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Epithelial Cell Rests of Malassez Modulate Cell Proliferation, Differentiation and Apoptosis via Gap Junctional Communication under Mechanical Stretching in vitro

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Abstract

Epithelial cell rests of Malassez (ERM) are involved in the maintenance and homeostasis of the periodontal ligament. The objective of this study was to investigate the effect of mechanical stretching on cell growth, cell death and differentiation in the ERM. Cultured porcine ERM were stretched for 24 hr in cycles of 18% elongation for 1 sec followed by 1 sec relaxation. The numbers of cells and TUNEL-positive cells were then counted. The expression of mRNAs encoding gap junction protein α1 (Gja1), ameloblastin, bone morphogenetic protein 2 (BMP2), bone morphogenetic protein 4 (BMP4) and noggin were evaluated using quantitative real-time PCR. The number of cells in the stretching group was approximately 1.3-fold higher than that in the non-stretching controls at 24 hr (p<0.01). Apoptotic cells ranged from 1.9–2.5% in the stretching group at 24 hr, but were only 0.6% in the control group (p<0.01). The expression of Gja1, ameloblastin and noggin mRNAs in the stretching group was decreased at 24 hr compared with in the non-stretching group (p<0.01), whereas the expression of BMP2 and BMP4 mRNAs in the stretching group was significantly higher than that in the control group (p<0.01). Incorporation of 18α-glycyrrhetinic acid (18GA, a gap junction inhibitor) promoted proliferation and apoptosis and confirmed both the increase of BMP2 and BMP4 and the decline of Gja1, ameloblastin and noggin in ERM. Thus, the ERM modulate cell proliferation and apoptosis, and inhibit differentiation by reducing expression of Gja1 under mechanical stretching.

Key words: Epithelial cells—Dental stress analysis—Periodontal ligament—Gja1—Ameloblastin—BMPs

This paper was a thesis submitted by Dr. K. Haku to the Graduate School of Tokyo Dental College.
Introduction

Epithelial cell rests of Malassez (ERM) are located in the periodontal ligament (PDL) tissue and are derived from the Hertwig’s epithelial root sheath (HERS) fragments during advancing root development. The ERM are usually found in the inner zone of the PDL near the root cementum, and can be seen, not as isolated groups of cells, but as a network, similar to a fishnet surrounding the root. The ERM had been believed to be quiescent epithelial remnants of the HERS throughout life in the PDL based on electron microscopic findings. The ERM have a number of functions, such as to prevent root resorption, induce cementum formation and maintain the homeostasis of the PDL. On the other hand, ERM can be stimulated to proliferate in response to injury in rats. When stimulated by inflammatory cytokines, ERM can proliferate and differentiate into the lining epithelium of periapical cysts. These earlier reports suggest that ERM become active under various stress conditions. Although ERM are continuously subjected to mechanical stress caused by occlusion and mastication and are thought to be active, few studies have examined the effect of mechanical stretching on ERM. Thus, there is still insufficient evidence to fully reveal the extent of the characteristics of ERM under mechanical stretching.

Gap junctions (GJs) are transmembrane proteins that allow for direct exchange of signaling molecules and metabolites between adjacent cells. Connexins comprise a family of GJ structural proteins, and the gap junction protein α1 (Gja1) gene encodes a 43-kDa gap junction protein termed connexin43. Gap junctions have multiple and diverse roles regulating various aspects of cell signaling, communication, differentiation, proliferation, polarity and development. Upstream regulation via GJs controls the bone morphogenetic protein 2 (BMP2) and bone morphogenetic protein 4 (BMP4) pathway involved in regulating morphogenesis. However, little is known about the role of GJs in ERM, although GJs are well developed in ERM at the ultrastructural level.

Ameloblastin is an abundant non-amelogenin enamel matrix protein that plays important roles in the differentiation of ameloblasts and the induction of cementoblasts, and it has synergistic functions with amelogenin. Ameloblastin may promote the differentiation of ameloblasts via Gja1. The ERM also express ameloblastin, however, the exact function of ameloblastin in the ERM remains unknown.

