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Active Matrix Metalloproteinases Are Present in Cartilage During Immune Complex-Mediated Arthritis: A Pivotal Role for Stromelysin-1 in Cartilage Destruction

Joyce van Meurs,* Peter van Lent,1* Astrid Holthuysen,* Dimitri Lambrou,* Ellen Bayne,† Irwin Singer,† and Wim van den Berg*†

The involvement of immune complexes during experimental arthritis in induction of metalloproteinases (MMP)-induced neoepitopes in aggregan in cartilage, as well as the role of stromelysin-1 (SLN-1) in the induction of this neoepitope, was investigated. Passive immune complex arthritis was induced, and generation of the MMP-specific cleavage product (VDIPEN) was investigated by immunolocalization. The role of SLN-1 was studied with use of SLN-1-deficient (SLN-1KO) mice. VDIPEN expression was studied in vitro by exposing the cartilage to IL-1 and subsequent activation of latent MMPs. Immune complex arthritis was characterized by an acute inflammation, with influx of mainly polymorphonuclear cells into the joint cavity. Expression of VDIPEN neoepitopes was consistently found in areas extensively depleted from proteoglycans. SLN-1KO mice did not show expression of the VDIPEN neoepitope, although inflammation and proteoglycan depletion was comparable to wild-type mice. In addition, erosions of cartilage were absent in SLN-1KO mice, but were present in wild-type mice, suggesting an important role for SLN-1 in cartilage destruction. In vitro studies showed that SLN-1 is also pivotaly involved in IL-1-induced MMP activity. Stimulated polymorphonuclear neutrophils were able to activate latent MMPs present in the cartilage. Neutrophil elastase was also capable of activating IL-1-induced latent MMPs, which identifies elastase as a possible activator for latent VDIPEN-inducing MMPs. This study suggests that IC are important in the activation of latent MMPs in cartilage, possibly through polymorphonuclear neutrophil activation on the cartilage edge. SLN-1 is a pivotal enzyme in overall MMP-activity in cartilage during immune complex-mediated arthritis. The Journal of Immunology, 1999, 163: 5633–5639.

Degradation of cartilage is a major feature of rheumatoid arthritis (RA).2 It occurs through enzymatic cleavage of cartilage constituents. Two major cleavage sites have been identified in the first interglobular domain of aggrecan. The first cleavage site is mediated by aggrecanase. This protease is a member of the ADAM (a disintegrin and metalloproteinase) family that cleaves aggrecan at the Glu73–Ala74 bond (1). It results in the neoepitope sequence NITEGE left in the cartilage after cleavage. The second site is mediated by metalloproteinases (MMPs) that cleave aggrecan at the Asn341–Phe342 site, and the neoepitope VDIPEN is the result of cleavage at this site (2–6).

In synovial fluid, as well as in cartilage of arthritis patients, neoepitopes resulting from both enzyme activities were found (7–9). Also, in animal models of arthritis, both neoepitopes were induced in cartilage (10, 11). We recently studied the occurrence of VDIPEN during Ag-induced arthritis (AIA) (12). IL-1 was found to be a pivotal mediator in VDIPEN expression, because IL-1 receptor antagonist prevented the formation of the VDIPEN epitope. IL-1 is a known inducer of pro-MMPs, but these latent MMPs need further activation by other enzymes to become active. Involvement of VDIPEN-inducing MMPs was only found during the late phase of cartilage degradation, and not during early proteoglycan (PG) depletion, which suggests linkage of MMP activity to advanced cartilage damage (12, 13). Furthermore, we have found that the collagenase-induced cleavage site in type II collagen fully colocalizes with VDIPEN epitopes in this model (14, 15). This further suggests that VDIPEN is linked to severe and maybe even irreversible cartilage damage because collagen damage is difficult to repair (16). Stromelysin-1 (SLN-1) is pivotally involved in this colocalization of MMP neoepitopes in aggrecan and collagenase neoepitopes in type II collagen (14, 15), because SLN-1-deficient (SLN-1KO) mice showed no VDIPEN expression during AIA, nor did they show COL2–3/4C (Gly-Pro-Hyp-Gly-Pro-Gln-Gly) staining. This link is probably through activation of collagenases by SLN-1, because in vitro studies have shown that SLN-1 is capable of activating collagenase-1, -2, and -3 (17–19).

The linkage of VDIPEN occurrence to severe cartilage damage was further substantiated by studies in two other murine arthritis models (13). On the one hand, zymosan-induced arthritis (ZIA), which is induced by local injection of yeast particles, showed almost no expression of VDIPEN neoepitopes. On the other hand, collagen-induced arthritis (CIA), which is based on an autoimmune response to type II collagen, showed widespread and persistent VDIPEN expression. Cartilage damage that is induced during ZIA is fully reversible, whereas full destruction of cartilage is noted in CIA.

