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Effects of Tension Force on Proliferation and Differentiation of Human Periodontal Ligament Cells Induced by Lipopolysaccharides

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Abstract

Human periodontal ligament cells (hPDLCs), with the potential for multi-directional differentiation and reproduction, are the target cells of orthodontic tooth movement. The aim of this study was to examine the effect of mechanical tension force and lipopolysaccharides (LPS) on hPDLCs and whether they induce proliferative and differentiated characters *in vitro*. Tension force was applied to hPDLCs stimulated with and without LPS for 24 hrs. Real-time polymerase chain reaction (qPCR) was carried out to analyze the mRNA expression of Cyclin 2 (CCND2), WNT1 inducible signaling pathway protein 1 (WISP1), runt-related transcription factor 2 (RUNX2) and alkaline phosphatase (ALP). Analysis of variance (ANOVA) was used for statistical analysis. Significant differences were indicated by $P < 0.05$. The results showed that tension force promoted the mRNA expression of both the proliferation-related genes (CCND2 and WISP1) and differentiation-related genes (RUNX2 and ALP), and that both were enhanced by the simulation of LPS. In addition, the relative expression ratios CCND2/RUNX2 and CCND2/ALP both increased significantly after the application of tension, and this effect was further enhanced by LPS. All results indicated that with the assessed level of mechanical force loading, tension could promote both the proliferation and differentiation of hPDLCs, which could be enhanced by LPS, and that proliferation is promoted to a greater extent than differentiation. These findings may be valuable for understanding the importance of the application of suitable mechanical force in periodontal remodeling, especially in the process of orthodontic tooth movement with inflammation.

Keywords

Human Periodontal Ligament Cells, Tension Force, Lipopolysaccharides, Proliferation, Differentiation

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1. Introduction

Orthodontic tooth movement is mediated by bone resorption and deposition on the compression and tension side, mainly due to delicate changes within the periodontal ligament (PDL), which is functionally heterogeneous and contains a subpopulation of cells able to receive mechanical signals of orthodontic force loading and transduce them into biological signals [1] [2]. The regenerative potential of the periodontium is believed to be related to the functions of periodontal ligament cells (PDLCs) [3].

The activation of specific transcription factors is essential for cellular proliferation and commitment to a differentiation lineage, which are also affected by mechanical force application [2]. As a member of the Cyclin family, Cyclin D2 (CCND2) is a key component for facilitating the G1-to-S-phase transition and subsequently increased cell proliferation [4]. The WNT1 inducible signaling pathway protein 1 (WISP1), a member of the secreted, cysteine-rich CCN family and a connective tissue growth factor, exerts diverse biological effects such as the proliferation of fibroblasts and smooth muscle cells [5]. In terms of hPDLC differentiation, many experiments demonstrated the important role of runt-related transcription factor 2 (Runx2) in regulating osteogenic differentiation, which may be the key to the different signaling pathways involved in mechanotransduction, and can induce the synthesis of alkaline phosphatase (ALP) [6] [7]. ALP, which is produced by PDLCs, can initially respond to force loading with gene expression detected after 24 hrs [8]. ALP activity is involved in the process of calcification in various mineralizing tissues, and is found at much higher levels in PDL than in other connective tissues [9].

Many adults with orthodontic treatment demands have dental problems that involve inflammation such as periodontitis, which aggravates periodontal problems during orthodontic tooth movement if the inflammation is not well controlled [10]. This inflammatory disease leads to gingival connective tissue destruction and irreversible alveolar bone resorption. PDLCs are the target cells of the inflammation. Lipopolysaccharides (LPS) partly comprise the cell wall of periodontal pathogens, and may contribute to alveolar bone loss and connective tissue degradation in periodontal disease [11].

Thus, there is great interest in the tension force involved in the regulation of the expression of hPDLC proliferation and differentiation in terms of LPS inducement. In this study, we evaluated the mRNA production of CCND2, WISP1, RUNX2 and ALP in hPDLCs induced by tension force and their changes in expression after LPS addition. This information may clarify the importance of suitable mechanical force in periodontal remodeling, especially in the process of orthodontic tooth movement with inflammation.

