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Mechanical Force Induces Type I Collagen Expression in Human Periodontal Ligament Fibroblasts Through Activation of ERK/JNK and AP-1

Sung-Ho Kook,[1,2] Jung-Min Hwang,[2,3] Jong-Sun Park,[2,4] Eun-Mi Kim,[2,3] Jung-Sun Heo,[3] Young-Mi Jeon,[2,4] and Jeong-Chae Lee[2,3,4*]

[1] *Division of Biological Sciences, Chonbuk National University, Jeonju 561-756, South Korea*

[2] *Institute of Oral Biosciences and 21 Century Education Center for Advanced Public Dental Health (BK 21 program), Chonbuk National University, Jeonju 561-756, South Korea*

[3] *Department of Bioactive Materials and Research Center of Bioactive Materials, Chonbuk National University, Jeonju 561-756, South Korea*

[4] *Department of Orthodontics, School of Dentistry, Chonbuk National University, Jeonju 561-756, South Korea*

ABSTRACT

Type I collagen (COL I) is the predominant collagen in the extracellular matrix of periodontal ligament (PDL), and its expression in PDL fibroblasts (PLF) is sensitive to mechanical force. However, the mechanism by which PLF induces COL I to respond to mechanical force is unclear. This study examined the nature of human PLF in mediating COL I expression in response to centrifugal force. Signal transduction pathways in the early stages of mechanotransduction involved in the force-driven regulation of COL I expression were also investigated. Centrifugal force up-regulated COL I without cytotoxicity and activated extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 kinase. ERK and JNK inhibitor blocked the expression of COL I but p38 kinase inhibitor had no effect. Centrifugal force activated activator protein-1 (AP-1) through dimerization between c-Fos and c-Jun transcription factors. ERK and JNK inhibitors also inhibited AP-1-DNA binding, c-Fos nuclear translocation, and c-Jun phosphorylation that were increased in the force-exposed PLF. Further, transfecting the cells with c-Jun antisense oligonucleotides almost completely abolished the force-induced increase of c-Jun phosphorylation and COL I induction. Our findings suggest that mechanical signals are transmitted into the nucleus by ERK/JNK signaling pathways and then stimulate COL I expression through AP-1 activation in force-exposed human PLF. J. Cell. Biochem. 106: 1060–1067, 2009.

KEY WORDS: CENTRIFUGAL FORCE; HUMAN PERIODONTAL LIGAMENT FIBROBLASTS; TYPE I COLLAGEN; MITOGEN-ACTIVATED PROTEIN KINASES; ACTIVATOR PROTEIN-1

Orthodontic treatment is achieved by applying a prolonged mechanical force to the tooth, which causes bio-physical changes to the periodontal tissue comprising the alveolar bone, periodontal ligament (PDL), and gingiva [Reitan, 1985]. The PDL is a band of dense connective tissue located between the tooth-root cementum and the alveolar bone, and PDL fibroblasts (PLF) are the most abundant cells in the PDL [Sodek and Limeback, 1979; Berkovitz, 1990]. PLF maintain PDL integrity and play important roles in cellular responses to physiological and mechanical stresses [Lekic and McCulloch, 1996].

The PDL contains many different types of collagen including types I, III, V, VI, XII, and XIV [Bornstein and Sage, 1980; Dublet et al., 1988; Zhang et al., 1993]. PLF can induce collagen molecules to respond to mechanical stimuli in the PDL [Wang et al., 2003], and this is an essential event in the process of alveolar bone remodeling induced by mechanical force [Bumann et al., 1997]. The induction of collagen fibers and their turnover in PDL occur very rapidly [Sodek and Limeback, 1979; Sodek and Ferrier, 1988]. Importantly, type I collagen (COL I) is the predominant collagen in PDL. COL I forms solid fibers anchored to cementum and alveolar bone and protects PDL from tensile strength and masticatory loading [Bumann et al., 1997; Wu et al., 2006].

There are a few studies that elucidate PLF's role in the expression of collagen molecules in response to mechanical force. For example,

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*Correspondence to: Prof. Jeong-Chae Lee, Department of Orthodontics, Institute of Oral Biosciences and Research Center of Bioactive Materials, Chonbuk National University, Jeonju 561-756, South Korea.
E-mail: leejc88@chonbuk. ac.kr

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the induction of collagens in response to orthodontic stimulation appeared to be higher in the compression rather than in the tension side, where COL I was the highest collagen type synthesized in both zones [Bumann et al., 1997]. Application of centrifugal force to PLF in vitro also increased significantly the expression of COL I [Wei et al., 2008]. However, Garlet et al. [2007] demonstrated that the tension side presented higher expression of COL I, while compression side did not exhibit such increases. Further, centrifugal force increased mRNA levels of matrix metalloproteinase-1 with almost no effect on COL I in PLF [Redlich et al., 2004]. We therefore postulated that expression patterns of COL I in response to mechanical force may differ according to the experimental conditions and the types of mechanical stimuli exposed.

