Differences in Synovial Fluid Levels of Matrix Metalloproteinases Suggest Separate Mechanisms of Pathogenesis in Lyme Arthritis before and after Antibiotic Treatment


The cause of persistent arthritis in patients with Lyme disease who have received standard antibiotic therapy remains an area of debate. In this study, synovial fluid levels of matrix metalloproteinases (MMPs) were compared in persons with untreated and antibiotic-resistant Lyme arthritis. Levels of MMP-1 and MMP-3, as determined by ELISA, were higher in untreated patients (P = .0064 and P = .002, respectively), whereas levels of MMP-8 and MMP-9 were higher in antibiotic-resistant patients (P = .0002 and P = .0014, respectively). In vitro studies of chondrocyte cultures infected with *Borrelia burgdorferi* revealed induction of MMP-1 and MMP-3 but not of MMP-8 or MMP-9. Neither *Staphylococcus aureus* nor lipopolysaccharide stimulated MMP-1 or MMP-3 release from these cells. The mechanism of recognition of *B. burgdorferi* may be through CD14 and toll-like receptor–2, which were up-regulated in the presence of *B. burgdorferi*. These findings suggest different stimuli for MMP induction in untreated and antibiotic-resistant Lyme arthritis.

Oligoarticular arthritis is a prominent feature of late-stage Lyme disease in North America [1]. When left untreated, infection with *Borrelia burgdorferi* can result in intermittent or chronic arthritis that may progress to an erosive arthritis, primarily affecting the knees, with histopathologic similarities to rheumatoid arthritis [2]. Treatment with antibiotics resolves the arthritis in ~90% of patients. However, the remaining 10% continue to have persistent arthritis—often despite multiple courses of antibiotics [3]. The cause of arthritis in patients with post-treatment disease remains an area of intense debate. The 2 major hypotheses are that antibiotics are unable to completely eradicate *B. burgdorferi*, resulting in persistent arthritis, and that persistent disease is the result of autoimmune phenomena that develop after contact with *B. burgdorferi* but does not require the continued presence of the organism. There is some evidence to support each of these hypotheses [4–6]. There is little current understanding of specific pathways and enzymes involved in cartilage degradation for either untreated or antibiotic-resistant Lyme arthritis. In septic arthritis caused by bacteria, such as *Staphylococcus aureus*, a combination of bacterial proteases and host proteases from inflammatory cells leads to rapid destruction of articular cartilage. In contrast, both untreated and antibiotic-resistant Lyme arthritis are slowly progressing processes, in which synovial fluid studies typically reveal only moderate elevations of inflammatory cells [3]. There has been a single report that stated that lysates of *B. burgdorferi* possess a collagenolytic enzyme >200 kDa [7]. However, we and others have found that *B. burgdorferi* does not secrete any enzymes capable of digesting collagen, laminin, or gelatin [8, 9]. In addition, a search of the *B. burgdorferi* genome showed no genes with significant identity to those of other bacterial collagenases [10].

Matrix metalloproteinases (MMPs) are enzymes produced by host cells that are capable of digesting many of the components of cartilage tissue [11]. They have been implicated in the pathogenesis of other slowly progressive arthritides, such as rheumatoid arthritis and osteoarthritis [12–15]. We showed elsewhere that the presence of *B. burgdorferi* can cause degradation of cartilage explants, as measured by release of glycosaminoglycans and hydroxy-proline from cartilage explants [16]. This breakdown is inhibited by batimastat, a specific inhibitor of MMPs, which confirms the role of MMPs in *B. burgdorferi*-induced cartilage degradation. Here we present data on the individual MMPs found in the synovial fluid of patients with untreated and antibiotic-resistant Lyme arthritis.

