

# Effect of Mechanical Stress on the Proliferation and Expression of Cell Cycle Regulators in Human Periodontal Ligament Cells

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## I. Introduction

Periodontal ligament(PDL) cells are major fibroblastic cells in periodontal connective tissue. They serve in maintenance and remodeling of the periodontal ligament. Much is known regarding the regulation of proliferation, differentiation, and activity of the cells in the periodontal ligament during normal function. But less is known about alterations on their phenotypic expression under specific conditions like a mechanical stress. It is likely that PDL cells stimulated by the mechanical forces produce some important local factors that participate in the maintenance and remodeling of the ligament itself. It has been reported that PDL cells increase the levels of prostaglandin E<sup>1,2)</sup>, interleukin - 1<sup>3,4)</sup>, cyclic adenosine monophosphate(cAMP)<sup>2,5)</sup>, DNA synthesis<sup>6)</sup>, and collagen synthesis<sup>6)</sup> in response to mechanical stress. PDL cells also activate the plasminogen activator in response

to tension force<sup>7)</sup>. In contrast, alkaline phosphatase activity and its mRNA level were decreased by tension force<sup>8)</sup>. So it is likely that mechanical stress evokes biochemical responses in PDL cells.

In order to understand both the development and the differentiation of normal tissues, it is necessary to study the nature and regulation of the normal cell cycle. Increasing evidences suggest that the growth of tissues in both health and disease is a balance between cell proliferation and cell death. Most reproductive cells begin the process that lead to cell division whose objectives are to produce a pair of identical daughter cells. There are two closely related families of proteins that are involved in regulating the cell cycle: the cyclin dependent kinases(cdks) and the cyclins themselves<sup>9,10)</sup>. The cdks are serine/threonine protein kinases. They are functional only when combined with one of the cyclins, and each mammalian cyclin can interact

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with multiple cdks. Cyclin function is primarily controlled by changes in cyclin levels while cdks are activated by phosphorylation of a conserved threonine residue. The cyclins regulate the activities of the cdks and control their ability to modulate the enzymes involved in driving the cell cycle<sup>11</sup>). Hence, the assembly, activation, and disassembly of the cyclin - cdk complex are the critical events in controlling the cell cycle. The cyclins undergo synthesis and degradation during each cycle of the cell. There are two main classes of cyclins. One is the G1 cyclins that bind cdks during the G1 phase and are required for entry into the S phase. The other is the mitotic cyclins that bind cdks during the G2 to permit the entry of the cell into M phase<sup>12</sup>). The G1 cyclin complexes exist as the quaternary structures of cyclins, cdks, the universal cdk inhibitor p21<sup>WAF1/CIP1</sup>, and proliferating cell nuclear antigen(PCNA)<sup>13 - 20</sup>. Progression from G1 to S phase requires coordinated and independent activation of both cyclin D complexes in early G1 and cyclin E complexes in later G1<sup>21 - 24</sup>). D - type cyclins(D1, D2, D3) are associated with either cdk 4 or cdk 6, while cyclin E is associated with cdk 2. These cyclins play distinct roles in the regulation of the G1 - S phase transition. Both cdk and cyclin are once associated with each other and activated by cyclin - activating kinase<sup>25 - 27</sup>).

Cyclin dependent kinase inhibitors are proteins that bind and inhibit the activity of cdks. One of them to be identified is p21<sup>WAF1/CIP1</sup> which binds and inhibits G1 cyclin - cdks complexes<sup>28</sup>). p21<sup>WAF1/CIP1</sup> is a constant part of G1 cyclin complexes, but

only acts as a cdk inhibitor when its stoichiometric quantities increase from one to several/unit complexes<sup>18 - 20</sup>). In addition to its kinase inhibitor activity, p21<sup>WAF1/CIP1</sup> is able to inhibit DNA replication in the S phase of the cell cycle by interaction with PCNA, blocking the ability of PCNA to activate DNA polymerase<sup>29,30</sup>).

