Hyaluronan enhances proteoglycan4 expression in synovial cells

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Abstract

Synovial fluid contains various molecules such as hyaluronan (HA) and proteoglycan4 (PRG4) that play important roles in maintaining proper joint lubrication. However, the interaction between HA and PRG4 remains unclear. We aimed to investigate the effect of high molecular weight hyaluronan (HMW-HA) on the expression of PRG4 and signaling pathways in cultured human fibroblast-like synoviocytes (HFLS).

HFLS were treated with hyaluronidase (HAase) prior to addition of HMW-HA. PRG4 expression was significantly increased by HA and significantly down-regulated by HAase treatment; however, the effect of HAase was inhibited by the presence of HMW-HA. HFLS were also treated with an anti-CD44 neutralizing antibody or MEK1/2 inhibitor (U0126) prior to addition of HMW-HA. PRG4 mRNA expression was significantly down-regulated by addition of the anti-CD44 neutralizing antibody or U0126, when compared to the untreated control. Co-treatment with HMW-HA prevented the inhibitory effect of the anti-CD44 antibody but could not prevent the inhibitory effect of U0126 on PRG4 mRNA expression. Thus HMW-HA regulates the expression of PRG4 by a slight interaction with CD44 through MAPK-ERK signaling pathway.

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KEYWORDS

Fluid lubrication, temporomandibular

disorders, osteoarthritis, MAPK signaling, synovial membrane cells

1. Introduction

Recent studies suggest that osteoarthritis (OA) is an inflammatory disease and that activated synoviocytes play important roles in the progression of OA in humans. Articular cartilage exerts buffering and lubricating functions when mechanical stress is applied to the joint surfaces during movement. Synovial fluid has been suggested to be essential for the lubrication and protection of articular cartilage (Rainer and Ribitsch 1985). Synovial fluid is composed of various molecules such as hyaluronan (HA) and proteoglycan4 (PRG4) that play important roles in maintaining proper joint lubrication and in providing a protective barrier for the cells in the deep zone of cartilage (Buckwalter and Mankin 1997).

HA is a glycosaminoglycan with repeated disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine connected by β-linkages, and is distributed throughout the extracellular space of connective tissue. In normal synovial joints, HA is present at a concentration ranging from 1 to 4 mg/mL, and its molecular weight ranges continuously from 4 kDa to approximately 8 MDa. HA plays some essential roles in joint movements; it is the major factor for joint lubrication due to its high viscoelasticity (Schmidt et al. 2007), and it contributes to fluid lubrication. HA is critical for the structural organization and function of cartilage and the large size and high negative charge of HA contribute to its physiological features. On the other hand, the viscosity of synovial fluid is reduced due to the decrease in both concentration and molecular weight of HA in joints with OA (Kawai et al., 2004)

PRG4 is a high molecular weight (~345 kDa) glycoprotein with small amounts of keratan and chondroitin sulfate substitution, and it is present at a concentration ranging from 0.05 to 0.5 mg/mL (Schmid et al. 2002). The PRG4 molecule is homologous to the molecules referred to as lubricin and superficial zone protein (SZP) (Flannery et al. 1999, Jay et al. 2001, Schumacher et al. 2005). PRG4 is involved in synovial fluid function, and is also detected in the surface layer of articular cartilage (Rees et al. 2002), the synovium (Bothner and Wik 1987). PRG4 contributes to boundary lubrication properties, facilitating low friction levels at interfacing surfaces of articular cartilage (Ohno et al. 2006).

It has been reported that human fibroblast-like synoviocytes (HFLS) secrete both HA and PRG4 into the synovial fluid (Blewis et al. 2010) Recent studies have shown that HA and PRG4 can functionally interact in synovial fluid. The combination of HA and PRG4 induces a greater decrease in the friction coefficient than either HA or PRG4 alone (Kwiecinski et al. 2011). Moreover, an HA-PRG4 complex produced a greater boundary-lubricating effect than either constituent alone (Hua et al. 1993). Taken together, it appears that both PRG4 and HA are critical to the lubricating function, however, the mechanism by which HA and PRG4 interact remains unknown.

