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変形性関節症の発症原因

福井　尚志**

Pathogenesis of Osteoarthritis

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Key words：osteoarthritis(変形性関節症)，pathogenesis(原因)

変形性関節症(OA)は運動器領域の代表的な common disease であるため疾患の進行のメカニズムについては多くの研究がなされ，様々な知見が得られている．しかし OA の狭義の発症のメカニズムについては研究が困難なこともあり，まだ不明な点が多く残されている．本稿では疫学的研究により OA 発症の危険性を増すことが知られている年齢，肥満，性について疾患発症とどのように関連するのか現在までに知られていることを述べ，さらに最近新たな知見が相次いで報告されている OA の遺伝的要因についても触れる．

変形性関節症(OA)は運動器領域の代表的な common disease であるため，病態についてもさまざまな角度から解明が進められており，今までに多くの知見が得られている．OA の病態は一般に OA が発症するメカニズムと発症した後に疾患が進行するメカニズムとに分けて考えたほうが理解が容易である．本稿では前者，すなわちなぜ OA が発症するのかに焦点を絞り，現在までにわかっている知見をまとめてみたい．

OA の発症のメカニズムを考える場合，疫学的研究により明らかになった OA 発症の確率を増加させる因子(危険因子)に着目するのが有用である．従来の研究から外傷や関節炎の既往，年齢，肥満，性，の 4 つが OA の明らかな危険因子であることが知られている[3,4]．本稿では以下にこのうち年齢以下の 3 つの要因についてそれぞれ OA の発症とどのように関連するのかを述べ，最後に最近新たな知見が相次いで報告されている OA の遺伝的要因についても触れる．

加齢と OA の発症

外傷や関節炎などの既往のない一次性の OA の場合，年齢は発症の最大の危険因子であり，OA の発症率は年齢が高くなるに従い顕著に上昇する．では年齢に伴って OA が発症しやすくなるのはなぜだろうか？　これに対するはっきりした回答はまだ得られていないが，軟骨の基質と細胞の両方に加齢に伴う変化が生じることが知られており，これが OA 発症の引き金となるのではないかと考えられている．

軟骨中にある細胞は軟骨細胞だけであり，軟骨基質の維持は軟骨細胞の代謝に大きく依存する．したがって軟骨細胞の挙動の変化は直接に軟骨の変化を引き起こすことになる．高齢者の軟骨細胞は一般に軟骨基質を産生する能力が低下しており[16]，これが OA の発症に関連している可能性がある．また高齢者から採取した軟骨細胞は，タンパク分解酵素の発現や細胞の障害を引き起こす nitric oxide(NO)を産生する傾向が強まってお

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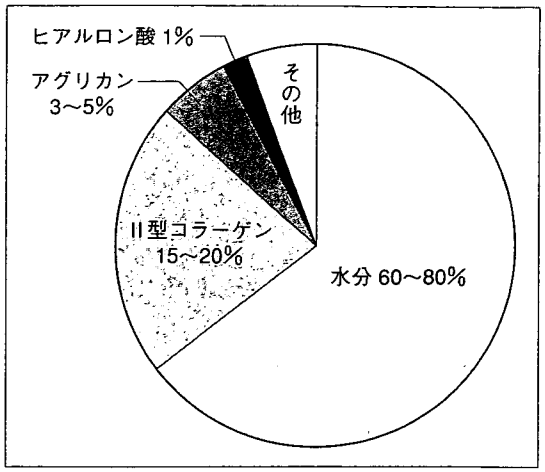

図 1　関節軟骨の組成
軟骨はその重量の 60〜80%が水分である．水分以外の構成要素としてはII型コラーゲンが最も多く，アグリカンがこれに次ぐ．

り[8]，これも OA 発症の一因となっているのかもしれない．ただし OA では疾患の進行に伴って軟骨細胞の基質産生は正常軟骨の何倍にも亢進するようになることから，加齢に伴う軟骨細胞の基質産生能の低下は不可逆的なものではなく，適切な刺激により回復可能なものであると考えられる．

一方，加齢に伴う軟骨基質の性質の変化は基質の変性に直接関連する要因の 1 つである．とくに荷重関節では軟骨に常に大きな荷重が加わっているため，軟骨基質の特性は軟骨の変性に密接に関連する．基質の変化はまた軟骨細胞の挙動に影響を与えることにより間接的にも OA の発症に関与する．これは軟骨細胞が力学的な環境の変化にきわめて敏感な細胞であるためで，たとえば軟骨基質の性質が変化して細胞に加わる力学的な負荷が変わることで軟骨細胞の基質産生量が変化する可能性が指摘されている．以下に加齢に伴う軟骨基質の変化がどのような機序によって起こるのかについて考えてみたい．

軟骨基質はII型コラーゲンを中心とするコラーゲンと，アグリカンをはじめとするプロテオグリカンによって構成される．コラーゲンは軟骨の乾燥重量のおよそ 60%を占め，そのうち 90%がII型コラーゲンである．一方，プロテオグリカンもその 90%はアグリカンである(図 1)．軟骨中のII型コラーゲン，アグリカンにはいずれにも加齢に伴う変化が生じるが，その内容はそれぞれ異なる．

加齢とともにII型コラーゲンに生じる変化の中で最も重要なものは advanced glycation end product(AGE：最終糖化反応物)の形成である．

AGE はタンパク分子に糖分子が直接に化合することによって生じる化合物で，体内の種々の組織でタンパク分子の存在する期間に比例して AGE の量が増すことがわかっている．軟骨の特徴の 1 つは，これを構成するマトリクスの代謝回転が極めて遅いことで，たとえばII型コラーゲンの場合その半減期は 100 年以上とされ[17]，このため高齢者の軟骨中のII型コラーゲンには高い密度で AGE が形成されている[2]．高齢者の関節軟骨はとくに病的変化がなくてもしばしば黄色を呈するが，これは AGE の形成によるものである．AGE の形成は単に軟骨の色調の変化をきたすだけではなく，軟骨の機械的な特性を変化させる．AGE にはいくつかの種類があるが，その 1 つペントシジンは分子の間に架橋構造を形成する性質があり(図 2)，その形成に伴って組織は硬く，脆くなる．また軟骨細胞は AGE に対するリセプターを持っており，軟骨基質中の AGE の濃度が高くなると，軟骨細胞ではこれに応じて炎症性サイトカインや matrix metalloproteinase(MMP)の産生が亢進する[15]．

軟骨基質のもう 1 つの主成分であるアグリカンには加齢に伴い分子の短縮という別の変化が生じる．アグリカン分子はコア・プロテインと呼ばれる直鎖上のタンパク分子にグリコサミノグリカンの側鎖が多数結合した構造を有し，軟骨内ではヒアルロン酸に結合して存在する(図 3)．いろいろな年齢のヒトの関節軟骨からアグリカンを抽出して調べたところ，高齢者ほど切断され分子量が小さくなったアグリカンが増加していることが明らかになった[18](図 4)．アグリカン分子はヒアルロン酸との結合部分を保っているため，短縮した後も軟骨基質内にとどまる．アグリカン分子の第一の機能は，その側鎖が持つ強い負の電荷によって電解質や水分子を軟骨基質内にひきつけ，これによって軟骨の弾性を維持することにある．このため分子の短縮が起こり側鎖の数が減少すると，

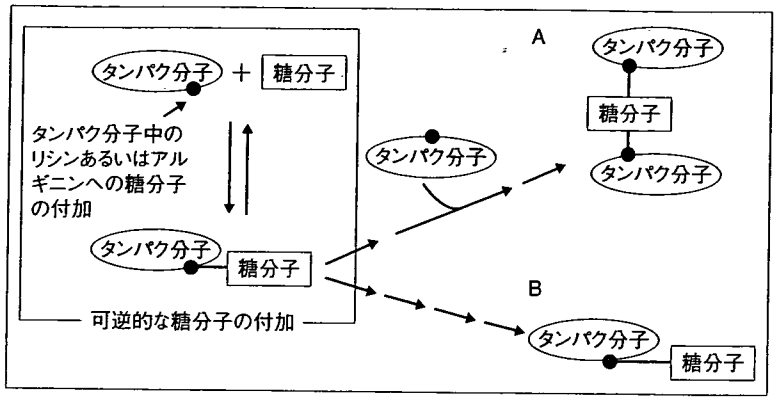

図 2　AGE による分子間の架橋形成の概念図

AGE はタンパク分子中のリシンあるいはアルギニン分子にショ糖や果糖などの糖分子が化学的に結合することにより形成が開始される．はじめこの結合は可逆的であるが，いくつかのプロセスを経て不可逆的な結合へと変化する．この過程で形成される AGE の種類により，糖分子を介して他の分子との間に架橋を形成する場合(A)と糖分子が単にタンパク分子に付加するだけで架橋を形成しない場合(B)とに分かれる．

それに伴い軟骨の弾性も低下することになる．高齢者における軟骨の物性の変化には，前述の AGE の形成とともにこのアグリカン分子の短縮が大きく関与していると考えられる．つまり高齢者の軟骨は基質内の AGE の蓄積とアグリカン分子の短縮という 2 つの機序によって OA 変化が生じやすい状態になっていると言えよう．

