図7　軟骨周囲にみられる破軟骨細胞

破軟骨細胞は破骨細胞に類似した多核巨細胞である。破骨細胞はよく知られているが，破軟骨細胞の存在はあまり知られていない。

（筆者ら提供）

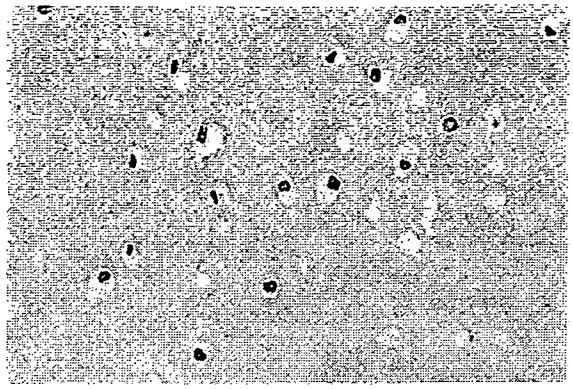

図8　RAの軟骨に発現するNO

変性しつつある軟骨の軟骨細胞には，NOの発現が認められる。

NO：nitric oxide，RA：関節リウマチ

（文献13より）

2．軟骨の変性

軟骨の変性，破壊には2つの様式がある。一つはパンヌスによる吸収であり，この中には浸潤してくる炎症性細胞およびFLCから産生されるタンパク分解酵素による変性，あるいは破軟骨細胞による吸収などがあげられる（図7）。特に前者のタンパク分解酵素には多くの種類の酵素があげられている。最近注目されているのはMMP，中でもMMP-3であるが，カテプシンL（Cat L）やカテプシンK（Cat K）などもかなり強力なタンパク分解酵素である。

また，軟骨の分解，変性に関与するもう一つの大きな要素は，軟骨自らがnitric oxide（NO）やタンパク分解酵素などを発現して，基質を分解しながらアポトーシスに陥り自滅していくことである（図8，9）[15][16]。しかし，なぜ基質に囲まれた軟骨細胞に酵素の発現やNOの発現がみられるようになるのか，その機序は明らかにされていない。また，軟骨細胞が未分化な間葉系細胞，線維芽細胞にtransformしていることを示す変化もみられる。

タンパク分解酵素については，周囲の肉芽組織のみにみられる酵素，軟骨細胞のみに発現される酵素，そして肉芽組織と軟骨細胞いずれにも産生される酵素がそれぞれ存在することが明らかになった。例えば，Cat Lなどは周囲の肉芽組織と軟骨細胞自身にみられるが，MMP-9は破軟骨細胞や単球系細胞には発現するものの軟骨細胞自身に発現することはない[17]。このように，軟骨組織は関節液，周囲のパンヌス，軟骨細胞自らの自滅によって吸収が進行していく。

軟骨基質に関しても，破壊されるというよりは吸収される形で失われていく。それを示すマーカーにはヒアルロン酸，Ⅱ型コラーゲン，コンドロイチン硫酸などがあげられているが[18][19]，我々はヒアルロン酸が軟骨の破壊された結果ではなく，破壊されつつある際の有力なマーカーになり得ることを証明している[20][21]。

骨吸収への進展

骨破壊というと一部では骨が壊されると思われているようであるが，実際は骨梁が細くなる吸収

Cat K：カテプシンK，Cat L：カテプシンL，NO：nitric oxid

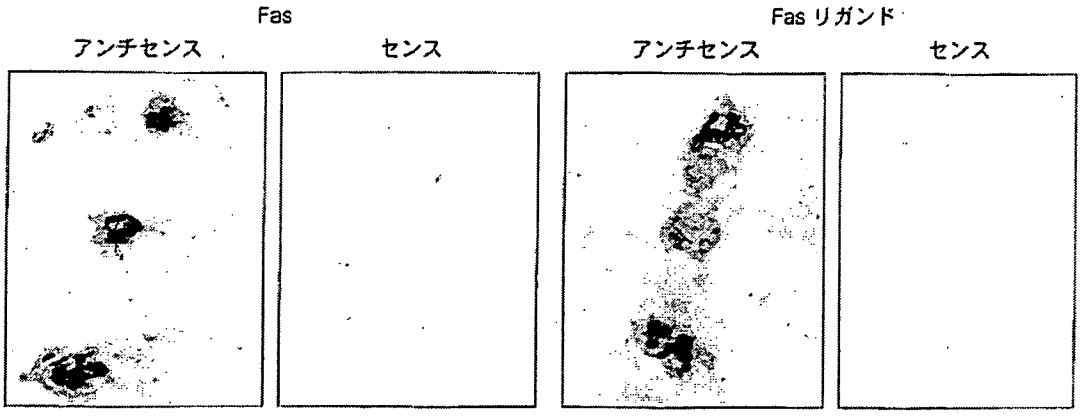

図9　RAの軟骨に発現するアポトーシス関連分子

変性しつつある軟骨細胞には，細胞にアポトーシス（細胞死）のシグナルを伝える際に重要とされるFasおよびFasリガンドが発現し，アポトーシスに至る。
RA：関節リウマチ

（筆者ら提供）

の形をとる。骨の吸収に関しては破骨細胞のほかに単球，FLCなどの関与も考えられるが，このほか，炎症に伴う閉塞性動脈炎など循環障害の影響もある（図10）。

パンヌスによる骨吸収は，ほとんど軟骨の吸収に連続した形で進行する。骨吸収部では破骨細胞が多数，骨縁に沿ってみられるが，破骨細胞のマーカーである酒石酸抵抗性酸ホスファターゼ（TRAP）やマクロファージマーカー（CD68）などで検討すると，破骨細胞以外にも多くの単球系細胞が陽性になっており，前破骨細胞であることを示唆している。

破骨細胞の周囲では水素イオン（H^+）による酸性プロテアーゼの活性化も作用している。破骨細胞の胞体内にはH^+を産生するcarbonic anhydrase Ⅱ（CAⅡ）が証明され，さらに産生されたH^+を胞体外にくみ出すプロトンポンプの液胞型アデノシントリホスファターゼ（V-ATPase）が同一細胞で証明されている。また，酸性下で初めて

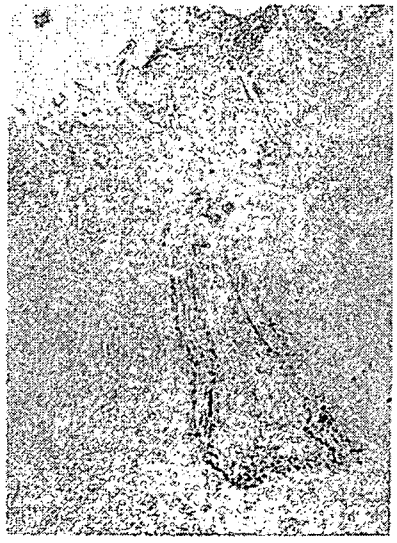

図10　RAにみられる骨壊死と周囲の動脈の閉塞

RAでは動脈の閉塞という循環障害によって骨壊死が起こる。これはあまり注目されていない所見であるが，骨の変性には循環障害も大きな影響を与える。
RA：関節リウマチ

（筆者ら提供）

CAⅡ：carbonic anhydrase Ⅱ，H^+：水素イオン，TRAP：酒石酸抵抗性酸ホスファターゼ，V-ATPase：液胞型アデノシントリホスファターゼ

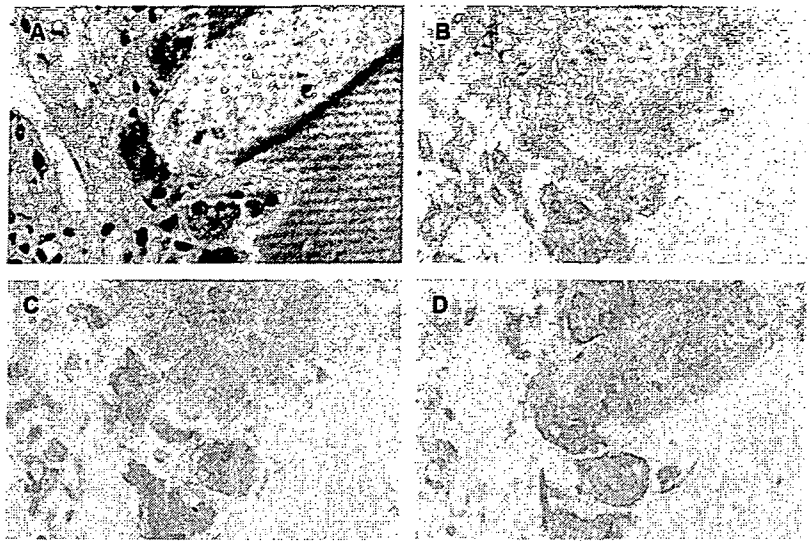

図 11　破骨細胞にみられる酸性プロテアーゼの活性機構

A：HE，B：Cat D，C：CAⅡ，D：V-ATPase

吸収骨の周囲にみられる破骨細胞では，CAⅡによりプロトンを産生し，V-ATPaseによりプロトンが細胞外に放出され，Cat Dのような酸性プロテアーゼが活性化される。破骨細胞周囲はかなり酸性であると考えられる。

CAⅡ：carbonic anhydrase Ⅱ，Cat D：カテプシンD，HE：ヘマトキシリン-エオジン染色，V-ATPase：液胞型アデノシントリホスファターゼ

