本研究の限界は、まず、サンプルサイズ小さいことで、統計学的パワーが低いことがあげられる。次に、介護施設高齢者におけるFES評価の限界である。介護施設ではFESの項目のうち「簡単な食事の用意をする」は実際に行う必要がない。実際に行わない動作は「やろうと思えばどのくらい転ばずに自信をもってできますか」で回答するが、その際に対象者が"Fear of falling"ではなく、"likelihood of falling"を答えていた可能性は否定しきれない。3つ目にQOL評価の感度の問題である。SF-8は短時間で回答でき使用しやすく、SF-36の間で同じ概念を測定する下位尺度得点間の相関も高く、SF-8の信頼性は支持されている。しかし、本研究のような高齢の対象者へのSF-8使用の信頼性が十分確立されているわけではない。また、二次的解析の結果、コントロール群のMMSE20点以下のPCSに有意な変化が認められており、認知機能が低下すると自身の身体的な健康感を問う調査では、結果が変動しやすいかもしれない。

今後は転倒恐怖の分布や関連要因に性差が報告されていることから男性を含めた検討も必要と考えている。

Table 1. Baseline characteristics in hip protectors group ,control group, and all subjects

Characteristic	Hip protectors group (21 subjects in 26 nursing homes)		Control group (21 subjects in 17 nursing homes)		Total		*P* value
	Mean or (No)	SD† or (%)	Mean or (No)	SD† or (%)	Mean or (No)	SD† or (%)	
Age	86.9	6.7	85.3	6.4	86.1	6.5	0.442
Height(cm)	146.6	6.7	142.4	7.2	144.5	7.2	0.056
Weight(kg)	46.6	8.9	44.4	7.2	45.5	8.0	0.384
Body-mass index	21.6	3.6	22.0	3.4	21.8	3.5	0.782
History of hip fracture	(3)	(14.3)	(8)	(38.1)	(11)	(26.2)	0.132
Fall in the past year	(5)	(23.8)	(10)	(47.6)	(15)	(35.7)	0.156
Total number of complicating conditions	1.8	1.2	2.1	1.2	2.0	1.2	0.366
MMSE‡	22.0	4.3	22.3	4.5	22.2	4.4	0.862
FIM§ motor items	71.6	14.8	64.3	9.4	68.0	12.8	0.064
Falls Efficacy Scale(FES)	34.8	24.8	45.0	24.3	39.9	24.8	0.185
SF-8‖							
Physical Component Score (PCS)	44.0	11.2	45.1	10.3	44.6	10.6	0.740
Mental Component Score (MCS)	52.6	6.9	49.5	8.8	51.1	8.0	0.213
Physical Activities¶							
Steps	909.8	597.1	829.9	984.7	878.7	744.0	0.832

*statistically significant with t test or χ^2 test
†standard deviation
‡Mini-Mental State Examination
§Functional Independence Measure
‖MOS 8-Item Short-Form Health Survey
¶Physical Activities は FIM5 以上のヒッププロテクター群 11 名、コントロール群 7 名のみ測定

Table 2. Change in FES, PCS, MCS and steps in hip protector and control groups

	Hip protectors group (21 subjects in 26 nursing homes)								
	Baseline		3 months		6 months		12 months		*P*value
	Mean	SD†	Mean	SD†	Mean	SD†	Mean	SD†	
FES‡	34.8	24.8	41.9	22.9	40.0	21.3	41.7	21.4	0.202
SF-8									
PCS§	44.0	11.2	43.7	11.6	47.6	8.4	47.4	7.7	0.209
MCS‖	52.6	6.9	54.1	6.6	50.8	7.3	53.2	4.8	0.298
Physical Activities¶									
Steps	909.8	597.1	1299.0	1399.5	1022.1	998.2	1091.8	973.8	0.554

	Control group (21 subjects in 17 nursing homes)								
	Baseline		3 months		6 months		12 months		*P*value
	Mean	SD†	Mean	SD†	Mean	SD†	Mean	SD†	
FES‡	45.0	24.3	46.6	18.7	43.5	20.5	47.9	22.0	0.824
SF-8									
PCS§	45.1	10.3	47.5	5.9	44.8	7.8	46.6	5.8	0.526
MCS‖	49.5	8.8	50.7	6.3	51.9	6.2	51.4	6.1	0.536
Physical Activities¶									
Steps	829.9	984.7	758.4	847.5	810.6	1234.8	926.9	1237.9	0.568

