とがわかった．これらの結果から本装具が即時的に膝OA患者に運動学，および運動力学的な影響と，主観的な疼痛の減少の効果を与えることがわかった．今後は症例数を増やし，主観的な効果に加え，客観的なパラメータに対する効果の検討を長期的に追っていく必要があると考える．

おわりに

臨床では膝装具を装着するのみでは求めている結果が得られないことも多く，装具の性能をさらに引き出すためには義肢装具士，理学療法士と検討しながら，適切な評価を行い，患者に応じた装具を選択する必要がある．装具療法を開始する場合には装具装着による姿勢や歩行を始めとした動作への影響を観察し，身体機能が適切に制御されるように装具の調整，さらに筋力低下，他関節や体幹などの代償を考慮して運動療法を併行して行うことが重要である．装具療法に加え各種保存療法を組み合わせることで膝OAの病態，症状の進行，腰部や足・股関節などの他の身体部位の関節疾患の発生を防ぐことで初めて膝OA患者の病態進行の抑制，症状の緩和が可能になると考える．

今後も膝OA患者用の膝装具の開発が行われると推察されるが，前述のようにその効果に関する根拠が統一されておらず，またその長期的な効果に関しての見解も少ないのが現状である．新たな装具の開発と装具療法を行うにあたり，膝装具の効果と身体に与える影響など，厳密な検討とさらに長期的効果を今後も詳細に分析することが必要である．

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Kinematics MRI

運動器の画像診断では，「動き」や「荷重」などの動的かつ機能的な画像診断が望ましい．MRIは，高磁場化と撮像シーケンスの進歩により空間分解能が向上し，運動器疾患での形態診断では非常に重要となっているが，非荷重の静止肢位での撮影である点に限界がある．今後，動的な機能診断が求められる．

本項では，より動態に近い機能を解析する目的で筆者らが開発している，撮影肢位を変えた撮影と解析方法および荷重負荷での撮影解析法を紹介し，MRIでの新しい画像診断法につき解説する[1-3]．

MRIによる関節組織の三次元動態解析

MRI撮影方法と工夫

撮像装置と体表コイル

オープンMRI装置，または，ガントリーが大きいMRI装置を用いて，関節の肢位を変えて撮像する（❶-a，❷-a）．撮影部位に応じて適切な体表コイルを用いて，肢位を変えても良いMR画像が得られることを確認する（❸，❹-a, b，❺-a, b）．

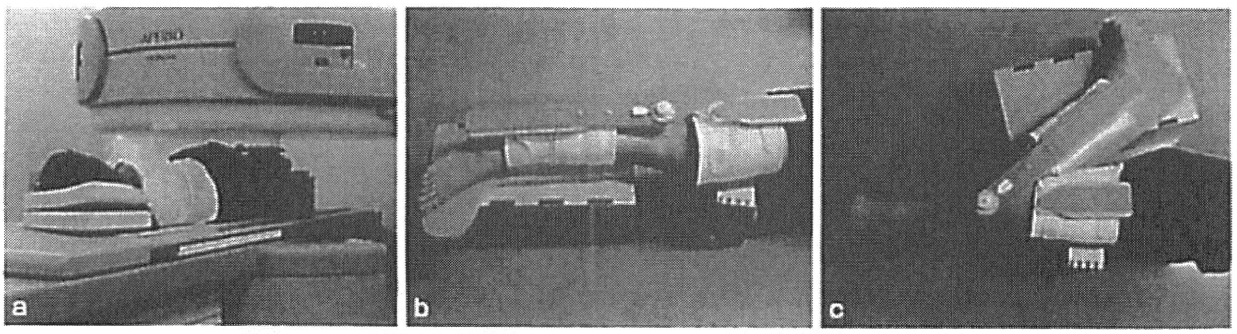
得られたMR画像が良好でないと定量的な解析に適さないため，前撮影にて条件を十分に検討する必要がある．X線撮影技師や放射線科医との，撮影目的や撮像方法の相談，協力が不可欠である．

撮影ポジション

関節角度を変えて異なる肢位の数ポジションで撮像する．なるべくMRI装置の磁場中心付近で良い像が得られるようにする．

撮像中に動かないように仰臥位や側臥位や立位などの体位を工夫することと，固定用装具やパッドなども用いるが金属性のものはMRI装置内で使えないため自作しなければならないことが多い（❶-b, c，❷-b）．

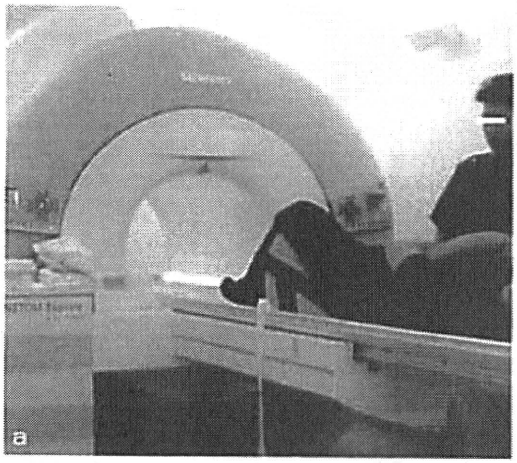
多くのポジションで撮像すると細かな動態解析が可能になるが，一方，撮像にかかる時間が長くなるため，解析に必要な撮像ポジション数を検討する必要がある．実際にかかる撮像の時間は，MRI装置や撮像条件によって異なるので，いくつかの条件を試し検討する．

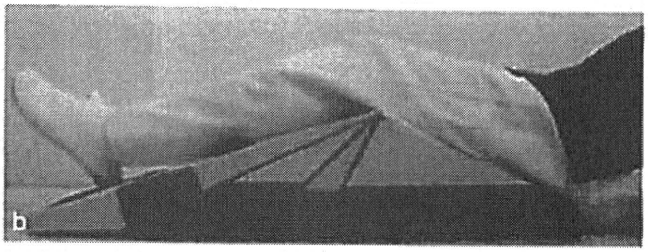


❶ オープンMRI装置を用いた膝関節動態撮影

a：被検者は測定する膝関節を上側にして横型オープンMRI装置で側臥位にて撮像する．

b, c：膝関節を伸展0°（b）から屈曲150°（c）まで曲げた状態で，MRI撮像中に動かないように固定する装具を装着する．

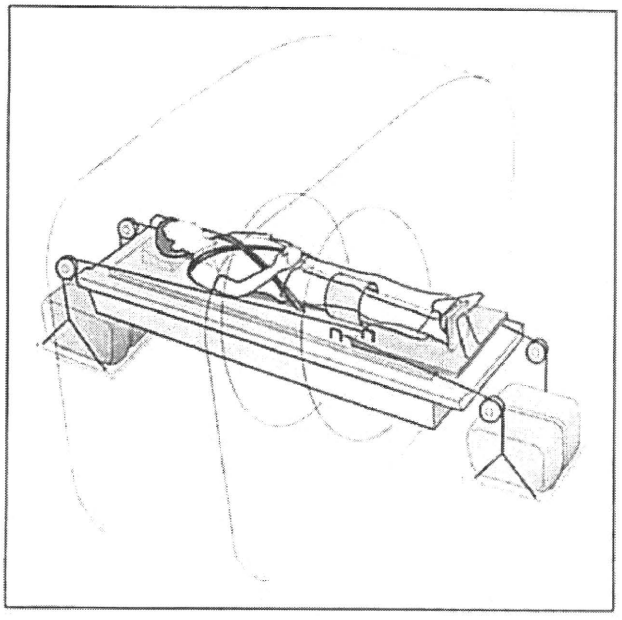




❷ 通常の MRI 装置を用いた膝関節動態撮影

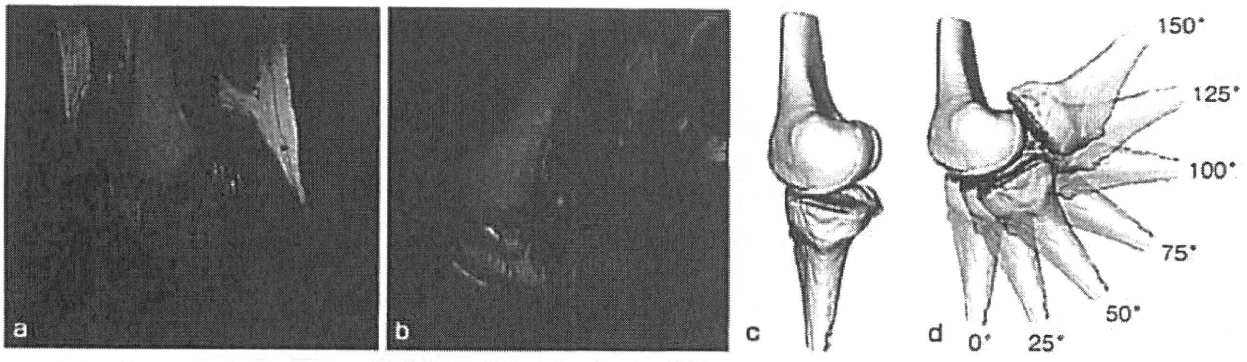
a：被検者は MRI 装置のコイルの中で膝関節をやや屈曲角度で保持できるようにして撮像する．

