Table 2. Effect of PD98059 or SP600125 on the enhancement by PDGF-BB of FGF-2-stimulated VEGF release in MC3T3-E1 cells

Inhibitor	PDGF-BB	FGF-2	VEGF (pg/ml)
–	–	–	<7.8
–	–	+	1068 ± 40*
–	+	–	<7.8
–	+	+	2910 ± 155**
PD98509	–	–	<7.8
PD98509	–	+	521 ± 30**
PD98509	+	–	<7.8
PD98509	+	+	1205 ± 121***
SP600125	–	–	<7.8
SP600125	–	+	629 ± 38**
SP600125	+	–	<7.8
SP600125	+	+	1124 ± 95***

The cultured cells were pretreated with 30 μM PD98059, 10 μM SP600125 or vehicle for 60 min, and then incubated with 30 ng/ml PDGF-BB or vehicle for 60 min. The cells were subsequently 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. *$P < 0.05$, compared to the control; **$P < 0.05$, compared to the value of FGF-2 alone; ***$P < 0.05$, compared to the value of FGF-2 with PDGF-BB pretreatment

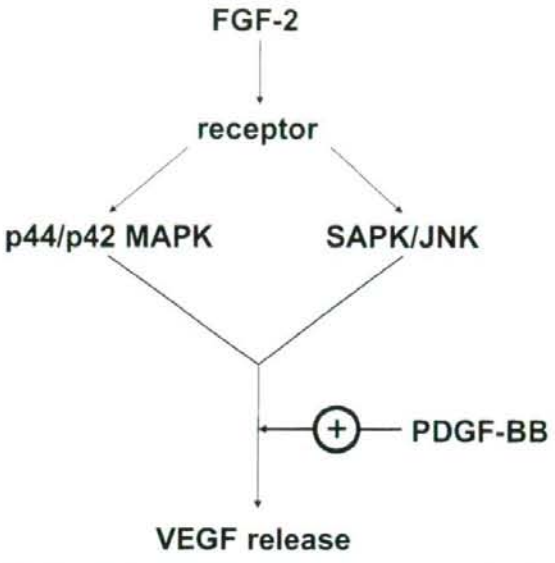

Fig. 5. Schematic illustration of the signaling pathways of VEGF release stimulated by FGF-2 and PDGF-BB in osteoblasts. *FGF-2*, fibroblast growth factor-2; *MAPK*, mitogen-activated protein kinase; *SAPK/JNK*, stress-activated protein kinase/c-*Jun* N-terminal kinase; *PDGF-BB*, platelet-derived growth factor-BB; *VEGF*, vascular endothelial growth factor; +, amplification

induced by FGF-2 and its enhancement by PDGF-BB in osteoblasts.

We next examined the effect of AG1295, a specific inhibitor of PDGF receptor kinase [30], on the amplification by PDGF-BB. AG1295 significantly reduced the PDGF-BB-induced enhancement of VEGF release without affecting the FGF-2-stimulated VEGF release. AG1295 (0.7 μM) caused about 40% inhibition of the PDGF-BB potentiation on FGF-2-induced VEGF release in MC3T3-E1 cells. AG1295 reportedly inhibits the PDGF effects in Swiss 3T3 cells and in porcine aorta endothelial cells with 50% inhibitory concentrations below 5 and 1 μM, respectively [30]. Thus, it seems that our present result is consistent with the previous report. In addition, we found that AG1295 truly attenuated the PDGF-BB-induced phosphorylation of PDGF receptor-β in these cells. Taking these findings into account, it is most likely that the activation of PDGF receptor potentiates the FGF-2-stimulated VEGF release in osteoblast-like MC3T3-E1 cells. Indeed, we did not exchange the medium at the start of FGF-2 stimulation; in turn, PDGF existed in the medium during the stimulation by FGF-2 under the experimental conditions. As indicated, the pretreatment with PDGF was 60 min before the stimulation, and PDGF by itself had little effect on VEGF release up to 24 h. Thus, it is likely that the simultaneous stimulation with PDGF and FGF-2 could reproduce the enhancement of VEGF release, although the time course should be influenced.

We next investigated the mechanism of PDGF-BB underlying the potentiation of VEGF release in MC3T3-E1 cells. In our previous studies [17,18], we have shown that p44/p42 MAP kinase and SAPK/JNK act as positive regulators in FGF-2-induced VEGF release. Thus, we tried to clarify the relationship between PDGF-BB signaling and these MAP kinases in the FGF-2-stimulated VEGF release in these cells. However, PDGF-BB failed to strengthen the phosphorylation of p44/p42 MAP kinase and SAPK/JNK. Therefore, it seems unlikely that PDGF-BB signaling pathway upregulates the FGF-2-stimulated release of VEGF at a point upstream from p44/p42 MAP kinase or SAPK/JNK in osteoblast-like MC3T3-E1 cells. On the other hand, we showed that PD98059 [31] and SP600125 [32] markedly reduced the amplification by PDGF-BB of FGF-2-induced VEGF release. PDGF-BB caused about 200% enhancement of FGF-2-induced VEGF release without SP600125 pretreatment; however, the enhancement decreased less than 100% with SP600125. It is likely that SP600125 not only inhibits the effect of FGF-2 on VEGF release but also reduces the enhancement by PDGF-BB. Therefore, our findings suggest that PDGF-BB upregulates the FGF-2-stimulated VEGF release at a point downstream from SAPK/JNK. Based on our findings, it is probable that PDGF-BB upregulates the FGF-2-stimulated VEGF release as an amplifier at a point downstream from p44/p42 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells. The potential mechanism of PDGF-BB in FGF-2-stimulated VEGF release in osteoblasts shown here is summarized in Fig. 5. Further investigations are necessary to clarify the exact mechanism of PDGF-BB in the amplification of VEGF synthesis in osteoblasts.

It is well recognized that angiogenesis and capillary outgrowth are essential for bone remodeling [10]. Our present findings indicate that PDGF-BB signaling in osteoblasts plays an important role in the control of the release of VEGF, one of the potent key factors of bone metabolism.

Because VEGF is a specific mitogen of vascular endothelial cells [11], our results lead us to speculate that PDGF-BB-amplified VEGF levels act as a positive feedback regulator of the microvasculature development in bone. Moreover, it has been reported that VEGF is involved in trabecular bone formation and expansion of the hypertrophic chondrocyte zone in epiphyseal growth plate of mouse [12], supporting the significance of VEGF in bone metabolism. The mitogenic activities of PDGF and its release by platelets suggest a pivotal role in wound healing and bone fracture repair [23]. In addition, PDGF is well recognized to be expressed by a variety of malignant cells including osteosarcoma cells, suggesting the involvement of tumorigenesis [23]. It has been reported that FGF-2 synthesized by osteoblasts is embedded in bone matrix [2,3]. Taking these findings into account, it is probable that PDGF-BB-enhanced VEGF release in the presence of FGF-2 from osteoblasts plays a crucial role in the process of bone remodeling or tumorigenesis via upregulating the proliferation of capillary endothelial cells. It is possible that PDGF-BB cooperatively enhances FGF-2-induced VEGF release, essential for the development of microvasculature in the process of bone repair or the pathogenesis of osteosarcomas. Additionally, in this study, we investigated the effect of PDGF-BB on the FGF-2-stimulated VEGF release utilizing osteoblast-like MC3T3-E1 cells only. Therefore, to confirm the generalization of our findings shown here, further investigations in other osteoblast cell lines are required.

In conclusion, our results strongly suggest that PDGF-BB potentiates FGF-2-stimulated VEGF release at a point downstream from p44/p42 MAP kinase and SAPK/JNK in osteoblasts.

Acknowledgments We are very grateful to Yoko Kawamura and Seiko Sakakibara for their skillful technical assistance. This investigation was supported in part by Grant-in-Aid for Scientific Research (16590873 and 16591482) for the Ministry of Education, Science, Sports and Culture of Japan, the Research Grants for Longevity Sciences (15A-1, 15C-2 and 17A-3), Research on Proteomics and Research from the Ministry of Health, Labour and Welfare of Japan.

