

Role of Aggrecanase 1 in Lyme Arthritis

Aruna K. Behera,¹ Ethan Hildebrand,¹ Jon Szafranski,² Han-Hwa Hung,²
Alan J. Grodzinsky,² Robert Lafyatis,³ Alisa E. Koch,⁴ Robert Kalish,¹ George Perides,¹
Allen C. Steere,⁵ and Linden T. Hu¹

Objective. Arthritis is one of the hallmarks of late-stage Lyme disease. Previous studies have shown that infection with *Borrelia burgdorferi*, the causative agent of Lyme disease, results in degradation of proteoglycans and collagen in cartilage. *B burgdorferi* do not appear to produce any exported proteases capable of digesting proteoglycans and collagen, but instead, induce and activate host proteases, such as matrix metalloproteinases (MMPs), which results in cartilage degradation. The role of aggrecanases in Lyme arthritis has not yet been determined. We therefore sought to delineate the contribution of aggrecanases to joint destruction in Lyme arthritis.

Methods. We examined the expression patterns of aggrecanases 1 and 2 (ADAMTS 4 and 5, respectively) in *B burgdorferi*-infected primary human chondrocyte cell cultures, in synovial fluid samples from patients with active Lyme arthritis, and in the joints of mice by real-time quantitative reverse transcription–polymerase

chain reaction and immunoblotting techniques. Bovine cartilage explants were used to determine the role of aggrecanases in *B burgdorferi*-induced cartilage degradation.

Results. ADAMTS-4, but not ADAMTS-5, was induced in human chondrocytes infected with *B burgdorferi*. The active forms of ADAMTS-4 were increased in synovial fluid samples from patients with active Lyme arthritis and were elevated in the joints of mice infected with *B burgdorferi*. Using cartilage explant models of Lyme arthritis, it appeared that the cleavage of aggrecan was predominantly mediated by “aggrecanases” rather than MMPs.

Conclusion. The induction of ADAMTS-4 by *B burgdorferi* results in the cleavage of aggrecan, which may be an important first step that leads to permanent degradation of cartilage.

Oligoarticular arthritis is a prominent feature of late-stage Lyme disease in North America (1). When left untreated, infection with *Borrelia burgdorferi*, the causative agent of Lyme disease, can result in intermittent or chronic arthritis that may progress to an erosive arthritis with certain histopathologic similarities to rheumatoid arthritis (RA) (2). The progression of erosions in Lyme arthritis is quite delayed in comparison with the septic arthritis caused by other bacterial agents. This may be due to the fact that cartilage degradation in response to *B burgdorferi* occurs as a result of induction of host proteases rather than bacterial proteases (3). Elevations in the levels of host matrix metalloproteinases (MMPs) have been found in the synovial fluid (SF) of patients with Lyme arthritis, and *B burgdorferi* has been shown to induce specific MMPs from chondrocytes (4). Inhibitors of MMPs have been shown to decrease *B burgdorferi*-induced cartilage degradation (3).

Articular cartilage consists of an extracellular matrix that is synthesized and maintained by chondrocytes, the resident cells of the tissue. Aggrecan, which

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¹Aruna K. Behera, PhD, Ethan Hildebrand, BS, Robert Kalish, MD, George Perides, PhD, Linden T. Hu, MD: Tupper Research Institute, Tufts University School of Medicine, Boston, Massachusetts; ²Jon Szafranski, PhD, Han-Hwa Hung, BS, Alan J. Grodzinsky, PhD: Massachusetts Institute of Technology, Cambridge, Massachusetts; ³Robert Lafyatis, MD: Boston University School of Medicine, Boston, Massachusetts; ⁴Alisa E. Koch, MD: Veterans Affairs Healthcare System, and University of Michigan Medical School, Ann Arbor, Michigan; ⁵Allen C. Steere, MD: Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts.

Address correspondence and reprint requests to Linden T. Hu, MD, New England Medical Center, Box 41, 750 Washington Street, Boston, MA 02111. E-mail: lhu@tufts-nemc.org.

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consists of a protein core backbone substituted with many highly sulfated glycosaminoglycans (GAGs), is the major proteoglycan present within articular cartilage (5). The high negative-charge density of the GAG chains present on aggrecan provides cartilage with the ability to resist mechanical compression. In arthritic diseases characterized by cartilage destruction, aggrecan is one of the first matrix components to be degraded. While many enzymes have been demonstrated to be capable of cleaving the protein backbone of aggrecan, the predominant activity responsible for the degradation of aggrecan in cartilage is aggrecanase (6–11). Five sites along the aggrecan core have been shown to be susceptible to aggrecanase cleavage. These are located within the interglobular domain of the core protein between amino acid residues TEGE³⁷³ and ³⁷⁴ARGS, and within the chondroitin sulfate attachment region between amino acids GELE¹⁴⁸⁰ and ¹⁴⁸¹GRGT, KEEE¹⁶⁶⁶ and ¹⁶⁶⁷GLGS, TAQE¹⁷⁷¹ and ¹⁷⁷²AGEG, and VSQE¹⁸⁷¹ and ¹⁸⁷²LGQR (7–9,12).

Although MMPs may be involved in the cleavage and release of aggrecan from the cartilage matrix, studies in other diseases, including RA, osteoarthritis (OA), and after joint injury, have shown that the majority of aggrecan fragments found in SF are generated by aggrecanases, which cleave aggrecan at sites different from those used by MMPs (13–16). ADAMTS-4 (aggrecanase 1) and ADAMTS-5 (aggrecanase 2) have been identified as the known enzymes that are most efficiently capable of cleaving aggrecan (17). ADAMTS-4 and ADAMTS-5 are members of the family of proteins known as a disintegrin and metalloproteinase with thrombospondin motifs. Aggrecanase-like activity in SF from patients with Lyme arthritis has previously been reported (3). However, the induction of aggrecanases and the relative contributions of aggrecanases and MMPs in *B burgdorferi*-induced cartilage destruction have not previously been reported.