*statistically significant with repeated measures ANOVA
†standard deviation
‡Falls Efficacy Scale
§Physical Component Score
‖Mental Component Score
¶Physical Activities は FIM5 以上のヒッププロテクター群 11 名、コントロール群 7 名のみ測定

Table 3 . Change in FES, PCS, MCS and steps in hard type of hip protector and soft type of hip protector

	Hip protectors group: 硬質群 (9 subjects in 14 nursing homes)								
	Baseline		3 months		6 months		12 months		*P* value
	Mean	SD†	Mean	SD†	Mean	SD†	Mean	SD†	
FES‡	42.9	28.7	49.8	22.4	48.0	25.1	43.3	24.8	0.430
SF-8									
PCS§	40.8	12.1	38.1	12.7	43.7	10.5	44.5	8.8	0.434
MCS‖	52.1	7.2	53.0	9.1	50.8	8.4	53.0	4.3	0.882
Physical Activities¶									
Steps	944.5	894.8	722.5	541.4	662.3	556.6	630.5	256.9	0.819

	Hip protectors group: 軟質群 (12 subjects in 12 nursing homes)								
	Baseline		3 months		6 months		12 months		*P* value
	Mean	SD †	Mean	SD †	Mean	SD †	Mean	SD †	
FES‡	28.8	20.8	35.9	22.3	34.1	16.6	40.5	19.5	0.212
SF-8									
PCS§	46.5	10.3	47.9	9.1	50.5	5.3	49.5	6.4	0.496
MCS‖	53.0	6.9	54.9	4.2	50.7	6.8	53.4	5.4	0.295
Physical Activities¶									
Steps	890.0	438.9	1628.4	1664.1	1227.7	1170.5	1355.4	1150.8	0.392

*statistically significant with repeated measures ANOVA
†standard deviation
‡Falls Efficacy Scale
§Physical Component Score
‖Mental Component Score
¶Physical Activities は FIM5 以上の硬質群 4 名、軟質群 7 名のみ測定

Table 4 . Change in FES, PCS, MCS and steps in MMSE≧21and MMSE≦20

	Hip protectors group MMSE21点以上 (13 subjects in 26 nursing homes)								
	Baseline		3 months		6 months		12 months		*P*-value
	Mean	SD†	Mean	SD†	Mean	SD†	Mean	SD†	
FES‡	38.8	26.8	41.1	25.4	42.2	25.4	38.9	24.4	0.797
SF-8									
PCS§	43.0	11.1	42.7	12.7	45.9	9.2	47.2	8.6	0.382
MCS‖	52.6	6.0	53.3	7.4	51.8	7.5	53.6	3.7	0.855
Physical Activities¶									
Steps	837.29	696.72	806.14	489.93	632.14	399.39	994.43	1033.63	0.645

	Hip protectors group MMSE20点以下 (8 subjects in 26 nursing homes)								
	Baseline		3 months		6 months		12 months		*P*-value
	Mean	SD†	Mean	SD†	Mean	SD†	Mean	SD†	
FES‡	28.4	21.3	43.1	19.6	36.6	12.8	46.3	15.9	0.074
SF-8									
PCS§	45.7	11.8	45.4	10.1	50.3	6.6	47.6	6.7	0.588
MCS‖	52.7	8.5	55.3	5.3	49.1	7.1	52.7	6.6	0.275
Physical Activities¶									
Steps	1036.8	428.7	2161.5	2118.9	1704.5	1423.5	1262.3	981.4	0.288

	Control group MMSE21点以上 (12 subjects in 17 nursing homes)								
	Baseline		3 months		6 months		12 months		*P*-value
	Mean	SD†	Mean	SD†	Mean	SD†	Mean	SD†	
FES‡	55.7	23.3	51.8	17.9	42.8	16.7	53.6	21.2	0.247
SF-8									
PCS§	40.0	10.8	45.1	5.1	46.6	8.4	46.0	4.5	0.177
MCS‖	48.7	11.3	50.1	7.3	50.6	7.4	49.6	6.2	0.905
Physical Activities¶									
Steps	1085.0	1289.5	894.5	1071.6	1164.8	1626.1	1321.5	1589.1	0.329

