Ⅲ. 資　料

日常臨床で測定する骨量及び骨代謝マーカーと海綿骨組織形態計測の関連性の検討

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研究要旨

骨粗鬆症の診療では、椎体骨折診断のためのX線撮影、DXAなどによる骨量(骨密度)測定、骨代謝マーカー測定が基本的な検査となっている。一方、生検腸骨の骨形態計測では骨量や骨代謝回転ばかりでなく骨梁構築、類骨量、骨形成と骨吸収の状態、石灰化速度などが解り、骨代謝性疾患の診断や治療判定に有用である。しかし、日常臨床で測定する骨量や骨代謝マーカーと生検腸骨の海綿骨形態計測値との関連性は明らかにされていない。今回、生検腸骨の海綿骨形態計測値と日常臨床で測定する骨量や骨代謝マーカーとの関連性を検討した。

股関節疾患で人工股関節置換術を行う患者で、骨代謝性疾患の合併や骨代謝に影響する薬剤の使用がない52人(38～81歳[平均61歳]、女性47人、男性5人、股関節疾患は変形性股関節症46人、大腿骨頭壊死症4人、その他2人)で、インフォームドコンセントを得た後、手術時に腸骨生検を施行し、海綿骨組織形態計測を行った。骨量の代表的指標であるBV/TVと、日常臨床で計測する骨量(腰椎骨密度、両股関節X線前後像でのBarnett & Nordinのfemur scoreとNobleのcanal flare index)との間に有意な相関がなかった。骨組織形態計測の中で骨代謝回転の代表的指標であるBFR/BSと骨代謝マーカー(BAP、OC、NTX、DPD)との間に有意な相関がなかった。

これらの結果は、日常臨床で測定する骨量や骨代謝マーカーが、海綿骨の組織学的変化を必ずしも反映していないことを意味している。対象患者の中で原発性骨粗鬆症の診断基準に合致したのは3人だけであった。今後、骨粗鬆症症例を増やして、骨粗鬆症診療で測定する骨量と骨代謝マーカーの骨組織形態学的意義を検討することが必要と考える。

A. 研究目的

骨粗鬆症の診療では、椎体骨折診断のためのX線撮影、DXAなどによる骨量(骨密度)測定、骨代謝マーカー測定が基本的な検査となっている。一方、生検腸骨の骨形態計測では骨量や骨代謝回転ばかりでなく骨梁構築、類骨量、骨形成と骨吸収の状態、石灰化速度などが解り、骨代謝性疾患の診断や治療判定に有用である。しかし、日常臨床で測定する骨量や骨代謝マーカーと生検腸骨の海綿骨形態計測値との関連性は明らかにされていない。今回、生検腸骨の海綿骨形態計測値と日常臨床で測定する骨量や骨代謝マーカーとの関連性を検討した。

B. 研究方法

股関節疾患で人工股関節置換術を行う患者で、骨代謝性疾患の合併や骨代謝に影響する薬剤の使用がない者に、インフォームドコンセントを得た後、手術時に腸骨生検を行った。生検に先立ってテトラサイクリンを2回投与した。腸骨生検部位(前上腸骨棘より後方へ2cmの腸骨稜より末梢へ2cm)に同側股関節疾患が骨代謝学的影響を及ぼさないことは、先の研究で確認している。生検腸骨は70%アルコール固定、Villanueva骨染色、メチルメタクリレート包埋を行った後、約5μm厚の非脱灰薄切標本を作製した。半自動骨形態計測システムで骨形態計測を行った[1]。なお、股関節病変が腸骨生検部に影響を及ぼさないこ

とは、先の研究で定量的骨シンチグラフィーによって確認している[2]。

術前検査時に、腰椎(L2-4)DXA 骨密度測定を行った。両股関節X線前後像でBarnett & Nordinのfemur score(大腿骨骨幹部での骨皮質幅の外径に対する割合で同部の骨量の指標)[3]と、Noble の canal flare index(大腿骨皮質が菲薄化し髄腔が拡大した寸胴な髄腔形状の指標)[4]を計測した。また、骨代謝マーカー(血清骨型アルカリフォスファターゼ[BAP]、オステオカルシン[OC]、尿中 I 型コラーゲン架橋 N-テロペプチド[NTX]、デオキシピリジノリン[DPD])の測定も行った。

52 人の患者より腸骨生検を行った。年齢が 38～81 歳(平均 61 歳)で、女性が 47 人で男性が 5 人で、股関節疾患は変形性股関節症 46 人、大腿骨頭壊死症 4 人、その他 2 人であった。患者活動性は、Gustilo I(sedentary)1 人、II(non-strenuous)13 人、III(moderately strenuous)29 人、IV(very active)9 人であった。3 人に胸腰椎の X 線像で椎体骨折があったが、腰椎骨密度の T 値は 67%、71%、90%であった。残りの 50 人には骨折の既往がなく、腰椎骨密度の T 値は 73～115%であった。従って、原発性骨粗鬆症の診断基準に当てはまる例は 3 人であった。

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C. 研究結果

日常臨床で測定する骨量と骨代謝マーカーの結果を**表1**に、生検腸骨骨形態計測結果を**表2**に示す。

表1. 日常臨床で測定する骨量と骨代謝マーカー

計測項目	平均値	SD
[骨量]		
腰椎 L2-4 骨密度	0.914 g/cm^2	0.122
T 値	90%	12.0
Z 値	109%	14.5
Femur score	53	7.0
Canal flare index	3.7	0.75
[骨代謝マーカー]		
BAP	27 U/L	5.7
OC	7.6 ng/ml	3.3
NTX	87 nmolBCE/mmol・Cr	48.3
DPD	7.2 nmol/mmol・Cr	2.5

表2. 生検腸骨の海綿骨組織形態計測値

計測項目	平均値	SD
BV/TV	12.7%	5.6
OV/BV	1.6%	1.3
Tb.Th	120 μ m	39
OS/BS	10.7%	8.2
ES/BS	10.6%	5.9
BFR/BS	0.0069mm3/mm2/yr	0.0105

骨組織形態計測の中で骨量の代表的指標である BV/TV と日常臨床で計測する骨量との相関を調べたが、有意な相関がなかった(**表3, 図1**)。

表3. BV/TV と日常臨床での測定する骨量との相関

骨量の指標	相関係数	p 値
腰椎 L2-4 骨密度	0.005	0.97
T 値	0.001	0.99
Z 値	0.014	0.93
Femur score	−0.027	0.85
Canal flare index	−0.20	0.17

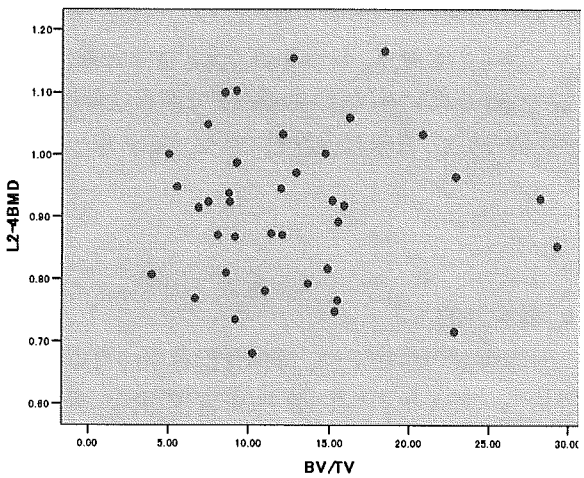

図1. BV/TV と L2-4BMD の散布図

骨組織形態計測の中で骨代謝回転の代表的指標である BFR/BS と骨代謝マーカーとの相関を調べたが、有意な相関がなかった(**表4**)。

表4. BFR/BSと骨代謝マーカーとの相関

骨代謝マーカー	相関係数	p値
BAP	−0.39	0.88
OC	0.027	0.91
NTX	0.34	0.41
DPD	0.14	0.57

対象を変形性股関節症患者46人に絞って同様の検討を行ったが、いずれの組合せでも有意な相関はなかった。

D. 考察

生検腸骨の骨形態計測では骨量や骨代謝回転ばかりでなく骨梁構築、類骨量、骨形成と骨吸収の状態、石灰化速度などが解り、骨代謝性疾患の診断や治療判定に有用であると考えられてきている。ビスフォスフォネートなどの骨粗鬆症治療薬が骨組織学的異常を惹起していないか検証する手段として用いられてきた。

