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 insulin-like growth factor-I, platelet-derived growth factor and cytokines [12]. Akt regulates biological functions such as gene expression, survival and oncogenesis [12]. Akt is a downstream target of phosphatidylinositol 3-kinase (PI3-kinase) [13,14]. Akt containing a pleckstrin homology domain is recruited to the plasma membrane by the lipid product of phosphatidylinositol 3-kinase and activated. As for osteoblasts, insulin-like growth factor-I and platelet-derived growth factor induce translocation of Akt to the nucleus [15]. Recently, Akt is reportedly activated by cyclic stretch or androgen [16,17]. We have recently shown that Akt plays an important role in insulin-like growth factor-I-stimulated alkaline phosphatase activity in MC3T3-E1 cells [18]. However, the correlation between TNF-$\alpha$ and PI3-kinase/Akt in osteoblasts has not yet been clarified.

In the present study, we have investigated whether PI3-kinase/Akt signaling pathway plays a role in the TNF-$\alpha$-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells. We show here that TNF-$\alpha$-stimulated IL-6 synthesis through the PI3-kinase/Akt pathway in addition to p44/p42 MAP kinase in these cells.

## Materials and Methods

### Materials

TNF-$\alpha$ was obtained from Funakoshi Pharmaceutical Co. (Tokyo, Japan). IL-6 ELISA 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], wortmannin, LY294002, PD98059 and rapamycin were obtained from Calbiochem. Co. (La Jolla, CA). Phospho-specific Akt antibodies, Akt antibodies, phosphor-specific p44/p42 MAP kinase and p44/p42 MAP kinase were purchased from New England BioLabs, 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 rapamycin were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1 %, which did not affect assay for IL-6 activity or Western blotting analysis.

### Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [19] were maintained as previously described [20]. Briefly, the cells were cultured in $\alpha$-minimum essential medium ($\alpha$-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 or 90 mm diameter dishes in $\alpha$-MEM containing 10 % FCS. After 5 days, the medium was exchanged for $\alpha$-MEM containing 0.3 % FCS. The cells were used for experiments after 48 hours.

Freshly isolated osteoblasts were obtained from the calvaria of new-born (1 or 2-day-old) balb/c mice as previously described [21]. They were seeded into 90 mm diameter dishes (25×104 cells) in $\alpha$-MEM containing 10 % FCS. The medium was changed every 3 days until the cells had reached confluence at about the 5the day. Then, the medium was exchanged for $\alpha$-MEM containing 0.3 % FCS. The cells were used for experiments after 48 hours.

### Assay for IL-6

The cultured cells were stimulated by TNF-$\alpha$ in 1 ml of $\alpha$-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. Unless otherwise indicated, the cells were pretreated with Akt inhibitor, wortmannin, LY294002, PD98059 or rapamycin, for 60 minutes.

### Analysis of Western blotting

The cultured cells were stimulated by TNF-$\alpha$ in $\alpha$-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 according to Laemmli [22] in 10 % polyacrylamide gel. Western blotting analysis was performed as described previously [23] by using phospho-specific Akt antibodies, Akt antibodies, phosphor-specific p44/p42 MAP kinase, or p44/p42 MAP kinase with peroxidase-labeled antibodies raised in goat against rabbit IgG as second antibodies. Peroxidase activity on PVDG membrane was visualized on X-ray film by means of the ECL Western blotting detection system. Unless otherwise indicated, the cells were pretreated with Akt inhibitor, wortmannin, LY294002 or PD98059 for 60 minutes.

### Determinations

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).

### cDNA Microarray

The cultured cells were washed twice with PBS, and total RNA was isolated using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Using the obtained total RNAs, cDNA microarray analysis (GeneChip® Expression Analysis; AFFYMETRIX) was performed once by KURABO Industries (Osaka, Japan) with the authorization of AFFYMETRIX Japan K.K. (Tokyo, Japan). The arrays were scanned and the array image was analyzed with GeneChip® operating software (AFFYMETRIX).

### 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 statistically significant. All data are presented as the mean ± SEM of triplicate determinations. Each experiment was repeated three times with similar results.

## Results

### Effects of TNF-α on the phosphorylation of Akt in MC3T3-E1 cells

To investigate whether TNF-$\alpha$ activates Akt in osteoblast-like MC3T3-E1 cells, we examined the effects of TNF-$\alpha$ on the phosphorylation of Akt. TNF-$\alpha$ significantly induced the phosphorylation of Akt in a time dependent manner (Fig. **1**). The phosphorylation of Akt reached its peak at 15 min, and decreased thereafter.

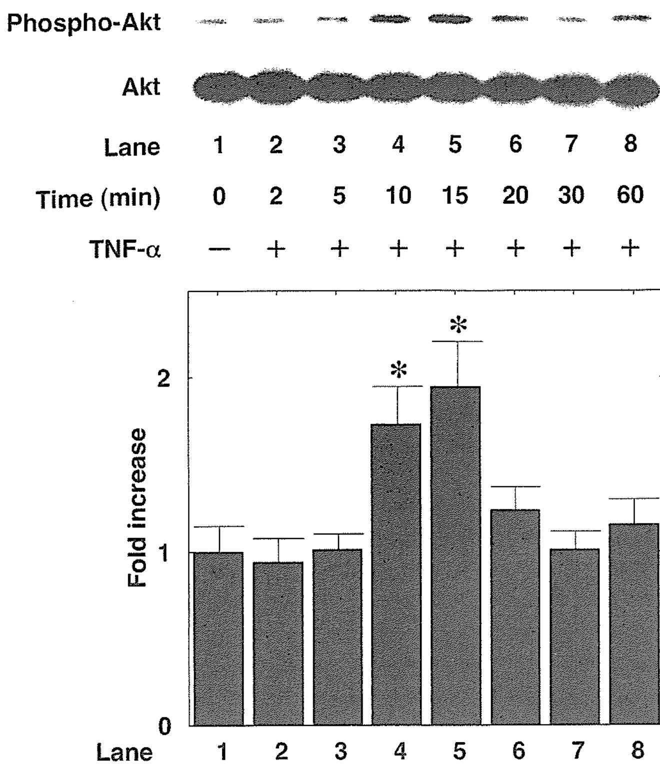


Fig. 1 Effect of TNF-α on the phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were stimulated with 30 ng/ml TNF-α for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. The histogram shows quantitative representations of the levels of TNF-α-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Similar results were obtained with two additional and different cell preparations. $^{*}p < 0.05$, compared to the value of control.

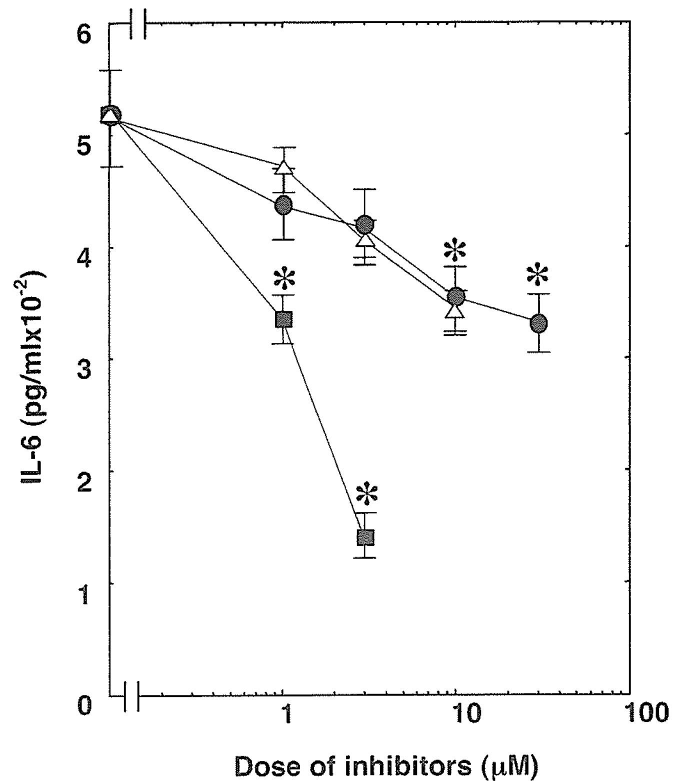


Fig. 2 Effect of LY294002, wortmannin, or Akt inhibitor on the TNF-α-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Akt inhibitor (●), LY294002 (■), or wortmannin (△) for 60 min, and then stimulated by 30 ng/ml TNF-α for 48 h. In the absence of TNF-α all IL-6 levels were approximately 25 pg/ml and did not vary with any inhibitor. 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 TNF-α alone.

**Effect of Akt inhibitor, LY294002, or wortmannin on the TNF-α-induced IL-6 synthesis in MC3T3-E1 cells**

We next examined the effect of Akt inhibitor, 1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate [24], on the TNF-α-stimulated IL-6 synthesis in order to investigate whether Akt is involved in the IL-6 synthesis in MC3T3-E1 cells. Akt inhibitor, which alone did not affect the basal levels of IL-6, significantly suppressed the TNF-α-induced IL-6 synthesis. The inhibitory effect of Akt inhibitor on the IL-6 synthesis was partial and dose dependent in the range between 1 and 30 μM (Fig. **2**). The maximum inhibitory effect of Akt inhibitor at 30 μM caused about 40% reduction in the TNF-α-effect. It is generally known that Akt acts as a downstream effector of PI3-kinase [13,14]. We next examined the effect of LY294002, a specific inhibitor of PI3-kinase [25], on the TNF-α-stimulated IL-6 synthesis in MC3T3-E1 cells. LY294002 significantly reduced the synthesis of IL-6 stimulated by TNF-α in a dose-dependent manner between 1 and 3 μM (Fig. **2**). Furthermore, the effect of wortmannin, another inhibitor of PI3-kinase [26], on the IL-6 synthesis was examined. The TNF-α-stimulated synthesis of IL-6 was markedly reduced by wortmannin in a dose-dependent manner between 1 and 10 μM (Fig. **2**).

**Effect of Akt-inhibitor on TNF-α-stimulated IL-6 synthesis in primary culture of osteoblasts**

We investigated the effect of Akt-inhibitor on TNF-α-stimulated IL-6 synthesis in primary culture of osteoblasts. Akt-inhibitor also significantly suppressed the TNF-α-induced IL-6 synthesis (Fig. **3**). The inhibitory effect of Akt inhibitor on the IL-6 synthesis was partial and dose dependent in the range between 1 and 30 μM (Fig. **3**). The maximum inhibitory effect of Akt inhibitor at 30 μM caused about 25% reduction in the TNF-α-effect.

**Effects of LY294002 or wortmannin on the phosphorylation of Akt induced by TNF-α in MC3T3-E1 cells**

We found that the phosphorylation of Akt induced by TNF-α was markedly attenuated by LY294002 (Fig. **4A**). According to the densitometric analysis, LY294002 (10 μM) caused almost complete reduction of the TNF-α-effect on the Akt phosphorylation. In addition, wortmannin significantly reduced the Akt phosphorylation induced by TNF-α (Fig. **4B**).

