

骨粗鬆症治療

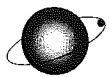
別刷

MMP

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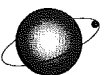
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はじめに

生体組織はさまざまな細胞外マトリックス (Extracellular matrix : ECM) 成分により構築されている。ECMは、遺伝子レベルで制御された産生量と蛋白分解酵素により代謝回転がコントロールされており、ECMの分解には、マトリックスメタロプロテアーゼ (Matrix metalloproteinase : MMP) が重要な役割を担っている。MMPは、サイトカイン、細胞増殖因子とともに個体発生や生体の恒常性維持に必要であるとともに、循環器疾患、呼吸器疾患、脳疾患、関節リウマチや変形性関節症などの骨、軟骨変性疾患、癌の浸潤、転移、血管新生、創傷治癒機転など非常に多くの病態でECM代謝に関与しているとされている。

本稿では、MMPの種類、発現制御、活性化機構について解説したい。



MMPsの種類と分類

蛋白分解酵素は活性中心の触媒残基によって、アスパラギン酸プロテアーゼ、システインプロテアーゼ、セリンプロテアーゼ、メタロプロテアーゼ (MMP) に分類さ

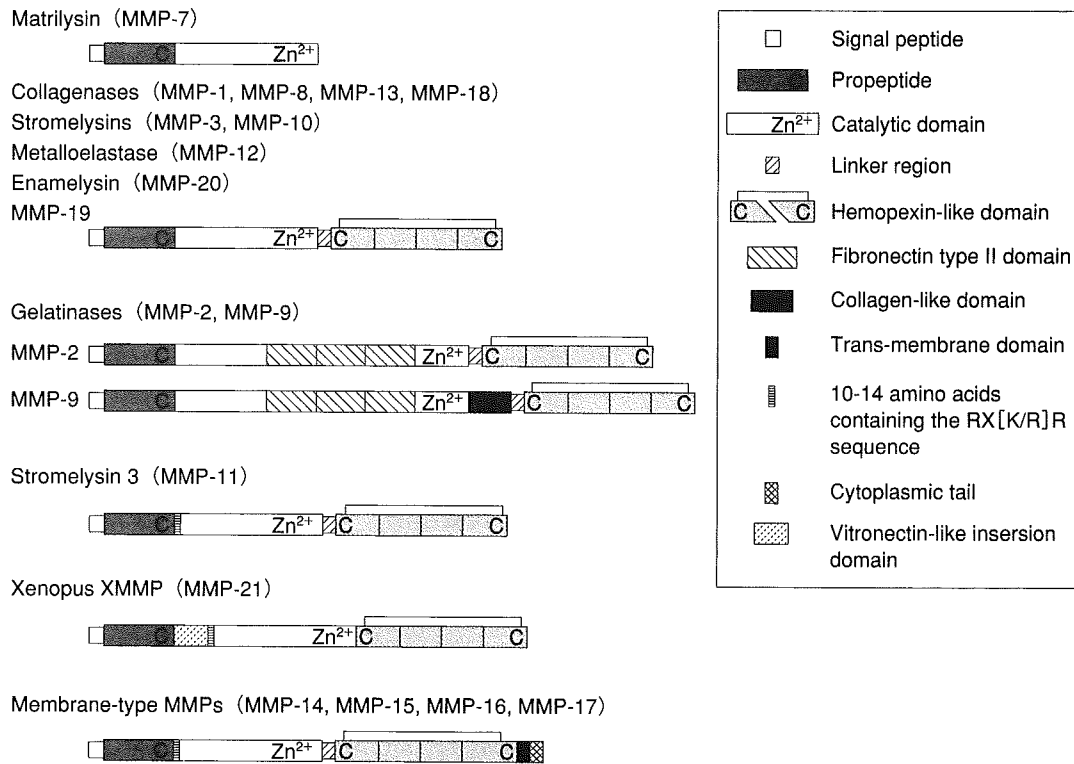
れるが、MMPは Zn^{2+} を有する金属プロテアーゼであり、MMPの基本構造は酵素の潜在性を保持するための propeptide鎖、酵素活性部位を含む catalytic domain、ヘモペキシンと相同性のある hemopexin-like domain から構成される (図①)。

MMPは共通した性質として、①活性中心に Zn^{2+} を有し酵素活性に Ca^{2+} を必要とすること、②潜在型として分泌され細胞外で活性化されること、③アミノ酸配列において高い相同性を有すること、④ECM成分に対し基質特異性を有し、分解すること、⑤共通のインヒビターである tissue inhibitor of metalloproteinases (TIMPs) によって活性が阻害されること、があげられる。

また、MMPは一次構造と基質特異性の違いから5群に分別できる (表①)。MMPの多くは分泌型酵素であり、さまざまな基質特異性があることから、組織における広範なECM分解にそれぞれが協調しながら関与していると考えられている。コラゲナーゼ群には、間質細胞がもに産生するコラゲナーゼ-1/MMP-1、好中球由来のコラゲナーゼ-2/MMP-8、コラゲナーゼ-3/MMP-13が含まれる。MMP-13は骨芽細胞や軟骨細胞において発現しており、関節リウマチにおける骨、軟骨破壊への関与が考えられている。ゼラチナーゼ群には、ゼラチナーゼA/MMP-2、ゼラチナーゼB/MMP-9、がある。ゼラチナーゼA/MMP-2は線維芽細胞や種々の癌細胞において、ゼラチナーゼB/MMP-9はマクロファージに発現している。これらの酵素はゼラチンやIV型コラーゲンに対し分解活性が強く、癌浸潤と関連がある。またゼラチナーゼ

関連語

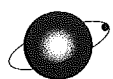
- ・ 蛋白分解酵素
- ・ 細胞外マトリックス (ECM)



図① MMPの種類と分類

(Woessner JF, Nagase H : Matrix Metalloproteinases and TIMPs, Oxford University press, 2000)

A/MMP-2, ゼラチナーゼB/MMP-9はマクロファージや破骨細胞系の細胞にも発現しており骨吸収にも関与している¹⁾。ストロメライシン群には、ストロメライシン1/MMP-3, ストロメライシン2/MMP-10がある。これらのストロメライシンはアグリカンやフィブロネクチンなどに対し分解活性を示すだけでなく、潜在型 (pro) MMP-1,8,9,13の活性化酵素である。膜型MMP群には、MT1-MMP/MMP-14, MT2-MMP/MMP-15, MT3-MMP/MMP-16, MT4-MMP/MMP-17, MT5-MMP/MMP-24, MT6-MMP/MMP-25がある。これら膜型MMPは細胞膜上に発現することより、細胞近傍のECM分解に関与することで細胞増殖や細胞の運動性に関与すると考えられている。



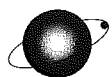
MMPsの遺伝子発現制御機構

MMPは組織由来細胞, 癌細胞, 炎症細胞など多くの細胞から産生される。この産生誘導はサイトカイン, 増殖因子, ホルモンあるいは環境因子などさまざまな刺激

群	種類	MMP番号
コラゲナーゼ群	組織コラゲナーゼ	MMP-1
	好中球コラゲナーゼ	MMP-8
	コラゲナーゼ3	MMP-13
ゼラチナーゼ群	ゼラチナーゼA	MMP-2
	ゼラチナーゼB	MMP-9
ストロメライシン群	ストロメライシン-1	MMP-3
	ストロメライシン-2	MMP-10
膜貫通型MMP群	MT1-MMP	MMP-14
	MT2-MMP	MMP-15
	MT3-MMP	MMP-16
	MT4-MMP	MMP-17
	MT5-MMP	MMP-24
	MT6-MMP	MMP-25
その他	マトリライシン	MMP-7
	ストロメライシン-3	MMP-11
	メタロエラスターゼ	MMP-12
	新規MMP	MMP-19
	エナメライシン	MMP-20
	マトリライシン-2	MMP-26

