骨粗鬆症　治療　生活療法

運動指導・転倒防止のリハビリテーション

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Exercise for fall prevention and osteoporosis treatment

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Abstract

The recent meta-analyses of many randomized controlled trials that examined the effectiveness of exercise on the fall frequency and the bone mineral density were reviewed. All of the various exercise programs showed the significant reduction of the number of falls. In addition, the bone mineral density also increased with aerobics, resistance training, and walking in the lumbar spine and increased with walking in the femoral neck. Although exercise programs have not yet showed evidence for fracture prevention, it seemed to reduce the risk of fall and osteoporosis.

Key words: exercise, fall prevention, osteoporosis, bone mineral density, fracture prevention

はじめに

高齢者は誰もが死を迎える前に自立が低下して介護を要する一定の時期(先死期)を過ごす．この先死期をいかに短くするかは，大多数の高齢者，その家族，そして社会全体の願いであると考えられる．この時期に自立が低下する原因となる老年病の三大主徴は，‘動けない’，‘わからない’，‘失禁’である．これらが単独で，あるいは複合して高齢期の自立損失をもたらしている．したがって，先死期を少しでも短縮するためには，各徴候の病因のうち可変性の要因については，できるだけ減らし，かつ改善して，現れる徴候の量を少しでも減らし，程度を少しでも軽くすることが肝要である．なかでも，三大主徴を引き起こしている多数の病因のうちで，メカニズムが明らかになっていて実際にリスクを除去，あるいは軽減する医学的方法が可能な部分には，積極的に介入をすべきであると思われる．

さて，‘動けない’状態を高齢者にもたらす原因の主なものには，脳血管疾患やParkinson病などの中枢神経疾患と骨折・転倒，関節疾患や脊柱管狭窄症などの運動器疾患がある．平成13年度の国民生活基礎調査によれば，これらの運動器疾患の中で要介護者の原因となった割合が最も高いのは，骨折・転倒である[1]．特に下肢の骨折，とりわけ大腿骨頸部骨折は，その頻度の高さ，重篤さ，機能回復の不良さの点から高齢者の運動機能にとって最大の脅威となっている．大腿骨頸部骨折の直後に完全な歩行不能まで著しく低下する運動機能は，治療によっ

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て経時的に改善するが，受傷前と同じレベルまで最終的に回復する率は高くなく，受傷前に歩行可能であった大腿骨頸部骨折患者の半数が最終的に自立した歩行能力を再獲得できず，受傷前に自立生活可能であった患者のやはり半数が，最終的に長期の介護・支援を受けるようになり，介護施設入所のリスクが1/3以上で高まるとされる[2]．このように高齢者が'動けない'状態になる確率が非常に高い大腿骨頸部骨折は，大腿骨頸部骨密度の1SD低下によりそのリスクが2.6倍になるとされ[3]，欧米でも我が国でも転倒が発生原因の80-90%を占めることはよく知られており，これらの事実が示すように，防ぐべき高齢期の骨折の主要因は，骨強度の低下と転倒による外力の2つであることがわかる．

本稿の主題である運動を主体としたリハビリテーションの目的も，この2種類のリスクを改善して骨折予防に結びつけることにある．そこで以下に，転倒リスク，骨粗鬆症リスク，骨折リスクに分けて，これらに対する運動療法について概説する．

1. 転倒リスクに対する運動療法

転倒リスクは外的リスクと内的リスクに分けられる．外的リスクは，つまずきやすい段差や滑りやすい床や暗い照明などの生活環境における物理的障害であり，運動が介入する余地は少ない．一方，低い運動機能，転倒歴，転倒関連の疾病・薬物などが含まれる内的リスクのうちで，低下した運動機能は，運動療法による改善が見込める格好の要因である．転倒に直接関連する運動機能には，最大筋力，持続筋力，反応時間，姿勢反射，平衡機能，運動速度などがあるが，低下したこれらの運動機能を鍛えることで向上できれば，それに応じて転倒が減るはずと容易に想像できるが，実際にはどうであろうか．

これまでに，運動療法による転倒予防のための臨床試験は，単独因子に対する介入から多因子に対する包括的介入プログラムまで多様な運動介入試験が多数行われてきているが，その研究レベルには差違が大きい．ここでは，そのうちで無作為対照比較試験(RCT)に関するメタアナリシス[4-9]を紹介する(図1)．

米国における老人ホームでの2試験と地域在宅高齢者での5試験に行われたメタアナリシスでは，短期間の運動療法が平均年齢73-88歳の高齢者(2,328例)において転倒を減少させるかを検討した結果，運動療法は転倒率を10%減少させた．運動療法の内容は多様であったが，バランス訓練は17%減少と特に有効で，高齢者に対する運動療法は転倒リスクを減少すると結論している[4]．更に，11試験を検討したGardnerらのメタアナリシスでは，運動療法は選択条件に合致した中等度の自立障害のある高齢者においても効果的かつ安全に転倒リスクを低下させると結論づけている．すなわち，筋力強化，バランス訓練，持久力訓練，太極拳などが，60歳以上の地域在宅住民(4,933例)に大きな有害事象なく，転倒率を減少させたとしている[5]．また，ニュージーランドの質的均一性の高い4試験を検討したRobertsonらは，平均82歳の地域在宅住民(1,016例)に，転倒予防のために家庭で個別に実施された筋力強化とバランス訓練のプログラムが，転倒数を35%減らし，オッズ比0.67とリスク抑制に成功している．転倒による外傷数も35%減らし，オッズ比が0.56と報告している．加えて，80歳以上とそれ未満で比べると，運動プログラムによる転倒数減少には差はないものの，傷害性転倒数は80歳以上群の方が少なかった．更に，1年以内の転倒歴の有無による解析を加えると，家庭での個別運動プログラムは，転倒歴のある80歳以上高齢者において転倒や傷害性転倒のリスクを最もよく抑制できていた．これは，自立運動機能を喪失しかけているような対象では，筋力とバランスのわずかな改善でさえも，大きな改善に結びつくことになることを示唆している[6]．更に，Cochrane Libraryのメタアナリシスは，在宅，施設，入院など様々な住居環境の高齢者に対する転倒予防を目的とした運動指導プログラムの効果を解析し，転倒減少率は，筋力強化とバランス改善の家庭個別指導プログラム(566例)で20%，太極拳(200例)で49%，家庭環境

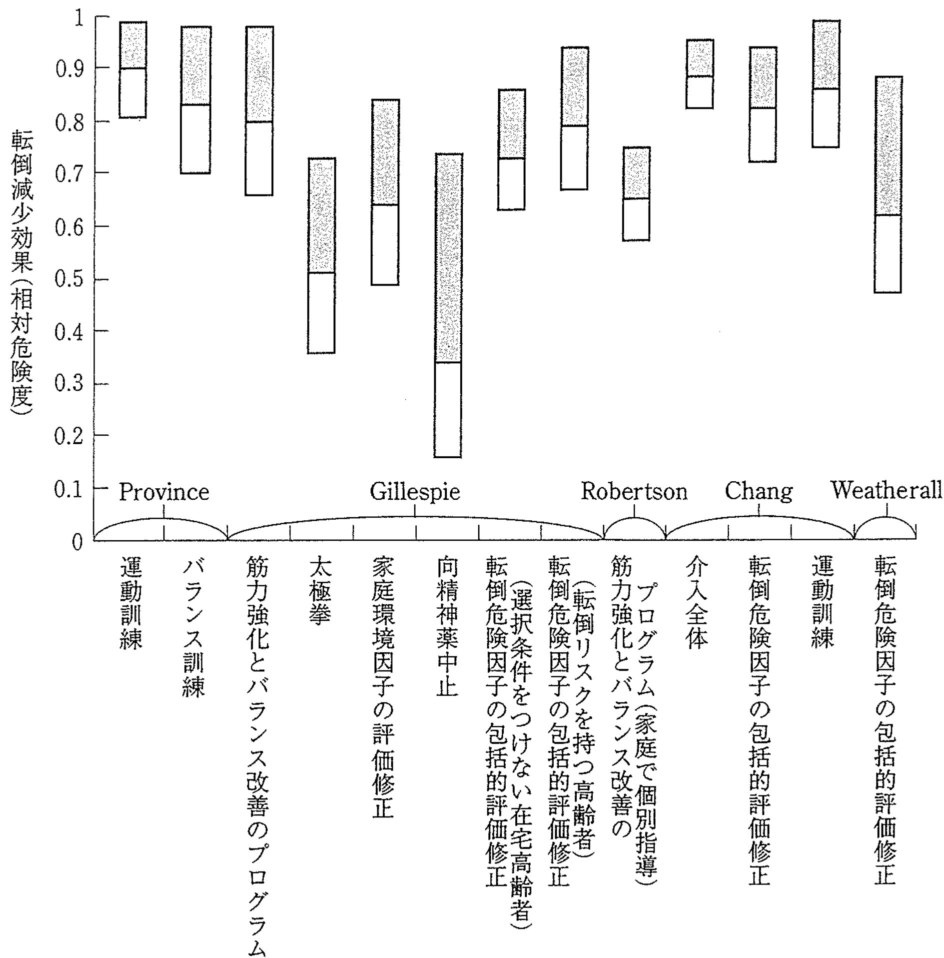

図1 運動療法の転倒に対する効果(文献[4-9]より引用)
白色部と黒色部は95%信頼区間の下位と上位の範囲を示す.多種多様な運動プログラムはそれぞれ有効に転倒を抑制した.

