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Rho-kinase inhibitors decrease TGF-β-stimulated VEGF synthesis through stress-activated protein kinase/c-*Jun* N-terminal kinase in osteoblasts

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ABSTRACT

We have previously reported that transforming growth factor-β (TGF-β) stimulates the synthesis of vascular endothelial growth factor (VEGF) through p44/p42 mitogen-activated protein (MAP) kinase, p38 MAP kinase and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) in osteoblast-like MC3T3-E1 cells. In order to investigate whether Rho-kinase is involved in the TGF-β-stimulated VEGF synthesis in these cells we examined the effects of Rho-kinase inhibitors on the VEGF synthesis. TGF-β time-dependently induced the phosphorylation of myosin phosphatase targeting subunit (MYPT-1) which is a well known substrate of Rho-kinase. Y27632 and fasudil, Rho-kinase inhibitors, significantly reduced the TGF-β-stimulated VEGF synthesis as well as the MYPT-1 phosphorylation. Y27632 and fasudil failed to affect the TGF-β-induced phosphorylation of p44/p42 MAP kinase, p38 MAP kinase or Smad2. On the contrary, Y27632 as well as fasudil markedly suppressed the TGF-β-induced phosphorylation of SAPK/JNK. Taken together, our results strongly suggest that Rho-kinase regulates TGF-β-stimulated VEGF synthesis via SAPK/JNK activation in osteoblasts.

1. Introduction

Vascular endothelial growth factor (VEGF) is a potent mitogen displaying high specificity for vascular endothelial cells [1]. VEGF, produced and secreted from a variety of cell types, increases capillary permeability and stimulates proliferation of endothelial cells [1]. The bone metabolism is regulated mainly by two functional cells, osteoblasts and osteoclasts, responsible for bone formation and bone resorption, respectively [2]. During bone remodeling, the microvasculature is provided by capillary endothelial cells. It is currently recognized that the activities of osteoblasts, osteoclasts, and capillary endothelial cells are

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closely coordinated and regulate bone metabolism [3]. These functional cells are considered to influence one another via humoral factors as well as by direct cell-to-cell contact. As for bone metabolism, it has been reported that an inactivation of VEGF causes complete suppression of blood vessel invasion concomitant with impaired trabecular bone formation and expansion of hypertrophic chondrocyte zone in mouse tibial epiphyseal growth plate [4]. Evidence is accumulating that osteoblasts among bone cells produce and secrete VEGF in response to various physiological agents such as insulin-like growth factor-I and bone morphogenetic protein [4]. In our previous studies [5,6], we have reported that transform growth factor-β (TGF-β) stimulates VEGF synthesis in osteoblast-like MC3T3-E1 cells, and that the synthesis is positively regulated by p44/p42 mitogen-activated protein (MAP) kinase, p38 MAP kinase and stress-activated protein kinase/c-*Jun N*-terminal kinase (SAPK/JNK), members of the MAP kinase superfamily [7]. Based on these findings, VEGF secreted from osteoblasts may couple angiogenesis to bone formation by adjusting the angiogenic response to osteoblastic activity [4]. It is currently recognized that VEGF is a major regulator of bone growth and repair. However, the exact mechanism underlying VEGF synthesis in osteoblasts and its release from these cells is not precisely clarified.

It is generally recognized that Rho and the down-stream effector, Rho-associated kinase (Rho-kinase) play important roles in a variety of cellular functions such as cell motility and smooth muscle contraction [8–10]. Regarding about osteoblasts, it has been demonstrated that Rho and p38 MAP kinase are involved in the endothelin-1-induced expression of prostaglandin endoperoxide G/H synthase mRNA in osteoblasts [11]. In addition, it has been shown that the Rho/Rho-kinase pathway stimulates osteoblast proliferation whereas it inhibits osteoblast differentiation [12]. In our previous study [13], we have reported that Rho-kinase functions as a positive regulator in endothelin-1-induced synthesis of interleukin-6, a potent bone resorptive agent, in osteoblast-like MC3T3-E1 cells. However, the exact role of Rho-kinase in osteoblasts has not yet been fully elucidated.

In the present study, we investigated the involvement of Rho-kinase in the TGF-β-stimulated VEGF synthesis in osteoblast-like MC3T3-E1 cells. We here show that Rho-kinase regulates TGF-β-stimulated VEGF synthesis through SAPK/JNK activation in these cells.

