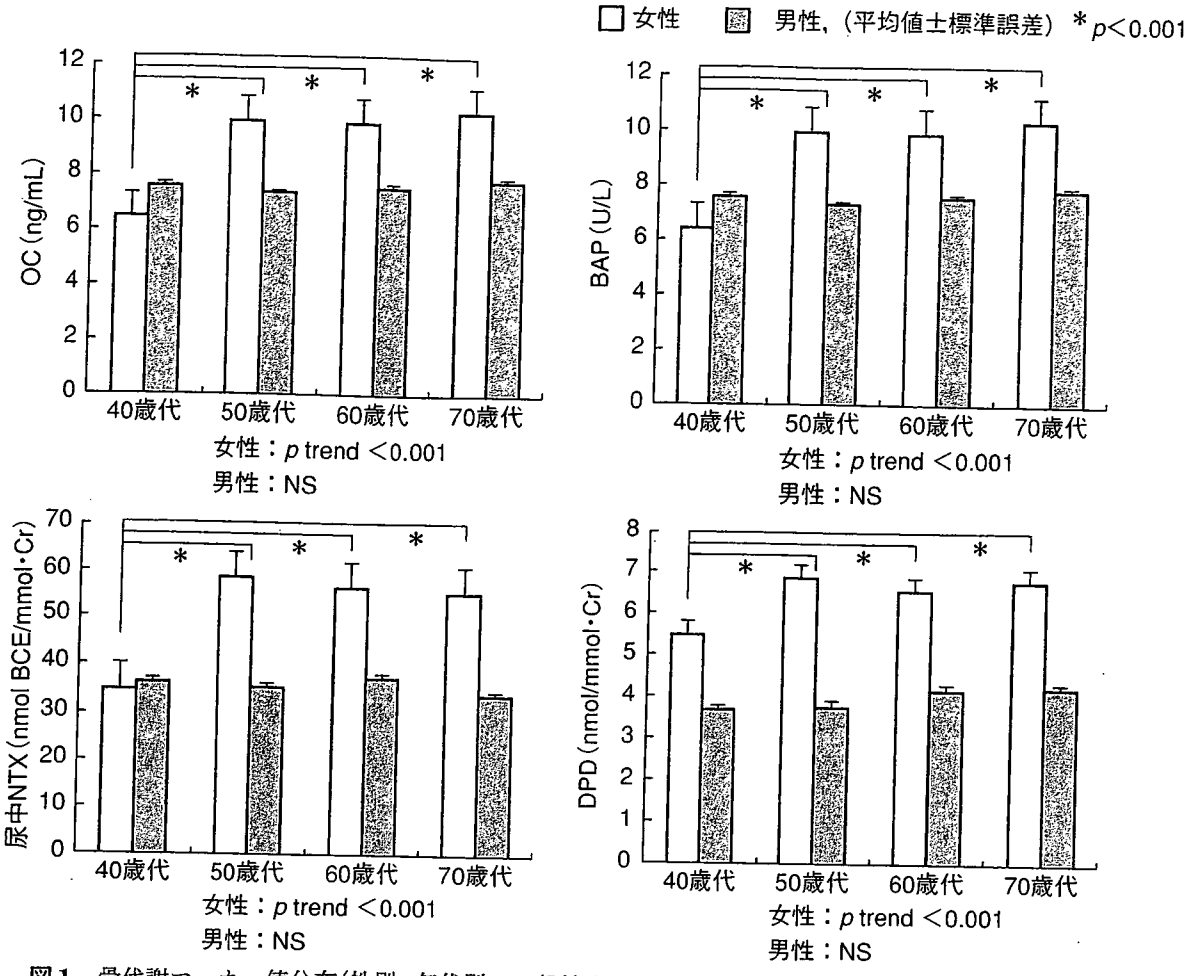

図1 骨代謝マーカー値分布(性別，年代別：一般線形モデルによる多重比較およびトレンド検定)

表2 骨代謝マーカー 1SD 上昇による骨粗鬆症/骨量減少有病発生のオッズ比

腰椎	女性	男性
	オッズ比(95%信頼区間)	オッズ比(95%信頼区間)
OC	1.32 (1.01～1.72)*	1.28 (0.88～1.86)
BAP	1.35 (1.03～1.76)*	1.37 (0.97～1.94)
NTX	1.56 (1.21～2.02)*	1.09 (0.74～1.62)
DPD	1.27 (0.99～1.63)	1.26 (0.88～1.78)

大腿骨頸部	女性	男性
	オッズ比(95%信頼区間)	オッズ比(95%信頼区間)
OC	1.18 (0.93～1.43)	1.22 (0.99～1.50)
BAP	1.413 (1.11～1.81)*	1.36 (1.10～1.66)*
NTX	1.387 (1.10～1.74)*	1.07 (0.87～1.32)
DPD	1.281 (1.03～1.60)*	1.12 (0.95～1.42)

*$p<0.05$

表3　骨代謝マーカー1SD上昇による骨粗鬆症/骨量減少有病発生のオッズ比(女性閉経別)

腰　椎	未閉経	閉経後
	オッズ比(95%信頼区間)	オッズ比(95%信頼区間)
OC	1.05 (0.75～1.48)	1.63 (1.14～2.33)*
BAP	1.57 (1.07～2.30)*	1.34 (0.96～1.88)
NTX	1.62 (1.17～2.34)*	1.48 (1.05～2.07)*
DPD	1.17 (0.80～1.72)	1.31 (0.94～1.83)

大腿骨頸部	未閉経	閉経後
	オッズ比(95%信頼区間)	オッズ比(95%信頼区間)
OC	0.77 (0.48～1.24)	1.54 (1.15～2.10)*
BAP	0.88 (0.58～1.34)	1.73 (1.25～2.39)*
NTX	1.04 (0.71～1.52)	1.60 (1.18～2.16)*
DPD	1.07 (0.72～1.58)	1.43 (1.07～1.90)*

*$p < 0.05$

の関連についての研究で，重相関係数が0.52であったと報告したのに始まる。閉経後白人女性を対象としたGarneroら[8]は，4年間の前腕骨骨密度変化率とOC, BAP, I型プロコラーゲン-C-プロペプチド(PICP)，I型プロコラーゲン-N-プロペプチド(PINP)，尿中NTX，尿中I型コラーゲン架橋Cテロペプチド(CTX)との四分位解析を行った研究で，BAPおよびPICP以外の各マーカーの各群間に有意差を認めたと報告した。Rogersら[9]は，49歳から62歳の閉経後女性60人を対象にNTX，総DPD，BAP，PICP，PINPと2～4年間の腰椎骨密度変化率との関連についての研究で，−0.35から−0.53の有意な相関を報告した。

またわが国では，茶木ら[10]は46歳から75歳の健常日本人女性を対象に，各種骨代謝マーカーと腰椎骨密度の検討を行った研究で，未閉経女性では有意な相関はなく，閉経女性においては尿中NTXと骨密度変化率に有意な負の相関を追跡開始時から3年間までは認めたが，4年以降はなかったと報告している。35歳以上の日本人女性を対象にした伊木ら[11]の研究では，骨代謝マーカーと2年間の腰椎骨密度変化との間に，未閉経女性では有意な相関はなかったが，閉経女性ではBAPと有意な負の相関があったと述べている。これまでの報告では，骨代謝マーカーによる骨密度変化予測は，比較的短期に限れば期待できる可能性があると考えられている。

今回我々は，骨代謝マーカーと6年間という比較的長期の将来の骨量減少/骨粗鬆症発生について両者の相関を求めた。骨量減少/骨粗鬆症判定を腰椎骨密度で行った場合，男性では有意な結果は得られなかったが，未閉経女性ではBAP，NTXが，閉経女性ではOC，NTXで有意な負の相関を認めた。大腿骨頸部判定の場合には，男性ではBAPが有意であった。閉経女性では測定したすべての骨代謝マーカーで有意な結果が得られたが，未閉経女性に有意な結果はえられなかった。骨代謝マーカーによる，10年後の新規骨粗鬆症発生の予知について検討した吉村ら[12]の報告では，骨粗鬆症を腰椎で診断した場合，男性は有意な結果は得られなかったが，女性ではPINP，β-CTXで有意な関連を示した。大腿骨頸部診断の場合，男性はOC, PICPが，女性ではDPDで有意な関連を示した。

骨粗鬆症の治療目標は「骨折の予防」である。低骨密度は骨折危険因子の一つであることはよ

く知られており，骨粗鬆症発生のハイリスク群を早期に選別して予防・治療介入していくことが，臨床上極めて重要と考える。今回の研究結果より，中～長期後の骨量減少/骨粗鬆症発症を骨代謝マーカーが予測する可能性が示唆された。

また，骨代謝マーカーは，値が高値であると骨密度の減少が大きく，骨折のリスクが上昇すると報告され，骨折予測因子としても期待されている[13,14]。今後，骨折発生をエンドポイントとした検討を進めていく必要がある。

ま と め

本研究では地域在住一般住民を対象に，骨代謝マーカーの分布について検討した。女性の骨代謝マーカーは，加齢に伴い高値になる傾向であった。また，骨代謝マーカー値が将来の骨量減少/骨粗鬆症を予測できうるか検討した。その結果，予測に反映される骨代謝マーカーは性別，部位別で異なっており，臨床応用には骨代謝マーカーの用途に沿った選択が必要と考えられた。