In the present study, we examined the role of ADAMTS-4 and ADAMTS-5 in cartilage degradation following *B burgdorferi* infection in patients with Lyme disease, as well as in both in vitro and in vivo models of Lyme disease.

MATERIALS AND METHODS

Primary cell cultures and infection with *B burgdorferi*.

Primary human chondrocytes derived from a healthy donor were purchased from Cambrex (Walkersville, MD) and maintained in chondrocyte growth medium (Cambrex). Primary human dermal fibroblasts and primary human pulmonary artery smooth muscle cells were purchased from Cell Appli-

cations (San Diego, CA). Cell cultures were maintained and infected as previously described (18). For inhibitor experiments, various inhibitors of MAPK and JAK/STAT pathways were added to the cells in fresh serum-free medium 2 hours prior to infection with *B burgdorferi* and then harvested at 24 hours after infection. The inhibitor concentrations we used (20 μ M SP600125, 3 μ M SB203580, 10 μ M U0126, and 30 μ g/ml of a JAK-3 inhibitor I [JAK-3I]) were selected on the basis of previous dose-finding studies, both by our group and by others, showing no visible cytotoxic effect on the human chondrocyte, as judged by trypan blue exclusion (19). Cells were washed and harvested in cold phosphate buffered saline, and the cell pellets were stored at -70°C until used.

Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was purified from human chondrocytes with TRIzol (Invitrogen, San Diego, CA) according to the manufacturer's instructions. First-strand synthesis of complementary DNA (cDNA) from total RNA was performed using ImProm II reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions. Control reactions performed in the absence of reverse transcriptase were used to check for contamination with genomic DNA. The cDNA samples that had been contaminated by genomic DNA were discarded, and the original RNA was treated with DNase before the reverse transcriptase reaction was repeated. Quantitation of cDNA from specific messenger RNA (mRNA) transcripts was accomplished by real-time quantitative RT-PCR (iCycler; Bio-Rad, Hercules, CA) using SYBR Green technology (QuantiTect SYBR Green PCR kit; Qiagen, Valencia, CA) as previously described (18).

The following primers were used for PCR amplification: for human ADAMTS-4, 5'-CACGCTGGGTATGGCTGATG-3' (forward) and 5'-CATGACATGGCGAGAGGTGC-3' (reverse); for mouse ADAMTS-4, 5'-CCCGAGTCCCATTTCCCGCA-3' (forward) and 5'-GGGTCTGGCGGCACTTGGC-3' (reverse); for bovine ADAMTS-4, 5'-GCCTCAAGCACCCGAGCAT-3' (forward) and 5'-CATCCTCCACAATGGCGCAG-3' (reverse); for bovine ADAMTS-5, 5'-TGTCCGAGGACGGTGTGTGA-3' (forward) and 5'-CAGGGCTAAGTAGGCAGGGAATC-3' (reverse); and for bovine GAPDH, 5'-CATGTTTGTGATGGGCGTGA-3' (forward) and 5'-GCGCCAGTAGAAGCAGGGAT-3' (reverse). The human β -actin and mouse nidogen primer sequences we used have been described elsewhere (18,20). All of the primers we used were validated for amplification efficiency according to the method of Livak and Schmittgen (21), and the absolute value of the slope of the log template amount versus the difference in threshold cycle (ΔC_t) for each primer combination was <0.1 . Calculations of expression were normalized to β -actin (human) or nidogen (mouse) using the $\Delta\Delta C_t$ method, where the amount of target, normalized to an endogenous reference and relative to a calibrator, is given by $2^{-\Delta\Delta C_t}$, where C_t is the cycle number of the detection threshold.

Immunoblot analysis. After incubation with *B burgdorferi*, total cellular protein extracts were prepared from chondrocyte cultures as described previously (18,19). Equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane. Immunoblotting was performed with polyclonal antibodies to human ADAMTS-4 (1:500 dilution; Santa Cruz Biotechnology, Santa

Cruz, CA) and horseradish peroxidase-conjugated anti-goat secondary antibody (1:1,000 dilution; Southern Biotechnology, Birmingham, AL).

Patients. SF was obtained from 9 patients with untreated Lyme arthritis and 9 patients with persistent Lyme arthritis after antibiotic therapy. The samples used have been previously described (4,18,22). Use of the patient samples was reviewed and approved by the Institutional Review Board of the Tufts–New England Medical Center. All patients were infected when they were in the northeastern US. All had oligoarticular arthritis involving one or both knees. All patients in both groups met the Centers for Disease Control and Prevention (CDC) clinical criteria for the diagnosis of Lyme disease (23). Patients had mono- or oligoarticular arthritis affecting at least 1 knee, accompanied by positive findings on a serum IgG Western blot test for Lyme disease, as interpreted according to the CDC/Association of State and Territorial Public Health Laboratory Directors criteria (24). SF samples from all 18 patients with untreated Lyme arthritis tested positive for *B burgdorferi* DNA by PCR, performed as described by Nocton et al (25). All SF samples from patients with persistent Lyme arthritis tested negative for *B burgdorferi* DNA by PCR.

The mean duration of symptoms at the time of sample collection was 2.2 years in the untreated group (range 6 months to 6 years) and 1.7 years in the posttreatment group (range 3 months to 6 years). All patients in the posttreatment group received at least 4 weeks of treatment with an antibiotic that is active against *B burgdorferi* (median number of courses of antibiotic was 2). Samples were obtained a minimum of 2 months after patients had completed their antibiotic courses.

All specimens were divided into aliquots and stored at -70°C until used. An equal volume of each sample ($1\ \mu\text{l}$) was used for immunoblotting. All blots were normalized to standards, which were included in every blot to allow comparison between blots.

SF samples from patients with other arthritic diseases (13 with RA, 12 with OA, 4 with gouty arthritis, and 2 with reactive postinfectious arthritis [ReA]) were collected from discarded specimens obtained during the course of standard patient care. All SF was obtained during active disease from an involved knee joint.

