Oral Diseases (2006) 12, 171–175. doi: 10.1111/j.1601-0825.2005.01179.x

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ORIGINAL ARTICLE

Mechanical stress induces expression of cytokines in human periodontal ligament cells

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OBJECTIVE: Periodontal tissue has a unique structure in that the human periodontal ligament (hPDL) lies between the hard tissues of cementum and alveolar bone. Although the role of cytokines in hPDL function is not clearly understood, we investigated the effect of mechanical stress as hydrostatic pressure (HP) on cytokine expression in hPDL cells.

MATERIALS AND METHODS: The hPDL cells were obtained from a healthy maxillary third molar. After the third to fourth passage, the cells were exposed to HP ranging from 1 to 6 MPa as previously described. Total RNA was extracted and the expression of cytokine mRNA was determined by RT-PCR.

RESULTS: The exposure to 6 MPa of HP caused no morphological changes of hPDL cells, and did not affect the cellular viability. No expression of IL-1β, IL-6, IL-8, TNF-α, receptor activator of NF-λB (RANK), receptor activator of NF-λB ligand (RANKL), or osteoprotegerin mRNA was observed in the control cells under atmospheric pressure, whereas, in hPDL cells treated with HP, a pressure-dependent enhancement of IL-6, IL-8, RANKL, and OPG mRNA expression was observed between 10 and 60 min after the exposure to HP.

CONCLUSION: These results suggest that hPDL cells may play a role in the production of cytokines in response to mechanical stress *in vivo*.

Oral Diseases (2006) **12**, 171–175

Keywords: periodontium; immunology; pathogenesis

Introduction

Periodontal tissues comprise gingiva epithelia, cementum, periodontal ligament, and alveolar bone, and have a unique structure in that the human periodontal ligament (hPDL) lies between the cementum and alveolar bone. The hPDL functions as a cushion mitigating the mechanical forces under mastication, and in so doing might be subjected to such forces. It is well known that a local immune response is caused by inflammation and bone absorption by mechanical stress in hPDL cells (Saito *et al*, 1991; Yamaguchi and Shimizu, 1994; Kanzaki *et al*, 2002). It has now been reported that gene expression was induced by mechanical stress in hPDL cells (Myokai *et al*, 2003). In addition, these cells do not show specific markers (Kawashima, 2001), and it is difficult to analyze the function of hPDL cells *in vitro*. Although hPDL cells undergo biological changes in response to mechanical stress, it is not clear whether mechanical stress of a certain magnitude has a mastication-like effect on these cells. Hydrostatic pressure (HP) as mechanical stress that includes tension force, compressive force, and stretching force, has an effect on osteoblasts (Haskin and Cameron, 1992; Haskin *et al*, 1993) and odontoclasts (Rubin *et al*, 1997); and is used in the orthopedic surgery. hPDL cells produced prostaglandin E and intercellular cyclic AMP (cAMP), in response to HP (Yousefian *et al*, 1995), however, the roles of cytokines in hPDL cells are not clearly understood. In the present study, an attempt was made to examine whether HP can induce the expression of cytokines in hPDL cells.

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Received 8 June 2005; accepted 20 June 2005

Materials and methods

Preparation of hPDL cells

The hPDL cells were obtained from a healthy erupted maxillary third molar from three donors (21-year-old female, 23-year-old male, and 24-year-old female) for orthodontic reasons with informed consent. The tissue was minced and cultured as explants in DMEM containing 10% FBS and antibiotics as previously described (Yamamoto *et al*, 2004a,b). After three to four passages, the cells were used for experiments. This experimental procedure was approved by the Ethics Committee, Kyoto Prefectural University of Medicine.

Hydrostatic pressure apparatus

The apparatus used in this study (Figure 1a,b) allows sterile manipulation and can apply up to 6 MPa of HP to cultured cells at a constant level and temperature.

