CONCISE REPORT

Anti-apoptogenic function of TGFβ1 for human synovial cells: TGFβ1 protects cultured synovial cells from mitochondrial perturbation induced by several apoptogenic stimuli

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Objective: To investigate anti-apoptogenic mechanism of transforming growth factor β1 (TGFβ1) towards synovial cells.

Methods: Isolated synovial cells, treated or not with TGFβ1, were cultured in the presence or absence of anti-Fas IgM, proteasome inhibitor Z-Leu-Leu-Leu-aldehyde (LLL-CHO), etoposide, or C2-ceramide. After cultivation, apoptosis of synovial cells was examined by the presence of hypodiploid DNA$^+$ cells, the presence of terminal deoxy (d)-UTP nick end labelling$^+$ cells (TUNEL$^+$ cells), activation of caspases, and disruption of mitochondrial transmembrane potential (ΔΨm).

Results: Activation of caspase-9 and ΔΨm was found in anti-Fas IgM treated synovial cells. The increment of both hypodiploid DNA$^+$ cells and TUNEL$^+$ cells accompanied by the activation of caspase-8 and caspase-3 was also determined in anti-Fas IgM treated synovial cells. These hallmarks for apoptosis induced by anti-Fas IgM were significantly suppressed in TGFβ1 treated synovial cells. LLL-CHO, etoposide, and C2-ceramide also caused ΔΨm, the increment of both hypodiploid DNA$^+$ cells and TUNEL$^+$ cells, and the activation of both Leu-Glu-His-Asp ase (LEHDase; caspase-9 like activity) and Asp-Glu-Val-Asp ase (DEVDase; caspase-3 like activity) in synovial cells. As determined in anti-Fas IgM treatment, TGFβ1 significantly reduced apoptotic cell death of synovial cells induced by the above chemicals.

Conclusions: The protective effect of TGFβ1 for mitochondrial homoeostasis may be important in the anti-apoptogenic function of TGFβ1 for synovial cells.

Cytokines and growth factors present in rheumatoid synovial tissues are important factors which regulate an apoptotic process of synovial cells.[1 2] Transforming growth factor β1 (TGFβ1) is highly expressed in rheumatoid synovial tissues,[4] and found to possess an anti-apoptogenic effect for synovial cells; this was demonstrated by the experimental results showing that TGFβ1 inhibits Fas mediated apoptosis as well as proteasome inhibitor induced apoptosis in cultured synovial cells.[2 3] TGFβ1 not only suppresses Fas expression, but increases the expression of Bcl-2 and Bcl-xL in cultured synovial cells.[2 3] the latter finding implies that TGFβ1 protects synovial cells from apoptogenic stimuli through a mitochondria dependent mechanism.

We show in this study that mitochondrial perturbation as well as both DNA fragmentation and the activation of caspases in cultured synovial cells, induced by several apoptogenic stimuli, are significantly suppressed by TGFβ1 treatment, which may be closely associated with the anti-apoptogenic function of TGFβ1.

MATERIALS AND METHODS

Synovial cell culture

Synovial cells were isolated from synovial tissues obtained from 18 patients with rheumatoid arthritis (RA) who met the American College of Rheumatology criteria for RA[7] at the time of orthopaedic surgery, as we previously described.[2 3] In some experiments, synovial cells isolated from patients with osteoarthritis were also used in this study. The adherent synovial cells used in this study at third to fifth passages were less than 1% reactive with monoclonal antibodies, including CD3, CD68, CD20, and von Willebrand factor, which are defined as fibroblast-like synovial cells.

Induction of synovial cell apoptosis by several apoptogenic stimuli

Synovial cells were cultured with or without recombinant human TGFβ1 (5 ng/ml; R&D Systems Inc, Minneapolis, MN) for 48 hours in RPMI 1640 containing 2% bovine serum albumin. After incubation, apoptosis sensitivity in untreated or TGFβ1 treated synovial cells was examined by further incubation with anti-Fas IgM (1 μg/ml for 12 hours; MBL, Nagoya, Japan), Z-Leu-Leu-Leu-aldehyde (LLL-CHO, 10 μM for 24 hours, Peptide Institute, Osaka, Japan), etoposide (50 μM for 24 hours; TopoGen, Inc, Columbus, Ohio), or C2-ceramide (50 μM for 24 hours; Sigma). Apoptosis of synovial cells was quantified by the presence of hypodiploid DNA$^+$ cells, the presence of terminal deoxy (d)-UTP nick end labelling$^+$ cells (TUNEL$^+$ cells), activation of caspases, and disruption of mitochondrial transmembrane potential (ΔΨm) as previously described.[3 5 8 9]

DNA fragmentation was estimated by the presence of hypodiploid DNA$^+$ cells and TUNEL$^+$ cells, determined by flow cytometry (Epics XL, Beckman Coulter, Hialeah, FL). Detection of hypodiploid DNA$^+$ cells was done by propidium iodide staining (100 μg/ml; Sigma Chemical Co, St Louis, MO), and TUNEL was examined by Mebstain Apoptosis Kit (MBL, Nagoya, Japan).

Activation of caspases in synovial cells was studied by western blot analysis, colorimetric protease assay and flow cytometry. Western blot analysis was done by

Abbreviations: DEVDase, Asp-Glu-Val-Asp ase; DiOC6, 3, 3'-dihexyloxacarbocyamine iodide; Δψm, disruption of mitochondrial transmembrane potential; IETDase, Ile-Glu-Thr-Asp ase; LEHDase, Leu-Glu-His-Asp ase; LLL-CHO, Z-Leu-Leu-Leu-aldehyde; RA, rheumatoid arthritis; TGFβ1, transforming growth factor β1; TUNEL, terminal deoxy (d)-UTP nick end labelling

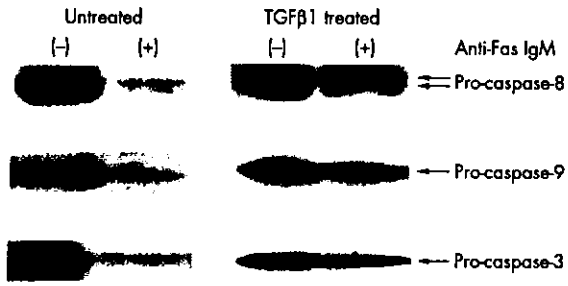

Figure 1 Western blot analysis for the activation of caspase-3/-8/-9 in synovial cells induced by anti-Fas IgM, which is inhibited by TGFβ1. Synovial cells isolated from the rheumatoid synovial tissues were cultured with or without TGFβ1 for 48 hours, washed, and further incubated with control mouse IgM or anti-Fas IgM for 12 hours. After cultivation, the expression of procaspase-3/-8/-9 in synovial cells was examined by western blotting as described in the text. Note that the disappearance of procaspase-3/-8/-9 in anti-Fas IgM treated synovial cells, which indicates the activation of each caspase, was significantly inhibited by TGFβ1 treatment. Results are representative data from six determinations. anti-Fas IgM (−); addition of control mouse IgM.

enhanced chemiluminescence system (Amersham, Arlington Heights, IL, anti-caspase-3; Transduction Laboratories, Lexington, KY, anti-caspase-8; MBL, anti-caspase-9; MBL). Decrement of procaspase expression and/or the appearance of cleaved products indicate the activation of each caspase.[10] In addition to western blotting, increment of an enzymatic activity of Asp-Glu-Val-Asp ase (DEVDase: caspase-3 like activity), Ile-Glu-Thr-Asp ase (IETDase: caspase-8 like activity), and Leu-Glu-His-Asp ase (LEHDase: caspase-9 like activity) was used for detection of activation in each caspase.

Enzymatic activity of DEVDase (intracellular DEVDase$^+$ cells) was detected by flow cytometry (Epics XL) by the use of DEVD substrate (OncoImmunin, Inc, College Park, MD) as previously described.[4] Enzymatic activity of both IETDase and LEHDase was examined by colorimetric protease assay kit (MBL), and the activity of IETDase and LEHDase was evaluated by a spectrophotometer at an optical density of 405 nm (Multiskan JX, LABSYSTEMS, Tokyo, Japan), according to the manufacturer's protocol.

Expression of Bcl-2 (anti-Bcl-2; Dako Japan, Kyoto, Japan), Bcl-xL (anti-Bcl-xL; Trevigen, Gaithersburg, CA), and Bax (anti-Bax; Santa Cruz Biotechnology, Santa Cruz, CA) in synovial cells was studied by western blotting, and a relative expression ratio of Bcl-2 to Bax and of Bcl-xL to Bax was calculated by the software NIH Image (1.61) as follows: density of Bcl-2 or Bcl-xL/density of β-actin to density of Bax/density of β-actin. β-Actin (anti-β-actin; Sigma) was used as an internal control protein in western blotting.

Mitochondrial perturbation in synovial cells was examined by $\Delta\Psi m$.[9] The cells were reacted with saturating amount of DiOC6 (3,3'-dihexyloxacarbocyamine iodide, Fluoreszenz-technologie, Grottenhofstr, Austria) at 37°C for 15 minutes, washed, and analysed by flow cytometry. In some experiments, synovial cells were cultured in the presence of Z-Val-Ala-Asp-CH_2DCB (caspase inhibitor, 200 μM; Phoenix Pharmaceuticals, Inc, Mountain View, CA), and apoptosis of these cells was also examined.

Statistical analysis

Data were expressed as mean (SD). Differences between groups were tested for statistical significance using the Student's *t*-test. A p value <0.05 was considered significant.

RESULTS

Inhibition of Fas mediated mitochondrial perturbation in cultured synovial cells by TGFβ1

Although we did not find the cleaved products of each caspase, activation of caspase-3/-8/-9 in synovial cells by anti-Fas IgM was strongly suggested by western blotting (fig 1), which was confirmed by an enzymatic activity assay for DEVDase, IETDase, and LEHDase (table 1). Mitochondrial perturbation with DNA fragmentation in synovial cells was also clearly induced by anti-Fas IgM (table 1). Expression of procaspase-3/-8/-9 in synovial cells was not changed by TGFβ1 (fig 1), but TGFβ1 treatment significantly suppressed $\Delta\Psi m$, activation of caspase-3/-8/-9, and DNA fragmentation of synovial cells induced by anti-Fas IgM (fig 1, table 1).

