VDIPEN, A Metalloproteinase-generated Neoepitope, Is Induced and Immunolocalized in Articular Cartilage during Inflammatory Arthritis

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Abstract

The destruction of articular cartilage in immune inflammatory arthritic disease involves the proteolytic degradation of its extracellular matrix. The role of activated matrix metalloproteinases (MMPs) in the chondrodestructive process was studied by identifying a selective cleavage product of aggrecan in murine arthritis models initiated by immunization with either type II collagen or proteoglycan. We conducted semiquantitative immunocytochemical studies of VDIPEN^{341} using a monospecific polyclonal antibody requiring the free COOH group of the COOH-terminal Asn for epitope detection. This antibody recognizes the aggrecan G1 domain fragment generated by MMP [i.e., stromelysin (SLN) or gelatinase A] cleavage of aggrecan between Asn^{341}-Phe^{342} but does not recognize intact aggrecan. VDIPEN was undetectable in normal mouse cartilage but was observed in the articular cartilage (AC) of mice with collagen-induced arthritis 10 d after immunization, without histological damage and clinical symptoms. This aggrecan neoepitope was colocalized with high levels of glycosaminoglycans (GAGs) in pericellular matrices of AC chondrocytes but was not seen at the articular surface at this early time. Digestion of normal (VDIPEN negative) mouse paw cryosections with SLN also produced heavy pericellular VDIPEN labeling. Computer-based image analysis showed that the amount of VDIPEN expression increased dramatically by 20 d (70% of the SLN maximum) and was correlated with GAG depletion. Both infiltration of inflammatory cells into the synovial cavity and early AC erosion were also very prominent at this time. Analysis of adjacent sections showed that both induction of VDIPEN and GAG depletion were strikingly codistributed within sites of articular cartilage damage. Similar results occurred in proteoglycan-induced arthritis, a more progressive and chronic model of inflammatory arthritis. These studies demonstrate for the first time the MMP-dependent catabolism of aggrecan at sites of chondrodestruction during inflammatory arthritis. (*J. Clin. Invest.* 1995. 95:2178–2186.) Key words: arthritis • articular cartilage • aggrecan • neoepitope • immunostaining

Introduction

RA is a chronic joint disease characterized by articular cartilage (AC)[1] destruction and synovial membrane inflammation associated with loss of joint function. Cartilage erosion results from cleavage of its dominant extracellular matrix (ECM) components: large aggregating proteoglycan (aggrecan) and type II collagen (CII). Aggrecan is a high buoyant density proteoglycan (PG) consisting of a protein core (M_r 2.0–2.5 × 10^5) to which chondroitin sulfate and keratan sulfate glycosaminoglycan (GAG) side chains and N-linked and O-linked oligosaccharides are attached (1, 2). The core protein contains three globular domains (G1, G2, and G3) and an interglobular domain (between G1 and G2). The G1 domain mediates the binding of aggrecan to hyaluronan and the formation of large aggregates that are stabilized by link protein. Several matrix metalloproteinases (MMPs) have been implicated in the catabolism of cartilage ECM: stromelysin (SLN; EC 3.4.24.17), collagenase (CLN; EC3.4.24.7), gelatinase A (GLN-A; EC3.4.24.24), and gelatinase B (GLN-B; EC3.4.24.35) (3–6). SLN appears to be important in both inflammatory and degenerative arthritides, because it can directly degrade aggrecan (7) and link protein (8), type II collagen telopeptide, and type IX collagen (9), is elevated in synovial fluids and cartilage of RA and osteoarthritic (OA) patients, and may be up-regulated in synoviocytes and chrondrocytes (10–14).

Experimental animal models of inflammatory arthritis have provided important insights for understanding human arthritis. Although the pathological mechanisms of type II collagen-induced (CIA) and PG-induced (PGIA) arthritis are different and not fully understood, both appear to be sustained by genetically controlled autoimmune responses to cartilage ECM components (15–21). These models share many similarities with human RA and ankylosing spondylitis, such as humoral and cellular immune responses to immunizing and self-antigens, mononuclear cell infiltration of the synovium, formation of an erosive pannus, and progressive loss of PG and destruction of articular cartilage (18–20, 22). The pathogenesis of CIA and PGIA appears to be dependent on binding of PG or CII antibodies to joint cartilage followed by C5a release (23–25), activation of autoreactive T cells to matrix components (21, 26–28), and induction of the cytokines IFNγ, TNFα, and IL-1β (20, 29–

