Increased vulnerability of postarthritic cartilage to a second arthritic insult: accelerated MMP activity in a flare up of arthritis

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Abstract

Objective—Murine antigen induced arthritis (AIA) is a chronic, smouldering inflammation. Flares of arthritis can be induced by antigen rechallenge or exposure to inflammatory mediators like interleukin 1 (IL1). These flares are characterised by a fast and marked proteoglycan (PG) depletion if compared with the initial arthritis. This study investigated the involvement of metalloproteinases in both the initial and the flare phase of arthritis.

Methods—Murine AIA was induced and a flare up of arthritis was induced by injection of 10 ng of IL1β. Messenger RNA levels of MMP-1 and -3 were studied by RT-PCR. MMP activity in cartilage, during both primary AIA as well as the flare up of arthritis, was studied by immuno detection of MMP specific neoepitopes in aggrecan (VDIPEN). Cartilage just before flare induction was analysed for presence of MMPs at the mRNA level as well as at the protein level by zymography.

Results—At the onset of AIA, a fast upregulation of mRNA for stromelysin and collagenase was noted. However, no VDIPEN epitopes were detected during this early phase of arthritis. They appeared when PG depletion was severe at day 7 of arthritis and disappeared when cartilage was repaired. IL1 injection into a knee joint at week 4 of AIA caused a flare up of arthritis, coinciding with a fast and marked PG degradation. This degradation was characterised by accelerated expression of VDIPEN epitopes if compared with the expression in primary AIA. Analysis of cartilage at week 4 of AIA showed still increased mRNA levels of MMP-1 and -3. Moreover, increased levels of latent MMPs were present as well, as APMA activation induced profound VDIPEN epitope. In vitro exposure to IL1 did show increased PG breakdown but no VDIPEN expression, suggesting that factors in addition to IL1 are needed to cause the in vivo VDIPEN expression.

Conclusions—The fast and marked PG depletion seen in a flare up of AIA coincides with accelerated expression of MMP induced neoepitopes compared with expression during primary AIA. This accelerated expression is probably linked to increased levels of latent enzyme, which were found to be present in the cartilage before induction of a flare up.

The destruction of joint cartilage is a characteristic feature in rheumatoid arthritis (RA). Metalloproteinases (MMPs) have been implicated in this process, but lately involvement of MMPs in cartilage destruction has been a matter of debate. Although high amounts of MMPs are found in synovial tissue and synovial fluid of arthritis patients most of the enzymes found are in the inactive proform. Analysis of proteoglycan (PG) fragments in synovial fluid of RA patients showed products resulting from two major cleavage sites in aggrecan. One results from cleavage at the Asp 341-Phe342 bond and is specific for MMP8. The other is a result of cleavage at the Glu373-Ala374 bond and this site is caused by an up to now unidentified enzyme activity named aggrecanase. Involvement of MMPs in cartilage degradation during RA has been demonstrated by detection of MMP specific neoepitopes both in synovial fluid as well as in cartilage of RA patients. We have recently shown that blocking interleukin 1 (IL1) during murine antigen induced arthritis reduces flares 

References

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the IL1 induced flare up of arthritis. This was done by studying mRNA levels of two important MMPs in the cartilage layer: MMP-1 and -3. MMP activity was furthermore studied by detection of the MMP specific neoepitope VDIPEN.

Methods

ANIMALS

Male 8–10 week old C57Bl/6 mice were used. They were kept in cages with a wood chip bedding. The animals were maintained on a commercial pellet diet (Hope Farms, Deventer, the Netherlands); tap water was provided ad libitum.

INDUCTION OF ANTIGEN INDUCED ARTHRITIS

Mice were immunised with 100 µg of methylated bovine serum albumin (mBSA, Sigma) emulsified in 100 µl Freud’s complete adjuvant (FCA). Injections were divided over both flanks and footpads of the forelegs. Heatkilled Bordetella pertussis (2 × 10¹⁰ organisms) was administered intraperitoneally as an additional adjuvant. Two subcutaneous booster injections with 50 µg mBSA/FCA were given in the neck region one week after the initial immunisation, without further boosting with Bordetella pertussis. Two weeks after these injections, arthritis was induced by intra-articular injection of 60 µg mBSA in 6 µl saline into the right knee joint, resulting in semi-chronic arthritis.

IL INJECTIONS

Mice were injected intra-articularly with 10 ng recombinant murine IL1α once every day for four days. One day after the last injection, mice were killed and knee joints were dissected for histological examination.

