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Disruption of periodontal integrity induces expression of apin by epithelial cell rests of Malassez

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Background and Objective: It has been suggested that epithelial cell rests of Malassez (ERM) may express enamel matrix proteins and play an important role in periodontal regeneration. Two novel proteins, apin (APIN) and amelotin (AMTN), produced by maturation-stage ameloblasts and junctional epithelium, have recently been identified. The objective of this study was to evaluate whether the ERM express APIN and AMTN under normal conditions and after periodontal challenge.

Material and Methods: Gingivectomy and orthodontic tooth movement were carried out on the left side of the maxillae of rats. The control group included the untreated contralateral side of these animals and the maxillae of normal, untreated rats. Animals were sacrificed by intracardiac perfusion on days 3 and 5 after the experimental procedures and maxillary molars were decalcified and processed for paraffin embedding. Immunohistochemistry was used to evaluate the expression of various ameloblast products, including APIN, AMTN, ameloblastin (AMBN) and amelogenin (AMEL).

Results: At 3 and 5 days after periodontal challenge, ERM were more evident in the periodontal ligament along the root surface and in the root furcations. Immunodetection of APIN, but not of the other three proteins, was observed in the ERM following the disruption of periodontal integrity. No immunolabeling for APIN, AMTN, AMBN and AMEL was detected in the ERM under normal conditions.

Conclusion: The expression of APIN at an early time-point following disruption of periodontal integrity suggests that this protein may be part of the cascade of events leading to the activation of ERM during periodontal healing and regeneration.

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The epithelial cell rests of Malassez (ERM) are developmental residues of Hertwig's epithelial root sheath (HERS), which localize in the periodontal ligament (PDL) along the radicular cementum (1,2). Morphologically, ERM are characterized as clusters of epithelial cells which have a high nuclear : cytoplasmic ratio with condensed nuclei and poorly developed protein synthetic organelles (3–5). Although the morphological characteristics of ERM are well known, their function is still a subject of debate. Several studies have shown that ERM express, under certain conditions, proteins produced by ameloblasts (6–8) and that they may play an important functional role not only in preserving a normal periodontium, but also contributing to its regeneration (2,4,9).

Recently, efforts to characterize the secretome of enamel organ cells have led to the cloning of two novel proteins called apin (APIN) (10) and amelotin (AMTN) (11,12), which are produced by ameloblasts during the maturation stage of amelogenesis. Both proteins were unexpectedly

Table 1. Expression of proteins produced by ameloblasts in the epithelial cell rests of Malassez (ERM) and Hertwig's epithelial root sheath (HERS) cells entrapped in cementum in normal periodontium and after disruption of periodontal integrity

	Proteins produced by ameloblasts			
ERM/HERS cells	APIN	AMTN	AMBN	AMEL
ERM – normal periodontium	–	–	–	–
ERM – disrupted periodontal integrity	+	–	–	–
HERS cells – normal periodontium	+	+	+	+
HERS cells – disrupted periodontal integrity	+	+	+	+

APIN, apin; AMTN, amelotin; AMBN, ameloblastin; AMEL, amelogenin

found to be expressed in the junctional epithelium (JE) (10,12), and northern blotting analyses revealed that APIN (13) and AMTN (12) mRNAs were also present in the PDL. While AMTN localizes in the basal lamina at the interface between ameloblasts and JE cells with the tooth surface, APIN is particularly interesting because it is also present between the cells of the JE, which is believed to be an incompletely differentiated epithelium (14). In addition, APIN has been found to be upregulated in some neoplasias, such as gastric cancer (15) and cancer of the cervix (16) and in human calcifying epithelial odontogenic tumors (17). The fact that APIN is expressed by normal, incompletely differentiated cells in the JE and by dedifferentiated cancer cells, suggests a relationship with differentiation status.

The objective of this study was to determine, using immunolabeling, whether APIN and AMTN, similarly to other proteins produced by ameloblasts, can be expressed by ERM. The results show that APIN is distinctively expressed soon after periodontal challenge and implicate this protein in the early response to periodontal insult.