References

1. Nijweide PJ, Burger EH, Feyen JHM (1986) Cells of bone: proliferation, differentiation, and hormonal regulation. Physiol Rev 86:855–886
2. Baylink DJ, Finkleman RD, Mohan S (1993) Growth factor to stimulate bone formation. J Bone Miner Res 8:S565–S572
3. Hurley MM, Abreu C, Harrison JR, Lichtler AC, Raisz LG, Kream BE (1993) Basic fibroblast growth factor inhibits type I collagen gene expression in osteoblastic MC3T3-E1 cells. J Biol Chem 268: 5588–5593
4. Bolander ME (1992) Regulation of fracture repair by growth factors stimulate tyrosine kinase activity *in vivo*. Proc Soc Exp Biol Med 200:165–170
5. Mari PJ (2003) Fibroblast growth factor signaling controlling osteoblast differentiation. Gene (Amst) 316:23–32
6. Suzuki A, Shinoda J, Kanda S, Oiso Y, Kozawa O (1996) Basic fibroblast growth factor stimulates phosphatidylcholine-hydrolyzing phospholipase D in osteoblast-like cells. J Cell Biochem 63:491–499
7. Kozawa O, Niwa M, Matsuno H, Ishisaki A, Kato K, Uematsu T (2001) Stimulatory effect of basic fibroblast growth factor on induction of heat shock protein 27 in osteoblasts: role of protein kinase C. Arch Biochem Biophys 388:237–242
8. Brighton CT, Hunt RM (1991) Early histological and ultrastructural changes in medullary fracture callus. J Bone Joint Surg [Am] 73:832–847
9. Parfitt AM (1994) Osteonal and hemi-osteonal remodeling: the spatial and temporal framework for signal traffic in adult human bone. J Cell Biochem 55:273–286
10. Erlebacher A, Filvaroff EH, Gitelman SE, Derynck R (1995) Toward a molecular understanding of skeletal development. Cell 80:371–378
11. Ferrara N, Davis-Smyth T (1997) The biology of vascular endothelial growth factor. Endocr Rev 18:4–25
12. Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N (1999) VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med 5:623–628
13. Goad DL, Rubin J, Wang H Jr, Tashijian AH, Patterson C (1996) Enhanced expression of vascular endothelial growth factor in human SaOS-2 osteoblast-like cells and murine osteoblasts induced by insulin-like growth factor I. Endocrinology 137:2262–2268
14. Wang DS, Yamazaki K, Nohtomi K, Shizume K, Ohsumi K, Shibuya M, Demura H, Sato K (1996) Increase of vascular endothelial growth factor mRNA expression by 1,25-dihydroxyvitamin D_3 in human osteoblast-like cells. J Bone Miner Res 11:472–479
15. Schalaeppi JM, Gutzwiller S, Finlenzeller G, Fournier B (1997) 1,25-Dihydroxyvitamin D_3 induces the expression of vascular endothelial growth factor in osteoblastic cells. Endocr Res 23: 213–229
16. Widmann C, Gibson S, Jarpe MB, Johnson GL (1999) Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev 79:143–180
17. Tokuda H, Kozawa O, Uematsu T (2000) Basic fibroblast growth factor stimulates vascular endothelial growth factor release in osteoblasts: divergent regulation by p44/p42 mitogen-activated protein kinase and p38 mitogen-activated protein kinase. J Bone Miner Res 15:2371–2379
18. Tokuda H, Hirade K, Wang X, Oiso Y, Kozawa O (2003) Involvement of SAPK/JNK in basic fibroblast growth factor-induced VEGF release in osteoblasts. J Endocrinol 177:101–107
19. Zelzer E, Olsen BR (2005) Multiple roles of vascular endothelial growth factor (VEGF) in skeletal development, growth, and repair. Curr Top Dev Biol 65:169–187
20. Heldin CH, Westermark B (1999) Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev 79:1283–1316
21. Heldin CH, Eriksson U, Ostman A (2002) New members of the platelet-derived growth factor family of mitogens. Arch Biochem Biophys 398:284–290
22. Heldin CH, Johnsson A, Wennergren S, Wernstedt C, Betsholtz C, Westermark B (1986) A human osteosarcoma cell line secretes a growth factor structurally related to a homodimer of PDGF A-chains. Nature (Lond) 319:511–514
23. Canalis E, Rydziel S (2002) Platelet-derived growth factor and the skeleton. Principles Bone Biol 2:817–824
24. Kozawa O, Suzuki A, Watanabe Y, Shinoda J, Oiso Y (1995) Effect of platelet-derived growth factor on phosphatidylcholine-hydrolyzing phospholipase D in osteoblast-like cells. Endocrinology 136:4473–4478
25. Hanai Y, Tokuda H, Ohta T, Matsushima-Nishiwaki R, Takai S, Kozawa O (2006) Phosphatidylinositol 3-kinase/Akt autoregulates PDGF-BB-stimulated interleukin-6 synthesis in osteoblasts. J Cell Biochem 99:1564–1571
26. Sudo H, Kodama H, Amagai Y, Yamamoto S, Kasai S (1983) In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol 96:191–198
27. Kozawa O, Tokuda H, Miwa M, Kotoyori J, Oiso Y (1992) Cross-talk regulation between cyclic AMP production and phosphoinositide hydrolysis induced by prostaglandin E_2 in osteoblast-like cells. Exp Cell Res 198:130–134
28. Laemmli LK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond) 227: 680–685
29. Kato K, Ito H, Hasegawa K, Inaguma Y, Kozawa O, Asano T (1996) Modulation of the stress-induced synthesis of hsp27 and

alpha B-crystallin by cyclic AMP in C6 rat glioma cells. J Neurochem 66:946–950

30. Kovalenko M, Gazit A, Bohmer A, Rorsman C, Ronnstrand L, Heldin CH, Waltenberger J, Bohmer FD, Levitzki A (1994) Selective platelet-derived growth factor receptor kinase blockers reverse sis-transformation. Cancer Res 54:6106–6114
31. Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR (1995) PD98059 is a specific inhibitor of the activation of mitogen-activated protein kinase in vitro and in vivo. J Biol Chem 270: 27489–27494
32. Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW (2001) SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci U S A 98:13681–13686

(-)-Epigallocatechin Gallate Inhibits Basic Fibroblast Growth Factor-stimulated Interleukin-6 Synthesis in Osteoblasts

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Key words
- catechin
- basic fibroblast growth factor
- interleukin-6
- MAP kinase
- osteoblast

Abstract

We previously showed that basic fibroblast growth factor (FGF-2) activates the mitogen-activated protein (MAP) kinase superfamily in osteoblast-like MC3T3-E1 cells and that p38 MAP kinase functions as a positive regulator in the FGF-2-stimulated synthesis of interleukin-6 (IL-6), a potent bone-resorptive agent, in these cells. In the present study, we investigated the exact mechanism of IL-6 and the effects of (−)-epigallocatechin gallate (EGCG), one of the major green tea flavonoids, on the synthesis of IL-6. PD98059, an inhibitor of MEK, but not SP600125, an inhibitor of stress-activated protein kinase/c-Jun N-terminal kinase, suppressed FGF-2-stimulated IL-6 synthesis. EGCG significantly reduced the IL-6 synthesis stimulated by FGF-2 in a dose-dependent manner. EGCG attenuated the FGF-2-induced phosphorylation of p44/p42 MAP kinase and p38 MAP kinase. These results strongly suggest that EGCG inhibits the FGF-2-stimulated synthesis of IL-6 at least partly via suppression of the p44/p42 MAP kinase pathway and the p38 MAP kinase pathway in osteoblasts.