（文献 22 より）

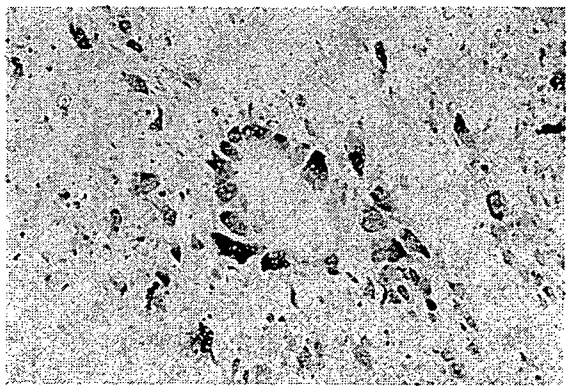

図 12　破骨細胞が産生する MMP-9

破骨細胞の周囲は酸性環境であるとされているが，MMP-9のような中性プロテアーゼも産生，放出される。MMPは完全に中性でなくても活性を示すという報告がある。

MMP：マトリックスメタロプロテアーゼ

（筆者ら提供）

活性化されるCat L，Cat Dなどの酸性プロテアーゼも同様の分布を示すことが知られている（図 11）[22,23]。

破骨細胞周囲はこのように酸性化されているが，同細胞はMMP-9などの中性プロテアーゼも産生しており（図 12）[24]，中性プロテアーゼが酸性環境下でも活性化され得ることが示唆される。また，パンヌスを電子顕微鏡で観察すると，破骨細胞のほかに紡錘形をした細胞が認められるが，これらはHLA-DR陽性であるだけでなく，さまざまな酵素を産生，分泌している。

骨基質の面からみた骨破壊の様式は，カルシウム，リンが同じように減少して骨粗鬆症の形をとり，骨梁はもろくなる。RAの骨は，手で押すと簡単に崩れそうなほど柔らかいものであるが，組織学的には骨粗鬆症の形をとる。

このようにしてRA関節の骨は吸収されるが，

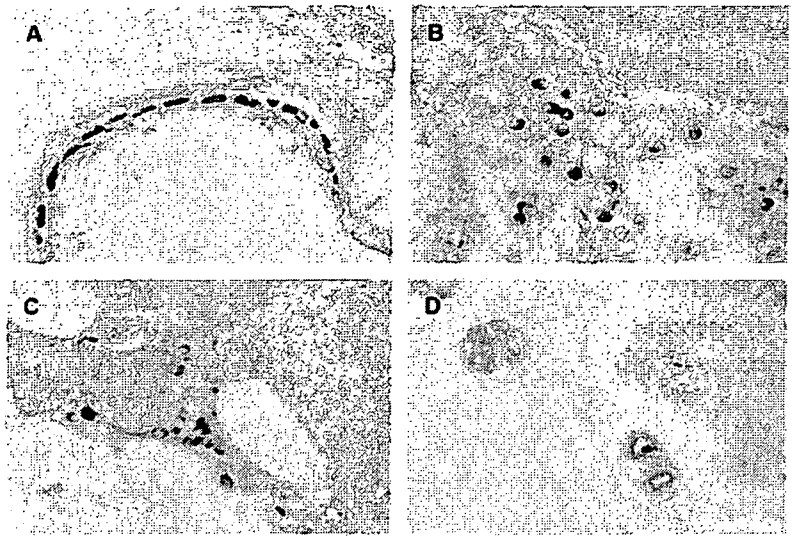

図13　骨周囲にみられるRANKLの発現

A：骨芽細胞，B：リンパ球，C：Mφ，D：軟骨細胞

破骨細胞の分化，誘導を惹き起こすRANKLは，従来考えられていた骨芽細胞だけでなく，リンパ球，マクロファージ，軟骨細胞でも発現しており，破骨細胞の分化誘導以外の機能も有していることが示唆される。

RANKL：receptor activator of NF-κB ligand

（筆者ら提供）

膝関節などでは関節面の軟骨が消失して関節腔が消失し，骨面が直接摺り合うようになると骨には間葉細胞の反応性巣状増殖あるいは線維性軟骨からなるfibrocartilagenous plugを形成するようになる。一方，炎症が長期化すると，靱帯の付着部に嚢胞を形成したり[25]，軟骨・骨の吸収に加えて，靱帯の断裂や関節包の線維化などによる無理な外力が作用することによって，関節は大きく変形して運動の機能障害を惹き起こす。また，軟骨が消失したために二次的変化としての変形性関節症（OA）変化が進行して疼痛に結びつく。

軟骨・骨破壊と滑膜の炎症との関係

RAの治療では，何を標的にして関節の変形を防ぐのが最も有効であろうか。これまでみてきた中で，RAの根本的な原因に対する治療は別として，破骨細胞の分化誘導も含めて機能を抑えることがターゲットになり得るものと思われる。また，破骨細胞の分化誘導因子，タンパク分解酵素の合成を促進させる因子，分解酵素の分泌や活性の因子などを個々に抑制するべきかということも問題になる。

破骨細胞の分化誘導因子としては，receptor activator of NF-κB ligand（RANKL）（図13）あるいはさまざまなサイトカインが知られているが，その中で最近最も注目されているのは，tumor necrosis factor（TNF）-αである。これらの因子は破骨細胞だけではなく，さまざまな細胞で産生されており[17]，サイトカインの刺激によってタンパク分解酵素が誘導され，軟骨細胞自体のアポトーシスが惹き起こされる可能性もある[17]。一方，TNF-α単独での細胞障害性はなく，他の因

OA：変形性関節症，RANKL：receptor activator of NF-κB ligand，TNF：tumor necrosis factor

子と協調してアポトーシスを誘導するという報告もあり，TNF-αはアポトーシスの必要条件であっても十分条件ではない可能性もあり，今後の検討課題である[26]。

骨吸収は破骨細胞だけで行われるものではないが，RAの組織を観察するとOAなどの非RA例に比較してFLCの増殖が特徴的であり，このFLCがRAの骨吸収に関与しているのではないかと思われる。一般的に，RAを代表する滑膜の組織像というとリンパ濾胞の形成や滑膜の絨毛状増生がよく知られているが，筆者らは，RAの最も大きな特徴はFLCの増殖であろうと考えている。この細胞は紡錘形で線維芽細胞様でもあるが，電子顕微鏡で観察するとライソゾームを保有しており，Cat Lなどのタンパク分解酵素を産生する一方で，MMP-9などの非ライソゾーム型のタンパク分解酵素も認められており[27]，この細胞が直接あるいは間接的に骨破壊に関与していることは間違いない。

このFLCは骨髄にあるCD34陽性のnurse cell由来であり，それがFLCとなり[28][29]，さらに骨破壊に関与するのではないかという報告もある。もしそうであれば，さまざまなサイトカインやタンパク分解酵素と同時に，この細胞の活性を抑制することがRAの治療につながるものと思われる。

RAの炎症は，免疫異常を基礎とした炎症と非特異的炎症の2つに特徴づけられる。極論すると，前者を代表する炎症性細胞はリンパ球を中心としたものであり，後者は顆粒球と言えるが，後者の肉芽組織が骨吸収につながるのではないかと思われる。この非特異的な肉芽組織の活性を抑え，ここに含まれるサイトカイン，タンパク分解酵素の産生や活性を抑えることが骨吸収の抑制に繋がるのではないかと思われる。

おわりに

以上，病理組織学からみて軟骨・骨破壊を取り上げてきたが，現在，未だにはっきりしていないのが滑膜の炎症から軟骨・骨吸収に至る過程である。この点についてはFLCを中心とした詳細な分析が今後の課題と言える。

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Downregulation of inhibitor of apoptosis proteins in apoptotic human chondrocytes treated with tumor necrosis factor-alpha and actinomycin D

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Summary

Objective: Apoptosis of chondrocytes plays a pivotal role in cartilage degeneration. Tumor necrosis factor-alpha (TNF-α) is a proinflammatory cytokine and has been assumed to cause the degradation of human cartilage. To investigate the mechanisms of TNF-α-mediated apoptosis of human chondrocytes from a point of view of the balance between the caspase-cascade and the expression of inhibitor of apoptosis proteins (IAPs), although both of them are induced with TNF-signals.

Methods: The expression of TNF-receptors (TNF-Rs) in normal human articular chondrocyte (NHAC-kn) was examined with immunocytochemistry. Subconfluent cultures of NHAC-kn were tested with TNF-α and/or actinomycin D (actD), and the induction of apoptosis was evaluated by the frequency of apoptotic cells visualized with nuclear staining using Hoechst 33342. The activation of caspases and the expression of IAPs were examined with Western blot analyses.

Results: NHAC-kn expressed TNF-R1 and -R2. When NHAC-kn was treated with TNF-α (10 ng/ml) and actD (0.2 μg/ml) for 24 h, the frequency of apoptotic cells increased to more than 25%. TNF-α alone, however, induced the apoptosis insufficiently (up to 8.3%), even when used at the concentration of 100 ng/ml for 48 h. In apoptotic human chondrocytes induced with TNF-α (10 ng/ml) and actD (0.2 μg/ml), the caspase-3, -8, and -9 were activated and the protein expression of XIAP and c-IAP1 decreased.