b：臥位にて膝を角度を変えて保持する台．MRI で磁気干渉しないよう発泡スチロールにて取り外し可能にして，膝関節角度を変えられるようにした．



❸ 臥位で荷重負荷での MRI 撮像

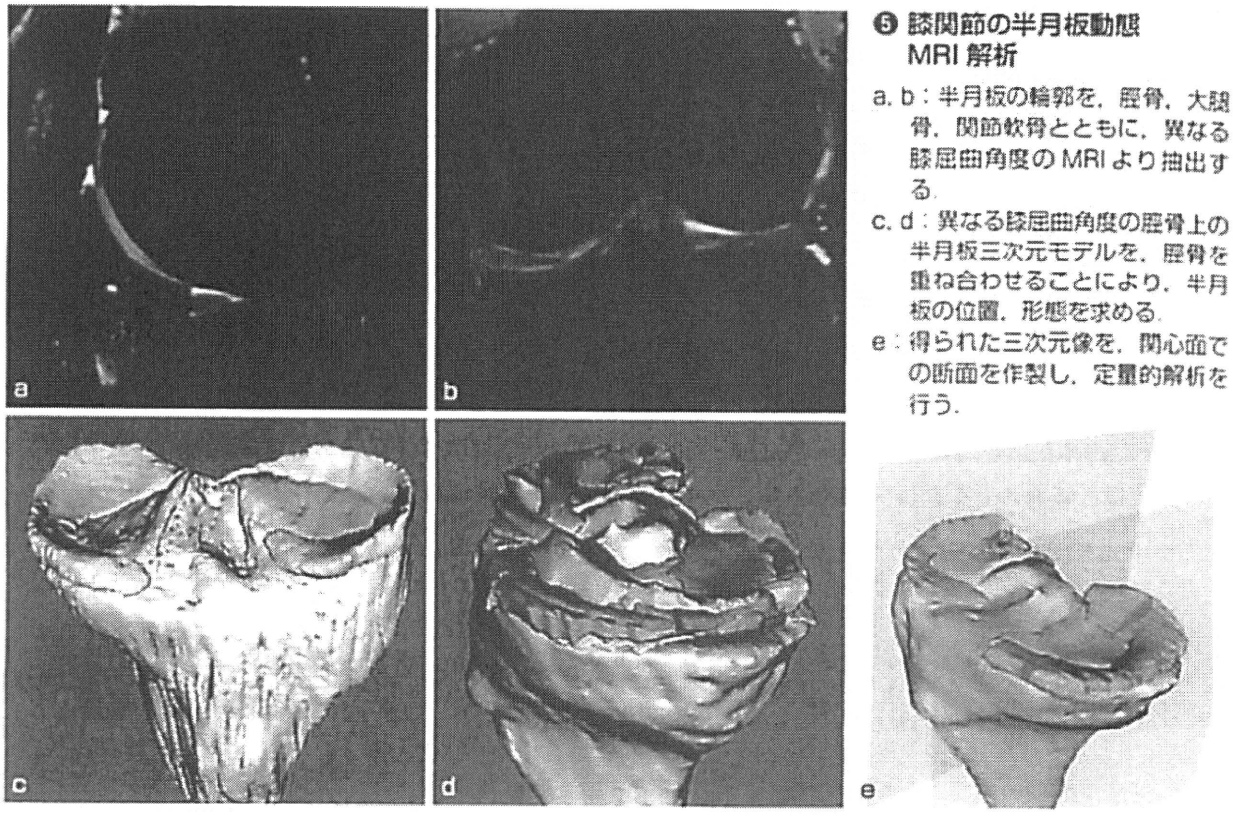
臥位で両肩と足部の踏み板に，水をいれたタンクを錘りとして，足部，膝関節，股関節，脊椎に荷重がかかるように工夫して撮像する．膝関節の観察の場合には，膝表面コイルを用いる．



❹ 膝関節屈曲の動態 MRI 解析

a, b：膝関節を屈曲して撮像した MR 画像から，大腿骨，脛骨，関節軟骨の輪郭を各スライスで抽出する．

c, d：各スライスの抽出した画像から三次元モデルを作製し，異なる角度の三次元モデルを大腿骨を重ね合わせることで，脛骨の位置を解析する．



❺ 膝関節の半月板動態 MRI 解析

a, b：半月板の輪郭を，脛骨，大腿骨，関節軟骨とともに，異なる膝屈曲角度の MRI より抽出する．

c, d：異なる膝屈曲角度の脛骨上の半月板三次元モデルを，脛骨を重ね合わせることにより，半月板の位置，形態を求める．

e：得られた三次元像を，関心面での断面を作製し，定量的解析を行う．

撮影画像のコンピュータへの取り込み

得られた MRI データを DICOM データから画像化し，対象とする組織の輪郭を抽出する（❹-a, b, ❺-a, b）．

輪郭が適切に抽出できるためには，組織の輪郭が鮮明で境界のコントラストが十分であること，病的状態や関節の動きに伴う組織の変形や力学的負荷により画質が大きく変わらないことが必要である．

関節の動きに伴って組織の画像の鮮明度，コントラストが大きく変わるようであれば組織形状の抽出には適さないので，MRI 撮像条件を検討する．

三次元バーチャルモデルの作製（❹-c, ❺-c）

MR 画像の各スライスから，解析対象とする組織とその周囲組織の輪郭を抽出し，三次元構築して組織のバーチャル三次元像を作製する．

三次元のマッチング（❹-d, ❺-d）

骨を基準に重ね合わせ（マッチング）することにより，半月板や関節唇，その他軟部組織の三次元モデルの異なる肢位での変位，変形を評価する．

アニメーション作製による動態の把握，評価，解析を行う．

信頼度，再現性の確認

得られたデータの測定者間，または，同一測定者での再現性（inter-observer error, intra-observer error）を測定し，定量性を確認する．この方法での骨の位置計測では，1mm 以下の精度が得られている[1]．

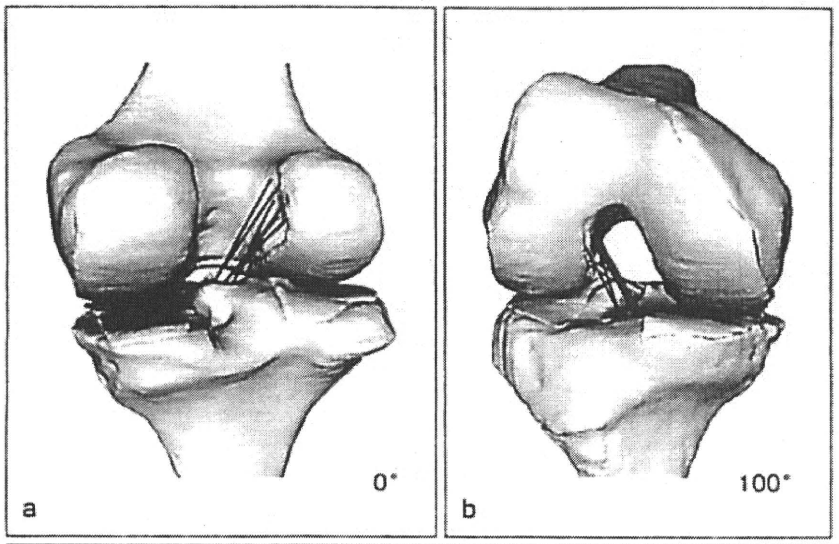
定量的解析（❺-e, ❻）

三次元モデルの動態を，ある特定の断面での評価や特徴点を抽出することにより定量的解析を行う．

具体的な定量的解析例

膝前十字靭帯（ACL）の“機能的長さ”の解析[1]

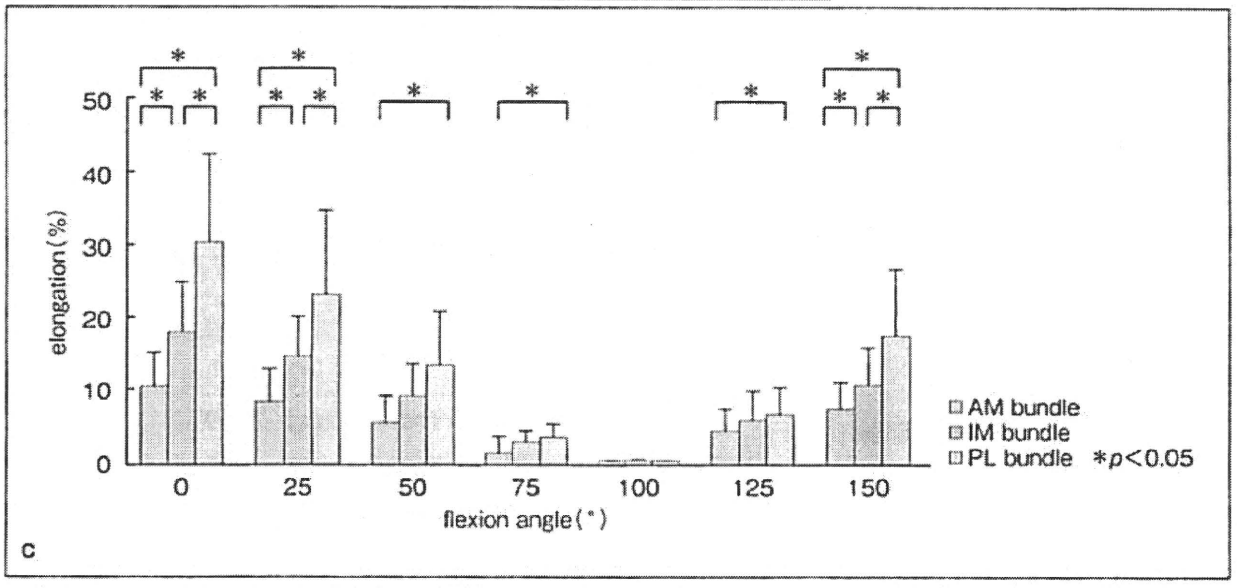
健常人 9 人の膝関節の動態解析（0～150° 屈



❻ 膝前十字靭帯（ACL）の"機能的長さ"の解析例

a, b：ACLの異なる線維束の大腿骨側，脛骨側の付着部を結ぶ線分の長さを"機能的長さ"として，膝屈曲角度0°から150°までそれぞれ求める．

c：膝屈曲100°の"機能的長さ"が最短となり，伸展，屈曲によりそれぞれの線維束は，異なる割合で長くなる．



曲までの25°ずつ7ポジション）をオープンMRI装置にて行った（❹）．

ACLの3つの線維束（AM（前内側線維束），IM（中間線維束），PL（後外側線維束））の大腿骨，脛骨のそれぞれの付着部中央を結ぶ線分の長さを各膝屈曲角度における"機能的長さ"として計測した（❻-a, b）．