Introduction

Compounds in foods such as vegetables and fruits have beneficial properties to human beings. Among them, it has been reported that flavonoids possess antioxidative, antibacterial, and antitumor effects [1,2]. Catechins are one of the major flavonoids, which are present in various species of plants such as green tea [2]. It is well recognized that two functional cells, osteoblasts and osteoclasts, strictly regulate bone metabolism, the former being responsible for bone formation and the latter for bone resorption [3]. The formation of bone structures and bone remodeling results from the coupling process, that is, bone resorption by activated osteoclasts with subsequent deposition of a new matrix by osteoblasts. In bone metabolism, it has been shown that catechin suppresses bone resorption [4]. As for osteoblasts, it has been shown that catechin stimulates alkaline phosphatase activity, a mature osteoblast phenotype, and reduces apoptosis in osteoblast-like MC3T3-E1 cells [5]. It has recently been reported that EGCG increases the formation of mineralized bone nodules and alkaline phosphatase activity in human osteosarcoma SaOS-2 cells while it decreases Runx 2 [6]. We have recently reported that catechin amplifies prostaglandin $F_{2\alpha}$-stimulated synthesis of vascular endothelial growth factor in these cells [7]. However, evidence about the effects of catechin on osteoblasts is not sufficiently accumulated.

Interleukin-6 (IL-6) is a multifunctional cytokine that has eminent physiological effects on a wide range of functions, such as promoting B-cell differentiation and T-cell activation and inducing acute-phase proteins [8–11]. In bone metabolism, IL-6 is one of the most potent osteoclastogenic factors [10,11]. Bone resorption is mediated by the increased local production of inflammatory cytokines such as tumor necrosis factor-α and IL-1. In osteoblasts [12–14], it has been reported that bone-resorptive agents such as tumor necrosis factor-α and IL-1 stimulate the synthesis of IL-6. As for bone metabolism, IL-6 has been shown to stimulate bone resorption and induce osteoclast formation [10–12, 15]. Therefore, evidence is accumulating that IL-6 secreted from osteoblasts plays a key role as a downstream effector of bone-resorptive agents. In our recent studies [16], we have reported that basic fibroblast growth factor (FGF-2) stimulates IL-6 synthesis at least in part through p38 MAP kinase, a member of the MAP kinase superfamily [17], in osteo-

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Bibliography
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blast-like MC3T3-E1 cells. However, the exact mechanism of FGF-2 underlying IL-6 synthesis in osteoblasts remains to be clarified.

In the present study, we investigated the effects of (−)-epigallocatechin gallate (EGCG), one of the major green tea flavonoids, on FGF-2-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells and the mechanism underlying it. Here we show that EGCG reduces FGF-2-stimulated IL-6 synthesis at least partly via attenuation of p44/p42 MAP kinase and p38 MAP kinase in these cells.

Materials and Methods

Materials

FGF-2 and IL-6 ELISA kits were purchased from R&D Systems, Inc. (Minneapolis, MN). EGCG was 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, and p38 MAP kinase antibodies were purchased from Cell Signaling Technology (Beverly, MA). The ECL Western blotting detection system was purchased from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources.

Cell culture

The cloned osteoblast-like MC3T3-E1 cells, which were derived from newborn mouse calvaria [18], were maintained as previously described [19]. 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 diameter dishes (5×10^4/dish) or 90-mm diameter dishes (5×10^5/dish) in α-MEM containing 10% FCS. After five days, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 48 hours.

Assay for IL-6

The cultured cells were stimulated by FGF-2 in 1 ml α-MEM containing 0.3% FCS and then incubated for the indicated periods. The conditioned medium was collected, and IL-6 in the medium was then measured by an IL-6 ELISA kit. When indicated, the cells were pretreated with various doses of EGCG for 60 minutes.

Analysis of Western blotting

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% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol, and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125000×g for 10 minutes at 4 °C. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by Laemmli [20] in 10% polyacrylamide gel. Western blotting analysis was performed as described previously [21] by using 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. The peroxidase activity on PVDF membrane was visualized on X-ray film by means of the ECL Western blotting detection system. When indicated, the cells were pretreated with various doses of EGCG for 60 minutes.

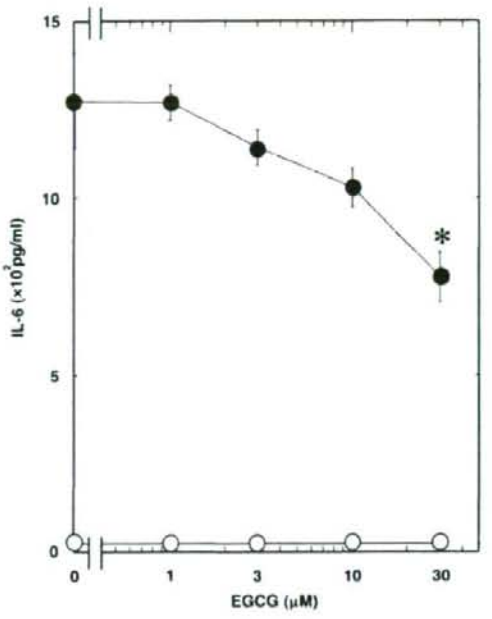

Fig. 1 Effect of EGCG on the FGF-2-stimulated IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of EGCG for 60 min and then stimulated by 70 ng/ml FGF-2 or vehicle for 24 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$p < 0.05$ compared with the value of FGF-2 alone.

Determinations

The absorbance of enzyme immunoassay samples was measured at 450 nm with the 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). The cell viability was assessed by the trypan blue dye exclusion test.

Statistical analysis

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

Results

Effects of EGCG on FGF-2-stimulated IL-6 synthesis in MC3T3-E1 cells

We previously found that FGF-2 stimulates IL-6 synthesis in osteoblast-like MC3T3-E1 cells [16]. We first examined the effects of EGCG on FGF-2-stimulated IL-6 synthesis. EGCG, which alone had little effect on IL-6 levels, significantly reduced the FGF-2-stimulated synthesis of IL-6 in a dose-dependent manner in doses between 1 μM and 30 μM (☞ **Fig. 1**). EGCG (30 μM) caused about a 40% reduction in the FGF-2 effect. We confirmed that the viability of the cells incubated at 37 °C for 24 hours in

Table 1 Effect of PD98059 or SP600125 on FGF-2-stimulated IL-6 synthesis in MC3T3-E1 cells

Inhibitors	FGF-2	IL-6 (pg/ml)
–	–	26 ± 5
–	+	1275 ± 51*
PD98059	–	27 ± 10
PD98059	+	929 ± 85**
SP600125	–	25 ± 10
SP600125	+	1350 ± 49

The cultured cells were pretreated with 50 μM PD98059, 10 μM SP600125, or vehicle for 60 minutes and then stimulated by 70 ng/ml FGF-2 for 24 hours. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.
*$p < 0.05$ compared with the value of control
**$p < 0.05$ compared with the value of FGF-2 alone

the presence of 30 μM EGCG was more than 90% compared with that of the control cells. To determine whether EGCG could affect cell proliferation, we counted cell numbers before and after the 24-hour incubation with 30 μM EGCG. We confirmed that EGCG did not affect the cell number at a dose of 30 μM. These findings suggest that EGCG at 30 μM hardly affects the viability or the proliferation of osteoblast-like MC3T3-E1 cells after up to 24 hours' incubation.

Effects of PD98059 and SP600125 on FGF-2-stimulated IL-6 synthesis in MC3T3-E1 cells

In our previous study [16], we showed that p38 MAP kinase acts as a positive regulator of FGF-2-stimulated IL-6 synthesis in MC3T3-E1 cells. We have already demonstrated that FGF-2 activates p44/p42 MAP kinase and stress-activated protein kinase/c-*Jun* N-terminal (SAPK/JNK) in addition to p38 MAP kinase in these cells [22, 23]. Therefore, in order to investigate the involvement of these MAP kinases in IL-6 synthesis, we next examined the effects of PD98059, a specific inhibitor of MEK upstream kinases that activates p44/p42 MAP kinase [24], and SP600125, a specific inhibitor of SAPK/JNK [25], on IL-6 synthesis. PD98059 (50 μM) significantly suppressed the FGF-2-stimulated synthesis of IL-6 (**Table 1**), suggesting that p44/p42 MAP kinase, in addition to p38 MAP kinase, is involved in FGF-2-stimulated IL-6 synthesis. On the other hand, the FGF-2-induced IL-6 synthesis was hardly affected by SP600125 (10 μM) in these cells (**Table 1**).