HA function is enhanced by activation of HA receptors such as CD44 (Laurent et al. 1992) It has been reported that stimulation with HA increases the mRNA expression level of CD44. Moreover, high molecular weight (HMW)-HA down regulates the gene expression of human OA cytokines and enzymes in HFLS (Wang et al. 2006). Another study has indicated that HMW-HA has effects on the distribution and movement of proteoglycan around chondrocytes (Kikuchi et al. 2001). We therefore assumed that HA affects the expression of PRG4 by activation of CD44. The aim of this study was to investigate the effect of HA on the expression of PRG4 and signaling pathways in cultured HFLS.

2. Materials and Methods

2.1 Cell culture

HFLS were purchased from Cell Applications Inc. (San Diego, CA, USA) and were seeded at a density of 1×10^6 cells/well in six-well culture plates (35 mm diameter; Corning Inc., NY, USA). The cultures were maintained in 2 ml of α-Modified Eagle's Medium (α-MEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Biological Industries, Kibbutz Bet-Haemek, Israel), 60 μg/mL kanamycin (Meiji Seika, Tokyo, Japan) and 96 U/mL penicillin (Meiji Seika). The medium was changed every other day. HFLS cells were subcultured when they reached confluence. All the cultures were maintained at 37 °C in a humidified 5% CO_2 incubator.

2.2 Treatment of HFLS with HMW-HA and pharmacological inhibitors

HFLS were seeded in 6-well plates (Corning Inc., New York, NY, USA) at a density of 1×10^6 cells/well. On day 1 of culture, 12 h before treatment, cells were deprived of serum in a gradient fashion using a serum gradient from 10, 5, and 1 to 0%. HFLS were then starved in serum-free medium overnight.

On day 2, the cells were treated for 12 h with Suvenyl® (1.0 mg/mL) (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan), which was used as a HMW-HA. The weight average molecular weight of Suvenyl is 2700 kDa (its viscosity average molecular weight is 1900 kDa). Some cultured cells were treated for 12 h with bovine testicular hyaluronidase (HAase) (10 units/mL) in the presence or absence of 1.0 mg/mL HMW-HA (Wako Pure Chemical Industries, Osaka, Japan). For other experiments, cells were pre-treated for 2 h with anti-CD44 neutralizing antibody (IM7.8.1; Thermo Fisher Scientific, Waltham, MA, USA; 5.0 μg/mL) prior to stimulation with 1.0 mg/mL HMW-HA.

For ERK1/2 inhibition experiments, U0126 (Cell Signaling Technology, Danvers, MA, USA) was used as an upstream inhibitor of ERK1/2. The cells were pretreated with 10 μM U0126 for 1h, then with 1.0 mg/mL HMW-HA for 12h in the presence or absence of each inhibitor.

2.3 Quantitative real-time PCR analysis of PRG4 mRNA level

The mRNA level of PRG4 was examined by

quantitative real-time polymerase chain reaction (PCR) analysis, using a Light Cycler® Quick system 350S (Roche Diagnostics, Tokyo, Japan) and the QuantiTect SYBR® Green PCR Master Mix (QIAGEN, Tokyo, Japan) (Table 1).

Table 1. Nucleotide sequences of primers used for the real-time polymerase chain reaction

Gene		**Sequence 5'-3'**
PRG4	Forward	**TCA ATA TTC ACC TGC CAG ACT**
	Reverse	**TGA TGT TGG GCA GTG ATA TAG**
GAPDH	Forward	**CCA CTC CTC CAC CTT TGA**
	Reverse	**CAC CAC CCT GTT GCT GTA**

Cultured HFLS were washed in PBS and total RNA was then isolated by lysis in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from total RNA (1 μg) by reverse-transcription using an Oligo (dT) 20 primer (Toyobo, Osaka, Japan) and Rever Tra Ace-α (Toyobo). The primer sequences are listed in Table 1. The mRNA signal was evaluated in a quantitative manner, relative to the signal of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Normalized cycle threshold (Ct) values are expressed relative to those of the controls.