で調節されている。各MMPの発現様式, プロモーター構造から, 大きく2群に分けることができる。1つは, イ

インターロイキン-1(IL-1), 腫瘍壊死因子(tumor necrosis factor: TNF)- α , 腫瘍プロモーターである tissue polypeptide Antigen(TPA)などにより発現が促進されるもので, MMP-1,-3,-7,-9が含まれ, これらのMMP遺伝子のプロモーター領域には, TPA responsible element(TRE)が存在し, IL-1, TNF- α , TPAにより誘導されたc-Jun, c-Fosなどのactivating protein-1 (AP-1)ファミリーの転写因子が結合し, 転写活性が上昇する. この転写活性はAP-1ファミリーの転写因子のみで制御されているのではなく, Etsファミリーの転写因子, nuclear factor- κ B(NF- κ B)など多くの転写因子により調節されている. もう1つの群は, 外部からの刺激に反応することなく, 組織や細胞特異的に恒常的発現パターンを示すものであり, MMP-2, MT1-MMP/MMP-14が含まれる. MMP-2はサイトカインや増殖因子, TPAの制御を受けない. MMP-2のプロモーター領域には, TATA boxやTREが存在せず, AP-2やp53などの転写活性促進により調節されている. MT1-MMP/MMP-14ではプロモーター領域に, early growth response(Egr)ファミリーのErg-1やNF- κ Bなどの結合部位が存在し, これらにより発現調節がおこなわれている.



MMPsの活性化機構

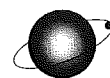
MMPは活性化の違いから, ①潜在型酵素(proMMP)として分泌され, 細胞外で活性化されるMMP(細胞外活性化型MMP), ②細胞内で活性化され, 活性型MMPとして細胞表面で発現または細胞外へ分泌されるMMP(細胞内活性化型MMP), ③細胞膜表面で活性化を受けられるMMP(細胞膜上活性化型MMP), に分類できる.

細胞外で活性化されるMMPは, MMP-1,-3,-7,-8,-9,-10,-12,-13が含まれる. proMMPのN末端側にはpropeptide鎖があり, このpropeptide鎖に存在するMMP共通配列PRCG (V/N)PDのシステインが-SH基を介して活性中心のZn²⁺に配置して潜在性を維持している. このシステインとZn²⁺の相互作用を切断することにより活性型MMPに変換される. proMMPの活性化酵素として, トリプシン, プラスミンや好中球エラスターゼなどのセリンプロテアーゼが知られているが, 活性型MMPもproMMPを

活性化する. MMP-3はproMMP-1,-7,-8,-9を活性化し, MMP-7,-11もproMMP-1を活性化する. MMP-7はproMMP-9を部分的に活性化する. MMP-10はproMMP-1,-8,-9を活性化しMMP-7を部分的に活性化する²⁾.

細胞内で活性化されるMMPは, MT-MMPsおよびMMP-11である. これらのMMPのpropeptide鎖中のRXKR配列は細胞内プロセシング酵素であるフリンの認識部位であり, 合成されたproMT-MMPsおよびproMMP-11はフリンにより細胞内で活性化される³⁾⁴⁾. したがって, MT-MMPsは細胞膜上に存在し, MMP-11は細胞外に活性型として存在する.

細胞膜上で活性化されるMMPは, MMP-2である. MT1-MMPは細胞膜上でTIMP-2の介在のもとにproMMP-2を活性化する. MT1-MMPとTIMP-2がそれぞれのN末端ドメインを介して複合体を形成する. さらにTIMP-2のC末端ドメインとproMMP-2のC末端ドメインが結合し細胞膜上でヘテロ三量複合体を形成する. そして, この複合体近傍のTIMP-2と結合していないMT1-MMPが複合体中のproMMP-2を活性化する⁴⁾⁵⁾.



おわりに

近年の研究の進歩により, MMPは, 関節軟骨破壊, 癌浸潤, 転移, のみならず, 呼吸器疾患, 心血管疾患, 肝臓疾患, 眼疾患, 皮膚疾患, 神経疾患と非常に多くの病的状態でのかかわりが示されている. その一方でMMP阻害剤の開発も進んでおり, すでに多くの開発薬の試験がおこなわれている. 近い将来, MMP阻害剤を用いた治療の実現が期待される.



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整形外科看護

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3 エタネルセプト

関節リウマチ（以下、RA）の患者では、TNF- α 、IL-6、IL-1といった炎症を惹起する物質が過剰状態にあります。エタネルセプト（エンブレル®皮下注用25mg）は、その過剰なTNFの働きを抑える可溶性TNFレセプター製剤です。

① 効き目は？

RA患者では「だるい」「疲労感がある」といった全身の症状とともに、関節には腫脹と疼痛が生じ、関節構造が破壊されるにいたります。エタネルセプトはCRP・ESRといった炎症マーカーを減少させるとともに、患者自身が「体が軽くなった、痛みや腫れがよくなった」などの病状改善を実感することの多い薬剤です。また手や足の末梢関節で関節破壊の進行抑制効果が確認され¹⁴⁾、このような生物学的製剤の登場によって、RAの治療は、炎症を抑えるだけではなく、関節破壊の出現・進行抑止による運動機能維持を目指して考えるようになってきています。エタネルセプトには表1のような特徴があります。

表1 エタネルセプト（エンブレル®皮下注用25mg）の特徴

- 週2回の皮下注射
- 患者による自己注射が可能
- 効果発現が速い
投与開始後2週間ごろから効果が期待できる
- 単剤での効果が期待できる
メトトレキサートの併用が必須でない
- 脱落例が少ない
中和抗体ができない

② 注意点は？

エタネルセプトは感染症に対する宿主防御に影響を及ぼすことが考えられており、副作用として重篤な感染症や結核の危険性があります。医療従事者が本剤の特性と注意点（表2、3）を十分理解するとともに、患者への十分な教育・指導（有害事象の注意すべき症状・自己注射手技）も重要となります。

A) 投与前の注意事項

エタネルセプト投与前には感染症や結核に対するスクリーニングが必要です。具体的には過去の既往歴などの十分な問診・胸部X線検

表2 頻度の高い副作用

- | | |
|---------|-------------------|
| ●注射部位反応 | 注射部位の、癢痒・腫脹・熱感・発赤 |
| ●感染症 | カゼ様症状 |

表3 重い副作用症状から疑うべき病態

症状	疑うべき病態
発熱・微熱	感染症・結核・血液障害
咳・空咳・長引く咳	感染症・間質性肺炎・結核
呼吸困難	カリニ肺炎・間質性肺炎
倦怠感	血液障害・結核
体重減少	結核
口内炎	血液障害
咽頭痛	感染症・血液障害
発熱・腰背部痛・排尿痛	尿路感染症
発疹・かゆみ	アレルギー反応
血液障害	汎血球減少症、顆粒球減少、血小板減少など

査・ツベルクリン反応検査・血液学的検査が必要になります。

B) 使用上の注意点

週2回の皮下注射です。有効性と安全性を確認するために、初期1カ月は毎回通院が必要ですが、その後は主治医の判断によって自己注射に移行することができます。

自己注射（ご家族でも可能）に移行すると、患者の通院による負担を軽減することができます。患者自身の注射手技（清潔操作・薬剤の調整・薬剤の投与・注射器具の廃棄など）については十分な教育訓練が必要です。エタネルセプトはゆっくり溶かす（泡立てない）、皮下注射後はもまないことが注意点です。

C) 投与中に注意すべき副作用

表2, 3のような副作用が出現する可能性があります。特に表3に列挙した症状とその際に考えられる重篤な病態をよく知ったうえで、患

者にこのような症状が出ていないかどうかの注意を促すとともに、その症状を的確に拾い上げて対応することが重要です。（橋本 淳）

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Adiponectin increases bone mass by suppressing osteoclast and activating osteoblast

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Abstract

Adiponectin, an adipose-derived hormone, exhibits various biological functions, such as increasing insulin sensitivity, protecting hypertension, and suppression of atherosclerosis, liver fibrosis, and tumor growth. Here, we report the role of adiponectin on bone metabolism. C57BL/6J mice were treated with adenovirus expressing lacZ or adiponectin, and their bones were analyzed by three-dimensional microcomputed tomography. Adiponectin-adenovirus treatment increased trabecular bone mass, accompanied by decreased number of osteoclasts and levels of plasma NTx, a bone-resorption marker. In vitro studies showed that adiponectin inhibited M-CSF- and RANKL-induced differentiation of mouse bone marrow macrophages and human CD14-positive mononuclear cells into osteoclasts and also suppressed the bone-resorption activity of osteoclasts. Furthermore, adiponectin enhanced mRNA expression of alkaline phosphatase and mineralization activity of MC3T3-E1 osteoblasts. Our results indicate that adiponectin exerts an activity to increase bone mass by suppressing osteoclastogenesis and by activating osteoblastogenesis, suggesting that adiponectin manipulation could be therapeutically beneficial for patients with osteopenia.

