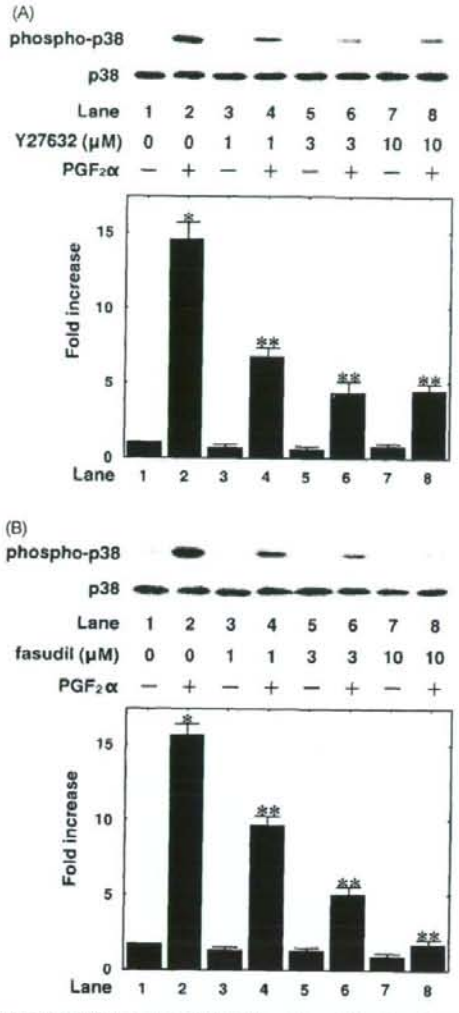

Fig. 7. Effects of Y27632 or fasudil on the $PGF_{2\alpha}$-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 (A) or fasudil (B) for 60 min, and then stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 10 min. Twenty micrograms of cytosolic protein were subjected to SDS-PAGE with subsequent Western blot analysis with antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. The histogram shows quantitative representations of the levels of $PGF_{2\alpha}$-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. Values represent the amount of phospho-p38 MAP kinase divided by those of total p38 MAP kinase. $^{*}p < 0.05$, compared to the control (lanes 1). $^{**}p < 0.05$, compared to the value of $PGF_{2\alpha}$ alone (lanes 2).

MC3T3-E1 cells, using phospho-specific MYPT-1 (Thr850) antibodies. MYPT, which is a myosin-binding subunit of myosin phosphatase and regulates the interaction of actin and myosin, is well known to be a downstream target of Rho-kinase (Fukata et al., 2001; Ito et al., 2004). Additionally, we next demonstrated that Y27632 and fasudil, inhibitors of Rho-kinase (Shimokawa and Rashid, 2007), truly suppressed the $PGF_{2\alpha}$-induced phosphorylation of MYPT-1. Y27632 also reduced the basal MYPT-1 phosphorylation, whereas fasudil did not suppress it. It has been reported that Y27632 also inhibits other kinases like PKCδ with a similar potency to that for Rho kinase 2 (Davies et al., 2000; Eto et al., 2001). The discrepancy between the effects of Y27632 and fasudil on basal phosphorylation of MYPT-1 might be due to the unselectivity of Y27632. Therefore, our results indicate that $PGF_{2\alpha}$ elicits the activation of Rho-kinase in osteoblast-like MC3T3-E1 cells.

We next investigated the involvement of Rho-kinase in the $PGF_{2\alpha}$-stimulated IL-6 synthesis or not in osteoblast-like MC3T3-E1 cells. Y27632 (Shimokawa and Rashid, 2007) significantly reduced the $PGF_{2\alpha}$-stimulated synthesis of IL-6. This finding suggests that the $PGF_{2\alpha}$-activated Rho-kinase functions as a positive regulator in the IL-6 synthesis in these cells. In addition, we showed that the IL-6 synthesis stimulated by $PGF_{2\alpha}$ was inhibited by fasudil (Shimokawa and Rashid, 2007) as well as Y27632. Therefore, taking our results into account, it is most likely that $PGF_{2\alpha}$ activates Rho-kinase, resulting in up-regulation of IL-6 synthesis in osteoblast-like MC3T3-E1 cells.

With regard to IL-6 synthesis in osteoblasts, we have previously reported that the activation of p44/p42 MAP kinase is involved in the $PGF_{2\alpha}$-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells (Tokuda et al., 1999). Additionally, we investigated the relationship between Rho-kinase and p44/p42 MAP kinase in the $PGF_{2\alpha}$-stimulated IL-6 synthesis in these cells. However, Y27632 or fasudil had little effect on the $PGF_{2\alpha}$-induced phosphorylation levels of p44/p42 MAP kinase. Based on our findings, it seems unlikely that Rho-kinase affects the $PGF_{2\alpha}$-stimulated IL-6 synthesis through the modulation of p44/p42 MAP kinase in osteoblast-like MC3T3-E1 cells. It is generally recognized that SAPK/JNK and p38 MAP kinase in addition to p44/p42 MAP kinase are known as central elements used by mammalian cells to transduce the various messages (Widmann et al., 1999). We have shown that $PGF_{2\alpha}$ stimulates the activation of SAPK/JNK and p38 MAP kinase in MC3T3-E1 cells (Tokuda et al., 2007). In the present study, the IL-6 synthesis induced by $PGF_{2\alpha}$ was suppressed by SB203580, a specific inhibitor of p38 MAP kinase (Cuenda et al., 1995) but not SP600125, a specific inhibitor of SAPK/JNK (Bennett et al., 2001). It has been reported that SB203580 inhibits other than p38 MAP kinase protein kinases with similar or even greater (receptor-interacting protein 2, RIP2) potency (Godl et al., 2003). As RIP2-mediated NF-κB activation reportedly leads to increase IL-6 (Sarkar et al., 2006), it is possible that SB203580-induced suppression of IL-6 synthesis is due to RIP2 blocking by SB203580. Thus, we further examined the effect of BIRB0796 (Bain et al., 2007) on the $PGF_{2\alpha}$-stimulated IL-6 synthesis in MC3T3-E1 cells, and found that BIRB0796 significantly suppressed the $PGF_{2\alpha}$-stimulated IL-6 synthesis. These results strongly suggest that p38 MAP kinase as well as p44/p42 MAP kinase plays a part in the $PGF_{2\alpha}$-stimulated IL-6 synthesis. Next, we tried to elucidate the relationship between Rho-kinase and p38 MAP kinase in the $PGF_{2\alpha}$-stimulated IL-6 synthesis in these cells. The $PGF_{2\alpha}$-induced phosphorylation levels of p38 MAP kinase were markedly suppressed by Y27632 and fasudil. Therefore, it is probable that Rho-kinase regulates the $PGF_{2\alpha}$-stimulated IL-6 synthesis via p38 MAP kinase. In the present study, the maximum effect on the phosphorylation of MYPT-1, a well-known downstream target of Rho-kinase (Fukata et al., 2001), was observed within 2 min after the $PGF_{2\alpha}$ stimulation. In our previous study (Tokuda et al., 2007), we have shown that the phosphorylation of p38 MAP kinase reach the peak at 10 min after the stimulation of $PGF_{2\alpha}$ in MC3T3-E1 cells. The time course of the $PGF_{2\alpha}$-induced phosphorylation of MYPT-1 seems to be faster than that of p38 MAP kinase. Therefore, it seems reasonable that $PGF_{2\alpha}$-induced activation of p38 MAP kinase subsequently occurs after the Rho-kinase activation. Taking our findings into account as a whole, our results strongly suggest that Rho-kinase acts at a point upstream from p38 MAP kinase in the $PGF_{2\alpha}$-stimulated IL-6 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 (Fukata et al., 2001; Riento and Ridley, 2003; Shimokawa and Rashid, 2007). In bone metabolism, it has been reported that the activation of Rho-kinase suppresses the differentiation of osteoblasts and induces their proliferation (Harmey et al., 2004). Our present results suggest that Rho-kinase in osteoblasts acts as positive regulator in the synthesis of IL-6, one of the central modulators of bone metabolism. It is well known that IL-6 produced by osteoblasts is a potent bone resorptive agent and induces osteoclast formation (Ishimi et al., 1990; Kwan Tat et al., 2004). In addition, it is recognized that $PGF_{2\alpha}$, an inducer of IL-6, acts as a bone resorptive agent in bone metabolism (Nijweide et al., 1986; Pilbeam et al., 1996). During the fracture healing, once mineralized cartilage resorption is completed and woven bone formation has occurred, a subsequent phase of bone resorption takes place in which IL-6 increases in their expression (Louis and Einhorn, 2006). Therefore, our present findings lead us to speculate that $PGF_{2\alpha}$-activated Rho-kinase in osteoblasts functions as a positive regulator of bone resorption via the fine-tuning of the local cytokine network, such as induction of IL-6. The Rho-kinase pathway in osteoblasts might be a new candidate as a molecular therapeutic target of fracture healing, bone formation and bone metabolic disease such as osteoporosis. The exact mechanism of Rho-kinase activation in osteoblasts is still not precisely known. Further investigations are required to elucidate the exact role of Rho-kinase in bone metabolism.

Taken together, our results strongly suggest that Rho-kinase regulates the $PGF_{2\alpha}$-stimulated IL-6 synthesis via p38 MAP kinase in osteoblasts.