2.4 Western blot analysis

HFLS were lysed in × 5 sodium dodecyl sulfate (SDS) sample buffer (125 -mM Tris–HCl (pH 6.8), 4% SDS and 10% glycerol). Protein concentration was determined using a bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA). After the addition of 10% 2-mercaptoethanol and 0.01% bromophenol blue, samples were boiled at 100 °C for 3 min and 30 μg of protein was resolved on 10% SDS-polyacrylamide gels. Proteins were transferred to PVDF membranes using an iBlot gel transfer system (Invitrogen). Membranes were blocked with 5% nonfat dry milk at room temperature on a shaker for 30 min. The primary antibodies were incubated with the membranes at room temperature for 2 h. Primary antibodies used at 1:1,000 dilutions were: anti-lubricin monoclonal antibody (Affinity BioReagentsTM, Golden, CO, USA), anti-phospho ERK1/2 antibody (sc-7383; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-phospho-NF-kB, anti-phospho-p38 and anti-phospho-c-Jun N-terminal kinase (JNK) antibody (all from Cell Signaling Technology). Anti-β-actin antibody (AC-15; Sigma-Aldrich) was used at 1:5,000 dilutions. After primary antibody incubation, the membranes were washed three times for 5 min per wash with 15 mL of PBS and 0.1% Tween-20 before addition of the secondary antibody conjugated to a

fluorescent label: IRDye 800CW-conjugated goat anti-mouse IgG (dilution 1: 15,000, LI-COR, Cambridge, UK) or IRDye 680CW-conjugated goat anti-rabbit IgG (dilution 1: 15,000, LI-COR) in LI-COR blocking buffer. At the end of the incubation period, membranes were washed twice for 5 min per wash with 15 mL of PBS and 0.1% Tween-20 and once with 15 mL of PBS. Membranes were dried, visualized, and analyzed using the Odyssey® IR imaging system (M&S Techno Systems, Osaka, Japan).

2.5 Statistical analysis

All experiments were repeated an additional 3 times. Reproducibility of the results was confirmed by at least two sets of experiments executed in the same manner. Means and standard deviations were calculated from the data obtained, and statistical comparisons of the means were performed by one-way analysis of variance (ANOVA) with a Bonferroni's modification of the Student's *t* test as a post-hoc test. Resulting *p* values of less than 0.05 and 0.01 were defined as significant.

3. Results

3.1 Effects of HMW-HA on PRG4 expression in cultured HFLS

The effect of treatment of cultured HFLS with HMW-HA on PRG4 expression was analyzed using Western blotting. The expression of PRG4 was up-regulated ($p < 0.01$) by HMW-HA treatment compared with the untreated control (Fig. 1).

3.2 Effect of HAase and an anti-CD44 neutralizing antibody on the mRNA expression of PRG4 in cultured HFLS

The effect of treatment of cultured HFLS with HAase or combination of HMW-HA, on PRG4 mRNA expression was analyzed. The mRNA expression of PRG4 was significantly down-regulated ($p < 0.01$) by HAase treatment (Fig. 2A). This effect of HAase was inhibited if HMW-HA was added to the cells together with HAase.

We next determined if the HA receptor CD44 was involved in the expression of PRG4 enhanced by HMW-HA. The mRNA expression of PRG4 was significantly down-regulated ($p < 0.01$) by the addition of an anti-CD44 neutralizing antibody (Fig. 2B). The addition of HMW-HA significantly restored the expression of PRG4, which decreased with the treatment of anti-CD44 neutralizing antibody.

3.3 Effects of HMW-HA on MAPK cascade and NF-kB signaling in cultured HFLS

We next determined the effect of HMW-HA addition on MAPK cascade and NF-kB signaling by Western blot analysis of activated forms of signaling proteins in these pathways. Western blot analysis showed enhanced phosphorylated (p)- ERK1/2 levels and decreased p-JNK levels at 30 and 60 mins following the addition of HMW-HA (Fig. 3), whereas there were no significant changes in the levels of p-p38 or p-NF-□B expression. These findings suggested that

HMW-HA activates the ERK1/2 mitogen-activated protein kinase (MAPK) pathway in cultured HFLS.

3.4 Effects of the MEK1/2 inhibitor on the HA-induced mRNA expression of PRG4 in cultured HFLS

In order to determine if the ERK1/2 pathway was involved in mediating HMW-HA induced PRG4 expression, we used U0126 (10 μM), a specific inhibitor of MEK1/2, the upstream activator of ERK1/2. The mRNA expression of PRG4 was significantly down-regulated ($p < 0.01$) to 0.51 of the level of that of the control group by treatment with U0126 alone (Fig. 4). HMW-HA addition could not increase PRG4 mRNA expression in the presence of U0126. The combined data indicate that HMW-HA regulates the expression of PRG4 through the ERK-MAPK pathway.