Effect of TGFβ1 for synovial cell apoptosis induced by other apoptogenic stimuli

We next examined whether TGFβ1 treatment protects mitochondrial perturbation induced by other apoptogenic stimuli. LLL-CHO, etoposide, and C2-ceramide, the chemicals triggering apoptosis in a mitochondria dependent fashion,[11 12] induced $\Delta\Psi m$ with the presence of DNA fragmentation toward synovial cells (table 2). Z-Val-Ala-Asp-CH_2DCB did not inhibit $\Delta\Psi m$ of synovial cells in the process (data not shown), which supported the importance of the mitochondrial pathway in synovial cell death induced by LLL-CHO, etoposide, and C2-ceramide. Activation of both LEHDase and DEVDase was also clearly found in synovial cells treated with the chemicals, and TGFβ1 treatment significantly suppressed the above hallmarks for apoptosis (table 2). As we previously described,[3] the relative expression ratio of Bcl-2 or Bcl-xL to Bax in synovial cells was increased by TGFβ1 (untreated synovial cells: Bcl-2 to Bax 0.56 (0.05) and Bcl-xL to Bax; 0.12 (0.01); TGFβ1 treated synovial cells: Bcl-2 to Bax 0.95 (0.07)* and Bcl-xL to Bax 0.88 (0.06)*; *$p<0.01$ *v* untreated synovial

Table 1 Inhibition of Fas mediated apoptosis of synovial cells by TGFβ1

Stimuli		Apoptosis					
Anti-Fas IgM	TGFβ1	Hypodiploid DNA (%)	TUNEL (%)	ΔΨm (%)	DEVDase (%)	IETDase (OD)	LEHDase (OD)
−	−	1.4 (0.1)	2.1 (0.1)	1.0 (0.1)	1.6 (0.1)	0.32 (0.03)	0.34 (0.09)
−	+	1.3 (0.2)	2.1 (0.2)	1.0 (0.1)	1.5 (0.1)	0.29 (0.03)	0.31 (0.04)
+	−	60.3 (5.9)*	45.8 (3.9)*	61.5 (5.6)*	62.5 (6.1)*	0.96 (0.07)*	0.92 (0.07)*
+	+	14.2 (1.1)*	12.5 (1.4)*	14.1 (1.4)*	12.9 (1.4)*	0.44 (0.03)*	0.39 (0.03)*

Rheumatoid synovial cells were cultured with or without TGFβ1 for 48 hours, washed, and further incubated with anti-Fas IgM for 12 hours. After cultivation, the percentage of hypodiploid DNA$^+$ cells, percentage of TUNEL$^+$ cells, ΔΨm, DEVDase activity, IETDase activity, and LEHDase activity were examined as described in the text. Note that apoptotic cell death of synovial cells induced by anti-Fas IgM was significantly suppressed by TGFβ1.
Results are mean (SD) from six individual experiments.
Anti-Fas IgM (−): addition of control mouse IgM. OD of IETDase and LEHDase was described as OD/μg protein.
*$p<0.01$.

Table 2 TGFβ1 mediated inhibition in synovial cell apoptosis induced by LLL-CHO, etoposide and C2-ceramide

		Apoptosis				
Stimuli	TGFβ1	Hypodiploid DNA (%)	TUNEL (%)	ΔΨm (%)	DEVDase (%)	LEHDase (OD)
LLL-CHO	–	23.2 (1.8)*	19.0 (1.8)*	22.7 (2.5)*	25.1 (2.0)*	0.68 (0.05)*
	+	5.9 (0.4)*	5.0 (0.4)*	5.7 (0.8)*	6.8 (0.8)*	0.39 (0.03)*
Etoposide	–	20.1 (2.2)*	18.2 (1.7)*	19.6 (1.7)*	19.7 (1.9)*	0.69 (0.06)*
	+	5.1 (0.5)*	5.2 (0.4)*	5.2 (0.7)*	5.7 (0.6)*	0.43 (0.04)*
C2-ceramide	–	22.5 (1.5)*	19.5 (1.7)*	22.4 (2.2)*	23.9 (2.1)*	0.72 (0.06)*
	+	6.5 (0.7)*	5.4 (0.3)*	6.3 (0.7)*	6.5 (0.7)*	0.42 (0.04)*

Rheumatoid synovial cells were cultured with or without TGFβ1 for 48 hours, washed, and further incubated with LLL-CHO, etoposide, or C2-ceramide for 24 hours. After cultivation, the percentage of hypodiploid DNA+ cells, percentage of TUNEL+ cells, ΔΨm, DEVDase activity, and LEHDase activity were examined as described in the text. Note that apoptotic cell death of synovial cells induced by LLL-CHO, etoposide, and C2-ceramide was significantly suppressed by TGFβ1. Results are mean (SD) of five individual experiments.
OD of LEHDase was described as OD/μg protein. Each parameter in untreated and TGFβ1 treated synovial cells cultured in the absence of LLL-CHO, etoposide, or C2-ceramide was the same as in table 1.
*$p<0.01$.

cells. Results are the mean (SD) of five individual experiments. We found no difference in TGFβ1 induced inhibition for synovial cell apoptosis induced by anti-Fas IgM, LLL-CHO, etoposide, and C2-ceramide between rheumatoid synovial cells and synovial cells isolated from patients with osteoarthritis (data not shown).

DISCUSSION

Our series of studies have indicated that the growth promoting activity of TGFβ1 for synovial cells can, in part, be mediated by inhibition of synovial cell apoptosis.[2 3] As reported in other cell types,[14 15] activation of caspase-3/-8/-9 as well as ΔΨm was found in anti-Fas monoclonal antibody treated synovial cells, whereas these hallmarks for apoptosis were markedly suppressed in TGFβ1 treated synovial cells.

Mitochondrial dysfunction in synovial cells was also induced by LLL-CHO, etoposide, and C2-ceramide, leading to the activation of caspase-3/-9 and DNA fragmentation. TGFβ1 treatment significantly suppressed apoptogenic activity of LLL-CHO, etoposide, and C2-ceramide. As we previously reported,[3] relative expression of Bcl-2 and Bcl-xL was increased by TGFβ1, which may prevent the release of cytochrome *c* and apoptotic cell death of synovial cells induced by the above chemicals. TGFβ1 mediated protection for the mitochondria may also be effective in Fas induced apoptosis in synovial cells.

Impaired apoptosis of synovial cells in rheumatoid synovial tissues is supposed to have an important role in the progression of synovial cell hyperplasia of patients with RA, but recent investigations have suggested its characteristic may not be an intrinsic property of rheumatoid synovial cells, but that the rheumatoid synovial microenviroment affects the sensitivity of synovial cell apoptosis.[1 5 12] One factor which may inhibit apoptosis of synovial cells is TGFβ1, but rheumatoid synovial tissue contains a variety of cytokines and growth factors other than TGFβ1, which also inhibit apoptotic cell death of synovial cells, including basic fibroblast growth factor and interleukin 1β.[1 4] The present data are obtained only under bovine serum albumin culture conditions, thus, the in vivo role of TGFβ1 in rheumatoid synovial tissues remains obscure. Further investigations, including interactions between TGFβ1 and other humoral factors, are necessary to clarify the in vivo role of TGFβ1 in the regulation of apoptotic cell death of synovial cells of patients with RA.

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抗 CCP 抗体の経時的評価の有用性に関する検討

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Key words : Rheumatoid arthritis, Anti-CCP antibody, Disease activity

はじめに

数十年前より抗核周囲因子あるいは抗ケラチン抗体が関節リウマチ(RA)に比較的特異的とされていたが、詳細は不明であった[1]。1990 年代に入り、フィラグリンが対応抗原として同定され、さらに citrulline とその周囲がエピトープと同定され[2)3)]、2000 年には citrulline を含む環状ペプチドを抗原とした測定系が開発された。その結果、抗 cyclic citrullinated peptide(CCP)抗体が感度、特異度ともに高い優れた血清指標であることが明らかにされた。しかし、抗 CCP 抗体を経時的に測定して有用性を検討した報告はまれであり今回検討した。

対象および方法

対象は、最低 8 週間以上の間隔で経時的に血清を採取・保存できた当科の RA59 例(男性 4 例、女性 55 例)、129 検体で、明らかな感染症状を有する時期の検体は除外した。各臨床経過における抗 CCP 抗体の推移を、CRP やリウマトイド因子 (RF) の推移と対比して検討した。抗 CCP 抗体の測定法は ELISA で、基準値は 5 U/ml 以下である。測定範囲を超えた検体は希釈して再測定した。RF の測定はラテックス免疫比濁法で、基準値は 10 IU/ml 以下である。

経時的な検体採取については、2 検体が得られた 49 例では 49 経過を評価し、3 検体が得られた 9 例では前半と後半の二つの経過を評価し、4 検体が得られた 1 例では 4 検体による 3 つの間隔(経過)を評価し、合計 59 例、70 の経過、平均間隔 17 ヶ月における検査値の変動を評価した。さらに、70 の臨床経過を CRP の変動で 3 群に大別した。すなわち、CRP の変動が 1.0mg/dl 未満の不変群、CRP が 1.0mg/dl 以上上昇した上昇群、1.0mg/dl 以上低下した低下群とした。

結　　果

抗 CCP 抗体は 84.5%、RF は 82.9%の陽性率で、両者陽性が 101 例、両者陰性が 14 例、抗 CCP 抗体のみ陽性 8 例、RF のみ陽性 6 例であり、両者の乖離例は合計 14 例、10.9%であった。抗 CCP 抗体と RF とは、弱いながら正相関した(r=0.234、p<0.01)。129 検体を RF の値で 5 群に分けて、抗 CCP 抗体の値を比較したが、RF 陰性群は抗 CCP 抗体も低値で、他の群に比べて有意に低い結果であった(図 1)。

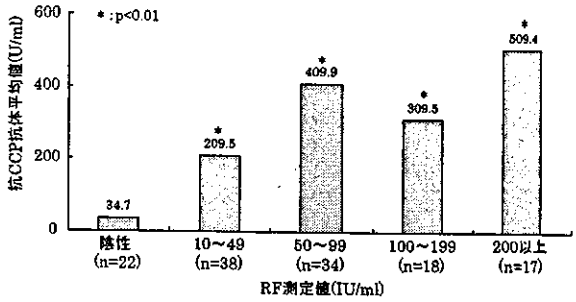

図1　RF測定値別の抗CCP抗体の比較

抗 CCP 抗体と CRP、RF と CRP とでは、いずれも有意の相関は認めなかった。CRP の値が 1.0mg/dl 未満か 1.0 以上かで 2 群に分けて両抗体を比較したが、抗 CCP 抗体(U/ml)(mean±SD)が順に 277±394 vs 295±370、RF(IU/ml)(mean±SD)が順に 93±141 vs 151±266 であり、いずれも群間で有意差は認めなかった。

経過中の CRP の変動で大別した不変群 32 例、上昇群 16 例、低下群 22 例の CRP を表 1 に示すが、上昇群と低下群でそれぞれ有意の変動が認められた。なお、経過間隔(月)(mean±SD)は不変群、上昇群、低下群の順に 12.5±17.7、28.1±22.0、15.5±16.1 であり、上昇群が他群に比べて有意に長期であった。

不変、上昇、低下の 3 群で、抗 CCP 抗体の推移を比

群(n)	定義	実際のCRPの変動 (mean±SD)
●不変群 (32例)	1.0mg/dl 未満の増減	0.71 ± 0.62 → 0.73 ± 0.63 n.s.
●上昇群 (16例)	1.0mg/dl 以上の上昇	1.42 ± 2.27 → 4.60 ± 4.43 p<0.01
●低下群 (22例)	1.0mg/dl 以上の低下	5.00 ± 4.48 → 1.20 ± 1.52 p<0.001

表1　経過におけるCRPの変動による群分け

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CLINICAL SIGNIFICANCE OF ANTI-CYCLIC CITRULLINATED PEPTIDE ANTIBODY IN PATIENTS WITH RA

MASAAKI NAKANO et al : Dept. of Med. Technology, School of Health Sciences, Fac. of Med. Niigata Univ.

