(投与前CRP) であり投与後2週のCRPは投与前CRPに有意に相関していた (R2=0.4731) (**図3**)。インフリキシマブで寛解に入った症例は2004年9月15日から2006年7月22日までの間にインフリキシマブ投与した68例中8例11.8%寛解であり，そのうち早期RAでは28例中7例寛解であり寛解率25%であった。すなわち早期RAは全体と比較して約2倍の寛解率を示した。76例のインフリキシマブ投与の投与継続率はKaplan-Meier法で1年，82%，2年で71%，3年で48%であった (**図4**)。この継続に関する因子を，投与前CRP，投与後CRP，年齢，罹患期間，MTX量，ステロイド量についてCox回帰分析を行ったところ投与前CRP (p=0.041) と年齢 (p=0.034) が有意に関係を示した (**表2**)。このことは投与前CRPが高

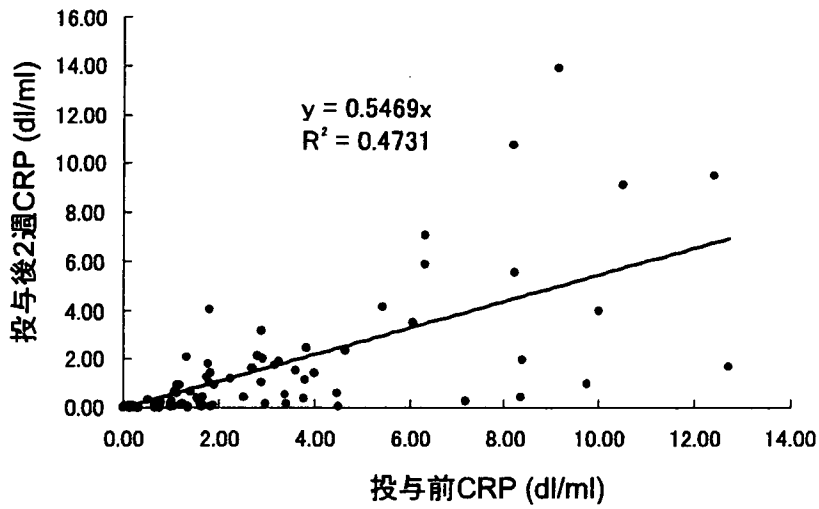

図3　投与後CRPの早期改善度

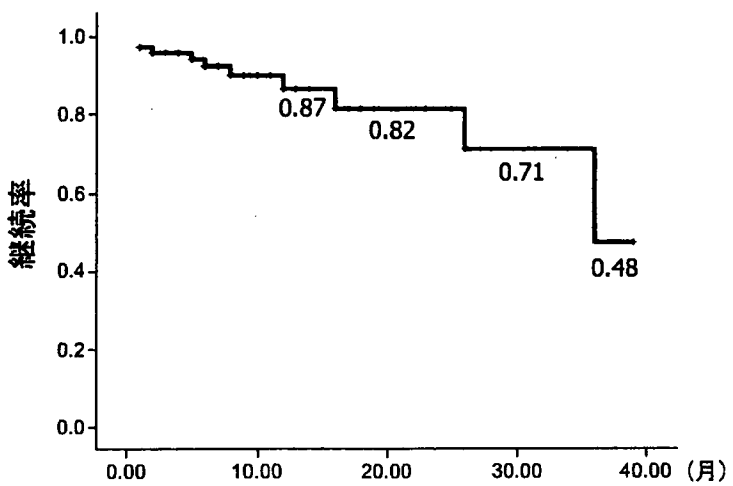

図4　インフリキシマブの継続率，Kaplan-Meier法 (N=76)

表2　インフリキシマブ中止による薬剤投与の変化と中止時の検査値

症例	インフリキシマブ投与前	インフリキシマブ（中止時）	RAPA（中止時）	CRP mg／dl（中止時）	MMP-3 ng／ml（中止時）
K.S.	MTX 6 mg	MTX 4 mg	40未満（−）	0.03	47.3
T.A.	MTX 4 mg，NSAID	MTX 6 mg	40	0.05	51.1
S.M.	MTX 4 mg，ブシラミン，PSL 1 mg	MTX 2 mg	40	0.01	37.5
Y.H.	MTX 6 mg，PSL 5 mg，NSAID	MTX 4 mg	40未満（−）	0.01	39.8
M.M.	MTX 6 mg，PSL 5 mg	MTX 4 mg	40	0.21	56.3
M.N.	MTX 6 mg，PSL 5 mg	MTX 2 mg	40未満（−）	0.11	94.2
D.M.	MTX 6 mg，NSAID	MTX 4 mg	40	0.19	40.7
S.T.	MTX 6 mg，NSAID	MTX 6 mg	40未満（−）	0.08	34.7

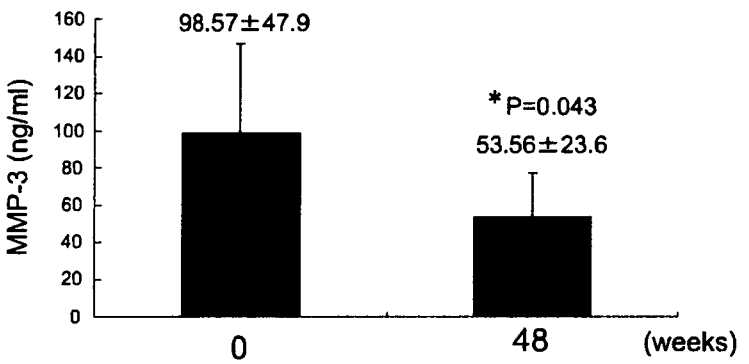

図5　寛解症例の投与後1年のMMP-3の変化

いほど，あるいは年齢が高いほどインフリキシマブは脱落する症例が多かったことを示す。寛解症例のMMP-3は投与前98.57±47.9ng／mlから53.56±23.6ng／mlに有意に減少を示した（P＝0.043）（図5）。寛解症例の経時的CRPの推移を見ると，症例5以外の全例はCRP陰性が継続しているが，症例5は投与中止して18週後よりCRP 0.45mg／dlと陽性化したがMTX 4 mgのまま継続し，その8週後に再び陰性化した（図6）。症例4は中止後1年でMTXを中止してCRPは陰性化を継続しており治癒の可能性がある。DAS28は投与前平均6.1（5.07〜7.55）が投与中止後平均2.06（1.88〜2.3）に低下していた。手ＸＰにおいて8例とも骨萎縮が改善し，症例5においては骨びらんの改善が投与中止後1年にて継続して認められた（図7）。インフリキシマブ投与前の薬剤は寛解例8例のうちステロイドを服用していた4例は，全例ステロイドを中止できMTXと葉酸にて治療継続している（表2）。

考　　察

RAの寛解については1948年Shortらによってはじめて報告されて以来，寛解基準については多くの議論がされているが，現在ではDAS28が2.6以下を寛解基準と決めている場合が多い[8]。しかし，抗リウマチ薬のみでの寛解は臨床上寛解でもMRI上，滑膜炎が持続して