Patients and Methods

**Patients.** Synovial fluid was obtained from 18 patients with untreated Lyme arthritis (ULA) and 15 patients with persistent
Lyme arthritis (PLA) after antibiotic therapy. All patients were infected in the northeastern United States and had oligoarticular arthritis involving 1 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. They had monoarticular or oligoarticular arthritis affecting ≥1 knee that was accompanied by a positive IgG Western blot test for Lyme disease, which was interpreted according to CDC/Association of State and Territorial Public Health Laboratory Directors criteria [17].

All 18 ULA patients had positive polymerase chain reaction (PCR) results for B. burgdorferi DNA in synovial fluid (done as described in [18]). All 15 PLA patients tested negative for B. burgdorferi by a positive IgG Western blot test for Lyme disease, which was described in [18].

Infection of cell cultures with B. burgdorferi. Primary human chondrocyte cultures from a healthy donor and primary human synoviocyte cultures from a patient with rheumatoid arthritis were obtained from Cell Applications. Cells were grown to confluence in 6-well trays in either chondrocyte or synoviocyte culture media (Cell Applications) containing 10% fetal calf serum (FCS). At 24 h before infection with B. burgdorferi, the culture medium was changed to either chondrocyte or synoviocyte culture medium without FCS. Low-passage (passage 4–8) B. burgdorferi strain N40 was cultured in Barbour-Stoenner-Kelly (BSK) medium, as described elsewhere [19, 20].

For experiments with killed bacteria, 10^7 B. burgdorferi (N40) or S. aureus were sonicated for 2.5 min (Branson Sonifier 450) or were heated at 100°C for 10 min and added to cell cultures, as described above. Lipidated outer surface protein (Osp) A of B. burgdorferi was generated as a recombinant fusion protein to maltose-binding protein, as described elsewhere [21].

MMP assays. Sandwich ELISAs for MMP-1, MMP-3, MMP-8, and MMP-9 were obtained from Oncogene Research and were done according to the manufacturer’s instructions. All samples were run in duplicate. Samples that showed intra-assay variability >10% were repeated. Selected samples were tested in 3 separate experiments to verify interexperiment reliability. Variability between experiments was <10%. For calculation purposes, samples that fell below the limit of detection were assigned values at the limit of detection. Gelatinolytic zymography also was performed to assess levels of MMP-2 and MMP-9. 0.1 µL of synovial fluid was subjected to zymography in 7.5% polyacrylamide gels containing 0.1% gelatin as a substrate, as described elsewhere [22].

Reverse-transcriptase (RT) PCR. Total RNA was obtained from cells by use of RNeasy (Qiagen), according to the manufacturer’s instructions. DNaseI (Gibco) was added to the preparation to remove any DNA contamination. RT-PCR was performed by using Superscript (Gibco), according to the manufacturer’s instructions. Primers for CD14 (5′-AGACTTATCGACCATGAGC-3′ and 5′-GCCCTACCAGTAGCTGAGCAG-3′) [23], TLR-2 (5′-AC- TTTGTTGATGGTGGTGGTTGCTTG-3′ and 5′-AGATGGTTCC- CCTAGGTTTTGTGAAGGA-3′), and TLR-4 (5′-TACAAAA- TCCCGAACACCTCCCT-3′ and 5′-AACGATGGAATTCT- AAACCGCCAGA-3′) produced predicted products of 333, 420, and 637 bp, respectively. PCR products were electrophoresed on 1% agarose gels. Quantitative comparison of bands was done by using scanning densitometry.

Results

Comparison of synovial fluid MMPs in patients with ULA and antibiotic-resistant PLA. We examined synovial fluid from 18 patients with ULA and from 15 patients with PLA for the presence of MMP-1, MMP-2, MMP-3, MMP-8, and MMP-9, using ELISA or gelatinolytic zymography. Because there is minimal synovial fluid in healthy joints, comparison with normal synovial fluid could not be made. All patients with ULA had evidence of B. burgdorferi in their synovial fluid, as determined by PCR, for the OspA gene of B. burgdorferi. All patients with PLA were negative for B. burgdorferi DNA, using PCR. By using ELISA, we found that levels of MMP-1 and MMP-3 were significantly elevated in patients with ULA, compared with those in patients with PLA (figure 1). Levels of MMP-8 and MMP-9 were reversed, with significantly higher levels in the synovial fluid of patients with PLA than that of patients with ULA. In fact, the data for MMP-9 underestimate the true difference between the groups, because 13 of the 18 ULA samples fell below the level of detection of the assay and were assigned values equal to the level of detection (0.15 µg/mL) for calculation. Levels of MMP-2 and MMP-9 also were determined by gelatinolytic zymography. Densitometry measurements from zymograms for MMP-9 were comparable with ELISA results. No significant differences in levels of synovial fluid MMP-2 were seen between the groups (data not shown).