の評価改善(530例)で36%,向精神薬中止(93例)で66%,包括的プログラムで,転倒リスク有無不明の在宅者(1,973例)において27%,転倒リスクを有する者(713例)において21%であった.各介入はそれぞれ転倒予防に有効で,実践できるものであると結論されている[7].

ほかに最近発表されたメタアナリシスでは,転倒予防に最も効果的な介入法は,包括的プログラムで,運動だけでも有効であるとしている[8,9].

2. 骨粗鬆症リスクに対する運動療法

運動療法は,骨粗鬆症に対する様々な介入法の効果における主要な間接的尺度となっている骨密度にどう影響するであろうか.Cochrane Libraryによるメタアナリシスで,選択された18のRCTから閉経後女性における骨粗鬆症予防・治療に関する運動療法の効果が検討されている(**図2**)[10].そこでは,骨密度への効果は変化率(%)の差で評価されており,エアロビクス(856例)では,骨密度測定部位は腰椎7試験,大腿骨頸部5試験,前腕骨2試験で,変化率差は腰椎で0.83,前腕で1.12と有意にエアロビクスで増加したが,大腿骨頸部では−0.7と無効であった.コンプライアンスは39−83%までであった.抵抗運動は個別筋の訓練(158例)で,骨密度への効果をみるためにはより強い抵抗運動が行われた研究群の検討がなされ,骨密度測定部位は腰椎3試験,大腿骨頸部3試験,前腕骨が1試験で,変化率差は腰椎では2.50と有意な増加を得たが,前腕では−0.28,大腿骨頸部では0.41と有意な増加はなかった.コンプ

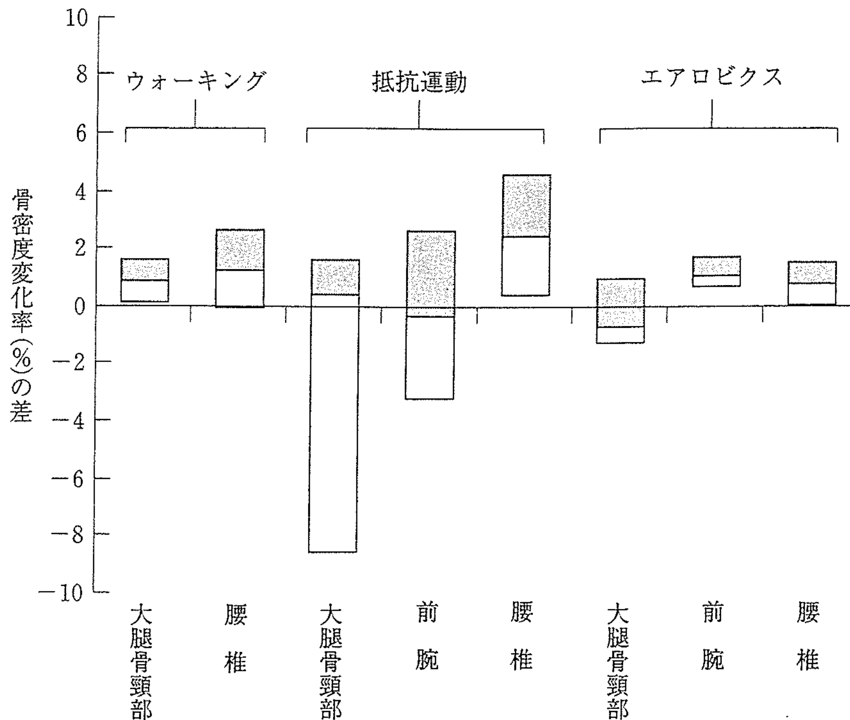

図2 運動療法の骨密度に対する効果(文献[10]より引用)
白色部と黒色部は95%信頼区間の下位と上位の範囲を示す．骨密度は，腰椎では3種の運動すべてで増加し，大腿骨頸部ではウォーキングで増加した．

ライアンスは高く，79-100%であった．更に，ウォーキング(156例)は通常歩行より若干速い程度のものからトレッドミルによるエアロビクスに近いものまで負荷の幅が広いが，骨密度の変化率差は，腰椎で1.31，大腿骨頸部では0.92と有意な増強効果がみられた．

結論として，エアロビクス，抵抗運動はいずれも閉経女性の腰椎骨密度の増強効果を有することが示され，加えて，ウォーキングは大腿骨頸部でも有効であるとされた．このように，運動療法と骨密度部位の一部の組み合わせ以外では，運動療法が骨密度を有意に増加させる効果を有することは明らかで，特に特殊な器具が必要なく，コンプライアンスも一般的に良好と考えられているウォーキングが推奨されている．ただし，長期効果については，この検討では，追跡期間が短いことや扱った試験の方法論的質が決して高くはなく，特に割付秘匿と盲検化において低いことなどから不明とされている．

3. 骨折リスクに対する運動療法

これまで見てきたように，運動療法には骨密度の増強効果と転倒の減少効果もある．ここから期待される点は，やはり転倒による重度外傷，特に骨折の予防効果であるが，これまでのところその点に関してのエビデンスは不十分である．Provinceらによるメタアナリシスでも，傷害性転倒に対しては有意な効果はなかった．これは，頻度がより低い傷害性転倒を評価するには統計学的パワーが低いためとされている[4]．また，Gardnerらによるメタアナリシス[5]や，運動療法による80歳以上での傷害性転倒の減少効果がいっそう優れることを指摘したRobertsonらの解析[6]でも，大腿骨頸部骨折はもちろん，骨折を有意に抑制したという明確な記載はない．結局，骨強度リスクと転倒リスクという骨折リスクの両輪を改善する点までは確認されているが，骨折抑制の明確な証拠は得られていないのが現状である．

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骨粗鬆症，骨折における性差医療

Gender Differences in Osteoporosis and Osteoporotic Fracture

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Key Words　骨粗鬆症（osteoporotis），骨折（fracture），性差（gender differences），高齢者（elder），閉経（menopause）

はじめに

高齢期になると，医療のあり方は多様化して明らかに成人期におけるそれとは異なったものとなり，医療の目的は，生存そのものより生存中の生活の質，自立度などに向けられるようになる。その観点から骨粗鬆症は大変重要な意義を有する疾患である。その理由は，骨が骨粗鬆症に陥ると脆弱性が増して，骨が果たすべき運動器の支柱として機能に破綻を来たす骨折が発生するからである。その結果，洗顔，食事，排便，排尿，トイレ動作，移乗，移動，更衣，入浴などの日常生活の基本的動作が障害されて，生活の質と自立が損なわれてしまう。

この骨粗鬆症と骨粗鬆症性骨折には大きな性差が存在し，女性における頻度が大変高いことはよく知られている。とりもなおさず，男性より長い女性の生前の要介護期間（先死期）の状況に大きな影響を及ぼしていると言える。厚生労働省の国民生活基礎調査の結果が示す性差がそのことを端的に示唆している（図1）。

この要介護の原因疾患における性差をみると，男性においては脳血管疾患が最重要であるのに対して，女性においては脳血管疾患だけでなく，認知症，関節疾患そして転倒・骨折の予防が同じような割合で必要であることが示唆されている。特に後期高齢期ではその傾向はより顕著となり，その年代の女性では年齢とともに脳血管疾患と関節疾患の割合は低下するが，転倒・骨折の割合はむしろ上昇傾向を示す（図2）。

このように，女性が人生の最後に迎える要介護期間において介護の量をできるだけ少なく，質をできるだけよくするためには，骨粗鬆症と骨折に対する理解を深めることが重要であると考えられ，本稿ではその解説を行う。

1．骨粗鬆症における性差

骨粗鬆症は，「骨量の減少と微細構造の劣化によって骨強度が低下し，骨折の危険性が高まった全身性疾患」と定義されている[1]。骨強度を低下させる一方の要因である骨量減少は骨密度減少で，臨床で使用できる検査指標である。他方の微細構造の劣化は，最近の解釈で骨そのものに起因する骨折リスクのうち，骨密度で説明できない部分を表す言葉として使われる骨質に該当するものであるが，臨床では使用できない。したがって，骨粗鬆症の診断に当たっては，骨折リスクをよりよく抽出するための診断基準として骨密度のみを用いた世界保健機構（WHO）の診断基準がグローバルスタンダードとなっている。しかし，骨密度だけに頼るため，骨折予測能に

