あり，アグリカン合成を反映するマーカーと考えられている[2]．とくに846エピトープを有するアグリカンはHAとの凝集能を有する完全なアグリカンであることが知られている[11]．

3．ADAM-TSやMMPによるネオエピトープ

ADAM-TSやMMPの作用によってIGDから生じた新しい断端（ネオエピトープ）は関節液をセシウムクロライド溶液中で超遠心した後に，ネオエピトープに特異的な抗体を使用してSDS-PAGE後にwestern blottingを行うことにより検出されている[12]．ADAM-TS4によるネオエピトープに対してはIGD領域における N 末端シークエンス ^{374}ARGS-を認識する抗体を使用する．MMPによるIGD領域での切断ネオエピトープについては，^{342}FFGV-を認識する抗体が使用される．

4．HA結合能を有するアグリカンフラグメント

プラスティックプレートに固定された抗ケラタン硫酸抗体によって補足されたアグリカン分子に抗G1ドメイン抗体を反応させることによってケラタン硫酸とG1ドメインの両者を有するアグリカン分子を選択的に測定する手法が開発されている[13]．前述したように，この測定法はケラタン硫酸とHA結合部分をあわせもつという関節軟骨に直接，由来した可能性がきわめて高いアグリカン分子を選択的に測定しており，生体内に残存する関節軟骨量をよく反映している．

5．マーカーの生理的変動

関節軟骨を構成するマトリックスであるアグリカンに由来する分子は荷重ストレスや運動によって軟骨組織からの遊離が増加するので，歩行や運動を行った後には関節液中や血清濃度が増加する．また，日内変動としては，ほとんどのマーカーは夜間にピークをもつ．よって早朝でかつ非運動時の検体採取を心がけるべきである．また，マーカー分子の最終的代謝過程を考慮すると，肝や腎疾患のある患者では高値を示す可能性があり注意を要する．

臨床的意義

1．病態評価の診断マーカーとして

OA患者の関節液中に存在するアグリカンに由来するGAGについては健常軟骨に比較的特異性が高いとされるC6Sと変性軟骨や滑膜に由来するC4Sが代表的である．股関節OAの関節液の検討では，C6Sの関節液中濃度は残存軟骨の総量を反映してX線病期の進行とともに減少傾向を示した．ただしOA関節液では貯留する関節液量による希釈の影響により，マーカーの濃度は大きく変動する．この関節液量の影響を小さくするためには，マーカー間の比率をとることが簡便な手法である．股関節OAにおけるC6S/C4Sは病期の進行とともに有意差をもって低下しており，関節液量の影響を受けにくい指標として有用であった（図3）[8]．関節液中のアグリカン由来フラグメントは，軟骨細胞による合成と破壊の結果，関節液中に放出されてくるので，これらのマーカーは残存する軟骨量のほかに，そのマトリックスの代謝回転を総合的に反映している．

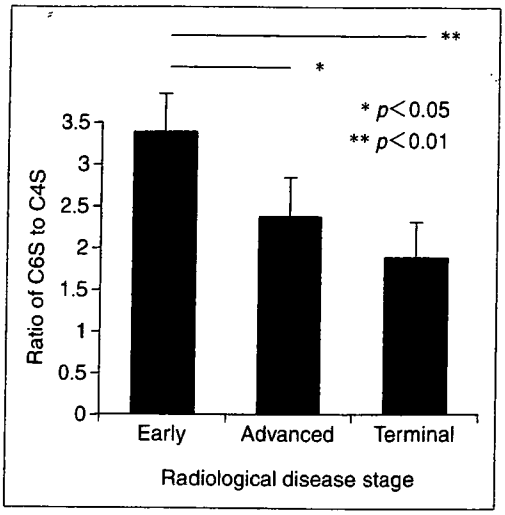

図3　股関節OAの関節腋中C6SとC4Sの比率（病期間の比較）
変形性股関節症の関節液中C6SとC4Sを高速液体クロマトグラフィーにより測定した．X線上の病期の進行に伴いC6S/C4Sは低下する．（文献[8]より引用改変）

膝十字靱帯や半月などの損傷に際しては軟骨損傷の合併が多く，将来的にOAを発症する可能性がある．よって，これらの膝外傷後の軟骨病態を把握することは臨床上，重要な課題である．これらの関節外傷後の関節液中C6S，C4Sを測定した結果，受傷後早期にきわめて高値を示し，以後すみやかに漸減することが報告されている（図4）[14]．とくに受傷後の急性期におけるC6Sの上昇は，損傷軟骨から直接，遊離したものであ

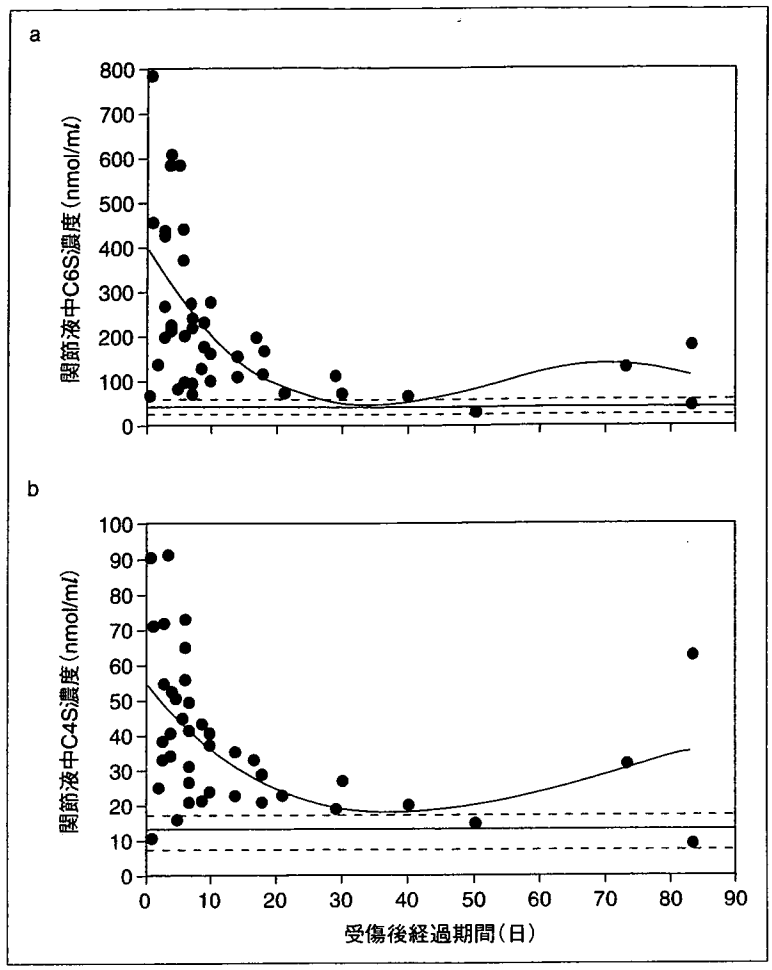

図4 膝十字靱帯損傷後の関節液中C6SおよびC4S
a：C6S，b：C4S．横実線：変形性関節症における中央値(a：50.75nmol/ml，b：12.7nmol/ml)，横破線：変形性関節症における25%および75%分布域．
(文献[14]より引用改変)

り，主に軟骨マトリックスの破壊の程度を反映するものと推察している．

エピトープ3-B-3(−)と846はアグリカン合成のマーカーとされている．これらのエピトープは正常人ではほとんど検出されないが，OA軟骨で増加し，疾患重傷度との相関も認められている．エピトープ846の関節液中濃度は，Ⅱ型コラーゲンの合成マーカーであるコンドロカルシン濃度と相関を示し，アグリカンとコラーゲンの修復，合成がリンクしていることを示唆している[15]．

2．治療効果予測のマーカーとして

関節マーカーの臨床応用のひとつに治療効果の予測がある．OAはきわめて有病率の高い疾患であるので，治療開始前に，その治療による有効性を予測できれば医療経済上も有用である．膝OAに対する代表的な保存療法であるHAの注入療法開始前の関節液中に存在するHA結合型アグリカン分子を前述の方法により測定した結果，注入前アグリカン濃度と注入後4，8週後の膝JOAスコア改善度とのあいだには有意の正の相関が認められた．すなわちアグリカン濃度が高い症例ほどJOAスコア改善が良好であった(図5)[16]．関節液中のHA結合型アグリカンレベルは残存軟骨量とその代謝活性に依存しているので，本結果はHA注入療法が有効であるためにはアグリカン代謝が維持されていること，すなわち軟骨が

