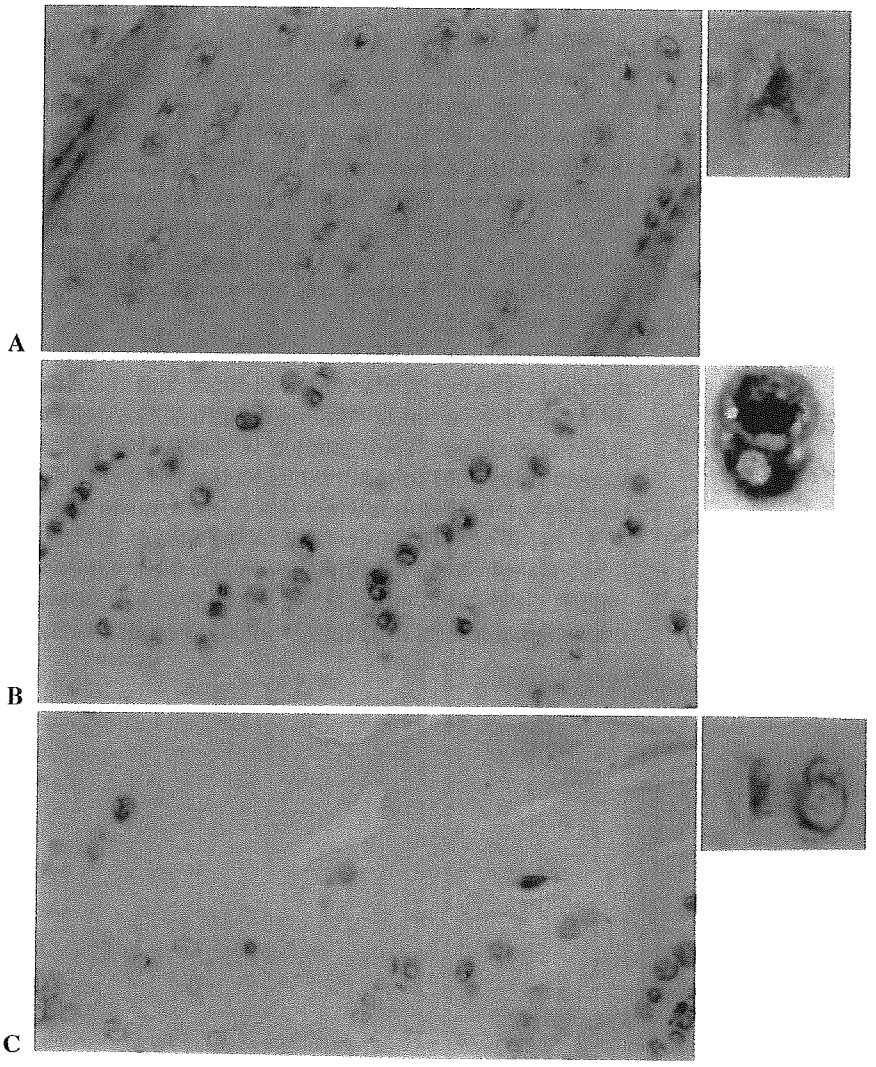

Fig. 10. In situ hybridization of type II collagen mRNA in articular cartilage 2 weeks after surgery. mRNA expression is stronger in the chondrocytes of the para-degenerative region than in those of the intact and central-degenerative regions. **A** Intact region. **B** Para-degenerative region. **C** Central-degenerative region. **A–C** ×100. **Insets** ×400

analyzed the relation between the progression of OA and the metabolism of type II collagen. When the pCOL II-C level in synovial fluid from the knee joints of humans with OA was analyzed, the progression of early OA to moderate OA was found to be associated with an elevated concentration of pCOL II-C in the synovial fluid; as the OA increased in severity, however, the pCOL II-C level decreased.[4,5,12] Related to these findings, Kobayashi et al. reported that the degree of pCOL II-C staining and the synovial fluid concentration decreased in accordance with the progression of articular cartilage regeneration observed after surgical treatment using a high tibial osteotomy for human OA.[13]

A conclusion similar to that made by Nakajima et al. can be drawn from the histopathological findings of the present study using a rabbit OA model. The present study revealed that the main site of pCOL II-C expression shifts from the center of degeneration toward the para-degenerative region, suggesting that the potential to synthesize type II collagen is higher in the para-degenerative region than in the most degenerated central-degenerative region. During the early stages of degeneration, when the extent of degeneration is mild even in the central-degenerative region, the synthesis of type II collagen is enhanced at the center of degeneration. As the degeneration increases in severity, however, the synthesis of type II collagen increases in the peripheral regions (where degeneration is still relatively mild) and decreases in the central-degenerative region (where the degeneration has already become severe).

None of the previously reported studies simultaneously analyzed the responses to immunostaining for pCOL II-C and the expression of type II collagen mRNA in the articular cartilage of either OA-affected humans or OA animal models. Of the studies reported to date, reports on the distribution of type II collagen synthesis in the articular cartilage of humans with OA

