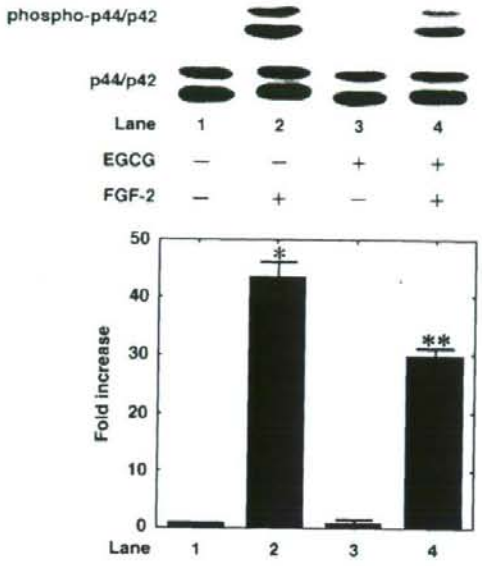

Fig. 3 Effect of EGCG on the phosphorylation of p44/p42 MAP kinase induced by FGF-2 in MC3T3-E1 cells. The cultured cells were pretreated with 100 μM EGCG or vehicle for 60 min and then stimulated by 70 ng/ml FGF-2 or vehicle for 3 min. The extracts of cells were subjected to SDS-PAGE with subsequent 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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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

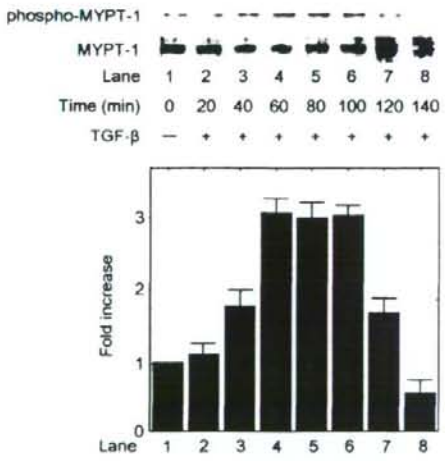

Fig. 1 – Effect of TGF-β on the phosphorylation of MYPT-1 in MC3T3-E1 cells. The cultured cells were stimulated by 3 ng/ml TGF-β for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific MYPT-1 or MYPT-1.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

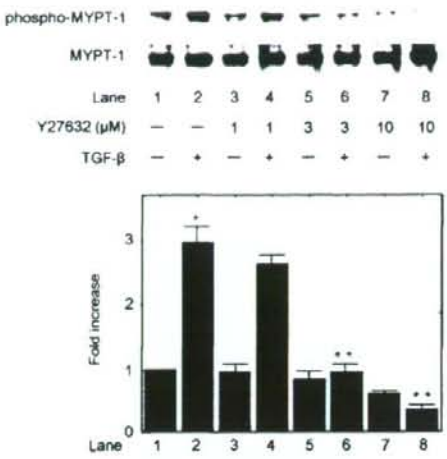

Fig. 2 – Effect of Y27632 on the TGF-β-induced phosphorylation of MYPT-1 in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 for 60 min, and then stimulated by 3 ng/ml TGF-β or vehicle for 60 min. The histogram shows quantitative representations of the levels of TGF-β-induced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

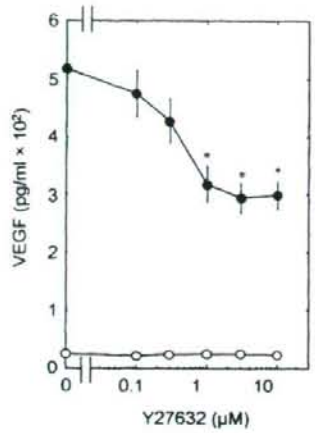

Fig. 3 – Effect of Y27632 on the TGF-β-induced VEGF synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 48 h. Each value represents the mean ± S.D. of triplicate independent determinations. Similar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

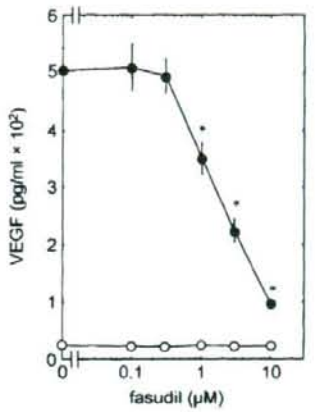

Fig. 4 – Effect of fasudil on the TGF-β-induced VEGF synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of fasudil for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 48 h. Each value represents the mean ± S.D. of triplicate independent determinations. Similar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

