転倒及び転倒不安に対するヒッププロテクターの効果の検討

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研究要旨

日本で介護を必要とするようになった原因の第1位は脳血管障害、第2位は老衰、第3位が転倒・骨折によるものである。転倒による骨折の予防は、要介護者を減少させるためにも、非常に重要な問題である。本研究では、硬性および軟性のヒッププロテクター2製品を選んで2つの介入群を設定し、コントロール群との間で比較試験を行う。研究デザインは、施設別無作為比較試験である。対象は、介護施設入所の介助車椅子レベル以上の高齢者で、39施設、392名である。開始時調査項目は、一般病歴、転倒・骨折歴、身体測定、超音波骨評価、血液骨代謝マーカー、血液中の骨代謝制御ホルモン・生理的骨代謝活性化因子、筋力、バランス、歩行速度、反応時間、ADL評価（FIM）、失禁評価、Mini-Mental State Examination (MMSE)、転倒不安評価である。主要アウトカムは、大腿骨頚部骨折率、他の骨折率、転倒率、転倒不安感、時間帯別コンプライアンス、介護者負担で、コントロールと2製品の間で比較する。転倒不安感、介護者負担は開始前からの経時変化も解析する。本年度は、ヒッププロテクター装着の試験期間を終了していないために、エントリー開始時のデータより、転倒既往と関連ある因子の検討を行った。その結果、転倒の既往と有意に関連がみられたのは、ADLが低いこと（FIMの低値）、MMSEが低いこと、低体重である。従来、転倒リスクとしていわれている既往歴、薬剤の内服と転倒の既往とは関連がみられなかった。また、血液骨代謝マーカーや血液学的データと転倒の既往とは有意な関連はみられなかった。

A. 研究目的

高齢者においては、自立度を維持することが高齢者自身のQuality of life （QOL）の維持や医療・介護の面からも重要である。日本の高齢者は国際的にも類をみないレベルでの長寿命化がすすんでいるが、一方で寝たきりなど医療・介護の必要な人の割合が高いことが指摘されている。日本で介護を必要とするようになった原因の第1位は脳血管障害、第2位は老衰、第3位が転倒・骨折によるものである。転倒による骨折の予防は、非常に重要な問題である。

高齢者においては立位能力・歩行能力が低下し、転倒の危険性が高くなっている。65歳以上の高齢者の約1/3が1年間に1回あるいはそ

れ以上、転倒経験があるといわれている。また受傷すると日常生活動作に著しく障害をきたしやすい大腿骨頸部骨折の 90%は転倒によって生じると報告されている。転倒の経験は身体的・精神的に悪影響を及ぼし、健やかな老後生活の妨げとなり、高齢者の QOL を著しく低下させる要因となる。

本年度は、ヒッププロテクター装着の試験期間を終了していないために、エントリー開始時のデータより、転倒および骨折の既往と関連ある因子の検討を行った。

B. 研究方法

本研究のデザインは施設別無作為比較試験で無作為化はコンピュータによって行った。対象は、愛知県、三重県、岐阜県、静岡県、長野県、大阪府の 39 施設の介護施設入所者 392 名が、エントリーした。参加基準は介助車イスレベル以上の移動能力のある参加同意者で、認知症の有無は問わない。試験期間は 1 年である。使用するヒッププロテクターは、我が国で販売されている製品のうち、タイプが異なり対照的と考えられる T 社製品と D 社製品を選択した。3 群に無作為に分け、介入は、一つの群には T 社製硬質タイプヒッププロテクター（T 群）、もう一つの群には D 社製軟質タイプヒッププロテクター（D 群）を装着させた。残りの群はコントロールとして介入なしで観察のみを行った。

開始時調査項目としては、一般病歴、転倒歴、骨折歴、服薬状況、視力障害、体重、身長、踵骨超音波骨評価、血液による骨代謝と転倒に関連する測定（BAP, NTx, CTx, Vitamin D, Vitamin A, intact PTH）及び一般内科的評価、筋力（大腿四頭筋伸展力、握力）、バランス、歩行速度、ADL 評価(FIM)、失禁評価、MMSE、転倒不安評価(Fall Efficacy Scale:FES)を行った。ほかに介護者に対してリサーチ PT がヒッププロテクター装着前 1 週間の介護負担評価を行う。ヒッププロテクター装着開始後は、毎日ヒッププロテクターの装着状況、装着の感想、転倒と転倒傷害の有無、転倒時装着状況を介護者が観察記録した。試験終了時には、血液による骨代謝と転倒に関連する測定（BAP, NTx, CTx, Vitamin D, Vitamin A, intact PTH）及び一般内科的評価、FIM、転倒不安評価を行う予定である。主要アウトカムは、大腿骨頸部骨折率、他の骨折率、転倒率、転倒不安感、時間帯別コンプライアンス、介護者負担でこれらを製品間で比較する。転倒不安感、介護者負担は開始前からの経時的変化も解析する。転倒を集計し、ヒッププロテクターの転倒抑制効果を解析する。同時に身体機能と FES を用いた転倒不安感にヒッププロテクター装着が与える影響も検討する。

本年度は、ヒッププロテクター装着に試験期間を終了していないために、エントリー開始時のデータより、転倒の既往と関連ある因子の検討を行った。

倫理面での配慮として、1）インフォームドコンセントに基づき、同意を得た場合に調査を行う。2）調査結果については秘密を厳守し、被検者本人から要請があった場合にのみ直接本人に知らせる。3）被検者のプライバシーを尊重し、いかなる個人情報も外部に漏れないように細心の配慮を行う。4）専門学会あるいは学会誌に発表する場合は被検者個人の情報としてではなく、結果全体のまとめとして発表を行うこととした。本研究は、国立長寿医療センターの倫理委員会の承認を受けた。

C. 研究結果

解析を行ったのは、愛知県、三重県、岐阜県、静岡県、長野県、大阪府の 39 施設の介護施設入所者 392 名である。平均年齢は、86.5±6.7 歳である。転倒歴と身体計測値との間では、身長では有意な差がみられなかったが、転倒あり群では有意に体重が低かった。ADL との関係では、転倒あり群で FIM 値は有意に低値であった。MMSE は、転倒あり群で 14.6 点、 非転倒群で 17.51 点で、転倒あり群は有意に MMSE が低値であった。FES との関係は、転倒あり群で 60.97、非転倒群で 66.7 で有意差はみられなかった。