Effects of EGCG on the FGF-2-induced phosphorylation of p44/p42 MAP kinase and p38 MAP kinase in MC3T3-E1 cells

In order to clarify the inhibitory mechanism of EGCG underlying the FGF-2-stimulated IL-6 synthesis in these cells, we examined the effects of EGCG on the FGF-2-induced phosphorylation of p44/p42 MAP kinase and p38 MAP kinase. EGCG markedly reduced the FGF-2-induced phosphorylation of p44/p42 MAP kinase (☞ **Fig. 2**). According to the densitometric analysis, EGCG caused about a 30% reduction in the FGF-2 effect.

In addition, EGCG, which by itself had little effect on the phosphorylation levels of p38 MAP kinase, significantly suppressed FGF-2-induced p38 MAP kinase phosphorylation (☞ **Fig. 3**). According to the densitometric analysis, EGCG caused about a 70% reduction in the FGF-2 effect.

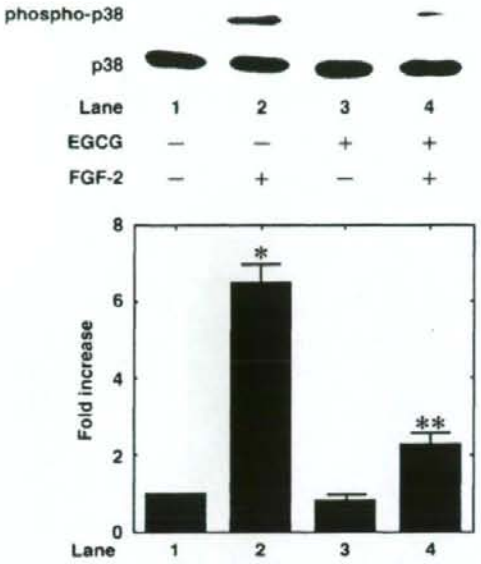

Fig. 2 Effect of EGCG on the phosphorylation of p38 MAP kinase induced by FGF-2 in MC3T3-E1 cells. The cultured cells were pretreated with 100 μM EGCG or vehicle for 60 min and then stimulated by 70 ng/ml FGF-2 or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. The histogram shows quantitative representations of the levels of FGF-2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$p < 0.05$ compared with the value of FGF-2 alone.

Discussion

In the present study, we demonstrated that EGCG significantly reduced the FGF-2-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells. We next investigated the mechanism of EGCG behind the suppressive effect on IL-6 synthesis. It is well known that the MAP kinase superfamily plays an important role in cellular functions including proliferation, differentiation, and survival in a variety of cells [17]. Three major MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK, are known as central elements used by mammalian cells to transduce the diverse messages [17]. We have previously shown that p38 MAP kinase is involved in FGF-2-stimulated IL-6 synthesis in MC3T3-E1 cells [16]. In addition, we already demonstrated that FGF-2 activates p44/p42 MAP kinase and SAPK/JNK as well as p38 MAP kinase in these cells [22, 23]. In this study, PD98059 [24] but not SP600125 [25] suppressed FGF-2-stimulated IL-6 synthesis. It appears that the inhibitor SP600125 increases FGF-2-stimulated IL-6 levels (**Table 1**). However, the p-value was 0.11, compared with the value of FGF-2 alone. Thus, we could not find any statistical differences. We have shown that 10 μM SP600125 markedly attenuates the FGF-2-induced phosphorylation of SAPK/JNK [22]. Therefore, it seems unlikely that SAPK/JNK is involved in the FGF-2-induced IL-6 synthesis in these cells. Although the potential for some nonspecific effect of PD98059 still remains, based

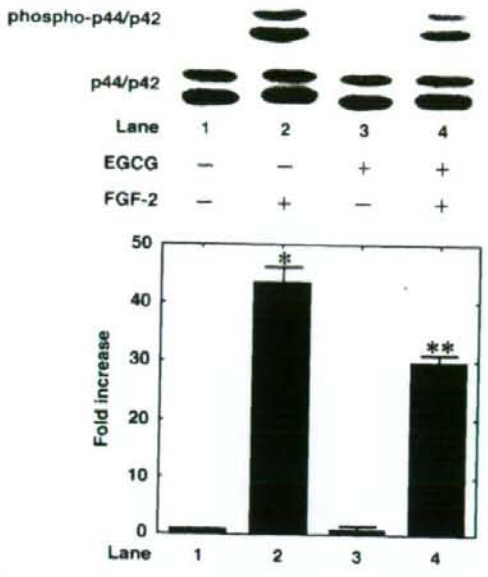

Fig. 3 Effect of EGCG on the phosphorylation of p44/p42 MAP kinase induced by FGF-2 in MC3T3-E1 cells. The cultured cells were pretreated with 100 μM EGCG or vehicle for 60 min and then stimulated by 70 ng/ml FGF-2 or vehicle for 3 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p44/p42 MAP kinase or p44/p42 MAP kinase. The histogram shows quantitative representations of the levels of FGF-2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$p<0.05$ compared with the value of FGF-2 alone.

on our findings it is most likely that FGF-2 stimulates IL-6 synthesis via p44/p42 MAP kinase and p38 MAP kinase, but not via SAPK/JNK, among the MAP kinase superfamily in osteoblast-like MC3T3-E1 cells.

We next investigated the involvement of p44/p42 MAP kinase and p38 MAP kinase in the suppressive effect of EGCG in MC3T3-E1 cells. Here we showed that EGCG significantly attenuated the FGF-2-induced phosphorylation of p44/p42 MAP kinase. In addition, the FGF-2-induced phosphorylation of p38 MAP kinase was markedly suppressed by EGCG. These results suggest that EGCG downregulates the FGF-2-stimulated activation of both p44/p42 MAP kinase and p38 MAP kinase. Taking our findings into account, it is most likely that EGCG inhibits FGF-2-stimulated IL-6 synthesis via suppression of the p44/p42 MAP kinase pathway and the p38 MAP kinase pathway in osteoblast-like MC3T3-E1 cells. We recently reported that EGCG suppresses endothelin-1-stimulated IL-6 synthesis via suppression of the p44/p42 MAP kinase pathway in osteoblasts [26]. However, EGCG shows little effect on the p38 MAP kinase pathway stimulated by endothelin-1, which is quite different from that stimulated by FGF-2. Therefore, it is likely that the effects of EGCG on MAP kinases depend upon the species of stimuli. Further investigation is necessary to clarify the detailed mechanism of catechin underlying the suppression of IL-6 synthesis in osteoblasts.

It is generally known that IL-6, which is synthesized from osteoblasts, regulates a variety of bone cell functions [10]. In bone metabolism, IL-6 secreted from osteoblasts acts as an autocrine/paracrine factor, which induces osteoclast formation and stimulates its bone-resorption activity [11, 12]. On the other hand, it has been reported that catechin exerts an inhibitory effect on bone resorption [4]. Additionally, it was recently shown that catechin increases cell viability of osteoblast-like MC3T3-E1 cells and alkaline phosphatase activity, a marker of the mature osteoblast phenotype, and that apoptosis of these cells is suppressed by catechin [5]. Moreover, catechin reportedly induces apoptotic cell death of osteoclasts [27]. Taking our results into account as a whole, in bone metabolism it is probable that catechin potently antagonizes bone resorption through the suppression of IL-6 synthesis in osteoblasts, resulting in the attenuation of osteoclastogenesis and its bone-resorbing activity, in addition to the induction of osteoclast apoptosis and the reduction of osteoblast apoptosis. In the present study, we found that EGCG suppresses FGF-2-stimulated IL-6 synthesis at a dose over 3 μM. It has been reported that the pharmacokinetics of EGCG in human volunteers taking a single dosage of 1600 mg/day shows a rapid absorption with a maximum plasma concentration value of 11.08 μM and that the time to reach maximum plasma concentration is 2.2 hours, with the terminal elimination half-life ranged between 1.9 and 4.6 hours [28]. It seems that the concentration of EGCG used in the present *in vitro* study is likely to be achievable *in vivo*. Therefore, it is possible that intake of catechin-containing beverages such as green tea could prevent the progression of postmenopausal osteoporosis. Further investigation is required to elucidate the exact role of catechin in bone metabolism.