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Keywords: Bone mass; Adiponectin; Adipose tissue; Adipocytokines; Osteoclasts; Osteoblasts; Bone metabolism; Osteopenia

Osteoporosis and related bone fractures are growing medical problems especially with the enhanced longevity in industrial countries [1,2]. Therefore, it is important to know the factors that regulate bone mass and to develop effective therapeutic methods. Bone and bone marrow consist of various cells, including osteoblasts, osteoclasts, hematopoietic cells, and adipocytes. Bone adipocytes share a common mesenchymal precursor with

osteoblasts and chondrocytes, and their numbers in bone marrow are altered in various pathophysiological conditions [3,4], but their roles in bone biology have not been clarified.

Several studies of adipocyte function have revealed that adipose tissue is not merely an energy-storing organ but it secretes a variety of biologically active molecules, which we conceptualized as “adipocytokines,” including plasminogen activator inhibitor-1, tumor necrosis factor- α , resistin, leptin, and adiponectin [5–8]. Recent studies suggested that leptin, an anti-satiety adipocytokine, might have enhancing effects on bone mass

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[9,10]. Administration of leptin partially prevented bone loss in ovariectomized rats [11] and increased bone mineral density in leptin-deficient mice [12,13].

Adiponectin is a fat-specific secretory factor that was identified by our group in human fat cDNA [14]. The mouse homologue of adiponectin was independently cloned as adipoQ and Acrp30 [15,16]. We and others have shown that adiponectin has various biological functions, such as increasing insulin sensitivity in the liver and skeletal muscle, and protecting vascular walls from atherosclerosis, hence low plasma adiponectin levels in obesity might contribute to insulin resistance, diabetes, and atherosclerosis [17–21]. Furthermore, we recently showed that adiponectin inhibited liver fibrosis by suppressing proliferation and activity of hepatic stellate cells producing fibrotic collagen and transforming growth factor- β [22]. Others also reported that adiponectin have tumor growth inhibitory properties [23]. On the other hand, receptors of adiponectin were cloned and found to be expressed ubiquitously [24–26], suggesting that adiponectin might also play certain roles in bone biology.

The present study was designed to determine the effects of adiponectin on bone metabolism. The results showed that adiponectin increased bone mass by suppressing osteoclastogenesis and by activating osteoblastogenesis, suggesting that adiponectin could be potentially useful therapeutically for patients with reduced bone mass.

Materials and methods

Animals. All animals were purchased from Clea Japan (Tokyo, Japan) and housed in a room under controlled temperature (23 ± 1 °C) and humidity (45–65%), and had free access to water and chow (Oriental Yeast). All animal experiments were conducted in accordance with the Institutional Guidelines for the Care and Use of laboratory animals.

Adiponectin adenovirus. Adenovirus producing the full-length mouse adiponectin was prepared as described previously [27]. Then, 2×10^8 plaque-forming units of adenovirus-adiponectin (Ad-adipo) or adenovirus-lacZ (Ad-lacZ) were injected into the jugular vein of 8-week-old C57BL/6J male mice. Mice were sacrificed on day 14 after virus injection.

Skeletal morphology. Three-dimensional microcomputed tomography (3D- μ CT) scan was undertaken and the trabecular bone area (percentage of bone volume [BV] per tissue volume [TV]) was measured using a composite X-ray analysis system (Shimadzu, SMX-100CT-SV, Kyoto, Japan). Bones were fixed in 10% buffered formalin, decalcified in 14% ethylenediaminetetraacetic acid (EDTA), and embedded in paraffin. The sections were stained with tartrate-resistant acid phosphate (TRAP) and the number of TRAP-positive cells was counted in five sections per mouse.

Bone marker measurement. The blood samples were collected on day 14 after virus injection and processed within 30 min of collection, and aliquots of plasma were kept frozen at -80 °C until analyzed. Plasma cross-linked N-telopeptide of type 1 collagen (NTx), which is a marker of bone resorption, was measured by using an enzyme immunoassay (EIA). NTx concentrations were expressed as nanomoles of bone collagen equivalents (BCE) per liter (nM BCE/L).

Culture of mouse bone marrow macrophages. Mouse bone marrow macrophages (M-BMMs) were obtained as reported previously [28].

Briefly, whole bone marrow cells prepared from the femur and tibia of 5-week-old C57BL/6J male mice were suspended in α -minimal essential medium (α MEM) containing 10% heat-inactivated fetal bovine serum (FBS) and recombinant human macrophage colony-stimulating factor (M-CSF, 100 ng/ml) at 5×10^6 cells in a 10-cm culture dish. After 3 days in culture, the cells were washed, harvested with 0.02% EDTA in phosphate-buffered saline (PBS), and seeded at 3×10^5 cells into another 10-cm culture dish. After a further 3 days in culture, the cells were harvested, plated at a density of 1.5×10^4 cells/cm² in 48-well plates, and maintained for 5 days in the presence of recombinant human soluble receptor activator of nuclear factor- κ B ligand (RANKL, 50 ng/ml) and M-CSF (100 ng/ml) with or without recombinant human adiponectin [29].

Culture of human CD14⁺ peripheral blood mononuclear cells. Human CD14-positive peripheral blood mononuclear cells (PBMCs) were prepared as reported previously [30]. Briefly, peripheral blood was obtained from healthy male volunteers and was carefully layered on the Ficoll-Paque PLUS solution (Amersham Biosciences, USA), and centrifuged at 1500 rpm for 30 min. PBMC layer was collected and washed twice in PBS. CD14-positive cells were selected using a magnetic isolation procedure (MACS CD14 Microbeads, Miltenyi Biotec, Germany). The cells were plated at a density of 1×10^5 cells/cm² in 48-well plates in α MEM containing 10% FBS and 1% penicillin/streptomycin, and cultured for 7 days in the presence of M-CSF (25 ng/ml) and RANKL (40 ng/ml) with or without recombinant human adiponectin.

Tartrate-resistant acid phosphate staining. TRAP staining was performed using a commercial TRAP staining kit (Hokudo, Sapporo, Japan). The number of TRAP-positive multinuclear (>3 nuclei) cells in each well was counted.

Calcium resorption assay. Calcified matrix-resorption activity of osteoclasts was determined using BD BioCoat osteologic calcium hydroxyapatite-coated 16-well chamber slides (BD Biosciences, Bedford, MA). Human CD14-positive monocytes were seeded at a density of 1×10^5 cells/cm² with M-CSF (25 ng/ml) and RANKL (40 ng/ml). At day 0, 3 (pre-osteoclasts) or day 7 (mature osteoclasts), recombinant human adiponectin was added to the culture medium. At day 14 cells were removed by vigorous washing, and resorption area was measured using Win Roof software version 3.5 (Mitani, Fukui, Japan).