Conclusions: In apoptotic human chondrocytes induced with TNF-α and actD, the balance between caspase activation and IAPs' expression lay with the executioner caspase (caspase-3) and led to decreased expression of XIAP and c-IAP1.

Key words: Chondrocyte, Apoptosis, Tumor necrosis factor-alpha (TNF-α), Actinomycin D, Inhibitor of apoptosis proteins.

Introduction

Apoptosis of chondrocytes plays a pivotal role in cartilage degeneration. The apoptosis of chondrocytes is observed in the cartilage of patients of rheumatoid arthritis (RA) and osteoarthritis (OA)[1,2]. Various stimuli such as nitric oxide (NO) and Fas-signals induce the apoptosis in chondrocytes[3,4]. Proinflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α) have been assumed to cause the degradation of human cartilage[5,6]. TNF-α is a multifunctional cytokine that initiates a wide variety of biological responses, and as one of these functions, may play a role in the course of apoptosis of chondrocytes. Nowadays, several anti-TNF-α therapies have been reported to be effective on RA[7,8]. Although there are a few reports describing TNF-α-mediated apoptosis of human chondrocyte, the detailed signaling mechanisms are still unknown[9–12].

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Through binding to the major TNF-receptor (TNF-R), TNF-R1, TNF-α activates two intracellular signaling pathways. One is apoptosis pathway involving caspase-cascade, and the other is anti-apoptosis survival signals, an opposite function, mediated by nuclear factor-κB (NF-κB)[13,14]. As the outcome of opposite signals, many cellular lineages, including chondrocyte, are resistant to programmed cell death mediated by TNF superfamily, including TNF-α, Fas, and TNF-related apoptosis-inducing ligand (TRAIL)[9–12,15–18]. These cells, however, undergo apoptosis in the presence of actinomycin D (actD), which inhibits DNA-dependent RNA polymerases[10,12,15–18]. At low concentration (less than 0.1 μg/ml) actD induces the segregation of the nucleolar components and impairs r-RNA synthesis, and the treated cells display the early apoptosis features. When used at high concentration (more than 1 μg/ml), on the other hand, actD induces massive apoptosis[19]. In many cellular lineages except chondrocytes, TNF superfamily in combination with actD inhibits NF-κB activation[15], increases the expression of Fas-associating death domain (FADD)[16], and downregulates FADD-like IL-1 converting enzyme (ICE) (FLICE)-inhibitory protein (FLIP)[17] and survivin expression[18]. In apoptotic human chondrocytes induced with TNF-α in combination with actD, on the other hand, the caspases and c-Jun N-terminal kinase (JNK) are activated, but the activation of NF-κB is not inhibited[10,12]. Furthermore, actD alone does not affect the

expression of apoptosis-related protein (Bcl-2, Bax, FLIP)[10]. But there are many still unidentified points about the details of an apoptosis-related signal.

Nowadays, inhibitor of apoptosis proteins (IAPs) has been identified as a new category of regulators of apoptosis. In 1993, the first IAP's family member was identified in SF21 insect cells[20]. XIAP is identified in a search for mammalian genes homologous to the insect IAPs[21], which binds and inhibits caspase-3, -7, and -9 with nanomolar affinity, but does not bind and inhibit caspase-8[22,23]. XIAP also activates NF-κB through the phosphorylation and degradation of I-κB[24]. c-IAP1 and c-IAP2 are identified as the proteins interacting with TNF-R-associated factors (TRAFs), which are signaling complexes on the cytoplasmic tail of the second TNF-R, TNF-R2[25]. These two IAPs bind and inhibit caspase-3, -7, and -9, albeit less strongly than XIAP[22]. TNF-α-induced anti-apoptotic signals through both TNF-R1 and TNF-R2 require the interaction between c-IAPs and TRAFs[26,27], and then, activate NF-κB. Furthermore, the activation of NF-κB induces expression of XIAP, c-IAP1 and c-IAP2[26,28]. Therefore, this pathway would work as positive feedback mechanism on the inhibition of TNF-α-induced apoptotic signals.

In the current study, we investigated the mechanisms of TNF-α-mediated apoptosis of human chondrocytes from points of view of the expression of TNF-R, the identification of apoptosis by morphologic examination, and the activation of caspase-cascade. Furthermore, we examined the change of expression of IAPs in human chondrocytes in the course of apoptosis induced with the TNF-α and actD.

Materials and methods

CELL CULTURE AND REAGENTS

Normal human articular chondrocyte (NHAC-kn) was purchased from Cambrex (Walkersville, MD). The cells were cultured in the growth medium specifically supplied by the manufacturer. Recombinant human TNF-α was purchased from Pepro Tech (London, UK). actD was purchased from Sigma–Aldrich (St. Louis, MO).

IMMUNOCYTOCHEMICAL DETECTION OF TNF-R

NHAC-kn was cultured in 2-well Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL), and fixed in acetone at room temperature for 10 min. Cells were washed with phosphate-buffered saline (PBS), incubated in 10% normal horse serum (Vector laboratories Inc., Burlingame, CA) in PBS at room temperature for 30 min, and then incubated with primary antibodies at 4°C overnight. Primary antibodies used in this study were mouse monoclonal anti-human TNF-R1 (Austral Biologicals, San Ramon, CA) and TNF-R2 (Austral Biologicals). After washing with PBS, slides were dipped in 0.3% H_2O_2 for 30 min, and washed with PBS again, and cells were incubated with 2% biotinylated anti-mouse-IgG made in horse (Vector laboratories Inc.) at room temperature for 30 min. After washing with PBS, cells were further incubated with ABC kit (Vector laboratories Inc.) at room temperature for 30 min, and signals were generated with 3,3-diaminobenzidine tetrahydrochloride.

NUCLEAR STAINING WITH HOECHST 33342

Subconfluent culture of NHAC-kn in 6-well culture plates was used. After the treatment with apoptosis-inducing reagents for indicated time periods, Hoechst 33342 (Sigma–Aldrich) was added to the culture at the final concentration of 20 μg/ml.

After incubation at 37°C for 30 min, cells were examined under fluorescence microscopy and fragmented apoptotic nuclei were identified. In each preparation, 300 cells were examined and apoptosis rates were calculated.

IMMUNOBLOT ANALYSIS

Subconfluent culture of NHAC-kn in 10 cm culture dishes was tested with 10 ng/ml of TNF-α and/or 0.2 μg/ml of actD for 24 h in total. Cells were detached with 0.025% of trypsin and 0.01% ethylenediaminetetraacetic acid (EDTA), counted by using trypan-blue dye exclusion, and collected by centrifugation at 7000 rpm for 5 min. Cells were lysed in Tris–NaCl-EDTA (TNE) buffer [50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 50-fold diluted protease inhibitor cocktail for use with mammalian cells (P8340; Sigma–Aldrich), 1% Triton X-100], incubated at 4°C for 30 min with gentle rotation, sonicated, and followed by further incubation at 4°C for 30 min. The cell lysates were mixed with the equal volume of 2× Laemmli sample buffer with 2-mercaptoethanol, and boiled for 5 min. Cell lysates were separated in 12% sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Hybond-P; Amersham Biosciences, Buckinghamshire, UK). After incubation in 5% nonfat dried milk in Tris-buffered saline (TBS), pH 7.6 containing 0.1% Tween 20 (TBST) at room temperature for 2 h. Membranes were incubated with primary antibodies at 4°C overnight or at room temperature for 1 h. Antibody solutions used in this study were mouse monoclonal antibody to β-actin (Sigma–Aldrich) and rabbit polyclonal antibody to human c-IAP1 (R&D Systems, Minneapolis, MN) diluted with 5% bovine serum albumin (Fraction V; Sigma–Aldrich) in TBST, and mouse monoclonal antibodies to human caspase-8 (Cell Signaling, Beverly, MA) and to human XIAP (BD Biosciences, San Diego, CA), and rabbit polyclonal antibodies to human caspase-3, caspase-9 (Cell Signaling) and to human c-IAP2 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted with solution-1 (Can get signal; Toyobo, Tokyo, Japan). After washing with TBST, membranes were incubated at room temperature for 1 h with horseradish peroxidase (HRP)-linked F(ab')2 fragment of anti-mouse immunoglobulins (Igs) made in sheep (Amersham Biosciences) or with HRP-linked F(ab')2 fragment of anti-rabbit Igs made in donkey (Amersham Biosciences). After washing with TBST and subsequently with TBS, signals were generated with ECL Plus detection reagents (Amersham Biosciences) by chemiluminescence and exposed at room temperature according to the protocols suggested by the manufacturer.

Results

EXPRESSION OF TNF-R1 AND TNF-R2 IN CHONDROCYTE

Before investigating the effects of TNF-α on human chondrocytes, we examined the expression of TNF-Rs in human chondrocytes by immunocytochemistry. Figure 1(a and b) shows the expression of two TNF-Rs, TNF-R1 and TNF-R2, in cultured human chondrocytes. The receptors are diffusely expressed in the cytoplasm, which is probably consistent with the localization of membrane receptors. Slides treated without the primary antibody exhibited no staining (results not shown).