2. Materials and methods

2.1. Materials

TGF-β and mouse VEGF enzyme immunoassay (ELISA) kit were purchased from R&D Systems, Inc. (Minneapolis, MN). Y27632 was obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Hydroxyfasudil (fasudil) was purchased from Sigma (St. Louis, MO). Phospho-specific MYPT-1 antibodies were purchased from Upstate (Lake Placid, NY). MYPT-1 antibodies were obtained form Santa Crus Biotechnology, Inc. (Santa Crus, CA). Phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific Smad2 antibodies and Smad2 antibodies were purchased from Cell Signaling, Inc. (Beverly, MA). ECL Western blotting detection system was purchased from Amersham Biosciences (Piscataway, NJ). Other materials and chemicals were obtained from commercial sources. Y27632 was dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect the assay for VEGF or Western blot analysis.

2.2. Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [14] were maintained as previously described [15]. 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_2/95% air. The cells were seeded into 35-mm (5×10^4/dish) or 90-mm (25×10^4/dish) diameter dishes in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

2.3. VEGF assay

The cultured cells were pretreated with various doses of Y27632 or fasudil for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle in the presence of inhibitors in 1 ml of α-MEM containing 0.3% FCS for 48 h. The conditioned medium was collected at the end of the incubation, and the VEGF concentration was measured by ELISA kit.

2.4. Western blot analysis

Western blotting analysis was performed as described previously [16] as follows. The cultured cells were pretreated with various doses of Y27632 or fasudil for 60 min, and then stimulated by TGF-β in the presence of inhibitors in α-MEM containing 0.3% FCS for the indicated periods. The cells were washed twice with phosphate-buffered saline and then lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris–HCl; pH 6.8, 3% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125,000 × *g* for 10 min at 4 °C. SDS-polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli [17] in 10% polyacrylamide gel. The protein (20 μg) was fractionated and transferred onto an Immun-Blot PVDF Membrane (Bio-Rad, Hercules, CA). Membranes were blocked with 5% fat-free dry milk in Tris-buffered saline-Tween (TBS-T; 20 mM Tris–HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) for 2 h before incubation with the primary antibodies. The rabbit polyclonal phospho-specific MYPT-1 antibodies, MYPT-1 antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific Smad2 antibodies or Smad2 antibodies were used as primary antibodies. Peroxidase-labeled antibodies raised in goat against rabbit IgG were used as second antibodies. The first and second antibodies were diluted at 1:1000 with 5% fat-free dry milk in TBS-T. Peroxidase activity on the membrane was

visualized on X-ray film by means of the ECL Western blotting detection system.

2.5. Determination

The absorbance of enzyme immunoassay samples was measured at 450 nm with EL 340 Bio Kinetic Reader (Bio-Tek Instruments, Inc., Winooski, VT). The densitometric analysis of the bands on the film was performed using Molecular Analyst/Macintosh (Bio-Rad Laboratories, Hercules, CA).

2.6. Statistical analysis

The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs, and a $p < 0.05$ was considered significant. All data are presented as the mean ± S.D. of triplicate independent determinations. Each experiment was repeated three times with similar results.

3. Results

3.1. Effects of TGF-β on the phosphorylation of MYPT-1 in MC3T3-E1 cells

Myosin phosphatase targeting subunit (MYPT-1), which is a component of myosin phosphatase, is well known as a down-stream substrate of Rho-kinase [9,18]. In order to clarify whether TGF-β activates Rho-kinase in osteoblast-like MC3T3-E1 cells, we examined the effect of TGF-β on the phosphorylation of MYPT-1. TGF-β markedly elicited the phosphorylation of MYPT-1 in a time-dependent manner (Fig. 1). The effect of TGF-β on the phosphorylation of MYPT-1 reached its maximum at 60 min, sustained up to 100 min, and decreased thereafter (Fig. 1).

