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ORIGINAL ARTICLE

M Takano
M Yamaguchi
R Nakajima
S Fujita
T Kojima
K Kasai

Effects of relaxin on collagen type I released by stretched human periodontal ligament cells

Authors' affiliation:
M. Takano, M. Yamaguchi, R. Nakajima, S. Fujita, T. Kojima, K. Kasai, Department of Orthodontics, Nihon University School of Dentistry at Matsudo, Chiba, Japan

Correspondence to:
Masaru Yamaguchi
Department of Orthodontics
Nihon University School of Dentistry at Matsudo
2-870-1 Sakaecho-Nishi
Matsudo City
271-8587 Chiba
Japan
E-mail: yamaguchi.masaru@nihon-u.ac.jp

Structured Abstract

Authors – Takano M, Yamaguchi M, Nakajima R, Fujita S, Kojima T, Kasai K

Objectives – Relapse of teeth that have moved during orthodontic treatment is a major clinical issue with respect to the goals of successful treatment. Such relapse is a physiologic response of the supporting tissues to application of force, and is mainly attributed to occlusal instability and increased mechanical tension exerted by the periodontal ligament (PDL). Relaxin, a member of the insulin/relaxin family of structurally related hormones, has an influence on many physiologic processes, such as collagen turnover, angiogenesis, and antifibrosis. Therefore, relaxin may also affect orthodontic tooth movement through alterations of the PDL, though little is known regarding the relationship between relaxin and stretched human PDL (hPDL) cells. In the present study, we investigated the effects of relaxin on the expression of collagen type I (Col-I) and matrix metalloproteinase 1 (MMP-1) in stretched hPDL cells *in vitro*.

Materials and Methods – The release and gene expression of Col-I, as well as those of MMP-1 in stretched hPDL cells treated with relaxin were investigated using enzyme-linked immunosorbent assay and real-time PCR methods.

Results – Relaxin decreased the release and gene expression of Col-I, and increased those of MMP-1 by stretched hPDL cells in a magnitude-dependent manner.

Conclusion – Our results indicate that relaxin modulates collagen metabolism in stretched hPDL cells via the release and expression of Col-I and MMP-1. This hormone may be useful to prevent orthodontic relapse following orthodontic treatment.

Key words: collagen type 1; matrix metalloproteinase 1; periodontal ligament cell; relaxin; rotational force

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Introduction

Relapse of teeth that have moved during orthodontic treatment is a major clinical issue with respect to the goals of successful treatment (1, 2). Such relapse is a physiologic response of the supporting tissues to application of force, and is mainly attributed to occlusal instability and increased mechanical tension exerted by the periodontal ligament (PDL).

In particular, the trans-septal fibers, the main constituents of the PDL, which travel from the cementum to adjacent cementum and from the cementum to the gingival papillae (3) and consist of collagen and oxytalan fibers (4–6), are held responsible. These fibers have been shown to be important for tooth position stabilization and provide the source of pressure that leads to relapse of moved teeth (5, 7, 8).

The PDL is composed of an abundance of types I and III collagen (Col-I and III). The PDL homeostasis causes both intensive and subtle transcriptional and translational regulation of collagen and matrix metalloproteinase (MMP) genes, which are a family of zinc-dependent enzymes that have the capacity to degrade nearly all components of the extracellular matrix (9). Precise regulation of MMP gene expression in relation to collagen gene expression is critical for tissue repair and homeostasis, because otherwise it might lead to pathologic events. For examples, an overexpression of Col-I leads to keloids, and an overexpression of MMP-1 leads to ulcerative skin lesions (10, 11).

Relaxin is a member of the insulin/relaxin family of structurally related hormones and has been shown to bind to receptors that are part of the leucine rich repeat G-protein receptor family (LGR7 and LGR8) (12). This hormone is produced in a variety of mammals during pregnancy, and has been shown to promote cervical softening and elongation of the interpubic ligaments in mice and cattle (13). Furthermore, relaxin is related to many other physiologic processes, such as collagen turnover, angiogenesis, and antifibrosis in both males and females.