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口演発表3 ● 転倒と疾患・その他

施設入所高齢者の転倒恐怖とQOL，ADL，身体活動量との関連

加藤智香子[1)]・猪田邦雄[2)]・長屋政博[3)]・徳田治彦[3)]・原田 敦[3)]

【はじめに】

高齢者が，転倒恐怖を有すると活動性が低下し，身体機能の虚弱化が加速して，活動範囲が狭くなり，ひいてはQOLが低下するという悪循環に陥る。転倒恐怖感の測定には直接恐怖感を問う方法と転倒自己効力感尺度（falls efficacy scale：FES）[1)]を用いる方法がある。FESを用いて地域高齢者を対象として，activity of daily life (ADL)-instrumental ADL (IADL)[2)]，quality of life (QOL)[3)]，身体活動量[2)]との関連が報告されているが，施設入所高齢者におけるFESに関する報告はみられない。そこで，施設入所高齢者を対象に転倒恐怖をFESにて調査し，QOL，ADL，身体活動量との関連を検討した。

【方 法】

1）対 象

愛知県近隣の介護老人保健施設での70歳以上の女性におけるヒッププロテクター臨床試験参加者（35施設342名）のうち，mini-mental state examination（MMSE）15点以上でインフォームドコンセントが十分とれる認知機能を有する133名（平均年齢85.6歳，平均介護度2.1，平均歩行レベルは軽介助）の横断的評価を対象とした。

2）横断的評価項目

年齢，body mass index（BMI），要介護度，大腿骨頸部骨折歴，過去1年間の転倒歴，転倒・骨折リスクに関連する疾患（心疾患，高血圧，脳卒中，糖尿病，関節疾患，眼疾患），MMSE，FES[1)]，The MOS 8-item short-form health survey (SF-8)[4)]，functional independence measure (FIM)運動項目，身体活動量を評価した。

①FES：FESは10のADL項目において，転ばずに自信をもってできるかを，大変自信がある（1点）～全く自信がない（10点）で評価する（計10～100点）[1)]。よって，点数が低いほど高い転倒自己効力感を意味する。なお，本研究では，施設入所者を対象としたので施設内におけるADLに当てはめて使用した。

②SF-8[4)]：SF-8はSF-36の短縮版調査票である。8つの下位尺度からphysical component score (PCS) とmental component score (MCS) を求めた。

③FIM運動項目：FIM運動項目は13項目からなり，全介助1点から完全自立7点で評価する（計7～91点）。なお，本研究では，歩行項目に車椅子移動は含まず，歩行のみで判定した。

④身体活動量：歩行が監視以上の83名には身体活動量として，ライフコーダEX（㈱スズケン）を使用して1週間の平均歩数/日を計測した。

3）統計学的検定

すべての統計はSPSS14.0Jを用いて行われた。有意水準は5%未満とした。FESの合計点により4つの層に分類［低得点層，平均－standard deviation (SD) 層，平均+SD層，高得点層］して比較検討した。名義変数にはχ^2検定を，連続変数に対してはone-way ANOVA（One-way analysis of Variance）およびポストホック比較としてTurkey's HSD検定を用いた。

4）倫理的配慮

名古屋大学および国立長寿医療センター倫理委員会の承認を受けて実施した。

【結 果】

FESの合計点は平均45.01±22.32点であった。そこでFESを4つの層［10～20点21名，21～45点46名，46～70点48名，71～100点18名］

[1)] 名古屋大学医学部保健学科，[2)] 名古屋大学名誉教授，[3)] 国立長寿医療センター病院

表1　FES層別による比較

	転倒自己効力感 高			低	p value[a]
	10～20点 (n=21)	21～45点 (n=46)	46～70点 (n=48)	71～100点 (n=18)	
年齢	87.29	84.63	85.98	84.94	0.37
BMI[b]	21.02	20.85	21.65	20.64	0.66
要介護度	2.00	1.96	2.13	2.33	0.62
頸部骨折歴	(23.8)	(28.3)	(31.3)	(33.3)	0.91
転倒歴	(14.3)	(56.5)	(50.0)	(38.9)	0.01*
転倒・骨折リスクに関連する疾患					
心疾患	(23.8)	(26.1)	(31.3)	(11.1)	0.42
高血圧	(42.9)	(50.0)	(47.9)	(44.4)	0.95
脳卒中	(23.8)	(43.5)	(39.4)	(55.6)	0.23
糖尿病	(19.0)	(17.4)	(14.6)	(16.7)	0.97
関節疾患	(23.8)	(23.9)	(16.7)	(22.2)	0.83
眼疾患	(19.0)	(28.3)	(33.3)	(22.2)	0.61
MMSE[c]	21.24	21.02	23.31	24.00	0.02*
SF-8[d]					
PCS[e]	49.32	44.12	38.46	33.21	0.00*
MCS[f]	51.82	50.28	50.51	46.75	0.28
FIM[g]運動項目	73.71	67.91	66.77	56.28	0.01*
身体活動量					
歩数[h]	1188.73	1480.03	824.63	487.29	0.14

Mean or (%)　*p<0.05

[a] one-way analysis of variance (ANOVA) および χ^2 検定, [b] body-mass index, [c] mini-mental state examination, [d] MOS 8-item short-form health survey, [e] physical component score, [f] mental component score, [g] functional independence measure, [h] 歩数は監視歩行以上の83名（10～20点=16名，21～45点=31名，46～70点=30名，71～100点=7名）が実施。

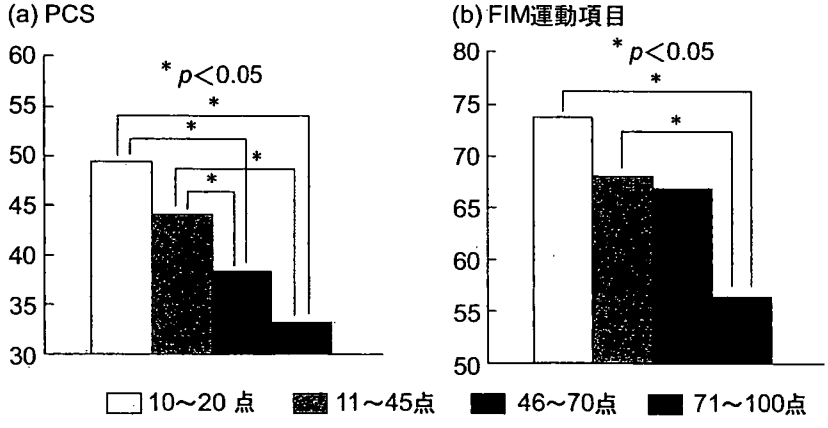

図1　physical component score (PCS) (a)と functional independence measure (FIM) 運動項目(b)のポストホック比較 (turkey's HSD 検定)

に分類し比較検討した（表1）。4層間で年齢，BMI，要介護度，頸部骨折歴，疾患については有意な差はみられなかったが，低得点層（高い転倒自己効力感を有する層）はその他の層（21～100点）に比して転倒歴が少なかった（14.3% vs 38.9%～56.5%）。SF-8では，転倒自己効力感が低下すると（FESの得点は高くなる）PCSは段階的に有意な低下がみられたが（図1(a)），MCS

には差がみられなかった。FIM 運動項目も転倒自己効力感が低下すると有意に低下がみられたが（図 1(b)），歩数には有意な差がみられなかった。

【考　　察】

施設入所高齢者の FES は，地域在住高齢者の報告[2,3]と同様に過去1年間の転倒歴，PCS，FIM 運動項目に有意な関連がみられた。Tinetti らによる地域在住高齢者の報告では身体活動量の指標として質問紙票を用い，FES と相関ありとしている[2]。今回，施設入所高齢者におけるわれわれの調査では身体活動量の指標として歩数を用いたところ，FES との関連はみられなかった。そこで，同じ歩数という指標で地域在住高齢者（女性5名，平均年齢 77.6±2.1 歳）を調査したところ，平均歩数は 7,112 歩であった。一方，本研究における施設入所高齢者の歩数は 487～1,188 歩，平均 1;107 歩と非常に少なかった。このように，もともとの歩数が少ない施設入所高齢者では転倒恐怖による影響が歩数に反映されにくいと考えられた。施設入所高齢者の転倒恐怖による身体活動量への影響を検討するには，より詳細な調査が必要であろう。

【結　　論】

施設入所高齢者において転倒自己効力感が低下すると，ADL，身体的な QOL には段階的な低下がみられたが，精神的な QOL や身体活動量（歩数）には差がみられなかった。

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Limitation by p70 S6 kinase of platelet-derived growth factor-BB–induced interleukin 6 synthesis in osteoblast-like MC3T3-E1 cells

Shinji Takai[a], Haruhiko Tokuda[a,b], Yoshiteru Hanai[a,b], Osamu Kozawa[a,*]

[a]*Department of Pharmacology, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan*
[b]*Department of Clinical Laboratory, National Hospital for Geriatric Medicine, National Center for Geriatrics and Gerontology, Obu, Aichi 474-8511, Japan*

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Abstract

It has been reported that platelet-derived growth factor–BB (PDGF-BB) stimulates interleukin 6 (IL-6) in osteoblasts. In the present study, we investigated the mechanism of IL-6 synthesis induced by PDGF-BB in osteoblast-like MC3T3-E1 cells. Platelet-derived growth factor–BB time-dependently induced the phosphorylation of p44/p42 mitogen-activated protein (MAP) kinase, p38 MAP kinase, stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK), and p70 S6 kinase. PD98059 (an inhibitor of MAP kinase/extracellular signal-regulated kinase kinase [MEK]), SB203580 (an inhibitor of p38 MAP kinase), or SP600125 (an inhibitor of SAPK/JNK) suppressed the IL-6 synthesis induced by PDGF-BB. Rapamycin, an inhibitor of p70 S6 kinase, significantly enhanced the PDGF-BB–stimulated IL-6 synthesis. The PDGF-BB–induced phosphorylation of p70 S6 kinase was suppressed by rapamycin. Rapamycin failed to affect the PDGF-BB–induced phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, or SAPK/JNK. These results strongly suggest that PDGF-BB stimulates IL-6 synthesis through activation of 3 MAP kinases in osteoblasts and that p70 S6 kinase negatively regulates the IL-6 synthesis.