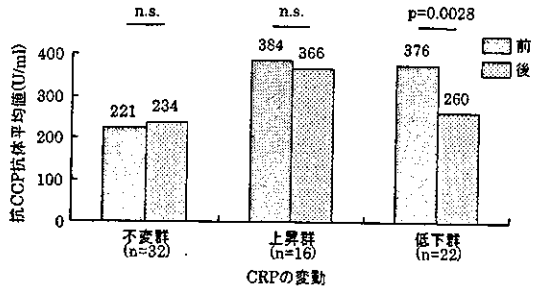

図2 CRPの変動別の抗CCP抗体の推移

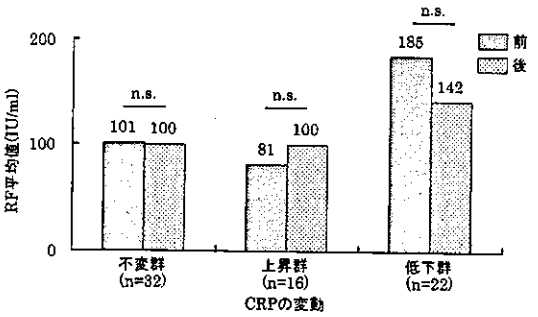

図3 CRPの変動別のRFの推移

較した結果、不変群、上昇群では前後で有意差は認めなかったが、低下群では抗 CCP 抗体も有意に低下した(図2)。同じく RF の変動を比較したが、低下群を含めて、経過における RF の有意の変動は認めなかった(図3)。

70 の臨床経過における抗 CCP 抗体と RF の変動様式を比較した結果、抗 CCP 抗体は RF に比べて高いレベルで大幅に変動する傾向が認められた（表2)。

	抗CCP抗体	RF
3桁 ⇔ 4桁の変動	6(9%)	2(3%)
3桁間での変動	28(40%)	10(14%)
2桁 ⇔ 3桁の変動	5(7%)	13(19%)
2桁以内での変動	31(44%)	45(64%)

$p<0.001$

表2 経過における抗CCP抗体とRFの変動様式

考　　案

近年、抗 CCP 抗体の測定が広く行われるに至り、RA に対する感度、特異度ともに高い、きわめて優れた血清マーカーであることが認められてきており、さらには予後の判定要因や早期診断としての意義も検討されている[4) 5)]。今回の検討では、CRP の低下群において、抗 CCP 抗体も有意に低下しており、特に新たな薬物の治療効果が出現するような場合の疾患活動性を反映する指標としての測定意義の可能性も示唆された。CRP 低下群では RF も低下傾向を示したが、RF は抗 CCP 抗体に比べて低い値で推移するため、有意差が認められなかったものと思われる。一方、CRP 上昇群では有意の自己抗体の変動がみられなかったが、この群の間隔は平均 28 ヶ月以上と長期であり、この期間における検査値異常に影響を与える他の要因の影響により自己抗体が疾患活動性を反映しない結果になった可能性が考えられた。いずれにしても、抗 CCP 抗体が RA の疾患活動性を反映するか否かについては、より症例を重ねて検討する必要があると思われる。

ま　と　め

(1) 抗CCP抗体の陽性頻度は免疫比濁法による RF とほぼ同様で、両者の乖離例は 10.9%と低頻度であった。

(2) 抗 CCP 抗体と RF は弱いながら有意に正相関した。

(3) 抗 CCP 抗体および RF と CRP とでは有意の相関は認めなかった。

(4) CRP 低下群では、抗 CCP 抗体が有意に低下したが、RF は有意の変動を認めず、他の群では両抗体とも有意の変動はなかった。

(5) 抗 CCP 抗体は RF に比べて、高いレベルで大幅に変動する傾向を認めた。

結　　語

抗 CCP 抗体は免疫比濁法による RF に比べて、CRP の低下に伴って低下する傾向があり、RA の疾患活動性の指標としての可能性も示唆された。

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CONCISE COMMUNICATION

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Association between autoantibodies to the Ku protein and DPB1*

The Ku protein, a heterodimer consisting of 70-kd (p70) and 80-kd (p80) polypeptide subunits, binds free ends of double-stranded DNA (dsDNA). Once associated with DNA it creates a binding site for the catalytic subunit of the holoenzyme known as DNA-dependent protein kinase. This enzyme is essential for repairing dsDNA breaks that occur during radiation injury and V(D)J recombination (1).

Autoantibodies to the Ku protein were identified originally in 9 individuals among a randomly selected group of 330 Japanese patients (3%) with various connective tissue diseases studied with a classic immunodiffusion assay. Six of the patients who tested positive for autoantibodies came from a subgroup of 11 individuals (55%) with polymyositis–scleroderma (PM–scleroderma) overlap syndrome (2).

A somewhat different picture of anti-Ku autoantibodies emerged from studies of patients in the US. Reeves observed anti-Ku autoantibodies in the sera of 39% of patients with systemic lupus erythematosus (SLE), 55% of patients with mixed connective tissue disease, and 40% of patients with scleroderma, using an enzyme-linked immunosorbent assay (3). These antibodies also appear to be much more common among African American patients than white patients with SLE (4). Using immunoprecipitation assays, Francoeur et al observed anti-Ku antibodies in 10% of patients with SLE and in no samples obtained from patients with scleroderma (5). These observations suggest that anti-Ku antibodies have unique clinical associations in different racial groups, but further studies applying the same assay systems to different populations simultaneously will be required to confirm this speculation.

In the last several years, it has become clear that autoantibodies to nucleoproteins are antigen driven and require T helper cell support. Therefore, variations of autoantibody correlations in different patient groups seem likely to reflect racial differences in distribution of major histocompatability complex (MHC) phenotypes and the pattern of peptide antigens that are presented to T cells. We have now explored this idea through a genotypic analysis of all patients with anti-Ku autoantibodies at our institution in Japan.

A total of 750 Japanese patients were screened for autoantibodies in a radioimmunoprecipitation assay (6), and 21 were found to have anti-Ku autoantibodies. The presence of these antibodies was confirmed in an immunoblot assay using extracts of HeLa cells. The clinical diagnosis was established from a review of the medical record (Table 1). None of these patients had familial relationships. Clinically, 13 patients had PM or overlap syndromes with myositis (5 had PM–scleroderma, 4 had PM–scleroderma–SLE overlap, 2 had PM–SLE overlap, and 2 had PM), 5 had SLE, 2 had autoimmune hepatitis, and 1 had scleroderma according to established classification criteria (7–10). Forty-six healthy unrelated Japanese individuals served as control subjects. The HLA class II (DRB1, DQA1, DQB1, and DPB1) alleles were identified from restriction fragment length polymorphisms of polymerase chain reaction–amplified genomic DNA (11).

The HLA class II genotypes of all 21 patients are shown in Table 1. DRB1*0901 (62% of subjects versus 28% of controls; $P = 0.009$, odds ratio [OR] = 4.1), DQA1*0302 (62% versus 59%), and DQB1*0303 (62% versus 30%) were elevated in the study group, but none of these associations were statistically significant. However, DPB1*0501 was present in all patients with anti-Ku autoantibodies, compared with 59% of control subjects. This association was significant ($P = 0.0016$, OR 30) and remained significant ($P = 0.03$) when corrected for the number of alleles examined. Thirteen of the 21 patients (62%) with anti-Ku antibodies had myositis. Ten of these individuals (77%) had the class II haplotype of DRB1*0901-DQA1*0302-DQB1*0303, compared with 38% of anti-Ku–positive patients without myositis and 28% of controls ($P = 0.004$, OR 8.5). Four patients were homozygous for DRB1*0901, DQA1*0302, and DQB1*0303, but we found no indication of more severe disease in this group.

Studies of HLA associations with anti-Ku autoantibodies are limited. Yaneva and Arnett reported that the HLA class II antigen DQw1 was present in 17 of 19 anti-Ku positive patients (89%), compared with its frequency in local white (58%) and African American (61%) controls ($P = 0.01$, relative risk 5.8) (12). Although this allele occurs at increased frequency in patients with SLE, it is not associated with myositis and scleroderma. In the present study, the most striking finding is the universal occurrence of DPB1*0501 in 21 consecutive patients with anti-Ku autoantibodies. The DRB1*0901-DQA1*0302-DQB1*0303 haplotype also correlates with myositis in this patient cohort. Both DPB1*0501 and the DRB1*0901-DQA1*0302-DQB1*0303 haplotype are more common in the Japanese population than in the white population (13). It should be noted that DPB1*0501 is also a risk factor for Graves' disease in Japan (14). These findings suggest that there is a common immunogenetic background for Graves' disease and the anti-Ku autoimmune response. Therefore, these associations help to rationalize the earlier findings that anti-Ku autoantibodies are more clearly associated with myositis among the Japanese population.

Among the patients studied here, 9 had PM–scleroderma overlap syndrome with anti-Ku antibodies but none had the anti–PM-Scl, specificity. In the US population, ~10% of patients with this syndrome develop anti–PM-Scl. We have examined >100 patients with this overlap syndrome, but none have had anti–PM-Scl, nor have any of the >3,000 patients screened in our clinical diagnostic laboratory. Therefore we believe this autoantibody is rare among Japanese individuals. An explanation may be that anti–PM-Scl antibodies have been linked with DR3, a phenotype that is uncommon in the Japanese population (13). In any case, the MHC phenotype appears to exert a stronger influence over expression of specific autoantibodies than over the emergence of individual autoimmune syndromes. Further studies including analysis of MHC-restricted T cell responses could provide important clues for understanding mechanisms of onset of the PM–scleroderma overlap syndrome and the expression of anti-Ku antibodies.

