Tensile strength suppresses the osteogenesis of periodontal ligament cells in inflammatory microenvironments

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Abstract. The present study aimed to investigate the role of orthodontic force in osteogenesis differentiation, matrix deposition and mineralization in periodontal ligament cells (PDLCs) cells in inflammatory microenvironments. The mesenchymal origin of PDLCs was confirmed by vimentin and cytokeratin staining. PDLCs were exposed to inflammatory cytokines (5 ng/ml IL-1β and 10 ng/ml TNF-α) and/or tensile strength (0.5 Hz, 12% elongation) for 12, 24 or 48 h. Cell proliferation and tensile strength-induced cytokine expression were assessed by MTT assay and ELISA, respectively. Runx-related transcription factor 2 (RUNX2) and type I collagen (COL-I) expression were analysed by reverse transcription-quantitative polymerase chain reaction and western blot analysis. Additionally, alkaline phosphatase activity was measured, and the mineralization profile was evaluated by alizarin red S staining. PDLCs exposed to tensile strength in inflammatory microenvironments exhibited reduced proliferation and mineralization potential. Treatment with the inflammatory cytokines IL-1β and TNF-α increased RUNX2 expression levels; however, decreased COL-1 expression levels, indicating that bone formation and matrix deposition involve different mechanisms in PDL tissues. Notably, RUNX2 and COL-1 expression levels were decreased in PDLCs exposed to a combination of an inflammatory environment and loading strength. The decreased osteogenic potential in an inflammatory microenvironment under tensile strength suggests that orthodontic force may amplify periodontal destruction in orthodontic patients with periodontitis.

Introduction

The periodontal ligament (PDL) is a complex, highly specialized cellular connective tissue that supports the teeth by surrounding and anchoring them to the alveolar bone. In addition, periodontal ligament cells (PDLCs) facilitate the bone-regenerative potential of periodontium (1). The versatility of the PDL is primarily due to the ability of PDLCs to adapt to various microenvironments, including inflammatory and loading conditions (2).

An increasing number of patients with malocclusion and malpositioned teeth are opting for orthodontic therapy to correct these conditions and improve aesthetics and function. The adult periodontium is exposed to a variety of physical forces, including those caused by orthodontic therapy. Orthodontic force serves as biomechanical stress that promotes the remodelling process and metabolism of bone matrix (3). Earlier studies and models have investigated the biochemical responses evoked by biomechanical forces, including static compression (4), dynamic compression (5), centrifugal force (6) and tensile stress (7), on various oral tissues, including PDLs.

It is important to understand the physiological and homeostatic response of the periodontium to mechanical and physical forces under various conditions, including an inflammatory microenvironment. It is understood that periodontitis is associated with an increase in pro-inflammatory cytokines, including interleukin-1 beta (IL-1β) and tumour necrosis factor α (TNF-α) (8,9). IL-1β and TNF-α mediate the pathogenesis of periodontitis, and their expression promotes periodontal tissue destruction (10,11). Elevated IL-1β and TNF-α levels have previously been detected in the serum and gingival tissues of patients with periodontitis (12). Additionally, primary cells cultured from inflammatory PDL tissues were demonstrated to secrete IL-1β and TNF-α levels that were similar to those from healthy donors stimulated with exogenous cytokines (13). These cytokines are primary chemical mediators in the inflammatory microenvironment and serve as markers of a large number of inflammatory diseases, including periodontitis (14,15). Notably, there appears to be ambiguity regarding the exact effects of these cytokines in different cell types. For
instance, studies on human mesenchymal stem cells (hMSCs) revealed that expression of the master transcription factor for osteoblast differentiation, runt-related transcription factor 2 (RUNX2), was enhanced in the presence of media pre-conditioned with increased pro-inflammatory cytokine levels from classically activated monocytes (16,17). By contrast, inflammatory stimuli additionally induced factors that facilitate bone resorption, including matrix metalloproteinases (MMPs) in PDL fibroblasts (18) and the anterior cruciate ligament (19). In vitro (20) and in vivo (21,22) studies have demonstrated that alveolar bone resorption is closely associated with MMP production. Notably, inflammatory cytokines appear to trigger cellular responses that facilitate bone apposition and bone resorption. Evidence regarding the mechanisms underlying the process of osteoblastogenesis in response to physical forces in inflammatory microenvironments is beginning to emerge. In particular, understanding the PDLC response to tensile strength and inflammatory status is pivotal for comprehending tooth movement in the inflammatory microenvironment.