Mice and infection with *B burgdorferi*. C3H/HeN, C57BL/6, and BALB/c mice were purchased from Charles River (Wilmington, MA) and from Taconic (Hudson, NY). The procedures used were reviewed and approved by the Institutional Animal Care and Use Committee of Tufts University. Five-day-old mice were infected intradermally by needle inoculation with *B burgdorferi* strain N40 (10^4 low-passage infectious organisms per mouse) or were sham infected. Mice were killed 2 weeks postinfection, at which time the *B burgdorferi*-infected mice were clearly distinguishable from the sham-infected mice by the visible swelling of the ankle joints. Cartilage was microdissected from the ankle joints by using a stereomicroscope to separate the cartilage from bone and adjoining synovial tissue (18), and total RNA was isolated using TRIzol. Successful infection of individual mice was confirmed by culturing ear samples in BSK-H medium and monitoring the growth of *B burgdorferi* by darkfield microscopy.

Preparation of bovine cartilage explants and infection with *B burgdorferi*. Bovine cartilage explants were prepared and maintained as previously described (26). Low-passage infectious *B burgdorferi* strain N40 organisms (10^7) were washed 3 times in culture medium and added to wells, each of which contained a single 3-mm explant. Batimastat (250 nM) (BB-94; British Biotech, Oxford, UK) or BAY 12-9566 (300 nM; Bayer Pharmaceuticals, Elkhart, IN) was added at the same time as the *B burgdorferi* in a final volume of $200\ \mu\text{l}$ of culture medium. The culture supernatant was collected after 48 hours and stored at -70°C until used.

To detect BC-3-immunoreactive fragments, culture supernatants from bovine cartilage explants were transferred to a PVDF membrane and detected with mouse monoclonal BC-3 antibody (1:100 dilution; Abcam, Cambridge, MA) and alkaline phosphatase-conjugated anti-mouse secondary antibody (1:10,000 dilution; Promega).

Measurement of sulfated GAGs. Quantitation of sulfated GAGs was performed using the dimethylmethylene blue (DMMB) method (27). Briefly, culture supernatants ($3\text{--}10\ \mu\text{l}$) from bovine cartilage explants were mixed with 1 ml of DMMB solution ($38.45\ \mu\text{M}$ 1,9-dimethylmethylene blue, $40.49\ \text{mM}$ glycine, $40.55\ \text{mM}$ NaCl, and $95\ \text{ml}$ of $0.1\ \text{M}$ HCl, pH 3.0). Optical density was measured at 525 nm within 30 minutes and was compared with the optical density of known concentrations of chondroitin sulfate A as a standard.

Statistical analysis. Each experiment was performed 2–4 times, as indicated. Statistical significance was analyzed using the nonparametric Mann-Whitney U test in the SPSS software package (SPSS, Chicago, IL). *P* values less than 0.05 were considered significant.

RESULTS

***B burgdorferi*-induced expression of ADAMTS-4 in primary human chondrocytes.** We first examined the expression pattern of aggrecanases in primary human chondrocytes following *B burgdorferi* infection. Cultures of human chondrocytes in serum-free medium were infected with *B burgdorferi* at various multiplicities of infection (MOI; the number of bacteria per cell) or were sham infected and harvested 24 hours later. The cDNA was synthesized using the total cellular RNA from these cells, and the expression of ADAMTS-4 and ADAMTS-5 was examined by real-time quantitative RT-PCR (Figure 1A). We found that ADAMTS-4 mRNA was induced in a dose-dependent manner following *B burgdorferi* infection. Expression of ADAMTS-4 was significantly increased in treated cells as compared with untreated cells (63-fold at an MOI of 1 and $>1,000$ -fold at an MOI of 100; $P < 0.05$). An MOI of 10 was used in all subsequent experiments in this study.

The change in expression of ADAMTS-4 was seen in as little as 0.5 hours after addition of *B burgdorferi* and remained high through 72 hours after infection

