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Amplification by (-)-epigallocatechin gallate of prostaglandin $F_2\alpha$-stimulated synthesis of osteoprotegerin in osteoblasts

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Abstract. (-)-Epigallocatechin gallate (EGCG) and chlorogenic acid (CGA), major flavonoids in green tea, and coffee, respectively, are recognized as possessing potential benefits in a multitude of human health conditions, including bone disorders. We have previously demonstrated that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), a potent bone remodeling mediator, stimulates the synthesis of osteoprotegerin (OPG) through the activation of p44/p42 mitogen-activated protein kinase (MAPK), p38 MAPK and stress activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in osteoblast-like MC3T3-E1 cells. In the present study, the effects of EGCG and CGA on $PGF_{2\alpha}$-stimulated OPG synthesis in MC3T3-E1 cells were investigated. EGCG significantly upregulated $PGF_{2\alpha}$-stimulated OPG release, whereas CGA did not affect OPG release. The $PGF_{2\alpha}$-induced expression level of OPG mRNA was enhanced by EGCG. Regarding the intracellular signaling underlying the effect of EGCG, EGCG failed to affect $PGF_{2\alpha}$-stimulated phosphorylation of p44/p42 MAPK, p38 MAPK or SAPK/JNK. EGCG by itself markedly induced the phosphorylation of p44/p42 MAP kinase for up to 10 min and the status decreased subsequently, whereas EGCG did not significantly affect the phosphorylation status of p38 MAPK or SAPK/JNK within 60 min. These results indicated that EGCG, but not CGA amplifies the $PGF_{2\alpha}$-stimulated OPG synthesis in osteoblasts.

Introduction

Green tea and coffee are traditionally consumed as dietary beverages all over the world. It is generally recognized that polyphenolic compounds in green tea have potentially beneficial effects on human health including anti-inflammation, anti-oxidation and prevention of tumor development (1,2). In addition, polyphenolic compounds in coffee are proposed to potentiate human health benefits such as prevention of type 2 diabetes mellitus and Parkinson's disease (3). A major flavonoid of green tea is (-)-epigallocatechin gallate (EGCG), which is considered as one of the most active polyphenolic molecules relating to the health promoting properties of this beverage (4). On the other hand, chlorogenic acid (CGA) is a main phenolic compound in coffee (5). Regarding the beneficial properties of green tea and coffee for bone disorders, accumulating evidence indicates that consumption of green tea prevents both age-related bone loss and fracture in elderly people (6), and that coffee consumption reduces the risk of osteoporosis and osteoporotic fracture (3). However, the details underlying the beneficial effects of green tea and coffee on bone remain unclear.

Bone metabolism is mainly regulated by two types of functional cells, osteoblasts and osteoclasts (7). The former cells are responsible for bone formation, and the latter cells are for bone resorption. In the adult skeletal system, bone mass is continuously maintained by resorption of old bone and subsequent formation of new bone, so called bone remodeling. Disorder of this important process is considered to cause metabolic bone diseases, including osteoporosis and fracture healing distress. It is currently recognized that various humoral factors including cytokines, hormones, growth factors and prostaglandins (PGs) play important roles as bone remodeling mediators (8). As for the EGCG-effects on bone metabolism, it has been shown that EGCG attenuates bone resorption through the inhibition of osteoclast formation, resulting in suppression of osteoclastogenesis (5). In osteoblasts, EGCG reportedly increases alkaline phosphatase activity followed by increment of osteoblastic bone formation (5). On the other hand, it has been reported that CGA suppresses osteoclast-mediated bone resorption by downregulation of receptor activator of nuclear factor-κB (RANK) ligand-mediating effects (9). *In vivo* studies in rat model, CGA increases mineralization in the tibia and improve mechanical properties of the femoral diaphysis (10). We have previously reported that EGCG suppresses the interleukin-6 (IL-6) synthesis stimulated by platelet-derived growth factor-BB, basic fibroblast growth factor or endothelin-1 in

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osteoblast-like MC3T3-E1 cells (11-13). Additionally, we recently demonstrated that EGCG and CGA enhance the tumor necrosis factor-α (TNF-α)-stimulated IL-6 synthesis in these cells (14). However, the exact mechanism behind the effects of EGCG and CGA on bone remodeling has not yet been elucidated.