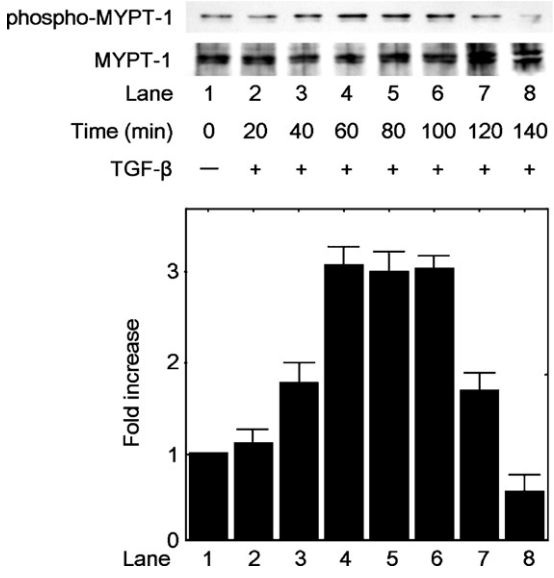

Fig. 1 – Effect of TGF-β on the phosphorylation of MYPT-1 in MC3T3-E1 cells. The cultured cells were stimulated by 3 ng/ml TGF-β for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific MYPT-1 or MYPT-1. The histogram shows quantitative representations of the levels of TGF-β-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.D. of triplicate independent determinations. Similar results were obtained with two additional and different cell preparations. *$p < 0.05$, compared to the value of control.

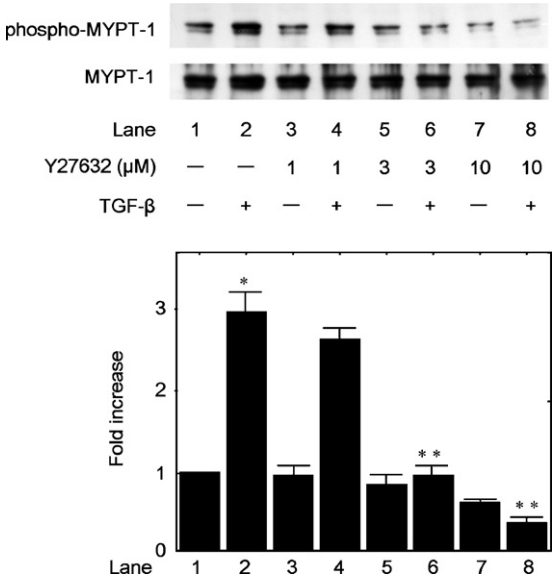

Fig. 2 – Effect of Y27632 on the TGF-β-induced phosphorylation of MYPT-1 in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 for 60 min, and then stimulated by 3 ng/ml TGF-β or vehicle for 60 min. The histogram shows quantitative representations of the levels of TGF-β-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.D. of triplicate independent determinations. Similar results were obtained with two additional and different cell preparations. *$p < 0.05$, compared to the control. **$p < 0.05$, compared to the value of TGF-β alone.

We confirmed that Y27632, a specific inhibitor of Rho-kinase [10], suppressed the TGF-β-induced phosphorylation levels of MYPT-1 in a dose-dependent manner in the range between 1 and 10 μM (Fig. 2). In addition, we found that fasudil, another inhibitor of Rho-kinase [10], attenuated the TGF-β-induced levels of MYPT-1 phosphorylation (data not shown).

3.2. Effects of Y27632 or fasudil on the TGF-β-stimulated VEGF synthesis in MC3T3-E1 cells

We previously showed that TGF-β stimulates VEGF synthesis in osteoblast-like MC3T3-E1 cells [5]. In order to investigate the involvement of Rho-kinase in the TGF-β-induced synthesis of VEGF in MC3T3-E1 cells, we next examined the effect of Y27632 on the synthesis of VEGF induced by TGF-β. Y27632, which by itself had little effect on the VEGF levels, significantly

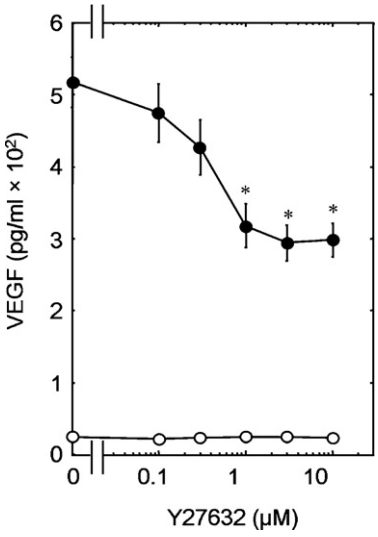

Fig. 3 – Effect of Y27632 on the TGF-β-induced VEGF synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 48 h. Each value represents the mean ± S.D. of triplicate independent determinations. Similar results were obtained with two additional and different cell preparations. *$p < 0.05$, compared to the value of TGF-β alone.