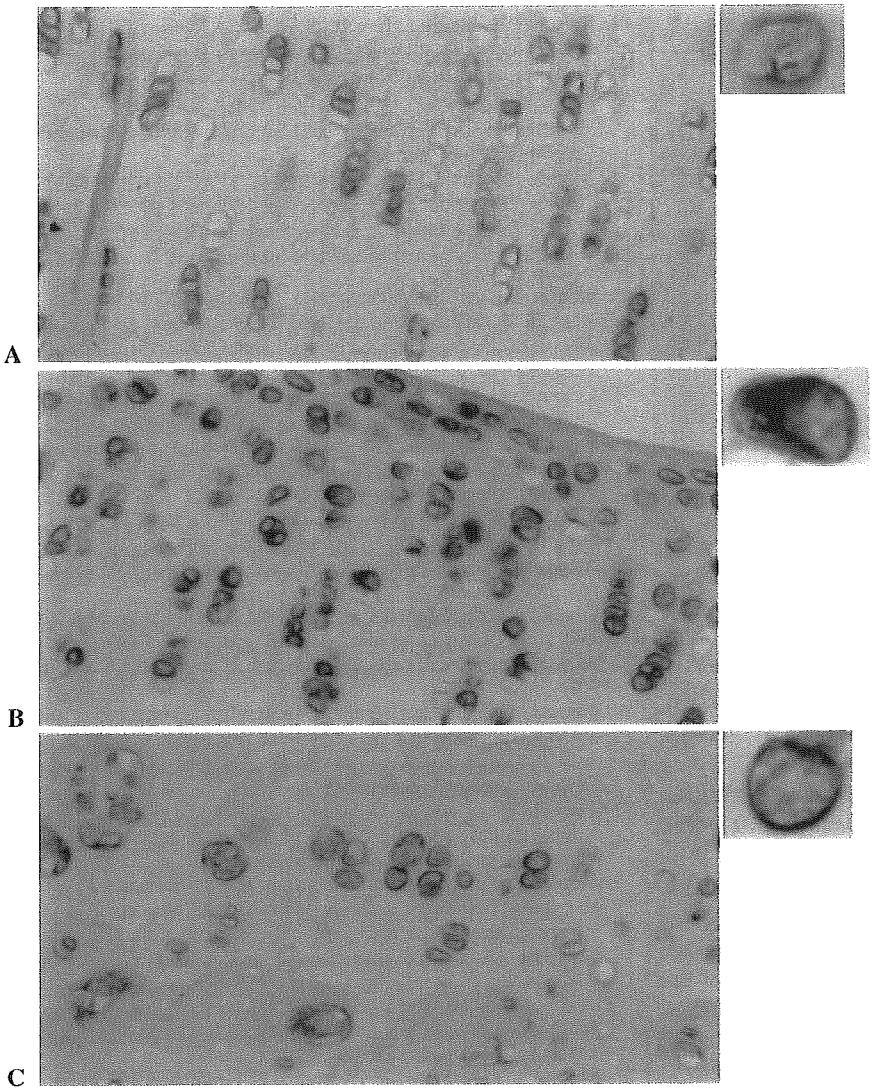

Fig. 11. In situ hybridization of type II collagen mRNA in articular cartilage 4 weeks after surgery. mRNA expression is stronger in the para-degenerative region than in the intact and central-degenerative regions. **A** Intact region. **B** Para-degenerative region. **C** Central-degenerative region. **A–C** ×100. **Insets** ×400

have revealed that in patients with moderate degeneration the expression of type II collagen mRNA in the chondrocytes increased from the intermediate to the deeper layers of articular cartilage[14] and that the area showing enhanced mRNA expression was approximately identical to that in the extracellular matrix region, which exhibited increased immunostaining for type II collagen.[15,16] Furthermore, an increase in type II collagen mRNA expression has been reported in the osteophytes of articular cartilage in humans with OA, and this increase in mRNA expression was related to an increased response of the extracellular matrix in the same region to immunostaining for type II collagen.[17,18] In OA models, the increase of immunostaining intensity of degenerated type II collagen has been also reported in the degenerated area of articular cartilage.[19–22] In the present study, the regions showing increased rates of pCOL II-C immunostaining in the chondrocytes were almost identical to the regions showing enhanced expression of type II procollagen mRNA. This finding provides additional support to the view that the increase in pCOL II-C staining in the chondrocytes reflects an increase in the synthesis of type II procollagen.

When examined using immunoelectron microscopy, gold particles, which represent the anti-pCOL II-C antibody, were detected on the rough endoplasmic reticulum (RER) of the chondrocytes. This finding suggests that this antibody is bound to the C-peptide of type II procollagen being synthesized on the RER. Gold particles were also observed in the nuclei, although the significance of this finding remains obscure. If this finding is interpreted as being suggestive of the active uptake of pCOL II-C by the nuclei, pCOL II-C may have some regulatory function, such as acting as a transcription factor. Nakata et al. reported that one of the DNA-binding proteins (binding to the type II collagen

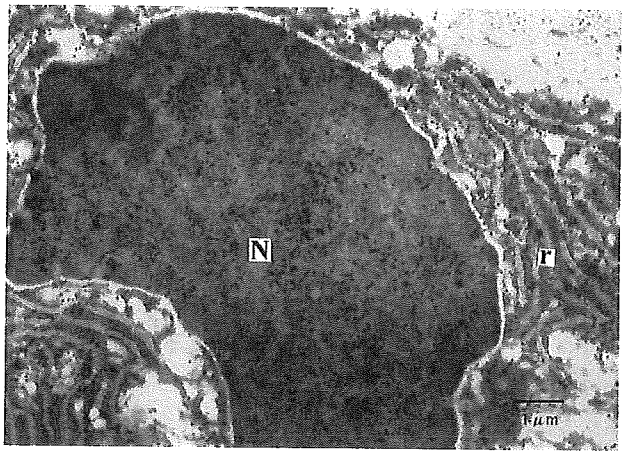

Fig 12. Immunoelectron microscopy image of the ultrastructural distribution of pCOL II-C in the chondrocytes of the articular cartilage in an OA-induced rabbit model 2 weeks after surgery. Gold particles are visible in the cytoplasm and nuclei along the rough endoplasmic reticulum. *N*, nucleus; *r*, rough endoplasmic reticulum. *Bar* 1 μm

enhancer) was identified as pCOL II-C and that pCOL II-C suppresses transcription of the type II collagen gene.[23]

Osteoarthritis may be understood as a condition where the balance between the destruction and repair of articular cartilage is biased toward destruction. The results of the present study using a rabbit model of OA also suggest that the repair potential of chondrocytes continues to exist even as cartilage destruction advances, and that this potential varies according to the location of the cartilage relative to the point of maximum destruction.