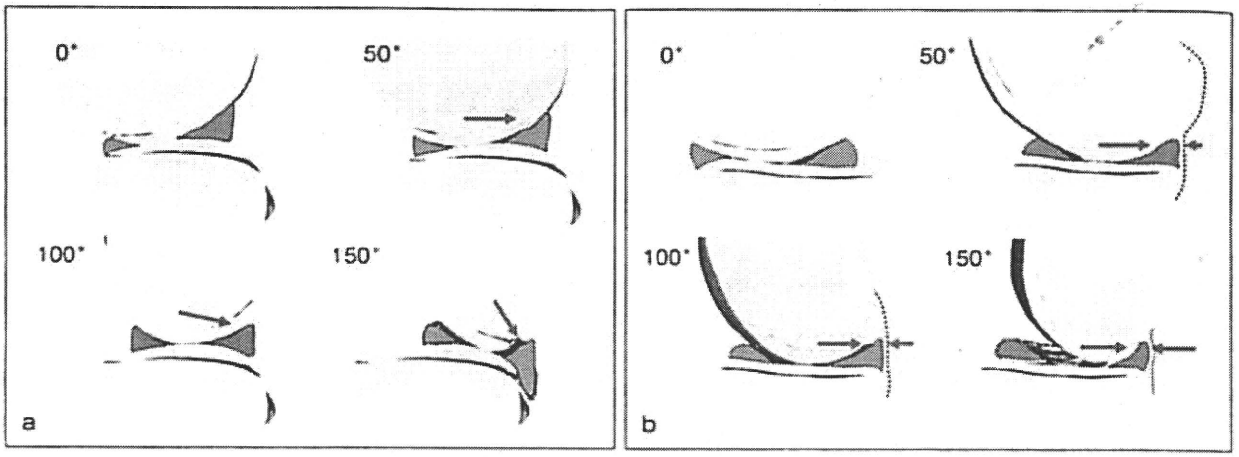
測定した9人のACL各線維束の"機能的長さ"は，AMでは31～34mm，IMでは28～33mm，PLでは21～27mmであり，伸展位で最大となり，屈曲100°で最小となった（❼）．

ACL各線維束の"機能的長さ"の変化率を，最小長となる膝屈曲100°を基準にすると，AM，IM，PLで25°以上の伸展，または，150°屈曲

❼ ACLの各線維束の異なる膝屈曲角度における"機能的長さ"

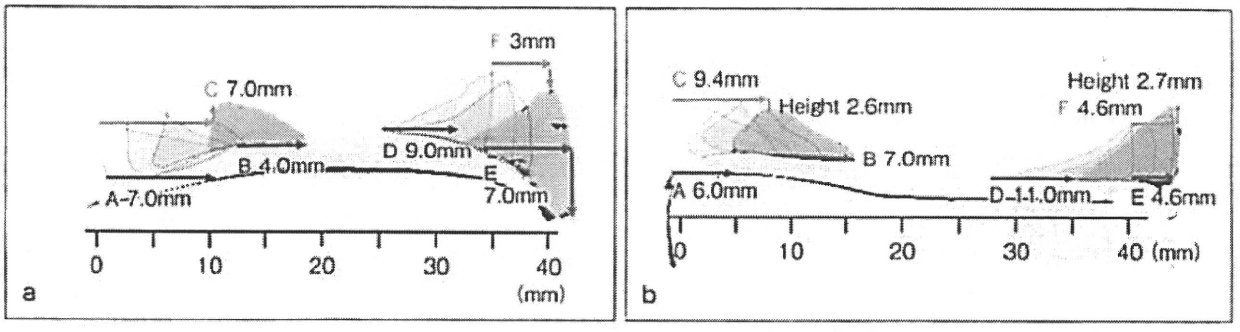
	AM	IM	PL
0°	34±1	33±1	27±2
25°	34±1	32±2	26±2
50°	33±1	31±1	24±2
75°	32±2	29±2	22±3
100°	31±2	28±2	21±3
125°	32±2	30±2	22±2
150°	33±1	31±2	25±2

The values are in mm (mean±SD).
AM：前内側線維束，IM：中間線維束，PL：後外側線維束．



❽ 膝半月板の三次元動態の画像解析

a：外側半月板，b：内側半月板．
膝関節 0° から 150° までの三次元動態解析を，断面で示した図（本文参照）．



❾ 膝半月板の三次元動態の定量的解析

a：外側半月板，b：内側半月板．
膝関節 0° から 150° までの動態を，内側，外側のコンパートメント内での断面を作製し，半月板の特徴点の位置，実質の形態につき定量的に解析した（本文参照）．

で，各線維束間に有意な差があり，AM が最も等長性があり，PL では膝伸展位で膝屈曲 100° に比べて 30％の "機能的長さ" の延長を認めた（❻-c）．

半月板の三次元動態解析[2]

半月板の動態の解析例を示す．健常人の半月板は，膝関節の伸展から屈曲 150° までに，内側半月，外側半月とも前節，後節は後方に移動して変形し，特に外側半月後節では変形が強く，屈曲 150° では関節軟骨面から亜脱臼するように後方に変位，変形する（❽）．

外側半月板では，膝関節 0° から 150° の屈曲に伴い前節は 4～7mm 後方に移動して半月の幅が 3mm 短縮し，後節は 7～9mm 後方に移動する（❾-a）．

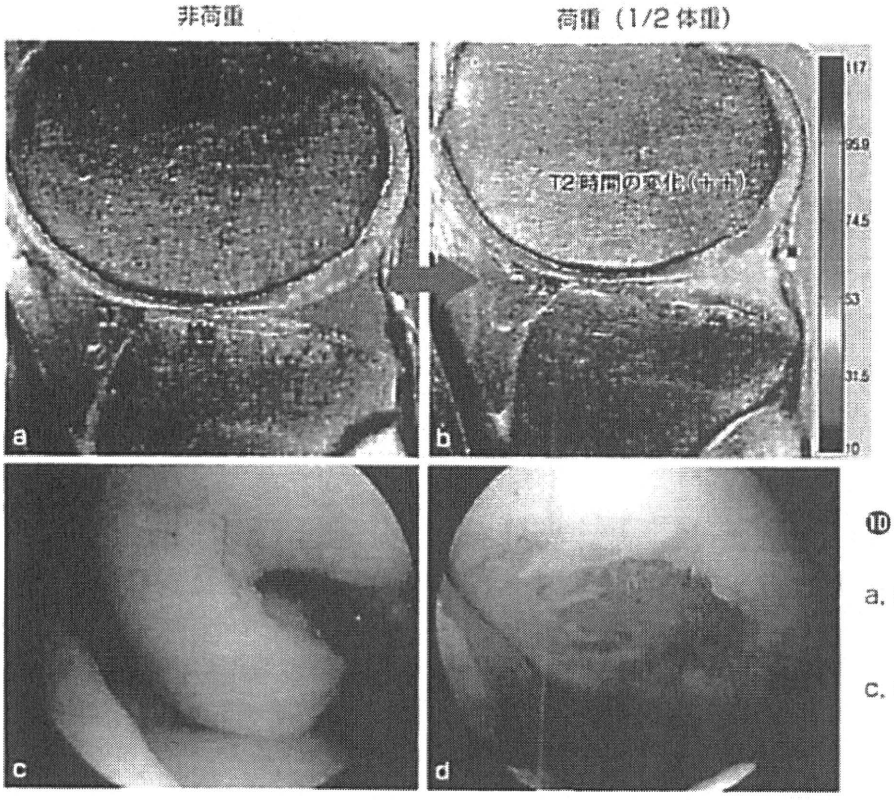
内側半月板では，前節は大腿骨側の動きが 9.4mm と脛骨側 6mm より大きく，後節では幅が短縮し，滑膜移行部での半月板の高さが増加し，半月実質が変形している（❾-b）．

荷重 MRI

撮影方法

荷重をかける装置の工夫

臥位で撮影する間に，足底と上体で保持した荷重をかける装置で，脊椎や股関節，膝関節，足関節など荷重関節に荷重した状態を再現する（❸）．



⑩ 膝関節軟骨の荷重時 MRI 解析

a, b：膝関節 3Tesla MRI を❸で示した荷重負荷にて撮像し，T2 時間で定量的解析を行った．

c, d：同一症例の膝関節鏡像．荷重 MR にて T2 時間の変化がみられた部位は，明らかに関節軟骨の異常を認めた（本文参照）．

定量的解析

MRI 撮影画像を定量評価するために，関心領域（ROI）を作製し，T1 マッピングや変位，変形などを求める（⑩）．

具体的な解析例

3 Tesla 高解像度 MRI 装置を用いて，荷重 MRI による T2 時間の変化を解析し，関節鏡像と比較した（⑩）．

外側半月板切除後の軟骨障害の例で，大腿骨外側顆部関節軟骨面に，非荷重にても関節軟骨の厚みの増大，T2 時間の異常を認め，荷重 MR にて，さらに正常ではみられない T2 時間の変化を認めた（⑩-a, b）．

関節鏡像にて大腿骨外側顆部関節軟骨面の膨隆を認め，プローブにて容易に剥離を認めた（⑩-c, d）．

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特集
変形性膝関節症の治療戦略

【装具療法】

変形性膝関節症に対する膝装具療法の最近の知見

Recent observation of treatment for knee osteoarthritis with knee orthosis.