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1. *Abbreviations used in this paper:* AC, articular cartilage; CII, type II collagen; CIA, type II collagen-induced arthritis; CLN, collagenase; ECM, extracellular matrix; GLN-A, gelatinase A; GLN-B, gelatinase B; GAG, glycosaminoglycan; HC, hyaline cartilage; MMPs, matrix metalloproteinases; OA, osteoarthritic; PC, pericellular; PG, proteoglycan; PGIA, proteoglycan-induced arthritis; SLN, stromelysin.

32). Of these, IL-1β appears to be very important because neutralizing antibodies ameliorate the histopathology and clinical symptoms in CIA (33–35) and because IL-1β induces SLN and CLN in putative target cells (36). SLN has been detected in CIA and in experimental arthritis induced by the intraarticular injection of IL-1β (22, 37, 38).

Because all MMPs are secreted as latent zymogens (10, 11, 39, 40) and the activities of mature MMPs are regulated by tissue inhibitors of MMPs (41–43), it is difficult to determine which of the expressed MMPs are active in RA. SLN and GLN-A cleave aggrecan in the interglobular domain between amino acid residues Asn^{341} and Phe^{342}, generating a hyaluronan-binding G1 fragment with the COOH-terminal sequence VDIPEN, which remains bound to hyaluronan in cartilage (7). Generation and accumulation of this VDIPEN neoepitope, which is common to both human and murine aggrecans (44, 45), can thus provide a convenient marker of MMP activity in cartilage. An antibody recognizing this COOH-terminal aggrecan neoepitope has been developed; it does not recognize the VDIPEN sequence when it is an integral part of the peptide VDIPENFFGVG (46, 47). Because of this specificity, anti-VDIPEN IgG does not recognize intact aggrecan but does detect the G1 fragment generated by SLN or GLN-A. The purpose of our experiments was to determine whether the VDIPEN neoepitope is induced and localized in articular cartilage lesions as an early indicator of MMP activity in autoimmune murine models of inflammatory arthritis.

Methods

Animals. CIA was generated in B10.RIII mice (bred at Mayo Clinic) by injecting a single dose of highly purified porcine CII in CFA subcutaneously, as previously described (48). Control animals were injected with CFA emulsified with an equal volume of acid extraction buffer. PGIA was induced in female BALB/c mice (Charles River, Portage, MI) by intraperitoneal injection of chondroitinase ABC-digested canine aggrecan in CFA, followed by boosting with PG in incomplete Freund's adjuvant during weeks 1 and 4. Controls were injected in the same manner but without antigen (18). CIA mice were killed 10 d after immunization and at day 20 when they showed stage 2 clinical signs (> 3 swollen toes per paw). PGIA animals were killed when the legs exhibited swollen digits for periods of 1–5 mo. Paws were removed above the ankle or wrist, quenched in LN_2, and stored at −70°C. Animals were maintained in American Association of Laboratory Animal Care accredited facilities, and the experiments were approved by the respective institutional animal care and use committees.

Anti-VDIPEN antibody. An antibody was prepared against a peptide conjugate corresponding to the carboxy-terminal sequence of the MMP-generated aggrecan G1 fragment ($FVDIPEN^{341}$) and characterized (47). Peptide mapping studies using this antibody in an RIA indicate that it requires the free carboxyl group of the COOH-terminal Asn for optimal recognition. If the COOH-terminal Asn is either removed from the sequence, substituted with closely related amino acids, or extended across the MMP cleavage site, there is a 40–10,000-fold loss in detection sensitivity. Further, this VDIPEN antibody detects an aggrecan G1 fragment with an M_r of 50,000 that is generated by the MMPs SLN and GLN-A using RIA or by Western blotting. In contrast, intact aggrecan is not recognized by this antibody. Under identical conditions, the closely related MMPs GLN-B and CLN, as well as cathepsin G, cathepsin B, and human leukocyte elastase, did not generate a G1 fragment recognized by this antibody.

