HSP70 inhibitor amplifies the bFGF-induced release of IL-6 in osteoblasts

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Abstract. Heat shock protein 70 (HSP70) functions as an ATP-dependent molecular chaperone under stress and is involved in protein homeostasis, folding and degradation. HSP70 inhibitors amplify TGF-β-stimulated VEGF synthesis in the mouse osteoblastic MC3T3-E1 cell line. Basic fibroblast growth factor (bFGF) stimulates IL-6 release via p38 MAPK in MC3T3-E1 osteoblast-like cells. In the present study, the effects of HSP70 on the bFGF-stimulated release of IL-6 was evaluated using MC3T3-E1 osteoblast-like cells. IL-6 release and mRNA expression levels were analyzed using ELISA and reverse transcription-quantitative PCR, respectively. Phosphorylation of p38 MAPK and HSP70 was assessed using western blotting. HSP70 inhibitor VER-155008 significantly increased the bFGF-stimulated release of IL-6 in both MC3T3-E1 osteoblastic cells and normal human osteoblasts. Furthermore, VER-155008 significantly enhanced the mRNA expression levels of IL-6 stimulated by bFGF. Western blotting demonstrated a significant increase in the bFGF-stimulated phosphorylation of p38 MAPK in VER-155008-treated MC3T3-E1 cells. A significant increase in the bFGF-stimulated phosphorylation of p38 MAPK was also demonstrated in MC3T3-E1 cells treated with YM-08, another HSP70 inhibitor. VER-155008 or YM-08 did not significantly affect the expression of HSP70 with or without bFGF stimulation. Finally, the specific p38 MAPK inhibitor SB203580 markedly suppressed the enhancing effects of VER-155008 on bFGF-stimulated release of IL-6. Taken together, these results indicated that HSP70 inhibitor amplified bFGF-stimulated release of IL-6 through p38 MAPK activation in the osteoblastic MC3T3-E1 cell line.

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Introduction

Heat shock proteins (HSPs) are induced by stressful environments such as high temperature or pathological conditions (1). HSPs are generally characterized as molecular chaperones that prevent protein aggregation and restore protein homeostasis (1). Based on their molecular weight, HSPs are now divided into seven categories as follows: HSPA family (HSP70), HSPH family (HSP110), HSPC family (HSP90), HSPD/E family (HSP60/HSP10), HSPB family (small HSPs), DNAJ (HSP40) and chaperonin containing tailless complex polypeptide 1 or tailless complex polypeptide 1 ring complex (1). Among these groups, HSP70 is expressed in unstressed cells and acts as an ATP-dependent molecular chaperone (2). HSP70 has been reported to serve an important role in promoting protein homeostasis and folding, and survival of cells under stressful conditions (3). Regarding the functions of HSP70 in bone metabolism, HSP70 increases both alkaline phosphatase activity and mineralization of human mesenchymal stem cells (hMSCs) (4). HSP70 also increases the expression of osteogenic markers such as runt-related transcription factor 2 (Runx2) and Osterix in hMSCs, which promotes osteoblastic differentiation (4). In bone cells, we previously reported that HSP70 was highly expressed in mouse calvaria-derived MC3T3-E1 osteoblastic cells (5). As for the functions of HSP70, we showed that TGF-β VEGF release was downregulated by HSP70 in MC3T3-E1 osteoblastic cells (6). However, the functions of HSP70 in bone metabolism, especially in osteoblasts, have not yet been fully elucidated.

Bone is a dynamic organ, in which osteoclasts reabsorb calcified bone and osteoblasts subsequently form new bone. These processes, termed bone remodeling, are tightly regulated to maintain proper bone mass under physiological conditions. Imbalance of bone formation and resorption leads to metabolic bone disorders, including osteoporosis (7). Interleukin-6 (IL-6) is a multifunctional cytokine that is

