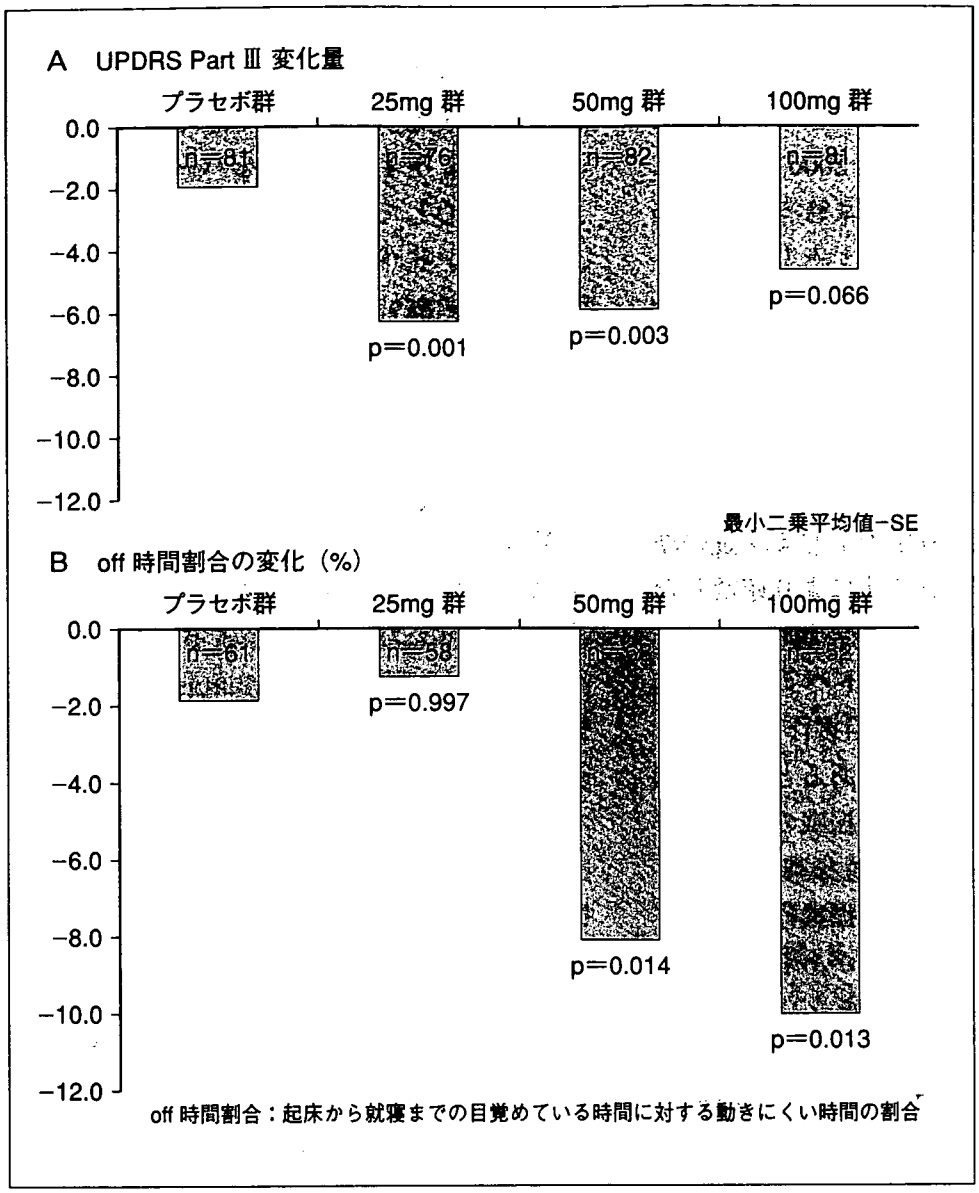
図1　ゾニサミドの効果



A：25mg, 50mg 群で有意な UPDRSⅢスコアの改善をみた.
B：50mg, 100mg 群で有意な off 時間割合の減少（50mg 群で平均 1.3 時間，100mg 群で平均 1.63 時間の減少）を認めた.

作用機序とその後の研究の展開

型どおり治験を進めながら，一方で基礎実験を進めた．比較的初期に行った実験で非常にうれしかったのは，MPTP サルでの ZNS の効果を見たときである．患者で効果があるのだから MPTP サルで効果があって当然なのではあるが，静注での実験であったこともあり，目の前で症状が改善していく，また，L-DOPA との併用により明らかに L-DOPA の効果の持続が延長するのを見ると，偽薬効果のない動物での結果であるだけに，確かに効果があることを実感できた．またこの静注での即時効果から，ZNS の抗パーキンソン作用には TH mRNA 合成を介さない，より速いタイムスケールでの機序もあることが明らかになった.

この薬剤の作用機序や新たな可能性を明ら

かにするためには多くの方の協力が必要であり，2003 年度より厚生労働省難治性疾患克服研究事業の研究費を頂き，「日本発の新しい抗パーキンソン作用薬ゾニサミドの臨床研究班」を立ち上げ，さらに 2006 年度から「新規抗パーキンソン病薬ゾニサミドの神経保護作用に関する臨床研究班」として研究を進めている．ZNS の作用機序などを明らかにすることは，ZNS を越える新たな抗パーキンソン病薬の開発につながると考えている．班員のご協力により多くの成果が得られている[7)8)]．線条体でのドパミン量の増加は，細胞内のみならず，細胞外液中のドパミン量の増加をマイクロダイアリシス法により確認した（愛媛大学 野元正弘教授ら）．現時点では，ZNS は被殻を作用点としてドパミン合成亢進作用，中等度の MAO-B 阻害作用を持ち，ドパミン取り込み阻害作用もあると考えられる．さらに ZNS は，静止時振戦のみならず姿勢に伴う粗大な振戦（本態性振戦も含めて）にも効果を認めるが，この抗振戦作用にはドパミン系を介さない作用機序があることが明らかになり，ZNS の抗パーキンソン作用発現にドパミン系以外の系の関与が初めて明らかになった（和歌山県立医科大学 近藤智善教授ら）．またサルのモデルで，ZNS がパーキンソン病で認める淡蒼球，視床下核の異常発火パターンの正常化作用を持つことが見いだされた（生理学研究所 南部 篤教授ら）．この正常化作用にドパミン系が関与するのか，あるいは ZNS がT型カルシウムチャネルブロッカーであることが関与するのか，などについては今後明らかにされる予定である．

さらに，最初に ZNS をパーキンソン病患者に投与するきっかけとなった神経保護作用について，班研究の中で素晴らしい成果が得られた．すなわち ZNS は，脳内グルタチオン増加作用とドパミン自動酸化系でのキノン体の速やかかつ強力な除去作用を持つことが明らかになった．これまで L-DOPA が毒と思われた最大の原因はこの自動酸化におけるキノン体合成であり，ZNS が速やかにこれらを消去するということは，ZNS を併用することにより L-DOPA をパーキンソン病初期から安心して使えることを示唆する．6-ヒドロキシドパミン（6-OHDA）によるモデルマウスにおいては，6-OHDA 投与 3 週間後の ZNS 投与でも黒質ドパミン神経の脱落は有意に減少しており，すでに発症している患者での効果も期待できる結果を得た（岡山大学 浅沼幹人准教授ら）．

培養細胞系では，ZNS が高濃度ドパミンおよび MPP^+ に対して保護効果を持つことを明らかにした．ZNS によってアポトーシス経路の PTEN（phosphatase and tensin homolog），akt1，FOXO3A（forkhead transcription factor）のリン酸化が増加することより，ZNS が Akt 経路の最終機転をリン酸化して不活性化することで細胞死抑制に作用しているものと考えられた（順天堂大学 服部信孝教授ら）．

ZNS の長期投与試験において，平均罹患期間 8.7 年の患者でも 1 年以上改善が維持され，しかも 40 週以降より改善する傾向にあることは，臨床的な神経保護効果を期待できる結果であると考えられる．現時点ではパーキンソン病の神経保護効果を評価する系が確立していないことから，まずその系を確立し，ZNS の臨床的な神経保護作用の評価を行いたいと考えている．

ところで，パーキンソン病患者の中には ZNS の効果が驚くほど高い患者が少なからず存在する．これらの人々の臨床的な特徴ははっきりせず，現在 1 塩基多型（SNP）タグチップを用いて検討を進めている．この研究がパーキンソン病のテーラーメード治療のきっかけになることを期待している（大阪大学 戸田達史教授ら）．

また，半減期の長いドパミン系刺激薬剤の1つとして，むずむず脚症候群への効果も少数例で確認され，この効果の評価を進める予定である（国立病院機構相模原病院 長谷川一子医長ら）．

おわりに

現在，まだダメ押しの追加の治験をしているところであるが，ZNS を使うことで手術例がかなり減ったという話も聞き，1日も早く使用承認が得られることを心待ちにしている．

ZNS が抗パーキンソン作用を持つことを発見したのは全くの偶然であった．しかし，ある病態に対する治療薬が別な作用を持つことは当然ありうることで，このような「発見」は，実は身近にあるのに気がついていないだけかもしれないとも思われる．注意深い臨床的な観察と，事実を科学的・論理的に分析すること，それに少しの思い込みにより，誰にも新たな「発見」のチャンスはあるものと思われる．

謝 辞

患者さんはじめ，治験や研究にご参加いただいた先生方，その他多くの皆さまのご協力でこれらの成果を挙げることができたことを，この場をお借りして深く感謝いたします．特に，終始ご指導いただいた金澤一郎国立精神・神経センター名誉総長，および大学院生として初期の基礎実験を一緒に進めてくれた現 国立病院機構相模原病院神経内科 堀内恵美子博士に深謝いたします．

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Development of the New Anti-parkinsonian Drug: Zonisamide