BMPs are multifunctional growth factors belonging to the transforming growth factor-β superfamily, and strong evidence exists that supports a role for both BMP2 and BMP4 as molecules critical to epithelial-mesenchymal signaling events required for enamel and dentin formation. In addition, BMP2 potently stimulates alveolar bone regeneration around teeth, as well as cementogenesis in periodontal wounds. Therefore, BMP signaling appears to be critical in regulating periodontal tissue formation in pre- and post-natal life. On the other hand, BMPs induce and regulate apoptosis during early tooth morphogenesis. The expression of BMPs is also detected in ERM, and apoptosis has been reported in ERM. However, little information is known about the role of BMPs in ERM.

Noggin is an antagonist of BMP and harmonious interactions between BMP4 and noggin regulate important functions in the PDL and HERS for proper periodontium and tooth root development.

In this study, we investigated cell growth, cell death and expression of Gja1, ameloblastin, BMP2, BMP4 and noggin mRNA in ERM under mechanical stretching to test the hypothesis that mechanical stress is associated with cell proliferation, differentiation and apoptosis via gap junctional intercellular communication (GJIC) in ERM.

Materials and Methods

1. Cell culture

Porcine ERM cells were provided by Prof. Yoshihiro Abiko (Department of Dental Sci-
ence, Institute of Personalized Medical Science University of Hokkaido, Japan). The method for obtaining ERM cells has been described previously by Liu et al.\textsuperscript{24}. ERM cells were cultured in 100-mm tissue culture dishes (Corning, Tokyo, Japan) with Dulbecco’s MEM (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Sigma, St. Louis, MO, USA) and 100 IU/ml penicillin-streptomycin (Sigma). The ERM cells were cultured by incubation in a humidified atmosphere of 95% air and 5% CO\textsubscript{2} at 37°C; ERM cells at the 4th passage were used in this study.

2. Mechanical stretching

The ERM cells were seeded (1.0 × 10\textsuperscript{5}/well) and cultured in 6-well flexible-bottomed tissue culture plates coated with type I collagen (BioFlex\textsuperscript{®} PLATES Collagen I; Flexcell International, McKeesport, PA, USA). After 2 days of culture, the ERM cells were stretched for 24 hr with cycles of 18% elongation for 1 sec followed by 1 sec relaxation, using a FX-4000™ Flexercell Tension Plus™ System (Flexcell International) according to the method of Koshihara et al.\textsuperscript{22} with modification. The flexible cell-covered elastomer membranes were stretched by applying an oscillating vacuum to the underside of the membranes and the duration, amplitude and frequency of the stretch applied were controlled by a computer. The vacuum manifold that held the plates was maintained at 37°C in a humidified incubator with 95% air and 5% CO\textsubscript{2}. The cells were collected 24 hr after the initiation of the stretching (stretching group); cells without stretching were used as controls (control group).

3. Cell growth

The ERM cells were seeded at 1 × 10\textsuperscript{5} cells/well into culture plates. The ERM cells were detached using 0.05% trypsin-EDTA at 24 hr with or without mechanical stretching, and were counted using a Coulter Counter (Beckman Coulter, Fullerton, CA, USA). Results and growth curves were then plotted using 6 wells per group; means and standard deviations (SD) were calculated and reported.