This study examined whether mechanical forces actually up-regulate COL I expression in mechanical force-exposed PLF. This study also determined the plausible mechanisms by which centrifugal force regulates COL I expression. Our study focused on understanding the roles of mitogen-activated protein (MAP) kinases and activator protein-1 (AP-1). MAP kinases tightly regulates the signal transduction pathways involved in mechanotransduction in osteoblastic cells [Liedert et al., 2006], and AP-1is a specific target of MAP kinases in the mechanical signaling [Kletsas et al., 2002].

MATERIALS AND METHODS

CHEMICALS AND LABORATORY WARE

Unless otherwise specified, all chemicals and laboratory ware were obtained from Sigma Chemical Co. (St. Louis, MO) and Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ), respectively. MAP kinase inhibitors, PD98059 (2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one), SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole), and SP600125 (anthra[1,9-cd]pyrazol-6(2*H*)-one) were purchased from TOCRIS (MI) and dissolved in either absolute ethanol or dimethylsulfoxide (DMSO) prior to use. The final ethanol or DMSO concentration did not exceed 0.5% (v/v) in any experiments.

CELL CULTURE

PLF were obtained from three male healthy individuals, aged 20–30 years who underwent molar extraction. PLF were cultured according to the methods described elsewhere with minor modifications [Howard et al., 1998]. Written informed consent for use of the periodontal tissues was obtained from all donors. This study was approved by the Review Board of Chonbuk National University Hospital (CNUH). All cultures were maintained at 37°C with a humidified gas mixture of 5% CO_2/95% air and switched to a fresh batch of medium every 3 days. After cells reached confluence, they were sub-cultured for several passages in T75 culture plates and spread onto 6-well or 96-well flat-bottomed plates before the application of centrifugal force. All the experiments were carried out at passages 4–7.

APPLICATION TO MECHANICAL FORCE

PLF cultures were exposed to mechanical force by applying centrifugal force to the cells, similar to a well-established in vitro compression model of centrifugation of cultured cells [Redlich et al., 1998]. This is useful in the evaluation of the direct effects of compression on cells without side effects [Redlich et al., 2004]. PLF were also treated with MAP kinase inhibitors for 1 h prior to centrifugation of culture plates at a magnitude of 50 g/cm^2 for 60 min using a horizontal microplate rotor (Universal 32 R, Hettich, Germany) as described previously [Redlich et al., 1998]. The applied force calculation was based on the following equation [Redlich et al., 2004]: $P = (m \times r \times rpm^2 \times \pi^2)/(A \times 9.8 \times 900)$, where P = kg pressure per cm^2 of cells, m = mass of medium (g), r = radius (0.12 m), rpm = revolution per min, A = area of contact between the medium and cells (cm^2). PLF were adjusted to the analyses for cytotoxicity, COL I expression at the mRNA level, and for the mechanisms involved in the force-induced up-regulation of COL I at various times after the force application.

MEASUREMENTS OF CYTOTOXICITY AND DNA FRAGMENTATION

This study examined whether centrifugal force induced cytotoxic effects on PLF using trypan blue staining and agarose gel electrophoresis. In trypan blue exclusion assay, the level of cytotoxicity was determined at various times (0–48 h) after the force and was calculated after counting 100 cells for each treatment as follows: % cytotoxicity = [(total cells − viable cells)/total cells] × 100. In addition, the force-exposed PLF were incubated with a lysis buffer [1% nonidet P (NP)-40 and 1% sodium dodecyl sulfate (SDS) in 50 mM Tris–HCl, pH 8.0] at 65°C for 1 h. DNA was extracted with phenol/chloroform/isoamyl alcohol, and the degree of fragmentation was analyzed using 1.5% agarose gel electrophoresis.

WESTERN BLOT ANALYSIS

Whole cell lysates from the control and force-exposed PLF were made in a NP-40 lysis buffer (30 mM Tris–Cl, pH 7.5, 1 mM EDTA, 150 mM NaCl, and 1% NP-40). Protein content was quantified using the Bradford method [1976]. Nuclear proteins were prepared as previously described [Maulik et al., 1998]. Equal amounts of protein extracts were separated by 12% SDS–PAGE and blotted onto poly vinyl difluoride membranes. The blots were probed with primary antibodies overnight at 4°C prior to incubation with secondary antibody in a blocking buffer for 1 h. The blots were developed with enhanced chemiluminescence (Amersham Pharmacia Biotech Inc.) and exposed to X-ray film (Eastman-Kodak, Rochester, NY). The polyclonal antibodies specific to extracellular signal-regulated kinase (ERK; SC-94), c-Jun N-terminal kinase (JNK; SC-9252), JunB (SC-73), c-Jun (SC-45), p-c-Jun (SC-7981), c-Fos (SC-52), FosB (SC-48) and Fra-2 (Q-20), and the monoclonal antibodies specific to p-ERK (SC-7383), p-JNK (SC-6254) and p-p38 (SC-7973) were purchased from Santa Cruz Biotechnology. The monoclonal antibodies specific to p38 (9217) were purchased from Cell Signaling (MA).