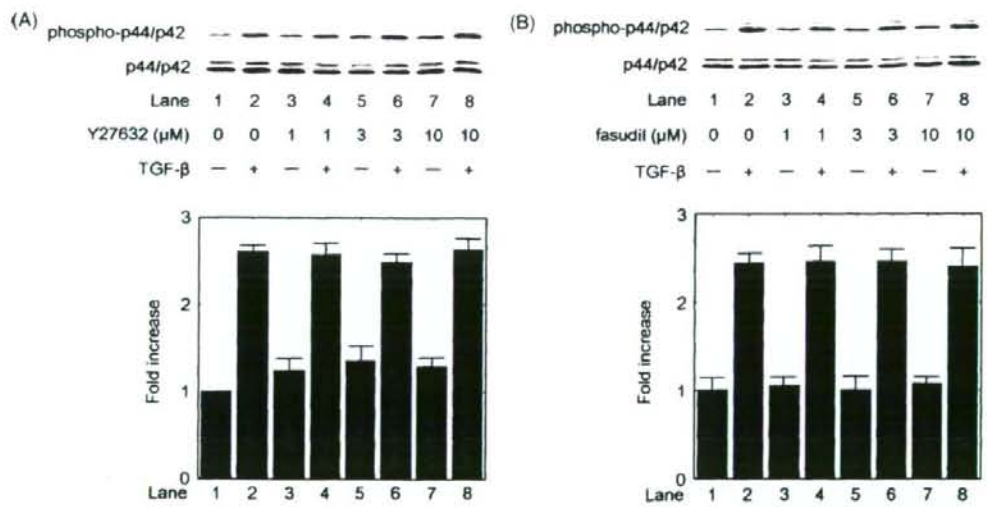

Fig. 5 – Effects of Y27632 or fasudil on the TGF-β-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 (A) or fasudil (B) for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 120 min. The histogram shows quantitative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

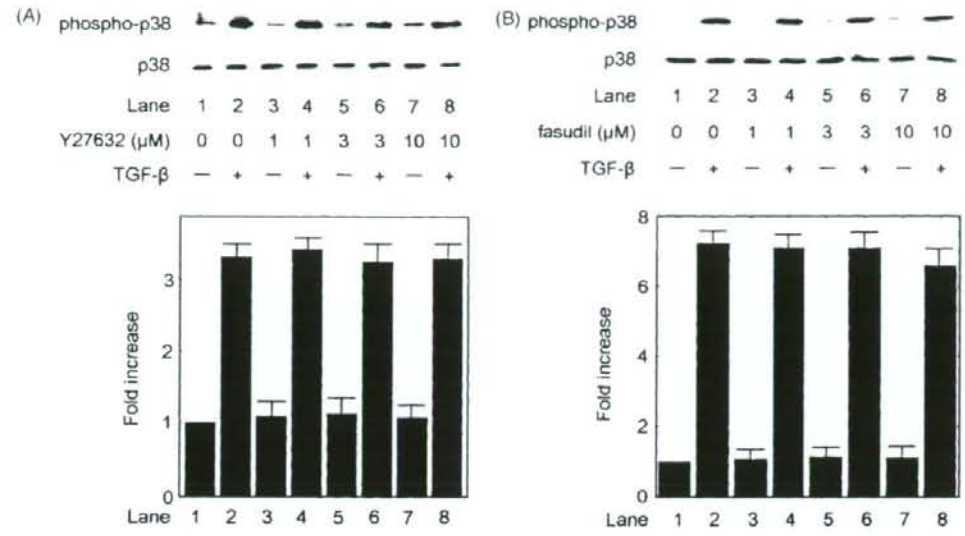

Fig. 6 – Effects of Y27632 or fasudil on the TGF-β-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 (A) or fasudil (B) for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 120 min. The histogram shows quantitative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