**Effects of Akt inhibitor, wortmannin or LY294002 on the phosphorylation of p44/p42 MAP kinase induced by TNF-α, and effect of PD98059 on the TNF-α-induced Akt phosphorylation in MC3T3-E1 cells**

We have previously shown that p44/p42 MAP kinase plays a part in the TNF-α-stimulated IL-6 synthesis in osteoblast-like MC3T3-

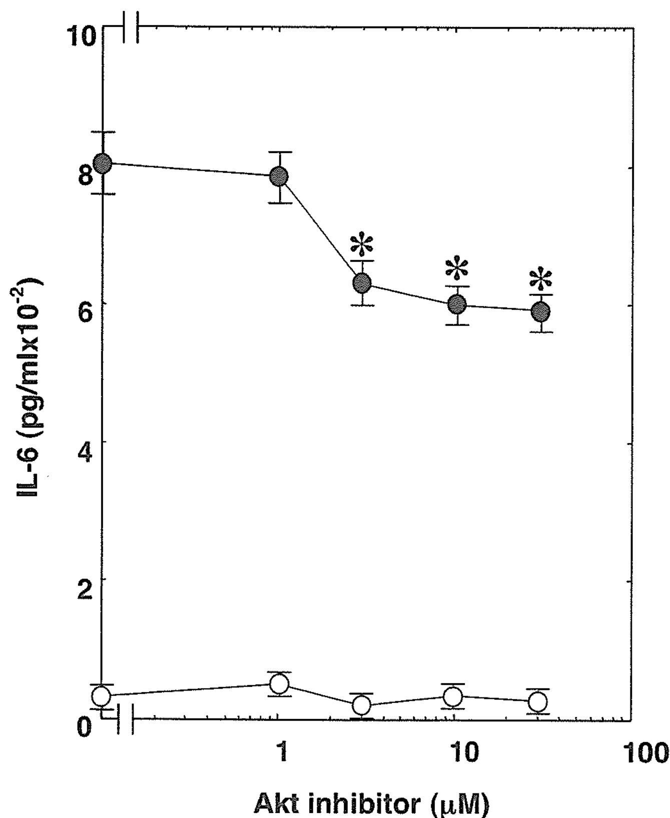


Fig. 3 Effect of Akt-inhibitor on TNF-α-stimulated IL-6 synthesis in primary osteoblasts. The cultured cells were pretreated with various doses Akt inhibitor for 60 min, and then stimulated by 30 ng/ml TNF-α (●) or vehicle (○) for 48 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}p < 0.05$, compared to the value of TNF-α alone.

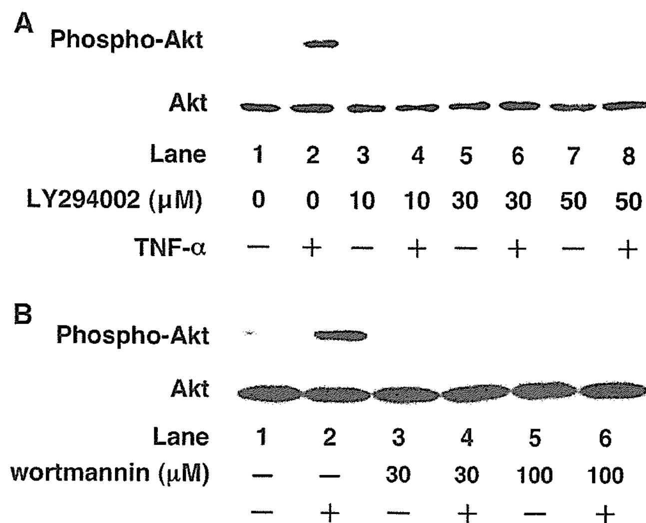


Fig. 4 Effect of LY294002 or wortmannin on the TNF-α-induced phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were pretreated with various doses of LY294002 (**A**) or wortmannin (**B**) for 60 min, and then stimulated by 30 ng/ml TNF-α or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. Similar results were obtained with two additional and different cell preparations.



E1 cells [10,11], which led us to investigate the relationship between p44/p42 MAP kinase and Akt in these cells. Akt inhibitor had little effect on the TNF-α-induced phosphorylation of p44/p42 MAP kinase (Fig. **5A**). In addition, the TNF-α-induced phosphorylation of p44/p42 MAP kinase was slightly suppressed by wortmannin (Fig. **5B**) or LY294002 (data not shown). These suppressive effects were not statistically significant. Furthermore, PD98059, a specific inhibitor of MEK, upstream kinase that activates p44/p42 MAP kinase [27] failed to affect the TNF-α-induced phosphorylation of Akt (Fig. **5C**).

### Effects of rapamycin on TNF-α-stimulated IL-6 synthesis in MC3T3-E1 cells

It is well known that mammalian target of rapamycin (mTOR) is a downstream target of Akt. We examined the effect of rapamycin on TNF-α-stimulated IL-6 synthesis in these cells. We found that rapamycin markedly increased the TNF-α-induced IL-6 release (25 ± 4 pg/ml for control; 45 ± 9 pg/ml for 10 μM rapamycin; 584 ± 63 pg/ml for 30 ng/ml TNF-α alone; and 1.062 ± 96 pg/ml for 30 ng/ml TNF-α with 10 μM rapamycin, as measured during the stimulation for 48 h).

### Effect of TNF-α on mRNA expression of IL-6 receptor and gp130

We next investigated the effect of TNF-α on mRNA expression of IL-6 receptor and gp130 by cDNA microarray. Approximately 45,000 genes were screened. As a result, 1,372 genes showed a greater than 2-fold change in expression level after TNF-α stimulation. The genes of neither IL-6 receptor nor gp130 were included in them. On the other hand, the gene of IL-6 was truly included. It is unlikely that TNF-α affects the IL-6 effect via up-regulation of the receptor or the signal transducer in osteoblasts.

### Combined effect of Akt inhibitor and PD98059 on TNF-α-stimulated IL-6 synthesis in MC3T3-E1 cells

We have shown that the suppressive effect of PD98059 (50 μM) on TNF-α-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells was partial [10]. We further examined the effect of a combination of Akt inhibitor and PD98059 on the TNF-α-stimulated IL-6 synthesis in MC3T3-E1 cells. Combining Akt inhibitor and PD98059 significantly suppressed the TNF-α-stimulated IL-6 synthesis in an additive manner (Table **1** ). We have previously confirmed that PD98059 at the concentration used in this experiment is not toxic but specific to MEK1/2 [28].

## Discussion

In the present study, we have demonstrated that TNF-α induces the phosphorylation of Akt in osteoblast-like MC3T3-E1 cells depending upon time. Akt mediates intracellular signaling of extracellular agonists and plays a crucial role in cellular functions such as proliferation and cell survival in a variety of cells [12]. Akt is also activated by phosphorylation of threonine and serine residues [13,14]. Taking these results into account, it is most likely that TNF-α activates Akt in osteoblast-like MC3T3-E1 cells.

We investigated whether Akt is involved in the TNF-α-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells. Herein, we

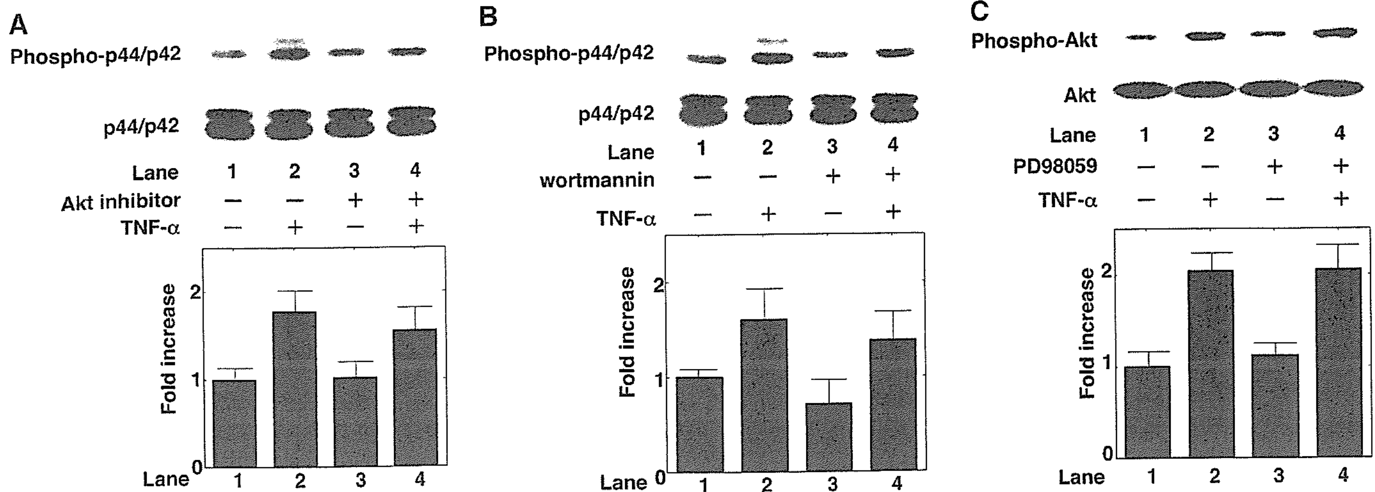


Fig. 5 Effect of Akt inhibitor or wortmannin on the TNF-α-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. Effect of PD98059 on the TNF-α-induced phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were pretreated with 50 nM Akt inhibitor (**A**) or wortmannin (**B**) for 60 min, and then stimulated by 30 ng/ml TNF-α or vehicle for 10 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. (**C**) The cultured cells were pretreated with 50 µM of PD98059 for 60 min, and then stimulated by 30 ng/ml TNF-α or vehicle for 10 min. 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 TNF-α-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.

Table **1** Effect of a combination of Akt inhibitor and PD98059 on the TNF-α-stimulated IL-6 synthesis in MC3T3-E1 cells

| *Akt inhibitor* | *PD98059* | *TNF-α* | *IL-6 (pg/ml)* |
|---|---|---|---|
| – | – | – | 23 ± 4 |
| – | – | + | 506 ± 30* |
| – | + | – | 25 ± 3 |
| – | + | + | 388 ± 18** |
| + | – | – | 23 ± 5 |
| + | – | + | 359 ± 12** |
| + | + | – | 25 ± 4 |
| + | + | + | 257 ± 20*** |

The cultured cells were pretreated with 30 µM Akt inhibitor, 50 µM PD98059 or vehicle for 60 min, and then stimulated by 30 ng/ml TNF-α for 48 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$p < 0.05$, compared to the control. **$p < 0.05$, compared to the value of TNF-α alone. ***$p < 0.05$, compared to the value of TNF-α with Akt inhibitor or PD98059.

show that a specific inhibitor of Akt [24], significantly suppressed the TNF-α-induced IL-6 synthesis in MC3T3-E1 cells. We also confirmed that Akt-inhibitor significantly suppressed the TNF-α-induced IL-6 synthesis also in primary culture of osteoblasts. Thus, it is probable that the activation of Akt is involved in TNF-α-stimulated IL-6 synthesis in osteoblasts. While Akt is a downstream target of PI3-kinase [12–14], we next examined the effects of PI3-kinase inhibitors on the TNF-α-stimulated synthesis of IL-6 in MC3T3-E1 cells. We found that wortmannin [26], significantly reduced the IL-6 synthesis stimulated by TNF-α and markedly attenuated the TNF-α-induced Akt phosphorylation. These findings suggest that PI3-kinase is implicated in TNF-α-stimulated IL-6 synthesis through Akt in MC3T3-E1 cells. We have shown earlier that the IL-6 synthesis induced by TNF-α was significantly suppressed by LY294002 [25]. LY294002 dose dependently reduced the TNF-α-stimulated phosphorylation of Akt. Our results suggest that Akt plays a crucial role as a positive regulator in TNF-α-stimulated IL-6 synthesis at a point downstream from PI3-kinase in osteoblast-like MC3T3-E1 cells.