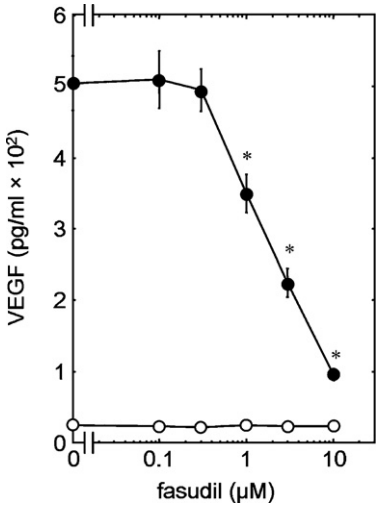

Fig. 4 – Effect of fasudil on the TGF-β-induced VEGF synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of fasudil for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 48 h. Each value represents the mean ± S.D. of triplicate independent determinations. Similar results were obtained with two additional and different cell preparations. *$p < 0.05$, compared to the value of TGF-β alone.

suppressed the TGF-β-induced synthesis of VEGF (Fig. 3). The inhibitory effect of Y27632 was dose-dependent in the range between 0.1 and 10 μM. Y27632 (10 μM) caused approximately 50 % inhibition in the TGF-β-effect.

Fasudil as well as Y27632, which alone failed to affect the VEGF levels, inhibited the TGF-β-stimulated VEGF synthesis in MC3T3-E1 cells (Fig. 4). The effect of fasudil on the VEGF synthesis was dose-dependent in the range between 0.1 and

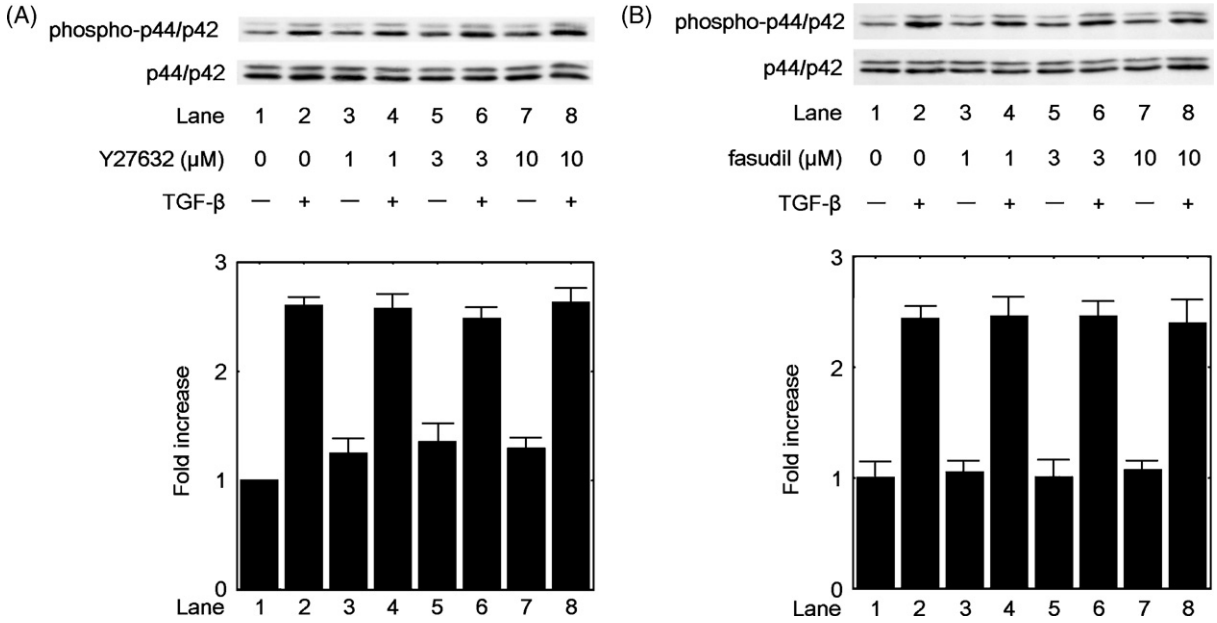

Fig. 5 – Effects of Y27632 or fasudil on the TGF-β-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 (A) or fasudil (B) for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 120 min. The histogram shows quantitative representations of the levels of TGF-β-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.D. of triplicate independent determinations. Similar results were obtained with two additional and different cell preparations.