Material and methods

Periodontal challenge procedures

Six adult male Wistar rats weighing 250 ± 50 g (Charles River Canada, St-Constant, QC, Canada) were used for orthodontic tooth movement and for gingivectomy. These procedures were carried out on the left side of maxilla and the contralateral side was left intact. The control group included the untreated right side of these animals as well as the maxillae/mandibles of normal, untreated rats. The animals were anesthetized with an intraperitoneal injection of a mixture of 50 mg/kg of ketamine hydrochloride (Ketaset®; Wyeth Canada, St Laurent, QC, Canada), 5 mg/kg of xylazine (Rompun®; Bayer Inc., Toronto, ON, Canada) and 1 mg/kg of acepromazine maleate (Acevet 10; Vétoquinol, Lavaltrie, QC, Canada). For tooth movement, an orthodontic elastic module (3M Unitek, St Paul, MN, USA) was placed between the first and the second maxillary molars (18). Removal of the gingiva and the JE along the maxillary molars (gingivectomy) was accomplished by scraping the tooth surface and extending 2 mm along the palate with periodontal curettes (SU 15/33, SL 5/6 and KS 1/2; Hi-Friedy, Chicago, IL, USA) (19).

Following experimental manipulations, the animals received an injection of buprenorphine hydrochloride (Temgesic®; Reckitt and Colman, Hull, UK) and were sacrificed by intracardiac perfusion 3 and 5 days later. The experimental protocol and all animal procedures were carried out in accordance with the guidelines of the Comité de déontologie de l'expérimentation sur les animaux of Université de Montréal.

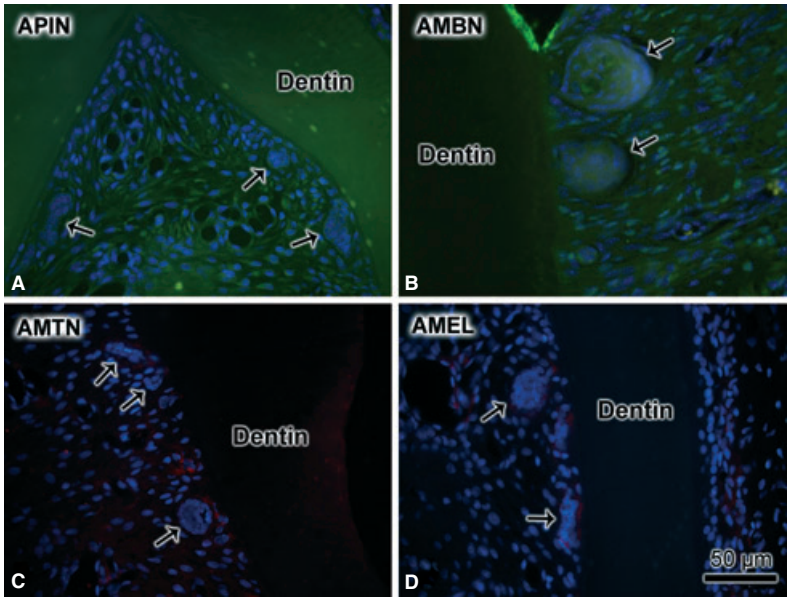

Fig. 1. The epithelial cell rests of Malassez (ERM) found in normal periodontal ligament (arrows) did not express apin (APIN) (A), ameloblastin (AMBN) (B), amelotin (AMTN) (C) and amelogenin (AMEL) (D).

Tissue processing

The animals were anesthetized and perfused, as previously described (20). Maxillae were dissected, immersed in the same fixative solution overnight at 4°C and decalcified for 30 days at 4°C in 4.13% disodium EDTA. Decalcified samples were washed for 24 h in 0.1 M cacodylate buffer (pH 7.2), processed for paraffin embedding and sectioned at 5 µm thickness. For morphological analyses, the sections were stained with hematoxylin and eosin.

