

## Original Article

# Inflammatory response in epithelial cells induced by mechanical stress is suppressed by hyaluronic acid

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Hyaluronic acid (HA) is a widely distributed component of the extracellular matrix that has been shown to play regulatory roles in inflammation. In the present study, we examined the inhibitory mechanism of HA related to mechanical stress-induced inflammatory response in mouse gingival epithelial GE1 cells. Application of compressive force in a three-dimensional cell culture system increased the expression of cyclooxygenase-2 (COX-2) mRNA in a force-dependent manner, while COX-2 protein and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) levels were also increased in a time-dependent manner. Interestingly, we found that HA suppressed the expression of COX-2 and production of PGE<sub>2</sub> under the same conditions. Western blotting analysis revealed that mechanical stress induced the phosphorylation of ERK and p38 MAPK at 60 minutes after loading, while that of I $\kappa$ B $\alpha$  was activated at 15 minutes and peaked at 30 minutes. In addition, I $\kappa$ B $\alpha$  degradation was activated at 30 minutes and recovered at 60 minutes. Furthermore, treatment with HA down-regulated the phosphorylation of p38 MAPK, ERK, and I $\kappa$ B $\alpha$  in GE1 cells. These results indicate that mechanical stress enhances the induction of COX-2 and PGE<sub>2</sub> in epithelial cells, while HA inhibits those mechanical stress-induced inflammatory responses via MAPKs and the NF- $\kappa$ B signaling pathway.

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## Introduction

Mechanical, physiological, and chemical stress during clinical treatment can cause damage and irritation in various tissues<sup>1)</sup>. Mechanical stress induces a variety of biochemical and morphological reactions in many cell types<sup>2-4)</sup>. With respect to the oral cavity, substantial knowledge has been acquired regarding the role of physical forces in regulating the functions of periodontal ligament (PDL) cells<sup>5)</sup>. It has been reported that mechanical stress regulated the gene expression of biological mediators, such as interleukin-6 (IL-6), IL-1 $\beta$ , matrix metalloproteinase and tissue inhibitor of metalloproteinase, plasminogen activator, cyclooxygenase-2 (COX-2), alkaline phosphatase activity, type 1 collagen, and osteocalcin in PDL cells<sup>6)</sup>.

It is well known that COXs catalyze the conversion of arachidonic acid to prostaglandin H<sub>2</sub> (PGH<sub>2</sub>), the immediate substrate for a number of cell specific PG and thromboxane syntheses, and induce the production and release of PGE<sub>2</sub> as well as other prostanooids, which contribute to inflammatory and immunomodulatory responses<sup>7-9)</sup>. Two isoforms, COX-1 and COX-2, have been identified and their expression shown to be differently regulated. COX-1 is constitutively expressed by most cell types and may be responsible for housekeeping functions, whereas the expression of COX-2, which is regulated at the transcriptional and post-transcriptional levels, is barely detectable in normal tissues, though rapidly induced in response to tumor promoters, oncogenes, and mitogens<sup>7-9)</sup>.

PGE<sub>2</sub> has been shown to have several kinds of biological activities, such as induction of bone resorption and attachment loss in periodontitis and peri-implantitis<sup>10)</sup>. *In vitro* studies demonstrated that gingival fibroblasts and epithelial cells produce PGE<sub>2</sub> via COX-2 in response to stimuli such as proinflammatory cytokines and lipopolysaccharide, suggesting that COX-2 may play critical role in PGE<sub>2</sub> production in periodontal tissue<sup>11)</sup>. However, there are few information about the role of PGE<sub>2</sub> in the induction of gingival epithelial cells with the mechanical stress. Hyaluronic acid (HA) is a large glycosaminoglycan composed of repeating disaccharides of D-glucuronic acid and N-acetyl-glucosamine, and belongs to the glycosaminoglycan family. Several studies have indicated the efficacy of HA for modulating acute and chronic inflammation. High molecular weight HA was found to inhibit macrophage proliferation and cytokine release, leading to decreased inflammation in early wounds in a preclinical postlaminectomy rat model<sup>12)</sup>. HA also enhances proteoglycan synthesis, reduces the production and activities of proinflammatory mediators, matrix metalloproteinases and alters the behavior of immune cells<sup>13)</sup>. Presently, high molecular

weight HA is widely used in the treatment of osteoarthritis via intra-articular injection to improve the symptoms of rheumatoid arthritis<sup>14)</sup>.