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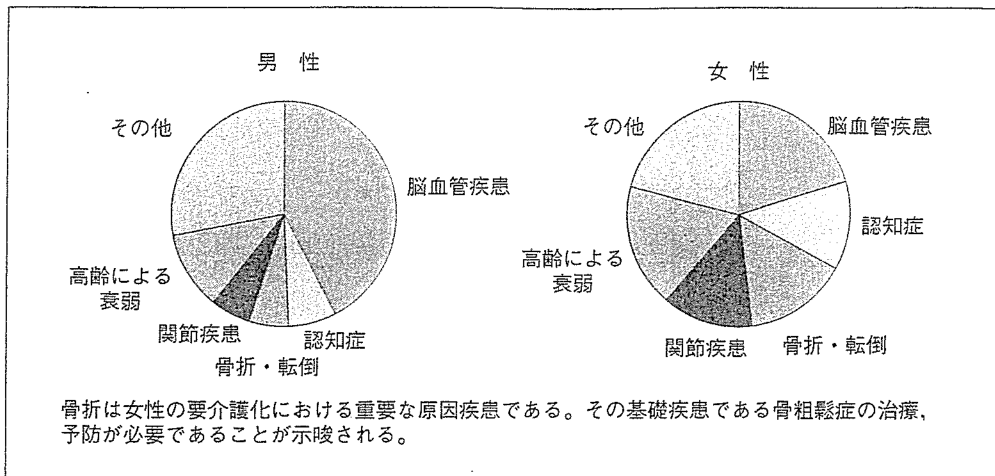

図1　要介護の原因疾患における性差（平成13年度国民生活基礎調査による）

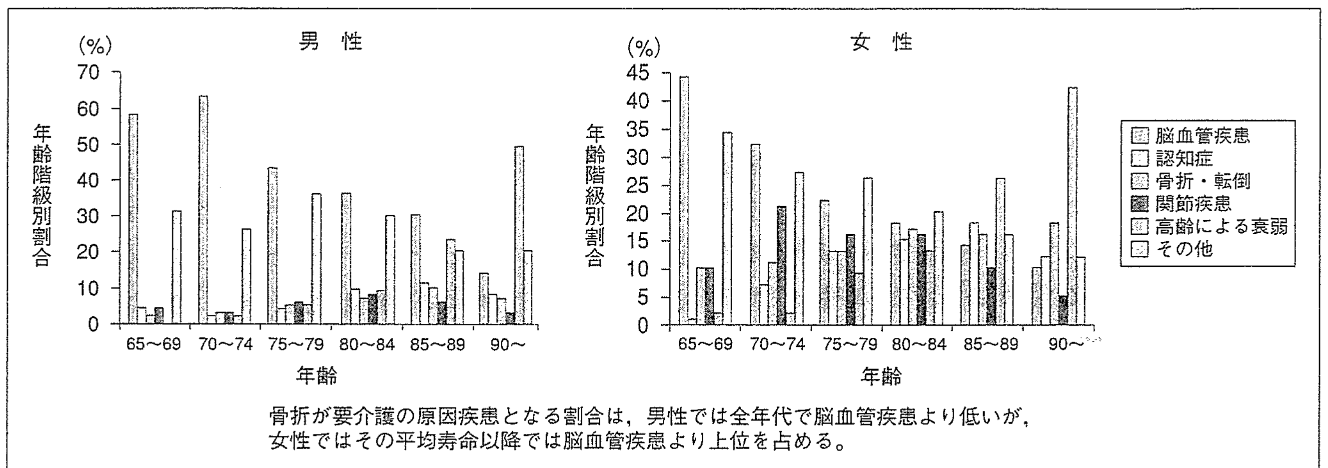

図2　要介護の原因疾患における年代別性差（平成13年度国民生活基礎調査による）

限界があり，わが国では，骨折既往歴の骨折予測能を採用して，脆弱性骨折と骨密度で判定する診断基準となっている。診断基準がダブルスタンダードとなっているため，骨粗鬆症の疫学データもどの診断基準を使うかで差が生じる状況であるが，WHOの診断基準による調査でみると，骨粗鬆症の有病率における性差は歴然としている。広島での藤原らによる調査では，50歳以上の男女を大腿骨頸部骨密度による診断で比較すると，女性では13%，男性では4％と女性の有病率は男性の3.4倍にも及んでいた[2]。この性差は欧米でも同様で，カナダで1.6倍，英国で3.9倍女性の骨粗鬆症有病率が高いと報告されている（表2）[3,4]。

当然，患者数そのものも性差が明らかで，わが国の2004年における年齢・性別人口構成で推計される50歳以上の女性と男性の人口と大腿骨頸部骨密度による有病率から見積もられる骨粗鬆症患者数は，50歳以上女性が367万人と，50歳以上男性が96万人と推計される[5]。

このような骨粗鬆症における性差は，エストロゲン欠乏による骨量減少の惹起という女性の側に特有な要因が存在することが大きく影響していると考えられる。すなわち，閉経前後から卵巣機能が低下してエストロゲンの不足が始まるのを契機に急速な骨代謝回転の亢進が，骨吸収が骨形成を上回る負のバランスのアンカップリングという形で起こるために急速な骨量減少を来たす。エス

表1　骨粗鬆症の有病率の性差（WHO診断基準による大腿骨頸部骨密度での診断）〔文献2～4より〕

年齢・性	国	有病率（%）	報告者	報告年
50歳以上女性	日本	12.8	藤原佐枝子ほか	1997
	英国	22.5	Kanis JA, et al	1994
	カナダ	7.9	Tenenhouse A, et al	2000
50歳以上男性	日本	3.8	藤原佐枝子ほか	1997
	英国	5.8	Kanis JA, et al	1994
	カナダ	4.8	Tenenhouse A, et al	2000

トロゲンには強い骨吸収抑制作用があるので，閉経による欠乏は骨吸収を著しく高め，それに応じて二次的に骨形成を亢進するものの骨量維持には足らない。閉経後の急速な骨量減少は5年ほど継続し，その後，減少カーブはいったん緩やかとなり，閉経の影響も消失していくが，前期高齢期（65～74歳）から再び加齢とともに減っていく。閉経によって中年期に早くも始まる骨量減少が，それ以降の骨折リスクにおける性差を決定づけるものである。

2．骨粗鬆症性骨折における性差

骨粗鬆症性骨折は，日本骨代謝学会の診断基準によれば，脆弱性骨折と呼ばれ，骨量低下がある状態で，軽微な外力にて発生した骨折と定義されている。軽微な外力とは立位からの転倒による程度のものを指す。この骨折は，全身のどの部位に起こりうるが，脊椎，大腿骨頸部，橈骨遠位端，上腕骨頸部，肋骨，骨盤などがその代表的部位である。ここでは，最も重篤な大腿骨頸部骨折と最も高頻度な脊椎骨折の性差について述べる。

1）大腿骨頸部骨折

この骨折は，歩行能力を奪うため，入院して90%以上が手術を受けており，しかも，その後の機能回復は必ずしも良好ではなく，大腿骨頸部骨折による寝たきりの発症は42%となるとされている[6]。このように，最も重篤な本骨折には著しい性差がみられる。この骨折は，骨粗鬆症による骨強度低下に陥った大腿骨に股関節の強い打撲が加わって起こっており，日本でも転倒が原因の71%を占めている。前述したように，女性は骨粗鬆症による骨折リスクが男性より高いのに加えて，転倒頻度も高く，転倒頻度が有意に上昇する75歳以上の後期高齢期で本骨折リスクが高まる[7]。

2002年の1年間における日本の大腿骨頸部骨折新規発生数は，女性が9万2,600例，男性が2万5,300例と推計され，女性が男性の約3.7倍であった[8]。発生率は，40歳代までは男性の方が高い傾向にあったが，これは女性にまだ閉経後骨粗鬆症の影響がなく，労働やスポーツなどでの外傷機会が男性に多いのが理由と考えられる。一方，60歳以降では女性が男性の2倍以上高率であった。閉経後すぐに女性の大腿骨頸部骨折発生率の性差がみられないのは，本骨折の直接の引き金となる転倒リスクが高くなるのが前期高齢期以降であるためと思われる。まだ発生率の性差は，15年間で男性は1.9倍の増加であったのに対し，女性では2.3倍も増加し，その格差は拡大した。年齢別発生率を前の2回の全国調査と比べると，男性では90歳以上で，女性では80歳以上でさらに発生率が高くなっていた。年齢別発生頻度（年間1万人当たり）は，男性では40～49歳が0.84，50～59歳が1.82，60～69歳が5.26，70～79歳が17.49，80～89歳が58.61，90歳以上が141.39であり，女性では40～49歳が0.58，50～59歳が2.41，60～69歳が9.11，70～79歳が41.07，80～89歳が156.10，90歳以上が315.52と70歳代から90歳代にかけて指数関数的増加を呈している（図3）[8]。

このように，女性は，大腿骨頸部骨折発生が著しく男性より高いものの，骨折後の死亡率は男性より低い。元来，大腿骨頸部骨折は生命予後に影響する唯一の骨折であり，女性は骨折後1年間に骨折のない同年代者より10～20%多く死亡するが，この死亡率悪化は男性におい