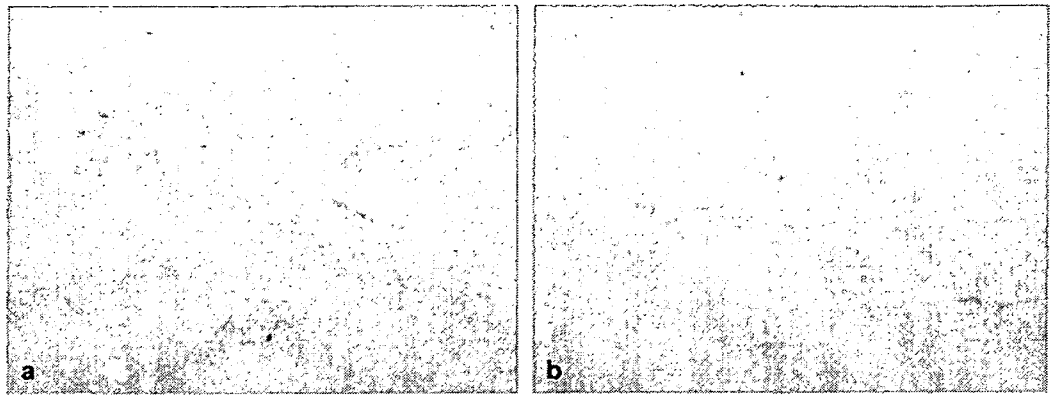

Fig. 1. Immunostaining for TNF-R1 (a) and TNF-R2 (b) of human cultured chondrocytes. Positive staining is visible in cytoplasm of all cells.

APOPTOSIS INDUCED BY TNF-α AND actD

When we treated, at first, the cultured chondrocytes with TNF-α alone at the concentration of up to 100 ng/ml for 24 h, the frequency of the cells with apoptosis features was low, up to 7.0%, and further reached to 8.3% with longer incubation periods up to 48 h [Figs. 2(a) and 3(a)]. Thus, this apoptosis-inducing protocol was insufficient for further biochemical analyses.

Therefore, we tested the combination of TNF-α and actD as an apoptosis-inducing stimulation. We added actD to the cell culture, incubated for 2 h, and further added TNF-α[10]. After 22 h incubation, 24 h in total as for actD treatment, the cells were examined for apoptotic features with Hoechst 33342 staining. The apoptosis-promoting effects of actD were negligible at the concentration of 0.02 μg/ml. The frequency of apoptosis significantly increased to more than 25% used at 0.2 μg/ml [Figs. 2(b) and 3(b)]. The cells were almost completely broken when treated with 2 μg/ml actD and 10 or 100 ng/ml TNF-α, thus the frequency of apoptosis was not exactly calculated. From these results, we selected the conditions of apoptosis induction, 10 ng/ml of TNF-α and 0.2 μg/ml of actD for 24 h in total, for the following experiments.

COMBINATION OF TNF-α AND actD INDUCES CASPASE CLEAVAGE

Activation of the cascade of caspase proteases is the major arm of the apoptotic machinery, because caspases are responsible for cleavage and degradation of important intracellular proteins. To determine whether the treatment of chondrocyte with TNF-α and actD causes activation of caspases, we performed Western blot analyses for the initiator caspases (caspase-8, -9) and executioner caspase (caspase-3) using antibodies recognizing both the proform and cleaved forms of these caspases. As shown in Fig. 4, TNF-α in combination with actD induced the cleavage of the 57-kDa procaspase-8 in human chondrocytes, and increased the accumulation of its cleaved forms of 43-, 41-, and 18-kDa. On the other hand, neither TNF-α nor actD alone induced the cleavage of procaspase-8. As for caspase-9, TNF-α in combination with actD induced the cleavage of the 47-kDa procaspase-9, and we observed its 37- and 35-kDa cleaved forms. Neither TNF-α nor actD alone did cause the cleavage of procaspase-9. Furthermore, as for executioner caspase (caspase-3), treatment with TNF-α in combination with actD causes the cleavage of the 32-kDa procaspase-3 to its 19- and 17-kDa cleaved

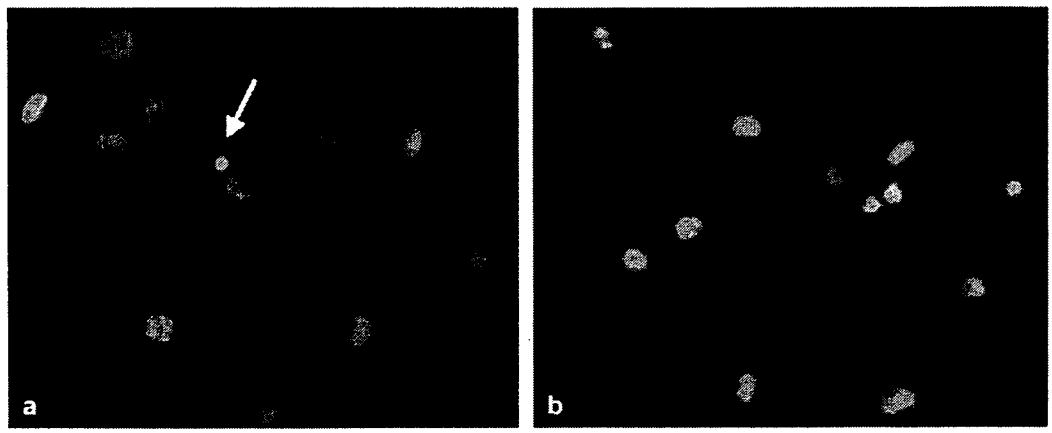

Fig. 2. Hoechst 33342 staining of chondrocytes incubated with 10 ng/ml of TNF-α (a), and with 10 ng/ml of TNF-α and 0.2 μg/ml of actD (b) for 24 h. One cell shows chromatin condensation (arrow) (a). Chromatin condensation and fragmentation are visible, indicating definite apoptosis (b).

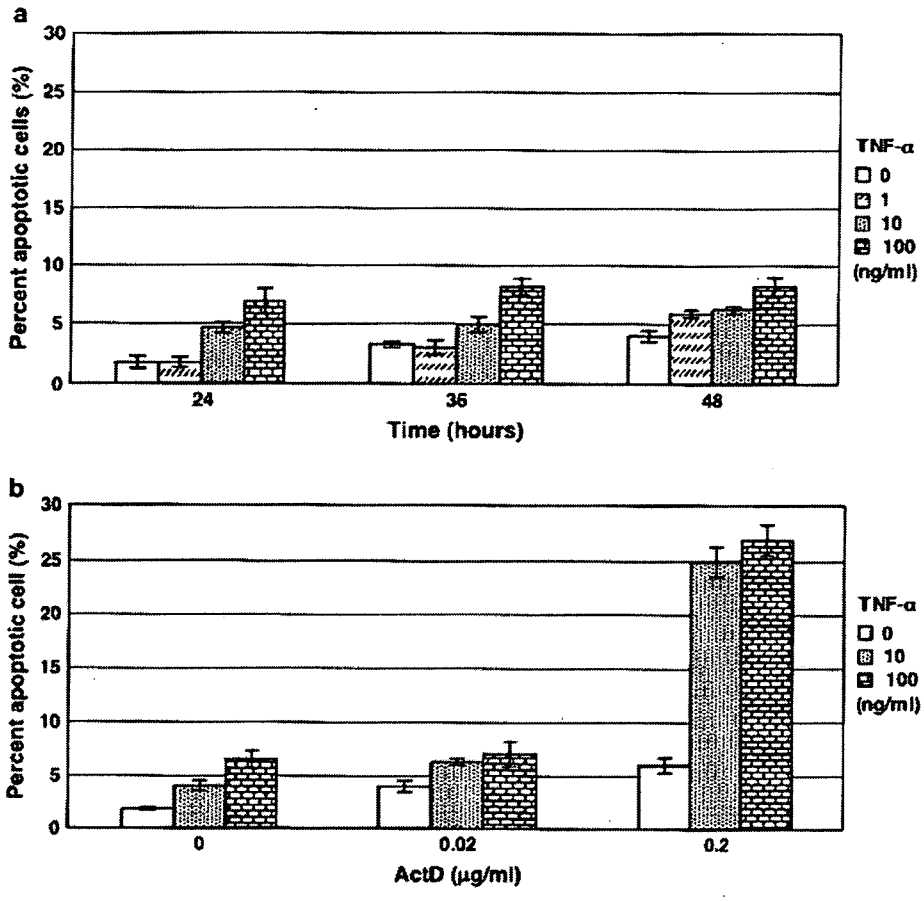

Fig. 3. Apoptosis-inducing effect of TNF-α and/or actD on human chondrocytes. Effect of TNF-α alone at different time points (a) and those of TNF-α in combination with actD at 24 h (b). After the indicated treatment, the cells were stained with Hoechst 33342 and percent apoptotic cells were calculated as described in Materials and methods. Results were presented as mean ± s.d. of duplicate wells of three independent experiments.

forms. Neither TNF-α nor actD alone did cause the cleavage of procaspase-3.

DECREASED PROTEIN EXPRESSION OF THE IAPS BY TNF-α IN COMBINATION WITH actD

IAP family proteins play a critical role in the inhibition of caspase-dependent cell death by binding to caspases. We examined the alteration of protein expression of the IAPs' families during TNF-α-induced apoptosis of human chondrocyte. The protein expression of the XIAP (57-kDa) and c-IAP1 (68-kDa) decreased by the 24-h treatment of TNF-α in combination with actD. TNF-α alone, on the other hand, slightly upregulated the expression of these two IAPs. In contrast, the treatment of TNF-α in combination with actD rather increased c-IAP2 (66-kDa) in human chondrocytes (Fig. 5).