Osteoprotegerin (OPG) is a cytokine which has an inhibitory effect on osteoclast functions. OPG is classified into a member of the TNF receptor family along with RANK (15). OPG, which is produced in osteoblasts and secreted, binds to RANK ligand (RANKL) as a decoy receptor and prevents RANKL from binding to RANK, resulting in the suppression of bone resorption through the reduction of both osteoclastgenesis and osteoclast activity (16). OPG-deficient mice and RANKL-overexpressing transgenic mice reportedly suffer from severe osteoporosis (17,18). Therefore, it is generally recognized that RANK/RANKL/OPG axis is a major regulatory system for bone remodeling (19). On the other hand, PGs are lipid mediators with various functions and play crucial roles in the pathophysiological responses of skeletal tissue (20). Among them, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is a potent stimulator of bone resorption and promotes osteoclast formation (20). In addition, accumulating evidence indicates that $PGF_{2\alpha}$ also takes part in the process of bone formation (21). We have previously shown that $PGF_{2\alpha}$ stimulates the synthesis of OPG through the activation of p44/p42 mitogen-activated protein (MAP) kinase, p38 MAP kinase and stress activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) in osteoblast-like MC3T3-E1 cell (22).

In the present study, we investigated the effects of EGCG or CGA on the $PGF_{2\alpha}$-stimulated OPG synthesis in osteoblast-like MC3T3-E1 cells. We herein show that EGCG but not CGA upregulates the $PGF_{2\alpha}$-stimulated OPG synthesis in osteoblasts.

Materials and methods

Materials. EGCG, CGA and $PGF_{2\alpha}$ were purchased from Sigma-Aldrich (St. Louis, MO, USA). A mouse OPG enzyme-linked immunosorbent assay (ELISA) kit was obtained from R&D Systems, Inc. (Minneapolis, MN, USA). 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 and SAPK/JNK antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The enhanced chemiluminescence (ECL) Western Blotting Detection system was obtained from GE Healthcare Life Sciences (Little Chalfont, UK). Other materials and chemicals were obtained from commercial sources. EGCG was dissolved in dimethyl sulfoxide. CGA and $PGF_{2\alpha}$ were dissolved in ethanol. The maximum concentration of dimethyl sulfoxide or ethanol was 0.1%, which did not affect either the assay for OPG or western blot analysis.

Cell culture. Cloned osteoblast-like MC3T3-E1 cells that have been derived from newborn mouse calvaria (23) were maintained as previously described (24). Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS) at 37˚C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 35-mm diameter dishes ($5x10^4$ cells/dish) or 90-mm diameter dishes ($2x10^5$ cells/dish) in α-MEM containing 10% FBS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FBS. The cells were used for experiments after 48 h.

Assay for OPG. The cultured cells were pretreated with various doses of EGCG or CGA for 60 min, and then stimulated by 10 μM of $PGF_{2\alpha}$ or vehicle in 1 ml of α-MEM containing 0.3% FBS for the indicated periods. The conditioned medium was collected at the end of incubation, and the OPG concentration was then measured using a mouse OPG ELISA kit according to the manufacturer's protocol.

Real-time (RT)-PCR. The cultured cells were pretreated with 10 μM of EGCG or vehicle for 60 min, and then stimulated by 10 μM of $PGF_{2\alpha}$ or vehicle in α-MEM containing 0.3% FBS for 6 h. Total RNA was isolated and transcribed into complementary DNA using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) and OmniScript Reverse Transcriptase kit (Qiagen, Inc., Valencia, CA, USA), respectively. RT-PCR was performed using a LightCycler system (version 3.5; Roche Diagnostics, Basel, Switzerland) in capillaries and FastStart DNA Master SYBR Green I provided with the kit (Roche Diagnostics). Sense and antisense primers for mouse OPG mRNA were purchased from Takara Bio, Inc. (primer set ID: MA026526; Otsu, Japan), while mouse GAPDH mRNA primers were synthesized based on the report of Simpson *et al* (25). The amplified products were determined using a melting curve analysis. The OPG mRNA levels were normalized to those of GAPDH mRNA.