Previously, two key factors important in PDL osteogenesis, RUNX2 and type I collagen (COL-I), have obtained increasing attention from researchers. RUNX2 is a transcription factor that serves an important role in the transcription of many osteogenesis regulating genes (23). COL-I is responsible for the formation of the primary fibril-forming collagen in the PDL, forms solid fibres located between the alveolar bone and the cementum, and functions as a cushion mitigating masticatory and orthodontic force loading (24,25). The present study investigated the combined effects of tensile strength, IL-1β and TNF-α on PDLCs. RUNX2 and COL-I mRNA and protein expression levels and alkaline phosphatase (ALP) activity were measured. The results obtained from this study will improve understanding of the risks associated with periodontitis in adult orthodontic patients, and the association between orthodontic force and periodontal inflammation.

Materials and methods

Isolation and identification of PDLCs. The present study was approved by the Ethics Committee of the School of Stomatology, Wenzhou Medical University (Wenzhou, China; no. WYKQ2013003). A total of 6 teeth were obtained from four systemically healthy patients (age, 18-28 years; two males and two females) during extraction as part of orthodontic treatment. According to the manufacturer's protocol, the absorbance was measured at a wavelength of 405 nm using a microplate reader (Tecan Group, Ltd., Mannedorf, Switzerland).

MTT assay. Proliferation of PDLCs following various treatments was determined by MTT assay (Amresco, LLC, Solon, OH, USA). A total of 320 µl MTT solution (5 g/l dissolved in ddH2O) was added to each well of a 6-well plate and cells were incubated at 37°C for 4 h. The MTT solution and culture medium was removed and 3,000 µl dimethyl sulfoxide was added. Complete dissolution of each sample was aided by 10 min of light-tight vibration. The absorbance was measured at a wavelength of 490 nm using a spectrophotometer. Pure culture medium without PDLCs was used as the blank control.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from PDLCs following treatment using TRIzol™ reagent (Invitrogen; Thermo Fisher Scientific, Inc.). A PrimeScript™ RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) was used to reverse transcribe cDNA from cellular RNA. qPCR was performed using a LightCycler® 480 Real-Time PCR Detection system (Roche Diagnostics, Basel, Switzerland) and the LightCycler® 480 SYBR-Green I Master qPCR kit (Roche Diagnostics) according to the following parameters: 40 cycles of 10 sec at 95°C, 10 sec at 60°C and 20 sec at 72°C. Primer sequences of osteogenic genes, including RUNX2, COL-I and GAPDH were synthesized by
Invitrogen; Thermo Fisher Scientific, Inc. GAPDH was employed as an internal reference. Primer sequences are listed in Table I.

Western blot analysis. PDLCs were washed three times with precooled PBS at 4˚C and promptly lysed in high intensity radioimmunoprecipitation assay lysis buffer with 1 mM phenylmethylsulfonyl fluoride, then scraped from the Bioflex plates. The concentration of total protein in the lysates was assayed using the BCA Protein Quantitation kit (Fudebio, Hangzhou, China) according to the manufacturer's protocol. Total protein from cell lysates (50 µg per lane) were separated per lane by 8% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% skimmed milk for 2 h and subsequently incubated with primary antibodies against RUNX2 (1:1,000; cat. no. 8486; rabbit; Cell Signaling Technology, Inc., Danvers, MA, USA), COL-1 (1:10,000; cat. no. sc-8785; goat; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and GAPDH (1:10,000; cat. no. KC-5G5; rabbit; KangChen Bio-tech, Inc., Shanghai, China) overnight at 4˚C. Following this, membranes were incubated for 2 h at room temperature with the following horseradish peroxidase (HRP)-conjugated secondary antibodies: Goat anti-rabbit IgG (1:5,000; cat. no. AP132P; EMD Millipore) and rabbit anti-goat IgG (1:10,000; cat. no. AP106P; EMD Millipore). Immunoreactive protein bands were detected by using Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore). Protein levels were quantified using ChemiDoc™ XRS+ (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and Quantity One software (version 4.6.2; Bio-Rad Laboratories, Inc.).

ALP activity assay. To evaluate the impact of tensile strength and the inflammatory microenvironment on ALP activity, an ALP assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used according to the manufacturer's protocol. The optical density was measured at a wavelength of 405 nm using a microplate reader (Tecan Group, Ltd.).