We have previously reported that the activation of p44/p42 MAP kinase is involved in the TNF-α-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells [10,11]. Hence, we investigated to clarify the relationship between p44/p42 MAP kinase and Akt in these cells. We found that Akt inhibitor- and wortmannin-caused minor reductions in TNF-α-induced phosphorylation of p44/p42 MAP kinase did occur in MC3T3-E1 cells, and a specific MEK inhibitor, PD98059 [27] failed to affect the TNF-α-induced phosphorylation of Akt. These results indicate that some cross talk may exist between the PI3-kinase/Akt and the p44/p42 MAP kinase systems, although mostly these are independent. It is not at all unexpected in that Akt will affect many things downstream which may indirectly affect the p44/p42 MAP kinase system. As for the involvement of mTOR, we found that rapamycin markedly increased the TNF-α-induced IL-6 synthesis in MC3T3-E1 cells. It is unlikely that TNF-α induces IL-6 synthesis through the PI3-kinase/Akt-dependent activation of mTOR. TNF-α is known to increase the expression of IL-6 genes through activation of nuclear factor-κB (NFκB) in osteoblast-like cells [29]. TNFR2 reportedly facilitates PI3-kinase-dependent NFκB activation [30]. It is possible that the inhibition of PI3-kinase/Akt pathway suppress TNF-α-induced IL-6 synthesis through NFκB inhibition. In addition, from cDNA microarray results, TNF-α truly induced the up-regulation of IL-6 mRNA expression, but influenced the mRNA levels of neither IL-6 receptor nor gp130 in MC3T3-E1 cells. It is unlikely that TNF-α affects the IL-6 effect via up-regulation of the receptor or the signal transducer in osteoblasts.

Additionally, we have shown that the inhibitory effect of PD98059 on the TNF-α-stimulated IL-6 synthesis was partial [10]. In this study, the TNF-α-stimulated IL-6 synthesis was partially reduced by Akt inhibitor (30 μM). We found that a combination of Akt inhibitor and PD98059 additively suppressed TNF-α-stimulated IL-6 synthesis. Based on our findings as a whole, it is most likely that TNF-α stimulates the synthesis of IL-6 via Akt and p44/p42 MAP kinase, and maybe mainly independent of each other in osteoblast-like MC3T3-E1 cells.

The PI3-kinase/Akt pathway plays a pivotal rôle in several cellular functions, such as proliferation and cell survival in a variety of cells [12]. Our present findings suggest that the PI3-kinase/Akt pathway in osteoblasts has an important role in the regulatory mechanism of the TNF-α-induced production of IL-6 in bone metabolism. IL-6 are potent bone resorptive agent and induces osteoclast formation [1, 4]. Therefore, our present results lead us to speculate that TNF-α-activated PI3-kinase/Akt signaling acts as a positive regulator directing toward bone resorption. It is possible that the PI3-kinase/Akt pathway in osteoblasts might be considered as a molecular target of bone resorption concurrent with various bone diseases. Both TNF-α and IL-6 are well recognized as inflammatory cytokines which play crucial roles in the process of acute and chronic inflammatory diseases. Our present findings might suggest that PI3-kinase/Akt pathway is involved in the process of pathological bone resorption especially in inflammatory bone diseases. In addition, *in vivo* and *in vitro* models of postmenopausal osteoporosis reportedly demonstrate that estrogen deficiency leads to an increase in the adaptive immune function that culminates in an increased production of TNF-α by activated T cells [31]. The signaling molecules contributed to the TNF-α-induced IL-6 synthesis in osteoblasts might be considerable as therapeutic targets of postmenopausal osteoporosis. Further investigation is required to clarify the exact role of PI3-kinase/Akt in osteoblast cell function and bone metabolism.

In conclusion, our results strongly suggest that TNF-α stimulated IL-6 synthesis via PI3-kinase/Akt in addition to p44/p42 MAP kinase in osteoblasts.





## Acknowledgements

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# Modulation by the Steroid/Thyroid Hormone Superfamily of TGF-β-Stimulated VEGF Release From Vascular Smooth Muscle Cells

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**Abstract** We previously reported that transforming growth factor-β (TGF-β) stimulates the release of vascular endothelial growth factor (VEGF) from aortic smooth muscle A10 cells via activation of p38 mitogen-activated protein (MAP) kinase. In the present study, we investigated whether nuclear hormone receptor superfamily members affect TGF-β-stimulated VEGF release from A10 cells. Retinoic acid or 1,25-dihydroxyvitamin $D_3$ enhanced TGF-β-induced VEGF release in a concentration-dependent manner, whereas dexamethasone or corticosterone suppressed TGF-β-induced VEGF release. 1,25-Dihydroxyvitamin $D_3$ and TGF-β stimulated phosphorylation of p38 MAP kinase in an additive manner. SB203580, an inhibitor of p38 MAP kinase, decreased the VEGF release induced by TGF-β or 1,25-dihydroxyvitamin $D_3$. However, retinoic acid, dexamethasone, or corticosterone did not affect phosphorylation of p38 MAP kinase. These results indicate that retinoic acid, 1,25-dihydroxyvitamin $D_3$, and glucocorticoids affect TGF-β-stimulated VEGF release from aortic smooth muscle cells. The stimulatory effect of 1,25-dihydroxyvitamin $D_3$ occurs, in part, via modification of TGF-β-induced activation of p38 MAP kinase. J. Cell. Biochem. 99: 187–195, 2006.
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**Key words:** steroid/thyroid hormone superfamily; TGF-β; VEGF; p38 MAP kinase; vascular smooth muscle cells

Regulation of vascular smooth muscle cell proliferation and differentiation is critical for vasculogenesis, angiogenesis, and the maintenance of homeostasis in mature vessel walls [Hungerford and Little, 1999; Ross, 1999]. Proliferation and differentiation of these cells are central importance in the pathogenesis of atherosclerosis, hypertension, and restenosis after procedural revascularization [Hungerford and Little, 1999; Ross, 1999]. Vascular smooth muscle cells and vascular endothelial cells interact with each other; for example, vasoactive agents produced by aortic smooth muscle cells, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β), and interleukin (IL)-1β, affect endothelial cell function [Hungerford and Little, 1999; Ross, 1999]. VEGF is a heparin-binding angiogenic growth factor that is highly specific for endothelial cells; VEGF binds to tyrosine kinase receptors expressed almost exclusively in endothelial cells and stimulates endothelial cell proliferation, migration, and inhibition of apoptosis [Gospodarowicz et al., 1989; Neufeld et al., 1999]. VEGF also induces angiogenesis, increases blood vessel permeability, and plays a central

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role in the regulation of vasculogenesis [Neufeld et al., 1999]. Deregulated VEGF expression contributes to the development of solid tumors by promoting tumor angiogenesis and contributes to the development of diseases characterized by abnormal angiogenesis [Neufeld et al., 1999]. Vascular smooth muscle cells are the predominant source of VEGF [Tischer et al., 1991]. Platelet-derived growth factor (PDGF) BB, TGF-β, hypoxia, endothelin, and bFGF induce VEGF production in these cells [Brogi et al., 1994; Stavri et al., 1995; Pedram et al., 1997]. However, the mechanisms underlying VEGF synthesis in vascular smooth muscle cells have not been fully elucidated.

The TGF-β superfamily comprises a large number of structurally related polypeptide growth factors, each capable of regulating an array of cellular processes including cell proliferation, lineage determination, differentiation, motility, adhesion, and death [Massagué, 1998; Miyazono et al., 2000]. In regard to the vascular system, TGF-β plays a pivotal role in promoting alterations in vessel structure [Massagué, 1998]. Recent evidence suggests that alterations in the local abundance of TGF-β in the arterial wall promote vascular cell transdifferentiation, vascular wall remodeling, arterial lesion growth, and lesion regression associated with apoptosis [Schulick et al., 1998]. In these processes, TGF-β stimulates VEGF synthesis in vascular smooth muscle cells and exerts angiogenic effects [Brogi et al., 1994; Stavri et al., 1995]. We previously reported that TGF-β stimulates the release of VEGF from aortic smooth muscle A10 cells at least in part via p38 mitogen-activated protein (MAP) kinase [Yamamoto et al., 2001].

Recently, the nuclear hormone receptor superfamily has been shown to modulate vascular tone and vascular smooth muscle cell proliferation and differentiation [Kornel, 1993; Miano and Berk, 2000; Mizuma et al., 2001; Dubey et al., 2002; Rebsamen et al., 2002]. This superfamily includes receptors for retinoids, vitamin D, steroid hormones, and thyroid hormone [Evans, 1988; Carlberg, 1995; Miano and Berk, 2000]. These receptors are ligand-activated transcription factors that bind discrete *cis* elements within the regulatory regions of a growing list of target genes [Evans, 1988; Carlberg, 1995; Miano and Berk, 2000]. We previously reported that among nuclear hormone receptor superfamily members, 1,25-dihydroxyvitamin $D_3$ or retinoic acid alone stimulates the release of VEGF from A10 cells [Yamamoto et al., 2002; Tanabe et al., 2004]. In the present study, we investigated the effect of nuclear hormone receptor superfamily members on TGF-β-induced VEGF release from A10 cells.

## MATERIALS AND METHODS

### Materials

A mouse VEGF enzyme-linked immunosorbent assay (ELISA) kit (recognizing both 120- and 164-amino acid forms of murine VEGF) and active TGF-β were purchased from R&D Systems (Minneapolis, MN). All-*trans* retinoic acid (retinoic acid), 9α-fluoro-16α-methylprednisolone (dexamethasone), 4-pregnene-11β, 21-diol-3, 20-dione (corticosterone), 4-pregnene-3,20-dione (progesterone), 4-androsten-17β-ol-3-one (testosterone), 1,3,5[10]-estratriene-3,17β-diol (estradiol), and 3,3′,5-triiodo-L-thyronine ($T_3$) were obtained from Sigma-Aldrich (St. Louis, MO). 1α,25-Dihydroxyvitamin $D_3$ (vitamin $D_3$) and SB203580 were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA). Antibodies against phospho-p38 MAP kinase and p38 MAP kinase were from New England BioLabs, Inc. (Beverly, MA). The ECL Western blot detection system was purchased from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. Retinoic acid, vitamin $D_3$, dexamethasone, corticosterone, progesterone, testosterone, and estradiol were dissolved in ethanol. $T_3$ was dissolved in 0.1 N NaOH. SB203580 was dissolved in dimethyl sulfoxide. The maximum concentration of ethanol or dimethyl sulfoxide was 0.1%, which did not affect VEGF immunoassay or Western blot results.

### Cell Culture

A10 cells derived from fetal rat aortic smooth muscle [Kimes and Brandt, 1976] were obtained from American Type Culture Collection (Manassas, VA). Cells were seeded into 35-mm ($1 \times 10^5$ cells) or 90-mm ($5 \times 10^5$ cells) diameter dishes and maintained at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal calf serum. After 5 days, the medium was replaced with serum-free DMEM. Cells were used for experiments 48 h thereafter.

