria-mediated apoptosis signaling molecules among the different types or subtypes of ameloblastomas.

Immunohistochemical reactivity

Immunohistochemical reactivity for mitochondria-mediated apoptosis signaling molecules in tooth germs and ameloblastomas is summarized in Table 2.

Table 2 Immunohistochemical reactivity for mitochondria-mediated apoptosis signaling molecules in tooth germs and ameloblastomas

	Cytochrome c	*APAF-1*	*Caspase-9*	*AIF*
Tooth germ ($n = 10$)				
Epithelial cells				
Inner enamel epithelium	+/++	++	++	++
Outer enamel epithelium	–	++	++	++
Stellate reticulum	–	++	++	–
Stratum intermedium	–	++	++	–
Dental lamina	–	++	++	++
Mesenchymal cells				
Dental papilla	+	+	++	–
Dental follicle	+	+	++	–
Ameloblastoma ($n = 39$)				
Neoplastic cells				
Peripheral cells	++	++	++	++
Central cells	–	++	++	+
Keratinizing cells	–	–/+	–	–
Granular cells	+	++	–/+	–/+
Basal cell variant	++	++	++	++
Desmoplastic variant	+	++	++	++
Stromal cells	+	+	++	–
Metastasizing ameloblastoma ($n = 2$)				
Neoplastic cells				
Peripheral cells	++	++	++	++
Central cells	–	++	++	+
Stromal cells	+	+	++	–
Ameloblastic carcinoma ($n = 3$)				
Neoplastic cells	++	++	++	–/++
Stromal cells	+	+	++	–

–, negative; +, scatteredly positive; ++, diffusely positive.
APAF-1, apoptotic protease-activating factor-1; AIF, apoptosis-inducing factor.

Immunoreactivity for cytochrome *c* was detected in the cytoplasm of cellular components in both normal and neoplastic odontogenic tissues (Fig. 2). In tooth germs, cytochrome *c* was expressed weakly and sporadically in inner enamel epithelium of enamel organs, especially evident near the basement membrane (Fig. 2a). Weak reactivity was found also in some endothelial cells of dental papillae and dental follicles. Ameloblastomas showed cytochrome *c* reactivity in peripheral columnar or cuboidal neoplastic cells, especially evident near the basement membrane (Fig. 2b). Keratinizing cells in acanthomatous ameloblastomas exhibited no reactivity for cytochrome *c*, whereas scattered granular cells in granular cell ameloblastomas were reactive with cytochrome *c*. Basal cell ameloblastomas showed cytochrome *c* reactivity in most neoplastic cells, and some neoplastic cells in desmoplastic ameloblastomas were positive for cytochrome *c*. Metastasizing ameloblastomas showed a cytochrome *c* expression pattern similar to that of benign ameloblastomas, whereas ameloblastic carcinomas exhibited cytochrome *c* reactivity in most neoplastic cells, especially evident near the basement membrane (Fig. 2c). In these epithelial odontogenic tumors, some stromal endothelial cells were also weakly reactive with cytochrome *c*.