In the present study, we investigated the mechanism involved in enhancement of inflammatory responses by epithelial cells induced by mechanical stress. We also examined the involvement of HA in the inhibition of those inflammatory responses.

## Materials and Methods

### 1) Reagents

High molecular weight HA (OPEGAN Hi; 2000 kDa) was kindly supplied by Seikagaku Corp. (Tokyo, Japan). Recombinant murine epidermal growth factor (EGF) was purchased from Pepro Tech EC Ltd. (London, UK). Anti-ERK monoclonal, anti-phospho-ERK polyclonal, anti-p38 MAPK polyclonal, anti-phospho p38 MAPK polyclonal, and anti-JNK antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA), while anti-phospho-JNK monoclonal, anti-I $\kappa$ B $\alpha$  polyclonal, anti-phospho-I $\kappa$ B $\alpha$  monoclonal, anti-COX-2 polyclonal, and anti-actin monoclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). A COX-2 selective inhibitor, NS-398, was purchased from Sigma (St. Louis, MO, USA).

### 2) Cell culture

The mouse-derived gingival epithelial cell line, GE1 was obtained from RIKEN CELL BANK (Ibaragi, Japan). The cells were maintained in SFM-101 medium (Nissui, Tokyo, Japan) supplemented with 1% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), penicillin G (100 U/ml), streptomycin (100  $\mu$ g/ml), and EGF (10 ng/ml) in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C.

### 3) Application of compressive force

To examine the effects of static compressive force, GE1 cells were cultured in a three-dimensional cell culture system<sup>6)</sup>. In brief, collagen gel cultures were assembled by mixing 7 volumes of 0.3% type I-A collagen solution (Nitta-gelatin, Osaka, Japan), 1 volume of 20 mM HEPES buffer containing 2.2% sodium bicarbonate and 0.05% sodium hydroxide, and 1 volume of cell suspension to provide a final cell density of 1 x 10<sup>6</sup> cells/ml. The gel mixture coating EGF (10 ng/ml) were cast in 24-well plates, and allowed to polymerize for 1 h. After polymerization, the gels were transferred to 6-well plates to promote nutrient diffusion from their surroundings. Three sets of gel mixtures in each well were cultured with 2 ml of SFM-101 containing 1% FBS and

EGF (10 ng/ml), and allowed to set for 24 h prior to force loading. In some experiments, NS-398 (0.001, 0.01, or 0.1  $\mu$ M) and HA (10, 50, or 100  $\mu$ g/ml) were mixed with the collagen gels. Compressive force was applied using a sterile titanium plate (32 mm in diameter) and a plastic cylinder placed over the gels, which was adjusted by adding lead granules to the cylinder.

#### 4) RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from GE1 cells embedded in the collagen gels was extracted using ISOGEN-LS (Nippon Gene, Tokyo, Japan). In brief, the collagen gels containing cells were washed extensively with phosphate-buffered saline (PBS; pH 7.2), minced in ISOGEN-LS, and then RNA was isolated according to the manufacturer's instructions. DNase-treated RNA was reverse transcribed using oligo-dT primers. PCR reactions were performed as follows: 9 min at 95°C, followed by 20–28 cycles of 40 sec at 94°C, 40 sec at the gene-specific annealing temperature, and 9 min at 72°C. RT-PCR reactions were carried out according to standard procedures using the following primers for COX-1, COX-2, and glyceraldehydes-3-phosphate dehydrogenase (GAPDH): COX-1, 5'-CTTCTCCACGATCTGGCTTC-3' and 5'-GCTGCAGGAAATAGCCACT, C-3'; COX-2, 5'-AGAAGGAAATGGCTGCAGAA-3' and 5'-GCTCGGCTTCCAG, TATTGAG-3'; and GAPDH, 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCA, CCCTGTTGCTGTA-3'. The PCR products were electrophoresed and using GelRed™ nucleic acid gel stain (Biotium, CA, USA) and visualized with ultraviolet light illumination.