4. **Discussion**

Synovial fluid contains HA and PRG4, which play important roles in maintaining proper joint lubrication and in providing a protective barrier for cells in the deep zone of cartilage. Under normal conditions, synovial fluid provides low-friction properties to synovial joints through a combination of lubrication mechanisms. Furthermore, both HMW-HA and PRG4 contribute to boundary lubrication, individually and in combination, at both physiological and pathophysiological concentrations (Schmidt et al. 2007).

OA is a degenerative joint disease characterized by destruction of articular cartilage, which leads to joint disability and dysfunction. There is a strong association between the loss of boundary-lubricating abilities of synovial fluid and damage of the articular cartilage following OA. Cytokines are produced during inflammation, leading to joint destruction. The concentration of IL-1β in the synovial fluid of OA patients was reported to be significantly higher than that in healthy joints (Kaneyama et al. 2002). Although the mechanism by which PRG4 expression is modulated has not been completely elucidated, it has been reported that PRG4 expression is regulated by several cytokines (Jones and Flannery 2007). Our previous study demonstrated that adequate mechanical stress is important for maintaining PRG4 synthesis, but that excessive mechanical stress from cyclic tensile loading also increased the mRNA levels of matrix metalloproteinases (MMPs) and proinflammatory cytokines such as IL-1β and TNF-α, resulting in an increase in HAase and low molecular weight (LMW)-HA in cultured articular chondrocytes (Honda et al. 2000). Furthermore, mechanical stimuli induce a reduction in PRG4 expression levels during the first stage of cartilage degradation (Kamiya et al. 2010).

HMW-HA is a major component of synovial fluid, and significantly increases the ability of synovial fluid to lubricate the cartilage boundary. HA is degraded in inflammation sites, resulting in accumulation of LMW-HA fragments (Teder P et al., 2002) LMW-HA causes a reduction in the viscosity of synovial

fluid and in inflammatory processes (Hodge-Dufour et al. 1997, McKee et al. 1997). The combined data suggest that the molecular weight of HA in pathological synovial fluids is lower than in normal synovial fluids (Takahashi T et al., 2004).

The mRNA expression of PRG4 was significantly upregulated by the addition of HMW-HA, however, down-regulated by the addition of HAase. Our previous study showed that treatment with exogenous HAase causes the degradation of HA in cultured chondrocytes. On the other hand, the mRNA expression of PRG4 was significantly up-regulated by the addition of HAase together with HMW-HA. Therefore, these results suggested that the HAase treatment might disrupt HA-cell interactions, resulting in down-regulation of PRG4 expression. Our previous study demonstrated that HAase treatment increases the frictional coefficient in TMJ (Asakawa-Tanne et al., 2015). It is also reported that the cartilage boundary lubricating ability of HA alone at physiological levels varies with molecular weight, however when combined with PRG4 at physiological levels the synergistic friction-reducing ability of HA-PRG4 did not vary with the molecular weight of HA (Kwiecinski et al., 2011).

On the other hand, decreased concentrations of HMW-HA also limited the cartilage boundary lubricating ability of HA+PRG4 solutions (Ludwig et al., 2015). These reports demonstrate that both PRG4 and HMW-HA are necessary contributors to the cartilage boundary lubricating ability of synovial fluid.

CD44 is the principal HA receptor, and is involved in important cellular functions including cell adhesion, migration, and the modulation of signals from cell surface receptors. While most of these CD44 functions are considered to involve HA, relatively little is known about the contribution of CD44 to HA maintenance and organization on the cell surface, and to the role of CD44 in HA synthesis and catabolism (Khalafi et al. 2007). In addition, it was reported that cell stimulation with HA increased the mRNA expression level of CD44 in a dose-dependent manner (Yamawaki et al. 2009). MAPKs, such as ERK, JNK, and p38 MAPK, are reported to be significantly activated in rheumatoid arthritis (RA) synovial tissue compared with their activation in degenerative joint disease (Schett et al. 2000). It has been reported that CD44 links to p38 signaling and that a separate, as yet unidentified receptor links to ERK signaling (Khaldoyanidi et al. 1999). Moreover, ERK has been shown to regulate the activation of NF-□B (Pillinger et al. 2004). We therefore assessed the role of the CD44 receptor, the activation of MAPK signaling pathways, and the effect of HMW-HA on the expression of PRG4 in HFLS.