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(−)-Epigallocatechin Gallate Enhances Prostaglandin $F_{2\alpha}$-Induced VEGF Synthesis Via Upregulating SAPK/JNK Activation in Osteoblasts

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Abstract Catechin, one of the major flavonoids presented in plants such as tea, reportedly suppresses bone resorption. We previously reported that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) stimulates the synthesis of vascular endothelial growth factor (VEGF) via p44/p42 mitogen-activated protein (MAP) kinase in osteoblast-like MC3T3-E1 cells. To clarify the mechanism of catechin effect on osteoblasts, we investigated the effect of (−)-epigallocatechin gallate (EGCG), one of the major green tea flavonoids, on the VEGF synthesis by $PGF_{2\alpha}$ in MC3T3-E1 cells. The $PGF_{2\alpha}$-induced VEGF synthesis was significantly enhanced by EGCG. The amplifying effect of EGCG was dose dependent between 10 and 100 μM. EGCG did not affect the $PGF_{2\alpha}$-induced phosphorylation of p44/p42 MAP kinase. SB203580, a specific inhibitor of p38 MAP kinase, and SP600125, a specific inhibitor of stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK), reduced the $PGF_{2\alpha}$-induced VEGF synthesis. EGCG markedly enhanced the phosphorylation of SAPK/JNK induced by $PGF_{2\alpha}$ without affecting the $PGF_{2\alpha}$-induced phosphorylation of p38 MAP kinase. SP600125 markedly reduced the amplification by EGCG of the SAPK/JNK phosphorylation. In addition, the $PGF_{2\alpha}$-induced phosphorylation of c-Jun was amplified by EGCG. These results strongly suggest that EGCG upregulate $PGF_{2\alpha}$-stimulated VEGF synthesis resulting from amplifying activation of SAPK/JNK in osteoblasts. J. Cell. Biochem. 100: 1146–1153, 2007. © 2006 Wiley-Liss, Inc.

Key words: catechin; $PGF_{2\alpha}$; VEGF; MAP kinase; osteoblast

It is generally recognized that compounds in foods such as vegetables and fruits have beneficial properties to human being. Among them, flavonoids reportedly show antioxidative, antibacterial, and antitumor effects [Jankun et al., 1997; Harbourne and Williams, 2000]. Catechins are one of the major flavonoids, which are present in various species of plants such as tea [Harbourne and Williams, 2000]. In bone metabolism, it has been reported that catechin suppresses bone resorption [Delaisse et al., 1986]. Bone metabolism is regulated by two functional cells, osteoblasts, and osteoclasts, responsible for bone formation and bone resorption, respectively [Nijweide et al., 1986]. The

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formation of bone structures and bone remodeling results from the coupling process, bone resorption by activated osteoblasts with subsequent deposition of new matrix by osteoblasts. As for osteoblasts, it has been shown that catechin stimulates alkaline phosphatase activity, a mature osteoblast phenotype and reduces bone-resorptive cytokine production in osteoblast-like MC3T3-E1 cells [Choi and Hwang, 2003]. However, the exact role of catechin in osteoblasts has not yet been clarified.

Vascular endothelial growth factor (VEGF) has been characterized as a heparin-binding angiogenic growth factor displaying high specificity for vascular endothelial cells [Ferrara and Davis-Smyth, 1997]. It is well recognized that VEGF, which is produced and secreted from a variety of cell types, increases capillary permeability and stimulates proliferation of endothelial cells [Ferrara and Davis-Smyth, 1997]. 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 [Gerber et al., 1999]. Accumulating evidence indicates that osteoblasts among bone cells produce and secrete VEGF in response to various humoral factors [Goad et al., 1996; Wang et al., 1996; Ferrara and Davis-Smyth, 1997; Schalaeppi et al., 1997]. During bone remodeling, capillary endothelial cells provide the microvasculature, and osteoblasts and osteoprogenitor cells, which locally proliferate and differentiate into osteoblasts, migrate into the resorption lacuna. It is currently recognized that the activities of osteoblasts, osteoclasts and capillary endothelial cells are closely coordinated via humoral factors as well as by direct cell-to-cell contact, and these cells cooperatively regulate bone metabolism [Erlebacher et al., 1995]. Thus, there is no doubt that VEGF secreted from osteoblasts plays an important role in the regulation of bone metabolism. However, the mechanism underlying VEGF synthesis in osteoblasts has not yet been fully clarified.

It is well known that prostaglandins (PGs) act as autocrine/paracrine modulators in osteoblasts and play crucial roles in the regulation of bone metabolism [Nijweide et al., 1986; Pilbeam et al., 1996]. Among them, $PGF_{2\alpha}$ is known as a potent bone-resorptive agent and stimulates the proliferation of osteoblasts and inhibits their differentiation [Pilbeam et al., 1996]. In our previous studies [Miwa et al., 1990; Kozawa et al., 1994], we have reported that $PGF_{2\alpha}$ stimulates both phosphoinositide-hydrolyzing phospholipase C (PI-PLC) and phosphatidylcholine-hydrolyzing phospholipase D (PC-PLD), recognized to be two major pathways of physiological protein kinase C (PKC) activation [Nishizuka, 1992; Exton, 1999], in osteoblast-like MC3T3-E1 cells. In addition, we have recently shown that $PGF_{2\alpha}$ stimulates the VEGF synthesis through PKC-dependent activation of p44/p42 mitogen-activated protein (MAP) kinase in these cells [Tokuda et al., 2003]. In the present study, we investigated the effect of (−)-epigallocatechin gallate (EGCG), one of the major green tea flavonoids, on the $PGF_{2\alpha}$-induced VEGF synthesis in osteoblast-like MC3T3-E1 cells. We here show that EGCG upregulates $PGF_{2\alpha}$-stimulated VEGF synthesis via enhancing SAPK/JNK activation among the MAP kinase superfamily in these cells.

MATERIALS AND METHODS

Materials

$PGF_{2\alpha}$ was purchased from Sigma Chemical Co. (St. Louis, MO). Mouse VEGF enzyme immunoassay kit was purchased from R&D Systems, Inc. (Minneapolis, MN). (−)-Epigallocatechin gallate (EGCG), SB203580, 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, and c-Jun antibodies were purchased from Cell Signaling Technology (Beverly, MA). ECL Western blotting detection system was purchased from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. $PGF_{2\alpha}$ was dissolved in ethanol. SB203580 and SP600125 were dissolved in dimethyl sulfoxide. The maximum concentration of ethanol or dimethyl sulfoxide was 0.1%, which did not affect the assay for VEGF or the analysis of Western blot.

Cell Culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [Sudo

et al., 1983] were maintained as previously described [Kozawa et al., 1992]. 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 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

VEGF Assay

The cultured cells were stimulated by $PGF_{2\alpha}$ in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pretreated with EGCG, SB203580, or SP600125 for 60 min. The reaction was terminated by collecting the medium, and VEGF in the medium was measured by a VEGF enzyme immunoassay kit.

Western Blot Analysis

The cultured cells were stimulated by $PGF_{2\alpha}$ in α-MEM containing 0.3% FCS for the indicated periods. The cells were washed twice with phosphate-buffered saline and then lysed, homogenized, and sonicated in a lysis buffer containing 62.5 mM Tris/HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol, and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125,000*g* for 10 min at 4°C. SDS–polyacrylamide gel electrophoresis (PAGE) was performed by Laemmli [1970] in 10% polyacrylamide gel. Western blotting analysis was performed as described previously [Kato et al., 1996] by using 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, or c-Jun antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. Peroxidase activity on the nitrocellulose sheet was visualized on X-ray film by means of the ECL Western blotting detection system. The pretreatment of EGCG or SP600125 was performed for 60 min before the addition of $PGF_{2\alpha}$.

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 was performed using Molecular Analyst/Macintosh (Bio-Rad Laboratories, Hercules, CA).

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 ± SEM of triplicate determinations. Each experiment was repeated three times with similar results.

RESULTS

Effect of EGCG on the $PGF_{2\alpha}$-Stimulated VEGF Synthesis in MC3T3-E1 Cells

We have previously shown that $PGF_{2\alpha}$ stimulates VEGF synthesis in osteoblast-like MC3T3-E1 cells [Tokuda et al., 2003]. We first examined the effect of EGCG on the $PGF_{2\alpha}$-stimulated VEGF synthesis. EGCG, which by itself had little effect on the VEGF levels, significantly enhanced the $PGF_{2\alpha}$-stimulated synthesis of VEGF (Table I). The amplifying effect of EGCG was dose dependent between 10 and 100 μM (Table I).

Effect of EGCG on the Phosphorylation of p44/p42 MAP Kinase Induced by $PGF_{2\alpha}$ in MC3T3-E1 Cells

We have previously reported that the $PGF_{2\alpha}$-stimulated VEGF synthesis is regulated by $PGF_{2\alpha}$-activated p44/p42 MAP kinase in MC3T3-E1 cells [Tokuda et al., 2003]. In order to investigate whether EGCG effect on the $PGF_{2\alpha}$-stimulated VEGF synthesis is mediated

TABLE I. Effect of EGCG on the $PGF_{2\alpha}$-Stimulated VEGF Synthesis in MC3T3-E1 Cells

EGCG (μM)	$PGF_{2\alpha}$	VEGF (pg/ml)
—	–	19 ± 10
—	+	580 ± 51
10	–	14 ± 10
10	+	630 ± 85
30	–	15 ± 10
30	+	2680 ± 167*
100	–	20 ± 10
100	+	4100 ± 249*

The cultured cells were pretreated with various doses of EGCG for 60 min, and then stimulated by 10 μM $PGF_{2\alpha}$ 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 $PGF_{2\alpha}$ alone.