Cell culture. Mouse pre-osteoblast MC3T3-E1 cells were cultured in α MEM supplemented with 10% FBS and 1% penicillin/streptomycin. For cell differentiation study, the cells were seeded at a density of 2×10^4 per well in 12-well plates. After the cultures reached confluence, the medium was changed to α MEM with 10% FBS containing 50 μ g/ml L-ascorbic acid phosphate magnesium (Wako Pure Chemical, Osaka, Japan) and 10 mM β -glycerophosphate (Wako Pure Chemical). Then, the cells were further cultured for indicated time with or without recombinant mouse adiponectin, which was generated in a manner similar to recombinant human adiponectin as described previously [31].

RNA analysis. Total RNAs were extracted from cells with an RNA-STAT-60 kit (Tel-Test “B”). The first-strand cDNA was synthesized using ThermoScript RT-PCR System (Invitrogen, San Diego, CA). Real-time polymerase chain reaction (PCR) was performed on a Light Cycler using the Fast Start DNA Master SYBR Green I (Roche Diagnostics) according to the protocol provided by the manufacturer [32]. Sequences of primers used for real-time PCR were as follows: alkaline phosphatase (ALP), 5'-GCC CTC TCC AAG ACA TAT A-3' and 5'-CCA TGA TCA CGT CGA TAT CC-3', 18S, 5'-CGG CTA CCA CAT CCA AGG AA-3' and 5'-GCT GGA ATT ACC GCG GCT-3'.

Mineralization assay. MC3T3-E1 cells were incubated at 34 °C for 24 h in culture medium, and then the medium was refreshed with culture medium with or without adiponectin. The degree of mineralization was determined in the 12-well plates using Alizarin Red staining, as described previously [33]. Briefly, the differentiated MC3T3-E1 cells were rinsed twice with PBS followed by fixation with 70% ethanol for 1 h at room temperature. Then cells were stained with 40 mM

Alizarin Red S (Wako Pure Chemical) at pH 4.0 for 10 min at room temperature, and were washed five times with deionized water and twice with PBS. Then, staining was released from the cell matrix by incubation in 10% cetylpyridinium chloride at pH 7.0 for 15 min. The degree of mineralization was determined by measuring the absorbance of supernatants at 562 nm.

Statistical analysis and ethical considerations. Results were expressed as means \pm SEM. Differences between groups were examined for statistical significance using Student's *t* test or analysis of variance with Fisher's protected least significant difference test. A *P* value less than 0.05 denoted the presence of a statistically significant difference. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine.

Results

Adenovirus-mediated overexpression of adiponectin in vivo

To investigate the *in vivo* role of adiponectin in bone metabolism, we treated C57BL/6J mice with adenovirus producing adiponectin (Ad-adipo) or lacZ (Ad-lacZ). Two weeks after injection, we estimated structural changes in the bone by analyzing cortical and trabecular bones with 3D- μ CT. Fig. 1A shows representative 3D- μ CT images of the proximal tibia of Ad-lacZ- and Ad-adipo-treated mice, demonstrating a significantly larger trabecular bone volume in Ad-adipo-treated mice than in Ad-lacZ-treated mice. The trabecular bone volume of the proximal tibia at two different areas located 50–250 and 500–700 μ m from the growth plates of the proximal tibia (representing the most active areas for bone remodeling, Fig. 1B) was significantly higher in Ad-adipo-treated mice than in the control Ad-lacZ-treated mice (Fig. 1B). Similar findings were observed in the femur, by 3D- μ CT analysis (data not shown).

To investigate whether adiponectin modulates osteoclastogenesis, we analyzed TRAP-stained sections of the distal femur in Ad-lacZ- and Ad-adipo-treated mice. Examination of these sections demonstrated fewer TRAP-positive osteoclasts in Ad-adipo-treated mice than in Ad-lacZ-treated mice (Fig. 2A). Quantitative analysis confirmed that the number of TRAP-positive osteoclasts was significantly lower in adiponectin-adenovirus-treated mice than in Ad-lacZ-treated mice (Fig. 2B). Furthermore, in Ad-adipo-treated mice, plasma levels of NTx, a marker of bone resorption, were significantly lower than in Ad-lacZ-treated mice (Fig. 2C). Taken together, our *in vivo* data indicated that adiponectin suppresses osteoclastogenesis.

Effects of adiponectin on osteoclast differentiation and activity

Next, we investigated the effects of adiponectin on the differentiation of osteoclasts *in vitro* by treating primary cultures of bone marrow stromal cells, which contain

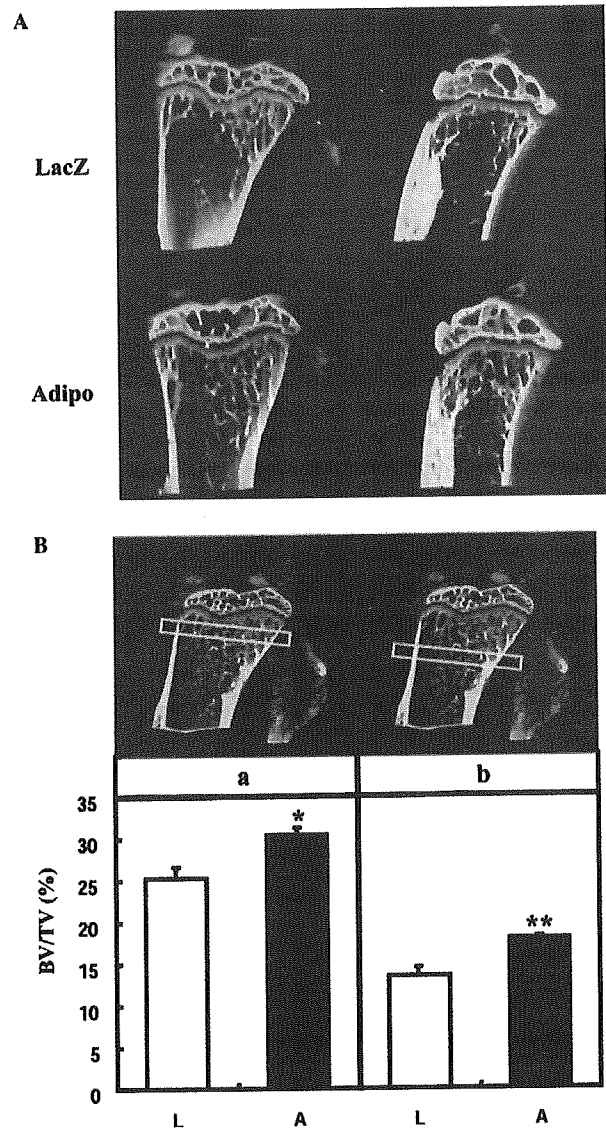


Fig. 1. Increased trabecular bone mass in adiponectin-adenovirus-treated mice. (A) Three-dimensional μ CT scan images of the proximal tibia of C57BL/6J mice treated with lacZ- (upper panels) or adiponectin-adenovirus (lower panels). The left-side panels show anterior–posterior view and the right-side panels show lateral–medial view. (B) Quantitative data of trabecular bone volume at indicated two areas; 50–250 μ m (a) and 500–700 μ m (b) from the distal end of the growth plate, in the proximal tibia of C57BL/6J mice treated with lacZ- (L, $n = 8$) and adiponectin-adenovirus-treated mice (A, $n = 8$). Data are expressed as percentage of total tissue volume (BV/TV [%]). Data are means \pm SEM. * $P < 0.05$, ** $P < 0.01$, compared with lacZ-adenovirus-treated mice.

osteoclast progenitor cells, with or without recombinant adiponectin. Previous studies reported that M-CSF and RANKL induced differentiation of progenitor cells into TRAP-positive osteoclasts [34,35]. In the present study, treatment of bone marrow stromal cells with adiponectin suppressed M-CSF/RANKL-induced differentiation of these cells into osteoclasts, in a dose-dependent