2. Materials and Methods

2.1. Cell Culture

This study was approved by the Ethics Committee of The University of Hong Kong (NO. UW13-120). The hPDLCs were obtained from three healthy individuals aged 13 - 18 years who had undergone premolar extraction for orthodontic treatment. Cells were obtained from the middle third of the root surfaces of healthy human premolars, as described previously [12]. The primary hPDLCs were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, USA) containing 10% foetal bovine serum (FBS; HyClone, Logan, USA) and antibiotic solution (100 U ml^{-1} penicillin and 100 U ml^{-1} streptomycin) at 37˚C in a 5% CO_2 incubator. After achieving confluence, cells were detached with 0.25% trypsin and subcultured in fresh DMEM. The hPDLCs were characterized by immunocytochemical staining for vimentin and cytokeratin. hPDLC suspensions (1×10^6 cells ml^{-1}) were plated onto special force-loading plates and cultured to confluence. Before the experiments, the cultured cells were serum starved for 12 hrs to be synchronized [13], and the medium was then changed to fresh DMEM containing 1.5% FBS with or without 0.1 μg/ml LPS (InvivoGen, San Diego, USA). All of the experiments were carried out at passages 4 - 7.

2.2. Force Application

The cells at the centers of the force-loading plates were loaded with cyclic uniaxial tension (2000 μ of strain, 0.5 Hz) for 24 hrs by a four-point bending system (SXG4201, University of Electronic Science and Technology of China, China) [13]-[15]. The cells in the control group were prepared using the same procedures as the experimental groups, except in terms of mechanical loading.

2.3. Real-time Polymerase Chain Reaction Analysis

The mRNA levels of CCND2, WISP1, RUNX2, and ALP were determined by system (MyiQ, Bio-Rad, Hercules, CA, USA). *β*-actin was analyzed as the housekeeping gene for the internal control. In brief, after the mechanical loading, the total RNA was extracted from the cells with Trizol reagent (Invitrogen, Carlsbad, USA) immediately and cDNA was reverse transcribed from mRNA using a SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Real-time PCR was performed with a Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). The sequences of the primers are listed in **Table 1**.

2.4. Statistical Analysis

All data were expressed as the mean standard deviations (SDs) from three independent experiments. Analysis of variance (ANOVA) was performed with the use of the SPSS 19.0 statistical software package (SAS Institute, Cary, NC, USA). Significant differences were indicated by $P < 0.05$.

3. Results and Discussion

3.1. Tension Induced CCND2, WISP1, RUNX2 and ALP mRNA

Mechanical loading is a fundamental determinant of bone formation and reconstruction. It can be converted into a cellular response involving rapid, kinase-mediated changes in gene expression [1]. Physiological strains reported for daily activities in human long bones are of the order of 2000 - 4000 μ strain [16]. Orthodontic tooth movement induced by mechanical stimuli is dependent on the remodeling capacity of the local periodontal ligament and alveolar bone. In this study, the magnitude (2000 μ strain) was chosen according to stress analysis of the periodontal ligament under various orthodontic loadings [17].

After tension application, there were significant increases in the mRNA expression level of the proliferation-related genes [CCND2 ($P < 0.05$) and WISP1 ($P < 0.05$)] and differentiation-related genes [RUNX2 ($P < 0.05$) and ALP ($P < 0.05$)] compared with the control group (**Figure 1**). This indicates that given the assessed level of mechanical force loading, tension might have promoted the proliferation and differentiation of the hPDLCs.

The effect of mechanical loading on the proliferation of hPDLCs is controversial. Some studies have shown that an appropriate mechanical force could induce hPDLC growth. Researchers reported cell proliferation on both the "tension" and "pressure" sides of the PDL by measuring 3 H-thymidine incorporation [18] [19]. Another study reported that a continuous force produced a three-stage proliferative response over 20 hrs [20]. Other researchers found no significant difference in cell proliferation between cells subjected to the tension force and those of control groups [9]. Our findings suggest that tension force could promote the proliferation of hPDLCs.