1. Introduction

Interleukin 6 (IL-6) is a pleiotropic cytokine that has important physiologic effects on a wide range of functions such as promoting B-cell differentiation and T-cell activation and inducing acute-phase proteins [1-3]. It is generally recognized that bone metabolism is regulated mainly by 2 functional cells, osteoblasts and osteoclasts, responsible for bone formation and bone resorption, respectively [4]. As for bone metabolism, IL-6 has been shown to stimulate bone resorption and promote osteoclast formation [2,3,5,6]. It has been reported that potent bone resorptive agents such as tumor necrosis factor α and IL-1 stimulate IL-6 synthesis in osteoblasts [5,7,8]. Currently, accumulating evidence indicates that IL-6 secreted from osteoblasts plays a pivotal role as a downstream effector of bone-resorptive agents.

It is well known that platelet-derived growth factor (PDGF) is a mitogenic factor, which mainly acts on connective tissue cells [9,10]. Platelet-derived growth factor occurs as 5 different isoforms [10]. Platelet-derived growth factor isoforms were originally isolated from platelets but have been shown to be produced and released from a variety of cell types including osteosarcoma cells [9,11]. As for stimulation of biologic activities in bone cells, PDGF-BB is a potent stimulator and induces osteoblast proliferation and collagen synthesis [12]. It is recognized that PDGF, released during platelet aggregation, has a pivotal role in fracture healing as a systemic factor and that PDGF also regulates bone remodeling as a local factor [12]. Platelet-derived growth factor receptor has an intrinsic protein tyrosine kinase activity and associates with SH-2 domain–containing substrates such as phospholipase C-γ and phosphatidylinositol 3-kinase [9]. We have previously reported that PDGF-BB activates phosphatidylcholine-hydrolyzing phospholipase D via tyrosine kinase activation, resulting in protein kinase C activation in osteoblast-like MC3T3-E1 cells [13]. It has been shown that PDGF-BB induces the transcription of IL-6 through the activator protein 1 complex and activating transcription factor 2 in primary cultured rat osteoblasts [14]. However, the exact mechanism underlying PDGF-BB–stimulated IL-6 synthesis in osteoblasts is not fully known.

It is generally recognized that p70 S6 kinase is a mitogen-activated serine/threonine kinase required for cell

* Corresponding author. Tel.: +81 58 230 6214; fax: +81 58 230 6215.
E-mail address: okozawa@cc.gifu-u.ac.jp (O. Kozawa).

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proliferation and G_1 cell-cycle progression [15]. As for osteoblasts, it has been shown that fluoroaluminate induces an increase in p70 S6 kinase phosphorylation [16]. In our previous study [17], we have reported that p70 S6 kinase plays as a positive regulator in bone morphogenetic protein 4–stimulated synthesis of vascular endothelial growth factor in osteoblast-like MC3T3-E1 cells. In addition, we recently demonstrated that p38 mitogen-activated protein (MAP) kinase, a member of the MAP kinase superfamily, functions at a point upstream from p70 S6 kinase in the synthesis of vascular endothelial growth factor in these cells [18]. However, the exact role of p70 S6 kinase in osteoblasts has not yet been fully clarified.

In the present study, we investigated the mechanism behind PDGF-BB–stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells. We here show that PDGF-BB stimulates IL-6 synthesis through activation of 3 MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK), in these cells, and that p70 S6 kinase concomitantly activated by PDGF-BB has an inhibitory role in the IL-6 synthesis.

2. Materials and methods

2.1. Materials

Platelet-derived growth factor–BB and mouse IL-6 and osteocalcin enzyme-linked immunosorbent assay (ELISA) kit were purchased from R&D Systems (Minneapolis, MN). Indomethacin was purchased from Sigma Chemical (St Louis, MO). PD98059, SB203580, SP600125, and rapamycin were obtained from Calbiochem-Novabiochem (La Jolla, CA). Phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific p70 S6 kinase antibodies (Thr389), and p70 S6 kinase antibodies were purchased from Cell Signaling (Beverly, MA). ECL Western blotting detection system was purchased from Amersham Biosciences (Piscataway, NJ). Other materials and chemicals were obtained from commercial sources. PD98059, SB203580, SP600125, or rapamycin were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect the assay for IL-6 or Western blot analysis.

2.2. 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 α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 35- or 90-mm diameter dishes in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 48 hours.

2.3. Interleukin 6 ELISA

The cultured cells were stimulated by various doses of PDGF-BB in 1 mL of α-MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pretreated

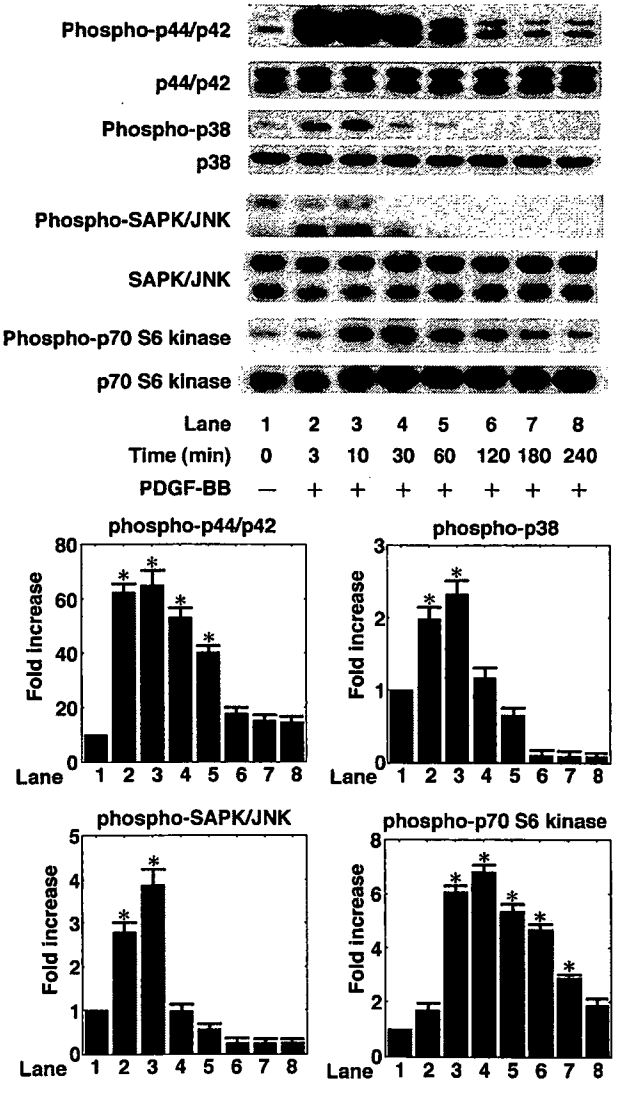

Fig. 1. Effects of PDGF-BB on the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, SAPK/JNK, or p70 S6 kinase in MC3T3-E1 cells. The cultured cells were stimulated by 50 ng/mL PDGF-BB for the indicated periods. The extracts of cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis with subsequent Western blotting 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, SAPK/JNK, phospho-specific p70 S6 kinase, or p70 S6 kinase. Similar results were obtained with 2 additional and different cell preparations. The histogram shows quantitative representations of the levels of PDGF-BB–induced phosphorylation obtained from laser densitometric analysis of 3 independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with 2 additional and different cell preparations. *$P < .05$ compared with the value of control.