These data suggest that activation of latent MMPs in cartilage is correlated to induction of irreversible cartilage damage and is possibly of importance in destruction of cartilage. The question

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*Abbreviations used in this paper: RA, rheumatoid arthritis; MMP, matrix metalloproteinases; PG, proteoglycan; SLN-1, stromelysin-1; KO, knockout; ZIA, zymosan-induced arthritis; CIA, collagen-induced arthritis; SLN-1KO, stromelysin-1 knockout; IC, immune complex; ICA, IC arthritis; PLL, poly-l-lysine; PMN, polymorphonuclear neutrophil; APMA, aminophenylmercuric acetate.
remains which mechanisms are involved in activation of pro-MMPs leading to VDIPEN expression. There are two major differences between the mechanisms of inflammation in ZIA on the one hand and AIA/CIA on the other hand. In both AIA and CIA, T cells and immune complexes (ICs) are involved, whereas these are not present in ZIA.

The objective of this study was to establish the role of ICs in the induction of VDIPEN epitopes. This was done in a model of passive IC arthritis (ICA). In this model, arthritis is induced by injecting lysozyme coupled to poly-L-lysine (PLL) in the knee joint of mice that previously were given anti-lysozyme Abs systemically. If induced in the knee joints of mice, which are prone to develop collagen type II autoimmune arthritis, a severe arthritis develops resulting in PG depletion and surface erosion of the cartilage matrix. This model has been described before and was shown to be dependent on IL-1 (20, 21). Furthermore, the role of SLN-1 in IC-mediated VDIPEN expression was studied with use of SLN-1KO mice. The activation mechanism of MMPs was further studied in vitro.

Materials and Methods

Animals

SLN-1KO mice were generated in congenic 129SvEv mice and outcrossed to C57BL/6 mice as described earlier (22). Homozygous C57BL/6/129SvEv KO mice were outcrossed to B10RIII mice. F1 outcross mice were twice backcrossed to B10RIII mice. KO mice showed no gross histopathological abnormalities. Age-matched congenic B10RIII and C57BL/6 mice were used as wild-type controls. Mice were ~10 wk of age. They were given a standard laboratory diet and tap water ad libitum.

Induction of ICA

PLL (Sigma, St. Louis, MO) was coupled to lysozyme as described earlier (20). PLL-lysozyme (3 μg) was injected into the right knee joint of mice that had previously been given specific antiserum directed against lysozyme (0.2 ml, i.v.). The antiserum, raised in rabbits, were demplemented by heating at 56°C for 30 min.

Histology

Knee joints were dissected and fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4) for 3 days. Subsequently, joints were decalcified in 10% EDTA (Merck, Rahway, NJ) in phosphate buffer (pH 7.4) and embedded in paraffin wax. Coronal whole knee joint sections were prepared. Sections were stained for PG with safranin O and counterstained with fast green.

Determination of PG content and amount of inflammation

Histologic parameters were scored in a blinded way by two independent observers. Cartilage PG content is reflected in safranin O staining intensity. PG depletion was visualized by diminished staining of the matrix. Safranin O staining was scored arbitrarily; scoring was from 0 to 3 (0, no PG depletion; 1, minimal; 2, moderate; and 3, complete PG depletion). Erosion of the cartilage matrix was scored by expressing the amount of cartilage loss as a percentage of the cartilage surface up to the tide mark and was graded on a scale from 0 to 3 (0%; 1, 0–30%; 2, 30–60%; and 3, 60–100%). Inflammation was scored for the amount of exudated and infiltrated cells. The amount of cells were graded on a scale from 0 to 3 (0, no cells; 1, minimal; 2, moderate; and 3, maximal number of cells).

Immunohistochemical VDIPEN staining

For immunohistochemical analysis, sections were deparaffinized, rehydrated, and digested with proteinase-free chondrointinase ABC (0.25 U/ml 0.1 M Tris-HCl, pH 8.0; Sigma) for 1 h at 37°C to remove chondroitin sulfates from the PG. Subsequently, sections treated with 1% H2O2 in methanol for 20 min and 5 min with 0.1% Triton-X-100 in PBS. After incubation with 1.5% normal goat serum for 20 min, sections were incubated with affinity-purified rabbit anti-VDIPEN IgG overnight at 4°C. This Ab has been extensively characterized before (7, 10, 11). Then sections were incubated with biotinylated goat anti-rabbit IgG, the binding of which was visualized using avidin-streptavidin-peroxidase and 3,3′-diaminobenzidine tetrahydrochloride as a chromogen (Elite kit; Vector Laboratories, Burlingam, CA). Development of the peroxidase staining was done using nickel enhancement. Counterstaining was done with 2% orange G for 5 min.