### VEGF Assay

Cultured cells were pretreated with various agents belonging to the nuclear hormone receptor superfamily members in serum-free DMEM for 9 h and were then stimulated with TGF-β for the indicated periods. When indicated, the cells were pretreated with SB203580 for 60 min prior to stimulation with vitamin $D_3$. The conditioned medium was then collected, and the VEGF in the medium was measured with a VEGF ELISA kit that recognizes rat VEGF (according to the manufacturer), as described previously [Seko et al., 1999].

### Western Blot Analysis of p38 MAP Kinase

Cultured cells were pretreated with retinoic acid, vitamin $D_3$, dexamethasone, or corticosterone in serum-free DMEM for 9 h and were then stimulated with TGF-β for 45 min. When indicated, the cells were pretreated with SB203580 for 60 min prior to stimulation with vitamin $D_3$. Stimulated cells were rinsed twice with phosphate-buffered saline, then lysed, homogenized, and sonicated in lysis buffer containing 62.5 mM Tris/Cl, 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. The supernatant was used for analysis of p38 MAP kinase by Western blotting. SDS–polyacrylamide gel electrophoresis (PAGE) was performed by the Laemmli method in 10% polyacrylamide gels [Laemmli, 1970]. Western blot analysis was performed as described previously [Yamamoto et al., 2001, 2002] with phospho-specific p38 MAP kinase antibody or p38 MAP kinase antibody with peroxidase-labeled goat anti-rabbit IgG as a secondary antibody. Peroxidase activity was visualized on X-ray film by ECL.

### Other Methods

The absorbance of ELISA samples at 450 nm was measured with a Multiskan JX ELISA reader (Thermo Labsystems, Helsinki, Finland). Absorbance was correlated with concentration by means of a standard curve. Densitometric analysis was performed with Molecular Analyst Software for Macintosh (BioRad, Hercules, CA).

### Statistical Analysis

Data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs. $P < 0.05$ was considered significant. All data are presented as mean ± SD of triplicate determinations from three independent experiments.

## RESULTS

### Effect of Retinoic Acid or Vitamin $D_3$ on TGF-β-Induced VEGF Release

We previously reported increased VEGF release by A10 cells after 12 h or more of TGF-β stimulation, and the effect of TGF-β was significant at concentrations greater than 1 ng/ml [Yamamoto et al., 2001]. In addition, retinoic acid alone significantly stimulates VEGF release in time-and concentration-dependent manners [Tanabe et al., 2004]. In the present study, retinoic acid and TGF-β additively induced VEGF release in a time-dependent manner up to 60 h (Fig. 1A), the effect being significant after 24 h or more of stimulation. The additive effect of retinoic acid on TGF-β-stimulated VEGF release was concentration-dependent from 0.1 nM to 0.1 μM (Fig. 1B). The effect was significant at concentrations greater than 10 nM.

We previously reported that vitamin $D_3$ alone at concentrations greater than 10 pM stimulates VEGF release from A10 cells [Yamamoto et al., 2002]. Vitamin $D_3$ and TGF-β synergistically induced VEGF release in a time-dependent manner up to 60 h (Fig. 1C), the effect being significant after 12 h or more of stimulation. The synergistic effect of vitamin $D_3$ was concentration-dependent from 0.1 to 10 nM (Fig. 1D). The effect was significant at concentrations greater than 0.1 nM.

### Effect of Dexamethasone or Corticosterone on TGF-β-Induced VEGF Release

Dexamethasone alone had little effect on the basal level of VEGF release but significantly decreased TGF-β-induced VEGF release in a time-dependent manner up to 60 h (Fig. 2A), the effect being significant after 12 h or more of stimulation. The inhibitory effect of dexamethasone was concentration-dependent from 0.1 to 10 nM (Fig. 2B). The effect was significant at concentrations greater than 1 nM.

Corticosterone, another glucocorticoid, which alone did not affect the basal level of VEGF release, significantly decreased TGF-β-stimulated VEGF release. The inhibitory effect of corticosterone was concentration-dependent

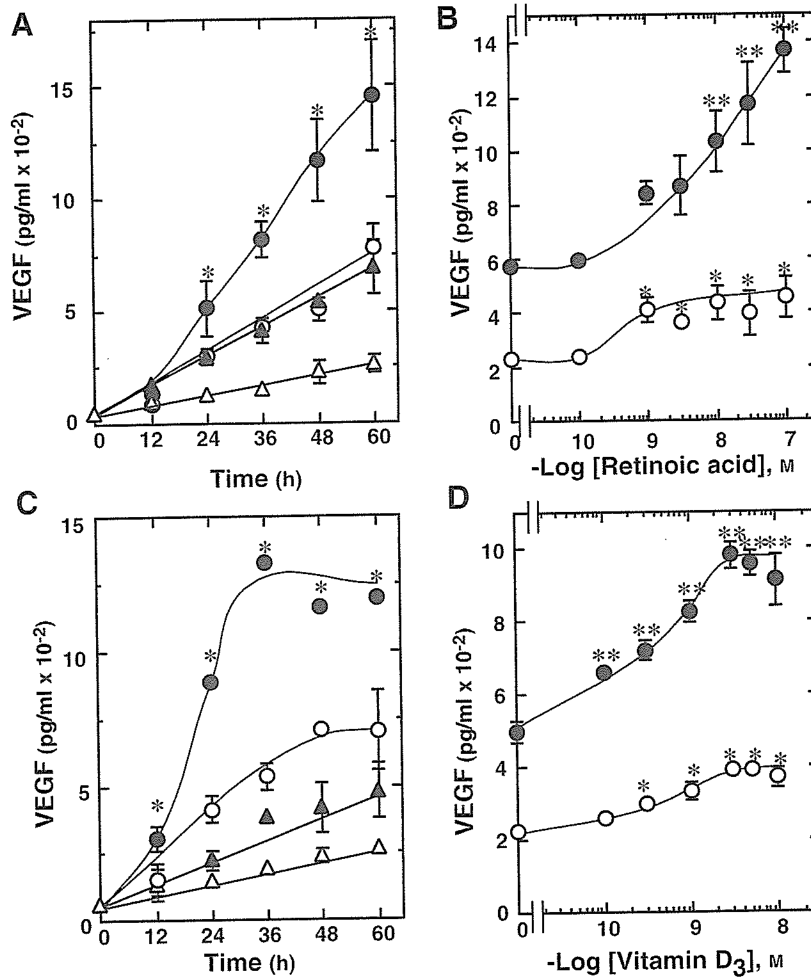


**Fig. 1.** Effects of retinoic acid or vitamin $D_3$ on the TGF-β-induced vascular endothelial growth factor (VEGF) release from A10 cells. **A**: Time-course of VEGF release after TGF-β-stimulation. Cultured cells were treated by 0.1 μM retinoic acid (●, ▲) or vehicle (○, △) for 9 h, and then stimulated by 5 ng/ml TGF-β (circles) or vehicle (triangles) for the indicated periods. $*P < 0.05$ compared with TGF-β or retinoic acid alone. **B**: Dose-dependent effect of retinoic acid on the TGF-β-induced VEGF release. Cultured cells were treated by various doses of retinoic acid for 9 h, and then stimulated by 5 ng/ml TGF-β (●) or vehicle (○) for 48 h. $*P < 0.05$ compared with vehicle alone. $**P < 0.05$ compared with TGF-β or retinoic acid alone. **C**: Time-course of VEGF release after TGF-β-stimulation. Cultured cells were treated by 10 nM vitamin $D_3$ (●, ▲) or vehicle (○, △) for 9 h, and then stimulated by 5 ng/ml TGF-β (circles) or vehicle (triangles) for the indicated periods. $*P < 0.05$ compared with TGF-β or vitamin $D_3$ alone. **D**: Dose-dependent effect of vitamin $D_3$ on the TGF-β-induced VEGF release. Cultured cells were treated by various doses of vitamin $D_3$ for 9 h, and then stimulated by 5 ng/ml TGF-β (●) or vehicle (○) for 48 h. $*P < 0.05$ compared with vehicle alone. $**P < 0.05$ compared with TGF-β or vitamin $D_3$ alone. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations.

from 0.1 to 10 nM (Fig. 2B). The effect was significant at 10 nM.

### Effect of Sex Hormones or $T_3$ on TGF-β-Induced VEGF Release

Other hormones of the steroid/thyroid hormone superfamily, including the sex hormones progesterone, testosterone, and estradiol and $T_3$, had no affect on TGF-β-induced VEGF release from A10 cells (Fig. 2C,D).

### Effect of Retinoic Acid, Vitamin $D_3$, Dexamethasone, or Corticosterone on TGF-β-Induced Phosphorylation of p38 MAP Kinase

We previously reported that TGF-β stimulates the release of VEGF from A10 cells at least in part via activation of p38 MAP kinase, with a maximum effect at 5 ng/ml TGF-β [Yamamoto et al., 2001]. Therefore, we investigated whether retinoic acid, vitamin $D_3$, dexamethasone, or

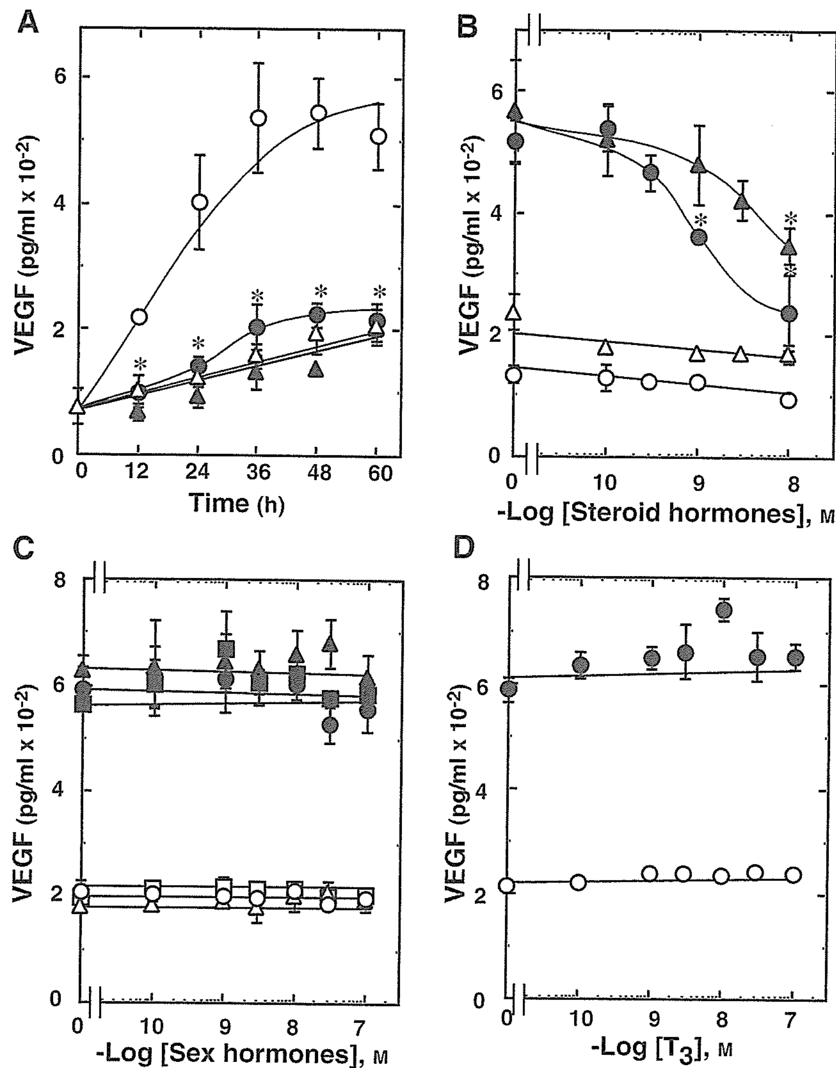


**Fig. 2.** Effects of dexamethasone, corticosterone, progesterone, testosterone, estradiol, or $T_3$ on the TGF-β-induced VEGF release from A10 cells. **A**: Time-course of VEGF release after TGF-β-stimulation. Cultured cells were treated by 10 nM dexamethasone (●, ▲) or vehicle (○, △) for 9 h, and then stimulated by 5 ng/ml TGF-β (circles) or vehicle (triangles) for the indicated periods. **B**: Dose-dependent effects of dexamethasone or corticosterone on the TGF-β-induced VEGF release. Cultured cells were treated by various doses of dexamethasone (circles) or corticosterone (triangles) for 9 h, and then stimulated by 5 ng/ml TGF-β (●, ▲) or vehicle (○, △) for 48 h. *$P < 0.05$ compared with TGF-β alone. **C**: Cultured cells were treated by various doses of progesterone (circles), testosterone (triangles), or estradiol (squares) for 9 h, and then stimulated by 5 ng/ml TGF-β (closed symbols) or vehicle (open symbols) for 48 h. **D**: Cultured cells were treated by various doses of $T_3$ for 9 h, and then stimulated by 5 ng/ml TGF-β (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations.

