Unphosphorylated heat shock protein 27 suppresses fibroblast growth factor-2-stimulated vascular endothelial growth factor release in osteoblasts

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Abstract. Heat shock protein 27 (HSP27) also known as heat shock protein β1 (HSPB1) is a member of the family of small heat shock proteins ubiquitously expressed in all tissues. It has previously been demonstrated that HSP27 regulated the synthesis of osteocalcin and interleukin-6 in osteoblast-like MC3T3-E1 cells. In the present study, the effect of HSP27 on basic fibroblast growth factor (FGF-2)-stimulated vascular endothelial growth factor (VEGF) synthesis in MC3T3-E1 cells, was observed. The levels of VEGF release stimulated by FGF-2 in the HSP27-overexpressing MC3T3-E1 cells were significantly lower compared with those in the control cells. In addition, the levels of VEGF release stimulated by FGF-2 in the phosphomimic HSP27-overexpressing cells were significantly higher compared with those in the non-phosphorylatable HSP27-overexpressing cells. Furthermore, no significant differences were observed in the FGF-2-induced phosphorylation levels of p44/p42 mitogen-activated protein (MAP) kinase, p38 MAP kinase, stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) or p70 S6 kinase among the four types of transfected cells. These results suggested that unphosphorylated HSP27 attenuated the FGF-2-stimulated VEGF synthesis in osteoblasts.

Introduction

Bone metabolism is predominantly regulated by osteoblasts and osteoclasts, which are responsible for bone formation and bone resorption, respectively (1). It is well known that osteoblasts synthesize basic fibroblast growth factor (FGF-2), which is embedded in the bone matrix (2). During fracture repair, high levels of FGF-2 expression are detected in osteoblasts (3). Data suggest that FGF-2 is important in fracture healing, bone remodeling and osteogenesis. There are four FGF receptor subtypes with a high structural affinity (FGF receptors 1-4) (4) and it has been determined that FGF-2 activated the FGF receptors 1 and 2 in osteoblast-like MC3T3-E1 cells (5). It has also been observed that FGF-2 stimulated the release of vascular endothelial growth factor (VEGF), a mitogen that is highly specific for vascular endothelial cells responding to various physiological stimulants including insulin-like growth factor-1 and bone morphogenetic protein (6). VEGF is important in bone remodeling to stimulate angiogenesis inducing the formation of bone microvasculature (7). Regarding the signaling mechanisms in osteoblasts, it has been demonstrated that FGF-2-induced VEGF release is positively regulated by p44/p42 mitogen-activated protein (MAP) kinase and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK). However, it is negatively regulated by p38 MAP kinase and p70 S6 kinase (8-10).

Heat shock proteins (HSPs) are induced by numerous types of stress such as heat and chemical stress (11,12). HSPs have been classified into groups including HSPA (HSP70), HSPB (small HSPs), HSPC (HSP90) and HSPH (HSP110) (12). High-molecular-weight HSPs such as HSPA (HSP70), HSPC (HSP90) and HSPH (HSP110) have been investigated and were demonstrated to act as molecular chaperones that prevent the aggregation of unfolded proteins and exert a cytoprotective function (12). Small HSPs (HSPB) with a monomer molecular mass of 12-43 kDa such as HSP27 (HSPB1), αB-crystallin (HSPB5) and HSP20 (HSPB6), are constitutively expressed in unstimulated cells and tissues such as skeletal, smooth and cardiac muscle. Members of the HSPB group are currently considered to be involved in essential functions such as protein intracellular transport and the cytoskeletal architecture (12). It has been demonstrated

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that the expression levels of HSP27 were low in unstimulated osteoblasts (13). In addition, certain bone modulating agents such as prostaglandins and transforming growth factor-β are able to induce the expression of HSP27 via intracellular signaling systems such as the activation of MAP kinase in osteoblast-like MC3T3-E1 cells (13-18). It has been observed that HSP27 is involved in regulating the balance between the differentiation and apoptosis of osteoblasts (19,20). However, the precise function of HSPBs has yet to be elucidated, unlike that of the high-molecular-weight HSPs.