Recently, we reported that relaxin decreased the release and expression of Col-I, and increased the expression of MMP-1 in human PDL (hPDL) cells (14). In dog experimental tooth movement, relaxin stimulated the velocity of tooth movement *in vivo*. Therefore, it is considered that this hormone may have an influence on orthodontic tooth movement through alterations of the PDL (15). In contrast, Madan et al. (16) reported that relaxin has no effect on tooth movement. Little is known regarding prevention of the effects of relaxin on relapse in patients who have received orthodontic treatment. In the present study, we investigated the effects of relaxin on the release and expression of Col-I and MMP-1 by stretched hPDL cells *in vitro*.

Materials and methods

Cell culture

Human PDL cells were prepared according to a modification of the method of Somerman et al., as described previously (17, 18). Briefly, PDL tissues were taken from the roots of premolars extracted from six healthy young volunteers (three males, three females; 14- to 16-year old) during the course of orthodontic treatment, after obtaining informed consent from the donors. The study protocol was reviewed by the Ethics Committee of Nihon University School of Dentistry at Matsudo (#04-021). The PDL tissues were placed in 35-mm tissue culture dishes and covered with a sterilized glass cover slip. The medium used was α-MEM (Gibco, Grand Island, NY, USA), which was supplemented with 100 μg/ml of penicillin-G (Sigma Chemical Co., St Louis, MO, USA), 50 μg/ml of gentamicin sulphate (Sigma), 0.3 μg/ml of amphotericin B (Flow Laboratories, McLean, VA, USA), and 10% fetal calf serum (FCS; Cell Culture Laboratories, Cleveland, OH, USA). The cultures were kept at 37°C in a humidified incubator (Forma CO_2 Incubator MIP-3326; Sanyo Electric Medica System Co., Tokyo, Japan) in the presence of 95% air and 5% CO_2. When cells growing from each explant had reached confluence, they were detached with 0.05% trypsin (Gibco) in phosphate-buffered saline for 10 min and sub-cultured in culture flasks. Those cells still attached to the bottom of the flask were discarded to avoid contamination by epithelial cells. The hPDL cells (7×10^6 cells/well) were transferred to the 10 cm^2 STREX-chamber (STREX Co. Osaka, Japan). For the experiments, hPDL cells were used at passages 6–9.

Application of a tension–force

In order to reproduce the tension required to rotate an individual tooth during orthodontic tooth movement, we used the *in vitro* method reported by Long et al. (19). Briefly, hPDL cells (7×10^6 cells/well) were transferred to the 10 cm^2 STREX-chamber named ST-CH-10 (STREX Co.) and cultured for 3 days until confluent. The medium was then replaced with the medium described above, except that it contained 1% instead of 10% FCS. The hPDL cells were placed under continuous tension with a STREX system (STREX Co.) as a model of tooth rotation during orthodontic

movement. STREX-chambers were designed for use in the tension unit driven by manual device (STB-10; STREX Co.) that allowed variation in magnitude (5%, 10%). We applied the tension (5% or 10%) for the hPDL cells using the cell mechanical stretch device (STB-10-10; STREX Co.). After that, we hold the chambers with continuous tension by chamber stands (STB-CH-10ST; STREX Co.) for 12 h. Static tension–force is thought to mimic that found *in vivo* during orthodontic treatment. Previous studies have shown that tension stress can be effectively applied by the method utilized in the present study (19). We used the cells treated without tension, as a control.

Treatment of hPDL cells with relaxin

To examine the effects of relaxin (PHOENIX Co., Belmont, MA, USA) on the expression of Col-I and MMP-1, approximately 7×10^6 hPDL cells were transferred to the 10 cm^2 STREX-chamber. Confluent-stage cells were subjected to 5% or 10% tension–force for 12 h in culture medium containing 1% FCS, after which they were incubated for 48 h in the presence of relaxin (100 ng/ml) added to the culture medium. For this experiment, we used the relaxin concentration described previously (14). We used the cells treated without relaxin and tension, as a control.