転倒歴と血液骨代謝マーカーや血液学的データとの関連では、総タンパク、アルブミン、ALP、カルシウム、骨型 ALP 、AST、ALT、総コレステロール、尿素窒素、クレアチニン、無機リン、NTx, Vitamin D、PTH、オステオカルシン、音速、透過指標、音響的骨評価価値は、転倒歴との間に有意な関連は認められなかった。転倒の既応と、既応歴との関連では、視力障害、心疾患、高血圧、脳卒中、糖尿病、パーキンソン病、不整脈、てんかん発作、変形性関節症、関節リウマチ、眼疾患などに関して、転倒の有無で有意な差はみられなかった。

転倒の既往と内服薬の関連では、精神安定剤、催眠剤、抗うつ剤、抗けいれん剤、降圧利尿剤、非ステロイド消炎鎮痛剤、抗パーキンソン病薬の内服と転倒の有無で有意な差はみられなかった。

E. 結論

本年度は、ヒッププロテクター装着の試験期間を終了していないために、エントリー開始時のデータより、転倒既往と関連ある因子の検討を行った。その結果、転倒の既往と有意に関連がみられたのは、ADL が低いこと(FIM の低値)、MMSE が低いこと、低体重である。従来、転倒リスクとしていわれている既往歴、薬剤の内服と転倒の既往とは関連がみられなかった。また、血液骨代謝マーカーや血液学的データと転倒の既往とは有意な関連はみられなかった。

F. 健康危険情報

本年度の研究では、健康危険情報は特に認められなかった。

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分担研究報告書

ヒッププロテクター研究参加者の骨代謝と老年学的解析

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研究要旨

高齢女性における骨代謝環境を明らかとするため、ヒッププロテクター研究参加者から研究参加時に血清試料を採取し、ALP、Ca、P、骨型 ALP、NTｘ、オステオカルシン、1α, 25-(OH) 2D3 および副甲状腺ホルモン(PTH intact)の測定を実施した。栄養状態の評価として総コレステロール、総蛋白およびアルブミンを、肝・腎機能の評価として AST、ALT、BUN およびクレアチニン(Cr)を測定・解析した。対象の平均年齢は 86.2 ± 6.7 歳、有効データの総数は 354 件であった。骨代謝マーカーである骨型 ALP、NTx およびオステオカルシンの平均値はそれぞれ 32.9 ± 15.5 IU/l、16.8 ± 7.1 nmol BCE/l および 8.19 ± 3.8 ng/ml であった。1α, 25-(OH) 2D3 および PTH intact の平均値はそれぞれ 43.8 ± 17.0 pg/ml および 54.3 ± 28.2 pg/ml でこれらはいずれも基準値内であった。骨代謝マーカーは相互に正の相関関係を認めたが、NTx とオステオカルシンの相関が最も強かった。PTH intact はこれらとの間に正の相関を示し、1α, 25-(OH) 2D3 は NTx との間に負の相関を示した。Cr は NTx、オステオカルシンおよび PTH intact との間に正の相関を、1α, 25-(OH) 2D3 との間に負の相関を示した。同様の相関は BUN においても認められた。Ca は、PTH intact との間に負の相関を示す一方、アルブミンあるいは総コレステロールとの間に正の相関を示した。なお年齢と Cr との間には正の相関が認められた。以上の結果より、後期高齢女性においては消化管からの Ca 吸収の低下や肝臓・腎臓におけるビタミン D 水酸化能の低下等により、潜在的な PTH 分泌亢進状態および骨代謝亢進状態にある可能性が強く示唆された。

A. 研究目的

大腿骨頚部骨折の予防対策としてヒッププロテクターの有効性が知られている。本研究では、その製品差について検討することとしており、その信頼性を高めるためには、被検者の骨代謝環境を知ることが有用であると考えられる。

近年の検討により高齢期における骨粗鬆症では高代謝回転を呈するものが少なくないことが示唆されているが、その詳細は未だ判然としていない。そこで本分担研究では、エントリーの時点における骨代謝マーカーや副甲状腺ホルモン等について栄養状態・肝機能・腎機能を踏まえて詳細に解析し、後期高齢女性の骨代謝環境について評価することとした。

B. 研究方法

本研究への施設参加について同意を得られた施設のうち、国立長寿医療センター臨床検査部に当日検体搬送が可能である施設への入所者 355 名について、研究参加に際して採血を実施し、検体到着後直ちに血清の分離を施行した。院内で測定が可能な項目（総蛋白、アルブミン、ALP、AST、ALT、総コレステロール、BUN、Cr、Ca、P）については即日解析を実施し、骨型 ALP、NTx、オステオカルシン、1α, 25-(OH) 2D3 および PTH intact については、三菱化学ビーシーエル株式会社（東京）に測定を委託した。相関関係を含む統計的解析には Stat View ver. 5.0 (SAS Inst. Inc. Cary, NC) を用い、P<0.001 をもって有意な相関を認めるものとした。

(倫理的配慮)

研究計画については倫理委員会に諮り、承認を得た。

C. 研究結果

参加者の年齢（平均値 ± SD）は 86.2 ± 6.7 歳で全例が女性であり、有効データ総数は 354 件であった。総蛋白、アルブミンおよび総コレステロールはそれぞれ 6.91 ± 0.45 g/dl、3.90 ± 0.36 g/dl および 199.4 ± 38.9 mg/dl であった。ALP、AST および ALT はそれぞれ 323.5 ± 113.2 IU/l、19.76 ± 8.19 IU/l および 12.29 ± 7.72 IU/l であった。BUN および Cr はそれぞれ 18.34 ± 6.84 mg/dl および 0.700 ± 0.303 mg/dl で、3 件が Cr>2.0 mg/dl を示した。Ca および P はそれぞれ 8.80 ± 0.42 mg/dl および 3.60 ± 0.44 mg/dl であった。骨型 ALP、NTx およびオステオカルシンはそれぞれ 32.9 ± 15.5 IU/l、16.8 ± 7.1 nmol BCE/l および 8.19 ± 3.8 ng/ml であった。1α, 25-(OH) 2D3 および PTH intact はそれぞれ 43.8 ± 17.0 pg/ml および 54.3 ± 28.2 pg/ml であった。これらはいずれも基準値内であった。

