Mechanisms for Asporin Function and Regulation in Articular Cartilage*

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Osteoarthritis (OA), the most prevalent form of skeletal disease, represents a leading cause of disability following middle age. OA is characterized by the loss of articular cartilage; however, the details of its etiology and pathogenesis remain unclear. Recently, we demonstrated a genetic association between the cartilage extracellular matrix protein asporin and OA (Kizawa, H., Kou, I., Iida, A., Sudo, A., Miyamoto, Y., Fukuda, A., Mabuchi, A., Kotani, A., Kawakami, A., Yamamoto, S., Uchida, A., Nakamura, K., Notoya, K., Nakamura, Y., and Ikegawa, S. (2005) Nat. Genet. 37, 138–144). Furthermore, we showed that asporin binds to transforming growth factor-β (TGF-β), a key cytokine in OA pathogenesis, and inhibits TGF-β-induced chondrogenesis. To date, functional data for asporin have come primarily from mouse cell culture models of developing cartilage rather than from human articular cartilage cells, in which OA occurs. Here, we describe mechanisms for asporin function and regulation in human articular cartilage. Asporin blocks chondrogenesis and inhibits TGF-β1-induced expression of matrix genes and the resulting chondrocyte phenotypes. Small interfering RNA-mediated knockdown of asporin increases the expression of cartilage marker genes and TGF-β1; in turn, TGF-β1 stimulates asporin expression in articular cartilage cells, suggesting that asporin and TGF-β1 form a regulatory feedback loop. Asporin inhibits TGF-β/Smad signaling upstream of TGF-β type I receptor activation in vivo by co-localizing with TGF-β1 on the cell surface and blocking its interaction with the TGF-β type II receptor. Our results provide a basis for elucidating the role of asporin in the molecular pathogenesis of OA.

Osteoarthritis (OA‡; OMIM accession number 165720) is the most prevalent disease affecting bones and joints, and it represents a leading cause of disability in aging populations. OA is characterized by the loss of articular cartilage, which is composed of abundant extracellular matrix (ECM) proteins, including proteoglycan and type II collagen (1). Genetic factors have been implicated in the onset and development of OA (2–4), and several susceptibility genes have been reported (5, 6); however, the etiology and pathogenesis of OA remain unclear. Recently, we demonstrated the genetic association of a functional polymorphism in the gene encoding asporin (ASPN) with knee and hip OA in the Japanese population (7). This association is replicated in knee OA in the Han Chinese population (8). Asporin is an ECM protein that is abundantly expressed in OA articular cartilage, and its expression increases with progressive cartilage degeneration (7, 9). Asporin belongs to a family of small leucine-rich proteoglycans (SLRPs), which compose a major non-collagen component of the ECM (10). Although SLRPs play known roles in biological processes, such as skeletal growth, craniofacial structure, and collagen fibrillogenesis (11), the exact role of asporin is not known. Previously, we showed that asporin binds directly to transforming growth factor-β1 (TGF-β1) in vitro (7). Abundant in cartilage ECM, TGF-β1 regulates proliferation, differentiation, and matrix production of chondrocytes and their progenitor cells (12, 13). We have also demonstrated that asporin inhibits TGF-β1-induced expression of cartilage matrix genes in ATDC5 cells (7), an in vitro mouse model for chondrogenesis (14, 15). These results strongly suggest that asporin acts as a negative regulator of TGF-β in cartilage, playing a critical role in the etiology and pathogenesis of OA. However, functional data for asporin have been obtained primarily from mouse cartilage cells rather than from human articular cartilage cells, in which OA occurs. In addition, the mechanism by which asporin inhibits TGF-β function, as well as the regulation of asporin in articular cartilage, is still undetermined.