In conclusion, our present results strongly suggest that catechin inhibits the FGF-2-stimulated synthesis of IL-6 at least partly via suppression of the p44/p42 MAP kinase pathway and the p38 MAP kinase pathway in osteoblasts.

Acknowledgments

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References

1 *Jankun J, Selman SH, Swiercz R, Skrzypczak-Jankun E.* Why drinking green tea could prevent cancer. Nature 1997; 387: 561
2 *Harbourne JB, Williams CA.* Advances in flavonoid research since 1992. Phytochemistry 2000; 55: 481–504
3 *Nijweide PJ, Burger EH, Feyen JHM.* Cells of bone: proliferation, differentiation, and humoral regulation. Physiol Rev 1986; 66: 855–886
4 *Delaisse JM, Eeckhout Y, Vaes G.* Inhibition of bone resorption in culture by (+)-catechin. Biochem Pharmacol 1986; 35: 3091–3094
5 *Choi E-M, Hwang J-K.* Effects of (+)-catechin on the function of osteoblastic cells. Biol Pharm Bull 2003; 26: 523–526
6 *Vali B, Rao LG, El-Sohemy A.* Epigallocatechin-3-gallate increases the formation of mineralized bone nodules by human osteoblast-like cells. J Nutr Biochem 2007; 18: 341–347
7 *Tokuda H, Takai S, Matsushima-Nishiwaki R, Akamatsu S, Hanai Y, Hosoi T, Harada A, Ohta T, Kozawa O.* (−)-Epigallocatechin gallate enhances prostaglandin $F_{2\alpha}$-induced VEGF synthesis via up-regulating SAPK/JNK activation in osteoblasts. J Cell Biochem 2007; 100: 1146–1153
8 *Akira S, Taga T, Kishimoto T.* Interleukin-6 in biology and medicine. Adv Immunol 1993; 54: 1–78
9 *Heymann D, Rousselle AV.* gp130 Cytokine family and bone cells. Cytokine 2000; 12: 1455–1468
10 *Kwan Tat S, Padrines M, Theoleyre S, Heymann D, Fortun Y.* IL-6, RANKL, FGF-2-alpha/IL-1: interrelations in bone resorption pathophysiology. Cytokine Growth Factor Rev 2004; 15: 49–60
11 *Blair HC, Robinson LJ, Zaidi M.* Osteoclast signalling pathways. Biochem Biophys Res Commun 2005; 328: 728–738
12 *Ishimi T, Miyaura C, Jin CH, Akatsu T, Abe E, Nakamura Y, Yamaguchi Y, Yoshiki S, Matsuda T, Hirano T, Kishimoto T, Suda T.* IL-6 is produced by osteoblasts and induces bone resorption. J Immunol 1990; 145: 3297–3303
13 *Helle M, Brakenhoff JPJ, Groot ER De, Aarden LA.* Interleukin-6 is involved in interleukin-1-induced activities. Eur J Immunol 1988; 18: 957–959
14 *Littlewood AJ, Russil J, Harvey GR, Hughes DE, Russel RGG, Gowen M.* The modulation of the expression of IL-6 and its receptor in human osteoblasts in vitro. Endocrinology 1991; 129: 1513–1520
15 *Roodman GD.* Interleukin-6: an osteotropic factor? J Bone Miner Res 1992; 7: 475–478
16 *Kozawa O, Tokuda H, Matsuno H, Uematsu T.* Involvement of p38 mitogen-activated protein kinase in basic fibroblast growth factor-induced interleukin-6 synthesis in osteoblasts. J Cell Biochem 1999; 74: 479–485
17 *Kyriakis JM, Avruch J.* Mammarian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev 2001; 81: 807–869
18 *Sudo H, Kodama H, Amagai Y, Yamamoto S, Kasai S.* In vivo differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol 1983; 96: 191–198
19 *Kozawa O, Suzuki A, Tokuda H, Uematsu T.* Prostaglandin $F_{2\alpha}$ stimulates interleukin-6 via activation of PKC in osteoblast-like cells. Am J Physiol 1997; 272: E208–E211
20 *Laemmli UK.* Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680–685
21 *Kato K, Ito H, Hasegawa K, Inaguma Y, Kozawa O, Asano T.* Modulation of the stress-induced synthesis of hsp27 and αB-crystallin by cyclic AMP in C6 glioma cells. J Neurochem 1996; 66: 946–950
22 *Tokuda H, Kozawa O, Uematsu T.* Basic fibroblast growth factor stimulates vascular endothelial growth factor synthesis in osteoblasts: divergent regulation by p42/p44 MAP kinase and p38 MAP kinase. J Bone Miner Res 2000; 15: 2371–2379
23 *Tokuda H, Hirade K, Wang X, Oiso Y, Kozawa O.* Involvement of SAPK/JNK in basic fibroblast growth factor-induced VEGF release in osteoblasts. J Endocrinol 2003; 177: 101–107
24 *Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR.* PD98059 is a specific inhibitor of the activation of mitogen-activated protein kinase in vitro and in vivo. J Biol Chem 1995; 270: 27489–27494
25 *Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW.* SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci USA 2001; 98: 13681–13686
26 *Tokuda H, Takai S, Hanai Y, Matsushima-Nishiwaki R, Hosoi T, Harada A, Ohta T, Kozawa O.* (−)-Epigallocatechin gallate suppresses endothelin-1-induced interleukin-6 synthesis in osteoblasts: Inhibition of p44/p42 MAP kinase activation. FEBS Lett 2007; 581: 1311–1316
27 *Nakagawa H, Wachi M, Woo JT, Kato M, Kasai S, Takahashi F, Lee IS, Nagai K.* Fenton reaction is primarily involved in a mechanism of (−)-epigallocatechin-3-gallate to induce osteoclastic cell death. Biochem Biophys Res Commun 2002; 292: 94–101
28 *Ullmann U, Haller J, Decourt JP, Girault N, Girault J, Kichard-Caudron AS, Pinceau B, Weber P.* A single ascending dose study of epigalocatechin gallate in healthy volunteers. J Int Med Res 2003; 31: 88–101

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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

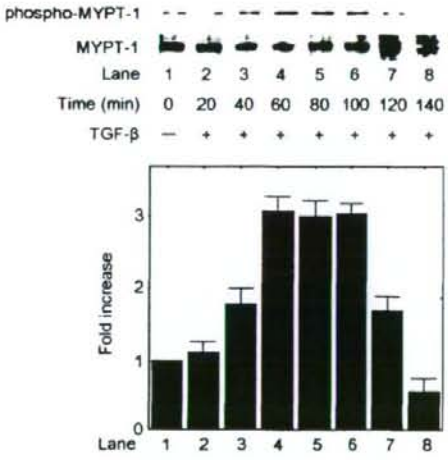

Fig. 1 – Effect of TGF-β on the phosphorylation of MYPT-1 in MC3T3-E1 cells. The cultured cells were stimulated by 3 ng/ml TGF-β for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific MYPT-1 or MYPT-1. 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.

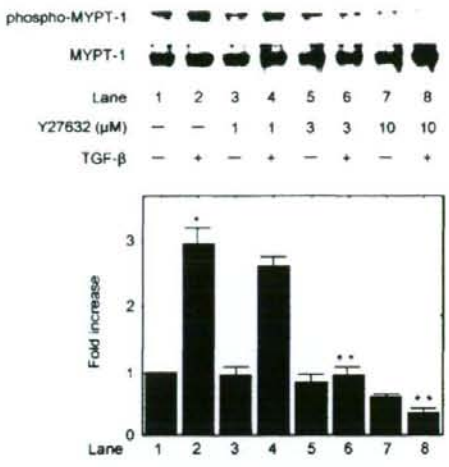

Fig. 2 – Effect of Y27632 on the TGF-β-induced phosphorylation of MYPT-1 in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 for 60 min, and then stimulated by 3 ng/ml TGF-β or vehicle for 60 min. The histogram shows quantitative representations of the levels of TGF-β-induced 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.