	Control group MMSE20点以下 (9 subjects in 17 nursing homes)								
	Baseline		3 months		6 months		12 months		*P*-value
	Mean	SD†	Mean	SD†	Mean	SD†	Mean	SD†	
FES‡	30.9	18.4	39.7	18.4	44.6	25.8	40.2	21.8	0.210
SF-8									
PCS§	52.1	3.1	50.7	5.6	42.5	6.4	47.5	7.4	0.003*
MCS‖	50.7	3.9	51.5	4.9	53.6	3.8	53.8	5.4	0.228
Physical Activities¶									
Steps	489.7	332.9	577.0	588.1	338.3	149.6	400.7	287.7	—

*statistically significant with repeated measures ANOVA
†standard deviation
‡Falls Efficacy Scale
§Physical Component Score
‖Mental Component Score
¶Physical Activities は FIM5 以上の硬質群 4 名、軟質群 7 名のみ測定

E. 結論

・介護施設高齢者におけるヒッププロテクター装着による効果を、転倒恐怖としての転倒自己効力感、QOL、身体活動量の 3, 6 ヶ月に続き、12 ヶ月までの経時的な変化から検討した。

・ヒッププロテクター群、コントロール群とも転倒自己効力感、QOL、歩数の有意な変化はみられなかった。

・ヒッププロテクター群を硬質群、軟質群に分けて検討しても同様な結果が得られたことから、使用したヒッププロテクターの種類の影響は少ないと考えられた。

・介護施設高齢者において、ヒッププロテクターの使用が転倒恐怖や QOL、身体活動量に好影響を及ぼすという間接的効果は認められなかった。

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ORIGINAL ARTICLE

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Potentiation by platelet-derived growth factor-BB of FGF-2-stimulated 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. In the present study, we investigated the effect of platelet-derived growth factor-BB (PDGF-BB) on FGF-2-induced VEGF release in MC3T3-E1 cells. PDGF-BB significantly enhanced the FGF-2-stimulated VEGF release. The amplifying effect of PDGF-BB was dose dependent in the range between 0.1 and 30 ng/ml. AG1295, a selective inhibitor of PDGF receptor kinase, which reduced the autophosphorylation of PDGF receptor-β, suppressed the enhancement by PDGF-BB without affecting the FGF-2 effect. PDGF-BB failed to strengthen the FGF-2-induced phosphorylation of p44/p42 MAP kinase or SAPK/JNK. The amplification by PDGF-BB of FGF-2-stimulated VEGF release was reduced by PD98059, a specific inhibitor of MEK, or SP600125, a specific inhibitor of SAPK/JNK. These 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.

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Key words FGF · PDGF · MAP kinase · VEGF · osteoblast

Introduction

Bone metabolism is strictly regulated by two functional cells, osteoblasts and osteoclasts, which are responsible for bone formation and bone resorption, respectively [1]. It has been shown that osteoblasts synthesize basic fibroblast growth factor (FGF-2), and FGF-2 is embedded in bone matrix [2,3]. FGF-2 expression in osteoblasts is detected during fracture repair [4]. Therefore, it is recognized that FGF-2 may play a pivotal role in fracture healing, bone remodeling, and osteogenesis [5]. We have previously reported that FGF-2 autophosphorylates FGF receptors 1 and 2 among four structurally related high-affinity receptors, possessing an intrinsic protein tyrosine kinase activity in osteoblast-like MC3T3-E1 cells [6]. In addition, we reported that FGF-2 stimulates induction of heat shock protein 27 in these cells [7].

Bone remodeling carried out by osteoblasts and osteoclasts is accompanied with angiogenesis and capillary outgrowth [8,9]. Because capillary endothelial cells provide the microvasculature during bone remodeling, it is generally recognized that the activities of osteoblasts, osteoclasts, and capillary endothelial cells are closely coordinated and regulate bone metabolism [10]. These functional cells are considered to influence one another via humoral factors as well as by direct cell-to-cell contact. It is well known that vascular endothelial growth factor (VEGF) is an angiogenic growth factor highly specific for vascular endothelial cells [11]. As for bone metabolism, it has been shown that 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 [12]. Evidence is accumulating that osteoblasts among bone cells produce and secrete VEGF in response to various physiological agents [11,13–15]. We have previously shown that FGF-2

stimulates VEGF release in osteoblast-like MC3T3-E1 cells, and that among the mitogen-activated protein (MAP) kinase superfamily [16], p44/p42 MAP kinase and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) act as positive regulators in the VEGF release [17,18]. These findings led us to speculate that VEGF secreted from osteoblasts may play a pivotal role in the regulation of bone metabolism [10,19]. However, the exact mechanism underlying VEGF synthesis and its release in osteoblasts remains to be clarified.