エチドロネートを7年投与した46例の骨生検では、骨軟化症など、臨床的に問題となる骨代謝異常はなかった[5]。アレンドロネート(ALN)もしくはプラセボを2、3年投与した例の腸骨骨生検の形態計測では、ALN投与により類骨と骨代謝回転は減少するが、石灰化速度は保たれることが示された[6]。生検標本の定量的マイクロラジオグラフィーでは海綿骨と皮質骨の石灰化の増加がみられ[7]、走査電子顕微鏡学的解析では石灰化粒子のサイズや特性を損ねることなく石灰化が増加することと皮質骨の多孔性が減少することが示された[8]。Reckerらは、骨組織形態計測とμCT解析を行い、2〜3年のALN投与はプラセボと比べ、骨量、骨梁幅、骨梁数を増し、骨代謝回転と骨梁間隙を減らすことを示し、ALNによる微細構造変化が骨質改善に寄与していると述べている[9]。VERT-NAのリゼドロネート(NE)かプラセボ3年投与各31例の骨組織形態計測では、NEによって骨代謝回転が約50%低下していたが、石灰化障害や異常骨髄は見られず、皮質の厚さと多孔性が増加していた[10]。NEかプラセボ投与1年[11]と3年[12]の生検腸骨のμCTによる比較では、NE群の方が骨量、骨梁の幅と数とプレート様/ロッド様骨梁割合が高値で、骨梁間距離、髄腔star volumeが低値となっていた。従って、ALNもNEも骨量ばかりでなく、骨の微細構造(骨質)も改善することで、骨強度向上、すなわち骨折予防に寄与していることが示された。

骨粗鬆症の診療では、既存骨折とともに、骨量(骨密度)と骨代謝マーカーは、新規骨折発生を予測する重要な検査項目となっている。しかし、日常臨床で測定する骨量や骨代謝マーカーと生検腸骨の海綿骨形態計測値との関連性は明らかにされていない。

今回の研究では、生検腸骨の海綿骨形態計測でのBV/TVと日常臨床で測定する骨量の間に相関がなく、BFR/BSと骨代謝マーカーの間にも相関がなかった。このことは、日常臨床で測定する骨量や骨代謝マーカーが、海綿骨の組織学的変化を必ずしも反映していないことを意味している。今回の対象群は、腰椎骨密度が平均で0.914 g/cm^2、T値90%、Z値109%で、原発性骨粗鬆症の診断基準に合致したのは3人だけであった。今後、骨粗鬆症症例を増やして、骨粗鬆症診療で測定する骨量と骨代謝マーカーの骨組織形態学的意義を検討することが必要と考える。

E. 結論

生検腸骨の海綿骨形態計測値と日常臨床で測定する骨量や骨代謝マーカーとの関連性を検討した。股関節疾患で人工股関節置換術を行う患者で、骨代謝性疾患の合併や骨代謝に影響する薬剤の使用がない52人(38〜81歳[平均61歳]、女性47人、男性5人、股関節疾患は変形性股関節症46人、大腿骨頭壊死症4人、その他2人)で、手術時に腸骨生検を施行し、海綿骨組織形態計測を行った。

骨量の代表的指標であるBV/TVと、日常臨床で計測する骨量(腰椎骨密度、両股関節X線前後像でのBarnett & Nordinのfemur scoreとNobleのcanal flare index)との間に有意な相関がなかった。骨組織形態計測の中で骨代謝回転の代表的指標であるBFR/BSと骨代謝マーカー(BAP、OC、NTX、DPD)との間に有意な相関がなかった。

これらの結果は、日常臨床で測定する骨量や骨代謝マーカーが、海綿骨の組織学的変化を必ずしも反映していないことを意味している。対象患者の中で原発性骨粗鬆症の診断基準に合致したのは3人だけであった。今後、骨粗鬆症症例を増やして、骨粗鬆症診療で測定する骨量と骨代謝マーカーの骨組織形態学的意義を検討することが必要と考える。

F. 参考文献

1) Parfitt AM et al: Bone histomorphometry: standardization of nomenclature, symbols and units. J Bone Miner Res 2: 595-610, 1987
2) Kobayashi S et al: Trabecular minimodeling in human iliac bone. Bone 32: 163-169, 2003
3) Barnett E, Nordin BEC: The radiographic diagnosis of osteoporosis: a new approach. Clin Radiol 11: 166-174, 1960
4) Noble PC et al: The anatomic basis of femoral component design. Clin Orthop 235: 148-165, 1988
5) Miller PD, Watts NB, Licata AA et al: Cyclical etidronate in the treatment of postmenopausal osteoporosis: efficacy and safety after seven years of treatment. Am J Med 103: 468-476, 1997
6) Chavassieux PM, Arlot ME, Reda C, et al: Histomorphometric assessment of the long-term effects of alendronate on bone quality and remodeling in patients with osteoporosis. J Clin Invest 100: 1475-80, 1997
7) Boivin GY, Chavassieux PM, Santora AC, et al: Alendronate increases bone strength by increasing the mean degree of mineralization of bone tissue in osteoporotic women. Bone 27(5): 687-694, 2000
8) Roschger P, Rinnerthaler S, Yates J et al: Alendronate increases degree and uniformity of mineralization in cancellous bone and decreases the porosity in cortical bone of osteoporotic women. Bone 29(2): 185-191, 2001
9) Recker R, Masarachia P, Santora A, et al: Trabecular bone microarchitecture after alendronate treatment of osteoporotic women. Curr Med Res Opin 21(2): 185-194, 2005
10) Harris ST, Watts NB, Genant HK et al: Effects of risedronate treatment on vertebral and nonvertebral fractures in women with postmenopausal osteoporosis: a randomized controlled trial. JAMA 282 (14): 1344-52, 1999
11) Dufresne TE, Chmielewski PA, Manhart MD, et al: Risedronate preserve bone architecture in early postmenopausal women in 1 year as measured by three-dimensional microcomputed tomography. Calcif Tissue Int 73: 423-32, 2003
12) Borah B, Dufresne TE, Chmielewski PA, et al: Risedronate preserves bone architecture in postmenopausal women with osteoporosis as measured by three-dimensional microcomputed tomography. Bone 34: 736-746, 2004

G. 研究発表

1. 論文発表

1) 小林千益:股関節の機能解剖と疾患と外傷.日本義肢装具学会誌 21(1):8-12, 2005
2) 小林千益、白木正孝、高岡邦夫:骨粗鬆症の予防と管理:WHO テクニカルレポートをめぐって:併用療法の効果. Clinical Calcium 15(4): 661-5, 2005
3) 小林千益:人工関節置換術と骨質.Clinical Calcium 15(6): 970-6, 2005
4) 小林千益、久保俊一、高岡邦夫:特発性大腿骨頭壊死症に対する人工関節置換術の成績:人工骨頭置換術との比較. 別冊整形外科 48: 173-177, 2005
5) 小林千益:EBM に基づく骨粗鬆症の薬物療法:ビスフォスフォネート製剤の使い方. 特集:骨粗鬆症の薬物療法と予防. Modern Physician 35(11): 1368-1376, 2005

2. 学会発表

1) 小林千益、斎藤直人、堀内博志、天正恵治、小平博之、伊藤明美、山本智章、高橋榮明、高岡邦夫:日本人成人女性の生検腸骨の海綿骨形態計測. 第25回 日本骨形態計測学会、東京都、2005
2) 小林千益、堀内博志、天正恵治、高橋榮明、伊藤明美、高岡邦夫:日本人の生検腸骨の海綿骨形態計測. 第20回 日本整形外科学会基礎学術集会、伊勢市、2005
3) 小林千益:骨粗鬆症とその予防.平成16年度長野県医師会生涯教育講座, 教育研修講演, 松本市, 2005
4) 天正恵治、小林千益、斎藤直人、脇谷滋之、堀内博志、岡部高弘、加藤博之、縄田昌司、太田浩史:Spongiosa metal I・II の短・中期成績. 日本

人工関節学会(第 35 回) 宜野湾市, 2005

5) Kobayashi S: The quest for improved long-term survivorship of total hip arthroplasty: Studies with Dr. Eftekahr and their ramifications. Biennial Meeting of New York Orthopaedic Hospital Alumni Association (第 70 回), 教育研修講演, New York, 2005

6) Kobayashi S: C-stem Japanese Experience: Total hip arthroplasty using C-stem through direct lateral approach.中部整災(第 104 回) Cement THA イブニングセミナー: C-stem Japanese experience, 大阪市, 2005

7) 小林千益、久保俊一、山本卓明、樋口富士男、菅野伸彦、大園健二、進藤裕幸、大橋弘嗣、佛淵孝夫、松本忠美:人工股関節置換術の長期耐用性に関する多施設共同研究.日本整形外科学会学術総会(第 78 回), 横浜市, 2005

8) Kobayashi S, Horiuchi H, Tensho K, Saito N, Kodaira H, Nawata M: Cemented revision THAs by impaction bone grafting and uncemented revision THAs using solution prostheses: experience at Shinshu University Hospital. Senimor on Advanced Technologies in Hip and Knee Arthroplasties. Anderson Orthopaedic Research Institute, Alexisandria, Verginia, USA, 2005

9) Kobayashi S, Horiuchi H, Tensho K, Kodaira H, Saito N, Iorio R: Charnley total hip arthroplasty with structural autogenous acetabular bone grafting for developmental dysplasia of the hip. Annual symposium of the International Society for Technology in Arthroplasty (第 18 回), Symposium A7 『THA-long-term Results』, Kyoto, Japan, 2005

10) Saito N, Horiuchi H, Kobayashi S, Nawata M, Takaoka K: Cryotherapy for pain relief following total hip arthroplasty. Annuanl symposium of the International Society for Technology in Arthroplasty (第 18 回), Kyoto, Japan, 2005