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INTERNAL MEDICINE

□ CASE REPORT □

Raloxifene-Induced Acceleration of Platelet Aggregation

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Abstract

A 59-year-old postmenopausal woman diagnosed to have primary osteoporosis began to take 60 mg daily of oral raloxifene. The platelet aggregation induced by 1 μM adenosine diphosphate (ADP) and the α2-antiplasmin activity were accelerated significantly after 8 weeks from the beginning of raloxifene-treatment, and gradually deteriorated up to 24 weeks. ADP markedly caused the phosphorylation of Akt in the platelets obtained at 24 weeks. Although there were no subjective complaints at 24 weeks, the medication was stopped with her consent to avoid any adverse effects due to thrombus formation. The platelet hyper-aggregability and Akt phosphorylation induced by ADP disappeared at 4 weeks after the cessation of medication. These results strongly suggest that raloxifene caused the acceleration of platelet aggregation and subclinical thrombus formation through the Akt signal pathway in this case.

Key words: raloxifene, osteoporosis, Akt, platelet aggregation

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Introduction

Raloxifene, a selective estrogen receptor modulator (SERM), has been well recognized to be an efficient therapeutic agent for postmenopausal osteoporosis (1). Raloxifene reportedly increases the vertebra and femoral neck bone mineral density, and reduces the risk of vertebral fracture (1). In addition, raloxifene has been reported to significantly reduce the incidence of breast cancer (1). Raloxifene is thought to be a preferable medicine for postmenopausal women. However, the use of this drug as well as estrogen is associated with an increased risk of developing venous thromboembolus (2). Platelet aggregation and the coagulation system are important for the formation of the thrombus. Although significant attention has been paid to the coagulation system, the influence of raloxifene on platelet aggregation is not known.

Platelet aggregation is usually measured using either the optical density (OD) method (3) or the impedance method (4), both of which are indispensable for the clinical evaluation of platelet function. However, these methods provide little information about subtle but crucial changes in the number of platelet microaggregates in response to small changes in the aggregating stimuli or the proaggregatory status. The light-scattering (LS) method, which has primarily been used for experimental research, provides a tool with a greater sensitivity for detecting microaggregates of platelets than the conventional light transmittance method (4). A particle-counting method that employs LS has recently been developed (5), thus allowing identification of particle size in terms of light intensity and minimizing the interference by neighboring platelets, which may attenuate the high intensity light scattered by larger particles (6). Recently, an important role of platelet microaggregation in the circulation and a significant alteration of platelet microaggregation in pathogenesis were reported by the creation of a laser LS system for microaggregates (7-9). Therefore, an LS system was employed for the detection of microaggregates of platelets in our clinical study (10).

Adenosine diphosphate (ADP) is considered to be a weak physiological agonist for platelet aggregation by itself in comparison, for example, to thrombin or collagen (11). However, ADP is a necessary cofactor for normal activation

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of platelets by other agonists. Even if it is weak agonist for platelet aggregation, low-concentration ADP amplifies the effects (12). A recent investigation revealed that purinoreceptor P2Y12 is a principal receptor for mediating ADP-induced platelet aggregation and the thromboenbolism (13, 14). Phosphatidylinositol 3-kinase (PI3K) effectors, such as the serine/threonine kinase Akt, play important roles in platelet aggregation as an intracellular signal system downstream of the P2Y12 receptor (14). The current report describes the acceleration of low-dose ADP-induced platelet aggregation with the increasing Akt activation in a patient who was treated with a SERM, raloxifene.

Case Report

A 59-year-old postmenopausal woman, who had suffered from intermittent lumbago for several years, was referred to the outpatient service for osteoporosis. X-ray examination showed a vertebral fracture on T-12. Bone marrow depression (BMD) determined by dual-energy X-ray absorptiometry (QDR-2000) was: lumbar spine 0.803 g/cm^2 (T-score, -2.6); total femur 0.598 g/cm^2 (T-score, -2.8; Table 1). The blood analysis appeared to be in normal range except the elevation of alkaline phosphatase (ALP) and bone type ALP (Table 2). According to the diagnostic criteria for primary osteoporosis in Japan (15), she was diagnosed to have primary osteoporosis with a high turnover bone metabolism, and then was administered 60 mg daily of oral raloxifene. She agreed to undergo an assessment of her platelet function before and after the treatment, and signed an informed consent agreement as approved by the local institutional review board after receiving a detailed explanation of the protocol. Platelet-rich plasma (PRP) was chronologically obtained from freshly drawn venous blood samples. Platelet aggregation was assessed by PA-200 aggregometer (Kowa, Tokyo, Japan), which can determine the size of platelet aggregates based upon particle counting using light scatter (13). The platelets were preincubated for 2 minutes, then platelet aggregation was monitored for 5 minutes after the addition of various doses of ADP (0.3-30 μM). The percentage of transmittance of the isolated platelets was recorded as 0%, and that of the appropriate platelet-free plasma (blank) was recorded as 100%. ADP concentration that caused 50% of transmittance of the isolated platelets was considered as ED 50. Just after evaluating platelet aggregation, the remaining PRP was sufficiently washed with phosphate buffered saline, separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and a Western blot analysis was performed using antibodies against phospho-specific Akt (Thr308) to detect the activated form and with Akt antibodies for determination of the total amount, as previously described (16).

Figures 1, 2 show that the ED50 of ADP for the platelet aggregation gradually decreased after treatment with raloxifene. After 8 weeks after the initiation of treatment, less than 1 μM ADP caused the significant acceleration of the platelet aggregation, and it deteriorated up to 24 weeks. Considering these results, although she did not show any symptoms of thrombosis, the administration of raloxifene was terminated to avoid major clinical problems. Four weeks after the cessation of raloxifene, the level of the platelet aggregation was markedly improved to the level before the raloxifene-treatment. According to the analysis of the size of platelet aggregates, a decrease of small aggregates (9-25 μm) and the increase of large aggregates (50-70 μm) were observed after 2 weeks, and an increase of medium aggregates (25-50 μm) was subsequently observed after 4 weeks (Fig. 2), suggesting that the pathological change of platelet function occurred within at least 2 weeks of the raloxifene treatment. In addition, ADP (1 μM) markedly caused the phosphorylation of Akt in the platelets obtained at 24 weeks, but the ADP-induced Akt phosphorylation disappeared in the platelets obtained at 4 weeks after the cessation of raloxifene (Fig. 3).

It is well known that a lack of α2-antiplasmin (α2AP), a major component of fibrinolytic system, causes an excess of plasmin, and that plasmin plays an important role in the formation of platelet aggregates. Consequently, the reduction of α2AP could be a risk factor for the activation of platelets resulting in thrombus formation (9). The α2AP activity was examined in this case to determine the effect of raloxifene treatment on the fibrinolytic system. The α2AP activity gradually increased from the beginning of raloxifene-treatment, and it was sustained up to 4 weeks after the cessation of the medication (Fig. 4).

Table 1. Clinical Feature

Age	59 years
Sex	female
Height	152.9 cm
Weight	56.4 kg
Body mass index	24.12 kg/m^2
Lumber spine L2-L4 BMD	0.803 g/cm^2
YAM	72%

Footnote: YAM; young adult mean

Discussion

This report presents a case of primary osteoporosis showing platelet hyper-aggregability to ADP, closely related to the administration of the standard dose of raloxifene. This is probably the first report clearly showing raloxifene-induced unusual platelet aggregation, which was detectable using the LS method. Interestingly, the hyper-sensitivity was correlated with the enhancement of Akt phosphorylation induced by ADP in platelets. The platelets play an important role in normal homeostasis and abnormal activation of platelets thus leading to thrombosis. During vascular injury or conditions of high shear, exposure of the collagen-rich subendothelium

Table 2. Laboratory Data

Parameters		normal range
Peripheral Blood		
WBC	4600/μL	3500-9200/μL
RBC	431 x 10^4/μL	388-488 x 10^4/μL
Hb	13.3 g/dL	11.3-15.5 g/dL
Hct	39.9%	34.4-45.6%
Plt	16.4 x 10^4/μL	15.5-36.5 x 10^4/μL
Blood Chemistry		
Total protein	7.7 g/dL	6.3-8.1 g/dL
Albumin	4.7 g/dL	3.7-4.9 g/dL
AST	24 IU/L	9-38 IU/L
ALT	33 IU/L	4-36 IU/L
ALP	361 IU/L	60-201 IU/L
Urinary nitrogen	13 mg/dL	9-21 mg/dL
Creatinine	0.5 mg/dL	0.4-0.9 mg/dL
Serum calcium	9.5 mg/dL	8.4-9.7 mg/dL
Serum iP	3.5 mg/dL	2.5-4.5 mg/dL
Endocrinology		
free T_3	2.79 pg/mL	2.84-4.45 pg/mL
free T_4	1.19 pg/mL	0.82-1.72 pg/mL
TSH	0.73 mIU/mL	0.49-3.83 mIU/mL
Cortisol	14 ng/mL	7-25 ng/mL
High sensitivity PTH	212 pg/mL	90-270 pg/mL
Bone Metabolic Markers		
BAP	46.4 IU/L	7.9-29.0 IU/L
Serum NTx	13.3 nmolBCE/L	10.7-24.0 nmolBCE/L

Footnote: RBC: Red blood cell, WBC: White blood cell, Hb: Hemoglobin, Hct: Hematocrit, Plt: Platelet, AST: L-asparartate aminotransferase, ALT: L-alanine aminotransferase, ALP: Alkaline phosphatase, free T_3: free tri-iodothyronine, free T_4: free thyroxin, TSH: thyroid-stimulating hormone, PTH: parathyroid hormone, BAP: bone type alkaline phosphatase, Serum NTx: Serum cross-linked N-telopeptides of I type collagen.

activates the platelets and results in the formation of a stable thrombus due to the combined action of ADP secreted from platelet-dense granules and generated thrombin (14, 17). The present results suggest that raloxifene could induce hyperreactivity of platelet aggregation by increased sensitivity to ADP stimulation and by Akt activation. The hypersensitivity of platelets shown here might lead us to pay attention to the risk of arterial thrombosis, in addition to the increased hazard ratio of venous thromboembolism, under standard raloxifene treatment for osteoporosis.