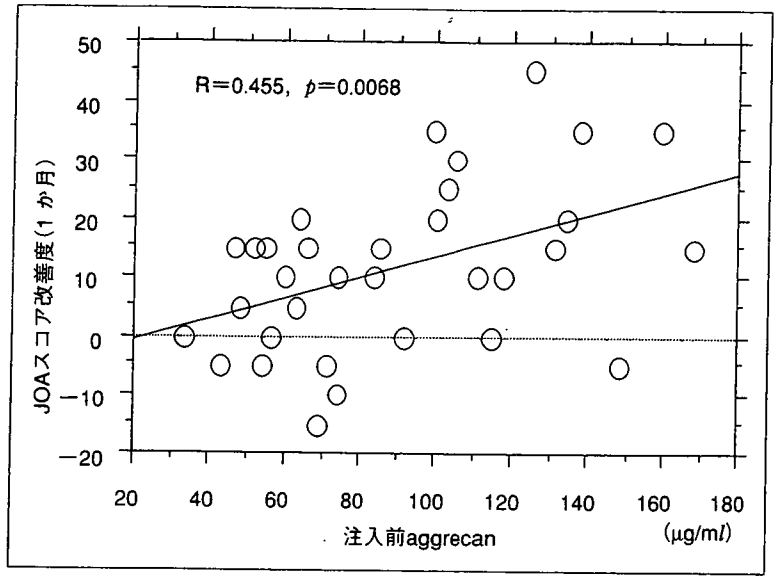

図5　膝OAに対するHA注入療法前の関節液中HA結合型アグリカン濃度と1か月後のJOAスコア改善度の関係
注入前のアグリカン濃度が高い症例ほどJOAスコア改善が良好であった．
（文献[16]より引用改変）

残存し，かつ活発に代謝を行っていることが前提になることを示唆している．以上の結果はアグリカン由来マーカーがHA注入療法の臨床的有効性予測に有用であることを示している．

おわりに

本稿ではアグリカン由来マーカーについて概説した．アグリカンは関節軟骨の機能維持上，重要なマトリックスであり，その関節軟骨における合成，異化は関節疾患の病態に密接に関係している．よってマーカーとしての価値は高い．アグリカン由来マーカーを臨床的に使用していく際には，対象としている関節軟骨に由来するアグリカン分子を選択的に測定する手法を確立していくことが重要である．

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変形性膝関節症の疫学

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Epidemiology of Knee Osteoarthritis

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臨整外 42：7～14，2007

Key words：knee osteoarthritis(変形性膝関節症)，epidemiology(疫学研究)，risk factors(危険因子)

変形性膝関節症(膝 OA)は，common disease であり，本症の発症と進行には多くの因子が関わっている．膝 OA の発症と進行のメカニズムやそれに関わる危険因子，さらに疾患自体の自然経過を明らかにする目的での疫学的研究は極めて重要であり，これまでに欧州や米国，そして日本でも様々な疫学調査が行われてきた．これらの研究から，膝 OA に影響する因子として，年齢，性別，人種，体重，膝外傷・手術の既往，下肢筋力，膝内反アライメントおよびスラスト運動，骨粗鬆症，性ホルモン，職業・日常活動性・生活習慣，喫煙，ビタミンなどの微量栄養素，代謝性疾患，遺伝子など多くの内容が挙げられている．しかし，これらの因子のうち影響のメカニズムを含めて明らかになっているものは少なく，今後多方面からのさらなる研究が必要と考えられる．

はじめに

変形性膝関節症(以下，膝 OA)によって生ずる関節変形，運動痛や可動域制限は，人間の起立歩行動作に大きく影響し QOL の低下に直結する．膝 OA の病態解明には，その自然経過や発症・進行に関わる危険因子を明らかにする目的での疫学研究は極めて重要であり，これまでに様々な調査が行われてきた．

本稿では，膝 OA に対する疫学研究とその結果として解明されている膝 OA の危険因子について概説する．なお，膝 OA の診断と病期は X 線所見により定義されるため，本稿における「膝 OA」も原則として X 線上診断された変形性膝関節症を意味する．

これまでに行われた代表的な疫学調査(表 1)

一般者を対象とした野外調査としては，欧米では Lawrence ら[20)]が 1958～1960 年に英国で 2,296 名の男女を対象に行った横断調査が最初の大規模な疫学調査である．その後，Davis による NHANES-I[2)]や Felson らによる Framingham study[9)]などが行われている．また，近年中国(Beijing study[41)])などアジア諸国において欧米と同様のプロトコールによる調査が行われ人種による差異が検討されている．一方，わが国でも中条ら[24)]が Lawrence らの調査とほぼ同時期に東北地区において野外調査を実施している．その後，いくつかの調査が行われているがいずれも小規模かつ 1 回の横断調査であり，さらに膝 OA の X 線評価に独自の基準を使用しているものが多く，欧米の研究と比較検討が困難であった．この中で筆者ら[25)]が行った松

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表 1　これまでに行われた膝 OA に関する代表的な疫学調査(次頁に続く)

a：欧米

研究者 (調査名称)	実施年	場所	対象者数	調査方法など
Lawrence JS	1966	英国 Leigh, Wensleydale	2,296 名 男性：1,098 名 女性：1,198 名	横断調査 X 線撮影，アンケート，身体診察
Davis MA (NHANES-Ⅰ)	1971- 1975	米国 全米各地	5,248 名 男性：2,457 名 女性：2,791 名	横断調査＋縦断調査(1982-84 年：NHFES-Ⅰ) X 線撮影(非荷重)，アンケート，身体診察，血液検査
Schouten JSA (Zoetermeer study)	1975- 1978	オランダ Zoetermeer	422 名(1975-78 年) 233 名(1988-89 年)	縦断調査(1988-89 年) X 線撮影(荷重)，アンケート，身体診察
Felson DT (Framingham study)	1983- 1985	米国 Framingham, MA	1,424 名 男性：591 名 女性：833 名	横断調査＋縦断調査(1992-93 年) X 線撮影(荷重)，アンケート，身体診察，血液検査
Hart DJ (Chingford study)	1988	英国 Chingford	1,003 名 全例女性	横断調査＋縦断調査(1994 年) X 線撮影(荷重)，アンケート，身体診察，血液検査
Zhang Y (Bejing study)	1996	中国 北京	1,202 名 男性：465 名 女性：737 名	横断調査 X 線撮影(荷重)，アンケート，身体診察

代膝検診は，1,000 人以上の母集団を 20 年以上の長期にわたって縦断的に評価し，さらに病期分類を Kellgren-Lawrence 分類に基づいているため欧米の研究との比較が可能であり，わが国における膝 OA の疫学研究としては特筆に価する内容となっている．

過去の疫学研究から考えられている膝 OA の危険因子(表 2)

膝 OA に関するこれまでの疫学研究から様々な危険因子が想定されているが，因果関係が証明されたものといまだ不明であるものとが混在するのが現状といえる．

1. 年齢および性別(図 1)

過去のいずれの疫学調査においても膝 OA の発生頻度は基本的に年齢とともに増加し，かつ 50 歳以降は女性で 1.5～2 倍頻度が高くなっている．ところが，30～40 歳代では逆に男性が女性よりわずかながら膝 OA の発生率が高い報告が多く，半月板損傷や軟骨損傷などの膝外傷の影響が示唆され興味深い．しかし，若年者の膝 OA の発生率や外傷との関連性についての研究は少なく不明な点が多い．

膝 OA の有症率

膝 OA の有症率については，発生率と同様に報告により差がみられる．男性では平均 10%以下で，40～70 歳代までは加齢とともに増加するが，それ以上になると逆に低下する報告が多い．これに対し，女性では加齢とともに増加し，80 歳以上でも 15～22%の有症率を示す場合が多い．

2. 人種

既述したように膝 OA の疫学調査では研究のデザインが同一ではないことが多く，人種間の相違を単純に比較するのは注意を要する．米国で行われた NHANES-Ⅰ[2]では，黒人は白人に比べて膝 OA に対する危険度が男性で 1.4 倍，女性で 2.8 倍大きくなっている．Zhang ら[42]は Framingham study と同一デザインで北京で調査を行い，中国人女性が白人女性に比べて有意に膝 OA が多いことを報告している．また，Yoshida ら[39]は長崎県で

