Mechanical Stress Modulates Expression of Toll-Like Receptors in Human PDL

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MECHANICAL STRESS MODULATES EXPRESSION OF TOLL-LIKE RECEPTORS IN HUMAN PDL CELLS

By

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A thesis submitted to the Faculty of the Graduate School,
Marquette University,
in Partial fulfillment of the Requirement for
the Degree of Master of Science

Milwaukee, Wisconsin
May 2012

ABSTRACT

MECHANICAL STRESS MODULATES EXPRESSION OF TOLL-LIKE RECEPTORS IN HUMAN PDL CELLS

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Orthodontic movement of teeth with compromised periodontium is risky because heavy orthodontic force could exacerbate the damaged periodontal status. Guidelines for moving periodontally-compromised teeth have not been established due to the lack of scientific evidence about the relationship between periodontal inflammation and mechanical force. In periodontitis, lipopolysaccharide (LPS) is recognized in the periodontal ligament (PDL) by toll-like receptors (TLR) which lead to destruction of periodontal tissues through inflammatory cascades. Recent studies have demonstrated that absence of TLR2 and TLR4 leads to reduced alveolar bone loss in mice. Therefore, reduction of TLR2 and TLR4 on PDLF cells can have a protective effect against the attack by periodontal pathogens and, accordingly, decrease susceptibility to periodontal disease.

The objective of the study was to explore the effect of mechanical stress on the expression of toll-like receptors on human periodontal ligament fibroblasts (hPDLF).Human periodontal ligament fibroblasts (hPDLF) were cultured on glass slides. Upon confluence, the cells were starved for 24 hours and then subjected to fluid shear stress (FSS) of 12 dynes/cm^2 for 1 hour. After FSS, the cells were lysed to test the phosphorylation of extracellular regulated kinase (ERK)1/2 and the expressions of TLR2 and TLR4. To explore the possible involvement of ERK1/2 signaling pathway, a specific ERK1/2 inhibitor PD98059 was added during the flow. The whole cell lysates from each group were immunoblotted with anti-TLR2 and anti-TLR4 antibodies. The signals of interest were determined using the ECL method. For quantification, densitometries of gel bands of interest were normalized to that of vinculin.

One-way ANOVA with Tukey's post hoc test was used to compare the results among the experimental groups, with *p* value being set at 0.05. As found, TLR4 but not TLR2 was abundantly expressed in hPDLF cells. Compared to the static controls, FSS significantly reduced the expression of TLR4. When PD98059 was added, the FSS-induced reduction of TLR4 was significantly recovered back to the control level. Conclusively, mechanical stress down-regulates the expression of TLR4, which is mediated by MAPK (ERK1/2) signaling pathway. Our findings suggest that FSS (mimicking light orthodontic force application) could possibly alleviate the compromised periodontal status via down-regulation of TLR4.

ACKNOWLEDGEMENTS

Yaroslav R Yarmolyuk, DDS

I would like to express my gratitude to all those who have helped and supported me throughout this research project. I would like to thank my committee members, Dr. Dawei Liu, Dr. T Gerard Bradley, Dr. Jose Bosio, Dr. Jeffrey Toth, and Dr. Arthur Hefti for their guidance and support during the selection of the research topic and the preparation of this thesis. I would like to extend my deepest appreciation and special thank you to Dr. Liu for serving as my thesis director and mentor throughout the research process. His complete and unyielding dedication to both his research projects and his residents is praiseworthy.

Last but not least, I would like to thank my wife Jane. I could not have done it without her constant support of my work and education. Throughout my undergraduate, dental, and post-graduate education she has been my partner, best friend, advisor, and financial provider. I would not have been able to achieve what I have without her by my side.

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CHAPTER 1

INTRODUCTION

Today, more and more adults are seeking orthodontic treatment to straighten their teeth, improve dento-facial appearance, or as a part of interdisciplinary treatment to control their overall oral health. However, the difference between traditional orthodontic teen patients and adult patients is that the latter are significantly more prone to periodontitis – a disease leading to destruction of periodontium (supportive tissues of teeth, including alveolar bone, PDL, cementum and gingiva). It has been reported that periodontal disease affects 8-30% of adult population (Papapanou 1989). According to the National Health and Nutrition Examination Survey (NHANES) done in 2004, 8.52% of U.S. population between the ages of 20 and 64 are affected by periodontal disease. The percentage ranges from 3.4% in adults from 20 to 34 years of age, to as high as 11.88 % in the 50-64 age group (NHANES 1999-2004). This is why it is highly likely for practicing orthodontists to encounter periodontally-compromised patients in their practice.

In orthodontics, moving teeth with destructed periodontal tissues is risky not only because the unhealthy periodontal tissues, but also because, if used inattentively, the mechanical force could easily exacerbate the damaged periodontal status. During orthodontic treatment, mechanical forces are applied to move the teeth to better functioning and more esthetic positions. Orthodontic forces are distributed through the teeth, to the periodontal ligament, and to the alveolar bone, producing a compression zone where the alveolar bone is resorbed and a tension zone where new bone is deposited during the tooth movement (Henneman 2008). A complex network of molecular signals produces

numerous cellular responses to resorb the alveolar bone to move teeth (Krishnan and Davidovitch 2009).

With modern orthodontic technique, periodontally-compromised teeth can be successfully managed. However, the indications and guidelines for moving periodontally-compromised teeth have not been fully established mainly due to the lack of scientific evidence. The relationship between bacterial invasion of periodontium and mechanical force in the periodontal tissues is not well understood.

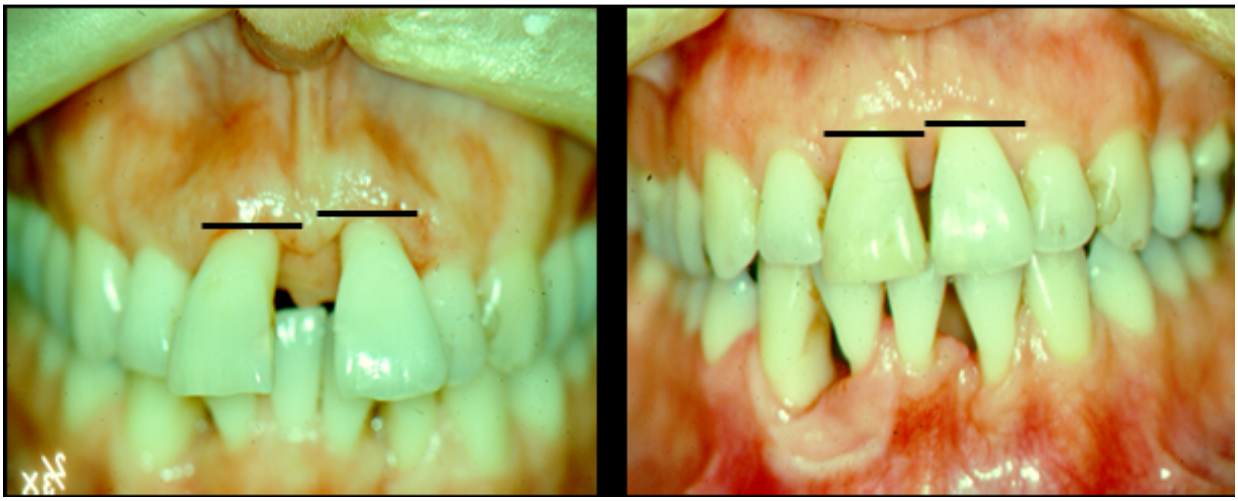


Figure 1. Clinical Case. If moved with light orthodontic forces, periodontally compromised teeth can be safely treated (intruded) and well maintained. Courtesy of Dr. Birte Melsen

CHAPTER II

REVIEW OF LITERATURE

Pathology of Periodontal Disease

Periodontal disease is a multifactorial bacterial disease leading to breakdown of periodontium, which in its advanced stages could cause mobility and eventual exfoliation of teeth (Haffajee and Socransky 1994). The direct cause of periodontal tissue destruction is the presence of periodontal bacteria. Because most of periodontal pathogens reside in the periodontal pockets and do not invade the periodontal tissues, the host immune system can never efficiently eliminate the bacteria. This leads to continued chronic inflammation of periodontal tissues. Continual bacterial stimulation of periodontal tissues causes periodontal tissue breakdown and hard tissue damage. In the presence of dental plaque in the periodontal pocket, inflammatory cells and osteoclasts are chronically activated, leading to attachment loss and destruction of alveolar bone (Okada 1998).