4. Detection of apoptosis

In order to detect apoptosis, TUNEL staining was carried out using the ApopTag\textsuperscript{®} Plus peroxidase in situ apoptosis detection kit (Chemicon International, Temecula, CA, USA) according to the manufacturer’s protocol. After stretching for 24 hr, the ERM cells were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 1 hr, and then were washed for 5 min in PBS. The cells were post-fixed and permeabilized in ethanol:acetic acid (2:1, −20°C) for 5 min and then washed in PBS. They were rinsed twice with PBS for 5 min, then immersed in equilibration buffer and incubated with Terminal deoxynucleotidyl Transferase (TdT) in a humidified chamber at 37°C for 1 hr. Reactions were terminated by incubation in PBS. The ERM cells were then incubated with horseradish peroxidase (HRP) conjugated anti-digoxigenin antibody for 30 min and finally incubated with 3,3’-diaminobenzidine for 5 min, after which they were counterstained with methyl green and observed using a light microscope. We observed 5 randomly selected fields and counted positive cells per 5000 cells in each well. The numbers of positive cells from six wells were counted and the positive ratio and SD expressed.

5. Quantitative RT-PCR

Total RNA was obtained from 6 wells in each group and reverse-transcribed into complementary DNA (cDNA) with TaqMan\textsuperscript{®} Reverse Transcription Reagent (Applied Biosystems, Foster City, CA, USA), and quantitative RT-PCR (QRT-PCR) with TaqMan MGB probes (Applied Biosystems) was carried out. The TaqMan MGB probes and primer sets for the genes (Gja1, ameloblastin, BMP2, BMP4, and noggin, with 18S gene as an internal control), were purchased from Applied Biosystems. Quantification of mRNA expression was analyzed with the ABI 7500 system software (Applied Biosystems) and compared using the ΔΔCt method. The reaction conditions consisted of a primary denaturation at 95°C for 20 sec, then cycling for 40 cycles at 95°C for 3 sec and at 62°C for 30 sec. PCR data are
reported compared with the corresponding control. QRT-PCR analyses were reproduced four times.

6. Incorporation of 18 α-glycyrrhetinic acid
In order to determine whether Gja1 plays a key role in stretching, we added 50 μM 18 α-glycyrrhetinic acid (18GA), a gap junction inhibitor, to the culture medium prior to 3 hr stretching. In the control group, dimethyl sulfoxide (DMSO) was added instead of 18GA.

7. Statistical analysis
Differences between the stretching group and the non-stretching group were analyzed statistically using the non-parametric Mann-Whitney U-test. Values of p<0.01 were considered significant.

Results

1. Cell growth
To determine whether mechanical stretching is involved in cell growth, ERM cells were cultured with or without mechanical stretching. Mechanical stretching elicited an increase in growth. The numbers of stretching and non-stretching ERM cells at 24 hr were (9.8 ± 0.7) × 10⁵ and (7.5 ± 0.6) × 10⁵, respectively. The growth rate of the stretching ERM cells was higher than that of the non-stretching ERM cells at 24 hr (Fig. 1). The number of cells in the stretching group was about 1.3-fold that in the non-stretching group at 24 hr, which was a significant difference (p<0.01).

2. Apoptosis
Apoptotic cells were detected using TUNEL staining. TUNEL-positive ERM cells were detected at 24 hr in the stretching group, while there were fewer in control group. Apoptotic cells were significantly different between stretching and non-stretching groups at 24 hr (p<0.01, Mann-Whitney U-test, n = 6).

3. QRT-PCR
1) Gja1
QRT-PCR confirmed a 2.0-fold reduction in Gja1 mRNA expression between the control and the stretching groups at 24 hr (Fig. 3A), which was statistically significant (p<0.01).

2) Ameloblastin
QRT-PCR demonstrated a 2.0-fold reduction in the expression of ameloblastin mRNA at 24 hr between the non-stretching and the stretching groups (Fig. 3B), which was statistically significant (p<0.01).
3) **BMP2** and **BMP4**

QRT-PCR indicated a 1.6-fold increase in the expression of BMP2 mRNA at 24 hr in the stretching group compared to the non-stretching group, a statistically significant difference (p<0.01, Fig. 3C). QRT-PCR showed a 1.4-fold increase in BMP4 mRNA expression in the stretching group compared to the controls, which was also statistically significant (p<0.01, Fig. 3D).