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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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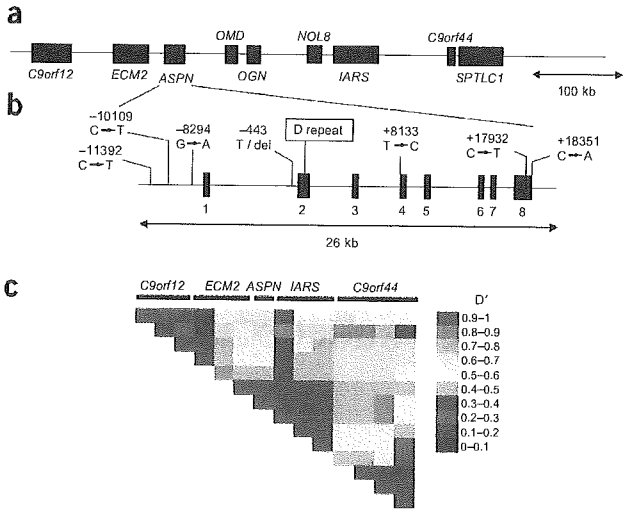

Figure 1 Genomic structure and LD of the *ASPN* region. (**a**) Gene map of the *ASPN* region. (**b**) Sequence variations in *ASPN*. Black boxes with numbers indicate exons. (**c**) Pairwise LD between 15 sequence variations in and around *ASPN* measured by the D' value. The entire *ASPN* is contained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

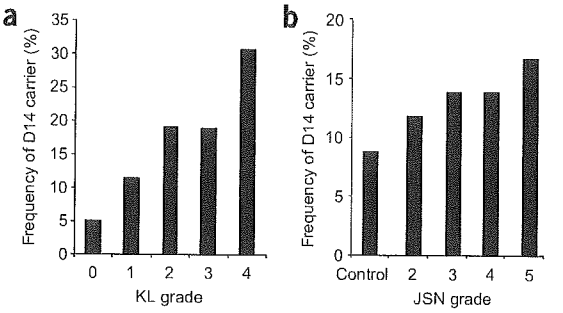

Figure 2 The frequency of individuals with the D14 variant of *ASPN* correlates with radiographic severity of knee osteoarthritis. (**a**) Cohort and (**b**) general osteoarthritis groups.

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.

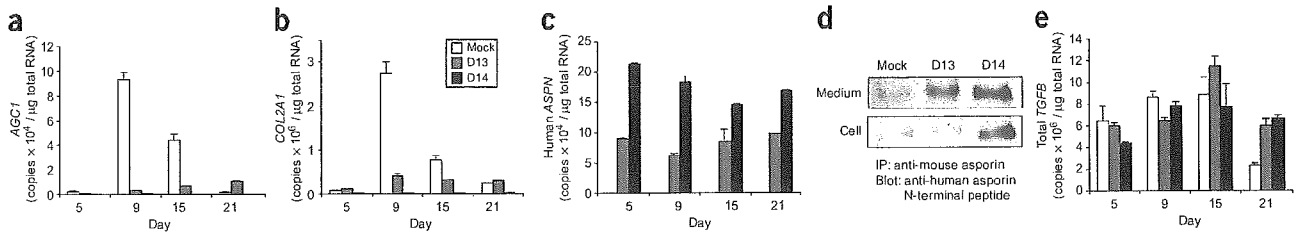

Figure 3 Effect of stable overexpression of asporin D13 and asporin D14 on the expression of cartilage marker genes during chondrogenic differentiation of ATDC5 cells. Corrected mRNA levels of (**a**) aggrecan (*AGC1*), (**b**) type II collagen (*COL2A1*), (**c**) human *ASPN* and (**e**) total *TGFB* (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).

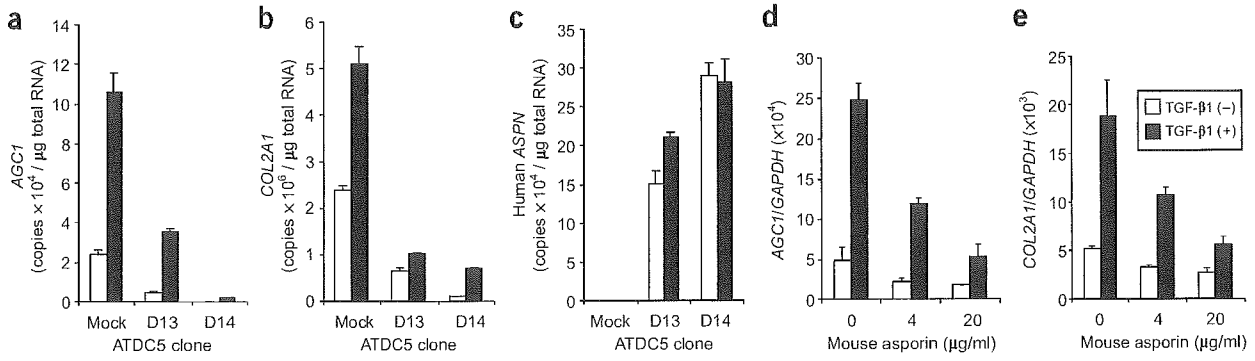

Figure 4 Asporin inhibits TGF-β1–induced and noninduced expression of cartilage matrix genes in ATDC5 cells. Effects of stable overexpression of asporin D13 and asporin D14 (**a–c**) and addition of recombinant mouse asporin (**d,e**) on TGF-β1 induction of *AGC1* (**a,d**) and *COL2A1* (**b,e**) in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