#### 5) PGE<sub>2</sub> measurement

The amounts of PGE<sub>2</sub> in the culture media were determined using a PGE<sub>2</sub> EIA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

#### 6) Western blotting analysis

GE1 cells in collagen gel were washed twice with PBS and lysed in cell lysis buffer (75 mM Tris-HCl containing 2% SDS and 10% glycerol, pH 6.8). Protein contents were measured using a DC protein assay kit (Bio-Rad). The samples were placed in 10% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes (Millipore Corp, Bedford, MA, USA). Nonspecific binding sites were blocked by immersing the membranes in 10% skim milk in PBS for 1 hour at room temperature and incubated with the primary antibodies. Subsequently, the membranes were incubated with the horseradish

peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (GE Healthcare, Little Chalfont, Buckinghamshire, UK). After washing the membranes, chemiluminescence was produced using an ECL Plus western blot detection system (GE Healthcare), according to the manufacturer's instructions. The blots were stained with Coomassie brilliant blue and we confirmed that all lanes contained similar amounts of protein extract.

#### 7) Statistical analysis

Statistical differences were determined using an unpaired Student's *t*-test. All data are expressed as the mean  $\pm$  standard deviation of three examinations, with similar results obtained in each experiment.

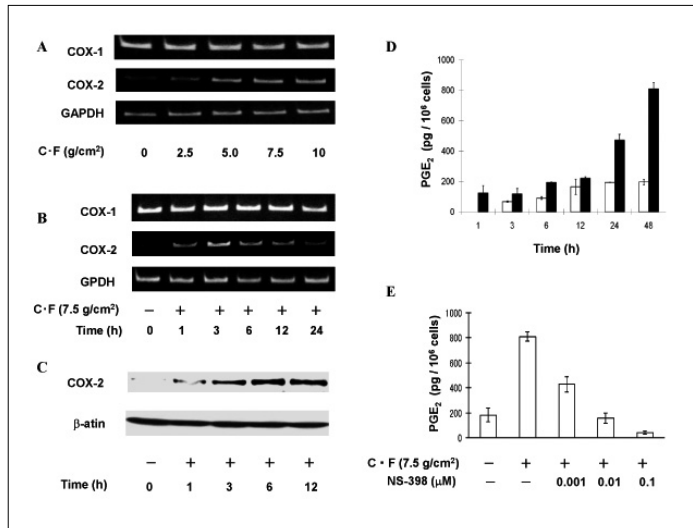
## Results

### 1) Mechanical stress up-regulated COX-2 and PGE<sub>2</sub> responses in GE1 cells

Under mechanical stress, the expression of COX-2 mRNA in GE1 cells was clearly detected at 5.0 g/cm<sup>2</sup> and increased in a force-dependent manner up to 10.0 g/cm<sup>2</sup> of loading force. In contrast, the expression of COX-1 mRNA was not changed (Fig.1A). Our time-course experiment revealed that the expression of COX-2 mRNA was detected at 1 h and increased up to 3 h, after which it was slightly reduced after 6 h. On the other hand, the expression of COX-1 mRNA did not change over time (Fig.1B). Western blotting analysis revealed that the expression of COX-2 protein was clearly detected from 3 to 6 h (Fig.1C). After GE1 cells were cultured for 48 h under mechanical stress, conditioned media were collected and the amounts of PGE<sub>2</sub> determined using a PGE<sub>2</sub> EIA kit, which revealed that PGE<sub>2</sub> was significantly increased in a time-dependent manner (Fig.1D). These results indicate that mechanical stress up-regulates both COX-2 expression and PGE<sub>2</sub> production in these cells. Next, GE1 cells were cultured with NS-398, a COX-2 selective inhibitor, for 24 h in collagen gel and subjected to 7.5 g/cm<sup>2</sup> of compressive force for 48 h. The amount of PGE<sub>2</sub> was completely inhibited by NS-398 at concentrations of 0.01 and 0.1  $\mu$ M (Fig.1E).

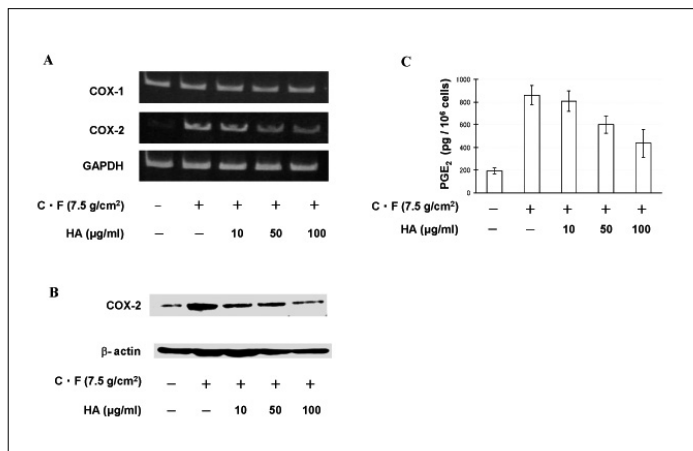
### 2) HA suppressed COX-2 and PGE<sub>2</sub> responses in GE1 cells under mechanical stress