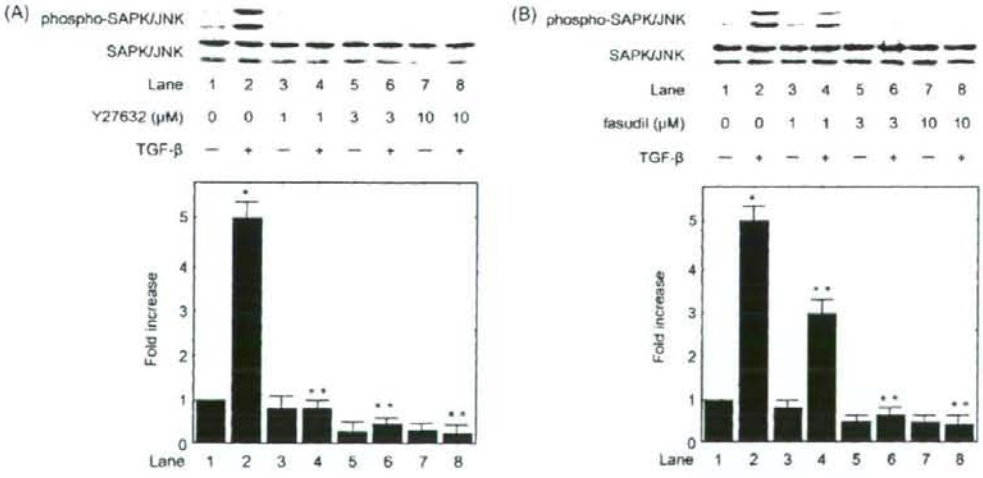

Fig. 7 – Effects of Y27632 or fasudil on the TGF-β-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 (A) or fasudil (B) for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 120 min. The histogram shows quantitative representations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

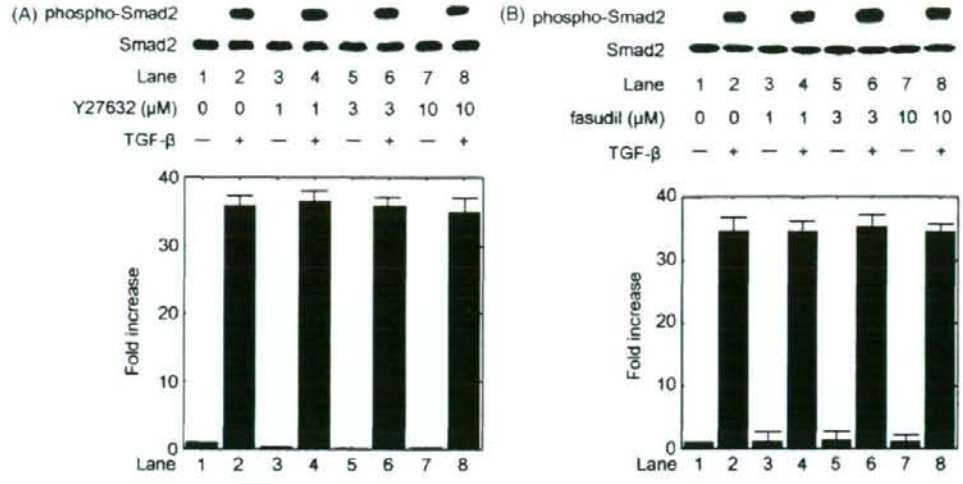

Fig. 8 – Effects of Y27632 or fasudil on the TGF-β-induced phosphorylation of Smad2 in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 (A) or fasudil (B) for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 120 min. The histogram shows quantitative representations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

Table 1 Attributes of all 133 subjects.