corticosterone affects TGF-β (5 ng/ml)-induced p38 MAP kinase phosphorylation in these cells. Retinoic acid (0.1 μM), which by itself had little effect on p38 MAP kinase phosphorylation, had no affect on TGF-β-induced phosphorylation of p38 MAP kinase (Fig. 3A). We previously showed that vitamin $D_3$ markedly induces p38 MAP kinase phosphorylation, with a maximum effect at 10 nM vitamin $D_3$ [Yamamoto et al., 2002]. TGF-β and vitamin $D_3$ (10 nM) induced phosphorylation of p38 MAP kinase in an additive manner (Fig. 3B). Dexamethasone (10 nM), which by itself had little effect on p38 MAP kinase phosphorylation, had no affect on TGF-β-induced phosphorylation (Fig. 3C). In addition, corticosterone (10 nM) had no effect on TGF-β-induced phosphorylation of p38 MAP kinase (data not shown).

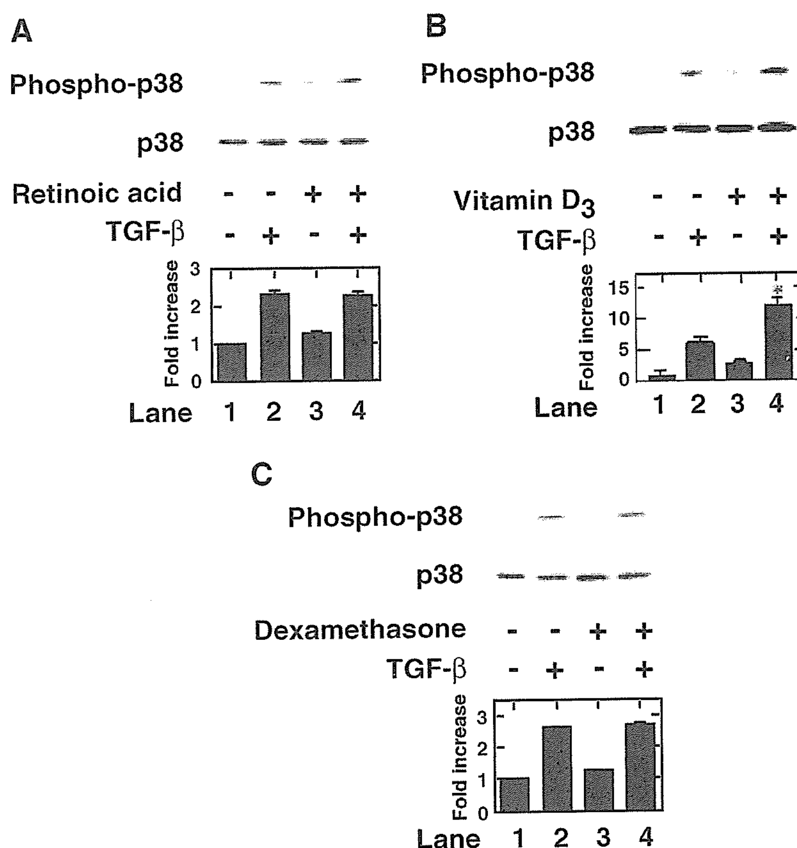


**Fig. 3.** Effects of retinoic acid, vitamin $D_3$, or dexamethasone on TGF-β-induced phosphorylation of p38 mitogen-activated protein (MAP) kinase in A10 cells. Cultured cells were treated with 0.1 μM retinoic acid (**A**), 10 nM vitamin $D_3$ (**B**), 10 nM dexamethasone (**C**), or vehicle for 9 h, and then stimulated by 5 ng/ml TGF-β or vehicle for 45 min. Cell lysates were subjected to SDS–PAGE followed by Western blot analysis using antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. The histogram shows quantitative representations of the phosphorylation level of p38 MAP kinase obtained from laser densitometric analysis. $*P < 0.05$ compared with the value in TGF-β alone. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations.

### Effect of SB203580 on TGF-β and Vitamin $D_3$-induced VEGF Release and Phosphorylation of p38 MAP Kinase

We examined the effect of SB203580, an inhibitor of p38 MAP kinase [Cuenda et al., 1995], on VEGF release and p38 MAP kinase phosphorylation induced by TGF-β in A10 cells in the absence or presence of vitamin $D_3$. SB203580 alone had little effect on the basal level of VEGF release but inhibited VEGF release induced by TGF-β or vitamin $D_3$. The enhancement of VEGF release induced by TGF-β plus vitamin $D_3$ was also inhibited by SB203580 (Table I). However, SB203580 alone had no affect on the basal level of phosphorylation of p38 MAP kinase and also had little effect on phosphorylation induced by TGF-β plus vitamin $D_3$ (Fig. 4).

**TABLE I. Effects of SB203580 on Vitamin $D_3$ or/and TGF-β-induced Vascular Endothelial Growth Factor (VEGF) Release From A10 Cells**

| SB203580 (30 μM) | Vitamin $D_3$(10 nM) | TGF-β (5 ng/ml) | VEGF (pg/ml) |
|---|---|---|---|
| − | − | − | 185 ± 11 |
| − | − | + | 583 ± 47* |
| − | + | − | 467 ± 35* |
| − | + | + | 1174 ± 88* |
| + | − | − | 180 ± 10 |
| + | − | + | 298 ± 28** |
| + | + | − | 302 ± 25** |
| + | + | + | 496 ± 38** |

Cultured cells were pretreated with 30 μM SB203580 or vehicle for 60 min and then stimulated by 10 nM vitamin $D_3$ for 9 h and/or 5 ng/ml TGF-β for 48 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations.
$*P < 0.05$ compared with vehicle alone.
$**P < 0.05$ compared with vitamin $D_3$ and/or TGF-β.

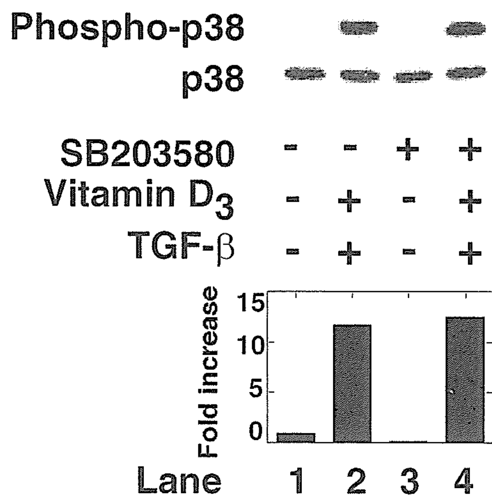


**Fig. 4.** Effect of SB203580 on a combination with TGF-β and vitamin $D_3$-induced phosphorylation of p38 MAP kinase in A10 cells. Cultured cells were treated with 30 μM SB203580 or vehicle for 60 min and then stimulated by 10 nM vitamin $D_3$ for 9 h and 5 ng/ml TGF-β for 45 min. Cell lysates were subjected to SDS–PAGE followed by Western blot analysis using antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. The histogram shows quantitative representations of the phosphorylation level of p38 MAP kinase obtained from laser densitometric analysis. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations.

## DISCUSSION

TGF-β family members are multifunctional agonists whose effects depend on the state of responsiveness of target cells [Massagué, 1998]. Alterations in the local abundance of TGF-β in the arterial wall promote vascular cell transdifferentiation, vascular wall remodeling, arterial lesion growth, and lesion regression associated with apoptosis [Schulick et al., 1998]. One possible explanation for the angiogenic effect of TGF-β is that TGF-β is an indirect mitogen that acts via the production of other angiogenic factors. TGF-β promotes VEGF synthesis in vascular smooth muscle cells and exerts angiogenic effects [Brogi et al., 1994; Stavri et al., 1995]. We previously reported that TGF-β stimulates the release of VEGF from aortic smooth muscle A10 cells via activation of the p38 MAP kinase pathway [Yamamoto et al., 2001].

In the present study, both retinoic acid and vitamin $D_3$, which alone stimulate VEGF release from A10 cells [Yamamoto et al., 2002; Tanabe et al., 2004], induced VEGF release with TGF-β in an additive manner. Retinoic acid or vitamin $D_3$ modulate growth and differentiation of vascular smooth muscle cells [Miano and Berk, 2000; Neuville et al., 2000; Rebsamen et al., 2002]. In vitro, it has been shown that in the absence of growth factor, retinoic acid stimulates vascular smooth muscle cell proliferation but attenuates growth factor-stimulated proliferation [Miano and Berk, 2000]. In addition, retinoic acid decreases the size of neointimal masses, elicits favorable remodeling, and increases lumen diameter and area of injured arteries in vivo [Neuville et al., 2000]. In vascular endothelial cells, retinoic acid enhances the expression of the TGF-β receptor, potentiates TGF-β-induced inhibition of fibrinolytic activity and cell proliferation, and modulates endothelial cell growth and morphology [Kojima and Rifkin, 1993; Yoshizawa et al., 1998; Miano and Berk, 2000]. VEGF also induces endothelial cell proliferation [Neufeld et al., 1999]. Thus, it is likely that retinoic acid, along with TGF-β, modulates endothelial cell growth. With respect to vitamin $D_3$, it plays an important role in regulatory calcium homeostasis, cell differentiation, and proliferation [Kato, 2000]. In the vascular system, vitamin $D_3$ contributes to the development of hypertension, induces vascular calcification, and modulates vascular smooth muscle cell growth [Rebsamen et al., 2002]. Vitamin $D_3$ also inhibits VEGF-induced angiogenesis by inhibiting vascular endothelial cell proliferation [Mantell et al., 2000]. Our results suggest that vitamin $D_3$ modulates endothelial cell function by working with TGF-β to stimulate VEGF release.