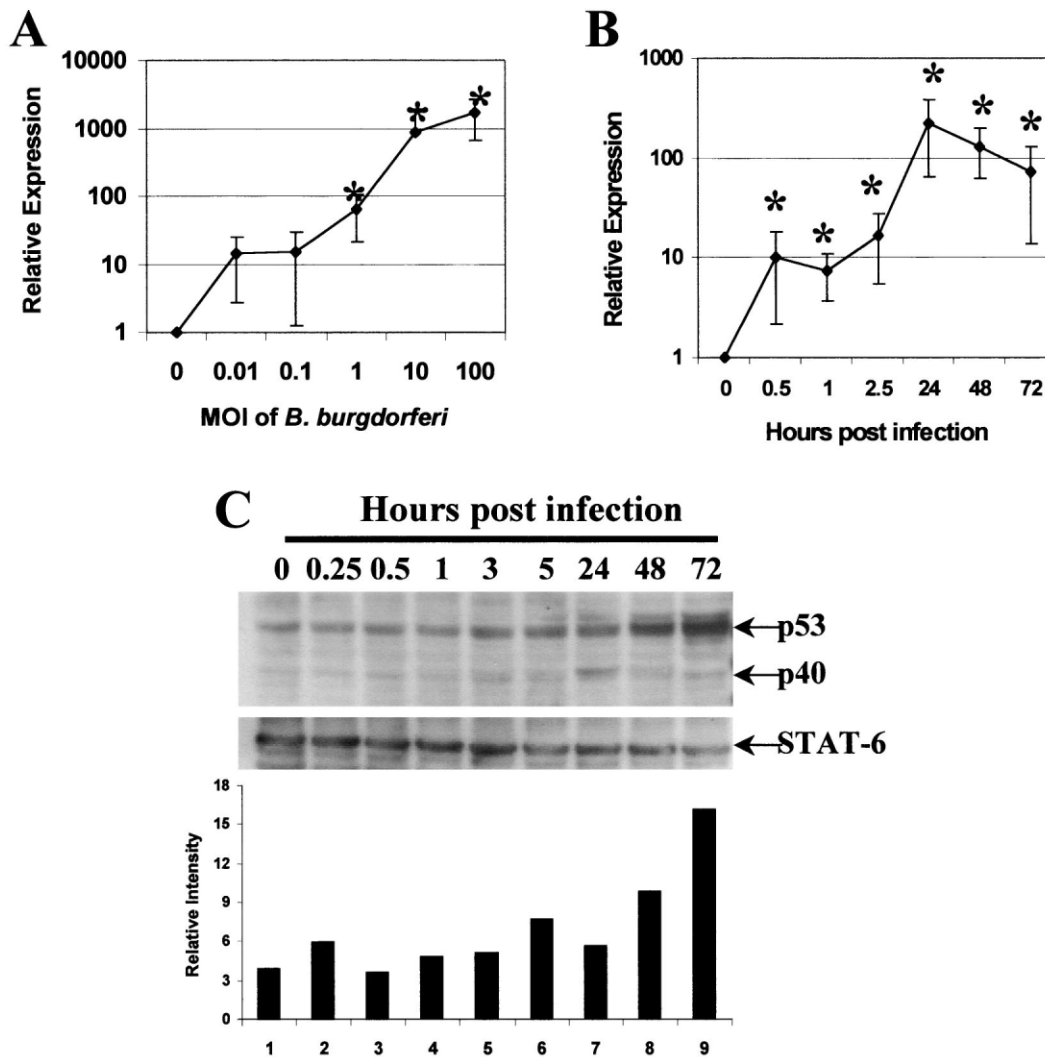


Figure 1. *Borrelia burgdorferi*-induced expression of ADAMTS-4 in primary human chondrocytes. **A**, Human chondrocytes were infected with *B. burgdorferi* at various multiplicities of infection (MOI), and expression of ADAMTS-4 was examined by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis at 24 hours postinfection. Expression of ADAMTS-4 was normalized to that of β -actin and the relative expression is shown. The time course of ADAMTS-4 induction in response to infection with *B. burgdorferi* (10 MOI) was measured by **B**, real-time quantitative RT-PCR and **C**, Western blotting (top). The active p53-kd band of ADAMTS-4 at each time point was densitometrically scanned, and the relative intensity of each band was normalized to the loading control, STAT-6 (bottom). Results are representative of 3–5 real-time quantitative RT-PCR experiments and 2 Western blot experiments. Values are the mean \pm SD. * = $P < 0.05$ versus chondrocytes not infected with *B. burgdorferi*, by nonparametric Mann-Whitney U test.

(Figure 1B). There was no change in the expression of ADAMTS-5 in infected human chondrocytes, as determined by real-time quantitative RT-PCR (data not shown).

To determine if the changes in gene transcription also paralleled increases in ADAMTS-4 protein expression, whole cell extracts of human chondrocytes, either

sham infected or infected with *B. burgdorferi* for different time periods, were analyzed by immunoblotting using an antibody raised against human ADAMTS-4. ADAMTS-4 is produced as a proenzyme that requires intracellular furin-mediated removal of the N-terminal pro domain (28), resulting in generation of the p68 form. While the p68 form can degrade the C-terminal chon-

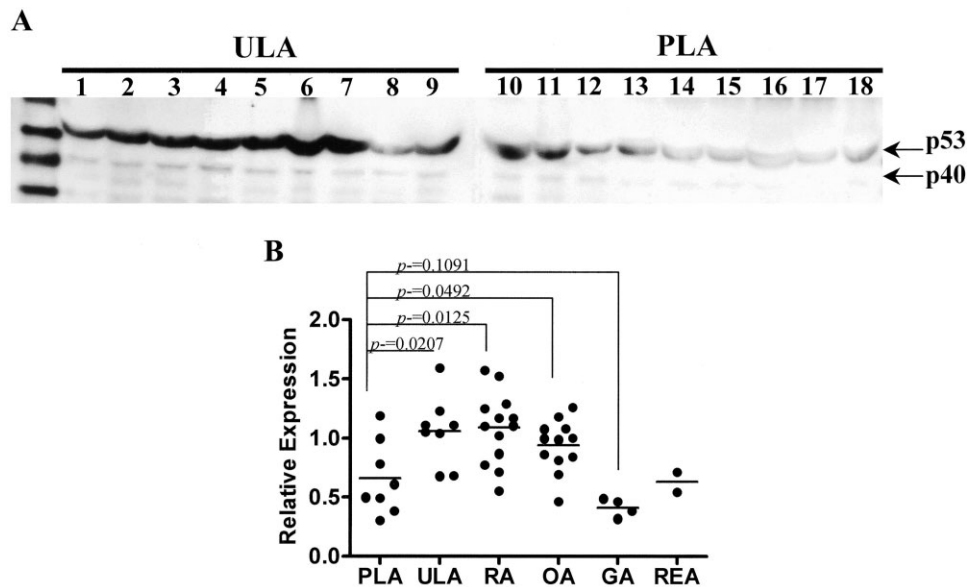


Figure 2. Expression of ADAMTS-4 in synovial fluid (SF) from patients with different forms of arthritis. **A**, SF samples from 9 patients with persistent Lyme arthritis after antibiotic treatment (PLA) and from 9 patients with untreated Lyme arthritis (ULA) were examined for the presence of ADAMTS-4 by Western blotting. Equal volumes of SF (1 μ l) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotted with an anti-ADAMTS-4 antibody. **B**, SF samples from 13 patients with rheumatoid arthritis (RA), 12 with osteoarthritis (OA), 4 with gouty arthritis (GA), and 2 with reactive postinfectious arthritis (ReA) were similarly analyzed for the expression of ADAMTS-4 by immunoblotting. The active p53-kd form of ADAMTS-4 was densitometrically scanned in a randomly selected subset of samples and normalized to a comparator sample (1 SF sample from a patient with persistent Lyme arthritis after antibiotic treatment) that was run on each gel (the value of which was arbitrarily set at 1), and the relative expression of ADAMTS-4 in each arthritis group was plotted. Bars show the mean. *P* values were determined by nonparametric Mann-Whitney U test.

droitin sulfate–bearing region, further C-terminal truncation of ADAMTS-4 is required for destructive cleavage of aggrecan in the interglobular domain. This truncation occurs through the activity of membrane type 4 MMP, resulting in the p53 and p40 forms of the proteinase. The p53 form acts as the major aggrecanase (29).