Haffajee and Socransky reported that over 400 different bacterial species have been shown to be capable of colonizing the oral cavity and, normally, any individual may harbor 150 or more of these species. Bacteria inhabit the human oral cavity from birth to death. They colonize soft and hard tissues such as cheeks, tongue, gingiva and teeth above and below gingival margins. Subgingival bacteria counts can range from about 10^3 in healthy, gingival sulci to more than 10^8 in deep periodontal pockets (Haffajee and Socransky 1994).

Multiple studies showed that characteristic bacteria found in dental plaque in the periodontal pocket include black-pigmented, gram-negative anaerobes such as *P. gingivalis* and *P. intermidia.* These bacteria are significantly more

prevalent in patients with periodontal disease and *P. gingivalis* is the strongest bacterial marker for destructive periodontal disease. Cell elements of *P. gingivalis,* such as cytoplasmic membrane and outer membrane proteins, peptidoglycans, and lipopolysaccharides induce excessive expression of inflammatory cytokines and lead to immunological responses (Offenbacher 1996, Genco 1984).

Destruction of periodontium is the result of innate and adaptive immune system responses to periodontal pathogens. A series of studies have demonstrated that the persistent host inflammatory immune response against the pathogens results in the destruction of soft and mineralized periodontal tissues (Graves 2008, Liu et al, 2010).

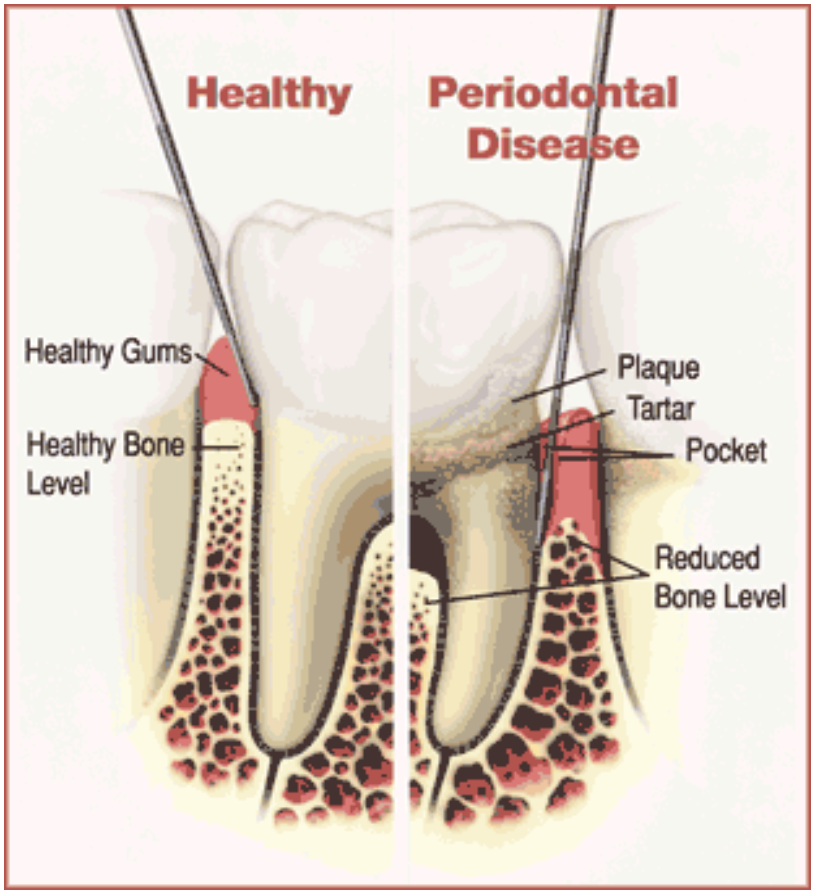


Figure 2. Periodontal Disease. In the presence of dental plaque in the periodontal pocket, inflammatory cells and osteoclasts are chronically activated, leading to attachment loss and destruction of alveolar bone. (Haffajee and Socransky 1994).

Lipopolysaccharides

Lipopolysaccharides (LPS) - a major bacterial component has been shown to play a key role in tissue destruction of periodontal tissue. LPS is a combination of lipid and polysaccharide and is a part of the outer membrane of gram-negative bacteria. LPS released from gram-negative bacteria, including *Porphyromonas gingivalis,* triggers a cascade of inflammatory responses that contribute to periodontal destruction. LPS induces inflammatory cytokines, such as inteleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) in periodontal ligament fibroblasts (Garlet 2010).

Toll-Like Receptors

Toll-like receptors (TLR) are pattern-recognition receptors that play a critical role in innate immune system. Medzhitov first described TLR receptors in 1997. TLR is a type I transmembrane protein, which consists of an extracellular domain made up of a leucine-rich repeat (LRR), and a cytoplasmic domain homologous to the cytoplasmic domain of the human interleukin (IL)-1 receptor (Medzhitov 1997). This domain is required for the interaction and recruitment of various adaptor molecules to activate the downstream signaling pathway. TLRs are expressed in distinct cellular compartments. TLR1, TLR2, TLR4, TLR5, TLR6 are expressed on cell the cell surface, whereas TLR3, TLR7, TLR8, and TLR9 are expressed in intracellular vesicles, such as the endosome and ER (endoplasmic reticulum) (Kumar 2009).

TLRs are able to recognize various molecules that are shared by many pathogens, known as pathogen-associated molecular patterns (PAMP). TLRs detect a wide range of PAMP including proteins, lipids, lipoproteins, nucleic acids, and lipopolysaccharides (Medzhitov et al, 1997, Yang et al, 1998). There are currently 12 known TLRs, each of which is able to recognize a specific set of PAMPS (Kumar et al., 2009). The two subtypes of TLRs explored in our study are TLR2 and TLR4. TLR2 recognizes many bacterial, fungal, and viral components such as lipoproteins, lipopeptides, and peptidoglycans. TLR4 recognizes lipopolysaccharides of gram-negative bacteria such as *Porphyromonas gingivalis* (Takeuchi 1999, 2001).

In the periodontal ligament, P. *gingivalis* LPS acts as a PAMP and is recognized by TLR2 and TLR4 that are expressed on PDL cells and leukocytes (Mahanonda 2007). Recent studies by Kikkert and Nussbaum describe a role of TLR2 and TLR4 in periodontal disease. Activation of TLRs leads to a diverse array of intercellular signaling pathways that dictate the magnitude, type, and duration of inflammatory response (Kikkert et al 2007, Nussbaum 2009). The result of TLR activation is recruitment of inflammatory cells, expression of cytokines and prostanoids, and subsequent osteoclast activation which leads to destruction of periodontal support by the process of osteoclastogenesis (Genco 1998, Gelani et al 2009, Lima 2010).

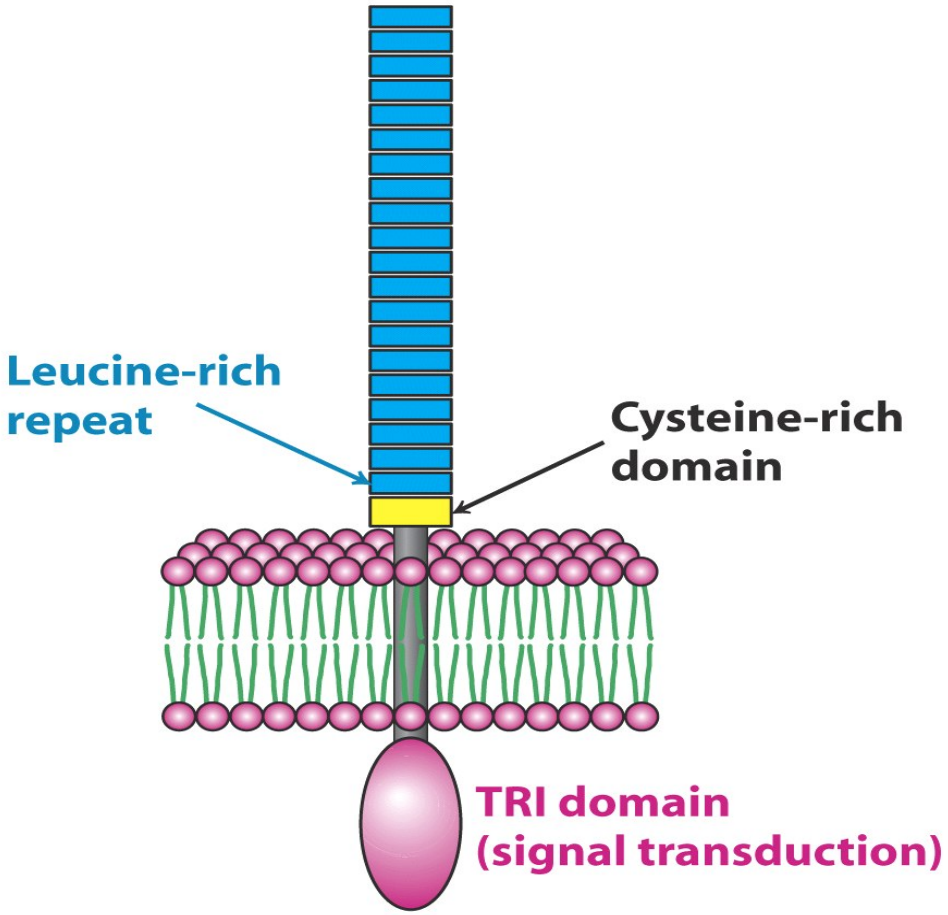


Figure 3. Schematic structure of TLR. TLR is a type I transmembrane protein which consists of an extracellular domain made up of a leucine-rich repeat (LRR), and a cytoplasmic domain homologous to the cytoplasmic domain of the human interleukin (IL)-1 receptor (Berg et al. 2006)