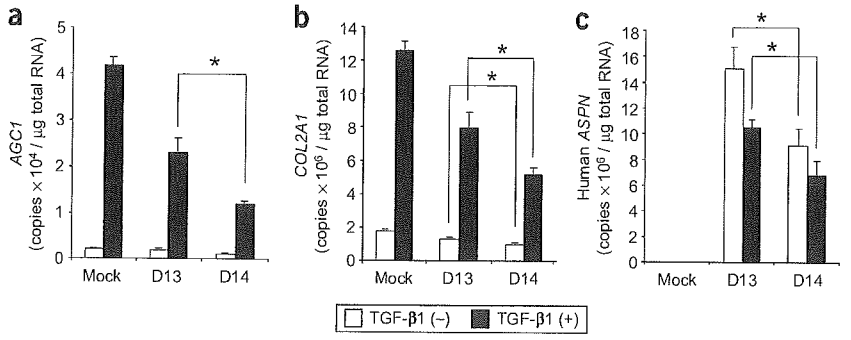

Figure 5 Asporin D14 inhibits TGF-β1–induced expression of cartilage marker genes more strongly than asporin D13. Effects of transient overexpression of asporin D13 and asporin D14 on TGF-β1 induction of *AGC1* (**a**) and *COL2A1* (**b**) in ATDC5 cells were compared. (**c**) Human asporin. $^*P < 0.05$ (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.

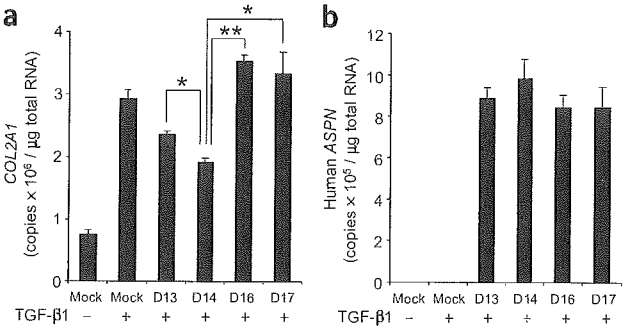

Figure 6 Enhanced inhibition of TGF-β signaling is unique to the D14 variant. Effect of transient overexpression of asporin D14 on TGF-β1 induction of *COL2A1* (**a**) in ATDC5 cells was compared with that of asporin D13, asporin D16 and asporin D17. (**b**) Human asporin. $^*P < 0.05$; $^{**}P < 0.01$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

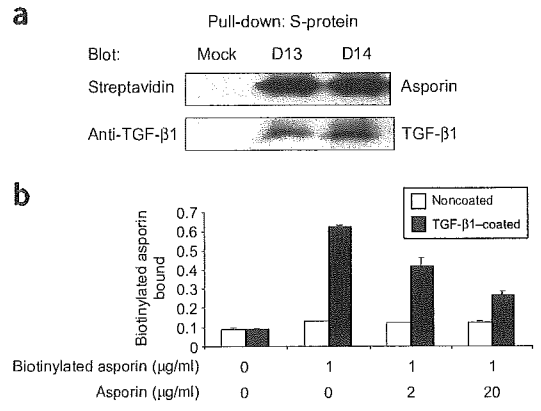

Figure 7 Asporin binds to TGF-β1 *in vitro*. (**a**) S-protein pull-down assay. *In vitro*–translated, S-tagged human asporin was incubated with recombinant human TGF-β1. Coprecipitated S-tagged biotinylated asporin was detected with streptavidin-HRP (upper panel), and TGF-β1 was detected with a biotinylated antibody to TGF-β1 (lower panel). (**b**) Solid-phase binding assay. Microplate wells coated or noncoated with recombinant human TGF-β1 were incubated with biotinylated and unlabeled recombinant mouse asporin purified from *E. coli*. Binding of biotinylated asporin to TGF-β1 was quantified by colorimetric assay at $A_{405\ nm}$ using streptavidin-HRP.

TGF-β is a multifunctional cytokine involved in numerous essential biological processes, including development, ECM synthesis, cell proliferation and differentiation, and tissue repair. Several lines of evidence suggest that TGF-β signaling is crucial for maintaining articular cartilage and preventing osteoarthritis, and that it may be a beneficial factor in cartilage repair. First, injection of TGF-β into naive murine knee joints increases the synthesis and accumulation of proteoglycan in articular cartilage[22]. Conversely, inhibition of endogenous TGF-β by treatment with recombinant soluble TGF-β type II receptor in experimental models of osteoarthritis markedly enhances proteoglycan loss in articular cartilage and reduces the thickness of articular cartilage[23]. In mouse skeletal tissue, overexpression of a dominant-negative TGF-β type II receptor promotes terminal chondrocyte differentiation and osteoarthritis[24]. Finally, targeted disruption of Smad3, the cardinal mediator of TGF-β signaling, produces degenerative joint disease mimicking human osteoarthritis, characterized by a progressive loss of articular cartilage and decreased production of proteoglycans[25]. These findings indicate that the TGF-β signal is crucial for maintaining articular cartilage and preventing osteoarthritis and that suppression of TGF-β signaling in chondrocytes probably leads to osteoarthritis.

TGF-β interacts with a number of cartilage ECM proteins, but the relationship between these proteins and TGF-β action in chondrocytes and its functional importance in chondrogenesis are unclear. But disruption of the regulatory relationship between ECM proteins and TGF-β activity contributes to several disease phenotypes. A relationship between dysregulation of TGF-β activity by fibrillin and pulmonary emphysema has been reported[26]. In addition, the SLRP family protein decorin acts as a negative regulator of TGF-β in the kidney and prevents glomerulonephritis in a rat model[27,28]. The ability of asporin to bind TGF-β directly and inhibit TGF-β–induced chondrogenesis provides another functional link between ECM proteins, TGF-β activity and disease. Our data indicate that TGF-β regulation by an ECM protein also accounts for the etiology and pathogenesis of osteoarthritis. Our data also suggest that agents controlling and modifying the TGF-β–ECM system are promising targets for treatment, offering a new therapeutic strategy for osteoarthritis.