Prior studies have shown that PI3K is a necessary component which plays a pivotal role in the signaling of purinoreceptor P2Y12 in platelets (14, 18). A potential downstream effector for PI3K is Akt, which contributes to fibrinogen receptor activation and platelet aggregation. In addition, PI3K/Akt signaling is also reportedly involved in the release of ADP-containing granules (18). The platelet-aggregated form consists of two types of platelet membrane receptors, glycoprotein (GP) IIb/IIIa and GPIb/V/IX. GPIIb/IIIa not only binds fibrinogen and von Willebrand factor (vWF) to mediate platelet aggregation and adhesion, but it also serves as a signaling receptor. ADP induces the signaling, which activates the receptor function of GPIIb/IIIa for soluble fibrinogen, and ADP-induced platelet aggregation promotes the

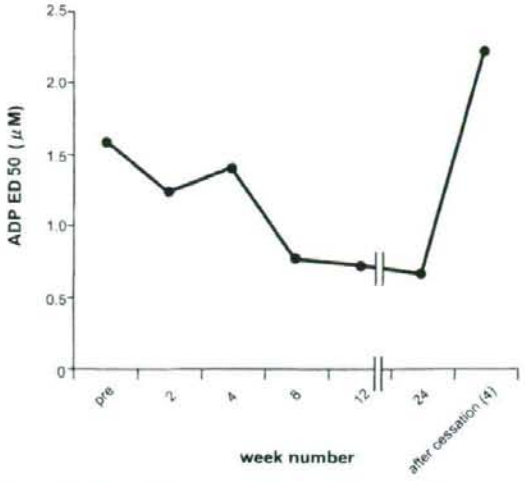

Figure 1. The change in ED50 for ADP-induced platelet aggregation. PRP samples were obtained during the treatment and 4 weeks after the cessation of raloxifene administration. PRP was chronologically obtained from freshly drawn venous blood samples. The ED50 for ADP was determined.

binding of fibrinogen to GPIIb/IIIa (19). The P2Y12 receptor is involved in the constitution of stable macroaggregates (irreversible change) through full activation of the GPIIb/IIIa (13, 20). In the present case, raloxifene probably increased the platelet sensitivity to ADP and induced the enhancement of Akt activity in platelets, thus suggesting that the up-regulation in signaling events of P2Y12 receptor occurred under raloxifene administration. Blockade of P2Y12 receptor signaling might be a possible therapeutic strategy to avoid the adverse effects of this useful agent. The accumulation of more such cases and further investigations will thus be required to clarify the above mechanism of action in detail.

On the other hand, an increase of α2AP activity was also observed in this case. Plasmin plays a crucial role in the formation of platelet aggregation. It is generally recognized that increase of α2AP, which is produced by the liver, causes the reduction of plasmin, resulting in the inhibition of fibrinolytic system. Thus, it seems that an inhibition of the fibrinolytic system occurred in parallel with the increase of platelet aggregation under the treatment with raloxifene. As plasmin is known to play an important role in the formation of platelet aggregation, it is possible that the increase of α2AP activity resulting in the reduction of plasmin in this case is a patho-physiological defensive mechanism against the raloxifene-induced platelet hyper-aggregability. In addition, the inhibition of fibrynolytic system might reduce the capacity of thrombolysis, resulting in the thrombus formation. In the present case, raloxifene treatment was continued for 6 months and the increased activity of α2AP sustained after the cessation of raloxifene. It seems that the 4 weeks cessation of raloxifen after 6 months administration is not sufficient for the recovery of fibrynolytic system. The monitoring of α2AP, in addition to the platelet microaggregates by a LS system, is probably a useful tool for the detection of the subclinical thrombus formation in the clinical use of raloxifene.

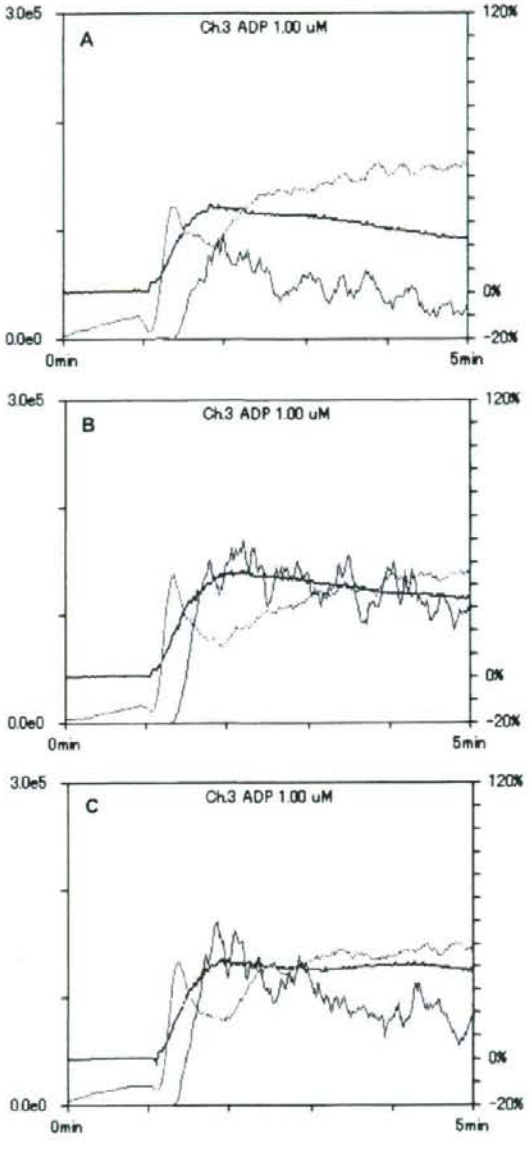

Figure 2. Platelet aggregability after the addition of ADP measured with the laser-light scattering method. Blue line indicates small aggregates; green line, medium aggregates; red line, large aggregates; and black line, optical density (%T). PRP samples were obtained before (A), after 2 weeks (B) and after 4 weeks (C) of raloxifene administration, and the ADP (1 μM)-induced platelet aggregation was determined using a PA-200 aggregometer.

In conclusion, a standard dosage of raloxifene, one type

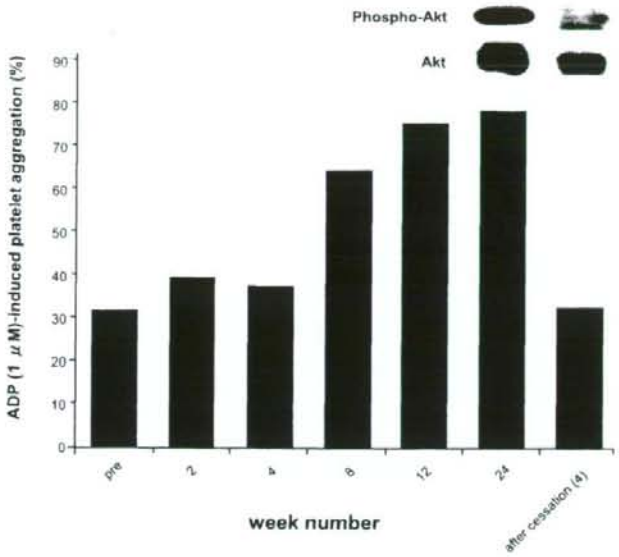

Figure 3. The change in ADP-induced platelet aggregation and the phosphorylation of Akt in the platelets under the treatment of raloxifene. Under the treatment of raloxifene and 4 weeks after the cessation, PRP sample were obtained at the indicated time. The platelet aggregation (%) induced by ADP (1 μM) was determined using a PA-200 aggregometer, and then with the same samples, the phosphorylation of Akt induced by ADP (1 μM) was examined at 24 weeks and 4 weeks after the cessation of the treatment. The platelet extracts were separated by SDS-PAGE and a Western blot analysis was performed with antibodies against either phospho-specific Akt or Akt.

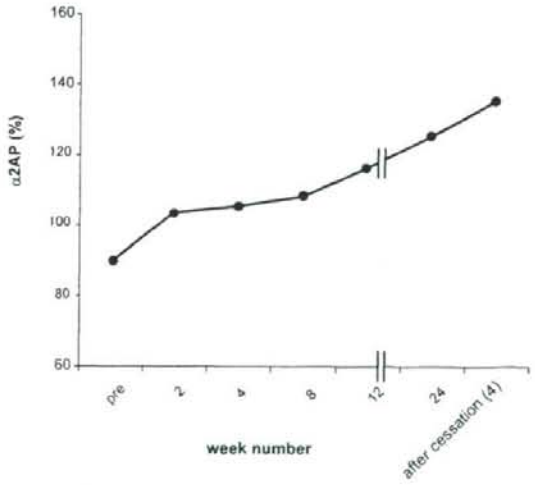

Figure 4. The change in the α2-antiplasmin (α2AP) activity. Under the treatment of raloxifene and 4 weeks after the cessation, PRP samples were obtained at the indicated week. PRP was chronologically obtained from freshly drawn venous blood samples.

of SERM, was found to induce platelet hyper-aggregability during the conventional therapeutic management for postmenopausal osteoporosis. The clinicians using SERM must be aware of the acceleration of platelet aggregation in addition to that of coagulation which may occur during the administration of this regimen.