TLR Signaling Pathways

Ligand recognition by TLRs leads to complex signaling pathways the final result of which is activation of AP-1 transcription factor. These transcription factors induce the transcription of inflammatory cytokines, type I interferon and chemokine. The TLR signaling pathways are categorized into MyD88-dependent and MyD88-independent (TRIF-dependent) pathways. The downstream event of Myd88-dependant pathway is induction of inflammatory cytokines and interferon, and an array of molecules involved in the regulation of inflammation and adaptive immunity. The major outcome of MyD88-independent signaling for TLR4 is the production of type I IFNs (Kumar 2009, Brown 2011).

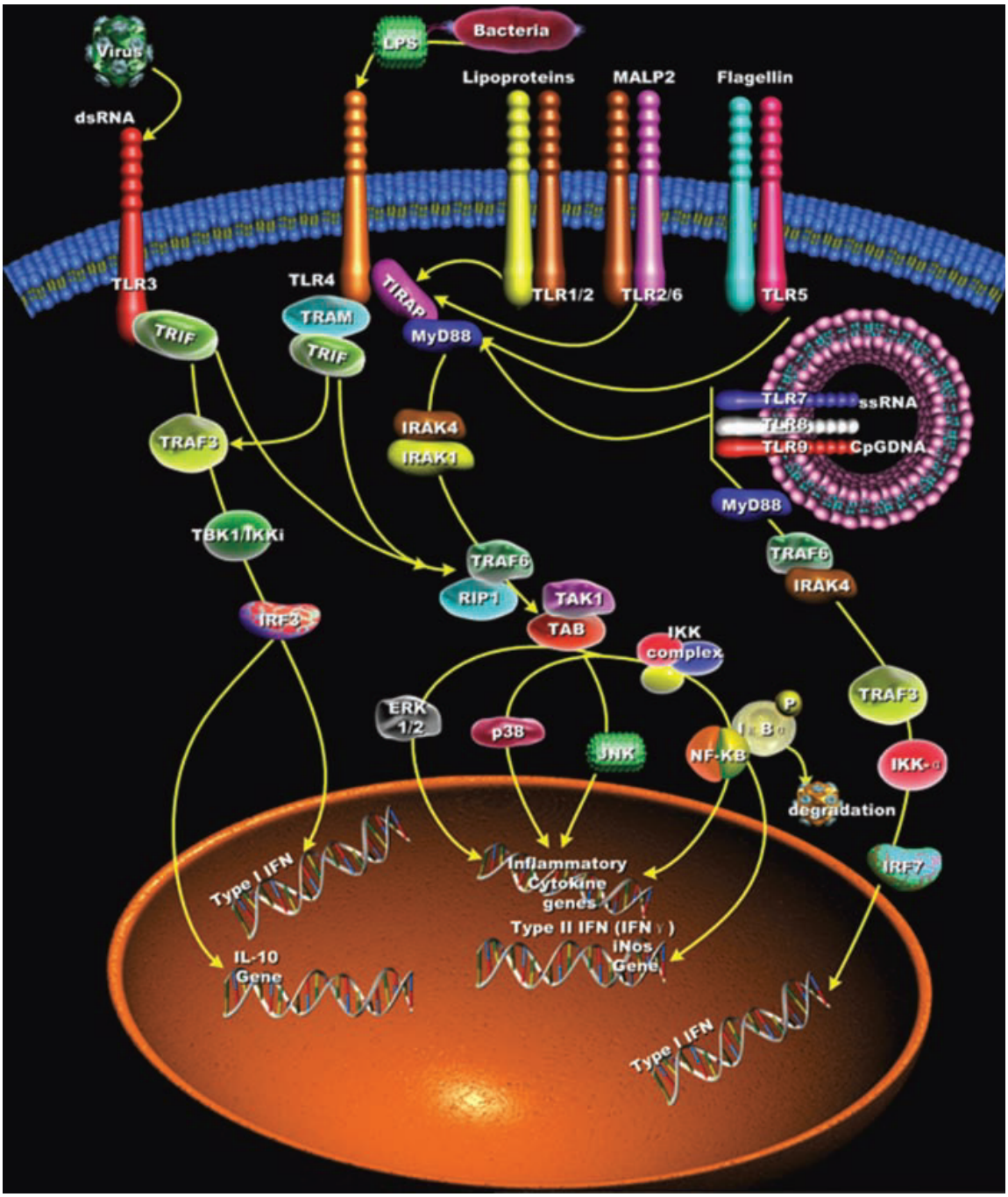


Figure 4. TLR adaptor molecules and signaling pathways. (Brown et al. 2011)

Multiple recent studies have demonstrated that absence of TLR2 and TLR4 leads to reduced loss of alveolar bone in mice after infection with *P. gingivalis* (Nakamura 2008, Costalonga 2009, Lima 2010). In 2009, Castalonga et al. studied the effect of TLR4 and TLR2 deficiency on the levels of alveolar bone loss in mice. Two strains of mice, either wild type, TLR4-deficient or TLR2-knockout, were infected orally four times, at 4 day intervals, with 109 colony forming units of *P. gingivalis*. At 47 days, the animals were sacrificed, jaws de-fleshed and stained, and photographed in a standardized position. The surface area of the root trunk was measured to assess the alveolar bone loss. The results demonstrated that in BALB/c (bone loss-susceptible) strain of mice, absence of TLR4 had a protective effect against alveolar bone loss in mice. TLR2 did not appear to have an effect on the amount of alveolar bone loss in vivo (Castalonge 2009)

In 2008, Nakamura et al. performed in vivo animal study to compare the change in bone resorption in the absence of TLR4. In the study, twenty five 7 week-old LPS-non-responsive mice and twenty five 7-week old LPS responsive mice were repeatedly injected with Actinobacillus actinomycetemcomitans LPS into their gingiva every 48 hours. Bone resorption was compared histomorphometrically between the two groups. The researchers found that bone resorption was significantly lower in the LPS-non-responsive group. The researchers concluded that TLR4 is vital for in vivo alveolar bone resorption (Nakamura 2008).

Human Periodontal Ligament Fibroblasts

The most common cell in the periodontal connective tissue is the human PDL fibroblast (hPDLF). Hassell reported that sixty-five percent of cells in gingival connective tissue are fibroblasts. Human PDL fibroblast cells (hPDLF) play an important role in the remodeling of soft tissues by production of structural components of PDL such as collagen, elastin, glycoproteins, and glycosaminoglycans (Hassell 1993). These cells may also function as regulators of the cytokine network as they have been shown to secrete various cytokines and immune mediators. Studies demonstrated that gingival fibroblasts secrete a variety of cytokines (IL-1, IL-13, IL-6, IL-8, TNF-α) and chemical mediators when they are activated with physiological and pathological stimuli in vitro. It has been reported that cultured gingival fibroblasts stimulated with LPS from *P. gingivalis* produce IL-1 and IL-6 (Garlet 2010, Takada 1991).

Recent studies showed that hPDLF cells are involved in periodontal tissue inflammation. It has been reported that hPDLF cells stimulated with *P. gingivalis* LPS induced the expression of inflammatory cytokine mRNA (Yamati 1995). Yamamoto et al. demonstrated that *P. gingivalis and P. intermedia* induce cytokine production not only at the mRNA level, but also at the protein level (Yamamoto et al 2006). Studies by Takada et al. show that *P. gingivalis* LPS stimulates fibroblasts to produce cytokines which subsequently activate the host cells involved in the immune-inflammatory processes (Takada 1991).