Attribute	Mean	SD or (%)
Age	85.6	6.1
Height (cm)	145.0	7.2
Weight (kg)	44.4	8.3
BMI	21.1	3.6
History of hip fracture		(29.3)
Fall(s) in past year		(45.1)
Complicating conditions		
Heart disease		(25.6)
Hypertension		(47.4)
Previous stroke		(40.6)
Diabetes mellitus		(16.5)
Parkinsonism		(6.8)
Arrhythmia		(2.3)
Epileptic seizure		(0.8)
Osteoarthritis		(21.1)
Rheumatoid arthritis or related condition		(3.0)
Eye disease (cataract or glaucoma)		(27.8)
Total number of complicating conditions	1.9	1.1
MMSE (range: 0–30)	22.3	4.4
SF-8		
Physical Component Summary (PCS)	41.4	10.8
Mental Component Summary (MCS)	50.1	8.4
Physical functioning (PF)	42.3	12.0
Role physical (RP)	41.7	12.6
Bodily pain (BP)	46.2	10.7
General health perception (GH)	47.5	7.4
Vitality (VT)	48.6	7.4
Social functioning (SF)	48.2	8.8
Role emotional (RE)	47.0	10.7
Mental health (MH)	48.7	7.9
FES (range:10-100)	45.0	22.3
FIM motor items		
Subscore of self-care (range: 6–42)	33.0	7.6
Subscore of sphincter control (range: 2–14)	11.2	3.2
Subscore of transfer (range: 3–21)	15.7	4.2
Subscore of locomotion (range: 2–14)	7.0	3.6

SD = standard deviation; BMI = Body-mass index;
MMSE = Mini-Mental State Examination;
SF-8 = MOS 8-Item Short-Form Health Survey;
FES = Falls Efficacy Scale; FIM = Functional Independence Measure.

Table 2 Spearman's rank correlation coefficient (rho) between PCS, MCS, subscales and other variables.

	PCS	MCS	PF	RP	BP	GH	VT	SF	RE	MH
FES	−0.50*	−0.08	−0.53*	−0.51*	−0.31*	−0.23*	−0.32*	−0.25*	−0.21*	−0.27*
Age	0.13	−0.08	0.14	0.13	0.07	−0.02	−0.10	0.07	0.01	0.01
BMI	0.05	0.07	0.08	0.00	0.10	0.05	0.07	0.06	−0.03	0.20*
History of hip fracture	0.06	−0.11	−0.03	0.04	0.08	−0.01	0.02	−0.03	−0.00	−0.16
Fall(s) in past year	−0.06	−0.11	−0.07	−0.14	−0.05	−0.03	−0.07	−0.11	−0.11	−0.08
Total number of complicating conditions	−0.20*	0.07	−0.08	−0.17	−0.21*	−0.10	−0.02	−0.16	−0.02	0.01
MMSE	−0.25*	0.10	−0.20*	−0.14	−0.24*	−0.09	−0.04	−0.15	0.05	−0.04
Subscore of self care	0.07	0.12	0.09	0.13	−0.03	−0.01	0.16	0.03	0.15	0.09
Subscore of sphincter control	0.04	0.03	0.04	0.07	−0.13	−0.02	0.05	−0.01	0.06	0.01
Subscore of transfer	0.18*	0.09	0.19*	0.23*	0.07	0.08	0.18*	0.02	0.13	0.16
Subscore of locomotion	0.27*	0.09	0.29*	0.37*	0.14	0.02	0.18*	0.12	0.21*	0.19*

FES = Falls Efficacy Scale; BMI = Body-mass index; MMSE = Mini-Mental State Examination.
*$p<0.05$

Table 3 Standardized partial regression coefficient (β) for PCS, MCS, and subscales as dependent variables by multivariate regression analysis.

	PCS	MCS	PF	RP	BP	GH	VT	SF	RE	MH
FES	−0.42*	−0.12	−0.42*	−0.42*	−0.27*	−0.25*	−0.30*	−0.24*	−0.27*	−0.27*
Age	0.08	−0.06	0.10	0.06	0.07	−0.04	−0.11	0.07	−0.04	0.01
BMI	0.06	0.07	0.07	0.02	0.15	0.05	0.05	0.11	−0.02	0.18*
Total number of complicating conditions	−0.13	−0.00	−0.03	−0.08	−0.19*	−0.13	−0.03	−0.16	−0.05	0.01
MMSE	−0.13	0.11	−0.08	−0.04	−0.17	−0.02	0.01	−0.05	0.11	−0.00
Subscore of transfer	0.04	0.01	0.04	0.05	−0.00	0.09	0.08	−0.05	−0.01	0.08
Subscore of locomotion	0.14	0.01	0.19	0.21*	0.02	−0.12	0.04	0.04	0.14	0.04
R^2	0.33	0.03	0.33	0.33	0.19	0.09	0.13	0.11	0.12	0.15

FES = Falls Efficacy Scale; BMI = Body-mass index; MMSE = Mini-Mental State Examination.
*$p<0.05$

the FES score).