We examined the effects of HA on the expression of COX-2 and production of PGE<sub>2</sub> using a three-dimensional cell culture system. GE1 cells were mixed with HA in the collagen gel and subjected to 7.5 g/cm<sup>2</sup> of compressive force, and the expression



**Fig.1** Mechanical stress induced COX-2 expression and PGE<sub>2</sub> production in GE1 cells

GE1 cells in collagen gels were subjected to 0 ~10 g/cm<sup>2</sup> for 3 h (A). In another experiment, the cells were subjected to 7.5 g/cm<sup>2</sup> for the indicated time periods (B,C). RT-PCR (A,B) and Western blotting (C) analyses were performed as described in Materials and Methods. Culture media from compressed GE1 cells were collected at the indicated times. □: without compressive stress. ■: with compressive stress (D). GE1 cells were cultured with NS-398 (0.001, 0.01, 0.1 μM) in collagen gels, and subjected to 7.5 g/cm<sup>2</sup> for 48 h. The amounts of PGE<sub>2</sub> in the culture media were determined as described in Materials and Methods. Each experiment was performed three times, with similar results obtained in each.



**Fig.2** Effects of HA on COX-2 expression and PGE<sub>2</sub> production in GE1 cells

GE1 cells were cultured with HA (10, 50, 100 μg/ml) in collagen gels and subjected to 7.5 g/cm<sup>2</sup> for 3 h. RT-PCR analysis was performed as described in Materials and Methods (A). GE1 cells were cultured with HA in collagen gels and subjected to 7.5 g/cm<sup>2</sup> for 6 h, after which whole lysates were subjected to Western blot analysis (B). GE1 cells were mixed with HA in collagen gels and compressed for 48 h, then the culture media were collected and amounts of PGE<sub>2</sub> determined as described in Materials and Methods (C). Each experiment was performed three times, with similar results obtained in each.

levels of mRNA and COX-2 protein were suppressed by HA in a dose-dependent manner (Fig.2A and 2B). Further, PGE<sub>2</sub> production was also suppressed by HA (100 μg/ml) (Fig.2C). These results indicate that HA inhibits COX-2 and PGE<sub>2</sub> production induced by mechanical stress in GE1 cells.

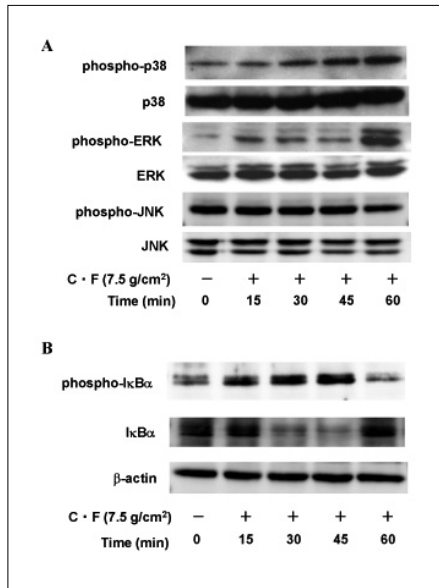
### 3) Mechanical stress activated MAPKs and NF-κB signaling pathway

Next, we examined the activation of MAPKs and NF-κB by mechanical stress activation in GE1 cells using the three-dimensional cell culture system. As shown in Fig.3A and 3B, the intensity of phosphorylation of p-38 MAPK and ERK was steadily increased for up to 60 min after loading of mechanical stress, whereas the phosphorylation of JNK was stably activated with or without mechanical stress. The phosphorylation of IκBα was

activated at 15 min after mechanical stress loading and peaked at 45 min, followed by a gradual decline. Interestingly, IκBα was degraded at 30 and 45 min after mechanical stress loading, and then recovered at 60 min. Taken together, these results suggest that mechanical stress induces inflammation via MAPKs and the NFκB signaling pathway.