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Function of Rho-kinase in prostaglandin D_2-induced interleukin-6 synthesis in osteoblasts

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ABSTRACT

We have previously reported that prostaglandin D_2 (PGD_2) stimulates interleukin-6 (IL-6), a potent bone resorptive agent, in osteoblast-like MC3T3-E1 cells. In the present study, we investigated whether Rho-kinase is implicated in the PGD_2-stimulated IL-6 synthesis in MC3T3-E1 cells. PGD_2 time-dependently induced the phosphorylation of myosin phosphatase targeting subunit (MYPT-1), a Rho-kinase substrate. Y27632, a specific Rho-kinase inhibitor, significantly reduced the PGD_2-stimulated IL-6 synthesis as well as the MYPT-1 phosphorylation. Fasudil, another inhibitor of Rho-kinase, suppressed the PGD_2-stimulated IL-6 synthesis. The PGD_2-stimulated IL-6 synthesis was reduced by PD98059, a MEK inhibitor, and SB203580, an inhibitor of p38 mitogen-activated protein (MAP) kinase, but not SP600125, an inhibitor of stress-activated protein kinase/*c-Jun N*-terminal kinase (SAPK/JNK). However, Y27632 and fasudil failed to affect the PGD_2-induced phosphorylation of p44/p42 MAP kinase. On the other hand, Y27632 as well as fasudil markedly attenuated the PGD_2-induced phosphorylation of p38 MAP kinase. In addition, PGD_2 additively induced IL-6 synthesis in combination with endothelin-1 which induces IL-6 synthesis through p38 MAP kinase regulated by Rho-kinase. These results strongly suggest that Rho-kinase regulates PGD_2-stimulated IL-6 synthesis via p38 MAP kinase activation in osteoblasts.

1. Introduction

It is well recognized that interleukin-6 (IL-6) is a multifunctional cytokine that has crucial effects on a wide range of functions such as promoting B-cell differentiation, T-cell activation and inducing acute phase proteins [1–3]. Bone homeostasis is maintained by the coordinated actions of osteoblast-mediated formation and osteoclast-mediated bone removal [4]. In the process of bone remodeling, IL-6 has been shown to stimulate bone resorption and promote osteoclast formation [2,3,5,6]. It has been reported that potent bone resorptive agents such as tumor-necrosis factor-α and interleukin-1 stimulate IL-6 synthesis in osteoblasts [5,7,8]. Currently, evidence is accumulating that IL-6 secreted from osteoblasts plays a pivotal role as a local downstream effector of bone resorptive agents in bone remodeling.

It is well known that prostaglandins act as local factors, autacoids, of osteoblasts [4,9]. Among them, prostaglandin D_2 (PGD_2) is recognized to be implicated in the control of osteoblast function and bone anabolism [9]. It has been reported that PGD_2 stimulates collagen synthesis during calcification of osteoblasts [10]. In addition, PGD_2 produced by osteoblasts reportedly modulates expression of osteoprotegerin and RANKL in osteoblasts [11]. In our previous study [12], we reported that PGD_2 stimulates IL-6 synthesis via Ca^{2+} mobilization in osteoblast-like MC3T3-E1 cells. However, the exact mechanism behind PGD_2-stimulated IL-6 synthesis in osteoblasts remains to be clarified.

Accumulating evidence suggests that Rho and the downstream effector, Rho-associated kinase (Rho-kinase) play crucial roles in a variety of cellular functions such as cell motility and smooth muscle contraction [13–15]. As for osteoblasts, it has been demonstrated that Rho and p38 mitogen-activated protein (MAP) kinase are involved in the endothelin-1 (ET-1)-induced expression of prostaglandin endoperoxide G/H synthase mRNA in osteoblasts [16]. In addition, it has been shown that the Rho/Rho-kinase pathway stimulates osteoblast proliferation whereas it inhibits osteoblast differentiation [17]. We have recently reported that Rho-kinase regulates ET-1-stimulated IL-6 synthesis through p38 MAP kinase

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in osteoblast-like MC3T3-E1 cells [18]. However, the exact role of Rho-kinase in osteoblasts has not yet been fully clarified.

In the present study, we further investigated the exact mechanism behind PGD_2-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells. We here show that Rho-kinase regulates PGD_2-stimulated IL-6 synthesis through p38 MAP kinase activation in these cells.

2. Materials and methods

2.1. Materials

PGD_2, ET-1 and mouse IL-6 enzyme immunoassay (ELISA) kit were purchased from R&D Systems, Inc. (Minneapolis, MN). Y27632, an inhibitor of Rho-kinase [15], PD98059, an inhibitor of MEK [19], SB203580, an inhibitor of p38 MAP kinase [20], and SP600125, an inhibitor of stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) [21], were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Hydroxyfasudil (fasudil), another inhibitor of Rho-kinase [15], was purchased from Sigma (St. Louis, MO). Phospho-specific myosin phosphatase targeting subunit (MYPT-1) antibodies were purchased from Upstate (Lake Placid, NY). MYPT-1 antibodies were obtained form Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies and p38 MAP kinase 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, PD98059, SB203580 and SP600125 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect the assay for IL-6 or Western blot analysis.

2.2. Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [22] were maintained as previously described [23]. 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 or 90-mm 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. IL-6 assay

The cultured cells were stimulated by 10 μM PGD_2, 0.1 μM ET-1, their combination or vehicle in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pretreated with various doses of Y27632, fasudil, PD98059, SB203580 or SP600125 for 60 min. The conditioned medium was collected at the end of the incubation, and the IL-6 concentration was measured by ELISA kit.

2.4. Western blot analysis

The cultured cells were pretreated with various doses of Y27632 or fasudil for 60 min, and then stimulated by PGD_2 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, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125,000g for 10 min at 4 °C. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by Laemmli [24] in 10% polyacrylamide gel. Western blotting analysis was performed as described previously [25] by using 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 or p38 MAP kinase antibodies with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. Peroxidase activity on the PVDF sheet 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 band on 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.E.M. of triplicate independent experiments. Each experiment was repeated three times with similar results.

3. Results

3.1. Effect of PGD_2 on the phosphorylation of MYPT-1 in MC3T3-E1 cells

It is well accepted that MYPT-1, which is a component of myosin phosphatase, is a downstream substrate of Rho-kinase [13,26]. In order to clarify whether PGD_2 activates Rho-kinase in osteoblast-like MC3T3-E1 cells, we examined the effect of PGD_2 on the phosphorylation of MYPT-1. PGD_2 markedly induced the phosphorylation of MYPT-1 in a time-dependent manner (Fig. 1A). The effect of PGD_2 on the phosphorylation of MYPT-1 reached its peak within 2 min and decreased thereafter (Fig. 1A).

Y27632, a specific inhibitor of Rho-kinase [15], suppressed the PGD_2-induced phosphorylation levels of MYPT-1 (Fig. 1B). In addition, fasudil, another inhibitor of Rho-kinase [15], reduced the PGD_2-induced levels of phosphorylated MYPT-1 (data not shown).

3.2. Effects of Y27632 or fasudil on the PGD_2-stimulated IL-6 synthesis in MC3T3-E1 cells

We previously showed that PGD_2 stimulates IL-6 synthesis in osteoblast-like MC3T3-E1 cells [12]. In order to investigate whether Rho-kinase is implicated in the PGD_2-induced synthesis of IL-6 in MC3T3-E1 cells, we next examined the effect of Y27632 on the synthesis of IL-6 induced by PGD_2. Y27632, which by itself had little effect on the IL-6 levels, significantly suppressed the PGD_2-induced synthesis of IL-6 (Fig. 2A). The inhibitory effect of Y27632 was dose dependent in the range between 0.1 and 10 μM. Y27632 (10 μM) caused approximately 80% inhibition in the PGD_2-effect.

In addition, fasudil as well as Y27632, which by itself had little effect on IL-6 synthesis, reduced the PGD_2-stimulated IL-6 synthesis in these cells (Fig. 2B). The effect of fasudil on the IL-6 synthesis was dose dependent in the range between 0.1 and

10 μM. Fasudil (10 μM) caused about 80% suppression in the PGD_2-effect.

3.3. Effect of a combination of PGD_2 and ET-1 on IL-6 synthesis in MC3T3-E1 cells

We have recently reported that Rho-kinase regulates ET-1-stimulated IL-6 synthesis through p38 MAP kinase in osteoblast-like MC3T3-E1 cells [18]. Therefore, we further examined the effect of a combination of PGD_2 and ET-1 on IL-6 synthesis in these cells. PGD_2 and ET-1 additionally induced IL-6 synthesis (Table 1).

3.4. Effects of PD98059, SB203580 or SP600125 on the PGD_2-stimulated IL-6 synthesis in MC3T3-E1 cells

It is currently known that three MAP kinases, p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK among the MAP kinase superfamily are known as central elements used by mammalian cells to transduce the various messages of extracellular stimuli [27]. We have previously reported that PGD_2 stimulates the activation of p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells [28,29]. To investigate the involvement of three MAP kinases in the PGD_2-stimulated IL-6 synthesis in these cells, we examined the effect of PD98059, a potent MEK inhibitor [19], SB203580, a specific inhibitor of p38 MAP kinase [20], or SP600125, a specific inhibitor of SAPK/JNK [21], on the IL-6 synthesis. PD98059 and SB203580 significantly suppressed the PGD_2-stimulated synthesis of IL-6 (Table 2). On the contrary, SP600125, a specific inhibitor of SAPK/JNK [21], failed to suppress the IL-6 synthesis stimulated by PGD_2 (data not shown).