4) **Noggin**

QRT-PCR demonstrated a 2.4-fold reduced expression of noggin mRNA in the stretching group compared to the control group (Fig. 3E), which was statistically significant (p<0.01).

5) **Effect of 18GA**

The numbers of stretching ERM cells treated with or without 18GA at 24 hr were (12.2 ± 0.3) × 10⁵ and (9.2 ± 0.4) × 10⁵, respectively, which was a significant difference (p<0.01, Fig. 4A).

TUNEL-positive ERM cells were detected at 24 hr in the stretching group, and ranged from 1.8 ± 0.4%. On the other hand, the number of TUNEL-positive ERM cells in the 18GA treated group increased, with the ratio reaching 2.8 ± 0.5% (Fig. 4B).

QRT-PCR demonstrated a 0.5- to 0.67-fold decrease in expression of Gja1, ameloblastin and noggin mRNAs at 24 hr between the 18GA-treated group and the control group (Figs. 4C, 4D, 4G), all being statistically significant (p<0.01). On the other hand, QRT-PCR indicated a 1.3-fold increase in the expression of BMP2 mRNA at 24 hr in the 18GA-treated
Group, a statistically significant level (p < 0.01, Fig. 4E). QRT-PCR showed a 1.8-fold increase in BMP4 mRNA expression following treatment with 18GA (p < 0.01, Fig. 4F).

Discussion

1. Gja1 expression and cell proliferation

The expression of Gja1 under mechanical

Gap junction inhibitor 18GA was incorporated into culture medium prior to 3 hr stretching.

(A) Cell growth: Number of stretching ERM cells treated with 18GA was higher than without 18GA, which was statistically significant (p < 0.01, Mann-Whitney’s U-test, n = 6).

(B) Apoptotic cells: Number of TUNEL-positive cells in 18GA-treated group increased compared to in DMSO treated (control) group (p < 0.01, Mann-Whitney’s U-test, n = 6).

(C–G) mRNA expression: QRT-PCR demonstrated a 1/2 to 1/3-fold decrease in expression of Gja1 (C), ameloblastin (D) and noggin (G) mRNAs between 18GA-treated group and control group, which was statistically significant (p < 0.01, n = 4). On the other hand, QRT-PCR showed a 1.3-fold increase in expression of BMP2 mRNA (E) compared with in 18GA-treated group, a statistically significant difference (p < 0.01, n = 4). QRT-PCR for BMP4 mRNA showed a 1.84-fold increase in expression as well as BMP2 (F).
stretching in osteogenic cells\textsuperscript{4,12,23} and in PDL cells\textsuperscript{33} has been investigated. However, whether mechanical stretching induces the activation of \emph{Gja1} in ERM remains unknown. In this study, we investigated \emph{Gja1} expression both in stretching and control groups. To our knowledge, this is the first study to investigate \emph{Gja1} expression in ERM cells in response to mechanical stress. An earlier study demonstrated an increased number of ERM after orthodontic dental movement \textit{in vivo}\textsuperscript{35}. Another study on ERM also showed proliferation and morphological changes against stimulation \textit{in vitro}\textsuperscript{41}, which suggested that mechanical stretching induces cell proliferation. In this study, the number of ERM cells increased after mechanical stretching, suggesting that this might be associated with a reduction of \emph{Gja1}. It has been shown that GJIC plays a major role in the maintenance of normal growth, and loss of GJs and reduced expression of connexin 43 have been frequently reported in various malignant tumors, where they lead to rapid cell proliferation\textsuperscript{37,42}. An earlier report also showed that transfection of the \emph{Gja1} gene resulted in slower cell proliferation \textit{in vitro}\textsuperscript{29}. Zhang \textit{et al.}\textsuperscript{43} demonstrated that connexin 43 binds S-phase kinase-associated protein (Skp2) to inhibit the degradation of the cell cycle regulator \emph{p27}. Therefore, \emph{Gja1} would be a negative regulator of the cell cycle, inhibiting excessive proliferation. The results of our study suggest that the reduction of \emph{Gja1} induced by mechanical stretching would lead to higher proliferation in the ERM.