It is generally observed that the functions of HSP27 (HSPB1) are regulated by post-translational modifications such as phosphorylation (12). HSP27, which normally exists as unphosphorylated oligomers (≤800 kDa), has three phosphorylatable serine residues (Ser-15, Ser-78 and Ser-82). When HSP27 is phosphorylated, a conformational change from the aggregated form to the dimer occurs (21,22). Prostaglandin D2 was observed to induce the phosphorylation of HSP27 through p44/p42 MAP kinase and p38 MAP kinase in the MC3T3-E1 cells (23). In addition, unphosphorylated HSP27 has a stimulatory effect on mineralization whereas phosphorylated HSP27 enhances tumor necrosis factor-α-stimulated interleukin-6 synthesis in these cells (24,25). These results led to the hypothesis that HSP27 is essential in the regulation of bone metabolism through the control of osteoblast functions. However, the exact role of HSP27 in bone metabolism remains to be fully elucidated. In the present study, the involvement of HSP27 in the VEGF synthesis stimulated by FGF-2 in osteoblast-like MC3T3-E1 cells was investigated.

Materials and methods

Materials. The mouse VEGF enzyme-linked immunosorbent assay (ELISA) kit and FGF-2 were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Antibodies against phospho-specific p44/p42 MAP kinase, p44/p42 MAP kinase, phospho-specific p38 MAP kinase, p38 MAP kinase, phospho-specific SAPK/JNK, SAPK/JNK, phospho-specific p70 S6 kinase and p70 S6 kinase were obtained from Cell Signaling, Technology, Inc. (Beverly, MA, USA). The enhanced chemiluminescence (ECL) western blotting system was obtained from GE Healthcare UK, Ltd. (Buckinghamshire, UK). The bicinchoninic assay (BCA) Protein Assay Reagent kit was purchased from Pierce Biotechnology, Inc., (Rockford, IL, USA). Other materials and chemicals were obtained from commercial sources.

Cell culture. Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria (26) were maintained as previously described (27). Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air.

Establishment of the cells stably transfected with HSP27. Mutant-HSP27 plasmids, in which serine residues (Ser-15, Ser-72 and Ser-82) were mutated to alanines to generate constitutively non-phosphorylatable HSP27 (3A cells), or were mutated to aspartic acid to generate constitutively phospho-mimicking HSP27 (3D cells), were provided by Dr C. Schafer (Klinikum Großhadern, Ludwig-Maximilians University, Munich, Germany). For stable transfections, MC3T3-E1 cells ($5x10^5$ cells) were cultured in 6-well plates. The cells were transfected with 2 μg of the wild-type (WT) or the mutant HSP27 plasmids expressing geneticin (G418; EMD Chemicals, Inc., San Diego, CA, USA) resistance using 12 μl of the UniFECTOR transfection reagent (B-Bridge International, Cupertino, CA, USA) in 1 ml α-MEM medium without FCS. Medium (1 ml) with 10% FCS was added 5 h following transfection. The cells were incubated in the presence of 400 μg/ml of G418. After 2 weeks, single G418-resistant colonies were obtained by serial dilution in 96-well plates. The colonies were maintained and analyzed individually for the expression of HSP27.

Western blot analysis. The cultured cells were stimulated with 30 ng/ml FGF-2 in α-MEM containing 0.3% FCS for the indicated periods. The cells were washed twice with phosphate-buffered saline, lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris/HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS) and 10% glycerol. SDS-polyacrylamide gel electrophoresis (PAGE) was conducted according to Laemmli (28) on 10% polyacrylamide gels. A western blot analysis was performed as previously described (13) using antibodies against phospho-specific p44/p42 MAP kinase, p44/p42 MAP kinase, phospho-specific p38 MAP kinase, p38 MAP kinase, phospho-specific SAPK/JNK, SAPK/JNK, phospho-specific p70 S6 kinase and p70 S6 kinase with peroxidase-labeled antibodies as the secondary antibodies. The peroxidase activity on the polyvinylidene fluoride (PVDF) membrane was visualized on X-ray film by the ECL western blotting system.