HISTOLOGICAL EXAMINATION

Knee joints were dissected and subsequently fixed in phosphate buffered formalin (pH7.4), decalcified in 5% formic acid, and embedded in paraffin wax. Semiserial frontal whole knee joint sections (7 µm) were stained with safranin O and fast green. Adjacent sections were used for immunohistochemical staining of the MMP induced neoepitope.

IMMUNOCOLocalisation of MMP Induced Neoepitope

For immunohistochemical analysis, sections were deparaffinised, rehydrated, and digested with chondroitinase ABC (Sigma, 0.25 U/ml 0.1 M TRIS-HCl pH 8.0) for one hour at 37°C, to remove chondroitin sulphate from the proteoglycans. Sections were then treated with 1% H2O2 in methanol for 20 minutes and subsequently five minutes with 0.1%/v/v triton X-100 in PBS. After incubation with 1.5%/v/v normal goat serum for 20 minutes, sections were incubated with affinity purified anti-VDIPEN IgG overnight at 4°C. These antibodies have been characterised before.22 24 In addition, sections were incubated with biotinylated goat anti-rabbit IgG and detected using avidine peroxidase staining (Elite kit, Vector Labs, Burlington, CA). Development of the peroxidase product was done using nickel enhancement and counterstaining was done with orange G (2%) for five minutes.

PROTEOGLYCAN DEGRADATION ASSAY

Whole murine patellas were dissected and prelabelled for three hours with 20 µCi/ml[35S]-sulphate in RPMI, which labels the newly synthesised proteoglycans. In addition, patellas were cultured for three hours in RPMI without any substitutions to process the labelled proteoglycans. After exposing the patellas to different mediators, the amount of labelled[35S]-sulphate left in the patellar cartilage was measured and expressed as a percentage of the[35S]-sulphate content of normal patellas cultured without these mediators.25

ISOLATION OF PATELLAR CARTILAGE

The cartilage layers from patellas were isolated after decalcification with 5% EDTA for four hours at 4°C. Subsequently, the patellas were washed in 0.9% NaCl and the cartilage layer was carefully removed from the underlying bone using forceps and a dissection microscope.

RNA ISOLATION AND RT-PCR

Messenger RNA quantification in murine cartilage was done as described earlier. Briefly, cartilage layers from patellas were obtained and total RNA was isolated using TRizol Reagent (Life Technologies). The total amount of mRNA was reverse transcribed into cDNA using Moloney murine leukaemia virus reverse transcriptase (Life Technologies). One twentieth of the total cDNA was used in one PCR. PCR was performed at a final concentration of 50 mM KCl, 20 mM TRIS pH8.4, 1.5 mM MgCl2, 200 mM dNTPs, 0.4 mM of each primer and 40 u/ml Taq-polymerase (Life Technologies). Semi-quantitative PCR was performed by removing aliquots (5 µl) of the PCR reaction after increasing number of cycles. The PCR products were electrophorised in a 1.6% agarose gel containing ethidium bromide. The cycle number at which the product was first detected on the agarose gel was taken as a measure for the amount of specific mRNA present in the originally isolated RNA. GAPDH was used to verify that equal amounts of RNA were put in the reaction.

ISOLATION OF MMPs AND ZYMOGRAPHY

Cartilage was extracted for 24 hours at 4°C in 10 mM cacodylate buffer (pH 6) containing 0.3 M NaCl, 5 mM CaCl2, 1 mM ZnCl2, 0.02%/v/v Na2EDTA, 0.1%/v/v TritonX-100, and 0.7 mg/ml pepstatin. Ten cartilage layers were extracted in 1 ml extraction buffer, after which this extract was dialysed against water and concentrated 10 times to a volume of 100 µl.

Gelatinase activity was determined by zymography according to modification of the method of Heussen and Dowdle.27 Slab gels containing 2 mg/ml gelatin (Difco Lab, Detroit, MI) were copolymerised in 10% polyacrylamide. After electrophoresis, SDS was removed from gels and subsequently incubated in 50 mM TRIS (pH7.5), 5 mM CaCl2, 1 mM ZnCl2, and 1%TritonX-100 for
18 hours at 37°C. Gels were stained in Coomassie blue. Enzymes were detected as clear bands within the blue background.