REVERSE TRANSCRIPTION-PCR (RT-PCR)

Total RNA was extracted according to the manufacturer's instructions (SV Total RNA Isolation System, Promega, Madison, WI). Reverse transcription and PCR amplification were also performed using an Access RT-PCR System (Promega) according to the

manufacturer's protocol. Primer sequences used to amplify COL I are as follows: COL I (~428 bp), 5′-AAA CTT TGC TCC CCA GCT GT-3′ (forward) and 5′-GAC CAG GAG CTC CAT TTT CA-3′ (reverse). The house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, ~190 bp) with the primer sequences 5′-AAC CTG CCA AAT ATG ATG AC-3′ (forward) and 5′-ATA CCA GGA AAT GAG CTT GA-3′ (reverse), was used as the positive control. PCR was performed for 30–35 cycles at 94°C for 30 s, at 50–55°C for 30 s, and at 72°C for 1 min in a DNA Thermal cycler (model; PTC-100, Waltham, MA). PCR products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining. Band intensity was calculated using a gel imaging system (model; F1-F2 Fuses type T2A, BIO-RAD, Segrate, Italy). PCRs were repeated in at least three independent RNA preparations.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Nuclear proteins from PLF were prepared at varying times after application of force, and the binding activity of AP-1 to its specific DNA was determined by EMSA as described elsewhere [Lee et al., 2003]. Briefly, DNA-protein binding reactions were carried out for 30 min at room temperature using 10–15 μg of the protein in a 20-μl volume containing 1 μg/ml albumin, 0.5 μg/μl poly (dI-dC), 5% glycerol, 1 mM DTT, 1 mM PMSF, 10 mM Tris–Cl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 30,000 cpm of [α-^{32}P] dCTP-labeled oligonucleotides, and the Klenow fragment of DNA polymerase. The samples were separated on 6% polyacrylamide gels, dried, and exposed to X-ray films. The oligonucleotide primer sequences of AP-1 used for EMSA were: 5′-AAG GGA TCC GGC TGA CTC ATC ACT AG-3′ and 3′-CTA GGC CGA CTG AGT AGT GAT CGG AA-5′.

TRANSFECTION WITH NONSENSE OR ANTISENSE c-JUN OLIGONUCLEOTIDES

PLF were seeded at 5×10^5 cells/well in 6 well plates and transfected with 1 μM nonsense (NSO, 5′-CAT CTT TGC AGT CAT-3′) or c-Jun antisense oligonucleotides (ASO, 5′-ATG ACT GCA AAG ATG-3′). The 15-mer antisense sequences for c-Jun were short enough to penetrate the cell with the longevity for their specificity [Urakami et al., 1997]. Each transfection was performed using Lipofectamine™ 2000 Transfection Reagent (Invitrogen, MD) according to the manufacturer's protocol. At 24 h thereafter, cells were exposed to centrifugal force and processed for the analyses of c-Jun, p-c-Jun, and COL I expression.

STATISTICAL ANALYSIS

Unless otherwise specified, all the data are expressed as the means ± standard deviation (SD) from triplicate experiments. A one-way ANOVA was used for multiple comparisons using SPSS version 15.0 software. A P value <0.05 was considered statistically significant.

RESULTS

CENTRIFUGAL FORCE UP-REGULATES COL I EXPRESSION WITHOUT A CYTOTOXIC EFFECT

We initially examined the ability of centrifugal force to cause cytotoxic effects on PLF. PLF were exposed to centrifugal force for 60 min, and at varying times (0–48 h) after the force the cells were adjusted to trypan blue staining and agarose gel electrophoresis. Centrifugal force neither caused significant cytotoxicity (Fig. 1A) nor induced DNA ladder formation (Fig. 1B) during the experimental periods.

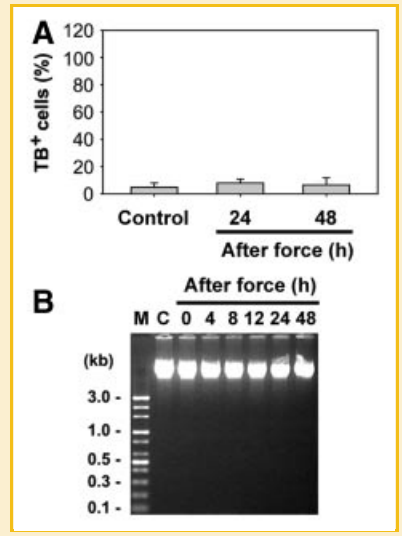

Fig. 1. Effects of centrifugal force on cytotoxicity and DNA fragmentation in PLF. PLF were exposed to centrifugal force at a magnitude of approximately 50 g/cm^2 for 60 min. Cytotoxicity and apoptosis were determined at varying times after initial force by trypan blue staining (A) and agarose gel electrophoresis (B). TB, trypan blue; M, molecular size maker; C, unforced control cells.