肥 満

一般に肥満は関節に加わる荷重を増やすことで OA の発症を促すと考えられている．しかし肥満している者では手指の関節のような非荷重関節においても OA の発症率が高いことが明らかになり[5,14]，肥満と OA の関係は単に荷重によるものだけではないことがわかってきた．最近の研究結果から，肥満者における OA の発症には脂肪組織で産生されるアディポカインと呼ばれるサイトカインに似た作用を持つ物質が関与しているのではないかと考えられている．なかでもアディポカインの一種レプチンは動物実験においてレプチンを関節内に投与したところ OA 様の変化が生じたこと，肥満している者において関節液中のレプチン濃度が肥満の程度に関連して上昇する傾向があったこと，などの理由で OA との関連がとくに注目されている物質である[10]．肥満傾向のある者では減量を行うことで OA の発症率，進行速度は明らかに減少する．このような減量の治療効果も一部はレプチンの産生低下によるものなのかもしれない．

性 差

OA の罹患率には明らかな性差がある．膝関節の場合，女性の OA 罹患率は同年代の男性の 1.5～2.0 倍であるとされ，膝以外の関節についても同様の性差が報告されている[3]．疫学的研究によって女性では閉経後に OA の発症が急増すること[6]，ホルモン補充療法を受けた女性では膝および股関節における OA の発症が減少すること[13,19]，またエストロゲン受容体の遺伝子多型が OA の発症と関連すること[1]などから，性差の原因はおもにエストロゲンにあると考えられている．しかし実際にエストロゲンがどのような機序で OA の発症を抑えるのかはまだ解明されていない．なお OA の発症を予防するためのエストロゲンの投与は，エストロゲンが OA の発症を抑止する効果が比較的弱いことと有害事象の問題から実

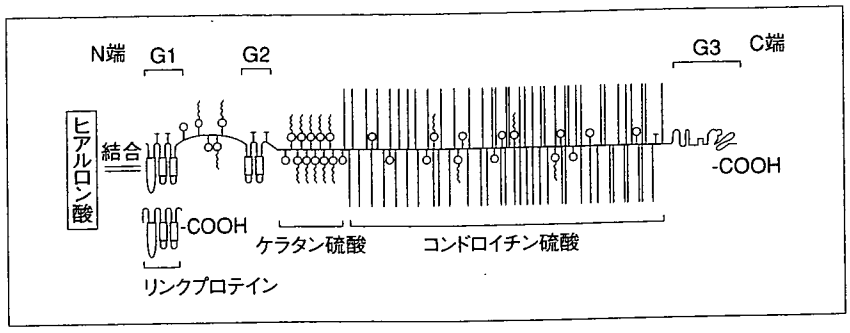

図 3　アグリカン分子の模式図

アグリカン分子は直鎖上のコア・プロテインにグリコサミノグリカンの側鎖が多数結合した構造をとる．アグリカンは軟骨基質中でヒアルロン酸に結合して存在し，網の目状の構造のコラーゲンの内部に閉じ込められた状態になっている．リンクプロテインはアグリカンとヒアルロン酸の結合を補強する働きがある．

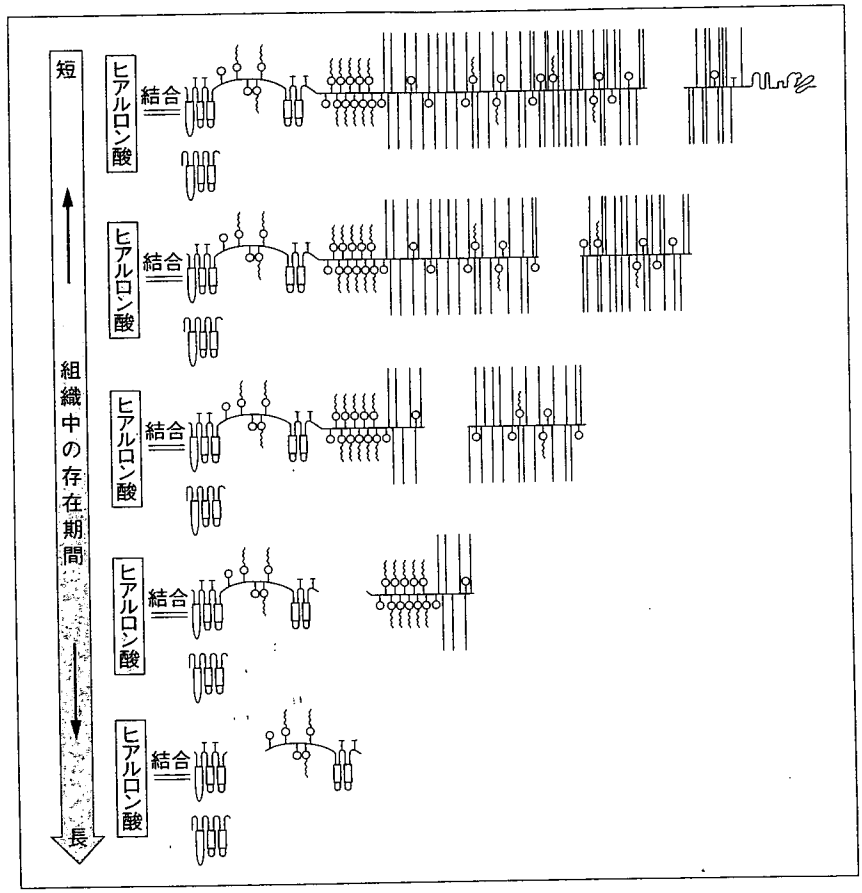

図 4　加齢に伴うアグリカン分子の変化

高齢者ではカルボキシル側が切断された，短いアグリカン分子が増えていく．これはOA 変化がなくても軟骨中でアグリカン分子がタンパク分解酵素による切断を受けるためと考えられる．アグリカン分子にはタンパク分解酵素の切断部位が複数あり，加齢とともに 1 つの分子が繰り返し切断を受けて徐々に短縮し，分子量を減じていくと考えられる．

際には行われていない．

遺伝的素因

OAの症例の中には全身の関節にOAが多発したり，家族集積性を示すものがあり，遺伝的素因の関与が考えられる場合がしばしばある．実際，一卵性双生児ではOAの発症が高い確率で一致することが知られている．このためOAの感受性遺伝子を捜す試みはかなり以前から行われてきた．当初，OAの原因遺伝子は軟骨を構成する成分にあるのではないかと考えられ，軟骨，骨を構成する遺伝子，代謝に関与する遺伝子について検索が精力的に行われたが，OAの発症に密接に関与する遺伝子は見出すことができなかった．

このため軟骨や骨との関連が明らかでない遺伝子も含めてすべての遺伝子の中からOAの発症に関与する遺伝子を探す試みが行われるようになった．この方法により最近同定されたOA感受性遺伝子として重要なものに*ASPN*，*CALM1*，*FRZB*の3つがあるが，このうち前2者は国内の研究グループによって明らかにされたものである．*ASPN*はアスポリンをコードする遺伝子である．アスポリンは軟骨中に含まれる小分子量のプロテオグリカン(小型プロテオグリカン)の1つで，成長因子の1つTGF-βと結合してその活性を変える性質を持つ．この性質がアスポリンの遺伝子型によって異なるため，一定の型のアスポリン遺伝子を持っている場合にOAの発症率が高くなると考えられている[7]．一方*CALM1*はカルモデュリンをコードする遺伝子である．カルモデュリンはカルシウムと結合する性質を持つ細胞内タンパクの1つで，軟骨細胞においてII型コラーゲンやアグリカンの発現を亢進させることが知られている．この遺伝子のプロモーター領域の特定の遺伝子型はカルモデュリンの発現を低下させることでOAの発症を引き起こすと考えられている[12]．なおアスポリンの遺伝子多型は膝関節，股関節のいずれのOAにも関連があることが知られているが，カルモデュリンは股関節のOAとのみ関連が知られている．一方，*FRZB*はSFRP3(secreted frizzled related protein 3)をコードする遺伝子である．SFRP3は軟骨細胞の肥大化・骨化を促す分泌型タンパクWntの活性を抑制する働きを持つタンパクであるが，*FRZB*の特定の遺伝子型ではこの分子が本来の機能を失っており，このためOAが高率で発症すると考えられる[9,11]．

以上，本稿ではOAの発症をもたらすメカニズムについての現在までの知見をまとめてきた．OAは日常の診療でしばしば単一の疾患として扱われるが，発症の機序や遺伝的素因の解析結果から明らかなようにその病態は実は極めて複雑である．今後発症や進行のメカニズムがさらに解明されていくことによって，OAの進行を抑止しうる治療法が確立されることが期待される．

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「読者の声」募集

弊誌では，読者の皆様の“ひとこと”(ご意見)を募集しています．

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- 診療，研究のなかでのエピソード，気づいた出来事，雑感
- 弊誌掲載論文，記事などに対する感想，意見
- 整形外科臨床，整形外科学(医学)教育，整形外科臨床研修などをめぐる諸問題
- 医療行政，社会保険制度などについての感想，意見

2）ご送付の際は弊誌Eメール(rinseige@igaku-shoin.co.jp)または綴じ込みハガキをご利用ください．(レイアウトの関係で字数等多少変更させていただくことがあります．)

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ORIGINAL ARTICLE

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Production of interleukin-6 and interleukin-8 by nurse-like cells from rheumatoid arthritis patients after stimulation with monocytes

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Abstract It has been reported that nurse-like cells (NLCs) play a critical role in the pathogenesis of rheumatoid arthritis (RA). The interaction between NLCs established from RA patients (RA-NLCs), and freshly isolated blood monocytes was analyzed to further elucidate the pathogenesis of RA. RA-NLC lines were established from the synovium of RA patients. The RA-NLCs were cultured with monocytes freshly isolated from peripheral blood of healthy donors, and induction of interleukin (IL)-6 and IL-8 as well as the mRNA expression of these cytokines was examined. The levels of IL-6 were over 400 times higher in the supernatant from coculture of RA-NLCs and monocytes than in those from cultures of RA-NLCs alone. Anti-tumor necrosis factor (TNF)-α monoclonal antibody inhibited the induction of both cytokine in a dose-dependent fashion, although there was no detectable level of TNF-α in the supernatant from coculture. In addition, coculture of RA-NLCs and monocytes without direct cell contact did not induce cytokine production. To determine IL-6 producing cells, RA-NLCs and monocytes were separated into each fraction after coculture for 24h. Cocultured RA-NLCs contained approximately 80 times higher IL-6 mRNA than the RA-NLCs cultured alone. The levels of IL-8 were also much higher (about 900 times) in the supernatant from coculture than in those from cultures of RA-NLCs alone. Cocultured RA-NLCs expressed IL-8 mRNA about 620 times higher than those cultured alone. These results indicate that NLCs produce high levels of IL-6 and IL-8 after cell–cell interaction with monocytes/macrophages via membrane-bound TNF-α, and that activation of NLCs by monocytes/macrophages may be involved in the pathogenesis of RA through maintenance of synovial inflammation.