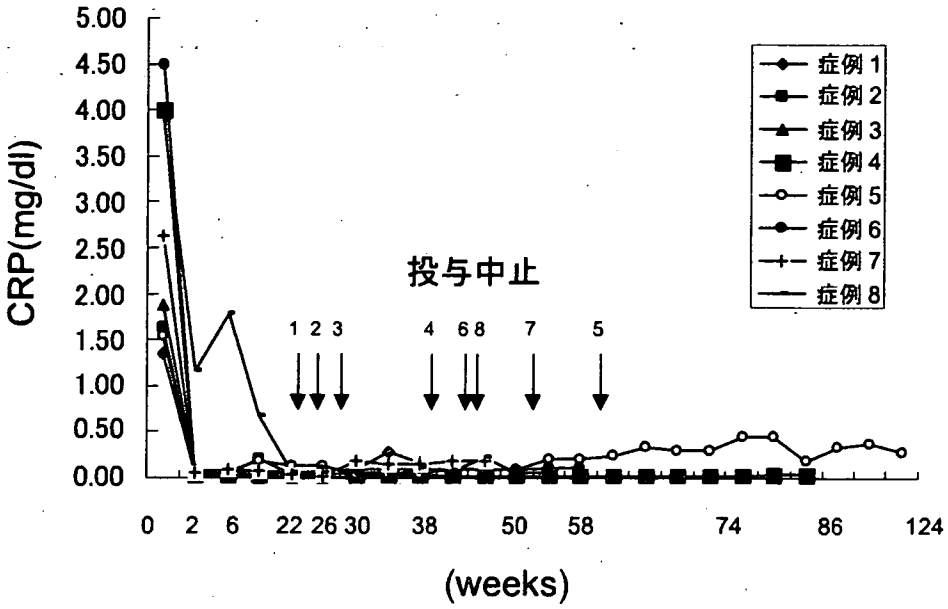

図6　寛解症例のインフリキシマブ投与中止後のCRPの推移

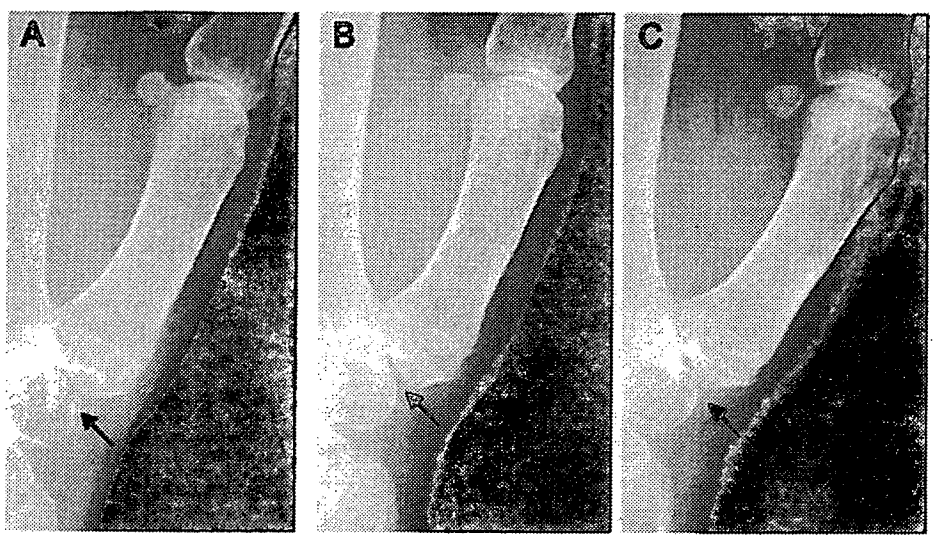

図7　インフリキシマブ中止できた寛解症例のXPの変化
A：投与前，B：投与後1年，C：中止後1年，矢印：骨破壊修復

いることが報告されており，DAS28のみでの寛解基準には疑問視される点もある[2,5]。現在のところ生物学的製剤を中止できる寛解についての基準はなく，本論文は本邦において初めての報告である。われわれは中止できる寛解基準をDAS28が2.6以下およびCRPが完全に陰性化して6ヵ月以上経過したものとした。この基準は今後本症例の長期経過観察において改良する可

表3：インフリキシマブの継続因子分析（Cox回帰分析の結果）

	B	標準誤差	Wald	自由度	有意確立	Exp (B)	Exp (B) の95.0% CI	
							下限	上限
CRP 前	−0.831	0.407	4.168	1	0.041	0.436	0.196	0.967
CRP 後	1.011	0.530	3.635	1	0.057	2.747	0.972	7.764
年　齢	0.243	0.115	4.472	1	0.034	1.274	1.018	1.596
罹患期間	−0.053	0.054	0.950	1	0.330	0.949	0.853	1.055
MTX	−0.426	0.302	1.990	1	0.158	0.653	0.362	1.180
steroid	0.184	0.166	1.234	1	0.267	1.202	0.869	1.664

能性があるが，少なくとも中止するまでの期間が長ければ長いほど患者に対する副作用や経済的負担が大きくなる可能性がある。Quinnらは早期RAに対してインフリキシマブを中止して1年後のMRIで滑膜炎が抑制され，2年後も機能的に効果が持続していたと報告している[7]。われわれの症例ではCRPだけでなくRAPAとMMP-3すべてが陰性化していないと再燃する可能性があることを経験した。インフリキシマブにおいて寛解による中止症例は本症例のごとく存在するが，エタネルセプトにおいて報告がないのはエタネルセプトにおいてCRPの陰性化が持続してもRAPAかMMP-3どちらか一方が陰性化していない症例が多かったためと筆者らは考えている。よって生物学的製剤の中止後再燃しない指標としてCRP，RAPAおよびMMP-3の3つが全て陰性が重要と思われる。

RAの治療はこれまでピラミッド式に行われることが多く、特に早期RAに対してはNSAIDやBucillamineなどで2～3年経過を見ることが多い。手指の変形が始まって初めてMTXなどの免疫抑制剤が使用されるが，間質性肺炎などの副作用の問題で使用されない症例もある。薬物治療ではClassの進んだRAにおいてはすでに肺などの全身の合併症を伴うことが多く，無理にMTXを増量したりすると心不全や肺炎を引き起こす危険性は高い。生物学的製剤はいかなる薬剤にも抵抗性のある症例に使用すべきと考えられる傾向があるが，その理由の一つとして生物学的製剤による副作用が重篤であるかもしれないとの医師側の危惧による。しかしながら，生物学的製剤のRAに対する適応を再検討すると，MTXに効果不十分な症例に限らず，早期RAにおいて少量のMTX（当科では4 mg／週）とインフリキシマブを用いて早く治療を行い，早く寛解に移行できれば生物学的製剤が中止可能であり，副作用の危険性はより少なくなる。薬物治療は長期間投与により蓄積された薬物の副作用と身体に与える負の影響を考慮すれば，最善の治療は薬剤中止できる治療と言える。今回，当科においてインフリキシマブで治療した193例のうち3年での継続率は48%であった。すなわち3年で約半数は脱落することを考慮すると，生物学的製剤は長期に使用するためのものでなくむしろ3年以内に中止できるような症例に使用すべきであると考える。これらは発症早期RAであり，当科では25%の高い寛解導入率を示した。

すなわち生物学的製剤の最もよい適応は早期RAに対する寛解導入療法であり，RAを早く発見して早く治療する最適の治療手段として力を発揮できる。早期RAに対しては海外でもインフリキシマブを用いて骨破壊を抑制したり，寛解をもたらすとの報告がある[9]。さらにはインフリキシマブを中止できた寛解の報告があるが，その詳細は明確ではない[3]。今回，われわれはインフリキシマブを中止できた寛解を追跡調査しStage Ⅲの症例5では投与中止して18週後より一時的にCRPが陽性化しており，この点に中止できうる寛解治療の限界がある可能

性がある。すなわちStage Ⅱ以下の手指の変形のないRAにおいてはこの寛解導入療法で安定したCRP陰性化をもたらすことができることが示唆される。今後5年以上の追跡調査が必要と思われる。

早期RAの診断では従来の診断基準においてもすでに進行していることがあり，当科では発症5年以内で手指MPあるいはPIPの腫脹と圧痛があり，CRP 0.5mg／dl以上，XPにて骨びらんあるいは骨萎縮を認め，両手に朝のこわばりがあるものを早期RAと診断し，Key drugとなるMTX 4 mgを開始する。患者と十分，RAの骨破壊について相談し，生物学的製剤を早期に開始する。自然寛解の可能性についても考慮するが，長期間経過観察しなければいけない点と，現在のところ本当に一生患者が自然寛解を継続できるかのデータはなく，経験上一時的に自然寛解に入っても3～5年すれば再びCRPやリウマチ因子が陽性になり，骨破壊が進む症例があることから自然寛解のエビデンスが確実でない以上，こうした中で少なくとも生物学的製剤により骨破壊抑制がもたらされ，CRP陰性，リウマチ因子陰性，MMP-3も陰性化できる寛解導入治療が現在存在することは患者側にとって十分治療のメリットがあるといえる。

生物学的製剤の効果減弱例に対して薬物を交替するよりもRAの病態の場である滑膜を除去して，サイトカイン産生を抑制し，生物学的製剤の効果を再現できることを筆者らは報告した[4]。寛解導入療法において今後Stage Ⅱ以下の関節破壊の少ないRAにおいて生物学的製剤の効果減弱例であっても関節鏡視下滑膜切除により寛解導入率が増加できる可能性があり整形外科的治療と生物学的製剤による集学的治療が発展する可能性がある。

