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

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Recently, mechanical stress has been found to be associated with the proliferation of human periodontal ligament (PDL) cells. Although the detailed mechanism for the proliferation of PDL cells by mechanical stress remains largely unknown, the effect may be due to the increased expression of cell cycle regulatory proteins. To investigate this possibility, we measured the growth pattern and determined the protein levels of cellular p53, $p21^{WAF1/CIP1}$, cyclin-dependent kinases (cdks), G_1 cyclins, and proliferating cell nuclear antigen (PCNA) expressed by PDL cells exposed to mechanical stress. Human PDL cells of passage number 6 were plated onto 55-mm Petriperm dishes (1×10^4 cells/dish) and exposed to mechanical stress (1 kg/dish) for 12 days. Mechanical stress markedly increased the proliferation of the PDL cells exposed to stress for 8-12 days, compared to the controls. It also increased the protein level of cellular PCNA in the PDL cells exposed to stress for 6-10 days. Mechanical stress also slightly increased the expression of cdks and cyclin D1 in these cells, but the protein levels of p53 and $p21^{WAF1/CIP1}$ were unchanged. The increased proliferation of PDL cells by mechanical stress may be due, in part, to the elevated cellular levels of cdks, without corresponding changes in p53 and $p21^{WAF1/CIP1}$ levels in human PDL cells.

Key words : human periodontal ligament cell, mechanical stress, cell proliferation, cell cycle regulator

Introduction

Much is known regarding the regulation of proliferation, differentiation, and the activity of periodontal ligament (PDL) cells during normal function. However, rather less is known of alterations in their phenotypic expression under specific conditions, such as those induced by mechanical stress. It is likely that PDL cells, when stimulated by mechanical force, produce some important local factors that participate in the maintenance and remodeling of the ligament itself. Previous studies have reported that human PDL cells increase the levels of prostaglandin E (Ngan *et al.*, 1990; Yamaguchi *et al.*, 1994), interleukin-1 (Saito *et al.*, 1991; Shimizu *et al.*, 1994), cyclic AMP (Ngan *et al.*, 1990: Yousefian *et al.*, 1995), DNA synthesis, and collagen synthesis (Kunz *et al.*, 1988) in response to mechanical stress. Human PDL cells also activate plasminogen activator in response to tension-force (Yamaguchi *et al.*, 1997). In contrast, alkaline phosphatase activity and its mRNA level were found to be decreased by tension-force (Yamaguchi *et al.*, 1996). Therefore, it is likely that mechanical stress evokes biochemical responses in PDL 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 (Nurse, 1994; Sherr, 1994). The cdks are serine/threonine protein kinases and functional only when combined with one of the cyclins. The cyclins regulate the activities of the cdks and control their ability to modulate the enzymes involved in driving the cell cycle (Kato *et al.*, 1994). Hence, the assembly, activation, and disassembly of the cyclin-cdk complex are critical events in terms of controlling the cell cycle. The cyclins undergo synthesis and degradation during each cycle of the cell. There are two main classes of cyclins: the G_1 cyclins bind cdks during the G_1 phase and are required for entry

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into the S phase, and the mitotic cyclins bind cdks during the G_2 phase, permitting entry of the cell into the M phase (Leake, 1996). The G_1 cyclin complexes exist as quaternary structures of cyclins, cdks, the universal cdk inhibitor $p21^{WAF1/CIP1}$, and the proliferating cell nuclear antigen (PCNA) (Xiong *et al.*, 1992; el-Deiry *et al.*, 1993; Gu *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993; Zhang *et al.*, 1993; Noda *et al.*, 1994; Zhang *et al.*, 1994). Progression from the G_1 to the S phase requires the coordinated and independent activation of both cyclin D complexes in early G_1 and cyclin E complexes in later G_1 (Lukas *et al.*, 1994; Tam *et al.*, 1994; Ohtsubo *et al.*, 1995; Resnitzky and Reed, 1995). D-type cyclins (D1, D2 and D3) are associated with either cdk 4 or cdk 6, whereas cyclin E is associated with cdk 2. These cyclins play distinct roles in the regulation of the G_1-S phase transition. Both cdk and cyclin are associated with each other and activated by cyclin-activating kinase (Fisher and Morgan, 1994; Makela *et al.*, 1994; Matsuoka *et al.*, 1994).