Key words Inflammatory cytokine · Monocyte · Nurse-like cell (NLC) · Rheumatoid arthritis (RA)

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Introduction

Rheumatoid arthritis (RA) is an inflammatory disease characterized by systemic arthritis with proliferation of synovium and destruction of bones and cartilage. It is known that macrophage-like synovial cells (type A cells) and fibroblast-like synovial cells (type B cells) as well as T and B lymphocytes produce a large amount of cytokines and contribute to the inflammation in the affected synovium.[1] The etiology of RA is, however, not clearly understood.

The nurse cell was originally reported by Weckerle et al. in 1980,[2,3] as a stromal cell from murine thymus holding thymocytes under itself in vitro. This phenomenon was named pseudoemperipolesis. Nurse cells are believed to play an important role in differentiation, maturation, and apoptosis of thymocytes. Human stromal cell, which demonstrates pseudoemperipolesis, was detected in the skin of healthy donors, RA synovium, and RA bone marrow by our group, and named nurse-like cell (NLC),[4–6] Nurse-like cells isolated from RA patients were designated as RA-NLC.

RA-NLCs demonstrate pseudoemperipolesis with T and B lymphocytes and interact with them. RA-NLCs promote the survival of T and B cells in vitro, activate them to produce cytokines, and induce production of immunoglobulin by B cells.[4,6,7] RA-NLCs are believed to contribute to the pathogenesis and persistence of inflammation in RA.[4]

Multiple inflammatory cytokines are known to be produced by various types of cells such as infiltrating T and B lymphocytes and monocytes/macrophages in the inflammatory synovial tissues.[8] Several studies have reported that interaction between synoviocytes and T lymphocytes promoted cytokine production.

Bombara et al.[9] reported that cell contact between fibroblast-like synoviocytes (FLSs) and T lymphocytes induced the expression of adhesion molecules, VCAM-1 (vascular cell adhesion molecule 1, CD106) and ICAM-1 (intercellular adhesion molecule-1, CD54) on FLSs and the production of tumor necrosis factor (TNF), interferon (IFN)-γ, and interleukin (IL)-6. Min et al.[10] reported that coculture of rheumatoid synovial fibroblasts and type II collagen-reactive T cells induced the expression of IL-8, monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein 1α (MIP-1α) mainly by cell–cell contact through CD40 ligand–CD40 engagement.

Interaction between RA synoviocytes and B lymphocytes has also been reported. Shimaoka et al.[7] reported that NLCs from bone marrow and synovium of RA patients promoted the survival of human B cells and enhanced the function. Takeuchi et al.[4] demonstrated that coculture of RA-NLCs and B cells induced the production of IL-1β and TNF-α, and enhanced the production of IL-6, IL-8, and granulocyte-colony stimulating factor (G-CSF), the proliferation of B cells, and Ig production. Reparon-Schuijt et al.[11] reported that survival of synovial B cells was regulated by VCAM-1 expressed on FLSs in RA patients. Takeuchi et al.[12] demonstrated VLA-4-dependent and -independent pathways in the proinflammatory cytokine production by synovial NLCs from RA patients through cell–cell contact with MC/car, a human B-cell line. Recently our group reported that B-cell clones, obtained when RA-NLCs were established, proliferated depending on the presence of RA-NLCs and that each clone produced immunoglobulin, which recognizes human stromal cell lines from various tissues.[13]

Rheumatoid arthritis synoviocytes and monocytes/macrophages also interact. Our group reported that monocytes cultured with RA-NLCs differentiated into osteoclast precursors, which became multinucleated bone resorbing cells, i.e., osteoclasts, when supplemented with IL-3, IL-5, IL-7, granulocyte macrophage-colony stimulating factor (GM-CSF), or a combination of receptor activator of nuclear factor-κ B ligand (RANKL) and macrophage-colony stimulating factor (M-CSF).[14]

In the present study, we analyzed the interaction between RA-NLCs and monocytes/macrophages. Interaction between RA-NLCs and monocytes/macrophages requires direct cell–cell contact and induces inflammatory cytokines probably via membrane-bound TNF-α. The results suggest that this interaction plays an important role not only in destruction of joints but in induction and persistence of inflammation in RA patients.

Patients and methods

Patients

Synovial tissues were collected with informed consent from patients with RA or osteoarthritis (OA) who had undergone arthroplasty at the National Hospital Organization Sagamihara National Hospital. All patients with RA satisfied the 1987 revised diagnostic criteria of the American College of Rheumatology (formerly the American Rheumatism Association).[15] The patients with OA were diagnosed according to the ACR clinical and radiographic criteria for OA of the knee.[16]

Establishment of NLCs and FLSs from synovial tissues

RA-NLCs were established from the synovium of RA patients according to the procedure previously reported.[4] Fibroblast-like synoviocytes were similarly established from synovium of OA patients and named as OA-FLSs. Briefly, tissue specimens were finely minced and digested with a cocktail of enzymes consisting of 0.1% hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA), 0.1% type II collagenase (Sigma-Aldrich), and 0.01% DNase (Sigma-Aldrich) for 1 h in a shaking water bath at 37°C. The digested tissue specimens were filtered with a 100-μm-diameter nylon filter (Cell Strainer; BD Biosciences Discovery Labware, MA, USA) and washed twice with Hanks' Balanced Salt Solution (HBSS; Invitrogen, Tokyo, Japan). The cell pellets were resuspended in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS; Thermo Trace, Melbourne, Australia), 100 units/ml penicillin G sodium, 100μg/ml streptomycin sulfate, and 2mM L-glutamine (Invitrogen), and seeded into a tissue-culture flask (Asahi Techno Glass, Tokyo, Japan). Nonadherent cells were removed and adherent cells were maintained in humidified air containing 7.5% CO_2 at 37°C. The medium was changed twice a week and the cells were passaged when they became confluent. Homogeneous populations of stromal cells were obtained during several passages. Twelve RA-NLC and five OA-FLS lines were established from the synovium from RA and OA patients, respectively. Two RA-NLC and three OA-FLS lines were selected and used after 3–6 passages in the experiments.

To examine the ability of pseudoemperipolesis, 1×10^4 RA-NLCs or OA-FLSs were cocultured with 4×10^5 MOLT17 cells, a human lymphoma cell line (American Type Culture Collection, Rockville, MD, USA) or MC/car cells, a human B-cell line (American Type Culture Collection). After 6h of coculture, the medium was changed gently to remove nonadherent cells. Pseudoemperipolesis was determined to be positive when more than three lymphoma cells were detected under one RA-NLC or OA-FLS.

Cells located beneath a synovial cell (an RA-NLC or an OA-FLS) (pseudoemperipolesis) looked like dark round cells inside of the outline of the synovial cell body, whereas cells which attached only to a cell body or a dendritic process of a synoviocyte looked like bright round cells and were easily washed out by a pipetting medium. Two hundred synoviocytes were counted in each experiment.

Coculture of RA-NLCs and peripheral blood cells

Peripheral blood samples were collected from RA patients and healthy adults with informed consent. The specimens were immediately heparinized, overlaid on 5 ml of Lymphocyte Separating Medium (LSM; ICN Biomedicals, Aurora, OH, USA), and centrifuged at 3000 rpm for 30 min at room temperature. Peripheral blood mononuclear cells (PBMCs) were collected and washed twice with HBSS. Monocytes, CD14-negative cells, and T and B lymphocytes were isolated from PBMCs using anti-CD14, -CD3, and -CD19 antibody-conjugated MACS beads (MACS; Miltenyi Biotech, Bergisch Gladbach, Germany), respectively, according to the manufacturer's instructions. The purity of each fraction was examined using FACSCalibur (Nippon Becton Dickinson, Tokyo, Japan) after staining with respective antibodies conjugated with phycoerythrin (PE) or fluorescein isothiocyanate (FITC) (CD14-PE, CD3-FITC, and CD19-PE; Nippon Becton Dickinson). Briefly, 5×10^5 cells of each fraction was resuspended in 100 µl of the medium, and 4 µl of respective fluorescent antibody was added and incubated on ice for 30 min. After centrifugation at 11 000 rpm for 10 s, the supernatant was removed. The cells were resuspended in 500 µl of medium for examination with FACSCalibur. The purities were greater than 95%.