The p53 tumor suppressor protein is a transcription factor required for the trans - activation of a number of genes involved in growth control<sup>31,32</sup>). Inactivation of wild - type p53 function can lead a growth advantage. It has been demonstrated that DNA damage activates p21<sup>WAF1/CIP1</sup> transcription in a p53 - dependent manner in human fibroblasts and epithelial cells<sup>33</sup>). In contrast, several studies on p53 - independent induction of p21<sup>WAF1/CIP1</sup> following DNA damage have also been reported<sup>34</sup>). It has also been shown that p53 can inhibit cell cycle progression without induction of p21<sup>WAF1/CIP1</sup> expression<sup>35</sup>), thus, p21<sup>WAF1/CIP1</sup> may arrest cell cycle progression following p53 - independent activation, and at the same time p53 may promote cell cycle arrest by activation of genes other than p21<sup>WAF1/CIP1</sup>.

PCNA is an essential component of the DNA replication machinery, functioning as the accessory protein for DNA polymerase  $\delta$ , required for processive chromosomal DNA synthesis, and DNA polymerase  $\epsilon$ , required for repair of nuclear DNA. PCNA is also required for DNA recombination and repair<sup>36</sup>). In addition, PCNA is shown to interact with cell cycle regulatory proteins, cdks, cyclin D, and p21<sup>WAF1/CIP1</sup><sup>13,27,37</sup>).

Mechanical stress is known to be associ -

ated with proliferation of PDL cells. Though detailed mechanisms for the proliferation of PDL cells by mechanical stress remain largely unknown, it may be due to increased expression of cell cycle regulatory proteins from the cells. The purpose of this study is to investigate on the growth pattern and expression of p53, p21<sup>WAF1/CIP1</sup>, cdk's, cyclins, and PCNA in PDL cells exposed to mechanical stress.

## II. Materials and Methods

### 1. Cell cultures

Human PDL cells were cultured from periodontal ligament explants of healthy premolar teeth extracted for orthodontic reason. The periodontal ligament tissues were teased from the mid - root surface, washed in Hanks' balanced salt solution, and plated onto 60 - mm culture dish. Separated tissues were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco/BRL, Grand Island, NY, U.S.A.) supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Confluent cells were subcultured up to six passages by trypsinization. The cultures with the passage number of 6 were used in this experiment.

### 2. Mechanical stress

Confluent cell monolayers in 100 - mm Petri dishes were trypsinized. The cells were suspended in culture medium and  $1 \times 10^4$  cells were plated onto 55 - mm Petriperm dishes with a hydrophilic flexible

plastic growth surface (Heraeus Instruments, South Plainfield, NJ, USA). The bottom of the dish was stretched by placing a template with a convex surface under the dish and placing a stainless steel weight (1kg/dish) on the top of the dish. Because the convex surface of dish is uniformly curved, stretching occurs evenly over bottom of the dish. The bottom surface curvature of the template is an arc of 36° and a radius of 7.6cm<sup>38</sup>).

### 3. Determination of Cell proliferation rate

Confluent cell monolayers in 100 - mm Petri dishes were trypsinized. The cells were suspended in culture medium, and  $1 \times 10^4$  cells were plated onto 55 - mm Petriperm dishes. The number of viable cells were counted after 0, 2, 4, 6, 8, 10 and 12 days of incubation under mechanical stress at 37 °C by trypan blue exclusion. There were six cultures in each group at each time.

### 4. Western blot analysis

Confluent cell monolayers in 100 - mm Petri dishes were trypsinized, and  $1 \times 10^4$  cells were plated onto 55 - mm Petriperm dishes. These cells were exposed to mechanical stress for 0, 2, 6, and 10 days. Protein was isolated from cells grown in 55 - mm Petriperm dishes using Tri - reagent solution (Molecular Research Center, Inc., Cincinnati, OH, U.S.A.) under the conditions recommended by the manufacturer. Protein concentration was deter -

mined by Lowry method<sup>39</sup>). The denatured supernatant containing 50  $\mu$ g of protein was electrophoresed in a 12.5% SDS - poly - acrylamide gel and transferred onto a nitrocellulose membrane(BioRad, Hercules, CA, U.S.A.). To reduce nonspecific anti - body binding, the membrane was incubated in a blocking solution(Zymed, San Francisco, CA, U.S.A.) for 1 h at room temperature. The membrane was exposed to a mouse anti - human monoclonal anti - body for p53(Ab - 2; Oncogene Science, Uniondale, NY, U.S.A.), a mouse anti - human monoclonal antibody for