Discussion

This is the first report describing the change of expression of IAPs in chondrocytes during the course of apoptosis induced with TNF-α and actD. In combination with actD, TNF-α-mediated signals decreased the expression of XIAP and c-IAP1 in human chondrocytes and led to apoptosis, while TNF-α-mediated activation of NF-κB signaling increases the expression of IAPs in other cell lineages[26,28].

Human chondrocytes express TNF-Rs[6,29]. In previous reports describing TNF-α-mediating apoptosis of chondrocytes, however, the expression of TNF-Rs is not examined[9-12]. In our apoptosis-inducing system, the cultured chondrocytes clearly expressed TNF-R1 and -R2, and thus, the TNF-α-signaling would be effective in our system.

We examined the activation of initiator caspases (caspase-8, -9) and executioner caspase (caspase-3) by Western blot analyses. Kim and Song reported that the apoptosis of human chondrocytes is induced through caspase activation, not inhibition of NF-κB activation by treatment with TNF-α and actD[10]. However, this report describes only the activation of caspase-3. In our current study, we demonstrated the activation of caspase-3, -8, and -9 during apoptosis induced by TNF-α and actD.

In nonchondrocyte cells, TNF-α signal activates NF-κB, resulting in the increased expression of IAPs[26,28]. IAPs suppress the activation of caspase-3, and lead to the inhibition of apoptosis[22,23]. Activated caspase-3, on the other hand, degrades XIAP and c-IAP1[25,30,31]. Therefore, the balance between the activation of caspase-3 and the expression level of XIAP and c-IAP1 would be a critical factor determining the fate of cells stimulated with TNF-α (Fig. 6). In our apoptosis-inducing system using actD, the balance lay with the activation of caspase-3 and led to decreased expression of XIAP and c-IAP1 proteins (Figs. 4 and 5). Since the activation of caspase-3 is

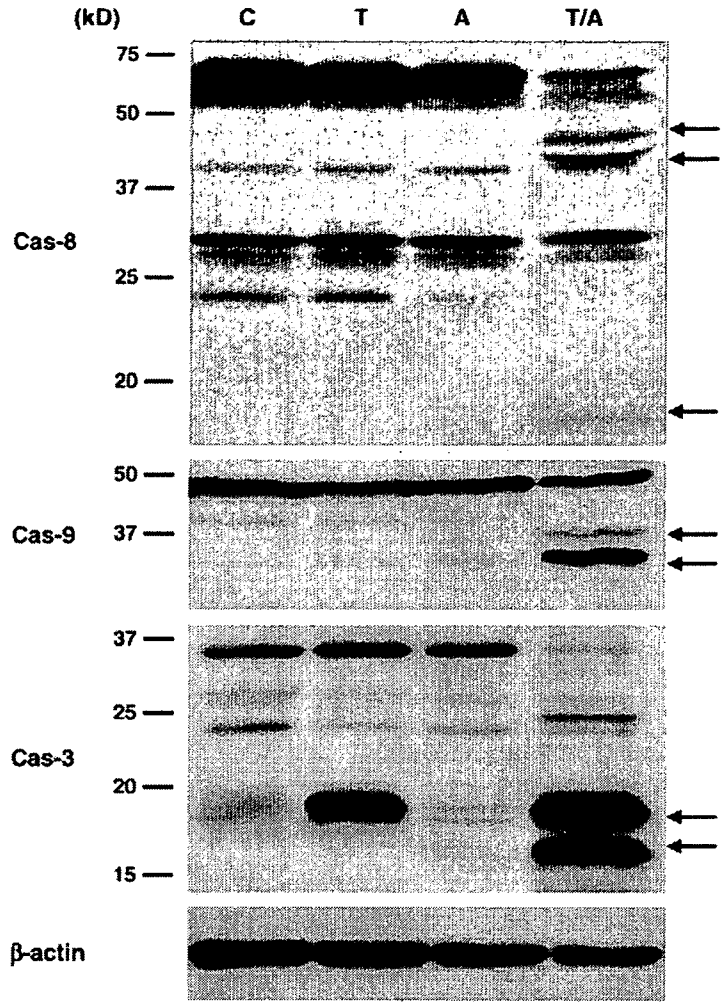

Fig. 4. Western blot for caspase-8, -9, and -3. Four hundred microliters of cell lysates was prepared from 4×10^5 cells, and a 30 μl aliquot of each sample was applied to each wall. After electrophoresis, proteins were transferred to a PVDF membrane, incubated with anti-caspase antibodies, and visualized as described in Materials and methods. The positions of molecular size markers are indicated (in kDa) on the left. Chondrocytes treated with TNF-α in combination with actD contain the cleaved form of caspases (arrows). C, medium only; T, TNF-α (10 ng/ml); A, actD (0.2 μg/ml); T/A, TNF-α (10 ng/ml) and actD (0.2 μg/ml); and Cas, Caspase.

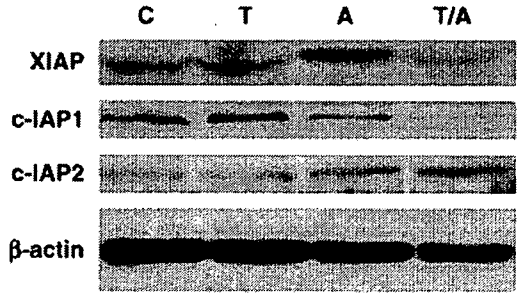

Fig. 5. Western blot for XIAP, c-IAP1, and c-IAP2. Four hundred microliters of cell lysates was prepared from 4×10^6 cells, and a 30 μl aliquot of each sample was applied to each wall. After electrophoresis, proteins were transferred to a PVDF membrane, incubated with anti-caspase antibodies, and visualized as described in Materials and methods. The amount of XIAP and c-IAP1 in apoptosis-induced chondrocytes decreases, and that of c-IAP2, on the other hand, rather increases. C, medium only; T, TNF-α (10 ng/ml); A, actD (0.2 μg/ml); and T/A, TNF-α (10 ng/ml) and actD (0.2 μg/ml).

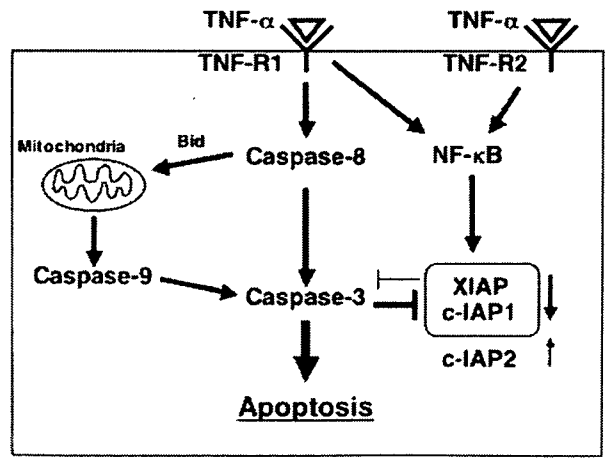

Fig. 6. Schematic diagram of TNF-α-induced apoptotic signals in human chondrocytes.

due to the activation of caspase-8 in our system (Figs. 4 and 6), activation of caspase-8 would be the pivotal event in the apoptosis induced with additional actD treatment. Because the caspase-9 was also activated in apoptotic chondrocytes (Fig. 4), the activation of initiator caspases would be critical in TNF-α-induced apoptosis of human chondrocytes. The mechanisms in which actD promotes the activation of initiator caspases in the presence of TNF-α are still unknown. In contrast with XIAP and c-IAP1, the protein expression of c-IAP2 was slightly increased in apoptotic chondrocytes (Fig. 5). The upregulation of c-IAP2 in TNF-α-mediated apoptotic cells was previously reported in human T-cell line[32], however, the precise mechanisms of this upregulation are still unknown.

In the patients with RA and OA, the apoptosis of chondrocytes is observed *in vivo*[33,34]. TNF-α induces the degradation of cartilage *in vivo*, however, TNF-α alone did not induce the apoptosis of human chondrocytes *in vitro*. Therefore, the inhibition of chondrocyte apoptosis might not be induced with anti-TNF-α drugs, although the anti-TNF-α therapy is effective in RA patients. The frequency of apoptotic chondrocytes in patients received with anti-TNF-α therapy needs to be examined.

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CASE REPORT

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Rheumatoid nodulosis during methotrexate therapy in a patient with rheumatoid arthritis

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Abstract We report a 62-year-old man with rheumatoid arthritis (RA) who developed nodulosis after methotrexate (MTX) treatment. The epithelioid cells of nodules were positive for matrix metalloproteinases (MMP)-2, MMP-3, MMP-9, and Ki67. The synovial tissues obtained from the same patient were negative for MMP-3, MMP-9, and Ki67. This study demonstrated that MTX-induced nodules are different from synovial tissues in terms of MMP expression, suggesting the presence of different pathologic mechanisms and differential MTX susceptibility.