Western blot analysis. The cultured cells were pretreated with various doses of EGCG for the indicated periods, and then stimulated by 10 μM of $PGF_{2\alpha}$ or vehicle in α-MEM containing 0.3% FBS 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. SDS-polyacrylamide gel electrophoresis (PAGE) was performed using the method of Laemmli (26) in 10% polyacrylamide gels. The proteins were fractionated and transferred onto an Immun-Blot polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked with 5% fat-free dry milk in Tris-buffered saline-Tween (TBS-T; 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) for 2 h prior to incubation with primary antibodies. A Western blot analysis was performed as described previously (27) using antibodies against phospho-specific p44/p42 MAP kinase, p44/p42 MAP kinase, phospho-specific p38 MAP kinase, p38 MAP kinase, phospho-specific SAPK/JNK, SAPK/JNK or GAPDH as primary antibodies at a dilution of 1:1,000 in 5% milk in TBS-T overnight at 4˚C. Goat anti-rabbit IgG horseradish peroxidase-labeled antibodies (KPL, Inc., Gaithersburg, MD, USA) were used as secondary antibodies at a dilution of 1:1,000 in 5% milk in TBS-T for 1 h at room temperature.

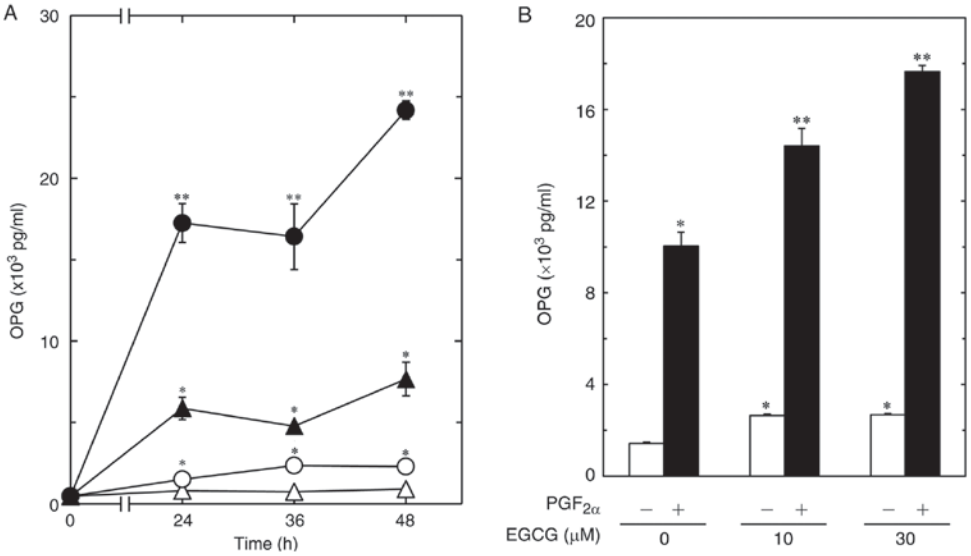

Figure 1. Effect of EGCG on the $PGF_{2\alpha}$-stimulated OPG release in MC3T3-E1 cells. (A) The cultured cells were pretreated with 30 μM of EGCG (●, ○) or vehicle (▲, Δ) for 60 min, and then stimulated by 10 μM of $PGF_{2\alpha}$ (closed symbols) or vehicle (open symbols) for the indicated periods. (B) The cultured cells were pretreated with various doses of EGCG for 60 min, and then stimulated by 10 μM of $PGF_{2\alpha}$ (closed bars) or vehicle (open bars) for 48 h. The OPG concentrations in the culture medium were determined using ELISA. Each value represents the mean ± SEM of triplicate determinations from three independent cell preparations. *P<0.05, compared to the value of control. **P<0.05, compared to the value of $PGF_{2\alpha}$ alone. EGCG, (-)-epigallocatechin gallate; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; OPG, osteoprotegerin; ELISA, enzyme-linked immunosorbent assay.