Enzyme-linked immunosorbent assay

Collagen type I and MMP-1 released into culture supernatants was measured using a Human Collagen Type I ELISA kit (Applied Cell Biotechnologies, Kanagawa, Japan) and MMP-1 ELISA Kit (R&D system, Minneapolis, MN, USA), according to the manufacturer's protocol.

Real-time RT-PCR

We extracted RNA from hPDL cells treated with tension–force and relaxin using an RNeasy Mini-kit (Qiagen Co., Tokyo, Japan), according to the manufacturer's protocol. RNA was amplified with an RT-PCR kit and 40 μl of purified total RNA was obtained, then total RNA was converted to cDNA using a Prime Script RT Reagent Kit (Takara Co., Shiga, Japan). Real-time PCR amplification was performed using SYBR Premix Ex Taq (Takara Co.) in a thermal cycler (TP-800 Thermal Cycler Dice; Takara Co.). After a hot start, the samples were denatured at 95°C for 5 s, then the primer was annealed at 60°C for 30 s 40 cycles. PCR primers for Col-I, MMP-1, and β-actin were purchased from Takara Co., and designed with reference to those respective cDNA sequences. The primers were designed as follows: Col-I, 5′-CCCGGGTTTCAGAGACAACTTC-3′ and 5′-TCCACATGCTTTATTCCAGCAATC-3′; MMP-1, 5′-ACAACTGCCAAATGGGCTTGA-3′ and 5′-CTGTCCCTGAACAGCCCAGTACTTA-3′; and β-actin, 5′-TGGCACCCAGCACAATGAA-3′ and 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′.

The levels of the real-time PCR products corresponding to β-actin were the same in the experimental groups. Therefore, it was considered acceptable to assume that the amount of real-time PCR products reflected the level of each mRNA. We used the real-time PCR products treated without relaxin and tension, as a control.

Statistical analysis

The values in each figure represent the mean ± standard deviation for each group. Intergroup comparisons of average values were evaluated by one-way ANOVA, followed by a Tukey test, with values of $p < 0.05$ considered to indicate a significant difference.

Results

Release and expression of Col-I in stretched hPDL cells treated with relaxin

When 5% and 10% tension–force were applied to hPDL cells for 12 h, the release (Fig. 1a) and gene expression (Fig. 1b) of Col-I were increased as compared with the control cells ($p < 0.05$, Tukey test), both of which occurred in a magnitude-dependent manner ($p < 0.05$, by one-way ANOVA). Relaxin (100 ng/ml, 48 h) decreased the release (Fig. 1a) and gene expression (Fig. 1b) of Col-I from both 5% and 10% stretched hPDL cells as compared with the corresponding control cells ($p < 0.05$, Tukey test), and this occurred in a magnitude-dependent manner ($p < 0.05$, by one-way ANOVA). However, as compared with those exposed to tension–force alone, the amount of Col-I released was about 20% less when hPDL cells were exposed to relaxin (Fig. 1a).

Time course effect of relaxin on release of Col-I in stretched hPDL cells

When stretched (10%) hPDL cells were treated with relaxin (100 ng/ml) for up to 48 h, Col-I release was decreased in a time-dependent manner as compared with the control cells ($p < 0.05$, by one-way ANOVA), with values after 48 h significantly different ($p < 0.05$, Tukey test) (Fig. 2).