When considering the balance between hPDLC proliferation and tissue-specific differentiation, the cessation of cell proliferation may indicate the onset of osteogenic lineage commitment. The application of mechanical force to the rat model suggested that PDLCs were primarily osteogenic under strained conditions [21]. Our previous study also showed that hPDLCs had osteogenic differentiation potential under mechanical tension loading [22]. Our data were consistent with previous reports stating that mechanical strain induces the expression of the osteogenic transcription factor RUNX2 at mRNA and protein levels [23]. hPDLCs possess high ALP activity, which is an indicator of hPDLC differentiation. However, Yamaguchi et al. found 10% and 42% decreases in the ALP activity of PDLCs exposed to low (9%) and high (24%) tension forces, respectively [9]. We speculate

Table 1. Primer sequences for CCND2, WISP1, RUNX2, ALP and *β*-actin.

Genes	Primer sequences (5'-3')	
	Forward	Reverse
CCND2	TGTGAACCAGACATGCCAAT	TGTGAACCAGACATGCCAAT
WISP1	CCACCGGGGCCTCTACT	CCACACCGACCACCTGT
RUNX2	ATCCAGCCACCTTCACTTACACC	GGGACCATTGGGAACTGATAGG
ALP	TATGTCTGGAACCGCACTGAAC	CACTAGCAAGAAGAAGCCTTTGG
β-actin	CACCCGCGAGTACAACCTTC	CCCATACCCACCATCACACC

that these decreases were dependent on the magnitude of the tension force.

3.2. LPS Enhanced the Effect of Tension

The stimulation of LPS at the same force-application time (24 hrs) promoted the mRNA expression of CCND2, WISP1, RUNX2 and ALP to a greater extent than tension alone (**Figure 1**).

LPS is a bacterial cell component that plays multifunctional roles in inflammatory reactions. Cell multiplication is often accompanied by inflammation. Junctional epithelium (JE) cells can enter the proliferating cell cycle when exposed to LPS, and the enhanced proliferating activity in the JE is an important factor in the deepening of the periodontal pocket [24]. One study reported that LPS enhanced the growth of hPDLCs at a concentration of 1 ug/ml [25]. The concept of orthodontic tooth movement as a kind of inflammatory process was revived along with the gradual confirmation of the neurotransmitters, inflammatory mediators, cytokines and P substances involved in periodontal remodeling in recent years [2] [26]. As such, the consistency of LPS and tension-force application for inflammation may lead to the enhanced proliferation of hPDLCs.

In terms of differentiation, previous studies showed that LPS from periodontopathic bacterium stimulated osteoclast formation in mouse bone marrow culture systems and diminished the ALP activity of hPDLCs [27]. This contradicts our data related to the increased osteogenic differentiation factor. We speculate that the difference might have been due to the concentrations of LPS and the addition of tension-force application.

