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

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

Introduction

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

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

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

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

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

Materials and methods

Materials. The FGF-2 and mouse VEGF enzyme immunoassay kits were purchased from R&D Systems, Inc. (Minneapolis, MN). Tacrolimus hydrate (FK506) was purchased from Sigma Chemical Co. (St. Louis, MO). Cyclosporine A and SP600125 were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific p70 S6 kinase antibodies and p70 S6 kinase antibodies were purchased from Cell Signaling, Inc. (Beverly, MA). The ECL Western blotting detection system was purchased from Amersham Biosciences (Piscataway, NJ). Other materials and chemicals were obtained from commercial sources. Tacrolimus, cyclosporine A and SP600125 were dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of DMSO was 0.1%, which did not affect the assay for VEGF or the Western blot analysis.

Cell culture. Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria (26) were maintained as previously described (27). Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37˚C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 35-mm ($5x10^4$) or 90-mm ($5x10^5$) diameter dishes in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were then used for experiments after 48 h.

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

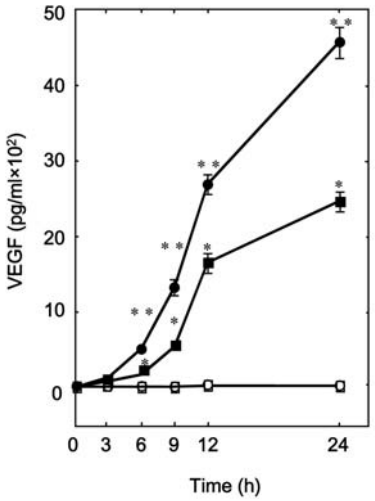

Figure 1. Effect of tacrolimus on the FGF-2-stimulated VEGF release in MC3T3-E1 cells. The cultured cells were pretreated with 300 ng/ml tacrolimus (circle symbols) or vehicle (square symbols) for 60 min, and then stimulated by 70 ng/ml FGF-2 (solid symbols) or vehicle (open symbols) for the indicated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

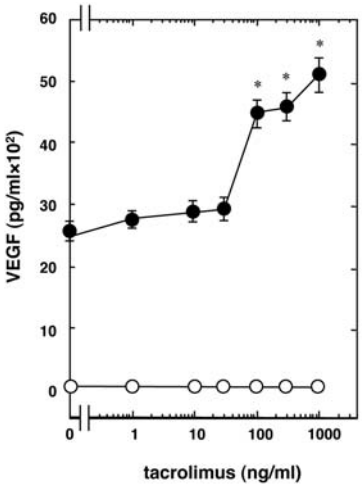

Figure 2. Dose-dependent effect of tacrolimus on the FGF-2-stimulated VEGF release in MC3T3-E1 cells. The cultured cells were pretreated with various doses of tacrolimus for 60 min and then stimulated by 70 ng/ml FGF-2 (●) or vehicle (○) for 24 h. Each value represents the mean ± SEM of triplicate determinations.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

Western blot analysis. The cultured cells were stimulated by FGF-2 in α-MEM containing 0.3% FCS for the indicated periods. The cells were washed twice with phosphate-buffered saline and then lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris/HCl, pH 6.8, 2%

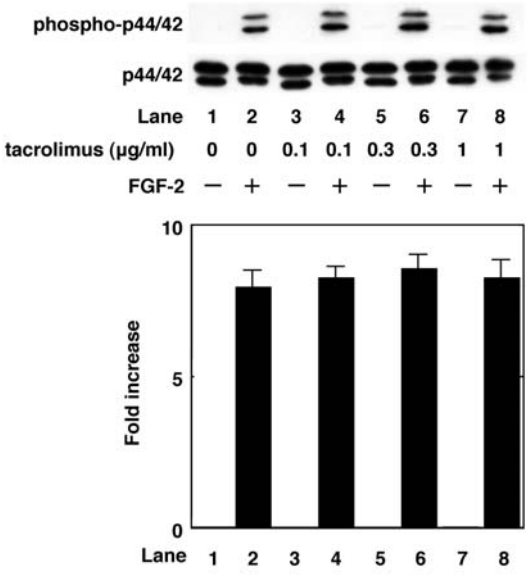

Figure 3. Effects of tacrolimus on the FGF-2-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of tacrolimus for 60 min, and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were analyzed by Western blotting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