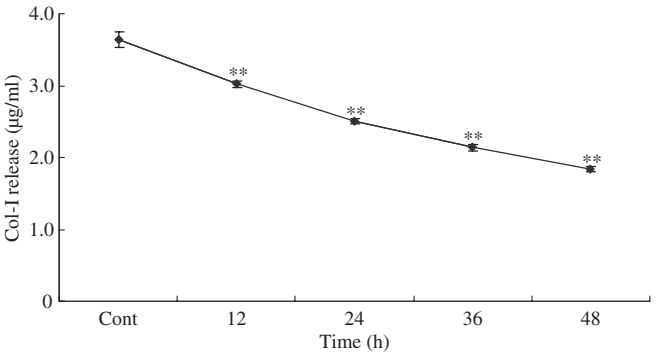

Fig. 2. Time course effect of relaxin on release of Col-I in stretched hPDL cells. When stretched (10%) hPDL cells were treated with relaxin, Col-I release was decreased in a time-dependent manner for 48 h ($p < 0.05$, by one-way ANOVA). The value for the stretched cells at 48 h was significantly decreased, as compared with that for the control cells ($p < 0.05$, Tukey test). **$p < 0.01$. The data shown are representative of four independent experiments using samples from six different patients.

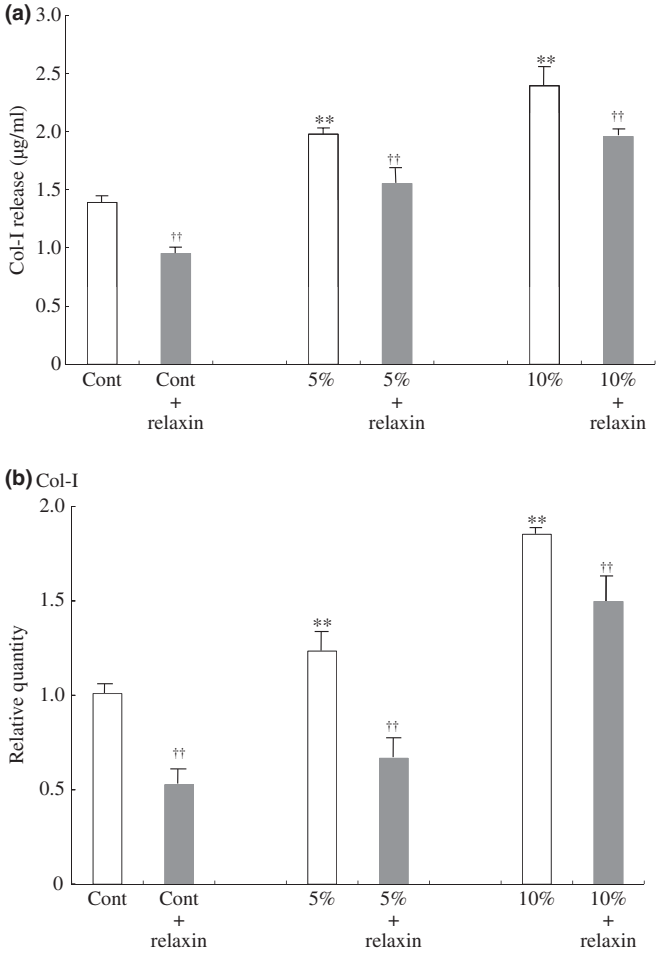

Fig. 1. Release and gene expression of Col-I in stretched hPDL cells treated with relaxin. The release (a) and gene expression (b) of Col-I were increased following application of 5% and 10% tension–force for 12 h as compared with the control cells ($p < 0.05$, Tukey test; **$p < 0.01$, which occurred in a magnitude-dependent manner ($p < 0.05$, by one-way ANOVA). Further, relaxin (100 ng/ml, 48 h) decreased the release (a) and gene expression (b) of Col-I from the 5% and 10% stretched hPDL cells as compared with the corresponding control cells ($p < 0.05$, Tukey test; ††$p < 0.01$, which also occurred in a magnitude-dependent manner ($p < 0.05$, by one-way ANOVA). The data shown are representative of four independent experiments using samples from six different patients.