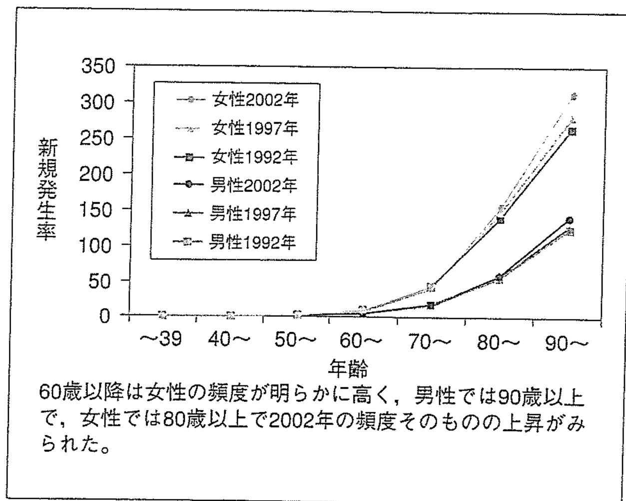

図3　日本の大腿骨頸部骨折新規発生率にみられる性差〔文献6より〕

てはさらに大きい[9]。

2）脊椎骨折

脊椎骨折は，大腿骨頸部骨折と比較して生命予後や機能予後への影響は少ないが，疼痛や脊柱後彎，身長低下などにより生活の質を低下させる。また，発生に転倒の関与する割合は少なく，痛みもなく脊椎X線写真で初めて見つかる無症候性の骨折が2/3を占める。転倒より骨強度低下そのものの関連が強いので，閉経後の骨粗鬆症による骨量減少にその発生率が反映しており，性差が生じている。

骨粗鬆症性骨折のなかで最も頻度が高いにも関わらず，上記のような理由で骨折発生の把握が困難であるため，日本の脊椎骨折の有病率などを推定できる調査は限られているが，その一つに広島の調査がある[10]。これは2,356人の日本人高齢者の平均4年のコホート縦断追跡で，男性では3.5%，女性では9.5%に新規脊椎骨折発生が確認され，男性と女性の年代別新規脊椎骨折発生率（1,000人年）は，Fujiwaraによれば，男性では50～59歳が2.71，60～69歳が7.51，70～79歳が40.15，80歳以上が84.28で，女性では50～59歳が6.6，60～69歳が13.77，70～79歳が40.15，80歳以上が84.28であった。このように，閉経後より女性の発生率が高くなり，60歳以上で年代とともにその差がさらに大きく開いている。

まとめ

骨粗鬆症と骨粗鬆症性骨折は明らかに女性に著しく多いという性差があり，それによる高齢者の要介護化にも大きな負の影を及ぼしている。女性は男性より平均余命が長いことも考えれば，このような差違によって自立を失う高齢女性数は今後大きく増加し続けると予想される。骨粗鬆症への関心は日々高まっているものの，その早期発見と治療は必ずしも十分になされていないのが現状であり，骨粗鬆症と骨折への対策に一層の啓発と実践が必要と考えられる。

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Phosphatidylinositol 3-Kinase/Akt Plays a Role in Sphingosine 1-Phosphate-Stimulated HSP27 Induction in Osteoblasts

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Abstract We previously reported that p38 mitogen-activated protein (MAP) kinase plays a part in sphingosine 1-phosphate-stimulated heat shock protein 27 (HSP27) induction in osteoblast-like MC3T3-E1 cells. In the present study, we investigated whether phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) is involved in the induction of HSP27 in these cells. Sphingosine 1-phosphate time dependently induced the phosphorylation of Akt. Akt inhibitor, 1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate, reduced the HSP27 induction stimulated by sphingosine 1-phosphate. The sphingosine 1-phosphate-induced phosphorylation of GSK-3β was suppressed by Akt inhibitor. The sphingosine 1-phosphate-induced HSP27 levels were attenuated by LY294002 or wortmannin, PI3K inhibitors. Furthermore, LY294002 or Akt inhibitor did not affect the sphingosine 1-phosphate-induced phosphorylation of p38 MAP kinase and SB203580, a p38 MAP kinase inhibitor, had little effect on the phosphorylation of Akt. These results suggest that PI3K/Akt plays a part in the sphingosine 1-phosphate-stimulated induction of HSP27, maybe independently of p38 MAP kinase, in osteoblasts. J. Cell. Biochem. 98: 1249–1256, 2006. © 2006 Wiley-Liss, Inc.

Key words: sphingosine 1-phosphate; heat shock protein; protein kinase; osteoblast

Sphingosine 1-phosphate is a metabolite of sphingomyelin. It is generally recognized that sphingomyelin is catalyzed by sphingomyelinase, resulting in the formation of ceramide, which is subsequently metabolized to sphingosine and sphingosine 1-phosphate [Spiegel and Merrill, 1996]. Accumulating evidence indicates that sphingosine 1-phosphate plays an important role in essential cellular functions such as proliferation, differentiation, and migration [Spiegel and Merrill, 1996; Spiegel and Milstein, 2003; Sanchez and Hla, 2004]. Bone metabolism is regulated by two functional cells, osteoblasts and osteoclasts, responsible for bone formation and bone resorption, respectively [Nijweide et al., 1986]. As for osteoblasts, it has been reported that sphingosine 1-phosphate prevents apoptosis via phosphatidylinositol 3-kinase (PI3K) in primary calvaria rat osteoblasts and human osteosarcoma SaOS-2 cells [Grey et al., 2002]. In our study [Kozawa et al., 1997a], we have previously reported that sphingosine 1-phosphate stimulates interleukin-6 synthesis in osteoblast-like MC3T3-E1 cells. However, the exact mechanism of sphingosine 1-phosphate in bone metabolism has not yet been precisely clarified.

Heat shock proteins (HSP) are expressed in both prokaryotic and eukaryotic cells in response to the biological stress such as heat stress and chemical stress [Hendrick and Hartl, 1993]. HSPs are classified into high-molecular-weight

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HSPs and low-molecular-weight HSPs based on apparent molecular sizes. Low-molecular-weight HSPs with molecular masses from 10 to 30 kDa, such as HSP27 and αB-crystallin have high homology in amino acid sequences [Inaguma et al., 1993; Benjamin and McMillan, 1998]. Though the functions of the low-molecular-weight HSPs are known less than those of the high-molecular-weight HSPs, it is generally accepted that they may have chaperoning functions like the high-molecular-weight HSPs [Inaguma et al., 1993; Benjamin and McMillan, 1998]. HSP27 becomes rapidly phosphorylated in response to various stresses, as well as to exposure to cyotokines and mitogens [Gaestel et al., 1991; Landry et al., 1992]. Under unstimulated conditions, HSP27 exists as a high-molecular weight aggregated form. It is rapidly dissociated as a result of phosphorylation [Kato et al., 1994; Rogalla et al., 1999]. The phosphorylation-induced dissociation from the aggregated form correlates with the loss of molecular chaperone activity [Kato et al., 1994; Rogalla et al., 1999]. In a previous study [Kozawa et al., 1999], we have shown that sphingosine 1-phosphate stimulates the induction of HSP27 in osteoblast-like MC3T3-E1 cells and that p38 mitogen-activated protein (MAP) kinase is involved in the HSP27 induction.

It is well known 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 insulin-like growth factor-I, platelet-derived growth factor (PDGF), and cytokines [Downward, 1995; Franke et al., 1995; Coffer et al., 1998]. It has been shown that Akt regulates biological functions such as gene expression, survival, and oncogenesis [Coffer et al., 1998]. Accumulating evidence suggests that PI3K functions at an upstream from Akt [Chan et al., 1999; Cantley, 2002]. Akt containing a pleckstrin homology domain is recruited to the plasma membrane by the lipid product of PI3K and activated. As for osteoblasts, it has been reported that IGF-I and PDGF induce translocation of Akt to the nucleus [Borgatti et al., 2000]. In addition, recently, Akt is reportedly activated by cyclic stretch or androgen [Danciu et al., 2003; Kang et al., 2004]. We have recently shown that Akt plays an important role in insulin-like growth factor-I-stimulated alkaline phosphatase activity in MC3T3-E1 cells [Noda et al., 2005]. However, the correlation between HSP27 and PI3K/Akt in osteoblasts has not yet been clarified.

In the present study, we investigated whether PI3K/Akt is involved in sphingosine 1-phosphate-stimulated phosphorylation of HSP27 in osteoblast-like MC3T3-E1 cells. We here show that PI3K/Akt pathway is involved in the sphingosine 1-phosphate-stimulated induction of HSP27, maybe independently of p38 MAP kinase, in these cells.

MATERIALS AND METHODS

Materials

Sphingosine 1-phosphate and β-actin antibodies were purchased from Sigma Chemical Co. (St. Louis, MO). Akt inhibitor (1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate), LY294002, wortmannin, and SB203580 were obtained from Calbiochem-Novabiochem (La Jolla, CA). Phospho-specific Akt antibodies, Akt antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific GSK-3β antibodies, and GSK-3β antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). HSP27 antibodies were obtained from R&D Systems, Inc. (Minneapolis, MN). An ECL Western blotting detection system was obtained from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. Sphingosine 1-phosphate, Akt inhibitor LY294002, wortmannin, and SB203580 were dissolved in dimethyl sulfoxide (DMSO). All inhibitors became soluble in the cell culture media after once dissolved in DMSO. The maximum concentration of DMSO was 0.1%, which did not affect Western blot analysis.

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., 1997b]. 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 90-mm diameter dishes (25×10^4/dish) in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h. When indicated, the cells were pretreated with Akt

inhibitor, wortmannin, LY294002, or SB203580 for 60 min prior to stimulation of sphingosine 1-phosphate.