表 1　これまでに行われた膝 OA に関する疫学調査

b：日本

研究者 (調査名称)	実施 年	場所	対象者数	調査方法など
中条　仁	1966	東北地区 山形県，宮城県	2,244 名	横断調査 X 線撮影，アンケート，身体診察
小松原良雄	1968	大阪府 (八尾市，富田林市) 和歌山県，福井県	5,256 名 男性：2,292 名 女性：2,964 名	横断調査 X 線撮影，アンケート，身体診察，血液検査
古賀良生 (松代膝検診)	1979	新潟県 (松代町)	1,327 名(初回検診者) 男性：252 名 女性：1,075 名	縦断調査(7 年間隔：1979～2000) X 線撮影(荷重)，アンケート，身体診察
末松典明	1986	北海道 (富良野地方)	347 名 男性：182 名 女性：165 名	横断調査 X 線撮影(荷重)，アンケート，身体診察
竹日行男	1989	群馬県 (草津町)	320 名 男性：102 名 女性：218 名	横断調査 X 線撮影，アンケート，身体診察
須藤啓広	1999	三重県 (宮川村)	597 名 男性：204 名 女性：393 名	横断調査 X 線撮影，アンケート，身体診察，骨量測定
Yoshida S	1999	長崎県 (肥前大島町)	586 名 全例女性	横断調査：米国 Framingham study と比較検討 X 線撮影，アンケート，身体診察
Yoshimura N	2002	和歌山県	202 名 全例女性	横断調査：Case-control study，英国と比較検討 X 線撮影，アンケート，身体診察

の調査を Framingham study と同一の方法で比較し，日本人女性は白人女性に比べて膝 OA の危険度が 1.9 倍高いと述べている．今後，同様の研究により人種間の相違や特徴が明らかになると思われる．

3. 肥満

過去の研究において肥満と膝 OA との有意な関連性を示す報告は多い．肥満の指標として BMI (body mass index)が用いられることが多く，NHANES- I では BMI>30 の場合，男性で 4.78 倍，女性で 3.87 倍危険度が増加するとしている．また，Schouten ら[31]はオランダで行った調査 (Zoetermeer study)で，BMI が 23 以上で 1.56 倍，25 以上で 3.82 倍に危険度が増すと報告した．わが国では，松代膝検診で BMI>25 の場合，男性でオッズ比が 2.63，女性で 3.11 となっており[4]，Yoshimura ら[40]や須藤ら[37]も BMI と膝 OA の有意な相関関係を述べている．また，肥満が膝 OA に与える影響のメカニズムについては，これまでの研究では高脂血症や高血圧，高血糖といった代謝性疾患による作用よりも膝関節にかかる荷重負荷による作用が大きい．

4. 代謝性疾患[14,22]

ピロリン酸カルシウム結晶(CPPD)の膝 OA との関連は古くから注目されており，膝 OA 患者の関節液では 50～60%に CPPD を含めた結晶性物質が存在し，OA の進行とともに増加すると言われている．しかし，全身的な高尿酸血症の影響については否定的な報告が多い．その他，高脂血症，血糖値，高血圧についても単因子，多因子解析を含めて様々な報告があるが一定の見解はなく，現時点では全身的な代謝性疾患の膝 OA への直接的

表 2　膝 OA のリスクファクターと相対危険度

リスクファクター	膝 OA 発症への影響	相対危険度
Occupational activity	heavy knee demand, heavy lifting work	1.7-3.4
Sports activity	high level, elite former athlete	1.3-6.5
	low level, recreational sports (↓risk)	insufficient data
Knee injury	ligament, cartilage, meniscus	5.2-14.0
	surgical menisectomy	2.6-4.8
Knee alignment	varus alignment (med knee OA)	4.0
	valgus alignment (lat knee OA)	2.0
Muscle strength	low quadriceps strength (↑risk)	insufficient data
Smoking	average smoking	0.7
Race	black women	2.1
	Japanese	1.9
	Chinese (women/men)	1.5/0.9
Obesity	high BMI	3.2-34.7
Bone	high BMD	1.1-2.3
Nutrients	Vitamin C intake	0.3
	betacarotine	0.4
	low vitamin D	1.02-2.9
Sex hormone	estrogen use	0.3-3.3

BMD：bone mineral density, BMI：body mass index

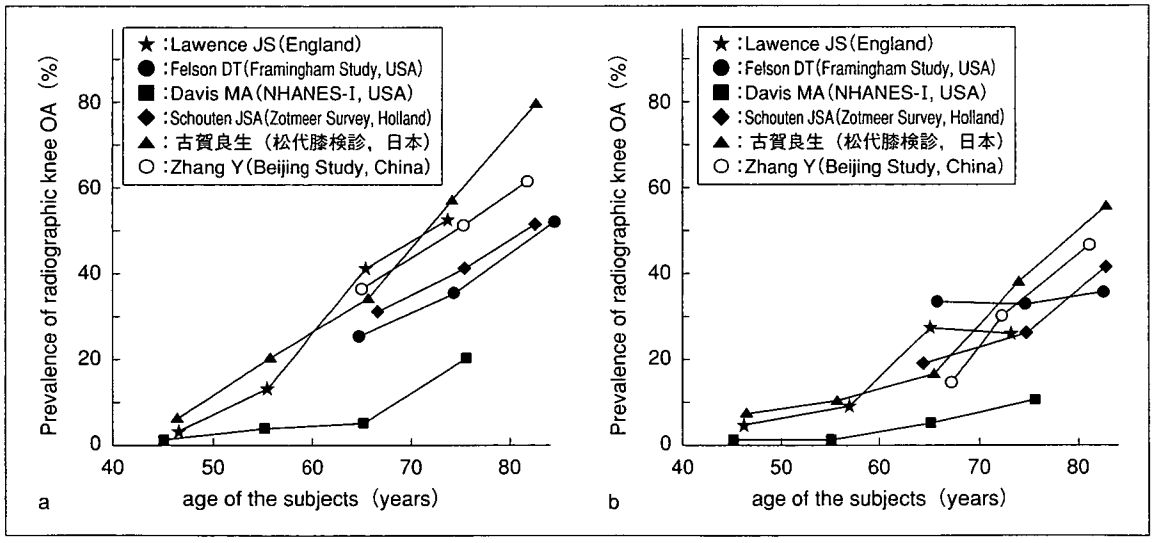

図 1　過去の疫学調査による膝 OA の発生率
a：女性　b：男性

な関与は明らかではない.

5. 喫煙

NHANES-I[2]や Framingham study[10]では喫煙習慣は膝 OA に予防的に作用することを示しており, Hart ら[15]は Chingford study の解析から喫煙は骨棘形成，関節裂隙狭小化のいずれにも影響しないと述べている．しかしながら，喫煙が膝 OA に及ぼす生物学的なメカニズムはほとんど解明されていない.

表 3　職業および日常活動性の膝 OA への影響

職業および日常活動性	膝 OA への影響
・炭鉱労働者[1]	男性で影響あり
・港湾労働者[2]	男性で影響あり
・膝屈曲を要する職業[3] （大工，トラック運転手など）	男性で 2.5，女性で 3.5(OR)*
・力を要する職業[3] （農夫，大工など）	男性で 1.8，女性で 3.1(OR)
・膝屈曲＋力仕事[4]	男性で 2.2，女性で 0.3(OR)
・しゃがみ込み動作(1 日 30 分以上)[5]	6.9(OR)
・膝つき動作(1 日 30 分以上)[5]	3.9(OR)
・階段昇降(1 日 10 段以上)[5]	2.7(OR)
・しゃがみ込み動作(1 日 1 時間以上)[6]	女性で 1.2(OR)
・階段昇降(1 日 30 段以上)[6]	女性で 1.19(OR)
・椅子の腰掛け(1 日 2 時間以上)[6]	女性で 0.77(OR)
・しゃがみ込み動作(1 日 2 時間以上)[7]	女性で 2.4(OR) 男性で 2.0(OR)

1) Kellgren JH, Lawrence JS(1952), 2) Partridge RE(1968), 3) Anderson JJ(1988)
4) Felson DT(1991), 5) Cooper C(1994), 6) Yoshimura N(2004), 7) Zhang Y(2004)
*OR：Odds Ratio

6. 職業，生活様式，日常活動性と運動(表 3)

炭鉱夫，港湾労働者，大工，農夫など重労働や膝屈伸を多用する仕事では，有意に膝 OA の発生率が高いという報告がみられる[1]．地域での生活習慣に関しては，エスキモー[5]やジャマイカの裸足生活者[3]が都市生活者に比べて膝 OA の頻度が高いという報告がある．また，日常生活動作では，しゃがみ込み動作や階段昇降は膝 OA を増加させ，逆に椅子の腰掛けは予防的に作用するという研究がみられる[40]．運動と膝 OA の関連については，ジョギングなど膝関節に対して軽度～中等度の負荷にとどまる運動の継続は膝 OA への影響は少ないとする報告が多い[27]．運動強度の高い種目については，後述する膝外傷との関連で検討されることが多く，Sandmark ら[30]はクロスカントリースキーやサッカー，アイスホッケーでは男性で 2.9 倍相対危険度が増すとしている．また，女性についても近年サッカーにおける前十字靱帯損傷後の膝 OA 発生との関連が報告されている[21]．