Cdk inhibitors are proteins that bind and inhibit the activity of cdks. $p21^{WAF1/CIP1}$ has been identified as one of this group and is responsible for binding and inhibiting G_1 cyclin-cdks complexes (Gartel *et al.*, 1996). $p21^{WAF1/CIP1}$ is a constant part of G_1 cyclin complexes, and only acts as a cdk inhibitor when its stoichiometric ratio increases from one to several per complex (Xiong *et al.*, 1993; Zhang *et al.*, 1993; Zhang *et al.*, 1994). In addition to its kinase inhibitor activity, $p21^{WAF1/CIP1}$ is able to inhibit DNA replication in the S phase of the cell cycle by interacting with PCNA, thereby blocking the ability of PCNA to activate DNA polymerase (Flores-Rozas *et al.*, 1994; Waga *et al.*, 1994). The p53 tumor suppressor protein is a transcription factor required for the transactivation of a number of genes involved in growth control (Clarke *et al.*, 1993; Lowe *et al.*, 1993). Inactivation of the wild-type p53 function can lead to growth advantages. It has been demonstrated that DNA damage activates $p21^{WAF1/CIP1}$ transcription in a p53-dependent manner in human fibroblasts and epithelial cells (Dulic *et al.*, 1994). In contrast, several studies on the p53-independent induction of $p21^{WAF1/CIP1}$ following DNA damage have also been reported (Akashi *et al.*, 1995). It has also been shown that p53 can inhibit cell cycle progression without inducing $p21^{WAF1/CIP1}$ expression (Hirano *et al.*, 1995). Thus, $p21^{WAF1/CIP1}$ 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 $p21^{WAF1/CIP1}$. PCNA is an essential component of the DNA replicative machinery, functioning as the accessory protein for DNA polymerase, which is required for processive chromosomal DNA synthesis and for the repair of nuclear DNA. PCNA is also required for recombination and repair (Kelman, 1997). In addition, PCNA has been shown to interact with the cell cycle regulatory proteins, cdks, cyclin D, and $p21^{WAF1/CIP1}$ (Xiong *et al.*, 1992; Matsuoka *et al.*, 1994; Pagano *et al.*, 1994).

Recently, mechanical stress has been found to be associated with the proliferation of human periodontal ligament (PDL) cells. Although the detailed mechanisms for the proliferation of PDL cells by mechanical stress are not well understood, the effect may be due to the increased cellular expression of cell cycle regulatory proteins. To investigate this possibility, we measured the growth pattern and determined the protein levels of cellular p53, $p21^{WAF1/CIP1}$, cyclin-dependent kinases (cdks), G_1 cyclins, and PCNA expressed from PDL cells exposed to mechanical stress.

Materials and Methods

Culture of primary PDL cells

Human PDL cells were obtained from a healthy lower premolar surgically extracted for orthodontic reasons. After extraction, the tooth was thoroughly washed three times with Hanks' balanced salt solution (HBSS; Gibco/BRL, Grand Island, NY). The mid-third portion of the periodontal ligament was collected carefully by scraping with a surgical scalpel and transferred into 60-mm culture dishes and then cultured with Dulbecco's Modified Eagle's Medium (DMEM, Gibco/BRL) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco/BRL) at 37°C in the presence of 5% CO_2 in air. Confluent cells were subcultured up to six passages and cultures at passage number 6 were used for this experiment.

Exposure of cells to mechanical stress

To determine the effect of mechanical stress on cell growth pattern, and intracellular PCNA and cell cycle regulator levels, PDL cells (passage number 6) were plated at 1×10^4 cells per 55-mm Petriperm dish with a hydrophilic flexible plastic growth surface (Heraeus Instruments, South Plainfield, NJ). Placing a template with a convex surface under the dish and a stainless steel weight (1 kg/dish) on the top of the dish stretched the flexible base of the dish (Fig. 1).