We also showed that dexamethasone and corticosterone inhibited TGF-β-induced VEGF release in A10 cells. Glucocorticoids inhibit angiogenesis [Auerbach and Auerbach, 1994], and dexamethasone, hydrocortisone, or cortisone inhibit PDGF-induced VEGF gene expression and VEGF release in vascular smooth muscle cells [Nauck et al., 1998]. Our present study is consistent with this report. Thus, it is likely that dexamethasone and corticosterone inhibit angiogenesis, at least in part, by inhibiting TGF-β-induced VEGF release from vascular smooth muscle cells.

We also investigated whether the stimulatory and inhibitory effects of retinoic acid, vitamin $D_3$, dexamethasone, or corticosterone on VEGF release are mediated via TGF-β-induced p38 MAP kinase activation in A10 cells. Retinoic

acid, dexamethasone, or corticosterone had no affect on TGF-β-induced phosphorylation of p38 MAP kinase. On the contrary, we showed that the maximal concentration of vitamin $D_3$ (10 nM) plus that of TGF-β (5 ng/ml) on p38 MAP kinase phosphorylation was additive. Therefore, it is likely that vitamin $D_3$ plus TGF-β stimulates VEGF release in A10 cells via enhancement of p38 MAP kinase activation. SB203580, a p38 MAP kinase inhibitor [Cuenda et al., 1995], inhibited TGF-β-induced VEGF release in A10 cells in the absence or presence of vitamin $D_3$. SB203580 did not affect p38 MAP kinase phosphorylation induced by TGF-β plus vitamin $D_3$. SB203580 is not an inhibitor of the upstream kinase of p38 MAP kinase but is a direct inhibitor of p38 MAP kinase. Therefore, it is likely that p38 MAP kinase, at least in part, mediates TGF-β-induced VEGF release in A10 cells in the absence or presence of vitamin $D_3$.

Other members of the nuclear hormone receptor superfamily (e.g., progesterone, testosterone, estradiol, and $T_3$) are known to modulate blood pressure and vascular smooth muscle cell growth [Mizuma et al., 2001; Dubey et al., 2002]. However, these factors had no affect on TGF-β-induced VEGF release from A10 cells. It has been reported that deoxycorticosterone and pregnenolone have no effect on VEGF gene expression or release in vascular smooth muscle cells [Nauck et al., 1998]. Thus, it is likely that retinoic acid, vitamin $D_3$, or glucocorticoids regulate the vascular system, at least in part, by modulating the release of endogenous and TGF-β-induced VEGF from vascular smooth muscle cells.

A10 cells derived from fetal rat aorta express many characteristics of vascular smooth muscle cells such as the production of spontaneous action potentials and increase myokinase and creatine phosphokinase activities [Kimes and Brandt, 1976]. VEGF which is synthesized and released predominantly by vascular smooth muscle cells plays a central role in the regulation of angiogenesis in both physiologic and pathologic states, such as wound healing and tumorigenesis, through the induction of vascular endothelial cell proliferation, migration, and inhibition of apoptosis [Neufeld et al., 1999]. It is likely that the modulation by steroid hormones of TGF-β-induced VEGF synthesis in vascular smooth muscle cells plays a role in pathophysiologic angiogenesis. However, discrepancies between in vivo and in vitro findings may occur. Further investigations are necessary to elucidate these effects in vivo.

In conclusion, nuclear hormone receptor superfamily members, including retinoic acid, vitamin $D_3$, and glucocorticoids, affect TGF-β-stimulated VEGF release from aortic smooth muscle cells. The stimulatory effect of vitamin $D_3$ occurs, in part, via up-regulation of TGF-β-induced p38 MAP kinase activation.

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# Monounsaturated Fatty Acid Modification of Wnt Protein: Its Role in Wnt Secretion

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## Summary

**The secretion and extracellular transport of Wnt protein are thought to be well-regulated processes. Wnt is known to be acylated with palmitic acid at a conserved cysteine residue (Cys77 in murine Wnt-3a), and this residue appears to be required for the control of extracellular transport. Here, we show that murine Wnt-3a is also acylated at a conserved serine residue (Ser209). Of note, we demonstrated that this residue is modified with a monounsaturated fatty acid, palmitoleic acid. Wnt-3a defective in acylation at Ser209 is not secreted from cells in culture or in *Xenopus* embryos, but it is retained in the endoplasmic reticulum (ER). Furthermore, Porcupine, a protein with structural similarities to membrane-bound O-acyltransferases, is required for Ser209-dependent acylation, as well as for Wnt-3a transport from the ER for secretion. These results strongly suggest that Wnt protein requires a particular lipid modification for proper intracellular transport during the secretory process.**

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## Introduction

The Wnt family of secreted signal proteins plays a key role in numerous aspects of embryogenesis, as well as in carcinogenesis (Logan and Nusse, 2004; Moon et al., 2004; Reya and Clevers, 2005). Most Wnt proteins transmit signals locally, presumably since their secretion and transport are under tight control. Although the molecular mechanism underlying their secretion and transport remains largely unknown, recent successes in identifying various molecules involved in these processes provide further clues. For instance, Wntless/Evi, a seven-pass membrane protein, plays an essential role in Wnt secretion (Banziger et al., 2006; Bartscherer et al., 2006), and a protein complex involved in intracellular membrane trafficking, called the retromer complex, participates in the long-range transport of Wnt protein (Coudreuse et al., 2006; Prasad and Clark, 2006). In addition, heparan sulfate-modifided proteoglycans are involved in Wnt signaling, possibly by regulating the extracellular transport of Wnt (Hacker et al., 2005; Lin, 2004).

One important step regulating the extracellular transport of various secreted signal proteins, including Wnt, Hedgehog (Hh), and Spitz (*Drosophila* Transforming Growth Factor $\alpha$), involves posttranslational modification with lipid moieties (Mann and Beachy, 2004; Miura et al., 2006; Nusse, 2003). A well-known example is cholesterol modification of the COOH terminus of the active form of Hh, which is generated by auto-proteolytic cleavage and is required for restricting the range of action of this protein (Lewis et al., 2001; Porter et al., 1996a, 1996b). In addition to cholesterol modification, a fatty acid modification, i.e., acylation, occurs with Hh, Wnt, and Spitz (Miura et al., 2006; Pepinsky et al., 1998; Willert et al., 2003). To date, at least three types of acylation are known to occur in eukaryotic cells: N-myristoylation, S-palmitoylation, and N-palmitoylation. N-myristoylation refers to the covalent modification with myristate at the N-glycine of proteins, S-palmitoylation refers to the reversible addition of fatty acids to cysteine residues through thioester linkages, and N-palmitoylation, first described for Hedgehog protein, involves modification at the N-terminal residues of proteins (Linder and Deschenes, 2003; Smotrys and Linder, 2004). In the case of Wnt, Nusse and coworkers reported that murine Wnt-3a is S-palmitoylated at a conserved cysteine residue at the 77$^{th}$ residue (Cys77) (Willert et al., 2003). A mutant form of mouse Wnt-3a, in which the palmitoylated Cys77 is substituted with alanine (C77A), shows a diminished ability to activate Wnt signaling, but is secreted normally into the culture medium (Willert et al., 2003). Thus, the authors proposed that palmitoylation of this cysteine residue may be required to produce an increased local concentration of Wnt on the plasma membrane. However, although their mass spectrometry analysis covered 85% of the primary amino acid sequence of Wnt-3a (Willert et al., 2003), there remains the possibility of additional acylation sites.

There is strong evidence to suggest that acylation is involved in the processing and intracellular trafficking

of Wnt prior to secretion. Genetic evidence suggests that Wnt-secreting cells require the action of specific genes, e.g., *porcupine* (*porc*) in *Drosophila* or its ortholog, *mom1*, in *C. elegans*, both of which encode proteins with structural similarities to those of a family of membrane-bound O-acyltransferases (MBOAT), which transfer acyl groups, such as a palmitoyl group, to substrates (Hofmann, 2000; Kadowaki et al., 1996; Thorpe et al., 1997; van den Heuvel et al., 1993). Porcupine (Porc) is localized at the endoplasmic reticulum (ER), and its overexpression in culture cells enhances the intracellular processing, for example, N-glycosylation, of Wingless (Wg: the *Drosophila* Wnt-1 ortholog) (Tanaka et al., 2002). In addition, treatment with a chemical inhibitor of acyltransferases produces defective intracellular trafficking of Wg (Zhai et al., 2004). Thus, *porc*-dependent acylation may regulate the processing and intracellular trafficking of Wnt, although acylation at Cys77 does not appear to be involved in these processes.

To resolve inconsistencies between studies examining the roles of Wnt acylation, and to better understand the biological significance and molecular mechanism of Wnt acylation, we carefully examined which amino acid residues are acylated, as well as the function of acylation. Our results indicate that another acylation with a monounsaturated fatty acid occurred at a conserved serine, and we suggest that Wnt-3a defective in acylation at this site does not exit the ER.

## Results

### Ser209 Is Required for Acylation of Wnt-3a, as Determined by Metabolic Labeling

We first examined acylation of Wnt-3a by metabolic labeling of cultured cells. In cultures of Wnt-3a-expressing mouse L cells (Shibamoto et al., 1998), specific labeling of secreted Wnt-3a protein with radiolabeled palmitic acid was observed, as previously reported. This labeling was detected after only 4 hr of incubation (Figure 1A), while labeling with radiolabeled cholesterol was not detected, even after 36 hr of incubation (data not shown). Palmitoyl moieties, when linked by thioester, but not oxyester, bonds, are known to be displaced from proteins by high concentrations of disulfide-reducing agents, such as 2-mercaptoethanol or dithiothreitol (Bizzozero, 1995). However, even though a previous report describes palmitoylation of Wnt-3a through a thioester linkage at Cys77 (Willert et al., 2003), we detected labeling even after incubation with a high concentration of a disulfide-reducing agent, i.e., 1.3 M 2-mercaptoethanol. We also observed that this labeling was resistant to neutral hydroxylamine (pH 7.0), which specifically cleaves thioester linkages (data not shown) (Bizzozero, 1995). These results suggest that acylation occurs at another site of Wnt-3a via an oxyester bond, in addition to the known acylation of Wnt-3a at Cys77 through a thioester linkage. Similarly, radiolabeled Wnt-5a was detected in cultures of Wnt-5a-expressing L cells under the same conditions, indicating that acylation commonly occurs among Wnt family members (Figure 1B). To confirm acylation of Wnt-3a at a site other than Cys77, we examined acylation of a mutant form of Wnt-3a, in which Cys77 was substituted by alanine (C77A). A significant reduction in acylation was not observed in C77A, indicating that another acylation site, besides Cys77, exists in Wnt-3a (Figure 1A).