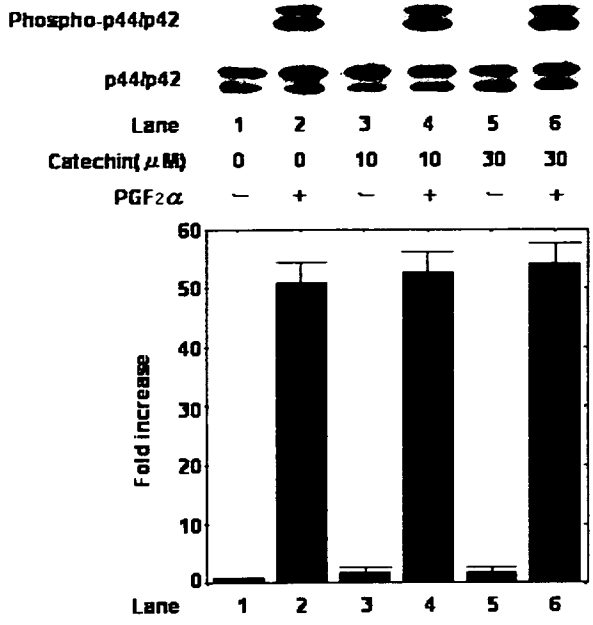

Fig. 1. Effect of EGCG on the phosphorylation of p44/p42 MAP kinase induced by $PGF_{2\alpha}$ in MC3T3-E1 cells. The cultured cells were pretreated with the indicated doses of EGCG or vehicle for 60 min, and then stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 30 min. The extracts of cells were subjected 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 $PGF_{2\alpha}$-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.

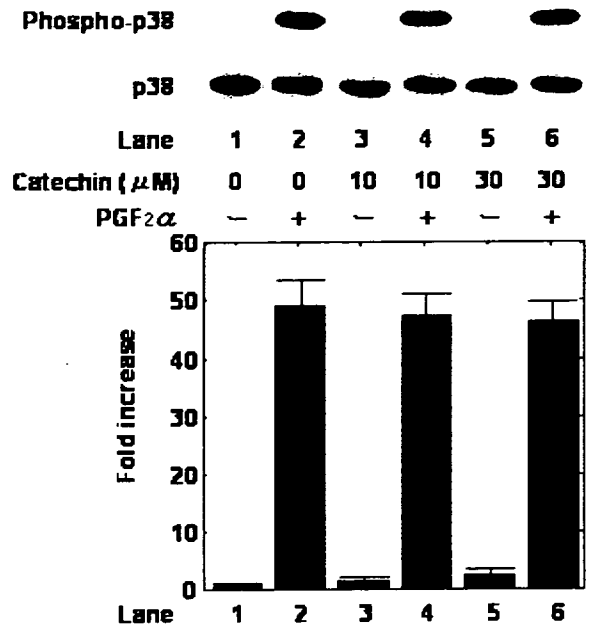

Fig. 2. Effect of EGCG on the phosphorylation of p38 MAP kinase induced by $PGF_{2\alpha}$ in MC3T3-E1 cells. The cultured cells were pretreated with the indicated doses of EGCG or vehicle for 60 min, and then stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 10 min. The extracts of cells were subjected 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 $PGF_{2\alpha}$-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.

through p44/p42 MAP kinase activation in MC3T3-E1 cells, we next examined the effect of EGCG on the $PGF_{2\alpha}$-induced phosphorylation of p44/p42 MAP kinase. However, EGCG hardly affected the $PGF_{2\alpha}$-induced phosphorylation of p44/p42 MAP kinase (Fig. 1).

Effect $PGF_{2\alpha}$ of on the Phosphorylation of p38 MAP Kinase and SAPK/JNK in MC3T3-E1 Cells

Among the MAP kinase superfamily, p38 MAP kinase and SAPK/JNK in addition to p44/p42 MAP kinase play a crucial role as intracellular components to transduce the various signals of agonists [Widmann et al., 1999]. Therefore, in order to investigate whether activates p38 MAP kinase or SAPK/JNK in MC3T3-E1 cells, we next examined the effect of $PGF_{2\alpha}$ on the phosphorylation of p38 MAP kinase and SAPK/JNK. $PGF_{2\alpha}$ induced both the phosphorylation of p38 MAP kinase (lane 2 in Fig. 2) and SAPK/JNK (lane 2 in Fig. 3).

Effects of SB203580 or SP600125 on the $PGF_{2\alpha}$-Stimulated VEGF Synthesis in MC3T3-E1 Cells

In order to clarify the involvement of the MAP kinases in the $PGF_{2\alpha}$-stimulated VEGF synthesis in MC3T3-E1 cells, we examined the effect of SB203580, a specific inhibitor of p38 MAP kinase [Cuenda et al., 1995] or SP600125, a specific SAPK/JNK inhibitor [Bennett et al., 2001], on the VEGF synthesis. SB203580 or SP600125, which by itself had little effect on the VEGF levels, significantly suppressed the $PGF_{2\alpha}$-stimulated synthesis of VEGF (Table II).

Effect of EGCG on the Phosphorylation of p38 MAP Kinase or SAPK/JNK Induced by $PGF_{2\alpha}$ in MC3T3-E1 Cells

In order to clarify whether p38 MAP kinase is involved in EGCG effect on the $PGF_{2\alpha}$-

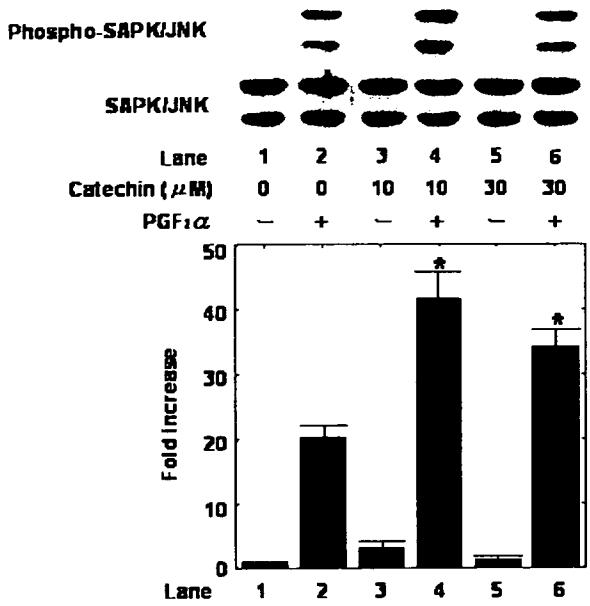

Fig. 3. Effect of EGCG on the phosphorylation of SAPK/JNK induced by $PGF_{2\alpha}$ in MC3T3-E1 cells. The cultured cells were pretreated with the indicated doses of EGCG or vehicle for 60 min, and then stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of $PGF_{2\alpha}$-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, compared to the value of $PGF_{2\alpha}$ alone.

stimulated VEGF synthesis in MC3T3-E1 cells, we examined the effect of EGCG on the $PGF_{2\alpha}$-induced phosphorylation of p38 MAP kinase. However, EGCG had little effect on the $PGF_{2\alpha}$-induced phosphorylation of p38 MAP kinase (Fig. 2). On the contrary, EGCG markedly enhanced the $PGF_{2\alpha}$-induced phosphorylation of SAPK/JNK (Fig. 3). According to the densitometric analysis, EGCG (10 μM) caused about 100% enhancement of the $PGF_{2\alpha}$ effect on the SAPK/JNK phosphorylation.

TABLE II. Effects of SB203580 or SP600125 on the $PGF_{2\alpha}$-Stimulated VEGF Synthesis in MC3T3-E1 Cells

Inhibitor (μM)	$PGF_{2\alpha}$	VEGF (pg/ml)
Vehicle	–	20 ± 10
Vehicle	+	559 ± 51
SB203580 (3)	–	13 ± 10
SB203580 (3)	+	221 ± 35*
SB203580 (10)	–	15 ± 10
SB203580 (10)	+	46 ± 12*
SP600125 (1)	–	15 ± 10
SP600125 (1)	+	399 ± 37*
SP600125 (30)	–	13 ± 10
SP600125 (30)	+	162 ± 29*

The cultured cells were pretreated with SB203580, SP600125, or vehicle for 60 min, and then stimulated by 10 μM $PGF_{2\alpha}$ 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 $PGF_{2\alpha}$ alone.

Effect of SP600125 on the Enhancement by EGCG of $PGF_{2\alpha}$-Induced Phosphorylation of SAPK/JNK in MC3T3-E1 Cells

SP600125 [Bennett et al., 2001], which by itself did not affect the basal levels of phosphorylation of SAPK/JNK, significantly reduced the enhancement by EGCG of $PGF_{2\alpha}$-induced SAPK/JNK phosphorylation (Fig. 4). The enhanced phosphorylation levels by EGCG of $PGF_{2\alpha}$-induced SAPK/JNK were suppressed by

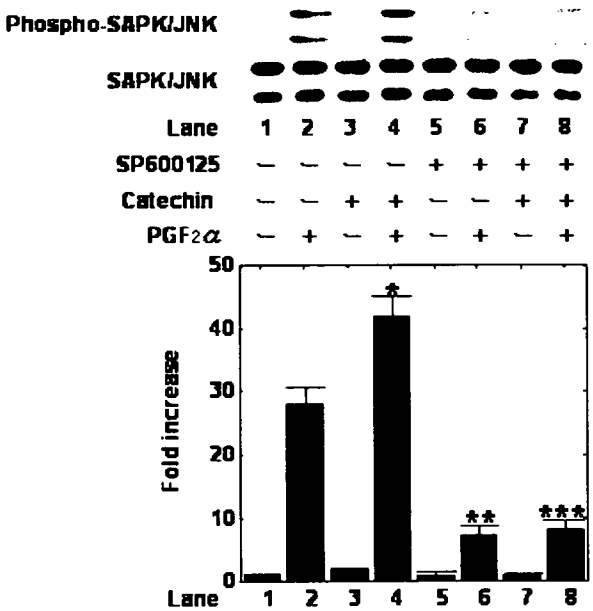

Fig. 4. Effect of SP600125 on the enhancement by EGCG of the $PGF_{2\alpha}$-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM SP600125 or vehicle for 60 min, and then incubated by 10 μM EGCG for 60 min. The cells were stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of $PGF_{2\alpha}$-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, compared to the value of $PGF_{2\alpha}$ alone. **$P < 0.05$, compared to the value of $PGF_{2\alpha}$ alone. ***$P < 0.05$, compared to the value of $PGF_{2\alpha}$ with EGCG pretreatment.