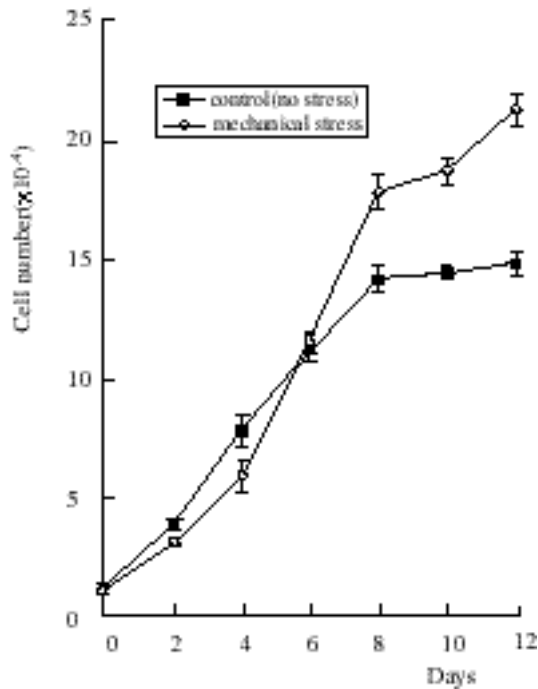


Figure 1. Growth curves of human PDL cells exposed to mechanical stress. Cells were plated at  $1 \times 10^4$  cells per 55 - mm Petriperm dish, and cultured for 0, 2, 4, 6, 8, 10 or 12 days in the presence of mechanical stress(1 kg/dish). Viable cells were counted with a hemocytometer by trypan blue exclusion. Values represent averages from six indepen -

p21<sup>WAF1/CIP1</sup>(Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), a rabbit anti - human polyclonal antibody for cdk 2(Santa Cruz Biotechnology), a rabbit anti - human polyclonal antibody for cdk 4(Oncogene Science), a rabbit anti - human polyclonal antibody for cdk 6(Santa Cruz Biotechnology), a mouse anti - human monoclonal antibody for cyclin D1(Santa Cruz Biotechnology), a mouse anti - human monoclonal antibody for cyclin E(Oncogene Science) and a mouse anti - human monoclonal antibody for PCNA(Oncogene Science) at room temperature for 90 min. After washing with PBS, the membrane was treated with anti - mouse or anti - rabbit IgG - horse raddish peroxidase conjugated

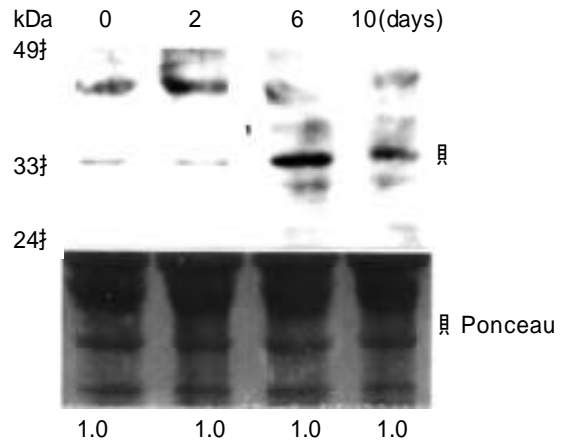


Figure 2. Western blot analysis for the intracel - lular PCNA level in human PDL cells exposed to mechanical stress for 0, 2, 6, or 10 days. Cell extract equivalent to 50 $\mu$ g of total cellular protein of PDL cells was electrophoresed by 12.5% SDS - PAGE and transferred to a nitro - cellulose membrane. The intracellular protein level of PCNA in PDL cells was probed with antibody diluted by 1:1000. After probing, the membrane was stained with 1X Ponceau S stain for 10 min to reveal the total cellular protein loaded per each lane. The densito -

secondary antibody for 1 h, and washed again with PBS. The membrane was then incubated in ECL western blotting detection reagent (Amersham, Buckingham - shire, U.K.), and was exposed to Hyperfilm - MP (Amersham) for a few min. The membrane was also stained with 1 × Ponceau S. The relative levels of proteins were determined by densitometric scanning of autoradiograms.