mRNA levels of the COL I gene in the force-exposed PLF were measured by RT-PCR (Figs. 2A,B). As shown in the figures, COL I expression increased from 1 h and peaked at 4 h after the force. Thereafter, COL I mRNA levels gradually decreased but still remained about 2.2-fold higher than basal levels at 16 h after

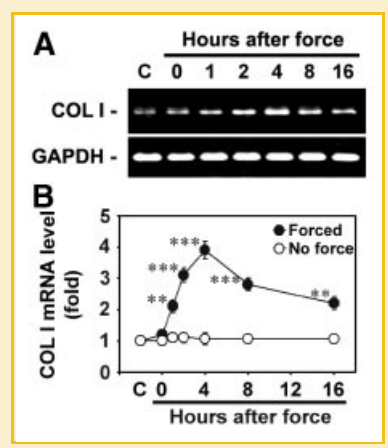

Fig. 2. Effects of centrifugal force on COL I expression in PLF. A: mRNA levels of COL I in force-exposed PLF were determined at varying times (0–16 h) after the force through RT-PCR. B: The data obtained from three independent assays were quantified by densitometry after normalizing the bands to GAPDH. $^{**}P<0.01$ and $^{***}P<0.001$ versus the unforced control values.

exposing the force. The COL I mRNA levels were not changed in unforced PLF (Fig. 2B).

CENTRIFUGAL FORCE INDUCES PHOSPHORYLATION OF MAP KINASES

MAP kinases play important roles in the signal transduction of mechanotransduction in osteoblastic cells [Liedert et al., 2006]. PLF have osteogenic potential and undergo osteoblastic differentiation in culture, depending on exposure to extracellular stimuli [Arceo et al., 1991; Basdra et al., 1995; Kletsas et al., 1998]. We examined the phosphorylated levels of MAP kinases using Western blot analyses. Centrifugal force induced the rapid and temporal activations of both ERK and JNK (Fig. 3A). p-ERK1/2 and p-JNK1/2 reached maximum level of 4.8 ± 0.4-fold and 3.8 ± 0.3-fold, respectively, and returned to basal levels at 2 h after the force, as compared with the non-forced control levels (Fig. 3B). In contrast, cellular levels of p-p38 increased significantly from 1 h after the force and maintained for up to 8 h. Maximum levels of force-induced p-p38 appeared lower than those of p-JNK or p-ERK.

ERK/JNK-MEDIATED SIGNALING ARE RELATED TO THE FORCE-INDUCED EXPRESSION OF COL I

We subsequently examined whether MAP kinase activation is involved in the force-induced up-regulation of COL I. The concentration of MAP kinase inhibitors used in this study was

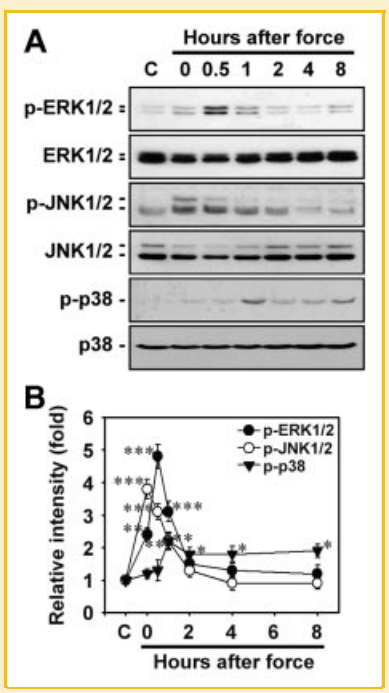

Fig. 3. Effects of centrifugal force on the phosphorylation of MAP kinases in PLF. A: Protein samples were prepared from the force-exposed PLF at the indicated times (0–8 h) after the force, and the levels of ERK1/2, JNK1/2, and p38 MAP kinases were measured by Western blot analysis. A representative result from triplicate assays was shown. B: Plots of densitometric results of MAP kinase phosphorylation after normalization of their whole protein bands. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus unforced control levels.