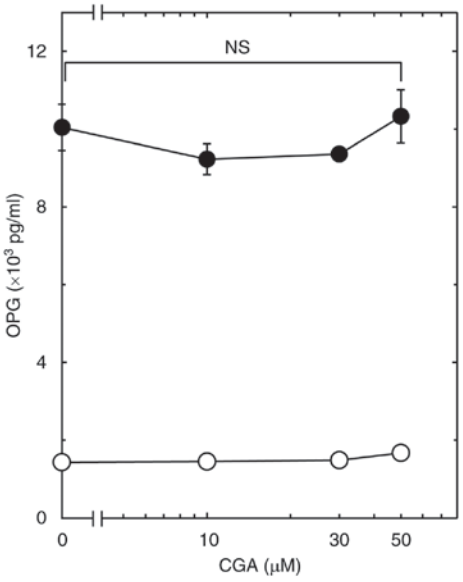

Figure 2. Effect of CGA on the $PGF_{2\alpha}$-stimulated OPG release in MC3T3-E1 cells. The cultured cells were pretreated with various doses of CGA for 60 min, and then stimulated by 10 μM of $PGF_{2\alpha}$ (•) or vehicle (○) for 48 h. The OPG concentrations in the culture medium were determined using ELISA. Each value represents the mean ± SEM of triplicate determinations from three independent cell preparations. N.S designates no significant difference between the indicated pairs. CGA, chlorogenic acid; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; OPG, osteoprotegerin; ELISA, enzyme-linked immunosorbent assay.

The peroxidase activity on the PVDF sheet was visualized on X-ray film by means of the ECL Western Blotting Detection system.

Determinations of the absorbance for ELISA and the densitometric analysis for western blotting. The absorbance of the ELISA samples was measured at 450 nm with the EL 340 Bio Kinetic Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). Densitometric analysis was performed using a scanner and image analysis software package (ImageJ version 1.48; National Institutes of Health, Bethesda, MD, USA). The phosphorylated protein levels were calculated as follows: the background-subtracted signal intensity of each phosphorylation signal was respectively normalized to the total protein signal and plotted as the fold increase in comparison with that of the control cells without treatment or stimulation.

Statistical analysis. The data were analyzed by an ANOVA followed by Bonferroni method for multiple comparisons between pairs, and a p-value <0.05 was considered to indicate statistically significant difference. All data are presented as the mean ± standard error of the mean (SEM) of triplicate determinations from three independent cell preparations.

Results

Effects of EGCG or CGA on the $PGF_2\alpha$-stimulated OPG release in MC3T3-E1 cells. In our previous study (22), we have shown that $PGF_{2\alpha}$ stimulates OPG synthesis in osteoblast-like MC3T3-E1 cells. Therefore, we first examined the effect of EGCG on the $PGF_{2\alpha}$-stimulated OPG release in MC3T3-E1 cells. EGCG significantly enhanced the $PGF_{2\alpha}$-stimulated OPG release time-dependently up to 48 h (Fig. 1A). The $PGF_{2\alpha}$-stimulated OPG release was significantly amplified by EGCG in a dose-dependent manner in the range between 10 and 30 μM. The maximum effect of EGCG on the release of OPG was observed at 30 μM, which caused an approximate 75% increase in the $PGF_{2\alpha}$-effect (Fig. 1B). On the contrary, CGA had little effect on the $PGF_{2\alpha}$-stimulated OPG release up to 50 μM (Fig. 2).