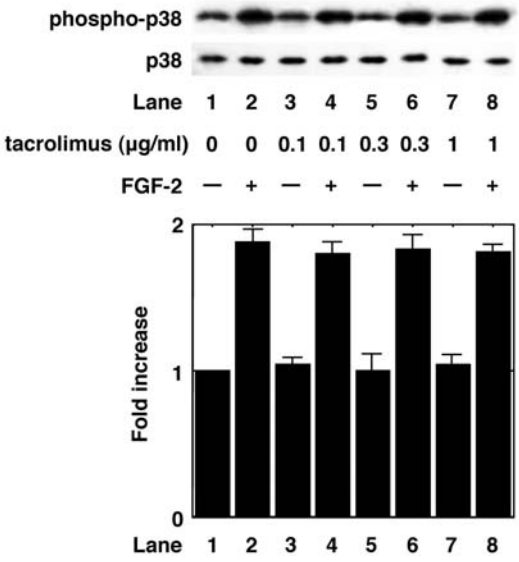

Figure 4. Effects of tacrolimus on the FGF-2-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of tacrolimus for 60 min and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were analyzed by Western blotting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

sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125,000 x g for 10 min at 4˚C. SDS-polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli (28) in 10% polyacrylamide gels. The Western blot analysis was performed as described previously (29) by using phospho-specific p70 S6 kinase antibodies, p70 S6 kinase antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies or SAPK/JNK antibodies, with peroxidase-labeled antibodies raised in goat anti-rabbit IgG being used as second antibodies. The peroxidase activity on the polyvinylidine difluoride membrane was visualized on X-ray film by means of an enhanced chemiluminescence Western blotting detection system.

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

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

Results

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

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

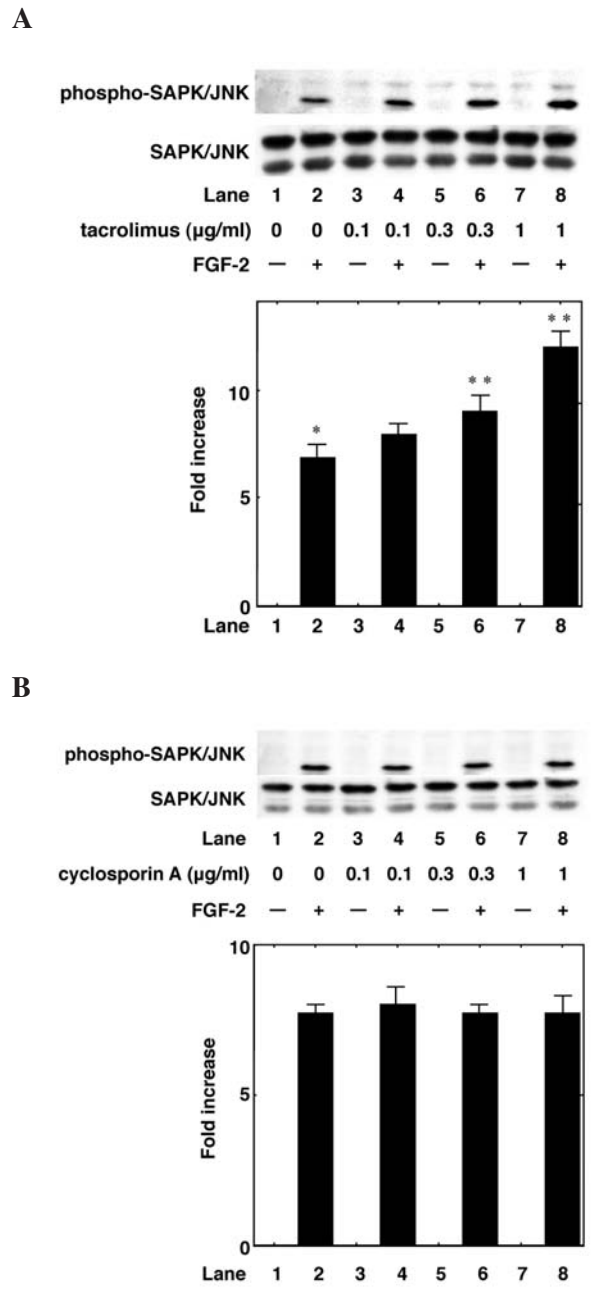

Figure 5. Effects of tacrolimus or cyclosporine A on the FGF-2-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with various doses of (A) tacrolimus or (B) cyclosporine A for 60 min and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells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

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

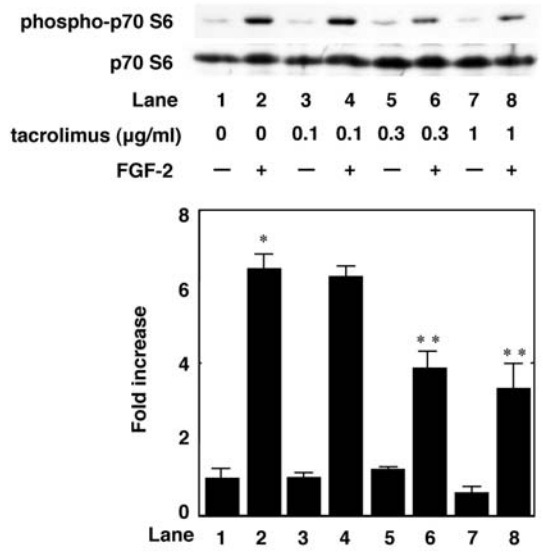

Figure 6. Effect of tacrolimus on the FGF-2-induced phosphorylation of p70 S6 kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of tacrolimus for 60 min, and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were analyzed by Western blotting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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

Acknowledgements

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