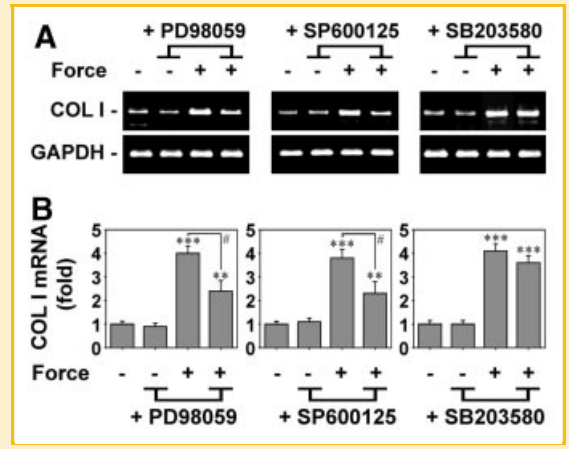

Fig. 4. Effects of MAP kinase inhibitors on the expression of COL I in centrifugal force-exposed PLF. A: PLF were treated with 10 μM of each MAP kinase inhibitor for 1 h prior to exposure to centrifugal force. COL I mRNA levels were determined by RT-PCR after 4 h of the force. A representative data from three independent experiments was shown, and GAPDH was used as control marker. B: The results from triplicate experiments were quantified by densitometry after normalizing the bands to GAPDH. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus the unforced control values. $^{\#}P < 0.05$ versus the experiments.

10 μM. At this dose, inhibitors did not affect basal phosphorylation levels of their specific target kinases and they significantly reduced MAP kinase levels that were increased after the force (data not shown). Inhibition of ERK with PD98059 significantly prevented COL I expression but was still higher than the non-forced control levels (Figs. 4A,B). Similarly, treatment of PLF with a JNK inhibitor, SP600125, significantly decreased COL I mRNA levels that were increased by application of force. However, SB203580, a p38 kinase inhibitor, did not affect COL I expression.

CENTRIFUGAL FORCE INDUCES DNA BINDING ACTIVATION OF AP-1

AP-1 regulates a broad range of cellular events such as proliferation, differentiation and apoptosis. MAP kinases have roles as upstream effectors of AP-1 by mediating the activation of c-Jun and c-Fos proteins which bind to the promoter of mechanosensitive genes [Franceschi et al., 2003; Xu et al., 2006]. We examined whether centrifugal force activated AP-1 binding to its specific target DNA. Centrifugal force activated AP-1 binding to its specific DNA in a transient fashion, where the activity peaked at 30 min to 1 h after the force, and decreased to basal levels by 4 h after application of force (Fig. 5A). In addition, PLF were treated with inhibitors of MAP kinases for 1 h prior to force application in order to clarify how MAP kinase affects AP-1 activation. Inhibitors of ERK and JNK significantly reduced the AP-1-DNA binding but a p38 inhibitor had no effect (Fig. 5B).

AP-1 IS TRANSCRIPTION FACTOR RESPONSIBLE FOR COL I UP-REGULATION BY CENTRIFUGAL FORCE

Fos and Jun proteins are the subfamily proteins of AP-1 and are sensitive to mechanical stress [Hipskind and Bilbe, 1998; Kletsas et al., 2002]. This study also revealed that centrifugal force increased AP-1 activity in human PLF, while ERK or JNK inhibitors

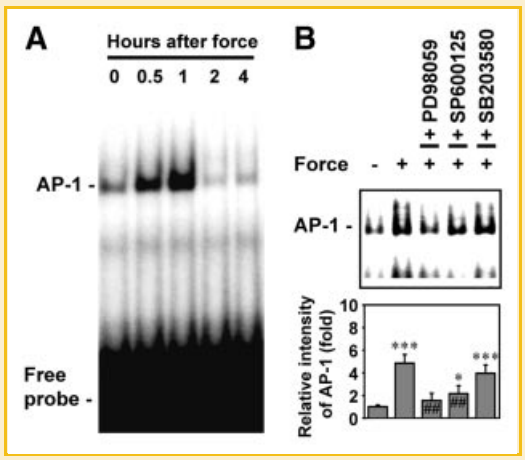

Fig. 5. Effects of centrifugal force on the DNA binding of AP-1 in PLF. A: Nuclear extracts were prepared from PLF at the indicated times after the force and were analyzed by EMSA. B: PLF were pretreated with MAP kinase inhibitors for 1 h prior to the application of force. Nuclear extracts were obtained at 1 h after the force and then used for the analysis of AP-1 DNA binding activity. Densitometric results of the AP-1 complex from three independent experiments were also shown. $^{*}P<0.05$ and $^{***}P<0.001$ versus the unforced control values. $^{\#\#}P<0.01$ versus the force-exposed PLF.