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).

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

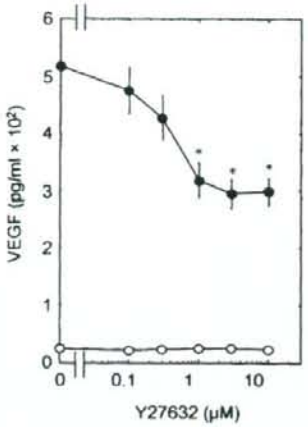

Fig. 3 – Effect of Y27632 on the TGF-β-induced VEGF synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 48 h. Each value represents the mean ± S.D. of triplicate independent determinations. Similar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

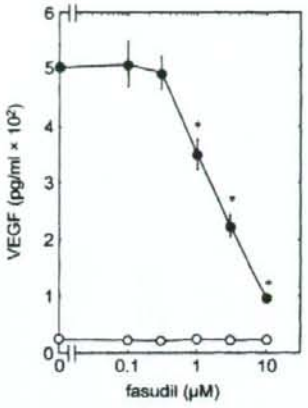

Fig. 4 – Effect of fasudil on the TGF-β-induced VEGF synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of fasudil for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 48 h. Each value represents the mean ± S.D. of triplicate independent determinations. Similar 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

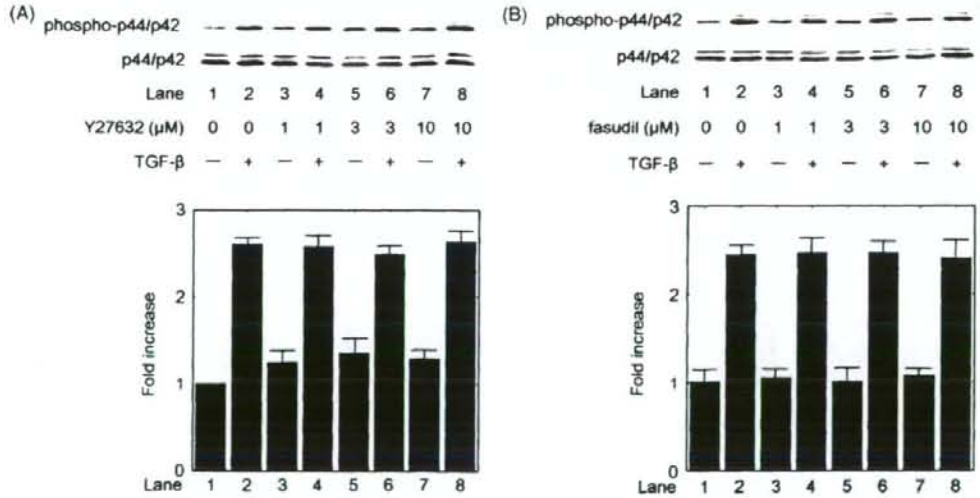

Fig. 5 – Effects of Y27632 or fasudil on the TGF-β-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 (A) or fasudil (B) for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 120 min. The histogram shows quantitative 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.

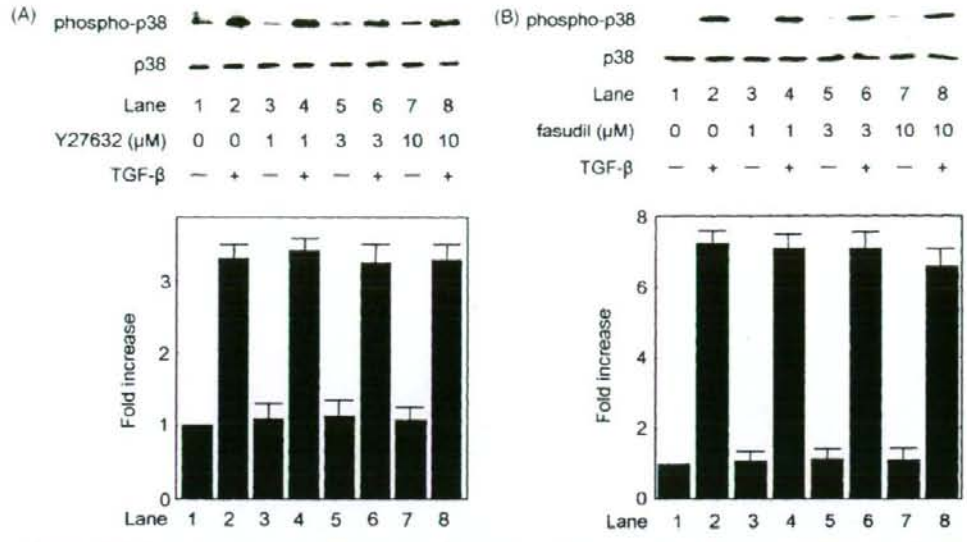

Fig. 6 – Effects of Y27632 or fasudil on the TGF-β-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 (A) or fasudil (B) for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 120 min. The histogram shows quantitative 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.

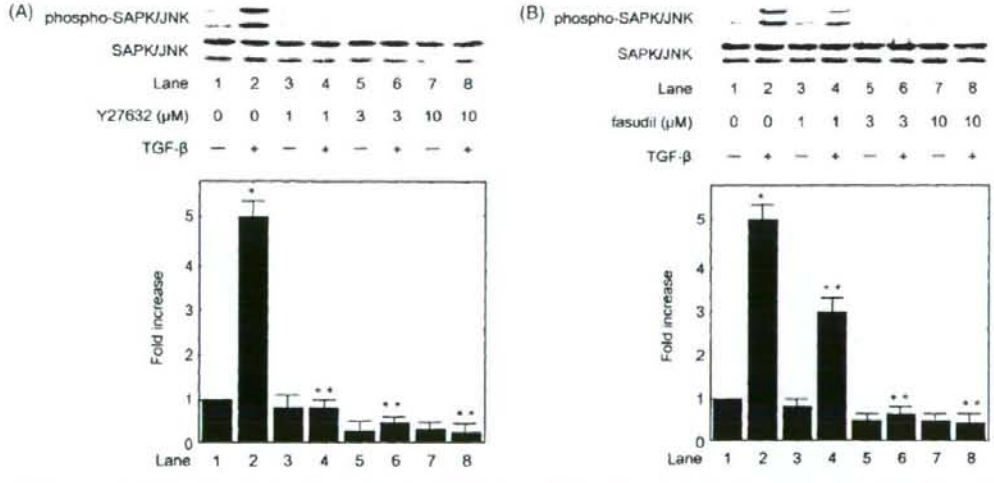

Fig. 7 – Effects of Y27632 or fasudil on the TGF-β-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 (A) or fasudil (B) for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 120 min. The histogram shows quantitative representations 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.

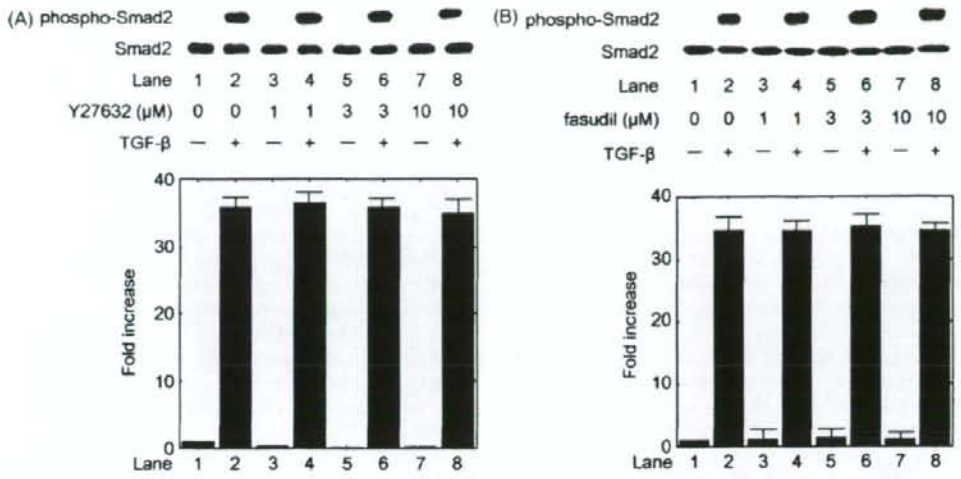

Fig. 8 – Effects of Y27632 or fasudil on the TGF-β-induced phosphorylation of Smad2 in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 (A) or fasudil (B) for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 120 min. The histogram shows quantitative representations 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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REFERENCES