Multiple studies demonstrated that hPDLF cells express TLR2 and TLR4, and their expression increases upon stimulation with *P. gingivalis* LPS

(Hatakeyama et al 2003, Wang 2000a,b, Tabeta 2000). Hatakeyama et. al. demonstrated that hPDLF expresses TLR4 and suggested that that LPS directly acts on these cells (Hatakeyama 2003). Wang et al. demonstrated that binding of *P. gingivalis* LPS to TLR4 on gingival fibroblasts activates second-messenger systems (Wang 2000a,b).

Mechanical Loading

A strong body of evidence shows that mechanical loading plays an important role in maintaining bone mass by triggering various signaling pathways leading to an anabolic change of bone remodeling (Duncan, 1995). Fluid shear stress (FSS) is a form of mechanical loading on cell level.

PDL cell are constantly subjected to fluid shear stress from mastication, speech, and orthodontic tooth movement. In 2011, Bergomy et. al. described the hydro-mechanical coupling model of PDL. The PDL's response to compression activates the compartments that incorporate fluids (the ground substance and the blood vessels). The model that describes compressive loads can be viewed as an interconnected system of voids through which fluid flows. Under compression, the fluids are forced through the voids and are relocated either to adjacent zones of the PDL or driven into the neighboring alveolar bone. This fluid movement creates the type of mechanical loading known as fluid shear stress (Bergomy 2011).

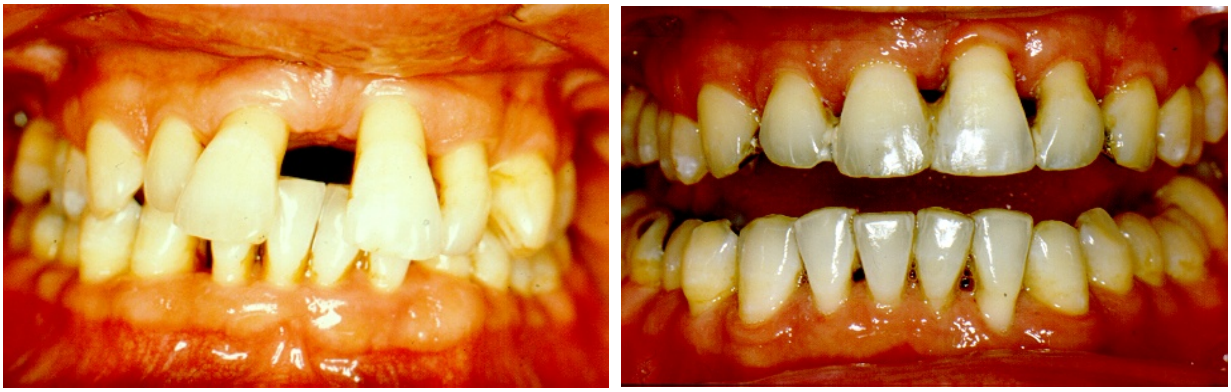
It has been shown that PDL cells are able to perceive and respond to mechanical stress. In vitro studies demonstrated that mechanical stress induces intracellular signals and alters gene expression in PDL cells (Pasavant 2011).

Studies show that FSS is able to reverse TNF-α induced apoptosis in osteoblasts *in vitro* (Pavalko 2003). FSS also plays a role in orthodontic tooth movement, however its mechanism remains unknown (Henneman 2008). Shimizu et al. showed that PDL cells respond to mechanical tension forces by elevated synthesis of IL-1β (Shimizu et al 1994). Previous experiments show that FSS produces anabolic changes in cementoblasts in culture and FSS activates MAPK signaling pathways in osteoblast (Liu 2006, Liu 2008).

Physiological levels of FSS have been established for long bones from 8-30 dynes/cm^2 (Pavalko 2003). Although the amount of FSS that occurs in the PDL is not known 12 dynes/cm^2 is the amount frequently used for studies examining molecular bone regulation (Chen et al 2000, Chen et al 2003, Pavalko 2003, Lee et al 2008, Liu 2008, Rangaswami et al 2009). The dyne is a unit of measurement often used to describe the surface tension in fluids. One dyne is the force required to cause a mass of one gram to accelerate at a rate of one centimeter per second squared in the absence of other force-producing effects. The application of the fluid flow model in research allows for the examination of cellular responses of cells to various environmental stimuli and will help further clarify the molecular regulation pathways involved in periodontal fibroblasts under mechanical loading.

The effect of mechanical force on the expression of TLRs in human has been the focus of a few studies. Liang et al. showed shear stress affected the expression of TLR4 in endothelial cells (Liang 2002). However, to date, there are

no known studies exploring the effect mechanical stress on the expression of TLRs in hPDLF cells.



Before Orthodontic Treatment After Orthodontic Treatment

Figure 5. Periodontally-compromised dentition. Orthodontic treatment greatly intrudes periodontally compromised maxillary incisors and rehabilitates oral function. Courtesy of Dr. Birte Melsen

Hypothesis

Knowing that a physiological level of FSS can affect TLR expression in other types of human cells, such as osteoblasts and osteoclast, our working hypothesis was that an equivalent level of FSS applied to hPDLF cells will produce a change in TLR expression.

Our first objective was to examine the expression of toll-like receptor TLR2 and TLR4 on hPDLF cells. The second objective was to study the change in the expression TRLs after the cells have been subjected to fluid shears stress application. We employed the use of a specific apparatus that was designed and fabricated to apply fluid shear stress to the cells in vitro. The third objective was to evaluate the possible involvement of MAPK signaling pathway by adding a specific ERK1 inhibitor - PD98059 during the flow.

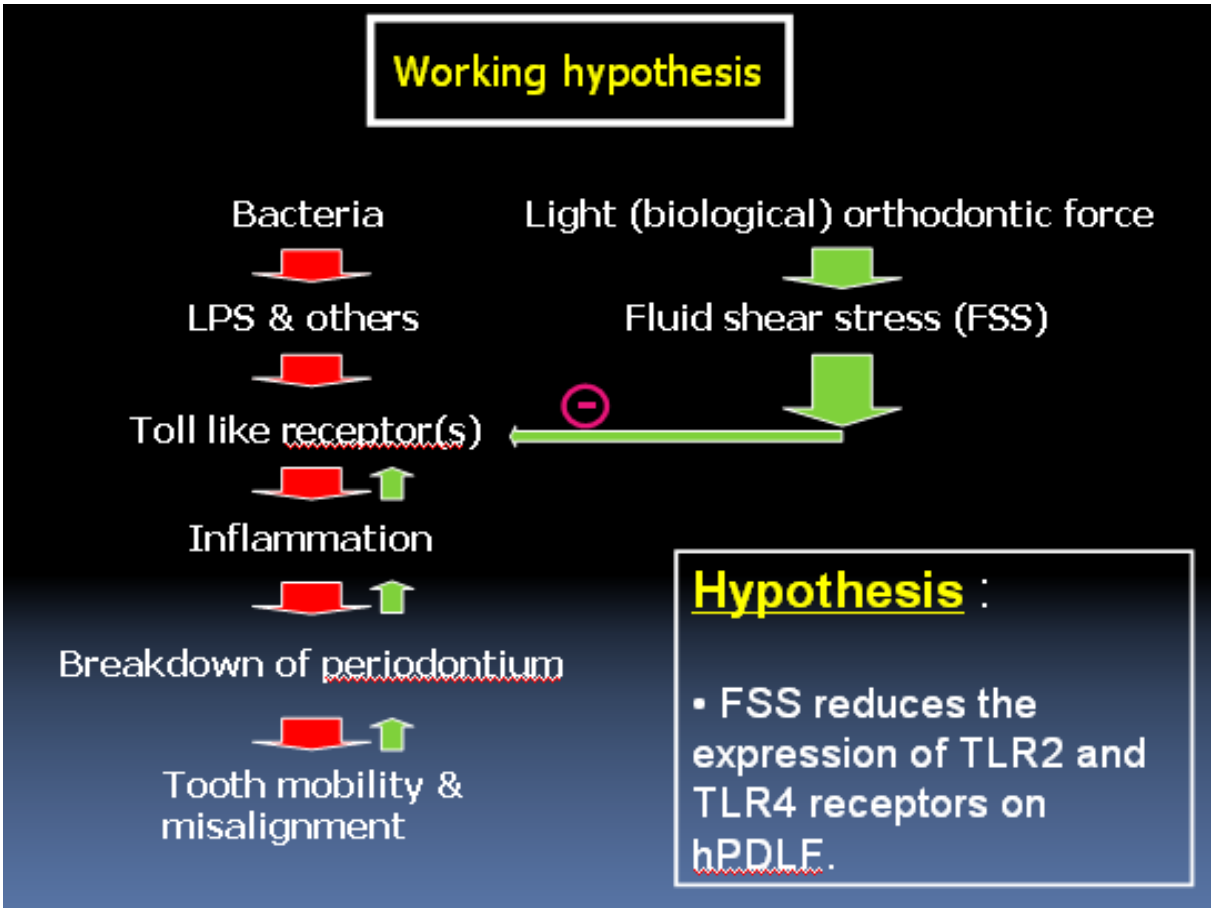


Figure 6. Working model. Fluid shear stress, which mimics light orthodontic force application, could negatively affect the expression of TLRs therefore reducing inflammation and destruction of periodontal tissues.