7. 膝外傷

疫学調査における膝外傷と膝 OA との関連性については，Gelber ら[11]は 1,321 名を対象とした 36 年間の調査で，膝外傷の既往がある場合，膝 OA 発症の相対危険度が 5.2 と報告しており，Yoshimura ら[40]は和歌山県での調査において女性の膝 OA のリスクファクターとして膝外傷の既往を挙げている．一方，患者を対象とした研究では，半月板切除と前十字靱帯損傷に関する検討が多い．半月板切除については，Fairbank[8]の報告以来膝 OA との有意な関連を示す研究が多く，Englund ら[7]は変性半月板の断裂と切除量が多いことが膝 OA 発症に影響するとしている．また，前十字靱帯損傷では Segawa ら[32]や Roos[29]が受傷後 12～14 年で半数近くに膝 OA が発症していると述べている．

8. 下肢筋力

Slemenda ら[34]は，膝 OA の女性では膝伸展力が対照群に比べて 15～18％低いと報告している．松代膝検診では，膝 OA の病期の進行に伴う大腿四頭筋力の低下がみられ，さらに縦断調査でスラスト(thrust)運動との関連が示されている[38]．最近の研究では，筋力の他に日常生活動作における大腿四頭筋の反応時間や膝屈筋とのバランス，関節位置覚や安定性との関連で膝 OA に影響するとした報告が散見される[17]．

表 4　膝 OA の候補遺伝子として報告されたもの

膝 OA の候補遺伝子として報告されたもの	膝 OA および類縁疾患との関連
COMP(cartilage oligomeric matrix protein)	偽性軟骨無形成症
COL11A1(human type-XI procollagen gene)	Stickler 症候群
COL2A1(human type-II procollagen gene)	軟骨形成不全，脊椎骨端異形成症など多数
VDR(vitamin D receptor gene)	骨粗鬆症，骨棘形成
Aggrecan	手指 OA
COL9A1(human type-IX procollagen gene)	股関節 OA
COL9A3(human type-IX procollagen gene)	股関節 OA
IGF1(insulin-like growth factor 1)	手指 OA，脊椎 OA
CRTL1(cartilage matrix protein gene 1)	手指 OA，股関節 OA
ER(estrogen receptor)	骨粗鬆症
PAPSS2 (3'-phosphoadenosine 5'-phosphosulfate synthase)	脊椎骨端異形成症
ASPN(Asporin)	膝 OA
AnK	CPPD 沈着
CALM1(calmodulin1)	股関節 OA
FRZB(serected frizzeled-related protein-3)	股関節 OA(女性)
IL-1	股関節 OA
MATN3(matrilin 3)	手指 OA
IL-4L	股関節 OA
ADAM12(metalloprotease)	股関節 OA

CPPD：ピロリン酸カルシウム結晶

9. 下肢アライメント，スラスト運動

Sharma ら[33]は膝 OA 患者を調べ，膝内反・外反アライメントが内側・外側型膝 OA を有意に進行させると報告し，われわれも松代膝検診において膝内反アライメントが膝 OA 発症の危険因子であることを明らかにしている[26]．また，立脚歩行初期にみられる膝の急激な内反運動であるスラスト運動は膝 OA の有力な危険因子と考えられており，その関連性が松代膝検診や Chang ら[6]によって示されている．

10. 骨粗鬆症

膝 OA に関する過去の疫学調査では，変形性関節症と骨粗鬆症は逆の作用を持つという仮説に基づいて研究が行われた．その結果，Framingham study[12]や Chingford study[13]では高骨密度と膝 OA の関連性が示され，わが国でも須藤ら[37]が同様の結果を報告している．しかし，近年の研究では高骨密度は膝 OA 発症に影響するが膝 OA の進展には低骨密度が関連するという報告もあり，現時点では骨粗鬆症と膝 OA の関連性は明らかであるが，その作用機序については今後の研究が待たれている．

11. 性ホルモン

Framingham study[41]や Chingford study[36]では，エストロゲン補充療法(ERT)は膝 OA に予防的に作用する結果が示されたが有意ではなかった．近年，ERT とアレンドロネートの併用が膝 OA の軟骨下骨変性に予防的に作用することが示されており[16]，今後疫学研究においても大規模な前向き調査が必要と考えられる．

12. 微量栄養素

Sowers ら[35]は，抗酸化物質としてのビタミン A，C，E およびベータカロチンは膝 OA の発症には影響しないものの進行および疼痛の軽減に有効であると述べており，McAlindon ら[23]は血中 25-hydroxy vitamin D の低下が膝 OA の進行を助長すると報告している．

13. 遺伝子(表 4)

膝 OA の遺伝形式は多因子遺伝であり，原因遺伝子よりも感受性遺伝子として研究される場合が多い．膝 OA の遺伝性については，Kellgren ら[19]が全身性関節症(GOA)の報告以後，軟骨形成不全症や Stickler 症候群の原因遺伝子として同定された COMP や COL2A1 を足がかりにして多くの遺伝子多形が発見された．さらに，これらの遺伝子多形の相関解析が行われているが，現在まで明らかな膝 OA の候補遺伝子として特定されたものはない[18,28]．本疾患の複雑な病態を考えると今後大規模な集団での解析が必要と考えられる．

おわりに

膝 OA は common disease であり，その発症と進行には多くの因子が関与している．これらのメカニズムおよびその自然経過を明らかにするためには，大規模集団に対する長期間の疫学的縦断研究は極めて重要である．

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Biochemical and Biophysical Research Communications 354 (2007) 846–851
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Impaired bone fracture healing in matrix metalloproteinase-13 deficient mice

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Received 19 December 2006
Available online 19 January 2007

Abstract

Vascular and cellular invasion into the cartilage is a critical step in the fracture healing. Matrix metalloproteinase-13 (MMP-13) is a member of the zinc-dependent endopeptidase family and plays an important role in remodeling of extracellular matrix. Therefore we investigated the possible involvement of MMP-13 in a murine model of stabilized bone fracture healing. Repair of the fracture in MMP-13 deficient (MMP-13$^{-/-}$) mice was significantly delayed and characterized by a retarded cartilage resorption in the fracture callus. Immunohistochemistry indicated severe defects in vascular penetration and chondroclast recruitment to the fracture callus in MMP-13$^{-/-}$ mice. Consistent with the observations, the chondrocyte pellets cultured from the MMP13$^{-/-}$ mice exhibited diminished angiogenic activities when the pellets were co-cultured with endothelial cells. These results suggest that MMP-13 is crucial to the process of angiogenesis during healing of fracture, especially in the cartilage resorption process.

Keywords: MMP-13; Fracture healing; Extracellular matrix; Angiogenesis; Chondroclast

Bone fracture triggers a steady cascade of bone regeneration. Under optimal conditions, fractured bone heals without scar formation and fully recovers its morphological and biomechanical properties. This reparative process consists of a variety of molecular and cellular events, which recapitulate several aspects of skeletal development [1]. Although various growth factors and cytokines that participate in fracture healing have been identified [2], the precise mechanism behind these processes has not been fully elucidated.

Matrix metalloproteinases (MMPs) constitute a family of zinc-dependent proteinases which have essential roles in degradation of extracellular matrix (ECM) components such as collagens and proteoglycans. They are involved in normal development and tissue dysfunction under various pathophysiological conditions including wound healing, arthritis, and tumor development [3]. Among the members of secreted-type MMPs, MMP-9, and MMP-13 are thought to be important to normal skeletal development in mice [4–6]. MMP-13 is primarily expressed in osteoblasts and hypertrophic chondrocytes, while MMP-9 is mainly expressed in osteoclasts [7]. In contrast to the difference in their expression patterns, MMP-9 deficient (MMP-9$^{-/-}$) mice and MMP-13 deficient (MMP-13$^{-/-}$) mice exhibited similar skeletal phenotypes characterized by the elongation of hypertrophic cartilage zone in the growth plates [4–6]. The skeletal defects in MMP-9$^{-/-}$ mice are explained by

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0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.12.234

the impaired angiogenesis as vascular invasion of calcified cartilage is a crucial step for endochondral bone formation [8]. Because of the critical involvement of MMP-13 in endochondral bone development, we conducted the following experiments to analyze the role of MMP-13 in fracture healing.