Western Blot Analysis

Cultured cells were stimulated by sphingosine 1-phosphate in serum-free α-MEM for the indicated periods. Cells were washed twice with phosphate-buffered saline and then lysed, homogenized, sonicated, and immediately boiled in a lysis buffer containing 62.5 mM Tris/Cl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol, and 10% glycerol. The sample was used for the analysis by Western blotting. SDS–polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli [Laemmli, 1970] in 10% polyacrylamide gel. Western blot analysis was performed as described previously [Kato et al., 1996], using phospho-specific Akt antibodies, Akt antibodies, HSP27 antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific GSK-3β antibodies, or GSK-3β antibodies with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. Peroxidase activity on PVDF membranes was visualized on X-ray film by means of the ECL Western blotting detection system and was quantitated using NIH image software. All of Western blot analyses were repeated at least three times in independent experiments.

Statistical Analysis

The data were analyzed by ANOVA followed by Bonferroni method for multiple comparisons between pairs, and a $P<0.05$ was considered significant. All data are presented as the mean ± SD of triplicate determinations.

RESULTS

Time-Dependent Effects of Sphingosine 1-Phosphate on the Phosphorylation of Akt in MC3T3-E1 Cells

Sphingosine 1-phosphate significantly stimulates the phosphorylation of Akt in osteoblast-like MC3T3-E1 cells in a time-dependent manner (Fig. 1). The phosphorylation of Akt was markedly observed at 5 min after the sphingosine 1-phosphate-stimulation. The phosphorylation reached its peak at 15 min after the stimulation and decreased thereafter.

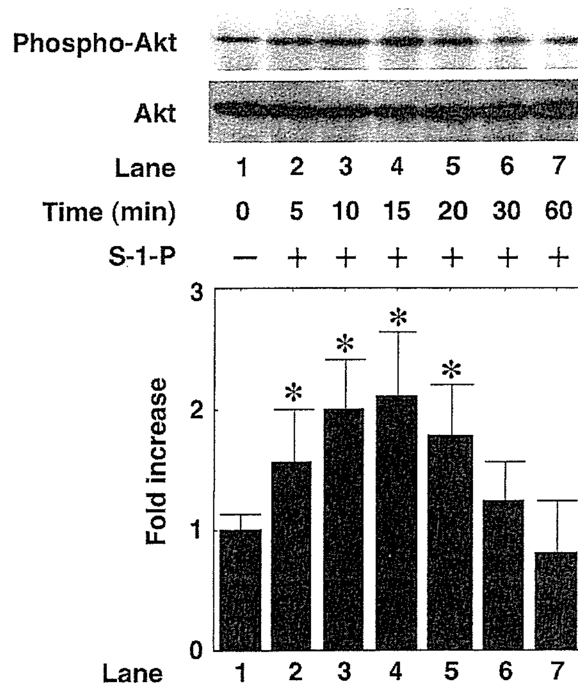
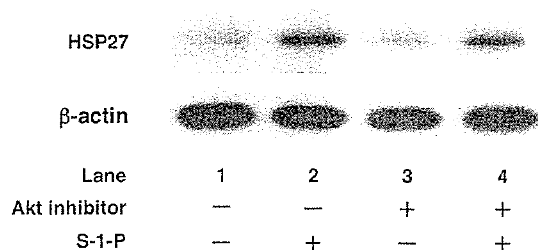


Fig. 1. Effect of sphingosine 1-phosphate (S-1-P) on the phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were stimulated with 30 μM S-1-P for the indicated periods. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. The histogram shows quantitative representations of the levels of S-1-P-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Similar results were obtained with two additional and different cell preparations. Each value represents the mean ± SD of triplicate determinations from triplicate independent cell preparations. *$P<0.05$, compared to the value of control.

Effect of Akt Inhibitor on the Induction of HSP27 in MC3T3-E1 Cells

Then we 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 sphingosine 1-phosphate-stimulated induction of HSP27. Akt inhibitor partially suppressed the sphingosine 1-phosphate-induced up-regulation of HSP27 levels (Fig. 2). Akt inhibitor (50 μM) caused about 40% reduction in the sphingosine 1-phosphate-effect.

We have previously shown that sphingosine 1-phosphate stimulates HSP27 induction at least in part via p38 MAP kinase in osteoblasts [Kozawa et al., 1999]. However, Akt inhibitor did not influence the sphingosine 1-phosphate-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells (data not shown). It is well known that GSK-3β is one of



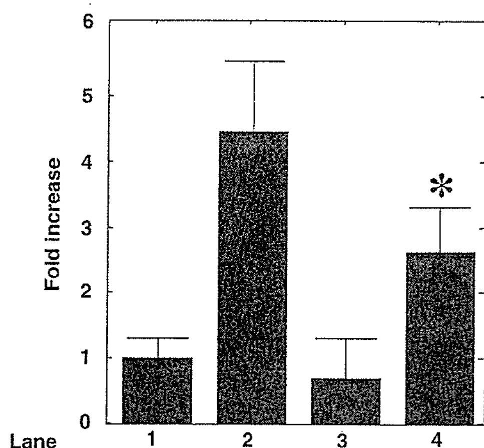
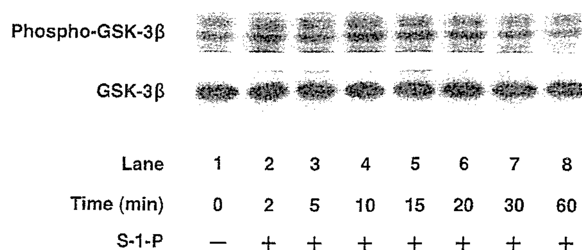


Fig. 2. Effect of Akt inhibitor on the sphingosine 1-phosphate (S-1-P)-induced levels of HSP27 in MC3T3-E1 cells. The cultured cells were pretreated with 50 μM Akt inhibitor for 60 min, and then stimulated by 30 μM of S-1-P or vehicle for 6 h. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against HSP27 or β-actin. The histogram shows quantitative representations of the levels of S-1-P-induced HSP27 after normalization to levels of β-actin. Similar results were obtained with two additional and different cell preparations. Each value represents the mean ± SD of triplicate determinations from triplicate independent cell preparations. $^*P < 0.05$, compared to the value of S-1-P.



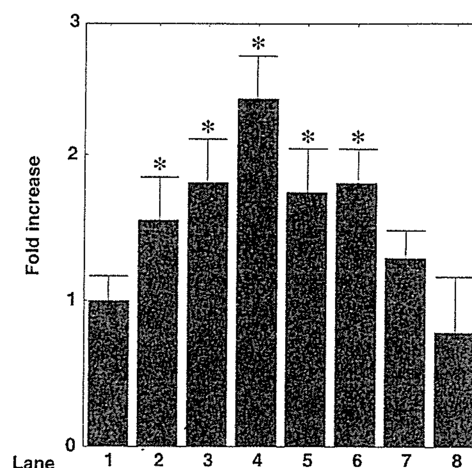


Fig. 3. Effect of sphingosine 1-phosphate (S-1-P) on the phosphorylation of GSK-3β in MC3T3-E1 cells. The cultured cells were stimulated with 30 μM S-1-P for the indicated periods. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific GSK-3β or GSK-3β. The histogram shows quantitative representations of the levels of S-1-P-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Similar results were obtained with two additional and different cell preparations. Each value represents the mean ± SD of triplicate determinations from triplicate independent cell preparations. $^*P < 0.05$, compared to the value of control.

the Akt substrates [Cross et al., 1995]. We found that GSK-3β was time dependently phosphorylated by sphingosine 1-phosphate (Fig. 3). In addition, Akt inhibitor attenuated the sphingosine 1-phosphate-induced phosphorylation of GSK-3β, suggesting that the Akt-mediating pathway actually functions in sphingosine 1-phosphate-stimulated MC3T3-E1 cells (Fig. 4). Akt inhibitor (50 μM) caused about 50% reduction in the sphingosine 1-phosphate-effect.

Effects of LY294002 and Wortmannin on the Sphingosine 1-Phosphate-Induced Phosphorylation of Akt in MC3T3-E1 Cells

In order to clarify whether PI3K acts at a point upstream from Akt, we examined the effect of LY294002, a specific inhibitor of PI3K [Vlahos et al., 1994], on the sphingosine 1-phosphate-induced phosphorylation of Akt. LY294002 dose dependently suppressed the sphingosine 1-phosphate-induced Akt phosphorylation (Fig. 5A). LY294002 (10 μM) caused almost complete reduction in the sphingosine 1-phosphate-effect. Wortmannin, another PI3K inhibitor [Arcaro and Wymann, 1993], also suppressed the phosphorylation of Akt (Fig. 5B). Wortmannin (10 μM) caused about 40% reduction in the sphingosine 1-phosphate-effect. However, LY294002 did not affect the sphingosine 1-phosphate-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells (data not shown). In addition, SB203580, a specific inhibitor of p38 MAP kinase [Cuenda et al., 1995] failed to affect the sphingosine 1-phosphate-induced phosphorylation of Akt (data not shown).