Western blot analysis of aggrecan fragments. The skin and superficial muscle were dissected from mouse paws, and the remaining tissues were homogenized in buffer (see below) with or without 100 μg of human recombinant SLN, overnight at 37°C. Active SLN was generated from recombinant human prostromelysin (2 mM) in 25 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.5, using trypsin (80 nM) for 30 min at 37°C. Trypsin was then inhibited with soybean trypsin inhibitor-agarose for 15 min at room temperature and the soybean trypsin inhibitor-agarose bound trypsin was removed from the sample by centrifugation. After digestion with SLN, aggrecan fragments were extracted from the homogenate using 4 M guanidine hydrochloride in 10 mM EDTA, 0.1 M 6-aminohexanoic acid, 50 mM benzamidine hydrochloride, 1 mM phenylmethyl sulfonylfluoride, 50 mM N-ethylmaleimide, and 1 mg/ml pepstatin. The extract was centrifuged at 3,000 × g for 30 min at 4°C. The supernatent containing the aggrecan was brought to 50 μg/ml with human umbilical cord hyaluronan (ICN Biologicals, Costa Mesa, CA) and dialyzed in a 3,000 mol wt cutoff membrane for 24 h at 4°C against 0.1 M sodium acetate, pH 6.0, containing the above proteinase inhibitors. After dialysis, the sample was centrifuged and the aggrecan/hyaluronan complex containing supernatent fractionated through an associative cesium chloride density gradient (starting density 1.5 gm/ml) (49). The bottom fourth of the gradient (A1) was harvested and digested with protease-free chondroitinase ABC and keratanase II (Seigagaku America, Rockville, MD) as follows. Samples (100 μl) in 0.1 M sodium acetate buffer, pH 8.0, were brought to 10 mM EDTA and treated with 0.02 U protease-free chondroitinase ABC overnight at 37°C. Keratanase II (0.1 U) was then added to each sample and incubated at 37°C for 2 h. Samples (1 paw per lane) were then electrophoresed through 4–20% SDS polyacrylamide Tris-glycine gels under reducing conditions and transferred to nitrocellulose. To eliminate nonspecific binding of antibodies, the nitrocellulose was incubated in 5% nonfat dry milk for 1 h at room temperature. The membranes were incubated in a 1:3,000 dilution of anti-VDIPEN antiserum for 1 h at room temperature. The membranes were then washed and incubated with a 1:1,000 dilution of biotinylated-goat anti-rabbit IgG for 1 h at room temperature. Blots were washed and incubated with 1:1,000 dilution of alkaline phosphatase-streptavidin followed by 5-bromo-4-chloro-3-indolyl phosphate nitroblue tetrazolium (Kirkegaard and Perry, Inc., Gaithersburg, MD) to detect immunoreactive bands.

Immunoperoxidase microscopy. Cryofixation was selected as the primary fixation procedure to minimize epitope denaturation that usually accompanies chemical cross-linking and to limit PG extraction resulting from prolonged exposure of cartilage to aldehyde fixatives (50). Mid-sagittal 5-μm cryostat sections were cut through the fully calcified unfixed hind paws with a carbide knife. Cryosections were then mildly fixed for 20 min with Nakane solution (periodate/lysine/paraformaldehyde) (51) and treated with 3% H_2O_2 in methanol to inactivate endogenous peroxidases, followed by 0.1% Triton-X 100 in PBS to increase permeability. To facilitate antibody penetration into cartilage (52), some sections were also digested with protease-free chondroitinase ABC (0.02 U, 30 mM Na acetate, 0.1 M Tris-HCl, pH 8.0; Seigagaku America) before fixation. Because chondroitinase ABC has a pH optimum of 8.0 for removal of chondroitin sulfate from proteoglycans, whereas that for hyaluranan digestion is 6.0, use of an alkaline pH greatly reduces hyaluronan cleavage and consequently circumvents possible release of aggrecan or aggrecan fragments from the sections (see Results). The VDIPEN neoepitope was labeled with rabbit anti-VDIPEN IgG (46) that was affinity-purified on VDIPEN-conjugated Reactigel (Pierce, Rockford, IL). An optimal staining concentration of 10 μg/ml IgG was determined in titration experiments conducted on SLN-treated cryosections of normal murine paws. Bound antibodies were detected via immunoperoxidase microscopy using the ABC technique (Elite kit, Vector Labs., Inc., Burlingame, CA). Peroxidase reaction product was developed with a glucose oxidase/diaminobenzidine/nickel method to provide maximum sensitivity (53); 1% Orange G was used as a counterstain. For specificity controls, the primary antibody was incubated with either the neoepitope peptide (H_2N-YTGEDFVDIPEN-COOH) or a peptide that spans the neoepitope cleavage site (H_2N-YTGEDFVDIPENFFGV-COOH) and clarified before staining. Peptides were synthesized on an Applied Biosystems 430A peptide synthesizer and purified