WBC counts and synovial fluid MMP levels. One possible source of MMPs in the synovial fluid of these patients is inflammatory cells. We had data for synovial fluid WBC counts for 17 samples from patients with ULA and for 10 samples from patients with PLA. Although the patients with ULA generally had a higher WBC count than did the patients with PLA (mean, 24,900 and 13,200 cells/µL, respectively), this did not reach significance (P = .072). There was no correlation between WBC counts and levels of MMP-1, MMP-3, or MMP-8 in patients with ULA or of MMP-1 and MMP-3 in patients with PLA. Values for MMP-9 in patients with ULA were too low for meaningful analysis, as only 4 values were above the limit of detection. WBC counts did correlate with levels of MMP-8 and MMP-9 in patients with PLA. The correlation coefficient...
was 0.82 ($P = 0.003$) for WBC counts and MMP-8 and 0.69 ($P = 0.028$) for WBC counts and MMP-9.

**Induction of MMPs from chondrocyte and synoviocyte cell cultures.** Since there did not appear to be a correlation between WBC counts and MMPs in the synovial fluid of patients with ULA, we next sought to determine whether *B. burgdorferi* could induce production of these MMPs from intrinsic joint cells. We added *B. burgdorferi* ($10^7$ organisms), tumor necrosis factor (TNF)-α (4 ng/mL), and *Escherichia coli* lipopolysaccharide (LPS; 10 ng/mL) to cultures of human chondrocytes and synoviocytes. Addition of TNF-α increased secretion of MMP-1 and MMP-3 from both chondrocytes and synoviocytes and MMP-9 from synoviocytes (figure 2). MMP-8 was not detectable in any of the supernatants (data not shown). LPS did not induce any of the 4 MMPs from either chondrocytes or synoviocytes. The addition of *B. burgdorferi* resulted in only minimal increases in MMP-1 and in no increases in MMP-3, MMP-8, or MMP-9 from synoviocytes. However, addition of *B. burgdorferi* resulted in a 230-fold increase in MMP-1 and a 103-fold increase in MMP-3 from chondrocytes. No induction of MMP-8 or MMP-9 by *B. burgdorferi* from chondrocytes was seen. The induction of MMP-1 and MMP-3 from chondrocytes was dose related (figure 3). As would be predicted by a lack of response to LPS, the addition of polymyxin B did not inhibit the MMP responses to *B. burgdorferi* (not shown).

**Characterization of MMP-1 and MMP-3 induction from chondrocytes.** We next sought to test whether the response of chondrocytes to *B. burgdorferi* is dependent on the viability of the spirochetes. We have found that *B. burgdorferi* grown in coculture with chondrocytes in cell culture medium remain viable for up to 1 week (i.e., can be recultured in BSK medium), although they will not grow or multiply in this medium. We added heat-killed and sonicated *B. burgdorferi* to chondrocyte cultures. Both heat-killed and sonicated spirochetes were capable of inducing MMP-1, although the response to heat-killed spirochetes was diminished (figure 4). Sonicated, but not heat-killed, *B. burgdorferi* were capable of inducing MMP-3 from chondrocytes. The *B. burgdorferi* lipoprotein OspA also was capable of inducing MMP-1 and MMP-3 release in a dose-related pattern. In contrast, sonicated *S. aureus* was incapable of inducing MMP-1 or MMP-3 from chondrocytes. Live *S. aureus* could not be studied because it grew rapidly in the culture medium, resulting in death of the chondrocytes.