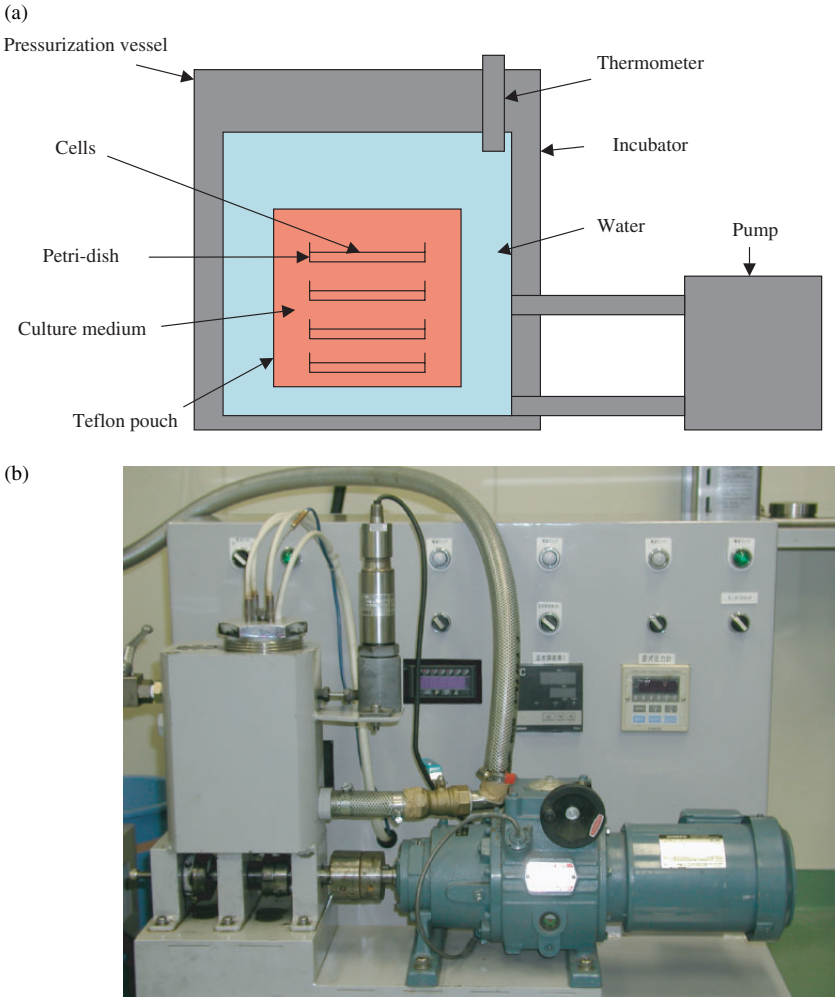

Figure 1 Schematic diagram (**a**) and photograph (**b**) of the special hydrostatic pressure (HP) apparatus. The apparatus was used to apply arbitrary HP to cells. The Petri dishes (35 × 10 mm) were placed in a deformable Teflon pouch. Water is used as the pressure medium and pressure is transmitted to the cells through the packed Teflon pouch. The culture medium is in a gas-free environment. The temperature is maintained at 37°C by using a thermostat placed in the pressure vessel

The Petri dishes were placed in a deformable Teflon pouch that was filled with serum-free DMEM. The pouch was placed in a stainless-steel pressurization vessel. Water was used as the pressure medium, and pressure was transmitted to the cells through the packed Teflon pouch in the culture medium in a gas-free environment. The temperature was maintained at 37°C by using a thermostat placed in the pressure vessel. A great advantage of this HP apparatus is the separation of the pressure medium from the air. In addition, because the pressure was transmitted to the cells by water, there was less contamination than in our previous studies (Takahashi *et al*, 1997; Ishida, 1998; Kubo *et al*, 1998) and the reproducibility was improved in an aseptic environment.

Loading of hydrostatic pressure

The hPDL cells were seeded onto Petri dishes at a concentration of 1×10^6 cells dish^{-1}. The cells reached confluence following approximately 1 week of culture, and then were exposed to HP of 1 MPa for 10 min, 1 MPa for 60 min or 6 MPa for 60 min. We confirmed that the pH of the DMEM inside the Teflon container was constant at 7.4 before and after the experiment. Cells seeded in Petri dishes and placed in the same apparatus under the same conditions were used as non-pressurized controls.

