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ORIGINAL ARTICLE

Cyclic stretching force-induced early apoptosis in human periodontal ligament cells

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OBJECTIVE: Human periodontal ligament (PDL) cells occur changes in morphology and express relative protein by stretching force. However, whether stretching force, especially excessive stretching force, induces PDL cell apoptosis is not yet clearly understood. In the present study we investigated the relationship between early apoptosis and stretching force in human PDL cells *in vitro*.

MATERIALS AND METHODS: The human PDL cells were obtained from healthy premolars. After three to five passages, the cells were stretched by strain 1%, 10% and 20% for 30 min, 1 h, 6 h and 12 h, then early apoptosis were detected through annexin fluorescein isothiocyanate (V-FITC) binding by flow cytometry and confocal laser scanning microscopy.

RESULTS: The experiments indicated that human PDL apoptotic cells in the early stage increased in a time- and force-dependent manner in response to stretching strain within 6 h, and then apoptosis decreased at 12 h. Human PDL cells which stretched inclined parallel to each other and aligned their long axis perpendicular to the stretching force vector, but in the centre of the disc, cells showed minimal deformation and unidirectional alignment of PDL cells.

CONCLUSION: The overall results suggested that stretching force not only influenced morphology but also induced early apoptosis in human PDL cells.

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Keywords: early apoptosis; human periodontal ligament cells; stretching force

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Introduction

During occlusal load or orthodontic tooth movement, the cells in the periodontal ligament (PDL) are directly subject to mechanical stress. The reaction to mechanical stress is an essential biological reaction (Pavlin and Gluhak-Heinrich, 2001). Prediction of tooth mobility under functional loads is a classical issue in dental biomechanics which becomes increasingly important in the development of new solutions in dental restorations, prosthodontic and orthodontic treatments. The understanding of tooth mobility requires the mechanical characterization of the PDL and to a lower extent, the much stiffer alveolar bone and dentine. The PDL is a complex soft tissue that connects the teeth to the surrounding bone and a common assumption is that it acts as the major element in tooth mobility and stress distribution to the supporting tissues (Pini *et al*, 2004). In general, mechanical stress mainly includes stretching and compressive force. Researchers found that a 1.4- to 1.6-fold increase in proliferation of vascular smooth muscle cells (SMCs) was induced when cells were stretched cyclically by a vacuum-operated downward flexion of the culture dish bottom in a stretch force-dependent manner in the range of 5–15% elongation, 30 cycles min^{-1} for 24 h (Li *et al*, 1997). And human PDL cells were cultured on flexible-bottomed plates and placed on a Flexercell Strain Unit. Cells were flexed at six cycles min^{-1} (5 s strain, 5 s relaxation) at six levels of tension-force (9%, 12%, 15%, 18%, 21%, and 24% increase in surface area) for 5 days. There was no significant difference in cell proliferation between the cells subjected to the tension-force and the controls. There was a 10% and 42% decrease, respectively, in the alkaline phosphatase (ALP) activity in PDL cells exposed to low (9%) and high (24%) tension forces, and these decreases were dependent on the magnitude of the tension force (Yamaguchi *et al*, 1996). However, another experiment observed the proliferation of periodontal ligament fibroblasts (PDLFs) under mechanical stretching by 6 cycles min^{-1} with 12% strain for 24, 48

and 96 h. Results showed that PDLF proliferation can be promoted by proper stretch treatment (Feng *et al*, 2003). The above phenomena may be caused by different stretch-loading units and/or loading time.