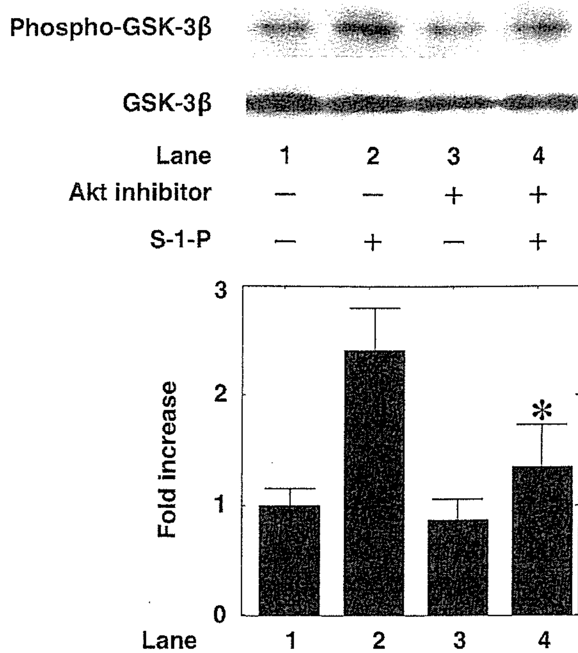
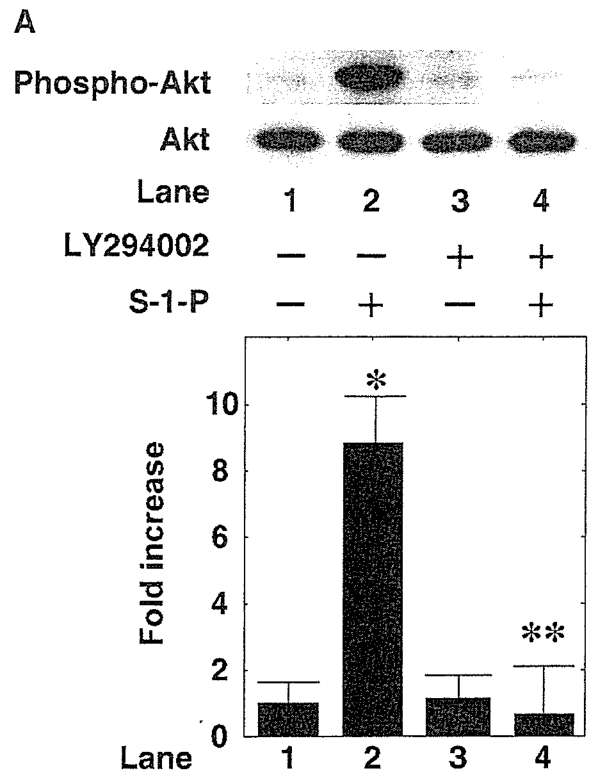


Fig. 4. Effect of Akt inhibitor on the sphingosine 1-phosphate (S-1-P)-induced phosphorylation of GSK-3β in MC3T3-E1 cells. The cultured cells were pretreated with 50 μM Akt inhibitor for 60 min, and then stimulated by 30 μM S-1-P or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific GSK-3β or GSK-3β. The histogram shows quantitative representations of the levels of S-1-P-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Similar results were obtained with two additional and different cell preparations. Each value represents the mean ± SD of triplicate determinations from triplicate independent cell preparations. *$P < 0.05$, compared to the value of S-1-P.

Effects of LY294002 and Wortmannin on the Sphingosine 1-Phosphate-Stimulated Induction of HSP27 in MC3T3-E1 Cells

LY294002 significantly suppressed the sphingosine 1-phosphate-stimulated induction of HSP27 in a dose dependent manner between



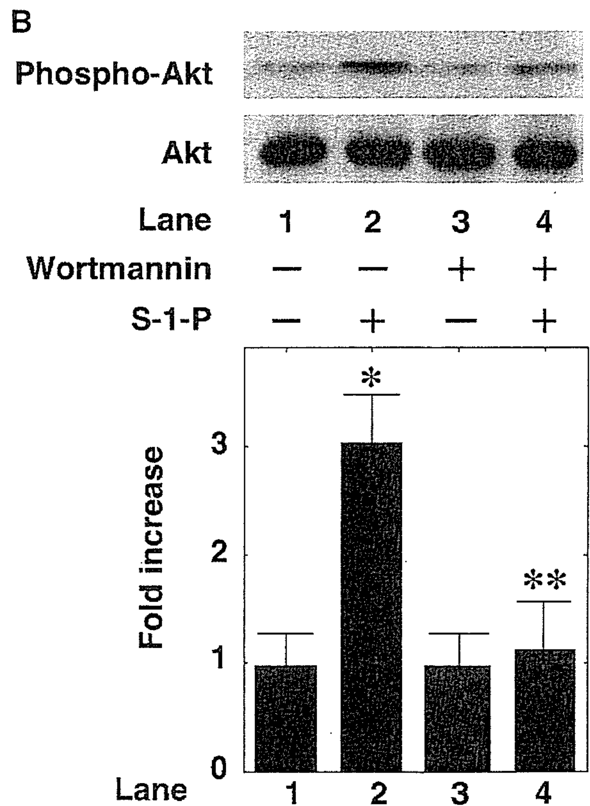


Fig. 5. Effects of LY294002 or wortmannin on the sphingosine 1-phosphate (S-1-P)-induced phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were pretreated with 10 μM LY294002 (**A**) or 10 μM wortmannin (**B**) for 60 min, and then stimulated by 30 μM S-1-P or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. The histogram shows quantitative representations of the levels of S-1-P-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Similar results were obtained with two additional and different cell preparations. Each value represents the mean ± SD of triplicate determinations from triplicate independent cell preparations. *$P < 0.05$, compared to the value of control (without agonist and inhibitor). **$P < 0.05$, compared to the value of S-1-P alone.

10 and 50 μM (Fig. 6A). Additionally, wortmannin markedly reduced the induction of HSP27 similarly to LY294002 (Fig. 6B).

DISCUSSION

We have previously shown that sphingosine 1-phosphate stimulates induction of HSP27 in osteoblast-like MC3T3-E1 cells and that p38 MAP kinase takes a part in the sphingosine 1-phosphate-effect [Kozawa et al., 1999]. In the present study, we first demonstrated that sphingosine 1-phosphate stimulated the phosphorylation of Akt in a time-dependent manner in MC3T3-E1 cells. In addition, we showed that PI3K inhibitors such as LY294002 and wortmannin, suppressed the sphingosine 1-phopshate-induced phosphorylation of Akt, suggesting that Akt functions at a point downstream from PI3K in these cells. PI3K is recruited upon growth factor receptor activation and produces 3′ phosphoinositide lipids [Dudek et al., 1997; Katso et al., 2001]. The lipid products of PI3K act as second messengers by binding to and activating diverse cellular target proteins. These events constitute the start of a complex signaling cascade, which ultimately results in the mediation of cellular activities such as proliferation, differentiation, chemotaxis, and survival. The PI3K/Akt signaling pathway is currently considered to play a critical role in mediating survival signals in a wide range of cell types. The recent identification of a number of substrates for the serine/threonine kinase Akt suggests that it blocks cell death by both impinging on the cytoplasmic cell death machinery and by regulating the expression of genes involved in cell death and survival. In addition, recent experiments suggest that Akt may also use metabolic pathways to regulate cell survival [Brunet et al., 2001; Masuyama et al., 2001].

Therefore, we next examined the correlation between the sphingosine 1-phosphate-stimulated induction of HSP27 and PI3K/Akt in osteoblast-like MC3T3-E1 cells. In the present study, the sphingosine 1-phosphate-stimulated HSP27 induction was reduced by Akt inhibitor. As for Akt inhibitor, 1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate [Hu et al., 2000], we found that it blocked the phosphorylation of GSK-3β, one of the Akt substrates [Cross et al., 1995]. In addition, we showed that PI3K inhibitors also suppressed

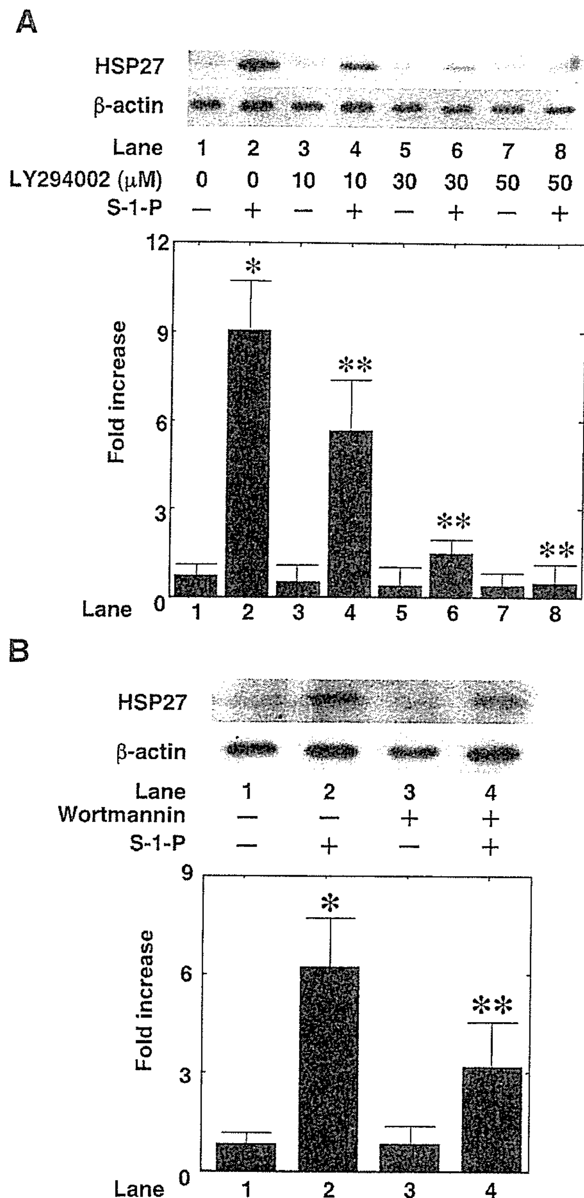


Fig. 6. Effects of LY294002 or wortmannin on the sphingosine 1-phosphate (S-1-P)-induced levels of HSP27 in MC3T3-E1 cells. The cultured cells were pretreated with various doses of LY294002 (**A**) or 50 μM wortmannin (**B**) for 60 min, and then stimulated by 30 μM S-1-P or vehicle for 6 h. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against HSP27 or β-actin. The histogram shows quantitative representations of the levels of S-1-P-induced HSP27 after normalization to levels of β-actin. Similar results were obtained with two additional and different cell preparations. Each value represents the mean ± SD of triplicate determinations from triplicate independent cell preparations. *$P < 0.05$, compared to the value of control. **$P < 0.05$, compared to the value of S-1-P alone.