released from numerous types of cells and serves important roles in biological processes, such as immunity, hematopoiesis and bone metabolism (8). During bone metabolism, IL-6 activates signal transduction pathways in osteoblasts via the gp130 receptor and induces the expression of receptor-activator of nuclear factor-κB-ligand (RANKL) (9). RANKL attaches to RANK expressed on osteoclast precursors and induces differentiation into mature osteoclasts (9). Therefore, IL-6 has been generally considered as a bone resorptive cytokine. By contrast, it has been reported that IL-6 is essential in the process of fracture healing (10,11). Therefore, IL-6 is known as an osteotropic factor that may modulate bone formation under bone turnover-accelerating conditions (12). Basic fibroblast growth factor (bFGF or FGF-2) is the most abundant FGF and is known as an angiogenic factor (13). During bone metabolism, bFGF serves crucial roles in bone regeneration and fracture repair (12). bFGF increases vascularization during the early stages of fracture healing and promotes the proliferation and differentiation of bone marrow stromal cells (14). We previously reported that bFGF stimulated IL-6 release through p38 MAPK in mouse MC3T3-E1 osteoblastic cells (15,16). However, the roles of HSP70 in the bFGF-stimulated IL-6 release in osteoblasts are still unknown. The present study evaluated whether HSP70 serves a role in the bFGF-stimulated release of IL-6 and the mechanism involved in this, using osteoblastic cells.

Materials and methods

Materials. bFGF was purchased from Roche Diagnostics GmbH. HSP70 inhibitors, VER-155008 and YM-08, were purchased from MilliporeSigma. SB203580 (cat. no. 559389) were purchased from Calbiochem; Merck KGaA. Phosphorylated (p)-p38 MAPK (cat. no. 4511S) and p38 MAPK (cat. no. 9212S) antibodies were purchased from Cell Signaling Technology, Inc. HSP70 antibodies (cat. np. ADI-SPA-812) were purchased from Enzo Life Sciences, Inc. GAPDH antibodies (cat. no. sc-25778) were purchased from Santa Cruz Biotechnology, Inc. Peroxidase-labeled anti-rabbit IgG antibodies (cat. no. 5220-0336; SeraCare Life Sciences, Inc.) and peroxidase-labeled anti-mouse IgG antibodies (cat. no. #7076; Cell Signaling Technology, Inc.) were used as secondary antibodies. An ECL western blot detection system (cat. no. RPN2106) was purchased from Cytiva. Mouse (cat. no. M6000B) and human (cat. no. D6050) IL-6 ELISA kits were purchased from R&D Systems, Inc.

Cell culture. Cloned MC3T3-El osteoblast-like cells established from neonatal mouse calvaria (17) were donated by Dr M Kumegawa (Department of Dentistry, Graduate School of Dentistry, Meikai University, Sakado, Japan), and maintained in α-minimum essential medium (α-MEM; MilliporeSigma) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific Inc.) at 37˚C in a humidified atmosphere of 5% CO_2-95% air, as previously described (18). For western blot analyses, the MC3T3-E1 osteoblastic cells were seeded into 90-mm diameter dish plates ($2x10^5$ cells/dish) in α-MEM supplemented with 10% FBS. For ELISA and RT-PCR analysis, the cells were seeded into 35-mm diameter dish plates ($5x10^4$ cells/dish) in α-MEM supplemented with 10% FBS. The culture medium was changed for α-MEM supplemented with 0.3% FBS after 5 days. These cells were used for the experiments after 48 h.

Normal human osteoblasts, which were originally derived from human samples from patients who provided informed consent, were purchased from Cambrex Bio Science Rockland, Ltd. (cat. no. CC-2538; passage 2) and used in the present study. The cultured normal human osteoblasts were cultured under the same conditions as those used for mouse MC3T3-E1 osteoblastic cells. These normal human osteoblasts were seeded into 35-mm diameter dish plates ($5x10^4$ cells/dish) in α-MEM supplemented with 10% FBS. The medium was changed to α-MEM supplemented with 0.3% FBS after 17 days. These cells were used for ELISA experiments after a further 48 h (19).

Measurement of IL-6. MC3T3-E1 osteoblast-like cells and normal human osteoblasts were pre-treated with 1, 10 and 30 μM VER-155008 in 1 ml α-MEM supplemented with 0.3% FBS at 37˚C for 60 min. These cells were subsequently treated with 30 ng/ml bFGF or vehicle and incubated for 48 h without washing away the VER-155008. Pre-incubation with 10 μM SB203580 was performed for 60 min prior to the VER-155008 stimulation. The conditioned medium was collected at the end of the incubation, and the IL-6 concentration in the medium was assessed using mouse and human IL-6 ELISA kits (16,19).