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Key words
●変形性膝関節症(knee osteoarthritis)
●保存療法(conservative treatment)
●機能的膝装具(functional knee brace)

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はじめに

近年，日本では急速な高齢化に伴い，運動器疾患の罹患人口は増加の一途を辿っている。とくに膝関節などの荷重関節ではメカニカルストレスにより退行性変性が生じるため，変形性膝関節症(膝OA)が発症，進行しやすい。将来的にも膝OA患者は増加していくことが容易に予測でき，その治療にかかる医療費の増大は社会的な問題であり，早急な解決が必要な課題である。

膝OA治療の主な目的は除痛，身体機能の改善，およびアライメント異常の修正であり，手術療法と保存療法に大別される。一般的に進行した重症例の場合は手術療法が行われるが，圧倒的多数は保存療法の適応となる。保存療法は運動療法，物理療法などを含めた理学療法，薬物療法，そして装具療法がある。

本稿では，装具療法のなかでもとくに膝装具についての膝OA治療における知見を述べる。

膝装具の種類

膝OAでは膝関節の内反変形を呈し，内側関節面への圧縮ストレスが増大することで関節軟骨の摩耗を引き起こし，変形が助長されるという悪循環が生じる。その結果，疼痛や関節運動制限や活動制限が生じ，ADLやQOLの低下につながる。これらに対する膝装具の役割として，

①変形の矯正と予防
②関節の運動制限と固定
③関節運動の補助
④免荷

があげられる[1]。膝装具にはいくつかの種類があり，それぞれに異なる機能と効果をもつ。

軟性装具(図1)

局所的な保温作用と，着用による安心感という心理的作用が主な目的で使用される。安価で

かつ簡易的に装着が可能なため高齢者は好んで着用するが，サポータータイプの場合，膝関節に対する直接的な矯正力はなく，精神的な安定感が疼痛を軽減していることが推測される。過去の報告として関節固有感覚の向上や姿勢制御機能の改善[2),3)]などの報告があるが，これらは膝関節全体を覆うことにより膝関節周囲筋を圧迫，刺激し，固有感覚受容器や身体機能に影響を与えたことが考えられる。

機能的膝装具(brace)(図2)

braceには膝関節を安定化させる作用があり，歩行立脚期における不安定性が制動され，関節の安定性と除痛効果を得ることができる。また筋電図による下肢の筋活動の評価では，膝関節周囲の筋活動が変化することで疼痛が軽減しているという報告もある[4)]。臨床的には中，長期的な治療結果が国内外で報告されており，その効果の持続性が報告されている[5)]。しかし，その機能的なメリットの反面，軟性装具と比較すると高価で，またその重量感，着用感から患者のコンプライアンスが低く，汎用性に欠けるともいえる。しかし，最近では膝OA患者の大半である高齢者向けに開発され，意匠性と実用性を配慮された装具も徐々に出現してきている。

braceの効果

外反矯正のメカニズム

膝OAでは内側関節面への圧縮ストレス増大が病態と密接に関係しており，その指標として三次元動作解析により計測される外部膝関節内反モーメント(膝内反モーメント)が注目されている。braceは外反矯正力(①)を加えることで膝関節にかかるストレスを減少させる(②)が，そのメカニズムは装具の種類によって異なる。たとえば，内側継ぎ手付き装具の場合，内側の支柱の大腿部分と下腿部分による圧迫と，外側のストラップによる(③)膝関節裂隙部分における圧迫によって三点支持機構をなしており，これによって外反矯正力を加える**(図3)**。

図1 支柱付き軟性膝装具

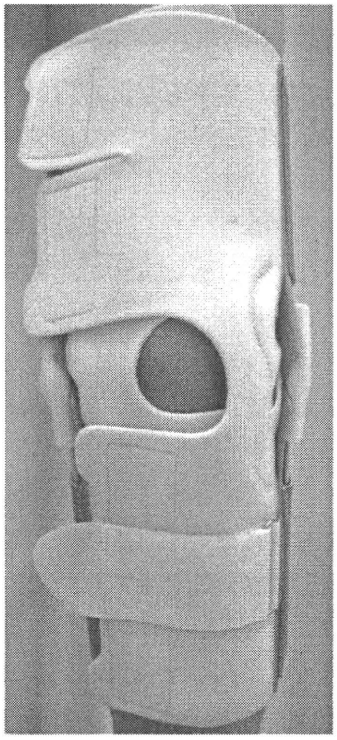


図2 機能的膝装具(brace)

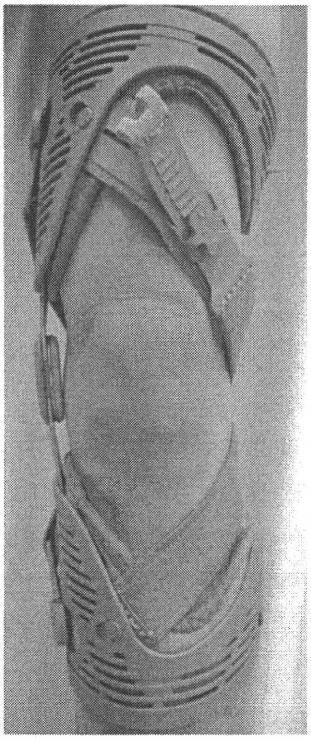
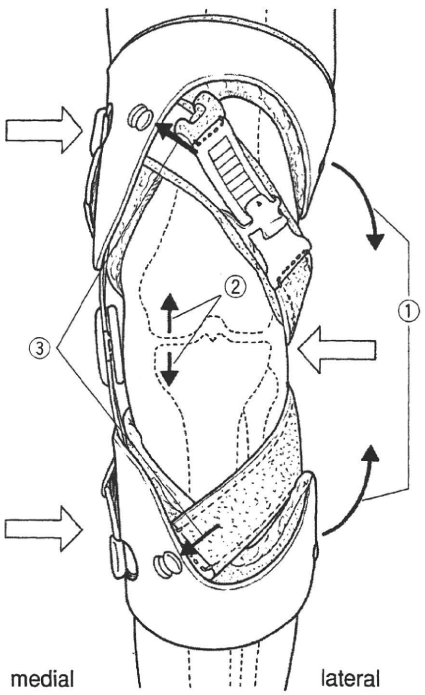


図3 三点支持機構のメカニズム



白矢印：三点支持

バイオメカニクス

過去の研究においてbraceによる膝内反モーメントへの影響に関してはさまざまな報告があり，その多くにおいて膝内反モーメントを減少させることが示されている[5]。また動的な下肢アライメントに対する影響としては，歩行中の踵接地時の関節裂隙の拡大を認めたという報告がある[6]。

著者らは最近開発されたbrace装着前後での歩行への影響に関する調査を行っている**(図4)**。braceを装着した際，装着した下肢の立脚期に骨盤が装具側へ傾斜しており，体幹もやや側屈している。またとくに高齢者の場合はbrace装着状況に適応するため，顔が下を向き，視野が狭まるため転倒のおそれもある。

このようにbraceを装着することで通常とは異なる状況におかれた患者では，環境に適応しようと動作を変化させることがあり，その歩容の変化もさまざまである。もし，この瞬時的な動作の変化が長期にわたって残存する場合は，ほかの筋骨格系に影響を及ぼす可能性があり，単にbraceを処方するだけでなく，理学療法士と連携をとりながら，歩行動作の改善も視野に含めた全身的なアプローチも考慮することが必要である。

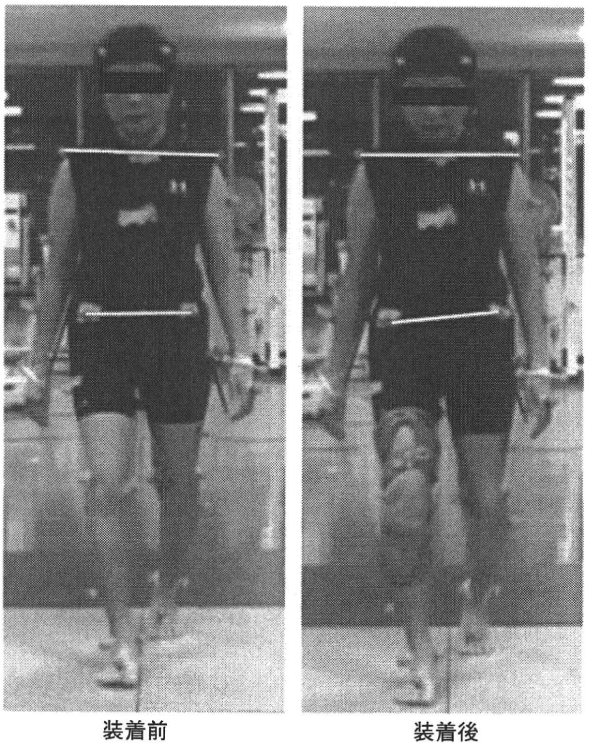
臨床効果

braceを装着することにより，非荷重位では関節裂隙に変化はみられないが，荷重位では関節裂隙の拡大がみられ，立位や歩行時など荷重活動時にbraceの有効性が出現すると予測される**(図5〜7)**。braceによる臨床的評価に関する過去の研究では，12カ月の介入後に大腿脛骨角(FTA)が改善したという報告がある一方で[7]，3カ月間ではX線像上での変化がなかったという報告も存在する[8]。装具の種類にもよるが，長期間のbraceの使用によってアライメント異常に対する効果が出現する可能性が考えられる。

また臨床症状に関する介入の報告も多くなされており，著者らはbrace装着による主観的症状とQOLに対する影響を調査している。

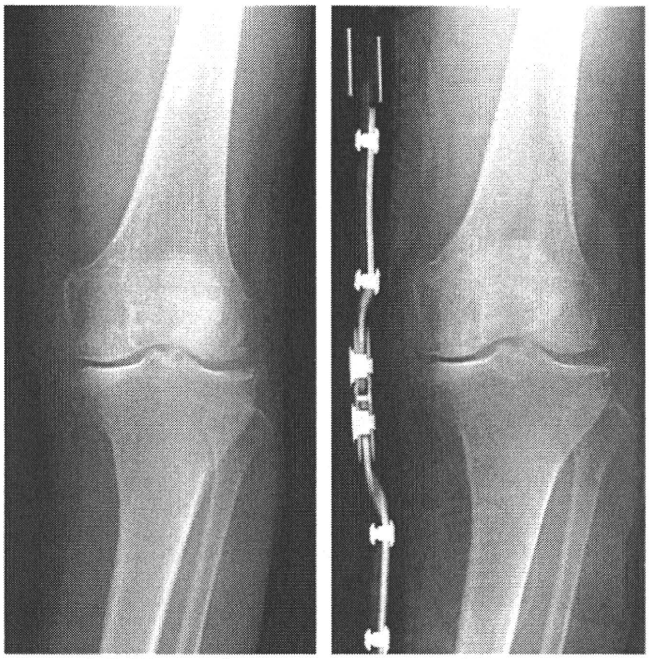
図4 brace装着前後での歩行の変化

装着により骨盤と体幹の傾斜が出現し，視線が下方に向いている。



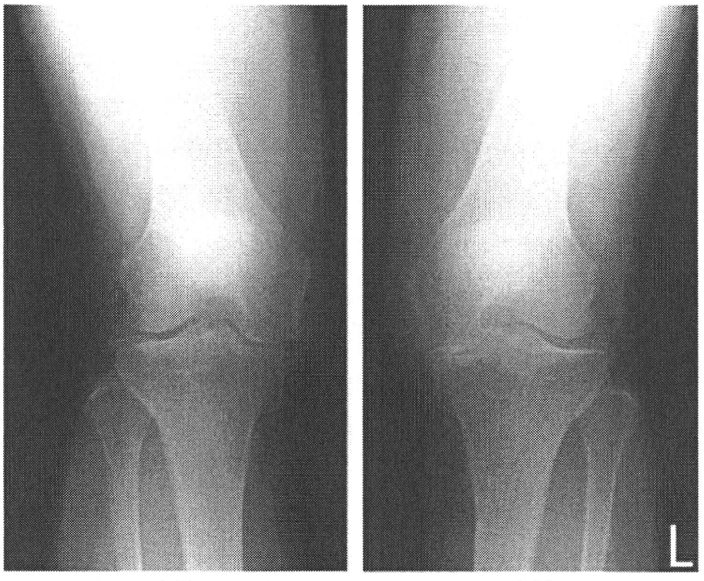
装着前　　装着後

図5 非荷重位での正面像
関節裂隙に違いはみられない。



左足(装具なし)　　左足(装具あり)