Cr が高値を示した 3 例を除外し、以下の解析を行った。骨代謝マーカーは相互に正の相関を認めた。骨型 ALP と NTx、骨型 ALP とオステオカルシンおよび NTx とオステオカルシンの間の相関係数 r はそれぞれ 0.334、0.328 および 0.504 であり NTx とオステオカルシンの相関が最も強かった。PTH intact はこれらとの間に正の相関を示した (r=0.254、骨型 ALP; r=0.405、NTx; r=0.440、オステオカルシン)。1α, 25-(OH) 2D3 は NTx との間に負の相関を示した (r=-0.200)。Cr は NTx、オステオカルシンおよび PTH intact との間に正の相関を (r=0.313、NTx; r=0.405、オステオカルシン; r=0.390、PTH intact)、1α, 25-(OH) 2D3 との間に負の相関を示した (r=-0.353)。同様の相関は BUN においても認められた (r=0.211、NTx; r=0.313、オステオカルシン; r=0.180、PTH intact; r=-0.247、1α, 25-(OH) 2D3)。Ca は、PTH intact との間に負の相関を示す一方、アルブミンあるいは総コレステロールとの間に正の相関を示した (r=-0.240、PTH intact; r=0.562、アルブミン; r=0.203、総コレステロール)。ALP は AST、ALP、骨型 ALP、NTx、オステオカルシンおよび PTH intact との間に正の相関を認めた (r=0.338、AST; r=0.286、ALT; r=0.798、骨型 ALP; r=0.306、NTx; r=0.198、オステオカルシン; r=0.196、PTH intact)。なお年齢と Cr との間には正の相関を認めた (r=0.210)。

D. 考察

原発性骨粗鬆症は卵巣機能の低下に基づくエストロゲンの欠乏により一元的に説明されているが、高齢期においてはビタミンD活性化能の低下や慢性的なPTHの分泌亢進により、さらに骨量の減少が促進されることが知られている。今回、骨代謝マーカーとして骨型ALP、NTxおよびオステオカルシンについて検討し相互に相関関係を示したことから、後期高齢女性において骨形成系と骨吸収系の共役は保たれていると考えられた。また、骨吸収のマーカーとして基準値の明らかとされていない血清NTxを測定したが、十分解析に耐えるデータを得ることができた。今回の平均値は、高齢女性における基準値として参考になりうると考えられる。さらにオステオカルシンとNTxの相関が最も強かったことは、オステオカルシンが骨型ALPに比してより分化の進んだ骨芽細胞の指標であることから、骨リモデリングを反映した興味深い結果と考えられた。一方、骨型ALPはALPのみならずASTあるいはALTとも相関を示したことから、高齢者においては骨代謝マーカーとしての特異性が低い可能性が示唆される。一方、PTH intactとこれらの骨代謝マーカーとの相関が明らかとなったことから、後期高齢者においてはPTHの分泌促進により一層骨代謝が亢進することが示唆された。CaとPTH intactの間に負の相関が見られたことから、PTHの分泌亢進はCaの低下による二次性のものと考えられる。

今回、腎不全例を除いてもCrがNTx、オステオカルシンおよびPTH intactと相関を示したことから、高齢者における腎機能と骨代謝との関連の重要性が示唆された。Crと年齢との間の相関が見られたことは、このことを支持する知見であると考えられる。1α, 25-(OH)2D3とNTxおよびCrとの間の負の相関関係がみられたことから、腎臓におけるビタミンDの水酸化能の低下が骨代謝に悪影響を及ぼす可能性が示唆される。なお1α, 25-(OH)2D3とPTH intactの間の相関は統計学的に有意ではなかったが、傾向は認められ（r=-0.152、P=0.004）、ビタミンD活性化の低下がPTH分泌亢進の一因である可能性が示された。

Caがアルブミンおよび総コレステロールと相関を示したことは、Caの低下は栄養状態と関連する可能性を示唆する知見と考えられる。またアルブミンやコレステロールが肝臓で合成されることを考慮すると、肝臓におけるビタミンD水酸化能の低下による活性型ビタミンDの不足もCa低下の要因と考えられる。

以上のように後期高齢女性においては、Caの経口摂取量の低下、腎機能および肝機能の減退等に起因する活性型ビタミンDの低下によるCa吸収量の低下等を介してPTH分泌亢進が引き起こされ、ひいては骨代謝の亢進とそれに続く骨量減少が惹起されると考えられる。今回の結果を踏まえて、Crを指標としての活性型ビタミンDの投与が、後期高齢女性の骨代謝環境の是正に有用であると考えられた。

E. 結論

後期高齢女性においては消化管からのCa吸収の低下や肝臓・腎臓におけるビタミンD水酸化能の低下等により、潜在的なPTH分泌亢進状態および骨代謝亢進状態にある可能性が強く示唆された。

F. 健康危機情報

なし

G. 研究発表

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H. 知的財産権の出願・登録状況

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

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研究成果の刊行物・別刷

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Tiludronate inhibits prostaglandin $F_{2\alpha}$-induced vascular endothelial growth factor synthesis in osteoblasts

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Abstract

We have previously reported that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) activates p44/p42 mitogen-activated protein (MAP) kinase through protein kinase C (PKC), resulting in the synthesis of vascular endothelial growth factor (VEGF) in osteoblast-like MC3T3-E1 cells, and that incadronate, a bisphosphonate, amplifies the VEGF synthesis. In the present study, we investigated the effects of tiludronate and etidronate, other bisphosphonates, on the $PGF_{2\alpha}$-stimulated VEGF synthesis in these cells. Tiludronate reduced the synthesis of VEGF induced by $PGF_{2\alpha}$. The $PGF_{2\alpha}$-stimulated phosphorylation of p44/p42 MAP kinase was suppressed by tiludronate. On the other hand, etidronate affected neither the VEGF synthesis nor the phosphorylation of p44/p42 MAP kinase elicited by $PGF_{2\alpha}$. Tiludronate attenuated the phosphorylation of both Raf-1 and MEK1/2 induced by $PGF_{2\alpha}$. The VEGF synthesis stimulated by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a direct activator of PKC, was suppressed by tiludronate. The TPA-induced phosphorylations of Raf-1, MEK1/2 and p44/p42 MAP kinase were inhibited by tiludronate. These results strongly suggest that tiludronate but not etidronate suppresses the $PGF_{2\alpha}$-stimulated VEGF synthesis in osteoblasts, and that the effect of tiludronate is exerted at the point between PKC and Raf-1.

Keywords: Bisphosphonate; Prostaglandin $F_{2\alpha}$; Vascular endothelial growth factor; Osteoblast

1. Introduction

Bone metabolism is well recognized to be regulated by two types of functional cells, osteoblasts and osteoclasts, the former is responsible for bone formation and the latter for bone resorption (Nijweide et al., 1986). Accumulating evidence indicates that bone resorptive agents such as parathyroid hormone and 1,25-$(OH)_2$ vitamin D_3 upregulate RANKL (receptor activator of nuclear factor κB ligand) expression through binding specific receptors on osteoblasts, suggesting that osteoblasts play pivotal roles in the regulation of bone resorption (Suda et al., 1999). The bone remodeling results from the finely coordinated process, bone resorption by activated osteoclasts coupled with subsequent deposition of new matrix by osteoblasts. During the process, capillary endothelial cells provide the microvasculature, and osteoblasts and osteoprogenitor cells, which locally proliferate and differentiate into osteoblasts, migrate into the resorption lacuna. Thus, it is currently recognized that the activities of osteoblasts, osteoclasts and capillary endothelial cells are closely coordinated via humoral factors as well as by direct cell-to-cell contact, and that these cells cooperatively regulate bone metabolism (Erlebacher et al., 1995).