Reverse transcription-quantitative (RT-q)PCR analysis. MC3T3-E1 osteoblast-like cells were pre-treated with 30 μM VER-155008 for 60 min, and then stimulated with 30 ng/ml bFGF in α-MEM supplemented with 0.3% FBS for 3 h. Total RNA was isolated using TRIzol® reagent (Invitrogen; Thermo Fischer Scientific, Inc.) and reverse transcribed into cDNA at 37˚C for 60 min using an Omniscript Reverse Transcriptase kit (Qiagen, Inc.). RT-qPCR was performed using a Light Cycler system with Fast Start DNA Master SYBR Green I according to the manufacturer's standard protocol (Roche Diagnostics). Samples were subjected to the following PCR thermocycling conditions: Initial denaturation at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 1 sec, annealing at 60˚C for 5 sec and elongation at 72˚C for 7 sec. IL-6 and GAPDH primers were purchased from Takara Bio, Inc., with sequences as follows: IL-6 forward (F), 5'-CCACTTCAC AAGTCGGAGGCTTA-3' and reverse (R), 5'-GCAAGTGCA TCATCGTTGTTCATAC-3'; and GAPDH F, 5'-AACGACCCC TTCATTGAC-3' and R, 5'-TCCACGACATACTCAGCAC-3'. All measurements were analyzed using the $2^{-\Delta\Delta Cq}$ method (20).

Western blotting. MC3T3-E1 cells were pre-incubated with 30 μM of VER-155008 or 10 and 20 μM YM-08, and then stimulated using 30 ng/ml bFGF in α-MEM containing 0.3% FBS for 10 min. For western blot analysis, MC3T3-E1 osteoblast-like cells were rinsed twice with phosphate-buffered saline, and lysed and sonicated in 800 μl lysis buffer containing 62.5 mM Tris/HCl, 50 mM dithiothreitol, 2% SDS and 10% glycerol. Proteins were separated on a 10% gel using SDS-PAGE and transferred to a PVDF membrane (21). Although the mass of protein per lane was not quantified, the same number of cells ($2x10^5$ cells/dish) was seeded in each dish. The days of treatment, conditions, and lysis buffer dose were

also the same for each dish. Lysates (10 μl) were applied per lane of all SDS-PAGE gels. The membrane was subsequently incubated at 4˚C overnight with primary antibodies against p38 MAPK, p-p38 MAPK, HSP70 and GAPDH (1:1,000) followed by incubation with the appropriate secondary antibodies (1:1,000) at room temperature for 1 h. Following the detection of specific antibodies, the same membranes were stripped with WB stripping solution (cat. no. 46430, Thermo Fisher Scientific Inc.) for 15 min at room temperature and reprobed. An ECL western blotting kit was used for the detection as previously described (22).

Densitometric analysis. Densitometric analysis of the western blots was performed using a scanner and ImageJ analysis software (version 1.48; National Institutes of Health). The phosphorylated protein levels were calculated as follows: The background subtracted signal intensity of each phosphorylation signal was normalized to the respective intensity of total protein and plotted as the fold increase compared with that in the control cells without stimulation.

Statistical analysis. All data were analyzed using Mini StatMate (version 2.01; ATMS Co., Ltd.). ANOVA followed by Bonferroni's significant difference test was used for multiple comparisons. All measurements were performed in triplicate from dependent cell preparations and analyzed. Data are presented as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

VER-155008 increases the bFGF-stimulated IL-6 release in MC3T3-E1 osteoblast-like cells and normal human osteoblasts. We previously reported that HSP70 was highly expressed in unstimulated MC3T3-E1 osteoblast-like cells (5,23). In the present study, the roles of HSP70 in bFGF-stimulated IL-6 release using VER-155008, a HSP70 inhibitor (24), were analyzed in MC3T3-E1 osteoblast-like cells. As a result, the bFGF-stimulated release of IL-6 was significantly increased by VER-155008 at each time point compared with the respective control (Fig. 1A). By contrast, the IL-6 release was not affected by treatment with VER-155008 alone. Furthermore, the effects of VER-155008 on the bFGF-induced release of IL-6 tended to be dose-dependent in the range between 1 and 30 μM (Fig. 1B).

Normal human osteoblasts were used to analyze the role of HSP70 inhibitors in different osteoblast types. As a result, it was confirmed that IL-6 release was significantly increased by bFGF in normal human osteoblasts compared with the control. Moreover, bFGF-stimulated release of IL-6 was significantly enhanced by VER-155008 compared with the bFGF only group. VER-155008 alone did not significantly affect the release of IL-6 in normal human osteoblasts (Fig. 2A).