11) 平林洋樹、小林千益、加藤博之、伊藤一人、山田誠司、北側恵史、宮 正彦、佐藤新司、春日和夫、大和俊彦:閉経後骨粗鬆症に対するリゼドロネート併用療法の検討. 日本骨粗鬆症学会(第 7 回), 大阪市, 2005

12) 小池達也、折戸芳紀、多田昌弘、洲鎌亮、豊田宏光、小林千益、高岡邦夫:ヒッププロテクターは大腿骨頚部骨折ハイリスク集団の頚部骨折を抑制する. 日本骨粗鬆症学会(第 7 回), 大阪市, 2005

13) 小林千益、斎藤直人、堀内博志、天正恵治、小平博之:人工股関節再置換術で impaction 同種骨移植により再建した Charnley 大腿骨コンポーネント周囲の長期骨密度変化. 日本股関節学会学術集会(第 32 回), 新潟市, 2005

14) 片桐佳樹、小林千益、斎藤直人、堀内博志、太田浩史、天正恵治、小平博之、脇谷滋之、加藤博之:セメント人工股関節とセメントレス人工股関節のステム周囲の骨密度変化の比較. 日本股関節学会学術集会(第 32 回), 新潟市, 2005

15) 小林千益、斎藤直人、堀内博志、天正恵治、小平博之、寺山和雄:股関節におけるセメント固定の現況と展望: Charnley 人工股関節置換術長期成績より. 日本人工関節学会(第 36 回), シンポジウム『人工関節インプラントの固定の現状と展望』, 京都市, 2006

H. 知的所有権の取得状況

1. 特許の取得
   なし
2. 実用新案登録
   なし
3. その他
   なし

閉経後女性での転倒率に対するVitamin D内服の影響に関する検討

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[背景・目的]

古くより筋力維持にはビタミンDが重要であることが基礎的・臨床的研究で報告されていた（Grady D, et al. J Clin Endocrinol Metab. 1991）。またビタミンDとカルシウム剤併用で高齢女性でのhip fradtureの防止効果があることが1992年に報告（Chapuy MC, et al. N Engl J Med. 1992）されて以降、転倒と関連するbody swayや筋力がビタミンD投与や血中のビタミンD濃度との関連すること、ビタミンDとカルシウムの併用により転倒が減少することなど(Pfeifer M, et al.Trends Endocrinol Metab. 1999、Pfeifer M, et al.　Exp Clin Endocrinol Diabetes. 2001、Pfeifer M, et al. J Bone Miner Res. 2000、Glerup H, et al. Calcif Tissue Int. 2000、Bischoff HA, et al. J Bone Miner Res. 2003、Ｌａｒｓｅｎ et al. JBMR 2002 supple) ビタミンDの転倒抑止効果を示す多くの研究結果が報告されてきている。しかし、日本人高齢女性でも同様の効果がみられるのかどうかに関してはまだ明らかになっていない。そこでこの研究は、骨粗鬆症外来通院中の通常歩行可能な閉経後女性を対象として、ビタミンDの内服の転倒抑止効果がどの程度期待できるかを明らかとすることを目的とする。

[対象・方法]

骨粗鬆症外来に定期的に通院し、年1度の定期的骨密度測定を行なっている女性患者を対象とする。パーキンソン病や関節リウマチなど歩行能力の低下を来たす疾患患者は除外する。毎年の骨密度測定時に転倒回数を問診で調査を行ない、定期的な骨密度測定の間の骨粗鬆症の薬物療法の変更は行わず、薬剤の変更は骨密度検診、転倒に関する問診調査の後に行う。調査は2000年より始めている転倒調査を基に、各年のビタミンD内服状況との関連を、解析する。調査期間中に継続的にビタミンD内服のなかった群と、継続的にビタミンDの内服をしていた群との比較は対応のない二群間比較を行い転倒率の比較をANOVAで統計解析を行う。

[結果]

対象患者数 183 名、転倒経験患者数　41 名

ビタミン D 投与期間のみの観察例：113 名

ビタミン D 非投与期間のみの観察例：７０名

	ビタミンD投与例	非投与例	p
n	113	70	
年齢	69.5±9.7	70.4±7.3	0.5
身長	153.0±6.1	152.8±5.4	0.82
BMI	21.1±2.3	21.4±2.6	0.47
転倒経験患者数	27	14	
観察期間(日)	607±503	652±453	0.54
年平均転倒回数	0.23±0.60	0.15±0.34	0.33

[考察]

外来通院中の通常歩行が可能な閉経後女性では骨粗鬆症治療目的で使用したビタミン D の転倒への影響をみた。その結果ビタミン D 使用群と非使用群の間に転倒率に全く差は見られなかった。この結果はこれまでのいくつかの報告と異なる。年齢や歩行能力、活動性、食事摂取状況。居住環境などの対象患者の状況により、このような検討結果は影響されると考えられるが、ビタミン D の転倒防止効果はあるとしてもこのような他の因子の影響により差がとらえられなくなる程度のものと考えられ、転倒防止効果を目指した薬物療法としてはさらに効果の強い薬剤の開発が必要であると考える。

Ⅳ. 研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kanis JA, Johnell O, Oden A, Johansson H, De Laet C, Eismans JA, Fujiwara S, Kroger H, McCloskey EV, Mellstrom D, Melton LJ, Pols H, Reeve J, Silman A, Tenenhouse A.	Smoking and fracture risk: a meta-analysis.	Osteoporosis Int	16	155-62	2004
Hagino H, Fujiwara S, Nakashima E, Nanjyo Y, Teshima R	Case-control study of risk factors for fractures of the distal radius and proximal humerus among the Japanese population.	Osteoporosis Int	15	226-230	2004
Kanis JA, Johnell O, De Laet C, Johansson H, Oden A, Delmas P, Eismans JA, Fujiwara S, Garnero P, Kroger H, McCloskey EV, Mellstrom D, Melton LJ, Pols H, Reeve J, Silman A, Tenenhouse A	A meta-analysis of previous fracture and subsequent fracture risk.	Bone	35	375-382	2004
Taguchi A, Fujiwara S, Masunari N, Suzuki G.	Self-reported number of remaining teeth is associated with bone mineral density of the femoral neck, but not of the spine, in Japanese men and women.	Osteoporosis Int	15	842-846	2004
Minamoto A, Taniguchi H, Yoshitani N, Mukai S, Yokoyama T, Kumagami T, Tsuda Y, Mishima K, Amemiya T, Nakashima E, Neriishi K, Hida K, Fujiwara S, Suzuki G, Akahoshi M.	Cataract in atomic bomb survivors.	Int J Radiat Biol	80	339-345	2004
Urano T , Shiraki M, Fujita M, Hosoi T Orimo H, Ouchi Y, Inoue S.	Association of a single nucleotide polymorphism in the lipoxygenase ALOX15 5'-flanking region (-5229G/A) with bone mineral density.	J Bone Mineral Metab	23	226-230	2005
Matsumoto T, Miki T, Hagino H, Sugimoto T, Okamoto S, Hirota T, tanigawa Y, hayashi Y, Fukunaga M, Shiraki M, Nakamura T.	A new active vitamin D, ED-71, increases bone mass in osteoporotic patients under vitamin D supplementation: A randomized, double-blind, placebo-controlled clinical trial.	J Clin Endocr Metab	90	5031-5036	2005
Uchida S, Taniguchi T, Shimizu T, Kakikawa T, Okuyama K, Okaniwa M, Arizono H, nagata K, Santora AC, Shiraki M, Fukunaga M, Tomomitsu T, Ohashi Y, Nakamura T.	Therapeutic effects of alendronate 35 mg once weekly and 5 mg once daily in Japanese patients with osteoporosis: a double-blind, randomized study.	J Bone Miner Metab	23	382-388	2005
Tsugawa N, Shiraki M, Suhara Y, Kamao M, Tanaka K, Okano T.	Vitamin K status of healthy Japanese women: age-related vitamin K requirement for gamma-carboxylation of osteocalcin	Am J Clin Nutr	83	380-386	2006
Yamada M, Wong FL, Fujiwara S, Akahoshi M, Suzuki G.	Noncancer disease incidence in atomic bomb survivors, 1958-1998.	Radiat Res.	161	622-632	2004
Cologne JB, Pawel D, Sharp G, Fujiwara S.	Uncertainty in estimating probability of causation in a cross-sectional study: joint effects of radiation and hepatitis-C virus on chronic liver disease.	J Radiol Prot	24	131-145	2004