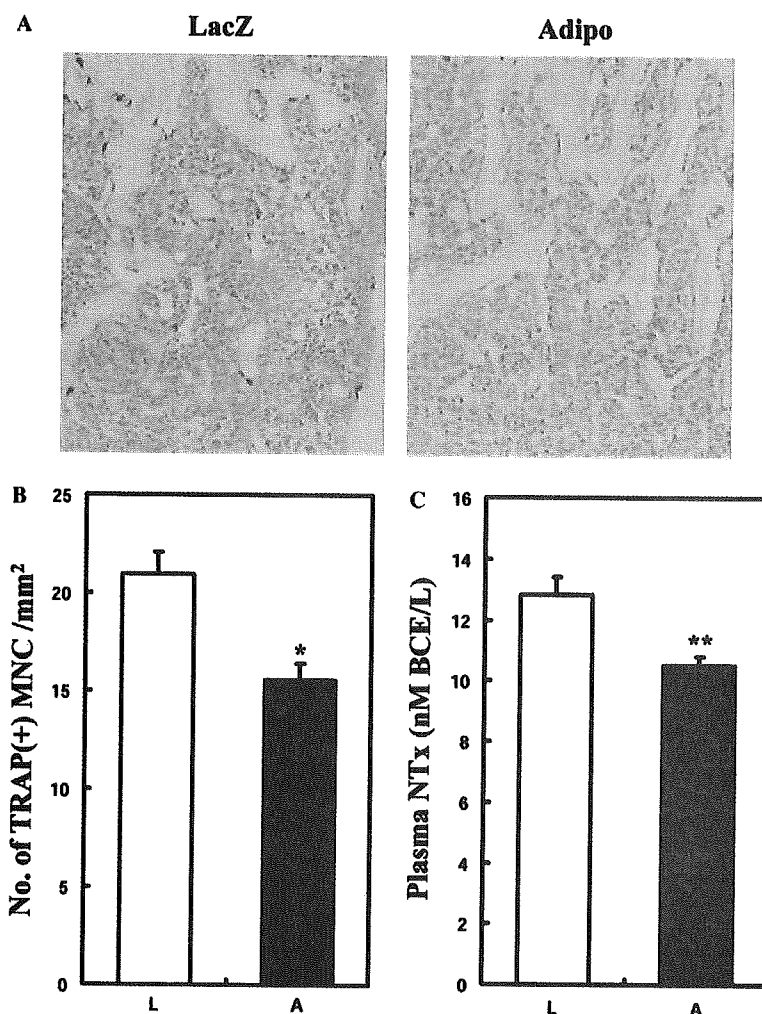


Fig. 2. Reduction of TRAP-positive osteoclasts in mice treated with adiponectin-adenovirus. (A) Histological examination of the distal femur with TRAP staining (left; lacZ, right; adiponectin). TRAP-positive osteoclasts are stained red. (B) Quantitative analysis of the number of TRAP-positive osteoclasts in C57BL/6J mice treated with lacZ- (L, $n = 8$) and adiponectin-adenovirus (A, $n = 8$). Data are means \pm SEM. * $P < 0.05$, compared with lacZ-adenovirus-treated mice. (C) Plasma NTx levels of C57BL/6J mice treated with lacZ- (L, $n = 8$) and adiponectin-adenovirus (A, $n = 8$). Data are means \pm SEM. ** $P < 0.01$, compared with lacZ-adenovirus-treated mice. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

manner (Fig. 3A). Similarly, adiponectin also dose-dependently suppressed differentiation of human CD14-positive PBMCs into osteoclasts (Fig. 3B).

Next, we performed bone-resorption analyses with or without adiponectin treatment, using human CD14-positive cells. Treatment of these cells with adiponectin for 14 days (days 0–14) after M-CSF- and RANKL-induced differentiation resulted in a significant reduction of the resorption area in a dose-dependent manner (Fig. 3C). Furthermore, a significant reduction of the resorption area was also noted with adiponectin treatment from day 3 or 7 when human CD14-positive cells were already differentiated (days 3–14, 7–14) (Figs. 3D and E). These results suggest that adiponectin could suppress osteoclastogenesis and bone-resorption activity of osteoclasts.

Effects of adiponectin on MC3T3-E1 osteoblasts

To investigate the effects of adiponectin on osteoblasts, we treated MC3T3-E1 osteoblasts with adiponectin for 18 days and measured mRNA levels of alkaline phosphatase (ALP) and the mineralization activity of MC3T3-E1 osteoblasts. Treatment with adiponectin significantly increased ALP mRNA level on day 12 and 18 compared with the control (Fig. 4A), suggesting that adiponectin might enhance the differentiation of osteoblasts. Previous reports indicated increased mineralization activity of MC3T3-E1 cell matrix in the presence of ascorbic acid [33,36]. Treatment of these cells with adiponectin significantly increased mineralization of the matrix (Fig. 4B).

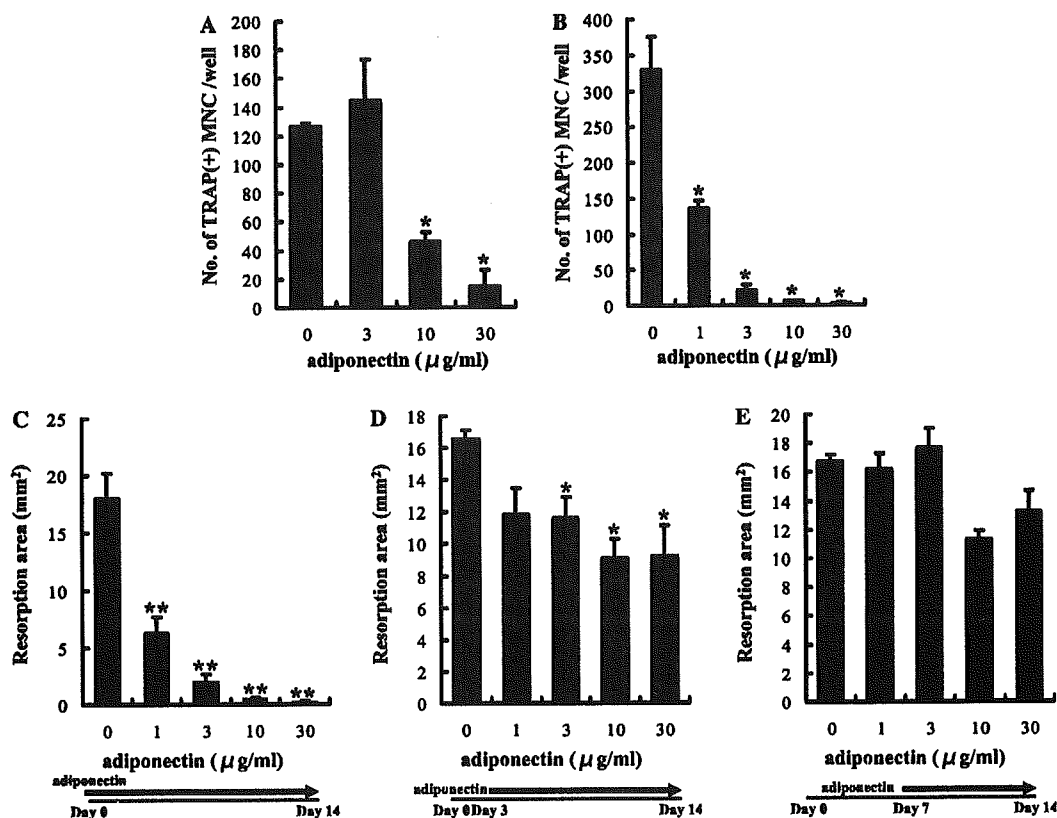


Fig. 3. Effects of adiponectin on primary cultures of osteoclasts. (A,B) TRAP assay of primary cultures of mouse bone marrow macrophages (A) and human CD14-positive PBMCs (B) stimulated by M-CSF and RANKL and treated with the indicated amounts of adiponectin. Data are expressed as means \pm SEM. * $P < 0.05$, compared with cells that were not treated with adiponectin (0–30 $\mu\text{g/ml}$). (C, D, and E) Bone-resorption assay of human CD14-positive PBMCs stimulated by M-CSF and RANKL, and treated with the indicated amounts of adiponectin from day 0 to 14 (C), from day 3 to 14 (D), and from day 7 to 14 (E). Data are means \pm SEM. * $P < 0.05$, ** $P < 0.01$, compared with cells that were not treated with adiponectin (0–30 $\mu\text{g/ml}$).