VEGF assay. The cultured cells were stimulated by 30 and 50 ng/ml of FGF-2 in 1 ml α-MEM containing 0.3% FCS for the indicated periods. The conditioned medium and cell lysates were collected at the end of the incubation period. The VEGF concentration was measured by a VEGF ELISA kit. The absorbance of the ELISA samples was measured at 450 and 560 nm with an EL 340 Microplate Reader BioKinetic plate (Bio-Tek Instruments, Inc., Winooski, VT, USA). The levels of VEGF release were adjusted for the respective whole cell lysates. The protein levels of the cells were measured by a BCA Protein Assay Reagent kit according to the manufacturer's instructions.

Statistical analysis. The data were analyzed by analysis of variance (ANOVA) followed by the Bonferroni method for multiple comparisons between pairs. $P<0.05$ was considered to indicate a statistically significant difference. Data are presented as the mean ± SEM of triplicate independent determinations.

Results

Effect of overexpressed HSP27 on FGF-2-stimulated VEGF release in MC3T3-E1 cells. It has previously been demonstrated that FGF-2 stimulated VEGF release in osteoblast-like MC3T3-E1 cells (8). In order to investigate the involvement of HSP27 on the FGF-2-induced VEGF release, its effect was investigated in WT HSP27 cDNA-transfected MC3T3-E1 cells. As previously observed (24), the 'empty' cells, which

were transfected with an empty vector, represented the normal parental MC3T3-E1 cells and the WT cells represented the MC3T3-E1 cells overexpressing the wild type-HSP27. FGF-2 (30-50 ng/ml) significantly stimulated VEGF release in a dose-dependent manner in the WT and empty cells (Fig. 1). The levels of VEGF release induced by FGF-2 were markedly lower in the WT cells compared with the empty cells (Fig. 1). The levels of VEGF induced by FGF-2 (50 ng/ml) in the WT cells were ~40% lower compared with those in the empty cells.

Effects of FGF-2 on intracellular signaling in the WT HSP27-transfected MC3T3-E1 cells and the empty vector-transfected MC3T3-E1 cells. In a previous study it was demonstrated that VEGF release stimulated by FGF-2 was positively regulated by p44/p42 MAP kinase and SAPK/JNK but negatively regulated by p38 MAP kinase and p70 S6 kinase in osteoblast-like MC3T3-E1 cells (8-10). The effects of FGF-2 on the intracellular signaling in the WT and empty cells were observed. FGF-2 markedly induced the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, SAPK/JNK and p70 S6 kinase in the WT (Fig. 2A) and empty cells (Fig. 2B). However, no significant differences were noted in the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, SAPK/JNK or p70 S6 kinase between the WT and empty cells.

Effect of phosphorylated HSP27 on FGF-2-stimulated VEGF release in MC3T3-E1 cells. To elucidate whether the effect of FGF-2 on VEGF release is affected by the phosphorylation status of HSP27 in osteoblast-like MC3T3-E1 cells, the effects of FGF-2 on VEGF release in the 3D cells compared with that in the 3A cells was observed. As previously demonstrated (24), HSP27 was overexpressed in the 3A and 3D cells, and the phosphorylation levels of HSP27 (Ser-82) in the 3D cells were much greater than those in the 3A cells. FGF-2 significantly stimulated VEGF release in a dose-dependent manner with a range of 30-50 ng/ml in the 3A and 3D cells (Fig. 3). However, the levels of VEGF released in the 3D cells were significantly higher than those in the 3A cells (Fig. 3). The levels of VEGF induced by FGF-2 (30 ng/ml) in the 3A cells were ~50% lower compared with those in the 3D cells.