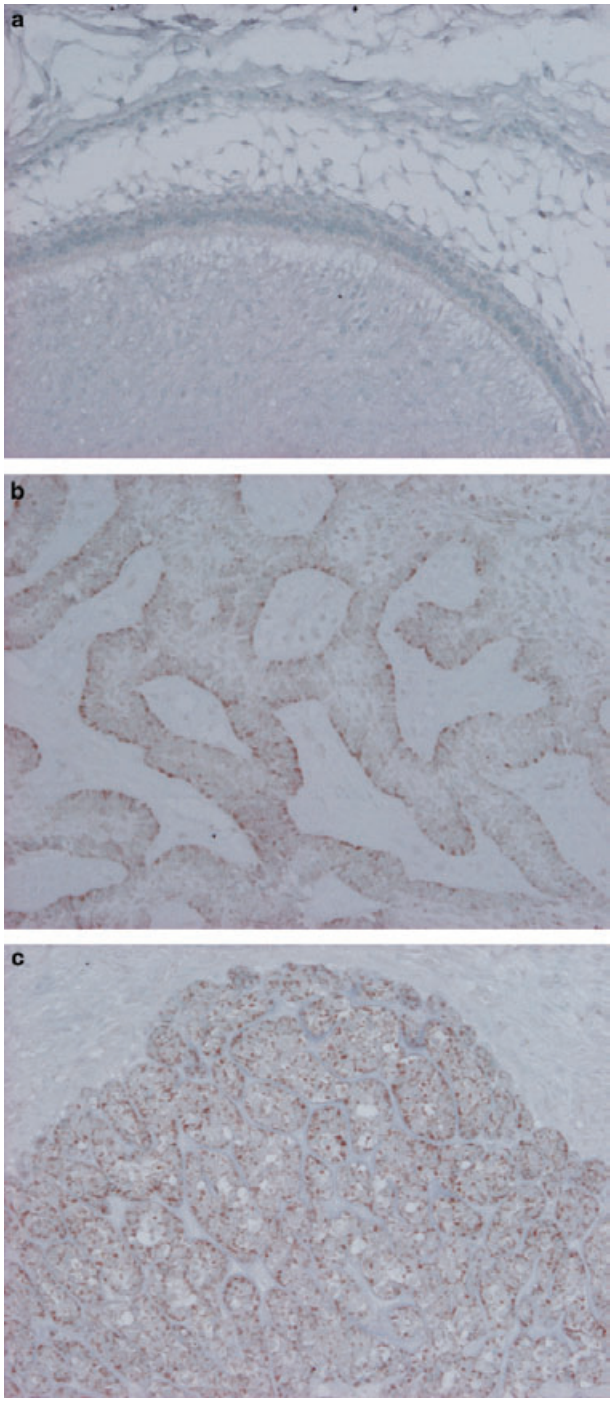

Figure 2 Immunohistochemical reactivity for cytochrome *c*. (a) Tooth germ showing weak reactivity in inner enamel epithelium (×125). (b) Plexiform ameloblastoma showing reactivity in peripheral cuboidal cells (×125). (c) Ameloblastic carcinoma showing reactivity in most neoplastic cells (×125).

The APAF-1 and caspase-9 were detected in the cytoplasm of cellular components in normal and neoplastic odontogenic tissues (Figs 3 and 4). In tooth germs, APAF-1 and caspase-9 were expressed in epithelial cells of enamel organs and dental laminae, and reactivity in inner enamel epithelium was slightly stronger than that in other epithelial components (Figs 3a and 4a). Weak APAF-1 reactivity was found also in some endothelial cells of dental papillae and dental follicles, and caspase-9 expression was also detected in mesenchymal cells of dental papillae and endothelial cells of dental papillae and dental follicles. Benign and malignant ameloblastomas showed APAF-1 and caspase-9 reactivity in most neoplastic cells. In benign and metastasizing ameloblastomas, reactivity for APAF-1 and caspase-9 in peripheral columnar or cuboidal neoplastic cells was slightly stronger than that in central polyhedral cells (Figs 3b and 4b). Acanthomatous ameloblastomas showed decreased APAF-1 reactivity in keratinizing cells (Fig. 3c). Caspase-9 expression was not detected in keratinizing cells in acanthomatous ameloblastomas, and granular cell ameloblastomas showed decreased caspase-9 reactivity in granular cells (Fig. 4c). In these epithelial odontogenic tumors, scattered stromal endothelial cells were also reactive with APAF-1 and caspase-9.