CHAPTER III

MATERIALS AND METHODS

Cell Culture

Human periodontal ligament cell line #2630 was purchased from ScienCell Research Laboratories (San Diego, CA). The cells were cultured in α-MEM containing 10% fetal bovine serum (FBS) and antibiotics. HPDLF #2630 cells were maintained in a humidified incubator at 37℃ a erating 5% CO_2 in air. The cells were routinely passaged at confluence. Passages 10-15 were used for the experimentation. For fluid shear stress experiment, HPDLF #2630 cells were plated at a density of 5 x $10^4/cm^2$ on 75 x 38 mm^2 glass slides coated with type I collagen from rat tail and grown up to 90% confluence. All cell culture materials were purchased from Sigma (St. Louis, Missouri, USA) unless indicated otherwise. Prior to FSS experimentation, cells were serum starved with 0.2% FBS containing medium for 24 hours in order to synchronize cell cycles and attain a basal level of metabolic activity.

Pharmacologic Reagents

ERK1/2 inhibitor, PD98059 was added 60 minutes prior to the onset of flow and remained for the duration of the experiment. The working concentration was 15 μM for PD98059 (in ethanol) (*Shimo et al. 2007*).

Fluid Shear Stress (FSS) Application

FSS was applied to the cell monolayer in a parallel plate flow chamber using a closed flow loop (Cytodyne, San Diego, California, USA). This setup (**Figure 7**) used a constant hydrostatic pressure head to subject the cell monolayer to a steady laminar flow stress of 12 dynes/cm^2 for 1 hour. The apparatus was maintained at 37℃ in a thermo box and the medium was aerated with 5% CO_2 in air throughout the duration of the experiment. (Figure 7)

In inhibitory studies, the ERK1/2 – inhibitor PD98059 was applied 1 hour prior to FSS and remained during FSS. Cells in control groups were exposed to identical experimental conditions but without flow treatment. Three samples were used in each experimental run, and each experiment was repeated three times.

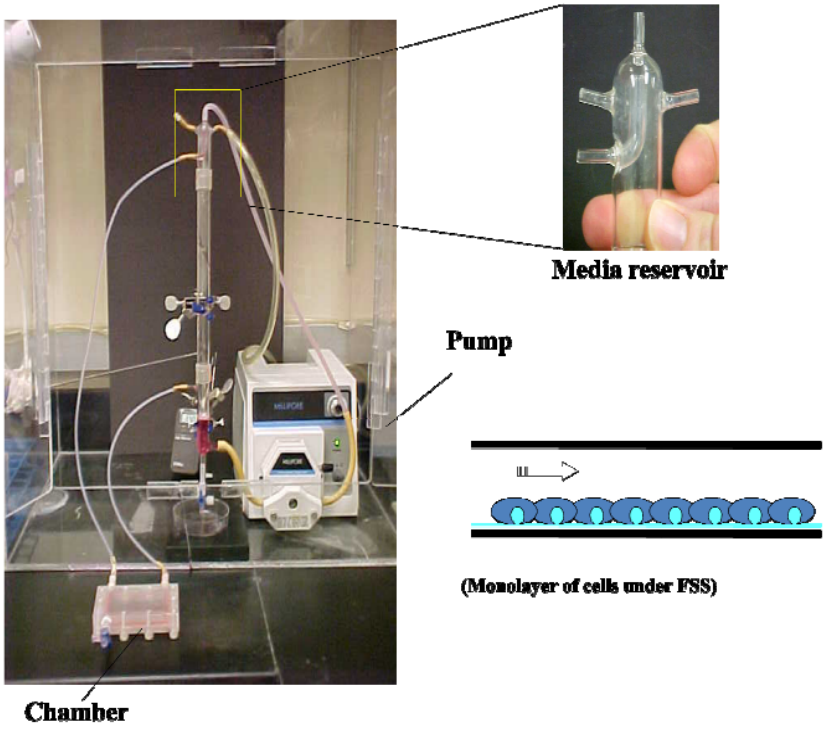


Figure 7. Fluid shear stress (FSS) system showing the experimental apparatus used for applying a mechanical load to the cells, including the parallel plate chamber for holding the glass slide with monolayer of cells, the medium reservoir for loading flow buffer, and the pump that provides the continuous fluid flow.

Calculating FSS Levels

The amount of FSS applied to the monolayer of cells during the FSS experiment varies depending on the height of the column set-up in the FSS apparatus. The height of the chamber was 21.75 cm tall and was 64 cm from the stand base. To calculate the actual levels of FSS that were used for the experiment, the apparatus was set up identical to experimental conditions described above. Fluid from the system was collected in a glass beaker for a measured period of time. This was done three times for each level of FSS used. Volume of medium was measured by pipette. The amount of volume collected per minute was calculated and averaged (**Table 1**). Using the FSS calibration chart, the amount of dynes/cm^2 was determined to be 12 dynes/cm^2 (**Figure 8** provided by Dr. Robling – Indiana University).

Determination of FSS force level

Time (sec)	15.7	15.5	15.7	**Avg (sec)**	15.63
Volume (ml)	14.42	14.21	14.96	**Avg (ml)**	14.53
				ml/min	55.78
				Dynes/cm^2	12.0

Table 1. Calculating FFS force level. Time and volume output data from FSS apparatus during fluid flow experiment used to calculate force levels.

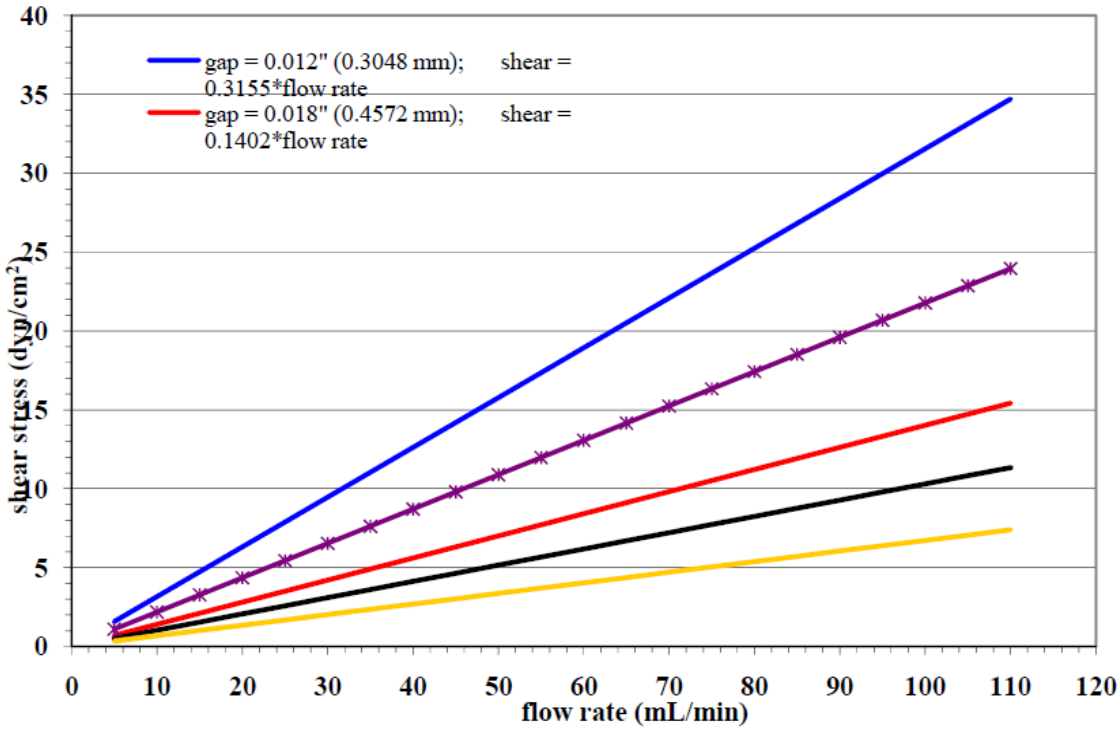


Figure 8. Fluid shear stress calculation chart. Chart used to calculate actual amount of force (dynes/cm^2) applied to cells by fluid shear stress during experiment using ml/min of fluid output generated by FSS apparatus. Based on the size of the flow chamber used in our experiment, the purple line was used for calculating FSS levels (Provided by Dr. Robling – Indiana University).