In this study, we investigated the function of asporin in articular cartilage and its mechanism for TGF-β inhibition during chondrogenesis. Asporin blocks chondrogenesis at both the early and late stages, inhibiting TGF-β1-induced gene expression and the resulting chondrocyte phenotypes. Knockdown of asporin by small interfering RNA (siRNA) increases the expression of cartilage marker genes, confirming its physiological role as a negative regulator of chondrogenesis. Asporin inhibits TGF-β/Smad signaling by co-localizing with TGF-β1 on the cell surface and inhibiting its binding to the TGF-β type II receptor (TβRII). TGF-β1 stimulates asporin expression, suggesting that asporin and TGF-β1 form a regulatory feedback loop.

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4 The abbreviations used are: OA, osteoarthritis; ECM, extracellular matrix; SLRPs, small leucine-rich proteoglycans; TGF-β1, transforming growth factor-β1; siRNA, small interfering RNA; TβR, transforming growth factor-β receptor; NHAC, normal human articular chondrocyte; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum.

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**Experimental Procedures**

**Human Articular Cartilage Samples**—Articular cartilage samples were obtained during surgery with written informed consent: knee OA cartilage was obtained from total knee arthroplasties, and normal cartilage was obtained from knee and hip joints. None of the control subjects had a clinical history of joint diseases or showed radiographic signs of OA. For RNA extraction, samples were frozen in liquid nitrogen immediately after resection and stored at −80 °C. For immunohistochemistry, the samples were fixed in 10% buffered formaldehyde and embedded in paraffin.

**Cell Culture**—We purchased normal human articular chondrocyte (NHAC) cells from Cambrex. To obtain a monolayer culture, NHAC cells were maintained in a standard chondrocyte growth medium (Cambrex) at 37 °C under 5% CO₂. To redifferentiate chondrocytes and to maintain the differentiated phenotype, we carried out three-dimensional culture using alginate beads (Cambrex) according to the manufacturer’s instructions. Unless noted otherwise, we used primary or secondary cells after redifferentiation in alginate bead culture for experiments. To examine the effects of asporin on TGF-β1-induced expression of various genes, NHAC cells were plated and cultured in a 12-well plate at a density of 5 × 10⁴ cells/well in Dulbecco’s modified Eagle’s medium (DMEM)/nutrient mixture F-12 containing 10% fetal bovine serum (FBS) until they reached confluence. At this point, the medium was replaced with DMEM/nutrient mixture F-12 containing 0.2% FBS. After 12 h, cells were treated with recombinant mouse asporin (5 μg/mL). After 12 h, cells were treated with TGF-β1 (40 ng/mL) for 12 h. To examine induction of asporin mRNA by TGF-β1, NHAC cells were cultured in a 12-well plate at a density of 1 × 10⁴ cells/well in DMEM/nutrient mixture F-12 containing 10% FBS until they reached confluence. The medium was then replaced with DMEM/nutrient mixture F-12 containing 0.2% FBS. After 24 h, cells were treated with TGF-β1 (10 ng/mL) for 12 h.

Chondrogenic ATDC5 cells were obtained from the RIKEN Cell Bank. ATDC5 cells were cultured in DMEM/F-12 containing 5% FBS at 37 °C under 5% CO₂ as described (14, 15). For induction of chondrogenesis, confluent cells were cultured in medium containing 10 μg/ml insulin, 10 μg/ml transferrin, and 3 × 10⁻⁸ M selenium (ITS; Sigma), and the medium was changed every 2 or 3 days.

**Real-time Quantitative PCR Assays**—We extracted total RNA from cells using Isogen (Wako Pure Chemical Industries, Ltd.) and purified them using the SV total RNA isolation system (Promega Corp.). We synthesized random-primed cDNA using MultiScribe reverse transcriptase (Applied Biosystems). Real-time PCR was carried out on an ABI PRISM 7700 sequence detection system (Applied Biosystems) using a QuantiTect SYBR Green PCR kit (Qiagen Inc.) in accordance with the manufacturer’s instructions.

**High Density Oligonucleotide Microarray**—We carried out high density oligonucleotide microarray analysis as described previously (7).