### 4) HA suppressed MAPKs and NF-κB signaling pathway

We also examined the effects of HA on MAPKs and the NF-κB signaling pathway in GE1 cells subjected to compressive force. GE1 cells were mixed with 2000-kDa HA (10, 50, and 100 μg/ml) in collagen gels and subjected to 7.5 g/cm<sup>2</sup> of compressive force. The levels of phosphorylated p-38 MAPK and ERK were significantly down-regulated at 60 min after treatment with 50 and 100 μg/ml of HA, while that of phosphory-



**Fig.3** Mechanical stress enhanced phosphorylation of MAPKs and IκBα in GE1 cells

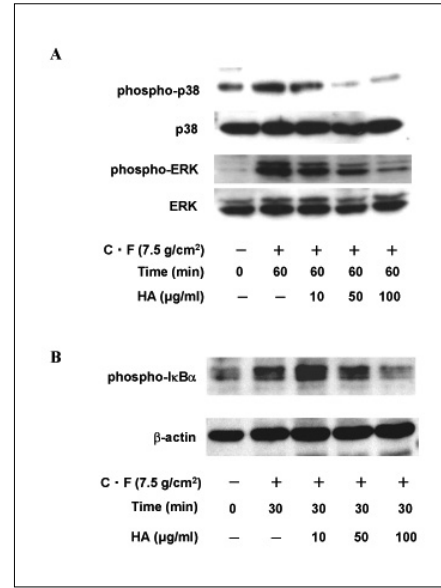
GE1 cells were placed in collagen gels and subjected to 7.5 g/cm<sup>2</sup> of compressive force for the indicated time periods. The expression levels of phosphorylated p38 MAPK, ERK, and JNK (A), and phosphorylated IκBα (B) were examined by western blotting analyses. Whole cell lysates were prepared at the indicated times. Each experiment was performed three times, with similar results obtained in each.

lated IκBα was down-regulated at 30 min after treatment with 100 μg/ml of HA (Fig.4A and 4B).

## Discussion

It has been reported that mechanical stress functions as a critical regulatory factor in bone cell biology, and is a postnatal determinant of bone homeostasis and skeletal morphology<sup>15</sup>. In addition, several studies have noted that mechanical stresses in the oral cavity, such as occlusal pressure and orthodontic force, have effects on remodeling, repair, and regeneration of periodontal tissue<sup>6,16</sup>. Although ankylosed teeth directly connected with alveolar bone without the intervention of periodontal ligaments are often seen in patients with dental implants, oral epithelial cells are thought to play an important role in the good adhesion of the surrounding tissue to the biomaterial to prevent such ankylosis<sup>17</sup>.

Recently, Araujo et al. developed an *in vitro* three-dimensional cell culture system that mimics *in vivo* conditions, which they used to analyze gene profiles in PDL cells regulated by static



**Fig.4** Effects of HA on phosphorylation of MAPKs and IκBα in GE1 cells

GE1 cells were cultured with HA (10, 50, 100 μg/ml) in collagen gels, and subjected to 7.5 g/cm<sup>2</sup> of compressive force for 60 min to detect the phosphorylation of p38 MAPK and ERK (A), and 30 min to detect that of IκBα (B). Whole cell lysates were prepared at the indicated times and the expressions were examined by Western blotting analyses. Each experiment was performed three times, with similar results obtained in each.

compressive force<sup>6</sup>. In the present study, we modified their culture system to examine the effects of titanium and static compressive force in oral epithelial cells. Titanium is a suitable biomaterial for dental implants used in dental treatment. However, static compressive force enhanced the expression of COX-2 and production of PGE<sub>2</sub> in the present three-dimensional culture system. Further, a COX-2 selective inhibitor, NS-398, completely suppressed PGE<sub>2</sub> production from epithelial cells subjected to 7.5 g/cm<sup>2</sup> of compressive force (Fig.1). These findings indicate that compressive force increased the production of PGE<sub>2</sub> via COX-2 in the present three-dimensional cell culture system.

MAPK family members have been shown to be praline-directed serine/threonine kinases important for the induction of biological responses, and studies have revealed that MAPKs are activated by phosphorylation of threonine and tyrosine in response to external stimuli, and classified them into the ERK, JNK, and p38 MAPK groups<sup>18,19</sup>. It has also been reported that MAPKs play crucial roles in the generation of inflammatory mediators including PGE<sub>2</sub> in IL-1 β-treated human synovial fibroblasts<sup>20</sup>.