ま と め

関節リウマチに対する生物学的製剤インフリキシマブによる寛解導入療法を行ない8例のインフリキシマブを中止した寛解の臨床経過を追跡調査した。Stage Ⅲの症例は中止後CRPの陽性化を一時的に認めたが，Stage Ⅱ以下の症例でインフリキシマブ中止時にCRP,リウマチ因子，MMP-3すべてが陰性化している症例は再燃を認めなかった。また寛解導入療法にてインフリキシマブ中止後1年でMTXも中止できた症例もあり，これは関節リウマチの治癒達成にむけた一歩前進した治療とも考えられ今後の長期報告が期待される。

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骨関節破壊の進行とQOL

神戸 克明* 井上 和彦**

関節リウマチ(RA)の病態の中心は関節機能障害であり，骨関節破壊により進行する。骨関節破壊の進行は各症例により一律でないが，発症5年以内に75%が起こるとされている。RAの早期からの骨関節破壊を，治療によりいかに最小限に抑えるかが患者のquality of life (QOL) につながる。近年，生物学的製剤の治療によりRAの骨関節破壊修復について注目されている。我々の臨床研究では，早期RAで関節破壊が乳頭状滑膜と有意に関係していることが認められている。この乳頭状滑膜増生をいかに抑えられるかがRAの骨関節破壊を食い止める鍵となる。骨関節破壊の抑制は，生物学的製剤で効果減弱した症例などの増生した乳頭状滑膜を除去することにより確実なものとできる。今後，生物学的製剤の骨関節破壊抑制について詳細な解析が必要である。これにより患者QOL向上により具体的な治療選択が明解となる。

Progression of bone and articular destruction and QOL.

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The joint dysfunction caused by bone and articular destruction is the most important pathology in rheumatoid arthritis (RA) patients. The progression of bone and articular destruction starts within five years from disease onset, however depending each case inflammation. Quality of life (QOL) of RA patients needs early stage treatment to prevent joint destruction. We recognized villonodular synovial proliferation is significantly correlated with early stage RA. Therefore to decrease those synovium leads to prevent joint destruction. Biological therapy itself can not inhibit villonodular synovial proliferation in effect attenuation cases. Arthroscopical synovectomy is effective to remove those synovium and restore the effect of biological therapy. It is needed that detail analysis of improvement of joint destruction by biological therapy near future to lead the improvement QOL of RA patients.

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はじめに

近年，関節リウマチ（RA）の治療が生物学的製剤により大きく変化してきている中で，その骨関節破壊の抑制効果は関節機能障害を予防しquality of life（QOL）改善につながる。しかし生物学的製剤を使用してもすべての症例で有効なわけではなく，未だ骨関節破壊抑制の詳細な解析はなされていない。日本においてどのような症例に骨関節破壊抑制効果があるか，詳細な検討が必要であると思われる。RA自体が単一なものでなくさまざまなstageにおいて骨関節破壊の程度が違っており，QOLを考える意味で生物学的製剤使用のみでは現在のところ不十分である。リウマチ治療薬と合わせて，関節鏡視下滑膜切除や人工関節等の手術療法にて関節機能障害を改善することにより，患者のQOL向上を目指す。以下，生物学的製剤による骨関節破壊のQOL改善だけでなく，手術療法についても述べる。

骨関節破壊の進行

Masiらは初期RAを5年間経過観察した報告によると，RA患者の経過は①炎症が数年間で鎮静化し，骨破壊も進行しない単相型（monocyclic）20%，②炎症の増悪を繰り返しながら経過し，骨破壊も進行する多相型（polycyclic）70%，③炎症が高く，骨破壊が進行性に経過し，寝たきりになっていく進行型（progressive）10%の3つの型に分けられると報告している[1]。メトトレキサート（MTX）による内服治療においても，関節破壊は現在のところ止めることはできない（**図1**）。

Yamamotoらは，発症1年以内の患者を2年間観察して，2年以内に寛解となる群15%，治療によって日常生活には不自由のない群50%であり，この2つの群の合計である65%は治療によ

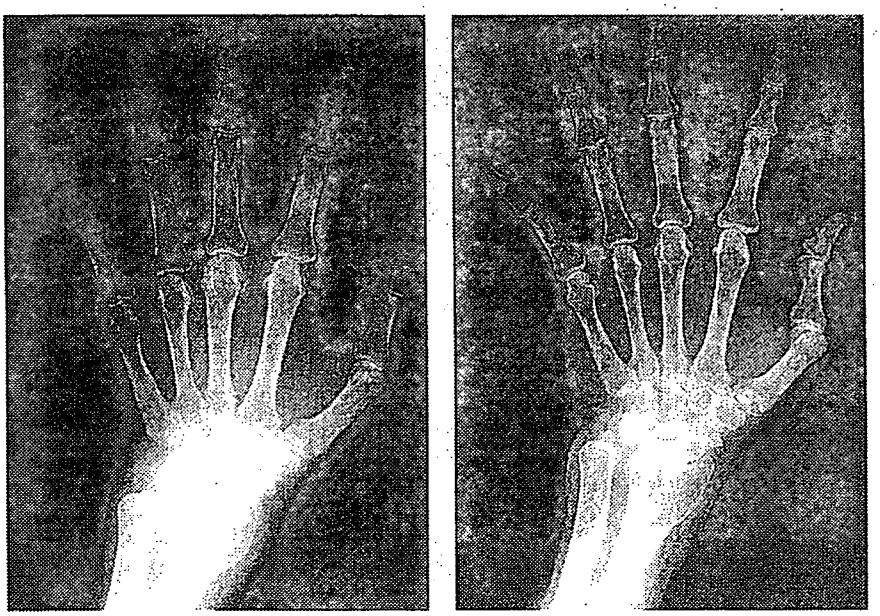

図1　関節破壊の進行

MTX内服のみでは関節破壊の進行は止めることができない。

（筆者ら提供）

MTX：メトトレキサート，QOL：quality of life，RA：関節リウマチ

り十分コントロールできるのでcontrol群と分類している[3]。治療するにもかかわらず身体機能障害者となるprogressive群は35%でuncontrol群とした。

Ochiらは、罹患期間10～15年の240例の破壊関節数の分布より、RA患者の関節破壊の広がりの自然経過に基づく病型を3つのタイプに分類した[3]。I群 小関節破壊型病型（LES）65%，II群 多関節破壊型病型（MES）30%，III群 ムチランス型病型（MUD）5%とし、II，III群には明らかな寛解はないと報告している。日常生活において、この患者がどの病型に属し、将来とるであろう経過を予測し、どのような薬物治療を選択するか、手術の時期の決定を早くするかどうかが重要な問題点である。

RAの骨関節破壊の原因として考えられるのは、病態の場が関節を中心としており滑膜増生および全身の免疫学的異常にかかわることである。そこで我々は関節滑膜の性状の病的変化に注目すべきと考え、17例の関節滑膜切除時に採取した滑膜所見を、組織学的に滑膜増生、乳頭状、血管増生、フィブリン析出、リンパ球浸潤の5項目に分類し、患者の罹患期間を従属変数にとった重回帰分析により、乳頭状滑膜が早期RAと有意に関係していることをつきとめた（$p = 0.018$）。関節破壊を起こす時期は発症して5年以内に75%であるとされることから、罹患期間の少ない時期に多く見られるこの乳頭状滑膜に骨関節破壊の原因があることも理論的に考えられる。

しかしながら、乳頭状滑膜がどのようにして骨関節破壊を起こすかは、サイトカインやケモカインおよびmatrix metalloproteinase（MMP）などの液性因子[4]のほか、滑膜内の破骨細胞の存在あるいは分化促進、さらには形態学的に関節周囲の関節包付着部の軟骨欠損部いわゆるベアエリアに入り込みやすいことも考えられる。浸入した乳頭状滑膜がやがてベアエリアを骨びらんへと進展させ骨関節破壊を促進する。

RAが発症して早期の関節で、C-reactive protein（CRP）上昇が持続する症例では特に、乳頭状滑膜の増生がみられる。さらに、RAが進行していても関節変形が少なく骨破壊が少ない関節で、CRPが上昇している症例では乳頭状滑膜が見られる。すなわち乳頭状滑膜は、発症罹患期間に統計学的には有意に関係するが、特に個々の関節破壊進行の早期と関係があると考えられる。よって骨破壊をくいとめて個々の関節破壊進行をとめるには、乳頭状滑膜増生を抑制することが鍵となる。