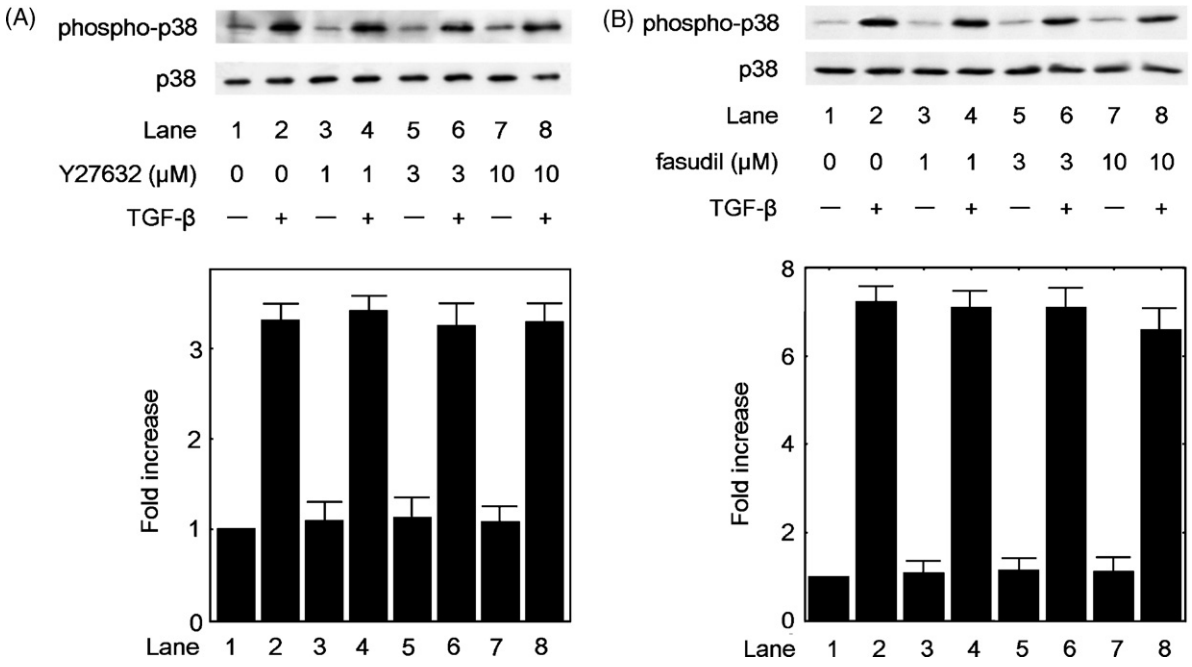

Fig. 6 – Effects of Y27632 or fasudil on the TGF-β-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 (A) or fasudil (B) for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 120 min. The histogram shows quantitative representations of the levels of TGF-β-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.D. of triplicate independent determinations. Similar results were obtained with two additional and different cell preparations.