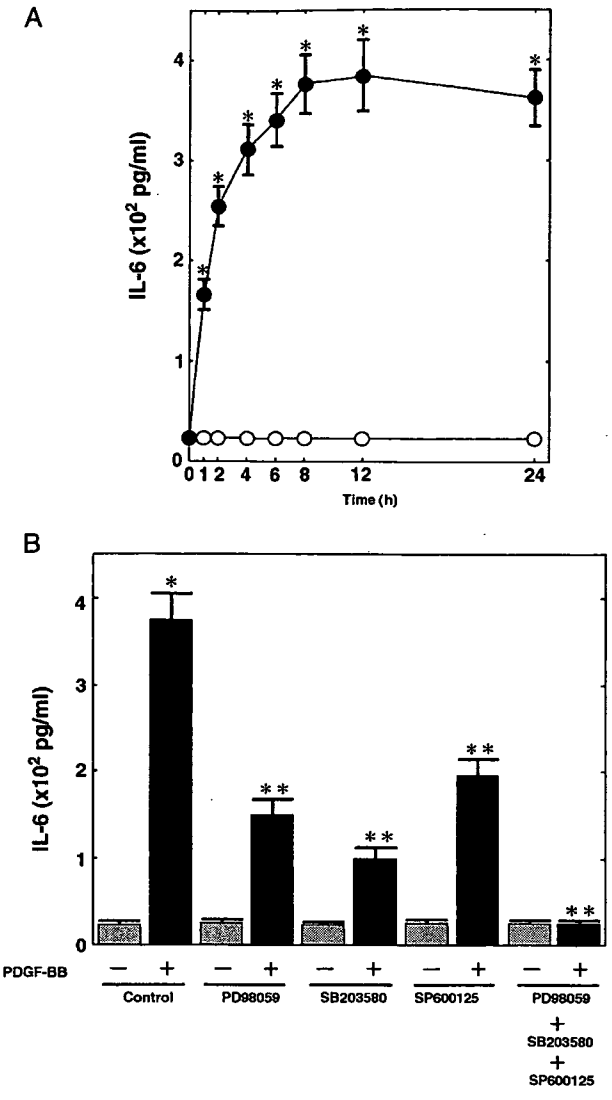

Fig. 2. Time course of PDGF-BB–induced IL-6 synthesis from MC3T3-E1 cells and effects of PD98059, SB203580, or SP600125 on the IL-6 synthesis by PDGF-BB in MC3T3-E1 cells. A, The cultured cells were stimulated by 50 ng/mL PDGF-BB (●) or vehicle (○) for the indicated periods. B, The cultured cells were pretreated with 3 μmol/L PD98059, 3 μmol/L SB203580, 3 μmol/L SP600125, or vehicle for 60 minutes and then stimulated by vehicle (gray bar) or 50 ng/mL PDGF-BB (black bar) for 24 hours. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with 2 additional and different cell preparations. *$P < .05$ compared with the value of control. **$P < .05$ compared with the value of PDGF-BB alone.

with PD98059, SB203580, SP600125, indomathacin, or rapamycin for 60 minutes. The conditioned medium was collected at the end of the incubation, and the IL-6 concentration was measured by ELISA kit.

2.4. Osteocalcin ELISA

The cultured cells were pretreated with various doses of rapamycin for 60 minutes and then stimulated by 50 ng/mL PDGF-BB or vehicle for 24 hours. The conditioned medium was collected at the end of the incubation, and the osteocalcin concentration was measured by ELISA kit.

2.5. Western blot analysis

The cultured cells were stimulated by PDGF-BB in α-MEM containing 0.3% FCS for the indicated periods. The cells were washed twice with phosphate-buffered saline and then lysed, homogenized, and sonicated in a lysis buffer (pH 6.8) containing 62.5 mmol/L Tris/HCl, 2% sodium dodecyl sulfate, 50 mmol/L dithiothreitol, and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125 000*g* for 10 minutes at 4°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by Laemmli [21] in 10% polyacrylamide gel. Western blotting analysis was performed as described previously [22] by using phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific p70 S6 kinase antibodies, or p70 S6 kinase antibodies, with peroxidase-labeled antibodies raised in goat-against-rabbit immunoglobulin G being used as second antibodies. Peroxidase activity on the polyvinylidene difluoride (PVDF) sheet was visualized on x-ray film by means of the ECL Western blotting detection system.

2.6. Determination

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

2.7. Statistical analysis

The data were analyzed by analysis of variance followed by the Bonferroni method for multiple comparisons between pairs, and $P < .05$ was considered significant. All data are presented as the mean ± SEM of triplicate determinations. Each experiment was repeated 3 times, with similar results.

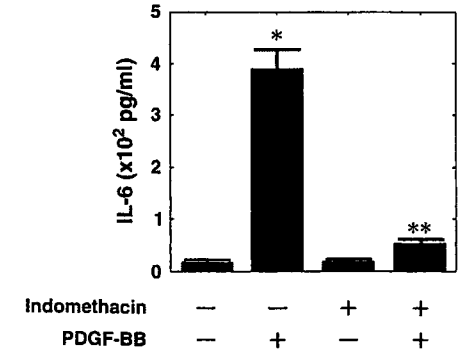

Fig. 3. Effect of indomethacin on the PDGF-BB–stimulated IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with 10 μmol/L indomethacin or vehicle for 60 minutes and then stimulated by 50 ng/mL PDGF-BB or vehicle for 24 hours. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with 2 additional and different cell preparations. *$P < .05$ compared with the control. **$P < .05$ compared with the value of PDGF-BB alone.

3. Results

3.1. Effects of PDGF-BB on the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, or SAPK/JNK in MC3T3-E1 cells

It is well recognized that 3 MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK, are known as central elements used by mammalian cells to transduce the various messages of a variety of agonists [23]. To investigate whether PDGF-BB activates MAP kinases in osteoblast-like MC3T3-E1 cells, we examined the effect of PDGF-BB on the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, or SAPK/JNK. Platelet-derived growth factor–BB time-dependently induced the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK. The effect of PDGF-BB on the p44/p42 MAP kinase phosphorylation reached its peak at 10 minutes and continued to 60 minutes after the stimulation of PDGF-BB (Fig. 1). On the other hand, the effect on the phosphorylation of p38 MAP kinase reached its peak at 10 minutes and diminished within 30 minutes after the stimulation of PDGF-BB (Fig. 1). In addition, the maximum effect on the SAPK/JNK phosphorylation was observed at 10 minutes and diminished within 30 minutes after the stimulation of PDGF-BB (Fig. 1).

3.2. Effects of PD98059, SB203580, or SP600125 on the PDGF-BB–stimulated IL-6 synthesis in MC3T3-E1 cells

It has been reported that PDGF-BB induces IL-6 transcription in osteoblasts from fetal rat calvariae [14]. We found that PDGF-BB time-dependently stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells (Fig. 2A). To

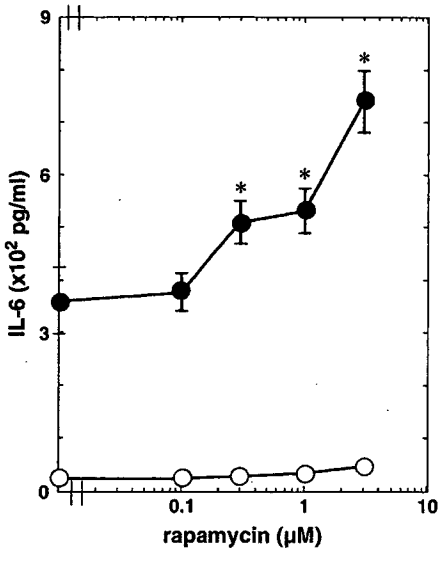

Fig. 4. Effect of rapamycin on the PDGF-BB–stimulated IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of rapamycin for 60 minutes and then stimulated by 50 ng/mL PDGF-BB (●) or vehicle (○) for 24 hours. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with 2 additional and different cell preparations. *$P < .05$ compared with the value of PDGF-BB alone.

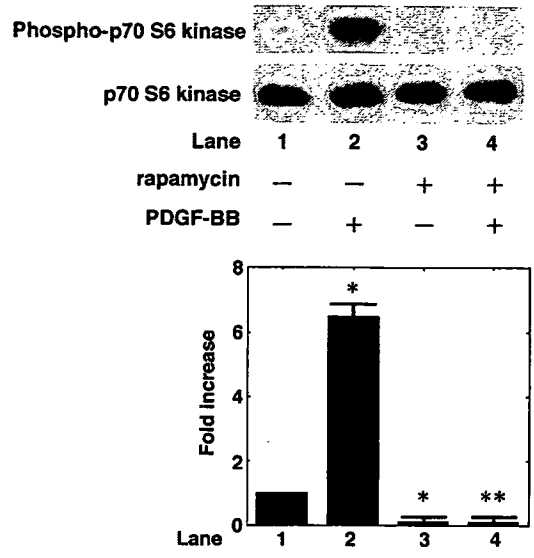

Fig. 5. Effect of rapamycin on the PDGF-BB–induced phosphorylation of p70 S6 kinase in MC3T3-E1 cells. The cultured cells were pretreated with 30 μmol/L rapamycin for 60 minutes and then stimulated by 50 ng/mL PDGF-BB or vehicle for 30 minutes. The extracts of cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis with subsequent Western blotting analysis with antibodies against phospho-specific p70 S6 kinase or p70 S6 kinase. The histogram shows quantitative representations of the levels of PDGF-BB–induced phosphorylation obtained from laser densitometric analysis of 3 independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with 2 additional and different cell preparations. *$P < .05$ compared with the value of control. **$P < .05$ compared with the value of PDGF-BB alone.

clarify the involvement of the MAP kinase pathway in the PDGF-BB–stimulated IL-6 synthesis in these cells, we first examined the effect of PD98059, a specific inhibitor of MAP kinase/extracellular signal-regulated kinase kinase (MEK, an upstream kinase that activates p44/p42 MAP kinase) [24], on the IL-6 synthesis. PD98059, which by itself had little effect on the IL-6 levels, significantly suppressed the PDGF-BB–stimulated synthesis of IL-6 (Fig. 2B). Similarly, the IL-6 synthesis stimulated by PDGF-BB was markedly reduced by SB203580, a specific inhibitor of p38 MAP kinase [25], or SP600125, a specific SAPK/JNK inhibitor [26] (Fig. 2B). In addition, a combination of PD98059, SB203580, and SP600125 completely suppressed the PDGF-BB–stimulated synthesis of IL-6 (Fig. 2B). To determine whether these inhibitors themselves could affect cell survival, or cell number, the cell viability had been assessed by trypan blue dye exclusion test. We confirmed that the viability of the cells incubated at 37°C for 24 hours in the presence of 3 μmol/L PD980590, 3 μmol/L SB203580, or 3 μmol/L SP600125 was more than 90% compared with that of the control cells.