[1] Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 2004;25:581–611.
[2] Nijweide PJ, Burger EH, Feyen JHM. Cells of bone: proliferation, differentiation, and hormonal regulation. Physiol Rev 1986;86:855–86.
[3] Erlebacher A, Filvaroff EH, Gitelman SE, Derynck R. Toward a molecular understanding of skeletal development. Cell 1995;80:371–8.
[4] Zelzer E, Olsen BR. Multiple roles of vascular endothelial growth factor (VEGF) in skeletal development, growth, and repair. Curr Top Dev Biol 2005;65:169–87.
[5] Tokuda H, Hatakeyama D, Akamatsu S, Tanabe K, Yoshida M, Shibata T, et al. Involvement of MAP kinases in TGF-β-stimulated vascular endothelial growth factor synthesis in osteoblasts. Arch Biochem Biophys 2003;415:117–25.

[6] Kanno Y, Ishisaki A, Yoshida M, Tokuda H, Numata O, Kozawa O. SAPK/JNK plays a role in transforming growth factor-β-induced VEGF synthesis in osteoblasts. Horm Metab Res 2005;37:140–5.
[7] Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev 2001;81:807–69.
[8] Fukata Y, Amano M, Kaibuchi K. Rho-Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells. Trends Pharmacol Sci 2001;22:32–9.
[9] Riento K, Ridley AJ. Rocks: multifunctional kinases in cell behaviour. Nat Rev Mol Cell Biol 2003;4:446–56.
[10] Shimokawa H, Rashid M. Development of Rho-kinase inhibitors for cardiovascular medicine. Trends Pharmacol Sci 2007;28:296–302.
[11] Windischhofer W, Zach D, Fauler G, Raspotnig G, Kofeler H, Leis HJ. Involvement of Rho and p38 MAPK in endothelin-1-induced expression of PGHS-2 mRNA in osteoblast-like cells. J Bone Miner Res 2002;17:1774–84.
[12] Harmey D, Stenbeck G, Nobes CD, Lax AJ, Grigoriadis AE. Regulation of osteoblast differentiation by *Pasteurella multocida* toxin (PMT): a role for Rho GTPase in bone formation. J Bone Miner Res 2004;19:661–70.
[13] Tokuda H, Hanai Y, Matsushima-Nishiwaki R, Yamauchi J, Doi T, Harada A, et al. Rho-kinase regulates endothelin-1-stimulated IL-6 synthesis via p38 MAP kinase in osteoblasts. Biochem Biophys Res Commun 2007;362:799–804.
[14] Sudo H, Kodama H, Amagai Y, Yamamoto S, Kasai S. In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol 1983;96:191–8.
[15] Kozawa O, Tokuda H, Miwa M, Kotoyori J, Oiso Y. Cross-talk regulation between cyclic AMP production and phosphoinositide hydrolysis induced by prostaglandin E_2 in osteoblast-like cells. Exp Cell Res 1992;198:130–4.
[16] Kato K, Ito H, Hasegawa K, Inaguma Y, Kozawa O, Asano T. Modulation of the stress-induced synthesis of hsp27 and alpha B-crystallin by cyclic AMP in C6 rat glioma cells. J Neurochem 1996;66:946–50.
[17] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[18] Ito M, Nakano T, Erdodi F, Hartshorne DJ. Myosin phosphatase: structure, regulation and function. Mol Cell Biochem 2004;259:197–209.
[19] Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev 1999;79:143–80.
[20] Miyazono K, Kusanagi K, Inoue H. Divergence and convergence of TGF-β/BMP signaling. J Cell Physiol 2001;187:265–76.
[21] Moustakas A, Heldin C-H. Non-smad TGF-β signals. J Cell Sci 2005;118:3573–84.
[22] Minamitani C, Otsuka T, Takai S, Matsushima-Nishiwaki R, Adachi S, Hanai Y, et al. Involvement of Rho-kinase in prostaglandin $F_{2\alpha}$-stimulated interleukin-6 synthesis via p38 mitogen-activated protein kinase in osteoblasts. Mol Cell Endocrinol 2008;291:27–31.
[23] Davies S, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 2000;351:95–105.
[24] Eto M, Kitagawa T, Yazawa M, Mukai H, Ono Y, Brautigan. et al. Histamine-induced vasoconstriction involves phosphorylation of a specific inhibitor protein for myosin phosphatase by protein kinase Cα and δ isoforms. J Biol Chem 2001;276:29072–8.
[25] Bonewald LF. Tranforming growth factor-β. In: Bilezikian JP, Raisz LG, Rodan GA, editors. Principles of bone biology. 2nd ed., San Diego: Academic Press; 2002. p. 903–18.

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RELATION OF FALLS EFFICACY SCALE (FES) TO QUALITY OF LIFE AMONG NURSING HOME FEMALE RESIDENTS WITH COMPARATIVELY INTACT COGNITIVE FUNCTION IN JAPAN

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ABSTRACT

The purpose of this study was to investigate the relation of the Falls Efficacy Scale (FES) to quality of life (QOL) among nursing home residents. The subjects were 133 institutionalized women aged 70 years or older. They had comparatively intact cognitive function, with a Mini-Mental State Examination (MMSE) score of 15 or more, and could provide sufficient informed consent for a questionnaire survey. We evaluated their age, height, weight, body-mass index, history of hip fracture, history of fall(s) within the past year, complicating conditions, MMSE, Medical Outcomes Study 8-Item Short-Form Health Survey (SF-8), FES, and their subscores for Functional Independence Measure (FIM) motor items (self care, sphincter control, transfer, locomotion). There was a significant relationship between the Physical Component Summary (PCS) of SF-8 and FES. In each subscale, FES showed significant relations that were especially close in physical functioning (PF) and role physical (RP), with those relations proving stronger than those of the subscores of transfer and locomotion. In conclusion, the present results suggested that taking account of mental confidence is important for physical QOL, and that falls self-efficacy, including not only physical activity per se but also mental confidence, should be given prominence in the physical QOL of the institutionalized elderly.

Key Words: Falls Efficacy Scale, Fear of falling, Quality of life, Institutionalized elderly

INTRODUCTION

Although people live longer as a result of advances in economic development and medicine, a greater proportion of the population in aging societies is afflicted with chronic disease. Improving quality of life (QOL) through various interventions is thus a worthy goal. Efforts to

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prevent falls and fall-related trauma are one way to accomplish this goal. Falls and fractures are the third leading cause of the need for care in Japan, and this trend is particularly marked in elderly women.[1] Falls and fractures tend to turn "mobile" elderly into "immobile" elderly, and while their impact can significantly change QOL, that impact is not limited to the direct physical trauma; there are also long-term psychological effects, such as a fear of falling and depression.[2,3] Fear of falling was defined by Tinetti *et al.*[4] as a level of anxiety associated with falls sufficient to prompt people to avoid certain activities of daily living even though they are capable of performing them. Fear of falling in the elderly also leads to a downward spiral of decreased activity, accelerated deterioration of physical functioning, and a narrower range of activity,[2,5] and overall QOL will also be diminished.

There are two methods of measuring fear of falling: asking people directly about their fear, and the use of falls self-efficacy. The latter is represented by the Falls Efficacy Scale (FES),[6] which is a method of assessment that was developed based on the self-efficacy theory proposed by Bandura.[7] Although the method of asking directly about fear of falling is a simple one, neither its reliability nor validity has been sufficiently established. On the other hand, FES has proved to be both reliable and valid.[8] There have been studies on the relation between FES and QOL in the community-dwelling elderly.[9,10] Falls tend to occur more often among elderly people in Japan living in nursing homes (10–40%) than among those still residing in their own community (10–20%).[11] Among the nursing home elderly who experience many falls,[11] the fear of falling is greater,[2] and QOL will predictably be further diminished.