Adequate mechanical stress maintains homeostasis of PDL cells, Cathepsin is a typical and well-characterized lysosomal cysteine protease that, under pathological conditions, is involved in tissue destruction. A recent immunocytochemical study demonstrated that cathepsins B (CAB) and L (CAL) were localized in the PDL of the rat molar, and they were expressed in compressed sites during experimental tooth movement. PDL cells were subjected to 0.5, 1.0, 2.0, or 3.0 g cm^{-2} of compression force or an increase in surface area by tension force of 0.28%, 0.95%, 1.72%, or 2.50% for 24 h. Results showed that compression and tension significantly increased the secretions of both CAB and CAL in PDL cells, which were exhibited in a time- and force magnitude-dependent manner (Yamaguchi *et al*, 2004). In the study of the relationship between occlusal stimuli and a hypofunctional PDL structure, nitric oxide (NO) produced by NO synthase was considered a factor for vascular and immune system control, and it increased according to mechanical stimuli, then NO significantly decreased in occlusal hypofunction compared with the control group and increased close to normal in an occlusal recovery group (Watarai *et al*, 2004). RGD-CAP, a member of the fasciclin family, is expressed in the PDL, and the adhesive functions of RGD-CAP may contribute to the maintenance or regeneration of PDL architecture. During experimental tooth movement, the expression of RGD-CAP was significantly enhanced in the PDL. *In vitro* experiments with cultured PDL cells also showed that the expression of RGD-CAP mRNA was significantly enhanced by mechanical tensile force of 15.4 kPa for 48 h. These findings suggested that RGD-CAP in response to mechanical stimuli played an important role in modulating the homeostasis of PDL (Doi *et al*, 2003).

While the application of excessive mechanical stress, irrespective of the stretching or compression force, results in cell death in the PDL cells (Reitan and Rygh, 1994). Cells undergo death by two major mechanisms: necrosis, in which primary damage to the metabolic or membrane integrity of the cell occurs, or apoptosis, which is an internal suicide programme contained in all cells (Majno and Joris, 1995). Programmed cell death (apoptosis) (Lockshin and Williams, 1965; Kerr *et al*, 1972) plays a key role in the regulation of tissue turnover in long-lived mammals that must integrate multiple physiological as well as pathological death signals. Several published studies revealed that the pressure applied during orthodontic tooth movement or in neurones *in vitro* (Kosnosky *et al*, 1995; Agar *et al*, 2000) caused apoptosis and then led to tissue necrosis (Rygh, 1974; Hatai *et al*, 2001; Rana *et al*, 2001; Mabuchi *et al*, 2002).

According to the above retrospection, we found a close relationship between PDL cells and mechanical stress, and apoptosis is also an important phenomenon to organisms. However, only one experiment *in vivo* investigated apoptosis of PDL cells at the compressed area during tooth movement by using TUNEL assay (Hatai *et al*, 2001). Furthermore, we tended to detect whether stretching forces induced apoptosis in human PDL cells *in vitro*. Apoptosis is a process which is divided into early apoptosis and late apoptosis, ultimately to necrosis. To clarify the phenomenon, we first examined whether early apoptosis of human PDL cells occurred by cyclic stretching force *in vitro*, and apoptosis were labelled with annexin V-FITC/PI by flow cytometry and confocal laser scanning microscope.

Materials and methods

Primary culture of human PDL cells

The human PDL cells were obtained from healthy premolars from five donors: two 13-year-old (female and male), two 16-year-old (female) and one 15-year-old (female) donor, after obtaining informed consent. Two teeth from each donor, totalling 10 teeth, were obtained. Pieces of PDL were obtained only from the middle of tooth roots with ophthalmic scissors carefully so as to exclude the intermixture of gingivae and dental pulp. Pieces of two teeth from one donor were attached to a flank in one culture bottle. They were then cultured in DMEM (Gibco, Grand Island, NY, USA) containing 20%(v/v) fetal bovine serum (FBS; Hyclone, Logan, Utah, USA) and fivefold reinforced antibiotics (500 U ml^{-1} penicillin, 500 mg ml^{-1} streptomycin) in 25 ml primary culture bottles. Cells that grew out from the extracts were passaged in 10% FBS–DMEM supplemented with antibiotics (100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin) (Chiba and Mitani, 2004). After three to five passages, the cells were used for experiments. This experimental procedure was approved by the Ethics Committee, Medical College of Shanghai Jiaotong University.

Cell stress unit (CSU)

The CSU used in this study (Figures 1 and 2) included a strainer, controller and personal computer (PC). The strain was produced by Spherical cap, according to formula: $\varepsilon(strain) = \frac{(2\pi RH - \pi r^2)}{\pi r^2} = \frac{(2RH - r^2)}{r^2}$, where R is the