3.3. Effect of indomethacin on the PDGF-BB–stimulated IL-6 synthesis in MC3T3-E1 cells

Because we have previously reported that prostaglandins (PGs) increase IL-6 synthesis in MC3T3-E1 cells [27-30], to address whether endogenous PGs are involved in the

PDGF-BB–induced IL-6 synthesis in MC3T3-E1 cells, we examined the effect of indomethacin, an inhibitor of cyclooxygenase [31], on the IL-6 synthesis. Indomethacin, which by itself had no effect on the IL-6 levels, significantly reduced the PDGF-BB–induced synthesis of IL-6 (Fig. 3). These findings suggest that PGs mediate the stimulatory effect of PDGF-BB on IL-6 synthesis in these cells.

3.4. Effect of PDGF-BB on the phosphorylation of p70 S6 kinase in MC3T3-E1 cells

To clarify whether PDGF-BB activates p70 S6 kinase in MC3T3-E1 cells, we next examined the effect of PDGF-BB on the phosphorylation of p70 S6 kinase. p70 S6 kinase was time-dependently phosphorylated by PDGF-BB (Fig. 1). The maximum effect on the p70 S6 kinase phosphorylation was observed at 30 minutes after the stimulation of PDGF-BB, and the PDGF-BB effect continued 180 minutes after the stimulation.

3.5. Effect of rapamycin on the PDGF-BB–stimulated IL-6 synthesis in MC3T3-E1 cells

To investigate whether p70 S6 kinase is involved in the PDGF-BB–induced synthesis of IL-6 in MC3T3-E1 cells, we examined the effect of rapamycin, a specific inhibitor of p70 S6 kinase [32,33], on the synthesis of IL-6 induced by PDGF-BB. Rapamycin, which alone failed to affect the IL-6 levels, significantly enhanced the PDGF-BB–induced synthesis of IL-6 (Fig. 4). The amplifying effect of rapamycin was dose-dependent in the range between 0.1 and 3 μmol/L. Rapamycin at 3 μmol/L caused approximately 110% enhancement in the PDGF-BB effect.

3.6. Effect of rapamycin on the PDGF-BB–induced phosphorylation of p70 S6 kinase in MC3T3-E1 cells

We examined the effect of rapamycin on the PDGF-BB–induced phosphorylation of p70 S6 kinase. Rapamycin, which itself significantly suppressed the phosphorylation of p70 S6 kinase in itself, truly suppressed the PDGF-BB–induced phosphorylation of p70 S6 kinase (Fig. 5).

3.7. Effect of rapamycin on the proliferation or the differentiation of MC3T3-E1 cells

To determine whether rapamycin could affect cell survival, or cell number, the cell viability had been assessed by trypan blue dye exclusion test. We confirmed that the viability of the cells incubated at 37°C for 24 hours in the

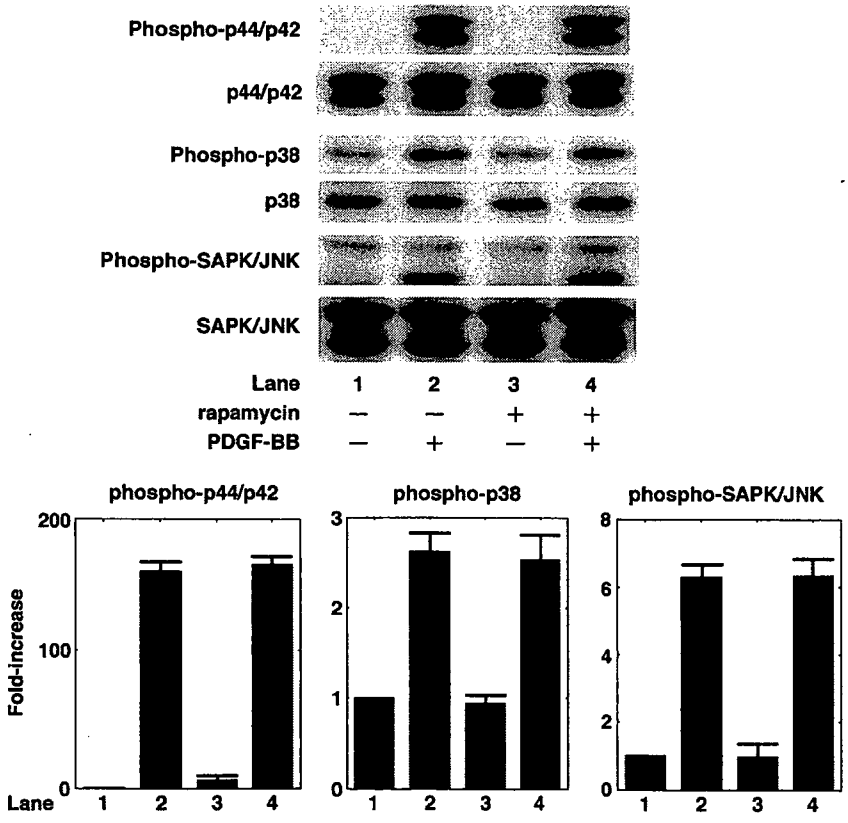

Fig. 6. Effects of rapamycin on the PDGF-BB–induced phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, or SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 30 μmol/L rapamycin or vehicle for 60 minutes and then stimulated by 50 ng/mL PDGF-BB or vehicle for 10 minutes. The extracts of cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis with subsequent Western blotting 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 representations of the levels of PDGF-BB–induced phosphorylation obtained from laser densitometric analysis of 3 independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with 2 additional and different cell preparations.

presence of 3 μmol/L rapamycin was more than 90% compared with that of the control cells. To determine whether rapamycin could affect the cell proliferation, we counted the cell number before and after the 24-hour incubation with rapamycin. We confirmed that rapamycin did not affect the cell number at a dose of 3 μmol/L ($9.7 \pm 1.1 \times 10^5$ cells/mL for control; $15.6 \pm 1.6 \times 10^5$ cells/mL for 50 ng/mL PDGF-BB alone; $10.1 \pm 1.4 \times 10^5$ cells/mL for 3 μmol/L rapamycin alone; and $14.7 \pm 1.6 \times 10^5$ cells/mL for 50 ng/mL PDGF-BB with 3 μmol/L rapamycin, as measured during the stimulation for 24 hours).

Next, to determine whether rapamycin affects the differentiation of these cells, we examined the effect of rapamycin on the production of osteocalcin, a mature osteoblast phenotype [34], in MC3T3-E1 cells. Platelet-derived growth factor–BB or rapamycin did not induce osteocalcin production in MC3T3-E1 cells (2.7 ± 0.3 ng/mL for control; 2.6 ± 0.3 ng/mL for 50 ng/mL PDGF-BB alone; 2.5 ± 0.4 ng/mL for 3 μmol/L rapamycin alone; and 2.5 ± 0.3 ng/mL for 50 ng/mL PDGF-BB with 3 μmol/L rapamycin, as measured during the stimulation for 24 hours). These findings as a whole suggest that rapamycin hardly affects the proliferation and the differentiation of osteoblast-like MC3T3-E1 cells within 24 hours.

3.8. Effects of rapamycin on the PDGF-BB–induced phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, or SAPK/JNK in MC3T3-E1 cells

To investigate whether rapamycin's effect on the PDGF-BB–stimulated IL-6 synthesis is dependent on the activation of p44/p42 MAP kinase, p38 MAP kinase, or SAPK/JNK, we next examined the effect of rapamycin on the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, or SAPK/JNK induced by PDGF-BB in these cells. However, rapamycin failed to affect the PDGF-BB–induced phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, or SAPK/JNK (Fig. 6).

4. Discussion

In the present study, we found that PDGF-BB time-dependently induced the phosphorylation of p70 S6 kinase in osteoblast-like MC3T3-E1 cells, using phospho-specific p70 S6 kinase (Thr389) antibodies. It is generally recognized that the activity of p70 S6 kinase is regulated by multiple phosphorylation events [15]. It has been shown that phosphorylation at Thr389 most strongly correlates with p70 S6 kinase activity [15]. Taking these results into account, it is most likely that PDGF-BB activates p70 S6 kinase in osteoblast-like MC3T3-E1 cells. To the best of our knowledge, this is probably the first report showing the PDGF-BB–induced p70 S6 kinase activation in osteoblasts.