Platelet-derived growth factor (PDGF) is a mitogenic factor, which mainly acts on connective tissue cells [20,21]. PDGF occurs as five different isoforms [21]. PDGF isoforms were initially isolated from human platelets, but have been shown to be synthesized and released from a variety of cell types including osteosarcoma and osteoblasts [20,22,23]. As for bone metabolism, PDGF-BB reportedly stimulates osteoblast proliferation and induces bone resorption [23]. It is recognized that PDGF, released during platelet aggregation, plays an important role in fracture healing as a systemic factor and that PDGF also regulates bone remodeling as a local factor [23]. We have previously reported that PDGF-BB activates phosphatidylcholine-hydrolyzing phospholipase D via tyrosine kinase activation, resulting in protein kinase C activation in osteoblast-like MC3T3-E1 cells [24]. In addition, we recently showed that phosphatidylinositol 3-kinase negatively regulates the PDGF-BB-stimulated interleukin-6 synthesis in these cells [25]. However, the exact role of PDGF-BB in osteoblasts has not yet been fully clarified.

In the present study, we investigated the effect of PDGF-BB on FGF-2-stimulated VEGF release in osteoblast-like MC3T3-E1 cells. We here show that PDGF-BB upregulates FGF-2-stimulated VEGF release in MC3T3-E1 cells, and that PDGF-BB acts at a point downstream from p44/p42 MAP kinase and SAPK/JNK.

Materials and methods

Materials

FGF-2, PDGF-BB, and mouse VEGF enzyme immunoassay kit were purchased from R&D Systems (Minneapolis, MN, USA). AG1295 (6,7-dimethyl-2-phenylquinoxaline), PD98059, and SP600125 were obtained from Calbiochem-Novabiochem (La Jolla, CA, USA). Phospho-specific PDGF receptor-β antibodies, PDGF receptor-β antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies, and SAPK/JNK antibodies were purchased from Cell Signaling (Beverly, MA, USA). The ECL Western blotting detection system was purchased from Amersham Biosciences (Piscataway, NJ, USA). Other materials and chemicals were obtained from commercial sources. AG1295, PD98059, 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 Western blot analysis.

Cell culture

Cloned osteoblast-like MC3T3-E1 cells, which have been 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- (5×10^4) or 90-mm- (5×10^5) 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 used for experiments after 48 h. When indicated, the cells were pretreated with AG1295, PD98059, or SP600125 for 60 min.

VEGF assay

The cultured cells were pretreated with PDGF-BB for 60 min, and then stimulated by various doses of FGF-2 in 1 ml α-MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pretreated with AG1295, PD98059, or SP600125 for 60 min. The conditioned medium was collected at the end of the incubation, and the VEGF concentration was measured by enzyme-linked immunoassay (ELISA) kit.

Western blot analysis

The cultured cells were stimulated by FGF-2 or PDGF-BB 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 min at 4°C. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by Laemmli [28] in 10% polyacrylamide gel. Western blotting analysis was performed as described previously [29] by using phospho-specific PDGF receptor-β antibodies, PDGF receptor-β antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies, or SAPK/JNK antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. Peroxidase activity on the polyvinylidene fluoride (PVDF) sheet was visualized on X-ray film by means of the ECL Western blotting detection system. When indicated, the cells were pretreated with PDGF-BB for 60 min.

Determination

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

Statistical analysis

The data were analyzed by analysis of variance (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 ± SEM of triplicate determinations. Each experiment was repeated three times with similar results.

Results

Effect of PDGF-BB on FGF-2-induced VEGF release in MC3T3-E1 cells

PDGF-BB, which by itself did not affect the VEGF levels, significantly potentiated the FGF-2-stimulated VEGF release in a time-dependent manner in osteoblast-like MC3T3-E1 cells (Fig. 1). The amplifying effect of PDGF-BB on the VEGF release was dose dependent in the range between 0.1 and 30 ng/ml (Fig. 2). PDGF-BB (30 ng/ml) caused about 200% enhancement of the FGF-2 effect on VEGF release.