3.5. Effects of Y27632 or fasudil on the PGD_2-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells

In order to elucidate whether Rho-kinase effect on the PGD_2-stimulated IL-6 synthesis is due to the activation of p44/p42 MAP kinase or not in MC3T3-E1 cells, we next examined the effect of Y27632 on the phosphorylation of p44/p42 MAP kinase by PGD_2. However, Y27632 hardly affected the PGD_2-induced phosphorylation of p44/p42 MAP kinase (Fig. 3A). Additionally, the phosphorylation of p44/p42 MAP kinase by PGD_2 was not reduced by fasudil (Fig. 3B).

3.6. Effects of Y27632 or fasudil on the PGD_2-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells

Furthermore, we examined the effect of Y27632 on the PGD_2-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. Y27632 markedly suppressed the PGD_2-induced levels of p38 MAP kinase phosphorylation (Fig. 4A). The inhibitory effect of Y27632 on the phosphorylated levels was dose dependent in the range between 1 and 10 μM. Y27632 (10 μM) caused more than 90% inhibition in the PGD_2-effect (Fig. 4A).

Fasudil suppressed the PGD_2-induced levels of phosphorylated-p38 MAP kinase (Fig. 4B). The suppressive effect of fasudil as well as Y27632 was dose dependent in the range between 1 and 10 μM. Fasudil (10 μM) reduced the PGD_2-induced levels almost to the control levels (Fig. 4B).

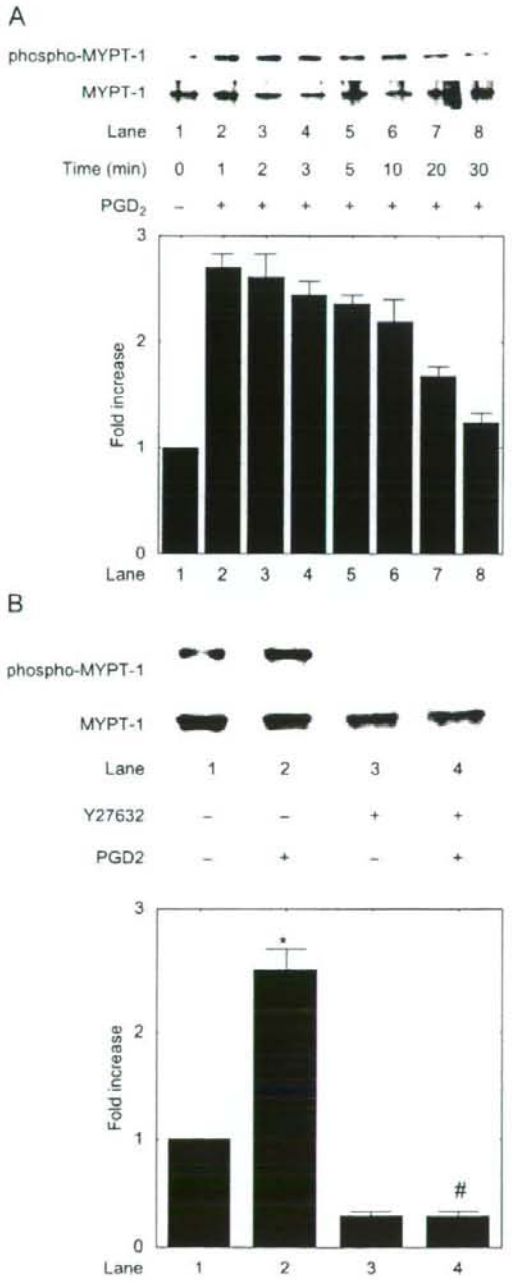

Fig. 1. Effect of Y27632 on the PGD_2-induced phosphorylation of MYPT-1 in MC3T3-E1 cells. (A) The cultured cells were stimulated by 10 μM PGD_2 for the indicated periods. (B) The cultured cells were pretreated with 10 μM Y27632 or vehicle for 60 min, and then stimulated by 10 μM PGD_2 or vehicle for 2 min. 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 PGD_2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate independent experiments. Similar results were obtained with two additional and different cell preparations. *$p < 0.05$, compared with the control. #$p < 0.05$, compared with the value of PGD_2 alone.

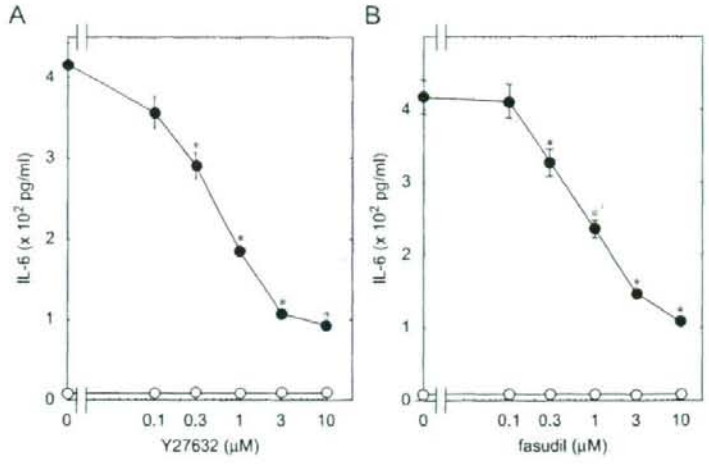

Fig. 2. Effects of Y27632 or fasudil on the PGD_2-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 (A) or fasudil (B) for 60 min, and then stimulated by 10 μM PGD_2 (●) or vehicle (○) for 24 h. Each value represents the mean ± S.E.M. of triplicate independent experiments. Similar results were obtained with two additional and different cell preparations. *$p < 0.05$, compared to the value of PGD_2 alone.

Table 1
Effect of a combination of PGD_2 and ET-1 on IL-6 synthesis in MC3T3-E1 cells

ET-1	PGD_2	IL-6 (pg/ml)
–	–	< 7.8
–	+	427 ± 25*
+	–	296 ± 18*
+	+	991 ± 41**

The cultured cells were stimulated by 0.1 μM ET-1, 10 μM PGD_2, their combination or vehicle for 24 h. Each value represents the mean ± S.E.M. of triplicate independent experiments. 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 PGD_2 alone.

Table 2
Effects of PD98059 or SB203580 on the PGD_2-stimulated IL-6 synthesis in MC3T3-E1 cells

Inhibitors	PGD_2	IL-6 (pg/ml)
–	–	< 7.8
–	+	447 ± 29*
PD98059	–	< 7.8
PD98059	+	282 ± 16**
SB203580	–	< 7.8
SB203580	+	78 ± 5**

The cultured cells were pretreated with 30 μM PD98059 or 30 μM SB203580 for 60 min, and then stimulated by 10 μM PGD_2 or vehicle for 24 h. Each value represents the mean ± S.E.M. of triplicate independent experiments. 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 PGD_2 alone.

4. Discussion

In the present study, we demonstrated that PGD_2 time-dependently induced the phosphorylation of MYPT-1 in osteoblast-like MC3T3-E1 cells, using antibodies against phospho-MYPT-1 (Thr850). MYPT, which is a myosin-binding subunit of myosin phosphatase and regulates the interaction of actin and myosin, is well known to be a downstream target of Rho-kinase [13,26]. In addition, we next found that Y27632 and fasudil, inhibitors of Rho-kinase [15], truly attenuated the PGD_2-induced phosphorylation of MYPT-1. Based on our results, it is most likely that PGD_2 induces the activation of Rho-kinase in osteoblast-like MC3T3-E1 cells.

We tried to clarify whether Rho-kinase functions in the PGD_2-stimulated IL-6 synthesis or not in osteoblast-like MC3T3-E1 cells. The PGD_2-stimulated synthesis of IL-6 was significantly suppressed by Y27632 [15] in a dose-dependent manner. This finding suggests that Rho-kinase activation is involved in the PGD_2-stimulated IL-6 synthesis in MC3T3-E1 cells. In addition, we demonstrated that fasudil [15] as well as Y27632 inhibited the IL-6 synthesis stimulated by PGD_2. Therefore, taking our findings into account, it is most likely that the PGD_2-activated Rho-kinase functions as a positive regulator in the IL-6 synthesis in osteoblast-like MC3T3-E1 cells.

Regarding about intracellular signaling system of PGD_2 in osteoblasts, we have previously reported that three MAP kinases, p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK among the MAP kinase superfamily are activated by PGD_2 in osteoblast-like MC3T3-E1 cells [28,29]. Therefore, we investigated the roles of three MAP kinases in the PGD_2-stimulated IL-6 synthesis in MC3T3-E1 cells. However, SP600125 [21] failed to reduce the PGD_2-stimulated IL-6 synthesis. Based on our finding, it seems unlikely that PGD_2 stimulates IL-6 synthesis through the activation of SAPK/JNK in osteoblast-like MC3T3-E1 cells. On the contrary, in the present study, we showed that the IL-6 synthesis induced by PGD_2 was suppressed by PD98059 [19] or SB203580 [20]. Thus, our results suggest that p44/p42 MAP kinase and p38 MAP kinase play a part in the PGD_2-stimulated IL-6 synthesis in these cells.