SP600125 similar to the levels by $PGF_{2\alpha}$ with SP600125 treatment.

Effect of EGCG on the Phosphorylation of c-Jun Induced by $PGF_{2\alpha}$ in MC3T3-E1 Cells

It is well known that c-Jun acts as a downstream effector of SAPK/JNK [Widmann et al., 1999; Weston and Davis, 2002]. Thus, in order to investigate whether EGCG effect on the $PGF_{2\alpha}$-stimulated VEGF synthesis is mediated through c-Jun activation in MC3T3-E1 cells, we next examined the effect of EGCG on the $PGF_{2\alpha}$-induced phosphorylation of c-Jun. We found that $PGF_{2\alpha}$ time-dependently phosphorylated c-Jun and that SP600125 suppressed the $PGF_{2\alpha}$-induced phosphorylation of c-Jun in these cells (data not shown). EGCG markedly amplified the $PGF_{2\alpha}$-induced phosphorylation of c-Jun (Fig. 5). According to the densitometric analysis, EGCG (10 μM) caused about 80% enhancement of the $PGF_{2\alpha}$ effect on the c-Jun phosphorylation.

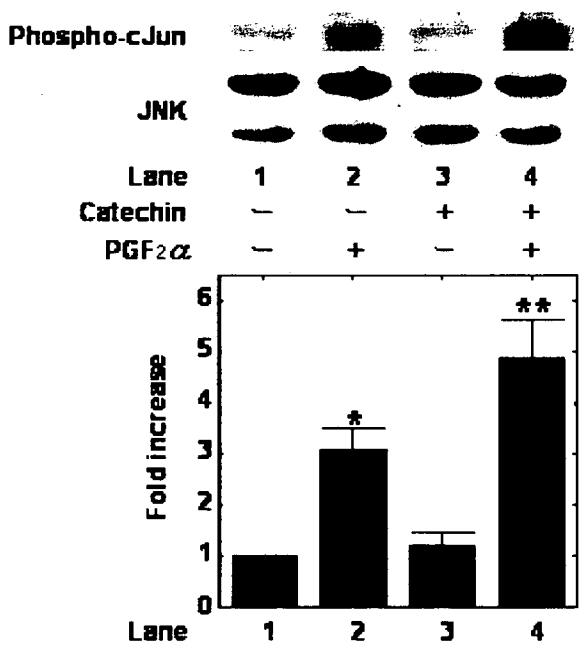

Fig. 5. Effect of EGCG on the phosphorylation of c-Jun induced by $PGF_{2\alpha}$ in MC3T3-E1 cells. The cultured cells were pretreated with the indicated doses of EGCG or vehicle for 60 min, and then stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against c-Jun or SAPK/JNK. The histogram shows quantitative representations of the levels of $PGF_{2\alpha}$-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}P < 0.05$, compared to the control value. $^{**}P < 0.05$, compared to the value of $PGF_{2\alpha}$ alone.

DISCUSSION

In the present study, we demonstrated that EGCG, which alone did not affect the levels of VEGF, significantly enhanced the $PGF_{2\alpha}$-stimulated VEGF synthesis in osteoblast-like MC3T3-E1 cells. We next investigated the mechanism of EGCG behind the amplifying effect on the VEGF synthesis. It is well recognized that the MAP kinase superfamily mediates intracellular signaling of extracellular agonists and plays a crucial role in cellular functions including proliferation, differentiation, and cell death in a variety of cells [Widmann et al., 1999]. Three major MAP kinase, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK are known as central elements used by mammalian cells to transduce the diverse messages [Widmann et al., 1999]. In our previous study [Tokuda et al., 2003], we have shown that $PGF_{2\alpha}$-activated p44/p42 MAP kinase acts as a positive regulator in $PGF_{2\alpha}$-induced VEGF synthesis in osteoblast-like MC3T3-E1 cells. In the present study, we showed that EGCG failed to affect the $PGF_{2\alpha}$-induced phosphorylation of p44/p42 MAP kinase. Thus, it seems unlikely that EGCG amplifies the $PGF_{2\alpha}$-induced VEGF synthesis through upregulating the activation of p44/p42 MAP kinase in osteoblast-like MC3T3-E1 cells.

We further investigated the involvement of p38 MAP kinase and SAPK/JNK in the amplifying effect of EGCG. We found that $PGF_{2\alpha}$ induced both the phosphorylation of p38 MAP kinase and SAPK/JNK. It has been shown that MAP kinases are activated by phosphorylation of threonine and tyrosine residues by dual specificity MAP kinase kinases [Widmann et al., 1999]. Therefore, our findings strongly suggest that $PGF_{2\alpha}$ activates p38 MAP kinase and SAPK/JNK in addition to p44/p42 MAP kinase in osteoblast-like MC3T3-E1 cells. In addition, we showed that the $PGF_{2\alpha}$-stimulated VEGF synthesis was suppressed by a specific p38 MAP kinase inhibitor, SB203580 [Cuenda et al., 1995], or a specific SAPK/JNK inhibitor, SP600125 [Bennett et al., 2001]. Based on our findings, it is probable that $PGF_{2\alpha}$ stimulates the synthesis of VEGF via the three MAP kinases in osteoblast-like MC3T3-E1 cells.

However, EGCG had little effect on the $PGF_{2\alpha}$-induced p38 MAP kinase phosphorylation. Therefore, it seems unlikely that the EGCG-induced enhancement of $PGF_{2\alpha}$-stimulated VEGF synthesis is due to the upregulation of p38 MAP kinase activation. On the contrary, we showed that the $PGF_{2\alpha}$-induced SAPK/JNK phosphorylation was markedly amplified by EGCG. Furthermore, SP600125 [Bennett et al., 2001] markedly reduced the enhancement by EGCG almost to the levels of $PGF_{2\alpha}$ alone with SP600125 in the phosphorylation of SAPK/JNK. These results suggest that EGCG upregulates the $PGF_{2\alpha}$-stimulated activation of SAPK/JNK. Additionally, we demonstrated that EGCG strengthened the $PGF_{2\alpha}$-induced phosphorylation of c-Jun, well known as a downstream effector of SAPK/JNK [Widmann et al., 1999; Weston and Davis, 2002], as well as SAPK/JNK. Taking our findings into account as a whole, it is most likely that EGCG upregulates $PGF_{2\alpha}$-stimulated VEGF synthesis through enhancing the activation of SAPK/JNK in osteoblast-like MC3T3-E1 cells. Further investigations are necessary to clarify the precise mechanism of catechin behind the amplification of VEGF synthesis in osteoblasts.

It is generally recognized that the expansion of capillary network providing microvasculature is an essential process of bone remodeling [Erlebacher et al., 1995]. Since VEGF is a specific mitogen of vascular endothelium [Ferrara and Davis-Smyth, 1997], it is speculated that VEGF synthesized by osteoblasts functions as a pivotal intercellular mediator between osteoblasts and vascular endothelial cells. 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 [Gerber et al., 1999], supporting the importance of VEGF in bone metabolism. On the other hand, it has been shown that catechin exerts an inhibitory effect on bone resorption [Delaisse et al., 1986]. Additionally, in osteoblasts, production of bone-resorptive cytokines such as tumor necrosis factor-α and interleukin-6 has been reported to be suppressed by catechin [Choi and Hwang, 2003]. Based on our results as a whole, it is probable that catechin-enhanced VEGF synthesized from osteoblasts acts a crucial role in the process of bone remodeling via regulating the capillary endothelial cells proliferation. Further investigations are required to elucidate the role of catechin in bone metabolism.

In conclusion, our present results strongly suggest that catechin upregulates $PGF_{2\alpha}$-stimulated VEGF synthesis via enhancing activation of SAPK/JNK among the MAP kinase superfamily in osteoblasts.