the sphingosine 1-phosphate-stimulated HSP27 induction thorough the reduction of the sphingosine 1-phosphate-induced Akt phosphorylation. Therefore, based on our findings, it is most likely that the sphingosine 1-phosphate-stimulated induction of HSP27 is regulated by PI3K/Akt in osteoblast-like MC3T3-E1 cells.

We have previously reported that the activation of p38 MAP kinase is involved in HSP27 induction by sphingosine 1-phosphate in osteoblast-like MC3T3-E1 cells [Kozawa et al., 1999]. Therefore, we investigated to clarify the relationship between p38 MAP kinase and PI3K/Akt in these cells. Akt inhibitor and PI3K inhibitor, LY294002 [Vlahos et al., 1994], failed to influence the sphingosine 1-phosphate-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells, and p38 MAP kinase inhibitor, SB203580 [Cuenda et al., 1995], had little effect on the sphingosine 1-phosphate-induced phosphorylation of Akt. In addition, the inhibitory effect of Akt inhibitor or wortmannin on the sphingosine 1-phosphate-stimulated HSP27 induction was partial. We have previously shown that the suppressive effect of SB203580 on the HSP27 induction was partial [Kozawa et al., 1999]. These findings suggest that PI3K/Akt pathway plays a role at least in part in addition to p38 MAP kinase pathway in the sphingosine 1-phosphate-stimulated HSP27 induction in MC3T3-E1 cells. Taking these results into account as a whole, it is most likely that sphingosine 1-phosphate stimulates the induction of HSP27 probably via two independent pathways, PI3K/Akt and p38 MAP kinase, in osteoblast-like MC3T3-E1 cells.

It is recognized that HSP27 is present at two forms, an aggregated form and a dissociated small form in unstressed conditions [Benjamin and McMillan, 1998]. It has been shown that HSP27 is constitutively expressed at high levels in various tissues and cells, especially in skeletal muscle cells and smooth muscle cells [Benjamin and McMillan, 1998]. Post-translational modifications such as phosphorylation and oligomerization are crucial regulators of its functions [Benjamin and McMillan, 1998]. In our previous study [Kato et al., 1994], we have reported that HSP27 is dissociated concomitantly with the phosphorylation of the aggregated form of HSP27. In addition, we have shown that conversion from the non-phosphorylated, aggregated form of HSP27 to the phosphorylated, dissociated form results in decreased tolerance to heat stress [Kato et al., 1994]. It has been shown that estrogen-induced resistance to osteoblast apoptosis is associated with increased HSP27 expression [Cooper et al., 2000]. We speculate that expression of HSP27 via p38 MAP kinase and PI3K/Akt in osteoblasts might be related to the maintenance of the number of viable osteoblasts in bone tissue. Interestingly, sphingosine 1-phosphate reportedly prevents apoptosis in primary rat osteoblasts and human osteosarcoma SaOS-2 cells [Grey et al., 2002]. Taking our findings into account, it is probable that sphingosine 1-phosphate directly affects osteoblasts through the induction of HSP27 through PI3K/Akt and p38 MAP kinase. However, the physiological significance of HSP27 in osteoblasts has not yet been precisely clarified. Further investigations are necessary to clarify the exact roles of HSP27 in osteoblasts.

In conclusion, these results strongly suggest that sphingosine 1-phosphate stimulates the induction of HSP27 via PI3K/Akt pathway in addition to p38 MAP kinase in osteoblasts.

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Involvement of p44/p42 MAP kinase in insulin-like growth factor-I-induced alkaline phosphatase activity in osteoblast-like-MC3T3-E1 cells

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Abstract

It has been shown that insulin-like growth factor-I (IGF-I) stimulates the activity of alkaline phosphatase, a marker of mature osteoblast phenotype, in osteoblasts. In the present study, we investigated the involvement of the mitogen-activated protein (MAP) kinase superfamily in the IGF-I-stimulated alkaline phosphatase activity in osteoblast-like MC3T3-E1 cells. IGF-I-stimulated alkaline phosphatase activity dose dependently in the range between 1 nM and 0.1 μM. IGF-I induced the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase but not stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK). PD98059 and U0126, specific inhibitors of the upstream kinase that activates p44/p42 MAP kinase, significantly suppressed the IGF-I-induced alkaline phosphatase activity. On the contrary, SB203580 and PD169316, specific inhibitors of p38 MAP kinase, failed to affect the activity induced by IGF-I. Specific inhibitors for phosphatidylinositol 3-kinase (PI3K)/Akt pathway (LY294002 and wortmannin) also had no significant effect on IGF-I-induced p44/p42 MAP kinase phosphorylation. The phosphorylation of p44/p42 MAP kinase induced by IGF-I was reduced by U0126. These results strongly suggest that p44/p42 MAP kinase among the MAP kinase superfamily plays a role in the IGF-I-stimulated alkaline phosphatase activity in osteoblast-like MC3T3-E1 cells.
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Keywords: IGF-I; Alkaline phosphatase; p44/p42 MAP kinase; Osteoblast

1. Introduction

It is well recognized that insulin-like growth factor-I (IGF-I) plays a crucial role in the regulation of growth and bone metabolism (Conover, 2000; Olney, 2003). IGF-I, which is mainly synthesized and secreted from liver, mediates a variety of the actions of growth hormone that is secreted from pituitary gland under the control of the hypothalamus. Accumulating evidence suggests that IGF-I is necessary for fracture healing (Trippel, 1998). Bone metabolism is regulated mainly by two functional cells, osteoblasts and osteoclasts, the former responsible for bone formation and the latter for bone resorption (Nijweide et al., 1986). As for osteoblasts, it has been reported that IGF-I stimulates the proliferation of these cells and synthesize bone matrix proteins (Conover, 2000). We have previously demonstrated that IGF-I induces DNA synthesis synergistically with protein kinase C activation in osteoblast-like MC3T3-E1 cells (Kozawa et al., 1989). In addition, IGF-I reportedly stimulates alkaline phosphatase activity, a marker of mature osteoblast phenotype (Robinson et al., 1973), in osteoblasts (Schmid et al., 1984). It is recognized that IGF-I is also produced by osteoblasts (Olney, 2003). In a previous study (Kozawa et al., 1992b), we have shown that osteoblast-like MC3T3-E1 cells secrete IGF-I resulting in inducing mineralization, and protein kinase C activation suppresses the secretion of IGF-I. Based on these findings, there is no doubt that IGF-I secreted from osteoblasts plays a pivotal role in the regulation of bone metabolism.

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The mitogen-activated protein (MAP) kinase superfamily is well recognized to play crucial roles in the intracellular signaling of variety of agonists (Widmann et al., 1999). Three MAP kinases, p44/p42 MAP kinase, p38 MAP kinase and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK), are known as central elements used by mammalian cells to transduce the various messages (Widmann et al., 1999). It has recently been reported that IGF-I up-regulated expression of core binding factor α1 through MAP kinase pathway in osteoblast-like MC3T3-E1 cells (Pei et al., 2003). However, the exact role of the MAP kinase superfamily in IGF-I-effect on osteoblasts has not yet been clarified.

In the present study, we investigated whether the MAP kinase superfamily is involved in the IGF-I-induced alkaline phosphatase activity in osteoblast-like MC3T3-E1 cells. We here show that IGF-I activates p44/p42 MAP kinase and p38 MAP kinase in these cells, and that p44/p42 MAP kinase plays a part in the IGF-I-stimulated alkaline phosphatase activity.

2. Materials and methods

2.1. Materials

IGF-I was purchased from R&D Systems, Inc. (Minneapolis, MN). Specific inhibitors for MEK (PD98059 and U0126), p38 MAP kinase (SB203580 and PD169316) and phosphatidylinositol 3-kinase (PI3K) (LY294002 and wortmannin) were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies and SAPK/JNK antibodies were purchased from New England BioLabs, Inc. (Beverly, MA). ECL Western blotting detection system was purchased from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. PD98059, U0126, SB203580 and PD169316 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect assay for alkaline phosphatase activity or the analysis of MAP kinases.