Immunohistochemical reactivity for AIF was detected in the cytoplasm of normal and neoplastic odontogenic epithelium (Fig. 5). In tooth germs, AIF was weakly expressed in inner and outer enamel epithelium and dental laminae (Fig. 5a). Ameloblastomas showed weak AIF reactivity in many peripheral columnar or cuboidal cells and some central polyhedral cells (Fig. 5b). AIF expression was not detected in keratinizing cells in acanthomatous ameloblastomas, and granular cell ameloblastomas showed decreased reactivity in granular cells. Basal cell ameloblastomas and desmoplastic ameloblastomas exhibited weak AIF reactivity in most neoplastic cells (Fig. 5c). Metastasizing ameloblastomas showed an AIF expression pattern similar to that of benign ameloblastomas, whereas ameloblastic carcinomas exhibited no or very weak reactivity for AIF.

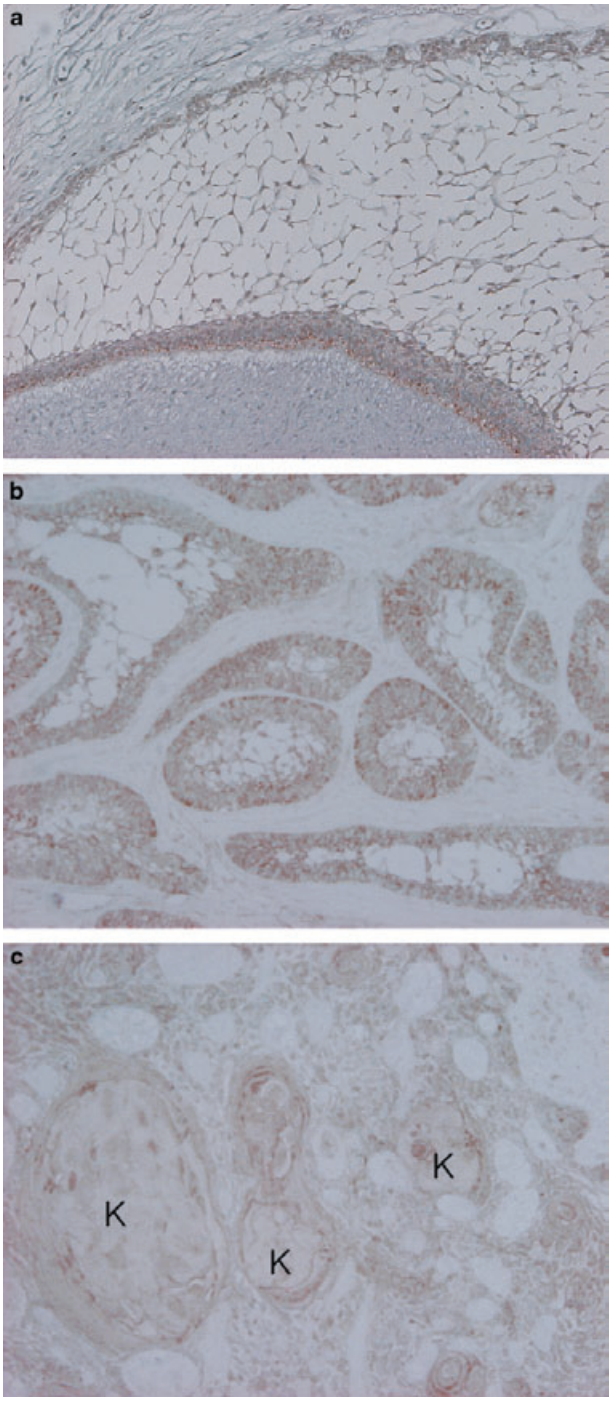

Figure 3 Immunohistochemical reactivity for apoptotic protease-activating factor-1 (APAF-1). (a) Tooth germ showing diffuse reactivity in enamel organ (×85). (b) Follicular ameloblastoma showing diffuse reactivity in neoplastic cells (×120). (c) Acanthomatous ameloblastoma showing decreased reactivity in keratinizing cells (K; ×120).