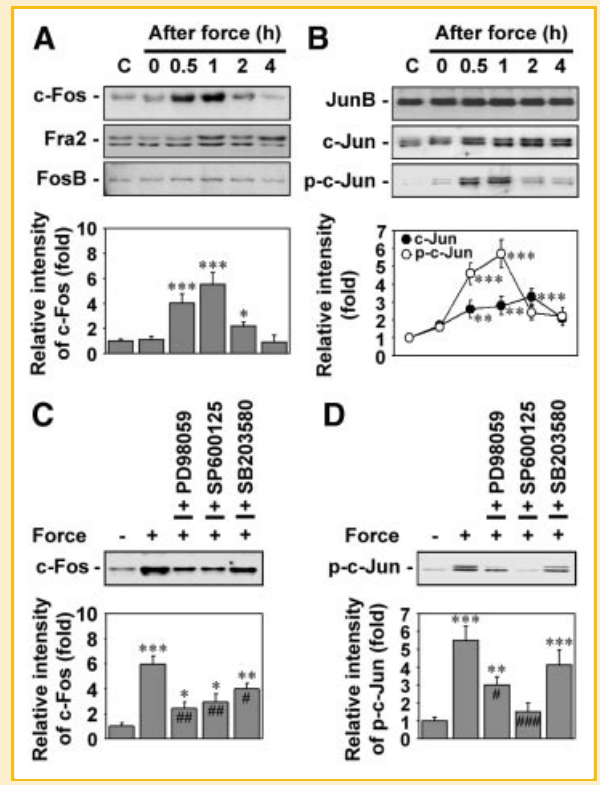

Fig. 6. Centrifugal force stimulated the binding of AP-1 to its specific DNA through ERK/JNK-mediated signaling. Effects of centrifugal force on the induction of c-Fos (A) and c-Jun subfamily (B) transcription factors were analyzed by immunoblotting using the same nuclear extracts. The bands of these proteins were quantified by densitometry. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ versus controls. Effects of MAP kinase inhibitors on c-Fos induction (C) and c-Jun phosphorylation (D) in the force-exposed PLF were also determined. PLF were pretreated with 10 μM of each MAP kinases for 1 h prior to the force, and the nuclear extracts were prepared at 1 h of the force and then analyzed by Western blot analysis. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ versus the unforced control values. $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$ versus the force-exposed positive controls.

prominently inhibited AP-1 activity. We therefore determined how centrifugal force influenced the induction of Jun and Fos proteins using Western blot analysis. Centrifugal force resulted in the nuclear increase of c-Fos protein but not of Fra2 and FosB (Fig. 6A). It also increased the nuclear induction of c-Jun and induced a 5.7-fold enhancement in the level of p-c-Jun at 1 h after the force (Fig. 6B).

We next used specific inhibitors of MAP kinases to address the participation of upstream signaling pathways in c-Fos and c-Jun induction after application of centrifugal force. Nuclear extracts were prepared from PLF at 1 h after the force, because this time produced almost maximum induction of c-Fos, c-Jun, and AP-1-DNA binding activity. Centrifugal force increased c-Fos induction (Fig. 6C). However, the increase was significantly inhibited in the presence of MAP kinase inhibitors, with differing efficacies. ERK and JNK inhibitors reduced the nuclear induction of c-Fos in the force-exposed PLF, and the p38 kinase inhibitor did so with less efficacy. The effect of these inhibitors on c-Jun phosphorylation was also examined under the same conditions (Fig. 6D). Inhibitors of ERK and JNK reduced the phosphorylated level of c-Jun that was augmented in force-exposed PLF. In particular, JNK inhibitor blocked the force-mediated c-Jun phosphorylation. However, the p38 inhibitor did not significantly decrease the p-c-Jun level.

To more understand the functional role of AP-1 in the force-mediated COL I expression, we determined the effects of transfection with the c-Jun antisense oligonucleotides. Centrifugal force increased dramatically the levels of c-Jun and p-c-Jun in PLF as expected, whereas these levels were significantly reduced by transfecting the cells with antisense oligonucleotides (Fig. 7, left panel). Blockage of c-Jun expression also reduced the force-induced increase of COL I expression to the basal level (Fig. 7, right panel). Down-regulation of c-Jun by the transfection alone did not induce cytotoxicity in a significant level during the experimental periods (data not shown). These data suggest that AP-1 is the main transcription factor which mediates the COL I overexpression in the centrifugal force-applied PLF.

DISCUSSION

In addition to orthodontic treatment, PLF are exposed to continuous mechanical loading derived from bite forces. The ability of PLF to receive mechanical loads is related to their potential to induce various bone tissue components required in the processes of bone remodeling and tooth movement. Collagen expression by PLF is one of the most critical events that occur in the process [Bumann et al., 1997]. Therefore, it is important to elucidate whether PLF actually up-regulates collagen expression in response to a compressive force. This study demonstrated that centrifugal force up-regulated COL I expression without any cytotoxicity in PLF (Figs. 1 and 2). This is not consistent with a previous report [Redlich et al., 2004], but suggests that the primary function of PLF in response to mechanical forces is