生物学的製剤を用いた骨関節破壊抑制効果は手足の小関節に比較的よく見られるが、時に膝などの加重関節にも認められることもある（図2）。

QOLからみた経過と予後

橋本らはArthritis Impact Measurment Scales, version 2（AIMS2）日本語版調査表を作成し、それを用いて11施設RA患者1,774例を調査した[5]。患者QOLに影響を及ぼす主要因子として、筋力、関節、慢性病期、貧血、炎症の因子の順で関与していた。また、MTX療法と過去2年以内の人工関節置換術は、QOLの改善に有意に貢献した。

居村らは、下肢大関節に人工関節置換を受けた患者358名のQOL調査を行った[6]。患者のQOLは人工関節置換術を受け改善されるが、長期的に見れば、手術以外の治療に負うところが大きい。また、ムチランス型破壊、2椎体以上の圧迫骨折の存在がQOLに大きな影響を持つと報告した。

患者の日常生活の中で、四肢筋力を保持させる運動や生活方法、在宅でのリハビリテーションの

AIMS2：Arthritis Impact Measurment Scales, version 2，CRP：C-reactive protein，LES：小関節破壊型病型，MES：多関節破壊型病型，MMP：matrix metalloproteinase，MUD：ムチランス型病型

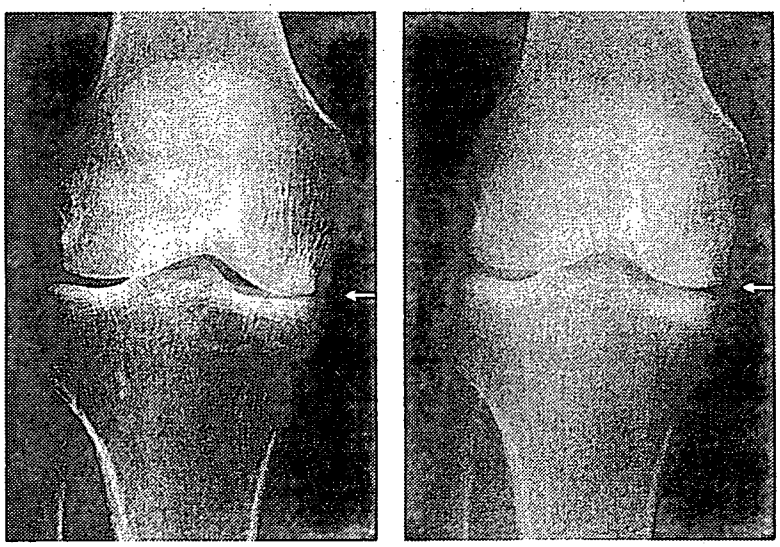

図2　骨関節破壊の改善

膝関節内側における関節裂隙の改善と，脛骨の骨萎縮の改善を認める。
（筆者ら提供）

指導，リハビリテーション施設の利用などの生活指導が必要である。また，薬物治療による炎症コントロールや下肢大関節の関節置換術の適応の有無を判定し，手術時期を逸しないように患者に伝えることが必要である。

生物学的製剤使用中の関節鏡視下滑膜切除の効果

生物学的製剤投与中に，患者本人から関節痛の痛みが緩和されたとの声をしばしば聞くことがある。しかしながら，膝や肩などの大関節においてはどうしても疼痛がとれないこともあり，こうした患者に対しては関節鏡視下滑膜切除術を行っている。これは，滑膜からのサイトカイン産生を減少させ骨関節破壊を抑制する意味がある。

インフリキシマブ投与にても改善が認められない関節滑膜では，血管増生を伴った乳頭状の滑膜が見られる（図3）。インフリキシマブ投与中における関節鏡視下滑膜切除術後の成績は良好で，CRPだけでなくdisease activity score 28（DAS28）の改善を導く。さらに，関節鏡視下滑膜切除術によって次回投与のインフリキシマブの効果が増強され，効果減弱例に対して有効な手段となり得る[7]。こうした生物学的製剤と手術療法の併用は，特に整形外科医にとって新しいRAに対する治療法として今後発展していく可能性がある。

生物学的製剤使用中の人工関節置換術

骨関節破壊の進行したRAに対して，生物学的製剤を使用して炎症症状は改善しても，既に進んだ関節破壊は改善することはできない。従って，人工関節手術の前に全身の炎症症状を抑えるという点では，生物学的製剤は有効な治療法である。ただし，間質性肺炎やインフュージョンリアクションなどの副作用を十分考慮しなければならない。初診時にCRP高値の患者と高齢者は，インフリキシマブの継続率が悪いことが分かっている。

DAS28：disease activity score 28

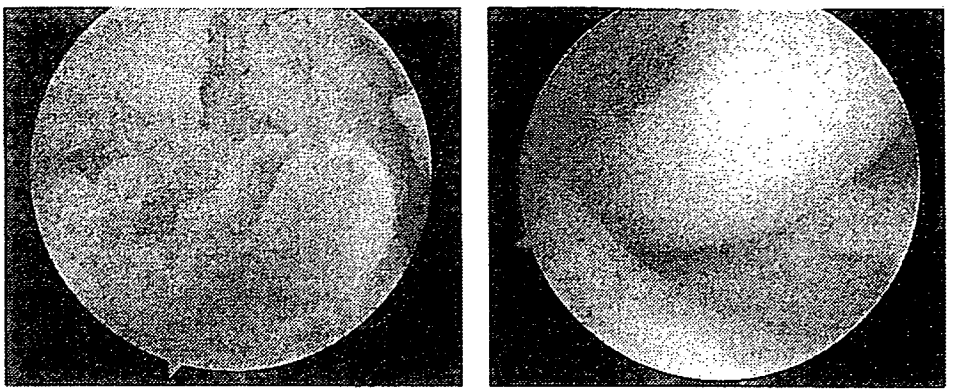

図3　インフリキシマブ効果減弱例の関節鏡視下滑膜切除術

膝関節内の血管新生に富む乳頭状滑膜を認めた。滑膜切除により再びインフリキシマブの効果が持続できる。（筆者ら提供）

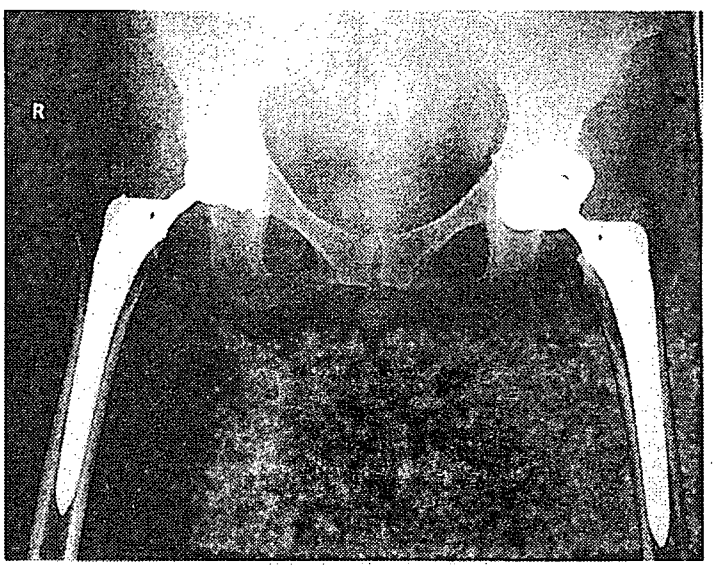

図4　インフリキシマブ投与中の人工股関節置換術

感染の危険性上昇なく，通常の手術と同様に行った。（筆者ら提供）

人工関節には足趾、足、膝、股関節、肘、肩、手指関節があるが、関節部位と生物学的製剤使用の特別な注意点と言えばやはり術後感染であり、これには十分注意を要することは言うまでもない（図4）。人工関節には生物学的製剤を使用したからといって通常の人工関節手術と特別な違いはないが、糖尿病との合併患者には注意を要する。

ただし、現実には生物学的製剤と人工関節置換術の術後感染の割合は非常に低く（東京女子医科大学東医療センター整形外科において43例中2例4.67%：1例足趾形成術後表層感染，1例脊椎後方固定術の血腫形成で軽微なもの），その理由として以下のことが考えられる。