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Fujiwara S,Sone T, Yamazaki K, Yoshimura N, Nakatsuka K, Masunari N, Fujita S, Kushida K, Fukunaga M	Heel bone ultrasound predicts non-spine fracture in Japanese men and women.	Osteoporosis Int	16	2107-2112	2005
Johnell O, Kanis JA, Oden A, , Johansson H, De Laet C, Delmas P, Eismans JA, Fujiwara S, Kroger H, Mellstrom D, Meunier PJ, Melton LJ, O'Neill, Pols H, Reeve J, Silman A, Tenenhouse A	A Predictive value of BMD for hip and other fractures.	J Bone Miner Res	20	1185-1194	2005
Nawada H, Soen S, Takayanagi R, Tanaka I, Takaoka K, Fukunaga M, Matsmoto T, Suzuki Y, Tanaka H, Fujiwara S, Miki T, Sagawa A, Nishizawa Y, Seino Y	Guideline on the management and treatment of glucocorticoid-induced osteoporosis of the Japanese Scoity for Bone and Mineral Research.	J Bone Miner Metab	23	105-109	2005
Hakoda M, Maaunari N, Yamada M, Fujiwara S, Suzuki G, Kodama K, Kasagi F	Serum uric acid concentration as a risk factor for cardiovascular mortality: A longterm cohort study of atomic bomb survivors.	J Rheumatol	32	906-12	2005
Yamada M, Wong FL, Fujiwara S, Tatsukawa Y, Suzuki G	Smoking and alcohol habits as risk factors for benign digestive diseases in Japanese population: The Radiation Effects Research Foundation Adult Health Study.	Digestion	71	231-237	2005
Wong FL, Yamada M, Tominaga T, Fujiwara S, Suzuki G	Effects of radiation on the longitudinal trends of hemoglobin levels in the Japanese Atomic Bomb survivors.	Radiat Res	164	820-827	2005
Hakoda M, Oiwa H, Kasagi F, Masunari N, Yamada M, Suzuki G, Fujiwara S.	Mortalitu of rheumatoid arthritis in Japan: Alongitudinal cohort study.	Annals Rheumatic Disease	64	1451-1455	2005
Tada M., Inui K., Koike T., Takaoka K.	Use of local electroporation enhances methotrexate effects with minimum dose in ajuvant-induced arthritis.	Arthritis Rheum	52	637-641	2005
Ito Y, Sakai T, Tomo H, Nakao Y, Inui K, Koike T, Nakatsuchi T, Takaoka K.	Computerized assessment of Bankart lesions under tension with magnetic resonance arthrography.	J Shoulder Elbow Surg.	14	247-251	2005
Nakatsuchi T., Otani M., Osugi H., Koike T	The necessity of chest physical therapy for thoracoscopic oesophagectomy.	The Journal of International Medical Research	33	434-441	2005
H. Toyoda, Y. Ito, H. Tomo, Y. Nakao, T. Koike, K. Takaoka	Evaluation of rotator cuff tears with magnetic resonance arthrography.	Clin Ortho Related Res	439	109-115	2005
Nishii T, Sugano N, Miki H, Hashimoto J, Yoshikawa H.	Does alendronate prevent collapse in osteonecrosis of the femoral head?	Clin Orthop Relat Res.	443	273-279	2006
Tsuboi H, Udagawa N, Hashimoto J, Yoshikawa H, Takahashi N, Ochi T.	Nurse-like cells from patients with rheumatoid arthritis support the survival of osteoclast precursors via macrophage colony-stimulating factor production.	Arthritis Rheum.	52	3819-3828	2005
Oshima K., Nampei A., Matsuda M., Iwaki M., Fukuhara A., Hashimoto J., Yoshikawa H., Shimomura I.	Adiponectin increases bone mass by suppressing osteoclast and activating osteoblast.	Biochem Biophys Res Comm	331	520-526	2005

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Sugita A, Hashimoto J, Maeda A, Kobayashi J, Hirao M, Masuhara K, Yoneda M and Yoshikawa H	Heterotopic ossification in bilateral knee and hip joints after long-term sedation.	Journal of Bone and Mineral Metabolism	23	329-332	2005
Watanabe T, Tomita T, Fujii M, Hashimoto J, Sugamoto K, Yoshikawa H.	Comparison between mobile-bearing and fixed-bearing knees in bilateral total knee replacements.	Int Orthop.	29	179-181	2005
藤原佐枝子	危険因子の民族差	カレントテラピー	22	59-61	2004
藤原佐枝子	脊椎骨折の発生率	日本臨床　増刊号	62	201-204	2004
藤原佐枝子	骨折リスクの予測因子	日本臨床　増刊号	62	583-586	2004
藤原佐枝子	腰椎変形とQOL	骨粗鬆症治療	3	32-37	2004
藤原佐枝子	脆弱性骨折の背景因子	ホルモンと臨床	52	279-283	2004
藤原佐枝子	脊椎骨折の位置付け	整形外科看護	9	17-19	2004
藤原佐枝子	骨折とEBM Ⅱ脊柱骨折	骨粗鬆症治療	3	70-73	2004
藤原佐枝子	骨粗鬆症の疫学	性差と医療	1	295-299	2004
藤原佐枝子	骨粗鬆症・骨折の疫学	Clinical Calcium	11	13-18	2004
藤原佐枝子	骨粗鬆症による椎体・非椎体骨折リスクのEBM	医学のあゆみ	212	139-142	2005
藤原佐枝子	QUS使用の実際　臨床応用　骨折のリスク評価	Osteoporosis Japan	13	43-44	2005
Fujiwara S.	Epidemiology of osteoporosis in Japan.	J Bone Miner Metab	23	81-83	2005
藤原佐枝子	骨粗鬆症の疫学と危険因子	日本内科学会雑誌	94	614-618	2005
藤原佐枝子	骨折の危険因子を知る	ホルモンと臨床	53	433-438	2005
藤原佐枝子	骨粗鬆症性脊椎圧迫骨折の診断と治療	Orthopaedics		181-185	2005
藤原佐枝子	骨粗鬆症の疫学	WHOテクニカル			
藤原佐枝子	骨粗鬆症と脊椎圧迫骨折	J Clinical Rehabilitation	11	984-988	2005
藤原佐枝子	ステロイドによる骨折リスク	骨粗鬆症治療	5	22-26	2006
藤原佐枝子	骨量測定・骨粗鬆症検診の有効性	地域保健におけるエビデンスに基づく骨折・骨粗鬆症予防ガイドライン（伊木雅之編）日本公衆衛生協会		68-72	2004
藤原佐枝子	骨粗鬆症検診・個別健康教育の進め方	地域保健におけるエビデンスに基づく骨折・骨粗鬆症予防ガイドライン（伊木雅之編）日本公衆衛生協会		92-96	2004
藤原佐枝子	骨密度減少率	基礎から臨床まで最新骨塩定量法（福永仁夫監修）メデカル　レビュー社		125-130	2004
藤原佐枝子	骨粗鬆症と骨折	(松本俊夫監修)ファーマ・ナビゲーター　メディカルレビュー社		42-49	2005
藤原佐枝子、増成直美、鈴木元、福永仁夫	超音波骨量測定値による骨折予知　骨密度による予知との比較	Osteoporosis Japan	12	73-75	2004
白木正孝	骨粗鬆症の薬物療法（最新情報）：治療薬の骨折予防効果に関する多剤比較試験	日老医誌	43	1の3	2006
小池達也、高岡邦夫	BMPs 薬理作用と生理作用-骨形成促進作用-	日本臨床	63	426-430	2005
小池達也	WHOテクニカルレポートをめぐって運動についての評価	Clinical Calcium	15	673-677	2005

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
渡邊具子、折戸芳紀、豊田宏光、洲鎌亮、多田昌弘、高岡邦夫、小池達也	ヒッププロテクターによる大腿骨頸部/転子部骨折の予防	整形外科看護	11	298-304	2006
渡邊具子、折戸芳紀、豊田宏光、洲鎌亮、多田昌弘、高岡邦夫、小池達也	ヒッププロテクターによる腿骨頸部骨折の予防	Osteoporosis Japan	14	88-90	2006
小池達也	ヒッププロテクターによる大腿骨頸部骨折の予防	Geriatric Medicine	44	187-193	2006
小池達也、折戸芳紀、多田昌弘、洲鎌亮、豊田宏光、小林千益、高岡邦夫	ヒッププロテクターは大腿骨頸部骨折ハイリスク集団の頚部骨折を抑制する	Osteoporosis Japan	14	42-45	2006
小林千益	レッグ-カルベ-ペルテス病(単純性股関節炎を含む) Legg-Calve-Perthes Disease (including Simple Coxitis).	今日の治療指針2005年版 (Volume 47), 山口 徹、北原光夫(総編集)、医学書院		749-750	2005
小林千益	ビスフォスフォネートと活性型ビタミンD3, ビタミンK2との併用は可能でしょうか.	松本俊夫(監), 水沼英樹, 萩野 浩(編) ファーマナビゲーター：ビスフォスフォネート編、メデイカルレビュー社		188-189	2005
小林千益	各種人工股関節置換術の要点	岩本幸英(監), 久保俊一(編) 股関節外科の要点と盲点., 文光堂		133-136	2005
小林千益	セメント人工股関節置換術のコツ	岩本幸英(監), 久保俊一(編) 股関節外科の要点と盲点, 文光堂		242-248	2005
小林千益	人工骨頭・人工股関節置換術：合併症予防のコツ	岩本幸英(監), 久保俊一(編) 股関節外科の要点と盲点, 文光堂		272-273	2005
小林千益	股関節の機能解剖と疾患と外傷	日本義肢装具学会誌	21	8-12	2005
小林千益、白木正孝、高岡邦夫	骨粗鬆症の予防と管理：WHOテクニカルレポートをめぐって：併用療法の効果	Clinical Calcium	15	661-665	2005
小林千益	人工関節置換術と骨質	Clinical Calcium	15	970-976	2005
小林千益、久保俊一、高岡邦夫	特発性大腿骨頭壊死症に対する人工関節置換術の成績：人工骨頭置換術との比較.	別冊整形外科	48	173-177	2005
堀内博志、五明広樹、中島滋郎、若林真司、斎藤直人、小林千益、縄田昌司、橋本博史、津田裕士、深沢徹、谷口俊一郎、高岡邦夫	特発性大腿骨頭壊死症におけるグルココルチコイド受容体の遺伝子多型解析	別冊整形外科	48	51-53	2005
小林千益	EBMに基づく骨粗鬆症の薬物療法：ビスフォスフォネート製剤の使い方. 特集：骨粗鬆症の薬物療法と予防	Modern Physician	35	1368-1376	2005