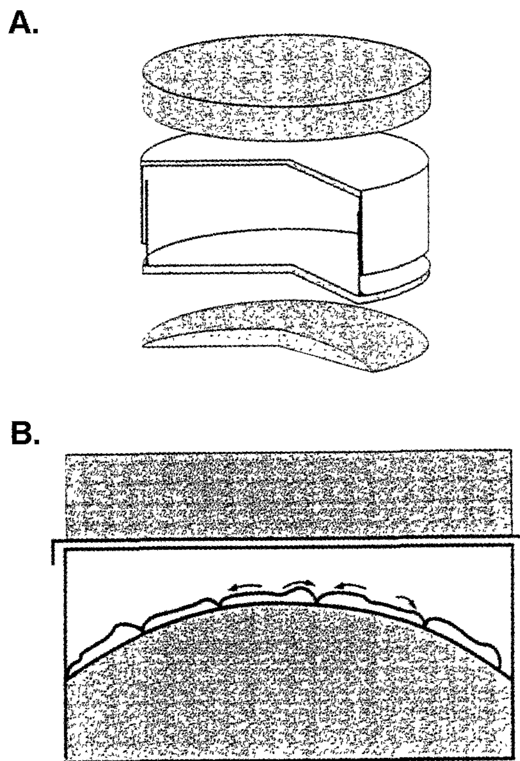

Fig. 1. A schematic representation of the mechanical stress apparatus. (**A**) Upper section, iron weight (1 kg/each); middle section, 55-mm Petriperm dish; lower section, template. (**B**) Cells were cultured on the flexible membrane of a Petriperm dish, which was placed over a template with a convex surface. A weight placed on the top of the dish (1 kg/dish) forced the membrane and the tightly attached cells to be stressed.

Because the convex surface of the dish was uniformly curved, stretching occurred evenly over the bottom of the dish. The bottom surface curvature of the template could be represented by an arc of 36° and a radius of 7.6 cm (Hasegawa *et al.*, 1985).

Determination of cell growth

To determine the effect of cell proliferation, cell growth was assayed. Cells were plated at 1×10^4 cells per 55-mm Petriperm dish and exposed to mechanical stress 12 h after cell seeding as described above. The cultures were harvested by trypsinization after 2, 4, 6, 8, 10, and 12 days of incubation under mechanical stress to count the number of viable cells after trypan blue exclusion. Average numbers and standard deviations were calculated from six independent experiments.

Western blot analysis

Western blot analyses were performed as previously described (Min *et al.*, 1995), using anti-human p53 (PAb 1801) monoclonal antibody, anti-human cyclin E (HE12) monoclonal antibody, anti-human PCNA (PC10) monoclonal antibody, anti-human cdk4 (Ab-1) polyclonal antibody (Oncogene Science, Uniondale, NY), anti-human $p21^{WAF1/CIP1}$ (187) monoclonal antibody, anti-human cdk2 (M2) polyclonal antibody, anti-human cdk6 (C-21) polyclonal antibody, and anti-human cyclin D1 (R-124) monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After probing with the respective antibodies, the membrane was stained with 1X Ponceau S stain for 10 min to reveal the total protein amount loaded per lane. The relative levels of proteins were determined by densitometric scanning of autoradiographs obtained from two different experiments.

Results

Mechanical stress increases proliferation of human PDL cells

To determine the effect of mechanical stress on the cell growth of human PDL cells, we cultured the cells in the absence or presence of stress. Before day 6, the growth pattern of the stressed PDL cells was similar to that of the non-stressed control cells. In contrast, relative growth stimulation was observed in the stressed PDL cells after day 6 (Fig. 2A). Additionally, the protein level of the intracellular PCNA, a molecular marker of proliferation, was similar on day 2, but was significantly increased on days 6 and 10 in the stressed PDL cells (Fig. 2B). These results indicate that mechanical stress stimulates the proliferation of human PDL cells.

Mechanical stress does not alter the expression of p53 and $p21^{WAF1/CIP1}$ proteins

To investigate whether human PDL cells acquire growth stimulation by mechanical stress through the altered expression of cell cycle-related genes, we determined the expression of p53 and $p21^{WAF1/CIP1}$ proteins by the stressed PDL cells. The intracellular levels of p53 and $p21^{WAF1/CIP1}$ proteins of the stressed cells were generally similar to each other when densitometrically analyzed (Fig. 3). These results indicate that growth stimulation by mechanical stress of human PDL cells is not linked to the downregulation of the expression of p53 and $p21^{WAF1/CIP1}$ proteins in such cells.