**Preparation of Recombinant Mouse Asporin**—Recombinant mouse asporin expressed in Escherichia coli was purified as described previously (7). The coding sequence for the mature mouse asporin protein was cloned into the pTriEx4 vector (Novagen). Recombinant mouse asporin was also expressed in COS-7 cells and purified from cell lysates using S-protein-agarose (Novagen) according to the manufacturer’s instructions.

**Pulldown Assay**—Pulldown assays to demonstrate direct interactions between asporin and TGF-β proteins were performed as described previously (7). Briefly, purified recombinant mouse asporin (5 μg) was incubated with 0.1 μg of recombinant TGF-β proteins (R&D Systems) for 1 h at 4 °C in 0.3 ml of binding buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1% Triton X-100). We added 12.5 μl of S-protein-agarose to the reactions and incubated them for 30 min at 25 °C. The precipitates were washed three times with binding buffer and subjected to SDS-PAGE. The proteins were blotted and visualized with the corresponding antibody. Each blot was then treated with horseradish peroxidase-conjugated S-protein (Novagen) to confirm the precipitation of recombinant mouse asporin.

**Safranin O Staining**—After redifferentiation in alginate bead culture, cells were plated on a Lab-Tek II chamber slide (Nunc) and cultured in DMEM/nutrient mixture F-12 containing 5% FBS and ITS in the absence or presence of TGF-β1 (40 ng/ml) with or without asporin (20 μg/ml) for 7 days. Cells were washed twice with phosphate-buffered saline, fixed with methanol, and stained with 0.1% safranin O for 5 min at room temperature. Sections of articular cartilage from individuals with OA were stained with safranin O/fast green. Safranin O-positive and -negative areas were defined as normal and degenerated areas, respectively.

**Alcian Blue Staining and Measurement of Staining Intensity**—At day 21 of culture, cells were washed with phosphate-buffered saline, fixed with 4% paraformaldehyde for 10 min, and stained with Alcian blue solution (pH 1.0) (Sigma) overnight at 25 °C. The stained culture plates were rinsed with distilled water and extracted with 6 M guanidine HCl for 2 h at 25 °C. The optical density of the extracted dye was measured at 650 nm with a spectrophotometer.

**siRNA**—We selected three siRNAs (Si1, Si9, and Si16) directed against human asporin using the siRNA design support system available on the Takara Bio web site. These siRNAs were synthesized and obtained from Takara Bio Inc. Among them, Si9 (target sequences 5’-UCCCUUCAGGAAUACCAGATT-3’ and 5’-UCUGGUAUAUCUGAAGGGATT-3’) showed the strongest knockdown effect in NHAC cells. As a control, we used a scrambled siRNA against Si1. We transfected siRNAs into NHAC cells using TransIT-TKO (Mirus Bio Corp.) and cultured the cells for 24 h in DMEM/nutrient mixture F-12 containing 0.2% FBS and 10 ng/ml TGF-β1 in the presence or absence of TGF-β-neutralizing antibodies (MAB240, R&D Systems).

**Western Blotting**—Cells were plated at 5 × 10⁴ cells/well in 12-well plastic tissue culture plates in medium containing ITS. After 24 h, the medium was changed to DMEM/F-12 containing 0.2% FBS and ITS. 16 h later, cells were treated with 10 ng/ml TGF-β1 for 5 min at 37 °C. Recombinant mouse asporin was added 1 h prior to TGF-β1 treatment. After incubation, the cells were lysed using the M-PER protein extraction kit (Pierce) containing protease inhibitor mixture (Roche Applied Science).
Proteins in the cell lysate were separated by electrophoresis on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Amersham Biosciences). The membranes were incubated in 5% nonfat dry milk in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween 20 to block nonspecific binding. Primary antibodies against phosphorylated Smad2 (Cell Signaling Technology, Inc.) and Smad2 (Invitrogen) were used at 1:1000 dilution. Membranes were then incubated in horse-radish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology, Inc.) at 1:3000 dilution.