Effects of phosphorylated HSP27 on the intracellular signaling of FGF-2 in MC3T3-E1 cells. The effects of FGF-2 on the intracellular signaling in the 3A and 3D cells were investigated. FGF-2 markedly induced the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, SAPK/JNK and p70 S6 kinase in the 3A (Fig. 4A) and 3D (Fig. 4B) cells. However, no significant differences were noted in the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, SAPK/JNK or p70 S6 kinase between the 3A and 3D cells.

Discussion

In the present study, the effect of HSP27 on FGF-2-stimulated VEGF synthesis in osteoblast-like MC3T3-E1 cells was observed. The levels of FGF-2-stimulated VEGF release were significantly attenuated in the HSP27-overexpressing MC3T3-E1 cells compared with those in the empty vector-transfected MC3T3-E1 cells. It was previously demonstrated that the levels of HSP27 were low in the

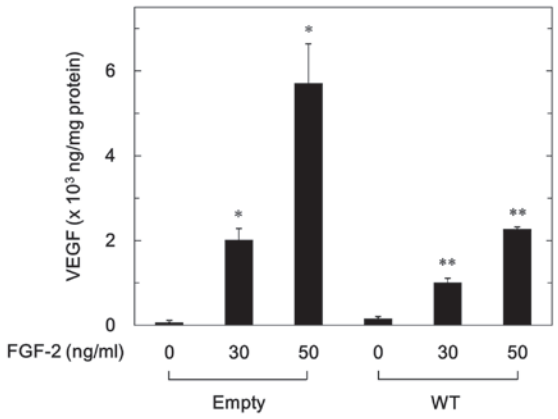

Figure 1. Dose-dependent effects of fibroblast growth factor (FGF)-2 on vascular endothelial growth factor (VEGF) release in the empty and wild-type (WT) cells. The cultured cells were stimulated by various doses of FGF-2 for 48 h. The levels of VEGF synthesis were corrected to the total protein levels. Values are presented as the mean ± SEM of triplicate independent determinations. *P<0.05, compared with the value of the vehicle in the empty cells. **P<0.05, compared with the value observed with the same dose of FGF-2 in the empty cells.

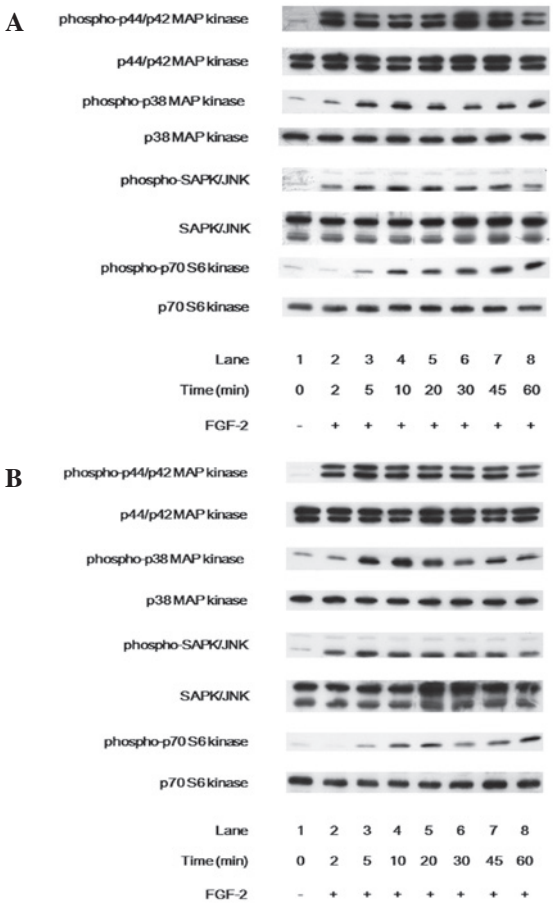

Figure 2. Effects of fibroblast growth factor (FGF)-2 on the phosphorylation of p44/p42 MAP kinase, p38 mitogen-activated protein MAP kinase, stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and p70 S6 kinase in the empty and wild-type (WT) cells. (A) The empty cells and (B) the WT cells were stimulated by 30 ng/ml of FGF-2 for the indicated time periods. The cell extracts were then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and a subsequent western blot analysis with antibodies against phospho-specific p44/p42 MAP kinase, p44/p42 MAP kinase, phospho-specific p38 MAP kinase, p38 MAP kinase, phospho-specific SAPK/JNK, SAPK/JNK, phospho-specific p70 S6 kinase and p70 S6 kinase.