Mechanical stress enhances the expression of G_1 cdks and cyclin D1

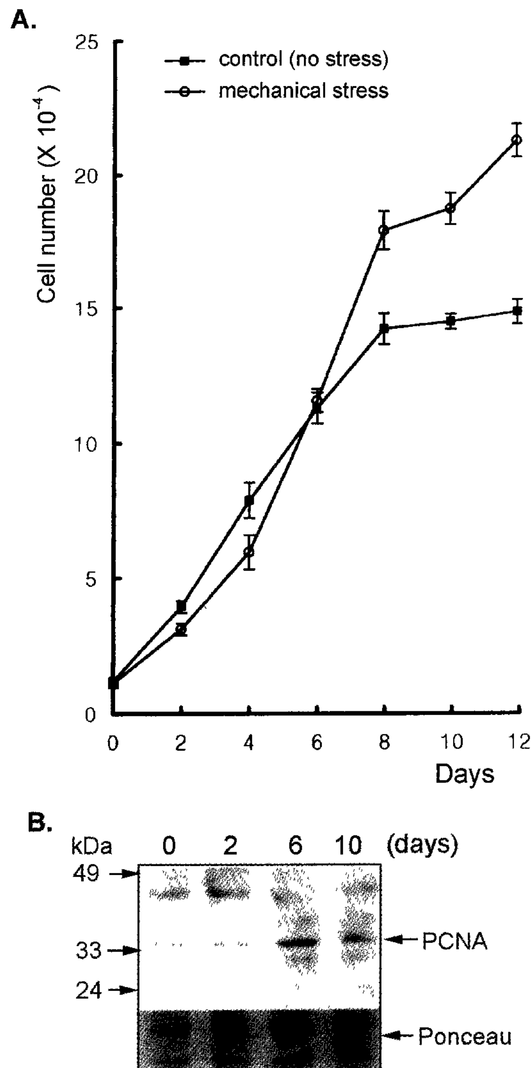

Fig. 2. Growth curves and intracellular PCNA protein levels of human PDL cells exposed to mechanical stress. **(A)** Cells were plated at 1×10^4 cells per 55-mm Petriperm dish, and cultured for 2, 4, 6, 8, 10 or 12 days in the presence of mechanical stress (1 kg/dish). Viable cells were counted with a hemocytometer by trypan blue exclusion. Average cell numbers and standard deviations were calculated from six independent experiments. **(B)** Western blot analysis of the intracellular PCNA protein in human PDL cells exposed to mechanical stress for 2, 6, or 10 days. The membrane was stained with Ponceau S stain to reveal the total protein loaded per lane. A structural protein stained with Ponceau S served as the internal control and accounts for the loading error (lower panel).

Since cdks and the cyclins of the G_1 phase are involved in regulating the cell cycle progression (Nurse, 1994; Sherr, 1994), we measured the expression levels of these proteins from stressed PDL cells to determine whether human PDL cells acquire growth stimulation by mechanical stress through the

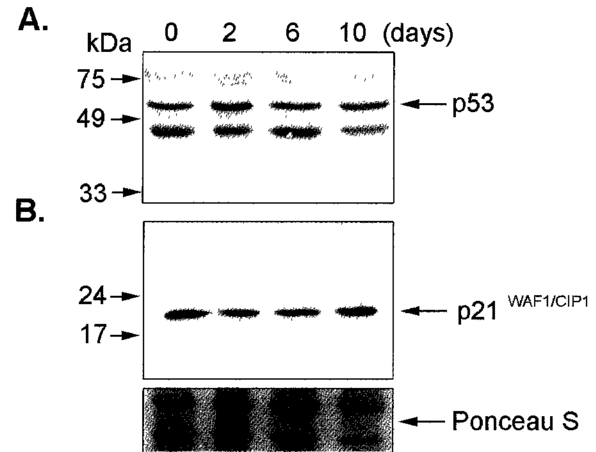

Fig. 3. Western blot analysis for the intracellular p53 **(A)** and $p21^{WAF1/CIP1}$ **(B)** protein levels in human PDL cells exposed to mechanical stress for 2, 6, or 10 days. The membrane was stained with Ponceau S stain to reveal the total protein loaded per lane. A structural protein stained with Ponceau S served as the internal control and accounts for the loading error (lower panel).

upregulation of cell cycle-related proteins. Human PDL cells in the absence or presence of mechanical stress expressed G_1 cdks and cyclins. The protein levels of cellular cdk4 and cdk6 on day 2 in the stressed PDL cells were generally similar to that of the non-stressed control cells; however, these were