In the current study, we generated a stabilized bone fracture at the middle of the tibia in MMP-13$^{-/-}$ mice and the healing process was then compared with those of WT mice. Cartilage formation and the angiogenic activity of chondrocytes derived from MMP-13$^{-/-}$ and WT mice were also analyzed using an *in vitro* chondrocyte culture system. The lack of MMP-13 leads to a severe delay in fracture healing, which is characterized by prolonged absorption of the fracture callus. In addition, chondrocytes derived from MMP-13$^{-/-}$ mice exhibit diminished angiogenic activity. These observations deepen our understanding of fracture healing and further underscore the importance of MMP-13 during this pathological condition.

Materials and methods

Animals. MMP-13$^{-/-}$ and WT male littermates in a C57BL/6J and 129/Sv hybrid background were generated from the intercross between heterozygous MMP-13$^{+/-}$ mice. The generation of MMP-13$^{-/-}$ mice is described elsewhere [9]. All experiments were performed according to the protocol approved by the Laboratory Animal Care and Use Committee of Keio University School of Medicine.

Fracture model. Bone fractures were generated essentially as previously described [10]. Thirty 8-week-old mice were used in each group. Briefly, under general anesthesia with xylazine (0.2 mg /10 g body weight, Bayer) and ketamine (0.5 mg/10 g body weight, Sankyo), an anterior knee incision was made, and a transverse osteotomy was performed at the middle of the tibia with a bone saw (Volvere GX, NSK Nakanishi). Fractured bones were repositioned and stabilized by inserting the inner pin of a 23-gauge spinal needle intramedullary. The mice were euthanized by cervical dislocation at designated time points and their tibiae were excised.

Radiological analysis. Bone radiographs were taken with a soft X-ray instrument (CMB-2, SOFTEX). Microarchitecture of the fracture callus was evaluated by using a micro-CT system (Scan Xmate-A100S40, Comscantecno). Calcified area and bone mineral content (BMC) of the entire tibiae were measured by a single energy X-ray absorptiometry utilizing a bone mineral analyzer for small animals (PIXImus, LUNAR), and gain of the calcified area and % gain of the BMC were calculated.

Histological analysis. The harvested tibiae were fixed in 4% paraformaldehyde, decalcified in 0.5 M EDTA (pH 7.4), embedded in paraffin, and then cut into 4-μm sections. Alcian blue and van Gieson stainings were performed according to the standard procedure. Ratio of cartilage area and bone area to total callus area was measured by a planimetric method using NIH Image.

Histochemical detection of tartrate resistant acid phosphatase (TRAP) and immunohistochemistry of type II collagen, type X collagen, MMP-9, CD31, and cathepsin K were performed as previously described [11,12].

Cell cultures. Rib chondrocytes were isolated from neonatal MMP-13$^{-/-}$ and WT littermates, and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics. Human umbilical vein endothelial cells (HUVEC, Kurabo) were cultured in HuMedia-EG2 medium (Kurabo). All the cells were maintained at 37 °C in a humidified CO_2 incubator. Any chemicals if not mentioned otherwise were purchased from Sigma.

Cell differentiation assay. To induce chondrogenic differentiation, chondrocyte 3D culture was performed as previously described [13]. Briefly, chondrocytes were suspended in atelopeptide collagen solution (0.5% atelopeptide collagen (Kawaken Fine Chemicals)/5 mM NaOH/26 mM $NaHCO_3$/20 mM Hepes/one volume of 10 times concentrated α-minimal essential medium (αMEM)) at a density of 1×10^7 cells/ml. Each 20 μl of the mixture was placed into the bottom of 15 ml conical tubes (Falcon) and incubated for 1 h at 37 °C to form a gel. DMEM with 200 ng/ml recombinant human bone morphogenetic protein-2 (kindly provided by Astellas Pharma Inc.), 5 μg/ml insulin and antibiotics (chondrogenic medium) was gently poured onto the gel at a volume of 1 ml. The paraffin sections of the pellets were made after fixation with 4% paraformaldehyde and stained with Alcian blue.

Angiogenesis assay. For the *in vitro* angiogenesis assay, the chondrocyte pellets that had been cultured in chondrogenic medium for 3 weeks were co-cultured with HUVEC. HUVEC were suspended in a collagen gel solution (Cellmatrix type IA (Nitta Gelatin)/5 mM NaOH/26 mM $NaHCO_3$/20 mM Hepes/one volume of 10 times concentrated αMEM) at a density of 1×10^6 cells/ml, and 400 μl of the mixture was poured into each well of 24-well plates. The chondrocyte pellets were then dropped at the center of each well and incubated for 30 min at 37 °C to form a gel. These cells were cultured together in HuMedia-EG2 medium and the angiogenic reactions were monitored for 2 weeks.

Statistical analysis. Means of groups were compared by analysis of variance, and significance of differences was determined by post hoc testing using Bonferroni's method.

Results

MMP-13$^{-/-}$ mice exhibit delayed fracture healing

To investigate the effect of MMP-13 on fracture healing, we generated a stabilized tibial fracture model in mice and assessed the healing process by radiological evaluation (Fig. 1A). In plain radiographs of WT mice, the calcified callus appeared at post-fracture week (PFW) 1, progressed to form a bony bridge by PFW3, and then gradually decreased in size by PFW10. In contrast, in the MMP-13$^{-/-}$ mice, a radiolucent zone was apparent in the fracture callus even at PFW3, suggesting that with the loss of MMP-13 the bony bridging was delayed. In axial CT images, the WT mouse callus contained a small noncalcified area at PFW2, which was subsequently replaced by calcification at PFW3. However, in MMP-13$^{-/-}$ mice, the callus consisted mainly of noncalcified tissue at PFW2, and although calcification progressed, there remained an area of noncalcified tissue at PFW3.

To quantify the extent of callus formation, the calcified area and BMC in the fractured and control tibiae were measured by a bone densitometer. As shown in Fig. 1B, both parameters increased for 3 weeks during the modeling phase and then decreased during the remodeling phase in WT mice. In contrast, MMP-13$^{-/-}$ mice showed a significant reduction in these parameters at PFW2-3 during the modeling phase. There were no differences in the calcified area and BMC between the two genotypes during the remodeling phase (PFW4-10). These results indicate that MMP-13 deficiency causes a delay in fracture healing through impaired bone modeling due to retarded calcification of the fracture callus.

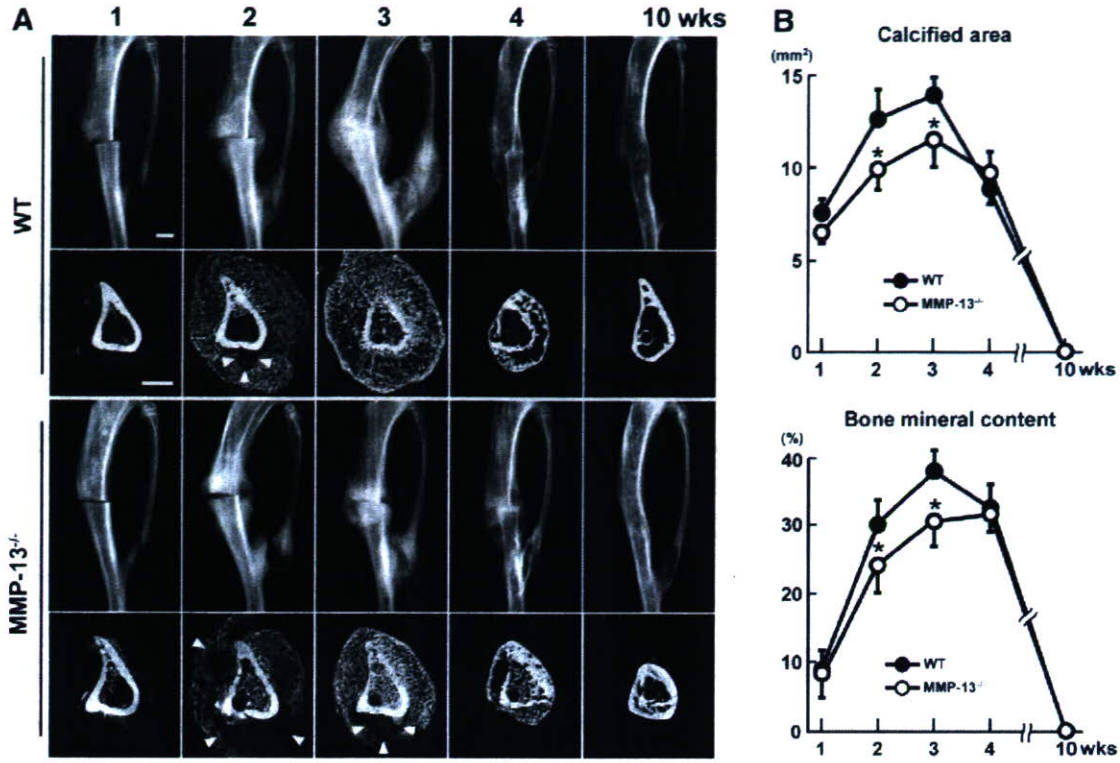

Fig. 1. Radiological analyses of bone fracture healing in WT and MMP-13$^{-/-}$ mice. (A) Plain radiographs (upper row) and CT images (lower row) of the representative fractured tibiae in WT and MMP-13$^{-/-}$ mice at PFWs 1–10. The arrowheads indicate non-calcified areas. Scale bar, 1 mm. (B) Time course changes of the calcified area and bone mineral content of the callus at the fracture site measured by single energy X-ray absorptiometry. Data are expressed as the means (symbols) ± SD (error bars) of 6 mice per genotype. $^{*}p < 0.05$ versus WT.