Discussion

In the present study, we investigated the role of adiponectin on bone formation *in vivo*, using an adiponectin-producing adenovirus. The major finding of the present study was that adiponectin supplement increased bone mass in trabecular bone. Analysis of the mechanism of this action revealed that adiponectin acts by reducing the differentiation and bone-resorption activity of osteoclasts, and possibly by enhancing the differentiation and mineralization activity of osteoblasts.

We observed the increase of bone mass only in trabecular bone, but not in cortical bone, of adiponectin-adenovirus-treated mice. The duration of adiponectin overproduction by adenovirus treatment is at most 2 weeks. This could explain, at least in part, the effect of adiponectin on trabecular bone and the lack of such effect on cortical bone, since the former has a higher remodeling activity than the latter [37]. We also observed a significant decrease in osteoclast count and plasma NTx levels in adiponectin-adenovirus-treated

mice, indicating that adiponectin inhibits bone resorption. These findings were confirmed in tissue culture experiments. In mouse bone marrow macrophages (M-BMMs) and CD14-positive human peripheral blood mononuclear cells (PBMCs), adiponectin inhibited the M-CSF- and RANKL-induced differentiation of these cells into osteoclasts, as well as the bone-resorption activity of mature osteoclasts. Furthermore, we found that both bone tissue and primary osteoclasts expressed both adipoR1 and R2 (Supplemental Fig. 1), suggesting that adiponectin directly targets osteoclast cells. These results demonstrate that adiponectin suppresses bone-resorption activity by inhibiting osteoclastogenesis.

In adiponectin-adenovirus infected mice, plasma glucose and insulin levels did not change compared to control-adenovirus infected mice, indicating that adenovirus treatment did not affect glucose metabolism systemically (data not shown). This is consistent with our previous report, in which we demonstrated that adiponectin-KO mice showed normal glucose and insulin levels in plasma, and adiponectin-adenovirus has no

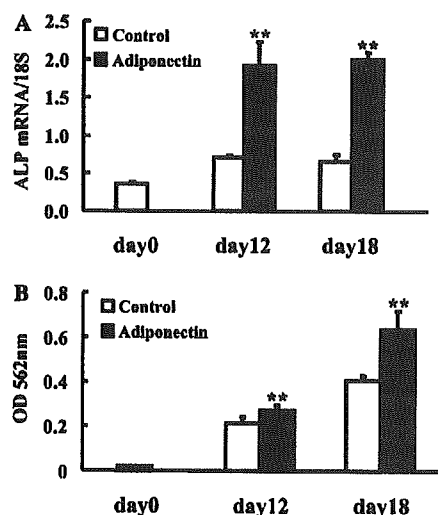


Fig. 4. Effects of adiponectin on MC3T3-E1 osteoblasts. (A) ALP mRNAs in MC3T3-E1 cells treated with or without adiponectin for 12 or 18 days were measured by real-time quantitative reverse transcriptase-PCR as described in Materials and methods. Data are normalized with 18S RNAs and expressed as means \pm SEM of three independent experiments. ** $P < 0.01$, compared with the control. (B) The mineralization activity of MC3T3-E1 cells treated with or without adiponectin for 12 or 18 days was measured as described in Materials and methods. Data are expressed as means \pm SEM of three independent experiments. ** $P < 0.01$, compared with the control.

effect on plasma glucose and insulin levels under control diet, although when adiponectin-KO mice are fed with high fat diet, their plasma glucose and insulin levels significantly elevate compared to wild-type mice and adiponectin-adenovirus reverses them to the level of wild-type mice [19]. Therefore, this effect of adiponectin should not be mediated by its systemic insulin-sensitizing effect in normal mice. However, whether it is mediated by local insulin-sensitizing effect remains to be elucidated.

The bone-forming activity of osteoblasts is also important in determining bone mass [37,38]. Our study revealed that adiponectin increased mRNA expression of ALP and mineralization activity of mouse MC3T3-E1 osteoblasts. We also investigated the effect of adiponectin on the osteoblastogenesis of primary mouse osteoblasts and mouse bone marrow cells. However, the effect was not significant on these cells (data not shown), indicating that adiponectin may have the potential to activate osteoblasts, but its effect may be dependent on the cell types.

In conclusion, we have demonstrated in the present study that adiponectin increases bone mass by suppressing osteoclastogenesis and possibly by activating osteoblastogenesis. These results suggest that increasing the concentration and/or enhancing the activity of adiponectin might be therapeutically beneficial for patients with reduced bone mass.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.03.210.

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CASE REPORT

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Heterotopic ossification in bilateral knee and hip joints after long-term sedation

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Key words Heterotopic ossification · Long-term sedation · Intact osteocalcin

Introduction

Heterotopic ossification (HO) is one of the disorders causing ankylosis. This syndrome is most commonly seen following neurological disorders such as traumatic brain or spinal-cord injury, or following joint surgery or severe burns [1–4]. HO occurring in a heavily sedated and immobilized patient in the absence of any anatomical central nervous system lesion is unusual (although Dellestable et al. [5] reported this in five patients undergoing artificial ventilation). Pathogenic mechanisms and appropriate treatments, including surgical timing for excision, have, therefore, not been defined for this form of HO. Herein we report procedures, including monitoring of ossification markers, which resulted in good prognosis, for a patient with HO following long-term sedation with artificial ventilation.

Case report

The patient was a 42-year-old man with limited joint motion of the bilateral hips and knees. He had undergone valve-

replacement surgery for aortic incompetence. He developed methicillin-resistant *Staphylococcus aureus* (MRSA) fibrous mediastinitis with swelling at the surgical site on postoperative day 14. Intermittent irrigation of the incision, under intubation anesthesia, was therefore performed, for 44 days, from postoperative days 16 to 59. After MRSA screenings yielded negative results, the patient displayed limited joint motion in the bilateral hips and knees, and was referred to our institute 3 months after the cessation of the long-term sedation.

On admission, passive and active range of motion (ROM) in the knee joints was 20°–40° flexion in the right knee and 10°–30° flexion in the left knee. The ROM for flexion/extension, adduction/abduction, and internal/external rotation in the right and left hip joints was 50°/–30°, 30°/10°, and 0°/20° in the right hip and 70°/–50°, 40°/0°, and 30°/–10° in the left hip, respectively. No pain was reported on motion in any joint, but he was unable to stand without assistance. Ambulatory ability was restricted to a slow gait over a short distance.

Radiography of the knee joints revealed HO from the medial epicondyle of the femur to the inferior facet of the patella (Fig. 1a–c). Radiography of the hip joints showed HO from the greater trochanter to the acetabular edge. Bone scintigraphy, using 99m technetium-labeled methylene diphosphonate, showed very high incorporation in the bilateral hip and knee joints, and slightly elevated incorporation in the left shoulder joint (Fig. 2).

Levels of the bone metabolic markers serum alkaline phosphatase (ALP), serum intact osteocalcin (iOC), and urinary deoxypyridinoline (DPD/creatinine [Cre]) were high, at: 488 IU/l (normal, 70–300 IU/l); 19.5 ng/ml (normal, <7 ng/ml); and 21.2 nmol/mmol Cre (normal, 2.1–5.4 nmol/mmol Cre), respectively (Fig. 3).