Construction of Plasmids—The SBE₄-luciferase plasmid (where SBE is Smad-binding element) was constructed as described previously (16). We cloned a cDNA encoding human TβRI (ALK5 (activin receptor-like kinase-5)) into the pcDNA3.1 vector (Invitrogen), and a constitutively active TβRI mutant (T204D) was engineered by PCR using mutagenic primers as described previously (17). Each construct was verified by DNA sequencing (Model 3700, Applied Biosystems).

Luciferase Assay—Cells were plated at a density of 2.5 × 10⁴ cells/well in 24-well plastic tissue culture plates and cultured in DMEM/F-12 containing 5% FBS. Cells were transiently transfected with the SBE₄-luciferase reporter plasmid and the pRL-TK vector (Promega Corp.) as an internal control using FuGENE 6 (Roche Applied Science) as a transfection reagent. After 24 h, cells were transferred to medium containing 0.2% FBS with or without recombinant mouse asporin and, after 1 h, treated with TGF-β1 for 24 h. To demonstrate the effect of asporin on ligand (extracellular TGF-β)-independent Smad activation, cells were transiently transfected with SBE₄-luciferase and pcDNA3.1 containing the constitutively active TβRI (T204D) mutant construct (17). After 24 h, cells were transferred to medium containing 0.2% FBS with or without recombinant mouse asporin and cultured for 24 h. Luciferase activity was measured using the PG-DUAL-SP reporter assay system (Toyo Ink Co.).

Affinity Cross-linking—Recombinant human TGF-β1 was iodinated using the chloramine-T method as described (18). ATDC5 cells were incubated for 3 h at 4 °C with 10 ng/ml ¹²⁵I-labeled TGF-β1 in binding buffer (phosphate-buffered saline containing 1 mM CaCl₂, 0.5 mM MgCl₂, and 1 mg/ml bovine serum albumin). After incubation, the cells were washed with binding buffer without bovine serum albumin, and cross-linking was performed in buffer containing 0.27 mM disuccinimidyl suberate (Pierce) for 15 min at 4 °C. The cells were then washed once with 10 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 10% glycerol, and protease inhibitor mixture. Cells were scraped, centrifuged, and resuspended in solubilization buffer (1% Triton X-100, 1% deoxycholate, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and protease inhibitor mixture), followed by incubation on ice for 30 min. Cell lysates were mixed with an equal amount of SDS sample buffer (100 mM Tris-HCl (pH 6.8), 0.01% bromophenol blue, 36% glycerol, and 4% SDS) containing 10 mM dithiothreitol and analyzed on 7% SDS-polyacrylamide gels. The gels were dried and subjected to analysis using a BAS-2500 imaging analyzer (FujiFilm Corp.).

Immunohistochemistry—Immunohistochemistry was carried out as described previously (19). Sections were stained with an anti-asporin antibody (2229-B01; 1 μg/ml) or an anti-

**FIGURE 1. Direct interaction of asporin with TGF-β.** A, S-tagged recombinant mouse asporin was incubated with TGF-β₁, -β₂, or -β₃ or insulin-like growth factor I (IGF-I), followed by treatment with 5-protein-agarose. Co-precipitates were detected using the corresponding antibodies (upper panels). Each blot was further developed with S-protein to confirm the presence of precipitated asporin. Asporin bound to all TGF-β proteins directly. Lanes P, positive control (10 ng of each ligand was loaded). B, E. coli and eukaryotic (COS-7) cells expressing recombinant mouse asporin proteins were incubated with TGF-β₁, and S-tag pulldown assays were performed as described for A. C, quantitation of S-tag pulldown assays in B. The TGF-β₁/asporin ratios were determined by quantitation of the TGF-β₁ and asporin proteins detected on the immunoblot.