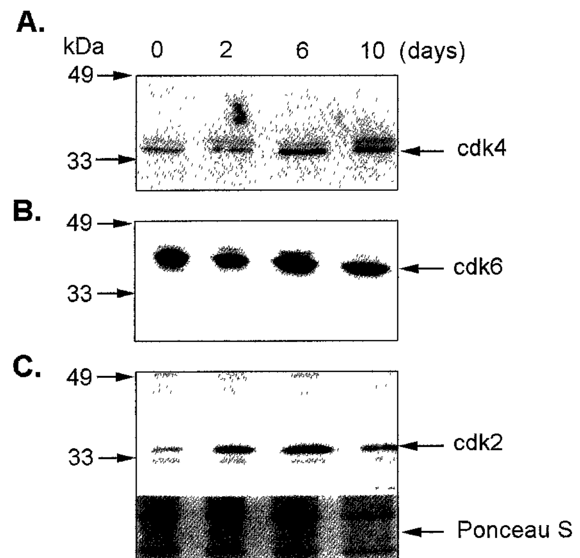

Fig. 4. Western blot analysis of the intracellular cdk4 **(A)**, cdk6 **(B)**, and cdk2 **(C)** protein levels in human PDL cells exposed to mechanical stress for 2, 6, and 10 days. The membrane was stained with Ponceau S stain to reveal the total protein loaded per lane. A structural protein stained with Ponceau S served as the internal control and accounts for the loading error (lower panel).

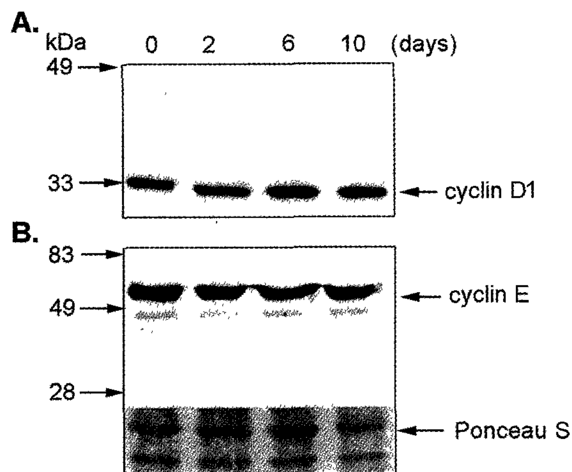

Fig. 5. Western blot analysis for the intracellular cyclin D1 (**A**) and cyclin E (**B**) protein levels in human PDL cells exposed to mechanical stress for 2, 6, and 10 days. The membrane was stained with Ponceau S stain to reveal the total protein loaded per lane. A structural protein stained with Ponceau S served as the internal control and accounts for the loading error (lower panel).

significantly increased on days 6 and 10 in the stressed PDL cells (Fig. 4). Interestingly, the level of cellular cdk2 protein was significantly higher from day 2 to day 10 in the stressed PDL cells. Because alteration in cdk protein expression was observed, we further investigated G_1 cyclin levels from the stressed PDL cells. Similarly, the protein level of cellular cyclin D1 on days 6 and 10 in the stressed PDL cells was higher than that in the non-stressed control cells (Fig. 5A). The cyclin E level was not changed under similar conditions (Fig. 5B). These results indicate that the increased levels of G_1 cdks and cyclin D1 proteins in stressed PDL cells may be linked to growth stimulation of human PDL cells by mechanical stress.