There was a basal level of ADAMTS-4 protein expression in primary human chondrocytes, which increased within 48 hours following the addition of *B burgdorferi* and continued to increase up to 72 hours after infection (Figure 1C). Both the p53 and p40 forms, representing activated ADAMTS-4, were found in these cells. Although the transcript levels of ADAMTS-4 peaked at 24 hours after infection, protein levels continued to increase through the later time points, as measured by densitometry (Figures 1B and C). The induction of ADAMTS-4 was due to the addition of *B burgdorferi*, since uninfected samples collected at match-

ing time points did not show induction of ADAMTS-4 (data not shown).

Cell-type specificity of ADAMTS-4 induction following *B burgdorferi* infection. To determine the specificity of ADAMTS-4 induction in human chondrocytes following *B burgdorferi* infection, we infected primary human dermal fibroblast cultures, which may contact *B burgdorferi* during the initial entry into humans, and primary human pulmonary artery smooth muscle cell cultures, which are found in an area where no pathology has been reported in Lyme disease. There was no induction of ADAMTS-4 from either cell type, as determined by real-time quantitative RT-PCR (data not shown).

Expression of ADAMTS-4 in SF samples from patients with treated and untreated Lyme arthritis. In order to determine the presence of ADAMTS-4 in the SF of patients with Lyme arthritis, SF samples from 9 patients with untreated Lyme arthritis were examined by

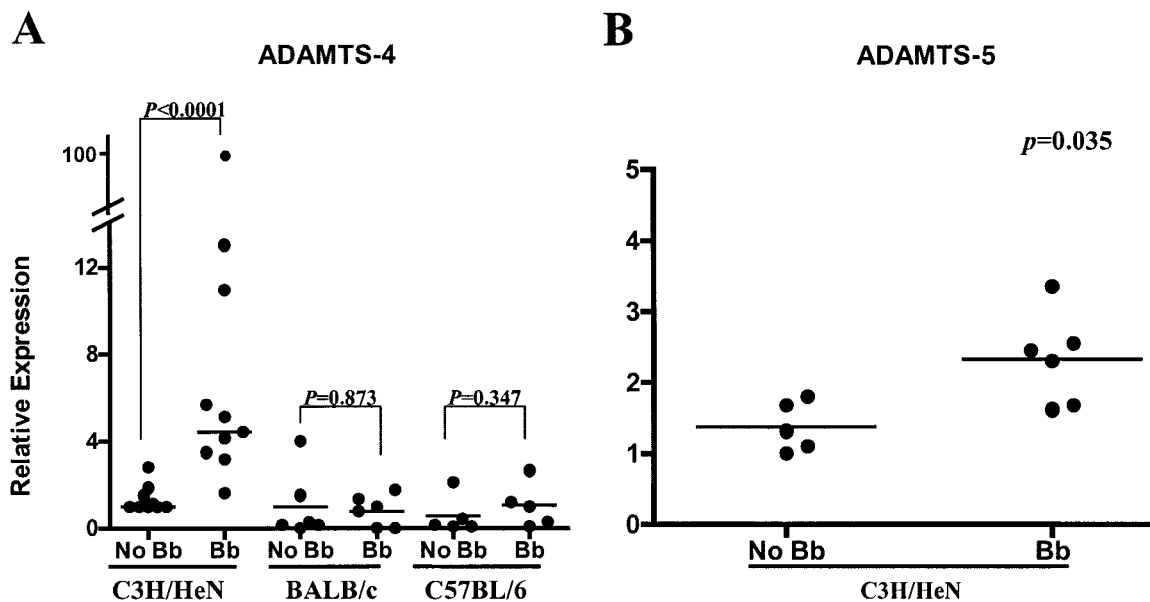


Figure 3. Induction of ADAMTS-4 and ADAMTS-5 in murine cartilage by *Borrelia burgdorferi*. Five-day-old C3H/HeN, BALB/c, and C57BL/6 mice were infected with *B burgdorferi* (Bb; 10^4 low-passage infectious organisms per mouse) or were sham infected. Mice were killed 2 weeks postinfection, ankle joint cartilage was microdissected, and total RNA was isolated. The cDNA generated from total RNA was analyzed for ADAMTS-4 or ADAMTS-5 expression by real-time quantitative reverse transcription–polymerase chain reaction analysis. The expression of ADAMTS-4 or ADAMTS-5 was normalized to that of nidogen, and the relative expression is shown. Bars show the median. *P* values were determined by nonparametric Mann-Whitney U test. **A**, Expression of ADAMTS-4 in C3H/HeN ($n = 10$), BALB/c ($n = 6$), and C57BL/6 ($n = 5$) mice. **B**, Expression of ADAMTS-5 in C3H/HeN mice ($n = 5$ sham-infected mice; $n = 6$ *B burgdorferi*-infected mice).

immunoblotting. Because normal SF is difficult to obtain in amounts sufficient for testing, we used for comparison SF samples from 9 patients with persistent Lyme arthritis after antibiotic therapy in whom no SF *B burgdorferi* was detectable, as well as SF samples from 13 patients with RA, 12 with OA, 4 with gouty arthritis, and 2 with ReA. The ADAMTS-4–specific band was detected in all patients. The level of expression of activated ADAMTS-4 (p53) was significantly higher ($P = 0.0207$) in patients with untreated Lyme arthritis than in those with persistent Lyme arthritis after antibiotic therapy (Figures 2A and B). SF levels of ADAMTS-4 were also elevated in RA ($P = 0.0125$) and OA ($P = 0.0492$) patients as compared with patients with persistent Lyme arthritis after antibiotic therapy and were similar to those in patients with untreated Lyme arthritis (Figure 2B). Expression of ADAMTS-4 in SF samples from patients with untreated Lyme arthritis ($P = 0.004$), RA ($P = 0.0039$), and OA ($P = 0.0092$) were also significantly higher than those in SF samples from patients with gouty arthritis. Expression of ADAMTS-4 in SF from patients with ReA was also lower and was similar to that in patients with persistent Lyme arthritis after

antibiotic therapy, but because of the sample size ($n = 2$), statistical analysis was not done.