Vascular endothelial growth factor (VEGF) is known as a potent angiogenic factor that induces endothelial cell pro-

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liferation, angiogenesis and capillary permeability (Ferrara and Davis-Smyth, 1997). VEGF is recognized to be produced and secreted from various types of cells (Ferrara and Davis-Smyth, 1997). It has been reported that inactivation of VEGF causes impaired trabecular bone formation and expansion of the hypertrophic chondrocyte zone in mouse tibial epiphyseal growth plate associated with the complete suppression of vascular invasion (Gerber et al., 1999). It has been shown that osteoblasts produce and secrete VEGF in response to various physiological agonists (Ferrara and Davis-Smyth, 1997; Harada and Thomas, 2002). On the other hand, it is well recognized that prostaglandins (PGs) act as autocrine/paracrine modulators in osteoblasts and play crucial roles in the regulation of bone metabolism (Nijweide et al., 1986; Pilbeam et al., 1996). Among them, $PGF_{2\alpha}$ is known as a potent bone-resorptive agent and stimulates the proliferation of osteoblasts and inhibits their differentiation (Pilbeam et al., 1996). We previously showed that $PGF_{2\alpha}$ stimulates both phosphoinositide-hydrolyzing phospholipase C (PI-PLC) and phosphatidylcholine-hydrolyzing phospholipase D (PC-PLD) (Miwa et al., 1990; Kozawa et al., 1994), recognized to be two major pathways of physiological protein kinase C (PKC) activation (Nishizuka, 1992; Exton, 1999), in osteoblast-like MC3T3-E1 cells. In addition, we have recently reported that $PGF_{2\alpha}$ stimulates the VEGF synthesis through PKC-dependent activation of p44/p42 mitogen-activated protein (MAP) kinase in these cells (Tokuda et al., 2003).

Bisphosphonates, stable analogues of pyrophosphate, are generally known as potent inhibitors of bone resorption (Fleisch et al., 2002). They are widely used for the treatment of various metabolic bone diseases associated with increased osteoclastic bone resorption such as Paget's disease, tumoral bone disease, and osteoporosis (Kozawa et al., 1994). Inhibition of osteoclast recruitment, osteoclastic adhesion to bone surface and osteoclast activity are recognized as the main mechanisms of the anti-bone resorptive actions of bisphosphonates (Kozawa et al., 1994). On the other hand, it has been reported that the osteoclast inhibiting action of bisphosphonates is mediated in part through its actions on osteoblasts (Sahni et al., 1993; Nishikawa et al., 1995). Ibandronate and alendronate reportedly induce the synthesis of an inhibitor of osteoclastic bone resorption in osteoblastic cell line CRP 10/30 (Vitte et al., 1996). We previously demonstrated that tiludronate inhibits interleukin (IL)-6 synthesis in osteoblast-like MC3T3-E1 cells (Tokuda et al., 1998). Etidronate, alendronate, pamidronate and olpadronate have been reported to prevent apoptosis of murine primary cultured osteoblasts through the activation of p44/p42 mitogen-activated protein (MAP) kinase (Plotkin et al., 1999). Pamidronate and zoledronate have recently been shown to enhance the differentiation and bone forming activities of primary cultured human fetal osteoblasts (Reinholz et al., 2000). It has also been reported that pamidronate and zoledronate increase osteoprotegerin mRNA in primary human osteoblasts (Viereck et al., 2002). Pamidronate and clodronate reportedly decrease receptor activator of nuclear factor κB ligand (RANKL) in UMR-106-01 osteosarcoma cells (Mackie et al., 2001). In addition, it has recently been reported that zoledronate upregulates osteocalcin and bone morphogenetic protein-2 (BMP-2) gene expression in human osteoblast-like cells (Pan et al., 2004b), and decreases membrane RANKL expression by up-regulating tumor necrosis factor-α converting enzyme (Pan et al., 2004a). Thus, it is no longer doubtful that the effects of bisphosphonates on bone metabolism are exerted through not only osteoclasts but also osteoblasts. We have recently demonstrated that incadronate enhances $PGF_{2\alpha}$-induced VEGF synthesis through the activation of p44/p42 MAP kinase in osteoblasts (Tokuda et al., 2003). However, the detailed mechanism of bisphosphonates in osteoblasts has not yet been fully clarified.

In the present study, we investigated the effects of tiludronate and etidronate, clinically used bisphosphonates, which are structurally different from incadronate, on the $PGF_{2\alpha}$-stimulated VEGF synthesis in MC3T3-E1 cells. Our results strongly suggest that tiludronate, but not etidronate, inhibits the $PGF_{2\alpha}$-stimulated VEGF synthesis in osteoblasts, and that the effect of tiludronate is exerted at the point between PKC and Raf-1.

2. Materials and methods

2.1. Materials

$PGF_{2\alpha}$ and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma Chemical Co. (St. Louis, MO). Tiludronate and etidronate were kindly provided by Meiji Seika Co. Ltd. (Kawasaki, Japan) and Sumitomo Pharmaceuticals Co. Ltd. (Osaka, Japan), respectively. Phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific MEK1/2 antibodies, MEK1/2 antibodies, phospho-specific Raf-1 antibodies and β-actin antibodies were purchased from New England Biolabs Inc. (Beverly, MA). Mouse VEGF ELISA kit was purchased from R&D Systems Inc. (Minneapolis, MN). ECL Western blotting detection system was purchased from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. $PGF_{2\alpha}$ was dissolved in ethanol. TPA was dissolved in dimethyl sulfoxide. The maximum concentration of ethanol or dimethyl sulfoxide was 0.1%, which did not affect the assay for VEGF or the analysis of Western blot.

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., 1997a,b). Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were

seeded into 35 mm (5×10^4) or 90 mm (2×10^5) diameter dishes in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

2.3. Assay for VEGF

The cells were pretreated with various doses of tiludronate, etidronate or vehicle for 8 h, and then stimulated by $PGF_{2\alpha}$ or TPA in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. The conditioned medium was collected, and VEGF in the medium was measured by VEGF ELISA kit.