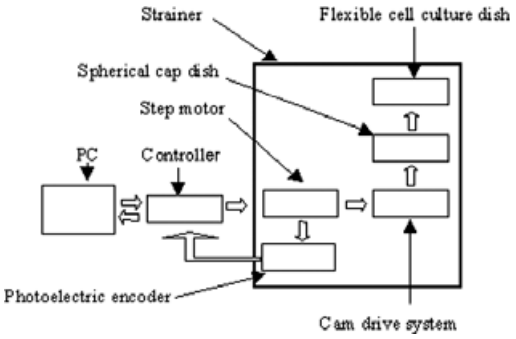

Figure 1 Outline drawing of the cell stress unit (CSU). Spherical cap moved up and down repeatly and was monitored by Photoelectric encoder exactly, the movement of spherical cap made the elastic membrane deformable, meanwhile, cells which attached to membrane of the flexible cell culture dish (radius 30 mm) were stretched. All changes in stretching strain and movement of the spherical cap were controlled by a PC

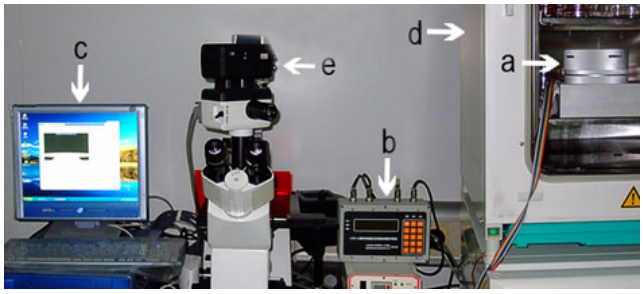

Figure 2 Photograph of the cell stress unit (CSU). (**a**) Strainer, (**b**) controller, (**c**) PC, (**d**) Cell incubator, (**e**) inverted phase-contrast microscopy

radius of the sphere, H, the height of the Spherical cap, r, the radius of the Sperical cap bottom. We can calculate the strain rate of elastic silicon rubber membrane which is deformed through Spherical cap. Strainer can be sterilized through ultraviolet ray and can provide stretching strain from 1% to 25% to cultured cells at a constant level in a humidified incubator at 37°C under 5% CO_2/air. Flexible cell culture dish whose bottom is elastic silicon rubber (Dow Corning Co., Midland, Michigan USA) allows sterile manipulation and is fixed in the Strainer. The Spherical cap moves up and down repeatedly that is monitored by a photoelectric encoder, the movement of the spherical cap deforms the elastic membrane while cells attached to the membrane were stretched. All changes in stretching strain and movement of the spherical cap are controlled by the controller and PC.

Loading of stretching force

The human PDL cells were seeded onto a flexible cell culture dish at a concentration of 1.5×10^6 cells per dish. The cells reached confluence following approximately 4 days of culture, and then were exposed to 1%, 10% and 20% stretching strain for 30 min, 1 h, 6 h, and 12 h respectively (total 16 groups including controls). One cycle of loading included that Spherical cap ascending to the highest point for 1 s, keeping the highest point for 4 s and descending for 1 s and loading frequency is 10 cycles min^{-1}. The treatments were repeated for three times in every group ($n = 3$). Human PDL cells seeded in a flexible cell culture dish placed in similar conditions were used as non-stretching controls.

Analysis of morphological changes

Before and after the loading of stretching strain, morphological changes of human PDL cells in each stretching and non-stretching group were observed using an inverted phase-contrast microscope (Leica DMRIRB, Bensheim Germany).

Measurement of early apoptosis by flow cytometry

Before and after the loading of stretching strain, cells were gently trypsinized and washed once with serum-containing media, and human PDL cells were collected (5×10^5) by centrifugation. Cells were resuspended in 500 μl of 1 × Binding Buffer, and 5 μl of annexin V-FITC and propidium iodide (PI) was added according to the manufacturer's instruction (Biovision, Inc., Mountain View, CA, USA). After incubated at room temperature for 5 min in the dark, analysed annexin V-FITC binding by flow cytometry (FACSCalibur, BD, Inc., San Jose, USA).

Measurement of apoptosis by confocal laser scanning microscopy

Human PDL cells stretched on the flexible membrane were incubated in 1 ml 1 × Binding Buffer containing 5 μl of annexin V-FITC and PI at room temperature for 5 min in the dark. The cells were observed under a confocal laser scanning microscope using a dual filter set for FITC.

Statistical analysis

Data were presented as the mean ± standard deviation (s.d.) of three separate experiments, and one-way ANOVA with Student–Newman–Keul's test comparison was used for statistical significance with $P \leq 0.05$.