Results

INVolvement of MMPs in PG Degradation during primary antigen induced arthritis

A mild form of AIA is characterised by an acute inflammation directly after antigen injection, followed by a chronic phase of smouldering arthritis. Mild cartilage degradation is already evident at day 1 of arthritis and increases further to maximal PG depletion at days 7 to 14 of arthritis. In the following two weeks, the synovial infiltrate declines and most of the cartilage is repaired. At week 4 of arthritis, PG content of the cartilage is restored. To study MMP involvement in cartilage degradation seen during the acute phase of AIA, first mRNA levels of stromelysin and collagenase were analysed. In addition induction of MMP induced neoepitopes was evaluated and compared with both MMP mRNA levels and PG depletion seen during AIA.

Stromelysin and collagenase mRNA levels during antigen induced arthritis

Stromelysin and collagenase mRNA levels were already increased in cartilage at 12 hours after arthritis induction and were still increased at week 2 of arthritis (fig 1). Upregulation of mRNA levels of stromelysin was pronounced, but collagenase mRNA showed only a small increase. In contrast with prolonged increase of mRNA of the two MMPs, IL1 mRNA showed a brief rise during the first two days of arthritis after which it returned to basal values. This identifies prolonged increase of MMP mRNA levels as a specific event not reflecting non-specific upregulation of all inflammation related genes.

PG Degradation and VDIPEN Expression during Antigen induced Arthritis

To study whether the increased mRNA levels resulted in MMP activity, MMP induced neoepitopes were studied in time and compared with PG depletion. At day 1 and 3 of AIA, PG depletion is already visible but no VDIPEN epitopes were seen (fig 2 A, B), suggesting no role of MMPs in PG depletion. PG depletion increased in the next few days and reached its maximum approximately one week after arthritis induction. At this time point VDIPEN was expressed and showed a distinct

Figure 1  Semiquantitative mRNA analysis in cartilage during antigen induced arthritis. At several time points after arthritis induction patellar cartilage was collected and RNA was isolated. Semiquantitive mRNA analysis was done using RT-PCR. PCR products were analysed on an ethidium coloured gel. The cycle number at which the product is first detected is taken as a measure for the amount of mRNA present in the original isolated RNA. GAPDH was used to verify that equal amount of RNA were used. Amounts of cycles gene of interest=amount of cycles GAPDH=cycle difference. Control cartilage is taken as a reference point. The experiment was done two times, and each PCR reaction was carried out twice with the same material. Variation between cycle differences was low, at most one PCR cycle.

Figure 2 Proteoglycan depletion and VDIPEN expression in primary antigen induced arthritis. Adjacent frontal knee joint sections were stained with safranin O (A, C) and immunostained with anti-VDIPEN (B, D). (A), (B) day 1 of AIA, (C), (D) day 7 of AIA. P=patella, F=femur, bar represents 200 µm. The figure shows absence of VDIPEN expression at day 1 of AIA (B), and pronounced VDIPEN staining at day 7 as indicated by the arrowheads in (D).
Figure 3 PG depletion and VDIPEN expression during IL1 induced exacerbation. Ten ng of IL1 was injected into a knee joint of day 28 AIA. Frontal whole knee joint sections were stained with safranin O (A–C) and anti-VDIPEN (D–F). At day 28 of AIA no PG depletion and VDIPEN staining was found (A, D). IL1 injection into such a knee joint induced marked PG depletion, which is illustrated in (B) by destaining of the upper cartilage layer, and widespread VDIPEN expression ((E), arrowheads). Repeated IL1 injection into a naive joint, resulted in mild PG depletion (C) and no VDIPEN expression (F). (A), (D) Knee joint of day 28 AIA, bar represents 150 µm. (B), (E) day 1 after IL1 injection into postarthritic knee joint, bar represents 150 µm. (C), (F) day 1 after IL1 injection into a naive knee joint, bar represents 350 µm. P=patella, F=femur.

Figure 4 Increased mRNA levels in postarthritic cartilage. RNA was isolated from cartilage at week 4 of AIA (postarthritic cartilage) and semiquantitative mRNA analysis of several genes was done using RT-PCR. The cycle number at which the product is first detected is taken as a measure for the amount of mRNA present in the original isolated RNA. GAPDH was used to verify that equal amount of RNA were used. Amounts of cycles gene of interest−amount of cycles GAPDH= cycle difference. Contralateral cartilage was used as a reference point. Depicted is the mean (SD) of three separate experiments.