Effect of AG1295 on the amplification by PDGF-BB of the FGF-2-induced VEGF release in MC3T3-E1 cells

PDGF receptor has an intrinsic protein tyrosine kinase activity [20]. To elucidate whether the upregulating effect of PDGF-BB on FGF-2-induced VEGF release is mediated through the activation of PDGF receptor kinase in MC3T3-E1 cells, we examined the effect of AG1295, a specific inhibitor of PDGF-receptor kinase [30], on the enhancement by PDGF-BB. AG1295 significantly suppressed the amplification by PDGF-BB of FGF-2-induced VEGF release whereas it had no effect on the FGF-2-induced VEGF release (Table 1). We found that AG1295, which by itself had little effect on the phosphorylation of PDGF receptor-β, actually attenuated the PDGF-BB-induced phosphorylation of PDGF receptor-β (Fig. 3).

Effects of PDGF-BB on the FGF-2-induced phosphorylation of p44/p42 MAP kinase or SAPK/JNK in MC3T3-E1 cells

We have previously shown that p44/p42 MAP kinase and SAPK/JNK play roles as positive regulators in the FGF-2-stimulated VEGF release in osteoblast-like MC3T3-E1 cells [17,18]. To clarify whether the enhancement by PDGF-BB of the FGF-2-stimulated VEGF release is an event via activation of p44/p42 MAP kinase or SAPK/JNK in these cells, we next examined the effect of PDGF-BB on the FGF-2-induced phosphorylation of p44/p42 MAP kinase or SAPK/JNK. However, PDGF-BB failed to affect the FGF-2-induced phosphorylation of p44/p42 MAP kinase (Fig. 4). In addition, the FGF-2-induced phosphorylation of SAPK/JNK was not enhanced by PDGF-BB (data not shown).

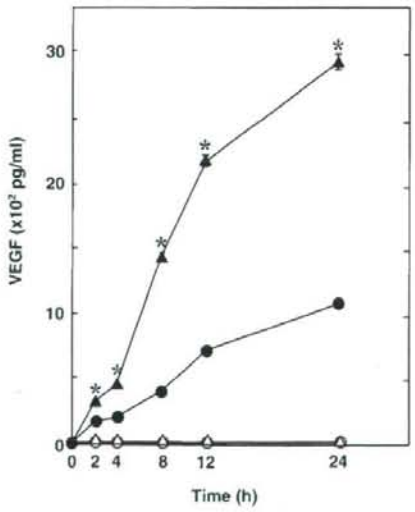

Fig. 1. Effect of platelet-derived growth factor-BB (PDGF-BB) on fibroblast growth factor (FGF)-2-stimulated vascular endothelial growth factor (*VEGF*) synthesis in MC3T3-E1 cells. The cultured cells were pretreated with 30 ng/ml PDGF-BB (▲, △) or vehicle (●, ○) for 60 min, and then stimulated by 70 ng/ml FGF-2 (●, ▲) or vehicle (○, △) for the indicated periods. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, compared to the value of FGF-2 alone

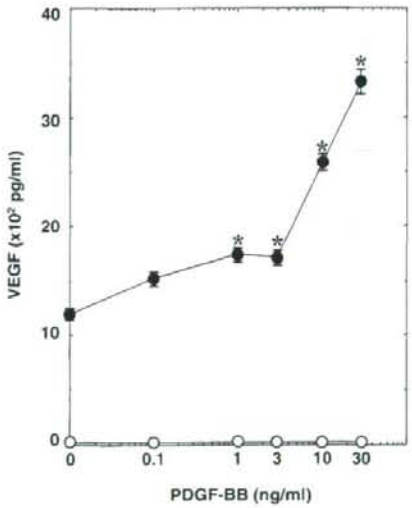

Fig. 2. Dose-dependent effect of PDGF-BB on FGF-2-stimulated VEGF synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of PDGF-BB for 60 min, and then stimulated by 70 ng/ml FGF-2 (●) or vehicle (○) for 24 h. Each value represents the mean ± SEM of triplicate determinations.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