Induction of ADAMTS-4 in murine cartilage.

Mice have been an invaluable model in the study of Lyme disease pathogenesis. Mice develop ankle and knee swelling that is clinically similar to that seen in humans with Lyme disease. Different strains of mice show variable degrees of arthritis in response to infection with *B burgdorferi*. In order to determine if ADAMTS-4 expression may play a role in the development of arthritis in a murine model of Lyme disease, we used real-time quantitative RT-PCR to evaluate the expression of ADAMTS-4 and ADAMTS-5 in the joints of C3H/HeN mice, a strain that is highly susceptible to arthritis. Expression of ADAMTS-4 was significantly increased 4.2-fold ($P < 0.0001$) in cartilage from *B burgdorferi*-infected mice as compared with cartilage from sham-infected mice (Figure 3A). There was a single outlier showing very high induction of ADAMTS-4 in the *B burgdorferi*-infected group; however, when the data for that animal were removed from the analysis, the expression of ADAMTS-4 remained statistically significant ($P = 0.0002$). There was minimal

increase (1.7-fold) in the expression of ADAMTS-5 between infected and sham-infected groups, although the difference did not reach statistical significance ($P = 0.035$). The biologic significance of this finding is not clear (Figure 3B).

In order to examine whether the difference in the severity of Lyme arthritis observed in different strains of mice could be correlated with the level of ADAMTS-4, we compared the expression of ADAMTS-4 in 2 well-characterized strains of mice that are less susceptible to arthritis, BALB/c and C57BL/6, with the expression in the C3H/HeN mice. None of the BALB/c mice showed any observable swelling of any joint as compared with the sham-infected mice. There was mild, but noticeable, swelling on both rear tibiotarsal joints in *B burgdorferi*-infected C57BL/6 mice, but the swelling was much less than that observed in C3H/HeN mice. No significant increase in the expression of ADAMTS-4 in the cartilage of either BALB/c or C57BL/6 mice was seen by real-time quantitative RT-PCR (Figure 3A).

Mechanism of *B burgdorferi*-induced ADAMTS-4 gene expression. We have previously shown that *B burgdorferi* induces activation of the MAPK and JAK/STAT pathways in human chondrocytes (19). To examine the role of these pathways in the induction of ADAMTS-4, we determined the expression of ADAMTS-4 in the presence of pathway-specific inhibitors. Inhibitors of ERK-1/2 (U0126) and JNK (SP600125) inhibited gene transcription of ADAMTS-4 by 81% ($P = 0.037$) and 80% ($P = 0.037$), respectively, as compared with the *B burgdorferi*-infected sample alone (Figure 4). However, inhibition of p38 MAPK with SB203580 had no effect on the expression of ADAMTS-4 following *B burgdorferi* infection. Inhibition of the JAK/STAT pathway by JAK-3I completely inhibited (100%) ($P < 0.01$) the expression of ADAMTS-4 (Figure 4).

Role of ADAMTS-4 in *B burgdorferi*-induced cartilage degradation. We next used a bovine articular cartilage explant model to determine the contribution of aggrecanases (including ADAMTS-4) in the degradation of the cartilage matrix following *B burgdorferi* infection. Bovine cartilage has been previously shown to produce MMPs in response to *B burgdorferi* in a manner consistent with that seen in human chondrocytes and identical to that seen in monkey cartilage. Although the use of human cartilage would be preferable, it is complicated because of the difficulty in obtaining sufficient and appropriate samples for study. The inability to select identically located cartilage tissue from different human subjects could lead to significant experimental variation,

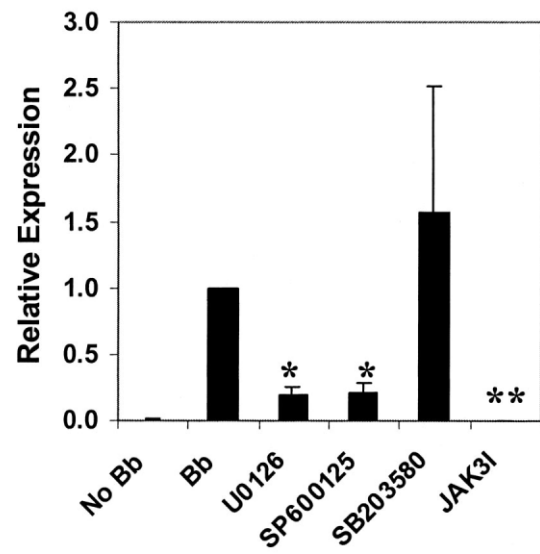


Figure 4. Role of p38 MAPK, ERK-1/2, JNK, and JAK/STATs in *Borrelia burgdorferi*-induced expression of ADAMTS-4 in primary human chondrocytes. Primary human chondrocyte cultures were treated with p38 MAPK inhibitor (SB203580), JNK inhibitor (SP600125), ERK-1/2 inhibitor (U0126), or JAK/STAT pathway inhibitor (JAK-3I) for 2 hours, and subsequently infected with *B burgdorferi* (Bb; 10:1 multiplicity of infection [Bb to cells]) or were sham infected. Cells were harvested at 24 hours postinfection, and ADAMTS-4 expression was examined by real-time quantitative reverse transcription-polymerase chain reaction analysis. The expression of ADAMTS-4 was normalized to that of β -actin. The expression of ADAMTS-4 in cells stimulated with *B burgdorferi* alone was arbitrarily set at 1, and the relative expression is shown. Values are the mean and SD of 3 experiments. * = $P < 0.05$; ** = $P < 0.01$ versus *B burgdorferi*-infected chondrocytes, by nonparametric Mann-Whitney U test.

since chondrocytes found in different types of cartilage and at different levels of cartilage differ significantly in their responses to stimuli. The bovine cartilage explants that were used were taken from identical locations in the knee joint, and this model has been widely used by other investigators in examinations of cartilage degradation (3,16,26,30).