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Table 1. HLA class II genes in Japanese patients with anti-Ku autoantibodies*

Patient no.	Diagnosis	DRB1*	DQA1*	DQB1*	DPB1*
1	PM/SSc	0405/1101	0303/0505	0401/0301	0501/0402
2	PM/SSc	0901/080302	0302/0103	0303/0601	0501/0202
3	PM/SSc	0901/080302	0302/0103	0303/0601	0501/0201
4	PM/SSc	0901/0405	0302/0303	0303/0401	0201/0501
5	PM/SSc	0901/0901	0302/0302	0303/0303	0501/0402
6	PM/SSc/SLE	0901/0901	0302/0302	0303/0303	0501/0402
7	PM/SSc/SLE	0901/1401	0302/0104	0303/0503	0501/0201
8	PM/SSc/SLE	0901/1502	0302/0103	0303/0601	0501/0901
9	PM/SSc/SLE	0901/0901	0302/0302	0303/0303	0501/0201
10	PM/SLE	0901/0901	0302/0302	0303/0303	0501/0201
11	PM/SLE	0405/0405	0303/0303	0401/0401	0501/0301
12	PM	0901/0802	0302/030101	0303/0302	0501/4101
13	PM	0405/1502	0303/0103	0401/0601	0501/0901
14	SLE	0901/1501	0302/0102	0303/0602	0501/0501
15	SLE	1501/0802	0401/0102	0302/0602	0201/0501
16	SLE	0405/080302	0303/0103	0401/0601	0501/0501
17	SLE	0901/080302	0302/0103	0303/0601	0501/0201
18	SLE	080302/1302	0103/0102	0601/0604	0501/0401
19	SSc	0405/0405	0303/0303	0401/0401	0501/0201
20	AIH	0802/0802	030101/030101	0302/0302	0201/0501
21	AIH	0901/0802	0302/030101	0303/0302	0501/0501

* PM = polymyositis; SSc = systemic sclerosis (scleroderma); SLE = systemic lupus erythematosus; AIH = autoimmune hepatitis.

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Ameliorative Effects of Follistatin-Related Protein/TSC-36/FSTL1 on Joint Inflammation in a Mouse Model of Arthritis

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Objective. **To clarify the in vivo function of follistatin-related protein (FRP)/TSC-36/FSTL1 in rheumatoid arthritis (RA), we investigated the roles of FRP in a mouse model of arthritis.**

Methods. **Arthritis was induced in BALB/c mice by injecting anti–type II collagen monoclonal antibody and lipopolysaccharide. Mice were treated with daily intraperitoneal injections of 20 μg of recombinant FRP. Development of arthritis was assessed by the clinical score and footpad swelling. Histologic examination of affected paws was performed on day 21 after the onset of arthritis. The gene expression profiles of affected paws in FRP-treated and untreated mice were compared using commercially available complementary DNA (cDNA) arrays. The difference in gene expression was confirmed by real-time quantitative reverse transcription–polymerase chain reaction.**

Results. **Treatment with recombinant FRP showed significant amelioration of the arthritis severity. Histologic analyses confirmed this finding and revealed the alleviation of cellular infiltration into the synovium as well as cartilage damage. The significant decrease in the amount of urinary deoxypyridinoline also indicated the ameliorative effect of FRP on joint destruction. Moreover, cDNA array analysis of the gene expression profile in FRP-treated arthritic lesions revealed a reduced expression of the c-*fos*, *ets*-2, *IL6*, *MMP3*, and *MMP9* genes, some of which are thought to be associated with synovial inflammation and joint destruction.**

Conclusion. **These findings from in vivo experiments suggest that FRP could be one of the key molecules in the treatment of inflammatory joint diseases such as RA.**

Rheumatoid arthritis (RA) is a chronic systemic disease characterized by destructive polyarthritis, and its etiology remains to be elucidated. In the pathogenesis of RA, an antigen-driven immune response involving synovial T cells and B cells is thought to play a central role in persistent joint inflammation (1). Therefore, several self antigens, such as type II collagen (CII), heat-shock protein 60, calpastatin, human cartilage glycoprotein 39, and glucose-6-phosphate isomerase, have been reported to be candidate target antigens of pathogenic autoantibodies in RA (2–6).

In an effort to find such antigens, we have cloned novel target proteins of autoantibodies from RA synovium with the use of RA synovial fluid IgG probes by an expression cloning method; one of these clones was follistatin-related protein (FRP) (7). FRP was originally isolated as the product of TSC-36, one of the genes found to be up-regulated by transforming growth factor β1 (TGFβ1) in the mouse osteoblastic cell line MC3T3-E1 (8). Rat and human homologs have been cloned from glioma cell lines (9).

FRP is a secreted glycoprotein, and it is expressed in all organs, except for peripheral blood leukocytes (7). FRP shares a characteristic structural module (the FS domain) with follistatin, an inhibitor of activin

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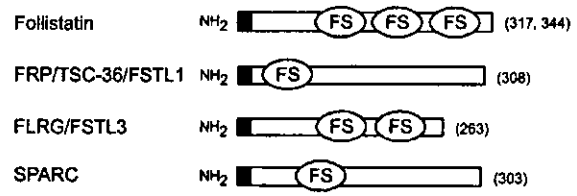

Figure 1. Schematic representation of follistatin and related FS domain–bearing proteins. Follistatin and follistatin-related gene protein (FLRG)/follistatin-like 3 (FSTL3) have more than 2 FS domains in the COOH terminus. Follistatin-related protein (FRP)/TSC-36/FSTL1 contains a single follistatin domain in the NH_2 terminus. Numbers in parentheses represent amino acid residues in the human proteins. Signal peptides are shown in the solid portions of the bars.

(10) (Figure 1). In addition to follistatin and FRP, other FS domain–bearing proteins, such as secreted protein, acidic and rich in cysteine (SPARC), SC1/hevin, testican, QR1, and follistatin-related gene protein (FLRG), have been cloned, and together, they compose the follistatin family (11). FLRG is a translated product of follistatin-related gene (*FLRG*), which was isolated from a B cell leukemia cell line (12). FRP and FLRG are different proteins (12,13) and are listed in the OMIM database under the names follistatin-like 1 (FSTL1) and follistatin-like 3 (FSTL3), respectively (13,14). Follistatin and FLRG have activin-binding activity and inhibit activin-mediated gene transcription (15). Other follistatin family proteins, including FRP, have not been demonstrated to bind activin or related TGFβ family proteins. SPARC, the best-studied of the group, is known to regulate cell–matrix interactions and thereby regulate tissue remodeling and homeostasis (11). FRP has been reported to play some roles in cell-cycle inhibition and to have negative regulatory effects on growth in human lung cancer cells (8,16). However, the exact physiologic function of FRP remains to be clarified.

We previously reported that serum autoantibodies to FRP were detected in 30% of patients with RA, and their appearance correlated with disease activity (7). Recently, our in vitro experiments demonstrated that FRP suppressed the production of matrix metalloproteinase 1 (MMP-1), MMP-3, and prostaglandin E_2 in synovial cells derived from patients with RA, and suggested that FRP could act as a joint defensive factor in RA (17).

To examine the in vivo effect of FRP on arthropathy in inflammatory joint diseases such as RA, recombinant FRP was administered in a mouse model of arthritis. In this experiment, we induced arthritis in mice with the use of anti-CII monoclonal antibodies and lipopolysaccharide (LPS) in order to achieve high reproducibility (18). We then examined the macroscopic and microscopic effects of FRP on arthritis. To obtain insight into the FRP signaling pathway, we analyzed the gene expression profiles of affected joint tissues using complementary DNA (cDNA) arrays.

MATERIALS AND METHODS

Recombinant human FRP. *Escherichia coli*–expressed glutathione S-transferase (GST)–FRP was prepared as previously described (7). The GST tag was removed from GST-FRP using a GSTrap FF column (Amersham Pharmacia Biotech, Uppsala, Sweden), and the protein was purified by an anion-exchange chromatography method with a Mono Q HR column (Amersham Pharmacia Biotech). Finally, the protein was filtered with a Zetapore Dispo filter (CUNO, Meriden, CT) to remove endotoxins.

Induction and evaluation of arthritis. Arthritis was induced in 7–8-week-old female BALB/c mice (Japan SLC, Hamamatsu, Japan) by intraperitoneal injection with 500 μl of phosphate buffered saline (PBS) containing 4 monoclonal antibodies to CII (500 μg of D1, D8, A2, and F10, respectively; Chondrex, Redmond, WA). After 72 hours, the mice were injected intraperitoneally with 100 μl of PBS containing 50 μg of LPS (Sigma, St. Louis, MO). The mice were maintained under specific pathogen-free conditions.

The severity of arthritis in each paw was estimated in a blinded manner. Paws were assessed by both visual inspection and measurement of limb swelling with calipers. Each paw was scored on a scale of 0–3, as previously described (19), where 0 = no swelling, 1 = swelling of finger joints or mild swelling of the ankle or wrist joint, 2 = severe inflammation of the entire paw, and 3 = deformity or ankylosis. The severity of arthritis in each mouse was the sum of the scores in the 4 paws (maximum possible score 12). Institutional approval of the bioethics of the study was obtained prior to these experiments (approval no. 02004).

Treatment with FRP. The mice were randomly assigned to 1 of 2 treatment groups before the onset of arthritis: FRP treatment or PBS treatment (control). The mice in the FRP treatment group were injected intraperitoneally with 20 μg (1 mg/kg) of recombinant human FRP daily for 10 days, starting 1 hour before injection of the monoclonal antibody. The mice in the PBS treatment group were injected intraperitoneally with 250 μl of PBS daily for 10 days.