by reversed-phase HPLC on a Waters C18 Deltapak column. All peptides were > 95% pure by reversed-phase HPLC, and the structure of each was confirmed by electrospray ionization mass spectrometry. Preimmune rabbit IgG served as an additional negative control. For positive controls, unfixed paw cryosections were digested with activated recombinant human SLN (100 μg/ml in 150 mM NaCl, 25 mM Tris-HCl, 10 mM $CaCl_2$, 0.05% Brij-35, pH 7.4) for 30 min at 37°C before staining with anti-VDIPEN IgG. The cartilage GAG content of adjacent sections was also determined by toluidine blue staining as previously described (54). Photomicrographs were made at 10–25× with a Leitz Vario Orthomat microscope.

Image analysis. Induction of VDIPEN epitope and depletion of GAGs in CIA and PGIA mouse hind paw AC were measured semiquantitatively at 10–20× on a Zeiss Axioscope fitted with a Kodak Megaplus camera (1,024 × 1,024 pixels) using the Presage CV-6 digital image analysis system (Advanced Imaging, Princeton, NJ). Areas exhibiting endogenous VDIPEN epitope were manually traced, and the mean pixel gray level (range of 0–255) of these regions was determined. Background densities, measured on adjacent sections stained using normal rabbit IgG as the primary antibody, were subtracted from each determination. Endogenous VDIPEN densities were expressed as a percent of the maximum VDIPEN staining obtained for uninvolved hyaline cartilage (HC) measured after exhaustive SLN digestion of adjacent sections. Similarly, GAG depletion was expressed as the mean pixel density of toluidine blue–labeled HC divided by the pixel density of normal toluidine blue–stained HC observed in nearby sections.

Results

Anti-VDIPEN IgG specifically labels a neoepitope in SLN-treated normal mouse cartilage. Normal murine articular cartilage contains a very thin hyaline zone that is rich in GAGs but lacks detectable staining with anti-VDIPEN IgG, even after digestion of the cryosection with chondroitinase-ABC (Fig. 1, *A–C*). However, SLN digestion of unfixed mouse paw cryosections induces intense anti-VDIPEN staining within the GAG-rich pericellular (PC) matrix of chondrocytes and at the articular cartilage surface (Fig. 1, *B* and *D*); moderate VDIPEN labeling is also induced in the interterritorial zones of the AC (Fig. 1 *D*). Preincubation of anti-VDIPEN IgG with 250 ng/ml YTGEDFVDIPEN peptide completely blocked this labeling, whereas similar quantities of the spanning peptide (YTGEDFVDIPENFFGV) failed to inhibit staining (Fig. 1, *E* and *F*). Sections of normal paws digested with chondroitinase-ABC followed by