RAに対する人工関節置換術の術後感染の比率は、血清CRPに比較的依存する。また、長期ステロイド使用患者には免疫力低下のため術後感染

に注意を要する。ところが生物学的製剤を使用すればステロイドなどを使用しなくてもCRPは改善していてむしろ比較的感染しにくい状態であり，人工関節置換術をしやすい環境であるとも言える。さらに，手術の時期であるが総じて生物学的製剤のインフリキシマブの投与と投与の中間で，具体的には投与後4週で行う場合が多く，生物学的製剤の影響は受けにくいことも考えられる。これらの人工関節などの手術を併用することによって，関節破壊による機能障害を改善し，患者のQOL向上を導くことができる。

おわりに

RAの骨関節破壊の進行とQOLについて生物学的製剤による治療を踏まえて述べた。骨関節破壊の進行を止めるためには十分な生物学的製剤を熟知した使用方法が必要であり，特に早期RAにおいては関節変形を食い止める手段となり得る。骨関節破壊の進行が既に進んでしまって日常生活が困難な場合には，生物学的製剤は炎症のコントロールとして用いられるが，単独使用では限界がある。従って，関節鏡視下滑膜切除や人工関節にて関節機能障害を改善することにより，患者のQOL向上を目指す。このように，手術も考慮した集学的治療が現在のRA治療では必要である。

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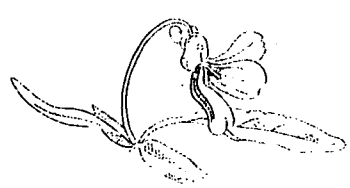

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ORIGINAL ARTICLE

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Identification of clock as a mechanosensitive gene by large-scale DNA microarray analysis: downregulation in osteoarthritic cartilage

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Abstract The chondrocytes of cartilage have so far been reported to include three mechanoresponsive genes: cartilage matrix protein (CMP, matrilin-1), type X collagen, and Indian hedgehog (Ihh). In fact, all of the genes identified in these chondrocytes are associated solely with mechanical stress. In this study we examined the how mechanical stress influenced the patterns of gene expression of chondrocytes in three-dimensional (3D) sponges in order to better understand the mechanisms that control the chondrocyte phenotype during the development of osteoarthritis. We cultured mouse chondrocytes in 3D sponge as a model of mechanical stress and isolated total RNA for a large-scale DNA microarray analysis covering 12000 genes. We analyzed the pattern of gene expression in relation to gene localization in cellular components such as the cytoplasm, cytoskeleton, and nucleus. Immunoblotting of osteoarthritis cartilage were performed using polyclonal anti-clock antibody. The biological rhythm of mRNA of clock and clock-related genes was analyzed by real-time polymerase chain reaction (PCR). Clock gene was confirmed by real-time PCR to validate the microarray data. Other clock-related genes such as Per1 and Per2 were also expressed in chondrocytes, exhibiting biological rhythm after serum shock. The large-scale DNA microarray indicated that clock and other genes functionally associated with mechanical stress play an essential role in regulating the biological rhythm of chondrocyte metabolism in osteoarthritis cartilage.

Key words Cartilage · Clock · Mechanical stress · Osteoarthritis

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Introduction

Mechanical stress is an important factor to consider in analyses of skeletal development and osteoarthritis. The genes that relate to mechanical stress overall remain to be identified, however. Conventional analyses of the regulation and functions of genes have been driven by highly focused studies targeting individual genes and proteins in a methodical, step-by-step fashion. In DNA microarray, DNA probes representing cDNA clones are arrayed onto glass slides and hybridized with fluorescence-labeled cDNA targets. The microarray technology provides a format for the simultaneous measurement of the expression levels of thousands of genes in a single hybridization assay. Our study was designed to determine changes of the gene expression profile in mouse chondrocytes undergoing mechanical-stretch. We compared the gene expression profiles of chondrocytes after mechanical stretch using cDNA microarrays containing 12000 sequence-verified mouse genes. The results identified an association in with mechanical stress in one gene, that is, clock. Clock, a gene expressed in the brain, heart, lung and peripheral tissue, is confirmed to be regulated by circadian biological rhythm and has a known influence on traits as diverse as body size and temperature on molecular clock.[1,2] Circadian rhythmicity plays important roles not only in basic cellular functions such as membrane excitation and energy metabolism, but also in behavioral events such as sleep–waking cycles. In mammals, all these circadian rhythms are generated at the cellular level by a circadian core oscillator composed of an autoregulatory transcription–translation-based feedback loop involving clock, per1, and per2 genes. Previous studies have not clarified whether the clock genes expressed in chondrocytes are functionally related to mechanical stress. Biological rhythm and associated gene expressions are crucial to the maintenance of a stable body metabolism. In this paper we analyze the role of the clock gene in the regulation of biological rhythm during the development of osteoarthritis.

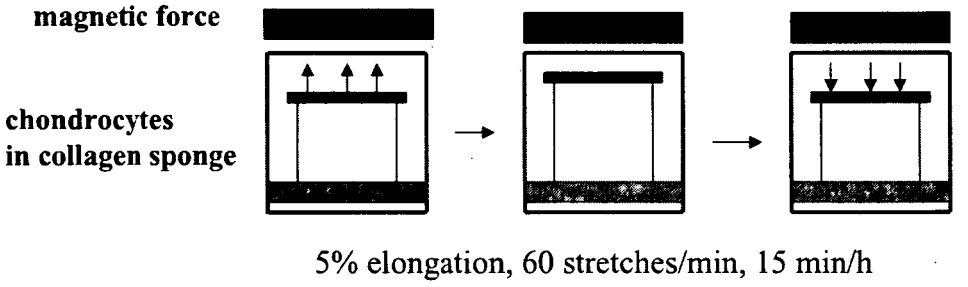

Fig. 1. Mechanical stress induced by magnetic force in chondrocytes in a 3D sponge

Materials and methods

Isolation and culture of newborn mouse chondrocytes

Chondrocytes were isolated from 8 newborn mice within 1 week of birth. After collecting the cells from rib cages digested in collagenase D (3 mg/ml) for 6 h, we seeded them onto plates (1×10^6 cells/plate) and cultured them overnight in DMEM containing 10% FCS and 1% penicillin-streptomycin to prepare a three-dimensional (3D) chondrocyte culture system for mechanical stimulation studies.

Mechanical stimulation of chondrocytes in the 3D chondrocyte culture system

Chondrocytes were cultured in 3D collagen sponges by the same method used in our earlier mechanical stress experiments.[3] The mechanical stress was applied by physiologically stretching the chondrocytes. Briefly, 100 µl of cell suspension was applied to 2 × 2× 0.25-cm Gelform sponges (Upjohn, Kalamazoo, MI, USA) presoaked with Hanks' balanced salt solution (HBSS). After overnight incubation, the sponges were stretched with an intermittent pattern (5% elongation, 60 stretches/min, 15 min/h) for 4 days with a Bio-stretch device (ICCT Inc., Markham, ON, Canada) for microarray in 10% FCS DMEM (Fig. 1). This procedure was found to generate matrix deformation to a degree comparable with that observed in vivo.[3] The chondrocytes were centrifuged and suspended in plating medium at 1×10^7/ml.

Real-time quantitative polymerase chain reaction (PCR)

Total RNA of chondrocytes cultured in stretched and nonstretched sponges was extracted with an RNeasy mini kit (Qiagen, Hilden, Germany). The mRNAs of clock were quantified by real-time quantitative reverse transcriptase (RT)–PCR with a Perkin–Elmer ABI Prism 7700 sequence detection system to validate the data from the large-scale DNA microarray using the same sample as the microarray. The following primers were used: clock, 5′-TGTCAGA TTAGTAACACCTC-3′ and 5′-TATTATTGGTGGTGC CCTGT-3′; Per1, 5′-ATGAGTGGTCCCCTAGAAGG-3′ and 5′-GCCATTGCTGTTTGCATCAG-3′; Per2, 5′-TGTGTGCGGCTTAGATT-3′ and 5′-TGCTGCTCTTG CACCTTGAC-3′; GAPDH, 5′-CTGGCGCTGATACG TCGGTG-3′ and 5′-GTTCACACCCATGACGAACA-3′. Osteopontin primers are 5′-TGGACGACGATGATGAC GAT-3′ and 5′-TTGGAGTGAAAGTGTCTGCT-3′.