**CD14 and toll-like receptor (TLR) expression by chondrocytes.** Reports by other investigators have suggested a role for CD14 and TLR-2 in cellular response to *B. burgdorferi* [24–26]. It appears that *B. burgdorferi* lipoproteins bind to CD14, which then interact with TLR-2 to signal for cellular activation [27]. We reported elsewhere that soluble (s) CD14 levels are increased in patients with untreated Lyme disease [28]. We examined expression of CD14, TLR-2, and the LPS receptor, TLR-4, by using semiquantitative RT-PCR. By using this method, we found that CD14 mRNA is transcribed by both chondrocytes and synoviocytes (figure 5A). Transcription of CD14 mRNA is minimally increased (~2–3-fold) from chondrocytes in the presence of *B. burgdorferi*, as measured by scanning densitometry of the amplicons. sCD14 measured by ELISA in chondrocyte culture supernatants was increased 2-
Figure 2. Induction of matrix metalloproteinase (MMP)-1, MMP-3, and MMP-9 from chondrocytes and synoviocytes. Levels are shown of MMP-1, MMP-3, and MMP-9 expression in supernatants from chondrocytes (A1–A3, respectively) and synoviocytes (B1–B3, respectively) after 5 days of incubation in presence of tumor necrosis factor (TNF)-α (4 ng/mL), lipopolysaccharide (LPS; 10 ng/mL), and Borrelia burgdorferi (BB; 10^7). No MMP-8 was detectable from chondrocytes or synoviocytes under any experimental conditions. Error bars, mean SEs of 3 experiments done in duplicate.

fold in the presence of B. burgdorferi. Neither CD14 mRNA transcription nor sCD14 in culture supernatants was increased in synoviocyte cultures in the presence of B. burgdorferi. TLR-2 is expressed by chondrocytes, and expression is increased >23-fold in the presence of B. burgdorferi (figure 5B). In contrast, we did not find expression of TLR-2 in our synoviocyte line.

Discussion

The pathways involved in the development of Lyme arthritis are just beginning to be explored. In this study, we found significant differences in the MMPs present in the synovial fluid of patients with ULA and PLA. Increases in synovial fluid MMP-1 and MMP-3 in patients with ULA, who have detectable B. burgdorferi DNA in their synovial fluid, are paralleled by the ability of the organism to induce these MMPs from chondrocytes in culture. B. burgdorferi are not capable of inducing MMP-8 or MMP-9, which are higher in PLA patients, from either synoviocytes or chondrocytes. The significant cor-
Figure 4. Matrix metalloproteinase (MMP)-1 (A) and MMP-3 (B) induction from chondrocytes by killed bacteria and borrelial lipoproteins. Chondrocytes were incubated in presence of 10^7 live *Borrelia burgdorferi* (Bb), 10^7 heat-killed *B. burgdorferi* (Bb heated), 10^7 sonicated *B. burgdorferi* (Bb sonicated), 10^7 sonicated *Staphylococcus aureus*, or recombinant *B. burgdorferi* outer surface protein A (OspA) at 5–5000 ng/mL. Cell culture supernatants were collected after 5 days and were tested by ELISA for MMP induction. Error bars, mean SEs of 3 experiments done in duplicate.