**RESULTS**

**TGF-β₁ Is Predominant among TGF-β Isoforms in Human Articular Cartilage**—Three TGF-β isoforms (β₁, β₂, and β₃) with similar functions exist in mammals, and all are expressed in ATDC5 cells (20). Upon finding that asporin bound to TGF-β₂ and TGF-β₃ as well as to TGF-β₁ (Fig. 1A), we evaluated the significance of each isoform in OA. Using microarray and real-time PCR, we examined the expression of all isoforms in cartilage samples from normal subjects and OA patients as well as in NHAC cells. Among the isoforms, TGF-β₁ was most abundantly expressed in OA and non-OA articular cartilage (Fig. 2, A and B) and in NHAC cells (Fig. 2C), and its expression levels were higher in OA cartilage than in non-OA samples (Fig. 2A). Increased expression of TGF-β₁ in the microarray analysis was not replicated in the real-time PCR analysis. These
results indicate that TGF-β1 is the most important of the three isofoms in human articular cartilage and OA.

Effect of Asporin on Cartilage Differentiation and TGF-β1-induced Expression of Cartilage Matrix Genes in Articular Cartilage—We demonstrated previously that asporin overexpression inhibits early chondrogenic differentiation in ATDC5 cells (7). To confirm the influence of asporin in this model, we measured the expression of chondrogenic differentiation marker genes (aggrecan and type II and X collagens) in the presence of recombinant mouse asporin. Asporin decreased the expression of marker genes in a dose-dependent manner (Fig. 3, A–C). Asporin also inhibited glycosaminoglycan accumulation measured at 21 days of culture (Fig. 3D). To verify that the TGF-β binding (Fig. 1A) and chondrogenesis (Fig. 3, A–D) inhibitory activities of recombinant asporin prepared from E. coli were comparable with those of native asporin, we examined these activities using recombinant asporin produced using a eukaryotic expression system (COS-7). As shown in Figs. 1 (B and C) and 3 (E and F), the activities were similar.

ATDC5 is an excellent model for chondrogenesis; sequential steps of chondrocyte differentiation are reflected in ATDC5 cells by increased expression of the chondrogenic differentiation marker genes type II and X collagens in the presence of insulin (14, 15); however, it most precisely represents growing cartilage in the mouse (21) rather than human articular cartilage, in which OA occurs. To clarify asporin function in human articular cartilage, we examined its effect on TGF-β1-induced changes in the cartilage phenotype of NHAC cells. Most TGF-β1-stimulated cells exhibited a spherical shape typical of chondrocytic cells, with areas of clustered cells staining...
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strongly with safranin O, a marker for proteoglycan synthesis (Fig. 4D). However, control (unstimulated) cells showed a fibroblastic morphology with no safranin O staining (Fig. 4C). Addition of asporin changed the morphology of TGF-β1-stimulated cells to a fibroblastic phenotype and reduced safranin O staining (Fig. 4E). Together, these results indicate that asporin reverses the TGF-β1-induced chondrocyte phenotype in human articular chondrocytes.

Endogenous Asporin Suppresses Expression of Cartilage Matrix Genes—To clarify the physiological role of asporin in human articular chondrocytes, we decreased endogenous asporin levels in NHAC cells via siRNA and measured the expression of cartilage marker genes. Knockdown of asporin significantly increased type II collagen and aggrecan mRNA expression (Fig. 5, A–C) as well as TGF-β1 (Fig. 5D), indicating that asporin negatively regulates cartilage matrix gene expression in articular cartilage under physiological conditions. The siRNA-induced increase in type II collagen and aggrecan mRNA expression was inhibited by TGF-β-neutralizing antibodies (Fig. 5E, F, and G), suggesting that increased matrix gene expression is due to increased available TGF-β1.