Figure 5 Increased gelatinolytic activity in postarthritic cartilage. Pooled extracts of five patellar cartilage layers, were subjected to zymography, using gelatin as a substrate. Postarthritic cartilage was taken at week 4 of AIA and compared with contralateral cartilage. Clear bands on the dark gel indicate gelatinolytic activities. Depicted is a representative example of five separate experiments.
Involvement of MMPs was studied by neoepitope detection. At day 1 after IL1 injection, VDIPEN epitopes were widely expressed in cartilage throughout the joint, clearly showing an accelerated expression of the MMP induced neoepitopes in comparison with the primary antigen induced arthritis (fig 3D, E). In contrast, when IL1 was injected into a naive knee joint four times, once daily, mild PG depletion was induced, but no VDIPEN epitopes were detected (fig 3F).

Presence of MMPs in cartilage of week 4 AIA

To understand the mechanism behind this accelerated breakdown and VDIPEN expression postarthritic cartilage was further analysed. At week 4 after arthritis induction, mRNA levels of stromelysin and collagenase were still increased (fig 4). The most pronounced was the stromelysin level, which showed an increase of at least 50 times compared with the level in normal cartilage (cycle difference is 7; 1.8>50). To study whether the prolonged increase of MMP mRNA levels also coincided with increased enzymatic activity in postarthritic cartilage, cartilage extracts were subjected to zymography. Figure 5 shows that gelatinolytic activity was increased in postarthritic cartilage. Two bands of ±60 kDa became more prominent, which probably correspond to different forms of gelatinase A. EDTA inhibited this gelatinolytic activity, indicating that the enzymes were MMPs. To detect activity of stromelysin, casein was also tried as a substrate for zymography, but no caseinolytic activity could be detected either in control or postarthritic cartilage. This indicates that stromelysin activity in cartilage of week 4 AIA is anyway below the detection limit of this assay.

When postarthritic cartilage was taken out of the joint and incubated with APMA, which activates latent MMPs, VDIPEN epitopes were strongly induced (fig 6), clearly identifying the presence of increased levels of latent MMPs in this cartilage. Such APMA activation did not induce VDIPEN epitopes in normal cartilage (results not shown).

Postarthritic cartilage is more vulnerable for IL1 induced degradation

To study whether such postarthritic cartilage shows increased vulnerability, it was exposed to IL1 in vitro. IL1 induced PG degradation was markedly higher in postarthritic cartilage as compared with normal cartilage (fig 7).

However, no VDIPEN expression could be detected in IL1 stimulated cartilage (fig 8), suggesting that factors in addition to IL1 are needed to provoke this expression in vivo.

Discussion

In this study we investigated the involvement of MMPs in cartilage degradation during the initial phase of AIA as well as during the IL1 induced flare up of arthritis. We demonstrated a fast and marked PG degradation during the flare up, if compared with the initial phase of arthritis. This correlated with accelerated expression of MMP induced neoepitopes. Furthermore, we found that mRNA levels of
MMP-1 and -3 in cartilage do not correlate with detectable MMP activity in cartilage, which suggests that translation or activation of latent MMP activity is not a key step in overall MMP activity in this model. The fact that APMA activation induced abundant neoepitope expression in postarthritic cartilage implies increased levels of latent enzyme as the mechanism behind accelerated VDIPEN expression in a flare up of arthritis.

During the initial phase of arthritis, early PG depletion is not accompanied by expression of MMP induced neoepitopes, which indicates that MMPs are not involved in the first phase of PG degradation. At later time points, when PG depletion is severe, VDIPEN epitopes appear, which implicates a linkage with advanced cartilage damage. In line with this finding is a recent study that showed that MMP activity correlated with IL1 induced collagen degradation and not with PG degradation.\(^\text{29}\) In a separate study, we found colocalisation of VDIPEN with collagenase cleavage sites in collagen (unpublished data), which couples VDIPEN inducing MMP activity to initiation of collagen damage. As collagen damage is thought to be a key event in irreversible cartilage damage,\(^\text{20}\) this further supports the linking of VDIPEN appearance in cartilage to severe cartilage damage.