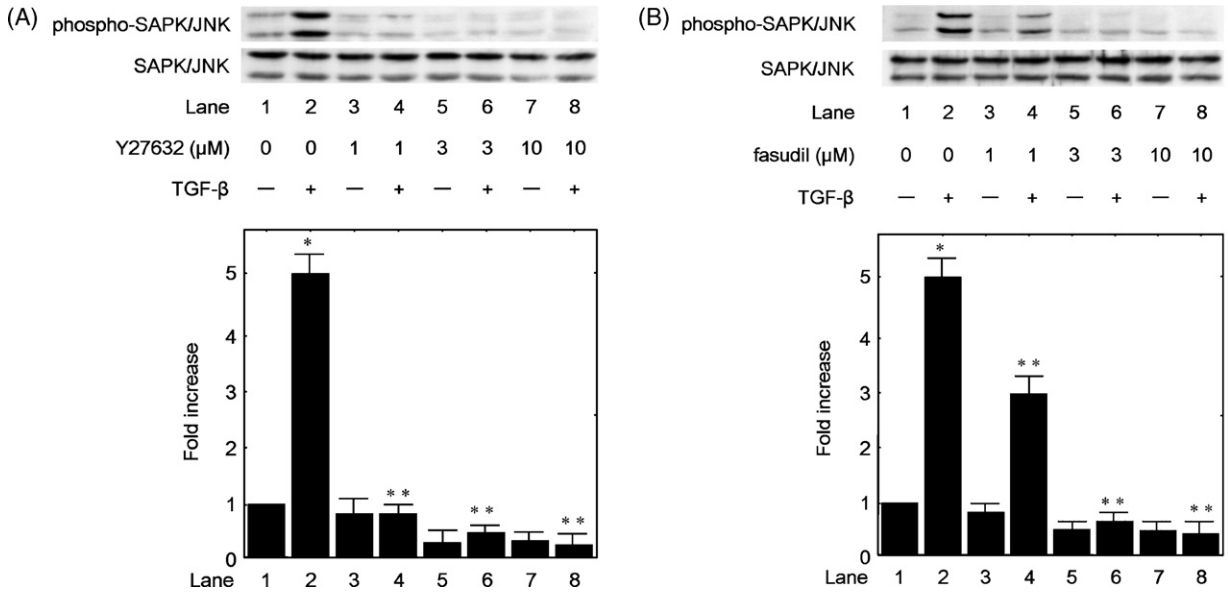

Fig. 7 – Effects of Y27632 or fasudil on the TGF-β-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 (A) or fasudil (B) for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 120 min. The histogram shows quantitative representations of the levels of TGF-β-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.D. of triplicate independent determinations. Similar results were obtained with two additional and different cell preparations. *$p < 0.05$, compared to the control. **$p < 0.05$, compared to the value of TGF-β alone.

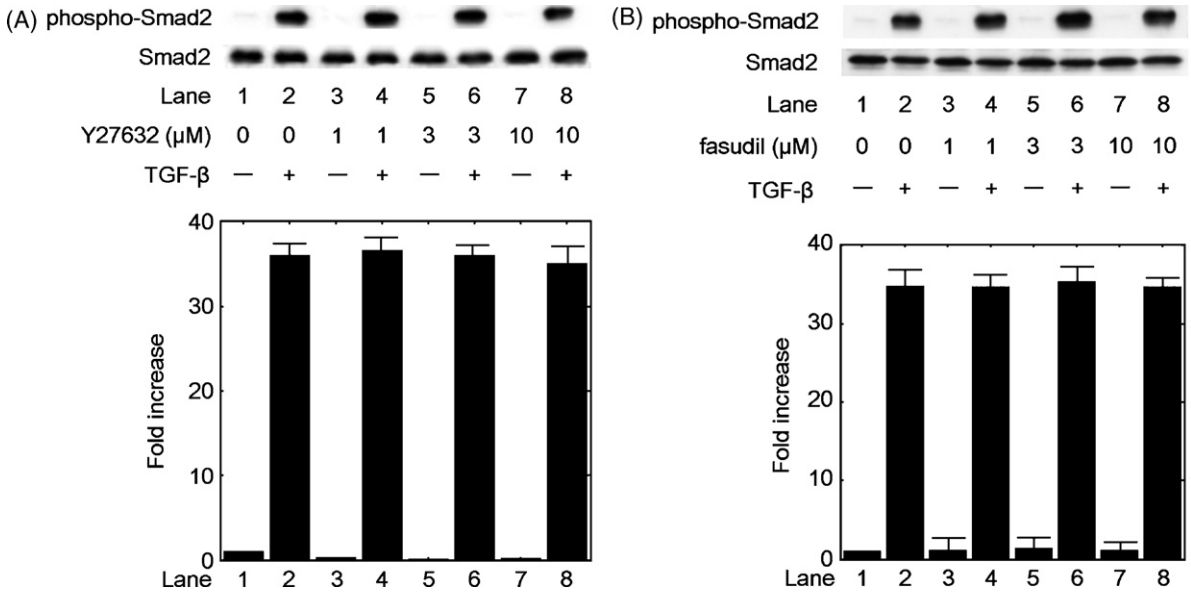

Fig. 8 – Effects of Y27632 or fasudil on the TGF-β-induced phosphorylation of Smad2 in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 (A) or fasudil (B) for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 120 min. The histogram shows quantitative representations of the levels of TGF-β-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.D. of triplicate independent determinations. Similar results were obtained with two additional and different cell preparations.