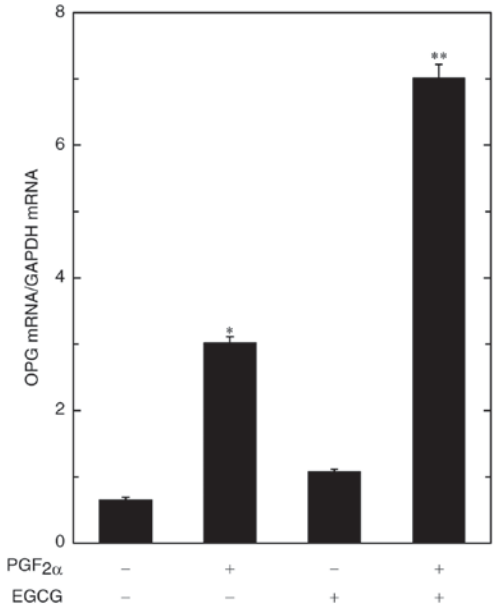

Figure 3. Effect of EGCG on the $PGF_{2\alpha}$-induced expression of OPG mRNA in MC3T3-E1 cells. The cultured cells were pretreated with 10 μM of EGCG or vehicle for 60 min, and then stimulated by 10 μM of $PGF_{2\alpha}$ or vehicle for 6 h. The respective total RNA was then isolated and quantified using RT-PCR. Each value represents the mean ± SEM of triplicate determinations from three independent cell preparations. *P<0.05, compared to the value of control. **P<0.05, compared to the value of $PGF_{2\alpha}$ alone. EGCG, (-)-epigallocatechin gallate; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; OPG, osteoprotegerin.

Effect of EGCG on the $PGF_2\alpha$-induced expression levels of OPG mRNA in MC3T3-E1 cells. In order to investigate whether the amplification by EGCG of the $PGF_{2\alpha}$-stimulated OPG release is mediated via transcriptional events in osteoblast-like MC3T3-E1 cells, we examined the effect of EGCG on the $PGF_{2\alpha}$-induced expression levels of OPG mRNA. EGCG at 10 μM significantly strengthened the $PGF_{2\alpha}$-induced OPG mRNA expression (Fig. 3).

Effects of EGCG on the $PGF_2\alpha$-stimulated phosphorylation of p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK in MC3T3-E1 cells. As for the intracellular signaling of $PGF_{2\alpha}$ in OPG synthesis, we have recently demonstrated that p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK act as positive regulators in osteoblast-like MC3T3-E1 cells (22). Therefore, we next examined the effects of EGCG on the $PGF_{2\alpha}$-stimulated phosphorylation of p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK in these cells. However, EGCG failed to strengthen the $PGF_{2\alpha}$-induced phosphorylation of p44/p42 MAP kinase (Fig. 4A), p38 MAP kinase (Fig. 4B) or SAPK/JNK (Fig. 4C) over the range 10 to 30 μM. EGCG by itself markedly induced the phosphorylation of p44/p42 MAP kinase up to 10 min, and the status decreased thereafter, whereas EGCG hardly affected the phosphorylation status of p38 MAP kinase or SAPK/JNK within 60 min (Fig. 5).

Discussion

In the present study, we demonstrated that EGCG, most abundant catechin in green tea, significantly enhanced the