Ⅴ. 研究成果の刊行物・別冊

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OSTEOARTHRITIS

A new biotechnology for articular cartilage repair: subchondral implantation of a composite of interconnected porous hydroxyapatite, synthetic polymer (PLA-PEG), and bone morphogenetic protein-2 (rhBMP-2)

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Summary

Objective: Articular cartilage repair remains a major obstacle in tissue engineering. We recently developed a novel tool for articular cartilage repair, consisting of a triple composite of an interconnected porous hydroxyapatite (IP-CHA), recombinant human bone morphogenetic protein-2 (rhBMP-2), and a synthetic biodegradable polymer [poly-D,L-lactic acid/polyethylene glycol (PLA-PEG)] as a carrier for rhBMP-2. In the present study, we evaluated the capacity of the triple composite to induce the regeneration of articular cartilage.

Methods: Full-thickness cartilage defects were created in the trochlear groove of 52 New Zealand White rabbits. Sixteen defects were filled with the bone morphogenetic protein (BMP)/PLA–PEG/IP-CHA composite (group I), 12 with PLA–PEG/IP-CHA (group II), 12 with IP-CHA alone (group III), and 12 were left empty (group IV). The animals were killed 1, 3, and 6 weeks after surgery, and the gross appearance of the defect sites was assessed. The harvested tissues were examined radiographically and histologically.

Results: One week after implantation with the BMP/PLA–PEG/IP-CHA composite (group I), vigorous repair had occurred in the subchondral defect. It contained an agglomeration of mesenchymal cells which had migrated from the surrounding bone marrow either directly, or indirectly via the interconnecting pores of the IP-CHA scaffold. At 6 weeks, these defects were completely repaired. The regenerated cartilage manifested a hyaline-like appearance, with a mature matrix and a columnar organization of chondrocytes.

Conclusions: The triple composite of rhBMP-2, PLA-PEG, and IP-CHA promotes the repair of full-thickness articular cartilage defects within as short a period as 3 weeks in the rabbit model. Hence, this novel cell-free implant biotechnology could mark a new development in the field of articular cartilage repair.

Key words: Articular cartilage repair, Interconnected porous hydroxyapatite (IP-CHA), BMP, PLA-PEG.

Introduction

To date, the myth "once destroyed, cartilage cannot be repaired" has yet to be dispelled[1]. Mature articular cartilage cannot heal spontaneously owing to its low mitotic activity, which contrasts to the rapid rate of chondrocytic mitosis during normal cartilage growth.

Recently, several researchers have attempted to utilize culture-expanded autologous chondrocytes in combination with collagen sponges or fibrin glue to effect the repair of cartilage defects[2,3]. However, the results were either unsatisfactory or, if satisfactory, were achieved only after a lengthy wait for the regeneration of hyaline cartilage[2,4]. These poor results may reflect the characteristics of the transplantation technique, which involves the application of cartilage-derived cells to the defect[5].

Mesenchymal stem cells (MSCs) isolated from bone marrow have the ability to differentiate into chondrocytes, osteoblasts and other connective tissue cells of mesenchymal origin when cultured under appropriate *in vitro* conditions[6,7]. In an effort to exploit the pluripotentiality of MSCs, MSC-based repair strategies have been instigated in rabbits and goats, but with limited success[8,9]. Clinically,

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surgical interventions, such as microfracturing, abrasion arthroplasty and osteochondral drilling, have been widely used and considered to be partially successful[10,11]. These techniques are based on the concept that intentional damage to the subchondral bone recruits MSCs to the defect, thereby promoting cartilage repair.

A potentially powerful alternative approach for cartilage regeneration is the local administration of bone morphogenetic proteins (BMPs), which are members of the transforming growth factor-β superfamily. BMPs have been shown to regulate and promote the growth and differentiation of chondrocytes, osteoblasts and MSCs[12,13]. Indeed, recombinant human bone morphogenetic protein-2 (rhBMP-2) can stimulate the *in vitro* synthesis of components of the chondrocytic matrix, such as proteoglycans and type-II collagen[14–16]. Furthermore, BMPs are known to induce the condensation of MSCs when administered *in vivo*[17–19].

Inorganic biomaterials, such as carbon fibers[20], collagen scaffolds[2,21], absorbable polymers[22,23], and hydroxyapatite[24,25], have been used for articular cartilage repair. Some success has been achieved in the repair of small osteochondral defects, but no widely accepted method exists for the complete healing of hyaline cartilage. The cause of the failure lies not in the nature of the biomaterial itself but in its structure, which is not regulated three-dimensionally.

In the present study, we attempted to combine two distinct approaches: the strong induction of subchondral bone regeneration, with a view to recruiting bone-marrow MSCs to the osteochondral defect; and the appropriate local delivery of rhBMP-2 to induce chondrocytic differentiation and to stimulate matrix production by the chondrocytes. To instigate these two approaches simultaneously, we developed a combined biomaterial, which consists of a synthetic hydroxyapatite with an interconnected porous structure (IP-CHA), and a synthetic bioabsorbable polymer, namely, PLA–PEG (poly-D,L-lactic acid–polyethylene glycol block copolymer). In this system, PLA–PEG serves as a drug-delivery carrier, which permits the ideal release of rhBMP-2 over a period of about 3 weeks[26–29]. IP-CHA is made from hydroxyapatite, which is a bioactive ceramic with osteoconductive properties[30,31]. In addition, IP-CHA has a finely organized, three-dimensional interconnecting pore structure. The material is highly porous (porosity: 75%) and the pore size (150 μm) is appropriate for bone formation. The large interconnecting channels (average diameter: 40 μm) permit the easy penetration of tissue into the deep pores[30]. Owing to these structural properties, IP-CHA can itself induce local bone repair processes[30,32,33]. The interconnecting pore structure of the material also permits its easy impregnation with cytokines or growth factors borne by an appropriate delivery system.

The rationale behind the selection of key experimental design parameters was as follows: Skeletally immature adolescent rabbits (4–6 months old and 2.5–3.0 kg in weight) were selected because the ability of articular cartilage to repair depends mostly on the bone-marrow MSCs, which are metabolically more active and have a higher capacity to induce repair in an immature model. The decision to use full-thickness defects with a diameter of 4 mm and a depth of 6 mm was based on the results of previous studies. In rabbits, partial-thickness defects do not heal spontaneously, whereas full-thickness ones with a diameter less than 3 mm do, and the repair tissue is composed either of hyaline- or of fibrocartilage[34–36]. Hence, it was necessary to establish a model in which this upper limit for spontaneous repair was exceeded.

In the present study, we demonstrate the capacity of the triple composite of rhBMP-2, PLA-PEG, and IP-CHA to effect articular cartilage repair. The goal was to achieve the repair of full-thickness articular cartilage defects in rabbits in as short a time as possible, with the ultimate view of inducing the repair of similar lesions in humans; specifically, those generated during osteoarthritis, rheumatoid arthritis, and osteochondritis dissecans.

Materials and methods

PREPARATION OF IMPLANTS

IP-CHA was synthesized by Toshiba Ceramics Co., Ltd. (Kanagawa, Japan), as previously described[30]. In short, we adopted a "foam gel" technique, which involves two unique steps: a foaming step and a crosslinking step. During the foaming step, the hydroxyapatite slurry is mixed with a foaming agent (polyethyleneimine, 40% by weight). During the crosslinking (polymerization) step, the foam-like hydroxyapatite slurry is rapidly gelatinized using a water-soluble crosslinking agent (a poly-functional epoxy compound)[30].

rhBMP-2, which is produced by the Genetics Institute (Cambridge, MA) and was given to us by Yamanouchi Pharmaceutical Co., Ltd. (Ibaraki, Japan), was dissolved in buffer (5 mM glutamic acid, 2.5% glycine, 0.5% sucrose, and 0.01% Tween 80) at a concentration of 1 mg/ml. The solution was then filter-sterilized. Two-hundred mg of PLA–PEG [molecular weight (MW) = 11,400, dispersity (Mw/Mn) = 1.1 (Taki Chemical Research Laboratory, Kanagawa, Japan)] was dissolved in 1 ml of acetone. To prepare a single implant sample, a 25 μl aliquot of the PLA–PEG mass (5 mg) was mixed with a 20 μl sample of rhBMP-2 (20 μg). The specimen of IP-CHA (4 mm in diameter and 4 mm in height) was immersed in the mixture and the solvent was evaporated in a centrifuge/evaporator. The resulting BMP/PLA–PEG/IP-CHA composite was sterilized with ethylene oxide gas for 24 h on the day preceding implantation.