Results

Stretching strain-induced morphological changes in human PDL cells

Morphological changes in human PDL cells were observed by an inverted phase-contrast microscopy. Human PDL cells in each group showed that cells inclined parallel to each other and aligned their long axis perpendicular to the stretching force vector; this status is more and more transparent with extension of time and stretching strain. Figure 3 shows the morphology of the stretched human PDL cells. Human PDL cells under non-stretching conditions were aligned multidirectionally (Figure 3a). Stretching strain induced morphological changes in the stimulated human PDL cells. Human PDL cells which stretched by strain 1% for 6 h assumed a spindle shape, and some cells aligned their long axis

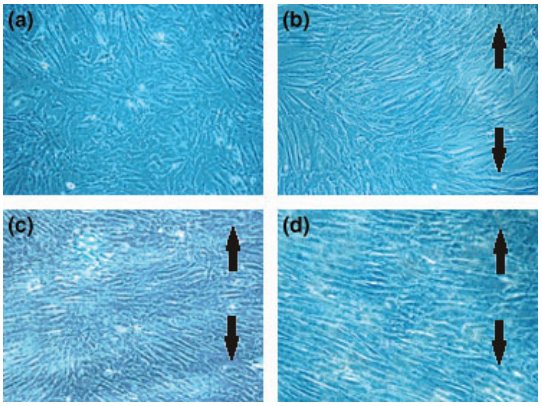

Figure 3 Stretching strain induced morphological changes in periodontal ligament cells. (**a**) Cells were cultured with non-stretching force. Cells aligned multidirectionally. (**b**) Cells were stretched by 1% strain for 6 h. Some cells were parallel to each other. (**c**) More cells were parallel (10% strain, 6 h). (**d**) Almost all cells aligned unidirectionally, and aligned their long axis perpendicular to the stretching force vector (20% strain, 6 h). Black arrow shows direction of stretching force [original magnifications ×200 (**a**–**d**)]

perpendicular to the stretching force vector (Figure 3b). At the marginal area (Figure 3c), more PDL cells which stretched by strain 10% for 6 h aligned in a manner similar to that observed in Figure 3b. The majority of PDL cells which stretched by strain 20% for 6 h were parallel to each other, and were elongated remarkably (Figure 3d). In the centre of the disc, cells showed minimal deformation and unidirectional alignment of PDL cells, similar to the cells in non-stretching controls.

Analysis of early apoptosis in human PDL cells by annexin V

Apoptotic cells were identified by double labelling with annexin V and PI. PI labels all dead cells, including necrosis and late stages of apoptosis, whereas cells entering early apoptosis are only stained by annexin V. Representative dot plots of annexin V staining are shown in Figure 4. The lower left quadrant indicates V-FITC−/PI− viable cells, the lower right quadrant reflects early stages of apoptosis (V-FITC+/PI−), the upper right quadrant indicates late stages of apoptosis (V-FITC+/PI+). When PDL cells stretched by 1%, 10% and 20% strain, the rate of apoptosis changed along with time increased. Time course analysis (10 cycles - min^{-1}) revealed a three- to fourfold increase in annexin V-FITC+/PI− cells in 6 h after stretching strain (1%, 10% and 20% strain) (Figure 5), reflecting an early stage of apoptosis. The rate of apoptosis enhanced in human PDL cells with stretching force increasing. It reached peak at 6 h, then decreased to non-stretching controls level at 12 h. Interestingly, the rate of apoptosis was lower than non-strteching controls at 30 min. Furthermore, experiments indicated a time- and force-dependent induction of early apoptosis of human PDL cells in response to stretching strain within 6 h, and then