Asporin Inhibits TGF-β1/Smad Signaling—We further investigated the influence of asporin on chondrogenesis by examining its effects on TGF-β1/Smad signaling. In ATDC5 cells, recombinant asporin inhibited TGF-β1-induced phosphorylation of Smad2 in a dose-dependent manner (Fig. 6A). Next, we measured the effect of asporin on TGF-β1-induced Smad3/4-specific reporter activity using a luciferase construct containing four tandem repeats of Smad-binding elements (SBE₄-luciferase) (16). Asporin significantly reduced TGF-β1-induced SBE₄-luciferase activity (Fig. 6B). Confirmation of these inhibitory effects in NHAC cells (Fig. 6, C and D) indicates that asporin negatively regulates TGF-β1/Smad signaling in articular cartilage.

Smad signaling is modulated not only by TβRI kinase, but also by various molecules, such as inhibitory Smad proteins, and by the ERK (extracellular signal-regulated kinase) MAPK (mitogen-activated protein kinase) and phosphatidylinositol 3-kinase/Akt pathways (25). To characterize the potential influence of asporin on Smad signaling, we used constitutively active TβRI (T204D), which activates Smad in the absence of TGF-β (17). Asporin did not inhibit TβRI-induced transcriptional activation of Smad (Fig. 6E), suggesting that it works upstream of TβRI activation and exerts no downstream effect following Smad activation. This result is consistent with our previous finding that asporin is an ECM protein that interacts with TGF-β1 (7). Therefore, we examined the effect of asporin on TGF-β1 binding to cell-surface receptors in vivo. Cells were incubated with ¹²⁵I-labeled TGF-β1 in the presence or absence of asporin, and then ¹²⁵I-labeled TGF-β1-receptor complexes were cross-linked and analyzed by SDS-PAGE. Addition of asporin reduced TGF-β1 binding to TβRI and TβRII in a dose-dependent manner (Fig. 6, F and G). Together, these findings suggest that asporin regulates TGF-β1 signaling by preventing interaction between TGF-β and its receptor on the cell surface.

Asporin Co-localizes with TGF-β1 in Vivo—Previously, we demonstrated that asporin and TGF-β1 directly interact in vitro (7). To investigate the interaction in vivo, we examined the localization of asporin in articular cartilage from individuals with OA by immunohistochemistry. Asporin appeared in clus-
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A

Asporin (µg/ml) 0 0 5 10 20 40 80 TGF-β1 (ng/ml) 0 10 P-Smad2 Smad2

B

C

Asporin (µg/ml) 0 0 20 40 TGF-β1

D

E

F

G

TGF-β1

Asporin

Smad2

Asporin

SBE4

Asporin

SBE4

TGF-β1

Asporin

P-Smad2

Smad2

TGF-β1

Asporin

Smad2

Asporin

TGF-β1

Asporin

Asporin

Asporin

FIGURE 6. Asporin inhibits TGF-β/Smad signaling. A, Western blot analyses for Smad2 and phosphorylated Smad2 (P-Smad2). Asporin inhibited phosphorylation of Smad2 in a dose-dependent manner. B, luciferase (Luc) assay using SBE4-luciferase. Asporin inhibited TGF-β–induced Smad transcriptional activity. C, asporin blocks TGF-β–induced phosphorylation of Smad2 in NHAC cells. D, asporin reduces TGF-β–induced Smad transcriptional activity in NHAC cells. NHAC cells were transiently transfected with SBE4-luciferase. Following treatment with asporin (40 µg/ml) for 12 h, cells were treated with TGF-β1 (40 ng/ml) for 24 h. *, p < 0.05 (Student’s t test). Data represent the mean ± S.E. from three samples. E, asporin inhibits TGF-β signaling upstream of TβRII activation. ATDC5 cells were transiently transfected with constitutively active TβRII (T204D) and SBE4-luciferase vectors. Cells were treated with recombinant mouse asporin for 24 h and subjected to luciferase assay. Asporin did not inhibit the Smad signal activated by constitutively active TβRII. Data represent the mean ± S.E. from three samples. F, asporin inhibits TGF-β binding to cell-surface receptors. ATDC5 cells were incubated with 125I-labeled TGF-β1 in the presence of recombinant mouse asporin, treated with the cross-linker disuccinimidyl suberate, lysed, and analyzed by SDS-PAGE. The positions of affinity-labeled TβRI and TβRII are indicated on the right. Binding of TGF-β to TβRII and TβRII was reduced by asporin. G, quantification of the binding of each affinity-labeled TβRI and TβRII by densitometry. The densities of the TβRI and TβRII bands in the asporin control were set to 1.