図6 Rosenberg view (装具なし)



右足　　左足

対象群(n=32)とbrace群(n=16)を設定し，6カ月経過した症例(各n=11，n=8)について3カ月後と6カ月後に評価を行ったところ，身体機能に関してbrace装着による効果が認められた(F<1,17>=6.418，p=0.021)。その後の検定では3カ月後と6カ月後ではbrace群が対象群よりも有意に身体機能スコアが高かった(**図8**，各p<0.05)。これらのことから，短期的ではあるがbraceによる身体機能の維持効果が期待できる。

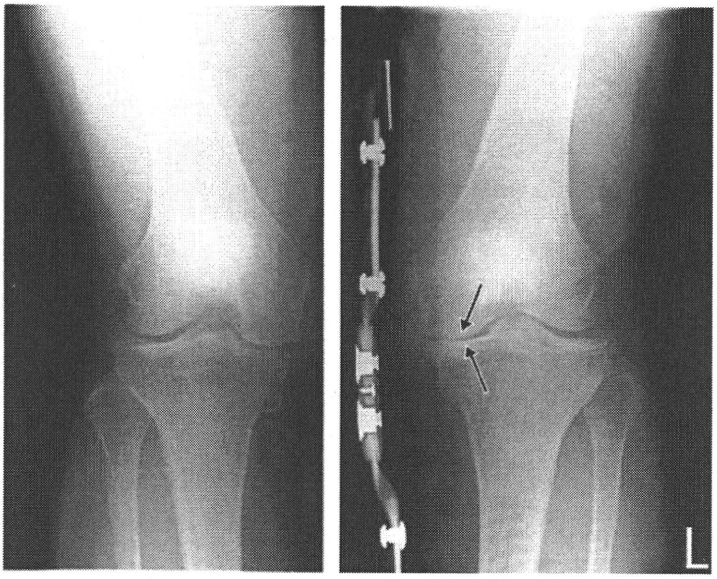
膝装具療法の問題点

使用感の問題と副作用

brace装着の際には装具がずれるなどのコン

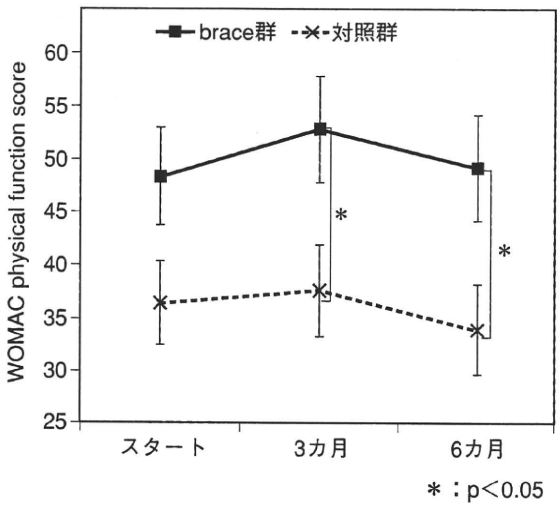
図7 Rosenberg view（装具あり）

荷重位では装具装着により図6より関節裂隙が拡大しているのがわかる（矢印）。



右足　　左足

図8 brace着用による身体機能への影響



＊：p＜0.05

プライアンスを低下させる諸問題がある[9]。また長期の使用で，皮膚刺激，機械的な問題，装具の不適合などが生じることもある[10]。さらに最も重要な問題として，まれに肺塞栓による呼吸困難を呈する[10]。

このためbraceによる治療を行う際は，既往歴や潜在的な心血管疾患のリスクなどを確認して慎重に処方を判断する必要がある。また患者にコンプライアンスを維持してもらうために，使用法の適切な指導と身体的な変化に対応するために適宜braceを調整する必要がある。

身体への影響

膝装具の8週間に及ぶ使用の影響として，筋力に変化はなかったものの，内側広筋の著明な萎縮，姿勢制御の反応劣化，関節固有感覚における誤認角度の増大が認められたとの報告がある[11]。この研究から，使用期間がさらに長期に及ぶ場合，筋力低下や運動制御機能の低下による転倒リスクが懸念される。これらを未然に防ぐために疼痛のない範囲で運動習慣を維持するように指導

し，必要に応じて薬物療法や理学療法を併用して行っていくことが重要である。

おわりに

保存療法では適切に疼痛や機能障害がコントロールできない場合，活動量の低下に合併して筋力低下を引き起こし，さらに活動量が減少するという悪循環が生じる可能性がある。膝装具療法は保存療法の1つとして身体活動向上の手助けとなるツールではあるが，それを処方するのみでは，必ずしも膝OAの治療としては成立しないと考える。装具の機能を最大限に発揮させるためには，義肢装具士や理学療法士と検討しながら，装具の種類の検討，装着による姿勢や歩行を始めとした動作への影響を観察し，適切な指導を行うことが必要である。膝装具療法に加え各種保存療法を組み合わせることで膝OAの病態，症状の進行や腰部や足・股関節などのほかの身体部位の関節疾患の発生を防ぐことが可能となり，そこで初めて膝OA患者の病態進行，症状の緩和が可能になると考える。

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In vitro cartilage formation using TGF-β-immobilized magnetic beads and mesenchymal stem cell-magnetic bead complexes under magnetic field conditions

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Abstract: We evaluated the efficacy of transforming growth factor (TGF)-β-immobilized magnetic beads for chondrogenesis *in vitro* using a mesenchymal stem cell (MSC) delivery system and an external magnetic force (EMF). MSCs isolated from the bone marrow of Sprague Dawley rats were mixed with carboxyl group-combined magnetic beads (Ferri Sphere 100C®) coated with anti-rat CD44 mouse monoclonal antibodies. TGF-β3 (10 and 1 ng/mL) was attached magnetically to such other Ferri Sphere 100C® beads via an amide bond formed between a primary amino group on the TGF-β3 and the carboxyl groups on the surface of the beads. MSC-magnetic bead complexes were centrifuged to form a pellet and cultured in chondrogenic differentiation medium (CDM) supplemented with either 10 or 1 ng/mL TGF-β-immobilized magnetic beads (10 or 1 ng/mL TGF-β-immobilized magnetic bead groups) or in CDM supplemented with 1 or 10 ng/mL TGF-β (1 or 10 ng/mL TGF-β group). TGF-β-immobilized magnetic beads were gathered effectively under an EMF. Chondrogenesis was achieved from the MSC-magnetic bead complexes in the presence of 1 ng/mL TGF-β-immobilized magnetic beads. © 2009 Wiley Periodicals, Inc. J Biomed Mater Res 92A: 196–204, 2010

Key words: transforming growth factor (TGF)-β; magnetic beads; magnetic force; mesenchymal stem cell; chondrogenic differentiation

INTRODUCTION

In recent years, autologous chondrocyte implantation or autologous osteochondral mosaicplasty have been widely performed to treat limited chondral and osteochondral lesions because articular cartilage has a limited potential to repair itself, and the cartilage defects treated by conventional surgical methods do not lead to hyaline cartilage regeneration.[1–5] However, the disadvantages of these procedures are that they involve undergoing an arthrotomy to harvest and transplant cartilage,[1] and they are limited by a lack of suitable donor sites from which large cartilage samples can be harvested.[5]

A treatment strategy that avoids undergoing an arthrotomy would ideally involve combining a group of multipotential cells and a controlled concentration of cytokines at a localized area with a single intra-articular injection. We originally developed a technique for cell-based cartilage repair in which cells were coupled with magnetic beads in association with an external magnetic force.[6] The underlying concept of this technique involves injection of cells coupled with magnetic beads into a joint and the use of an external magnetic force to position the transplanted cells in the desired location.[1,6] We isolated mesenchymal stem cells (MSCs) from bone marrow to use as our cell source and developed two types of MSC-magnetic bead complexes using anti-CD44 antibodies and a synthetic cell adhesion peptide (arginine-glycine-aspartic acid-serine, RGDS peptide).[6] Anti-CD44 antibody-bead complexes are useful for the identification of MSCs because the CD44 antigen is popularly regarded as a positive phenotypic maker of MSCs.[7] The RGDS peptide-bead complexes are useful for clinical applications as the RGDS peptide is a biodegradable material. We have also demonstrated that MSC-magnetic bead complexes could proliferate and differentiate into chondrocytes when supplemented with chondro-

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genic differentiation factors,[6] as first described by Johnstone et al.[8] We have shown that MSC-magnetic bead complexes that have been localized to a specific area by an external magnetic force could generate a chondrogenic matrix when cultured in a monolayer.[6] However, we have not examined whether MSC-magnetic bead complexes still possess chondrogenic potential under three-dimensional culture conditions against gravity and under the influence of an external magnetic force.