Quantitative analysis of immunohistochemical staining

Areas of immunostaining were measured using image analysis. Images of histologic sections were recorded with a Sony CCD color video camera (Tokyo, Japan) and displayed on a computer monitor. Immunostained areas were marked by hand, and marked areas were quantified in μm² by the computer. For every knee joint, three sections were measured, and the mean of these measurements was recorded.

In vitro cartilage assay

Whole murine patellae were isolated with a minimal amount of surrounding tissue. Patellae were cultured in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 0.25 μg/ml insulin-like growth factor and with or without 10 ng/ml IL-1β. Subsequently, patellae were incubated with several MMP-activating agents. Patellae specimens were then frozen in liquid nitrogen. Sagittal cryosections were prepared and stained with safranin O and immunostained anti-VDIPEN.

Polyomorphonuclear neutrophil (PMN) isolation

PMN were isolated from the blood of healthy volunteers. After dextran sedimentation, PMNs were separated from mononuclear cells on Ficoll-Paque (Pharmacia BV, Uppsala, Sweden). The isolation procedure yielded 95% pure PMN. The cells were suspended in RPMI 1640 and directly used for experiments.

PMN cartilage incubations

Purified PMNs were suspended in RPMI 1640 and added to patella specimens in 96-wells microtitre plates (1 patella per well, 5 × 10⁶ cells per well). PMNs were pelleted on the cartilage by centrifugation (5 min at 1200 rpm), PMA (100 ng/ml) was added to the culture, and the plate was incubated for 3 h. Subsequently the patellae were washed in 0.7% NaCl and then frozen in liquid nitrogen.

Results

ICA and VDIPEN expression

The involvement of ICs in the formation of VDIPEN epitopes was studied in passive ICA. Anti-lysozyme Abs were injected i.v. 16 h before arthritis induction. Arthritis was induced by injecting 3 μg PLL intrarticularly into the right knee joint. At day 2 of arthritis, knee joints were dissected and processed for histology. Safranin O staining showed that PG depletion was pronounced (Fig. 1A). Furthermore, many exudating cells were present in the synovial cavity, mainly consisting of PMNs. Some PMN adherence to the cartilage was consistently seen (Fig. 1B).

Immunolocalization showed that VDIPEN epitopes were present (Figs. 1, C and D). VDIPEN was found mainly in the femur and sometimes at the edges of the patella. Typically, these sites were also more depleted of PG.

SLN-1 determines VDIPEN expression and cartilage destruction

Our earlier study identified SLN-1 as a pivotal enzyme in expression of VDIPEN epitopes in AIA (13, 14). We studied whether this was also true in ICA. Special attention was given to occurrence of cartilage destruction. ICA was induced in SLN-1KO mice and the wild-type strains B10.RIII and C57BL/6. At day 2 of arthritis, histology was taken and studied for inflammation and PG depletion. Synovial infiltrate and exudate were comparable in all strains (Fig. 2). PG depletion, as studied with safranin O staining, was identical in SLN-1KO mice and the wild-type strain B10.RIII. However, C57BL/6 mice showed less PG depletion compared with the other two strains (Fig. 2). Strikingly, a clear difference between B10.RIII and the SLN-1KO was found with respect to erosions (Table I). B10.RIII mice showed beginning erosions of the cartilage (see Fig. 4A), whereas in SLN-1KO mice no cartilage destruction was found (see Fig. 4B). VDIPEN expression was studied by immunohistochemistry. Profound staining was detected in both C57BL/6 and B10RIII mice, but remarkably no VDIPEN epitopes were found in SLN-1KO mice (Fig. 3). At day 5 of arthritis, erosions had progressed in B10.RIII (illustrated in Fig. 4C and Table
I), but were still absent in SLN-1KO mice (Fig. 4D). Erosions were most often found in the femur and the edges of the patella. This localization correlated with VDIPEN expression in knee joints of B10.RIII at day 2 of arthritis. At day 5 of arthritis, SLN-1KO mice still did not show expression of the VDIPEN neoepitope.

Role of SLN-1 in IL-1-induced VDIPEN expression

To further evaluate the activation mechanism of MMPs, and possible involvement of PMNs in this process, in vitro studies were conducted.