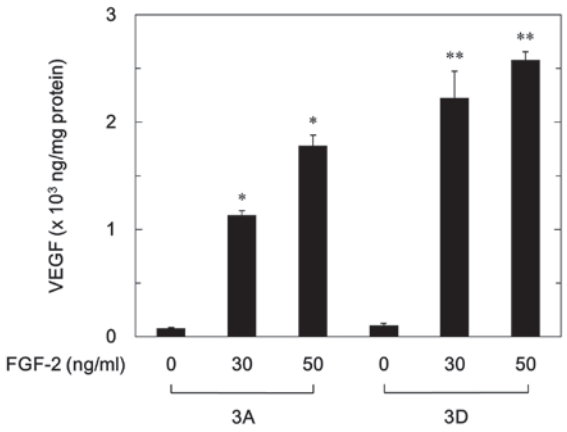

Figure 3. Effects of fibroblast growth factor (FGF)-2 on vascular endothelial growth factor (VEGF) release in constitutively non-phosphorylatable (3A) or constitutively phosphomimic (3D) HSP27-transfected MC3T3-E1 cells. The cultured cells were stimulated by various doses of FGF-2 for 48 h. The levels of VEGF synthesis were corrected to the total protein levels. Values are presented as the mean ± SEM of triplicate independent determinations. *P<0.05, compared with the value of the vehicle in the 3A cells. **P<0.05, compared with the value with the same dose of FGF-2 in the 3A cells.

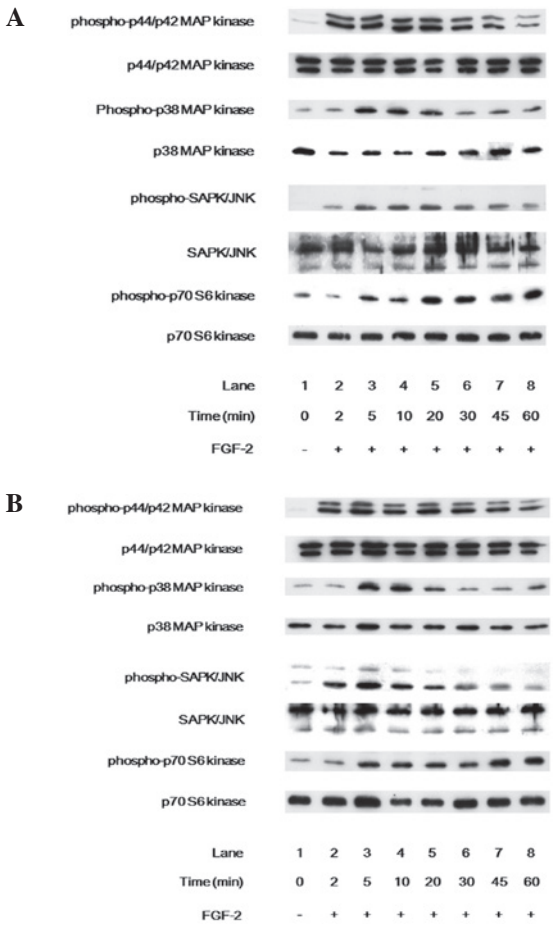

Figure 4. Effects of FGF-2 on the phosphorylation of p44/p42 mitogen-activated protein (MAP) kinase, p38 MAP kinase, stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and p70 S6 kinase in the 3A and 3D cells. (A) The 3A and (B) the 3D cells were stimulated by 30 ng/ml of FGF-2 for the indicated periods. The cell extracts were then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and a subsequent western blot analysis with antibodies against phospho-specific p44/p42 MAP kinase, p44/p42 MAP kinase, phospho-specific p38 MAP kinase, p38 MAP kinase, phospho-specific SAPK/JNK, SAPK/JNK, phospho-specific p70 S6 kinase and p70 S6 kinase.

unstimulated-MC3T3-E1 cells and that the expression of HSP27 is not significant in the empty cells (13,24). The results of these studies suggested that HSP27 exhibited an inhibitory effect on the FGF-2-induced VEGF synthesis in osteoblast-like MC3T3-E1 cells.