We next demonstrated that PDGF-BB induces the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK in these cells. It is well recognized that the MAP kinase superfamily mediates intracellular signaling of extracellular agonists and plays an important role in cellular functions including proliferation, differentiation, and apoptosis in a variety of cells [23]. Three major MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK, are known as central elements used by mammalian cells to transduce diverse messages [23]. It has been shown that MAP kinases are activated by phosphorylation of threonine and tyrosine residues by dual-specificity MAP kinases [23]. Therefore, our findings strongly suggest that PDGF-BB activates 3 MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK, in osteoblast-like MC3T3-E1 cells. In addition, we showed that the PDGF-BB–stimulated IL-6 synthesis was suppressed by a MEK inhibitor, PD98059 [24]; a specific p38 MAP kinase inhibitor, SB203580 [25]; or a specific SAPK/JNK inhibitor, SP600125 [26], in these cells. Thus, it is probable that PDGF-BB stimulates the synthesis of IL-6 via the 3 MAP kinases in osteoblast-like MC3T3-E1 cells. We have previously reported that PGs increase IL-6 synthesis in MC3T3-E1 cells [27-30]. In the present study, we found that indomethacin significantly reduced the PDGF-BB–induced synthesis of IL-6. These results suggest that PDGF-BB–induced IL-6 production is mediated, at least in part, by PDGF-BB–stimulated PG production in osteoblast-like MC3T3-E1 cells. In addition, we have previously shown that PGE_2, a major product of eicosanoids in osteoblasts, significantly stimulates IL-6 synthesis after 3 hours in MC3T3-E1 cells [30]. On the contrary, PDGF-BB significantly stimulated the IL-6 production within 3 hours. Taking our findings into account, it is quite likely that there will be PG-dependent and PG-independent effects of PDGF-BB–stimulated IL-6 synthesis, as has been demonstrated for so many growth factors and cytokines in bone cells, and it would be important to be define these. Therefore, experiments using PGE_2 itself instead of PDGF-BB are required.

We investigated whether p70 S6 kinase functions in the PDGF-BB–stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells. The PDGF-BB–stimulated synthesis of IL-6 was significantly amplified by rapamycin, a specific inhibitor of p70 S6 kinase [31,32]. We confirmed that rapamycin truly suppressed the PDGF-BB–induced phosphorylation of p70 S6 kinase. It seems that the activated p70 S6 kinase plays an inhibitory role in the IL-6 synthesis by PDGF-BB in osteoblast-like MC3T3-E1 cells. Therefore, taking our results into account, it is most likely that PDGF-BB activates p70 S6 kinase, resulting in down-regulation of IL-6 synthesis. It is probable that the p70 S6 kinase signaling pathway activated by PDGF-BB limits the PDGF-BB–stimulated IL-6 synthesis. As far as we know, our present finding is probably the first report to show that the activation of p70 S6 kinase leads to the negative-feedback regulation of IL-6 synthesis in osteoblasts.

We investigated the relationship between p70 S6 kinase and 3 MAP kinases in the PDGF-BB–stimulated IL-6 synthesis in MC3T3-E1 cells. However, rapamycin failed to enhance the PDGF-BB–induced phosphorylation levels of

p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK. Therefore, it seems unlikely that p70 S6 kinase signaling pathway affects the PDGF-BB–stimulated synthesis of IL-6 through the amplification of activities of 3 MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK, in osteoblast-like MC3T3-E1 cells.

The p70 S6 kinase pathway is recognized to play a crucial role in various cellular functions, especially cell-cycle progression [15]. Our present results indicate that the p70 S6 kinase pathway in osteoblasts has an important role in the control of the production of IL-6, one of the key regulators of bone metabolism. It is well known that IL-6 produced by osteoblasts is a potent bone resorptive agent and induces osteoclast formation [3,4]. The mitogenic activities of PDGF-BB and its release by platelets suggest an important role in wound healing and fracture repair [35]. It is also possible that PDGF-BB plays a role in acute bone repair after inflammation because the mitogenic actions of PDGF-BB are enhanced in the presence of cytokines [35]. Therefore, our present findings lead us to speculate that PDGF-BB–activated p70 S6 kinase acts as a negative regulator of bone resorption through the fine tuning of the local cytokine network. Thus, the p70 S6 kinase pathway in osteoblasts might be considered to be a new candidate as a molecular target of bone resorption concurrent with various bone diseases. On the contrary, we have previously shown that p70 S6 kinase acts as a positive regulator in bone morphogenetic protein-4–stimulated synthesis of vascular endothelial growth factor in MC3T3-E1 cells [17]. The physiologic significance of regulatory mechanism by p70 S6 kinase in osteoblasts still remains unclear. Further investigation is required to clarify the exact role of p70 S6 kinase in osteoblasts.

In conclusion, our results strongly suggest that p70 S6 kinase plays an important role in the regulation of PDGF-BB–stimulated, MAP kinase–mediated IL-6 synthesis in osteoblasts and may serve as a negative feedback mechanism to prevent from oversynthesizing IL-6 in these cells.

Acknowledgments

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

Shinji Takai [a], Haruhiko Tokuda [a,b], Yoshiteru Hanai [a,b], Osamu Kozawa [a,*]

[a] *Department of Pharmacology, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan*
[b] *Department of Clinical Laboratory, National Hospital for Geriatric Medicine, National Center for Geriatrics and Gerontology, Obu, Aichi 474-8511, Japan*

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Abstract

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

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

1. Introduction

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

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

* Corresponding author at: Yanagido1-1, Gifu 501-1194, Japan. Tel.: +81 58 230 6214; fax: +81 58 230 6215.
E-mail address: okozawa@gifu-u.ac.jp (O. Kozawa).

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

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

2. Materials and methods

2.1. Materials

Mouse VEGF enzyme immunoassay kit was purchased from R&D Systems, Inc. (Minneapolis, MN). Akt inhibitor (1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate), LY294002, wortmannin, PD98059 and SP600125 were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Actinomycin D was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Phospho-specific Akt antibodies, Akt antibodies, phospho-specific GSK-3β antibodies, GSK-3β antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies and SAPK/JNK antibodies were purchased from Cell Signaling, Inc. (Beverly, MA). ECL Western blotting detection system was purchased from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. Akt inhibitor, wortmannin, LY294002, PD98059 and SP600125 were dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of DMSO was 0.1%, which did not affect the assay for VEGF or Western blot analysis.

2.2. Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria (Sudo et al., 1983) were maintained as previously described (Kozawa et al., 1992). Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 35 or 90-mm diameter dishes in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 24 h.

Freshly isolated osteoblasts were obtained from the calvaria of newborn (1 or 2-day-old) balb/c mice as previously described (Yoshida et al., 2004). They were seeded into 90-mm-diameter dishes (25×10^4 cells) in α-MEM containing 10% FCS. The medium was changed every 3 days until the cells were reached confluence at about 5 days. Then, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

2.3. VEGF assay

The cultured cells were stimulated by FGF-2 in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. The cells were pretreated with Akt inhibitor, wortmannin, LY294002 or actinomycin D for 60 min. The reaction was terminated by collecting the medium, and then VEGF in the medium was measured by Quantikine® mouse VEGF enzyme immunoassay kit (R&D systems) according to the manufacture's instruction. The assay kit can detect the mouse VEGF in the range between 7.8 and 500 pg/ml. When the samples generate values higher than 500 pg/ml, the samples were adequately diluted with caliblator diluent provided with the kit, and re-assayed.

2.4. Western blot analysis

The cultured cells were stimulated by FGF-2 in α-MEM containing 0.3% FCS for the indicated periods. The cells were washed twice with phosphate-buffered saline and then lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris/HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at $125{,}000 \times g$ for 10 min at 4 °C. SDS–polyacrylamide gel electrophoresis (PAGE) was performed by Laemmli (1970) in 10% polyacrylamide gel. Western blotting analysis was performed as described previously (Kato et al., 1996) by using phospho-specific Akt antibodies, Akt antibodies, phospho-specific GSK-3β antibodies, GSK-3β antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies or SAPK/JNK antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. Peroxidase activity on the PVDF sheet was visualized on X-ray film by means of the ECL Western blotting detection system.

2.5. Determination

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

2.6. Statistical analysis

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

3. Results

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

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

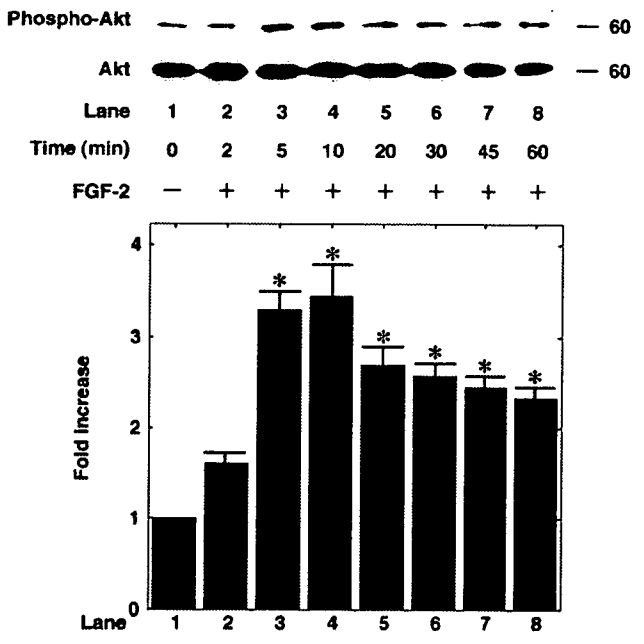

Fig. 1. Effect of FGF-2 on the phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were stimulated by 30 ng/ml FGF-2 for the indicated periods. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. The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