To induce differentiation of MSCs, cultivation under appropriate culture conditions and stimulation with several bioactive factors, including transforming growth factor-β (TGF-β), bone morphogenic protein (BMP), fibroblast growth factor (FGF), and insulin-like growth factor (IGF-I) are required.[7–11] In particular, TGF-β is essential to the *in vitro* chondrogenic differentiation of MSCs.[8] However, in terms of the clinical application of TGF-β, several investigators have shown that free injection of TGF-β into the joint cavity precipitates severe adverse effects such as osteophyte formation and inflammatory joint disease.[12,13] That is to say, because the injected TGF-β does not directly target and attach to the lesion, it affects the lesion and also extends into the normal intra-articular structure. Therefore, to assist this intra-articular cell transplantation without scaffolds and to solve the problems associated with injecting free TGF-β, we developed TGF-β-immobilized magnetic beads, which localized the TGF-β and enhanced our unique MSC delivery system.

On the basis of this information, this study had two main objectives. Firstly, we wanted to assess whether chondrogenesis using MSCs as a cell source could be induced under conditions against gravity and under the influence of an external magnetic force. Secondly, we hoped to evaluate a new cytokine delivery system involving TGF-β-immobilized magnetic beads and an external magnetic force. This included examining whether the localization of TGF-β-immobilized magnetic beads could lower the concentration of TGF-β necessary for chondrogenesis to occur from the MSC cell source.

MATERIALS AND METHODS

Animal experiments were performed in accordance with the Guide for Animal Experimentation, Hiroshima University, and the Committee of Research Facilities for Laboratory Animal Sciences, Graduate School of Biomedical Sciences, Hiroshima University.

Isolation and expansion of MSCs

A modification of Kotobuki's culture method,[9] as described previously,[6] was used for the isolation and *in vitro* expansion of MSCs. Briefly, the bone marrow of Sprague Dawley rats (12-weeks-old) was aspirated from the tibial marrow cavities with 24G injection needles and mixed in a culture medium consisting of high-glucose Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) with 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO) and penicillin-streptomycin-fungizone (Bio-Whittaker, ML). The cells, including buffy coat and red blood cells, were seeded onto 100-mm culture dishes (Falcon, BD Bioscience, Franklin Lakes, NJ) in culture medium, and incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The medium remained unchanged for the first 7 days, and was subsequently changed every 2–3 days. After 14–21 days, the cells had proliferated and reached confluence. The cells were then harvested by using 0.25% trypsin and 0.02% EDTA, and then rinsed twice with culture medium. To expand the MSCs, 2–3 × 10^5 of the harvested cells were seeded onto 100-mm culture dishes. On reaching confluence again, the cells were reseeded under the same conditions.

Carbodiimide-mediated immobilization of CD44 antibody to the magnetic beads by amide bond formation

Firstly, to examine the expression of the cell surface antigen CD44 in rat MSCs expanded in a monolayer culture, immunohistochemical staining was performed as described previously.[6] Then, according to Yanada's method,[6] we coated uniform, mono-sized magnetic beads composed of styrene-acryl polymers with a thin film of magnetic ferrite (diameter: 310 nm; density: 1.8 g/cm^3; the amount of magnetization: 27 emu/g; carboxyl groups introduced on the surface: 0.01–0.04 mol/mg; Ferri Sphere 100 C®, Nippon Paint, Tokyo, Japan). The coupling procedure involved the formation of an amide bond between a primary amino group of the CD44 antibody (Chemicon International, Temecula, CA) and the carboxyl groups on the surface of the magnetic beads, mediated by carbodiimide activation (Fig. 1). Because the intermediate product of the reaction between the carboxylic acid and the carbodiimide is very labile and hydrolyzed quickly, a less labile intermediate (NHS: *N*-hydroxy succinimide) was used. Briefly, after 3 mg of magnetic beads were washed twice with 500 μL 0.01*N* NaOH for 10 min with thorough mixing, they were washed three times with deionized water in the same manner to remove excess liquid. Then, 50 μL of EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) solution (50 mg/mL in 25 m*M* MES (2-[*N*-morpholino]ethane sulfonic acid), pH 5) and 50 μL NHS solution (50 mg/mL in 25 m*M* MES, pH 5) were added, mixed well, and incubated with slow tilt rotation at room temperature for 30 min. After incubation, the tube containing the magnetic beads was placed on a magnet for 4 min and the supernatant was removed. The beads were washed twice with 25 m*M* MES (pH 5). Twenty micrograms of CD44 antibody dissolved in 25 m*M* MES (pH 5) were added to the activated beads, making a total volume of 500 μL. The mixture was vortexed and then incubated for 3 h at 25°C with slow tilt rotation. After incubation, the

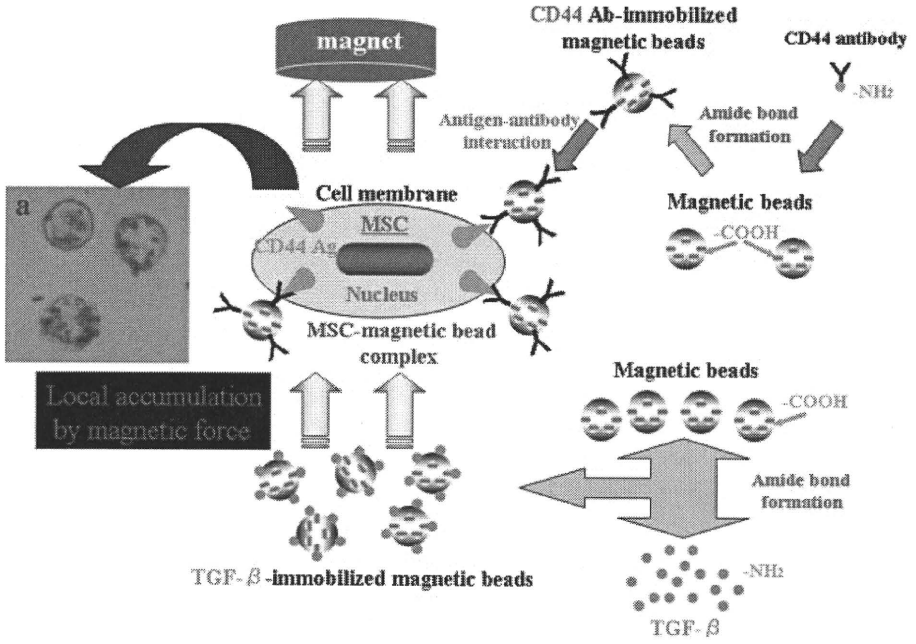

Figure 1. Schema with CD44 antibody-immobilized magnetic bead-conjugated MSC (MSC-magnetic bead complex) and TGF-β-immobilized magnetic beads. Light microscopic view (magnification ×400). Some massive bead conglomerates are attached to the surface of MSCs (a). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tubes were placed on a magnet for 4 min, and the supernatant was removed. Ethanolamine (0.05*M*) in PBS(-) (pH 8) was added and the magnetic beads were incubated for 1 h at room temperature with slow tilt rotation to quench nonreacted groups. Finally, the beads were washed four times with 0.5% BSA in PBS(-) and suspended in 0.5% BSA in PBS(-) at a concentration of 3 mg beads/mL. Thus, CD44 antibody-immobilized magnetic beads composed of magnetic beads and CD44 antibodies were prepared (Fig. 1).

Complexing CD44 antibody-immobilized magnetic beads to MSCs

Forty-five microliters of CD44 antibody-immobilized magnetic beads (225 μL beads) and 1×10^6 MSCs were mixed in 355 μL of 0.5% BSA in PBS(-) with slow tilting and rotation for 1 h at 4°C or at 37°C. The tube was then placed on a magnet for 4 min to collect the complexes. The assembled MSC-CD44 antibody-bead complexes were washed four times with 0.5% BSA in PBS(-) and resuspended in culture medium at 5×10^6 cells/mL (Fig. 1).

Carbodiimide-mediated immobilization of TGF-β3 to the magnetic beads by amide bond formation

To label TGF-β3 (Sigma-Aldrich, St. Louis, MO) magnetically, we used such other Ferri Sphere 100C® beads. The coupling procedure involved the formation of an amide bond between a primary amino group of TGF-β3 and the carboxyl groups on the surface of the magnetic beads, mediated by carbodiimide activation. After the magnetic beads were activated as described above, 50-μL of 1000 ng/mL TGF-β3 dissolved in 25 m*M* MES buffer (pH 5) was added to the activated beads, making a total volume of 500 μL. The mixture was vortexed and then incubated for 3 h at 25°C with a slow tilt rotation. After incubation, the tube was placed on a magnet for 4 min and the supernatant was removed. Ethanolamine (0.05*M*) in PBS(-) (pH 8) was added, and the magnetic beads were incubated for 1 h at room temperature with a slow tilt rotation to quench the nonreacted groups. Finally, the beads were washed four times with 0.5% BSA in PBS(-) and resuspended in 0.5% BSA in PBS(-) at a concentration of 1 mg beads/mL. The concentration of TGF-β3 was 100 ng/mg beads (Fig. 1).