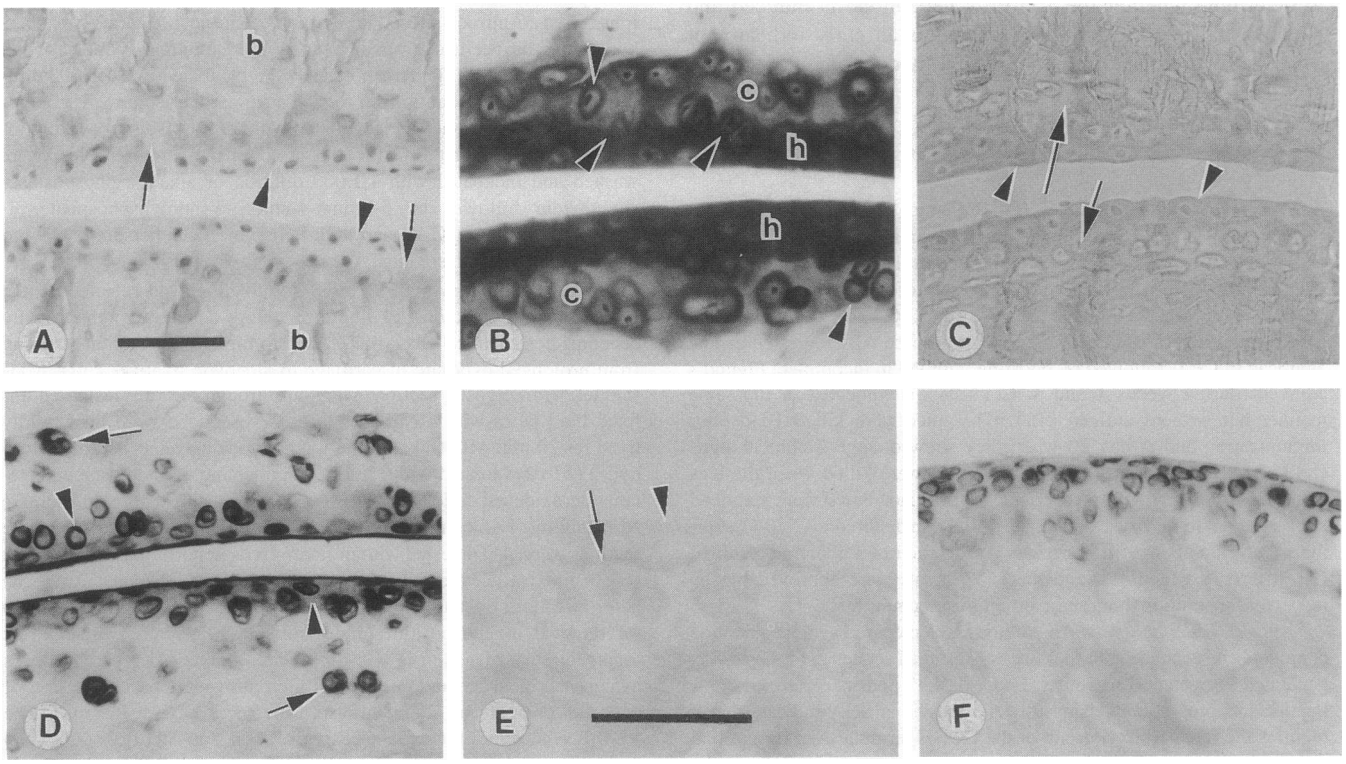

Figure 1. Anti-VDIPEN IgG specifically labels a SLN-induced neoepitope in cryosections of metatarsal articular cartilage from nonimmunized B10.R111 mice. (*A*) Joints exhibit a thin hyaline layer (*arrowheads*) distal to a calcified zone (*arrows*) of articular cartilage and bone (*b*); H & E staining. (*B*) GAGs in the hyaline cartilage (*h*) are intensely stained by toluidine blue, whereas the calcified cartilage (*c*) shows moderate GAG staining. Chondrocytes in both AC layers also exhibit dense GAG labeling in their pericellular matrix (*arrowheads*). (*C*) No VDIPEN epitope is detected in the hyaline layer (*arrowheads*) or in the calcified zone (*arrows*) of normal AC stained with anti-VDIPEN IgG after chondroitinase-ABC treatment; Orange-G counterstaining. (*D–F*) Sections of normal paws were digested with SLN before immunostaining and counterstaining with Orange-G. (*D*) Anti-VDIPEN IgG intensely stains the pericellular matrix of chondrocytes in the hyaline zone (*arrowheads*) and calcified zone (*arrows*) of the articular cartilage. The superficial region of the hyaline layer is also VDIPEN positive, and the territorial and interterritorial areas of the hyaline cartilage are moderately stained. (*E*) No immunostaining is observed in the hyaline (*arrowhead*) and calcified (*arrow*) layers of AC if anti-VDIPEN IgG is preabsorbed with 250 ng/ml YTGEDFVDIPEN immunogen. (*F*) Pretreatment of anti-VDIPEN IgG with 250 ng/ml of a peptide spanning the VDIPEN cleavage site (YTGEDFVDIPENFFGFG) does not prevent neoepitope labeling after SLN digestion of the cartilage. Bar for *A–D* and *F*, 50 μm; bar for *E*, 50 μm.