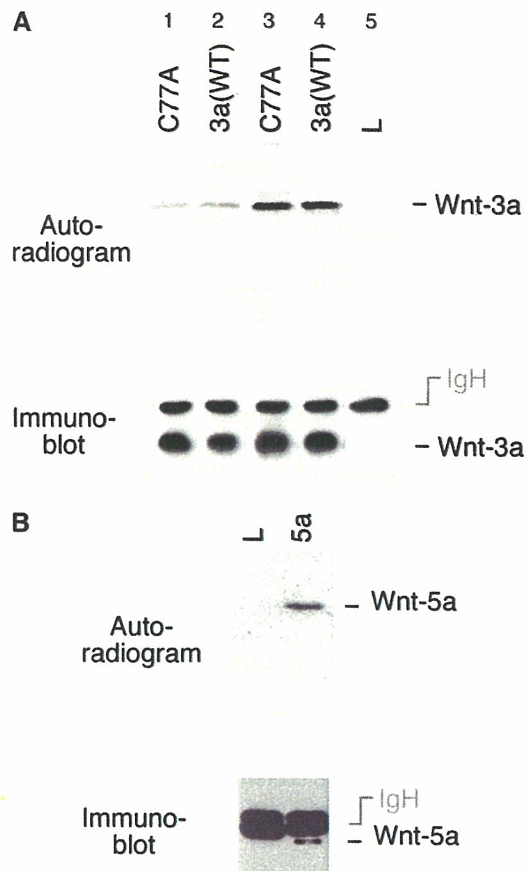


Figure 1. Acylation of Wnt-3a Protein Detected by Metabolic Labeling with Radiolabeled Palmitic Acid

(A) Acylation of wild-type and C77A forms of Wnt-3a proteins secreted from metabolically labeled cells was examined by autoradiography of anti-Wnt-3a immunoprecipitates (upper panel). Wild-type-producing (lanes 2 and 4), C77A-producing (lanes 1 and 3), and control (lane 5) L cells were incubated with $^{14}$C-palmitic acid for 4 hr (lanes 1 and 2) or 36 hr (lanes 3–5). Immunoprecipitated Wnt-3a protein from culture supernatant was identified by immunoblotting with anti-Wnt-3a antibody (lower panel). Immunoglobulin heavy chains (IgH) reactive with the secondary antibody were also detected.

(B) Acylation of Wnt-5a protein secreted from Wnt-5a-producing L cells incubated with $^{3}$H-palmitic acid for 36 hr was examined by immunoprecipitation by following the same procedure described in (A).

To map out the additional site of acylation within the amino acid sequence of Wnt-3a, we generated C-terminal-truncated forms of Wnt-3a protein and examined acylation (Figure 2A). Whereas Wnt-3a truncated at the 281$^{st}$ residue (ΔC-281) contained radiolabeled palmitic acid upon recovery from the cell lysate, as did the full-length protein (FL), Wnt-3a protein truncated at the 202$^{nd}$ residue (ΔC-202) did not contain the radiolabel (Figure 2B). Of note, in the region extending between the 203$^{rd}$ and 281$^{st}$ residues, numerous amino acid residues, including three serines and a threonine, were found to be highly conserved among members of the Wnt family (Figure 2A). Because serine and threonine may form oxyester linkages with acyl moieties, we next examined whether any of the conserved residues

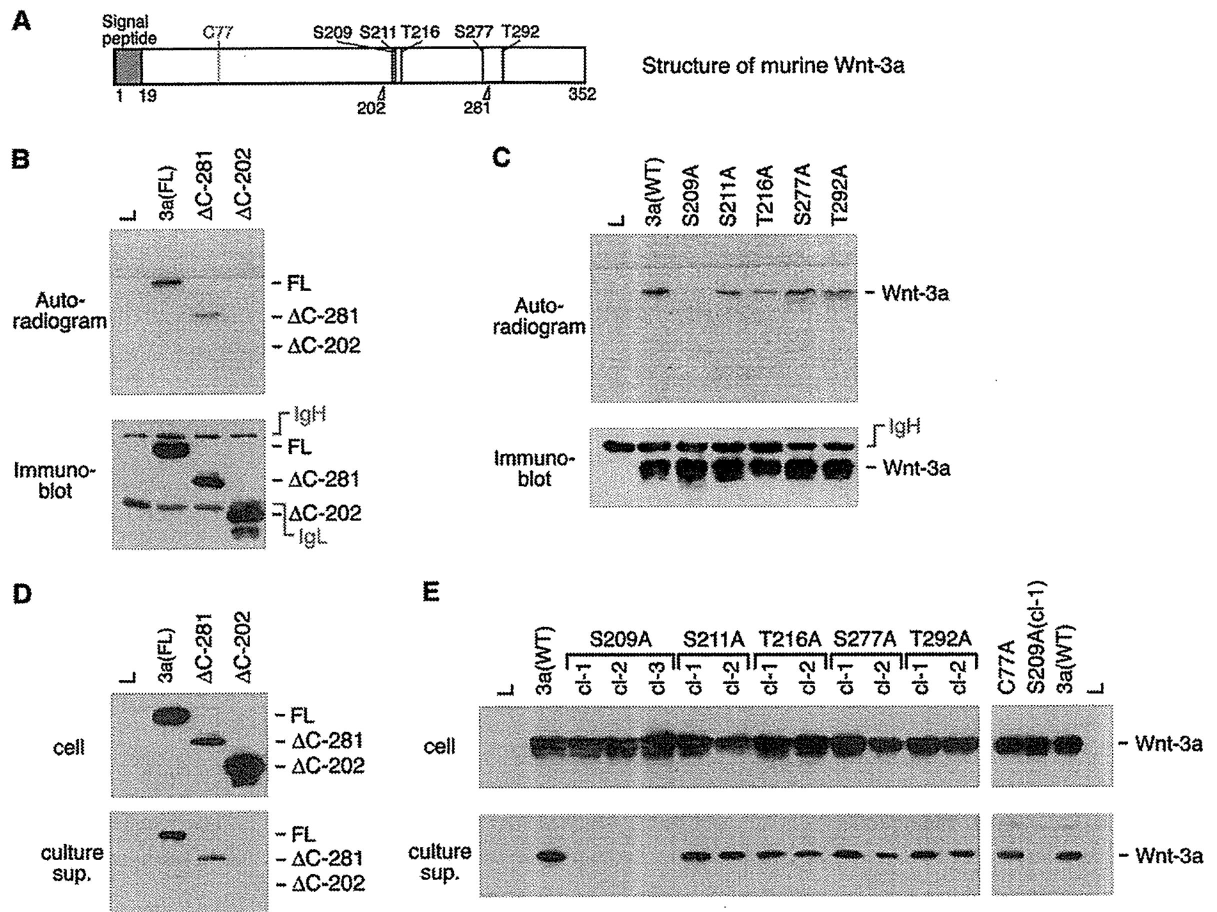


Figure 2. Serine 209 Is Required for the Acylation and Secretion of Wnt-3a in Cultured Cells

(A) Schematic representation of the sites truncated or mutated in this study.

(B) Acylation of truncated forms of Wnt-3a protein was examined by metabolic labeling by following the same procedure described in Figure 1A, except that samples were prepared from cell lysates. Control L cells and L cells expressing the HA-tagged full-length (FL) or one of two truncated (ΔC-281, ΔC-202) forms of Wnt-3a were incubated with $^3$H-palmitic acid for 36 hr. All immunoprecipitates were obtained from cell lysates by using anti-HA antibody (upper panel). Immunoprecipitation of Wnt-3a protein was confirmed by using anti-HA antibody (lower panel). Coprecipitation of immunoglobulin heavy chains (IgH) and light chains (IgL) was identified.

(C) Acylation of point-mutated forms of Wnt-3a protein was examined as shown in (B) by using anti-Wnt-3a antibody. As controls, analyses of immunoprecipitates from L cells producing wild-type Wnt-3a (3a(WT)) and from control L cells (L) are also shown.

(D) Secretion of the two truncated (ΔC-281, ΔC-202) forms of Wnt-3a protein, as well as the HA-tagged full-length (FL) Wnt-3a protein, into the culture medium was examined. The amount of Wnt-3a protein within cells (upper panel) and released into the culture medium (lower panel) was examined by immunoblotting with anti-HA antibody.

(E) Secretion of the five point-mutated forms of Wnt-3a proteins was examined by using anti-Wnt-3a antibody as shown in (D). For each point-mutated form, more than two independent cell clones (cl) are shown. The representative clones of each form shown in (C) are numbered "cl-1." As controls, secretion of wild-type and C77A forms of Wnt-3a was also examined.

might be required for acylation to occur, by substituting each of them with alanine (S209A, S211A, T216A, and S277A). A threonine-to-alanine mutation outside of this region (T292A) was also included in the analysis as a control. It was remarkable that no acylation was detected for S209A, while acylation of the four other mutated Wnt-3a proteins (S211A, T216A, S277A, and T292A) matched that of wild-type Wnt-3a (Figure 2C). Thus, we concluded that Ser209 was required for detectable acylation of Wnt-3a under these assay conditions. We should also note that despite a previous report indicating that overexpression of *porc*, a putative membrane-bound O-acyltransferase (Hofmann, 2000), enhances Wg glycosylation (Tanaka et al., 2002), no obvious defect in N-glycosylation was detected in the S209A mutant when compared with wild-type Wnt-3a, in which N-glycosylation produces two products of different sizes, both of which are sensitive to tunicamycin treatment (Figure S1; see the Supplemental Data available with this article online).

## Wnt-3a Is Modified with a Monounsaturated Fatty Acid at Ser209

Next we investigated whether acylation of Wnt-3a actually occurs at Ser209, as well as the structure of the attached moiety, by mass spectrometry (MS). FLAG-tagged Wnt-3a protein was purified from the conditioned media of the expressing cells (Figure S2), treated with trypsin, and then subjected to nano-flow reverse-phase liquid chromatography (LC) followed by MALDI-MS/MS. A number of peptides obtained after trypsin digestion, including 75% of the amino acid sequence