MMP-13 deficiency impairs the replacement of cartilage with bone in the fracture callus

Remodeling of bone requires the resorption of cartilage, which provides initial stabilization to the fractured bone [2]. To evaluate the turnover of cartilage to bone in the fracture callus, the ratio of cartilage area and bone area to total callus area (CA/TA and BA/TA, respectively) were measured utilizing a planimetric method in histological sections (Fig. 2). The cartilage area was stained blue with Alcian blue staining, and the bone area was stained red with van Gieson. In WT mice, CA/TA reached its peak value at PFW1 and then decreased by PFW4, whereas BA/CA increased to reach a plateau by PFW3. Compared to WT mice, MMP-13$^{-/-}$ mice exhibited higher CA/TA values and lower BA/TA values at PFW2-3, indicating that loss of MMP-13 interferes with cartilage-to-bone replacement.

MMP-13 deficiency impairs vascular and chondroclast invasion of cartilage

To further investigate the mechanisms underlying the impaired bone healing in MMP-13$^{-/-}$ mice, we performed immunohistochemical analysis of the fracture callus at PFW2 (Fig. 3). Type X collagen, a marker for hypertrophic chondrocytes, was more prevalent in the MMP-13$^{-/-}$ cartilage than in the WT cartilage, while type II collagen was equivalent between the genotypes (Fig. 3A–D). MMP-9 was expressed in hypertrophic chondrocytes and chondroclasts, and appeared to be up-regulated in the MMP-13$^{-/-}$ mice (Fig. 3E and F). In WT mice, the CD31-immunostained capillaries penetrated into the cartilage extending their cytoplasmic processes (Fig. 3G), and cathepsin K/TRAP-positive chondroclasts were directly attached to the cartilage matrix (Fig. 3I and K). In contrast, MMP-13$^{-/-}$ mice showed minimal cartilaginous breakage associated with capillary invasion (Fig. 3H), and the chondroclasts were not attached to the cartilage matrix (Fig. 3J and L). These findings suggest that the impaired cartilage resorption in the MMP-13$^{-/-}$ mouse callus is associated with the inability of capillaries and chondroclasts to invade the cartilage.

MMP-13 is required for the angiogenic activation of cartilage

In order to clarify the cellular mechanism underlying these abnormalities, we examined *in vitro* the differentiation of rib chondrocytes isolated from WT and MMP-13$^{-/-}$ littermates (Fig. 4). Calvarial osteoblasts cultured from MMP-13$^{-/-}$ mice exhibited no difference in proliferation and differentiation compared with those from WT mice,

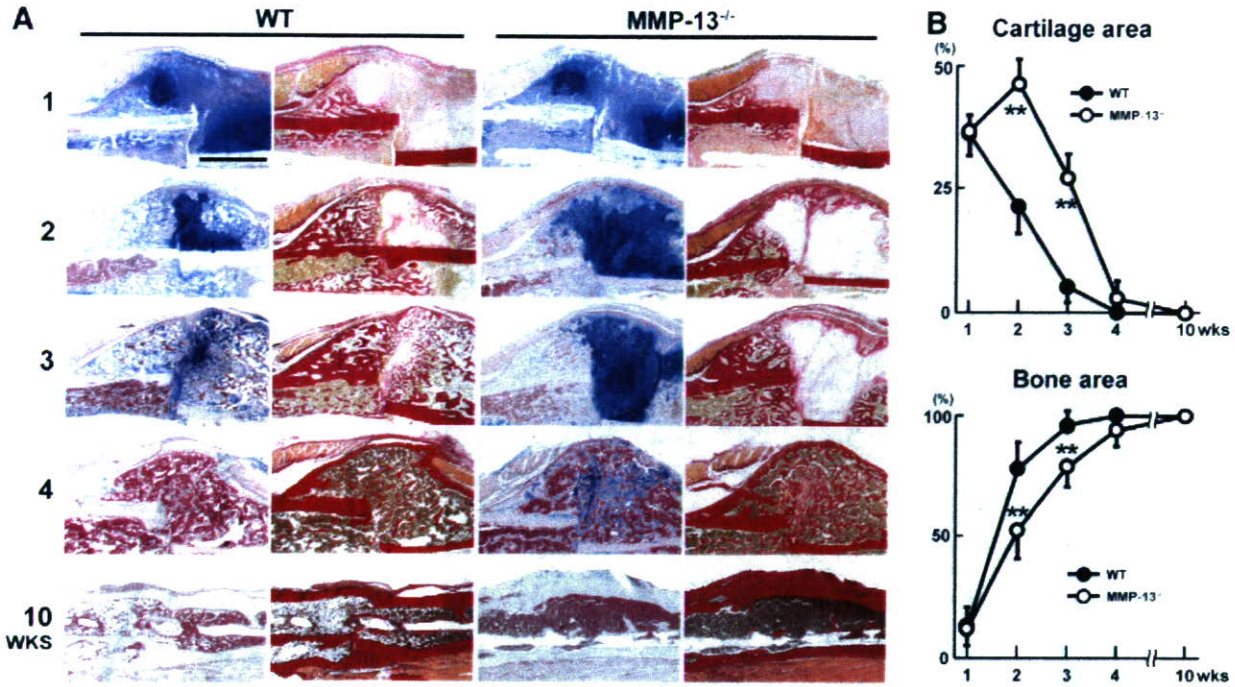

Fig. 2. Histomorphological analyses of bone fracture healing in WT and MMP-13$^{-/-}$ mice. (A) Alcian blue staining (right column) and van Gieson staining (left column) of the fractured tibia sections from WT and MMP-13$^{-/-}$ mice. Scale bar, 1 mm. (B) Ratios of cartilage area and bone area to total callus area of the fractured site, which were measured by a planimetric method. Data are expressed as the means (symbols) ± SD (error bars) of 6 mice per genotype. $^{**}p < 0.01$ versus WT.

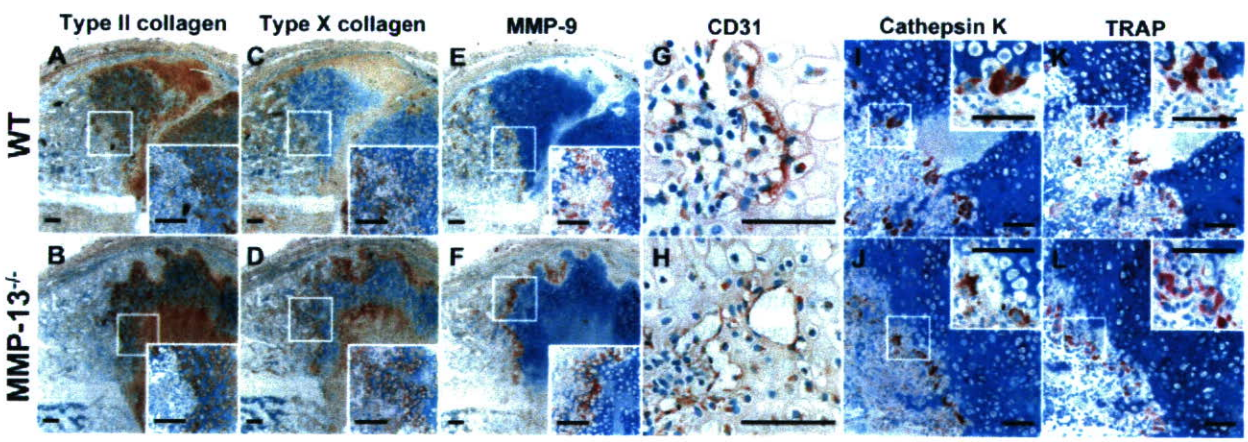

Fig. 3. Immunohistochemical and histochemical analyses of bone fracture healing in WT and MMP-13$^{-/-}$ mice. (A–J) Immunohistochemistry of type II collagen (A,B), type X collagen (C,D), MMP-9 (E,F), CD31 (G,H) and cathepsin K (I,J) in the fracture callus of WT and MMP-13$^{-/-}$ mice at PFW2. (K,L) Histochemistry for TRAP in the fracture callus of WT and MMP-13$^{-/-}$ mice at PFW2. All insets are higher magnification images of each figure. Note that the expression of type X collagen and MMP-9 in hypertrophic chondrocytes and/or chondroclasts appear to be up-regulated in MMP-13$^{-/-}$ mice compared to WT mice (C–F). Also note that and cathepsin K/TRAP-positive chondroclasts are unattached to cartilage matrix in MMP-13$^{-/-}$ mice (J,L). Scale bar, 80 μm.

and the cell proliferation rate of MMP-13$^{-/-}$ chondrocytes was also normal (data not shown).