The addition of an anti-CD44 antibody to cells could either directly block signaling by HA or could function to cluster CD44 on the cell membrane (Hua et al. 1993). In the present study, PRG4 mRNA expression was

significantly decreased after 12 h treatment with an anti-CD44 neutralizing antibody when compared to the untreated control. On the other hand, the addition of HMW-HA significantly but not completely restored the mRNA expression of PRG4. Thus, HA regulates the expression of PRG4 by a slight interaction with CD44. Our data indicates the involvement of another receptor in the expression of PRG4 regulated by HA, and therefore, influences of the interaction between HA and HA receptors to the expression of PRG4 should be clarified in future studies.

We also showed that p-ERK1/2 expression was significantly up-regulated by treatment of HFLS with HMW-HA. However, p-p38 and NF-kB were not affected by HMW-HA treatment of HFLS. Consistent with these results, PRG4 mRNA expression was also significantly decreased after 12 h treatment with the MEK1/2 inhibitor (U0126). In contrast with co-treatment with HMW-HA and anti-CD44, PRG4 mRNA expression remained downregulated following co-treatment with HMW-HA and U0126. These results suggested that HA affects PRG4 expression through an ERK (Akatsuka et al. 1993) related mechanism.

In conclusion, interaction between PRG4 and HA in articular cartilage may be critical for proper lubricating function on the surface of articular cartilage. HMW-HA regulates the expression of PRG4 by a slight interaction with CD44 through MAPK-ERK signaling pathway. Therefore, modulation of PRG4 metabolism would provide an insight into how cartilage degradation might be prevented and might contribute to the development of tissue engineering in the near future. Additionally, the use of purified HMW-HA and PRG4 formulations may be useful for the treatment of advanced OA.

5. References

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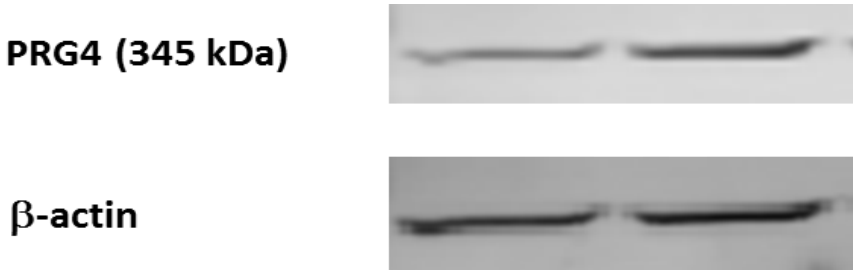

Figure 1.

Effects of HMW-HA on PRG4 expression in cultured HFLS .The expression of PRG4 protein was determined by Western blot analysis of lysed cells