IN VITRO RELEASE KINETICS OF RHBMP-2

The release of rhBMP-2 from the BMP/PLA–PEG/IP-CHA composite was measured using a quantitative sandwich enzyme immunoassay technique (AN'ALYZA®; BMP-2 immunoassay, TECHNE Co. MN, USA). The dose of rhBMP-2 used in the *in vivo* experiments (20 μg) was chosen for the release study. Twelve BMP/PLA–PEG/IP-CHA composites, which were prepared in the same way as those used for implantation in the rabbit model, were placed within 24-well plates together with 500 μl of phosphate-buffered saline [(PBS) Sigma] and incubated for 21 days at 37°C. The supernatant was removed and replaced with fresh PBS every day. The supernatants removed on days 1, 3, 7, 14 and 21 were analyzed for their concentrations of rhBMP-2 according to the enzyme-linked immunosorbent assay (ELISA) technique. The bioactivity of the composites maintained *in vitro* for 0, 7 and 21 days (four samples per time point) was also assessed (see next section).

IN VIVO BIOASSAY FOR THE BMP/PLA–PEG/IP-CHA COMPOSITE

To assess the biological activity of composites that were maintained *in vitro* for 0, 7 and 21 days, these, as well as

IP-CHAs without rhBMP-2 (controls), were implanted within the back muscles of 5-week-old male JCL: ICR mice (one composite per animal; four mice per group, i.e., control, day 1, day 7 and day 21) as previously reported[27,28]. The implants were harvested 2 weeks after implantation. They were then crushed, homogenized in 0.2% Nonidet P-40 containing 1 mM $MgCl_2$, and centrifuged at 10,000 rpm for 1 min at 4°C. The supernatants were assayed for alkaline phosphatase (ALP) activity using *p*-nitrophenyl phosphate as a substrate. The product was measured spectrophotometrically at an absorption wavelength of 410 nm ($n = 4$ per group)[37].

ANIMAL EXPERIMENTS

Fifty-two New Zealand White rabbits weighing 2.5–3.0 kg (4–6 months) were kept in cages and had free access to food pellets and water. The rabbits were anesthetized by the intravenous injection of 1 ml of pentobarbital [50 mg/ml (Nembutal®; Dainippon Pharmaceutical Co. Ltd., Osaka, Japan)] and the intramuscular injection of 1 ml of xylazine hydrochloride [25 mg/ml (Seractal®; Bayer, Germany)]. After shaving, disinfection, and draping, a straight 3-cm long medial parapatellar incision was made over the right knee and the patella was everted. Full-thickness articular osteochondral defects, 4 mm in diameter and 6 mm in depth, were created mechanically in the patellar groove of the right distal femur. Rabbit knees were divided into four implant groups: group I ($n = 16$ knees) received the BMP/PLA–PEG/IP-CHA composite; group II ($n = 12$ knees) received the PLA–PEG/IP-CHA composite (no rhBMP-2), group III ($n = 12$ knees) received IP-CHA alone; and group IV ($n = 12$ knees) underwent a sham operation with no implantation. In groups I, II, and III, all implants were placed at the subchondral bone level, 2 mm beneath the surface of the adjacent cartilage. The fascial layer was closed with absorbable sutures, and the skin with 4-0 nylon sutures. One week after surgery, four rabbits in group I were killed. At 3 weeks, 24 rabbits were killed (group I = 6, group II = 6, group III = 6, group IV = 6), and at 6 weeks 24 rabbits were killed (group I = 6, group II = 6, group III = 6, group IV = 6). The animals were killed by an intravenous injection of 5 ml of pentobarbital (Table I). All animal experiments were approved by the Animal Laboratory, Faculty of Medicine, Osaka University, Japan.

RADIOGRAPHIC AND HISTOLOGICAL EVALUATIONS

The harvested tissues were radiographed using a soft X-ray apparatus [35 kV; 300 μA; 300 s; MX20 (Faxitron X-ray Co., IL, USA)] and then fixed in 4% paraformaldehyde (pH 7.4) for 48 h at 4°C. Tissue samples were decalcified in 20% ethylenediaminetetraacetic acid (pH 7.4) at 4°C, dehydrated in a graded ethanol series and embedded in paraffin. Serial sections (5 μm in thickness) were cut sagittally through the center of the operative site and stained with hematoxylin and eosin (H&E) or with safranin-O. For the immunohistochemical analysis, paraffin sections were treated with 3% H_2O_2 to block endogenous peroxidase activity. They were pretreated with serum to block non-specific staining. The sections were then incubated with mouse monoclonal antibodies: anti-type-I collagen (I-8H5, Daiichi Fine Chemical Co., Ltd, Toyama, Japan), anti-type-II collagen (II-4C11, Daiichi Fine Chemical Co., Ltd, Toyama, Japan), and anti-CD105 [(Endoglin) 555722, BD Bioscience, NJ, USA]; and with the polyclonal antibody goat anti-Cbfa1 [(Runx2) C-19, Santa Cruz, CA, USA][38]. The specimens were treated with the appropriate biotinylated secondary antibodies, and then incubated with the streptavidin/horseradish peroxidase complex. The signal was visualized as the red reaction product of a 3-amino-9-ethyl carbazole liquid substrate chromogen (AEC, DAKO JAPAN Co., Ltd, Kyoto, Japan). To confirm the specificity of

Table I
Information respecting the deployment of the 52 rabbits used in this study

	Number of rabbits				Total number
	Group I	Group II	Group III	Group IV	
Materials					
IP-CHA	+	+	+	–	
rhBMP-2	+	–	–	–	
PLA–PEG	+	+	–	–	
Follow-up time					
1 week	4	0	0	0	4
3 weeks	6	6	6	6	24
6 weeks	6	6	6	6	24
Total number	16	12	12	12	52

Table II
*Histological scoring system**

Category	Points
Cell morphology	
Hyaline cartilage	4
Mostly hyaline cartilage	3
Mostly fibrocartilage	2
Mostly non-cartilage	1
Non-cartilage only	0
Matrix-staining (metachromasia)	
Normal	3
Slightly reduced	2
Markedly reduced	1
No metachromatic staining	0
Structural integrity	
Normal	2
Slight disruption	1
Severe disintegration	0
Surface regularity†	
Smooth	3
Moderate	2
Irregular	1
Severely irregular	0
Thickness of cartilage, %	
121–150	1
81–120	2
51–80	1
0–50	0
Regenerated subchondral bone	
Good	2
Moderate	1
Poor	0
Integration with adjacent cartilage	
Both edges integrated	2
One edge integrated	1
Neither edge integrated	0
Total maximum	18

*A modified version of the system described by Wakitani *et al.*[8].
†Total smooth area of repair cartilage compared with the entire area of the cartilaginous compartment of the defect.

the antibody under the adopted conditions and to confirm the specificity of the markers in target cells, all antibodies were tested for their reactivity in control tissues.

HISTOLOGICAL SCORING

To quantify the histological repair of articular cartilage defects, we employed a modified version of the grading scale described by Wakitani *et al.*[8]. This consists of seven categories and assigns a score ranging from 0 to 18 points (Table II). The following parameters were assessed: cell morphology (hyaline cartilage); metachromatic staining of the cartilage matrix; structural integrity of the regenerated cartilage; surface regularity of the tissue; thickness of the cartilage layer; regeneration of the subchondral bone; and integration of the tissue with adjacent cartilage.

STATISTICAL ANALYSES

Data pertaining to ALP activity were analyzed using an unpaired Student's *t* test. The histological scoring data were analyzed using the Kruskal–Wallis test, with a *post hoc* Bonferroni correction for non-parametric data.

Results

EVALUATION OF THE IMPLANTS

Scanning electron microscopy of IP-CHA samples revealed these to have a finely organized three-dimensional structure. Most of the IP-CHA pores were spherical, of similar size (approximately 100–200 μm in diameter) and uniformly interconnected via channels [10–80 μm in diameter; Fig. 1(B, C)]. Scanning electron microscopy of the BMP/PLA–PEG/IP-CHA composite revealed the BMP/PLA–PEG component to affect neither the pore size nor the interconnecting pore structure and to coat well the surface of the IP-CHA [Fig. 1(D, E)].