To evaluate the cartilage formation, chondrocytes were cultured in 3D collagen gel pellets. During the first 2 days the WT pellets shrunk and the cartilage matrix formation by the chondrocytes was observed at the pellet periphery on day 7. By 21 days cartilage maturation proceeded toward the center forming a mature cartilage pellet. In the MMP-13$^{-/-}$ pellets, the gel contraction rate on day 2 and matrix synthesis on day 7 were reduced compared to WT pellets, although the matured pellets exhibited no histological differences between genotypes on day 21.

To further investigate the angiogenic property of the cartilage pellets, we developed a co-culture system of the chondrocyte pellets with HUVEC. After 1 week of co-culture, extensive vascular sprouting was observed around the

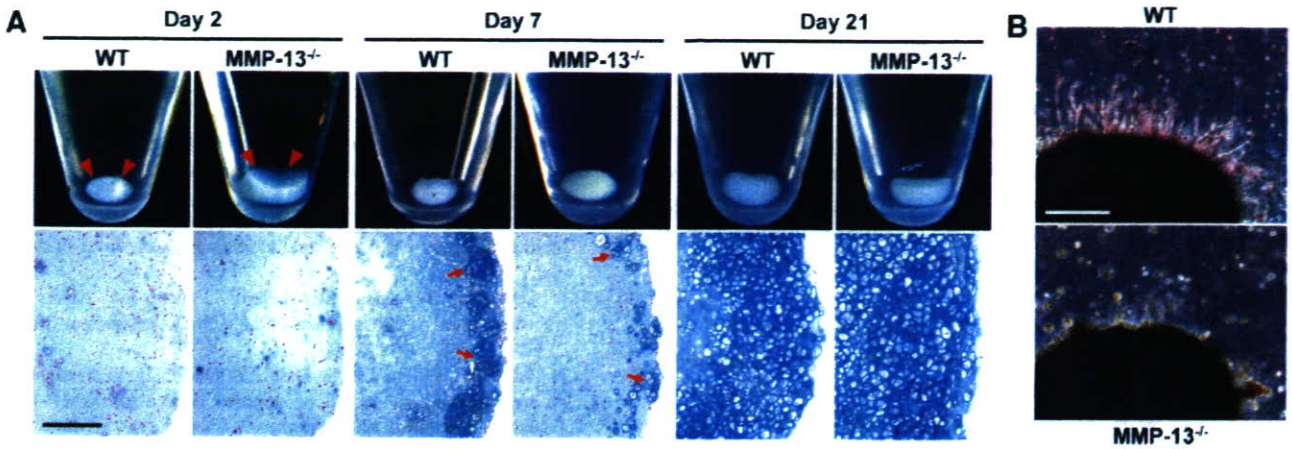

Fig. 4. Cultured chondrocytes from WT and MMP-13$^{-/-}$ mice. (A) Gross appearance (upper row) and Alcian blue stainings (lower row) of chondrocyte pellets on days 2, 7, and 21. Chondrocytes from WT and MMP-13$^{-/-}$ mice were cultured in 3D collagen gel pellets. Note that MMP-13$^{-/-}$ pellet is reduced in gel contraction rate on day 2 (arrowheads) and Alcian blue-positive matrix synthesis on day 7 (arrows). Scale bar, 500 μm. (B) *In vitro* angiogenesis assay. Chondrocyte pellets were co-cultured with HUVEC in collagen gel. Note that WT pellets are surrounded by numerous vascular sproutings, which are barely seen with the MMP-13$^{-/-}$ pellets. Scale bar, 250 μm.

WT pellets, although the MMP-13$^{-/-}$ pellets exhibited limited sprouting (Fig. 4B). These results suggest that MMP-13 is involved in the angiogenic activation of chondrocytes via matrix remodeling.

Discussion

MMP-13 is the major proteinase that cleaves interstitial fibrillar collagens (type I, II, and III collagens) [14]. Deficiency of MMP-13 causes a transient elongation of the hypertrophic zone in the growth plate during the early stages of growth and development, indicating a role for this enzyme in endochondral bone development [5,6]. Since the bone fracture healing process is thought to recapitulate skeletal development [1], and since MMP-13 is highly expressed in the fracture callus [7], we hypothesized that MMP-13 plays an important role in fracture healing. Utilizing a mouse fracture model and a chondrocyte pellet culture, the present studies demonstrate that the lack of MMP-13 leads to a significant delay in the fracture healing process, and that MMP-13 plays a critical role in the maturation of chondrocytes and the induction of angiogenesis.

During the bone fracture healing process, fracture callus consisting of cartilage tissue is transiently formed and subsequently resorbed and replaced with osseous tissue; hence the cartilage resorption process is an important step for fracture healing. In general, the vascular invasion is a critical rate-limiting determinant for bone formation [15], and at the chondroosseous junction there are three key players in this process: hypertrophic chondrocytes, capillaries, and chondroclasts [16]. Among them, hypertrophic chondrocytes are the major cell type producing MMP-13 in the fracture callus and, in MMP-13$^{-/-}$ mice, the cartilage resorption was delayed due to the impairment of the invasion of capillaries and chondroclasts into the cartilage. These data re-emphasize the role of vascular and chondroclast invasion during the fracture callus resorption and indicate that MMP-13 production by chondrocytes is a prerequisite for this process.

Since primary chondrocytes from the rib cartilage were not capable of effectively producing ECM in a monolayer culture, we utilized a 3D pellet culture model to mimic the *in vivo* environment. Cells/collagen gel composites are known to undergo shrinkage during culture [17], and early gel contraction and subsequent matrix synthesis in the MMP-13$^{-/-}$ chondrocyte pellets were both suppressed compared to those observed in WT controls, suggesting that cell–ECM and/or cell–cell interactions caused by gel contraction are important for chondrocyte development and that MMP-13 is involved in these processes. These findings are similar to those seen in mice deficient in α1 integrin, a key molecule for cell–ECM interaction, which exhibited reduced cartilage ECM synthesis during fracture healing [18]. Moreover, interactions of chondrocytes with their ECM are required for the expression of angiogenic activities of chondrocytes [19]. When chondrocyte pellets and endothelial cells from the MMP-13$^{-/-}$ mice were co-cultured, tubular formation was considerably reduced in the pellets from MMP-13$^{-/-}$ mice, indicating that MMP-13 is critical for the angiogenic activities of chondrocytes. These data strongly support the notion that MMP-13 contributes to the fracture healing process by regulating both chondrocyte development and vascular induction.

MMP-9 deficiency has been shown to cause delayed fracture healing [8] in a manner similar to that seen in the MMP-13$^{-/-}$ mice. MMP-9$^{-/-}$ mice exhibit hindered cartilage resorption mainly due to suppressed angiogenesis, which can be rescued by local injection of recombinant vascular endothelial growth factor. Similar to the MMP-13$^{-/-}$ mice, elongation of the hypertrophic cartilage zone is also observed in the MMP-9$^{-/-}$ mice [4–6], although the expression pattern of these enzymes are different; in bone tissue MMP-9 is expressed mainly in osteoclasts and chondroclasts while MMP-13 is expressed mainly in osteoblasts

and chondrocytes [7]. In line with the findings of the growth plates of MMP-13$^{-/-}$ mice in which MMP-9 mRNA expression is up-regulated [5], the intensity of MMP-9 expression in the fracture callus was higher in MMP-13$^{-/-}$ mice compared to that in WT mice. These findings indicate a possible compensatory/redundant mechanism between these two MMPs during cartilage resorption in both physiological and pathological conditions, where chondrocytes, endothelial cells, and chondroclasts are intricately regulated.