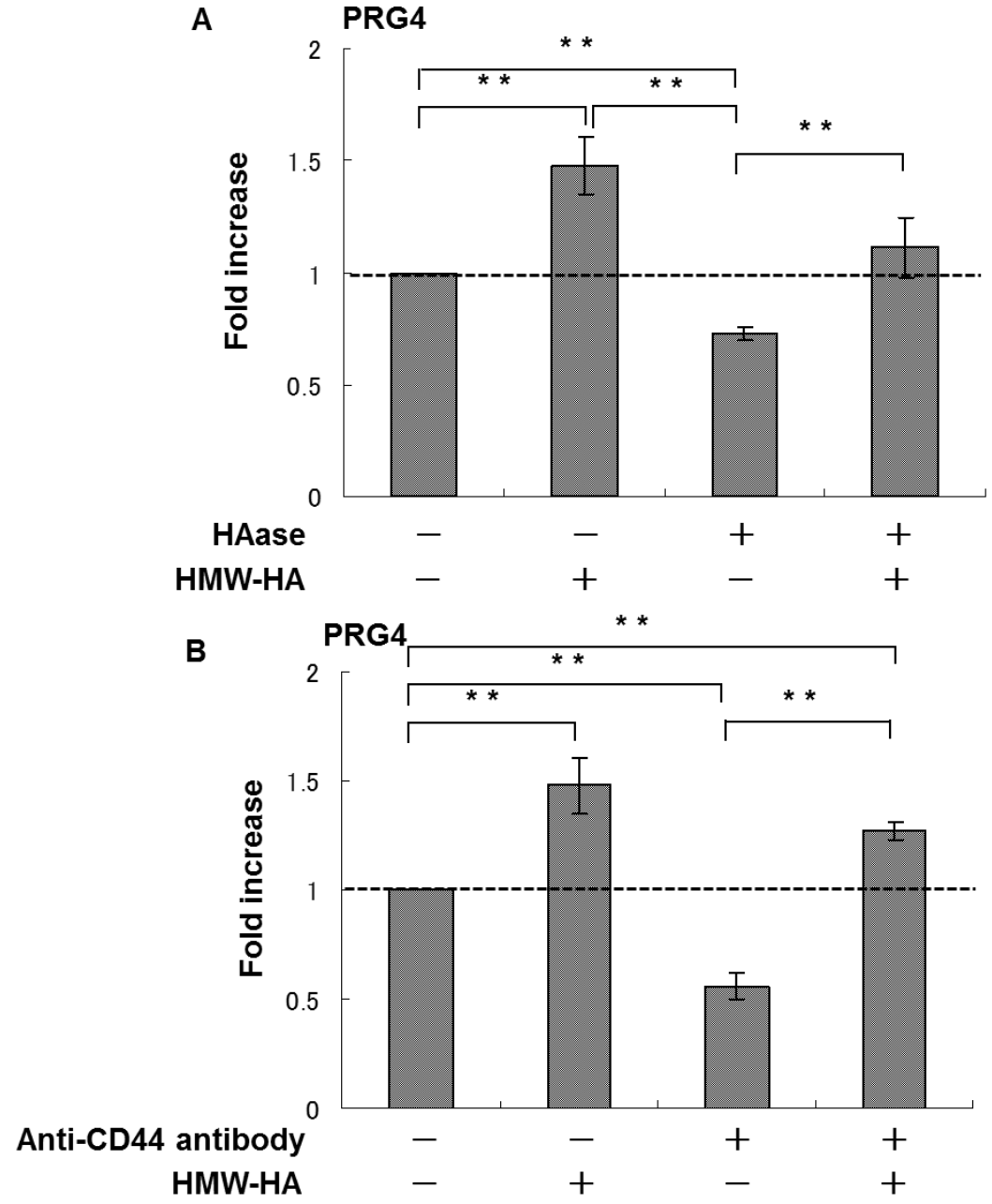

Figure 2.

Effects of HMW-HA, HAase and anti-CD44 neutralizing antibody on PRG4 expression in cultured HFLS Relative PRG4 mRNA levels in HFLS cultures treated with and without HAase(A) and anti-CD44 neutralizing antibody(B) as determined by real-time PCR analysis. The dotted line indicates the control PRG4 mRNA level. (** $P < 0.01$, mean ± SD, n = 3).