IN VITRO RELEASE KINETICS OF RHBMP-2 AND *IN VIVO* BIOASSAY FOR THE BMP/PLA–PEG/IP-CHA COMPOSITE

Based on ELISA, the BMP/PLA–PEG/IP-CHA composite released significant quantities of rhBMP-2 during the 21-day monitoring period [6.85 ± 1.31 μg/ml on day 1, 0.79 ± 0.22 μg/ml on day 3, 22.9 ± 0.62 ng/ml on day 7, 4.76 ± 1.13 ng/ml on day 14, and 2.71 ± 0.70 ng/ml on day 21; Fig. 2(A)].

To assess the bioactivity of the BMP/PLA–PEG/IP-CHA composites maintained *in vitro* for 0, 7 or 21 days, we implanted these, as well as IP-CHAs (control for the absence of rhBMP-2) within the back muscles of male ICR mice (a standard ectopic bone-formation model) and then analyzed the explanted material for its ALP activity. High levels of ALP activity could be detected even on day 21 [Fig. 2(B)], which accords with the rhBMP-2 release kinetics results *in vitro*.

MACROSCOPIC OBSERVATIONS OF CARTILAGE DEFECTS

Three weeks after implantation, the repaired defects in group I had a macroscopically smooth and glistening appearance and exhibited continuity with the surrounding host cartilage [Fig. 3(A)]. The controls (groups II–IV) revealed varying degrees of cartilage resurfacing with fibrous tissue [Fig. 3(B–D)].

At 6 weeks, the color and the glistening appearance of the repaired defects in group I were similar to those manifested by the adjacent host cartilage. The junction between the repaired tissue and the surrounding host cartilage was not clearly visible [Fig. 3(E)]. In contrast, the regenerated tissue in the control groups (groups II–IV) was

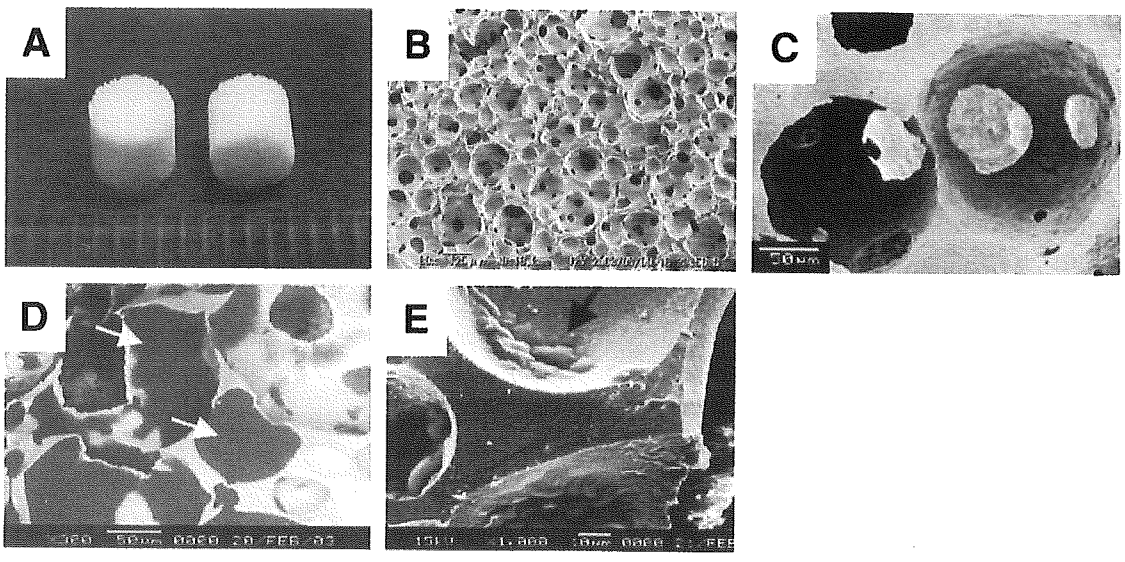

Fig. 1. Macroscopic photograph (A) and scanning electron micrographs (B–E) of IP-CHA specimens (4 mm in diameter and 4 mm in height). (A) Macroscopically, the surface of IP-CHA is slightly rough compared with that of other commercial porous hydroxyapatite materials, owing to its regular porous structure. (B, C) Scanning electron micrographs of IP-CHA, illustrating the regular arrangement of pores which are of similar size (100–200 μm in diameter), uniformly connected with each other, and separated by thin walls. (B) = 80×; (C) = 600×. (D, E) Scanning electron micrographs of the BMP/PLA–PEG/IP-CHA composite. (D) The dark areas lining the pores (white arrows) represent the BMP/PLA–PEG component. The introduction of BMP/PLA–PEG had no effect on either the pore size or the interconnecting pore structure. (E) Higher magnification of the lining of a pore (black arrow), revealing it to be well coated with BMP/PLA–PEG. (D) = 300×; (E) = 1000×.

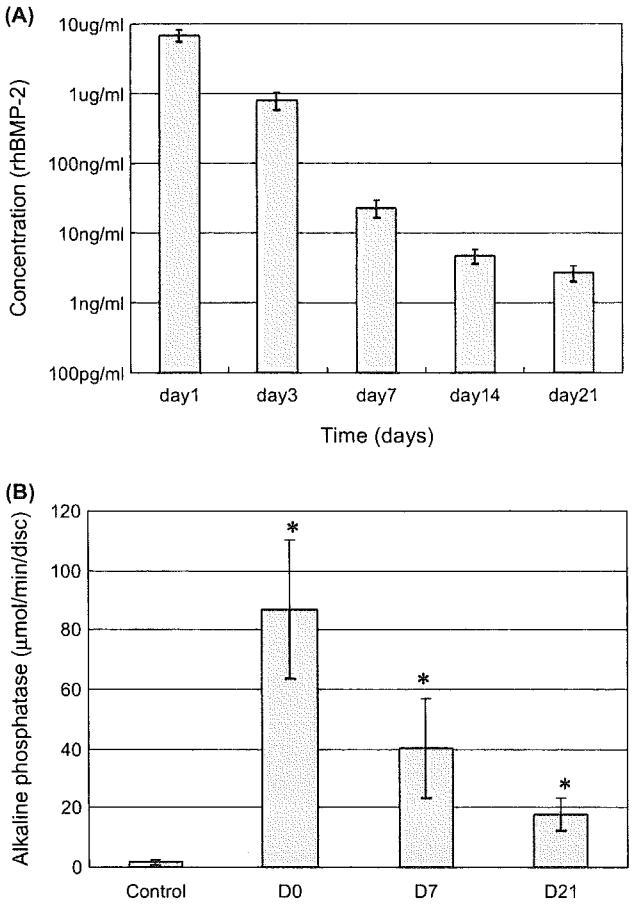

Fig. 2. Time course of rhBMP-2 release from the BMP/PLA–PEG/IP-CHA composite *in vitro* (A) and the bioactivity of the composites *in vivo* (B). (A) Release kinetics (measured by ELISA) of rhBMP-2 from the BMP/PLA–PEG/IP-CHA composite, illustrating significant quantities of rhBMP-2 during the 21-day monitoring period. The bar graph depicts the non-cumulative release at each time point. Mean values ± SD ($n = 4$) are represented. (B) The bioactivity of BMP/PLA–PEG/IP-CHA composites that were maintained *in vitro* for 0 (D0), 7 (D7), or 21 (D21) days was assessed 2 weeks after their implantation at an ectopic site in mice by monitoring the ALP activity of the explanted material. PLA–PEG/IP-CHA (no rhBMP-2) represented the control. High levels of ALP activity could be detected even on day 21 [86.8 ± 23.2 μmol/min/disc (day 0), 40.3 ± 16.8 μmol/min/disc (day 7), 17.7 ± 5.2 μmol/min/disc (day 21), 1.6 ± 0.8 μmol/min/disc (control)]. Mean values ± SD ($n = 4$) are represented. * = value is significantly different from the control ($P < 0.05$).

fibrous, and had a rough surface containing many fissures [Fig. 3(F–H)].

RADIOGRAPHIC EVALUATION

Six weeks after implantation, the soft X-ray analysis revealed defects treated with the BMP/PLA–PEG/IP-CHA composite (group I) to be consistently filled with newly formed bone, which was continuous with the surrounding intact subchondral bone [Fig. 3(I)]. In the control groups (groups II–IV), bone formation was incomplete and irregular [Fig. 3(J–L)].

HISTOLOGICAL EVALUATION

One week after implantation with the BMP/PLA–PEG/IP-CHA composite (group I), vigorous new bone formation was observed histologically within the pores of the IP-CHA scaffold [Fig. 4(B)], and about three-quarters of the defect depth above the IP-CHA had already been replaced with repair tissue. The central part of the repair tissue contained a fibrin clot and a few vessels. The lateral and lower regions consisted of granulation tissue, which was actively undergoing neovascularization and contained rounded fibroblast-like cells. These cells registered positive for Cbfa1 and/or CD105. They appeared to have infiltrated from the surrounding intact subchondral bone, either directly, or indirectly via the interconnecting IP-CHA pores, which were likewise filled with granulation tissue. Some of the pores in the peripheral 1-mm portions of IP-CHA blocks already contained newly formed bone (Fig. 4).