Furthermore, we tried to elucidate the relationship between Rho-kinase and these MAP kinase, p44/p42 MAP kinase and p38 MAP kinase in the PGD_2-stimulated IL-6 synthesis in MC3T3-E1 cells. However, Y27632 or fasudil did not affect the PGD_2-induced phosphorylation of p44/p42 MAP kinase. On the other hand, we demonstrated that the PGD_2-induced phosphorylation levels of p38 MAP kinase were markedly reduced by Y27632 and fasudil. Therefore, our findings suggest that Rho-kinase regulates the

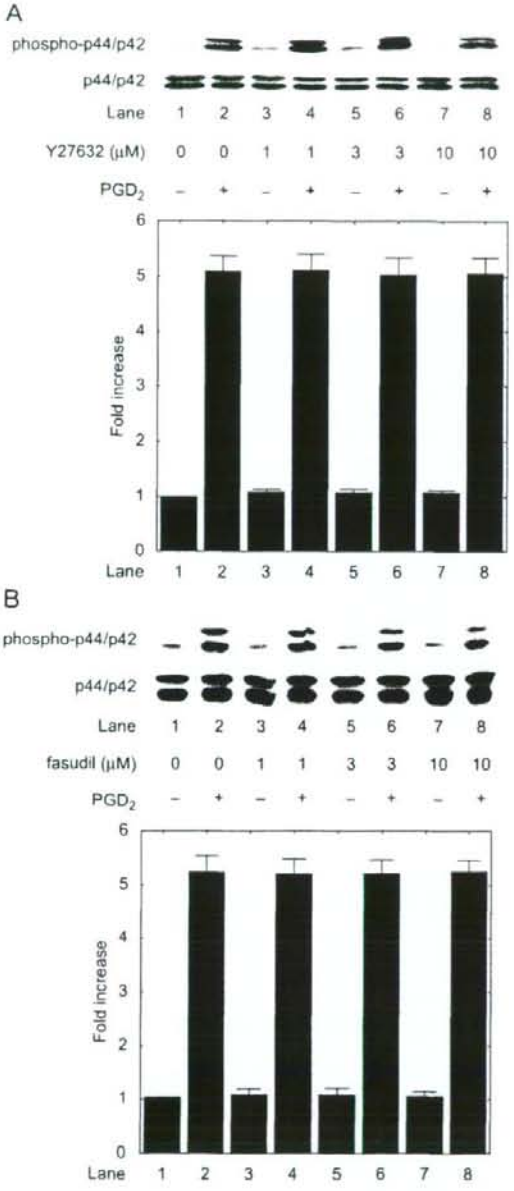

Fig. 3. Effects of Y27632 or fasudil on the PGD_2-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 (A) or fasudil (B) for 60 min, and then stimulated by 10 μM PGD_2 or vehicle for 20 min. The histogram shows quantitative representations of the levels of PGD_2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate independent experiments. Similar results were obtained with two additional and different cell preparations.

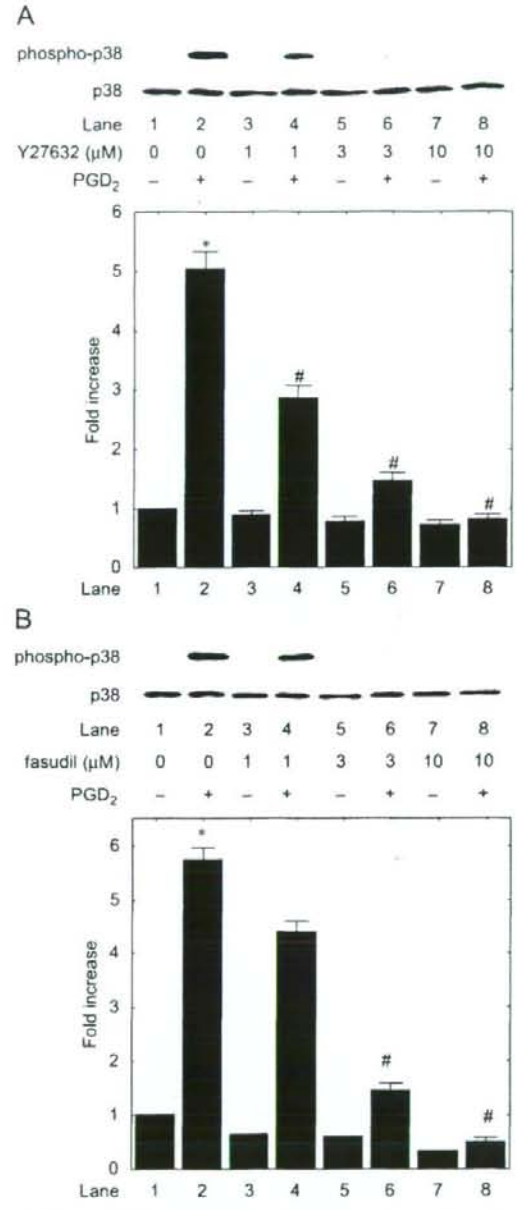

Fig. 4. Effects of Y27632 or fasudil on the PGD_2-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 (A) or fasudil (B) for 60 min, and then stimulated by 10 μM PGD_2 or vehicle for 10 min. The histogram shows quantitative representations of the levels of PGD_2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate independent experiments. Similar results were obtained with two additional and different cell preparations. $^*p < 0.05$, compared with the control. $^\#p < 0.05$, compared with the value of PGD_2 alone.

PGD_2-stimulated IL-6 synthesis via p38 MAP kinase but not p44/p42 MAP kinase. With regard to the time-dependent effect of PGD_2, we showed that the maximum effect of PGD_2 on the phosphorylation of MYPT-1, a well-known downstream target of Rho-kinase [13], was observed within 2 min after the stimulation. In our previous study [29], we have reported that the

phosphorylation of p38 MAP kinase reaches the peak at 20 min after the stimulation of PGD_2 in these cells. The time course of the PGD_2-induced phosphorylation of MYPT-1 appears to be faster than that of p38 MAP kinase. Thus, it is reasonable that PGD_2-induced activation of p38 MAP kinase subsequently occurs via the activation of Rho-kinase. In addition, we showed that PGD_2 additively induced IL-6 synthesis in combination with ET-1 which induces IL-6 synthesis through p38 MAP kinase regulated by Rho-kinase [18]. It seems that Rho-kinase-mediating signaling induced from different pathways could enhance the downstream signaling in osteoblasts, resulting in the amplification of IL-6 synthesis. Based on our results as a whole, it is most likely that PGD_2-activated Rho-kinase functions at a point upstream from p38 MAP kinase in the IL-6 synthesis in osteoblast-like MC3T3-E1 cells.

It is well known that Rho-kinase plays an important role in a variety of cellular functions, especially vascular smooth muscle contraction [13–15]. In bone metabolism, Rho-kinase reportedly plays a suppressive role in the differentiation of osteoblasts while it induces their proliferation [17]. Bone formation is recognized to be performed by differentiated mature osteoblasts, suggesting that Rho-kinase activation regulates bone formation toward the suppression. Our present results indicate that PGD_2 induces Rho-kinase activation in osteoblasts, resulting in synthesizing IL-6, a potent modulator of bone metabolism. IL-6 produced by osteoblasts is generally recognized to be a bone resorptive agent and induces osteoclast differentiation [3,5]. Taking our present findings into account as a whole, it is probable that PGD_2 plays a regulator of bone metabolism toward up-regulating bone resorption and down-regulating bone formation through the activation of Rho-kinase. It has recently been reported that human osteoblasts produce PGD_2, which acts on the DP receptor to decrease osteoprotegerin production and on the CRTH2 receptor to decrease RANKL expression [11], suggesting that PGD_2 might elicit divergent effects on osteoclastogenesis. Further investigations are necessary to elucidate the exact role of Rho-kinase in osteoblasts.

In conclusion, our results strongly suggest that Rho-kinase functions as a positive regulator in the PGD_2-stimulated IL-6 synthesis in osteoblasts.

Acknowledgments

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Tacrolimus but not cyclosporine A enhances FGF-2-induced VEGF release in osteoblasts

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Abstract. We previously reported that basic fibroblast growth factor (FGF-2) stimulates the release of vascular endothelial growth factor (VEGF) via p44/p42 mitogen-activated protein (MAP) kinase and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) in osteoblast-like MC3T3-E1 cells and that FGF-2-activated p38 MAP kinase negatively regulates VEGF release. In addition, p70 S6 kinase activated by FGF-2 negatively regulates VEGF release via SAPK/JNK. In the present study, we investigated the effects of tacrolimus (FK506) and cyclosporine A, well-known immunosuppressants, on the FGF-2-induced VEGF release in these cells. Tacrolimus, but not cyclosporine A which alone had no effect on VEGF basal levels, significantly enhanced FGF-2-stimulated VEGF release. Tacrolimus markedly enhanced FGF-2-induced phosphorylation of SAPK/JNK without affecting the phosphorylation of p44/p42 MAP or p38 MAP kinases. SP600125, a specific inhibitor of SAPK/JNK, reduced the amplification by tacrolimus of the FGF-2-induced VEGF release. The FGF-2-induced phosphorylation of p70 S6 kinase was suppressed by tacrolimus. These results strongly suggest that tacrolimus enhances FGF-2-stimulated VEGF release via up-regulation of SAPK/JNK through modulating p70 S6 kinase in osteoblasts.

Introduction

Vascular endothelial growth factor (VEGF) is an angiogenic growth factor displaying high specificity for vascular endothelial cells (1). VEGF, which is synthesized and secreted by a variety of cell types, stimulates proliferation of endothelial cells and increases capillary permeability (1). Bone metabolism is strictly regulated by osteoblasts and osteoclasts, responsible for bone formation and bone resorption, respectively (2). In addition, it is currently recognized that bone remodeling carried out by osteoblasts and osteoclasts is accompanied by new capillary extending (3,4). Capillary endothelial cells provide the microvasculature during bone remodeling. It is speculated that the activities of osteoblasts, osteoclasts, and capillary endothelial cells are closely coordinated and regulate bone metabolism (5). It is considered that these functional cells 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 the hypertrophic chondrocyte zone in the mouse tibial epiphyseal growth plate (6). It has been reported that osteoblasts among bone cells produce and secrete VEGF in response to various physiological agents (1,7-9). Based on these findings, it is well recognized that VEGF secreted from osteoblasts may play a crucial role in regulating bone metabolism (5,10). However, the exact mechanism underlying VEGF synthesis in osteoblasts and its release from these cells remains to be clarified.