In a resting state, HSP27 exists in an aggregated form (≤800 kDa), which is phosphorylated at three serine residues (Ser-15, Ser-78 and Ser 82). The phosphorylation is accompanied by a conformational change from the aggregated form to the dissociated dimer form (21). The effects of the phosphorylation status of HSP27 on FGF-2-stimulated VEGF synthesis using two types of mutant HSP27-transfected cells were demonstrated. The 3A cells overexpressed non-phosphorylatable HSP27 and the 3D cells overexpressed mutant-HSP27, mimicking the phosphorylated protein (24). The levels of FGF-2-stimulated VEGF release were significantly lower in the 3A cells compared with the 3D cells. In a previous study (24), it was observed that HSP27 was overexpressed in the WT cells but was not phosphorylated. Therefore it is likely that unphosphorylated HSP27 suppresses the FGF-2-induced VEGF synthesis in osteoblast-like MC3T3-E1 cells. In addition, the phosphorylation status of HSP27 may be able to induce the VEGF synthesis in osteoblasts.

Concerning the regulatory mechanism underlying FGF-2-stimulated VEGF synthesis in osteoblasts, it was previously observed that the FGF-2-stimulated VEGF release was positively regulated by the activation of p44/p42 MAP kinase and SAPK/JNK (8,9). However, it was negatively regulated by the activation of p38 MAP kinase and p70 S6 kinase in osteoblast-like MC3T3-E1 cells (8-10). In the present study, to investigate the effect of HSP27 expression on the activation of p44/p42 MAP kinase, p38 MAP kinase, SAPK/JNK and p70 S6 kinase, the FGF-2-induced phosphorylation levels of these intracellular signaling molecules in the HSP27-transfected MC3T3-E1 cells was also determined. However, the phosphorylation levels of these molecules were not significantly different among the four types of transfected MC3T3-E1 cells (empty vector, WT HSP27, non-phosphorylatable HSP27, and phospho-mimic HSP27). Therefore, it appears unlikely that HSP27 inhibited the FGF-2-stimulated VEGF synthesis through the activation of p44/p42 MAP kinase, p38 MAP kinase, SAPK/JNK and p70 S6 kinase, or at a point upstream of these molecules. Phosphorylated HSP27 was able to change its localization from the cytoplasm to the perinuclear area and acted as a functional regulator of the endoplasmic reticulum, contributing to the regulation of osteocalcin synthesis (24). Thus, it is probable that the localization change of HSP27 due to phosphorylation attenuated the inhibitory activity of HSP27 in the VEGF synthesis induced by FGF-2 in osteoblasts.

Osteoporosis is a predominant clinical problem in developed countries. The pathology of osteoporosis is a reduction of the bone mineral density, which is a risk factor for bone fractures (29). An increase in FGF-2 expression in osteoblasts is detected during fracture repair. In addition, VEGF induces the angiogenesis of the microvasculature in the bone tissue which is important in bone remodeling (3,6). Moreover, it has been observed that VEGF regulated the balance between osteoblast and adipocyte differentiation (30). The regulation of VEGF-related mechanisms, therefore, are predicted to determine novel aspects of bone remodeling adjustment. Thus,

the results demonstrating the involvement of HSP27 in osteoblast function may provide a novel therapeutic target for bone metabolic diseases such as osteoporosis. Further investigation is required to determine the precise mechanism of action of HSP27 in osteoblasts and in bone metabolism.

In conclusion, the results suggest that unphosphorylated HSP27 exerts an inhibitory effect on FGF-2-stimulated VEGF synthesis in osteoblasts.

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