Protein Extraction and Western Blot Analysis

Immediately, after FSS for 1 hour, the glass slide was removed from the parallel plate chamber and carefully rinsed with phosphate buffered saline (PBS) twice. The cells were then lysed with 100μL lysis buffer (5mM HEPES [pH 7.9], 150mM NaCl, 26% glycerol (v/v), 1.5mM $MgCl_2$, 0.2mM ethylenediaminetetraacetic acid (EDTA), 0.5mM dithiothreitol and 0.5mM phenylmethylsulfonyl fluoride). Slides were scrapped, and the lysis was transferred to a 1.5ml centrifuge tube and boiled for 5 minutes. The protein samples were mixed by vortex and boiled at 100℃ for 5 minutes to deactivate proteinases, then stored at -80℃ until analysis. Contro l samples were processed in the same manner as experimental samples. The protein samples were centrifuged at 14,000g for 10 minutes at room temperature, the supernatants were collected, and the whole cell lysates were quantified using the amido black method (Genetos et al, 2005). Fifty micrograms of whole cell lysate and a pre-stained molecular weight marker (Bio-RAD Laboratories, Hercules, California, USA) were revolved through 10% sodium dodecyl sulfate (SDS) gels, which were transferred to nitrocellulose membranes overnight. The membranes were blocked in Tris-buffered saline (TBS) containing 5% nonfat dry milk (Bio-Rad Laboratories, Hercules, California, USA) and 0.1% Tween-20 (TBST) for 2 hours at room temperature. Immunoblotting with 1stantibodies was performed on a shaker at 4ºC overnight. The 1st antibodies used in this study included anti-vinculin (1:10,000, Sigma, St. Louis, Missouri, USA), anti-TLR2, anti-TLR4 (Assay Designs, Ann Arbor, Michigan, USA), anti-ERK1/2, and anti p-ERK1/2

(Santa Cruz Biotechnology, CA). Following washes in TBST (1x), the membranes were incubated with goat anti-rabbit or goat anti-mouse IgG hydroperoxidase conjugated secondary antibodies (1:5000) for 1 hour at room temperature. The gel images were developed using enhanced chemiluminescence (ECL) method (Pierce, Rockford, Illinois, USA) and documented using a FUJIFILM LAS-1000 gel documentation system (Stamford, Connecticut, USA). Optical densitometries of gel bands of interest were normalized to that of vinculin (a cytoskeletal protein used as an internal loading control).

Statistical Analysis

SPSS version 17.0 software was used to complete the statistical analysis. The data are presented as the mean ± SD of three repeated experiments. One-way ANOVA was used to compare the means among the groups, with Tukey's post-hoc test to determine where the significance lies between the different groups. Statistical significance was accepted at $p<0.05$.

CHAPTER IV

RESULTS

TLR4 but not TLR2 is expressed in human hPDLF #2630 cells

As shown below, after electrophoresis and immunoblotting, TLR2 expression at about 90 kD level was minimal. However, TLR4 (a 100 kD protein) is highly expressed in human PDLF cells (Fig. 3-1).

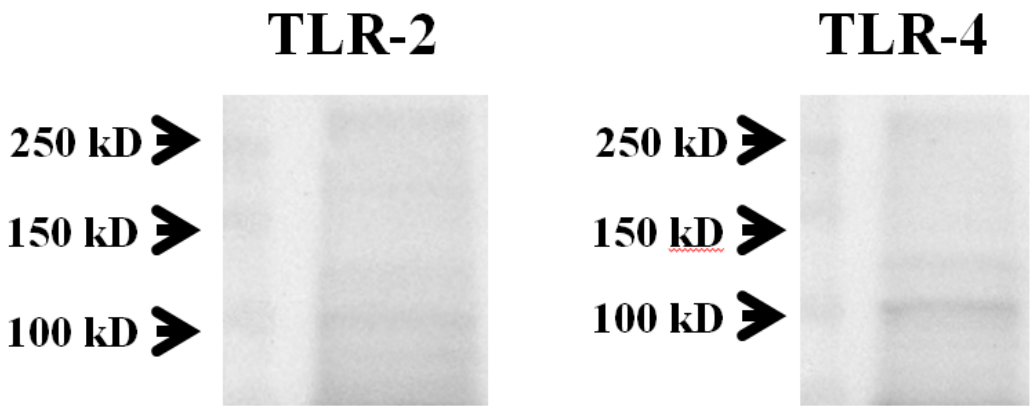


Figure 9. TLR western blot results. TLR4 (~100kD) but not TLR2 (~100kD) is strongly expressed in human PDL cells. The representative result of gel documentation from 3 repeated experiments.

FSS activates MAPK(ERK1/2) signaling pathway in hPDLF #2630 cells

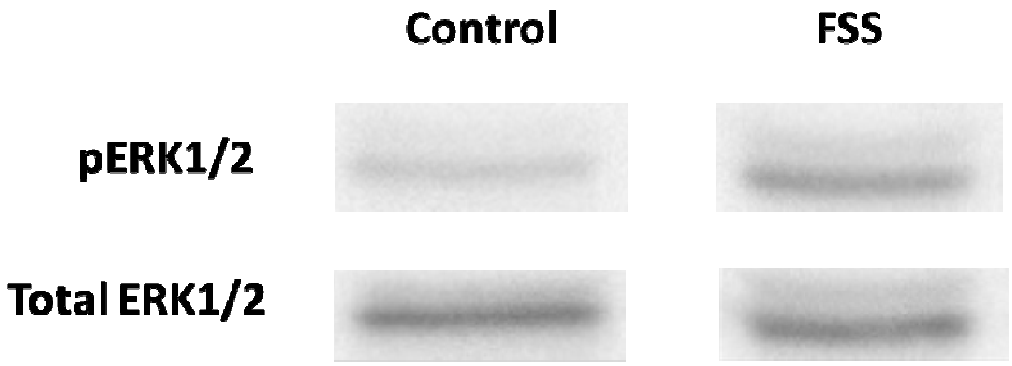


Figure 10. Phosphoryated ERK1/2 western blot results. FSS phosphorylates MAPK(ERK1/2) in hPDLF #2630 cells. Statically pERK1/2 keeps a lower basal level, which is increased by 1 hour of FSS.

FSS reduces the expression of TLR4 on human PDLF #2630 cells, mediated by MAPK(ERK1/2) signaling pathway

Compared to the basal level of TLR4 expression in the static controls, FSS significantly reduced the expression of TLR4 by about 20% (p=0.005, n=3, One-way ANOVA, SPSS version 17.0) (**Figure 11**). When PD98059 was added during FSS, the FSS-induced reduction of TLR4 was significantly recovered back to the basal level as seen in the static controls (p>0.05, n=3, One-way ANOVA, SPSS version 17.0) (**Figure 11**).

Groups	OD (VIN)	OD (TLR4)	OD ratio (TLR4/VIN)
Control-1	1620818	1869580	1.153479293
Control-2	1303116	1634613	1.254387944
Control-3	1468725	1600433	1.089675058
FSS-1	1442648	1353744	0.938374434
FSS-2	1552065	1531855	0.986978638
FSS-3	1848948	1612693	0.872221934
FSS+PD98059-1	1539921	1910625	1.240729232
FSS+PD98059-2	1568221	1902789	1.21334238
FSS+PD98059-3	1462240	1675692	1.145976037

Table 2. Measurements of the three experiments. Gel bands of interest were normalized to that of vinculin (housekeeping protein). FSS significantly reduces the expression of TLR4 by 20% (*$p < 0.01$), which was abrogated when PD98059 - an inhibitor of MAPK (ERK1/2) signaling was added.