To examine cytokine production, 1×10^3 RA-NLCs and 4×10^4 monocytes, T or B lymphocytes, or CD14-negative cells in 200 µl of the medium were dispensed to each well of a 96-well plate. In addition, to investigate if TNF-α was involved in the induction of cytokines, 1×10^3 of RA-NLCs and 4×10^4 monocytes were cultured in 200 µl of the medium in each well of a 96-well plate, with or without anti-TNF-α neutralizing monoclonal antibody at 0.01, 0.1, or 1 µg/ml (R&D Systems, Minneapolis, MN, USA). Furthermore, 1×10^4 RA-NLCs were cultured with or without 4×10^5 monocytes on a Millicell culture plate insert (Nihon Millipore, 0.45-µm pore; Kogyo, Yonezawa, Japan) or cocultured with the same number of monocytes without Millicell in each well of a 24-well plate. Cells were cultured for 72 h at 37°C in humidified air containing 7.5% CO_2, and the supernatant fluids were collected and stored at −20°C until use.

To quantitate the mRNA of cytokines, 3×10^5 RA-NLCs and 7×10^6 monocytes were dispensed into each well of a 6-well plate. Cells were cocultured or cultured alone for 24 h. The cells were collected after trypsin/EDTA treatment (Cambrex Bio Science Walkersville, Walkersville, MD, USA) and separated into two populations, monocytes and RA-NLCs, using CD14 antibody-conjugated MACS beads.

Quantification of cytokines

Levels of inflammatory cytokines, IL-6, IL-8, IL-1β, and TNF-α were determined in culture supernatant, using an enzyme-linked immunosorbent assay (ELISA) kit (BioSource International, Camarillo, CA, USA).

The levels of mRNA of IL-6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were assessed in RA-NLCs, monocytes (cultured alone, respectively), and a mixture of these cells after a coculture. A conventional reverse transcription–polymerase chain reaction (RT-PCR) procedure was performed using Premix Tag (TaKaRa, Shiga, Japan) and LightCycler Primer Set (search-LC, Heidelberg, Germany) of human IL-6 and human GAPDH with an annealing temperature of 60°C and amplification by 25 cycles for IL-6 and 20 cycles for GAPDH.

Moreover, the levels of mRNA of IL-6, IL-8, and GAPDH were assessed in RA-NLCs and monocytes by quantitative RT-PCR (LightCycler, Roche Diagnostics, Tokyo, Japan) using LightCycler Primer Set of human IL-6 and human GAPDH, LightCycler FastStart DNA Master^{PLUS} SYBR Green I (Roche Diagnostics), according to the manufacturer's instructions. GAPDH was used as an internal control.

Statistical analysis

The difference in the percentage of pseudoemperipolesis-demonstrating cells was compared between RA-NLCs and OA-FLSs by analysis of variance (ANOVA) and Bonferroni test. These statistical methods were also used to compare the levels of cytokine production among the RA-NLCs cultured alone and those cocultured with PBMCs or a fraction of PBMCs. The levels of IL-6 and IL-8 production were compared between cocultured RA-NLCs and RA-NLCs cultured alone, and between cultures with and without anti-TNF-α monoclonal antibody (mAb) by ANOVA and Bonferroni test. The levels of IL-6 production were compared between RA-NLCs and OA-FLSs by unpaired *t*-test. The cytokine levels were compared between coculture of RA-NLCs and monocytes with and without Millicell by ANOVA and Bonferroni test. A P value of less than 0.05 was considered statistically significant.

Results

Twelve RA-NLCs and five OA-FLSs were established from synovium from patients with RA and those with OA, respectively. RA-NLC lines demonstrated a higher percentage of pseudoemperipolesis (76% ± 12% with MOLT-17, 84% ± 19% with MC/car) than OA-FLS lines (5% ± 3% with MOLT-17, 7% ± 4% with MC/car) (Table 1).

Two RA-NLCs and three OA-FLSs were selected based on the average ability of pseudoemperipolesis and used after 3–6 passages in the experiments. RA-NLCs were cultured with PBMCs for 72 h, and the levels of IL-6 in the

culture supernatant were assessed. The levels of IL-6 were 10 times higher ($P < 0.01$) in the supernatant from coculture of RA-NLCs (RA275SY) and PBMCs than in those from cultures of RA-NLCs or PBMCs cultured alone (Fig. 1).

When RA-NLCs were cultured with CD14-negative cells, CD3-positive cells and CD19-positive cells, the levels of IL-6 in the culture supernatants were 10, 23, and 31 times higher ($P < 0.01$), respectively, than that in the culture supernatants from RA-NLCs alone (Fig. 1). When RA-NLCs were cultured with CD14-positive cells, the levels of IL-6 were 200–660 times higher than the culture of RA-NLCs alone (Figs. 1 and 2) and 12000–48000 times higher than the culture of monocytes alone (data not shown). Similar results were obtained using the PBMC fractions from four

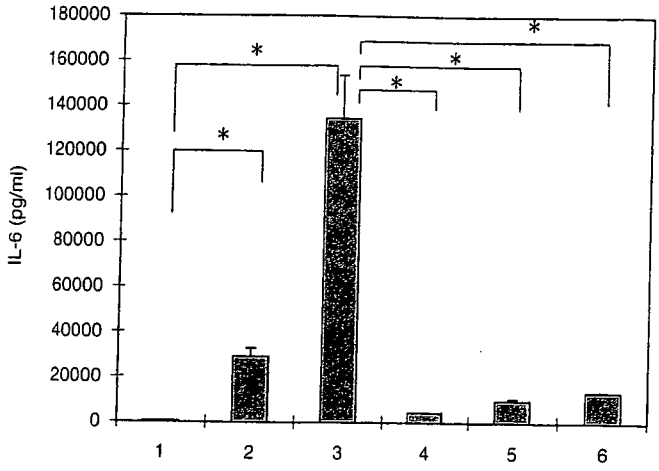

Fig. 1. Interleukin-6 (*IL-6*) production by nurse-like cells derived from rheumatoid arthritis synovium (RA-NLCs) cocultured with peripheral blood mononuclear cells (PBMCs) or fractionated cells. *1*, production of IL-6 by RA-NLCs cultured alone; *2–6*, production of IL-6 by RA-NLCs cocultured with PBMCs, CD14-positive cells, CD14-negative cells, CD3-positive cells, and CD19-positive cells, respectively. Levels of IL-6 are expressed as the mean ± SE ($n = 3$). RA275SY, one of the established cell lines, was used. 1×10^4 RA-NLCs and 2.5×10^5 cells isolated from PBMCs were cocultured or cultured alone in wells of a 24-well plate for 72h. Levels of IL-6 were assessed by an enzyme-linked immunosorbent assay (ELISA) kit. Data were statistically analyzed by analysis of variance (ANOVA) and Bonferroni test. *$P < 0.01$

Table 1. Pseudoemperipolesis of synoviocytes and lymphoma cell lines

Origin of synoviocytes	Cell lines	Pseudoemperipolesis (%) (mean ± SE)
RA ($n = 12$)	MOLT-17	76 ± 12*
OA ($n = 5$)	MOLT-17	5 ± 3
RA ($n = 12$)	MC/car	84 ± 19*
OA ($n = 5$)	MC/car	7 ± 4

1×10^4 synoviocytes established from rheumatoid arthritis (RA) and osteoarthritis (OA) as described in the text were cocultured with 4×10^5 human lymphoma cell line MOLT-17 or human B-cell line MC/car for 6h. The number of the synoviocytes was counted having more than three lymphoma cells per one synoviocyte beneath themselves. The data were examined using analysis of variance (ANOVA) and Bonferroni test