Immunohistochemistry

Sections were deparaffinized with d-limonene-based solvent (Citrisolv®;

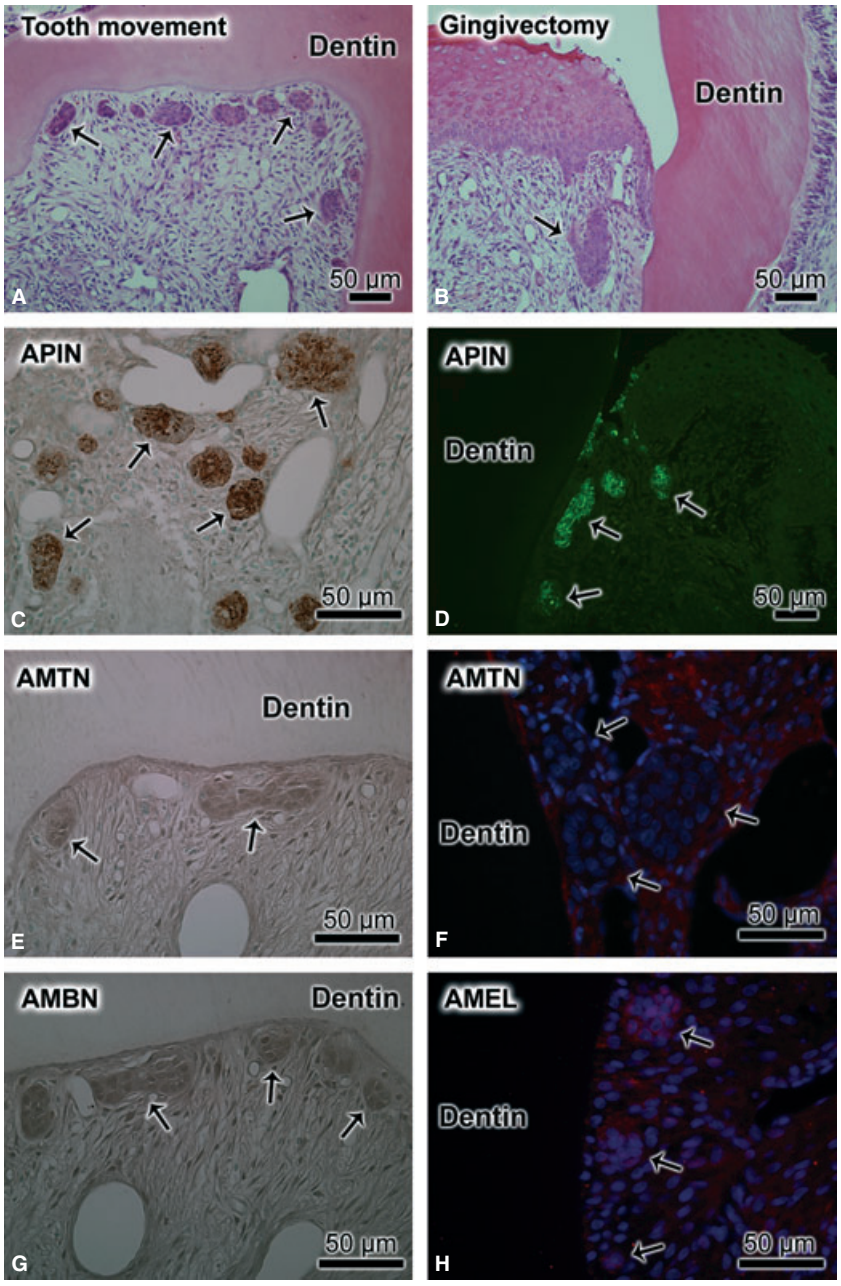

Fig. 2. Light microscopic view of the epithelial cell rests of Malassez (ERM) in the periodontal ligament in the root furcation (A) and cervical region (B) on day 5 following tooth movement (A,C,E,G) and gingivectomy (B,D,F,H). Immunoperoxidase (C,E,G) and immunofluorescence (D,F,H) preparations for apin (APIN), amelotin (AMTN), amelogenin (AMEL) and ameloblastin (AMBN) for the different experimental groups. Strong expression of APIN in the ERM was observed when periodontal integrity was disrupted (C,D). However, no evidence of immunolabeling for AMBN (G), AMEL (H) and AMTN (E,F) was detected in the ERM of the treated samples.