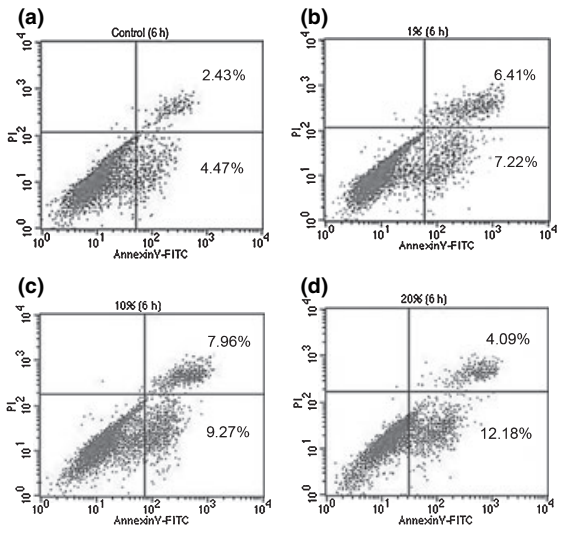

Figure 4 Dot plots of annexin V staining using flow cytometry. The lower right quadrant reflects the early stages of apoptosis, the upper right quadrant indicates the late stages of apoptosis. (**a**) Non-stretching group (6 h). (**b**) 1% strain (6 h). (**c**) 10% strain (6 h). (**d**) 20% strain (6 h). Results show that the rate of apoptosis increases with enhancing force. Significant differences were found between control, 1%, 10% and 20% groups ($P < 0.05$)

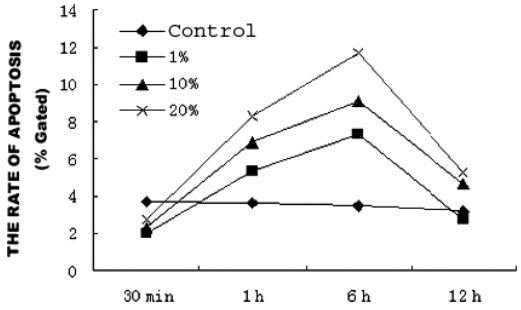

Figure 5 Human PDL cells labelled by annexin V-FITC+/PI. The early apoptosis increased time- and force-dependently from 30 min to 6 h in response to stretching strain, and decreased at 12 h. In contrast to control group, apoptosis increased about threefold in strain 20%, 6 h group.

Table 1 Statistical analysis between three strain groups

1% groups		*10% groups*		*20% groups*	
Groups	*P*	*Groups*	*P*	*Groups*	*P*
1 and 2	>0.05	1 and 2	>0.05	1 and 2	>0.05
1 and 3	>0.05	1 and 3	<0.01	1 and 3	<0.01
1 and 4	<0.01	1 and 4	<0.01	1 and 4	<0.01
1 and 5	>0.05	1 and 5	<0.05	1 and 5	<0.01
2 and 3	>0.05	2 and 3	<0.01	2 and 3	<0.01
2 and 4	<0.01	2 and 4	<0.01	2 and 4	<0.01
2 and 5	>0.05	2 and 5	<0.05	2 and 5	<0.01
3 and 4	<0.01	3 and 4	<0.01	3 and 4	<0.01
3 and 5	>0.05	3 and 5	<0.01	3 and 5	<0.01
4 and 5	<0.01	4 and 5	<0.01	4 and 5	<0.01

1: control group; 2: 30 min group, 3–1 h, 4–6 h, 5–12 h in 1%, 10% and 20% strain groups.

Table 2 Statistical analysis between four time groups

30 min groups		*1 h groups*		*6 h groups*		*12 h groups*	
Groups	*P*	*Groups*	*P*	*Groups*	*P*	*Groups*	*P*
1 and 2	>0.05	1 and 2	<0.01	1 and 2	<0.01	1 and 2	>0.05
1 and 3	>0.05	1 and 3	<0.01	1 and 3	<0.01	1 and 3	<0.05
1 and 4	>0.05	1 and 4	<0.01	1 and 4	<0.01	1 and 4	<0.05
2 and 3	>0.05	2 and 3	<0.01	2 and 3	<0.01	2 and 3	<0.05
2 and 4	>0.05	2 and 4	<0.01	2 and 4	<0.01	2 and 4	<0.05
3 and 4	>0.05	3 and 4	<0.01	3 and 4	<0.01	3 and 4	>0.05

1: control group; 2: 1%, 3–10%, 4–20% strain groups in 30 min, 1 h, 6 h and 12 h groups.

apoptosis decreased at 12 h (Figure 5). The statistical analysis is presented in Tables 1 and 2.