Histopathologic evaluation. Pairs of arthritic hind paws from representative mice in each group (n = 6 animals, 12 paws) were removed at the end of the experiment (day 21), fixed in 10% buffered formalin, decalcified in 10% formic acid, and then embedded in paraffin. Serial sagittal sections of whole hind paws were subjected to hematoxylin and eosin staining for histologic analyses and to Safranin O and fast green/iron hematoxylin staining for estimation of the loss of cartilaginous proteoglycan. Synovial lesions were scored on a scale of 0–3, where 0 = normal synovium, 1 = mild synovial hyperplasia and/or mild synovial infiltration, 2 = moderate synovial hyperplasia and/or moderate synovial infiltration, and 3 = severe synovial hyperplasia and/or severe synovial infiltra-

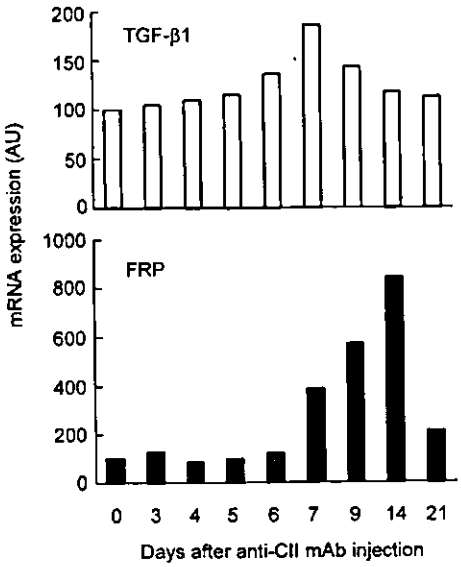

Figure 2. Changes in expression levels of transforming growth factor β1 (TGFβ1) and follistatin-related protein (FRP) mRNA during the development of arthritis. Levels of TGFβ1 and FRP mRNA expression (in arbitrary units [AU]) were quantified by real-time reverse transcription–polymerase chain reaction. Anti-CII = anti–type II collagen; mAb = monoclonal antibody.

tion. Cartilage depletion was identified by the presence of diminished Safranin O staining of the matrix and was scored on a scale of 0–3, where 0 = no cartilage destruction (full staining with Safranin O), 1 = localized cartilage erosions, 2 = more extended cartilage erosions, and 3 = severe cartilage erosions (depletion of entire cartilage). Histologic analyses were performed in a blinded manner.

Quantitative reverse transcription–polymerase chain reaction (RT-PCR) for TGFβ1 and FRP messenger RNA (mRNA) expression. Groups of mice were killed on days 0, 3, 4, 5, 6, 7, 9, 14, and 21, and total RNA was obtained from 6 arthritic hind paws in each group by mixing with TRIzol reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. Five micrograms of total RNA was subjected to RT using the SuperScript First Strand Synthesis system (Invitrogen, Carlsbad, CA). Each reaction was run in duplicate using SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The primer pairs used in these reactions were as follows: for TGFβ1, 5′-AGA-TCT-CCC-TCG-GAC-CTG-CT-3′ and 5′-GGG-TCT-CCC-AAG-GAA-AGG-TA-3′; for FRP, 5′-GGA-AGA-GAG-CGT-GAA-GGA-CA-3′ and 5′-CGG-AGG-CTC-AAA-GAA-AGG-AT-3′; and for GAPDH, 5′-CGC-TGA-GTA-CGT-CGT-GGA-GT-3′ and 5′-GGT-GCT-AAG-CAG-TTG-GTG-GT-3′. Amplification reactions, data acquisition, and analysis were performed with the ABI Prism 7900 HT instrument (PE Applied Biosystems), and relative levels of gene expression were normalized based on those of the GAPDH gene.

Urinary deoxypyridinoline levels. Mouse urine was collected on day 9, and urinary levels of deoxypyridinoline were measured with an Osteolinks DPD enzyme-linked immunosorbent assay kit (Metra Biosystems, Mountain View, CA).

Complementary DNA array analysis. Arthritic mice treated with FRP (20 μg/day) and untreated mice were killed on day 10, and total RNA was prepared from 3 hind paws by mixture in each group as described above. Moreover, mRNA was purified from the total RNA with a MagExtractor mRNA kit (Toyobo, Osaka, Japan). The Gene Navigator cDNA Array system (Toyobo) was used in this analysis. The cDNA array consisted of 561 species of immunologically related mouse cDNA fragments (20). (The Microsoft Excel file can be downloaded from http://www.toyobo.co.jp/seihin/xr/product/mouse%20immunology.xls) The biotin-labeled probes were prepared from the sample mRNA with biotin-16-dUTP with the Gene Navigator cDNA Amplification system (Toyobo). Signals produced by the hybridized probe–avidin–biotinylated alkaline phosphatase complex and substrate (Vistra ECF substrate; Amersham Pharmacia Biotech) were detected with the Imaging High Chemifluorescence detection kit (Toyobo). The chemifluorescence image of the cDNA array filter was analyzed using ImaGene software version 4.2 (Takara Bio, Otsu, Japan). The net signal intensity of genes was calculated from the measured raw signal intensity minus that of the background. Expression of individual genes was evaluated by their net signal intensity ratio compared with the standard housekeeping gene.

Confirmation of differentially expressed genes by quantitative RT-PCR. Expression levels of mRNA were estimated by real-time quantitative PCR using SYBR Green I. We obtained cDNA from the hind paws of FRP-treated (n = 3) and PBS-treated (n = 3) mice killed on day 10 as described above. Each reaction was run in triplicate using SYBR Green PCR Master Mix according to the manufacturer's protocol. The primer pairs used in these reactions were as follows: for c-Fos, 5′-CTA-TGC-AGC-AGA-CTG-GGA-GC-3′ and 5′-ACA-GCC-TGG-TGT-GTT-TCA-CG-3′; for Ets-2, 5′-CTT-CCA-AAA-GGA-GCA-ACG-AC-3′ and 5′-GTC-CTG-GCT-GAT-GGA-ACA-GT-3′; for interleukin-6 (IL-6), 5′-CAG-AGG-ATA-CCA-CTC-CCA-ACA-3′ and 5′-CCA-GTT-TGG-TAG-CAT-CCA-TC-3′; for MMP-3, 5′-CGA-TGG-ACA-GAG-GAT-GTC-AC-3′ and 5′-CCC-TCG-TAT-AGC-CCA-GAA-CT-3′; for MMP-9, 5′-ACT-TTC-CCT-TCA-CCT-TCG-AG-3′ and 5′-GAA-CAG-GCT-GTA-CCC-TTG-GT-3′; and for GAPDH, 5′-CGC-TGA-GTA-CGT-CGT-GGA-GT-3′ and 5′-GGT-GCT-AAG-CAG-TTG-GTG-GT-3′. The amplification reactions, data acquisition, and analysis were performed with the ABI Prism 7900 HT instrument, and relative levels of gene expression were normalized against those of the GAPDH gene.

Statistical analysis. Differences between experimental groups were tested using the Mann-Whitney U test and Student's unpaired *t*-test. *P* values less than 0.05 were considered significant.

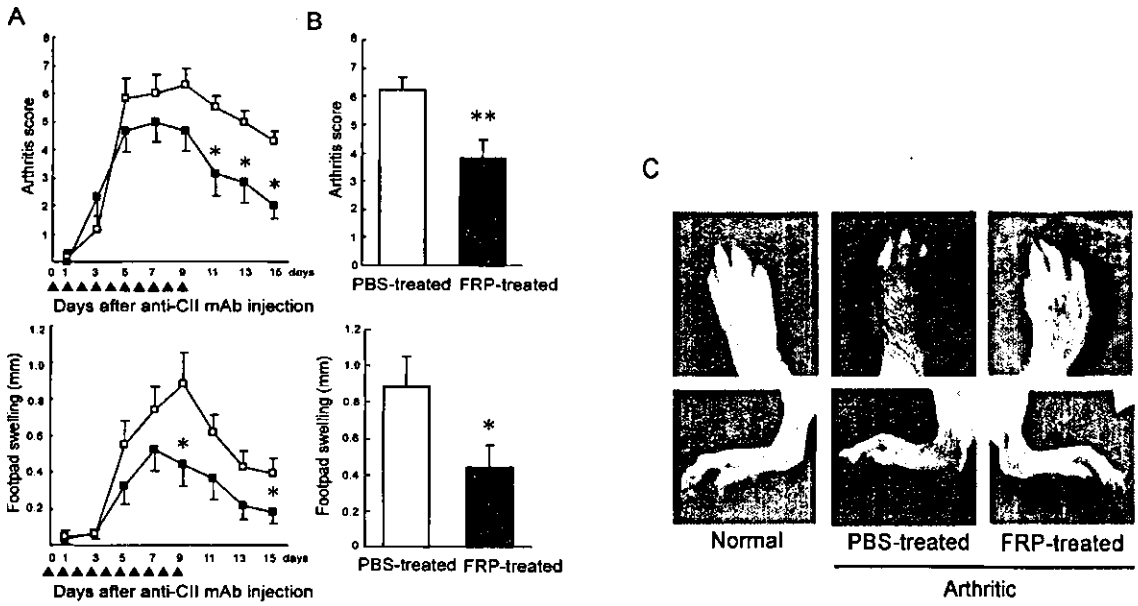

Figure 3. Amelioration of disease in arthritic mice treated with recombinant follistatin-related protein (FRP). **A,** Disease progress estimated by the arthritis score and footpad swelling (n = 6 mice per group). Anti–type II collagen (anti-CII) monoclonal antibodies (mAb) were injected on day 0, and 50 μg of lipopolysaccharide was injected on day 2. Recombinant FRP (■) or phosphate buffered saline (PBS; vehicle control) (□) was administered daily for 10 days, beginning on day 0 (arrowheads). **B,** The arthritis score and footpad swelling in both the PBS-treated (n = 12) and the FRP-treated (n = 11) groups were evaluated at the peak of arthritis (day 9). Bars show the mean and SEM. * = $P < 0.05$; ** = $P < 0.01$, by Mann-Whitney U test. **C,** Photographs showing representative paws of mice from each group on day 9.

RESULTS

First, to assess the regulation of FRP and its synovial inducer TGFβ1 in arthritis, we examined time course changes in their mRNA expression levels in arthritic paws from monoclonal antibody–injected mice (Figure 2). Depending on arthritis activity, both TGFβ1 and FRP were elevated temporarily. The elevation of TGFβ1 preceded up-regulation of FRP. Interestingly, after TGFβ1 began to decrease, FRP continued to rise for a time. Thus, some molecule besides TGFβ1 may regulate FRP expression.

To investigate the antiarthritic properties of FRP in vivo, we studied its effect on mouse arthritis using anti-CII antibodies and LPS. In this mouse model, severe arthritis occurs about 24 hours after LPS injection and persists for more than 3 weeks (18). As shown in Figure 3, FRP treatment demonstrated a significant ameliorative effect on the arthritis by reducing the clinical score and preventing the development of footpad swelling. On day 9, we could not detect a significant difference in the arthritis score in the small number of mice shown in Figure 3A (n = 6), but a significant difference was detected in the larger sample shown in Figure 3B (n = 11). Macroscopically, redness and swelling of the fore paws and hind paws were milder in the FRP-treated mice than in the PBS-treated mice (Figure 3C). The 10 daily 20-μg doses of FRP produced no significant change in body weight and no macroscopically abnormal findings in FRP-treated mice.