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(−)-Epigallocatechin gallate suppresses endothelin-1-induced interleukin-6 synthesis in osteoblasts: Inhibition of p44/p42 MAP kinase activation

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Abstract **We previously showed that endothelin-1 (ET-1) stimulates the synthesis of interleukin-6 (IL-6), a potent bone resorptive agent, in osteoblast-like MC3T3-E1 cells, and that protein kinase C (PKC)-dependent p44/p42 mitogen-activated protein (MAP) kinase plays a part in the IL-6 synthesis. In the present study, we investigated the effect of (−)-epigallocatechin gallate (EGCG), one of the major flavonoids containing in green tea, on ET-1-induced IL-6 synthesis in osteoblasts and the underlying mechanism. EGCG significantly reduced the synthesis of IL-6 stimulated by ET-1 in MC3T3-E1 cells as well primary cultured mouse osteoblasts. SB203580, a specific inhibitor of p38 MAP kinase, but not SP600125, a specific SAPK/JNK inhibitor, suppressed ET-1-stimulated IL-6 synthesis. ET-1-induced phosphorylation of p38 MAP kinase was not affected by EGCG. On the other hand, EGCG suppressed the phosphorylation of p44/p42 MAP kinase induced by ET-1. Both the IL-6 synthesis and the phosphorylation of p44/p42 MAP kinase stimulated by 12-*O*-tetradecanoylphorbol 13-acetate (TPA), a direct activator of PKC, were markedly suppressed by EGCG. The phosphorylation of MEK1/2 and Raf-1 induced by ET-1 or TPA were also inhibited by EGCG. These results strongly suggest that EGCG inhibits ET-1-stimulated synthesis of IL-6 via suppression of p44/p42 MAP kinase pathway in osteoblasts, and the inhibitory effect is exerted at a point between PKC and Raf-1 in the ET-1 signaling cascade.**

Keywords: Catechin; Endothelin-1; Interleukin-6; MAP kinase; Osteoblast

1. Introduction

Interleukin-6 (IL-6) is a pleiotropic cytokine that has important physiological effects on a wide range of functions such as promoting B cell differentiation, T cell activation and inducing acute phase proteins [1–4]. The bone metabolism is regulated by two functional cells, osteoblasts and osteoclasts, responsible for bone formation and bone resorption, respectively [5]. The formation of bone structures and bone remodeling results from the coupling process, bone resorption by activated osteoclasts with subsequent deposition of new matrix by osteoblasts. It is well recognized that IL-6 is one of the most potent osteoclastogenic factors in the bone metabolism [3,4]. Bone resorption is mediated by the increased local production of inflammatory cytokines such as tumor necrosis factor-α and IL-1. In osteoblasts [6–8], bone resorptive agents such as tumor necrosis factor-α and IL-1 have been reported to stimulate the synthesis of IL-6. As for the bone metabolism, IL-6 has been shown to stimulate bone resorption and induce osteoclast formation [3,4,6,9]. Therefore, accumulating evidence indicates that IL-6 secreted from osteoblasts plays a key role as a downstream effector of bone resorptive agents. In previous studies [10,11], we have shown that endothelin-1 (ET-1) induces the activation of p44/p42 mitogen-activated protein (MAP) kinase, a member of the MAP kinase superfamily [12], via protein kinase C (PKC), resulting in the IL-6 synthesis in osteoblast-like MC3T3-E1 cells. However, the mechanism of ET-1 behind the IL-6 synthesis in osteoblasts has not yet been precisely clarified.

It is well known that compounds in foods such as vegetables and fruits have beneficial properties on human beings. Among them, it has been reported that flavonoids possess antioxidative, antibacterial and antitumor effects [13,14]. Catechins are one of the major flavonoids, which are present in various species of plants such as green tea [14]. In bone metabolism, catechin has been shown to suppress bone resorption [15]. As for osteoblasts, it has been shown that catechin stimulates alkaline phosphatase activity, a mature osteoblast phenotype [5], and reduces apoptosis in osteoblast-like MC3T3-E1 cells [16]. However, the precise mechanism of catechin in osteoblasts is not fully known.

In the present study, we investigated whether (−)-epigallocatechin gallate (EGCG), one of the major flavonoids containing in green tea, affects the ET-1-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells and the mechanism behind it. We herein show that EGCG reduces the ET-1-stimulated

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IL-6 synthesis via attenuation of p44/p42 MAP kinase pathway in these cells.

2. Materials and methods

2.1. Materials

ET-1 and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) were purchased from Sigma Chemical Co. (St. Louis, MO). IL-6 ELISA kit was purchased from R&D Systems, Inc. (Minneapolis, MN). EGCG, SB203580 and SP600125 were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific MEK1/2 antibodies, MEK1/2 antibodies, phospho-specific Raf-1 antibodies and β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA). ECL Western blotting detection system was purchased from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. SB203580 and SP600125 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect either the assay for IL-6 activity or the Western blotting analysis.

2.2. Cell culture

The cloned osteoblast-like MC3T3-E1 cells, which have been derived from newborn mouse calvaria [17], were maintained as previously described [18]. 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 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

Primary cultured mouse osteoblasts were obtained from neonatal balb/c mouse by sequential collagenase digestions as previously described [19]. In brief, the calvarias of the neonatal mice were aseptically dissected from neonatal mice. The calvarias which were divided into small pieces were sequentially digested with 5 ml of PBS containing 0.1% collagenase (Sigma, MO) for 5 min at 37 °C. The cells isolated in fractions 2–6 were seeded into 90-mm diameter dishes in α-MEM containing 10% FCS at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The medium was exchanged at every 3 days. After reaching confluence, the cells were subcultured. After trypsinization using trypsin-EDTA (0.05%/0.53 mM), the cells were rinsed three times in 10 ml of PBS. The cells (1×10^5) were seeded into 35-mm diameter dishes in 2 ml of α-MEM containing 10% FCS. After reaching confluency, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h. We confirmed that the cultured cells possessed the high alkaline phosphatase activity, a mature osteoblasts phenotype [20].

2.3. Assay for IL-6

The cultured cells were stimulated by ET-1 in 1 ml of 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 EGCG, SB203580 or SP600125 for 60 min.

2.4. Analysis of Western blotting

The cultured cells were stimulated by ET-1 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 $125000 \times g$ for 10 min at 4 °C. SDS–polyacrylamide gel electrophoresis (PAGE) was performed by Laemmli [21] in 10% polyacrylamide gel. A Western blotting analysis was performed as described previously [22] by using phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific p44/p42 MAP kinase or p44/p42 MAP kinase with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. The peroxidase activity on PVDG 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 min.

2.5. 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).

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 means ± S.E.M. of triplicate determinations. Each experiment was repeated three times with similar results.

3. Results

3.1. Effects of EGCG on the ET-1-stimulated IL-6 synthesis in MC3T3-E1 cells and primary cultured mouse osteoblasts

We have previously demonstrated that ET-1 stimulates IL-6 synthesis in osteoblast-like MC3T3-E1 cells [10]. We first examined the effect of EGCG on the ET-1-stimulated IL-6 synthesis. EGCG, which alone had little effect on the IL-6 levels, reduced the ET-1-stimulated synthesis of IL-6 in a dose-dependent manner in the range between 1 and 100 μM (Table 1). In addition, we also examined the effect of EGCG in primary cultured mouse osteoblasts, and found that EGCG (30 μM) significantly reduced the IL-6 synthesis induced by ET-1 (Table 2). EGCG (30 μM) caused about a 55% reduction in the ET-1-effect.

3.2. Effects of SB203580 or SP600125 on the ET-1-stimulated IL-6 synthesis in MC3T3-E1 cells

In our previous study [11], we reported that p44/p42 MAP kinase plays a part in the ET-1-stimulated IL-6 synthesis in MC3T3-E1 cells. Among the MAP kinase superfamily, p38

Table 1
Effects of EGCG, SB203580 or SP600125 on the ET-1-stimulated IL-6 synthesis in MC3T3-E1 cells

EGCG or inhibitor	ET-1	IL-6 (pg/ml)
Vehicle	–	16 ± 5
Vehicle	+	468 ± 38*
EGCG (1 μM)	–	15 ± 10
EGCG (1 μM)	+	419 ± 35
EGCG (10 μM)	–	17 ± 8
EGCG (10 μM)	+	414 ± 30
EGCG (50 μM)	–	15 ± 10
EGCG (50 μM)	+	347 ± 28**
EGCG (100 μM)	–	23 ± 8
EGCG (100 μM)	+	69 ± 12**
SB203580	–	10 ± 7
SB203580	+	85 ± 12**
SP600125	–	15 ± 10
SP600125	+	456 ± 29

The cultured cells were pretreated with various doses of EGCG, 10 μM SB203580, 10 μM SP600125 or vehicle for 60 min, and then stimulated by 0.1 μM ET-1 or vehicle for 24 h. Each value represents the means ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.
*$P < 0.05$, compared to the control value.
**$P < 0.05$, compared to the value of ET-1 alone.

Table 2
Effects of EGCG on the ET-1-stimulated IL-6 synthesis in primary cultured mouse osteoblasts

EGCG	ET-1	IL-6 (pg/ml)
–	–	248 ± 39
–	+	354 ± 26*
+	–	216 ± 8
+	+	263 ± 26**

The cultured cells were pretreated with 30 μM EGCG or vehicle for 60 min, and then stimulated by 0.1 μM ET-1 or vehicle for 24 h. Each value represents the means ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.
*$P < 0.05$, compared to the control value.
**$P < 0.05$, compared to the value of ET-1 alone.

MAP kinase and stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) in addition to p44/p42 MAP kinase, which together play a crucial role as intracellular components to transduce the various signals of agonists [12]. We have previously shown that ET-1 also activates p38 MAP kinase and SAPK/JNK [23,24]. In order to clarify the involvement of the MAP kinases in the ET-1-stimulated IL-6 synthesis in these cells, we examined the effect of SB203580, a specific inhibitor of p38 MAP kinase [25], or SP600125, a specific SAPK/JNK inhibitor [26], on the IL-6 synthesis. SB203580, which by itself had little effect on the IL-6 levels, significantly suppressed the ET-1-stimulated synthesis of IL-6 (Table 1). On the other hand, SP600125 hardly affected the IL-6 synthesis induced by ET-1 (Table 1).

3.3. Effect of EGCG on the phosphorylation of p38 MAP kinase by ET-1 in MC3T3-E1 cells

In order to clarify whether p38 MAP kinase is involved in EGCG-effect on the ET-1-stimulated IL-6 synthesis in MC3T3-E1 cells, we examined the effect of EGCG on the ET-1-induced phosphorylation of p38 MAP kinase. However, EGCG had little effect on the ET-1-induced phosphorylation of p38 MAP kinase (Fig. 1).