The D-repeat polymorphic variants of asporin differ in their ability to suppress TGF-β signaling during chondrogenesis. The D repeat in asporin lies adjacent to a Zn^{2+}-binding domain that is important to conformational regulation in decorin[29]. Decorin binds fibrinogen, fibronectin and types I, IV and V collagens in a Zn^{2+}-dependent manner[30]. We therefore speculate that the D-repeat polymorphism in asporin may mediate conformational changes that consequently produce functional differences. Alternatively, the D repeat itself might affect asporin function; a D repeat contained in osteopontin functions as a Ca^{2+}-binding domain[31]. Determining structure-function relationships in asporin and assessing the contribution of the D repeat to asporin function will be the focus of future work.

The basic role of asporin in the pathogenesis of osteoarthritis remains to be examined. The reduced responsiveness to TGF-β caused by asporin may cause a mild chondrodysplasia with a consecutive osteoarthritis phenotype, with the main lesion occurring during cartilage development. Alternatively, asporin may have a major role in cartilage matrix homeostasis in the adult as a modulator of cell and ECM regeneration. There is no evidence for chondrodysplasia in our populations, and we found that asporin had a similar effect in adult articular cartilage chondrocyte as in ATDC cells. These observations support the latter hypothesis. We speculate that growth factors including TGF-β would be induced in the cellular response to tissue damage and would participate in regeneration of cartilage. In this process, asporin would act as a crucial modulator of TGF-β, fine-tuning its function. Derangement of the control mechanism (*e.g.*, too much inhibition of TGF-β activity by the disease-associated allele) would progress osteoarthritis more quickly. The allelic difference in the biological effects of asporin may result from the difference in binding intensity to TGF-β or from its effects on TGF-β receptors and not on TGF-β itself. Asporin might hinder the binding of TGF-β to its receptors or the ligand-induced dimerization of the receptors. Clarifying the mechanism would lead to the understanding of the functional link between ECM proteins, TGF-β activity and disease.

METHODS

High-density oligonucleotide microarray analysis. We carried out GeneChip analysis in accordance with a standard protocol (Affymetrix). We purchased total RNAs from osteoarthritis and normal cartilage from Direct Clinical Access and used 5–10 μg of total RNAs to prepare biotinylated cRNAs for hybridization. We hybridized biotinylated cRNAs to GeneChip Array Sets U-95 and U-133 (Affymetrix) using standard conditions. We scanned arrays with a Confocal Scanner (Molecular Dynamics) and normalized the array-hybridization signal using the median value among the signal intensities of all genes identified as 'present' by GeneChip analysis software.

Human articular cartilage samples. We obtained osteoarthritis cartilage from knee of eight individuals with osteoarthritis during surgery (total knee arthroplasty). We obtained normal cartilage from the femoral head of nine control subjects during surgery for femoral neck fractures. None of the control subjects had a clinical history of joint diseases or any radiographic sign of osteoarthritis. The samples were immediately frozen in liquid nitrogen after resection and stored at −80 °C.

Subjects. All individuals recruited for this study were Japanese and received clinical and radiographic examinations by orthopedic specialists. Osteoarthritis was diagnosed on the basis of clinical and radiographic findings. Rheumatoid arthritis and polyarthritis associated with autoimmune diseases were excluded, as were post-traumatic osteoarthritis and infection-induced osteoarthritis. Individuals who had clinical and radiographic findings suggestive of skeletal dysplasias, including overt short stature, multiple symmetric involvements of

epiphyses and a definitely positive mendelian family history, were also excluded from the study.

We recruited a population-based cohort (n = 371) from inhabitants of Miyagawa village in Mie prefecture, which is located in the middle of mainland Japan (Honshu). For each individual, we took standard three-direction (*i.e.*, antero-posterior, lateral and skyline view) knee radiographs. Using the grading system of Kellgren and Lawrence (KL grade)[2], we classified the subjects as having (2–4 KL grade; n = 137, 72% female; mean age ± s.d. = 75.3 ± 5.1 years) or not having (0–1 KL grade; n = 234, 61% female; mean age ± s.d. = 73.6 ± 5.3 years) knee osteoarthritis. We also examined clinical parameters that have been reported to confound results[3,4,32,33], including family history, body mass index and complications of clinical hand osteoarthritis (Heberden's node; **Supplementary Table 3** online).

We recruited case-control subjects from individuals who lived in or around Tokyo, also located in mainland Japan, and visited the participating clinical institutions. We studied a total of 393 individuals with knee osteoarthritis (84% female; mean age ± s.d. = 72.5 ± 7.4 years), 593 individuals with hip osteoarthritis (93% female; mean age ± s.d. = 58.3 ± 10.1 years) and 374 controls (56% female; mean age ± s.d. = 28.8 ± 11.9 years). All individuals with osteoarthritis were symptomatic and were treated in participating institutions on a regular basis. For each individual with knee osteoarthritis, we took standard three-direction knee radiographs, and for each individual with hip osteoarthritis, we took antero-posterior radiographs; we assessed JSN and osteophytes in both groups. We graded individuals with knee osteoarthritis by the JSN scale, which was described previously as a modification of KL grade[16]. We included in the case group only those individuals who had knee osteoarthritis of JSN grade three or higher. The criteria for hip osteoarthritis were described previously[34].

The study protocol was approved by the ethical committees of the participating institutions (SNP Research Center of RIKEN, Mie University, The University of Tokyo, Kyorin University, Tokyo Teishin Hospital and Tokyo Metropolitan Geriatric Hospital), and written informed consent was obtained from each participant. We obtained blood samples from the participants and prepared genomic DNA from peripheral leukocytes in accordance with standard protocols.