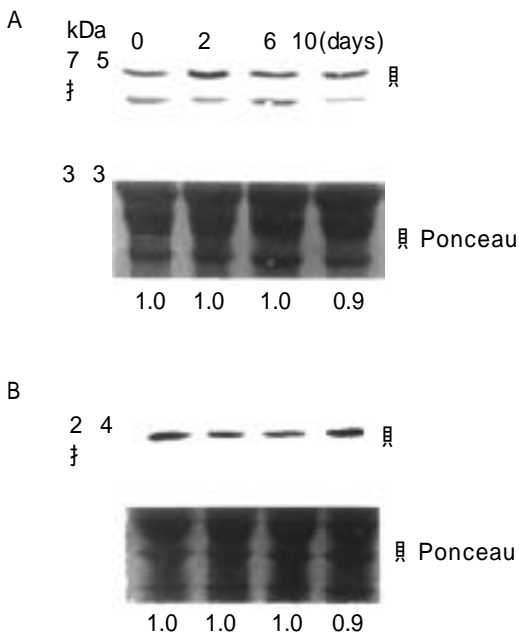


Figure 3. Western blot analysis for the intracellular p53 and p21WAF1/CIP1 levels in human PDL cells exposed to mechanical stress for 0, 2, 6, or 10 days. Cell extract equivalent to 50  $\mu$ g of total cellular protein of PDL cells was electrophoresed by 12.5% SDS - PAGE and transferred to a nitrocellulose membrane. The intracellular protein levels of p53 and p21<sup>WAF1/CIP1</sup> in PDL cells were probed with respective antibodies diluted by 1:1000. After probing, the membrane was stained with 1X Ponceau S stain for 10 min to reveal the total cellular protein loaded per each lane. The densitometric ratio of the Ponceau S stain per

## 5. Statistical analysis

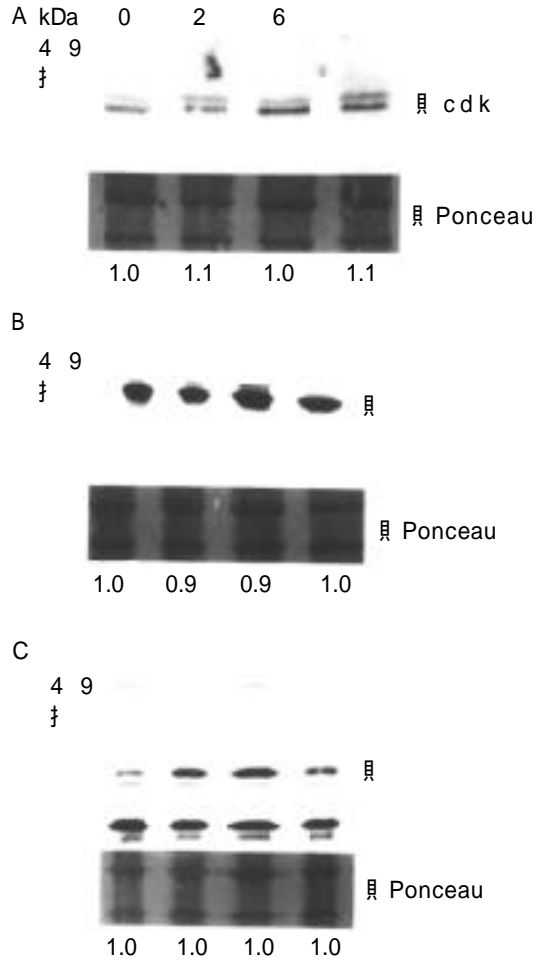


Figure 4. Western blot analysis for the intracellular levels of cdk in human PDL cells exposed to mechanical stress for 0, 2, 6, or 10 days. Cell extract equivalent to 50  $\mu$ g of total cellular protein of PDL cells was electrophoresed by 12.5% SDS - PAGE and transferred to a nitrocellulose membrane. The intracellular protein levels of cdk in PDL cells were probed with respective antibodies diluted by 1:1000. After probing, the membrane was stained with 1X Ponceau S stain for 10 min to reveal the total cellular protein loaded per each lane. The densitometric ratio of the Ponceau S stain per

Values were calculated as the mean  $\pm$  standard deviation(S.D.). Statistical significance was evaluated by one way analysis of variance(ANOVA) using SAS program of computer.