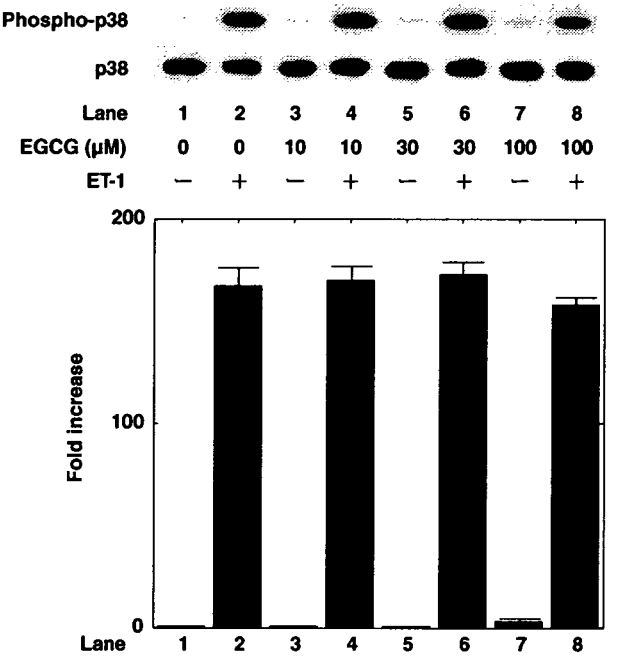

Fig. 1. Effect of EGCG on the phosphorylation of p38 MAP kinase induced by ET-1 in MC3T3-E1 cells. The cultured cells were pretreated with the indicated doses of EGCG or vehicle for 60 min, and then stimulated by 3 nM ET-1 or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE with a 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 ET-1-induced phosphorylation obtained from a laser densitometric analysis of three independent experiments. Each value represents the means ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

3.4. Effect of EGCG on the phosphorylation of p44/p42 MAP kinase induced by ET-1 in MC3T3-E1 cells

In order to investigate whether EGCG-effect on the ET-1-stimulated IL-6 synthesis is mediated through p44/p42 MAP kinase activation in MC3T3-E1 cells, we next examined the effect of EGCG on the ET-1-induced phosphorylation of p44/p42 MAP kinase. EGCG markedly attenuated the ET-1-induced phosphorylation of p44/p42 MAP kinase (Fig. 2). According to the densitometric analysis, EGCG (100 μM) caused about 80% reduction in the ET-1-effect.

3.5. Effects of EGCG on the TPA-stimulated IL-6 synthesis in MC3T3-E1 cells

We previously reported that PKC functions at a point upstream from p44/p42 MAP kinase in the ET-1-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells [11]. We next examined the effect of EGCG on the IL-6 synthesis stimulated by TPA, a direct activator of PKC [27]. In our previous study [28], we found that TPA alone stimulates IL-6 synthesis in MC3T3-E1 cells. EGCG, which by itself had little effect on the IL-6 levels, significantly reduced the TPA-stimulated synthesis of IL-6 (Table 3). EGCG (100 μM) caused about an 80% reduction in the TPA-effect.

3.6. Effect of EGCG on the phosphorylation of p44/p42 MAP kinase induced by TPA in MC3T3-E1 cells

In order to investigate whether EGCC-effect on the TPA-stimulated IL-6 synthesis is mediated through inhibition of p44/p42 MAP kinase activation in MC3T3-E1 cells, we next examined the effect of EGCC on the TPA-induced phosphorylation of p44/p42 MAP kinase. We previously found that TPA induces the phosphorylation of p44/p42 MAP kinase in these cells [29]. EGCC markedly suppressed the TPA-induced phosphorylation of p44/p42 MAP kinase (Fig. 3).

3.7. Effects of EGCG on the phosphorylation of MEK1/2 and Raf-1 induced by ET-1 or TPA in MC3T3-E1 cells

It is well known that p44/p42 MAP kinase is activated by an upstream kinase, MEK1/2, which is activated by Raf-1 [12]. To clarify the exact mechanism of EGCG behind the IL-6 synthesis, we further examined the effects of EGCG on the phosphorylation of MEK1/2 and Raf-1 induced by ET-1 or TPA in MC3T3-E1 cells. The levels of ET-1 or TPA-induced phosphorylation of MEK1/2 were reduced by EGCG (data not shown). EGCG suppressed the levels of both ET-1- and TPA-induced the phosphorylation of Raf-1 (Fig. 4).

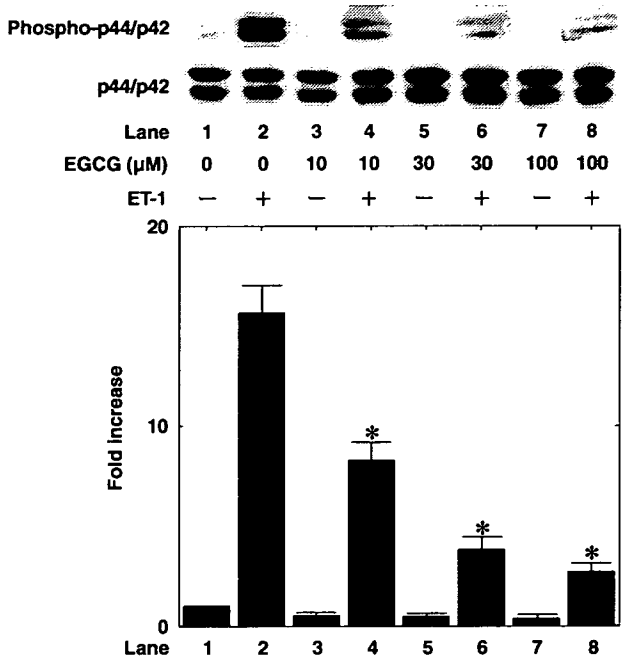

Fig. 2. Effect of EGCG on the phosphorylation of p44/p42 MAP kinase induced by ET-1 in MC3T3-E1 cells. The cultured cells were pretreated with the indicated doses of EGCG or vehicle for 60 min, and then stimulated by 3 nM ET-1 or vehicle for 5 min. The extracts of the cells were subjected to SDS–PAGE with a 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 ET-1-induced phosphorylation obtained from a laser densitometric analysis of three independent experiments. Each value represents the means ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}P < 0.05$, compared to the value of ET-1 alone.

Table 3
Effect of EGCG on the TPA-stimulated IL-6 synthesis in MC3T3-E1 cells

EGCG	TPA	IL-6 (pg/ml)
−	−	19 ± 7
−	+	415 ± 31*
+	−	29 ± 10
+	+	98 ± 17**

The cultured cells were pretreated with 100 μM EGCG or vehicle for 60 min, and then stimulated by 0.1 μM TPA or vehicle for 24 h. Each value represents the means ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.
*$P < 0.05$, compared to the control value.
**$P < 0.05$, compared to the value of TPA alone.

4. Discussion

In the present study, we showed that EGCG, which by itself had little effect on the levels of IL-6, significantly reduced the ET-1-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells. We found that EGCG reduced the IL-6 synthesis induced by ET-1 also in primary cultured mouse osteoblasts. It is likely that the inhibitory effect of EGCG on the IL-6 synthesis induced by ET-1 is not specific in a clonal osteoblast-like

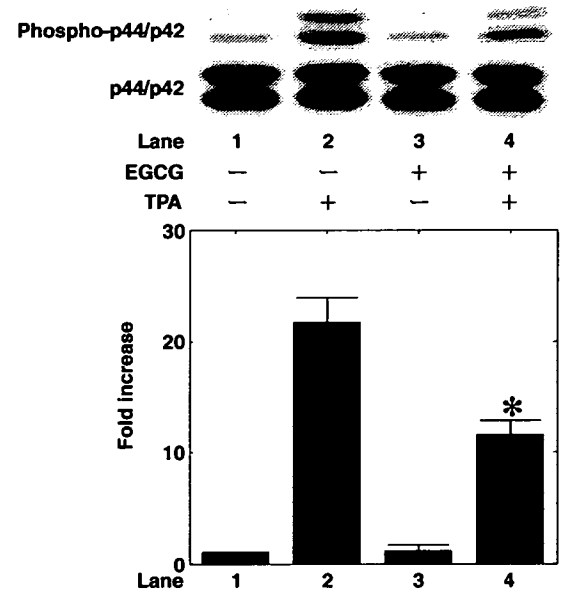

Fig. 3. Effect of EGCG on the phosphorylation of p44/p42 MAP kinase induced by TPA in MC3T3-E1 cells. The cultured cells were pretreated with 100 μM EGCG or vehicle for 60 min, and then stimulated by 0.1 μM TPA or vehicle for 60 min. The extracts of cells were subjected to SDS–PAGE with a subsequent 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 TPA-induced phosphorylation obtained from a laser densitometric analysis of three independent experiments. Each value represents the means ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}P < 0.05$, compared to the value of TPA alone.

MC3T3-E1 cells but it is common in osteoblasts. We next investigated the mechanism of EGCG underlying the suppressive effect on the IL-6 synthesis. It is generally known that the MAP kinase superfamily plays a pivotal role in cellular functions including proliferation, differentiation, and cell death in a variety of cells [12]. Three major MAP kinase, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK are known as central elements used by mammalian cells to transduce the diverse messages [12]. We have previously reported that ET-1-activated p44/p42 MAP kinase acts as a positive regulator in ET-1-induced IL-6 synthesis in osteoblast-like MC3T3-E1 cells [11]. In addition, we demonstrated that p38 MAP kinase and SAPK/JNK are activated by ET-1 in these cells [23,24]. In the present study, we showed that SB203580 [25], markedly suppressed the ET-1-induced IL-6 synthesis while SP600125 [26], failed to affect the IL-6 synthesis. We previously showed that ET-1-induced both synthesis of vascular endothelial growth factor and phosphorylation of SAPK/JNK are significantly suppressed by 10 μM SP600125 in MC3T3-E1 cells [24]. It thus seems unlikely that SAPK/JNK is involved in the ET-1-induced IL-6 synthesis in osteoblast-like MC3T3-E1 cells. Based on our results, it is probable that ET-1 stimulates the synthesis of IL-6 via p38 MAP kinase in addition to p44/p42 MAP kinase in osteoblast-like MC3T3-E1 cells.