Tarsocrural joints from normal mice had no synovial inflammation or cartilage erosion (Figure 4a). Joints from PBS-treated mice with an arthritis score of 2 had severe synovial inflammation, cartilage erosion, and bone resorption (Figure 4b), while joints from FRP-treated mice with an arthritis score of 1 had mild synovial inflammation and no cartilage or bone erosion (Figure 4c). The weak staining by Safranin O in the articular cartilage of the PBS-treated mice indicated a loss of proteoglycans from the cartilage matrix (Figure 4e). In contrast, Safranin O staining of articular cartilage from FRP-treated mice was equivalent to that in cartilage from normal mice (Figures 4f and d). Histologic

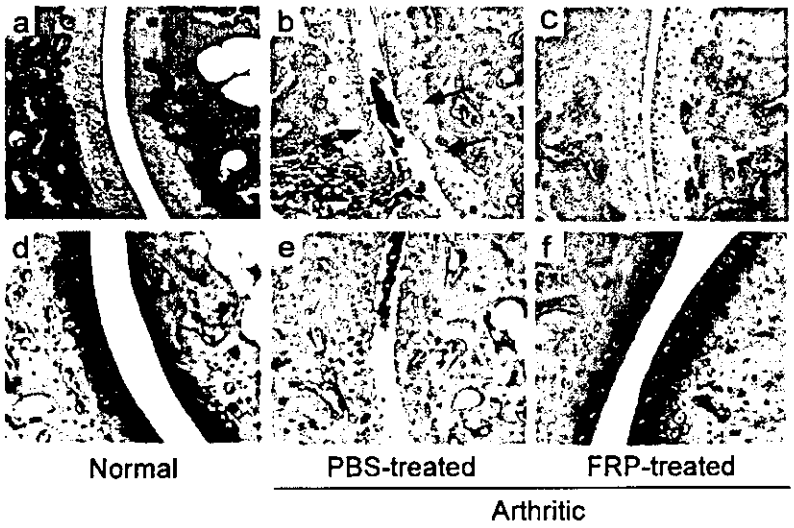

Figure 4. Histologic analysis of tarsocrural joints from normal and arthritic BALB/c mice. All specimens from normal, phosphate buffered saline (PBS)–treated, and follistatin-related protein (FRP)–treated mice were collected on day 21 after monoclonal antibody injection and stained with either hematoxylin and eosin (**a**, **b**, and **c**) or Safranin O (**d**, **e**, and **f**). **a** and **d**, Joints from normal mice exhibit normal histologic features, with intact articular cartilage and no evidence of inflammation or erosion. **b** and **e**, Affected joints from PBS-treated mice show severe synovitis, pannus formation (**p**), and cartilage/bone erosion (**arrows**). Absence of Safranin O staining indicates depletion of proteoglycans. **c** and **f**, In contrast, synovitis, cartilage/bone erosion, and loss of Safranin O staining of the cartilage were mild in FRP-treated arthritic mice. (Original magnification × 200.)

scores assessed on day 21 were significantly lower in the FRP-treated mice than in the PBS-treated mice. This confirmed the ameliorative effects of FRP on joint inflammation and cartilage destruction (Figure 5). Thus, FRP seemed to prevent cartilage breakdown in arthritis.

Because deoxypyridinoline is one of the common markers of bone resorption, we measured urinary levels of deoxypyridinoline on day 9 in arthritic mice treated with recombinant FRP or PBS. The urinary levels of deoxypyridinoline were markedly higher in the arthritic mice than in the normal mice. However, those in the FRP-treated arthritic mice were significantly lower than those in the PBS-treated mice (Figure 6). This suggested that FRP worked against the bone destruction in arthritis.

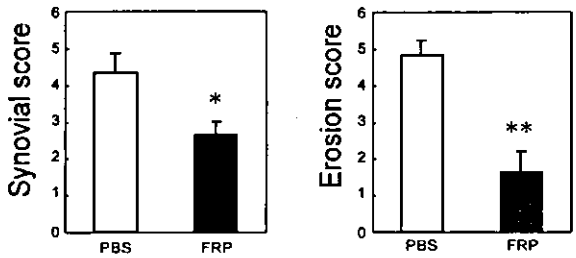

Figure 5. Comparison of histologic scores in phosphate buffered saline (PBS)–treated and follistatin-related protein (FRP)–treated mice on day 21. Bars show the mean and SEM (n = 6 mice per group). * = $P < 0.05$; ** = $P < 0.01$, by Mann-Whitney U test.

To identify the genes that are affected by FRP administration in this mouse model of arthritis, we examined the gene expression profiles by cDNA array techniques in joint tissues from normal, PBS-treated, and FRP-treated mice (n = 3 per group). We studied the expression of 561 genes blotted on the cDNA array, including 9 housekeeping genes and 2 negative control genes (see Materials and Methods). As a result, we found a change of >1.5-fold in the expression levels of 170 genes in the FRP-treated group versus the PBS-treated group. Five of these 170 genes (*c-fos*, *ets-2*, *IL6*, *MMP3*, and *MMP9*) were down-regulated in the FRP-treated group (Figure 7A); these genes have been

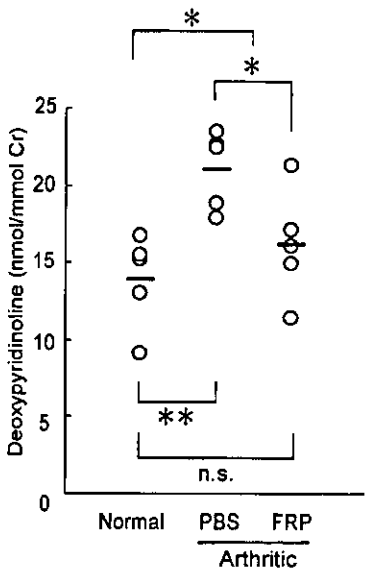

Figure 6. Urinary deoxypyridinoline levels in normal and arthritic mice. Urinary levels of deoxypyridinoline (expressed in nmoles/mmole of creatinine [Cr]) were significantly decreased in the follistatin-related protein (FRP)–treated arthritic mice compared with the phosphate buffered saline (PBS)–treated arthritic mice. Deoxypyridinoline levels were measured by enzyme-linked immunosorbent assay in samples collected on day 9 (n = 5 mice per group). * = $P < 0.05$; ** = $P < 0.01$, by Mann-Whitney U test. n.s. = not significant.

reported to be responsible for the destructive joint inflammation in RA (21–24).

To confirm whether the change in the expression of the 5 genes was in fact significant, we performed a real-time RT-PCR analysis using the same cDNA sources as those for the cDNA array. We found that the expression levels of the c-*fos*, *ets*-2, *IL6*, *MMP3*, and *MMP9* genes were significantly lower in paws from the FRP-treated mice than in the paws from the PBS-treated mice (Figure 7B). Therefore, FRP seemed to exert its preventive effect on the arthritis, in part, by down-regulating disease-promoting genes such as the 5 we identified.

DISCUSSION

In this study, we demonstrated that FRP had therapeutic effects on murine arthritis. Because of its rapid induction and high reproducibility, we used a mouse model of acute, destructive arthritis (resembling human RA) induced by anti-CII monoclonal antibodies and LPS. The expression of endogenous FRP and its synovial inducer TGFβ1 was higher in the arthritic paws of this model than in the normal paws. In addition, the expression of FRP was maintained at a high level even if the arthritis had peaked. A recent study also showed that the expression of FRP was increased at the synovium–bone erosion interface in early and late-phase collagen-

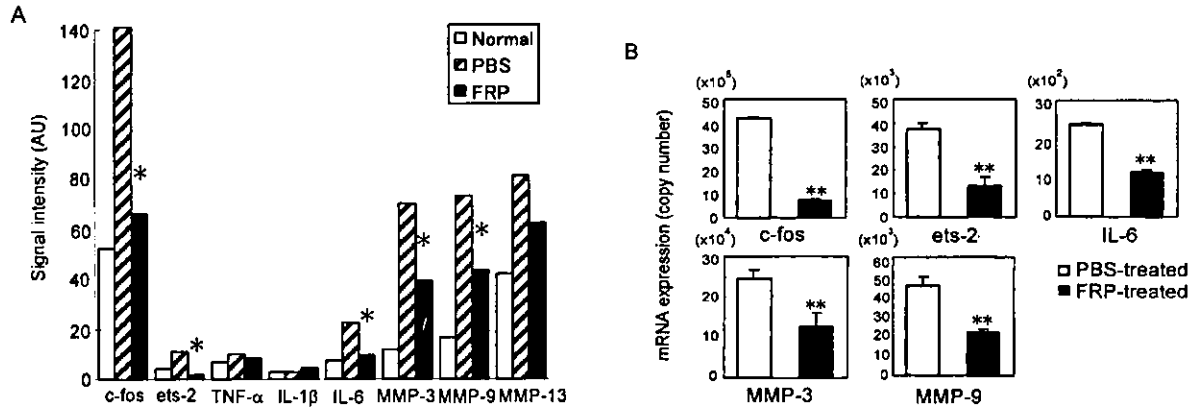

Figure 7. Gene expression in joint tissues of normal, phosphate buffered saline (PBS)–treated, and follistatin-related protein (FRP)–treated mice, as analyzed by cDNA arrays and real-time quantitative reverse transcription–polymerase chain reaction (RT-PCR). In both experiments, mRNA was extracted from joint tissues on day 10 of the experiment (n = 3 mice per group). **A,** The results of cDNA arrays analysis showed that the gene expression levels of c-*fos*, *ets*-2, *IL6*, *MMP3*, and *MMP9* were lower in the joints of FRP-treated mice than in the joints of PBS-treated mice. * = ratio of signal intensity (in arbitrary units [AU]) in PBS-treated mice to that in FRP-treated mice >1.5. TNF-α = tumor necrosis factor α. **B,** Real-time quantitative RT-PCR studies confirmed the significantly reduced levels of gene expression in FRP-treated mouse joints compared with PBS-treated mouse joints (control) for c-*fos*, *ets*-2, *IL6*, *MMP3*, and *MMP9*. ** = $P < 0.01$, by Student's unpaired *t*-test.

induced arthritis (25). Thus, we presumed that endogenous FRP was induced by TGFβ1 mainly from the synovium as the arthritis developed and may contribute to the regulation of arthritis. Our previous in vitro experiments suggested that FRP could prevent destructive arthritis (17). Therefore, we postulated that abundant exogenous FRP, in addition to feedback-regulated FRP, may improve arthritis.