Key words Immunohistochemical staining · Matrix metalloproteinase (MMP) · Methotrexate (MTX) · Rheumatoid arthritis (RA) · Rheumatoid nodulosis

Introduction

Rheumatoid nodules are characteristic extra-articular lesions often associated with severe or progressive rheumatoid arthritis (RA). We recently encountered a RA patient who developed multiple rheumatoid nodules despite improvement of joint symptoms by methotrexate (MTX) therapy. Occurrence of MTX-induced nodulosis has previously been reported,[1–6] but the histopathological analyses of such nodules have been limited. We obtained rheumatoid nodules and joint synovial tissues from a patient with MTX-induced accelerated nodulosis and compared their pathological features.

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Case report

The patient was a 62-year-old man who presented with RA in 1986. Gold salt and D-penicillamine therapies were started in 1988, the former being discontinued because of onset of proteinuria. Since his arthritis was not controlled satisfactorily, MTX (5 mg/week) therapy was started in October 1990. He showed remarkable clinical improvement after one month and received 2.5 mg/week of MTX after May 1993. In 1995, at a total cumulative MTX dose of ~1000 mg, multiple subcutaneous nodular masses became apparent, mainly in his extremities, and increased in size and number thereafter. He was also suffered from severe deformities of right toes and forefoot pain during walking. In March 2002, the patient was admitted to our hospital for resection of the nodules and resection arthroplasty of his right toes.

A total of 22 subcutaneous nodules were located on the bilateral elbows, fingers, buttocks, right knee, bilateral ankles, right heel, and bilateral feet. All of these nodules were firm and indolent. The patient had no vascular lesions or peripheral neuropathy.

Laboratory tests showed an erythrocyte sedimentation rate of 22 mm/h, white blood cell count of 6170/μl, red blood cell count of 454×10^4/μl, platelet count of 17.8×10^4/μl, and C-reactive protein level of 0.8 mg/dl, suggesting that there was little inflammation. The levels of CH50 and MMP-3 were within the normal range, being 35 U/ml and 98.8 ng/ml, respectively. However, the rheumatoid factor level was high (883 IU/ml). Human leukocyte antigen (HLA) typing showed that he was HLA-DRB1 *1502 and *0803 positive.

On X-ray, there were no destructive changes to the shoulder, elbow, hip, knee, or ankle joints, but there was marked erosion and deformity of the fingers and toe joints. Chest X-ray showed no abnormalities.

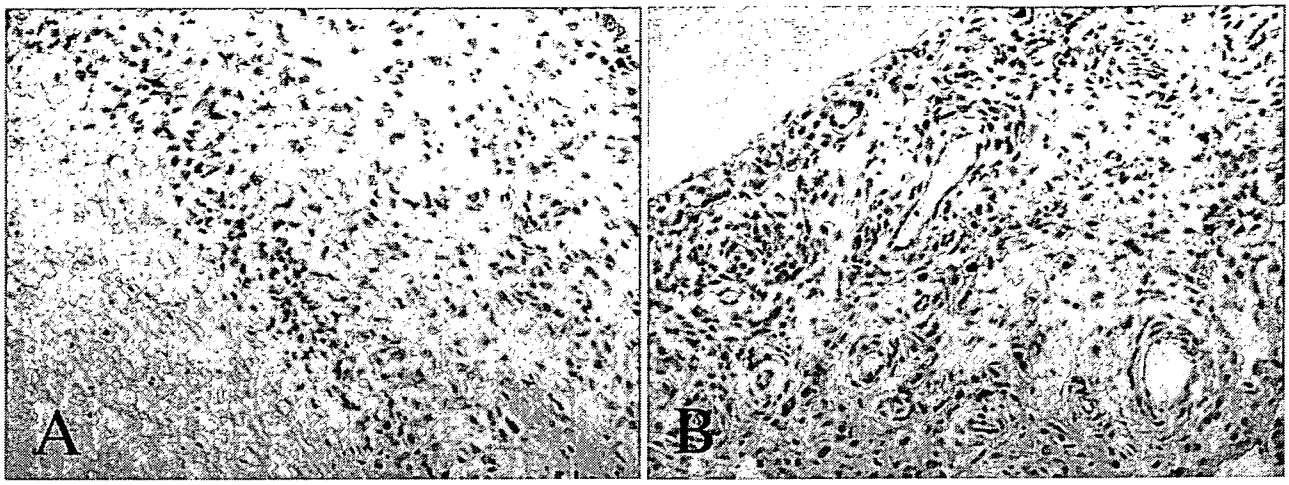

Fig. 1. **A** Photomicrograph of the resected subcutaneous nodules showed an amorphous necrotic substance containing neutrophils at the center of nodule, surrounded by epithelioid cells in a palisading pattern and lymphocytes. **B** Synovial tissue from the same patient showed multilayered synovial cells without pronounced villous proliferation. (**A,B** H&E stain, ×50)

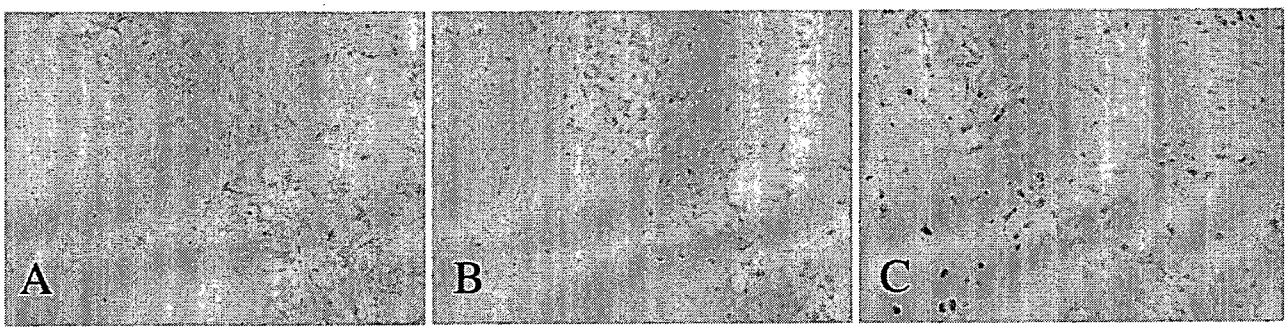

Fig. 2. **A** Immunohistochemical staining of the nodules showed that the epithelioid cells were positive for human leukocyte antigen-DR. Epithelioid cells in nodule were CD68-positive (**B**) and lysozyme-positive (**C**). (**A–C** ×50)

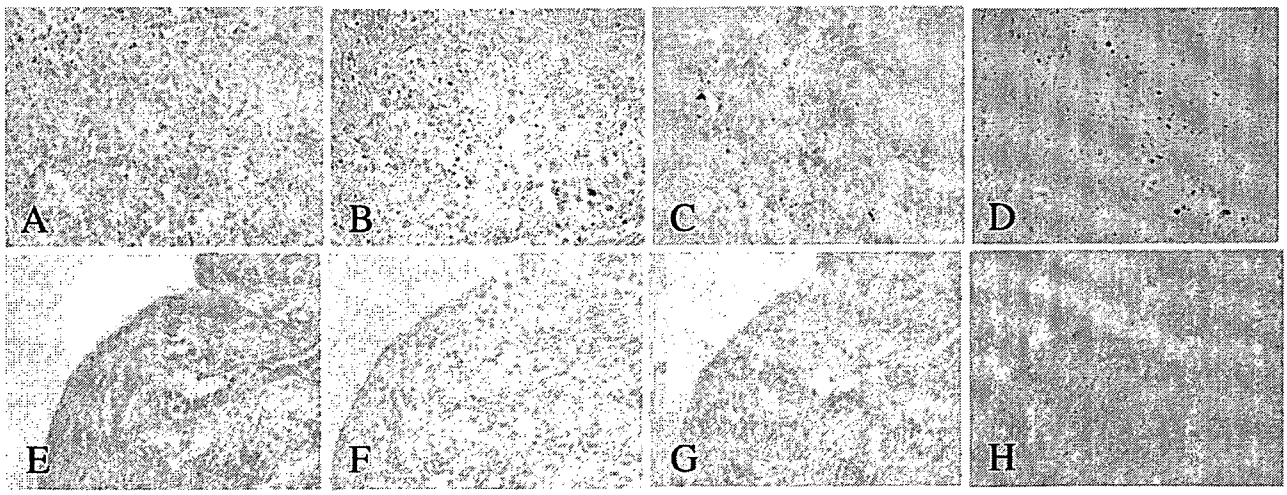

Fig. 3. **A** Epithelioid cells surrounding the necrotic center of the nodules expressed matrix metalloproteinase (MMP)-2 strongly. Some of the epithelioid cells were positive for MMP-3 (**B**), MMP-9 (**C**), and Ki67 (**D**). Immunohistochemical staining of synovial tissues showed MMP-2 expression (**E**), but MMP-3 (**F**), MMP-9 (**G**), and Ki67 (**H**) were not expressed. (**A–H** ×100)

All subcutaneous nodules were surgically excised. On gross examination at surgery, the nodules were grayish-white, firm, and adherent to the subcutaneous tissue.

Histopathologic study of the resected nodules showed amorphous necrotic substance surrounded by epithelioid cells in a palisading pattern, which is a typical picture of rheumatoid nodules. Lymphocytic infiltration was noted in the surrounding tissues (Fig. 1A). Synovial specimen harvested from the metatarsophalangeal (MTP) joint of the right great toe showed multilayered synovial cells. However, villous proliferation and cell infiltration were unremarkable (Fig. 1B).