Discussion

The data presented shows that mechanical stimulation of human PDL cells results in cell proliferation. This was evidenced by the increase in the proliferation of stressed PDL cells after day 6 and the increased protein levels of intracellular PCNA (the molecular marker of proliferation), on days 6 and 10 in the stressed PDL cells. When cells are stimulated by mechanical stress, biochemical characteristics and cell numbers change appreciably (Harell *et al.*, 1977; Leung *et al.*, 1977; Somjen *et al.*, 1980; Brunette, 1984; Binderman *et al.*, 1984; Binderman *et al.*, 1988; Kunz *et al.*, 1988; Ngan *et al.*, 1990; Saito *et al.*, 1991; Shimizu *et al.*, 1994; Yamaguchi *et al.*, 1994; Yousefian *et al.*, 1995; Yamaghchi *et al.*, 1996; Yamaguchi *et al.*, 1997). However, the effect of stretching on cell proliferation appears to be dependent upon cell types (Harell *et al.*, 1977; Leung *et al.*, 1977; Somjen *et al.*, 1980; Binderman *et al.*, 1984; Brunette, 1984; Binderman *et al.*, 1988). The biochemical mechanisms which are involved in the conversion of mechanical stimuli into biological response by signal transduction in cells are poorly understood. Although a mechanism for the detection and conversion of mechanical force into a biochemical signal has yet to be identified, several pathways have been proposed (Karin, 1992; Carvalho *et al.*, 1994; Banes *et al.*, 1995; Duncan and Turner, 1995; Brighton *et al.*, 1996). However, the precise relationship between proliferation and mechanical stress remains unclear and the mechanisms of proliferation induced by mechanical stress in human PDL cells are unknown. We have attempted to identify the relevant mechanisms by determining the expression of several genes which are associated with the cell cycle. Because wild-type p53 is involved in growth control (Clarke *et al.*, 1993; Lowe *et al.*, 1993), we measured the expression level of wild-type p53 and $p21^{WAF1/CIP1}$, a gene whose expression is regulated by wild-type p53, in many cell types. Moreover, the abrogation of wild-type p53 function results in the downregulation of $p21^{WAF1/CIP1}$ expression, the lack of which is associated with an extension of life span and the immortalization of cells (Tsang *et al.*, 1995; Iijima *et al.*, 1996). The cellular levels of p53 and $p21^{WAF1/CIP1}$ proteins of the stressed cells were generally similar to those of the non-stressed control cells. This data indicates that growth stimulation induced by mechanical stress in human PDL cells is not linked to the downregulation of the expression of wild-type p53 and $p21^{WAF1/CIP1}$ proteins in cells.

Numerous reports support the notion that cylin D and its major catalytic partners, cdk4 and cdk6, are involved in an early event of cell cycle initiation, and that cyclin E and cdk2 are involved in the later G_1 phase (Lukas *et al.*, 1994; Tam *et al.*, 1994; Ohtsubo *et al.*, 1995; Resnizky and Reed, 1995). Since the cell cycle phases in dividing mammalian cells are finely regulated by the sequential assembly and activation of key cell cycle regulators, namely the cyclins and cdks (Fisher and Morgan, 1994; Makela *et al.*, 1994; Matsuoka *et al.*, 1994), we investigated the expression of these key cell cycle

regulatory components in stressed PDL cells. A significant increase in the protein levels of cellular cyclin D1 and G_1 cdks in the PDL cells after 6 days of mechanical stimulation indicated that a definite regulation of cell cycle regulators occurred as a result of the applied mechanical stress. Each mammalian cyclin can interact with multiple cdks and the cyclin function is primarily controlled by changes in cyclin levels, whereas the cdks are activated by the phosphorylation of a conserved threonine residue (Kato *et al.*, 1994). Although we cannot conclude that enhanced protein levels of cdk4, cdk6, and cdk2 in the stressed PDL cells are directly linked to elevated cdk activity, it may be associated with the increased proliferation of human PDL cells induced by applied mechanical stress. Therefore, the collated data indicates that stress induced elevated levels of cellular cyclin D1 and G_1 cdks cause growth stimulation of human PDL cells.

Previous studies have shown that PCNA is localized in the nucleus of those cells which are in the S phase of the cell cycle (Bravo and Macdonald-Bravo, 1985; Bravo and Macdonald-Bravo, 1987). In normal cells, PCNA exists in multiple quaternary complexes, each containing a cdk, cyclin, and $p21^{WAF1/CIP1}$ (Xiong *et al.*, 1992; ei-Deiry *et al.*, 1993; Gu *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993; Zhang *et al.*, 1993; Noda *et al.*, 1994; Zhang *et al.*, 1994). The PCNA protein has been reported to play an important role in the regulation of the G_1 phase of cell cycle and in DNA synthesis (Madsen and Celis, 1985; Koff *et al.*, 1992). In the present study, the increase in the expression of cellular PCNA protein is regarded as an essential component of cell cycle progression, as well as being a molecular marker of cell proliferation. In summary, these results indicate that mechanical stress on human PDL cells causes an increase in the expression of both cyclin D1 and G_1 cdks without changing p53 or $p21^{WAF1/CIP1}$ levels or increasing the cell proliferation rate.

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