Mineralization assay. To investigate osteogenic differentiation potential, cells were initially grown under the conditions described above for 48 h. Following this, cells were immediately cultured in commercial osteogenic media supplemented with 50 µg/ml ascorbic acid, 0.1 µM dexamethasone and 10 mM β-glycerophosphate (Cyagen Biosciences, Santa Clara, CA, USA) for 14 days (13,30). Cells were subsequently fixed in 4% formalin for 30 min at room temperature and stained with alizarin red S (Cyagen Biosciences) for 30 min. To quantify mineralization, the mineral stain was solubilized in 5% SDS in 0.5 ml 0.5 N HCl for 30 min. The absorbance was measured at a wavelength of 405 nm using a microplate reader.

Statistical analysis. All experiments were performed at least three times and all data are expressed as the mean ± standard deviation. The Student's t-test was used for comparison between two groups and one-way analysis of variance was used for multiple comparisons, followed by Tukey's post hoc test. Statistical analysis was performed using SPSS 19.0 (IBM SPSS, Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Morphology and phenotype of PDLCs. To confirm that cells derived from the PDL were of mesodermal origin, the presence of vimentin and cytokeratin was detected by staining (28). The observation that the cells were positive for vimentin (Fig. 1A)
but negative for cytokeratin (Fig. 1B) was consistent with a mesodermal origin. Cells in PBS served as the negative control (Fig. 1C). PDLCs exhibited no particular morphological alterations and remained shuttle-shaped following stimulation with IL-1β+, TNF-α and/or tensile strength.
Tensile strength induces pro-inflammatory secretion with minimally increased proliferation. Tensile strength induced the secretion of pro-inflammatory cytokines in a time-dependent manner (Fig. 2) with a minimal increase in proliferation (Fig. 3). Continuous tensile strength up to 48 h led to the secretion of IL-1β (89.76±4.56 pg/ml) and TNF-α (77.52±3.51 pg/ml; Fig. 2). PDLCs treated with 5 ng/ml IL-1β and 10 ng/ml TNF-α exhibited a significant decrease in proliferative capacity compared with untreated controls. Tensile strength was demonstrated to have minimal influence on proliferation of PDLCs alone; however, it enhanced pro-inflammatory cytokine inhibition of PDLC proliferation (Fig. 3).

Tensile strength and a pro-inflammatory environment alters the osteogenic and matrix deposition potential of PDLCs. To investigate the osteogenic potential of PDLCs, the expression of osteogenic markers including RUNX2 and COL-I were determined. Tensile strength did not alter RUNX2 mRNA (Fig. 4A) or protein (Fig. 4B) expression levels in PDLCs. However, COL-I mRNA (Fig. 4C) and protein (Fig. 4D) expression levels were significantly reduced following tensile strength. Treatment with pro-inflammatory cytokines significantly increased mRNA and protein expression levels of RUNX2; however, decreased those of COL-I. The increase in RUNX2 induced by pro-inflammatory cytokines was markedly inhibited by tensile strength and was reduced below the levels observed in untreated controls. Similarly, downregulation of COL-I mRNA and protein expression levels induced by pro-inflammatory cytokines was accentuated by tensile strength. Combining tensile strength with pro-inflammatory cytokine exposure decreased COL-I mRNA and protein expression levels to markedly reduced levels compared with untreated PDLCs. Representative western blot images of COL-I and RUNX2 protein expression levels are presented in Fig. 4E.

ALP activity and mineralized nodule formation is decreased by tensile strength in an inflammatory environment in PDLCs. To determine the osteogenic and mineralization potential of PDLCs, the osteogenesis marker ALP and mineralization status were analysed. Cells were subjected to the conditions described above for 12, 24 or 48 h. ALP activity was reduced following pro-inflammatory cytokine treatment or tensile strength exposure. The greatest decrease in ALP activity was detected in cells treated with combined tensile strength and pro-inflammatory cytokines. The difference in ALP activity between the treatment groups was most marked 48 h post-exposure (Fig. 5). Increased mineralization in response to strength-induced osteogenic differentiation in the inflammatory microenvironment was measured using Alizarin Red S staining in PDLCs. A significant decrease in mineralization was observed in PDLCs treated with either pro-inflammatory cytokines or tensile strength followed by culturing in osteo-inductive medium for 14 days (Fig. 6A). Notably, a markedly significant decrease in Alizarin Red S staining was observed in cells exposed to the combination of pro-inflammatory cytokines and tensile strength (Fig. 6B).