Genotyping of the D-repeat polymorphism and SNPs. We separated 5′-FAM–labeled PCR products containing the D-repeat polymorphisms of asporin by size on an ABI PRISM 3700 DNA Sequencer (Applied Biosystems) against the Genescan-500LIZ size standard. We collected data using GeneScan Analysis 3.5 software (Applied Biosystems), and allelic assignment was semiautomated using Genotyper 3.7 software (Applied Biosystems). We genotyped SNPs using the TaqMan assay[35].

Statistical analysis. We assessed association and Hardy-Weinberg equilibrium by the χ^2 test. We calculated odds ratios and 95% confidence intervals with respect to the minor allele compared with the major allele. We calculated LD coefficients (D′) as described previously[36]. We estimated haplotype frequencies using the expectation-maximization algorithm[37].

Cell culture and transfections. We maintained ATDC5 cells in a standard medium of Dulbecco's modified Eagle medium (DMEM)-F12 containing 5% fetal bovine serum (FBS) at 37 °C under 5% CO2. For transient transfections, we plated 5 × 10^4 cells per well in a 12-well plate and grew them continuously in standard medium containing insulin-transferrin-sodium selenite media supplement (ITS, Sigma-Aldrich). After 24 h, we transfected cells with pcDNA3.1(−)-asporin containing D13, D14, D16 or D17 using FuGENE6 (Roche) in accordance with the manufacturer's instructions. We replaced the culture medium with DMEM-F12 containing 0.2% FBS and ITS 24 h after transfection. After 12 h, we treated cells with 10 ng ml^{-1} TGF-β1 for 16 h.

For stable transfections, we plated 6 × 10^4 cells per well in a 60-mm dish and grew them continuously in standard medium. After 24 h, we transfected cells with pcDNA3.1(−)-asporin containing D13 or D14 using FuGENE6. We replaced the culture medium with DMEM-F12 containing 5% FBS and G418 (500 μg ml^{-1}) 48 h after transfection. After 7 d, we diluted cells, plated them in a 96-well plate and cloned them by limiting dilution. We evaluated *ASPN* expression using real-time PCR. We selected the three clones showing the highest levels of expression of the asporin D13 and asporin D14 mRNAs. Because these clones showed the strongest inhibition of expression of *AGC1* and *COL2A1*, we used them for all subsequent experiments.

To examine the effects of asporin on TGF-β1–induced matrix gene expression, we cultured ATDC5 cells until they reached confluence. At this point, we replaced the standard medium with DMEM-F12 containing ITS, 0.2% FBS and G418 (500 μg ml^{-1}). After 12 h, we treated cells with TGF-β1 (10 ng ml^{-1}) for 16 h. For induction of chondrogenesis, we plated 4 × 10^4 cells per well in 12-well plates and grew them continuously in DMEM-F12 containing 5% FBS, ITS and G418 (500 μg ml^{-1}) at 37 °C under 5% CO2. We replaced the growth medium every other day.

Real-time PCR. We extracted total RNAs from cartilage samples or cells using Isogen (Nippongene) and purified them using an SV-Total RNA Isolation System (Promega). We synthesized random-primed cDNA using Multiscribe reverse transcriptase (PE Applied Biosystems). We carried out real-time PCR on an ABI PRISM 7700 (Applied Biosystems) using QuantiTect SYBR Green PCR (QIAGEN) in accordance with the manufacturer's instructions.

Immunoprecipitation. We cultured ATDC5 cells stably overexpressing asporin D13 and asporin D14 in the growth medium until they were ~50% confluent. We then discarded the growth medium and cultured the cells continually for 48 h with serum-free DMEM-F12 containing ITS and G418 (500 μg ml^{-1}). We centrifuged the culture media, concentrated the supernatants (conditioned media) with Amicon Ultra-15 10,000MWCO (MILLIPORE) and incubated them (0.4 ml) with 0.5 ml of BlockAce (Dainippon Pharmaceutical), 20 μl of polyclonal rabbit antibody to mouse asporin (2229-B01; recognizes mouse and human asporin) and 50 μl of Protein A-Sepharose CL-4B (Amersham Biosciences) in a rotator at 4 °C for 18 h. We collected the immunoprecipitated complex by centrifugation and washed it five times with ice-cold Buffer SNP (1% Nonidet P-40, 150 mM NaCl and 50 mM Tris-HCl (pH 8.0)). We eluted the bound proteins with 0.1 M glycine-HCl (pH 3.0). We neutralized eluted samples with 1:10 volumes of 1 M Tris-HCl (pH 8.0), separated them by SDS-PAGE and blotted them onto PVDF membranes.

Western blotting. As a primary antibody, we used a rabbit antibody against human asporin N-terminal peptide (NSLFPTREPRSHF; 2210-B02; recognizes mouse and human asporin). The antibody was biotinylated using Biotin Labeling Kit (Roche) in accordance with the manufacturer's instructions. To visualize the immune complex we used streptavidin–horseradish peroxidase (HRP) (Roche).

Measurement of cartilage proteoglycan. After 21 d of culture, ATDC5 cells overexpressing human asporin containing D13 or D14 were fixed with methanol and stained with 0.1% alcian blue (8GX, Sigma) in 3% acetic acid for 2 d at 4 °C. After washing them three times with distilled water, we photographed cells and then extracted them with 6 M guanidine-HCl. We measured the amount of dye associated with cartilage matrix at 630 nm using Ultramark Microplate Imaging System (Bio-Rad).

Expression and purification of recombinant mouse asporin. We cloned a cDNA encoding mature (lacking pre- and propeptide) mouse asporin into a pET29b expression vector and expressed it in the *Escherichia coli* Rosetta (DE3) pLys strain. We solubilized recombinant protein from inclusion bodies, renatured it using the Protein Refolding Kit (Novagen) and purified it by gel filtration using a Superose 12 column (Amersham Pharmacia).