VER-155008 enhances the bFGF-induced expression levels of IL-6 mRNA in MC3T3-E1 osteoblast-like cells. To evaluate whether the enhancing effect of the HSP70 inhibitor on the bFGF-stimulated release of IL-6 in MC3T3-E1 osteoblastic cells was mediated through transcriptional events, the effect of VER-155008 on the bFGF-induced expression of IL-6 mRNA was assessed. It was demonstrated that VER-155008 significantly increased the mRNA expression levels of IL-6 compared with the bFGF only group (Fig. 2B).

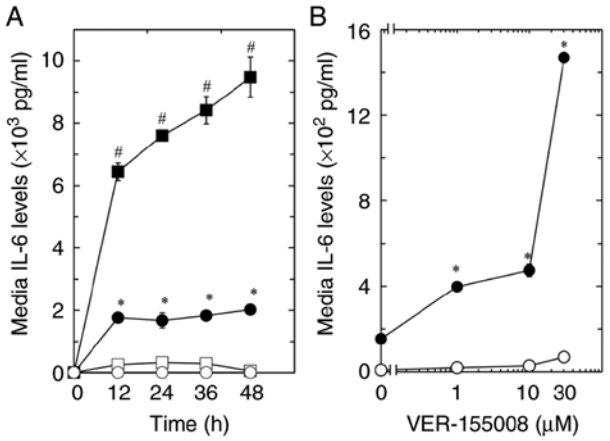

Figure 1. VER-155008 increases the bFGF-stimulated release of IL-6 in MC3T3-E1 osteoblast-like cells. (A) MC3T3-E1 cells were pretreated with 30 μM VER-155008 (squares) or vehicle (circles) for 60 min and then stimulated using 30 ng/ml bFGF (closed black symbols) or vehicle (open white symbols) for the indicated periods. (B) MC3T3-E1 cells were pretreated with certain concentrations of VER-155008 for 60 min and then stimulated using 30 ng/ml bFGF (closed black circles) or vehicle (open white circles) for 48 h. *P<0.05 vs. control. #P<0.05 vs. bFGF alone. bFGF, basic fibroblast growth factor; IL6, interleukin 6.

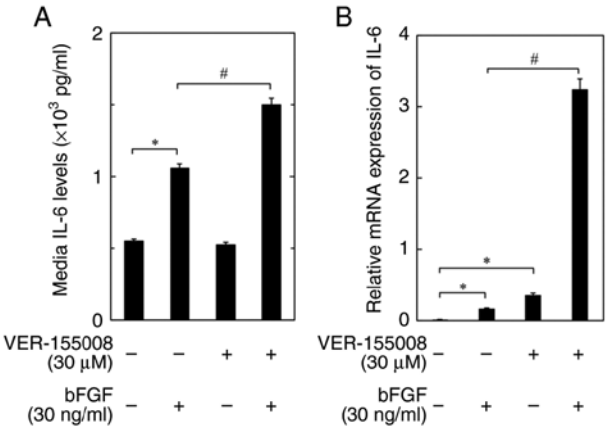

Figure 2. VER-155008 increases the bFGF-stimulated release of IL-6 in normal human osteoblasts and the mRNA expression levels of IL-6 in MC3T3-E1 osteoblast-like cells. (A) Normal human osteoblasts were pre-treated with 30 μM VER-155008 for 60 min and subsequently stimulated using 30 ng/ml bFGF or vehicle for 48 h. (B) MC3T3-E1 cells were pretreated with 30 μM VER-155008 or vehicle for 60 min and then stimulated using 30 ng/ml bFGF or vehicle for 3 h. The mRNA expression levels of IL-6 were quantified by reverse transcription-quantitative PCR. IL-6 mRNA expression levels were normalized to those of GAPDH mRNA. *P<0.05 vs. control. #P<0.05 vs. bFGF alone. bFGF, basic fibroblast growth factor; IL6, interleukin 6.