FIGURE 7. Asporin co-localizes with TGF-β1. A, co-localization of asporin and TGF-β1 in OA cartilage. Asporin-positive signals co-localized with TGF-β1-positive signals (arrowheads). B, co-localization of asporin and TGF-β1 in NHAC cells. Shown is the double staining of asporin and TGF-β1 using the anti-asporin antibody in the presence of biotinylated TGF-β1. Cells redifferentiated and maintained in chondrocyte growth medium on dishes for 3 days were plated on a Lab-Tek II chamber slide, cultured in DMEM/nutrient mixture F-12 containing 10% FBS, and used for immunostaining. Positive signals for asporin (red) and TGF-β1 (green) were detected with Alexa Fluor 594-conjugated goat anti-rabbit IgG and fluorescein isothiocyanate-labeled avidin, respectively. C, immunostaining of asporin and Smad2 without (–) and with (+) 0.2% Triton X-100 treatment. Asporin signal (red) was more intense in cells without Triton X-100 treatment. In contrast, Smad2 signal (red) was more intense in cells with Triton X-100 treatment. Nuclei were stained with 4’,6-diamidino-2-phenylindole (blue). Asporin localized in extracellular areas around NHAC cells.

tered chondrocytes in areas of severe degeneration, which were revealed by staining with safranin O, but was barely detectable in normal areas (Fig. 7A). TGF-β1 showed a similar localization pattern, suggesting that asporin and TGF-β1 are up-regulated and co-localize in chondrocytes within OA lesions of human articular cartilage. We further investigated the localization of asporin and TGF-β1 in NHAC cells by immunocytochemistry. Biotinylated TGF-β1– and endogenous asporin both were detected on clustered cells (Fig. 7B). Asporin staining was clearly detected on cells not permeabilized with Triton X-100, but was barely visible in permeabilized cells (Fig. 7C). In contrast, staining of Smad2, a marker for intracellular proteins, was stronger in permeabilized cells. These observations indicate that asporin localizes to the cell surface and, when there, interacts with TGF-β1.

Expression of Asporin mRNA Is Induced by TGF-β1—The expression of some negative regulators of TGF-β signaling, such as Smad7, is induced by TGF-β1 itself, forming a functional feedback loop (26). We therefore investigated whether TGF-β1 influences asporin expression. In NHAC cells, asporin mRNA was induced by addition of TGF-β1 (Fig. 8A) as early as 6 h after stimulation. If asporin induction likely occurs through TGF-β, then exogenous asporin should inhibit TGF-β1–induced expression of asporin. We confirmed this prediction in NHAC cells treated with asporin, observing inhibition of TGF-β1–induced expression of endogenous asporin in a dose-dependent manner (Fig. 8, B and C).


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Asporin and TGF-β1 form a functional feedback loop. A, stimulation of TGF-β1 (10 ng/ml)-induced expression of asporin mRNA in NHAC cells. B and C, effects of asporin on TGF-β1-induced expression of asporin and TGF-β1 mRNAs, respectively. Cells cultured for 2 weeks in alginate beads were seeded in 12-well plates and used for the experiment. Data represent the mean ± S.E. from duplicate assays.