Statistical Analysis

One-way ANOVA: GROUP1 (Control), GROUP2 (FSS), GROUP3 (FSS+PD98059)

TLR4/VIN

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	**.127**	**2**	**.064**	**15.146**	**.005**
Within Groups	**.025**	**6**	**.004**		
Total	**.152**	**8**			

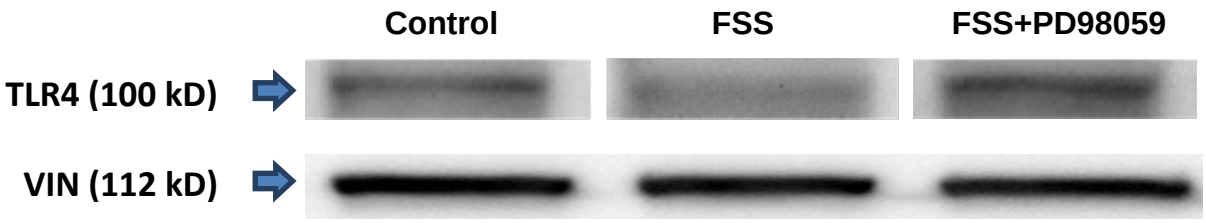
Multiple Comparisons

Tukey's Honestly Significant Difference

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	.23332*	.05290	.011	.0710	.3956
	3.00	-.03417	.05290	.801	-.1965	.1281
2.00	1.00	-.23332*	.05290	.011	-.3956	-.0710
	3.00	-.26749*	.05290	.006	-.4298	-.1052
3.00	1.00	.03417	.05290	.801	-.1281	.1965
	2.00	.26749*	.05290	.006	.1052	.4298

*. The mean difference is significant at the 0.05 level.

Table 3. Statistical analysis. One-way ANOVA was used to compare the means among the groups, with Tukey's post-hoc test to determine where the significance lies between the different groups. Statistical significance was accepted at $p<0.05$.



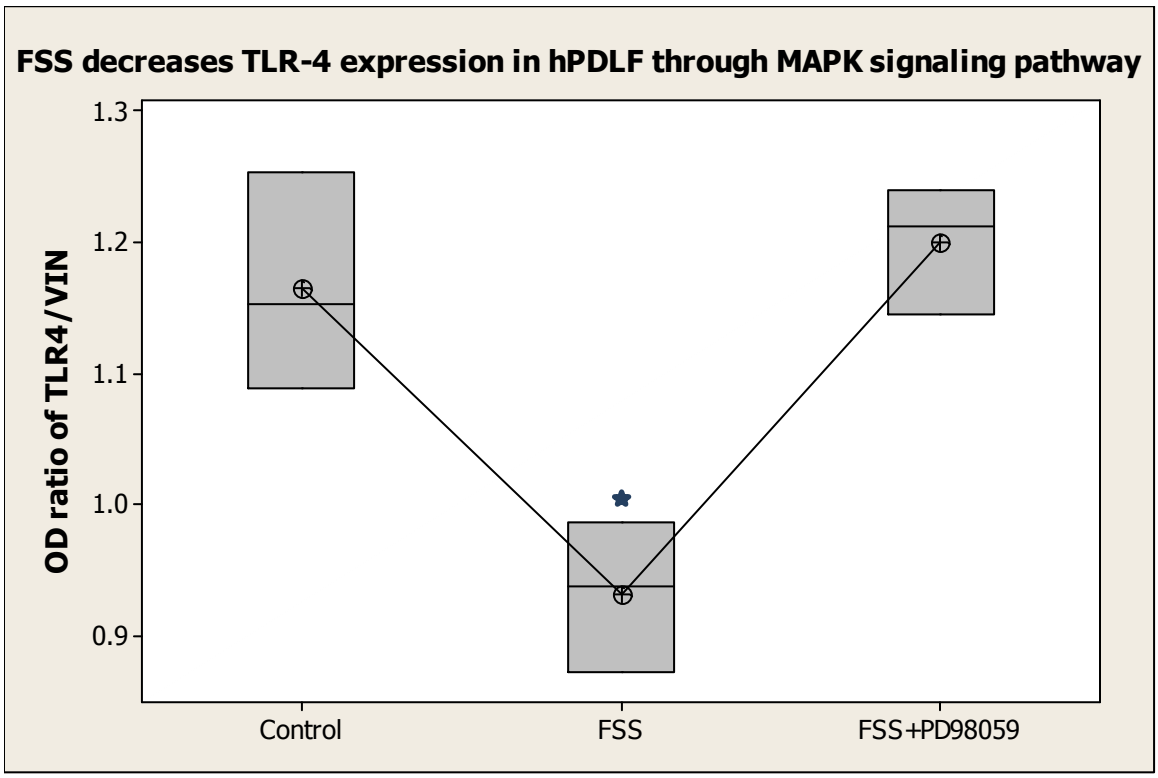


Figure 11. TLR4 expression. FSS significantly reduces the expression of TLR4 by 20% (*$p < 0.01$), which was abrogated when PD98059 - an inhibitor of MAPK (ERK1/2) signaling was added. The representative gel results are presented. Darker gel band represents higher protein expression. TLR4 expression was normalized to that of housekeeping protein vinculin with stable expression.

CHAPTER 4

DISCUSSION

Periodontitis is an oral infectious disease which may result in tooth mobility and loss. Periodontal disease is characterized by loss of alveolar bone resulting from host inflammatory response to presence of periodontal pathogens. Pathogenic periodontal bacteria including *P. gingivalis* contain multiple virulence factors, such as LPS, which activate the host immune response and initiate inflammatory cascades leading to alveolar bone resorption.

Akira et al. reported that LPS from gram-negative periodontal pathogens acts as a microbe-associated molecular pattern recognized through pattern-recognition receptors on immune and non-immune cells within the periodontium (Akira 2003) Studies by Genco and Listgarten showed that the immune response involves recruitment of inflammatory cells, generation of prostanoids and cytokines, elaboration of lytic enzymes, and osteoclast activation (Genco 1998, Listgarten 1995).

The host's immune response to microbial components is mediated by its innate immune system through the expression of a family of type I transmembrane receptor –TLRs (Medzhitov 1997). Each TLR is able to recognize a specific set of molecules. For example, TLR4 recognizes LPS of Gram-negative bacteria. TLR2 can heterodimerize with TLR1 or TLR6, and recognize peptidoglycan, lipopeptide, and lipoproteins (Takeuchi 1999, 2001).

In the first part of the project, the TLR expression on hPDLF was confirmed by Western Blot analysis. Previous studies showed that TLR2 and TLR4 are expressed on periodontal ligament fibroblasts. Sun et al. were able to show for the first time that the expression of certain genes increased or

decreased to regulate the signaling transduction under the stimulation of LPS in human periodontal ligament cells (Sun 2008). Among the activated genes was SITPEC, also known as ECSIT (evolutionarily conserved signaling intermediate in Toll pathways). Studies have revealed that knockdown of SITPEC with SITPEC-specific short hairpin RNA inhibits Toll signaling (Xiao 2000). Therefore, Sun et al. deduced that increased gene expression of SITPEC indicates activation of the Toll pathway. The same study also showed that anti-TLR4 MAb could inhibit lipopolysaccharide-induced IL-6 production in a dose-dependent manner, concluding that TLR4 is a key molecule controlling the production of IL-6 (Sun 2008).

In another study, Hatakeyama et al. compared responses of human periodontal ligament fibroblasts and human gingival fibroblasts in terms of the IL-8 responses of the cells to ultra-purified LPS, with special reference to the TLR system. The authors examined the effects of anti-TLR4 MAb on IL-8 production by fibroblasts in response to LPS. Their results showed that in human gingival fibroblasts, anti-TLR4 MAb significantly inhibited IL-8 secretion induced by LPS in a concentration-dependent manner. In human periodontal ligament fibroblasts, low IL-8 secretion induced by LPS was also inhibited by the anti-TLR4 MAb. This demonstrates that both human periodontal ligament and gingival fibroblasts express TLR4 receptors. The authors then examined the effects of anti-TLR2 MAb on IL-8 secretion by fibroblasts in response to bacterial LPS. In both human periodontal ligament fibroblasts and human gingival fibroblasts, anti-TLR2 MAb did not significantly inhibit IL-8 secretion induced LPS. These findings suggest

that LPS weakly activated human periodontal ligament fibroblasts and strongly activated human gingival fibroblasts through TLR4 and not TLR2 receptors (Hatakeyama et al. 2003).