\section*{2. Differentiation}

\textit{Ameloblastin} plays an important role in amelogenesis\textsuperscript{27} and in enamel formation\textsuperscript{7}. Furthermore, it has been reported that \textit{ameloblastin} in the ERM is involved in the differentiation and induction of cementoblasts\textsuperscript{13}. Shinmura \textit{et al.}\textsuperscript{30} reported that ERM cells could differentiate into ameloblast-like cells. However, the differentiation of ERM cells under mechanical stretching had not been studied. Therefore, we investigated the expression of \textit{ameloblastin} under mechanical stretching, and found that the expression of \textit{ameloblastin} was reduced under mechanical stretching. Our data suggest that mechanical stretching would reduce \textit{ameloblastin} expression and thus inhibit the differentiation of ERM cells into ameloblastic cells. Furthermore, in the present study, the reduced expression of \emph{Gja1} and \textit{ameloblastin} was seen synchronously following mechanical stretching. Moreover, the addition of 18GA reduced not only \emph{Gja1} expression but also \textit{ameloblastin} expression in ERM cells. Toth \textit{et al.}\textsuperscript{36} demonstrated that a reduction of \emph{Gja1} results in the dysregulation of ameloblast differentiation, and enamel hypoplasia. Our results suggest that the reduction of \textit{ameloblastin} might be directly associated with the inhibition of differentiation \textit{via} \emph{Gja1} in ERM cells under mechanical stretching.

\section*{3. Apoptosis}

\textit{BMPs} are multifunctional cytokines that are expressed in various tissues, and they play important roles in regulating morphogenesis and development\textsuperscript{20,21}. \textit{BMP2} and \textit{BMP4} are the most thoroughly investigated members of the TGF-\textit{\beta} superfamily for tooth and periodontal development and regeneration\textsuperscript{20,21}. Although \textit{BMPs} are expressed in the ERM\textsuperscript{39}, the expres-
sion of BMPs following mechanical stretching was unknown. On the other hand, He et al. demonstrated that over-expression of BMPs by inhibiting noggin induces apoptosis during palatogenesis. Furthermore, apoptosis of ERM cells has been reported in vivo. In this study, the number of TUNEL-positive cells increased after mechanical stretching, and therefore, we postulate that the increase of BMPs expression is involved in the apoptosis of ERM cells.

The reduced expression of noggin was detected under mechanical stretching in this study. Treatment with 18GA confirmed both the increase of BMP2 and BMP4 and the decline of noggin in ERM cells. These results suggest that an increase of BMPs mRNAs is involved in the reduction of noggin via Gja1 in ERM cells under mechanical stretching. In addition, He et al. reported that BMP is associated with noggin expression during palatogenesis. Furthermore, Wong et al. reported that the closure of GJs by the decoupling agent 18GA increased cell apoptosis. In this study, the number of TUNEL-positive cells in 18GA-treated group increased. Thus, the results of this study, taken together with that earlier study, suggest that GJs may correlate with apoptosis under mechanical stretching.

A hypothetical Gja1 cascade under mechanical stretching in ERM cells is shown in Fig. 5. The reduction of Gja1 expression induces proliferation of ERM cells. On the other hand, Gja1 would play an important role associated with cellular differentiation by down-regulating ameloblastin expression. In addition, reduced Gja1 expression down-regulates noggin expression, leading to cellular apoptosis by up-regulating BMPs. Furthermore, decrease of Gja1 would increase apoptosis. Thus, ERM cells seem to have multiple and diverse roles regulating various cell functions such as signaling, proliferation, differentiation and apoptosis.

Conclusion

In conclusion, ERM cells modulate cell proliferation and apoptosis, and inhibit differentiation due to reduced Gja1 expression under mechanical stretching. GJIC are involved in regulating the maintenance of number and differentiation of ERM cells.

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