VER-155008 and YM-08 stimulate bFGF-induced phosphorylation of p38 MAPK in MC3T3-E1 osteoblast-like cells. We have previously reported that P38 MAPK activation is related to the bFGF-stimulated release of IL-6 in MC3T3-E1 osteoblastic cells (16). Therefore, the effects of HSP70 inhibitors on the bFGF-induced phosphorylation of p38 MAPK was assessed in MC3T3-E1 osteoblastic cells. VER-155008

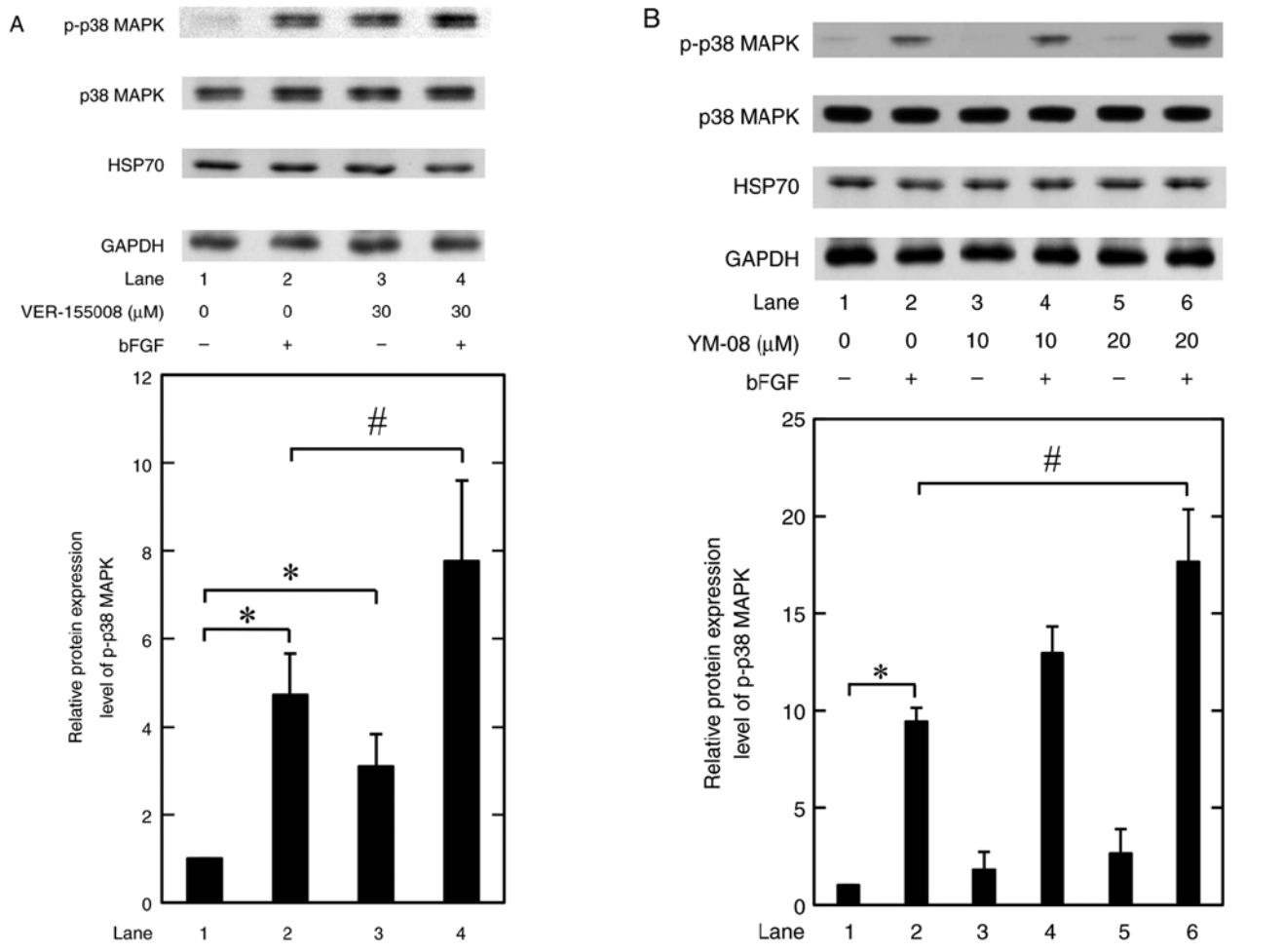

Figure 3. VER-155008 and YM-08 stimulate the bFGF-induced phosphorylation of p38 MAPK and HSP70 in MC3T3-E1 osteoblast-like cells. MC3T3-E1 cells were pretreated with various concentrations of (A) VER-155008 or vehicle, or (B) YM-08 or vehicle for 60 min and then stimulated using 30 ng/ml bFGF or vehicle for 10 min. The cell extracts subjected to western blotting with antibodies against p-p38 MAPK, p38 MAPK, HSP70 or GAPDH. The histogram presents the semi-quantified levels of p-p38 MAPK normalized to those of p38 MAPK. *P<0.05 vs. control. #P<0.05 vs. bFGF alone. bFGF, basic fibroblast growth factor; p, phosphorylated; HSP70, heat shock protein 70.