2.4. Analysis of p44/p42 MAP kinase, MEK1/2 or Raf-1

The cultured cells were pretreated with various doses of tiludronate, etidronate or vehicle for 8 h, and then stimulated by $PGF_{2\alpha}$ or TPA in 4 ml of 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% SDS, 50 mM dithiothreitol, and 10% glycerol. For the analysis of p44/p42 MAP kinase, MEK1/2 or Raf-1, the cytosolic fraction was collected as a supernatant after centrifugation at $125{,}000 \times g$ for 10 min at 4 °C. SDS-PAGE was performed as described by Laemmli (Laemmli, 1970) in 10% polyacrylamide gel. Western blotting analysis was performed as described previously (Kato et al., 1996) by using phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific MEK1/2 antibodies, MEK1/2 antibodies, phospho-specific Raf-1 antibodies or β-actin antibodies with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. Peroxidase activity on the nitrocellulose sheet was visualized on X-ray film by means of the ECL Western blotting detection system.

2.5. Determination

The absorbance of ELISA 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). Cell viability was assessed by trypan blue dye exclusion test.

2.6. Statistical analysis

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

3. Results

3.1. Effects of tiludronate or etidronate on the $PGF_{2\alpha}$-induced VEGF synthesis in MC3T3-E1cells

We have recently reported that $PGF_{2\alpha}$ induces the synthesis of VEGF in osteoblast-like MC3T3-E1 cells, and that incadronate amplifies the VEGF synthesis (Tokuda et al., 2003). Thus, we investigated the effects of tiludronate or etidronate on the $PGF_{2\alpha}$-induced VEGF synthesis in these cells. Tiludronate, which by itself did not affect the levels of VEGF, significantly suppressed the VEGF synthesis induced by $PGF_{2\alpha}$ (Table 1). The effect of tiludronate was significant at 50 μM, which caused about 35% inhibition in the $PGF_{2\alpha}$-effect (Fig. 1). Etidronate, which alone did not affect the VEGF synthesis, had little effect on the $PGF_{2\alpha}$-stimulated VEGF synthesis (Table 1). The viability of the cells incubated at 37 °C for 48 h in the presence of 50 μM tiludronate or 50 μM etidronate was more than 90% compared to that of the control cells.

3.2. Effects of tiludronate or etidronate on the $PGF_{2\alpha}$-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells

We have previously showed that $PGF_{2\alpha}$ activates p44/p42 MAP kinase in a PKC-dependent manner, resulting in the VEGF synthesis, in MC3T3-E1 cells (Pilbeam et al., 1996; Tokuda et al., 1999). We next examined the effect of tiludronate or etidronate on the phosphorylation of p44/p42 MAP kinase induced by $PGF_{2\alpha}$ in these cells. Tiludronate, which alone did not affect the phosphorylation of p44/p42 MAP kinase, significantly suppressed the $PGF_{2\alpha}$-induced p44/p42 MAP kinase phosphorylation (Fig. 2). Etidronate, which by itself had no effect on the phosphorylation of p44/p42 MAP kinase, had a limited affect on the phosphorylation of p44/p42 MAP kinase induced by $PGF_{2\alpha}$ (Fig. 2).

Table 1
Effects of tiludronate or etidronate on the $PGF_{2\alpha}$-induced VEGF synthesis in MC3T3-E1 cells

Bisphosphonate (50 μM)	$PGF_{2\alpha}$ (10 μM)	VEGF (pg/ml)
–	–	25 ± 3
–	+	1824 ± 146
Tiludronate	–	21 ± 2
Tiludronate	+	1222 ± 98*
Etidronate	–	23 ± 2
Etidronate	+	1788 ± 142

The cultured cells were pretreated with 50 μM bisphosphonate or vehicle for 8 h, and then stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 48 h. Each value represents the mean ± S.D. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. The cell viability after the treatments was more than 90% of the control cells.

* $P < 0.05$, compared to the value of $PGF_{2\alpha}$ alone.

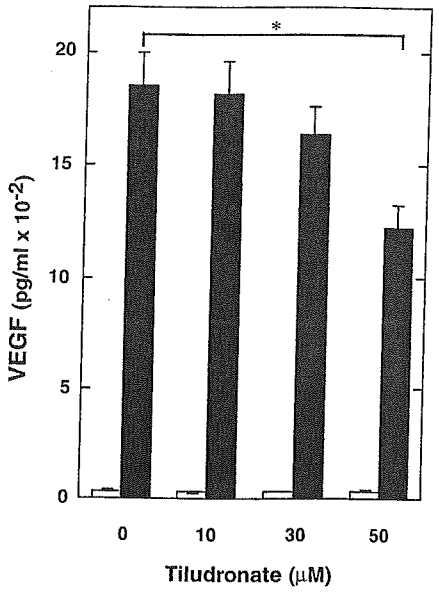

Fig. 1. Effect of tiludronate or etidronate on the $PGF_{2\alpha}$-induced VEGF synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of tiludronate for 8 h, and then stimulated by 10 μM $PGF_{2\alpha}$ (closed bars) or vehicle (open bars) for 48 h. Each value represents the mean ± S.D. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. The cell viability after the treatments was more than 90% of the control cells. $^{*}P < 0.05$, compared to the value of $PGF_{2\alpha}$ alone.

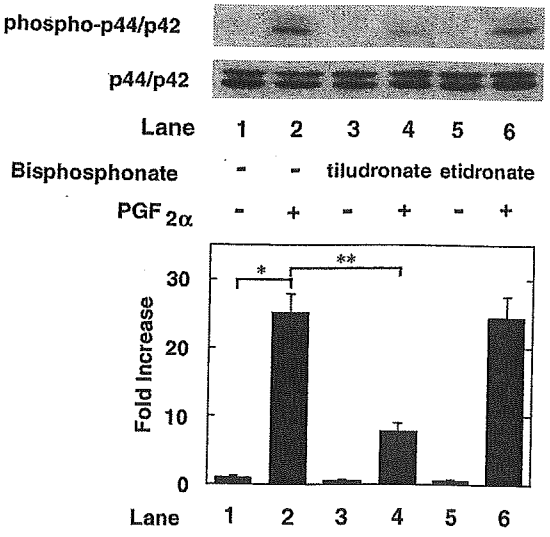

Fig. 2. Effects of tiludronate or etidronate on the $PGF_{2\alpha}$-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 50 μM tiludronate, 50 μM etidronate or vehicle for 8 h, and then stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 30 min. Extracts of cells were subjected to SDS-PAGE with subsequent Western blot analysis using antibodies against phospho-specific p44/p42 MAP kinase or p44/p42 MAP kinase. The histogram shows quantitative representations of p44/p42 MAP kinase phosphorylation obtained from laser densitometric analysis. Each value represents the mean ± S.D. of triplicate determinations from three independent experiments. $^{*}P < 0.05$, compared to the value of control; $^{**}P < 0.05$, compared to the value of $PGF_{2\alpha}$ alone.