The patient received oral administration of disodium etidronate, 1000 mg/day, and indomethacin, 50 mg/day, until the day before surgery was performed for HO. Exercises had been performed to maintain ROM. For 7 months from the time of admission to our institute, and for 4–10 months after his emergence from long-term sedation with artificial ventilation, no new development of HO was detected on

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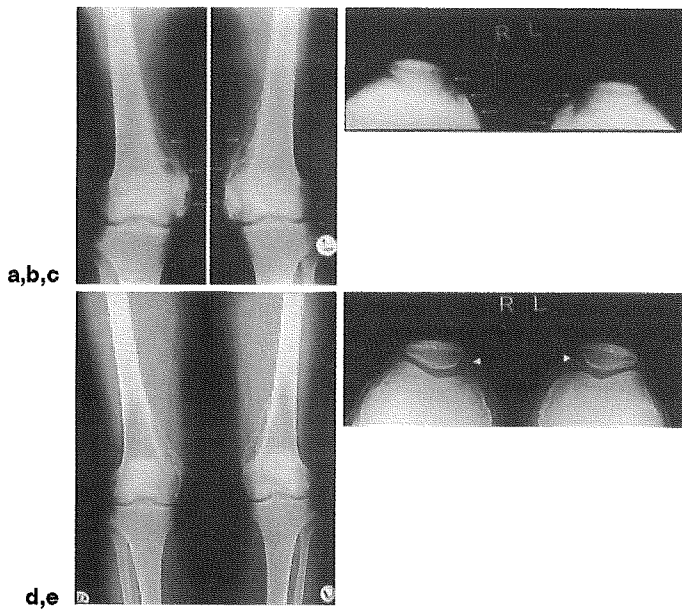


Fig. 1. Radiography of knee joints before and after resection. **a-c** Heterotopic ossification (HO; *arrows*) before resection, from the medial epicondyle of the femur to the inferior facet of the patella. **a** Anteroposterior (AP) view of right knee; **b** AP view of left knee; **c** skyline view. **d,e** No recurrence of HO (*arrowheads*) in **e** was detected in knee joints at 4.5 years postoperatively

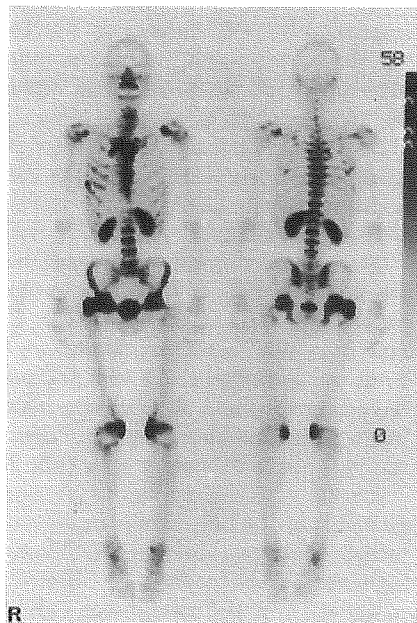


Fig. 2. Bone scintigraphy, using ^{99m} technetium-labeled methylene diphosphonate, before resection. high incorporation is apparent in the bilateral hip and knee joints, with slightly elevated incorporation in the left shoulder joint

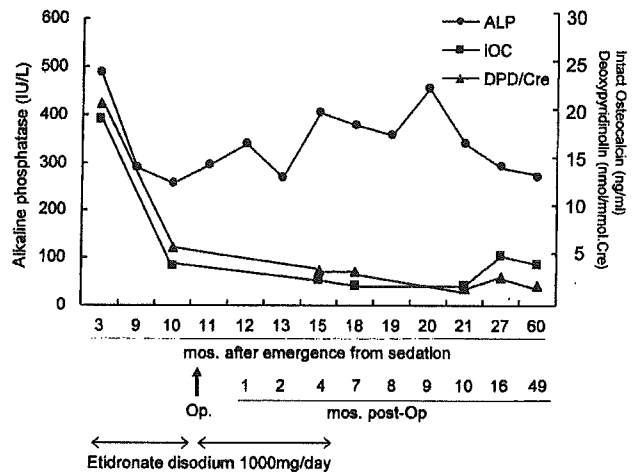


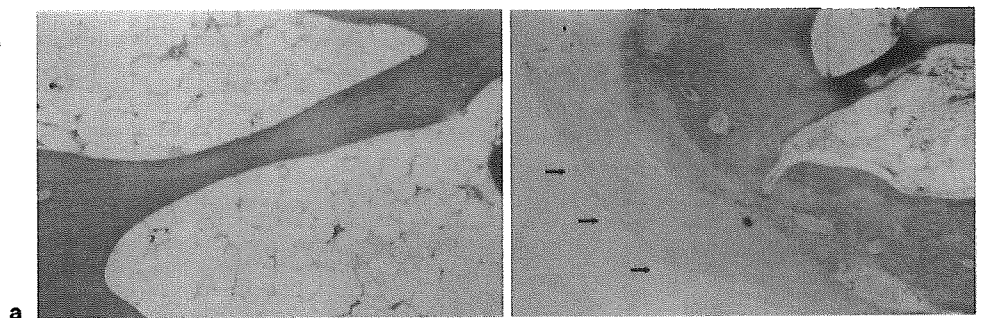
Fig. 3. Time course of biochemical markers of bone metabolism. Alkaline phosphatase (ALP) and serum intact osteocalcin (iOC) levels decreased to within normal ranges by 10 months after the cessation of long-term sedation. The urinary deoxypyridinoline/creatinine (DPD/Cre) level was still slightly higher than the normal range at 10 months. The ALP level increased temporarily at 4–10 months postoperatively (*post-Op*), and had normalized at 18 months postoperatively. Serum iOC and urinary DPD/Cre levels were within normal ranges throughout the follow-up period, *mos.*, months

radiography or computed tomography. Serum levels of ALP and iOC were normalized (serum ALP, 258 IU/L; serum iOC, 4.1 ng/ml) by 10 months after the cessation of long-term sedation. However, urinary DPD/Cre remained slightly above the normal range (6.1 nmol/mmol Cre) at this time. These data, showing no progression of HO and decreased levels of bone metabolic markers, implied the maturation of HO.

Areas of HO were thus resected at 11 months after the end of long-term sedation. Intraoperative macroscopic findings showed that areas of HO were located in the joint capsule and extracapsular muscle, and collateral ligaments were easily separated from the HO. Partial resection of the HO was performed until suitable ROM was achieved. This resulted in amelioration of the limited joint motion, in which ROM was changed from 115° to 0° of flexion in the left knee and from 110° to 0° of flexion in the right knee. Histological examination showed that most areas of resected HO were lamellar bone, with woven bone revealed in a few areas (Fig. 4a, b). No cartilage was present in the resected bone tissue.

Postoperative administration of disodium etidronate and indomethacin, and ROM exercises were employed from postoperative day 1 until 6 months after surgery. Postoperatively, the patient was able to stand unaided and he regained good ambulatory ability without any operative intervention at the hip joints. In view of the high risk of surgery-associated hemorrhage (due to warfarin sodium administration after the patient's aortic valve replacement) and the risk of development of thrombus if administration of warfarin sodium was suspended, resection of HO of the hip joints was not undertaken. The patient resumed office work 6 months after the knee operation. As of 4 years and

Fig. 4. Histological findings. Most areas of resected HO were lamellar bone (a), with woven bone in a few areas. Arrows show woven bone area. (b) (hematoxylin and eosin, $\times 100$ a and b)



6 months postoperatively, good ROM of the knee joints was maintained, with 110° to -5° of flexion in the left knee and 95° to -5° of flexion in the right knee. No recurrence of HO was detected in knee joints on radiography at 4.5 years postoperatively (Fig. 1d, e). ROM in the hip joints improved slightly without surgery, possibly due to increased daily motion of the hip joints. No progression of HO has been detected in hip joints on radiography. Serum ALP levels increased temporarily at 4–10 months postoperatively, and had normalized by 18 months postoperatively. Conversely, serum iOC and urinary DPD/Cre levels were within normal ranges throughout the follow-up period (Fig. 3).