at 30 mM significantly increased the phosphorylation levels of p38 MAPK induced by bFGF compared with the bFGF only group. Furthermore, 30 mM VER-155008 alone significantly increased the levels of p38 MAPK phosphorylation compared with the untreated control (Fig. 3A). YM-08, another type of HSP70 inhibitor (25), significantly increased the bFGF-induced phosphorylation of p38 MAPK, compared with the bFGF only group, at 20 mM; however it had no significant effect on the phosphorylation when used alone (Fig. 3B).

Neither VER-155008 nor YM-08 affect the expression of HSP70 with or without bFGF stimulation in MC3T3-E1 osteoblast-like cells. We previously reported that HSP70 was highly expressed in unstimulated MC3T3-E1 osteoblast-like cells (5,23). Whether VER-155008 and YM-08 with or without bFGF could affect the HSP70 expression in these cells was assessed in the present study. VER-155008 and YM-08 did not significantly affect the expression of HSP70 with or without bFGF stimulation (Fig. 3).

SB203580 inhibits the amplification of bFGF-elicited IL-6 release by VER-155008 in MC3T3-E1 osteoblast-like cells. Finally, the effect of SB203580, a specific inhibitor of p38 MAPK (26), on the amplification of the bFGF-elicited release of IL-6 by VER-155008 was assessed in MC3T3-E1 osteoblast-like cells. The results of the present study confirmed those of a previous report showing that SB203580 significantly inhibited the bFGF-elicited release of IL-6 (16). SB203580 almost completely inhibited the amplifying effects of VER-155008 on the bFGF-stimulated release of IL-6 in MC3T3-E1 cells (Fig. 4).

Discussion

The present study demonstrated that the HSP70 inhibitor, VER-155008, significantly increased the bFGF-induced release of IL-6 in mouse MC3T3-E1 osteoblastic cells and normal human osteoblasts. Furthermore, VER-155008 significantly increased the mRNA expression levels of IL-6 induced by bFGF in MC3T3-E1 osteoblastic cells, which suggested that the enhancing effects of VER-155008 on the bFGF-induced release of IL-6 may be mediated through a transcriptional event. Our previous study demonstrated that HSP70 inhibitors, VER-155008 and YM-08,

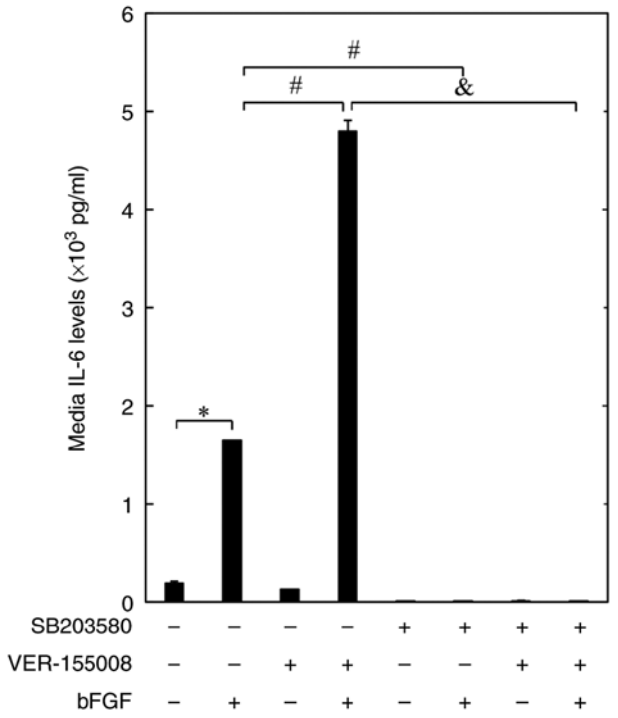

Figure 4. SB203580 inhibited the amplification of bFGF-elicited release of IL-6 by VER-155008 in MC3T3-E1 osteoblast-like cells. The cultured cells were pre-incubated with 30 μM SB203580 or vehicle for 60 min, subsequently pretreated with 10 μM VER-155008 or vehicle for another 60 min, and then stimulated using 30 ng/ml bFGF or vehicle for 48 h. IL-6 concentrations in the conditioned medium were assessed using ELISA. *P<0.05 vs. control. #P<0.05 vs. bFGF alone. &P<0.05 vs. bFGF and VER-155008. bFGF, basic fibroblast growth factor; IL6, interleukin 6.