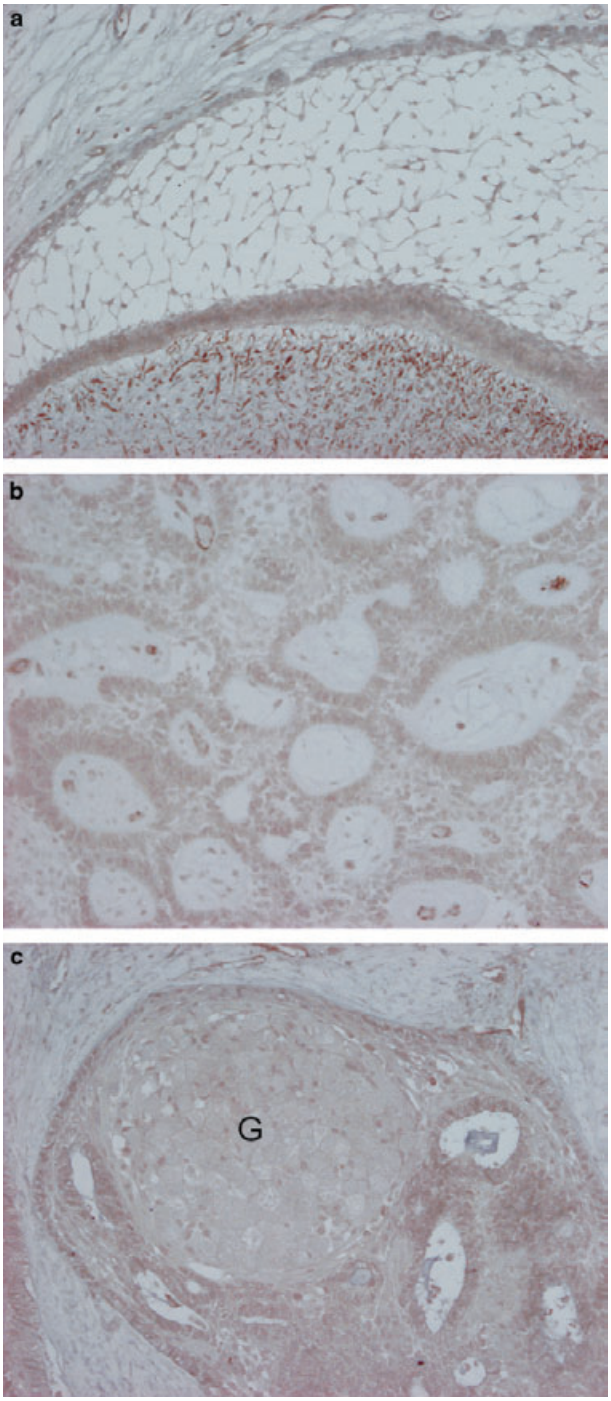

Figure 4 Immunohistochemical reactivity for caspase-9. (a) Tooth germ showing diffuse reactivity in enamel organ. Mesenchymal cells in dental papilla and endothelial cells in dental follicle are also reactive (×90). (b) Plexiform ameloblastoma showing diffuse reactivity in neoplastic cells. Stromal endothelial cells are also reactive (×110). (c) Granular cell ameloblastoma showing decreased reactivity in granular cells (G; ×100).

Discussion

Mitochondria-mediated apoptosis signaling molecules contribute to both caspase-dependent and caspase-independent processes in apoptotic cell death (7, 8, 24). Mice-deficient for *apaf-1* or *caspase-9* suffer a variety of developmental defects, such as brain overgrowth, craniofacial deformation, and retention of the interdigital webs, because of compromised apoptosis and die either in utero or shortly after birth (25–28). Investigation of embryonic stem cells with genetic