### III. Results

#### 1. Cell proliferation rate

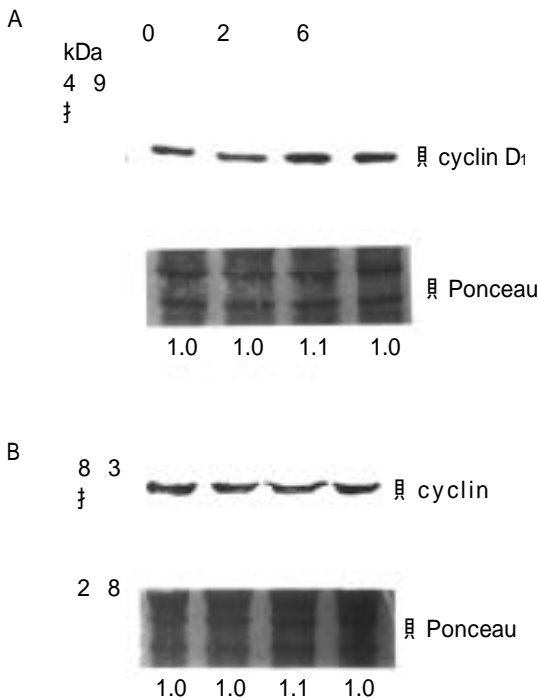


Figure 5. Western blot analysis for the intracellular levels of G1 cyclins(cyclin D1 and E) in human PDL cells exposed to mechanical stress for 0, 2, 6, or 10 days. Cell extract equivalent to 50  $\mu$ g of total cellular protein of PDL cells was electrophoresed by 12.5% SDS - PAGE and transferred to a nitrocellulose membrane. The intracellular protein levels of G1 cyclins in PDL cells were probed with respective antibodies diluted by 1:1000. After probing, the membrane was stained with 1X Ponceau S stain for 10 min to reveal the total cellular protein loaded per each lane. The densitometric ratio of the Ponceau S stain per each lane to that of

Human PDL cells proliferated well in DMEM supplemented with 10% FBS. There was a remarkable increase in the number of cells in the mechanically stressed cultures as compared to the non - stressed controls. Cell numbers of stressed and non - stressed periodontal ligament cells exhibited different proliferation rates in culture during a 12 - day period(Figure 1). Before day 6, the number of non - stressed PDL cells was slightly higher than that of stressed PDL cells. But after day 6, the proliferation rate of stressed PDL cells was notably higher than that of non - stressed PDL cells.

#### 2. Expression of PCNA

Because PCNA is a molecular marker of cell proliferation, the level of PCNA was studied. Expression of PCNA was increased after day 6 as compared to that of control in the same way as the cell proliferation rate discussed above(Figure 2). On day 6, the expression level was notably increased as compared to that of control. And on day 10, it was also increased as compared to that of control, but it was decreased when compared to that of day 6.

#### 3. Expression of p53 and p21<sup>WAF1/CIP1</sup>

Because p53 can regulate the expression of genes involved in cell cycle, and because the cdk inhibitory protein p21<sup>WAF1/CIP1</sup> can bind cyclin - cdk complexes and thereby inhibit their activities, the expression level of both proteins was investigated(Figure 3). The level of p53 and p21<sup>WAF1/CIP1</sup> protein in

stressed PDL cells was consistent throughout all the experimental periods.

#### 4. Expression of cyclins and cdk

Because the proteins involved in the middle G1 phase were cyclin D1 and cdk 4 or 6, and because regulatory proteins involved in the late G1 phase were cyclin E and cdk 2 in normal cells, western blot analysis was carried out to determine the nature of this complex in periodontal ligament cells. It revealed that cdk 4 was present at a low level on day 2, but it was notably increased on day 6 and 10 (Figure 4A). The increase rate was slightly reduced on day 10 when compared to day 6. It might be due to plateau of cell proliferation. The expression of cdk 6 (Figure 4B) and cyclin D1 (Figure 5A) was shown a slightly different pattern from that of cdk 4. There was a slightly increasing tendency on day 6 and 10 when compared to non-stressed cells. The level of cdk 2 was increased during the experimental periods when compared to that of control cells (Figure 4C), especially the expression on day 6 was notably increased as compared to that of any other experimental period. The degree of expression of cyclin E showed no change on day 6 and 10 (Figure 5B).