* $P < 0.05$ vs OA

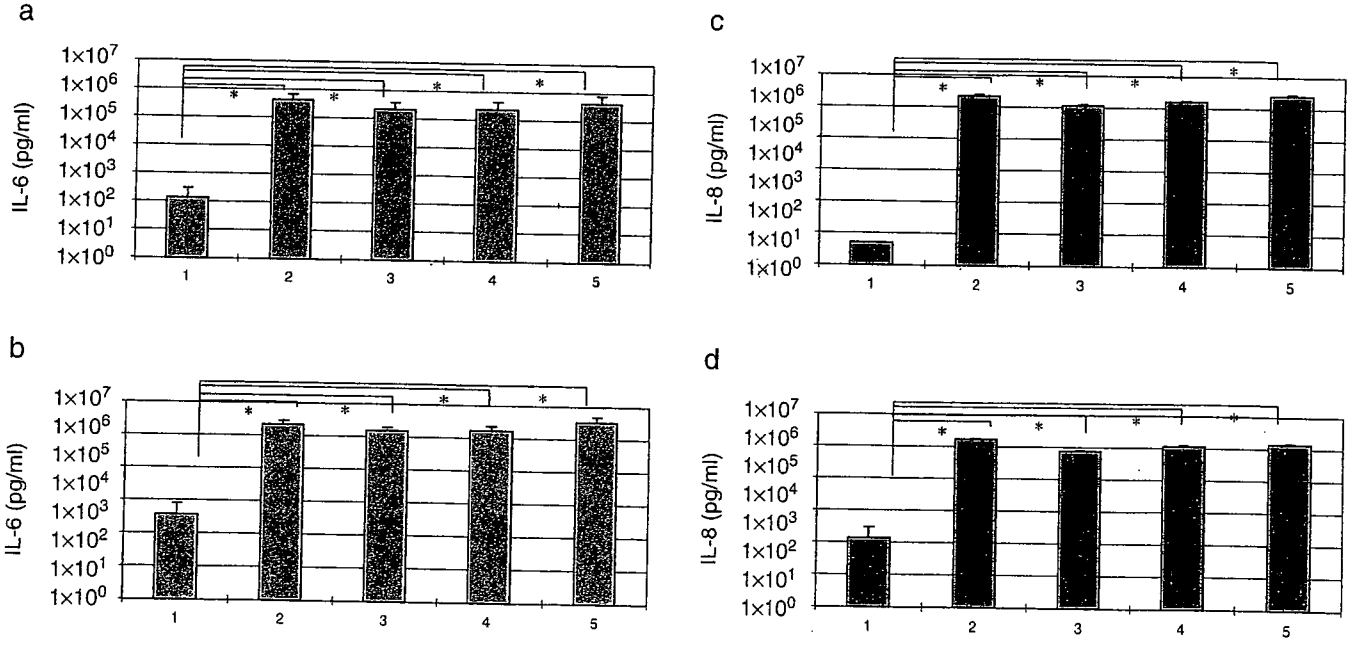

Fig. 2a–d. Production of interleukin-6 (*IL-6*) and interleukin-8 (*IL-8*) by nurse-like cells derived from rheumatoid arthritis synovium (RA-NLCs) cultured with monocytes (CD14-positive cells) from healthy donors. Levels of cytokines are expressed as the mean ± SE ($n = 3$). Data were statistically analyzed by analysis of variance (ANOVA) and Bonferroni test. **a** IL-6 production by RA275SY cultured alone (*1*) or by RA275SY cultured with monocytes from four healthy donors (*2–5*). **b** IL-6 production by RA615SY cultured alone (*1*) or RA615SY cultured with monocytes from four healthy donors (*2–5*). **c** IL-8 production by RA275SY cultured alone (*1*) or by RA275SY cultured with monocytes from four healthy donors (*2–5*). **d** IL-8 production by RA615SY cultured alone (*1*) or RA615SY cultured with monocytes from four healthy donors (*2–5*). *$P < 0.01$

donors. The levels of IL-6 were significantly higher in the coculture of RA-NLCs and monocytes than in RA-NLC culture ($P < 0.01$, Fig. 2a,b) or in monocyte culture (data not shown).

The culture supernatant was also examined for IL-8. The levels of IL-8 were also much higher in the coculture of RA-NLCs and monocytes than in RA-NLC culture and in monocyte culture, respectively (Fig. 2c,d, data not shown). Neither IL-1β nor TNF-α was detected in any of the culture supernatant fluids (data not shown).

In the next series of experiments, OA-FLSs were cultured with monocytes and the induction of IL-6 was examined. The levels of IL-6 were significantly higher in the coculture of OA-FLSs and monocytes than in OA-FLS culture ($P < 0.05$) and in monocyte culture ($P < 0.05$), respectively (Table 2). However, the levels were not as high as those in the coculture of RA-NLCs and monocytes (Table 2).

To elucidate the mechanism of cytokine production, RA-NLCs were cultured with monocytes in the presence of anti-human TNF-α mAb at 0.01, 0.1, or 1 μg/ml. Induction of IL-6 was inhibited by the mAb at 0.1 and 1 μg/ml mAb by 44% ($P < 0.05$) and 58% ($P < 0.01$), respectively. Interleukin-8 induction was also inhibited by the mAb at 0.01, 0.1 and 1 μg/ml by 44% ($P < 0.01$), 62% ($P < 0.001$), and 74% ($P < 0.001$), respectively. These results suggest that TNF-α plays a role in the induction of IL-6 and IL-8.

To examine whether direct contact is required for the interaction between RA-NLCs and monocytes, RA-NLCs and monocytes were cocultured with Millicells to inhibit contact. The level of IL-6 in the coculture of RA-NLCs and monocytes without direct contact was 285 ± 19 pg/ml, while the level in the supernatant from RA-NLCs alone was 255 ± 21 pg/ml ($P = 1.000$) (Table 3). The level of IL-8 in the

Table 2. Comparison of interleukin-6 (IL-6) production levels

Synovial cells	Monocytes	IL-6	
		pg/ml	mean ± SE
None	Healthy donor 1	5 90 5	33 ± 35
RA-NLCs			
RA615SY	None	181 280 253	238 ± 36*
	Healthy donor 1	126900 203400 331900	220733 ± 73252
OA-FLSs			
OA2823	None	63 70 71	68 ± 3*
	Healthy donor 1	451 784 814	683 ± 142
OA4615	None	5 42 5	17 ± 15*
	Healthy donor 1	588 1182 1888	1219 ± 460
OA8491	None	5 17 5	9 ± 5*
	Healthy donor 1	179 284 314	259 ± 50

Nurse-like cells derived from RA synovium (RA-NLCs) and fibroblast-like cells derived from OA synovium (OA-FLSs) were cultured without monocytes and with monocytes from healthy donors. Data were statistically analyzed by unpaired *t*-test
*$P < 0.05$ vs healthy donor 1

Table 3. Induction of interleukin-6 (IL-6) production by nurse-like cells derived from rheumatoid arthritis synovium (RA-NLCs) with or without direct interaction with monocytes

Synovial cells	Additional cells	IL-6, pg/ml (mean ± SE)
RA-NLCs	None	255 ± 21
RA-NLCs	Monocytes (separated)	285 ± 19*
RA-NLCs	Monocytes (mixed)	217000 ± 11800

1×10^4 RA-NLCs were cultured with medium in a 24-well plate with or without 4×10^5 monocytes on a Millicell culture plate insert or cocultured with the same number of monocytes without Millicell for 72 h. The data were analyzed with analysis of variance (ANOVA) and Bonferroni test
*$P < 0.05$ vs monocytes (mixed)

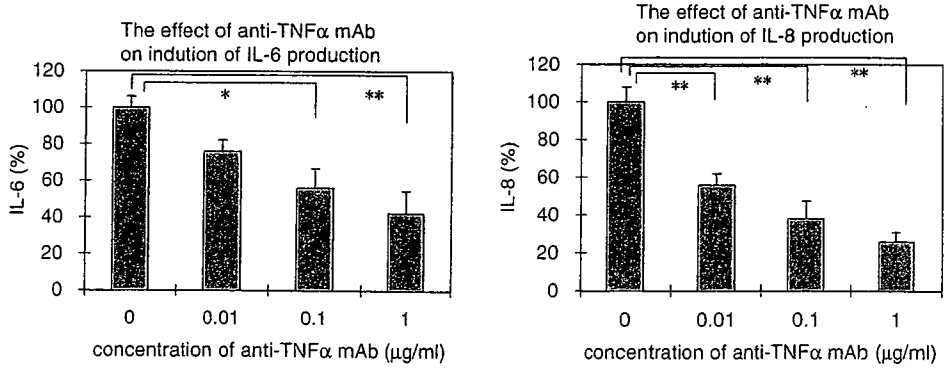

Fig. 3. Nurse-like cells derived from rheumatoid arthritis synovium RA615SY (1×10^3) and monocytes from peripheral blood mononuclear cells (PBMCs) of a healthy donor (4×10^4) were cocultured for 72 h in the presence of anti-human tumor necrosis factor α (*TNFα*) monoclonal antibody (*mAb*) at 0, 0.01, 0.1, or 1 μg/ml. Supernatant fluids were analyzed for the levels of IL-6 and IL-8 by an enzyme-linked immunosorbent assay (ELISA) kit. The levels of IL-6 and IL-8 were compared with those in the supernatant of the coculture without mAb. Data were analyzed using ANOVA and Bonferroni test. *$P < 0.05$, **$P < 0.01$

Table 4. Levels of interleukin (IL)-6 and interleukin (IL)-8 mRNAs in nurse-like cells derived from rheumatoid arthritis synovium (RA-NLCs) and monocytes that were fractionated after 24-h coculture

Cytokines	Cells	Ratio of mRNA	
		mRNA/GAPDH mRNA	Ratio
IL-6	RA-NLCs cultured alone	0.016	1
	RA-NLCs cultured with monocytes	1.189	76.5
	Monocytes cultured alone	0.025	1
	Monocytes cultured with RA-NLCs	0.131	5.2
IL-8	RA-NLCs cultured alone	0.025	1
	RA-NLCs cultured with monocytes	15.627	616
	Monocytes cultured alone	0.021	1
	Monocytes cultured with RA-NLCs	0.076	3.7

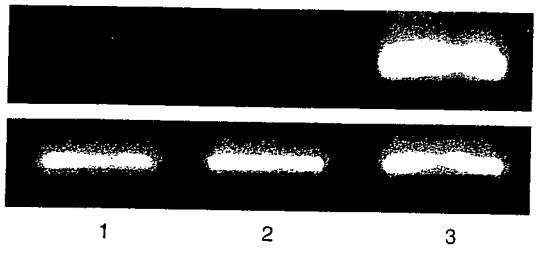

Fig. 4. Electrophoresis of polymerase chain reaction products of interleukin-6 (IL-6) (*top lane*) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*bottom lane*). The conventional revere transcription–polymerase chain reaction procedure was performed using Premix Tag (TaKaRa, Shiga, Japan) and LightCycler Primer Set (search-LC, Heidelberg, Germany) of human IL-6 and human GAPDH with annealing temperature at 60°C and amplification by 25 cycles for IL-6 and 20 cycles for GAPDH. Polymerase chain reaction products from monocytes cultured alone (*1*), nurse-like cells derived from rheumatoid arthritis synovium (RA-NLCs) cultured alone (*2*), and cocultured RA-NLCs and monocytes (*3*) are shown

coculture without direct contact was 347 ± 36 pg/ml, while that in the supernatant from RA-NLCs alone was 320 ± 25 pg/ml (P = 1.000) (Table 3). These results indicate that direct contact between RA-NLCs and monocytes is required for induction of cytokines.