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

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

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

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

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

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

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

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

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

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

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

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

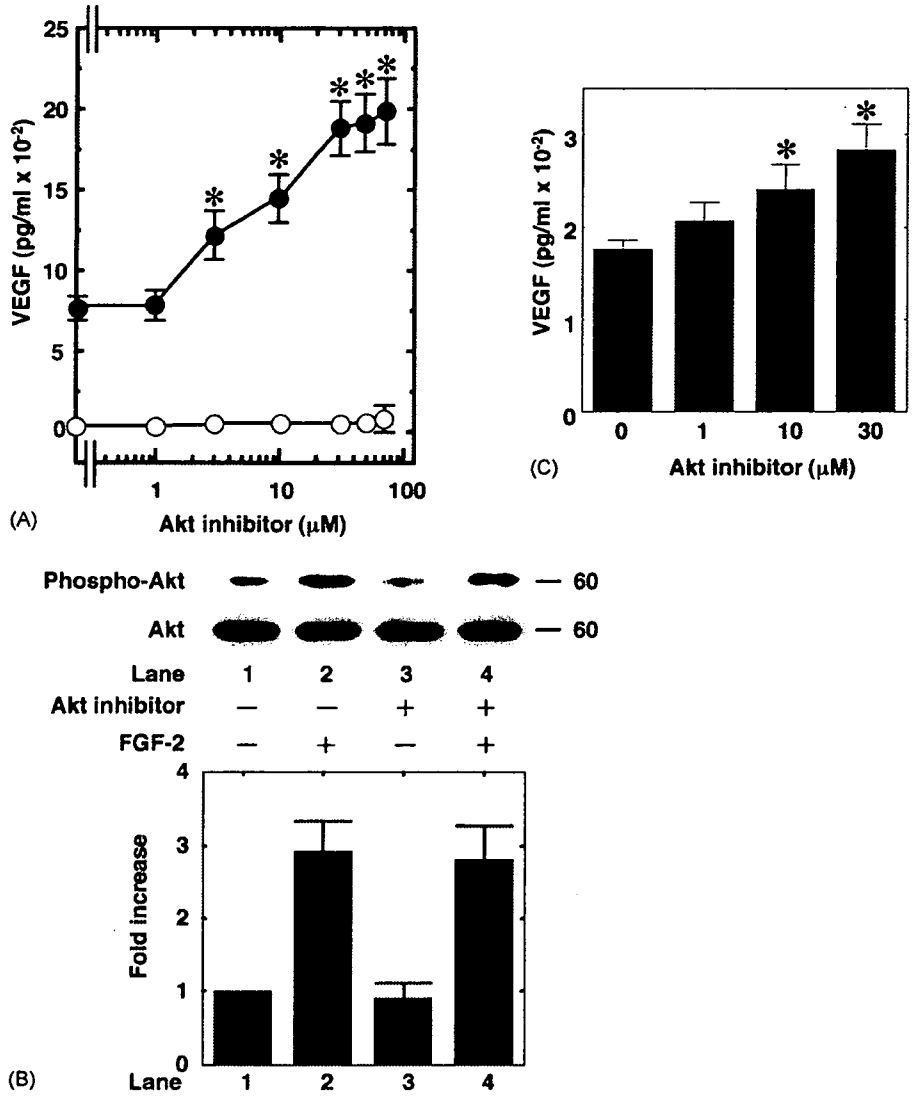

Fig. 2. Effects of Akt inhibitor on the VEGF release by FGF-2 or the FGF-2-induced phosphorylation of Akt. (A) Osteoblast-like MC3T3-E1 cells were pretreated with various doses of Akt inhibitor for 60 min, and then stimulated by 30 ng/ml FGF-2 (●) or vehicle (○) for 48 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}p < 0.05$, compared to the value of FGF-2 alone. (B) Osteoblast-like MC3T3-E1 were pretreated with 50 μM Akt inhibitor for 60 min, and then stimulated by 30 ng/ml FGF-2 or vehicle for 10 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. (C) Primary culture of osteoblast were pretreated with various doses of Akt inhibitor, and then stimulated by 30 ng/ml of FGF-2 or vehicle for 24 h. Values for FGF-2-unstimulated cells were subtracted to produce each data point. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}p < 0.05$, compared to the control value.

In order to investigate whether Akt inhibitor-effect on the FGF-2-induced VEGF release is dependent upon the activation of p44/p42 MAP kinase or SAPK/JNK, we next examined the effect of Akt inhibitor on the FGF-2-induced phosphorylations of p44/p42 MAP kinase or SAPK/JNK in these cells. However, Akt inhibitor failed to affect the phosphorylations of p44/p42 MAP kinase or SAPK/JNK induced by FGF-2 (Fig. 6A and B).

3.8. Effects of PD98059 or SP600125 on the FGF-2-induced phosphorylation of Akt in MC3T3-E1 cells

On the other hand, PD98059, a highly specific inhibitor of the upstream kinase that activates p44/p42 MAP kinase (Alessi et al., 1995), had little effect on the FGF-2-induced Akt phosphorylation (Fig. 7A). Furthermore, we found that the FGF-2-induced phosphorylation of Akt was not affected by SP600125, a highly specific inhibitor of JNK (Bennett et al., 2001) (Fig. 7B).

3.9. Effect of actinomycin D on the enhancement by Akt inhibitor of FGF-2-stimulated VEGF release in MC3T3-E1 cells

We examined the effect of actinomycin D, a transcriptional inhibitor (Reich, 1963), on the enhancement by the Akt inhibitor of FGF-2-induced VEGF release in osteoblast-like MC3T3-E1 cells. Actinomycin D, which by itself had no effect on the basal

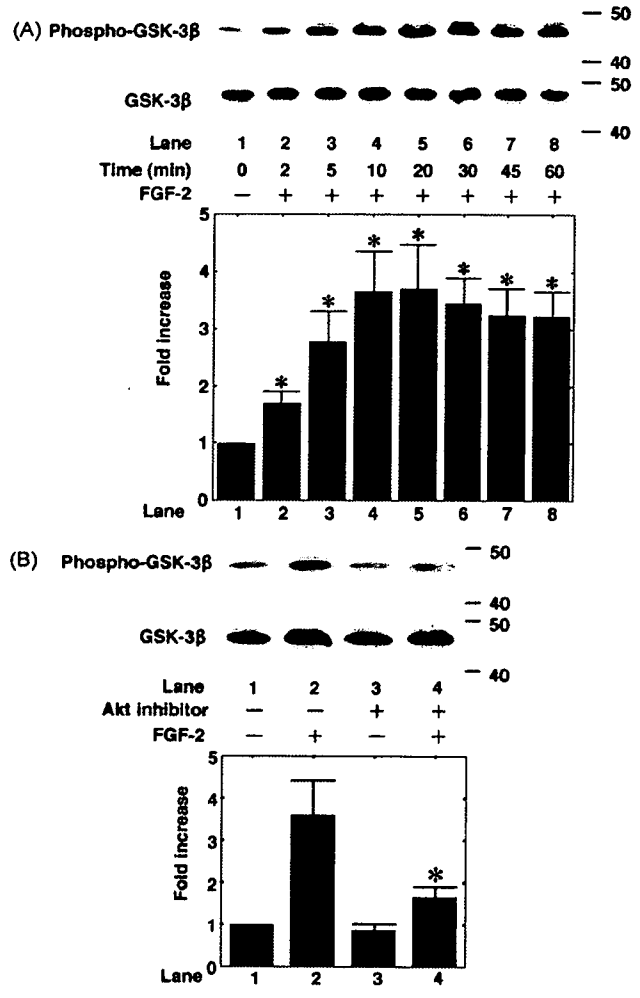

Fig. 3. Effect of FGF-2 on the phosphorylation of GSK-3β, and effect of Akt inhibitor on the FGF-2-induced phosphorylation of GSK-3β in MC3T3-E1 cells. (A) The cultured cells were stimulated by 30 ng/ml FGF-2 for the indicated periods. $^{*}p < 0.05$, compared to the value of control. (B) The cultured cells were pretreated with 50 μM Akt inhibitor for 60 min, and then stimulated by 30 ng/ml FGF-2 or vehicle for 10 min. $^{*}p < 0.05$, compared to the value of FGF-2 alone. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific GSK-3β or GSK-3β. The histogram shows quantitative representations of the levels of FGF-2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate determinations. Numbers on the right indicate molecular masses (kDa). Similar results were obtained with two additional and different cell preparations.