Immunohistochemical staining of the rheumatoid nodules showed that the epithelioid cells surrounding the necrotic center of each nodule were positive for HLA-DR (Fig. 2A). These cells consisted of numerous CD68-positive and lysozyme-positive macrophages (Fig. 2B,C). Analysis of matrix metalloproteinase (MMP) expression indicated that MMP-2 was strongly expressed by the epithelioid cells. MMP-3, MMP-9, and the cell-cycle related gene Ki67 were also detected in some of the epithelioid cells surrounding the necrotic center of the nodules (Fig. 3A–D), suggesting the presence of active inflammatory granulomatous process. Synovial tissues also showed MMP-2 expression; however, MMP-3, MMP-9, and Ki67 were not expressed by the synovial lining cells or interstitial cells (Fig. 3E–H).

Discussion

In 1986, Kremer and Lee[1] reported the occurrence of multiple subcutaneous nodules in 3 out of 29 patients on MTX therapy. Subsequently, MTX-induced multiple subcutaneous nodules were reported by various authors,[2-5] including cases accompanied by other extra-articular lesions such as vasculitis and skin ulcers, and bronchiolitis obliterans with organizing pneumonia (BOOP). Interestingly, multiple rheumatoid nodules developed following improvement of arthritis by MTX therapy in all reported cases, including the present patient.

Spontaneous rheumatoid nodule is a typical manifestation of aggressive RA and is considered to be a consequence, at least in part, of common pathologic mechanisms of the disease. Analysis of cytokine profiles[6] in such spontaneous rheumatoid nodule suggests that the nodule is a Th1 granuloma and that the damage to synovial joint tissues and subcutaneous tissue is caused by the same inflammatory mechanism. However, the MTX-induced rheumatoid nodules, as observed in the present patient, show marked contrast to spontaneous rheumatoid nodule, in that the MTX-induced nodules develop after effective suppression of synovial lesions.

In the present study, the MTX-induced nodules showed numerous activated epithelioid cells and expression of MMP-3 and MMP-9, suggesting the active nature of the granuloma in spite of regressed synovial lesion. The pathomechanism of such differential effects of MTX on the synovium and rheumatoid nodule in certain susceptible individuals still remains to be clarified. As suggested by Merrill et al.,[7] MTX increases adenosine concentration and shows adenosine A2 receptor-mediated anti-inflammatory effect within joint, whereas in extra-articular tissue, adenosine at relatively lower concentration may enhance nodule formation via ligation of A1 receptor on macrophages. It is thought that the inflammatory process mediated by adenosine A1 receptor induces inflammatory cytokines, such as tumor necrosis factor-α and interleukin-1β, and subsequently these cytokines enhance the expression of MMP-3 and MMP-9 in the epithelioid cells of nodules.

Segal et al.[3] reported that all of their patients who developed multiple rheumatoid nodules during MTX therapy were positive for HLA-DR4, and this finding was supported by Jeurissen et al.[4] Ahmed et al.[8] reported that HLA-DRB1*0401 was detected in patients who developed MTX-induced nodulosis (at a frequency of 71.4%). However, HLA-DRB1*0401 was not found in the present patient. Since most of the reports of MTX-induced accelerated nodulosis have been in Caucasians, the immunogenetic background association in non-Caucasians remains to be determined.

In this study, the MTX-induced nodules were different from the synovial tissues in terms of MMP and Ki67 expression, which strongly suggests the presence of different pathologic mechanisms. Possible immunogenetic factors associated with the susceptibility to MTX are thought to be important for mechanisms of accelerated nodulosis, and should be clarified.

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関節病変の病理

Key words: rheumatoid arthritis,
histopathology,
synovium,
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はじめに

関節リウマチ（Rheumatoid arthritis：RA）をはじめ，リウマチ性疾患において，関節・骨はもっとも病変頻度の高い臓器の一つである．他の膠原病でも程度の差はあれ，関節病変が生ずる場合が多い[1)2)10)13)]．また，感染症や他の全身疾患など膠原病以外の様々な疾患においても副所見として関節症状を示す場合があり，関節病変は多くの疾患との鑑別を必要とする病変である．

本稿ではRAの病理像を中心に他疾患との鑑別点および診断時の注意点について述べる．

肉眼像

様々な疾患においてもっとも早期に認められる滑膜病変は滑膜細胞の増生である．膠原病に限らず，関節内に刺激が加わると滑膜細胞の増生が起こり，滑膜は絨毛状の肉眼像を示すようになる．滑膜細胞の増生そのものは非特異的な反応なので，軽度の絨毛状増生はRA以外の他の疾患でも認められる．この段階の滑膜炎での特異的な病理診断は困難であり，特にRAの早期および他の膠原病の滑膜病変は類似しているため鑑別しがたい．RAでさらに炎症が高度になると滑膜の絨毛状増生は高度になり，文字どおり絨毛状になってくる．非炎症性疾患の変形性関節症（osteoarthritis：OA）の滑膜増生が「こん棒状」と表現されるのに対してRAの場合にはより細かい絨毛状を示す．また，OAでは浮腫や線維化を示すのに対してRAでは血管の増生を反映して肉眼的には赤味を帯びている．RAでは関節液中に滲出成分が多いために滑膜の表面にはフィブリンが付着し，しばしば不透明な白色を示す．また，滑膜細胞の増殖に対して血液の供給が追い付かないために滑膜の先端の部分が壊死になって関節液中に脱落するとも言われている．その他に米粒体（rice body）と呼ばれる米粒大の白色のdebris状のものが認められることもある．また，関節内の出血のために滑膜内にヘモジデリンの沈着がみられ，滑膜が茶褐色を示す場合もある．

軟骨や骨については最近ではMRIやCTで状態が把握できるようになり，RAやOAでは軟骨や骨にびらんが確認される．軟骨や骨が検体として提出されるような手術が行なわれた病期のRAやOAの患者においては軟骨組織や半月板に変性や欠損が認められ，骨が露出して

The histopathology of the arthritis.
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いる場合が多い．RAではパンヌスと呼ばれる肉芽組織が軟骨を破壊し，骨髄内に浸潤し骨破壊を示す．このパンヌスによる軟骨・骨破壊像はRAの特徴的な所見である．OAの場合の骨破壊はRAとは異なり物理的なストレスや荷重などが原因であるが，軟骨が失われ骨が露出しており関節面には高度な骨増生が認められ，骨梁は太くなっている．

病理組織像

1．関節リウマチ（RA）[12)4)-13)]

膠原病のうち，関節に主な病変が生じるものがRAである．他の膠原病でも関節に病変があらわれることがあるが，いずれもRAに比較すると関節炎の程度は軽微であり，骨破壊まで至るものはまれである．RAの初期からの進行過程を時間を追って観察すると，初期では滑膜組織の浮腫のほかに血管周囲にclass II抗原陽性の紡錘形細胞（線維芽細胞様細胞）が出現する．筆者らがアメリカリウマチ学会（ACR）の1987年の診断基準7項目中4項目以上を満たす早期RA患者の滑膜組織を検討した結果[3)]，もっとも早期の滑膜組織では浮腫や滑膜細胞の増生，血管周囲の紡錘形細胞の出現が起こり（図1），続いてTリンパ球が浸潤し，一部は集簇する．発症から約半年ぐらいのRAの滑膜組織内にはBリンパ球が出現するが，集簇する傾向を示す．

以上のようにRAを特徴づける病理組織像は，大きく分けるとa）滑膜細胞の増殖，b）リンパ球などの炎症性細胞の浸潤，c）血管新生，d）軟骨・骨破壊，の4つであるが，以下にその詳細を述べる．

1）滑膜細胞の増殖

正常の滑膜組織の表層は胸膜や腹膜と同様の中皮細胞系由来である1～2層の滑膜細胞でおおわれている（図2）．RAの滑膜組織の病理組織学的な変化のうちもっとも早期に認められるのが滑膜細胞の多層化である．滑膜表層細胞の多層化は他の様々な関節炎でも起こるとされ，モデル動物の関節内にバクテリアを注入すると滑膜には炎症性細胞の浸潤が生じ，数時間以内に滑膜細胞の増生が認められるようになるが，

図1　発症早期の関節リウマチの滑膜像

RA発症から非常に早い時期の滑膜組織では浮腫や滑膜細胞の増生，血管周囲の紡錘形細胞の出現が起こる．続いてTリンパ球が浸潤してくるが，この時点での炎症は軽度であり，他の関節炎との鑑別は困難である．

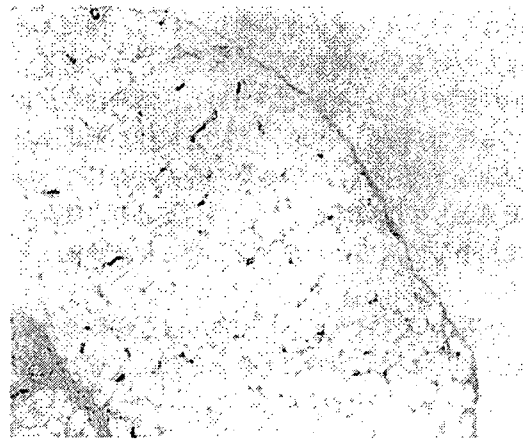

図2　正常の滑膜組織

正常の滑膜組織の表層は1，2層の滑膜細胞によって覆われており，その下はわずかな血管を含む脂肪組織からなる．

RAの滑膜では表層細胞の多層化は数層から20層程度にもなる．RAでは増殖した滑膜細胞自らがclass II抗原の他に多くの蛋白分解酵素やサイトカイン，およびそのインヒビターや増殖因子を産生し，さらにコラーゲンやヒアルロン酸[12)]などの細胞外マトリックスの合成も行っており，機能的には非常に多彩である[5)]．