Release and expression of MMP-1 in stretched hPDL cells treated with relaxin

When hPDL cells were subjected to 5% and 10% tension–force for 12 h, the release (Fig. 3a) and gene expression (Fig. 3b) of MMP-1 was increased as compared with the control cells ($p < 0.05$, Tukey test), which occurred in a magnitude-dependent manner ($p < 0.05$, by one-way ANOVA). Relaxin (100 ng/ml, 48 h) increased the release (Fig. 3a) and gene expression (Fig. 3b) of MMP-1 from both 5% and 10% stretched hPDL cells over 48 h as compared with the corresponding control cells ($p < 0.05$, Tukey test), and the increase occurred in a magnitude-dependent manner ($p < 0.05$, by one-way ANOVA) (Fig. 3).

Time course effect of relaxin on release of MMP-1 in stretched hPDL cells

When stretched (10%) hPDL cells were treated with relaxin (100 ng/ml) for up to 48 h, MMP-1 release was increased in a time-dependent manner as compared with the control cells ($p < 0.05$, by one-way ANOVA), with values after 48 h significantly different ($p < 0.05$, Tukey test) (Fig. 4).

Discussion

In the present study, hPDL cells were subjected to continuous tension–force (elongation by 5% or 10%) as

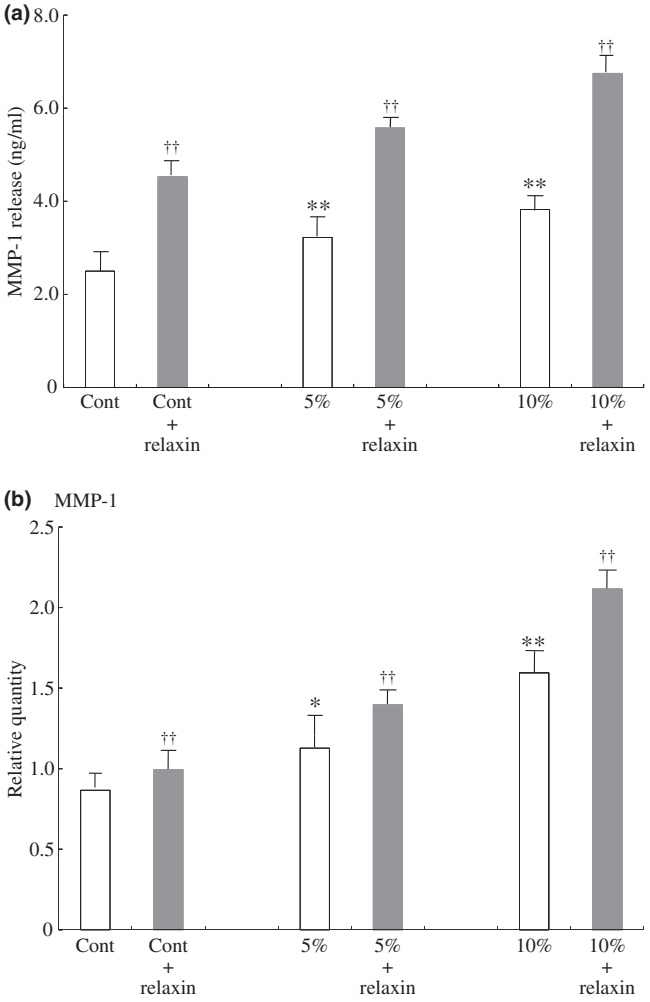

Fig. 3. Release and gene expression of MMP-1 in stretched hPDL cells treated with relaxin. The release (a) and gene expression (b) of MMP-1 was increased following application of 5% and 10% tension–force for 12 h as compared with the control cells ($p < 0.05$, Tukey test, $^{*}p < 0.05$; $^{**}p < 0.01$), which occurred in a magnitude-dependent manner ($p < 0.05$, by one-way ANOVA). Further, relaxin (100 ng/ml, 48 h) increased the release (a) and gene expression (b) of MMP-1 in the 5% and 10% stretched hPDL cells as compared with the corresponding control cells ($p < 0.05$, Tukey test, $^{\dagger\dagger}p < 0.01$), which also occurred in a magnitude-dependent manner ($p < 0.05$, by one-way ANOVA). The data shown are representative of four independent experiments using samples from six different patients.