***In vitro* translation and S-protein pull-down assay.** S-tagged human cDNAs encoding asporin D13 or asporin D14 were transcribed and translated *in vitro* by the TNT system (Promega) in the presence of Transcend Biotin-Lysyl-tRNA in accordance with the manufacturer's instructions. For each experiment, we incubated *in vitro*–translated, S-tagged asporin (10 μl) with 0.1 μg of recombinant human TGF-β1 (Genzyme) for 1 h at 4 °C in 0.3 ml of binding buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100 and Complete protease inhibitor cocktail (Roche)). We added 12.5 μl of S-protein–agarose (Novagen) to the reaction and incubated it for 30 min at room temperature. We washed precipitates three times with binding buffer and subjected them to

12.5% SDS-PAGE. We detected coprecipitated proteins by western blotting using biotinylated antibody to TGF-β1 (Genzyme) and streptavidin-HRP (R&D Systems).

Solid-phase binding assay. We coated Maxisorp ELISA plate (Nunc) wells with 100 μl of 1 μg ml^{-1} recombinant human TGF-β1 in 50 mM $NaHCO_3$ buffer (pH 9.6) at 4 °C overnight. We then blocked the wells with 200 μl of Blockace (Dainippon Pharm.) at 4 °C overnight, added unlabeled and biotinylated mouse asporin to the wells in a total volume of 100 μl of Blockace and incubated them at 4 °C overnight. We washed the wells twice with 20 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.05% Tween 20 and incubated them with streptavidin–alkaline phosphatase. After washing five times with 20 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.05% Tween 20, we assayed the bound phosphatase using Alkaline phosphatase Substrate Kit (Bio-Rad).

Accession numbers. Human asporin mRNA, NM_017680; mouse asporin mRNA, NM_025711.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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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, 100 μg/ml streptomycin sulfate, and 2 mM 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 6 h 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

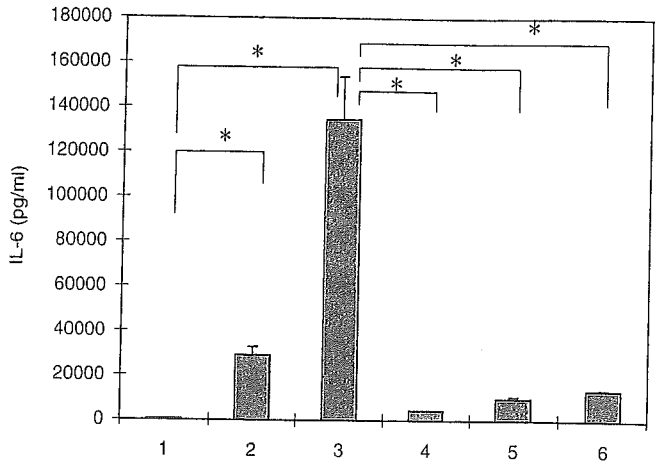

Fig. 1. Interleukin-6 (*IL-6*) production by nurse-like cells derived from rheumatoid arthritis synovium (RA-NLCs) cocultured with peripheral blood mononuclear cells (PBMCs) or fractionated cells. *1*, production of IL-6 by RA-NLCs cultured alone; *2–6*, production of IL-6 by RA-NLCs cocultured with 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 72 h. 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 6 h. 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

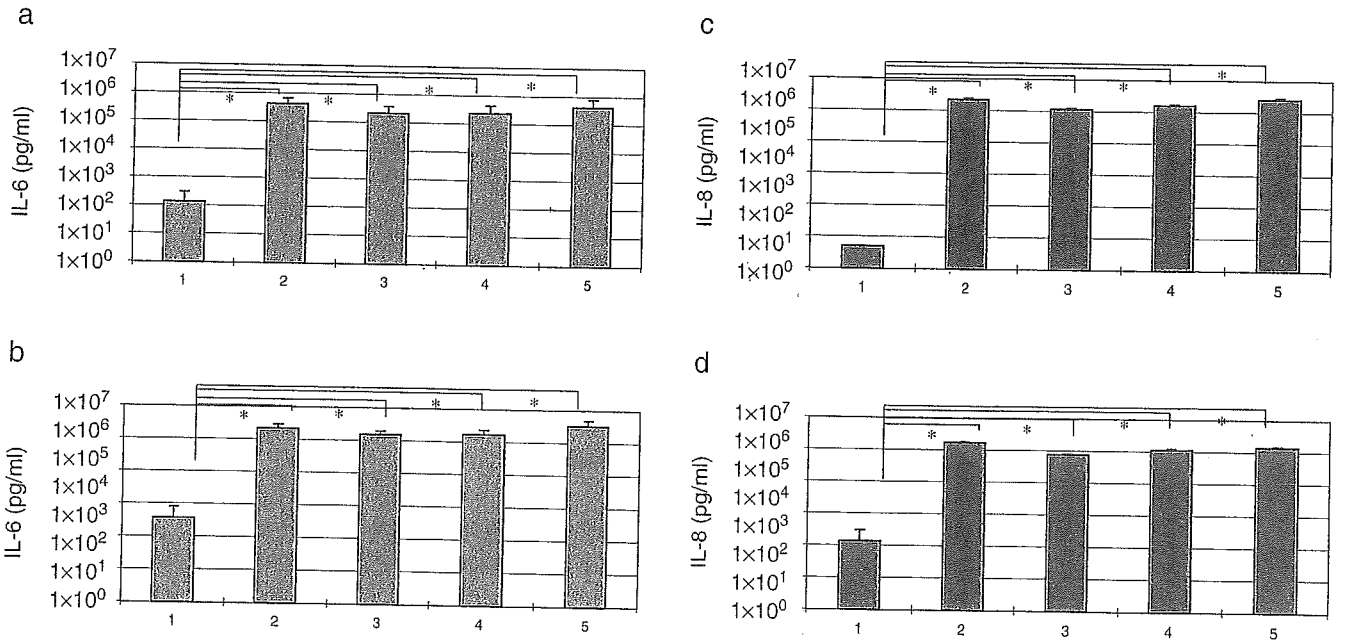

Fig. 2a–d. Production of interleukin-6 (*IL-6*) and interleukin-8 (*IL-8*) by nurse-like cells derived from rheumatoid arthritis synovium (RA-NLCs) cultured with monocytes (CD14-positive cells) from healthy donors. Levels of cytokines are expressed as the mean ± SE ($n = 3$). Data were statistically 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)