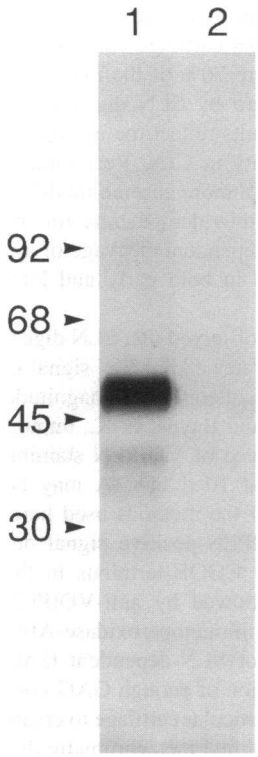

Figure 2. Anti-VDIPEN antibody detects a 50-kD aggrecan fragment in SLN-digested mouse paws. Extracts of paws from B10R.III mice were incubated with (lane *1*) or without (lane *2*) recombinant human SLN. The aggrecan fragments were extracted from the paw homoginate, fractionated through an associative cesium chloride density gradient, and evaluated by Western blotting using the anti-VDIPEN antibody. A single VDIPEN-positive band with a molecular mass of 50 kD is observed only after SLN treatment of the paw extract.

SLN produced a similar pattern and density of VDIPEN labeling (not shown), indicating that chondroitinase-ABC does not cause a loss of putative MMP-cleavage sites (VDIPEN-FFGVG) within aggrecan. Also, a single 50-kD aggrecan G1 fragment was detected with this anti-VDIPEN antibody in Western blots of SLN-digested mouse paw extracts (Fig. 2). This size indicates that the entire sequence from the NH_2-terminal Val through the COOH-terminal Asn^{341} of the neoepitope has remained intact (7). The 50-kD aggrecan fragment maintained its ability to bind to hyaluronan as indicated by its sedimentation at the bottom of an associative cesium chloride density gradient when incubated with hyaluronan. The anti-VDIPEN antibody did not detect any other fragments in this digested sample, or in extracts of untreated control paws (Fig. 2), indicating that its ability to detect the 50-kD aggrecan segment generated by SLN is specific. Together, these immunohistochemical and Western blotting results indicate that SLN generates the VDIPEN neoepitope on a single functional aggrecan G1 fragment in murine articular cartilage and that our antibody specifically detects this site only when its COOH-terminus is exposed.

VDIPEN is induced and GAGs are depleted during CIA. CIA mouse paws exhibit a well-defined chronological series of gross clinical changes after immunization (48). Although no outward symptoms were visible at day 10, many CIA mice showed redness and swelling in three or more digits of a given paw (clinical stage 2) by 15–20 d and ankylosis by day 28. Most 10-, 20-, and 28-d CIA mice exhibited VDIPEN staining in their hind paw AC, whereas vehicle-injected controls were negative (Table I). However, the patterns of VDIPEN induction and GAG depletion differed markedly between 10 and 20 d. Although limited foci of chondrocytes with pericellular VDIPEN labeling were observed in 10-d CIA mice (Fig. 3, *A–C*), GAG depletion of AC was not evident at this time. Levels of toluidine blue staining in AC were comparable with those of normal mice (data not shown, see Fig. 1 *B*), and induced pericellular VDIPEN labeling was strikingly codistributed with high concentrations of PC GAGs (compare Fig. 3 *B* with Fig. 1 *B*). Infiltrating inflammatory cells and cartilage erosion were also not detected at 10 d.

Table I. Detection of the VDIPEN Neoepitope in Hind Paw Articular Cartilage of CIA Mice via Immunoperoxidase Microscopy

Stage	No. positive mice	Total
10 d postimmunization	4	6
> 3 swollen digits per paw (15–20 d)	13	14
Ankylosis (> 28 d)	4	4
Controls	0	4

8-wk-old B10.RIII mice immunized intradermally with porcine CII in CFA. Midsagittal cryosections were cut through both hind paws of each animal.