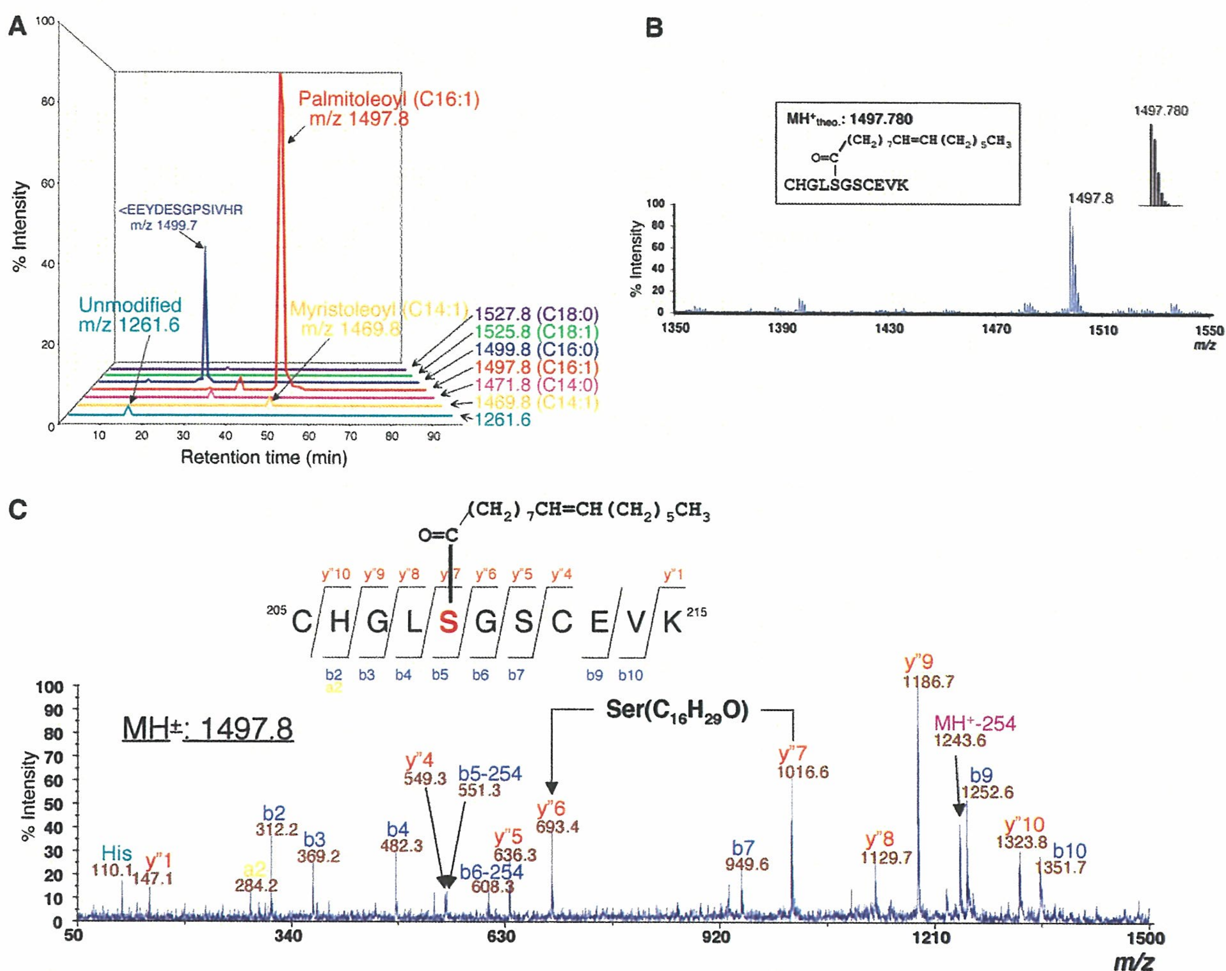


Figure 3. Lipid Modification of Wnt-3a Protein Probed by Nano-Flow LC/MALDI-MS and MS/MS

(A) The selected ion chromatogram reconstituted on the basis of m/z 1261.6, 1469.8, 1471.8, 1497.8, 1499.8, 1525.8, and 1527.8, corresponding to the respective theoretical $MH^+$ values of unmodified peptide and peptide modified with C14:1-, C14:0-, C16:1-, C16:0-, C18:1-, and C18:0-fatty acids. The ion intensities of each trace were normalized to that of the ion at m/z 1497.8. The peptide (m/z 1499.7) eluted at 32 min turned out to be a peptide (<EEYDESGPSIVHR, where <E is pyroglutamic acid) derived from γ-actin.

(B) The MALDI-MS spectrum of the peptide modified with C16:1-fatty acid. The $MH^+_{theo.}$ value and the theoretical isotopic ion distribution in the spectrum (inset) were calculated from the chemical structure indicated in the box.

(C) The MS/MS spectrum from the ion at m/z 1497.8. The spectrum was interpreted by the software *SeqMS* (see Experimental Procedures). The sequences from the N and C termini and the position of the modified residue were read out based on bm and y"l ions, respectively, where m and l denote the arbitrary positions counted from the N and C termini, which were produced by cleavage of peptide bonds during MS/MS. His, b-254 and $MH^+$254, respectively, in the spectrum denote the immonium ion of histidine and the fragment ions derived from β elimination of the lipid moiety ($C_{16}H_{29}O$) at Ser209 from the respective b or $MH^+$ ions.

of the secreted form of Wnt-3a, were recovered by using this procedure (Table S1). Unexpectedly, Ser209-containing peptides with the sequence CHGLS[209]GSCEVK were not obtained at m/z 1499.8, corresponding to a peptide modified with palmitate (C16:0), nor at m/z values of 1471.8, 1525.8, and 1527.8, corresponding to peptides modified with myristate (C14:0), oleate (C18:1), and stearate (C18:0), respectively (Figure 3A). It should be noted that the peptide prominently observed at m/z 1499.7 turned out to be a trypsin digestion product of γ-actin (42 kDa) on MS/MS analysis. Rather, the Ser209-containing peptides were obtained at m/z 1497.8, 1469.8, and 1261.6, corresponding to the theoretical $MH^+$ values of the peptides modified with palmitoleic acid (C16:1), myristoleic acid (C14:1), and the unmodified peptide, respectively (Figure 3A). Among the three peptides detected, the one observed at m/z 1497.8 (corresponding to modification with C16:1) was the predominant molecular species (Figures 3A and 3B), although the relative amounts of each peptide were not explicitly determined. MS/MS analysis of the predominant peptide revealed the site of modification to be Ser209, based on the prominent fragment ions, y"6 and y"7, as well as b4 and b5 − 254 (Figure 3C). Differences in the masses of these ions could be assigned to Ser (323.2) modified with a C16:1-fatty acid or dehydro-Ala (69.0), respectively, the latter of which is a product of β elimination of a lipid moiety from modified Ser209 during the MS/MS measurement.

Further characterization of this prominent peptide (at m/z 1497.8) was carried out by accurate mass measurement, which indicated a molecular mass of 1496.776.

Thus, the accurate mass of the modification was calculated to be 236.217. This value suggests a unique elemental composition, $C_{16}H_{28}O_1$ (theoretical mass: 236.214), indicating modification with a monounsaturated C16-fatty acid (C16:1). It is also noteworthy that the isotopic ion distribution observed for the ion at m/z 1497.8 almost completely matched the theoretical one calculated for a peptide modified with a C16:1-fatty acid (see Figure 3B and the inset). In addition, this isotopic ion distribution was partially shifted when deuterium-labeled palmitic acid ($CD_3(CH_2)_{14}COOH$) was added into culture of Wnt-3a-expressing L cells (data not shown), indicating that the deuterium-labeled palmitic acid (C16:0), as well as the radiolabeled one in the experiments described above, was metabolically processed to C16:1-fatty acid, which then bound to the peptide. Finally, to elucidate the position of the double bond within the C16:1-fatty acid moiety, the olefinic double bond of the prominent peptide (m/z 1497.8) was subjected to oxidative cleavage. The original peptide was mostly degraded into its oxidative products via the loss of $C_7H_{14}$ from the C16:1-fatty acid moiety (data not shown), suggesting that the double bond is located at $\Delta^9$, which is identical to the position within biosynthesized palmitoleic acid (C16:1). Based on these lines of evidence, we conclude that Wnt-3a is modified with a monounsaturated fatty acid, palmitoleic acid (C16:1), at Ser209.

### Ser209 Is Essential for Secretion of Wnt-3a

Next, we investigated whether Ser209-dependent acylation is involved in the secretion of Wnt-3a. We examined the secretion of the two C-terminal-truncated forms and five point-mutated forms of Wnt-3a proteins from L cells into their culture medium, as well as that of the wild-type and C77A forms of Wnt-3a. Interestingly, acylation was coupled with secretion for all Wnt-3a variants. Full-length (FL) and ΔC-281 forms of Wnt-3a demonstrated similar levels of secretion; however, secretion of the ΔC-202 form into the culture medium was not detected (Figure 2D). Furthermore, among the five point mutants, only S209A was not detected in the medium (Figure 2E).

Turnover of wild-type and S209A forms of Wnt-3a proteins was then examined during cycloheximide-mediated inhibition of de novo protein synthesis. S209A protein was stably retained in cells, while most wild-type Wnt-3a protein was secreted into the medium within 24 hr of treatment with cycloheximide (Figures 5A and 5B). Thus, the S209A mutation, preventing the palmitoleoyl modification, did not affect the stability of Wnt-3a protein, but rather blocked its secretion.

### Porcupine Is Required for the Acylation and Secretion of Wnt-3a

The gene *porc*, encoding a putative O-acyltransferase, is thought to be required for the secretion and intracellular transport of Wg in *Drosophila* (Hofmann, 2000; Kadowaki et al., 1996; van den Heuvel et al., 1993; Zhai et al., 2004). Thus, we examined whether *porc* is required for detectable Wnt-3a acylation under the conditions of this study. In a number of independent L cell transfectants, in which expression levels of *porc* were stably reduced by expression of siRNA specific for mouse *porc*, reduced acylation of Wnt-3a was observed, reflecting

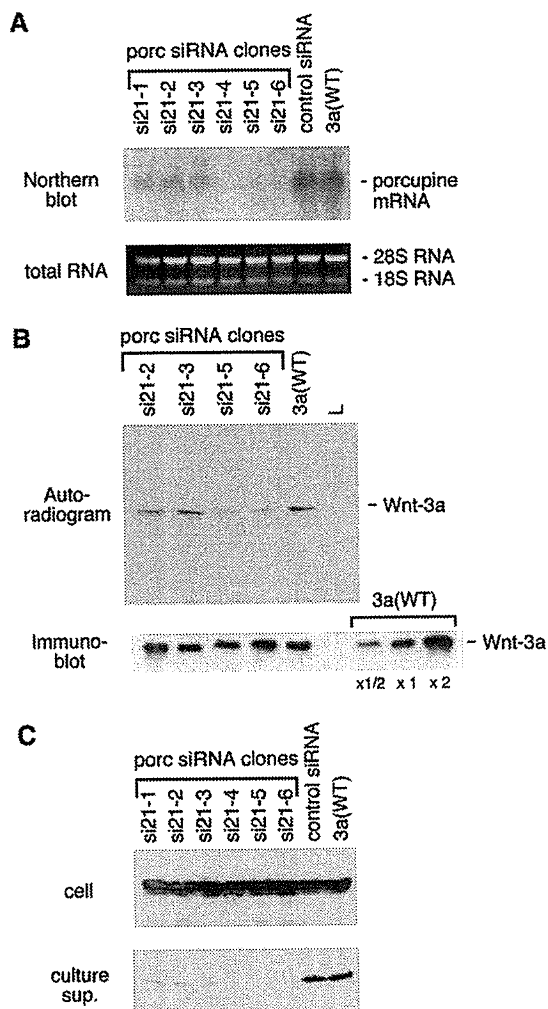


Figure 4. Porcupine Is Required for Palmitoleic Modification and Secretion of Wnt-3a

(A) Endogenous *porc* mRNA expression in clones expressing different levels of *porc*-specific siRNA (si21) is shown (upper panel). As controls, endogenous expression levels in cells expressing control siRNA and in parental Wnt-3a-expressing cells (3a(WT)) are also shown. Total RNAs loaded onto each lane are shown as well (lower panel).

(B) Acylation of Wnt-3a in *porc*-deficient clones, as well as in parental cells expressing Wnt-3a (3a(WT)), and control L cells (L) was analyzed as shown in Figure 2C. Standards to enable quantification of Wnt-3a were also loaded (right).

(C) Reduction in Wnt-3a protein secretion in *porc*-deficient cells was examined as shown in Figure 2E.

some residual *porc* expression, while the production and glycosylation of Wnt-3a appeared not to be perturbed (Figures 4A and 4B; data not shown). It is not certain whether the acylation remaining in *porc*-deficient transfectants was actually due to residual Porc activity. However, the possibility that the residual acylation was Cys77 palmitoylation, possibly detected even under this condition, can be excluded because levels of residual acylation were not reduced by substitution of Cys77 with alanine (Figure S3). Thus, *porc* is required for most of the acylation, which is also dependent on Ser209. Furthermore, as observed with S209A, Wnt-3a protein was retained in untreated *porc*-deficient cells and in