Among the community-dwelling elderly, FES showed a significant relation to PCS,[10] with PF showing an especially high correlation in each subscale, followed by SF, BP, VT, and RP.[9] This study suggested that among the institutionalized elderly, similar to the community-dwelling elderly, FES was significantly related to PCS, and that among the subscales the relation was especially strong with PF and RP.

The relation of FES to PF and RP, as items related to physical QOL, was stronger than the relations of the transfer or locomotion subscores. It was previously reported that there is a strong relation between PF and transfer or locomotion ability.[20] So, in people such as the institutionalized elderly whose physical ability had clearly deteriorated, it was predicted that the transfer or locomotion subscores might strongly relate to PF and RP rather than FES. Interestingly, the relation of FES to PF and RP was stronger than the relations of either transfer or locomotion subscores. The FES is based on both physical ability judged by disease/disability and by mental confidence (self-efficacy),[6] with the latter being affected by four main information sources: "enactive mastery experience," "vicarious experience," "verbal persuasion," and "physiological and affective states." This information influences mental confidence based on an individual's interpretation.[7] Since some type of care is needed in daily life for many nursing home residents, mental confidence tends to be readily influenced by the way a resident experiences that care. It is reported that interventions against fear of falling are effective among the community-dwelling elderly in the area of motor ability, particularly that which focuses on balance.[12] While it is important to attempt to reduce the fear of falling by improving physical function, it becomes more difficult to improve physical function in elderly people and chronic disease patients in care facilities. Therefore, for elderly care facility residents in particular, (a group with a high risk for falls that includes many people who require some type of care in daily life), considering mental confidence is important for physical QOL. We suggested 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. Although causal relationships could not be determined in this study since it was a cross-sectional analysis, we conjectured that raising falls self-efficacy might contribute to improving physical QOL.

In this study, as a secondary analysis, we conducted a similar multiple regression analysis with PCS and MCS for a group that had fallen in the past year and a group that had not. Friedman *et al.*[21] found that fear of falling is exacerbated by the experience of previous falls. It was predicted that the strength of the relation to PCS in the fall group would be greater than in the no-fall group. However, the relation of FES to PCS in the fall group was slightly weaker than in the no-fall group. Factors that have been suggested as related to fear of falling include the importance of life satisfaction[22] and decreased social activity.[10] Fear of falling may be influenced by various other factors in addition to the experience of falling. On the other hand, the possibility cannot be ruled out that FES excessively reflects psychological and social factors, while inadequately reflecting the fear of falling that accompanies falls.

Limitations of the present study include, first, the problem of sensitivity in evaluating QOL. In this study, SF-8, which can readily provide answers in a short time, was used to evaluate QOL. The correlation of the subscale score, which measures the same concept between SF-8 and SF-36, was as high as 0.56–0.87, thus supporting the reliability of SF-8.[16] Nevertheless, the accuracy of SF-8 measurements alone is undeniably inferior to that for SF-36. Next, There were also limits to FES evaluation of the institutionalized elderly in our study. Our subjects did not need to "prepare meals that required carrying heavy or hot objects," which was one of the standard FES items; moreover, there were other items the elderly could not actually perform. They were also asked to respond to the question: "If you try, how confident are you in performing an act

without falling?"[6] However, it is possible that some subjects, not wishing to admit to a "fear of falling," instead addressed the "likelihood of falling." In addition, since being female was a criterion for participation in the hip protector clinical trial, men were not analyzed. Differences between the sexes have been reported in the distribution and factors related to fear of falling,[22] so that the results of this study cannot be extrapolated to all elderly care institution residents.

In conclusion, FES was related to PCS, and that relation was particularly strong for the items of PF and RP, which were related to physical QOL. The strength of that relation was superior to that with the transfer or locomotion subscores. It becomes progressively more difficult to improve physical function in the institutionalized elderly because of their advanced age and chronic diseases. The results of the present study suggested that considering 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. We expect that evidence for the effectiveness of interventions to reduce fear of falling and improve QOL among the nursing home elderly will be forthcoming in the not too distant future.

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高齢者脊椎骨折の入院治療に関する施設特性別全国調査

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