We first confirmed that infection of bovine cartilage explants with *B burgdorferi* induced the expression of ADAMTS-4 (Figure 5A); expression was increased 57-fold as compared with that in uninfected explants ($P = 0.0286$). We next evaluated the role of ADAMTS-4 in cartilage degradation using different inhibitors of MMPs and ADAMTS. Batimastat (BB-94) is a hydroxamate inhibitor, which binds the zinc atom at the active site of the enzyme. It inhibits both MMPs and ADAMTS proteinases. As previously reported (3), the addition of

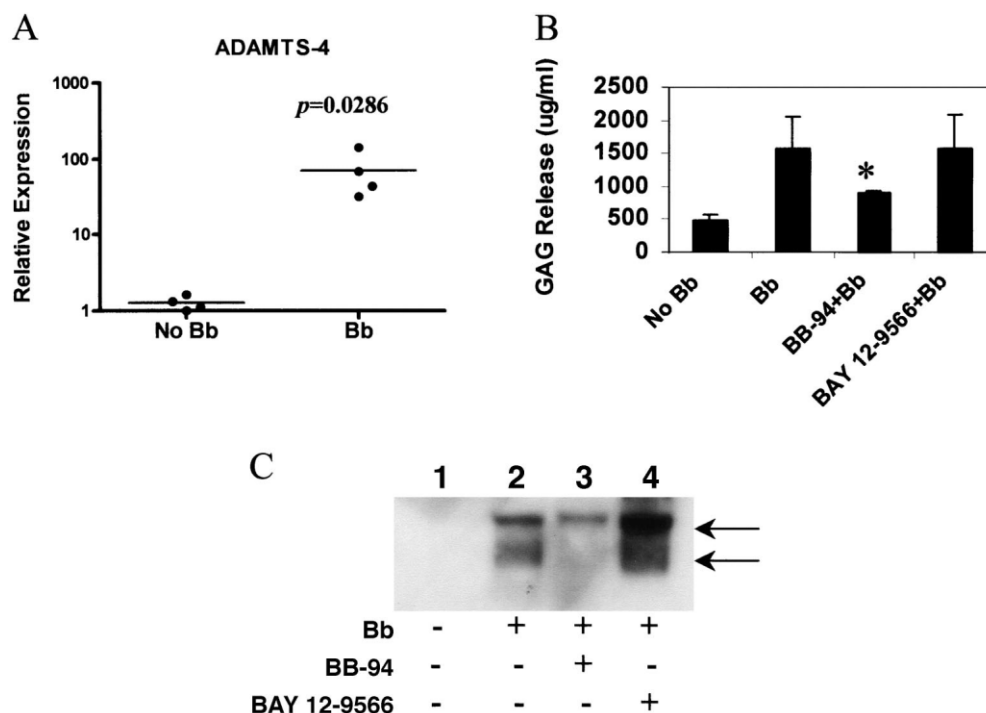


Figure 5. Role of ADAMTS-4 in *Borrelia burgdorferi*-induced cartilage degradation. **A**, Bovine cartilage explants ($n = 4$) were infected with *B burgdorferi* (Bb; 10:1 multiplicity of infection [Bb to cells]) or were sham infected for 2 days, and the expression of ADAMTS-4 was examined by real-time quantitative reverse transcription-polymerase chain reaction analysis. The expression of ADAMTS-4 was normalized to that of bovine GAPDH, and the relative expression is shown. Bars show the mean. The P value was determined by nonparametric Mann-Whitney U test. **B**, Bovine cartilage explants were infected with *B burgdorferi* alone or in the presence of 250 nM batimastat BB-94 (an inhibitor of both matrix metalloproteinases [MMPs] and ADAMTS proteinases) or 300 nM BAY 12-9566 (a selective inhibitor of MMPs), or were sham infected, and the supernatants were collected 48 hours postinfection. Levels of glycosaminoglycans (GAGs) in the supernatants were measured by the dimethylmethylene blue method. Values are the mean and SD of 3 experiments, each performed in triplicate. * = $P < 0.05$ versus *B burgdorferi*-infected explants, by nonparametric Mann-Whitney U test. **C**, Culture supernatants from the bovine cartilage explants were subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gels, and the aggrecanase breakdown fragments were detected using the BC-3 monoclonal antibody. Arrows indicate the aggrecanase-specific fragments, which typically run between 50 kd and 250 kd. A representative result from 3 experiments is shown.

BB-94 significantly inhibited *B burgdorferi*-induced GAG release ($P = 0.046$) (Figure 5B).

GAGs, including aggrecan, can be released from the cartilage matrix after cleavage with either MMPs or "aggrecanases" such as ADAMTS-4. The cleavage site recognized by MMPs differs from that recognized by aggrecanases, resulting in the generation of neoepitopes that can be distinguished by specific antibodies. Using an antibody raised against the neoepitope generated from aggrecan by cleavage with aggrecanases (BC-3 antibody), we found that BB-94 also significantly inhibited the generation of this neoepitope. We did not have access to a specific inhibitor of ADAMTS-4, so in order to determine whether the release of GAGs was due

predominantly to cleavage by MMP-type enzymes or by ADAMTS-type enzymes, we compared the effects of BB-94 with that of a more specific MMP inhibitor, BAY 12-9566. BAY 12-9566 is a selective nonpeptide biphenyl inhibitor of MMPs with nanomolar inhibitory activity against MMP-2, MMP-3, and MMP-9 (31,32), but no known ability to inhibit aggrecanase activity. As expected, using the BC-3 antibody, we found that BAY 12-9566 did not inhibit the amount of aggrecanase-cleaved aggrecan detected in supernatants after *B burgdorferi* infection, suggesting that it did not have an effect on ADAMTS activity (Figure 5C). Total GAG release in the presence of *B burgdorferi* was also not reduced by BAY 12-9566 (Figure 5B). This strongly suggests that

the majority of the *B burgdorferi*-induced GAG release is mediated by aggrecanases rather than by MMPs.