FRP is a genetically highly conserved protein, having 92% amino acid sequence identity between humans and mice (9), and our previous in vitro study strongly suggested that recombinant human FRP may function in other species (17). Accordingly, we used recombinant human FRP for the present in vivo experiments. Treatment of mice with recombinant FRP just before the onset of arthritis brought about a significant improvement in the clinical parameters of arthritis (i.e., arthritis score and footpad swelling), indicating that FRP had preventive effects on joint inflammation. In addition, the striking benefit of FRP administration was the prevention of joint destruction. Postmortem histologic examination of tarsocrural joints from the FRP-treated mice revealed not only reduced synovial cellular infiltration, but also retained cartilage proteoglycan. Moreover, the reduction in urinary levels of deoxypyridinoline, one of the metabolic markers of bone resorption in RA (26), confirmed that FRP protected joints against destructive arthritis by preventing cartilage degradation and bone resorption. Therefore, FRP has antidestructive as well as antiinflammatory effects, and therapeutic potential in inflammatory joint diseases such as RA.

FRP is considered to be an orphan molecule because its receptor has not yet been identified, and there has been no information about the signaling system of FRP. Thus, to investigate the molecular mechanisms through which FRP treatment ameliorated murine arthritis, we compared the gene expression profiles in FRP-treated and PBS (vehicle)–treated joints. Among 170 genes with significant changes in expression levels, 5 down-regulated genes, c-*fos*, *ets*-2, *IL6*, *MMP3*, and *MMP9*, have been reported to be closely associated with immune response and joint destruction in inflammatory arthritis. Their reduced expression in FRP-treated mice was confirmed by a real-time quantitative RT-PCR method.

The expression of *IL6* was significantly reduced in joints from FRP-treated mice, suggesting that inhibition of IL-6 could be one of the reasons for the antiinflammatory effects of FRP. IL-6 is thought to be one of the proinflammatory cytokines responsible for disease progression in RA, together with TNFα and IL-1 (23). Thus, FRP could be expected to have beneficial effects on joint inflammation in other animal models of arthritis and still greater effects in human diseases such as RA. However, a recent study indicated that in this mouse model of arthritis induced by anti-CII monoclonal antibodies and LPS, IL-6 was not as responsible for the progression of arthritis as TNFα and IL-1 were (27). There may be other mechanisms by which FRP ameliorates joint inflammation than by antagonism of IL-6.

The inhibitory effect of FRP on *MMP3* and *MMP9* was notable because these MMPs are thought to play a crucial role in the joint destruction of RA, where they are major direct effectors that degrade the extracellular matrix components of the joint structure, such as collagen and proteoglycan (24). Serum levels of MMP-3 in particular have been reported to be correlated with joint damage in RA (28). Synovial down-regulation of MMP-3 by FRP was also observed in our previous in vitro experiments (17). Gene expression of these MMPs is strongly up-regulated by proinflammatory cytokines, such as TNFα and IL-1, in the RA synovium (24), and it is considered that conditions in which the activities of the MMPs overwhelm the activities of the tissue inhibitors of metalloproteinases and MMP inhibitors, the joint destructive process is facilitated, as in RA (29). For these reasons, inhibition of MMPs has been considered as a new therapeutic approach to rheumatoid joint destruction (24). It has been reported that specific inhibition of individual MMPs has almost no effect in the treatment of murine arthritis, possibly due to another MMP compensation (30), but broad-spectrum MMP inhibition was shown to successfully reduce the clinical severity and cartilage destruction in a mouse model of RA (31,32). In our study, FRP inhibited the expression of *MMP3*, *MMP9*, and *MMP13*. The decrease ratio was significant in *MMP3* and *MMP9* (>1.5), but slight in *MMP13* (<1.5). Therefore, we hypothesized that one of the major mechanisms by which FRP prevented joint destruction was the down-regulation of several MMPs, such as MMP-3 and MMP-9.

How does FRP regulate MMP gene transcription? We found that 2 transcription factor genes, c-*fos* and *ets*-2, were down-regulated in FRP-treated joints. Their expression has been associated with a proliferative role in many tissues (21,33). It has been reported that c-*fos* and *ets*-2 expression was higher in rheumatoid synovial tissue than that in normal synovial tissue, suggesting that they may play an important role in the progression of RA (22,34,35). The nuclear oncogene c-*fos* and its product c-Fos bind to activator protein 1

sites on matrix-degrading genes, such as *MMP3* and *MMP9*. The gene *ets*-2 is one of the Ets family of transcription factors that binds to polyomavirus enhancer 3 (PEA-3) sites (36). Genes involved in the degradation of the extracellular matrix, such as *MMP3* and *MMP9*, contain PEA-3 sites in their regulatory sequences, and the binding of Ets-2 to PEA-3 sites accelerates their transcription (37). Moreover, Ets-2 cooperates with other transcription factors, including c-Fos, to stimulate the transcription of matrix-degrading proteases (37). Therefore, it is likely that FRP negatively regulates c-*fos* and *ets*-2 gene expression and, as a result, the transcription of *MMP3* and *MMP9* is suppressed.

In this series of experiments, for the limited amount of available recombinant FRP and the relatively short disease duration in this mouse model of arthritis, we performed preventive therapy for the arthritis by administering FRP just before the onset of disease. However, for further estimation of the therapeutic potential of FRP in arthritis, another series of experiments conducted according to the controlled curative therapy protocol will be required, involving the administration of FRP and control drugs during disease progression in other mouse models of chronic arthritis.

In conclusion, we demonstrated that the progression of murine arthritis can be inhibited by recombinant FRP treatment. Its beneficial effect was the prevention of joint destruction, which seemed to be mainly due to the reduction in MMP activities at the transcription level. In inflammatory joint diseases such as RA, FRP may be one of the key molecules that prevents the disease progression and a candidate as a new therapeutic biologic agent that may block the disease process in a manner different from that of TNFα, IL-1, and IL-6 antagonists.

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RAのMRI
－滑膜炎と骨変化の評価－

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Key words：rheumatoid arthritis, synovitis, erosion, bone marrow edema, early diagnosis, prognosis

はじめに

関節リウマチ(RA)を始めとする関節炎では滑膜炎が早期から認められるが，造影剤静注後のMRIでは滑膜炎の部位が造影効果を有する領域として明瞭に描出され，RAの早期診断や活動性判定に対する有用性が期待されている。また，造影剤急速静注後のdynamic studyは組織の血流増加を反映し，滑膜炎の活動性判定に有用であることがわかってきた。さらにX線写真で認識できない骨変化がMRIで捉えられることが明らかにされ，関節破壊や機能的予後との関係も注目されている。しかし現時点では，検査費用に見合った診断の有用性，あるいは検査結果が治療に及ぼす効果についての臨床的エビデンスは十分なものでない。本稿ではわれわれの行っている研究成果を紹介しつつ，RAの早期診断におけるMRIの有用性と限界について述べる。

MRIの基本的撮像法

MRIを活用するには撮像法についての基本的事項を知っておくことが望ましい。本稿を理解するために必要な撮像法について簡単にまとめる。

● T1強調像とT2強調像

撮像パルス系列の設定により，水(自由水)を低信号として描出するT1強調像，水を高信号として描出するT2強調像という異なった画像が得られる。多くの病変は自由水を多く含むため，T1強調像で低信号，T2強調像で高信号となる。

● 脂肪抑制画像

脂肪はいずれの画像でも高信号として描出されるが，本画像は，この信号を抑制して低信号として描出する方法である。T2強調像で病変と脂肪のコントラストを得たいとき(骨髄の病変など)，造影MRIにおける造影効果を明瞭にしたいときに用いられる(**図1**)。

選択的脂肪抑制法とSTIR (short time inversion

MR imaging of rheumatoid arthritis－Evaluation of synovitis and bone changes－

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recovery)法の2つに大別される。選択的脂肪抑制法は脂肪からの信号を特異的に抑制する方法であるが，低磁場の装置では脂肪抑制が得られにくく，磁場の不均一性に影響されやすい欠点がある。STIR法は低磁場の装置でも均一な脂肪抑制が得られやすいが，脂肪以外のT1強調像で高信号を示すもの(造影剤によるエンハンス効果，出血など)も信号が抑制されるために造影MRIには用いられない。

● 造影MRI

MRIの造影剤(Gd製剤)は静注で用いられ，造影効果のある部位がT1強調像において高信号として描出され，前述したように脂肪抑制法を併用することで明瞭にすることができる。造影効果は造影剤の濃度に比例し，組織の血流および血管の浸透性(血管内から組織への造影剤の移行度)を反映する。dynamic studyは，造影剤急速静注後の造影効果を経時的に観察する方法で，組織の血流を評価することが可能である。

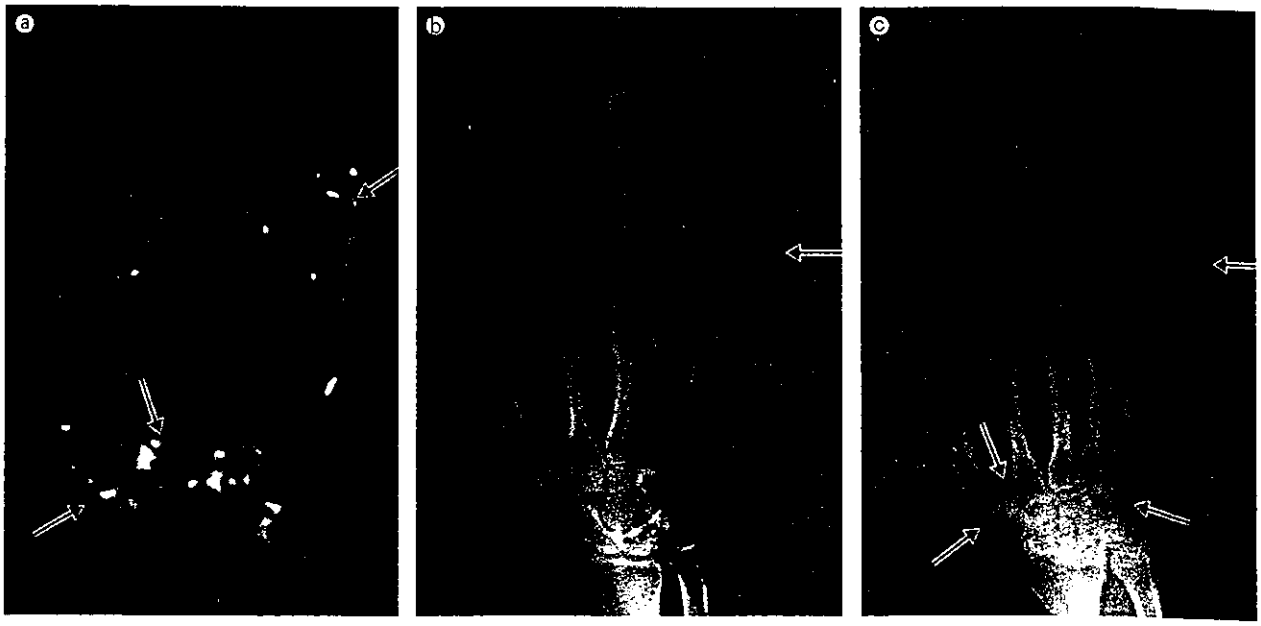
MRIによる関節病変の描出

● 滑膜炎

MRIは滑膜炎に伴う滑膜肥厚や関節炎を描出するのに優れ，肥厚した滑膜がT1強調像で低信号，T2強調像で低～高信号として描出される。T2強調像における信号の違いは線維化や滑膜の炎症の程度に左右され，線維化の強い慢性滑膜炎では比較的低信号に描出されることが多い。これに対して早期の滑膜炎で線維化の少ないものでは比較的高信号に描出され，通常のT1・T2強調像で関節液と区別がつかないことがあるが，造影MRIでは造影された滑膜と造影効果のない関節液を明瞭に区別することができる。とくに脂肪抑制法を併用することが望ましい[1] (図1)。ただし，造影剤は滑膜から次第に関節液に移行してくるため，造影剤投与後5分以内に撮像を行う必要がある[2]。