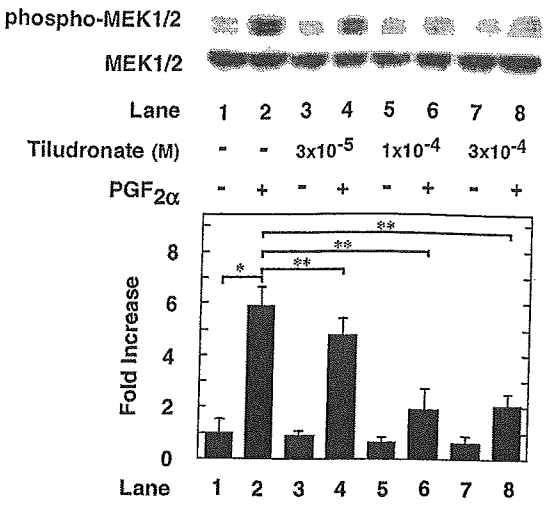

Fig. 3. Effects of tiludronate on the phosphorylation of MEK1/2 induced by $PGF_{2\alpha}$ in MC3T3-E1 cells. The cultured cells were pretreated with indicated doses of tiludronate or vehicle for 8 h, and then stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 30 min. Extracts of cells were subjected to SDS-PAGE with subsequent Western blot analysis using antibodies against phospho-specific MEK1/2 or MEK1/2. The histogram shows quantitative representations of MEK1/2 phosphorylation obtained from laser densitometric analysis. Each value represents the mean ± S.D. of triplicate determinations from three independent experiments. $^{*}P < 0.05$, compared to the value of control; $^{**}P < 0.05$, compared to the value of $PGF_{2\alpha}$ alone.

3.3. *Effects of tiludronate on the phosphorylation of MEK1/2 or Raf-1 induced by $PGF_{2\alpha}$ in MC3T3-E1 cells*

It is well recognized that the activation of p44/p42 MAP kinase is regulated by MEK1/2, activated by the upstream kinase known as Raf-1 (Widmann et al., 1999). We previously demonstrated that $PGF_{2\alpha}$ stimulates the phosphorylation of both MEK1/2 and Raf-1 in MC3T3-E1 cells (Tokuda et al., 2003). To know whether the inhibition by tiludronate of the $PGF_{2\alpha}$-induced phosphorylation of p44/p42 MAP kinase is due to the suppression of the upstream kinase or not, we next examined the effects of tiludronate on the phosphorylation of MEK1/2 or Raf-1 induced by $PGF_{2\alpha}$ in these cells. Tiludronate, which by itself had a limited affect on the phosphorylation of MEK1/2, significantly inhibited the phosphorylation induced by $PGF_{2\alpha}$ in a dose-dependent manner in the range between 30 and 300 μM (Fig. 3). The inhibitory effect of tiludronate reached almost plateau at 100 μM. The phosphorylation of Raf-1 was hardly affected by tiludronate alone. However, the $PGF_{2\alpha}$-induced phosphorylation of Raf-1 was significantly suppressed by tiludronate (Fig. 4).

3.4. *Effect of tiludronate on TPA-induced VEGF synthesis in MC3T3-E1 cells*

To clarify whether the inhibitory effect of tiludronate on the $PGF_{2\alpha}$-stimulated VEGF synthesis is exerted at a point downstream of PKC or not, we examined the effect of tiludronate on the VEGF synthesis elicited by TPA, a direct

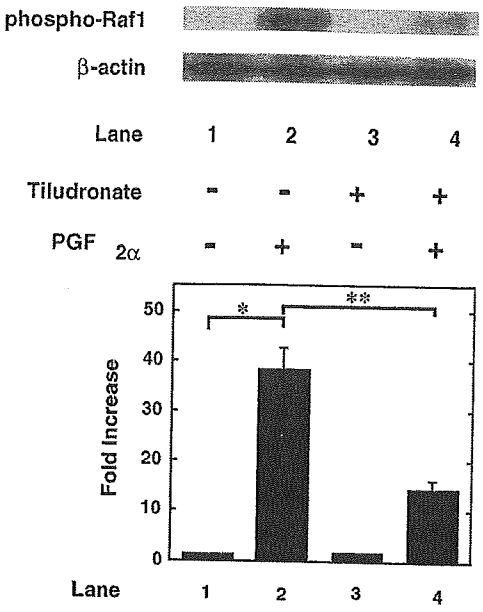

Fig. 4. Effects of tiludronate on the phosphorylation of Raf-1 induced by $PGF_{2\alpha}$ in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM tiludronate or vehicle for 8 h, and then stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 15 min. Extracts of cells were subjected to SDS-PAGE with subsequent Western blot analysis using antibodies against phospho-specific Raf-1 or β-actin. The histogram shows quantitative representations of MEK1/2 phosphorylation obtained from laser densitometric analysis. Each value represents the mean ± S.D. of triplicate determinations from three independent experiments. $^{*}P < 0.05$, compared to the value of control; $^{**}P < 0.05$, compared to the value of $PGF_{2\alpha}$ alone.

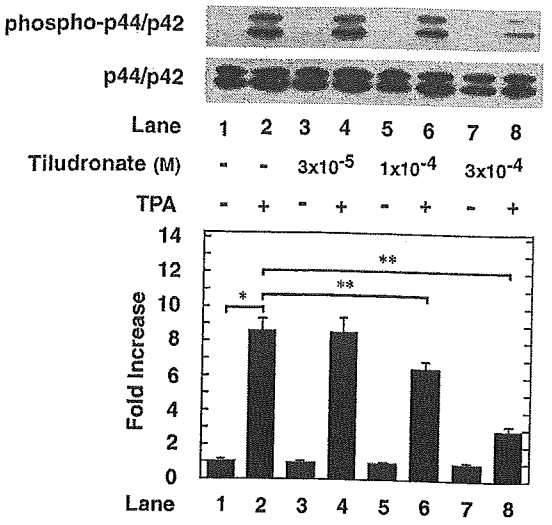

Fig. 5. Effects of tiludronate on the TPA-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with indicated doses of tiludronate or vehicle for 8 h, and then stimulated by 100 nM TPA or vehicle for 60 min. Extracts of cells were subjected to SDS-PAGE with subsequent Western blot analysis using antibodies against phospho-specific p44/p42 MAP kinase or p44/p42 MAP kinase. The histogram shows quantitative representations of p44/p42 MAP kinase phosphorylation obtained from laser densitometric analysis. Each value represents the mean ± S.D. of triplicate determinations from three independent experiments. $^{*}P < 0.05$, compared to the value of control. $^{**}P < 0.05$, compared to the value of TPA alone.

activator of PKC (Nishizuka, 1986), in MC3T3-E1 cells. As previously reported (Tokuda et al., 2003), TPA significantly stimulated the synthesis of VEGF, and the VEGF synthesis induced by TPA was markedly suppressed by tiludronate (Table 2).