Table 1. Effect of AG1295 on the enhancement by PDGF-BB of FGF-2-stimulated VEGF release in MC3T3-E1 cells

AG1295	PDGF-BB	FGF-2	VEGF (pg/ml)
–	–	–	<7.8
–	–	+	1191 ± 67*
–	+	–	<7.8
–	+	+	3335 ± 141**
+	–	–	<7.8
+	–	+	1158 ± 75
+	+	–	<7.8
+	+	+	2520 ± 135***

The cultured cells were pretreated with 0.7 μM AG1295 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
PDGF-BB, platelet-derived growth factor-B; FGF-2, fibroblast growth factor 2; VEGF, vascular endothelial growth factor

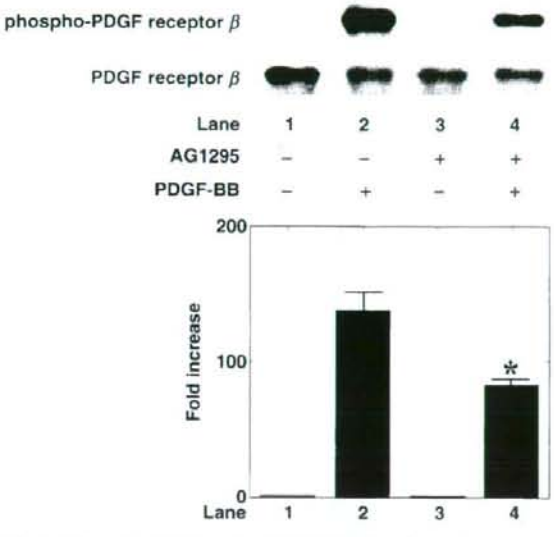

Fig. 3. Effect of AG1295 on the PDGF-BB-induced autophosphorylation of PDGF receptor-β in MC3T3-E1 cells. The cultured cells were pretreated with 0.7 μM AG1295 or vehicle for 60 min, and then stimulated by 30 ng/ml PDGF-BB or vehicle for 60 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific PDGF receptor-β or PDGF receptor-β. The histogram shows quantitative representations of the levels of PDGF-BB-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 to the value of PDGF-BB alone

Effects of PD98059 or SP600125 on the amplification by PDGF-BB of the FGF-2-induced VEGF release in MC3T3-E1 cells

We further examined the effect of PD98059, a specific inhibitor of MEK1/2, upstream kinases that activate p44/p42 MAP kinase [31], on the enhancement by PDGF-BB. PD98059 significantly suppressed the enhancement by PDGF-BB of the FGF-2-induced VEGF release in addition to the effect of FGF-2 alone. In addition, SP600125, a specific SAPK/JNK inhibitor [32], also reduced the enhancement by PDGF-BB of the VEGF release (Table 2).

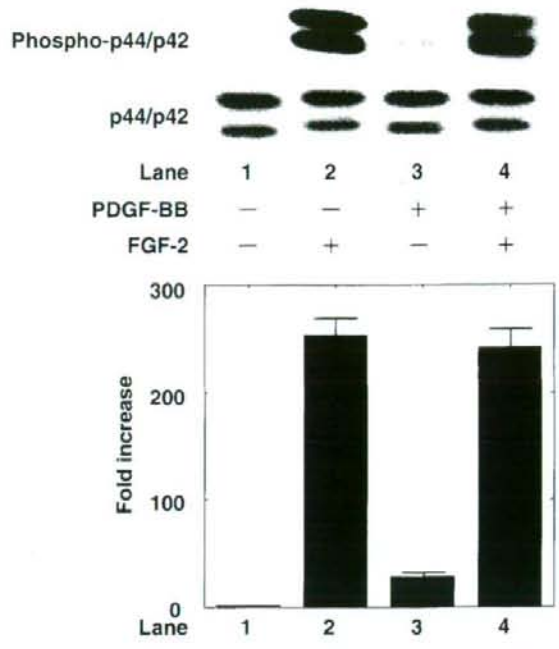

Fig. 4. Effect of PDGF-BB on FGF-2-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of PDGF-BB for 60 min, and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent 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