Expression of cytokine mRNA

Total RNA was extracted using ISOGEN® (Nippon gene, Tokyo, Japan) and the expression of cytokine mRNA was determined by the reverse transcription-polymerase chain reaction (RT-PCR) method. We analyzed the mRNA expression of interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor-α (TNF-α), receptor activator of NF-λB (RANK), receptor activator of NF-λB ligand (RANKL), and Osteoprotegerin (OPG). β-actin was used as an internal control. The primer sequences are shown in Table 1. For RT-PCR, we used our previously reported procedure (Kita *et al*, 1994). Briefly, total RNA was extracted with ISOGEN® and cDNA was produced using Superscript RNase H Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), oligo dT primer, and 2.5 mmol l^{-1} dNTP Mixture (Takara Shuzo, Otsu, Japan). PCR was performed for 35 cycles, each consisting of 1 min at 95°C for denaturation, 1 min at 50°C for annealing and 1 min at 72°C for extension. Ten microliters of each PCR product was analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide

Table 1 Polymerized chain reaction primers, sequences and predicted size

Cytokine	*Sequences*	*Predicted size*
IL-1β		
Sense	ATAAGCCCACTCTACAGCT	443
Antisense	ATTGGCCCTGAAAGGAGAGA	
IL-6		
Sense	GTACCCCCAGGAGAAGATTC	819
Antisense	CAAACTGCATAGCCACTTTC	
IL-8		
Sense	GCTTTCTGATGGAAGAGAGC	585
Antisense	GGCACAGTGGAACAAGGACT	
TNF-α		
Sense	TCGGGCCAATGCCCTCCTGGCCAA	468
Antisense	GTAGACCTGCCCAGACTCGGCAAA	
RANK		
Sense	TTAAGCCAGTGCTTCACGGG	497
Antisense	ACGTAGACCACGATGATGTCGC	
RANKL		
Sense	CAGCACATCAGAGCAGAGAAAGC	517
Antisense	CCCCAAAGTATGTTGCATCCTG	
OPG		
Sense	GTACGTCAAGCAGGAGTGCAATC	472
Antisense	TTCTTGTGAGCTGTGTTGCCG	
β-actin		
Sense	GTGGGGCGCCCCAGGCACCA	541
Antisense	CTCCTTAATGTCACGCACGATTTC	

IL, interleukin; TNF, tumor necrosis factor; RANK, receptor activator of NF-λB; RANKL, receptor activator of NF-λB ligand; OPG, osteoprotegerin.

(Bio-Rad Laboratories, Hercules, CA, USA), and the bands were visualized under UV light.

Morphologic changes of the cells
Before and after the loading of HP, morphologic changes of hPDL cells were observed using an inverted optical microscope (Olympus, Tokyo, Japan). The size of the cells was assessed at a simple glance. The viability of hPDL cells was examined using the trypan blue exclusion test.

Results

Expression of cytokine mRNA in hPDL cells
hPDL cells were exposed to HP ranging from 1 to 6 MPa. No expression of IL-1β, IL-6, IL-8, TNF-α, RANK, RANKL or OPG mRNA was observed in the control cells under atmospheric pressure, whereas in hPDL cells treated with HP, IL-6, and OPG mRNA were observed after the exposure to 1 MPa of Hp for 10 min. In addition, the expression of IL-8, TNF-α, and RANKL mRNA was specifically induced following exposure to 1 MPa of Hp for 60 min. After 6 MPa of Hp for 60 min, the expression of IL-6, IL-8, RANKL, and OPG mRNA was observed (Figure 2). Each experiment was performed eight to nine times. We carried out the same experiment using other hPDL cells, and obtained the same results.

Morphologic changes of the hPDL cells
The exposure to 6 MPa of HP caused no changes in the morphology of hPDL cells, and did not affect cellular viability (data not shown). hPDL cells definitely did not exhibit morphologic change (Figure 3).