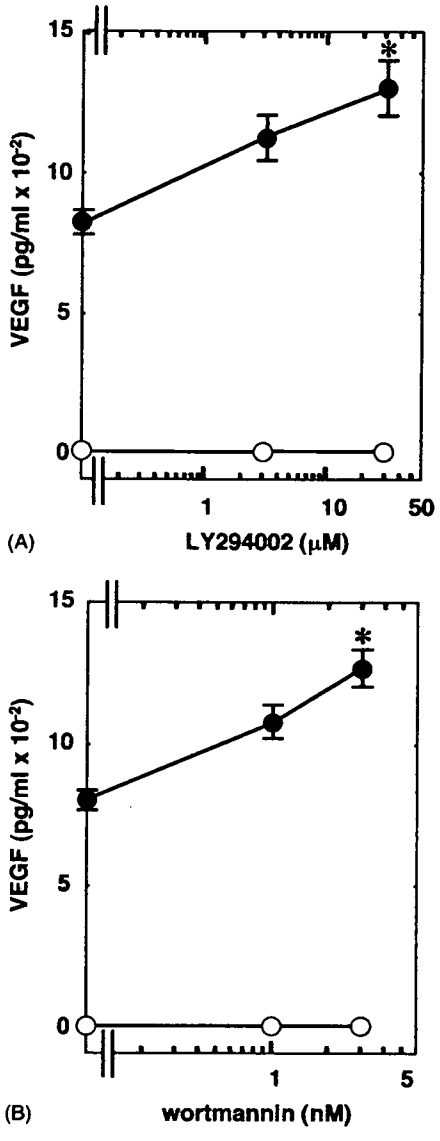

Fig. 4. Effects of wortmannin or LY294002 on the FGF-2-induced VEGF release in MC3T3-E1 cells. Osteoblast-like MC3T3-E1 cells were pretreated with various doses of LY294002 (A) or wortmannin (B) for 60 min, and then stimulated by 30 ng/ml FGF-2 (●) or vehicle (○) for 48 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}p < 0.05$, compared to the value of FGF-2 alone.

levels of VEGF, significantly reduced both the VEGF release induced by FGF-2 and the enhancement by the Akt inhibitor of FGF-2-stimulated VEGF release (Table 1).

4. Discussion

In the present study, we demonstrated that FGF-2 time dependently induced the phosphorylation of Akt in osteoblast-like MC3T3-E1 cells. It is generally known that Akt mediates intracellular signaling of various extracellular agonists and plays a crucial role in cellular functions such as proliferation and cell survival in a variety of cells (Coffer et al., 1998). According to the previous reports (Coffer et al., 1998; Chan et al., 1999), Akt is activated by phosphorylation of threonine and serine residues. Therefore, our present result suggests that FGF-2 truly activates Akt in osteoblast-like MC3T3-E1 cells. In addition, we next showed that PI3-kinase inhibitors such as LY294002 (Vlahos et al., 1994) and wortmannin (Arcaro and Wymann, 1993) attenuated the FGF-2-induced phosphorylation of Akt in MC3T3-E1 cells. PI3-kinase is recruited upon growth factor receptor activation and produces 3′ phosphoinositide lipids (Dudek et al., 1997; Katso et al., 2001). The lipid products of PI3-kinase act as second messengers by binding to and activating diverse cellular target proteins. In addition, it is well known that Akt is a downstream target of PI3-kinase (Chan et al., 1999; Cantley,

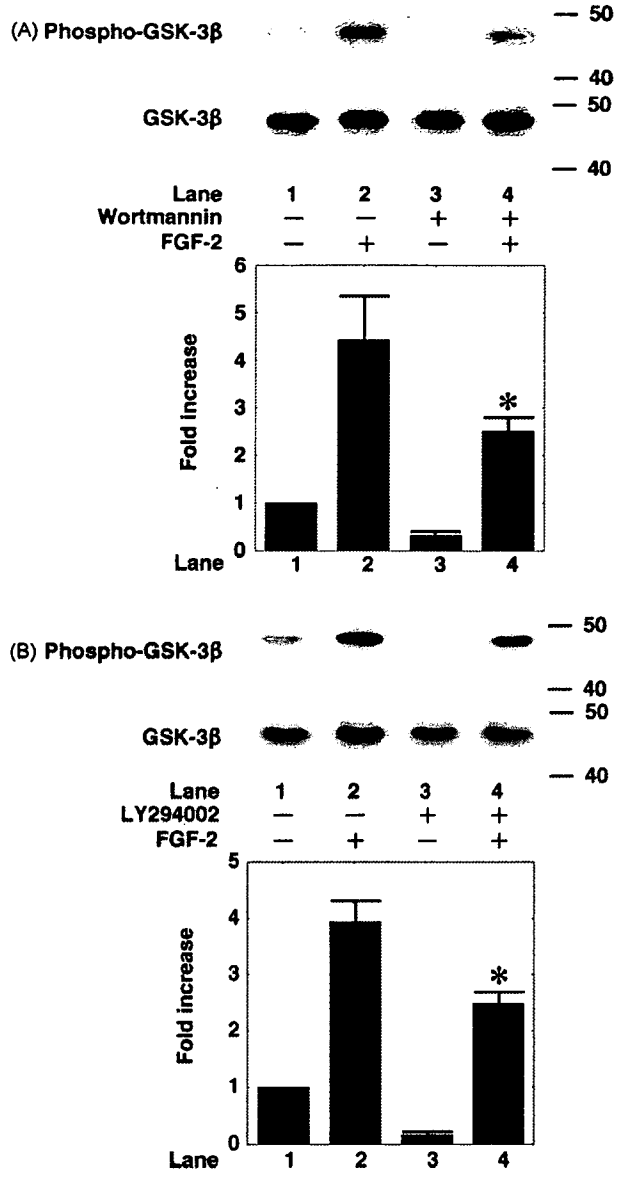

Fig. 5. Effects of wortmannin or LY294002 on the FGF-2-induced phosphorylation of GSK-3β in MC3T3-E1 cells. The cultured cells were pretreated with 0.1 μM of wortmannin (A), 30 μM of LY294002 (B) or vehicle for 60 min, and then stimulated by 30 ng/ml FGF-2 or vehicle for 10 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific GSK-3β or GSK-3β. The histogram shows quantitative representations of the levels of FGF-2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate determinations. Numbers on the right indicate molecular masses (kDa). Similar results were obtained with two additional and different cell preparations. $^{*}p < 0.05$, compared to the value of FGF-2 alone.

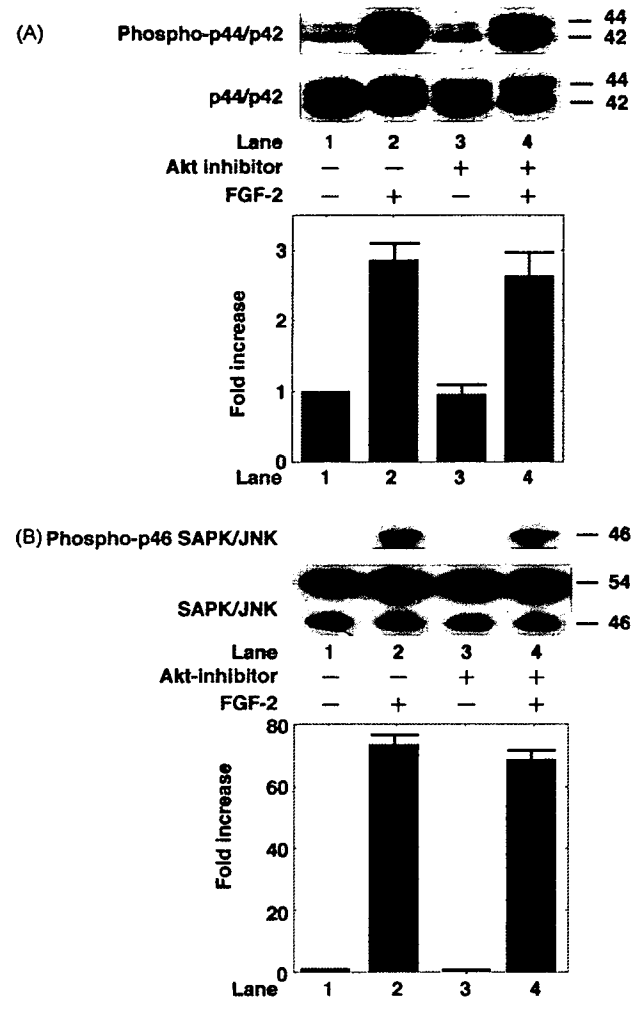

Fig. 6. Effects of Akt inhibitor on the FGF-2-induced phosphorylation of p44/p42 MAP kinase or SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 50 μM Akt inhibitor or vehicle for 60 min, and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against (A) phospho-specific p44/p42 MAP kinase or p44/p42 MAP kinase, or (B) phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of FGF-2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate determinations. Numbers on the right indicate molecular masses (kDa). Similar results were obtained with two additional and different cell preparations.

2002). Nowadays, the PI3-kinase/Akt signaling pathway is recognized to play a critical role in mediating survival signals in a wide range of cell types. Taking these findings into account, it is most likely that PI3-kinase/Akt pathway participates in the FGF-2 signaling in osteoblast-like MC3T3-E1 cells.

Hence, we have previously reported that FGF-2 stimulates the release of VEGF in osteoblast-like MC3T3-E1 cells (Tokuda et al., 2000), we next investigated whether PI3-kinase/Akt is involved in the FGF-2-induced VEGF release in MC3T3-E1 cells. First, Akt inhibitor (Hu et al., 2000) significantly enhanced the FGF-2-induced VEGF release in osteoblast-like MC3T3-E1 cells. In addition, we found that the Akt inhibitor significantly enhanced the FGF-2-induced VEGF release also in primary cultured osteoblasts. Therefore, it is probable that the negative regulation by Akt of FGF-2-induced VEGF release is a general phenomenon in osteoblasts. Although the phosphorylation of Akt was observed prior to the FGF-2 stimulation, and the Akt inhibitor alone did not show any effect on the VEGF release in osteoblast-like MC3T3-E1 cells. It is generally recognized that