DISCUSSION

MMPs and ADAMTS are both members of the broader family of metalloproteinases, the predominant host enzymes that cleave extracellular matrix proteins (11,33). Our understanding of the roles of each family member in different diseases is continuing to evolve. The expression of specific MMPs, including MMP-1, MMP-3, MMP-13, and MMP-19, is induced in joint tissue by infection with *B burgdorferi* (3,4,18). We show herein that *B burgdorferi* is capable of inducing ADAMTS-4, but not ADAMTS-5, in human chondrocytes.

The signaling pathways involved in the induction of ADAMTS-4 by *B burgdorferi* show some overlap with those responsible for the induction of MMPs, but notably, the p38 MAPK pathway does not seem to be involved. Previous studies have shown that activation of p38 MAPK is important both for the induction of MMPs and for the development of joint swelling in infected mice (19,34). This suggests that the pathways involved in the induction of ADAMTS-4, and by extension, aggrecan digestion and release, may be distinct from those involved in the mediation of inflammation and swelling, although they are likely to coexist in natural disease states. Our studies of inbred strains of mice with different susceptibilities to the development of Lyme arthritis appear to confirm this. While the most susceptible strain, C3H, exhibited a significant increase in ADAMTS-4 in infected joint tissue, the 2 more resistant strains, BALB/c and C57BL/6, were similar in their ADAMTS-4 responses (no significant increase) despite differences in joint swelling between the strains. Of note, in arthritic diseases such as RA, joint swelling and histologic progression of arthritis (e.g., permanent cartilage erosions) may be mediated by independent pathways; the effects of p38 inhibition on the development of histologic evidence of arthritis in *B burgdorferi*-infected mice has not been reported.

To determine whether ADAMTS-4 expression is elevated in Lyme arthritis in humans, we compared expression levels in SF samples from patients with untreated Lyme arthritis, persistent Lyme arthritis after antibiotic therapy, RA, OA, gouty arthritis, and ReA. The cause of persistent Lyme arthritis after antibiotic therapy remains a subject of controversy. Four basic hypotheses have been proposed as a mechanism for persistent Lyme arthritis after antibiotic therapy (35):

persistent infection, retained spirochetal antigens, infection-induced autoimmunity resulting from molecular mimicry, and nonspecific bystander activation. For the purposes of our study, the salient feature is that patients with persistent Lyme arthritis after antibiotic therapy have clearly lower amounts (if any) of bacteria present in the joints and SF.

We found that SF levels of ADAMTS-4 were significantly higher in patients with untreated Lyme arthritis as compared with patients with persistent Lyme arthritis after antibiotic therapy, which is consistent with our in vitro studies showing that *B burgdorferi* directly induces ADAMTS-4 production. Levels of expression of the active form of ADAMTS-4 (p53) were similar in SF from patients with untreated Lyme arthritis, RA, and OA. These levels were significantly higher than those in SF from patients with persistent Lyme arthritis after antibiotic therapy and patients with gouty arthritis. As with other inflammatory molecules, it is likely that ADAMTS-4 can be induced by different stimuli that result in similar activation of signaling pathways and the release of cytokines (either from chondrocytes or from migratory inflammatory cells). It is tempting to speculate that clinical progression and histologic changes are governed by the specific set of proteases that are induced and that similarities in histologic features of disease (e.g., Lyme arthritis and RA) are due to similarities in protease activity.

The catabolism of aggrecan with the subsequent loss of GAG-bearing aggrecan fragments from articular cartilage and their release into the SF is an early and persistent process during arthritic diseases. We have shown that GAG release in response to *B burgdorferi* in a bovine explant model appears to be predominantly mediated by aggrecanase activity and not by MMP activity. Whether this remains true in vivo, where there are many different factors, including the presence of activators and inhibitors generated from other cells, has not been ascertained. However, aggrecanase-type digestion fragments of aggrecan have previously been reported to be present in the joints of patients with Lyme arthritis (3), confirming that at a minimum, aggrecanases are active in Lyme arthritis.

Even if ADAMTS-4 plays the major role in cleaving aggrecan in Lyme arthritis, this does not preclude a role of MMPs in the development of arthritis. Cleavage of aggrecan attached to the collagen matrix is typically an early and fully reversible event in the development of arthritis. Irreversible damage to the cartilage matrix does not occur until collagen is cleaved and degraded. Aggrecan has been shown to protect

cartilage collagen from proteolytic cleavage (36). The keratan sulfate-rich region of aggrecan binds to type II collagen (37), thereby positioning the collagen fibril where it is protected by the highly sulfated chondroitin sulfate-rich regions of the aggrecan, preventing access to the fibrils (36). Thus, in the development of arthritis, cleavage and release of GAGs is a required early event that subsequently allows access of collagenases such as MMP-1 and MMP-13 to the collagen matrix. In Lyme arthritis in humans, both ADAMTS-4 and collagenases (MMP-1 and MMP-13) may be required for permanent damage to occur. In support of this, mice, including the most arthritis-susceptible C3H strains, do not have increased levels of interstitial collagenases in response to *B burgdorferi* infection and, in contrast to humans infected with *B burgdorferi*, do not develop permanent erosions, despite induction of ADAMTS-4 and noncollagenase MMPs such as MMP-3 and MMP-19 (18).

In conclusion, we have shown that *B burgdorferi* induces the expression of ADAMTS-4 in human chondrocytes, in susceptible strains of mice, and in patients with Lyme arthritis. Both in vitro and in vivo studies have shown that this protease is processed and found in its most active form—suggesting that it is likely having an effect—within the joint. Our current model for the pathogenesis of Lyme arthritis is that ADAMTS-4 cleaves aggrecan, thereby exposing the collagen matrix, which can then be processed by MMPs, leading to permanent cartilage degradation. Use of selective aggrecanase inhibitors may impart cartilage protection by preventing aggrecan degradation without some of the negative responses associated with more broad-spectrum MMP inhibitors. This will need to be determined by future studies.

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