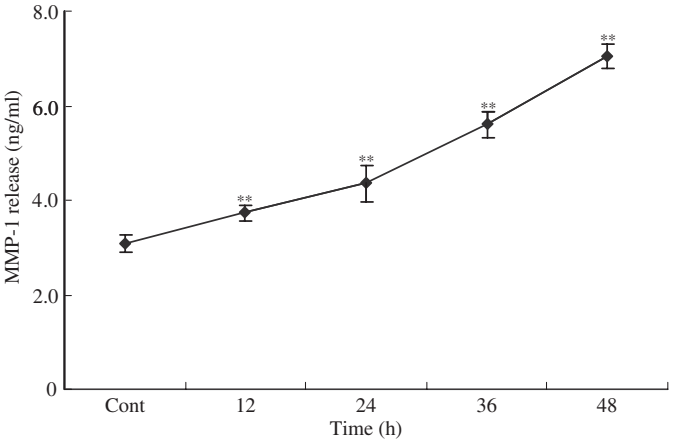

Fig. 4. Time course effect of relaxin on release of MMP-1 in stretched hPDL cells. When stretched (10%) hPDL cells were treated with relaxin, MMP-1 release was increased in a time-dependent manner for 48 h ($p < 0.05$, by one-way ANOVA). The value for the stretched cells at 48 h was significantly increased, as compared with that for the control cells ($p < 0.05$, Tukey test). $^{**}p < 0.01$. The data shown are representative of four independent experiments using samples from six different patients.

an *in vitro* model of tooth rotation during orthodontic movement. Although the etiology of orthodontic relapse is not well understood, it is likely to be multifactorial, with one of those factors considered to be the memory of PDL fibers, which have been shown to play a role in orthodontic relapse (5, 7, 8). In order to reproduce the tension required to rotate a tooth during orthodontic tooth movement, hPDL cells were continuously stretched by 5% or 10% for 12 h. Previous studies reported that only low-magnitude tensile strain elicited anti-inflammatory cytokine, such as interleukin (IL)-10, whereas higher magnitudes of strain (15–18%) induced inflammatory cytokine, such as IL-1β synthesis in PDL cells (20–22). Therefore, we used 5% and 10% stretching to reproduce the tension–force condition that causes rotation during orthodontic tooth movement.

Elongation of the cells by 5% and 10% increased the release and gene expression of Col-I, as well as those of MMP-1 (Figs 1 and 3). As for the relationship between mechanical stress and the synthesis of Col-I/MMPs, Howard et al. (23) reported that tension–force (5% biaxial strain) increased Col-I synthesis in hPDL cells, while Redlich et al. (24) found that centrifugal force increased the mRNA level of MMP-1 from hPDL cells. In addition, in their previously mentioned report, Howard et al. (23) concluded that collagen synthesis from hPDL cells in response to tension–force may be specific to the magnitude of orthodontic force. Therefore, tension–force may stimulate collagen turnover in hPDL cells.

Unemori et al. (25) showed that relaxin (1–100 ng/ml) inhibited the overexpression of interstitial Col-I and III in human lung fibroblasts mediated by transforming growth factor-beta by up to 45% in a dose-dependent manner. However, Henneman et al. reported the concentration of 250 ng/ml relaxin increased production of MMP-2 significantly in hPDL cells, with no significant effect reported seen at

100 ng/ml. Recently, we examined that effect of concentrations (1–100 ng/ml) of relaxin on release of Col-I and MMP-1 in hPDL cells. When hPDL cells were treated with relaxin (1–100 ng/ml), the release of Col-I was decreased in a dose-dependent manner ($p < 0.05$, by one-way ANOVA). Further, the release of MMP-1 was increased in a dose-dependent manner ($p < 0.05$, by one-way ANOVA) (14). Therefore, we used concentration of 100 ng/ml relaxin in this study.