In contrast, intense VDIPEN immunostaining was widespread at the articular surfaces of various tarsal and metatarsal joints of stage 2 paws at 20 d (Fig. 3, *D–K*). VDIPEN epitope appeared to be induced and heavily concentrated in the pericellular matrices of AC chondrocytes and at the eroding outer surface of the articular cartilage (Fig. 3, *D, I,* and *K*). Moderate VDIPEN labeling was also present in the interterritorial zones of AC (Fig. 3, *D* and *I*). Induction of intense VDIPEN staining correlated with marked depletion of GAGs throughout the AC of adjacent cryosections at this stage of CIA (Fig. 3, *D* and *E*). Erosion of the AC and infiltration with inflammatory cells and pannus were now very evident (Fig. 3, *D–G*). The invading pannus sometimes showed strong VDIPEN staining (Fig. 3 *G*), but it was often poorly labeled. Because intraarticular injection of SLN generates VDIPEN-bearing G1 fragments that are released into the synovial fluid (55), we believe that this synovial staining results from the differential uptake of VDIPEN fragments by macrophage-like cells of the pannus. Day 28 CIA specimens exhibited extensive cartilage and bone erosion, with small fragments of VDIPEN-positive AC remaining (not shown). These patterns of endogenous VDIPEN labeling were very similar to those observed after treatment of normal cartilage with SLN, except that endogenous VDIPEN immunostaining was not detected in the subadjacent calcified cartilage and bone (Figs. 1 *D,* and 3, *D* and *I*). SLN digestion of stage 2 paw cryosections did not diminish the VDIPEN labeling observed in damaged AC but induced VDIPEN neoepitope in the AC of other apparently normal joints present in the same section (as in Fig. 1 *D*). Predigestion of the sections with chondroitinase-ABC increased the staining intensity but did not alter the VDIPEN-staining pattern (not shown), indicating that this enzyme enhances accessibility of epitope to antibody and that it does not induce a loss of epitope-bearing aggrecan fragments. Endogenous VDIPEN staining was completely blocked by preincubating anti-VDIPEN IgG with YTGEDFVDIPEN (Fig. 3 *H*) but not by pretreatment with YTGEDFVDIPENFFGV (not shown); nonimmune rabbit IgG did not generate any immunoperoxidase labeling (Fig. 3 *J*). This pattern of VDIPEN staining

in CIA cartilage is highly specific, thus indicating that cross-reactive epitopes are probably not being generated by other proteases during advanced disease.

Comparison with PGIA mice. Because PGIA appears to be a more chronic model than CIA, with waxing and waning clinical symptoms that progressively worsen 1–5 mo after immunization (18), experiments were also conducted on paws of PGIA mice. One month after disease onset, intense VDIPEN staining was present in AC (Fig. 4 *A*) and was most concentrated in its pericellular matrix (Fig. 4 *B*). Marked GAG depletion was present in corresponding AC regions observed in adjacent sections (Fig. 4 *C*). Similar patterns were observed after 5 mo of disease, whereas normal BALB/c mouse AC lacked significant endogenous VDIPEN epitope (not shown).

Morphometry of VDIPEN induction and GAG depletion. Semiquantitative histomorphometric measurements were performed using digital image analysis to compare the degrees of articular cartilage VDIPEN induction and GAG depletion observed between days 10 and 20 in CIA with corresponding levels found in PGIA (Fig. 5). Quantities of endogenous VDIPEN labeling were expressed as a percent of the maximum anti-VDIPEN signal generated by exhaustive SLN digestion of normal AC, and GAG depletion was normalized to the GAG content of untreated AC. Although the mean staining density observed in VDIPEN-positive foci at 10 d in CIA was 21% of the SLN-treated maximum, it increased to 70% of the maximum at 20 d. GAG levels within corresponding AC regions were 95% of control values at day 10 but decreased to 38% of normal at 20 d. Similar levels of VDIPEN induction and GAG depletion were observed in paw AC of mice with PGIA for 1–5 mo.