10 μM. Fasudil (10 μM) caused about 80% inhibitions in the TGF-β-effect. There were not any differences between Y27632 or fasudil-treated cells and control cells in appearance through the experiments.

3.3. *Effects of Y27632 or fasudil on the TGF-β-induced phosphorylation of p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK in MC3T3-E1 cells*

It is generally recognized that three MAP kinases, p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK are known as central elements used by mammalian cells to transduce the various messages of a variety of agonists [7,19]. We have previously reported that TGF-β stimulates the synthesis of VEGF via p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells [5,6]. In order to clarify whether the suppressive effects of Rho-kinase inhibitors on the TGF-β-stimulated VEGF synthesis are dependent on the activation of three MAP kinases in MC3T3-E1 cells, we next examined the effect of Y27632 on the TGF-β-induced phosphorylation of p44/p42 MAP kinase. However, Y27632 did not affect the TGF-β-induced phosphorylation of p44/p42 MAP kinase in the range between 1 and 10 μM (Fig. 5A). Additionally, fasudil had little effect on the phosphorylation levels of p44/p42 MAP kinase (Fig. 5B). Furthermore, the TGF-β-induced phosphorylation of p38 MAP kinase was not suppressed by Y27632 (Fig. 6A) and fasudil in the range between 1 and 10 μM (Fig. 6B).

On the contrary, Y27632 markedly suppressed the TGF-β-induced phosphorylation of SAPK/JNK (Fig. 7A). One micromole of Y27632 elicited almost complete inhibition in the TGF-β-effect. Fasudil as well as Y27632 reduced the TGF-β-induced levels of phosphorylated-SAPK/JNK (Fig. 7B). The inhibitory effect of fasudil was dose-dependent in the range between 1 and 10 μM.

3.4. *Effects of Y27632 or fasudil on the TGF-β-induced phosphorylation of Smad2 in MC3T3-E1 cells*

It is well established that Smads such as Smad2 and Smad3 are principal mediators of intracellular signals from the receptors for TGF-β to the nucleus [20,21]. Therefore, we examined effect of Y27632 on the TGF-β-induced phosphorylation of Smad2 in MC3T3-E1 cells. However, Y27632 failed to affect the TGF-β-induced phosphorylation levels of Smad2 in the range between 1 and 10 μM (Fig. 8A). Fasudil as well as Y27632 had little effect on the TGF-β-induced phosphorylation of Smad2 (Fig. 8B).

4. Discussion

In the present study, we showed that TGF-β time-dependently induced the phosphorylation of MYPT-1 in osteoblast-like MC3T3-E1 cells, using phospho-specific MYPT-1 (Thr850) antibodies. MYPT, a myosin-binding subunit of myosin phosphatase, which regulates the interaction of actin and myosin, is well known to be a downstream target of Rho-kinase [14,23]. Additionally, we found that Y27632 and fasudil, inhibitors of Rho-kinase [16], truly reduced the TGF-β-induced phosphorylation of MYPT-1. Based on these findings, it is most likely that TGF-β elicits the activation of Rho-kinase in osteoblast-like MC3T3-E1 cells.

We next investigated the involvement of Rho-kinase in the TGF-β-stimulated VEGF synthesis in osteoblast-like MC3T3-E1

cells. Y27632, a specific inhibitor of Rho-kinase [16], which alone did not affect the basal levels of VEGF, significantly reduced the TGF-β-stimulated synthesis of VEGF. This finding suggests that the TGF-β-activated Rho-kinase is implicated as a positive regulator in the VEGF synthesis in these cells. In addition, we showed that the VEGF synthesis stimulated by TGF-β was markedly inhibited by fasudil, another inhibitor of Rho-kinase [16]. Therefore, our results suggest that TGF-β stimulates the activation of Rho-kinase in osteoblast-like MC3T3-E1 cells, resulting in up-regulation of VEGF synthesis.