Fisher Scientific, Ottawa, ON, Canada), rehydrated through a descending ethanol series and washed in distilled water. In order to avoid nonspecific sticking, the sections were blocked with 0.01 M phosphate-buffered saline (PBS, pH 7.2) containing 5% skim milk for 20 min at room temperature. Incubations with rabbit primary antibodies raised against rat APIN (1:2000 dilution) (10), AMTN (1:500 dilution) (10), ameloblastin (AMBN; 1:500 dilution, courtesy of P.H. Krebsbach) and amelogenin (AMEL; 1:1500 dilution, courtesy of H.C. Slavkin) were performed for 3 h at room temperature. The incubations with the secondary antibodies were performed at a dilution of 1:500, for 1 h at room temperature, with goat anti-rabbit IgG conjugated to AlexaFluor 488 (Molecular Probes/Invitrogen, Burlington, ON, Canada) for APIN and AMBN, and with goat anti-rabbit IgG conjugated to AlexaFluor 594 (Molecular Probes/Invitrogen) for AMTN and AMEL. Following each incubation, the slides were washed with 0.01 M PBS containing 0.05% (v/v) Tween 20, pH 7.4 (0.01 M PBS-Tween 20). Sections were mounted in ProLong Gold antifade reagent with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; Invitrogen) to counterstain nuclei in blue. Fluorescence was examined under a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany) equipped with an Olympus DP70 digital camera.

For immunoperoxidase preparations, the deparaffinized sections were blocked as described above, for immunofluorescence and incubated with rabbit primary antibody raised against rat APIN (1:4000 dilution), AMTN (1:4000 dilution), AMBN (1:2000 dilution) and AMEL (1:8000 dilution) for 3 h at room temperature. Sections were washed with 0.01 M PBS-Tween 20, followed by treatment with the Dako-Envision™ + System, horseradish peroxidase-labelled polymer anti-rabbit kit (Dako Corporation, Carpinteria, CA, USA) as recommended by the manufacturer. Visualization was performed with 3,3′-diaminobenzidine and sections were counterstained with 0.5% methyl green (Dako Corporation).

Immunolabeling of the sections with pre-immune antibody and omission of primary antibody were used as negative controls for both immunofluorescence and immunoperoxidase labelings.

Results

Based on qualitative survey of control (Fig. 1A-D) and treated (Fig. 2A-H, Table 1) groups revealed a noticeable

increase in the number of ERMs in the PDL along the root surface and in the root furcations following orthodontic tooth movement and gingivectomy.

The ERM in the control samples showed no detectable immunolabeling for APIN, AMBN, AMTN and AMEL under the conditions used in this study (Fig. 1A–D, Table 1). However, APIN was immunodetected in the ERM on day 3 after periodontal challenge (data not shown) and the expression increased by day 5 (Fig. 2C,D). On neither day was there evidence of immunolabeling for AMBN (Fig. 2G), AMEL (Fig. 2H) and AMTN (Fig. 2E,F) in the ERM following disruption of periodontal integrity.

In both treated and untreated samples, immunolabeling for APIN, AMBN, AMTN and AMEL was consistently found in the HERS cells entrapped in cementum (Fig. 3A–D, Table 1).

Incubation with pre-immune antibody and omission of primary antibody did not show immunolabeling for APIN, AMBN, AMTN and AMEL in the ERM or in the HERS cells entrapped in the cementum of treated and untreated samples (data not shown).

Discussion

It has been suggested that the ERM may play an important functional role in preserving the normal periodontal cellular elements (9), inducing acellular cementum formation, maintaining the periodontal membrane space (21) and preventing ankylosis (2,22). The fact that ERM have been reported to express extracellular matrix proteins, growth factors (23) and cytokeratins (8,24) suggests that these cells may also contribute to periodontal regeneration (4). In addition, the capability of the ERM to produce bone/cementum-related proteins, such as alkaline phosphatase, osteopontin (8) and bone sialoprotein (4), has led to the concept that ERM may also be associated with cementoblast development (4,8). Some studies have suggested that enamel matrix proteins, such as AMEL and AMBN, may be involved in a number of processes such as induction of mantle dentin, deposition of acellular and cellular cementum and maintenance of cementum integrity (7,25,26). A recent article by Bosshardt (27) reviewed the implication of the enamel matrix proteins as biological mediators during wound healing, new periodontal tissue formation and bone remodeling. Although Peters *et al.* (24) affirmed that ERM retain the major characteristics of epithelial cells throughout their differentiation from the HERS, the expression of enamel matrix proteins in HERS and ERM is still controversial. While there are several *in vitro* and *in vivo* studies showing that these proteins are expressed in the HERS (1,25,26,28), few studies have demonstrated that enamel matrix proteins are also synthesized by the ERM (6–8). In this study, although APIN, AMTN, AMBN and AMEL were expressed in the HERS cells entrapped in cementum, no evidence of these proteins was found in the ERM in normal periodontal conditions. In fact, the expression of APIN in the ERM was detectable only after its activation by the disruption of periodontal integrity. However, the mechanism of activation of ERM is not fully understood. Among other causes, the disruption of extracellular matrix and the inflammatory response of the PDL by gingivectomy and tooth movement may contribute to the activation of the ERM. Previous studies have already demonstrated that orthodontic dental movement (29) and experimental root resorption (7) generate an inflammatory reaction in the connective tissue and induce the proliferation of the ERM. Consistent with these previous reports, we also observed that the number of ERMs was increased by both periodontal challenge procedures.