Three weeks after implantation with BMP/PLA–PEG/IP-CHA, the defect space above the IP-CHA blocks (subchondral space) was filled with newly generated and vigorous bone tissue, which penetrated the interconnecting pores of this material [Fig. 5(A, F)]. The regenerated articular cartilage was more cellular and contained less extracellular matrix than normal cartilage. The regenerated cartilage was divided into three distinct zones: (1) a superficial one, which contained flattened hyperchromatic cells; (2) a middle one, which contained rounded chondrocytes; and (3) a zone of enchondral ossification. The cartilage-like layer was two-to-three times thicker than normal cartilage [Fig. 5(A, E)]. In each of the control groups (groups II–IV), the regenerated fibrous cartilage had a similar morphological appearance, irrespective of the absence or presence of an implant. Although the subchondral space in group II tended to be filled with more newly formed bone than did that in the other control groups (groups III and IV), the quantitative histological evaluation revealed no significant difference between them [Table III; Fig. 5(B–D)].

Six weeks after implantation, defects treated with the BMP/PLA–PEG/IP-CHA composite (group I) were filled with regenerated subchondral bone, which also penetrated the pores of the implant. The subchondral bone was covered with a layer of regenerated cartilage tissue of almost normal thickness. The hyaline nature of the cartilage was maintained, and the tissue was beginning to assume a columnar organization and a horizontal stratification into four distinct zones (superficial, middle, deep and calcified), as in normal cartilage [Fig. 6(A, E)]. Interestingly, no gaps could be distinguished microscopically between the host cartilage and the newly regenerated cartilage, which suggests that the tissues were functionally and biologically integrated [Fig. 6(F)]. Safranin-O staining was evident predominantly in the middle and deep zones. Immunoreactivity for type-II collagen tended to be weakest in the deep zone at the junction with host tissue. But generally, the matrix exhibited a hyaline-like cartilaginous phenotype [registering negative for type-I collagen; Fig. 6(J–L)]. In contrast, defects in the control groups (groups II–IV) were filled with a hypercellular type of fibrous tissue. No hyaline cartilage was detected, despite the presence of new bone above and within the implant; [Fig. 6(B–D, G–I)]. Histological sections were assessed quantitatively using a modified version of an established grading system, which measures the degree and quality of cartilage repair[8] (Table II). At each time point, the scores for the BMP/PLA–PEG/IP-CHA composite (group I) were significantly better than those for groups II, III and IV ($P < 0.01$). These findings accord with the macroscopic and histological observations.

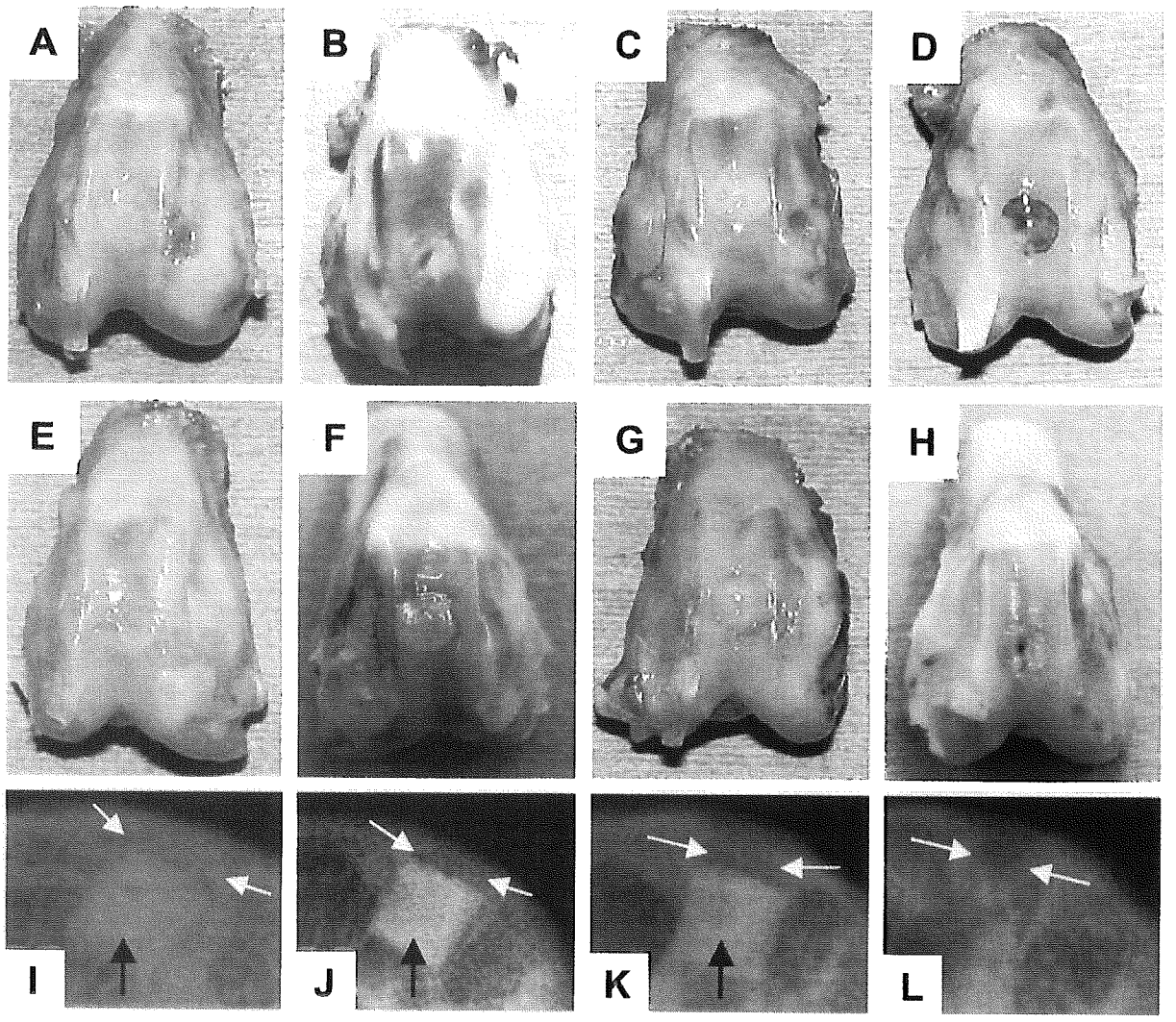

Fig. 3. Gross appearance (A–H) and soft X-ray photographs (I–L) of four specimens (one in each group) 3 weeks (A–D) and 6 weeks (E–L) after implantation. (A, E, I): BMP/PLA–PEG/IP-CHA composite (group I). (B, F, J): PLA–PEG/IP-CHA composite (group II). (C, G, K): IP-CHA alone (group III). (D, H, L): no implant (group IV). (A, E) In group I, reconstruction of the surface was good. At 3 weeks, the surface was still a little "white"; but at 6 weeks, it was smooth and glistening and exhibited continuity with the surrounding intact host cartilage. These macroscopic findings correspond with the histological results (Table III). (B, F) In group II, the articular cartilage defects were covered with "white" fibrous tissue with many fissures. (C, G) In group III, the regeneration of the defect looked better than those of other control groups (group II and IV), whereas the junction of the defects were still visible. (D, H) In group IV, in which the defects were left empty, at 3 weeks the defects were filled with red, semitransparent tissue with the margins sharply defined and the edges completely discernible (D). At 6 weeks, the defects were filled with irregular "white" tissue with pin-hole like fissure (H). (I–L) Representative soft X-ray photographs of the four specimens. The black arrows denote the implanted IP-CHA block. The white arrows above the IP-CHA block indicate the region where subchondral bone should be regenerated. A white, radiodense zone was observed above the IP-CHA block in group I (I); it denotes a vigorous regeneration of subchondral bone. This radiodense zone was not detected in groups II–IV (J–L).

Discussion

Several investigators have reported on the repair of articular cartilage defects using diverse tissue-engineering approaches. These include a gene-enhanced technique, the direct implantation of growth factors, and *in vitro* cell expansion[39–42]. BMPs have been shown to induce the differentiation of MSCs into chondrocytes both *in vitro* and *in vivo*. BMPs (BMP-2 and BMP-7) have also been used in conjunction with type-I collagen sponges to elicit the repair of osteochondral defects[43–45]. Cook *et al.*[40] have reported that type-I collagen sponges impregnated with rhBMP-7 can induce the repair of full-thickness osteochondral defects with hyaline-like cartilage in a dog model. The hyaline-like quality of the repair cartilage was still evident 52 weeks after surgery and the tissue had undergone no significant degradation. Sellers *et al.*[41] have demonstrated the capacity of rhBMP-2 to accelerate the healing of full-thickness articular cartilage defects and to improve the histological appearance and the biochemical characteristics of the repair cartilage. However, the tissue still differed from normal hyaline cartilage, both biochemically and structurally, and a long time elapsed before the defect area was completely filled with it. These suboptimal results probably reflect a limited recruitment of MSCs and/or a restricted delivery of cytokines, owing to the poor structural