Measurement of apoptosis by confocal laser scanning microscopy

Our experiments showed that after cells loaded stretching strain, the fluorescence of annexin V-FITC yielded positive labelling (fluorescence of annexin V is green), and PI staining was negative (fluorescence of PI is red). It can be found that the number of apoptotic cells with

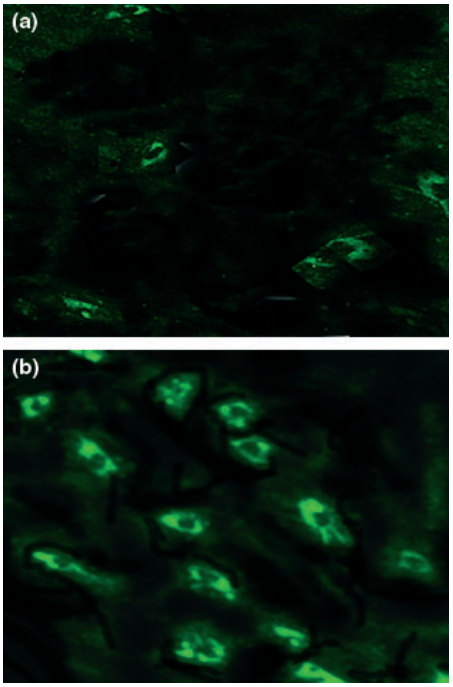

Figure 6 Human PDL cells observed under a confocal laser scanning microscope. (**a**) Apoptotic cells show less positive labelling (green fluorescence) by annexin V in non-stretching control. (**b**) Apoptotic cells increase distinctly by strain 20% for 6 h [original magnification ×63 (a,b)]

positive labelling in 20% strain, 6 h group were more than that in the non-stretching group (Figure 6).

Discussion

From the 1970s, PDL cells were separated and cultured successfully from PDL; nevertheless, it was difficult to purify PDL cells. PDL contains fibroblasts, cementoblasts, osteoblasts, undifferentiated mesenchymal cells, etc. (Arnold and Baram, 1972; Brunette *et al*, 1976; Blomlof and Otteskog, 1981; Ragnarsson *et al*, 1985; Adams *et al*, 1993). Fibroblasts are the predominant cells in PDL. Initially named as periodontal fibroblasts, these cultured cells contain not only periodontal fibroblasts but other cells (i.e. undifferentiated mesenchymal cells), therefore, they are usually called PDL cells nowadays.

Reports have indicated PDL breakdown during excessive occlusal loading *in vivo*, and observed osteopontin and osteoclast at the apical interradicular septum (Kaku *et al*, 2005). The mechanical force generated during tooth movement creates compressed and cell-free areas in the periodontal membrane, and compressed PDL cells generate apoptosis, and then are eliminated by using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nickend-labelling (TUNEL) method in the early phase of tooth movement (Hatai *et al*, 2001). PDL maintains homeostasis through occlusion including compressive and stretching force. At the same time apoptosis also operates in adult organisms to maintain normal cellular homeostasis. This is especially critical in long-lived mammals that must integrate multiple physiological as well as pathological death signals, which for example include regulating the response to infectious, the removal of growth factors, exposure to radiation, or administration of anti-cancer drugs (Haimovitz-Friedman *et al*, 1994; Boesen-de *et al*, 1998; Jilka *et al*, 1998).

Mechanical strain not only regulates structure and function in bones but also plays a crucial role in orthodontic tooth movement (Motokawa *et al*, 2005). To date, many investigators have studied the responses of bone cells to mechanical stresses such as stretch (Zhuang *et al*, 1996; Cheng *et al*, 1999; Basso and Heersche, 2002; Weyts *et al*, 2003), fluid flow (You *et al*, 2000), four-point bending (Kaspar *et al*, 2000) and hydrostatic pressure (Nagatomi *et al*, 2001). PDL fibroblasts which retain osteoblastic properties and high alkaline phosphatase levels are prominent sensors of this continuous stress and respond in a way culminating in periodontal tissue/alveolar bone regeneration (Egelberg, 1987). This process is likely to be influenced, and perhaps modulated, by TGF-β1, prostaglandin E, interleukin family, and express c-Fos, c-Jun (Karin, 1995; Salter *et al*, 1997; Chen and Bowden, 1999; Hatai *et al*, 2001; Bao *et al*, 2004; Yamamoto *et al*, 2006). The stretching forces exerted on the cells are probably analogous to the stresses exerted on PDL fibroblasts *in vivo* (Basdra *et al*, 1995).