In vitro induction of MMPs was performed by culturing cartilage with IL-1 for 24 h to induce latent MMPs and subsequent activation of these latent enzymes. Activation of latent MMPs was first done by incubating the cartilage with aminophenylmercuric acetate (APMA) (1 mM APMA in RPMI 1640), a well-known chemical activator of MMPs. This resulted in profound expression of VDIPEN (Fig. 5A) in IL-1 exposed cartilage, which was absent when cartilage was incubated with IL-1 alone or APMA alone (data not shown). When the same treatment was done with cartilage of SLN-1KO mice, no VDIPEN neoepitopes were found (Fig. 5B), which identified SLN-1 as a pivotal enzyme in IL-1-induced VDIPEN expression.

Role of PMNs in VDIPEN expression

Next, we studied whether PMNs are able to activate latent MMPs in cartilage from wild-type mice. Cartilage was first incubated with IL-1 for 24 h and subsequently with PMA-activated PMNs for 3 h. This resulted in profound VDIPEN expression (Fig. 6C), which

![FIGURE 1.](image)

**FIGURE 1.** PG depletion and VDIPEN epitope labeling in ICA. Frontal knee joint sections of day 2 of ICA were stained with safranin O, (A). Hematoxylin staining (B) identifies PMN adherence to the cartilage (arrowheads). Immunostaining with anti-VDIPEN (C), and control immunostaining with normal rabbit IgG (D). F, femur; T, tibia; C, cartilage.

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**FIGURE 2.** PG depletion and inflammation in SLN-1KO mice. On day 2 of arthritis, knee joints were dissected and prepared for histologic study. Synovial cell exudate and cellular infiltrate and PG depletion was scored arbitrarily, graded from 0 to 3 (3, most severe) (n = 10 for B10.RIII and C57BL/6; n = 20 for SLN-1KO mice).

![Graph]

**Table I.** Cartilage erosion in knee joints of SLN-1KO and control B10RIII during ICA

<table>
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<tr>
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<th>B10RIII</th>
<th>SLN-1KO</th>
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<tbody>
<tr>
<td>ICA:day 2</td>
<td>1.4 ± 0.5</td>
<td>0</td>
</tr>
<tr>
<td>ICA:day 5</td>
<td>2.6 ± 0.3</td>
<td>0</td>
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*Erosion was detected as loss of cartilage from the femoral surface. Loss of the cartilage surface up to the tide mark was determined using an arbitrary score from 0 to 3 (0, no loss; 1, 0–30%; 2, 30–60%; 3, 60–100%). Per knee joint, four serial sections spaced 140 μm were scored blindly by two independent investigators. Data represent the mean ± SD of six animals. Note that erosion was fully absent in arthritic knee joints of SLN-1KO mice.
did not occur when cartilage was not previously incubated with IL-1 (Fig. 6D). Full PG depletion was seen in both groups treated with PMNs (Figs. 6, A and B), showing the great potency of PMNs to degrade these cartilage components.

Previous studies identified elastase as the main cartilage PG-degrading enzyme in PMNs in vitro (23). The specific elastase inhibitor MAAPV-CMK (200 μM; Bachem, Bubendorf, Switzerland) was added to the medium during PMN stimulation and subsequent culturing of the cartilage. PG depletion was completely inhibited by this inhibitor. Additionally, induction of VDIPEN epitopes was also absent. Next, leukocyte elastase was tested for its ability to activate SLN-1 in this system. Incubation of cartilage with IL-1 and subsequently with elastase (10 μg/ml) resulted in intense PG depletion (Fig. 7C) and VDIPEN expression (Fig. 7D), identifying elastase as a possible activator for VDIPEN-inducing MMPs. Cartilage of SLN-1KO mice again did not show expression of the MMP neoepitope (Fig. 7B), despite the PG depletion also seen in SLN-1KO cartilage (Fig. 7A).