We further investigated the involvement of p38 MAP kinase and p44/p42 MAP kinase in the inhibitory effect of EGCG on the IL-6 synthesis. EGCG attenuated the ET-1-induced phosphorylation of p44/p42 MAP kinase without affecting the p38 MAP kinase phosphorylation. Therefore, our findings strongly

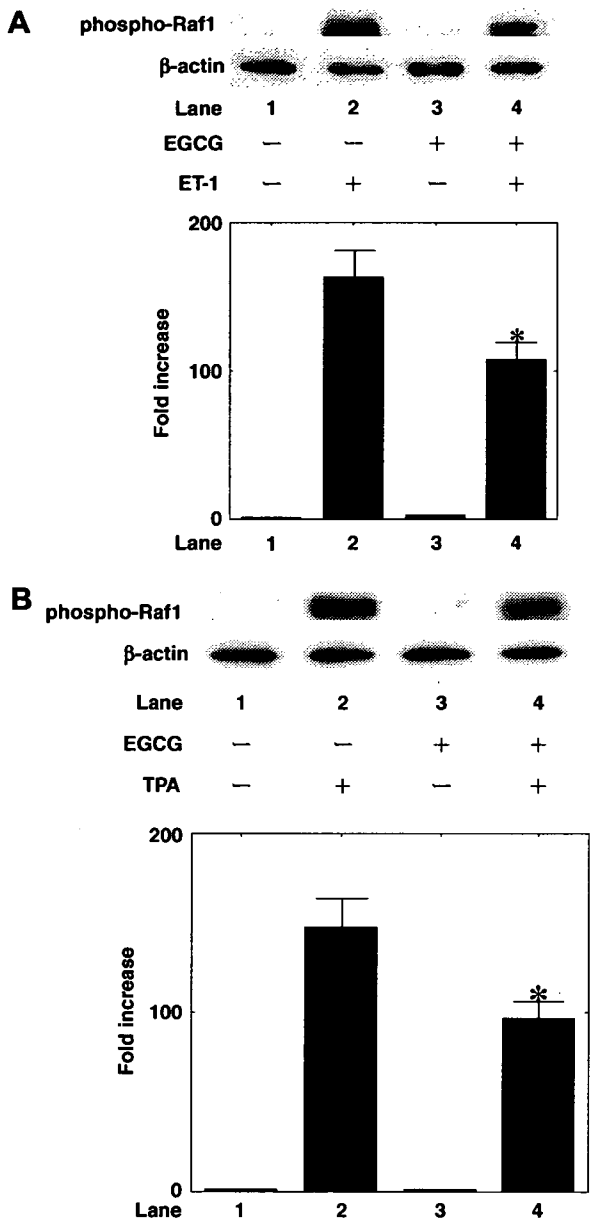

Fig. 4. Effects of EGCG on the phosphorylation of Raf-1 induced by ET-1 or TPA in MC3T3-E1 cells. The cultured cells were pretreated with 100 μM EGCG or vehicle for 60 min, and then stimulated by 0.1 μM ET-1 (A), 0.1 μM TPA (B) or vehicle for 60 min. The extracts of cells were subjected to SDS–PAGE with a subsequent Western blotting analysis with antibodies against phospho-specific Raf-1 or β-actin. The histogram shows quantitative representations of the levels of ET-1- (A) or TPA-induced phosphorylation (B) obtained from a laser densitometric analysis of three independent experiments. Each value represents the means ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^*P < 0.05$, compared to the value of ET-1 (A) or TPA (B) alone.

suggest that EGCG inhibits the ET-1-induced IL-6 synthesis via the down-regulating p44/p42 MAP kinase pathway in osteoblast-like MC3T3-E1 cells. In our previous studies [10,11], we demonstrated that p44/p42 MAP kinase acts at a point downstream of PKC in ET-1-stimulated IL-6 synthesis in MC3T3-E1 cells. To clarify whether the effect of catechin is exerted at a point downstream of PKC or not, we additionally examined the effect of EGCG on the IL-6 synthesis by TPA [27]. We have found that TPA alone stimulates IL-6 synthesis in MC3T3-E1 cells [28]. We herein presented that EGCG reduced the TPA-stimulated IL-6 synthesis as well as the TPA-induced phosphorylation of p44/p42 MAP kinase in these cells, suggesting that the effect of EGCG is exerted at a point upstream of p44/p42 MAP kinase. Furthermore, we found that EGCG actually suppressed the levels of both ET-1- and TPA-induced phosphorylation of MEK1/2 and Raf-1. Taking our findings into account as a whole, it is most likely that EGCG down-regulates ET-1-stimulated IL-6 synthesis in osteoblasts, and that the effect is exerted at a point between PKC and Raf-1. Further investigations are necessary to clarify the precise mechanism of catechin underlying the down-regulation of IL-6 synthesis in osteoblasts.

IL-6 is well recognized to be a potent bone resorptive agent and induces osteoclast formation [4,6]. On the other hand, catechin reportedly has an inhibitory effect on bone resorption [15]. In addition, it has recently been shown that catechin increases cell viability and alkaline phosphatase activity, a marker of mature osteoblast phenotype [5], in osteoblast-like MC3T3-E1 cells and apoptosis of these cells is suppressed by catechin [16]. Taking our results into account, it is probable that catechin-induced suppression of p44/p42 MAP kinase activation plays an effect against bone resorption via down-regulating IL-6 synthesis in osteoblasts. Our present data therefore provide new insight into the pharmacological effects of catechin action on bone cells. Further investigations are required to elucidate the exact role of catechin in the bone metabolism.

In conclusion, these results strongly suggest that catechin inhibits the ET-1-stimulated synthesis of IL-6 via suppression of p44/p42 MAP kinase pathway in osteoblasts, and this effect is exerted at a point between PKC and Raf-1 in the ET-1 signaling cascade.

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Activation of phosphatidylinositol 3-kinase/Akt limits FGF-2-induced VEGF release in osteoblasts

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Abstract

We previously reported that basic fibroblast growth factor (FGF-2) activates stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) and p44/p42 mitogen-activated protein (MAP) kinase, resulting in the release of vascular endothelial growth factor (VEGF) in osteoblast-like MC3T3-E1 cells. In the present study, we investigated the role of Akt/protein kinase B in the FGF-2-stimulated VEGF release in these cells. FGF-2 time-dependently induced the phosphorylation of Akt and GSK-3β, a downstream element of Akt. The Akt inhibitor, 1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-*methyl*-3-*O*-octadecylcarbonate, significantly amplified the FGF-2-induced VEGF release, in a dose-dependent manner between 1 and 70 μM, while it suppressed the FGF-2-induced phosphorylation of GSK-3β. The phosphorylation of Akt induced by FGF-2 was markedly attenuated by wortmannin and LY294002, inhibitors of phosphatidylinositol 3-kinase (PI3-kinase) in osteoblast-like MC3T3-E1 cells. Both wortmannin and LY294002 enhanced the FGF-2-induced VEGF release. In addition, Akt inhibitor had no significant effect on the FGF-2-induced phosphorylation of p44/p42 MAP kinase and SAPK/JNK. Furthermore, the FGF-2-induced Akt phosphorylation was not affected by PD98059, a MEK inhibitor, or SP600125, a SAPK/JNK inhibitor. Taken together, our findings strongly suggest that PI3-kinase/Akt plays an inhibitory role in FGF-2-induced VEGF release in osteoblasts.

Keywords: FGF-2; PI3K/Akt; VEGF; Osteoblast

1. Introduction

It is generally known that bone remodeling carried out by osteoclasts and osteoblasts is accompanied by angiogenesis and capillary outgrowth (Brighton, 1978; Brighton and Hunt, 1991; Parfitt, 1994). During bone remodeling, capillary endothelial cells provide the microvasculature. Thus, it is currently recognized that the activities of osteoblasts, osteoclasts and capillary endothelial cells are closely coordinated and regulate bone metabolism (Erlebacher et al., 1995). These functional cells influence one another via humoral factors as well as by direct cell-to-cell contact. Vascular endothelial growth factor (VEGF) is a heparin-binding angiogenic growth factor displaying high specificity for vascular endothelial cells (Ferrara and Davis-Smyth, 1997). VEGF that is produced and secreted from a variety of cell types, increases capillary permeability and stimulates proliferation of endothelial cells (Ferrara and Davis-Smyth, 1997). 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 (Gerber et al., 1999). Accumulating evidence indicates that osteoblasts produce and secrete VEGF in response to various humoral factors (Goad et al., 1996; Wang et al., 1996; Ferrara and Davis-Smyth, 1997; Schalaeppi et al., 1997). Therefore, it is thought that VEGF secreted from osteoblasts may play a crucial role in the regulation of bone metabolism (Erlebacher et al., 1995; Zelzer and Olsen, 2005). However, the exact mechanism behind VEGF synthesis and its release in osteoblasts is not fully understood.

Basic fibroblast growth factor (FGF-2) is embedded in bone matrix, and osteoblasts synthesize FGF-2 (Baylink et al., 1993; Hurley et al., 1993). FGF-2 expression in osteoblasts is detected during fracture repair (Bolander, 1992). Therefore, it is

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currently recognized that FGF-2 may play a pivotal role in fracture healing, bone remodeling and osteogenesis (Marie, 2003). We have previously reported that FGF-2 stimulates VEGF release in MC3T3-E1 cells, and that among the mitogen-activated protein (MAP) kinase superfamily (Widmann et al., 1999), p44/p42 MAP kinase and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) play as positive regulators in the VEGF release (Tokuda et al., 2000, 2003).