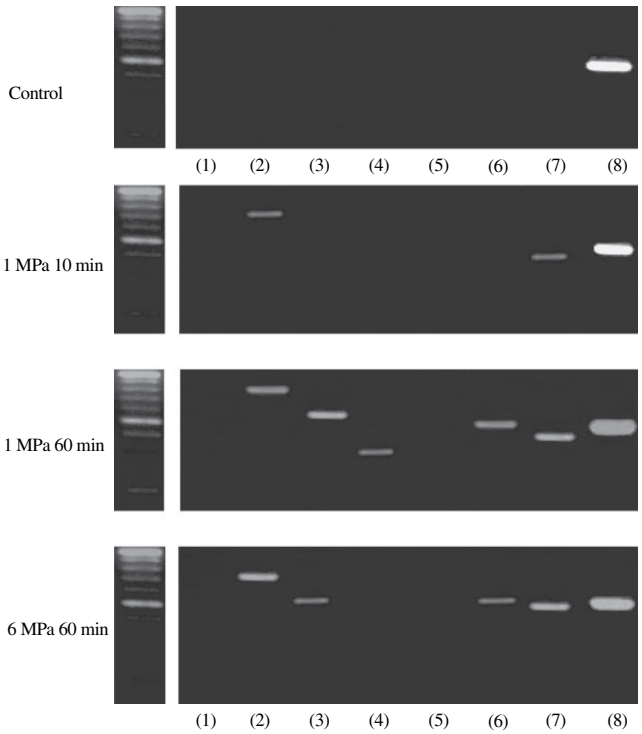

Figure 2 Expression of cytokine mRNA in human periodontal ligament (hPDL) cells after exposure to HP. The expression of interleukin (IL)-6, IL-8, tumor necrosis factor (TNF)-α, RANKL and OPG in hPDL cells after exposure to HP. Lanes from left to right: (1) IL-1β, (2) IL-6, (3) IL-8, (4) TNF-α, (5) RANK, (6) RANKL, (7) OPG, (8) β-actin. After the exposure to 1 and 6 MPa of Hp for 60 min, the expression of IL-6, IL-8, RANKL and OPG mRNA was augmented magnitude-dependently. There was no expression of TNF-α mRNA after exposure to 6 MPa

Discussion

Periodontal ligament maintains homeostasis through occlusion, with inflammation being caused by bruxism, traumatogenic occlusion and excessive orthodontic force, etc. and degeneration being caused by traumatogenic occlusion and orthodontic tooth movement. On the other hand, mechanical stress, i.e. compressive and tensile force, causes a local immune response, inflammation, and bone resorption (Saito *et al*, 1991; Yamaguchi and Shimizu, 1994; Kanzaki *et al*, 2002). In the present study, it was demonstrated that hPDL cells produced cytokines in response to HP as mechanical stress. Regarding inflammatory cytokines, it was reported that hPDL cells expressed IL-1β in response to tensile force (Saito *et al*, 1991), and endogenous IL-1β and PGE_2 production was reduced by ALP activity (Yamaguchi and Shimizu, 1994; Yamaguchi *et al*, 1996). hPDL cells also showed reduced expression of IL-1β, IL-6, and IL-8 mRNA in response to tensile force, and as a result, IL-1β-induced inflammation was suppressed. The production of inflammatory cytokines was also suppressed by IL-10 (Long *et al*, 2001). The present