正常関節にも軽度の造影効果を認めることがあ

図1　早期のRAにおける滑膜炎の描出と単純写真の経過
38歳，女性。
ⓐ：初診時の手関節造影MRI(脂肪抑制併用)。
矢印で示した部位に滑膜炎を示す造影効果が認められる。
ⓑ：ほぼ同時期の単純X線写真。
第5指のPIP関節の狭小化が認められるが(矢印)，このほかには異常は指摘できない。
ⓒ：約1.5年後の単純X線写真。
手根関節にも関節裂隙狭小化や骨侵食が出現しており(矢印)，この範囲は初診時の滑膜炎の範囲にほぼ一致している。



る。Ejbjergらの検討では，健常者の112中手指節(MP)関節のうち10関節(8.9%)，84手根関節のうち8関節(9.5%)に滑膜炎様の軽度の造影効果がみられた[3]。正常関節における造影効果の程度は弱く，dynamic studyにおける造影効果の立ち上がり速度は遅い。Ejbjergらによると，造影剤投与後60秒以内で造影前値の標準誤差の2倍を超える造影効果を認めることはなく，dynamic studyが正常と異常の区別に有用であることが示唆された。

● 腱鞘炎，滑液包炎

腱鞘炎(腱鞘滑膜炎)は，腱鞘内の液体貯留あるいは腱鞘滑膜の肥厚として描出される。肥厚した腱鞘滑膜の描出には造影MRIが有用である(図2)。われわれの造影MRIによる検討では早期RAにおける腱鞘炎の頻度はきわめて高く，RA患者の27例の53手関節のうち，初診時の検査で49関節(92%)に腱鞘炎を認めた。また従来より伸筋腱の腱鞘炎が多いと報告されているが，伸筋腱よりも屈筋腱の腱鞘炎の頻度が高かった(伸筋腱15%，屈筋腱75%，両方15%)。また，関節周囲の滑液包にもしばしば滑液包炎をきたし，滑膜肥厚や液体貯留を認める。

● 骨侵食および骨髄浮腫

単純X線写真でみられる骨変化としては，関節周囲の骨減少(periarticular osteopenia)や骨侵食像(erosion)がある。関節周囲の骨減少は比較的早期から認められる変化であるが，特異性に乏しい。X線写真における骨侵食像は進行例に認められ，早期診断における意義は少ない。

MRIにおける骨侵食は骨皮質欠損およびその近傍の骨髄における限局性の異常信号(T1強調像で低信号，T2強調像で等～高信号，STIRで高信号)で，造影MRIで造影効果がみられる(図3)。MRIは単純X線写真よりも早期から骨侵食を描出できることが報告されている[4)~7)]。McQueenらによると，MRIで描出された骨侵食のうち1年後および2年後の単純X線写真で認められた骨浸食はそれぞれ21% と26% であった[8]。逆にMRI上で骨侵食が認められない例でX線上の骨浸食が出現する頻度は少ない(negative predictive value 0.91)。しかし，X線上の骨侵食とMRI上の骨侵食が同じ病変かどうかについては議論のあるところであり，今後の検討が必要である。

骨髄浮腫は単純X線では認識できず，MRIで初めて認められる変化である。MRIではT1強調像で低信号，T2強調像で等～高信号を示し，STIR法で高信号を示す境界不明瞭な異常信号として認められ，造影効果を示す(図4)。これは骨髄内

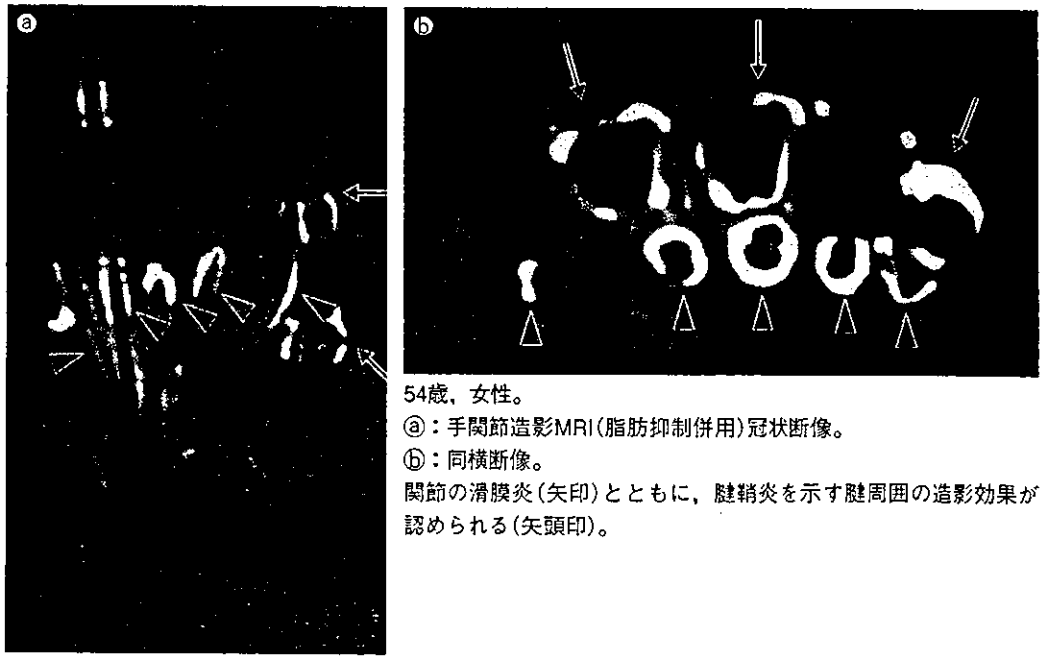


図2 RAにおける滑膜炎と腱鞘炎

54歳，女性。
ⓐ：手関節造影MRI(脂肪抑制併用)冠状断像。
ⓑ：同横断像。
関節の滑膜炎(矢印)とともに，腱鞘炎を示す腱周囲の造影効果が認められる(矢頭印)。

図3　単純X線写真よりも早期にみられるMRIの骨侵食像
57歳，女性。
ⓐ：初診時の手関節MRI T1強調像。
第5指の中手骨頭に骨侵食像を認める(矢印)。
ⓑ：ほぼ同時期の単純X線写真。
異常は指摘できない。
ⓒ：約4年後の単純X線写真。
中手骨頭の骨侵食が明らかで(矢印)，関節裂隙狭小化も出現している。

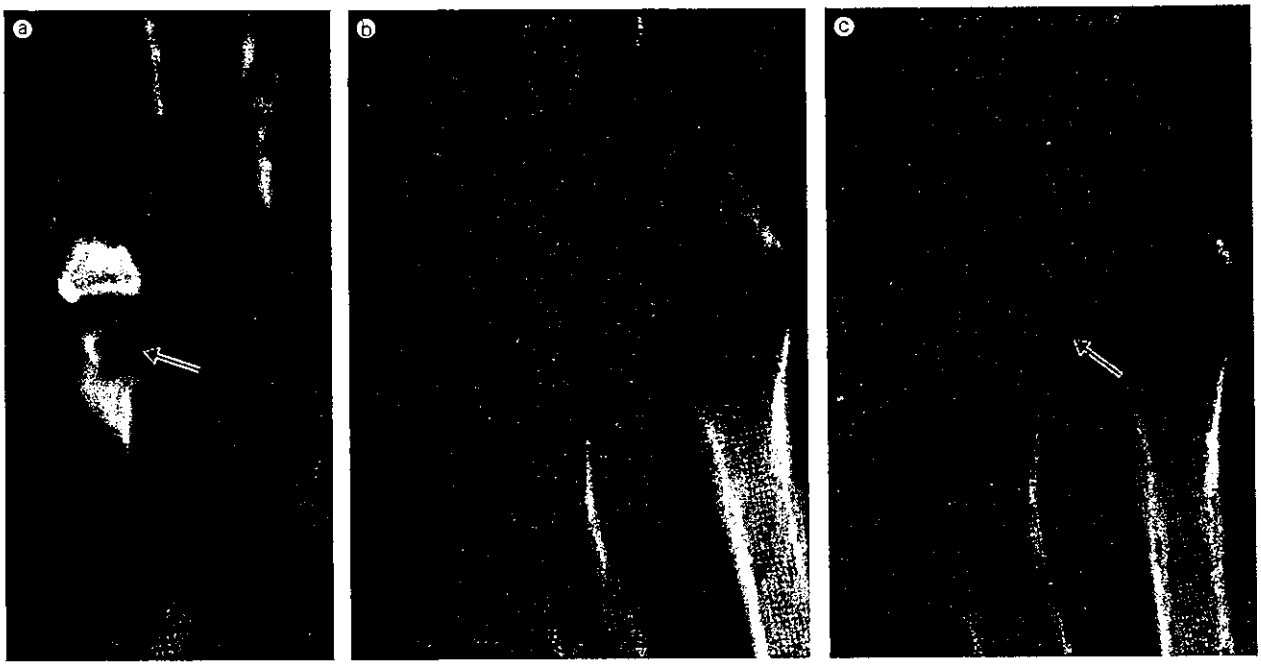


図4　滑膜炎の増強と骨髄浮腫
59歳，女性。
ⓐ：初診時の膝造影MRI(脂肪抑制併用)。滑膜炎を示す造影効果がみられる(矢印)。
ⓑ：4カ月後の造影MRI(脂肪抑制併用)。臨床所見で悪化あり。このときのMRIでは滑膜の造影効果の増強がみられ，骨髄に浮腫性変化が出現している(矢頭印)。