DNA microarray

Upon completing an RT reaction of 5 µg total RNA using aminoallyl dUTP, the cDNA were hydrolyzed and cleaned, then monofunctional dye NHS-esters Cy5 and Cy3 were coupled with cDNA for stretched chondrocytes and cDNA nonstretched chondrocytes (as control), respectively. Twelve thousand BMAP genes were used for the microarray. After removing quenched cye-dyes with a Qiaquick PCR purification kit, the cDNA were hybridized using 20× standard saline citrate, polyA (10 mg/ml), and 10% sodium dodecyl sulfate (SDS) in a microarray chamber overnight. Data collected by scanning with a GenePix 400A scanner were analyzed by IPLab software. Two gene tips were placed on each glass slide, and each experiment was performed in duplicate (i.e., two glass slides and four gene tips).

Biological rhythm by clock, Per1, and Per2 in chondrocytes

After treatment with 50% serum shock, we measured the mRNA expression levels of clock, Per1, and Per2 mRNA using real-time PCR.[4] Mouse chondrocytes (1×10^6) were seeded, washed with phosphate-buffered saline [PBS(−)] three times, incubated with 50% bovine serum for 30 min, washed again, and incubated with 10% Dulbecco's modified Eagle medium for 2, 4, 6, 12, 24, or 48 h. Total RNA was collected from the cultured chondrocytes using an RNeasy kit to analyze the mRNA levels of clock, Per1, Per2, and osteopontin as negative control by real-time PCR.

Immunoblotting

Articular cartilage was collected from eight osteoarthritis patients during total knee arthroplasties with informed consent. The patients were aged from 52 to 71 years old (mean 63.5 years). Four of the patients were assessed as grade II and the other four were assed as grade IV by Kellgren–Lawrence X-ray grading of osteoarthritis.[5] Immunoblotting with anti-clock polyclonal antibody was performed to

compare the levels of protein expressed by the clock gene in cell lysates collected from chondrocytes of the grade II and grade IV osteoarthritis samples. Proteins from the chondrocytes were applied in the same amounts by using anti-CD44 pAb (Bender MedSystems, Vienna, Austria) for control. The samples used for the non-reducing condition were mixed with standard 2× SDS gel loading buffer, while those used for the reducing condition were mixed with a loading buffer containing 5% beta-mercaptoethanol and 0.05M dithiothreitol. The samples were boiled for 10 minutes before loading onto 10% SDS–polyacrylamide gel electrophoresis (PAGE) gels. After electrophoresis, the proteins were transferred onto Immobilon-PVDF membrane (Millipore, Bedford, MA, USA) in 25mM Tris, 192mM glycine, and 15% methanol. The membranes were blocked in 2% bovine serum albumin fraction V (Sigma, St. Louis, MO, USA) in PBS for 30min and then probed with antibodies. The primary antibody used was an anti-clock polyclonal antibody (diluted 1:5000) (Affinity BioReagents, Golden, CO, USA). Horseradish peroxidase-conjugated goat antirabbit IgG (H + L) (Bio-Rad Laboratories, Melville, NY, USA) at a 1:3000 dilution was used as a secondary antibody. The immunoreactive proteins were visualized by applying ECL Western blotting detection reagents (Amersham, Arlington Heights, IL, USA) and exposing the membrane to Kodak X-Omat AR film. The molecular weights of the immunoreactive proteins were determined against two different sets of protein marker ladders.

Results

Large-scale DNA microarray data

Results from the microarray data analysis for upregulated genes on day 4 indicated the expression of Pak3 binding protein in membrane, ubiquitin-specific protease 25 in the cytoplasm, and transcription factor CA150 in the nuclei (Table 1). The analysis for downregulated genes at the same time point revealed the expression of collagen alpha 2(VI) chain precursor and glutamate receptor, NMDA1 in the membrane, and transcription factor 4 and clock genes in the nuclei (Table 2). Among these, the rate of clock gene downregulation was found to be 0.267 times that after the mechanical stress.

Table 1. Upregulated genes in chondrocytes after 4 days of mechanical stress (grouped by chondrocyte localization)

Location	Description	Ratio
Cytoplasmic	COA transferase	2.118
Cytoplasmic	SEC61a, transport protein	2.018
Cytoplasmic	Ubiquitin specific protease 25	2.002
Membrane	β2 microglobulin	2.154
Membrane	Pak3 binding protein	2.044
Nuclear	TBP-associated factor, RNA polymerase II	2.544
Nuclear	T-cell death associated gene	2.023

Validation of large-scale DNA microarray by real-time-PCR

Next, we conducted real-time PCR studies to validate the accuracy of the microarray data by comparing the mRNA of the clock gene in the nonstretched chondrocytes with that in the genes subjected to mechanical stress in the mRNA samples (0.298 times in the stretched chondrocytes). According to the data from the large-scale DNA microarray, the rate of downregulation of the clock gene in the stretched chondrocytes was 0.267 times that in the nonstretched chondrocytes (Fig. 2). We thus confirmed the accuracy of the microarray analysis for quantifying mRNA changes.

Biological rhythm by clock, Per1, and Per2 in chondrocytes

After the treatment with serum shock, we measured the mRNA expressions of clock, Per1, and Per2 using real-time PCR. We found, as a result, that clock expressed the biological rhythmic curve (Fig. 3a) to almost the same degree as Per1 (Fig. 3b) and Per2 (Fig. 3c). Among these three biological clock genes, Per2 in chondrocytes expressed the biological rhythm with exceptional accuracy over a 24-h period. Our findings thus confirm the function of these genes in regulating metabolism of chondrocytes by setting a biological rhythm. Findings on the mRNA expression of osteopontin in our control study did not suggest the development of biological rhythm in response to treatment of serum shock (Fig. 3d).

Immunoblotting revealed a suppression of the clock gene in severe osteoarthritic cartilage

We selected clock, a protein related to biological rhythm, to investigate whether mechanoresponsive genes are involved in osteoarthritis. The levels of clock protein indicated the expression in the four samples of cartilage with grade II osteoarthritic and downward expression in the four samples

Table 2. Downregulated genes in chondrocytes after 4 days of mechanical stress (grouped by chondrocyte localization)

Location	Description	Ratio
Matrix	Collagen α 2(VI) chain precursor	0.251
Membrane	Glutamate receptor, NMDA1	0.265
Membrane	Cation channel	0.271
Mitochondria	Adenylate kinase 2	0.196
Nuclear	Centromere autoantigen B	0.239
Nuclear	Rhombotin-1 (LIM)	0.257
Nuclear	Transcription factor 4	0.259
Nuclear	Orphan nuclear receptor	0.258
Nuclear	Retinoic acid repressible protein	0.263
Nuclear	Clock	0.267
Nuclear	Transcription factor NF-AT	0.278
Secreted	Semaphorin 3B	0.266

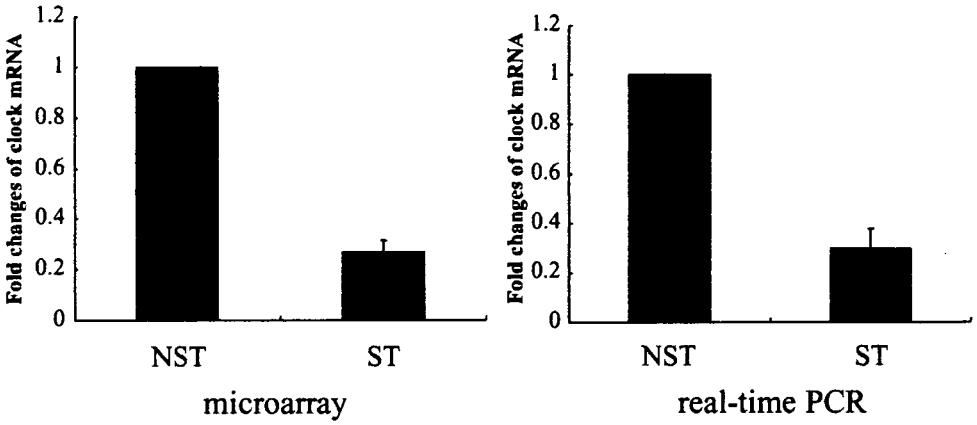

Fig. 2. Validation of large-scale DNA microarray data with clock compared to real-time polymerase chain reaction (*PCR*). *NST*, non-stretched culture; *ST*, stretched culture