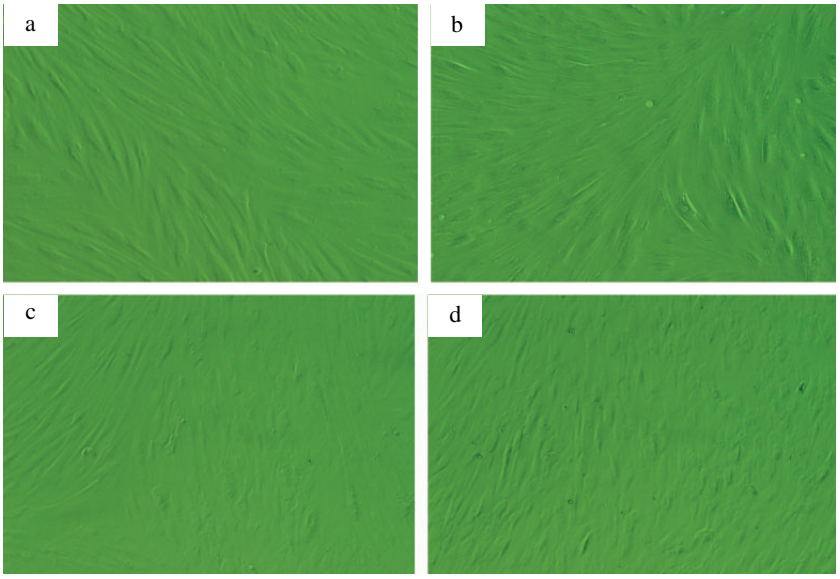

Figure 3 Photomicrographs of hPDL cells after exposure to hydrostatic pressure (HP); (**a**) control, (**b**) HP 1 MPa 10 min, (**c**) HP 1 MPa 60 min, (**d**) HP 6 MPa 60 min. hPDL cells were not influenced by exposure to HP. (Original magnification ×100)

results indicated that not IL-1β mRNA but IL-6 and IL-8 mRNA are expressed after loading of HP, similar to the finding made by Terada (2000). Interestingly, the expression of TNF-α mRNA was augmented time-dependently after exposure to 1 MPa of HP (Figure 2), suggesting that cytokine expression was magnitude-dependent as well as time-dependent. There was no expression of TNF-α mRNA after exposure to 6 MPa of HP. Thus, it will be necessary to quantify the cause of these alterations. These findings demonstrated that hPDL cells expressed inflammatory cytokines in response to HP and tensile force. Tominaga *et al* (1999) reported that the expression of inflammatory cytokines is augmented by excessive tensile force and periodontitic bacterium, and may promote destruction in periodontal tissue. It is possible that inflammatory cytokines act as the pathogenic factors in periodontal tissue.

It is not unusual for hPDL cells to be exposed to 6 MPa of HP. Nagai *et al* (2000) found the pressure from physiologic occlusion to be 6.9 $\pm$ 0.4 MPa, therefore, our model closely resembled the environment *in vivo*. In fact, the exposure to 6 MPa of HP caused no changes in the morphology of hPDL cells, and did not affect the cellular viability.

The advantages of this HP apparatus are (i) the environment is less aseptic than in our previous study, (ii) the pressure can be maintained, (iii) a different pressure can be gradually loaded and (iv) the pressure medium is separated from the air. Without such separation, the air dissolves into the culture medium, the pH of the medium then changes, and this pH change greatly affects the cells. Therefore, this apparatus is excellent for the analysis of pressure loading.

The expression of RANKL and OPG mRNA is involved in osteolysis at the hip joint (Tiba *et al*, 2002a) and rapidly destructive coxopathy (Tiba *et al*, 2002b). Both the up-regulation of RANKL expression and down-regulation of OPG expression have been demonstrated in marginal periodontitis (Liu *et al*, 2003). In response to compressive force, hPDL cells showed augmented expression of RANKL mRNA in a force- and time-dependent manner, and co-culture with PGE_2 induced production of RANKL (Kanzaki *et al*, 2002). Nukaga *et al* (2003) have reported that the expression of RANKL mRNA in hPDL cells was enhanced by IL-1β. In the present study, HP induced the expression of RANKL and OPG mRNA. The cytokines produced by hPDL cells in response to mechanical stress, especially HP, may affect bone metabolism. It was previously noted that the expression of TNF-α mRNA was associated with that of RANKL and OPG in radicular cyst walls (Yamamoto *et al*, 2004a,b), indicating a close correlation between inflammatory cytokines, RANKL and OPG.

In conclusion, the present study demonstrated that hPDL cells produced many kinds of cytokines in response to HP. These results suggest that a function of hPDL cells is to produce cytokines under mechanical stress. However, further studies are needed to characterize the roles of hPDL cells *in vivo*.

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