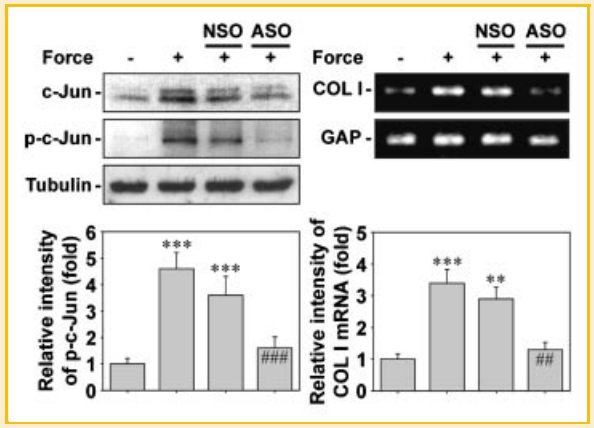

Fig. 7. Transfecting PLF with c-Jun antisense oligonucleotides inhibited the force-induced increase of c-Jun phosphorylation and COL I expression. PLF were transfected with the nonsense and antisense c-Jun oligonucleotides and after 24 h of incubation, they were exposed to centrifugal force for 1 h. The levels of c-Jun and p-c-Jun were determined by Western blot analysis at 1 h after the force (left panel). In addition, the COL I expression was analyzed by RT-PCR at 4 h after the force (right panel). NSO, nonsense oligonucleotides; ASO, antisense oligonucleotides. $^{**}P<0.01$ and $^{***}P<0.001$ versus the unforced control values. $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ versus the force-exposed positive controls.

in synthesis of extracellular matrix components such as collagens. We therefore assume that the force-mediated up-regulation of COL I is closely associated with osteogenic potentials, as in osteoblasts [Egelberg, 1987]. Further, the expression of osteoblast-specific genes in PLF is quite sensitive to mechanical stress [Franceschi, 1999; Wescott et al., 2007; Wei et al., 2008; Zhao et al., 2008].

Extracellular mechanical stresses are receptive to cellular messages by mechanoreceptors located in plasma membrane. These signals are transmitted to the nucleus through activation of various protein kinases, where MAP kinases are the most prominent kinases [Whitmarsh and Davis, 1996]. The present study showed that MAP kinases were activated in the force-exposed PLF (Fig. 3). This result is consistent with a report stating that application of cyclic strain to vascular smooth muscle cells resulted in a rapid and transient activation of all three types of MAP kinases [Li et al., 1999]. However, ERK or JNK, but not p38 kinase inhibitor, significantly blocked COL I expression stimulated by centrifugal force (Fig. 4). This suggests that the force-induced COL I up-regulation is mediated primarily by ERK/JNK-mediated signaling. AP-1 is a dimeric transcription factor comprised of members of the Fos and Jun sub-protein families. It is a key mediator of the early response of cells to external stimuli, where MAP kinases play important roles as upstream mediators [Angel and Karin, 1991; Karin et al., 1997]. Our present findings show that centrifugal force rapidly and transiently activated the DNA-binding capacity of AP-1 through ERK and JNK-mediated signaling in human PLF (Fig. 5).

Various types of extracellular stress can up-regulate c-Fos in diverse experimental systems [Peake et al., 2000; Kletsas et al., 2002; Li et al., 2007]. Mechanical stretching was associated with up-regulation of c-Fos induction in human PLF, and our results are consistent with this study [Kletsas et al., 2002]. A critical step in AP-1 activation in addition to c-Fos induction is ERK/JNK-mediated c-Jun activation followed by phosphorylation [Karin, 1995; Karin et al., 1997; Kletsas et al., 2002]. The nuclear translocation of c-Jun and its phosphorylation were induced rapidly in the force-applied PLF (Fig. 6). This is similar to the results obtained from other assay systems based on endothelial cells and cardiomyocytes exposed to mechanical stress [Nollert et al., 1992; Yamazaki et al., 1995]. In addition, Kletsas et al. [2002] reported that mechanical stretch of human PLF produced a 2.7- and 2-fold increase of c-Jun and p-c-Jun protein, respectively, within 2 h of stretching. We postulate that mechanical stress up-regulates c-Jun and its phosphorylation as well as c-Fos, although the response of cells to mechanical stress could, in part, differ according to the conditions of mechanical stimulus [Peverali et al., 2001].

We further investigated the mechanism of centrifugal force-mediated induction of c-Fos and c-Jun by treatment of PLF with MAP kinase inhibitors. This approach is postulated to evaluate the precise role of MAP kinases under more physiologic conditions than transfecting with siRNA or oligonucleotides [Cuenda and Alessi, 2000; Kletsas et al., 2002]. MAP kinase inhibitors reduced the increase of c-Fos and p-c-Jun with different efficacies according to the inhibitors used (Figs. 6C,D). c-Jun activation by phosphorylation is an important determinant of dimeric AP-1 activation and function [Karin, 1995; Whitmarsh and Davis, 1996; Karin et al., 1997]. It is accepted that JNK increases the AP-1 activity by phosphorylating the activation domain of c-Jun proteins while ERK stimulates the activity by inducing c-Fos synthesis and by enhancing c-Fos protein transactivation [Yoshioka et al., 1995; Davis, 2000]. We suggest that the ERK/c-Fos and JNK/c-Jun pathways are closely associated with the force-mediated up-regulation of COL I in human PLF. In addition, p38 is not the direct upstream effector of AP-1, although its specific inhibitor partially lowered the nuclear induction of c-Fos protein. To more understand the roles of AP-1 on the force-induced COL I up-regulation in PLF, the cells were transfected with c-Jun antisense oligonucleotides. This transfection almost completely abolished the force-induced increase of c-Jun phosphorylation and COL I expression (Fig. 7). This finding suggests that AP-1 is the critical factor involved in the force-induced COL I expression in PLF.