If the relation between fear of falling and QOL is strong, then it may be hoped that interventions to ease fear of falling would contribute to improving QOL. Such interventions among community-dwelling elderly are reportedly effective in the area of motor ability, particularly that which focuses on balance.[12] However, there are only a few reports on fear of falling in the institutionalized elderly[8,12] due to their often deteriorated cognitive function and physical infirmity. In Japan there are only reports dealing with motor functions,[13] but no reports that address the relation between fear of falling and QOL. Therefore, as a first step toward improving QOL through interventions against fear of falling among the institutionalized elderly, we have investigated that relation using the FES, the reliability and validity of which have been adequately demonstrated.

METHODS

Subjects

The subjects for this study were 133 institutionalized female elderly with comparatively intact cognitive function, who had a Mini-Mental State Examination (MMSE) score of 15 or more, and could provide sufficient informed consent for a questionnaire survey. All subjects were participants in a broader clinical trial of hip protectors in nursing homes in Aichi Prefecture, Japan. Inclusion criteria for the clinical trial were: female sex, 70 or more years of age, not bedridden, and with at least 1 risk factor for falls or a hip fracture.[14] Those risk factors were: a history of hip fracture, history of fall(s) in the past year, and complicating conditions that predispose an elderly person to falls or fractures, i.e., heart disease, hypertension, previous stroke, diabetes mellitus, parkinsonism, arrhythmia, epileptic seizure, osteoarthritis, rheumatoid arthritis or a related condition, and eye disease (cataract or glaucoma).

Cross-sectional evaluation items

This cross-sectional analysis was conducted from November 2004 to November 2005. The

cross-sectional evaluation items were age, height, weight, body-mass index (BMI), history of hip fracture, history of fall(s) in the past year, complicating conditions, MMSE,[15] Medical Outcomes Study 8-Item Short-Form Health Survey (SF-8),[16] FES,[6] and motor items on the Functional Independence Measure (FIM).[17]

SF-8—QOL was assessed in an interview using the Japanese version of the SF-8,[16] which is a shorter version of the SF-36 and is used as a comprehensive and multidisciplinary measure of health status. The Physical Component Summary (PCS) and Mental Component Summary (MCS) were calculated using eight subscales: physical functioning (PF), role physical (RP), bodily pain (BP), general health perception (GH), vitality (VT), social functioning (SF), role emotional (RE), and mental health (MH). It was reported that PF, RP, BP and GH showed a strong relation to PCS, and that SF, RE, and MH evidenced a strong relation to MCS. As for VT, it shows a medium relation to both PCS and MCS. The reliability of the eight subscales of the Japanese version of the SF-8 is reportedly 0.56–0.87, while that of PCS is 0.77 and that of MCS 0.73.[16]

Falls Efficacy Scale (FES)—The FES was designed to assess the degree of perceived efficacy at avoiding a fall during each of 10 relatively non-hazardous activities of daily living (Taking a bath or shower, Reaching into cabinets or closets, Preparing meals that do not require carrying heavy or hot objects, Walking around the house, Getting in and out of bed, Answering the door or telephone, Getting in and out of a chair, Getting dressed and undressed, Light housekeeping, and Simple shopping).[6] Each response was scored on a scale of 1 (completely confident) to 10 (no confidence), with a high score (possible total point range 10–100) indicating low falls self-efficacy. The internal consistency was reported to be 0.90 (Cronbach's α),[18] and the reliability 0.71 (Pearson's correlation coefficient).[6] However, since the present study was conducted with nursing home residents as subjects, the items used were arranged to correspond to ADL in a nursing home setting: walking around the house was equated with participant walking in the vicinity of the bed, light housekeeping with cleaning around the bed, and simple shopping as at stores or stands on the nursing home premises. In order to ascertain the influence of this modification, nine participants (mean age 85.2 years) were retested after 2 weeks, and internal consistency or reliability was confirmed (Cronbach's α=0.91, Pearson's correlation coefficient = 0.72, $p = 0.03$).

FIM motor items—ADL was evaluated using FIM motor items[17] comprised of 6 self care activities (eating, grooming, bathing, dressing (upper body), dressing (lower body), toileting), 2 sphincter control items (bladder management, bowel management), 3 transfer items (transfers to bed/chair/wheelchair, to toilet, and to tub or shower), and 2 locomotion items (ambulation, stairs). Four subscores (self care, sphincter control, transfer, locomotion) were calculated. Each item was graded from fully assisted (1 point) to completely independent (7 points). In the present study, only ambulation was judged, although ambulation or wheelchair movement indoors was judged in the original method.[17]

Statistical methods

The SPSS 14.0 program was used for all statistical analyses, with less than 0.05 as the level of significance. Dependent variables were PCS, MCS, and the subscales. First, we examined the correlation between dependent variables and other variables [FES, age, BMI, history of hip fracture, history of fall(s) in the past year, total number of complicating conditions, MMSE, and the subscores for FIM motor items (self care, sphincter control, transfer, and locomotion)] using Spearman's rank correlation coefficient (rho). Next, after adding significant variables to the correlation analysis and age to the multiple regression analysis (method of all possible combinations) with FES as explanatory variables, we calculated the standardized partial regression coefficient

(β) to investigate the strength of the relation between FES and QOL.

As a secondary analysis, to determine the influence of past falls on QOL, a similar multiple regression analysis was conducted with PCS and MCS as dependent variables for two groups, one with 60 subjects and one without 73 subjects falls in the past year.

Ethical considerations

All participants gave written informed consent, and their names were coded from the start of the study through data collection and analysis so that no single individual could be identified. This study was approved by the Ethics Committees of both the Nagoya University School of Health Sciences and the National Center for Geriatrics and Gerontology.

RESULTS

Informed consent to participate in the hip protector clinical trial was obtained from 342 women in 35 nursing homes. However, 7 later refused to participate, 12 left the nursing home in which they were living before the cross-sectional evaluation, 135 had MMSE scores of 15 or less, and 55, even though their MMSE was above 15, lacked sufficient cognitive ability to provide informed consent for surveys using questionnaires. The present study was therefore conducted with the remaining 133 subjects.

The attributes of all 133 subjects were shown in Table 1. As for the results of correlation analysis, PCS showed significant correlations with FES, the total number of complicating conditions, MMSE, the subscore of transfer, and locomotion. Moreover, all SF-8 subscales and FES were significantly correlated, and MH was significantly correlated with BMI (Table 2). Table 3 shows the results of multiple regression analysis. PCS and FES showed a significant relation, while MCS did not. In each subscale, all subscales and FES showed significant relations; these were especially close between PF and RP, and were stronger than those for the transfer and locomotion subscores.

In a secondary analysis, the relation of FES to PCS in the group that had fallen in the past year was slightly weaker than in the group that had not done so (β of fall group= –0.35 vs. β of no-fall group= –0.38).

DISCUSSION

In the present study, the subjects were 133 institutionalized female elderly with a comparatively intact cognitive function. Because so many elderly nursing home residents suffer a diminished cognitive function, it can be difficult to select participants for surveys using questionnaires. Our subjects were women who scored 15 or higher on MMSE, since it was reported that "for patients with of MMSE 15, test-retest coefficients were better (range 0.53–0.90)" in the SF-36.[19] Of the total 133 subjects, 45.1% had experienced a fall within the past year. A high-risk group with such a high incidence of falling is predicted to have a greater fear of falling than elderly people living at home,[2] which further decreases their QOL. However, since the relation of FES to QOL in a high-risk fall group has not been investigated, we made it the subject of the present study.

The mean FES of nursing home elderly was 45.0 ± 22.3, against the 18.56 ± 9.04 of those reported still residing in the community or in intermediate care facilities.[6] That result was in line with our prediction that the falls self-efficacy of the institutionalized elderly would be lower than that for those still residents of a community (the lower the falls self-efficacy is, the higher