### IV. Discussion

In the present study, when PDL cells were stressed by mechanical stimulation, cell proliferation and the levels of cell cycle progressing proteins, cyclin D1, cdk 4, 6 and 2, and PCNA, were increased after day

6. It was shown that increased expression of cdk

6 caused cell proliferation in PDL cells by mechanical stress. p53, a archetypical checkpoint regulator of cell cycle progression, and p21<sup>WAF1/CIP1</sup>, a universal negative regulator of cell cycle, however, had little effect on cell cycle progression in this experiment.

When cells are stimulated by mechanical stress, there are many changes in biochemical characteristics as well as in cell number. The effect of stretching on cellular proliferation appears to vary with cell type. Epithelial cells which were derived from the cell rests of Malassez exhibited a 92% increase in the labeling index<sup>40</sup>, but Leung et al. showed no consistent increase in DNA synthesis as a result of cyclic stretching of smooth muscle cells<sup>41</sup>. Somjen et al. reported that stretching caused a 45% increase in the amount of DNA synthesis in bone cell cultures<sup>42</sup>. And some investigators have shown an increase in the level of cAMP after mechanical stress was applied to bone cells<sup>43,44</sup>, but Harell et al. showed that a prolonged decrease followed an initial increase in the level of cAMP<sup>45</sup>. In the present study, an increase of PDL cells was observed as a whole, but there was a decrease in cell number at early time period. It might be because the number of cells was not large enough to be influenced by mechanical stress or because there was no direct effect of mechanical stress on PDL cells.

The biochemical mechanisms are poorly understood which were involved in the conversion of mechanical stimuli into biological response by signal transduction in

cells. Although the mechanism for the detection and conversion of mechanical force into a biochemical signal has yet to be determined, several pathways have been proposed: i) one possible transduction pathway is the extracellular matrix - integrin - cytoskeleton machinery, ii) mechanosensitive channels are candidates since no second messenger is required for channel activation, iii) another possible mechanism for mechanotransduction involves guanine nucleotide binding proteins, iv) specific regulation may occur at receptor tyrosine kinase by growth factor, v) and mechanical stress may induce a conformational change in a nonreceptor protein tyrosine kinase receptor<sup>46-50</sup>. The binding of ligand to receptor activates phospholipase C that hydrolyzes phosphatidyl inositol 4,5 - biphosphate into inositol 1,4,5 - triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). IP<sub>3</sub> causes the rapid release of Ca<sup>2+</sup> from intracellular stores of calcium, and DAG activates protein kinase C (PKC). The increased concentration of cytosolic Ca<sup>2+</sup> and PKC activates cAMP synthesis, and then causes the increase of cellular division and proliferation<sup>51,52</sup>.

Cylin D and its major catalytic partners, cdk 4 or cdk 6, are an early event in cell cycle initiation, and cyclin E and cdk 2 are involved in late G1 phase. In almost all of the studies, the expression level of cyclin D and E was associated with cdk 4 or 6, and 2. In the present study, the degree of expression of cyclin was changed along that of cdk. The increase in cdk 4 and cdk 6 was comparable to the increase in cyclin D1 as days go by. Although the expression

level of cyclin E was slightly increased during the experimental period, especially the expression of cdk 2 under mechanical stress condition was remarkably increased compared to that of control. The most abundant expression was shown on day 6 in cyclin and cdk. It might be due to the fact that contact inhibition of confluent cells occurred on day 10.

PCNA plays an important role in nucleic acid metabolism. The best understood function of PCNA is in DNA replication. PCNA plays an essential role in DNA replication as the auxiliary protein. PCNA is isolated as a protein with elevated levels during S phase. Studies have shown that the protein is localized to the nucleus only in cells that are in the S phase of the cell cycle<sup>53,54</sup>. In normal cells, PCNA exists in multiple quaternary complexes, each containing a cdk, cyclin, and p21<sup>WAF1/CIP1</sup><sup>13-20</sup>. In the present study, the increase in the expression of PCNA is regarded as an essential component of cell cycle progression as well as a molecular marker of cell cycle progression.