This study also showed that injection of IL1 into a postarthritic knee joint results in a marked PG depletion one day later. This depletion is accelerated if compared with the initial phase of AIA, in which the same kind of depletion is only reached a couple of days later. In line with that observation was the accelerated expression of VDIPEN epitopes, demonstrating early involvement of MMPs. In light of earlier stated coupling of VDIPEN with severe cartilage damage, these results indicate the destructive character of flare ups of arthritis and an increased vulnerability of cartilage that has just recovered from an arthritic insult. In agreement with this is an earlier study showing that human arthritic cartilage is more vulnerable for inflammatory mediators if compared to control cartilage.\(^\text{30}\)

Postarthritic cartilage showed an increased mRNA level of stromelysin and collagenase, which suggests a still activated stage of the chondrocytes. This might be caused by the smouldering inflammation in the joint at later stages of AIA, which keeps the chondrocyte in an activated condition. On the other hand, not all inflammation dependent genes showed a prolonged activation, as IL1 was only increased for a short period of time. This indicates that the increased level of MMPs is a specific event. Furthermore, we showed increased gelatinolytic activity present in postarthritic cartilage, probably in the inactive proform, as no VDIPEN epitopes were detected in this cartilage. Moreover, MMP activity could be induced by APMA incubation, which activates latent MMPs. The presence of latent proteinases provides a big risk for postarthritic cartilage, as a slight catabolic insult could directly trigger massive destruction to the cartilage.

The increased proteoglycan degradation after IL1 injection into a postarthritic knee joint can only partly be explained by a direct effect of IL1 on postarthritic cartilage. In vivo PG degradation during a flare up of arthritis is higher than the degradation caused by IL1 in vitro (own observations, results not shown). So, the reaction of the synovial (inflammatory) cells, is partly responsible for the high PG degradation seen in vivo. Resident synovial lining cells are probably involved, as selective depletion of these cells by cladronate loden liposomes significantly decreased the flare up of arthritis.\(^\text{31}\)

The involvement of MMPs in IL1 induced cartilage degradation has been a matter of debate. IL1 is known to increase mRNA levels of MMPs and also the amounts of enzyme synthesised.\(^\text{32-34}\) However, the produced enzymes are in the latent form and need further activation to be able to cleave the extracellular matrix. Neoepitope detection after IL1 incubation of normal cartilage showed that no degradation products could be detected that resulted from MMP cleavage.\(^\text{35}\) This suggests that in normal cartilage, MMPs are not involved in IL1 induced proteoglycan degradation. This holds also true for the in vivo situation, where repeated injections of IL1 into a naive knee joint failed to induce MMP activities. In case of the postarthritic knee joint, IL1 did induce MMP activities, but the production of MMP neoepitopes during IL1 induced exacerbations is not a result of direct activation of chondrocytes by IL1 alone, but are generated by cofactors originating from the inflammatory reaction.

This study emphasises the importance of activation of latent MMPs as a controlling step in overall MMP activity. Activation of MMPs occurs through proteolytic cleavage of a propeptide.\(^\text{36-38}\) A possible candidate for in vivo activation of pro-MMPs is plasmin. Plasminogen activators originating from inflammatory cells can activate plasminogen, the precursor of plasmin. A recent in vitro study showed that IL1 in combination with plasminogen causes collagen degradation via activation of IL1 induced pro-MMPs.\(^\text{39}\) Plasminogen is abundantly present in synovial fluid and serum of RA patients.\(^\text{40}\) However, no evidence has been found that cartilage is able to synthesise plasminogen. As our explant cultures are done in serum free conditions, this could explain the presence of MMP neoepitopes after IL1 challenge in vivo, and the absence of these epitopes after in vitro IL1 incubation of cartilage.

The fact that in vitro IL1 did induce increased PG degradation but no MMP epitopes, suggests that another enzyme activity is responsible for this increased breakdown. A probable candidate is aggrecanase activity, as this enzyme activity has been identified to be the cause of IL1 induced PG breakdown.\(^\text{41-44}\) We have tried to detect aggrecanase induced neoepitopes (NITEGE) in postarthritic cartilage, both before and after IL1 challenge. However, NITEGE epitopes were already present in very high amounts in
postarthritic cartilage if compared with control cartilage, and further increase could not be demonstrated. This high amount of aggrecanase epitopes in repairing cartilage indicates a role for aggrecanase in normal turnover, besides the role in cartilage degradation. The differences in vulnerability to degradative stimuli like IL1 between normal and postarthritic cartilage seen in this study, could be of importance when related to human disease. Classic RA manifests itself as a fluctuating disease, with periods of active exacerbations and remissions. During remissions, cartilage can recover from the arthritic insult, but becomes more vulnerable for the next arthritic insult, eventually leading to irreparable damage to the cartilage.

16. van Meurs JBJ, van Lent PLEM, Singer II, Bayne EK, van de Loo AAJ, van den Berg WB. Interleukin-1 receptor antagonist prevents expression of the metalloproteinases and generated neoepitope VDPiPEN in antigen-induced arthri-
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