Our results also demonstrated that relaxin (100 ng/ml, 48 h) decreased the release and gene expression of Col-I from stretched hPDL cells, while the amount of Col-I released from cells exposed to relaxin was decreased by about 20% in comparison subjected to tension–force alone (5% and 10%) (Fig. 1). In addition, the release of Col-I from hPDL cells stretched by 10% was decreased by treatment with relaxin in a time-dependent manner over a period of 48 h (Fig. 2). In contrast, the release and gene expression of MMP-1 was increased in stretched hPDL cells treated with relaxin after 48 h (Fig. 3). The release of MMP-1 from hPDL cells stretched by 10% was increased by treatment with relaxin in a time-dependent manner over a period of 48 h (Fig. 4). Previous studies have reported that relaxin downregulated collagen synthesis in human dermal fibroblasts and increased the gene expression of MMP-1 in human lung fibroblasts in a dose-dependent manner. Also, Unemori et al. (25, 26) and Masterson et al. (27) found that the antifibrotic effects of relaxin were related to downregulation of fibroblast activity and increases in collagenase synthesis. Relaxin was shown to have a role in connective tissue regulation by enhancing collagen turnover (28), and Nicozisis et al. (29) reported that the hormone disorganized and loosened the arrangement of the PDL from the tooth to the bone surface, and also dissolved Sharpey fiber insertions of the PDL *in vitro*. Furthermore, our recent study found that relaxin decreased the release and gene expression of Col-I, and increased the expression of MMP-1 in a dose-dependent manner (14). Finally, Stewart et al. (15) suggested that enhanced PDL fiber remodeling following relaxin treatment might reduce the rate or amount of relapse associated with orthodontic treatment by chemically mimicking the effects of a gingival fiberotomy. On the basis of the findings of the present study, it is possible that relaxin, taking the place of fiberotomy, prevents the relapse after orthodontic rotation movement. Further studies are necessary to confirm the inhibitory effects of relaxin on relapse after orthodontic rotation movement *in vivo*. In future, relaxin may be expected as an inhibitory medicine of relapse after orthodontic rotation movement.

PDL is constituted of Col-I, as well as other types of collagens such as Col-III, V, VI, XII, and XIV (30–32). In addition, Karimbux and Bumann (33, 34) reported that types I, III, V, VI, and XII were induced during experimental tooth movement. Therefore, relaxin may have an effect on the expression of these collagens in response to mechanical stress.

Tissue inhibitors of metalloproteinases (TIMPs) are important regulators of MMP activity. A previous study found that relaxin enhanced the expression of TIMP-1 and TIMP-2, and inhibited MMP-2 activity in pig uterine cervix specimens (35), which suggests its role in regulating the activities of TIMPs during growth and remodeling of reproductive connective tissue. Recently, Henneman et al. (36) reported that relaxin stimulated the expression of MMP-2 and α-smooth muscle actin by hPDL cells, and Ho et al. (37) found that relaxin induced MMP-9 expression in THP-1 cells, a human monocyte/macrophage cell line. Therefore, relaxin may have an effect on the expression of MMP-2 and 9, as well as that of TIMPs in hPDL cells, though additional studies are necessary to confirm the effects of relaxin on regulation of other types collagens and the MMP/TIMP complex in these cells.

In conclusion, our results indicate that relaxin decreases the release and gene expression of Col-I and increases that of MMP-1 in stretched hPDL cells. Thus, this hormone stimulates the collagen metabolism of PDL and may be useful to prevent orthodontic relapse following orthodontic treatment.

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