The fact that APIN is expressed in the ERM at early time-points following periodontal challenge is consistent with the evidence that this protein, unlike AMTN, is not only associated with the cell-tooth interface, but also with cells of the JE. In this context, the activation of APIN expression by ERM may reflect a change in the cellular status of the ERM. Although it has been demonstrated that AMBN (7) and AMEL (25) are expressed in epithelial cells that have similar structure to the ERM during periodontal regeneration, the absence of these enamel matrix proteins in the ERM in our study may reflect a temporal rela-

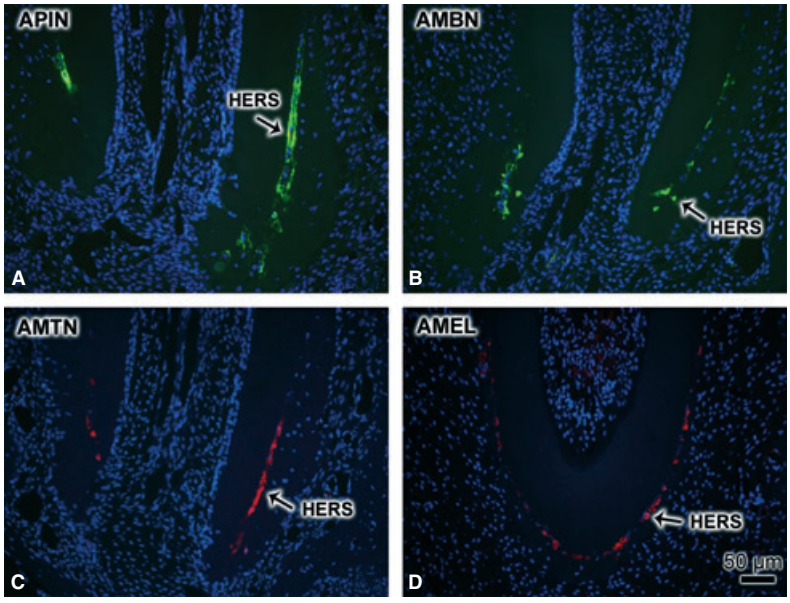

Fig. 3. In all cases, Hertwig's epithelial root sheath (HERS) cells entrapped in cementum (arrows) showed positive immunolabeling for (A) apin (APIN) and (B) ameloblastin (AMBN), illustrated here for samples following disruption of periodontal integrity at day 5, and (C) amelotin (AMTN) and (D) amelogenin (AMEL) in samples with normal periodontium.

tionship. Indeed, most studies were carried out at intervals longer than 14 days, while our longest time of observation was 5 days. Even though we detected AMBN and AMEL in the HERS cells entrapped in cementum, it cannot be excluded that the level of expression of these two proteins in the ERM is below the detectability level of immunohistochemistry under the conditions used in our study. Other methods, such as colloidal gold immunolabeling and *in situ* hybridization, could help to resolve this question.

In conclusion, the expression of APIN, but not other ameloblast products, by ERM following the disruption of periodontal integrity suggests that this protein may be involved in the initial events of periodontal regeneration and a possible association with cellular status. Modulation of APIN expression may open the door to controlled and targeted strategies for periodontal regeneration through the inherent regenerative capacity of the ERM.

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