3.5. Effects of tiludronate on the phosphorylation of p44/p42 MAP kinase, MEK1/2 or Raf-1 induced by TPA in MC3T3-E1 cells

We previously reported that TPA stimulates the phosphorylation of p44/p42 MAP kinase, MEK1/2 and Raf-1 in MC3T3-E1 cells (Tokuda et al., 2003). Therefore, we further examined the effect of tiludronate on the TPA-induced phosphorylation of these kinases. Tiludronate significantly reduced the TPA-induced phosphorylation of p44/p42 MAP kinase in a dose-dependent manner in the range between 30 and 300 μM (Fig. 5). The maximum inhibitory effect of tiludronate on the phosphorylation of p44/p42 MAP kinase induced by TPA was observed at 300 μM. In addition, tiludronate dose-dependently inhibited the TPA-induced phosphorylation of both MEK1/2 (Fig. 6). Moreover, the Raf-1 phosphorylation by TPA was attenuated by tiludronate (Fig. 7).

Table 2
Effects of tiludronate on the TPA-induced VEGF synthesis in MC3T3-E1 cells

Tiludronate (50 μM)	TPA (100 nM)	VEGF (pg/ml)
−	−	23 ± 3
−	+	563 ± 45
+	−	21 ± 2
+	+	45 ± 4*

The cultured cells were pretreated with 50 μM tiludronate or vehicle for 8 h, and then stimulated by 100 nM TPA or vehicle for 48 h. Each value represents the mean ± S.D. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

* $P < 0.05$, compared to the value of TPA alone.

4. Discussion

In the present study, we showed that tiludronate inhibited the $PGF_{2\alpha}$-induced VEGF synthesis in osteoblast-like MC3T3-E1 cells. To the best of our knowledge, this represents the first report showing the inhibitory effect of bisphosphonate on the VEGF synthesis in osteoblasts. In contrast to the findings presented here, we have recently reported that incadronate enhances the VEGF synthesis induced by $PGF_{2\alpha}$ in these cells (Tokuda et al., 2003). In addition, we showed here that etidronate had little effect on the VEGF synthesis stimulated by $PGF_{2\alpha}$ in MC3T3-E1 cells. Thus, it is probable that the inhibitory effect of tiludronate on the VEGF synthesis is the specific effect of this agent and is not the common effect of bisphosphonates. It has recently been reported

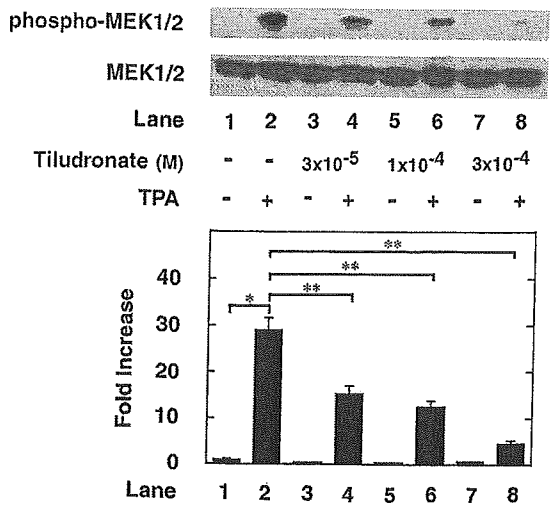

Fig. 6. Effects of tiludronate on the phosphorylation of MEK1/2 induced by TPA in MC3T3-E1 cells. The cultured cells were pretreated with indicated doses of tiludronate or vehicle for 8 h, and then stimulated by 100 nM TPA or vehicle for 60 min. Extracts of cells were subjected to SDS-PAGE with subsequent Western blot analysis using antibodies against phospho-specific MEK1/2 or MEK1/2. The histogram shows quantitative representations of MEK1/2 phosphorylation obtained from laser densitometric analysis. Each value represents the mean ± S.D. of triplicate determinations from three independent experiments. $^{*}P < 0.05$, compared to the value of control; $^{**}P < 0.05$, compared to the value of TPA alone.

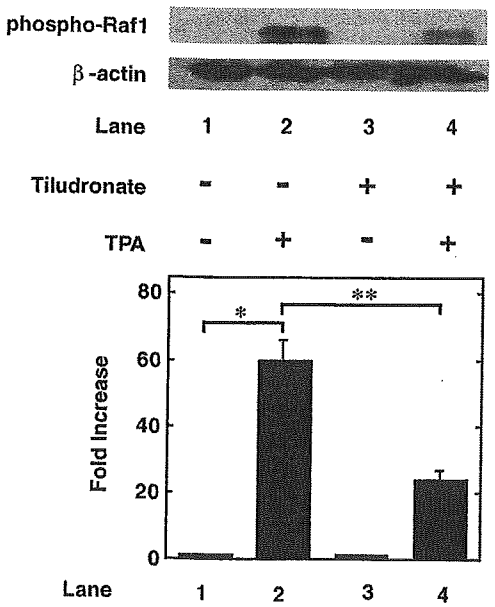

Fig. 7. Effects of tiludronate on the phosphorylation of Raf-1 induced by $PGF_{2\alpha}$ in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM tiludronate or vehicle for 8 h, and then stimulated by 100 nM TPA or vehicle for 60 min. Extracts of cells were subjected to SDS-PAGE with subsequent Western blot analysis using antibodies against phospho-specific Raf-1 or β-actin. The histogram shows quantitative representations of MEK1/2 phosphorylation obtained from laser densitometric analysis. Each value represents the mean ± S.D. of triplicate determinations from three independent experiments. $^{*}P < 0.05$, compared to the value of control; $^{**}P < 0.05$, compared to the value of TPA alone.

that pamidronate and zoledronate clinically induce avascular necrosis of the jaw (Marx, 2003; Carter and Goss, 2003). However, it has also been presented that no other bisphosphonates including tiludronate and etidronate are associated with avascular bone necrosis in the jaws (Marx, 2003). Thus, it is possible that the specific effects of each agent are involved in the clinical features, supporting our present findings about the agent-specific effect of bisphosphonates.