Levels of mRNA were examined after RA-NLCs and monocytes were cocultured for 24 h by conventional RT-PCR (Fig. 4) and by quantitative RT-PCR (Table 4). For quantitative RT-PCR, cytokine mRNA levels were normalized using GAPDH mRNA as an internal control (Table 4). Relative levels of IL-6 and IL-8 mRNA in RA-NLCs cocultured with monocytes were approximately 80 and 620 times higher than those in RA-NLCs cultured alone, respectively (Table 4). Levels of IL-6 and IL-8 mRNA in monocytes cocultured with RA-NLCs were approximately 5 and 4 times higher, respectively, than those in monocytes cultured alone (Table 4).

Discussion

Coculture of RA-NLCs established from the synovial tissues of RA patients and monocytes freshly isolated from PBMCs of healthy donors resulted in the induction of high levels of IL-6 and IL-8. The levels of IL-6 and IL-8 were much higher when RA-NLCs were cocultured with CD14-positive cells (i.e., monocytes)[17] than when cocultured with CD14-negative cells, CD3-positive cells (i.e., T lymphocytes),[18] or CD19-positive cells (i.e., B lymphocytes).[19] The levels of IL-6 and IL-8 mRNA in RA-NLCs were also increased when cocultured with monocytes. When cultured together, RA-NLCs were more activated than monocytes, determined by the levels of IL-6 and IL-8 mRNAs in respective fractions. These results suggest that monocytes are more potent stimulators to RA-NLCs, than they are to monocytes.

Multiple inflammatory cytokines are known to be produced by various types of cells such as infiltrating T and B lymphocytes and monocytes/macrophages in the inflammatory synovial tissues.[8] Several studies have reported that interaction between synoviocytes and T and B lymphocytes promoted cytokine production.

RA-NLCs also interact with monocytes/macrophages. Our group[14] reported that monocytes differentiated into osteoclasts in two steps: cultured in the presence of RA-NLCs and then supplemented with IL-3 and distorted bones. Recently we also induced osteoclasts from CD14-positive cells in synovial fluids (SFs) from RA patients and OA patients by culturing whole cells in each SF and then with supplement of IL-3, and found that osteoclasts derived from RA-SF were larger, had more nuclei, and had more capacity of resorption pit formation on dentine slice and of resorption area formation on osteologic discs than those induced from OA-SF.[20] Chomarat et al.[21] reported that interaction of monocytes and synoviocytes from RA patients induced the expression of adhesion molecules, VCAM-1 and ICAM-1. There were reports of IL-6 production in the coculture of synoviocytes from RA patients and monocytes.[21,22] One study demonstrated that coculture of U937, monocytic cell line, and FLSs leads to enhanced production of IL-6.[23] The levels of IL-6 were, however, only three times higher in the supernatant fluids from coculture of RA synoviocytes and U937 cells than in those from cultures of RA synoviocytes alone. As U937 is an established cell line, use of monocytes freshly isolated from PBMCs is more appropriate and will provide more physiological information. Chomarat et al.[24] reported that coculture of monocytes from healthy donors and synoviocytes from RA patients resulted in IL-6 production; the levels of produced IL-6 were, however, only 15–25 times higher than the sum of those produced by monocytes and synoviocytes cultured alone. Moreover, they compared the effect of coculture of monocytes from healthy donors and synoviocytes from RA patients and that of coculture of monocytes and synoviocytes obtained from patients with knee ligament symptoms. There was no difference in the amount of IL-6 production.

The present study demonstrated that coculture of NLCs from RA patients (RA-NLCs), not FLSs from OA patients (OA-FLSs), and monocytes resulted in production of high levels of IL-6 and IL-8. The results suggest that NLCs from RA patients may have a unique property to be activated more easily than OA-FLSs and that, for RA-NLCs, monocytes are more potent stimulators than T or B lymphocytes.

Our results also indicate that direct cell–cell contact is required for the interaction between RA-NLCs and monocytes. Cytokine induction through coculture of RA-NLCs and monocytes was inhibited by anti-human TNF-α mAb. No supernatant sample contained detectable levels of TNF-α by ELISA. Monocytes/macrophages are known to be a major producer of TNF-α.[25] Tumor necrosis factor α is produced as a membrane-bound, 26-kDa proform,[26] and the mature, 17-kDa TNF subunit is released from the proform by proteolytic cleavage.[27-31] The membrane-bound TNF-α has biological activities as soluble TNF-α: inducing apoptosis, proliferation, or cytokine induction.[32] Together, it is likely that interaction between RA-NLCs and monocytes is mediated by the membrane-bound TNF-α.

The present study also demonstrated that monocytes are more potent stimulators for RA-NLCs than T or B lymphocytes. The results suggest that production of a large amount of cytokines through the interaction between RA-NLCs and monocytes may be one mechanism in the pathogenesis and maintenance of arthritis in RA. Recently, infliximab,[33] a chimeric anti-TNF-α mAb, and etanercept,[34] a soluble TNF-α receptor conjugated to Fc fragment of IgG, have been clinically applied as therapeutic reagents to RA. It is expected that these will effectively inhibit the interaction between RA-NLCs and monocytes/macrophages in inflammatory synovium in RA.

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An aspartic acid repeat polymorphism in asporin inhibits chondrogenesis and increases susceptibility to osteoarthritis

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Osteoarthritis is the most common form of human arthritis. We investigated the potential role of asporin, an extracellular matrix component expressed abundantly in the articular cartilage of individuals with osteoarthritis, in the pathogenesis of osteoarthritis. Here we report a significant association between a polymorphism in the aspartic acid (D) repeat of the gene encoding asporin (*ASPN*) and osteoarthritis. In two independent populations of individuals with knee osteoarthritis, the D14 allele of *ASPN* is over-represented relative to the common D13 allele, and its frequency increases with disease severity. The D14 allele is also over-represented in individuals with hip osteoarthritis. Asporin suppresses TGF-β–mediated expression of the genes aggrecan (*AGC1*) and type II collagen (*COL2A1*) and reduced proteoglycan accumulation in an *in vitro* model of chondrogenesis. The effect on TGF-β activity is allele-specific, with the D14 allele resulting in greater inhibition than other alleles. *In vitro* binding assays showed a direct interaction between asporin and TGF-β. Taken together, these findings provide another functional link between extracellular matrix proteins, TGF-β activity and disease, suggesting new therapeutic strategies for osteoarthritis.

Osteoarthritis (OMIM 165720) is the most common joint disease in humans, and it is a primary cause of decreased activity in daily living and quality of life after middle age. Osteoarthritis affects more than 5% of adults worldwide[1] and more than 7 million individuals in Japan alone. Osteoarthritis is a polygenic disease controlled by genetic and environmental factors, but its precise etiology is unclear. Hence, fundamental treatment is lacking. Epidemiologic studies found that genetic factors are strong determinants of osteoarthritis[2–5]. Classic twin studies showed that the influence of genetic factors is between 39% and 65% in radiographic osteoarthritis of the hand and knee in women, ~60% in osteoarthritis of the hip and ~70% in osteoarthritis of the spine[5]. Linkage and association analyses have identified several loci associated with osteoarthritis susceptibility, including the interleukin-1 gene cluster[6–8], but their functional importance has yet to be confirmed.

Osteoarthritis is characterized by progressive loss of articular cartilage in the joint. The functional integrity of the joint is maintained through a delicate balance between degradation and synthesis of the cartilage extracellular matrix (ECM)[9] through mechanisms controlled by chondrocytes. The cartilage ECM consists primarily of type II collagen and aggrecan. Both proteins contribute to the viscoelasticity of cartilage: collagen provides tensile strength, and aggrecan retains water molecules through its polyanionic constituents. A breakdown of the cartilage ECM leads to osteoarthritis, causing pain and loss of joint function.

Asporin is a recently identified ECM protein that contains a unique D repeat in its N-terminal region[10,11]. It belongs to the small leucine-rich proteoglycan (SLRP) family, members of which bind to TGF-β, a key growth factor in cartilage metabolism, and to other ECM molecules of cartilage, including collagens[12]. Previous studies implicated SLRP family genes in the etiology of osteoarthritis. Mice with a single deficiency in fibromodulin[13] or a compound deficiency in fibromodulin and biglycan[14] develop mild and severe premature osteoarthritis, respectively. Asporin mRNA is expressed abundantly in osteoarthritis articular cartilage[10]. These observations prompted us to examine *ASPN* as a candidate gene for involvement in osteoarthritis.