In addition, Nagano et al. found that ERK and p38 MAPK are involved in LPS-induced COX-2 expression and PGE<sub>2</sub> production in neutrophils<sup>21</sup>. More recently, Zhang et al. reported that ERK and p38 MAPK pathways were involved in signaling COX-2 through NF- $\kappa$ B pathway in chronic inflammation diseases<sup>22</sup>.

HA is the most abundant glycosaminoglycan in tissues and plays an important role in biological responses including those related to inhibition of inflammation and pain<sup>23,24</sup>. In the present study, we found that HA suppressed not only COX-2 expression and PGE<sub>2</sub> production (Fig.2), but also the phosphorylation of p38 MAPK and ERK (Fig.4). These results are in accord with other results showing that mechanical stress enhances osteoblast differentiation through the activation of MAPK signal pathways<sup>25</sup>, and suggest that ERK and p38 MAPK play critical roles in the induction of inflammatory responses by epithelial cells under mechanical stress.

It is well known that NF- $\kappa$ B is normally sequestered in the cytoplasm of nonstimulated cells and translocates into the nucleus to regulate effector gene expression. Several researches have reported correlations between the levels of COX-2 mRNA and amount of NF- $\kappa$ B in the nuclear fraction of human brain cells<sup>26,27</sup>. Recently, Fiebich et al. demonstrated that IL-1 $\beta$  induced COX-2 protein synthesis and PGE<sub>2</sub> release in human neuroblastoma cells, and that p38 MAPK and NF- $\kappa$ B act as intracellular signal transducers, leading to IL-1 $\beta$ -induced expression of COX-2<sup>28</sup>. Interestingly, we found that mechanical stress enhanced COX-2 expression, PGE<sub>2</sub> production, and activation of MAPKs, suggesting a possible involvement of NF- $\kappa$ B in epithelial cells under mechanical stress.

NF- $\kappa$ B is well known to be regulators of immune and inflammatory responses<sup>29</sup>. In the present study, the involvement of the NF- $\kappa$ B pathway, we examined the expression level of I $\kappa$ B in GE1 cells. I $\kappa$ B, a family of inhibitory proteins against NF- $\kappa$ B, binds to NF- $\kappa$ B and masks its nuclear-localized signal domain, leading to regulation of NF- $\kappa$ B<sup>30</sup>. Among I $\kappa$ B members, I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$ , have common properties as well as significant differences. For example, TNF- $\alpha$  causes a transient activation of NF- $\kappa$ B and transient loss of I $\kappa$ B $\alpha$ , whereas treatment with IL-1 or lipopolysaccharide results in the degradation of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$ <sup>31</sup>. Further, the phosphorylation of I $\kappa$ B $\alpha$  at Ser-32 and Ser-36 plays a critical role in the ubiquitination and proteolysis of I $\kappa$ B $\alpha$ , which leaves NF- $\kappa$ B free to translocate to the nucleus<sup>32,33</sup>. However, little is known regarding the role of mechanical stress in the phosphorylation and degradation of I $\kappa$ B $\alpha$ . In the present study, we clearly demonstrated that mechanical stress

enhances the phosphorylation and subsequent degradation of I $\kappa$ B $\alpha$  in epithelial cells (Fig.3B). In addition, treatment with HA partly reduced the phosphorylation of I $\kappa$ B $\alpha$  (Fig.4B), suggesting that HA functions as an anti-inflammatory agent in epithelial cells under stress from compressive force. CD44 is first identified as an integral hyaluronan receptor which mediates HA signaling<sup>34</sup>. Lein *et al.* have reported that CD44 is important for bronchial epithelial cell binding to HA<sup>35</sup>. At present, further experiments are in progress to clarify the signaling pathway of HA through CD44 in gingival epithelial cells under the mechanical stress.

In conclusion, we found that compressive force stress enhanced the production of PGE<sub>2</sub>, a well-known inducer of inflammation in epithelial cells<sup>36,37</sup>, and up-regulated the COX-2 expression via activation of MAPK signal transduction pathways. Treatment with HA suppressed mechanical stress-induced inflammatory responses, as well as the phosphorylation of p38 MAPK and ERK in epithelial cells, suggesting that HA may be useful for suppression of inflammation caused by mechanical stress.

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