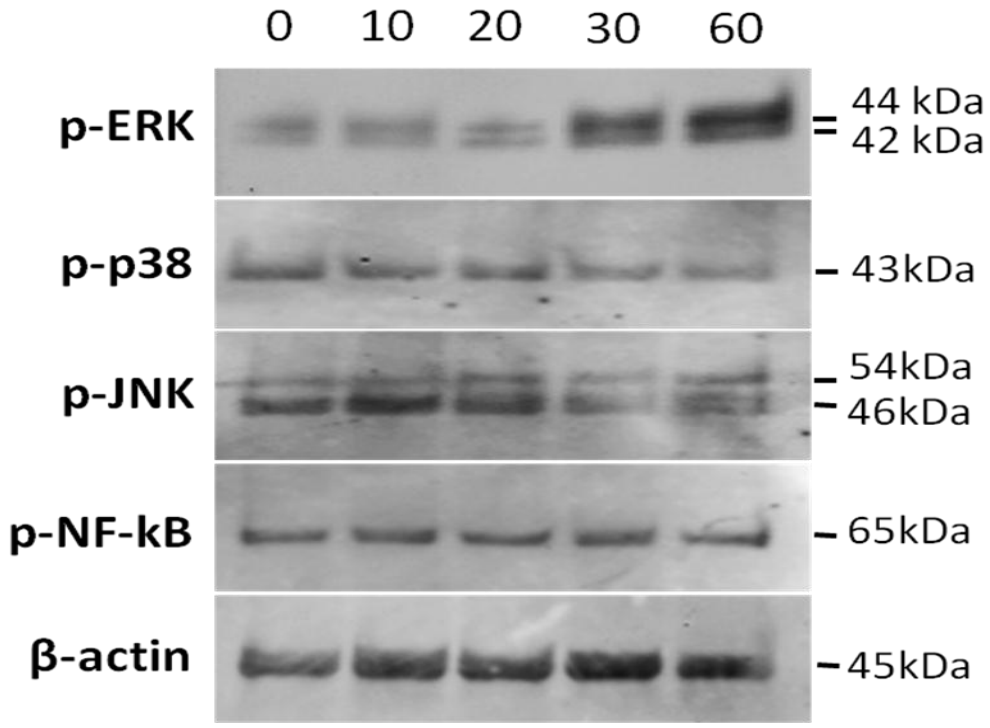

Figure 3

Effects of HMW-HA on MAPK cascade and NF-κB signaling in cultured HFLS. HFLS cultured in FBS-free α-MEM were treated with HMW-HA for 0-60 min. The expression of activated MAPKs (p-ERK/p-p38/p-JNK) and NF-κB was determined using Western blot analysis.

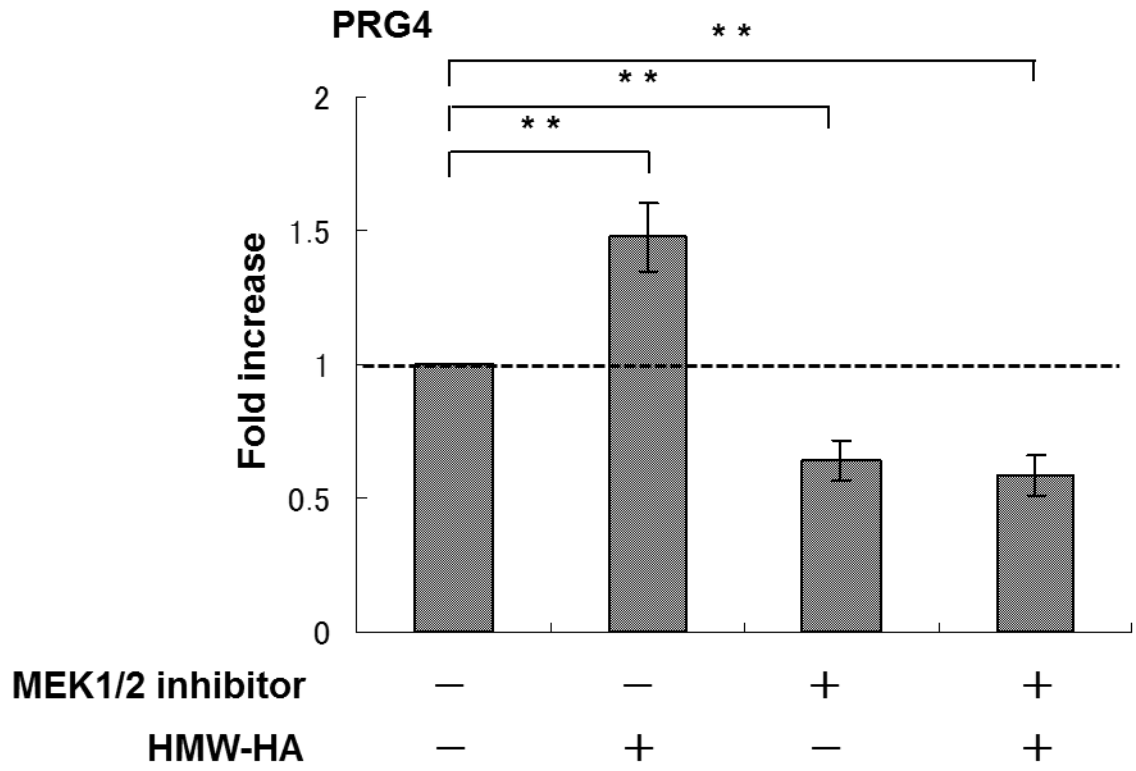

Figure 4.

Effects of the MEK1/2 inhibitor on PRG4 mRNA expression in cultured HFLS PRG4 mRNA levels in HFLS cultures treated with or without the MEK1/2 inhibitor U0126 (10 mM) /HMW-HA were determined by real-time PCR analysis. The dotted line indicates the control level of PRG4 mRNA. (** $P < 0.01$, mean ± SD, n = 3).