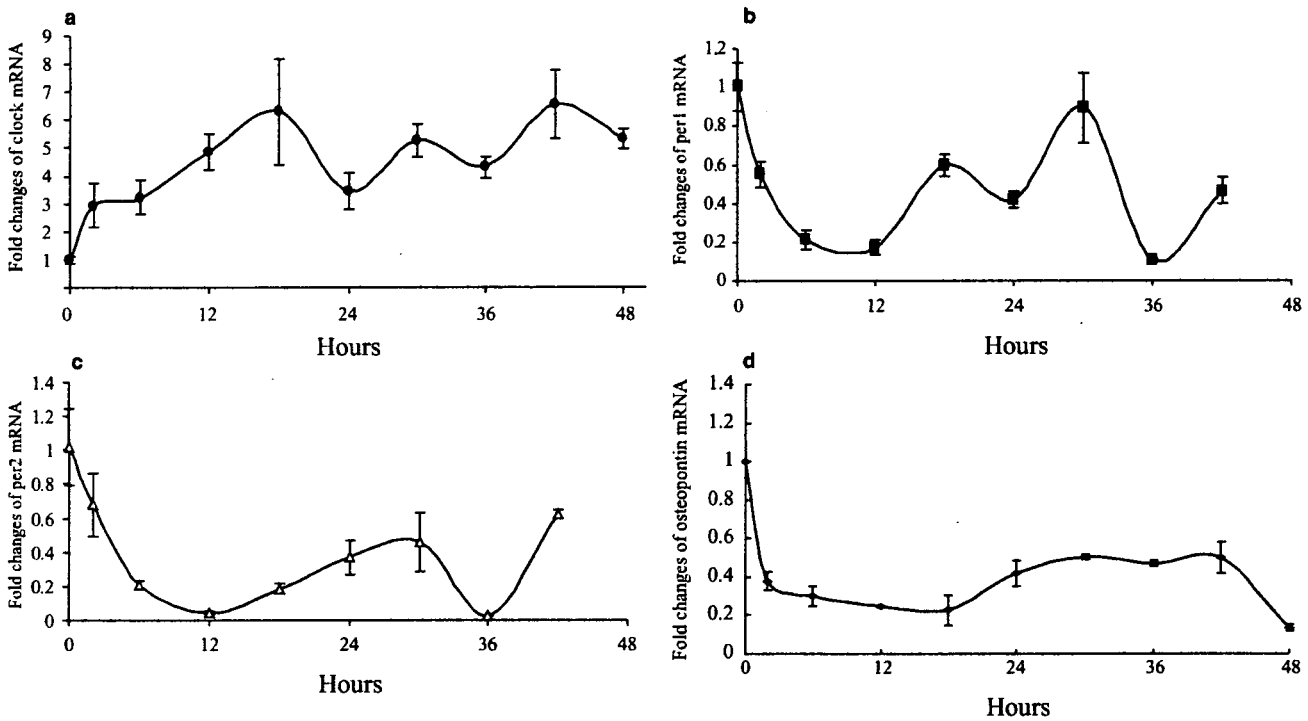

Fig. 3. **a** Biological rhythm of expression of clock mRNA by real-time reverse transcription–polymerase chain reaction (RT–PCR). **b** Biological rhythm of expression of per1 mRNA by real-time RT–PCR. **c** Biological rhythm of expression of per2 mRNA by real-time RT–PCR. **d** Negative control of biological rhythm with osteopontin mRNA by real-time RT–PCR

of cartilage with grade IV osteoarthritis. Thus, clock protein was confirmed to be more suppressed in the cartilage with severe osteoarthritis than in that with mild osteoarthritis cartilage (Fig. 4). Measurements of the densities of the blotting bands indicated a 75% downregulation in the protein level. These data suggest that the level of gene expression is similar to the level of protein expression.

Discussion

In this study we confirmed the role of the clock genes in functional responses to mechanical stress. Clock genes have an established involvement in the physiological changes accompanying jet lag. Perlecan, another biological-clock gene, may play a role in chondrocyte growth and differentiation. In animal studies, perlecan knockout mice have been confirmed to develop chondrodysplasia with dyssegmental ossification. Mice lacking molecular-clock components such as the per genes in osteoblasts display high bone mass, suggesting that bone remodeling may also be subject to circadian regulation.[6] Our group found that clock, a gene regulated by circadian rhythm, was downregulated in the chondrocytes of cartilage with severe osteoarthritis. We thus have reason to believe that the clock gene also plays an important role in maintaining the normal metabolism of chondrocytes.

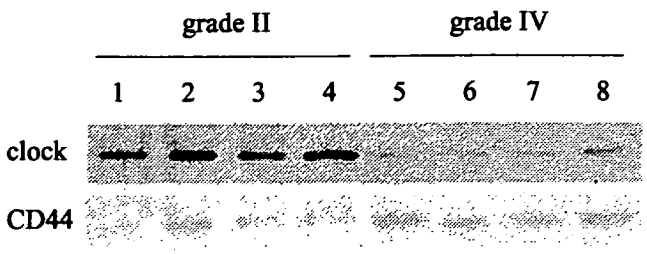

Fig. 4. Immunoblotting with polyclonal anti-clock antibody to cell lysate of osteoarthritis chondrocyte of Kellgren–Lawrence grade II and grade IV

The body is subjected to two types of mechanical stress, physiological and pathological. Having earlier observed a decrease in cell growth at 4 days after the induction of mechanical stress, our group simulated mechanical stress of the pathological type in this study using a bio-stretch machine. We observed downregulation of the clock gene after inducing this mechanical stress and then confirmed the finding by real-time PCR. Taken in sum, these findings suggest that the clock gene may play an important role in regulating physiological function in chondrocytes. In another study performed by mechanically stimulating human articular chondrocytes in vitro, the cells expressed enhanced levels of aggrecan mRNA and decreased levels of matrix metalloprotein (MMP)-3 mRNA.[7] The transduction process involves integrins, stretch-activated ion channels, and interleukin (IL)-4.[7] This chondroprotective response is absent in chondrocytes in osteoarthritis (OA) cartilage. Mechanical abnormalities leading to aberrant chondrocyte activity in diseased articular cartilage may be important in the progression of OA.[7] Other forms of mechanical stress such as hydrostatic pressure (HP) may also influence the cartilage metabolism in normal and pathological conditions, especially in weight-bearing areas of the skeletal system.[8] The incorporation of [^{35}S]sulfate into glycosaminoglycans (GAGs) was 1.3-fold higher and 1.4-fold higher than baseline control in samples exposed to constant HP and cyclic HP, respectively.[9] D'Lima et al. investigated apoptosis after mechanical stress on cartilage to elucidate the mechanisms behind the apoptosis of human chondrocytes observed in response to mechanical injury.[10] Another paper reported that inter-nucleosomal DNA fragmentation, an established marker of apoptosis, was detectable in 34% of chondrocytes at 96h after mechanical loading at 14MPa, versus only 4% of the chondrocytes of nonloaded explants.[11] Mechanical stress is known to induce osteoarthritis in articular cartilage. According to our data, clock was down regulated by mechanical stress and subsequently conferred an altered biological rhythm for the maintenance of chondrocyte metabolism. Thus, we found that the suppression of clock expression may contribute to the development of osteoarthritis by altering the cartilage metabolism.

Chondrocytes from deeper zones show higher rates of proliferation and collagen and proteoglycan synthesis than chondrocytes isolated from superficial and middle zones.[12,13] Furthermore, the proteoglycans synthesized by the chondrocytes from the deeper zones have higher keratin sulfate content, while those from the superficial zone are more sensitive to the catabolic effects of IL-1.[14] Progression through each of these phases is accompanied by profound changes in gene expression patterns.[15] Thus, the maintenance of the chondrocyte-specific phenotype plays a crucially important role in preserving the normal structure and biomechanical properties of articular cartilage, both under normal conditions and during repair of injured and diseased tissue. We found that clock was expressed in chondrocytes of the deeper zone by immunohistochemistry (data not shown). This site-specific expression of clock suggests that clock may be functionally involved in the maintenance of cartilage integrity via effects conferred on the chondrocyte metabolism. Among the current hypotheses to explain the changes in osteoarthritic chondrocytes, several investigators have proposed that phenotypic alterations of the cells occur in response to changing signals or matrix composition.[16,17] In histological findings, chondrocyte clusters were detected in the deeper zone of osteoarthritis cartilage. Clusters of chondrocytes in osteoarthritis cartilage have been shown to express type I and III collagens, proteins which can only be found at very low levels, if at all, in the normal articular chondrocytes.[18,19] The study of gene expression and histological change of OA cartilage may have implications for the understanding of the pathogenesis of osteoarthritis.