In conclusion, the current study provides *in vivo* and *in vitro* evidence for the critical role of MMP-13 in cartilage resorption during bone fracture healing. In addition, the finding that MMP-13 plays a major role in vascular invasion of cartilage provides an incentive to further study the mechanism underlying the pro-angiogenic function of MMP-13. Moreover, it will be interesting to examine the compensatory role of MMPs, such as MMP-9 and MMP-13, in fracture healing. Taken together, this study provides new insight into the role of MMP-13 in fracture healing, which in turn has implications for bone regenerative medicine.

Acknowledgments

The authors thank Dr. K. Horiuchi and Dr. J. Takito for critical discussions. We are grateful to Dr. K. Hoshi for sharing his expertise on the chondrocyte culture system. This study was supported by the National Institute of Aging (AG016994) and the Grants-in-Aid provided by the Ministry of Education, Science and Culture, Japan for General Scientific Research and Scientific Research on Priority Areas; by the Uehara Foundation and the General Insurance Association of Japan.

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J Bone Miner Metab (2007) 25:130–137
DOI 10.1007/s00774-006-0738-4

ORIGINAL ARTICLE

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Clinical effect of bisphosphonate and vitamin D on osteoporosis: reappraisal of a multicenter double-blind clinical trial comparing etidronate and alfacalcidol

Received: July 24, 2006 / Accepted: October 31, 2006

Abstract As inhibitors of bone resorption, bisphosphonates and vitamin D derivatives have been extensively used for the treatment of osteoporosis in various parts of the world, but the clinical effects of these two groups of agents have rarely been compared in detail. A multicenter, prospective, double-blind controlled study was started comparing the effects of etidronate and alfacalcidol (1-alpha-hydroxycholecalciferol) in 414 patients with established osteoporosis from 36 centers. Among these patients, 135 were given 400 mg etidronate daily at bedtime for 2 weeks followed by 10 weeks off treatment, and this cycle was repeated four times along with a placebo indistinguishable from the alfacalcidol capsule daily throughout the 48 weeks of study (Group A, High Dose Etidronate Group). In 133 patients, 200 mg etidronate was used instead of 400 mg (Group B, Low Dose Etidronate Group). In 138 patients, 1 μg alfacalcidol was given daily throughout the 48-week study period along with a placebo indistinguishable from the etidronate tablet in four separate periods of 2 weeks (Group C, Control Group). Dual-energy X-ray absorptiometry of the lumbar spine (L2–L4) was performed before the beginning of the study and every 12 weeks thereafter. Changes in spinal deformity were also assessed based on the lateral thoracic and lumbar spine X-ray films taken before and after the study. The lumbar spine bone mineral density (BMD) changes were +3.4% ± 0.6% (mean ± SEM) in Group A, +2.4% ± 0.5% in Group B, and −0.5% ± 0.4% in Group C, the former two being significantly higher than the last. New occurrence of spinal compression fracture was also significantly reduced in Group A compared to Group C. In patients without previous fracture at entry, incident fracture was 10.2% in Group C, but 0% in Groups A and B. In patients with prevalent fracture at entry, corresponding figures were 21.5% (Group C), 12.0% (Group A), and 13.2% (Group B), respectively. Alfacalcidol maintained lumbar spine BMD, preventing a decrease for 48 weeks, and etidronate significantly increased it further, demonstrating its usefulness in the treatment of established osteoporosis.

Key words etidronate · alfacalcidol · osteoporosis · DXA (dual energy X-ray absorptiometry) · fracture

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Introduction

Etidronate is the first bisphosphonate developed for the treatment of osteoporosis, based on its potent inhibitory effect on osteoclastic resorption [1–3]. Although several studies including prospective, placebo-controlled double

blind trials indicated positive effects of etidronate in preventing bone loss in osteoporosis [4–10], its effect has rarely been compared with those of other drugs. In addition to the well-known estrogen, calcitonin and vitamin D derivatives, especially alfacalcidol (1-alpha-hydroxycholecalciferol) have been extensively used for the treatment of osteoporosis in Japan [11,12], possibly because of the profound calcium deficiency caused by Japanese dietary habits. Vitamin D and its derivatives decrease vertebral fracture, and may also decrease nonvertebral fractures, according to a meta-analysis [13]. To evaluate the clinical effect of etidronate, alfacalcidol was therefore employed as the active control drug in a multicentered, prospective, double-blind program involving 414 patients with established osteoporosis at 36 centers in Japan.

Patients and methods

Test subjects

This study was started on 414 patients with established primary osteoporosis with scores higher than 4 according to the scoring method for the diagnosis of involutional osteoporosis established by the Osteoporosis Research Group sponsored by the Health and Welfare Ministry (Chairperson: Dr. Hajime Orimo) (Table 1). Patients with primary or secondary hyperparathyroidism, and secondary osteoporosis caused by renal failure, vitamin D deficiency, rheumatoid arthritis, bone metastases of malignant tumors, multiple myeloma, trauma or corticosteroid use, and osteomalacia, were excluded from the study. This study was conducted from July 1990 to June 1992.

Study design

Over a period of 8 weeks before the beginning of the study (washout period), specific treatments for osteoporosis including estrogens, calcitonins, vitamin D derivatives, and ipriflavone were withheld. Because no bisphosphonate had been therapeutically administered before this study and subsequent government authorization, no subjects had taken any bisphosphosphonate before their participation. A prospective, randomized, double-blind-controlled comparative study among the three groups was constructed as follows. Informed oral consent was obtained from each proposed test subject as to voluntary participation to this study.

The 414 test subjects were given either 400 mg etidronate (Group A), 200 mg etidronate (Group B), or 1 μg alfacalcidol (Group C) according to the preset randomized order. The key of the randomized samples was kept by the controller until the finalization of the data, when it was opened and the results were analyzed. To assure the blindness, a placebo with indistinguishable appearance was provided whenever the true drug was not given. The 48-week test period was divided into four equal parts consisting of 12 weeks each. Each morning, Groups A and B were given one alfacalcidol placebo. At bedtime, only during the first 2 weeks of each 12-week period, Group A was given two 200 mg etidronate tablets, Group B one etidronate placebo tablet and one etidronate 200 mg tablet, and Group C two etidronate placebo tablets. No calcium supplements were given in any of the three groups.

Table 1. Scoring method for the diagnosis of involutional osteoporosis

1. Decrease of bone mass: score 3	
Bone mineral density (AP spine by DXA) less than 2 SD of young adult mean and/or X-ray evidence of vertical trabecular loss	
2. Fracture	
One vertebra	1
More than two vertebrae	2
Proximal femur	3
Radius	1
3. Premenopausal female	−1
4. Backache	1
5. Serum Ca, P, and alkaline phosphatase	
Normal	1
One abnormal value	0
More than two abnormal values	−1

Each item is given scores specified, and evaluation is based on the total of the scores. In case the total is above 5, diagnosis of osteoporosis is definite. Total score 4 indicates that osteoporosis is likely. Total score 3 indicates that osteoporosis is suspected. In case the total is 2 on less, osteoporosis is unlikely

AP, antiroposterior; DXA, dual-energy X-ray absorptionetry

Measurement of efficacy

Each test subject was seen by the physician in charge of the project at each center every 2 weeks and asked for symptoms, especially backache, as well as those possibly related to side effects. X-ray pictures of the lumbar and thoracic spine were taken at anteroposterior and lateral projections at the beginning and end of the study. Vertebral fracture was defined by a decrease of the anterior height (wedge deformity) or middle height (biconcave deformity) to less than 80% of the intact posterior height. In case the posterior height was also decreased (flat deformity or crush fracture), the decrease of the anterior, middle, or posterior height to less than 80% of the posterior height of the adjacent intact vertebra was used as the criterion for the deformity. Incident fractures were analyzed by the logistic method in the whole series, and cases with and without fracture at the start of the test, to calculate the odds ratio.

For bone measurements, dual energy X-ray absorptiometry (DXA) of L2–L4 was performed at the beginning of the study and every 12 weeks thereafter using a QDR-1000 (Hologic), XR-26 (Norland), or DPX (Lunar). The results were expressed as percent (%) of the initial value, and the same method of measurement with the same apparatus was used for each patient throughout the study period. Subjects with compressive or osteophytic deformities, interfering with accurate bone mineral density (BMD) measurement in two adjoining vertebrae of L2–L4 were excluded from the series.

Biochemical measurements were carried out as follows. Vitamin D metabolites (25-hydroxyvitamin D and 1,25-