Discussion

In the present study, we demonstrated that PDGF-BB, which by itself had no effect on the levels of VEGF, significantly enhanced FGF-2-stimulated VEGF release in osteoblast-like MC3T3-E1 cells. FGF-2 significantly elicited VEGF release within 2 h after the stimulation, and the enhancement by PDGF-BB was also observed within 2 h. On the other hand, the levels of VEGF release induced by FGF-2 and the enhancement by PDGF-BB were stepped up after 8 h and sustained until 24 h after the stimulation. It is possible that both transcriptional processes and post-transcriptional reactions are involved in the VEGF release

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

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

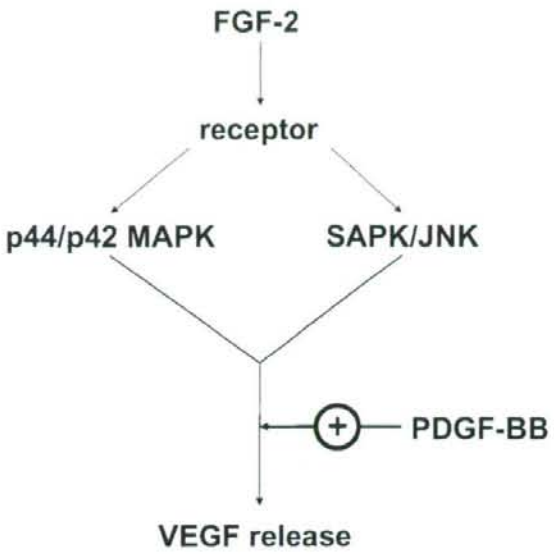

Fig. 5. Schematic illustration of the signaling pathways of VEGF release stimulated by FGF-2 and PDGF-BB in osteoblasts. *FGF-2*, fibroblast growth factor-2; *MAPK*, mitogen-activated protein kinase; *SAPK/JNK*, stress-activated protein kinase/c-*Jun* N-terminal kinase; *PDGF-BB*, platelet-derived growth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

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.

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

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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.

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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-

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.

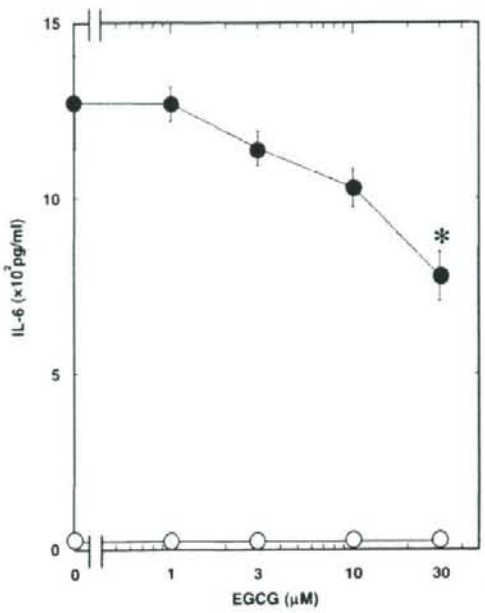

Fig. 1 Effect of EGCG on the FGF-2-stimulated IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of EGCG for 60 min and then stimulated by 70 ng/ml FGF-2 or vehicle for 24 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained 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 Interleukin-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.

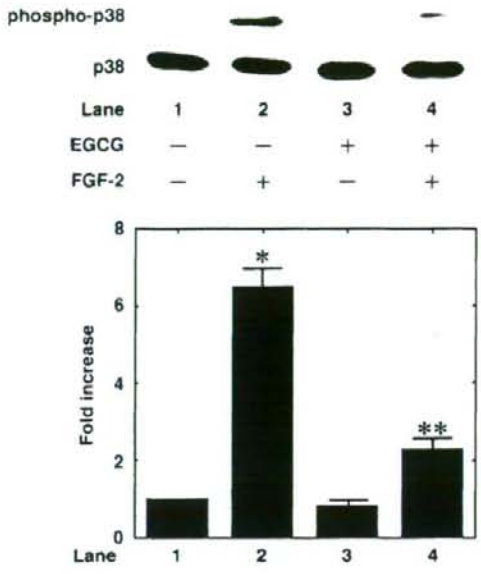

Fig. 2 Effect of EGCG on the phosphorylation of p38 MAP kinase induced by FGF-2 in MC3T3-E1 cells. The cultured cells were pretreated with 100 μM EGCG or vehicle for 60 min and then stimulated by 70 ng/ml FGF-2 or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western 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