p53 is associated with cell proliferation, DNA repair, maintenance of DNA integrity, and regulation of apoptosis. Transcription of the p21<sup>WAF1/CIP1</sup> gene can be activated by p53 and several p53 - independent mechanisms. In some cases p53 did not require induction of p21<sup>WAF1/CIP1</sup> to proliferate and differentiate cell. p53 - independent induction of p21<sup>WAF1/CIP1</sup> expression at the transcriptional level has been observed as an immediate early response to a variety of physiological and chemical inducers of differentiation. In the present study, p53 level



of expression showed little change for 2 to 10 days. This result indicates that p53 did not affect the progression of the mechanically stressed PDL cell cycle under the condition reported here. Previous studies have established that cdk inhibitor protein p21<sup>WAF1/CIP1</sup> has a role in regulating the G1 - S phase progression by inhibition of cyclin - cdk catalytic activity<sup>55,56</sup>. cdk inhibitors function by binding themselves to cyclin D - cdk complexes to inhibit their kinase activities. However, p21<sup>WAF1/CIP1</sup> does not seem to always function as a cyclin - cdk inhibitor because several findings showed that in proliferating cells the majority of p21<sup>WAF1/CIP1</sup> protein was found in active cyclin - cdk complexes<sup>20,57</sup>, and that despite the induction of cdk inhibitor p21<sup>WAF1/CIP1</sup>, cyclin D1 associated with cdk kinase remained activated and the cells grew essentially like that of their parent cells<sup>58</sup>. In the present study, western blot analysis of p21<sup>WAF1/CIP1</sup> in the mechanically stressed PDL cells illustrated that expressions of p21<sup>WAF1/CIP1</sup> showed little change, but the expression of cyclin - cdk complexes was slightly increased in the experimental period by mechanical stress. This result seems to be explained by the fact that the absolute ratio between p21<sup>WAF1/CIP1</sup> and the cyclin - cdk complexes determines the cell cycle inhibitory behavior of p21<sup>WAF1/CIP1</sup>. In more detail, although p21<sup>WAF1/CIP1</sup> level is increased in the stoichiometry between the cyclin D1 - cdk complex, but if p21<sup>WAF1/CIP1</sup> does not change, and a stable and active cyclin D1 - cdk - p21<sup>WAF1/CIP1</sup> complex is sustained, a progression of cell cycle may result under these conditions. If, however,

the increased level of p21<sup>WAF1/CIP1</sup> protein overcomes that of cyclin D1 - cdk, the ratio of the cyclin D1 - cdk complexes to p21<sup>WAF1/CIP1</sup> will be biased toward p21<sup>WAF1/CIP1</sup>. This situation would suppress the cell cycle progression<sup>58</sup>. In the present study, the results indicate that mechanical stress on human PDL cells causes the increase of expression of cdk without change of level of p53 and p21<sup>WAF1/CIP1</sup>, and the increase of cell proliferation rate.

## V. Conclusion

Mechanical stress is known to be associated with proliferation of periodontal ligament (PDL) cells. Though detailed mechanisms for the proliferation of PDL cells by mechanical stress remain largely unknown, it may be due to the increased expression of cell cycle regulatory proteins from the cells. To investigate this possibility, growth pattern and expression of p53, p21<sup>WAF1/CIP1</sup>, cyclin - dependent kinases (cdks), cyclins, and proliferating cell nuclear antigen (PCNA) were determined in PDL cells exposed to mechanical stress (1 kg/55 - mm Petriperm dish). Mechanical stress notably increased cell proliferation rate and expression of PCNA in the PDL cells exposed to stress for 6 - 10 days when compared to normal cells. Mechanical stress also slightly increased expression of cdk and cyclin D1 in these cells, but levels of p53 and p21<sup>WAF1/CIP1</sup> were not changed. These results indicate that the increase of cell proliferation by mechanical stress may be due to the increased expression of cdk without the change of p53 and p21<sup>WAF1/CIP1</sup>.

levels in human PDL cells.

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가 8 - 12 가 ,  
PCNA 가  
6 - 10 가 .  
cdk4, cdk6,  
cdk2 cyclin D1 가  
, p53 p21<sup>WAF1/CIP1</sup>  
가 .  
p21<sup>WAF1/CIP1</sup> p53  
cdks  
가

, 가 가

가

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: , , cyclin,  
cyclin - dependent kinase, p53, p21<sup>WAF1/CIP1</sup>

가

, p53,  
p21<sup>WAF1/CIP1</sup>, cyclin - dependent  
kinases(cdk), cyclins proliferating cell  
nuclear antigen(PCNA)

explantation  
culture 6

55 - mm Petriperm dish 1 × 10<sup>4</sup>  
, dish 1 kg

가 12