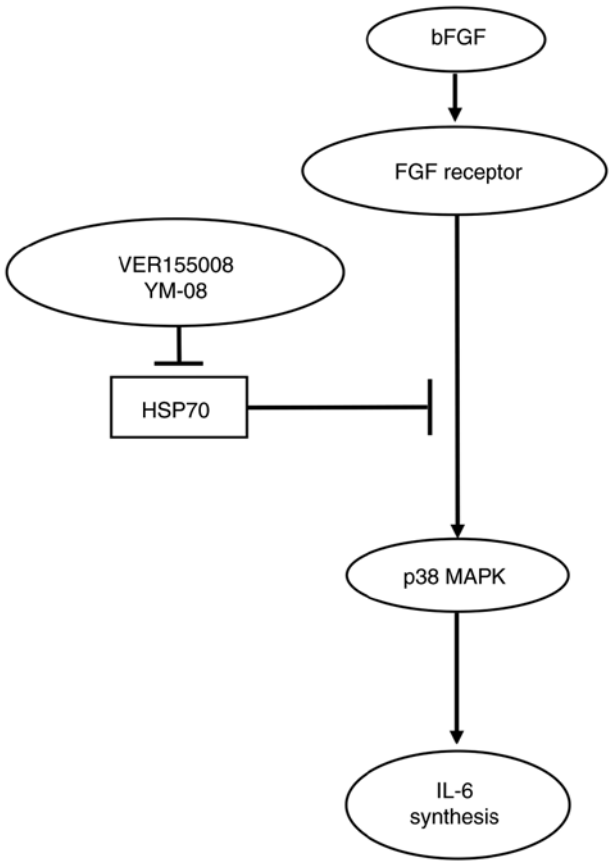

Figure 5. Schematic illustration of the regulatory mechanism of HSP70 on bFGF-induced IL-6 synthesis in osteoblasts. HSP70, which is inhibited by VER155008 or YM-08, negatively regulates bFGF-induced IL-6 synthesis in osteoblasts and the effect is exerted at a point upstream of p38 MAPK. bFGF, basic fibroblast growth factor; HSP70, heat shock protein 70; IL6, interleukin 6.

significantly increased TGF-β-stimulated VEGF release and the TGF-β-induced mRNA expression levels of VEGF in MC3T3-E1 osteoblast-like cells (6). These results indicated that HSP70 inhibitors were involved in VEGF synthesis, as well as IL-6 synthesis, in MC3T3-E1 osteoblastic cells.

Regarding the intracellular signaling exerted by bFGF in osteoblasts, our previous study demonstrated that bFGF elicits the synthesis of IL-6 via the activation of p38 MAPK in MC3T3-E1 osteoblastic cells (16). In the present study, both VER-155008 and YM-08 significantly increased the bFGF-elicited phosphorylation of p38 MAPK, which suggested that HSP70 inhibition upregulated bFGF-elicited activation of p38 MAPK in these cells. The inhibitors did not significantly affect the protein expression levels of HSP70, either with or without bFGF in these cells. Therefore, it is likely that the upregulation by HSP70 inhibitors of the activation of p38 MAPK causes the enhancement of IL-6 synthesis induced by bFGF in MC3T3-E1 osteoblastic cells. Treatment with VER-155008 alone, at 30 μM, caused a significant increase in the phosphorylation of p38 MAPK, which might be due to non-specific effects other than HSP70 inhibition. MAP kinase kinase kinases (MAP3Ks), including apoptosis signal-regulating kinase-1 (ASK-1) and MEK kinase (MEKK) are activated in response to numerous inflammatory cytokines. MAP3Ks activate MAP kinase kinases such as MKK3/6, and MKK3/6 subsequently activates p38 MAPK (27). HSP70 is known as a molecular chaperone that interacts with client proteins and modulates intracellular signal transduction. The inhibition of HSP70 chaperone function can cause inactivation and degradation of HSP70-dependent client proteins. The present study demonstrated that the HSP70 inhibitor VER-155008 (30 μM) significantly enhanced the phosphorylation of p38 MAPK. Although this remains to be confirmed as a genuine effect of the inhibitor, this result indicated that the activation of p38 MAPK could be elicited by HSP70 silencing. Therefore, HSP70 might downregulate p38 MAPK signaling through the chaperone effect on the upstream kinase(s) such as ASK-1, MEKK and MKK3/6 in MC3T3-E1 osteoblast-like cells.