The osteogenic differentiation of PLF is believed to have important roles in alveolar bone remodeling by orthodontic treatment. There are several transcription factors which required for osteogenic differentiation in response to mechanical forces. Osterix, a zinc finger-containing transcription factor, was up-regulated in the centrifugal force-exposed PLF. In addition to COL I, several osteogenic marker genes including osteopontin, osteocalcin, and bone sialoprotein were positively increased by transfecting the cells with Osterix expression vector [Zhao et al., 2008]. This leads to postulate that Osterix plays critical roles in mechanical force-mediated osteogenic differentiation of PLF. A recent report also demonstrated that activating transcription factor-4 (ATF4) is essential for the mechanical stress-induced differentiation of PLF to osteoblast-like cells. This was proven by the results showing that ATF4′s overexpression enhanced significantly the osteogenic marker genes [Wei et al., 2008]. Taken together, these reports indicate that both transcription factors, Osterix and ATF4, also play important roles in centrifugal force-mediated up-regulation of

COL I. More detailed experiments elucidating the direct effects of these transcription factors on COL I gene transactivation will be necessary to understand the precise mechanisms involved in force-induced COL I up-regulation in PLF.

Integrins connect cytoskeleton to extracellular matrix and mediate a variety of signaling cascades. They also transduce mechanical stimuli into biological signals. Mechanical stretch forces activate ERK2 and JNK1 in rat cardiac fibroblasts through mediation of $\alpha 4\beta 1$ and a non-$\alpha 5\beta 1$ integrin as well as extracellular matrix [MacKenna et al., 1998]. Mechanical force also induces MAP kinase activation through $\alpha 2$ and $\beta 1$-containing integrins in osteoblasts [Schmidt et al., 1998]. Furthermore, Rho family kinases are the downstream mechanosensors via integrins as well as the upstream effectors that regulate MAP kinase activation [Basdra et al., 1995; Whitmarsh and Davis, 1996; Hall, 2005]. It is therefore possible that integrins and MAP kinase-mediated signals cooperate to modulate signaling pathways in the mechanotransduction process in centrifugal force-exposed cells [Shyy and Chien, 1997; Loesberg et al., 2008].

Cyclic stretch has been known to induce persistent phosphorylation of p38 kinase [Kook et al., 2008]. Partial inhibition of p38 phosphorylation increased cellular levels of p-ERK in stretched cells, thereby suggesting that p38 kinase, not ERK, is the upstream signal transducer regulating cellular responses to mechanical stretch in muscle cells [Kook et al., 2008]. The activation of p38 kinase resulted in the up-regulation of c-Jun and c-Fos expression [Franceschi et al., 2003]. Kletsas et al. [2002] reported that the stretch-induced c-Fos and c-Jun up-regulation in human PLF is closely associated with p38 kinase activation. Therefore, p38 has important roles in transducing the mechanical signaling into the nucleus. However, the p38 signaling pathway does not appear to be associated with the AP-1 activation as well as COL I expression in the force-exposed PLF. We suggest that p38 kinase is also a mechanosensitive protein, and its role in mediating mechanical signals into the nucleus would differ according to direction and duration of the mechanical stimuli, and the type of cells examined.

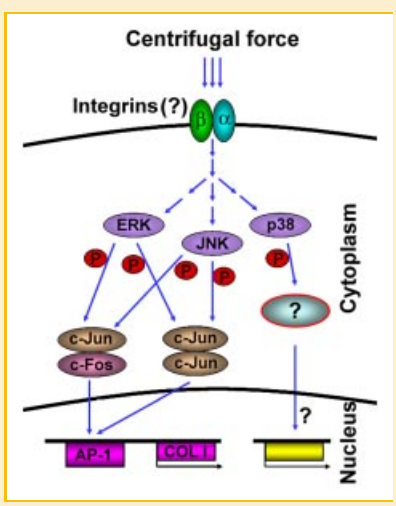

Fig. 8. Proposed model of the mechanotransduction pathways involved in COL I up-regulation in centrifugal force-exposed human PLF. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

In summary, current findings demonstrate that centrifugal force up-regulates the expression of COL I in human PLF, and provides plausible pathways of mechanotransduction involved in up-regulation of the COL I gene. We suggests that force-induced mechanical signals induce AP-1 activation and then stimulate COL I gene expression through ERK/JNK-mediated signaling (Fig. 8).

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