Basic fibroblast growth factor (FGF-2) is produced in osteoblasts and FGF-2 is embedded in the bone matrix (11,12). FGF-2 expression in osteoblasts is detected during fracture repair (13). Therefore, it is considered that FGF-2 plays a pivotal role in fracture healing, bone remodeling and osteogenesis (14). We demonstrated that FGF-2 auto-phosphorylates FGF receptors 1 and 2 among four structurally related high affinity receptors in osteoblast-like MC3T3-E1 cells (15). As for VEGF release, we previously reported (16,17) that FGF-2 stimulates VEGF release in MC3T3-E1 cells, and that release is positively regulated by p44/p42 mitogen-activated protein (MAP) kinase and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) among the MAP kinase superfamily (18), while FGF-2-activated p38 MAP kinase

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negatively regulates VEGF release. In addition, we recently demonstrated that p70 S6 kinase functions at a point upstream from SAPK/JNK and limits FGF-2-stimulated VEGF release via down-regulating SAPK/JNK (19).

Tacrolimus (FK506) and cyclosporine A are potent immunosuppressive agents which are used clinically to prevent tissue rejection in organ transplants and to treat autoimmune diseases (20,21). As for bone metabolism, it has been shown that immunosuppressants cause bone loss when systemically administered at high doses over the long term (22,23). Recently, though, these agents reportedly up-regulate alkaline phosphatase activity and osteocalcin levels, and promote bone formation (24,25). Therefore, it is speculated that immunosuppressants induce osteoblast differentiation. However, the exact role of immunosuppressive agents in bone metabolism and osteoblasts is not fully understood.

In the present study, we investigated the effects of tacrolimus and cyclosporine A on the FGF-2-stimulated VEGF release in osteoblast-like MC3T3-E1 cells. We showed that tacrolimus but not cyclosporine A amplifies VEGF release via up-regulation of SAPK/JNK in these cells.

Materials and methods

Materials. The FGF-2 and mouse VEGF enzyme immunoassay kits were purchased from R&D Systems, Inc. (Minneapolis, MN). Tacrolimus hydrate (FK506) was purchased from Sigma Chemical Co. (St. Louis, MO). Cyclosporine A and SP600125 were obtained from Calbiochem-Novabiochem Co. (La Jolla, 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 p70 S6 kinase antibodies and p70 S6 kinase antibodies were purchased from Cell Signaling, Inc. (Beverly, MA). The ECL Western blotting detection system was purchased from Amersham Biosciences (Piscataway, NJ). Other materials and chemicals were obtained from commercial sources. Tacrolimus, cyclosporine A and SP600125 were dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of DMSO was 0.1%, which did not affect the assay for VEGF or the Western blot analysis.

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_2/95% air. The cells were seeded into 35-mm ($5x10^4$) or 90-mm ($5x10^5$) 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 then used for experiments after 48 h.

Assay for VEGF. The cultured cells were stimulated by various doses of FGF-2 in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pretreated with tacrolimus, cyclosporine A or SP600125 for 60 min. The conditioned medium was collected at the end of incubation, and the VEGF concentration was measured by ELISA kit.

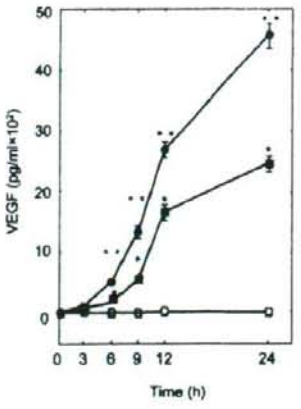

Figure 1. Effect of tacrolimus on the FGF-2-stimulated VEGF release in MC3T3-E1 cells. The cultured cells were pretreated with 300 ng/ml tacrolimus (circle symbols) or vehicle (square symbols) for 60 min, and then stimulated by 70 ng/ml FGF-2 (solid symbols) or vehicle (open symbols) for the indicated periods. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *p<0.05, in comparison to the control. **p<0.05, in comparison to the value of FGF-2 alone.

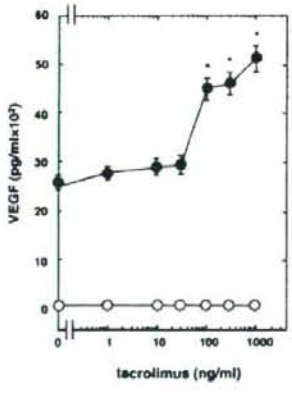

Figure 2. Dose-dependent effect of tacrolimus on the FGF-2-stimulated VEGF release in MC3T3-E1 cells. The cultured cells were pretreated with various doses of tacrolimus for 60 min and then stimulated by 70 ng/ml FGF-2 (●) or vehicle (○) for 24 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *p<0.05, in comparison to the value of FGF-2 alone.

Western blot analysis. The cultured cells were stimulated by FGF-2 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, 2%

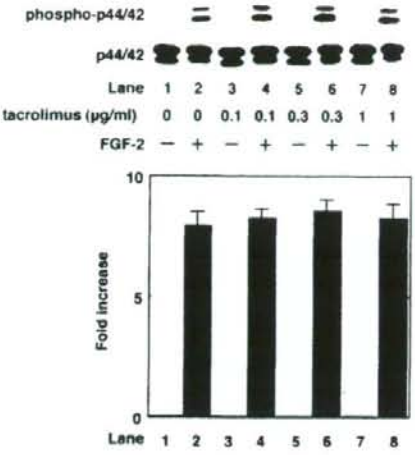

Figure 3. Effects of tacrolimus on the FGF-2-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of tacrolimus for 60 min, and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were analyzed by Western blotting using antibodies against phospho-specific p44/p42 MAP or p44/p42 MAP kinase. The histogram shows quantitative representations of the phosphorylation level for p44/p42 MAP kinase obtained from a laser densitometric analysis. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

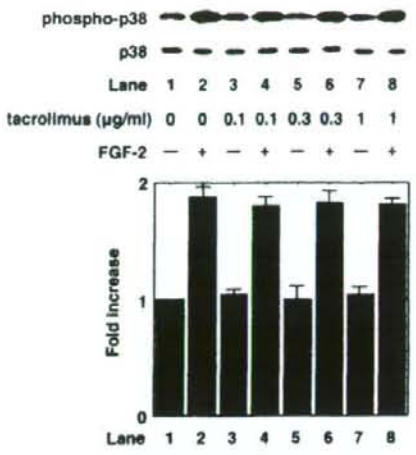

Figure 4. Effects of tacrolimus on the FGF-2-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of tacrolimus for 60 min and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were analyzed by Western blotting using antibodies against phospho-specific p38 MAP or p38 MAP kinase. The histogram shows quantitative representations of the phosphorylation level for p38 MAP kinase obtained from a laser densitometric analysis. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125,000 x g for 10 min at 4˚C. SDS-polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli (28) in 10% polyacrylamide gels. The Western blot analysis was performed as described previously (29) by using phospho-specific p70 S6 kinase antibodies, p70 S6 kinase 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 or SAPK/JNK antibodies, with peroxidase-labeled antibodies raised in goat anti-rabbit IgG being used as second antibodies. The peroxidase activity on the polyvinylidine difluoride membrane was visualized on X-ray film by means of an enhanced chemiluminescence Western blotting detection system.

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

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

Results

Effects of tacrolimus or cyclosporine A on the FGF-2-stimulated VEGF release in MC3T3-E1 cells. To investigate the effects of immunosuppressants on the FGF-2-induced release of VEGF in MC3T3-E1 cells, we examined the effect of tacrolimus and cyclosporine A on VEGF release. Tacrolimus, which alone did not affect basal levels of VEGF, significantly amplified the FGF-2-induced release of VEGF in a time-dependent manner (Fig. 1). The amplifying effect of tacrolimus was dose-dependent in the range between 1 and 50 ng/ml (Fig. 2). Tacrolimus at 1 μg/ml caused ~100% enhancement in the FGF-2 effect. However, cyclosporine A failed to strengthen the FGF-2-induced release of VEGF in the range between 1 ng/ml and 1 μg/ml (<7.8 pg/ml for control; <7.8 pg/ml for 1 μg/ml cyclosporine A; 2686±145 pg/ml for 70 ng/ml FGF-2 alone; 2343±185 pg/ml for 70 ng/ml FGF-2 with 1 μg/ml cyclosporine A, as measured during the stimulation for 24 h).