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., 1992a). Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 35-mm diameter dishes 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 h.

2.3. Assay for alkaline phosphatase activity

The cultured cells were pretreated with PD98059, U0126, SB203580 or PD169316 for 60 min, and then were stimulated by IGF-I in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. At the end of the incubation, the cells were harvested by scraping with a rubber policeman into 1 ml of 0.2% Nonidet P-40 and disrupted by sonication. After centrifugation at 1500 × *g* for 5 min of the homogenate, alkaline phosphatase activity of the supernatant was measured by the method of Lowry et al. (1954).

2.4. Analysis of mRNA expressions

The cultured cells were stimulated by IGF-I or vehicle in α-MEM containing 0.3% FCS for 0, 12, 24, 36, 48 and 60 h. The expression levels of mRNAs were estimated by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) according to the method previously described by Nakashima et al. (2005) and Ishisaki et al. (2004). In brief, at the end of each incubation period, the total RNA was extracted from the cells using Isogen (Nippongene, Toyama, Japan) and complementary DNA was synthesized with Omniscript reverse transcriptase (Qiagen, Valencia, CA) using an oligo$(dT)_{15}$ primer (1 μM) according to the manufacturer's instructions. Each PCR reaction was carried out in 50 μl of mixture containing 1 μl of cDNA, 5 μl of 10× Qiagen PCR buffer, 10 μl of 5× Q-solution PCR buffer, 1 μl of 10 mM each deoxynucleotide triphosphate mix, 0.1 μM each sense and antisense primers and 0.25 μl of Taq DNA polymerase (Qiagen). Each reaction consisted of initial denaturation at 94 °C for 3 min followed by three-step cycling: denaturation at 94 °C for 30 s, annealing at a temperature optimized for each primer pair for 30 s, and extension at 72 °C for 1 min. Amplification was stopped within linear range and the reaction underwent a final extension at 72 °C for 10 min. Twenty-five cycles were undergone. Amplification products were electrophoresed on 1.2% agarose gels and visualized by ethidium bromide staining followed by UV light illumination. Equal loading of RNA samples were checked by amplification of GAPDH cDNA. The primer sequences used for PCR amplifications, annealing temperature, and expected fragment size were as follows: osteocalcin, forward primer 5′-CTGAGTCTGACAAAGCCTTC-3′, reverse primer 5′-GCTGTGACATCCATACTTGC-3′, 55 °C and 312 bp; osteopontin, forward primer 5′-CGACGATGATGACGATGATGAT-3′, reverse primer 5′-CTGGCTTTGGAACTTGCTTGAC-3′, 60 °C and 495 bp; Runx2, forward primer 5′-AGCAACAGCAACAACAGCAG-3′, reverse primer 5′-GTAATCTGACTCTGTCCTTG-3′, 55 °C and 470 bp; collagen α1(I), forward primer 5′-TCTCCACTCTTCTAGTTCCT-3′, reverse primer 5′-TTGGGTCATTTCCACATGC-3′, 51 °C and 269 bp; glyceraldehydes-3-phosphate dehydrogenase (GAPDH), forward primer 5′-TTCATTGACCTCAACTACATG-3′, reverse primer 5′-GTGGCAGTGATGGCATGGAC-3′, 60 °C and 443 bp, respectively.

2.5. Analysis of MAP kinases

The cultured cells were stimulated by IGF-I in α-MEM containing 0.3% FCS for the indicated periods. The cells were washed twice with phosphate-buffered saline and then lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125,000 × *g* for 10 min at 4 °C. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by Laemmli (1970) in 10% polyacrylamide gel. Western blotting analysis was performed as described previously (Kato et al., 1996) by using phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies or SAPK/JNK 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. When indicated, the cells were pretreated with PD98059, U0126, PD169316, LY294002 or wortmannin for 60 min.

2.6. Determinations

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

2.7. 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 IGF-I on alkaline phosphatase activity in MC3T3-E1 cells

It has been shown that IGF-I induces alkaline phosphatase activity in osteoblasts (Schmid et al., 1984). We found that IGF-I significantly stimulates the activity of alkaline phosphatase in a dose-dependent manner in the range between 1 nM and 0.3 μM (Fig. 1). The maximum effect of IGF-I on the activity was observed at 0.3 μM. In addition, we investigated the effect of IGF-I treatment on the several known osteoblast differentiation markers (osteocalcin, osteopontin, Runx2 and collagen α1(I)) in MC3T3-E1 cells. GAPDH mRNA was served as a control. We found that IGF-I had no significant effect on up-regulation of these mRNA expressions at a dose (0.1 μM) that significantly induces ALP activation (data not shown).

3.2. Effects of IGF-I on the phosphorylation of the p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK in MC3T3-E1 cells

To investigate whether IGF-I activates the MAP kinase superfamily in osteoblast-like MC3T3-E1 cells, we examined the effects of IGF-I on the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK. Stimulation of IGF-I significantly induces the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase (Fig. 2). The phosphorylation of both MAP kinases reached the peak at 10 min. On the contrary, IGF-I did not substantially affect the phosphorylation of SAPK/JNK (data not shown).

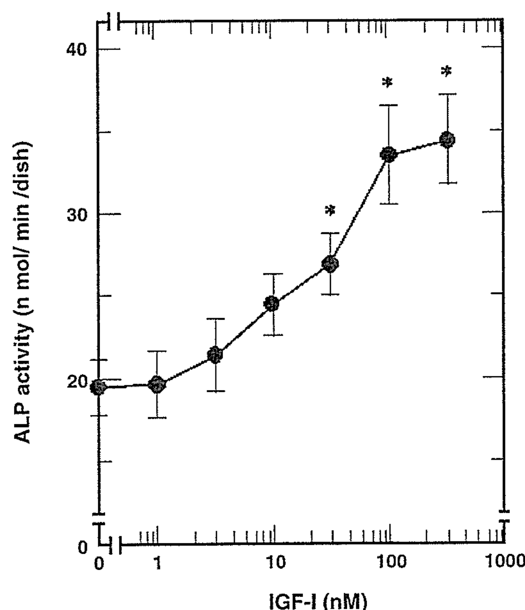

Fig. 1. Effect of IGF-I on alkaline phosphatase activity in MC3T3-E1 cells. The cultured cells were stimulated by various doses of IGF-I for 48 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$p < 0.05$, compared to the control value.

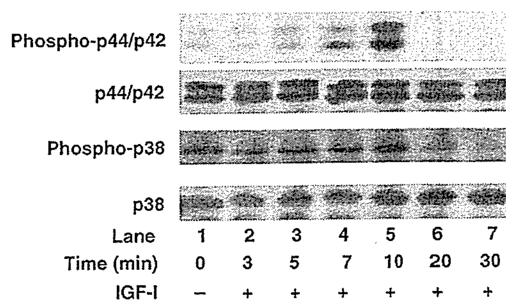

Fig. 2. Effects of IGF-I on the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 0.1 μM IGF-I for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p44/p42 MAP kinase, p44/p42 MAP kinase, phospho-specific p38 MAP kinase or p38 MAP kinase. Similar results were obtained with two additional and different cell preparations.

3.3. Effects of PD98059 and U0126 on the IGF-I-induced alkaline phosphatase activity in MC3T3-E1 cells

To investigate whether p44/p42 MAP kinase is involved in the IGF-I-stimulated alkaline phosphatase activity in MC3T3-E1 cells, we examined the effect of PD98059, a highly specific inhibitor of the upstream kinase that activates p44/p42 MAP kinase (Alessi et al., 1995), on the alkaline phosphatase activity. PD98059, which alone hardly affected the basal level of alkaline phosphatase activity, significantly reduced the IGF-I-induced alkaline phosphatase activity (Fig. 3). The maximum inhibitory effect of PD98059 at 30 μM caused about 65% reduction in the IGF-I-effect. In addition, U0126, another inhibitor of the upstream kinase that activates p44/p42 MAP kinase (Favata et al., 1998), dose dependently reduced the alkaline phosphatase

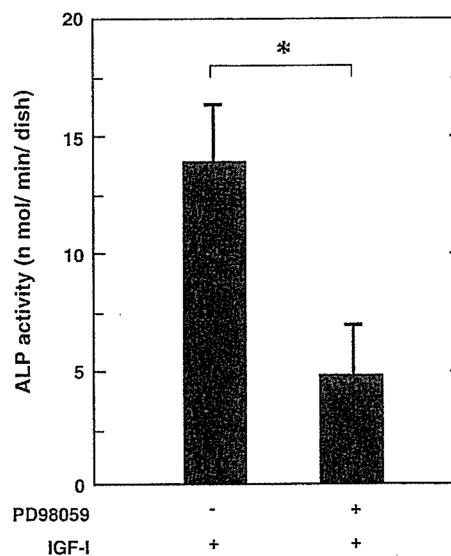

Fig. 3. Effect of PD98059 on the IGF-I-induced alkaline phosphatase activity in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM PD98059 for 60 min, and then stimulated by 0.1 μM IGF-I or vehicle for 48 h. Values for IGF-I-unstimulated cells were subtracted to produce each data point. Each 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 value of IGF-I alone.