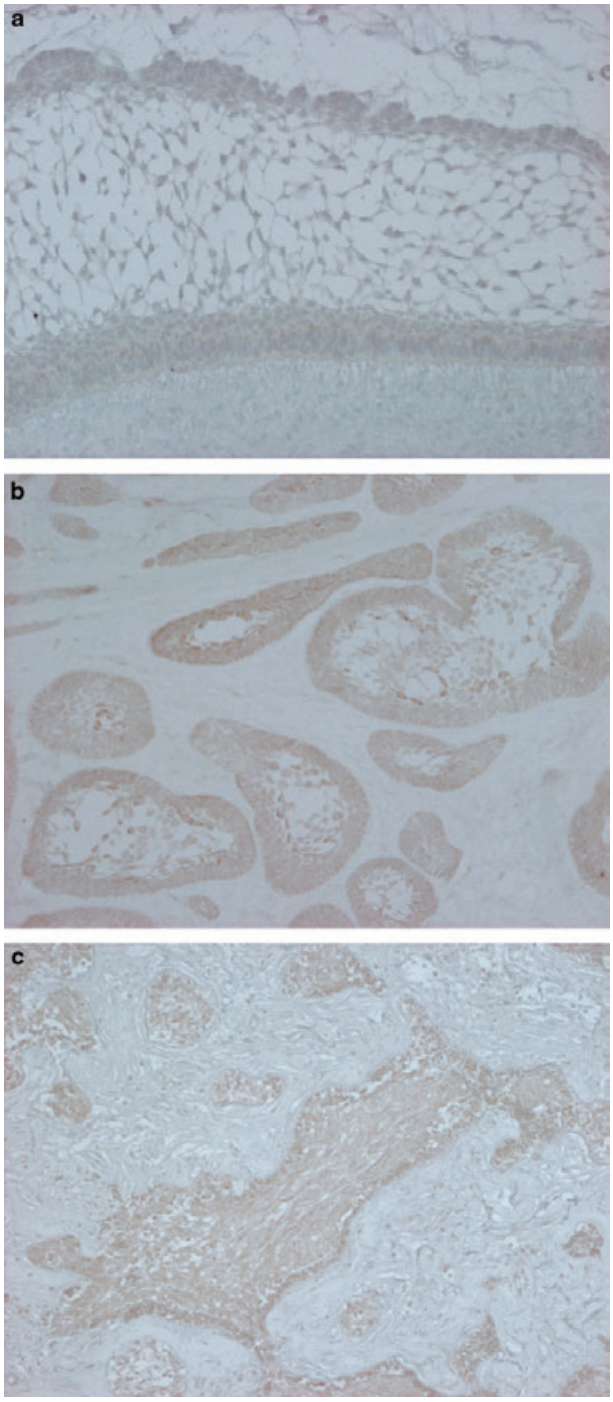

Figure 5 Immunohistochemical reactivity for apoptosis-inducing factor (AIF). (a) Tooth germ showing weak reactivity in inner and outer enamel epithelium (×115). (b) Follicular ameloblastoma showing weak reactivity in many peripheral columnar cells and some central polyhedral cells (×100). (c) Desmoplastic ameloblastoma showing weak reactivity in most neoplastic cells (×75).

inactivation of *AIF* has revealed that AIF is essential for cavitation of the embryoid body and controls apoptosis during early morphogenesis (29). Thus, the mitochondria-mediated apoptotic pathway has distinct roles in developmental processes (30). Apoptotic cell death is known to play an important role in tooth development (31, 32), and some apoptosis-related factors, such as bcl-2 family proteins and Fas/Fas ligand system, have been identified in tooth germ tissues (19, 20, 33). Our previous study revealed that tooth germs did not express caspase-8, a signaling molecule functioning in the death receptor-mediated apoptotic pathway (23). In the present study, expression of cytochrome *c*, APAF-1, caspase-9, and AIF was detected in tooth germs at the initial stage of crown mineralization, suggesting that the mitochondria-mediated apoptotic pathway plays a role in apoptotic cell death during tooth development in both caspase-dependent and caspase-independent manners.