Effects of tacrolimus on the FGF-2-induced phosphorylation of p44/p42 MAP kinase, p38 MAP kinase or SAPK/JNK in MC3T3-E1 cells. In our previous studies (16,17), we showed that p44/p42 MAP kinase and SAPK/JNK function as positive regulators in the FGF-2-stimulated VEGF release in osteoblast-like MC3T3-E1 cells and that FGF-2-activated p38 MAP kinase negatively regulates VEGF release. Therefore, in order to clarify whether the amplifying effect of tacrolimus on the FGF-2-stimulated VEGF release is through the activation of p44/p42 MAP kinase, p38 MAP kinase or

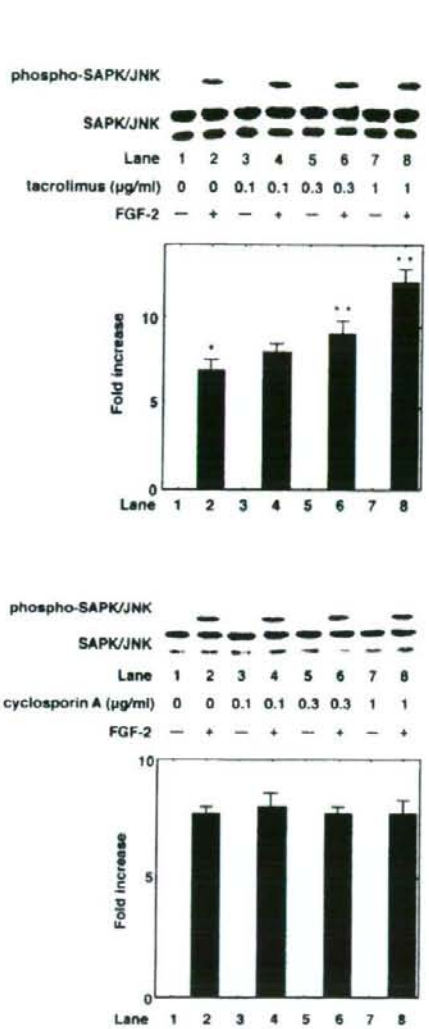

Figure 5. Effects of tacrolimus or cyclosporine A on the FGF-2-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with various doses of (A) tacrolimus or (B) cyclosporine A for 60 min and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were analyzed by Western blotting using antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the phosphorylation level for SAPK/JNK obtained from a laser densitometric analysis. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *p<0.05, in comparison to the value of FGF-2 alone.

SAPK/JNK in MC3T3-E1 cells, we next examined the effect of tacrolimus on the FGF-2-induced phosphorylation of p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK. The FGF-2-induced phosphorylation of p44/p42 MAP kinase (Fig. 3) and p38 MAP kinase (Fig. 4) was not affected by tacrolimus. On the contrary, tacrolimus, which by itself had no effect on the SAPK/JNK phosphorylation, significantly

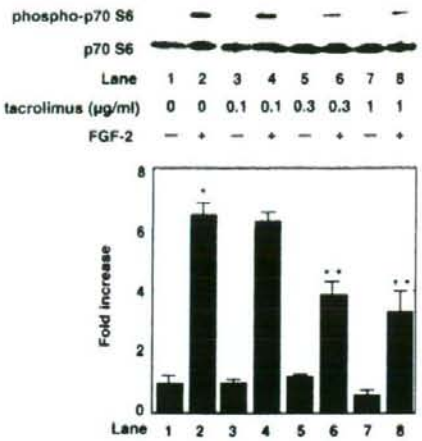

Figure 6. Effect of tacrolimus on the FGF-2-induced phosphorylation of p70 S6 kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of tacrolimus for 60 min, and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were analyzed by Western blotting using antibodies against phospho-specific p70 S6 kinase or p70 S6 kinase. The histogram shows quantitative representations of the phosphorylation level for p70 S6 kinase obtained from a laser densitometric analysis. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *p<0.05, in comparison to the value of FGF-2 alone.

enhanced the FGF-2-induced phosphorylation of SAPK/JNK (Fig. 5A). However, cyclosporine A had little effect on the phosphorylation of SAPK/JNK (Fig. 5B).

Effects of tacrolimus on the FGF-2-induced phosphorylation of p70 S6 kinase in MC3T3-E1 cells. We previously reported that p70 S6 kinase limits the FGF-2-stimulated release of VEGF via down-regulation of SAPK/JNK, composing a negative feedback system, in MC3T3-E1 cells (19). Thus, we examined the effect of tacrolimus on the FGF-2-induced phosphorylation of p70 S6 kinase. Tacrolimus markedly suppressed the FGF-2-induced phosphorylation of p70 S6 kinase (Fig. 6).

Effects of SP600125 on the amplification by tacrolimus of the FGF-2-induced VEGF release in MC3T3-E1 cells. SP600125, a specific SAPK/JNK inhibitor (30), which by itself did not affect basal levels of VEGF, significantly reduced the enhancement by tacrolimus of FGF-2-induced VEGF release (Table I). The enhanced levels by tacrolimus of FGF-2-induced VEGF release were reduced by SP600125 similar to the levels by FGF-2 with SP600125 treatment.

Discussion

We previously reported that FGF-2 stimulates the release of VEGF, which is an endothelial cell-specific mitogen and an angiogenic inducer (1) in osteoblast-like MC3T3-E1 cells (16). Accumulating evidence suggests that immunosuppressants modulate bone metabolism (22-25). Thus, we investigated the effects of tacrolimus and cyclosporine A,

Table I. Effect of tacrolimus on the enhancement by tacrolimus of the FGF-2-induced VEGF release in MC3T3-E1 cells.

SP600125	Tacrolimus	FGF-2	VEGF (pg/ml)
-	-	-	<7.8
-	-	+	2438±132[a]
-	+	-	<7.8
-	+	+	5733±221[b]
+	-	-	<7.8
+	-	+	1125±85[b]
+	+	-	<7.8
+	+	+	1208±103[c]

The cultured cells were pretreated with 10 μM SP600125 or vehicle for 60 min and then incubated with 0.3 μg/ml tacrolimus or vehicle for 60 min. The cells were stimulated by 70 ng/ml FGF-2 or vehicle for 24 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. [a]$p<0.05$, in comparison to the control. [b]$p<0.05$, in comparison to the value of FGF-2 alone. [c]$p<0.05$, in comparison to the value of FGF-2 with tacrolimus pretreatment.

well known and clinically used immunosuppressive agents (20,21), on the FGF-2-stimulated VEGF release in MC3T3-E1 cells. Tacrolimus markedly enhanced the FGF-2-stimulated release of VEGF in these cells. On the other hand, we found that cyclosporine A did not amplify VEGF release. Therefore, we investigated the mechanism of tacrolimus underlying the amplifying effect on the FGF-2-induced VEGF release in osteoblast-like MC3T3-E1 cells.

Previously, we demonstrated that FGF-2 induces the activation of p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells (16,17). The MAP kinase superfamily mediates intracellular signal transduction of a variety of extracellular factors and plays a central role in cellular functions including cell proliferation, differentiation, and apoptosis (18). It is well recognized that three major MAP kinases: p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK are central elements used by mammalian cells to transduce the diverse messages (18). In our previous studies (16,17), we demonstrated that p44/p42 MAP kinase and SAPK/JNK acted as positive regulators in the FGF-2-induced VEGF release in MC3T3-E1 cells. On the other hand, FGF-2-activated p38 MAP kinase functions as a negative regulator in VEGF release. In the present study, tacrolimus failed to affect the phosphorylation of p44/p42 MAP and p38 MAP kinases. Based on these results, it seems unlikely that tacrolimus affects the FGF-2-stimulated release of VEGF through up-regulating the activity of p44/p42 MAP kinase or down-regulating the activity of p38 MAP kinase in osteoblast-like MC3T3-E1 cells. On the contrary, we showed here that the phosphorylation levels of FGF-2-induced SAPK/JNK were markedly enhanced by tacrolimus. In addition, the amplification by tacrolimus of the FGF-2-stimulated VEGF release was suppressed by SP600125, a specific inhibitor of SAPK/JNK (30), similar to levels of FGF-2 with SP600125. In addition, we demonstrated that cyclosporine A had no effect on the phosphorylation levels of SAPK/JNK induced by FGF-2. In light of our findings, it is probable that tacrolimus enhances the FGF-2-stimulated VEGF release via strengthening the activity of SAPK/JNK in osteoblast-like MC3T3-E1 cells.

In our recent study (19), we showed that FGF-2-stimulated p70 S6 kinase functions at a point upstream of SAPK/JNK in osteoblast-like MC3T3-E1 cells and negatively regulated VEGF release by FGF-2. These findings led us to speculate that tacrolimus might modulate the FGF-2-induced p70 S6 kinase activation in MC3T3-E1 cells. Thus, we investigated the effect of tacrolimus on the FGF-2-stimulated activation of p70 S6 kinase. The FGF-2-induced phosphorylation of p70 S6 kinase was significantly attenuated by tacrolimus. Based on our collective results, it is most likely that tacrolimus modulates p70 S6 kinase-regulated SAPK/JNK in osteoblast-like MC3T3-E1 cells, resulting in enhanced FGF-2-stimulated VEGF release.

Tacrolimus (FK506) and cyclosporine A are well known immunosuppressants that are used mainly in clinical organ transplantation (20,21). It has recently been shown that these agents have significant effects on bone metabolism (22-25). The immunosuppressants that were employed at high doses after organ transplantation, reportedly caused the reduction in bone mineral density (22,23). On the contrary, evidence is accumulating that tacrolimus induces the promotion of osteoblastic differentiation *in vitro* (24,25,31). Our present results indicate that not cyclosporine A but tacrolimus plays a role in the control of the production of VEGF, one of the key regulators of bone metabolism. It is generally recognized that new capillary extending is essential for bone remodeling (5). Since VEGF is a specific mitogen of vascular endothelial cells (1), our present findings suggest that tacrolimus-enhanced VEGF release from osteoblasts play an important role in the pathophysiological process of bone remodeling under the medication of this agent. It is probable that tacrolimus positively regulates microvasculature development in bones. Therefore, tacrolimus might be considered to be a potent therapeutic agent useful for the disorder of bone metabolism. However, the details regarding immunosuppressants in bone metabolism still remain unclear. Further investigation is necessary to elucidate the exact mechanism of tacrolimus in osteoblasts and in bone metabolism.

In conclusion, our present results strongly suggest that tacrolimus enhances FGF-2-stimulated VEGF release via up-regulation of SAPK/JNK through modulating p70 S6 kinase in osteoblasts.

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