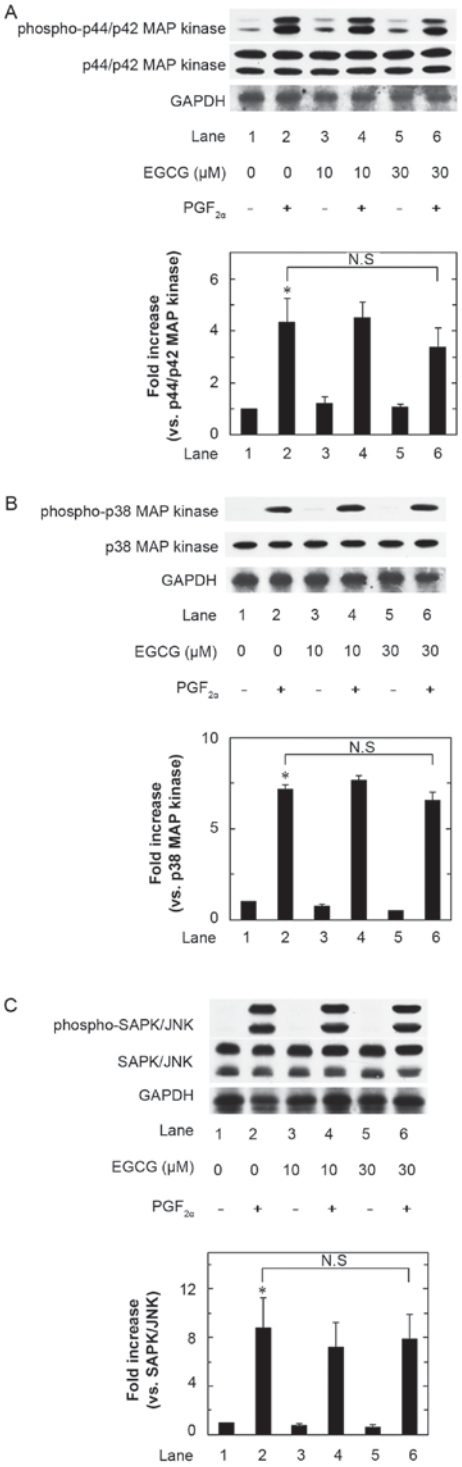

Figure 4. Effects of EGCG on the $PGF_{2\alpha}$-stimulated phosphorylation of p44/p42 MAP kinase (A), p38 MAP kinase (B) and SAPK/JNK (C) in MC3T3-E1 cells. The cultured cells were pretreated with various doses of EGCG for 60 min, and then stimulated by 10 μM of $PGF_{2\alpha}$ or vehicle for 20 min (A and C) or 10 min (B). The cell extracts were then subjected to SDS-PAGE with subsequent Western blot analysis with antibodies against phospho-specific p44/p42 MAP kinase, p44/p42 MAP kinase, phospho-specific p38 MAP kinase, p38 MAP kinase, phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representation of the levels of $PGF_{2\alpha}$-stimulated phosphorylation obtained from a laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations from three independent cell preparations. *P<0.05, compared to the value of control (lane 1). N.S designates no significant difference between the indicated pairs. EGCG, (-)-epigallocatechin gallate; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; MAPK, mitogen-activated protein kinase; SAPK/JNK, stress activated protein kinase/c-Jun N-terminal kinase

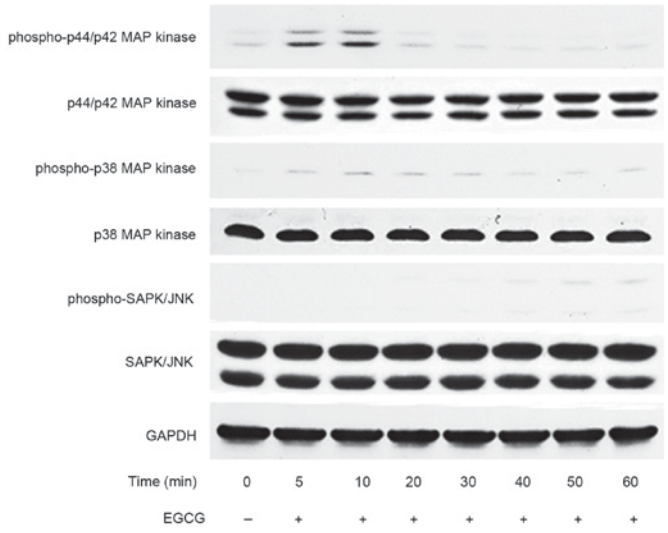

Figure 5. Effects of EGCG on the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK in MC3T3-E1 cells. The cultured cells were treated with 30 μM of EGCG or vehicle for the indicated periods. The cell extracts were then subjected to SDS-PAGE with subsequent western blot analysis with antibodies against phospho-specific p44/p42 MAP kinase, phospho-specific p38 MAP kinase, phospho-specific SAPK/JNK or GAPDH. EGCG, (-)-epigallocatechin gallate; MAPK, mitogen-activated protein kinase; SAPK/JNK, stress activated protein kinase/c-Jun N-terminal kinase.

$PGF_{2\alpha}$-stimulated OPG release in osteoblast-like MC3T3-E1 cells. In addition, we showed that EGCG increased the OPG mRNA expression levels induced by $PGF_{2\alpha}$. Based on these findings, it is most likely that the amplification by EGCG of the $PGF_{2\alpha}$-stimulated OPG release is mediated through a transcriptional event. To the best of our knowledge, this is probably the first report demonstrating the amplification of $PGF_{2\alpha}$-stimulated OPG synthesis by EGCG in osteoblast lineage. By contrast, CGA, which is contained abundantly in coffee, did not affect the $PGF_{2\alpha}$-induced OPG release in MC3T3-E1 cells. We have previously shown that CGA as well as EGCG enhances the TNF-α-stimulated IL-6 synthesis in these cells (14). Thus, it is likely that the effects of CGA on the IL-6 synthesis in osteoblasts would be different from EGCG in each stimulator or the responsive output.