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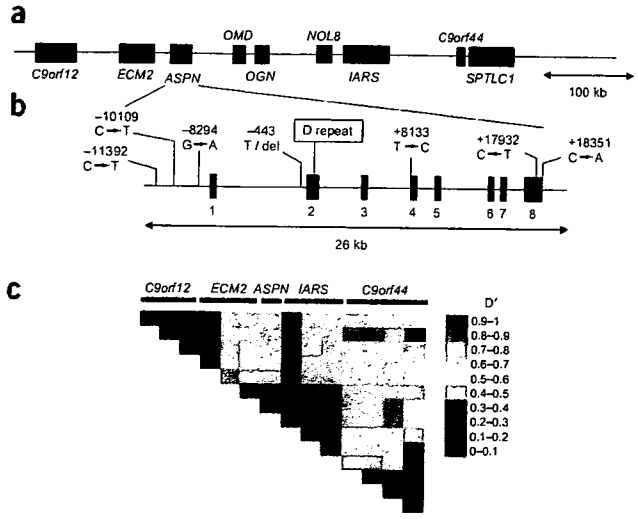

Figure 1 Genomic structure and LD of the *ASPN* region. (**a**) Gene map of the *ASPN* region. (**b**) Sequence variations in *ASPN*. Black boxes with numbers indicate exons. (**c**) Pairwise LD between 15 sequence variations in and around *ASPN* measured by the D′ value. The entire *ASPN* is contained within a single LD block.

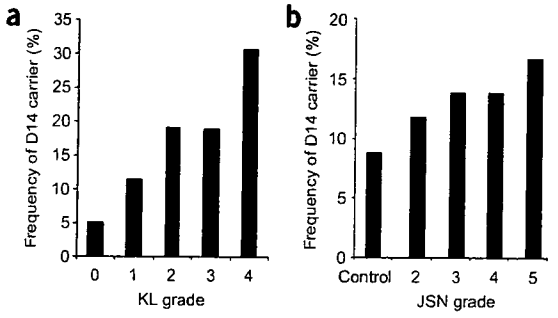

Figure 2 The frequency of individuals with the D14 variant of *ASPN* correlates with radiographic severity of knee osteoarthritis. (**a**) Cohort and (**b**) general osteoarthritis groups.

We carried out a case-control association study of *ASPN* in Japanese individuals with osteoarthritis and identified a significant association between *ASPN* and both knee and hip osteoarthritis. A functional polymorphism in *ASPN* affecting the D repeat confers susceptibility to osteoarthritis. Asporin inhibits TGF-β–mediated expression of the cartilage matrix genes, suggesting that it works as a negative regulator in chondrocyte differentiation and cartilage ECM formation.

RESULTS

Expression of *ASPN* in knee and hip joint cartilage

We examined the expression of *ASPN* in cartilage from individuals with osteoarthritis and from unaffected individuals using oligonucleotide microarray analysis. Asporin mRNA was expressed abundantly in knee and hip cartilage from individuals with osteoarthritis but was barely detectable in cartilage from unaffected individuals (**Supplementary Fig. 1** online). We reproduced these array data by real-time quantitative PCR analysis using articular cartilage samples from individuals with knee osteoarthritis ($n = 8$) and from unaffected individuals ($n = 9$) that were different from those used in the microarray analysis (**Supplementary Fig. 1** online).

Case-control association studies

To examine linkage disequilibrium (LD) in the *ASPN* region, we constructed a pairwise LD map by genotyping 658 controls for 15 SNPs in and around *ASPN* from the JSNP database[15]. We found that *ASPN* was contained completely within a single LD block (**Fig. 1**). Next, to identify sequence variations in *ASPN*, we sequenced all *ASPN* exons and their flanking regions. In genomic DNAs from 48 individuals with knee osteoarthritis, we identified 21 polymorphisms, 16 of which were new (data not shown).

We carried out two separate analyses to evaluate the association of *ASPN* with knee osteoarthritis. First, we examined a cohort containing 137 individuals with knee osteoarthritis and 234 unaffected control individuals. We genotyped eight polymorphisms that had a minor allele frequency of > 5%, including six SNPs and one deletion from the group of 21 polymorphisms identified in genomic DNA, as well as a unique D-repeat polymorphism that was previously reported[10]. The D-repeat polymorphism showed a positive association with knee osteoarthritis. We identified seven variants of this polymorphism (containing 12–18 D residues) in the cohort, the most common variant having 13 D residues (D13). In the osteoarthritis group, the variant with 14 D residues (D14) had a significantly higher frequency and the D13 variant had a significantly lower frequency (**Supplementary Table 1** online). We observed no differences in the frequencies of other alleles between the two groups and detected no associations between the other seven polymorphisms and knee osteoarthritis (**Supplementary Table 2** online). No particular haplotype showed a more significant association with osteoarthritis than the repeat polymorphism alone.

In the second analysis, we tested the association of the D-repeat polymorphism with knee osteoarthritis in an independent

Table 1 Association of D-repeat polymorphism in asporin with osteoarthritis of the knee and hip joints in Japanese individuals

	Genotype			Allele					
	D14* vs others			D14 vs others			D14 vs D13		
Groups compared	*P* value	OR	95% c.i.	*P* value	OR	95% c.i.	*P* value	OR	95% c.i.
Cohort KOA vs non-OA	0.0017	2.61	1.4–4.8	0.0013	2.49	1.4–4.4	0.00082	2.63	1.5–4.7
General KOA vs control	0.016	1.73	1.1–2.7	0.018	1.66	1.1–2.5	0.0089	1.77	1.1–2.7
Combined KOA vs non-OA + control	0.00025	1.95	1.4–2.8	0.00024	1.87	1.3–2.6	0.000066	2.00	1.4–2.8
HOA vs control	0.0039	1.84	1.2–2.8	0.0078	1.70	1.1–2.5	0.0081	1.71	1.1–2.6

*Number of individuals who are heterozygous or homozygous with respect to the allele encoding the D14 variant.
c.i., confidence interval; HOA, hip osteoarthritis; KOA, knee osteoarthritis; OA, osteoarthritis; OR, odds ratio.

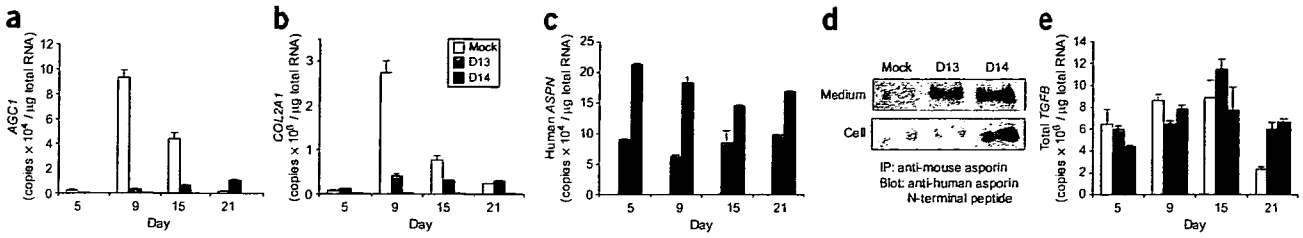

Figure 3 Effect of stable overexpression of asporin D13 and asporin D14 on the expression of cartilage marker genes during chondrogenic differentiation of ATDC5 cells. Corrected mRNA levels of (**a**) aggrecan (*AGC1*), (**b**) type II collagen (*COL2A1*), (**c**) human *ASPN* and (**e**) total *TGFB* (total quantity of TGF-β1, -β2 and -β3). Asporin inhibits chondrogenic differentiation without a decrease in TGF-β expression. Data represent the mean ± s.d. in duplicate assays. (**d**) Protein levels of asporins in the conditioned medium and in ATDC5 cells overexpressing asporin D13 and asporin D14, respectively. The asporin mRNA level corresponded to the asporin protein level in these cells.

case-control population, genotyping the polymorphism in 393 individuals with knee osteoarthritis and 374 unaffected control subjects. Both the genotypic (D14/− vs. others) and allelic (D14 vs. others) frequencies of the D14 variant were significantly higher in the osteoarthritis group (**Table 1**). The frequencies of the D14 variant in the control group of the cohort and in the general case-control population were similar. Thus, the association between asporin and knee osteoarthritis was reproduced in two studies using independent populations, indicating that the D14 variant is significantly over-represented relative to the D13 variant in individuals with osteoarthritis ($P = 0.000066$; **Table 1**).

We also examined the cohort and general populations to investigate the correlation between the frequency of the D14 variant and the severity of the knee osteoarthritis phenotype. In both populations, the frequency of individuals carrying the D14 variant increased with the grade of radiological severity of knee osteoarthritis[2,16] (**Fig. 2**). We then divided the cohort subjects into those with predominant osteophytes and little joint-space narrowing (JSN; $n = 135$) and those with predominant JSN ($n = 91$) and examined which trait was more strongly associated with *ASPN*. Frequencies of the D14 variant in the groups were 0.067 and 0.104, respectively. Therefore, JSN seems to contribute to the association more strongly. Stratification by gender showed no significant difference in genotype or allele frequency. There was no evidence of a consistent association with clinical hand osteoarthritis, although the phenotype was not well characterized.

To investigate the association between the D-repeat polymorphism and hip osteoarthritis, we genotyped 593 individuals with hip osteoarthritis. Both the genotypic and allelic frequencies of the D14 variant were significantly higher in individuals with hip osteoarthritis. The allelic and genotypic differences between the D14 and D13 variants were also significant (**Table 1**).