In conclusion, we have observed a profound change in the patterns of gene expression in chondrocytes cultured in 3D sponges and subjected to mechanical stress. Chondrocyte matrix, cytoplasmic genes, and nuclear genes were all expressed. The clock gene exhibited distinct properties among the genes investigated. In addition to being downregulated by mechanical stress in vitro, the clock gene was found to be downregulated in cartilage with severe osteoarthritis cartilage. Clock, an established regulator of biological rhythm, may therefore play a role in maintaining chondrocyte metabolism in tissues subjected to mechanical stress.

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BRIEF REPORT

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Efficacy of arthroscopic synovectomy for the effect attenuation cases of infliximab in rheumatoid arthritis

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Abstract To investigate whether arthroscopic synovectomy is effective for nonresponders to infliximab, anti-tumor necrosis factor-α antibody, for the treatment of rheumatoid arthritis (RA), we assessed seven patients including ten arthroscopic synovectomies in knee joint, in shoulder joint, and in ankle joints. We compared C-reactive protein (CRP) and DAS28 (ESR) before and after surgery at 6 and 50 weeks. After arthroscopic synovectomy, we continued the infliximab treatment with methotrexate in a routine manner. We detected synovium proliferation with vascular increase in patellofemoral joint and around the meniscus and femoral and tibial side of the anterior cruciate ligament in the knee joints. We also found synovial proliferation in rotator interval in the glenohumeral joint and fatty changing in subacromial bursa in the shoulder. In the ankle joint, we found synovial proliferation with white meniscoid between tibiofibular joint to develop impingement. Serum CRP was improved from 3.45±0.4 to 1.12± 0.2 at 6 weeks to 1.22±0.4 at 50 weeks after arthroscopic synovectomy. There is no severe side effect of arthroscopic synovectomy during infliximab treatment; however, one patient had slight rash that was improved. DAS28 was improved from 5.58±0.23 to 3.87±0.47 at 6 weeks to 2.58± 1.49 at 50 weeks after arthroscopic synovectomy. It is possible that arthroscopic synovectomy can be one of the effective methods to continue with the infliximab treatment when its efficacy decreased or in the nonresponders of infliximab for RA patients.

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Introduction

Even the treatment for rheumatoid arthritis (RA) with anti-tumor necrosis factor-α (TNF-α) therapy such as infliximab, a recombinant IgG1κ monoclonal antibody specific for tumor necrosis factor, including more than 6 mg of methotrexate (MTX) weekly, is the current treatment for RA, there are some cases that fails to control disease activity, prevent structural damage, and maintain quality of life. In patients with RA who have an incomplete response to anti-TNF-α therapy, the method such as the increase of methotrexate (MTX) or infliximab, or also the decrease of the interval period of injection with infliximab, is considerable. In our institute, we treated 35 cases by infliximab with MTX for RA. However, there is no clear strategy to control the disease if infliximab failed to control RA activity. We performed arthroscopic synovectomy in the middle of infliximab infusion interval, for example, 4 weeks after infusion of infliximab. Arthroscopic synovectomy is reported as effective method for early stage of RA [1]. However, degenerative change of RA could not be stopped by arthroscopic synovectomy only in long-term results [2]. We combined anti-TNF-α therapy and arthroscopic synovectomy for the patients who did not respond after around three to five times of infliximab infusion with MTX treatment. Arthroscopic synovectomy is safe and less painful compared with open synovectomy. Therefore, the hospitalization period of patients is relatively short after surgery. In regard to adverse events, infliximab has several adverse events such as high fever, high blood pressure, rash, headache for slight infusion reaction, and tuberculosis for severe adverse event. If we use these drugs too much for RA patients or for long period, it is possible to induce these adverse events, sometimes irreversibly, such as interstitial pneumonia with MTX. To avoid these adverse events, surgical treatment such as arthroscopic synovectomy is one

Table 1 Arthroscopic synovectomy (ASS) during infliximab treatment for RA

Case	Age (years)	Stage	Class	Operated joints
1. M.S.	61	II	II	Left-knee ASS
2. K.M.	62	IV	III	Right-shoulder ASS
3. Y.Y.	69	III	III	Left-ankle ASS
4. N.O.	49	II	II	Right-knee ASS, left-elbow ASS, left-wrist ASS, left-ankle ASS
5. T.I.	68	II	II	Right-knee ASS
6. S.N.	58	II	II	Right-shoulder ASS
7. T.H.	67	II	III	Left-knee ASS

of the options to treat RA with infliximab simultaneously. Efficacy of infliximab was already reported in long-term period, and it also prevented joint destruction by using sharp score [3]. However, there is no report of synovial finding in weak response to infliximab by arthroscopy during anti-TNF-α therapy. Here is the first report that synovial proliferation in the knee, the shoulder, and the ankle joints was less responsive to infliximab treatment. We investigated the combination of infliximab and arthroscopic synovectomy and also of DAS28 change to assess the efficacy of arthroscopic synovectomy in the short period for RA patients who had not responded well to infliximab.

Patients and methods

We performed arthroscopic synovectomy in 7 patients out of 35 infliximab and MTX cases for the treatment of RA. Infliximab (3 mg/kg) (Centcore, USA, Tanabe Co.) was administered at the rate of 60 ml/h for 30 min and 125 ml/h by infusion during 1-day admission. The patients include one male and six females from 49 to 68 years old, with average of 62 years old. Three patients underwent arthroscopic synovectomy after administration of infliximab for four times, two patients after five times, and two patients after six times. All patients initially responded to infliximab and MTX, but gradually, the effect decreased; the average of C-reactive protein (CRP) was 3.45±0.4 (2.7–5.6) mg/dl at the surgery. The indication for operation was that after treatment of infliximab, CRP was more than 2.5 mg/dl, and the numbers of arthritis joints were limited to within five joints of relatively large joints such as knee, shoulders, including ankles and wrists. The infliximab treatment included a diagnosis of RA based on the ACR (formerly, the American Rheumatism Association) criteria [4] and categorization by Steinbrocker et al. [5]. Five patients were categorized as stage II RA, one patient as stage III, and one patient as stage III. Four patients were class II, and three patients were class III. Three patients were given 6 mg of MTX during infliximab treatment, and

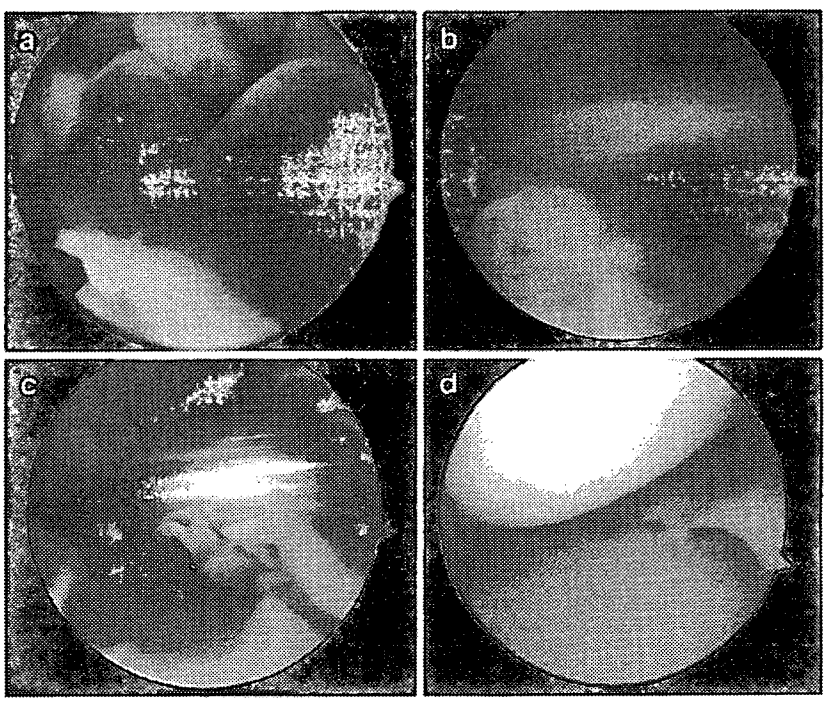

Fig. 1 Arthroscopic finding and synovectomy during infliximab treatment for RA. **a** Patellofemoral joint, **b** around medial disc, **c** shaving synovium around anterior cruciate ligament, **d** the view cleaned-up after arthroscopic synovectomy