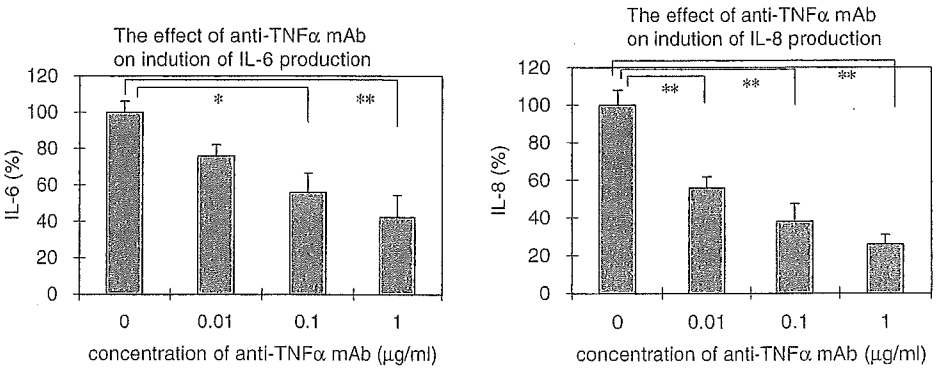

Fig. 3. Nurse-like cells derived from rheumatoid arthritis synovium RA615SY (1×10^3) and monocytes from peripheral blood mononuclear cells (PBMCs) of a healthy donor (4×10^4) were cocultured for 72 h in the presence of anti-human tumor necrosis factor α (*TNFα*) monoclonal antibody (*mAb*) at 0, 0.01, 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

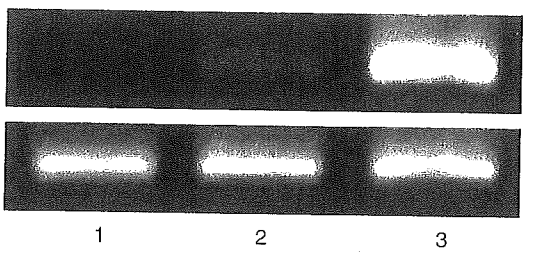

Fig. 4. Electrophoresis of polymerase chain reaction products of interleukin-6 (IL-6) (*top lane*) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*bottom lane*). The conventional revere transcription–polymerase chain reaction procedure was performed using Premix Tag (TaKaRa, Shiga, Japan) and 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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Light-induced gene transfer from packaged DNA enveloped in a dendrimeric photosensitizer

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The control of gene transfection in the body is a core issue in gene therapy. Photochemical internalization is a technology that allows light-induced delivery of DNA, drugs or other biological factors directly inside cells. Usually it requires that a photosensitizer be added to the drug-delivery system to photochemically destabilize the endosomal membrane. Here we present a system for *in vivo* DNA delivery in which these two components are assembled into one structure. This is a ternary complex composed of a core containing DNA packaged with cationic peptides and enveloped in the anionic dendrimer phthalocyanine, which provides the photosensitizing action. The ternary complex showed more than 100-fold photochemical enhancement of transgene expression *in vitro* with reduced photocytotoxicity. In an animal experiment, subconjuctival injection of the ternary complex followed by laser irradiation resulted in transgene expression only in the laser-irradiated site. This work demonstrates a new biomedical application for dendrimers, and the first success in the photochemical-internalization-mediated gene delivery *in vivo*.

There has been a strong incentive for the development of safe and effective gene vectors to achieve successful *in vivo* gene therapy[1–4]. Compared with viral vectors with an inherent risk for clinical use, non-viral synthetic vectors have received much attention owing to the advantages of safety, simplicity of use and ease of mass production[1–4]. A promising approach to the design of synthetic vectors is the use of cationic polymers and peptides. In general, the plasmid DNA (pDNA)/polycation complexes (polyplexes) are internalized by the cell through the endocytic pathway and need to be released from the endosome to deliver the genes to the nucleus. It is well known that this endosomal escape of the polyplexes is the main obstacle to obtaining efficient transfection[5]. Polycations possessing a buffering capacity, such as polyethylenimine (PEI), show a high *in vitro* transfection activity owing to the so-called proton sponge effect[5]; however, it is probable that the inherent cytotoxicity will impair their clinical utility as gene carriers. Hence, further efforts need to be devoted to the development of synthetic vectors especially for *in vivo* use.

In contrast, site-specific gene transfer to somatic cells is strongly desired; however, the existing vectors, including the viral and non-viral vectors, might have great difficulty in achieving *in vivo* transfection in a site-specific manner. In this regard, a different concept has been proposed[6–10], photochemical internalization (PCI): the cytoplasmic delivery of macromolecular compounds is enhanced by the photochemical disruption of the endosomal membrane using light and a hydrophilic photosensitizer. This smart concept is, in principle, applicable to the *in vivo* gene delivery in a light-sensitive manner[10]. However, the cytotoxicity is accompanied by photochemical reactions in the cell, and this might need to be reduced before considering further applications of this technology. Moreover, there is still room for optimization and modification