**DISCUSSION**

The SLRP family consists of 13 known members that can be divided into three distinct subfamilies based on their genomic organization, structure, and amino acid sequence similarity (10). Asporin belongs to the group of class I SLRPs that also includes decorin and biglycan (9, 27); unlike other family members, however, asporin lacks a glycosaminoglycan attachment site and hence is not a proteoglycan. Also unique to asporin is a polymorphic aspartic acid repeat in the N-terminal region, which ranges in length from 8 to 19 residues (7, 8, 28). This repeat shows a significant genetic association with knee and hip OA, with the D14 allele being over-represented in OA (7, 8). Our previous study using mouse chondrocytes demonstrated that asporin is a negative regulator of TGF-β function (7). This study confirms the inhibitory effect of asporin on TGF-β function during chondrogenesis in human articular cartilage chondrocytes.

Several SLRPs, including decorin, biglycan, and fibromodulin, have been shown to bind TGF-β proteins (29–31). Decorin inhibits TGF-β function; in vivo administration of decorin prevents fibrosis caused by overexpression of TGF-β in experimental animal models (32, 33). Two mechanisms for decorin inhibition of TGF-β have been reported, although neither was demonstrated in cartilage. In macrophages, decorin reverses the inhibitory effect of TGF-β by binding to it and preventing its interaction with the cell surface (34). It is likely that decorin blocks interaction of TGF-β with its cell-surface receptor(s), but this has not been confirmed. Inhibition of TGF-β by decorin can also occur through regulation of intracellular Smad and other signaling cross-talk (35). In human mesangial cells, decorin inhibits TGF-β-induced expression of plasminogen activator inhibitor-1 via activation of the Ca²⁺/calmodulin-dependent protein kinase II, which results in phosphorylation of Smad2 at Ser204, an important negative regulatory site. Our data argue against such cross-talk in asporin-mediated TGF-β inhibition.

We showed previously that asporin binds directly to the mature form of TGF-β and inhibits its biological effects on mouse chondrocytes (7). Here, we have shown that asporin co-localizes with TGF-β in the human articular cartilage extracellular space, inhibiting TGF-β signaling upstream of TβRI. Asporin shows no inhibitory effect on signaling downstream of TβRI, however. Through cross-linking experiments, we have demonstrated that asporin inhibits TGF-β binding to TβRII. These lines of evidence indicate that asporin in the ECM inhibits TGF-β signaling directly at the receptor level. A serum glycoprotein, fetuin, also inhibits TGF-β activity by competing with TGF-β for binding to TβRII (36). Fetuin and TβRII show homology in the TRH1 (TGF-β receptor II homology 1) domain, an 18–19-amino acid sequence that interacts with TGF-β. Fetuin binds directly to TGF-β1 via this domain and inhibits binding of TGF-β1 to the extracellular domain of TβRII. Cystatin C, which, like fetuin, belongs to the type 3 cystatin family, also inhibits TGF-β signaling by preventing the binding of TGF-β to TβRII, although physical binding of cystatin C to TGF-β has not been proven (37). We think, however, that such a mechanism is unlikely in asporin because it lacks a TRH1 domain. Further study is necessary to determine the precise inhibitory mechanism of the binding of TGF-β to TβRII by asporin.

In summary, we have created a more detailed picture of asporin function and regulation both in normal cell physiology and in OA. Asporin negatively regulates chondrogenesis in articular cartilage by blocking the TGF-β receptor interaction on the cell surface and inhibiting the canonical TGF-β/Smad signal. Furthermore, asporin and TGF-β1 form a functional feedback loop in human articular cartilage. TGF-β signaling is critical for maintaining healthy articular cartilage, and suppression of TGF-β signaling in chondrocytes leads to OA (38–40). Our studies suggest that asporin is a critical regulator of TGF-β in articular cartilage and thus plays an essential role in cartilage homeostasis and OA pathogenesis. Inhibition of asporin should then enhance cartilage regeneration by increasing TGF-β activity. More detailed study of the physical interface between the two molecules will be key to developing innovative treatments for OA, as it represents a promising target for agents that control and modify the asporin/TGF-β system.

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