Compressive force could induce PDL cell apoptosis and stretching force might influence function and correlative protein expression of PDL cells shown as previous studies (Hatai *et al*, 2001; Yamamoto *et al*, 2006). However, whether stretching force induced human PDL cells apoptosis remains unclear.

In this experiment human PDL cells were subjected to increasing stretching force and durations *in vitro* to investigate whether human PDL cells undergo apoptosis, and determine the adaptive stretching force and time-loading. When cells stretched by 1%, 10% and 20% strain for 30 min, the rate of apoptosis in human PDL cells was lower than the untreated controls; nevertheless, no significant difference was found between each of the experimental groups and untreated controls ($P > 0.05$) (Figure 5). As the duration of stretching increased, the rate of apoptosis augmented gradually, and higher than the untreated controls significantly. The rate of apoptosis reached peak when cells stretched by 20% strain for 6 h. Moreover, the rate of apoptosis approached maximum at 6 h in each of the different strain groups. A significant difference was found between the 6 h group and 30 min and 1 h groups ($P < 0.05$). However, when cells stretched for 12 h, the rate of apoptosis of each strain group decreased markedly, there was a significant difference between the 6 and 12 h group ($P < 0.05$). The reason for the above phenomenon could be that after stretching force induced human PDL cells to reach a peak in the early stage of apoptosis within 6 h, early apoptotic cells came into late apoptosis stage or necrosis stage, so the rate of early apoptosis decreases at 12 h. According to this experimental result, human PDL cells should not be stretched constantly beyond 6 h, and not beyond 10% strain, lest cells occur apoptosis redundantly, even necrosis.

Secondly, human PDL cells which were stretched occurred morphological changes. The cells were longer than non-stretching controls, but they were still spindle shaped and became aligned perpendicular to the stretching force vector. Researchers have reported that PDL cells show the same alignment (Howard *et al*, 1998; Hatai *et al*, 2001). Cyclic stretching induced orientation of microtubules of PDL cells *in vitro*. This orientation of PDL cells in response to cyclic stretching probably represents a self-protection mechanism in the cells, namely cells keep themselves from being elongated excessively, so as to avoid injury. However, an *in vivo* experiment showed that the cells were spindle shaped and oriented in the direction of the strain (Rygh, 1976). The collagen bundles are named as 'principle fibres', and the arrangement of the bundles is classified into five groups: gingival, crestal, horizontal, oblique and apical. Mastication may act as a stimulus to maintain the three-dimensional architecture of the PDL. Reduced PDL function or lack of occlusal stimuli induces PDL atrophy or a decrease in the thickness of the PDL, as the fibres decrease in number and density. Fibres in the PDL of the teeth without antagonists were reported to be aligned parallel to the surface of the dental root. During orthodontic retention, birefringent collagen fibre bundles running across the compressed and expanded PDL were observed, although they appeared to be thinner with less insertions into the alveolar bone or cementum in the experimental animals than in the controls. This suggests that the periodontal collagen fibres were partially reorganized and rearranged during retention. The PDL transmits external force applied to the teeth, for example by mastication, to stretching force. Therefore, when mechanical force was applied to the teeth, PDL cells proliferated and increased in number, which strengthened the physical properties of the PDL fibres, resulting in a widening of the PDL space. Thus, the alignment of PDL cells perpendicular to the strain force vector may be a characteristic essential to the maintenance of the PDL architecture (Fukui *et al*, 2003; Chiba and Mitani, 2004).

Finally, the main characteristics of CSU include: (1) the stretching force that exerted on the cells *in vitro* is presumably analogous to the one exerted on PDL fibroblasts *in vivo* to some degree (Basdra *et al*, 1995); (2) the CSU can produce stretching strain from 1% to 25%; (3) cyclic loading time of stretching strain can be set from 1 to 359 999 s continuously by program in PC; (4) photoelectric encoder in the CSU can monitor the ascending height of spherical cap real-timely, and feedback to PC, so as to ensure accuracy of stretching strain; (5) three-dimensional dynamic images real-timely simulate stretching strain of flexible membrane in PC so that we can observe it conveniently and directly. In this way, it can be believed that the CSU is adapt to study stretching strain of PDL cells.

In conclusion, this study found that certain stretching force can induce early apoptosis of human PDL cells significantly in a force- and time-dependent manner. However, it is necessary to further research the apoptosis mechanism of human PDL cells which are stretched.

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