Discussion

The demand for adult orthodontic treatment has grown exponentially in recent years (31). This increased demand has resulted in a proportional increase in the number of challenging
cases, particularly for orthodontists providing services to patients with periodontal diseases (32,33). Unfortunately, the risks involved in orthodontic therapy in patients with periodontal disease remain to be fully elucidated. The present study demonstrated alterations in the osteogenic potential of PDLCs in response to tensile strength in inflammatory environments.

Cellular behaviour, including proliferation, is highly dependent on biochemical and physical stimuli in the extracellular environment. The present study revealed PDLCs were sensitive to inflammatory mediators but insensitive to tensile strength, which was consistent with previous studies. A previous study demonstrated that conditioned medium from macrophages with Porphyromonas gingivalis-lipopolysaccharide (LPS) stimulation reduced the proliferation of osteoblasts (34). Another study demonstrated that tensile force loading did not influence the viability of PDLCs (35). However, the present study revealed that suppression of PDLC proliferation was enhanced following exposure to a combination of inflammatory and tensile loading microenvironments. These findings indicated that mechanical strength may amplify the inhibitory effects of inflammatory mediators.

Orthodontic tooth movement causes bone remodelling in areas under tensile strength, and this process is coupled with an inflammatory reaction (36). Previous in vivo studies have demonstrated that orthodontic tooth movement resulted in increased levels of pro-inflammatory cytokines in periodontal tissues (37,38). The present study confirmed an increase in IL-1β and TNF-α levels following exposure to tensile strength.

These results indicated that combined IL-1β and TNF-α exposure and tensile force loading significantly decreased RUNX2 expression in PDLCs. This result is consistent with a previous PDLC study that reported that combined IL-1β and tensile strain loading decreased RUNX2 expression (39). In the present study, combined IL-1β and TNF-α exposure upregulated RUNX2 in PDLCs. The differences between this study and previous investigations may be due to the presence of TNF-α. Studies in hMSCs using LPS-stimulated conditioned media reported high levels of secreted TNF-α and a robust enhancement of RUNX2 expression (16). There is evidence that activated monocytes and T lymphocytes produce inflammatory cytokines that serve an essential role in the process of osteogenic differentiation of mesenchymal tissue-derived cells (40,41).

Notably, IL-1β and TNF-α exposure robustly enhanced RUNX2 and suppressed COL-I expression in PDLCs. Inflammatory cytokines, including IL-1β and TNF-α are involved in the up-regulation of MMPs (18,19). MMPs mediate connective tissue breakdown (11) and the degradation of numerous extracellular matrix proteins (42). The results of the present study are consistent with those of a previous study that demonstrated that the expression of COL-I was inhibited in various cell types cultured in inflammatory media (13). These results demonstrated that COL-I expression by PDLCs was markedly suppressed following combined exposure to IL-1β, TNF-α and tensile force loading. Cytokine exposure exhibited an inhibitory effect on COL-I expression, and a stimulatory effect on RUNX2 expression. This may explain the different patterns of bone formation and matrix deposition in PDL tissue. Biomechanical strength imposed on PDLCs in an inflammatory microenvironment appears to be detrimental and results in progressive downregulation of RUNX2 and COL-I.

As biomechanical strength is involved in decreased bone formation and matrix deposition under inflammatory conditions, it was hypothesized that biomechanical strength may have a negative effect on ALP activity and the mineralization process. It was observed that ALP activity was significantly suppressed in PDLCs that were exposed to tensile strength combined with pro-inflammatory cytokines. Evidence from in vitro studies revealed that PDLCs and MSCs interact and respond in different ways to cytokines or biomechanical strength. For instance, increased ALP activity in response to IL-1β and TNF-α or biomechanical strength was observed in hMSCs (43,44). On the other hand, decreased ALP activity in response to cytokines or biomechanical strength in PDLCs has been reported by numerous studies (13,15,35). ALP activity and mineralization are interdependent and associated with osteoblast differentiation. The present study revealed that the level of mineralization in the I(+)T(+) group was significantly reduced compared with the I(-)T(-) group. This result suggested that biomechanical strength may serve as a key stimulus influencing PDLC tolerance of inflammatory microenvironments.

In conclusion, the present study demonstrated that mechanical stresses, including tensile strength, enhanced the suppression of osteogenesis, matrix deposition and mineralization in PDLCs that is induced by an inflammatory microenvironment. Therefore, it is important to assess the periodontal health status of adults prior to orthodontic therapy.

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