Cytochrome *c*, an essential component of the mitochondrial respiratory chain, was discovered to play a major role in bringing about cell death (9). Subsequently, APAF-1 and caspase-9 were identified as factors activating caspase-3, an executioner of apoptosis, in a cytochrome *c*-dependent fashion (11, 12). Inactivation or reduced expression of APAF-1 has been detected in malignant melanoma, ovarian cancer, and leukemia (13, 14, 34), and *caspase-9* gene is known to show various sequence polymorphisms in neuroblastoma (15). Our previous study revealed that caspase-8 expression was found in a limited number of ameloblastomas, but not in malignant ameloblastomas (23). In the present study, expression of cytochrome *c*, APAF-1, and caspase-9 was detected in all benign and malignant ameloblastomas, suggesting that the caspase-dependent mitochondria-mediated apoptotic pathway was associated with apoptotic cell death in these epithelial odontogenic tumors. Immunoreactivity for cytochrome *c* in benign and malignant ameloblastomas tended to be stronger than that in tooth germs. These results support the fact that apoptotic cells are more abundant in ameloblastomas than in tooth germs (18). Bcl-2 family proteins block release of cytochrome *c* from mitochondria, and IAP family proteins directly inhibit caspase family proteins, thereby preventing apoptosis (35–37). In the present study, cytochrome *c* was expressed predominantly in neoplastic cells near the basement membrane in benign and malignant ameloblastomas. Our previous studies showed that expression of bcl-2 family proteins, bcl-2 and bcl-x, and IAP family proteins, survivin and XIAP, was marked in neoplastic cells neighboring the basement membrane (19, 22). These features suggest that the mitochondrial apoptotic pathway is suppressed in neoplastic cells near the basement membrane in these epithelial odontogenic tumors. In our previous studies, increased apoptotic cell death and decreased expression of apoptosis suppressors were found in keratinizing cells in acanthomatous ameloblastomas and granular cells in granular cell ameloblastomas (18–20, 22). The present study showed distinct reactivity for cytochrome *c*, APAF, and caspase-9 in granular cells and markedly decreased expression of these mitochondrial apoptosis signaling molecules in keratinizing cells in these ameloblastoma variants.

The AIF was identified as the mitochondrial intermembrane protein fraction containing activity that

causes isolated nuclei to acquire apoptotic morphology (10). This factor does not only induce apoptotic cell death in a caspase-independent manner, but also triggers release of mitochondrial cytochrome *c* and subsequent caspase activation (24). Several investigators have demonstrated pathologic roles of AIF in lung carcinoma, leukemia, and neural tumors (16, 17, 38). In the present study, expression of AIF was detected at both mRNA and protein levels in benign and malignant ameloblastomas, suggesting that the caspase-independent mitochondrial apoptotic pathway might have a role in apoptotic cell death in these epithelial odontogenic tumors. Some bcl-2 family proteins inhibit mitochondrial release of AIF, as well as cytochrome *c* (10, 24). Expression of bcl-2 and bcl-x in ameloblastomas (19, 39) is also considered to suppress the function of AIF in neoplastic cells of these epithelial odontogenic tumors. In the present study, immunoreactivity for AIF was decreased in keratinizing cells or granular cells of ameloblastoma variants and neoplastic cells of ameloblastic carcinomas. These features suggest that AIF expression might be involved in cytodifferentiation and malignant transformation of odontogenic epithelium.

In conclusion, expression of cytochrome *c*, APAF-1, caspase-9, and AIF in tooth germs and ameloblastomas suggests that the mitochondria-mediated apoptotic pathway has a role in apoptotic cell death of normal and neoplastic odontogenic epithelium. Expression of these mitochondrial apoptosis signaling molecules might be involved in oncogenesis, cytodifferentiation, and malignant transformation of odontogenic epithelium.

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