Discussion

The involvement of MMPs in cartilage destruction during RA has been studied extensively over the last decade. Abs are developed against the MMP-specific cleavage product in aggrecan, VDIPEN (24–26). These neoepitope Abs enabled researchers to study MMP activity in cartilage. VDIPEN neoepitopes were found to be present in cartilage from RA and osteoarthritis patients (7, 8) and were also induced during murine CIA (10). In recent studies, we investigated the formation of VDIPEN epitopes in three different murine arthritis models (13). VDIPEN epitope formation was present in cartilage during CIA and AIA, both arthritis models in which IC formation is evident. In contrast, no VDIPEN was found in ZIA, in which no IC is present. In the present study, the role of IC in the induction of VDIPEN epitopes was established. Induction of VDIPEN epitopes was consistently found in passive ICA, indicating IC involvement in the induction of MMP activity. In addition, it was shown that SLN-1 is a pivotal enzyme in overall MMP activity during ICA.
The role of ICs in the formation of VDIPEN epitopes could involve PMN activation. ICs can provide an anchorage and a trigger for PMN activation (27). Igs, adherent to cartilage surfaces, have been identified in rheumatoid joints (28, 29). As a consequence, reactive oxygen species and proteolytic enzymes present in the PMNs will be released directly onto the surface of the cartilage, thereby escaping inhibitors present in the synovial fluid. PMNs need this close contact to the cartilage to inflict damage to the cartilage (30, 31). In the present study, stimulated PMNs were able to directly activate latent MMPs present in the cartilage after IL-1 exposure. This indicates that PMNs might be involved in the activation of latent MMPs through IC-targeted PMNs. This would fit with the absence of VDIPEN epitopes in ZIA, because no IC is found in this model. In agreement with this hypothesis is the presence of latent MMPs in the cartilage during ZIA (our unpublished observations, data not shown), which shows that MMPs are produced in the cartilage during ZIA, but are not activated.

ICA in SLN-1KO mice resulted in the absence of VDIPEN epitopes and erosions, but PG depletion was not inhibited. This implies that VDIPEN-inducing MMPs are not important in the PG degradation in this model. A likely mediator of early PG degradation is the putative enzyme activity aggrecanase (32–34). Earlier in vitro studies showed that IL-1-induced PG degradation is mediated by aggrecanase (34, 35). In line with these studies, we also found that in vitro incubation of murine cartilage with IL-1 only results in aggrecanase neoepitopes in cartilage, and not MMP-induced epitopes. When we studied the appearance of aggrecanase neoepitopes in vivo, we detected an increase of neoepitopes during early PG depletion (13). This suggests that aggrecanase is involved in the early phase of PG degradation during arthritis.

Earlier studies by our group revealed that the major PMN proteinase involved in PG degradation is elastase (30, 36). In this study, a specific inhibitor of leukocyte elastase completely blocked PMN-mediated PG degradation. Besides inflicting direct damage...
to the cartilage, elastase is also capable of activating latent MMP in vitro using recombinant enzymes (37). The present study showed that elastase is able to activate latent MMPs present in the cartilage, identifying elastase as a possible activator for MMPs in vivo.

In ICA, VDIPEN epitopes were only detected on sites extensively depleted from PGs, which suggests a correlation between VDIPEN expression and advanced cartilage damage, which had been shown in other models (13). In AIA, VDIPEN epitopes completely colocalized with collagenase-induced cleavage sites (COL2–3/4C) in type II collagen (14, 15). Degradation of type II collagen is thought to be a key event in cartilage degradation, which eventually leads to irreversible cartilage damage, such as surface erosions. Collagenase activity is considered to be of pivotal importance in the primary cleavage and subsequent denaturation of type II collagen in cartilage (38, 39). Several reports indicate that SLN-1 is able to activate several latent collagenases (17, 18) and might even be necessary for full activation of collagenase in vitro (19, 40). Our studies in SLN-1KO mice showed that SLN-1 is pivotally involved in induction of both VDIPEN and COL2–3/4C epitopes during AIA (14), which suggests that SLN-1 activates collagenase in vivo during AIA. In the present study, we showed that SLN-1 is also important in VDIPEN epitope induction during ICA. Moreover, no cartilage erosions were found in SLN-1KO mice, whereas B10.RIII wild-type mice showed widespread loss of cartilage. These results suggest an important role for SLN-1 in cartilage erosions in this arthritis model.

An earlier study of CIA in SLN-1KO mice showed clear VDIPEN expression and cartilage erosion in these mice (22). This seems contradictory with our present study in ICA. During ICA, only ICs that are not related to cartilage components are involved in pathology (20). In contrast, during CIA both cellular and humoral immune responses are directed against collagen type II, forming the main component of the cartilage matrix (22). This cartilage-directed attack by the immune system against the large depot of collagen type II may lead to a substantial overkill of other enzymes, overruling the prominent role of SLN-1 as is found in milder forms of arthritis like ICA, which lacks the T cell component in the inflammation. In the early phase of cartilage destruction during CIA, collagen damage might be SLN-1-dependent, but because of its severity its contribution might be overruled by other enzymes in a later phase.

In summary, this study indicates that ICs are involved in the activation of latent MMPs present in the cartilage. This occurs in the context of IL-1-mediated induction of latent MMPs and subsequent activation through PMN triggering. In addition, SLN-1 is a pivotal enzyme in overall VDIPEN-inducing MMP activity and cartilage erosions in this model.

References