Discussion

The etiology of HO formation is primarily categorized as neurogenic or traumatic. Neurogenic HO is commonly seen subsequent to traumatic brain or spinal-cord injuries, while traumatic HO typically occurs due to injury, burns, or hip-joint surgery [1–4,6]. Because the excision of immature HO is associated with high morbidity, due to recurrence and bleeding, allowing for maturation of the area of HO is important. Although the maturity of the HO was determined in the current patient by the lack of growth and marginal sharpening of HO on serial radiography, and by decreased uptake on serial bone scans, exact assessment may be difficult. The recommended interval after onset to allow for suitable maturation is 6 months for neurogenic HO and 12–18 months for traumatic HO [6,7]. In contrast, suitable periods for the maturation of HO in patients who have had long-term sedation with artificial ventilation remain unclear. However, two possible pathogenic processes seem to have been involved in our patient. One possible etiology is that sedation induced a pathogenic condition resembling neurogenic HO. The other is that long-term ventilation may cause changes in local tissue PO₂ and pH, which could subsequently result in HO formation [8,9].

Mysiwi et al. [10] showed that serum osteocalcin level was not a valuable adjunct in confirming a diagnosis of neurogenic HO after severe traumatic brain injury. However, some differences exist between the items assessed in our study and theirs. We assessed serum iOC, whereas Mysiwi

et al. [10] used serum osteocalcin. Serum iOC is more specific for bone formation than serum osteocalcin, as serum osteocalcin comprises accumulated heterogeneous fragments possibly produced as a result of catabolic breakdown in bones [11,12]. Another difference lies in items evaluated. The report by Mysiwi et al. [10] was an observational study examining the utility of OC for diagnosis, while our longitudinal data show changes useful for assessing the maturation of HO. The increased bone metabolic activity induced by fracture healing has already been described [13–15]. Akesson et al. [16] showed that increases in osteocalcin level were more pronounced than increases in serum ALP after fracture. Ohishi et al. [17] showed that the bone resorption marker, urinary DPD/Cre, increased more promptly than osteocalcin level after fracture. We therefore considered that iOC (which is more specific than osteocalcin) and urinary DPD/Cre might be useful as markers of HO maturation, although no previous reports have described the use of urinary DPD/Cre and iOC levels during HO after long-term sedation.

In the current patient, a 179% increase in serum iOC and a 292% increase in urinary DPD/Cre above upper normal limits were found at the initial measurement, 3 months after the cessation of sedation. A similar but less pronounced increase in serum ALP was evident, at 62%. This increase in bone metabolic markers may have resulted from both the compulsory bed rest during long-term sedation, and the HO formation. Kim et al. [18] reported that DPD/Cre increased significantly and that osteocalcin tended to decrease during bed rest. Conversely, Wilkinson et al. [19] demonstrated that patients with HO displayed significant elevations in osteocalcin, but not in DPD/Cre. These findings suggest that increases in iOC may reflect increased bone formation activity in HO, while increases in DPD/Cre reflect high bone turnover due to bed rest. After the administration of disodium etidronate, the bone metabolic markers gradually settled. This decrease in bone metabolic markers may have been the result of several mechanisms, including the natural course of HO maturation and changes accompanying increased physical activity after the cessation of sedation. Disodium etidronate has been widely used in patients with HO to prevent the progression and recurrence of HO [20]. In animal studies and experimental models of HO, disodium etidronate has been demonstrated to reduce bone formation [21,22]. The decrease in bone metabolic

markers may, thus, have been accelerated by disodium etidronate.

We decided to perform surgical resection of the HOs at 11 months after the cessation of the long-term sedation, as the patient's serum ALP, serum iOC, and urinary DPD/Cre levels had decreased to around normal ranges, and the progression of HO on imaging had stopped at 10 months after this cessation. Histopathological examination of HO specimens identified lamellar bone with no cartilage. No recurrence of HO has been found over the past 4 years, and the postoperative improvements in ROM and the normalized serum iOC levels have been maintained.

In the current patient, determination of serum iOC, along with serum ALP, serial radiography, and serial bone scintigraphy, facilitated the preoperative monitoring of HO maturation and the postoperative monitoring of remission. To the best of our knowledge, this report is the first to show the utility of serum iOC in assessing heterotopic bone maturation.

There are few reports of HO caused by inhibition of the central nervous system, without injuries or lesions, in ventilated patients receiving long-term sedation, while the functional immobility caused by joint contracture is well known. The use of sedation has been increasing in intensive care units with recent improvements in advanced clinical care in various fields. Clinical care physicians should be aware of this potential complication, as HO may lead to limited ROM in patients undergoing artificial ventilation for long-term sedation.

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Nurse-like Cells From Patients With Rheumatoid Arthritis Support the Survival of Osteoclast Precursors Via Macrophage Colony-Stimulating Factor Production

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Objective. To elucidate the role of nurse-like cells (NLCs) obtained from rheumatoid arthritis (RA) patients in bone loss during progressive synovial expansion.

Methods. CD14 monocytes were cocultured with NLCs for 4 weeks and collected as NLC-supported CD14 (NCD14) monocytes. To determine their ability to differentiate into osteoclasts, NCD14 monocytes were further cultured with macrophage colony-stimulating factor (M-CSF) together with RANKL or tumor necrosis factor (TNF). NCD14 monocytes were also cocultured with SaOS-4/3 cells, which were shown to support osteoclastogenesis in response to parathyroid hormone (PTH). CD14 monocytes were cocultured with SaOS-4/3 cells to elucidate how SaOS-4/3 cells and NLCs supported CD14 monocytes for a long period. Synovial expansion adjacent to bone in RA patients was examined immunohistochemically to detect osteoclast precursors such as NCD14 monocytes.

Results. NLCs supported the survival of CD14 monocytes for 4 weeks. NCD14 as well as CD14 monocytes differentiated into osteoclasts in the presence of M-CSF together with RANKL or TNF. NCD14 monocytes also differentiated into osteoclasts

in PTH-treated cocultures with SaOS-4/3 cells. SaOS-4/3 cells supported the survival of CD14 monocytes for 4 weeks in the presence, but not absence, of PTH. Treatment of SaOS-4/3 cells with PTH up-regulated the expression of M-CSF messenger RNA. Neutralizing antibodies against M-CSF inhibited the NLC-supported survival of CD14 monocytes. CD68 monocytes and M-CSF fibroblast-like synoviocytes were colocalized in regions adjacent to the destroyed bone of RA patients.

Conclusion. Our findings suggest that NLCs are involved in RA-induced bone destruction by maintaining osteoclast precursors via production of M-CSF.

Osteoclasts, the multinucleated cells that resorb bone, originate from the monocyte/macrophage lineage. Recent studies have established that bone-forming osteoblasts (or, bone marrow-derived stromal cells) are involved in the differentiation and function of osteoclasts (1–4). Macrophage colony-stimulating factor (M-CSF), which is produced by osteoblasts, is an essential cytokine for osteoclast formation. Osteoblasts also express RANKL, another cytokine involved in osteoclast differentiation, as a membrane-associated cytokine. Osteoclast precursors express RANK (the receptor for RANKL), recognize RANKL expressed by osteoblasts through cell–cell interaction, and differentiate into osteoclasts in the presence of M-CSF (1–4). Osteoprotegerin (OPG), which is produced mainly by osteoblasts, is a soluble decoy receptor for RANKL and blocks osteoclastogenesis by inhibiting RANKL–RANK interactions (5,6). Bone resorption–stimulating hormones and cytokines enhance the expression of RANKL in osteoblasts. Recent studies have shown that tumor necrosis factor (TNF) stimulates osteoclast differentiation in the presence of M-CSF through a mechanism independent of the RANKL/RANK system (7) and that

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