Figure 5. Expression of CD14, toll-like receptor (TLR)-2, and TLR-4 mRNA from chondrocytes and synoviocytes. A, CD14 expression in chondrocytes and synoviocytes: soluble (s) CD14 levels in cell culture supernatants, as measured by ELISA, from control and *Borrelia burgdorferi* (Bb)-infected cells and expression of CD14 mRNA compared by reverse-transcriptase polymerase chain reaction (RT-PCR) for control (lane C) and Bb-infected chondrocytes and synoviocytes. Primers for β-actin were used to ensure equal loading of cDNA. B, RT-PCR for expression of TLR-2 and TLR-4 from chondrocytes (origin by lane): lane 1, control cells; lane 2, cells incubated with tumor necrosis factor–α (4 ng/mL); lane 3, cells incubated with lipopolysaccharide (10 ng/mL); lane 4, cells incubated with Bb (10^7). No transcription of TLR-2 or TLR-4 mRNA was seen in synoviocytes (data not shown). Predicted product sizes were as follows: CD14, 333 bp; TLR-2, 420 bp; and TLR-4, 637 bp. Gels are representative of results of 3 experiments. Error bars, mean SEs of 3 experiments. Lane M, 1-kb DNA ladder.
B. burgdorferi recognition of consistent with a role for this system in MMP induction. Recognition of B. burgdorferi lipoproteins by the CD14–TLR-2 system is lost with deacylation and loss of lipid attachment [25, 27, 35]. The lack of MMP induction from chondrocytes by S. aureus furthers the concept that there is specificity in the recognition of B. burgdorferi and its products and in the subsequent development of chronic arthritis. The minimal expression of the LPS receptor TLR-4 on our cell lines is consistent with the lack of MMP response to LPS from chondrocytes and synoviocytes. S. aureus and LPS containing gram-negative bacteria cause acute arthritis characterized by rapid destruction of the joint through secretion of bacterial proteases and activation of WBC counts. The clinical and histologic development of ULA, with its slow progression to erosive arthritis, is more consistent with chronic arthritisides, such as rheumatoid arthritis or osteoarthritis, in which the induction of MMPs from joint tissue is thought to play an important role.

In contrast, PLA does not typically result in radiographically apparent joint erosions, even after longstanding disease (authors’ unpublished data). One possible explanation for this difference in phenotype is the difference in synovial fluid MMPs. Our data suggest that the stimulus for MMP induction in PLA is unlikely to be mediated through the innate immune response to B. burgdorferi in intrinsic joint cells. B. burgdorferi is not typically detectable in the synovial fluid of patients with arthritis after antibiotic therapy [36]. In the absence of borrelial lipoproteins in the joint, the activation of the innate immune response does not occur. Furthermore, the MMPs elevated in the synovial fluid of patients with PLA are not induced from chondrocytes or synoviocytes by B. burgdorferi.

One hypothesis to account for the ongoing joint inflammation is that, in a small percentage of genetically susceptible patients, there is inappropriate persistence of the antigen-specific response after antibiotic therapy. Gross et al. [6] found evidence for molecular mimicry between a dominant epitope of OspA and lymphocyte function–associated antigen–1 in patients with post–antibiotic treatment Lyme arthritis, suggesting an autoimmune pathogenesis of arthritis in these patients. The presence of a population of activated autoreactive T cells in the synovial fluid of PLA patients could explain the higher levels of MMP-8 and MMP-9, despite overall lower WBC counts, than in ULA patients. The signaling pathway by which molecular mimicry can lead to induction of MMP-8 and MMP-9 remains to be determined.

An understanding of the role of individual MMPs in the development of either ULA or PLA will require further study. Our in vitro studies do not address the impact of the various human MMP activators and inhibitors that may be coinduced by B. burgdorferi or the impact of the maturational stage of the cells and the complex cell-to-cell interactions that affect the induction of MMPs. However, the evidence for differences in the pathway to induction of these MMPs has important implications in the treatment of patients with these diseases. Patients with “chronic” Lyme disease continue to be treated with prolonged courses of antibiotics. Prolonged antibiotic therapy exposes patients to many risks, including adverse drug reactions, infections with resistant microorganisms, catheter-related infections, and, as recently reported for a patient receiving long-term therapy for chronic Lyme disease, even death [37]. Our study results argue that PLA should not be treated as a persistent B. burgdorferi infection. Understanding the basis for arthritis in PLA may lead to the development of more specific anti-inflammatory–based therapies for patients with persistent posttreatment disease.

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References

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