We have previously reported that $PGF_{2\alpha}$ activates both PI-PLC and PC-PLD via heterotrimeric GTP-binding protein in MC3T3-E1 cells (Miwa et al., 1990; Kozawa et al., 1994), recognized as two major physiological PKC-activating pathways (Nishizuka, 1992; Exton, 1999). Indeed, we have also shown that $PGF_{2\alpha}$ activates p44/p42 MAP kinase in a PKC-dependent manner in these cells (Tokuda et al., 1999). In addition, we previously demonstrated that TPA, a direct activator of PKC (Nishizuka, 1986), induces the phosphorylation of p44/p42 MAP kinase in these cells (Tokuda et al., 2003; Nishizuka, 1986). Moreover, we have recently reported that $PGF_{2\alpha}$ induces VEGF synthesis through the PKC-, probably PKCβI-dependent activation of p44/p42 MAP kinase in MC3T3-E1 cells (Tokuda et al., 2003). It is well known that p44/p42 MAP kinase is activated through the phosphorylation of threonine and tyrosine residues by the dual specificity MAP kinase kinase, known as MEK1/2 (Widmann et al., 1999). MEK1/2 is recognized to be activated through its phosphorylation induced by MAP kinase kinase, Raf-1 (Widmann et al., 1999). Thus, we further investigated the exact mechanism of tiludronate in the $PGF_{2\alpha}$ signaling in osteoblast-like MC3T3-E1 cells. Herein, we demonstrated that tiludronate inhibited the phosphorylation of p44/p42 MAP kinase induced by $PGF_{2\alpha}$. In addition, tiludronate suppressed the phosphorylation of MEK1/2 as well as that of Raf-1 induced by $PGF_{2\alpha}$, suggesting that the effect is exerted at a point upstream of Raf-1. On the other hand, we showed here that tiludronate inhibited the synthesis of VEGF induced by TPA. These findings suggest that the inhibitory effect of tiludronate on the VEGF synthesis is exerted at a point downstream of PKC. Furthermore, the phosphorylations of p44/p42 MAP kinase, MEK1/2, or Raf-1 promoted by TPA were thoroughly inhibited by tiludronate. Therefore, it is probable that the effect of tiludronate is exerted at the point between PKC and Raf-1. Taking these findings into account as a whole, it is most likely that tiludronate inhibits $PGF_{2\alpha}$-induced VEGF synthesis in osteoblasts, and the suppressive effect on the VEGF synthesis is exerted at the point between PKC and Raf-1. The potential mechanism of tiludronate in $PGF_{2\alpha}$-induced VEGF synthesis in osteoblasts shown here is summarized in Fig. 8.

The expansion of the capillary network providing microvasculature is thought to be an essential process for bone remodeling (Erlebacher et al., 1995). As VEGF is a specific mitogen of vascular endothelial cells (Ferrara and Davis-Smyth, 1997), the synthesis by osteoblasts is recognized to be an important intercellular mediator between the osteoblasts and the vascular endothelial cells. In fact, VEGF is reportedly involved in trabecular bone formation and expansion of the

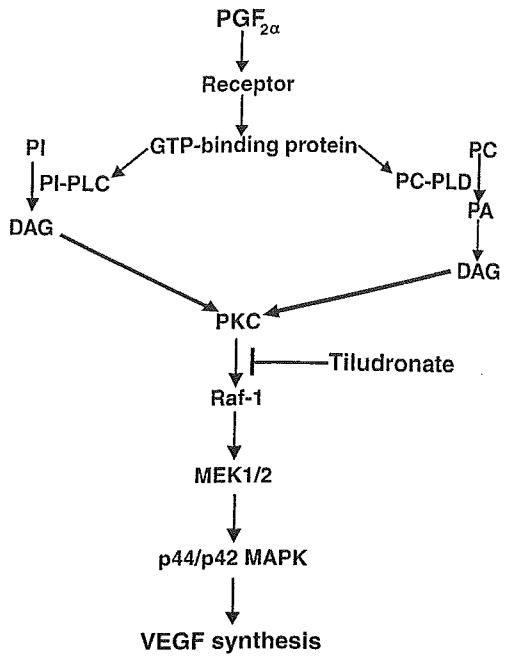

Fig. 8. Diagram of the regulatory mechanism of tiludronate on the $PGF_{2\alpha}$-induced VEGF synthesis in MC3T3-E1 cells (⊣, negative modulation; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; VEGF, vascular endothelial growth factor).

hypertrophic chondrocyte zone in epiphyseal growth plate of mouse (Gerber et al., 1999). In addition, it has recently been reported that Flk-1, a VEGF receptor, is expressed in osteoblasts (Karsenty and Wagner, 2002) and plays a role in bone formation synergistically with BMP-4 (Peng et al., 2002). Therefore, it is probable that VEGF synthesis by osteoblasts plays a role in the regulation of bone remodeling in an autocrine/paracrine fashion. We previously reported that incadronate, in opposition to tiludronate, amplifies $PGF_{2\alpha}$-induced VEGF synthesis in MC3T3-E1 cells and that the effect of incadronate is exerted at the point between PKC and Raf-1 (Tokuda et al., 2003). Interestingly, the opposite effects of two bisphosphonates on the $PGF_{2\alpha}$-stimulated VEGF synthesis in osteoblasts, the attenuation by tiludronate and the amplification by incadronate, are exerted at the similar point. As for the R2 side chain of these agents, tiludronate possesses (4-chlorophenyl)thiol-methylene structure (Fleisch et al., 2002). On the other hand, etidronate and incadronate possess cycloheptylamino-methylene and 1-hydroxyethylidene structures in their R2 side chains, respectively (Fleisch et al., 2002). Although the precise mechanism of the significant difference of the effects on $PGF_{2\alpha}$-induced VEGF synthesis remains unclear, it is probably due to the marked differences among R2 side chain of them. In addition, it is possible that the different effects of these bisphosphonates on the VEGF synthesis relate to the relative potency on the anti-bone resorptive activities of them. In metabolic bone diseases, it is certain that the rate of bone remodeling alters in each case. Our findings might allow us to speculate that we could select the class of bisphosphonate as a clinical tool for the metabolic bone diseases according to the specific effect of the agent on the bone forming cells. When compared with our previous finding of the VEGF-enhancing activity of incadronate (Tokuda et al., 2003), a new generation bisphosphonate, the significance of the inhibitory effect of tiludronate or the no effect of etidronate on the VEGF synthesis induced by $PGF_{2\alpha}$ shown here might be somewhat diminished. In addition, the clinical utility of the old generation bisphosphonates such as tiludronate and etidronate are limited by the greater toxicity. However, it is important to clarify the unique agent-specific effect(s) among bisphosphonates for the adequate therapeutics by these drugs. Therefore, our present findings could provide insight into the subtle differences between related bisphosphonates. Further investigation would be required to clarify the exact mechanism of bisphosphonates in bone cells.

In conclusion, our results strongly suggest that tiludronate inhibits the VEGF synthesis induced by $PGF_{2\alpha}$ in osteoblasts, and the inhibitory effect is exerted at the point between PKC and Raf-1.

Acknowledgments

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