Chondrogenesis of MSC-magnetic bead complexes using TGF-β-immobilized magnetic beads under an external magnetic force

To evaluate the effect of an external magnetic force on the chondrogenic potential of the MSC-magnetic bead complexes, a modification of Johnstone's pellet culture system was performed.[8] Approximately 2×10^5 MSCs, existing as complexes, were resuspended in chondrogenic differentiation medium (CDM) lacking TGF-β3. The CDM consisted of high-glucose DMEM supplemented with 10^{-8} M dexamethasone (Sigma), 50 μg/mL ascorbic acid-2-phosphate (Sigma), 40 μg/mL L-proline (Nacalai Tesque, Kyoto, Japan), ITS-A supplement (Invitrogen, 10 μg/mL insulin, 6.7 ng/mL sodium selenite, 5.5 μg/mL transferrin, 110 μg/mL sodium pyruvate), and 1.25 mg/mL BSA (Sigma). The MSC-magnetic bead complexes and non-

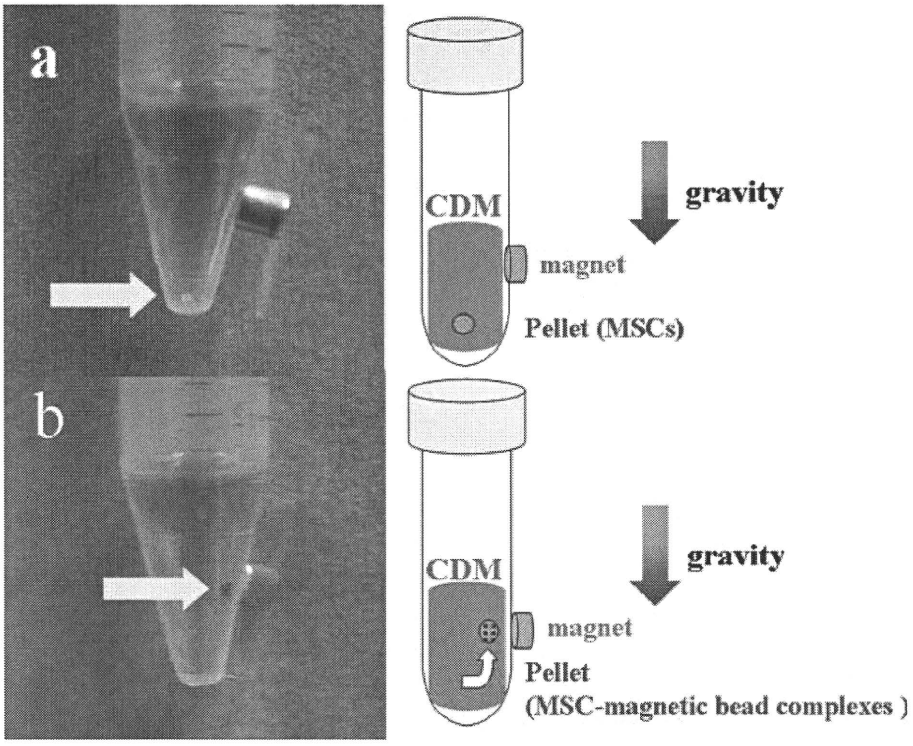

Figure 2. Pellet culture system in which a neodymium magnet was set on the lateral side of the polypropylene tube. Approximately 2×10^5 MSC-magnetic bead complexes (b) or MSCs alone (a) were centrifuged to form a pellet. Although the pellet of the MSCs alone remained on the bottom of the tube (a), the pellets of the MSC-magnetic bead complexes could be moved up against gravity by an external magnetic force (b). The pellets of the MSC-magnetic bead complexes (b) were cultured in CDM containing either 100 or 10 μL/mL TGF-β-immobilized magnetic beads (TGF-β concentration: 10 or 1 ng/mL, respectively) or 1 or 10 ng/mL TGF-β protein, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

labeled rat MSCs (MSCs alone) were centrifuged to form a pellet and a neodymium magnet (diameter: 5 mm; height: 5 mm; magnetic flux density: 0.43 Tesla (T))[6] was positioned on the lateral side of a 15-mL polypropylene tube [Fig. 2(a,b)]. The pellet of the MSCs alone remained on the bottom of the tube [Fig. 2(a)].

Next, to evaluate the effect of TGF-β-immobilized magnetic beads on the chondrogenic potential of the pelleted

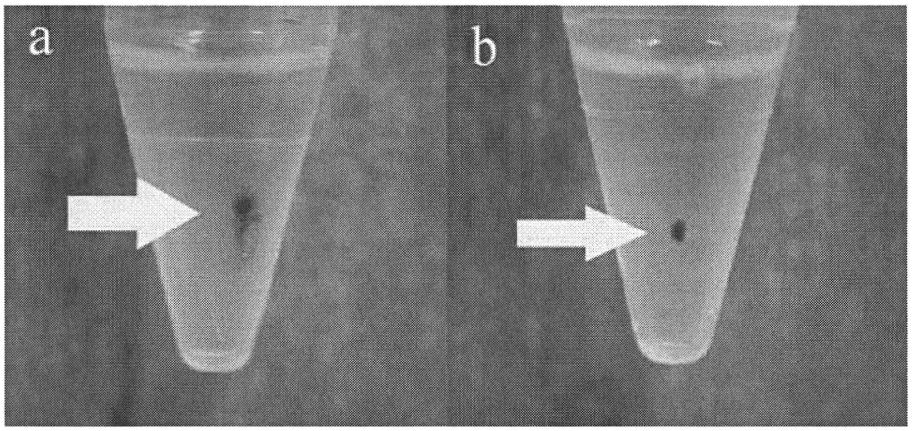

Figure 3. Macroscopic findings of the MSC-magnetic bead complexes pelleted after being cultured for 21 days. The pellet was transferred into PBS(-) under the influence of an external magnetic force, and these photographs were taken. Photograph (a) indicates the pellet cultured in the CDM lacking TGF-β, but supplemented with 10 μL/mL TGF-β-immobilized magnetic beads (1 ng/mL TGF-β) localized under an external magnetic force whenever the medium is changed. Photograph (b) indicates the pellet cultured in the CDM containing 1 ng/mL TGF-β. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I
Each Pellet Culture System of the Four Groups

	10 ng/mL TGF-β-immobilized magnetic bead group	1 ng/mL_TGF-β-immobilized magnetic bead group	1 ng/mL_TGF-β group	10 ng/mL TGF-β group
Cultured Cells	MSC-magnetic bead complexes	MSC-magnetic bead complexes	MSCs-magnetic bead complexes	MSC-magnetic bead complexes
TGF-β (ng/mL)	0	0	1	10
TGF-β-immobilized magnetic bead (ng/mL)	10	1	0	0

The basic CDM consisted of high-glucose DMEM supplemented with 10^{-8} M dexamethasone, 50 μg/mL ascorbic acid-2-phosphate, 40 μg/mL L-proline, ITS-A supplement (10 μg/mL insulin, 6.7 ng/mL sodium selenite, 5.5 μg/mL transferrin, 110 μg/mL sodium pyruvate), and 1.25 mg/mL BSA, and was applied under an external magnetic force.

MSC-magnetic bead complexes, TGF-β was labeled magnetically (10 ng/mL and 1 ng/mL TGF-β-immobilized magnetic bead groups) using the method described above (Fig. 1). The pelleted MSC-magnetic bead complexes were cultured at 37°C with 5% CO_2 and 95% air in 1 mL of CDM supplemented with either 100 μL/mL or 10 μL/mL TGF-β-immobilized magnetic beads (TGF-β concentration: 10 ng/mL or 1 ng/mL [Fig. 3(a)], respectively) and localized under an external magnetic force [Fig. 2(b)]. We have demonstrated previously that MSC-magnetic bead complexes had the ability to differentiate into the chondrogenic lineage in the presence of 10 ng/mL TGF-β.[6] To assess the influence of TGF-β concentrations lower than 10 ng/mL, the pellet from the MSC-magnetic bead complexes was cultured in CDM supplemented with 1 ng/mL TGF-β under an external magnetic force (1 ng/mL TGF-β group) [Fig. 3(b)]. As the positive control group, the pellet of the MSC-magnetic bead complexes was cultured for 21 days in CDM supplemented with 10 ng/mL TGF-β (10 ng/mL TGF-β group) and localized under an external magnetic force [Fig. 2(b)]. Table I is a list to clarify all the test groups. The medium was changed every 3 days and either 100 μL/mL or 10 μL/mL TGF-β-immobilized magnetic beads (TGF-β concentration: 10 ng/mL or 1 ng/mL, respectively) were added at the same time. In the 1 ng/mL TGF-β group and the 10 ng/mL TGF-β group, 1 ng/mL or 10 ng/mL TGF-β in solution was respectively added. After 14 and 21 days in culture, the chondrogenic ability of the four different groups was evaluated (Table I).

RNA preparation and reverse transcription-polymerase chain reaction analysis

Total RNA was prepared from the pellets of MSC-magnetic bead complexes using the RNeasy Micro kit (Qiagen, Tokyo, Japan). Prepared RNA was converted to cDNA using the Superscript™ First-Strand Synthesis System for reverse transcription-polymerase chain reaction (RT-PCR) (Invitrogen) according to the manufacturer's protocol. PCR was performed in a Minicycler (PTC-150, Bio-Rad, Hercules, CA). PCR amplification conditions for rat aggrecan, type II collagen, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: 94°C for 2 min followed by 35 cycles of 94°C for 15 s, 56°C (or for GAPDH: 58°C) for 30 s, and 68°C for 1 min. The reaction products were resolved by electrophoresis on a 2% agarose gel and visualized with ethidium bromide under UV illumination. The aggrecan and type II collagen primers have been described previously,[14] and the GAPDH primers were designed specifically for this study. The primer sequences used are as follows: aggrecan (forward): 5′-TAGAGAA GAAGAGGGGTTAGG-3′; aggrecan (reverse): 5′-AGCAG TAGGAGCCAGGGTTAT-3′; type II collagen (forward): 5′-GAAGCACATCTGGTTTGGAG-3′; type II collagen (reverse): 5′-TTGGGGTTGAGGGTTTTACA-3′; GAPDH (forward): 5′-GCCAAAAGGGTCATCATCTC-3′; GAPDH (reverse): 5′-GCCTGCTTCACCACCTTCTT-3′.

Histological evaluation

Pelleted MSC-magnetic bead complexes that had been cultured for 21 days were fixed with 4% paraformaldehyde and embedded in paraffin. After deparaffinization, sections (5 μm) were stained with toluidine blue and safranin O solution.

RESULTS

Assembly and proliferation of MSC-magnetic bead complexes

As described previously,[6] immunohistochemical staining for CD44 antigens revealed that CD44 antigens are expressed in ~90% of expanded MSCs up to at least passage four. We decided to use MSCs that had been expanded up to passage four for assembling MSC-magnetic bead complexes. Next, CD44 antibody-immobilized magnetic beads were combined with the expanded MSCs (Fig. 1). Electron and light microscopy demonstrated that some massive bead conglomerates had attached to the surface of the labeled MSCs (Fig. 1). In contrast, large bead conglomerates had not attached to the nonlabeled rat MSCs. We showed that MSCs could be combined with small magnetic beads via rat CD44 antibodies.