HSP70 is highly expressed in osteoblasts (5,23). HSP70 serves a central role in protein homeostasis by folding proteins into the correct form as a molecular chaperone and also prevents unfolded protein aggregation by binding to client proteins (28). In bone cells, it has been previously reported that HSP70 increases alkaline phosphatase activity and promotes hMSC mineralization, and that it also significantly upregulates the expression of Runx2 and Osterix under osteogenic induction conditions (29). Furthermore, overexpression of HSP family A member 1A, which encodes the cognate HSP70, stimulates osteoblastic differentiation of bone marrow stromal cells via the Wnt/β-catenin signaling pathway (30). Our previous study demonstrated that HSP70 inhibitors suppress the migration of MC3T3-E1 osteoblast-like cells induced by

insulin-like growth factor-I or epidermal growth factor (31,32). However, the precise mechanism by which HSP70, by itself or together with client proteins, influences bone metabolism has not yet been fully elucidated, and the exact functions of HSP70 in osteoblasts have not been fully reported. Our previous study reported that TGF-β-stimulated VEGF synthesis is upregulated by HSP70 inhibitors through activation of p38 MAPK in MC3T3-E1 cells (6). Therefore, the action of HSP70 against p38 MAPK in bFGF-stimulated MC3T3-E1 osteoblastic cells in this study seemed to be similar to the effect of HSP70 on p38 MAPK in TGF-β-stimulated same cells in our previous study. Furthermore, it was demonstrated that SB203580 almost completely inhibited the enhancement by VER-155008 of the bFGF-stimulated release of IL-6. Our previous study reported that SB203580 inhibited the enhancement, by VER-155008 and YM-08, of TGF-β-stimulated VEGF release (6). These results strongly indicated the involvement of p38 MAPK in the increased release of IL-6 and VEGF. Taking these findings into account, it is likely that HSP70 negatively regulates bFGF-stimulated synthesis of IL-6 as well as the TGF-β-stimulated synthesis of VEGF in MC3T3-E1 osteoblast-like cells, and that the effects of HSP70 are exerted by the inhibition of p38 MAPK activation. Furthermore, our recent study reported that HSP70 inhibitors upregulated prostaglandin E_1 (PGE_1)-stimulated synthesis of IL-6 through p38 MAPK in MC3T3-E1 osteoblastic cells (33). Although their receptors are quite different, the inhibition of HSP70 can cause upregulation of p38 MAPK in osteoblasts stimulated by both bFGF and PGE_1, which results in an increase in IL-6 synthesis. The potential mechanisms underlying regulation of bFGF-stimulated IL-6 release by HSP70 in osteoblasts is presented in Fig. 5. As HSP70 is an essential factor for cell survival, the knockdown of HSP70 by small interfering (si) RNA for >24 h is toxic to MC3T3-E1 cells, which has been indicated in our previous paper (6), but the results have not been fully published. The reason why only HSP70 siRNA, but not the HSP70 inhibitors, was toxic to cells was not reported. However, it can be hypothesized that the siRNA might suppress HSP70 function more completely than the inhibitors, which were demonstrated in the present study to not affect the expression of HSP70.

In bone metabolism, the multifunctional cytokine IL-6 is recognized as an osteotropic factor, which promotes bone formation under conditions of increased bone turnover, including during fracture healing (10,11). bFGF is known to be highly expressed during fracture healing as an angiogenic factor, which serves a crucial role in bone regeneration (13). However, HSP70 is constitutively expressed in non-stressed cells, including osteoblasts (2). Therefore, HSP70 may play an important role in bone metabolism by regulating the effects of bFGF and IL-6. The amplifying effects of the HSP70 inhibitors on the bFGF-induced release of IL-6 in osteoblast-like cells demonstrated in the present study indicated that the suppression of HSP70 might be a novel method to promote bone regeneration under conditions such as fracture healing.

In conclusion, the results of the present study suggested that HSP70 inhibitor amplified the bFGF-induced release of IL-6 in osteoblasts and that the inhibitory effect of HSP70 was exerted by inhibition of the activation of p38 MAPK.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GK and HT performed data analysis and interpretation, and wrote the manuscript. GK and HT confirm the authenticity of all the raw data. TH and RMN collated and analyzed the data. OK and HT conceived and designed the study, and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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