Asporin inhibits expression of cartilage marker genes

To examine the role of asporin in chondrocyte differentiation, we established stable clones of ATDC5 cells, an *in vitro* model for chondrogenesis[17,18], overexpressing asporin D13 or asporin D14. We selected the clonal cell lines expressing the most asporin D13 and asporin D14 mRNA. In these cell lines, recombinant asporin proteins were efficiently secreted into the medium, and protein contents in the medium were higher than those in the cells (**Fig. 3d**). The expression levels of human asporin mRNAs corresponded to those of human asporin proteins (**Fig. 3c,d**). We detected a faint band in mock-transfected cells, reflecting weak expression of the endogenous mouse asporin protein in ATDC5 cells (**Fig. 3d**). Using these cells, we examined the expression patterns of the marker genes aggrecan (*AGC1*) and type II collagen (*COL2A1*) during chondrocyte differentiation. In mock-transfected cells, expression of *AGC1* and *COL2A1* increased over time, with peak levels occurring at day 9 of culture. In the cells overexpressing asporin, however, expression of both genes was suppressed (**Fig. 3a,b**). We also investigated the effect of asporin overexpression on cartilage ECM at the protein level. At day 21 of culture, overexpression of asporin significantly inhibited proteoglycan accumulation in the ECM ($P < 0.05$; **Supplementary Fig. 2** online).

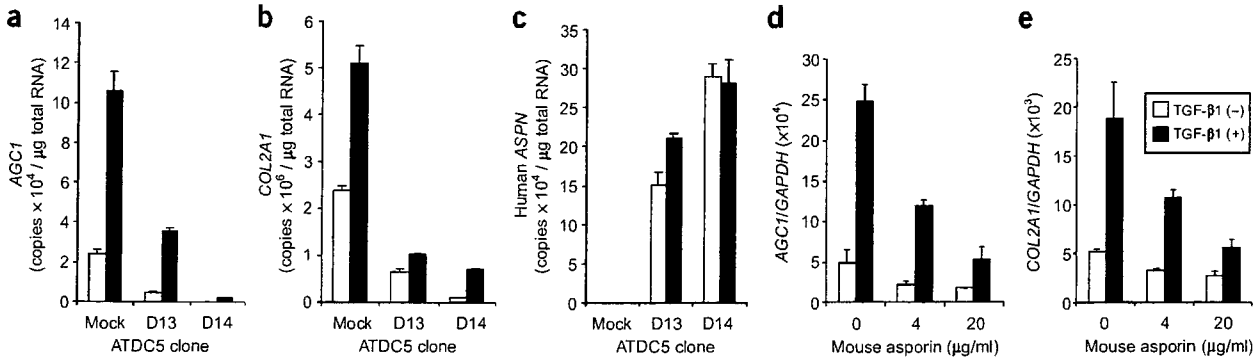

Figure 4 Asporin inhibits TGF-β1–induced and noninduced expression of cartilage matrix genes in ATDC5 cells. Effects of stable overexpression of asporin D13 and asporin D14 (**a–c**) and addition of recombinant mouse asporin (**d,e**) on TGF-β1 induction of *AGC1* (**a,d**) and *COL2A1* (**b,e**) in ATDC5 cells were investigated. Data represent the mean ± s.e.m. in quadruplicate assays.

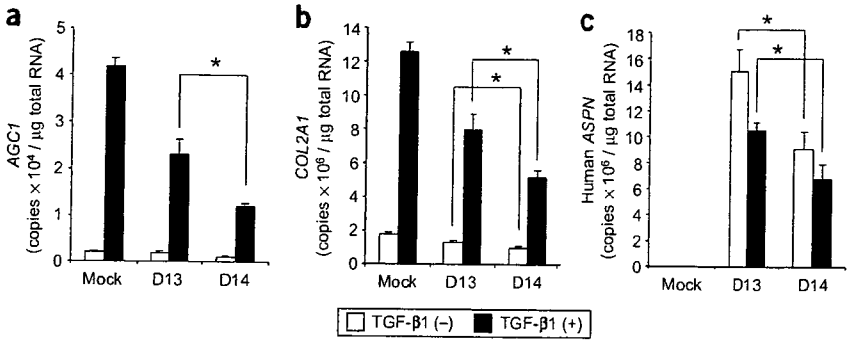

Figure 5 Asporin D14 inhibits TGF-β1–induced expression of cartilage marker genes more strongly than asporin D13. Effects of transient overexpression of asporin D13 and asporin D14 on TGF-β1 induction of *AGC1* (**a**) and *COL2A1* (**b**) in ATDC5 cells were compared. (**c**) Human asporin. $^*P < 0.05$ (Student's *t*-test). Data represent the mean ± s.e.m. in quadruplicate assays.

Asporin inhibits TGF-β–induced chondrogenesis

TGF-β is a key regulator of chondrocyte differentiation and proliferation and has an important role in the pathogenesis of osteoarthritis[9]. In differentiating ATDC5 cells, TGF-β rapidly induces transcription of the marker genes *AGC1* (ref. 19) and *COL2A1* (ref. 20). Because asporin expression downregulates these two genes in ATDC5 cells, we investigated the potential role of asporin in TGF-β–induced chondrogenesis. We measured expression levels of *AGC1* and *COL2A1* in asporin-expressing ATDC5 clones treated with TGF-β. In cells overexpressing either asporin D13 or asporin D14, TGF-β–mediated induction of *AGC1* and *COL2A1* was suppressed (**Fig. 4a–c**). In three independent clones for each asporin variant, suppression levels correlated with the expression level of asporin (data not shown), suggesting that asporin acts as a negative regulator of TGF-β signaling. There was little change in the expression of endogenous asporin mRNA between control cells and those overexpressing asporin D13 or asporin D14 (data not shown).

To investigate further the effect of asporin on TGF-β activity, we measured expression levels of *AGC1* and *COL2A1* in ATDC5 cells treated with recombinant mouse asporin in the presence or absence of exogenous TGF-β. Purified mouse asporin inhibited TGF-β–induced expression of the genes in a dose-dependent manner (**Fig. 4d,e**). These inhibitory effects of the recombinant asporin were reproduced in adult human articular cartilage chondrocyte (data not shown).

Asporin D14 has a stronger inhibitory effect on TGF-β induction

To evaluate functional differences between the D13 (osteoarthritis-protective) and D14 (osteoarthritis-susceptible) forms of asporin, we compared their inhibitory effect on TGF-β–induced expression of *AGC1* and *COL2A1* using a transient assay system. In ATDC5 cells transiently transfected with cDNA expression plasmids encoding asporin D13 or asporin D14, TGF-β induction of cartilage matrix genes was suppressed, and the D14 variant had a stronger inhibitory effect on TGF-β induction than did the D13 variant (**Fig. 5a,b** and **Supplementary Fig. 3** online). This functional difference did not result from asporin D14 having higher expression levels than asporin D13 (**Fig. 5c**).

Enhanced inhibition of TGF-β signaling is unique to asporin D14

To investigate whether the enhanced inhibition of TGF-β signaling was unique to the D14 variant, we compared the biologic activities of the D14 variant and of common larger variants (D16 and D17). These variants were less potent than the D14 variant at inhibiting TGF-β signaling (**Fig. 6**). Therefore, enhanced inhibition of TGF-β signaling is unique to the D14 variant and not simply associated with increasing length of the D repeat.

Asporin binds to TGF-β1 *in vitro*

Total levels of TGF-β expression differed little between mock-transfected cells and cells overexpressing asporin (**Fig. 3e**), indicating that asporin suppresses cartilage differentiation without affecting TGF-β expression. Observations of physical interactions between other SLRP family proteins (including decorin, biglycan and fibromodulin) and TGF-β1 (ref. 21) raises the possibility that asporin interacts with TGF-β as well. We investigated this possibility by testing the ability of *in vitro*–translated, S-tagged human asporin to bind TGF-β1. Both asporin D13 and asporin D14 coprecipitated specifically with TGF-β1 in the presence of S-protein agarose (**Fig. 7a**). We also assayed the ability of asporin to bind TGF-β1 in a solid-phase binding assay. Biotinylated recombinant mouse asporin bound effectively to TGF-β1 coated on microplate wells. In the presence of unlabeled competitor, asporin binding decreased in a dose-dependent manner (**Fig. 7b**). Taken together, these results confirm a direct *in vitro* interaction between asporin and TGF-β1.

DISCUSSION

Using a case-control association study, we identified *ASPN* as a susceptibility gene for osteoarthritis in the Japanese population. We showed that asporin expression is substantial in cartilage of individuals with osteoarthritis and is greater in these individuals than in cartilage from unaffected individuals. We also showed that asporin acts as a negative regulator of chondrogenesis *in vitro* by inhibiting TGF-β function, probably through a direct physical interaction with TGF-β.

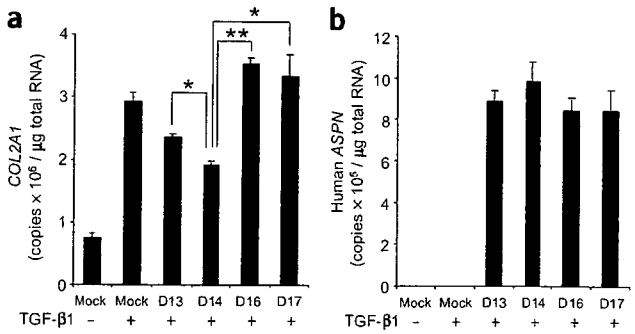

Figure 6 Enhanced inhibition of TGF-β signaling is unique to the D14 variant. Effect of transient overexpression of asporin D14 on TGF-β1 induction of *COL2A1* (**a**) in ATDC5 cells was compared with that of asporin D13, asporin D16 and asporin D17. (**b**) Human asporin. $^*P < 0.05$; $^{**}P < 0.01$ (Student's *t*-test). Data represent the mean ± s.e.m. in quadruplicate assays.