It is currently recognized that TGF-β exerts the effects on a variety of biological functions via Smad-independent signaling in addition to Smad-dependent signaling [20,21]. The MAP kinase superfamily such as p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK function as central elements used by mammalian cells to transduce the various messages [7,19]. With regard to VEGF synthesis in osteoblasts, we have previously reported that the activation of major three MAP kinases such as p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK is involved in the TGF-β-stimulated VEGF synthesis in osteoblast-like MC3T3-E1 cells [5,6]. Thus, we next investigated the relationship between Rho-kinase and p44/p42 MAP kinase in the TGF-β-stimulated VEGF synthesis in these cells. However, Y27632 or fasudil had little effect on the TGF-β-induced phosphorylation levels of p44/p42 MAP kinase. In addition, the TGF-β-induced phosphorylation level of p38 MAP kinase was not influenced by the Rho-kinase inhibitors. Based on these findings, it seems unlikely that Rho-kinase affects the TGF-β-stimulated VEGF synthesis through the modulation of p44/p42 MAP kinase or p38 MAP kinase in osteoblast-like MC3T3-E1 cells. As shown in Fig. 3, the maximum effect of Y27632 on the TGF-β-induced VEGF was observed at 3 μM, but the inhibitory effect was partial. We examined the dose-dependent effect of Y27632 on the TGF-β-induced phosphorylation of MYPT-1, and found that 3 μM Y27632 significantly reduced the TGF-β-induced phosphorylation of MYPT-1 without inhibiting the basal levels of MYPT-1 phosphorylation (Fig. 2). In addition, we have recently reported that fasudil at a dose up to 10 μM hardly affected the basal levels of MYPT-1 phosphorylation in MC3T3-E1 cells [22]. It has been reported that Y27632 also inhibits other kinases like PKCδ with a similar potency to that for Rho-kinase [23,24]. It is possible that some differences between Y27632 and fasudil about the selectivity might be existed in these cells.

Next, we tried to elucidate the relationship between Rho-kinase and SAPK/JNK in the TGF-β-stimulated VEGF synthesis in MC3T3-E1 cells. The TGF-β-induced phosphorylation level of SAPK/JNK was markedly suppressed by Y27632. Fasudil as well as Y27632 significantly reduced the phosphorylation levels. Therefore, it is probable that Rho-kinase regulates the TGF-β-stimulated VEGF synthesis via SAPK/JNK. On the other hand, the TGF-β-induced phosphorylation of Smad2 was not affected by the Rho-kinase inhibitors, Y27632 and fasudil. Thus, it seems unlikely that Rho-kinase regulates the TGF-β-stimulated VEGF synthesis via activation of Smads in these cells. Taking our findings into account as a whole, our results strongly suggest that Rho-kinase acts at a point upstream from SAPK/JNK among the MAP kinase superfamily in the TGF-β-stimulated VEGF synthesis in osteoblast-like MC3T3-E1 cells.

It is currently recognized that Rho-kinase plays an important role in a variety of cellular functions, especially vascular smooth muscle contraction [8–10]. In bone metabolism, the activation of Rho-kinase reportedly suppresses the differentiation of osteoblasts and induces their proliferation [12]. Our present results show that the Rho-kinase stimulated by TGF-β in osteoblasts acts as positive regulator in the synthesis of VEGF. VEGF produced by osteoblasts is a potent regulator of bone growth and repair, which provide the microvasculature via capillary endothelium [3,4]. Capillary network-providing microvasculature is an essential process in bone remodeling [3]. In addition, it is well known that TGF-β is synthesized in osteoblasts, stored abundantly in bone matrix in the latent form, and activated in the bone microenvironment [25]. During bone resorption, TGF-β is released and stimulates the recruitment and proliferation of osteoblasts. Therefore, our present findings lead us to speculate that TGF-β-induced VEGF acts as a positive regulator of bone remodeling via the activation of Rho-kinase in osteoblasts. In addition, the findings that not p44/p42 MAP kinase or p38 MAP kinase but SAPK/JNK is solely regulated by Rho-kinase, might suggest the importance of the fine tuning of these MAP kinase-mediating VEGF synthesis induced by TGF-β in bone remodeling. However, the exact role of Rho-kinase in osteoblasts is not precisely known. Further investigations including another osteoblast population would be necessary to elucidate the exact roles of Rho-kinase in bone metabolism.

In conclusion, our results strongly suggest that Rho-kinase inhibitors decrease the TGF-β-stimulated VEGF synthesis via suppression of SAPK/JNK in osteoblasts.

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