In addition, we next investigated the intracellular signaling mechanisms underlying the effect of EGCG on the $PGF_{2\alpha}$-stimulated OPG synthesis in osteoblast-like MC3T3-E1 cells. It is generally established that the MAP kinase superfamily plays a pivotal role in a variety of cellular functions including proliferation, differentiation and survival (28). Three major MAP kinases including p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK, are generally recognized as central elements used by mammalian cells to transducer diverse messages (29). We have previously shown that $PGF_{2\alpha}$ stimulates OPG synthesis via activation of p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells (22). However, we found that EGCG hardly affected the $PGF_{2\alpha}$-induced phosphorylation of p44/p42 MAP kinase, p38 MAP kinase or SAPK/JNK in these cells. It is firmly established that MAP kinases are activated through the phosphorylation of threonine and tyrosine residues induced by the responsible dual specificity kinase, also known as MAP kinase kinase (30,31). Thus, it seems unlikely that the modulations of these MAP kinases activities are involved in the enhancement by EGCG of the $PGF_{2\alpha}$-stimulated OPG synthesis in osteoblast-like MC3T3-E1 cells. Regarding IL-6 synthesis, it is likely that EGCG might act at a point downstream of the MAP kinases or another target, directing the amplification of IL-6 synthesis. Indeed, we found that EGCG by itself markedly induced the phosphorylation of p44/p42 MAP kinase up to 10 min, and the status decreased thereafter, whereas EGCG hardly affected the phosphorylation status of p38 MAP kinase or SAPK/JNK within 60 min. It seems likely that EGCG affects the cell function of osteoblast-like MC3T3-E1 cells through the p44/p42 MAP kinase-mediating signaling. In addition, in order to investigate the $PGF_{2\alpha}$-effects on OPG synthesis in EGCG-affected osteoblasts compared with those in naive osteoblasts, osteoblast-like MC3T3-E1 cells were pretreated with EGCG for 60 min before $PGF_{2\alpha}$ stimulation. Further investigations are necessary to elucidate the exact mechanism of EGCG in osteoblasts. Taking our findings into account as a whole, it is most likely that not CGA but EGCG enhances the $PGF_{2\alpha}$-stimulated OPG synthesis in osteoblast-like MC3T3-E1 cells, and that the amplifying effect of EGCG on the IL-6 synthesis is independent from the activation of p44/p42 MAP kinase, p38 MAP kinase or SAPK/JNK.

EGCG is a natural polyphenol abundantly found in green tea, and possesses numerous favorable effects on the health of human being through the actions of anti-oxidation (6). Accumulating evidence indicate that green tea consumption prevents both age-related bone loss and fracture in elderly people (6). It is well known that $PGF_{2\alpha}$ has multiple effects on bone metabolism, and that $PGF_{2\alpha}$ acts as a mediator of bone remodeling, resulting in the regulation of bone turnover (21). Osteoblast-producing OPG functions as a decoy receptor of RANKL, and blocks RANKL-RANK interaction which is critical for the osteoclastgenesis and the activation of osteoclasts (16,32). Based on our present findings demonstrating the upregulation by EGCG of OPG synthesis induced by $PGF_{2\alpha}$ in osteoblasts, it is possible that EGCG directs the bone metabolism toward the increase of formation but the suppression of resorption. Therefore, the effect of EGCG showing here might provide a novel potential aspect for the favorable actions of EGCG-containing beverages in bone health for the elderly peoples. Further investigations would be needed to clarify the detailed mechanism of EGCG underlying the OPG synthesis in osteoblasts.

In conclusion, our results strongly suggest that EGCG but not CGA enhances the $PGF_{2\alpha}$-stimulated OPG synthesis in osteoblasts.

Acknowledgements

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