As described previously,[6] the MSC-magnetic bead complexes did not proliferate during the first 3 days of culture although the complexes remained attached to the bottom of the well. However, microscopic

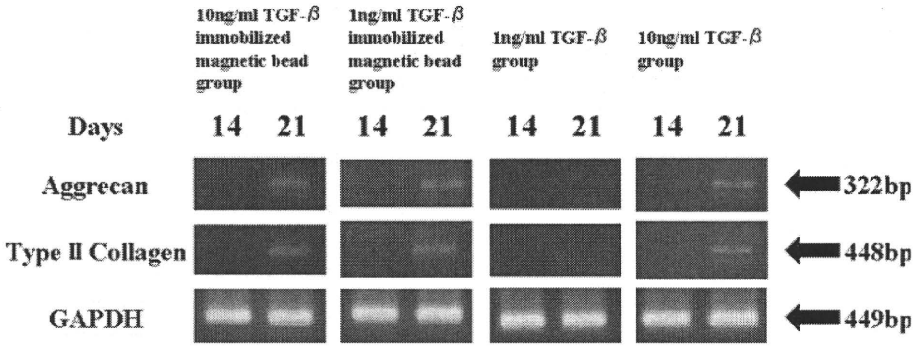

Figure 4. RT-PCR analysis of the 10 ng/mL and 1 ng/mL TGF-β-immobilized magnetic bead groups, 1 ng/mL and 10 ng/mL TGF-β groups. The expression of aggrecan and type II collagen mRNA could be detected in both the 10 ng/mL and 1 ng/mL TGF-β-immobilized magnetic bead groups after being cultured for 21 days, but not after being cultured for 14 days. The data shown are typical of four independent experiments (n = 6).

observation revealed that the cells proliferated after the CD44 antibody-immobilized magnetic beads separated from the surface of MSCs in the well. After 7 days of culture, cell proliferation of MSC-magnetic bead complexes was ~50% that of normal rat MSCs. The assembled MSC-magnetic bead complexes were able to proliferate after separating from the mediator-immobilized magnetic beads, although the proliferation of the complexes was slower during the early period of culture than that of nonlabeled rat MSCs.[6]

Macroscopic findings from the three-dimensional culture system of pellets influenced by an external magnetic force

All of the pellets in each group were attached to the wall of the tube by an external magnet after 21 days in culture [Fig. 3(a,b)]. Moreover, in the 10 ng/mL and 1 ng/mL TGF-β-immobilized magnetic bead groups [Fig. 3(a)], the TGF-β-immobilized magnetic beads gathered effectively under the influence of the external magnet.

Chondrogenesis of MSC-magnetic bead complexes in the three-dimensional culture system under the influence of an external magnetic force

RT-PCR analysis of the pellet cultures demonstrated that aggrecan and type II collagen mRNA expression could not be detected in the 1 ng/mL TGF-β group after either 14 or 21 days in culture (Fig. 4). On the other hand, mRNA expression of these two markers was not detected in the 10 ng/mL or 1 ng/mL TGF-β-immobilized magnetic bead groups or the 10 ng/mL TGF-β group after 14 days in culture (Fig. 4). However, after 21 days in culture, aggrecan and type II collagen mRNA expression could be detected (Fig. 4). The same RT-PCR analysis results were obtained in all six pellets (n = 6) from each group.

In addition, after 21 days in culture, MSC-CD44 antibody-bead complex-derived-MSCs were partially surrounded by a chondrogenic matrix in the 10 ng/mL and 1 ng/mL TGF-β-immobilized magnetic bead groups, as well as the 10 ng/mL TGF-β group (Fig. 5). This matrix stained metachromatically with toluidine blue and safranin O, albeit sparsely (Fig. 5). The complexes in the 1 ng/mL TGF-β-immobilized magnetic bead group had higher chondrogenic potential than that of 1 ng/mL TGF-β group based on these histological findings. The complexes in the 10 ng/mL TGF-β-immobilized magnetic bead group also had a higher chondrogenic potential than the complexes in the 10 ng/mL TGF-β group (Fig. 5). This pattern of histological findings was consistent in four of six pellets (n = 6) in each group.

DISCUSSION

This study demonstrated that MSC-magnetic bead complexes could differentiate along the chondrogenic lineage in the presence of 1 ng/mL TGF-β. This concentration of TGF-β is 10% lower than the 10 ng/mL generally used, and was made possible by using TGF-β-immobilized magnetic beads.

The chondrogenic differentiation of MSCs *in vitro* requires the addition of certain bioactive factors, such as TGF-β and dexamethasone. Several investigators have speculated that TGF-β initially increases the proliferation of cells with chondrogenic potential to the point where a critical number is reached and differentiation can then occur.[7,8,10,11] Johnstone et al.[8] reported that lowering the TGF-β concentration decreased chondrogenesis in the aggregates of rabbit MSCs, and that the optimal TGF-β concentration was 10 ng/mL. In addition, Bosnakovski et al.[15]

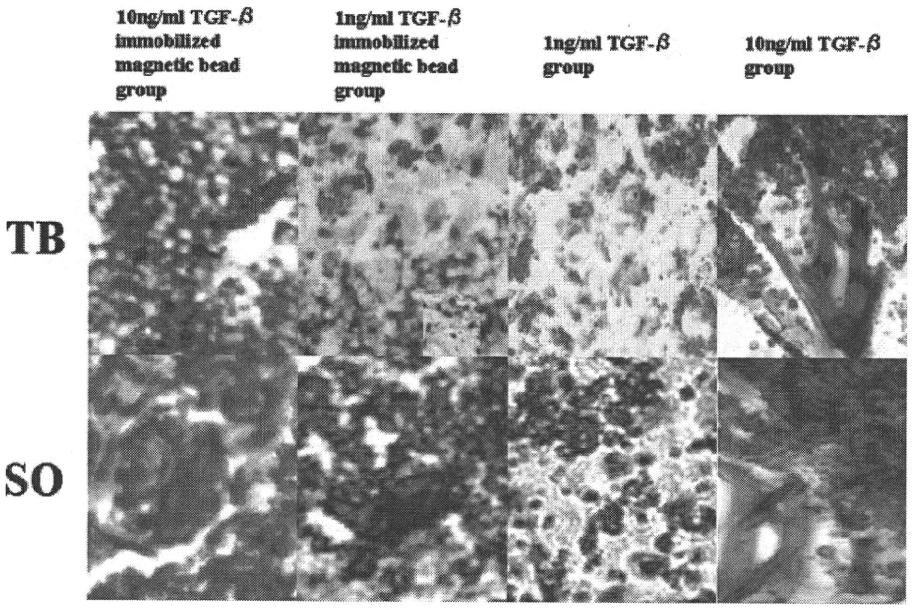

Figure 5. Histological results using light microscopy. Representative histology of cartilage pellets stained with Toluidine blue or safranin O under high (×200) magnification: the 10 ng/mL TGF-β-immobilized magnetic bead group; the 1 ng/mL TGF-β-immobilized magnetic bead group; the 1 ng/mL TGF-β group; and the 10 ng/mL TGF-β group. The histological results shown are typical of four independent experiments ($n = 6$).

reported that chondrogenic differentiation of bovine MSCs in monolayer culture appeared to be dose-dependent and time-dependent in relation to TGF-β. The optimal TGF-β concentration was >5 ng/mL, with higher concentrations able to promote chondrogenic differentiation of bovine MSCs better than lower concentrations. Several investigators[7,10,11] have reported that chondrogenic differentiation of human MSCs can be promoted by supplementing the cell cultures with 10 ng/mL TGF-β. Similarly, 10 ng/mL TGF-β was often added to the CDM when culturing rat MSCs *in vitro*.[16] Thus, regardless of these interspecies differences, TGF-β promotes chondrogenesis of MSCs *in vitro*, and the minimal and optimal TGF-β concentration required is 10 ng/mL in a number of species. In addition, the concentration of TGF-β in the medium correlates with the chondrogenic potential of the MSCs. However, there are some complications regarding the clinical application of TGF-β. Several investigators have demonstrated that injection of free TGF-β into the joint cavity precipitates severe adverse effects such as osteophyte formation, cartilage destruction, and inflammatory joint disease.[12,13,17] Therefore, to inject TGF-β into the joint at the lowest concentration possible for effective chondrogenic differentiation, we devised a method that allowed the accumulation of TGF-β at the site of interest only by immobilizing TGF-β to Ferri Sphere 100 C® magnetic beads (Fig. 1). That is, by using TGF-β-immobilized magnetic beads and an external magnetic force, we enabled TGF-β to act at the local site more effectively. Although we have already shown that MSC-magnetic bead complexes can differentiate along the chondrogenic lineage in both monolayer and three-dimensional cultures grown in CDM supplemented with 10 ng/mL TGF-β,[6] in this study, we established new culture conditions for chondrogenesis comprising of TGF-β-immobilized magnetic beads and an external magnetic force.

In this study model, we have developed a new pellet culture system in which a magnet was applied to the lateral wall of a tube to properly evaluate the effect of TGF-β-immobilized magnetic beads under an external magnetic force (Fig. 2). In the conventional pellet culture system described by Johnstone et al.,[8] the pellet is usually found on the bottom of the tube. Without an external magnetic force, TGF-β-immobilized magnetic beads should also gravitate to the bottom of the tube under the influence of gravity. Under these conditions, it is difficult to use the TGF-β-immobilized magnetic beads to their full capacity and the effects of the beads cannot be evaluated precisely. TGF-β-immobilized magnetic beads should be applied against gravity. Therefore, to prevent the pellet from falling to the bottom of the tube because of gravity, we made a pellet composed of MSC-magnetic bead complexes (Fig. 2) and showed that the pellet of MSC-magnetic bead complexes could be held up off the bottom of the tube under an external magnetic force for 21 consecutive days in