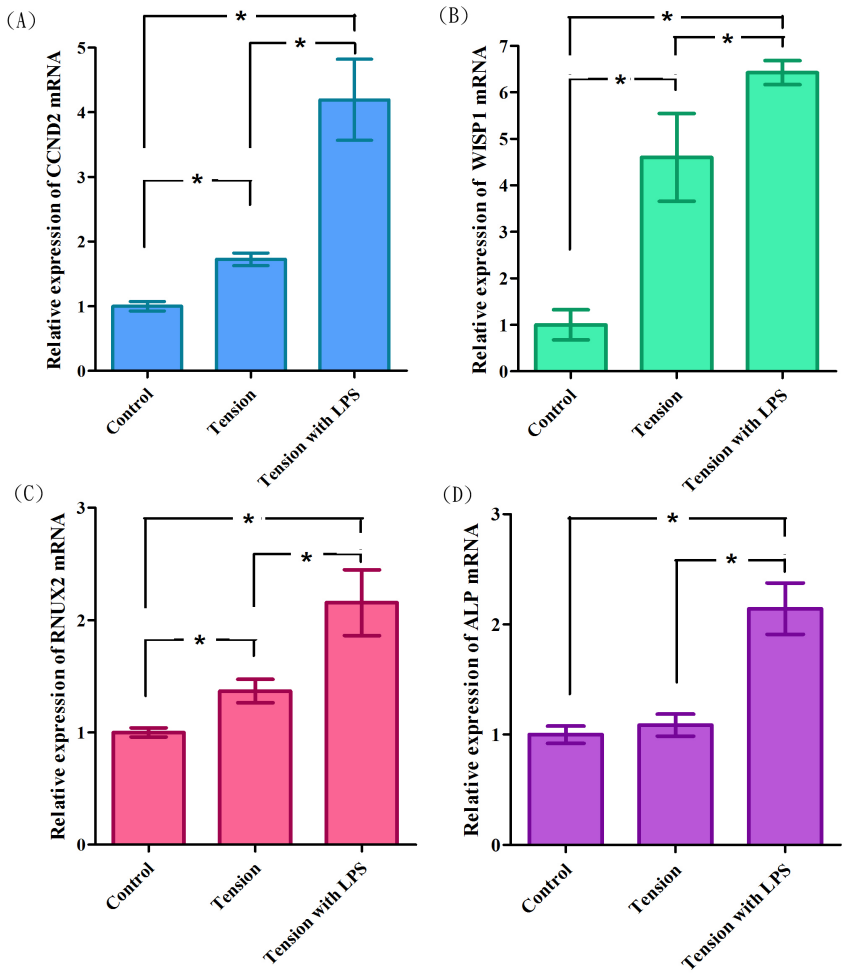

Figure 1. Relative mRNA expression of CCND2, WISP1, RUNX2 and ALP. Tension with and without LPS promoted relative mRNA expression of CCND2 (A), WISP1 (B), RUNX2(C) and ALP (D) in hPDLCs, and the effect of the tension was further enhanced by LPS (A-D). ($^{*}P < 0.05$ was considered statistically significant.)

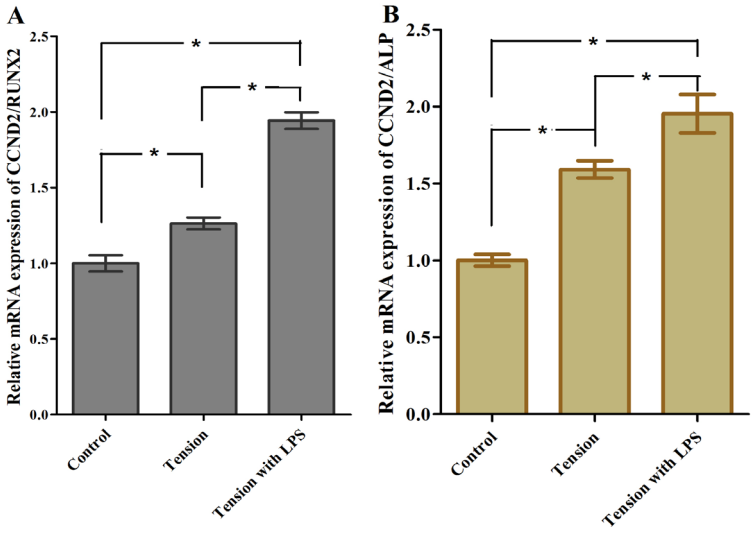

Figure 2. Relative mRNA expression of CCND2/RUNX2 and CCND2/ALP. Tension with and without LPS promoted the ratio of mRNA expression CCND2/RUNX2 (A) and CCND2/ALP (B), and LPS further enhanced the effect of the tension (A, B). ($^{*}P < 0.05$ was considered statistically significant.)

3.3. Proliferation was Promoted to a Greater Extent than Differentiation

Taking the ratio of the mRNA expression of CCND2 to RUNX2 or ALP into account, CCND2/RUNX2 and CCND2/ALP both increased significantly after tension was applied ($P < 0.05$). This effect was further enhanced by LPS ($P < 0.05$) (**Figure 2**), indicating that proliferation was promoted to a greater extent than differentiation in the hPDLCs by mechanical stimulation with or without LPS. This result was consistent with the observation that LPS is a major virulence factor involved in periodontal diseases that cause inflammatory proliferation [28]. In the future, different tension-force magnitudes should be tested to verify inclinations about hPDLC proliferation and differentiation.

4. Conclusion

Our results indicate that with the assessed level of mechanical force loading (2000 μ), tension can promote both hPDLC proliferation and differentiation, which could be enhanced by LPS. In addition, tension and LPS promotes proliferation to a greater extent than differentiation in hPDLCs. These findings may be valuable for understanding the importance of a suitable mechanical force in periodontal remodeling, especially in the process of orthodontic tooth movement with inflammation. However, further studies are needed to elucidate the relationship between the tension force and LPS in periodontal remodeling.

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