2）リンパ球などの炎症性細胞の浸潤

RA滑膜組織ではリンパ球の浸潤，集簇が高度である．滑膜組織に浸潤するリンパ球の多くは濾胞を形成するBリンパ球と濾胞周囲でび漫

性に浸潤するTリンパ球からなり，さらにTリンパ球の中でもCD4陽性のhelper/inducer Tリンパ球がCD8陽性のsuppressor/cytotoxic Tリンパ球より量的に優位である．これらの細胞の比率は滑膜の炎症の程度によって異なっており，同一患者でも時期によって異なる像を示す．また，この滑膜組織中のBリンパ球がリウマチ因子を産生すると言われている．炎症の初期および炎症が遷延して陳旧化してくるとリンパ濾胞はみられなくなるため，リンパ濾胞の有無は免疫反応の活動性を計るにはよい指標である．一般に骨破壊の病変部をみると，軟骨や骨の基質を分解する蛋白分解酵素を産生し破壊に関与するとされている細胞は好中球，マクロファージを中心とする非特異的な炎症性細胞であり，リンパ球はむしろRAにおける免疫学的反応に関与しているのではないかと思われる．つまり，RAの滑膜組織にみられる炎症はリンパ球を中心とする免疫学的反応による炎症と好中球中心の非特異的炎症の混在した像であり，それらが複雑に絡みあっている状態とみると理解しやすい．

マクロファージについては，本来の貪食機能を呈する他に免疫学的に抗原提示能を有し，サイトカインや増殖因子，接着分子の発現など多くの機能を有することが明らかにされている．また，顆粒球については，RAの初期や炎症の増悪期に好中球浸潤が目立っている．なお，RAでは長期にわたって炎症が持続しているため線維化も目立つ．一般的には濾胞を形成するリンパ球の浸潤と血管新生がRAの滑膜像の特徴と言われているが(図3)，線維芽細胞による線維成分の増殖がみられるのもRAの特徴である．

3）血管新生

RAの滑膜の特徴的な所見に血管新生がある．正常の滑膜組織では，脂肪組織および表層細胞直下には毛細血管ないしは小静脈が認められるのみであるが(図2)，RAになると，透過性亢進をみるとともに血管も増生し，増殖した血管の内皮細胞には種々の接着分子が発現する．また，深部にみられる血管周囲にも時間とともにリンパ球を主体とする炎症性細胞が集簇

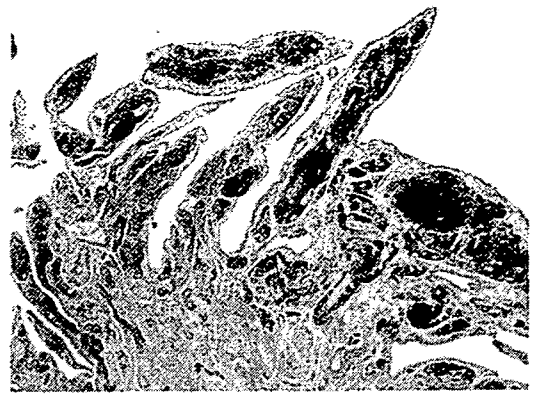

図3　典型的なRAの滑膜組織像

滑膜組織は絨毛状増殖を示しており，発達したリンパ濾胞の形成が高度に認められる．

してくるようになる．この時，滑膜組織では血管増殖因子が産生され，血管の内皮細胞には接着分子の発現，周囲の細胞にはそれぞれのリガンドの発現がみられるようになる[7]．この毛細血管は炎症とともに成長し，絨毛状に増殖した滑膜組織内に伸びていくが，リンパ濾胞を形成したり多様な機能を営んでいることが考えられる．

4）軟骨・骨破壊

RAでは，滑膜の炎症が軟骨・骨におよんで関節破壊に至るが，この滑膜におこった炎症は肉芽組織（パンヌス）となって浸潤していく．パンヌスの進行の仕方には2通りあり，一つは滑膜と骨膜の移行部分でポケットとよばれる若い間葉系組織のみられるbare areaからsubchondralに浸潤していく形であり(図4)，もう一つは軟骨の表層を這うように浸潤し，下方にむかって侵食していく形である(図5)．そこにはマクロファージ以外にも線維芽細胞様細胞(fibroblast-like cell)，破骨細胞も認められる．線維芽細胞様細胞はpannocyteとも呼ばれ，class II抗原の他に種々の蛋白分解酵素も発現する．

2．RAの炎症の程度

RAの関節病変の特徴は長期間にわたって持続する炎症と進行する骨破壊であるが，病変の程度も発症初期の軽度のものから骨破壊の著し

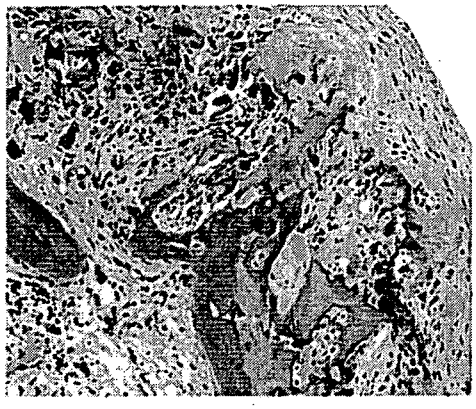

図4 RA 患者の骨破壊部（bare area）

炎症性パンヌスは滑膜と骨膜との移行部位（ポケットあるいは bare area と呼ばれる）から骨膜を越えて軟骨下骨の骨髄内へと骨破壊性に浸潤する．

図5 RA 患者の膝関節の骨破壊部のパンヌスの拡大像

図4と異なり，軟骨の表層を覆うようにパンヌスが浸潤し，下方にむかって侵食していく像．骨吸収部においては破骨細胞をはじめ，線維芽細胞様の紡錘形細胞や単核球がみとめられる．

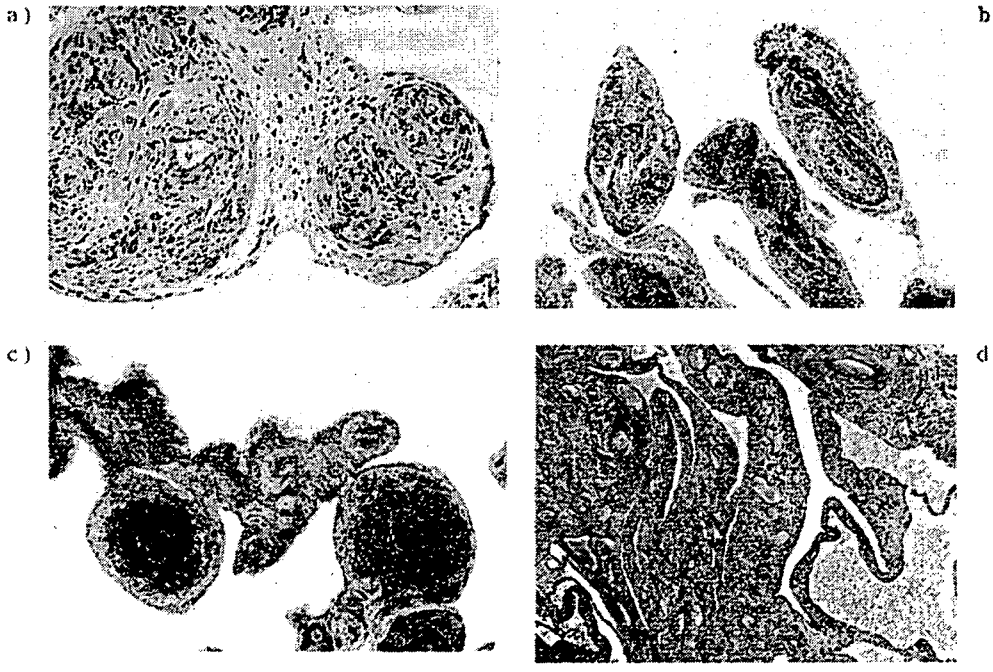

図6 RA の滑膜組織像

a）早期 RA の滑膜像：発症から5カ月の患者の滑膜組織．滑膜細胞の軽度の多層化と浮腫，わずかなリンパ球浸潤が認められる．この段階では他の疾患との鑑別は困難である．
b）炎症が中等度の時期の RA の滑膜像：a）に比較して絨毛状増生や滑膜細胞の多層化が目立ち，リンパ球を主体とする炎症性細胞浸潤もびまん性にみとめられる．血管の新生もさかんである．
c）炎症が高度な時期の RA の滑膜像：炎症が高度な時期の RA 滑膜では滑膜の絨毛状増生はさらに高度になり，滑膜細胞の多層化は20層にもおよぶことがある．また，胚中心を有するリンパ濾胞の形成も目立つ．RA との診断は比較的容易である．
d）炎症が沈静化した時期の RA の滑膜像：内科的治療で炎症がコントロールされていたり，罹病期間が長期化して炎症が落ち着いた RA 患者の滑膜では線維化が目立ち，リンパ球浸潤などは軽度となっている．