Furthermore, to study the distribution of TLR in human periodontal ligament fibroblasts and human gingival fibroblasts, Hatakeyama et al. examined mRNA expression by RT-PCR for TLR2, TLR4 in human periodontal ligament fibroblasts, human gingival fibroblasts, human fibroblasts prepared from skin and lung tissue, and human monocytic THP-1 cells. According to the authors, human periodontal ligament fibroblasts showed much higher levels of TLR2 mRNA expression than human gingival fibroblasts, which expressed TLR2 mRNA at a low level. No marked differences were observed in TLR4 expression between human periodontal ligament fibroblasts and human gingival fibroblasts (Hatakeyama et al. 2003).

The authors also examined TLR2 and TLR4 expression in human periodontal ligament fibroblasts and human gingival fibroblasts from the same donor by flow cytometry. Consistent with the mRNA expression results, human periodontal ligament fibroblasts and human gingival fibroblasts clearly expressed TLR4 to similar extents. Human periodontal ligament fibroblasts expressed TLR2 at a low level (Hatakeyama et al. 2003).

These results are consistent with the results of our experiment. In Western Blot protein staining, we demonstrated that hPDLF cells strongly express TLR4. TLR2, on the other hand, are expressed at a much lower level.

The second objective of the project was to study the effect of mechanical stress on the expression of TLRs in hPDLF. The effect of mechanical stress on fibroblasts has been extensively studied in the recent years, but to our knowledge no other studies looked at TLR expression. However, the change in TLR expression under mechanical loading has been studied in other cell types.

Liang et al. showed shear stress effected the expression of TLR4 in endothelial cells. The authors used RT-PCR and Northern hybridization to look at the changes in TLR4 expression on endothelial cells before and after FSS application. Liang et. al. subjected endothelial cells to 4.2 dyne/cm^2 fluid shear stress for 1 hour. The results showed increase in TLR4 expression after fluid shear stress application as compared to control cells.

Another study by Dunzendorfer et al. (2004) investigated the effect of chronic laminar flow on the expression of endothelial TLR2. In the study human coronary artery endothelial cells were subjected to fluid shear stress equal to10 dynes/cm^2. The cells were adapted to the flow for 12 hours and shear stress was maintained for another 12 hours. Afterwards, mRNA expression was assessed by semi-quantitative RT-PCR. The authors observed significant increases in TLR2 mRNA levels. No change was noticed in the expression levels of TLR4 mRNA.

In our study, hPDLF cells were subjected to 12 dynes/cm^2 of shear stress for the period of 1 hour. The shear stress of 12 dynes/cm^2 was chosen because it is the amount of flow most frequently used for studies examining molecular bone regulation mechanisms in osteoblasts, osteoclasts and fibroblasts (Chen et al

2000, Chen et al 2003; Pavalko 2003; Lee et al. 2008, Liu 2008, Rangaswami et al. 2009). The exact magnitude of FSS in PDL currently remains unknown.

A special apparatus, designed and fabricated to apply a fluid shear stress to the hPDLF cells, was used for mechanically loading the cells. Our results show that stimulation of hPDLF with FSS significantly reduced the expression of TLR4. The amount of TLR4 production was 20% less in FSS subjected cells compared to the control cells.

Absence of TLR2 and TLR4 has been shown to lead to decreased alveolar bone loss in mice infected with *P. gingivalis* (Nakamura, 2008; Costalonga, 2009; Lima, 2010). In their animal study, Nakamura et al. concluded that TLR4 is indispensable for LPS-induced bone resorption in vivo.

In line with the available information, we therefore theorize that lowered expression of TLR2 and TLR4 on hPDLF cells could reduce inflammatory response which results from TLR recognition of periodontal pathogens, and subsequently decrease the resulting periodontal destruction.

The third objective in our research was to further study the possible involvement of MAPK signaling pathway. MAPK is a family of protein kinases consisting of three isoforms: ERK1/2, JNK and p38 (Johnson et al 2002). These kinases have been shown to be important in the proliferation, growth, and differentiation in many cell types (Lai et al. 2001, Matsuda et al. 1998, Valledor et al. 2000, Xiao et al. 2000) including osteoblasts (Gabarin et al. 2001, Jessop et al. 2002). Activation of MAPK has been shown to be important in shear induced increases in OPN and COX-2 mRNA expression and cell proliferation (You et al.

2001, Wadhwa et al. et al. 2002, Jiang 2002). But no information has been known about whether MAPK is involved in the regulation of TLR4. In this study we found that FSS activated MAPK, which lead us to hypothesize that MAPK activation regulates TLR4 changes.

In our study, when ERK inhibitor (MAPK signaling pathway inhibitor) was added to cell medium during flow, the decrease in TLR4 expression was reversed. The amount of TLR4 expression in ERK inhibitor group was similar to the control group. This clearly demonstrated that MAPK pathway is involved in the regulation of TLR4 expression.

In a previous study, Liu et al. found that ERK1/2 (not JNK and P38) was activated by fluid shear, becoming phosphorylated within 5 min of the onset of flow with peak activation observed after 30 min (Liu et al. 2008). This observation is consistent with previous studies (Jiang et al 2002). However, other studies have shown that additional MAPK isoforms are sensitive to mechanical stimulation. You et al. reported that both ERK1/2 and p38 were activated in MC3T3-E1 cells that were exposed to oscillating fluid flow (You et al. 2001). These conflicting observations could result from differences in experimental procedure (i.e., serum concentrations) or design (steady versus oscillatory fluid shear). Alternately, p38 and JNK have been shown to be sensitive to oxygen pressure (Matsuda 1998), which could influence the results from cells exposed to oscillatory or steady fluid shear.

Limitations

The purpose of our study was to explore the effect of mechanical stress on the expression of toll-like receptors in human periodontal ligament fibroblasts and the possible involvement of MAPK signaling pathway in the process. While in vitro cellular studies are an unavoidable step to understanding the molecular processes, they need to be followed up by animal and clinical studies to provide complete understanding of the issue. In cell culture, cells exist in isolation without any external influence. This is a major disadvantage of all in vitro studies. Without the interactions that take place in vivo, the complex signaling pathways and mechanisms that contribute to cellular functions and molecular interactions cannot be fully understood. In addition, one must be careful not to over-interpret in vitro results, which could lead to flawed clinical conclusions.

Another limitation of our study is the focus on only two TLR subtypes. Although TLR2 and TLR4 are most commonly studied subtypes in relation to the pathology of periodontal disease, other TLRs could also be involved in the periodontal destruction.

In addition, because the magnitude of FSS in periodontal ligament is unknown at this time, the amount of 12 dynes/cm^2 used in this study might not be representative of the actual FSS created by orthodontic loading in the PDL. The magnitude of 12 dynes/cm^2 was chosen because it is the amount of flow most frequently used in studies examining molecular bone regulation.

Furthermore, the short-term 1 hour application of FSS was used in this project. The reason for using this period of FSS application was based on the

previous studies which demonstrated that 1-hour of FSS is sufficient for detecting molecular changes. However, clinically PDL cells would be subjected to long-term fluid flow which could result in different effects on molecular regulation.

Future Studies

To complement this study, several further studies have been planned, and one of them is currently in progress. To mimic bacterial invasion in PDL and its effect on fibroblasts, *P gingivalis* LPS will be added to cell medium before and during FSS application. From previous research by Wang et al. we know that the expression of TLR2 and TLR4 increases upon stimulation with *P. gingivalis* LPS (Wang, 2000). By applying LPS before and during FSS we would explore if similar reduction in LPS-stimulated TLR2 and TLR4 will be seen after flow.

Another study is planned to represent and explore the effects of bacterial inflammation during orthodontic treatment. In this experiment, LPS will be added to cell medium after the application of FSS. This study will explore the change in TLR4 expression after the initial FSS-induced reduction in the receptor production. This experiment will help understand the changes that occur in periodontal ligament of periodontally-healthy patients subjected to periodontal pathogens (poor oral hygiene) during orthodontic treatment.

CHAPTER 5

CONCLUSIONS

Human periodontal ligament fibroblasts strongly express TLR4 but not TLR2. Stimulation of hPDLF cells with fluid shear stress significantly reduces the expression of TLR4 which is mediated by MAPK signaling pathway. As TLR4 activation is the first step in LPS-induced periodontal inflammation, FSS-induced reduction in TLR4 expression could have a protective role against inflammatory periodontal destruction. Furthermore, FSS application mimics the loading on PDL cells under light orthodontic forces. Likely, light orthodontic force could favorably be used to move the periodontally compromised teeth without jeopardizing periodontal tissues.

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