It is well recognized that Akt, also called protein kinase B, is a serine/threonine protein kinase that plays crucial roles in mediating intracellular signaling of variety of agonists including FGF-2, insulin-like growth factor I (IGF-I) and platelet-derived growth factor (PDGF) (Coffer et al., 1998). It has been shown that Akt regulates biological functions such as gene expression, cell survival and oncogenesis (Coffer et al., 1998). Akt has been identified as a downstream target of phosphatidylinositol 3-kinase (PI3-kinase) (Chan et al., 1999; Cantley, 2002). As for osteoblasts, FGF-2 reportedly induces the cell survival via PI3-kinase/Akt (Chaudhary and Hruska, 2001; Debiais et al., 2004). We have recently shown that IGF-I stimulates activity of alkaline phosphatase, a mature osteoblast phenotype, via Akt in osteoblast-like MC3T3-E1 cells (Noda et al., 2005). These findings gave rise to our speculation that Akt in osteoblasts may play an important role in the regulation of bone metabolism. However, the relationship between VEGF release and PI3-kinase/Akt in osteoblasts remains unclear. In the present study, therefore, we investigated whether Akt is involved in the FGF-2-induced VEGF release in osteoblast-like MC3T3-E1 cells. We here show that PI3-kinase/Akt auto-regulates FGF-2-induced VEGF release in these cells.

2. Materials and methods

2.1. Materials

Mouse VEGF enzyme immunoassay kit was purchased from R&D Systems, Inc. (Minneapolis, MN). Akt inhibitor (1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate), LY294002, wortmannin, PD98059 and SP600125 were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Actinomycin D was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Phospho-specific Akt antibodies, Akt antibodies, phospho-specific GSK-3β antibodies, GSK-3β 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, Inc. (Beverly, MA). ECL Western blotting detection system was purchased from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. Akt inhibitor, wortmannin, LY294002, 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.

2.2. Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria (Sudo et al., 1983) were maintained as previously described (Kozawa et al., 1992). 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 or 90-mm diameter dishes in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 24 h.

Freshly isolated osteoblasts were obtained from the calvaria of newborn (1 or 2-day-old) balb/c mice as previously described (Yoshida et al., 2004). They were seeded into 90-mm-diameter dishes (25×10^4 cells) in α-MEM containing 10% FCS. The medium was changed every 3 days until the cells were reached confluence at about 5 days. Then, 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 stimulated by FGF-2 in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. The cells were pretreated with Akt inhibitor, wortmannin, LY294002 or actinomycin D for 60 min. The reaction was terminated by collecting the medium, and then VEGF in the medium was measured by Quantikine® mouse VEGF enzyme immunoassay kit (R&D systems) according to the manufacture's instruction. The assay kit can detect the mouse VEGF in the range between 7.8 and 500 pg/ml. When the samples generate values higher than 500 pg/ml, the samples were adequately diluted with caliblator diluent provided with the kit, and re-assayed.

2.4. 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% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at $125{,}000 \times g$ for 10 min at 4 °C. SDS–polyacrylamide gel electrophoresis (PAGE) was performed by Laemmli (1970) in 10% polyacrylamide gel. Western blotting analysis was performed as described previously (Kato et al., 1996) by using phospho-specific Akt antibodies, Akt antibodies, phospho-specific GSK-3β antibodies, GSK-3β 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 PVDF sheet was visualized on X-ray film by means of the ECL Western blotting detection system.

2.5. Determination

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

2.6. Statistical analysis

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

3. Results

3.1. Effect of FGF-2 on the phosphorylation of Akt in MC3T3-E1 cells

We examined the effect of FGF-2 on the phosphorylation of Akt in order to investigate whether FGF-2 activates Akt in MC3T3-E1 cells. FGF-2 time-dependently induced the phosphorylation of Akt up to 60 min (Fig. 1). The maximum effect of FGF-2 on the phosphorylation of Akt was observed at 10 min after the stimulation.

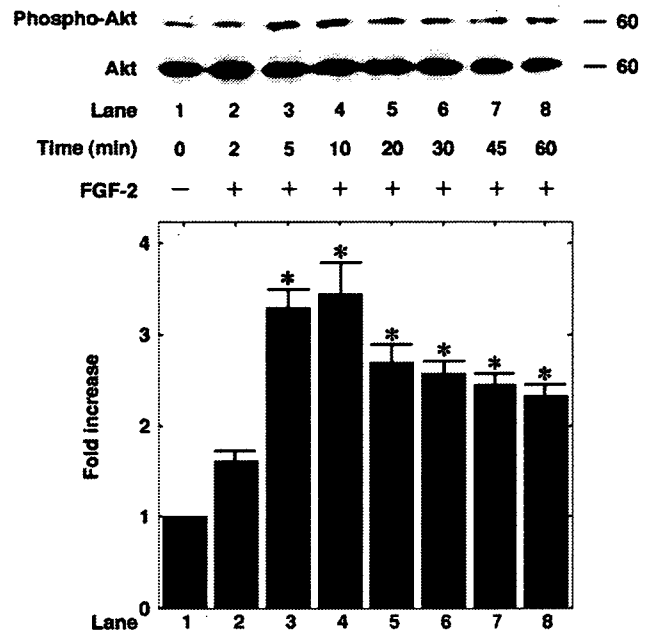

Fig. 1. Effect of FGF-2 on the phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were stimulated by 30 ng/ml FGF-2 for the indicated periods. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. The 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 ± S.E.M. of triplicate determinations. Numbers on the right indicate molecular masses (kDa). Similar results were obtained with two additional and different cell preparations. $^{*}p < 0.05$, compared to the value of control.

3.2. Effects of Akt inhibitor on the VEGF release by FGF-2 or the FGF-2-induced phosphorylation of Akt in MC3T3-E1 cells

In our previous studies (Tokuda et al., 2000, 2003), we have demonstrated that FGF-2 stimulates VEGF release in osteoblast-like MC3T3-E1 cells. In order to clarify whether or not Akt pathway is involved in the FGF-2-stimulated VEGF release in these cells, we first examined the effect of Akt inhibitor, 1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-*methyl*-3-*O*-octadecylcarbonate (Hu et al., 2000), on the VEGF release. The Akt inhibitor, which by itself had little effect on the VEGF levels, significantly amplified the FGF-2-induced release of VEGF (Fig. 2A). The amplifying effect of the Akt inhibitor on the VEGF release was dose-dependent between 1 and 70 μM (Fig. 2A). The Akt inhibitor at 70 μM caused about 140% enhancement in the FGF-2-effect. We next examined the effect of the Akt inhibitor on the phosphorylation of Akt induced by FGF-2 in MC3T3-E1 cells. The Akt inhibitor failed to affect the FGF-2-induced phosphorylation of Akt (Fig. 2B).

3.3. Effect of Akt inhibitor on the VEGF release by FGF-2 in primary culture of osteoblasts

We investigated the effect of Akt inhibitor on the FGF-2-induced VEGF release in primary culture of osteoblasts. We found that the Akt inhibitor significantly enhanced the FGF-2-induced VEGF release also in primary cultured osteoblasts (Fig. 2C). The amplifying effect of the Akt inhibitor on the VEGF release was dose-dependent in the range between 1 and 30 μM (Fig. 2C). The maximum effect of the Akt inhibitor (30 μM) caused about 60% enhancement in the FGF-2-effect.

3.4. Effect of Akt inhibitor on the phosphorylation of Akt and GSK-3β induced by FGF-2 in MC3T3-E1 cells

It is well recognized that GSK-3 is a critical downstream target molecule of the Akt cell survival pathway, and its activity can be inhibited by Akt mediated phosphorylation of GSK-3α at Ser21 and GSK-3β at Ser9 (Cross et al., 1995; Srivastava and Pandey, 1998). We found that FGF-2 truly induced the Akt mediated phosphorylation of GSK-3β time-dependent manner in MC3T3-E1 cells (Fig. 3A). Then, we examined the effect of Akt inhibitor on the phosphorylation of GSK-3β induced by FGF-2 in these cells. Akt inhibitor markedly suppressed the FGF-2-induced phosphorylation of GSK-3β (Fig. 3B). The inhibitor (50 μM) caused a reduction in FGF-2-effect of about 50%.

3.5. Effects of LY294002 or wortmannin on the FGF-2-induced VEGF release in MC3T3-E1 cells

To investigate whether or not PI3-kinase is correlated to Akt, we examined the effect of LY294002, a specific inhibitor of PI3-kinase (Vlahos et al., 1994), on the VEGF release by FGF-2 in MC3T3-E1 cells. LY294002, alone hardly affected the VEGF levels, significantly enhanced the FGF-2-induced VEGF release. The amplifying effect of LY294002 was dose-dependent between 3 and 30 μM (Fig. 4A). The LY294002 at 30 μM caused about 60% enhancement in the FGF-2-effect. Wortmannin, another PI3-kinase inhibitor (Arcaro and Wymann, 1993), as well as LY294002, also enhanced the FGF-2-induced VEGF release in the range between 1 and 3 nM without affecting VEGF release alone (Fig. 4B). Wortmannin at 3 nM caused about 60% enhancement in the FGF-2-effect.

3.6. Effects of wortmannin or LY294002 on the FGF-2-induced phosphorylation of Akt in MC3T3-E1 cells

We next examined the effects of wortmannin or LY294002 on the phosphorylation of Akt in MC3T3-E1 cells. Both wortmannin and LY294002 truly suppressed the FGF-2-induced phosphorylation of Akt (data not shown). Wortmannin (100 nM) caused a reduction in FGF-2-effect of about 80%. In addition, we found that the phosphorylation of GSK-3β induced by FGF-2 was significantly attenuated by wortmannin and LY294002 (Fig. 5A and B).

3.7. Effects of Akt inhibitor on FGF-2-induced phosphorylations of p44/p42 MAP kinase and SAPK/JNK in MC3T3-E1 cells

We have previously reported that FGF-2 stimulates VEGF release at least in part via p44/p42 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells (Tokuda et al., 2000, 2003).