Discussion

We have shown that the VDIPEN^{341} aggrecan neoepitope is undetectable in normal mouse cartilage but is induced within AC of CIA mice as early as 10 d after immunization, in the complete absence of measurable GAG depletion, AC erosion, inflammatory infiltrate, and clinical symptoms. The VDIPEN neoepitope was concentrated in and colocalized with high concentrations of PGs in pericellular matrices of AC chondrocytes but was not detectable at the articular surface, in the interterritorial matrix, or in the calcified layer of cartilage at this time. SLN digestion of normal mouse paw cryosections also produced heavy PC VDIPEN labeling in both articular and calcified cartilage and moderate staining in interterritorial zones of the AC. This anti-VDIPEN staining is very specific because: (*a*) it was inhibited by peptides with a VDIPEN^{341} COOH-terminus but not with peptides spanning the VDIPENFFGVG cleavage site, in both SLN-treated normal and arthritic AC; (*b*) it was not induced when sections of normal paws were untreated or predigested with highly purified chondroitinase-ABC, a glycanase that removes chondroitin sulfate from aggrecan but does not hydrolyze its core protein or cleave hyaluronan at pH 8.0; and (*c*) a single 50-kD VDIPEN-positive fragment was detected upon digestion of paw extracts with SLN.

The levels of VDIPEN expression increased dramatically in stage 2 CIA paws by 20 d postimmunization; they were correlated with GAG depletion at this time, as shown by image analysis. Infiltration of inflammatory cells into the synovial cavity and the beginning of AC erosion were also very evident at this stage. Analysis of adjacent sections also showed that both induction of VDIPEN neoepitope and GAG depletion were strikingly colocalized at sites of articular cartilage damage. Because the induced VDIPEN levels were 70% of the maximum quantity of neoepitope created in vitro by SLN digestion of normal AC, we surmise that these results reflect the up-regulation of intense local enzymatic activity in CIA. Very similar observations were obtained for PGIA, a more chronic model of inflammatory arthritis. These studies thus demonstrate for the first time the semiquantitative MMP-dependent cleavage of aggrecan at sites of chondrodestruction in both early and later stages of arthritis.

Using image analysis, we recently observed that SLN digestion of normal paw cryosections generates a VDIPEN signal in AC more readily than a GAG depletion of equivalent magnitude could be produced (Donatelli, S. A., and Bayne, E. K., unpublished data). Thus, the early appearance of VDIPEN staining before overt GAG depletion found at 10 d in CIA may be due to differences in the sensitivity of the methods used here. Appearance of a SLN-mediated VDIPEN-positive signal depends on generation of the VDIPEN COOH-terminus in the interglobular region of aggrecan, followed by anti-VDIPEN staining and amplification via the immunoperoxidase-ABC technique. In comparison, detection of SLN-dependent GAG depletion requires cleavage and diffusion of enough GAG-containing aggrecan fragments out of the articular cartilage to create a reduction in staining with an unamplified metachromatic dye (toluidine blue). It is therefore possible that some GAG depletion also occurs in the early stages of AC damage in CIA but may not be as readily detectable as VDIPEN induction.

When the MMPs SLN, CLN, GLN-A, and GLN-B were evaluated for VDIPEN generation using intact aggrecan as a substrate, only SLN and GLN-A generated an epitope recognized by VDIPEN antibody; non-MMPs did not produce a VDIPEN signal (46, 47). However, other MMPs such as GLN-B, interstitial or neutrophil CLN, and PUMP may also generate a VDIPEN COOH-terminus using a proteolyticially derived G1–G2 fragment of porcine aggrecan as a substrate (56–58). GLN-A is constituitively expressed in normal human synovia and is not up-regulated in disease, whereas SLN is dramatically induced in both RA and OA. These observations suggest that SLN may play a more dominant role than GLN-A in aggrecan catabolism in human arthritis (14). However, induction of VDIPEN staining in CIA and PGIA may reflect up-regulation of SLN and GLN-A activity during the early phases of matrix destruction. The question of whether one or both of these MMPs participate in VDIPEN induction may be answered when inhibitors specific for selected MMPs are developed and evaluated in these models of inflammatory arthritis.

These results do not rule out the possibility that additional enzymes could also be participating in aggrecan cleavage in CIA and PGIA. Recently, fragments consistent with another aggrecan cleavage site in the interglobular domain between Glu^{373} and Ala^{374} have been reported to accumulate in joint fluids of patients with inflammatory and noninflammatory joint diseases (59, 60). An enzyme, refered to as aggrecanase, has been proposed to generate this cleavage. We have prepared an antibody against the COOH terminus (NITEGE^{373}) of this additional G1 fragment (61) that would result from "aggrecanase" cleavage and have detected it in articular cartilage of animals with CIA (62). When reagents become available to other specific aggrecan cleavage fragments, it will be interesting

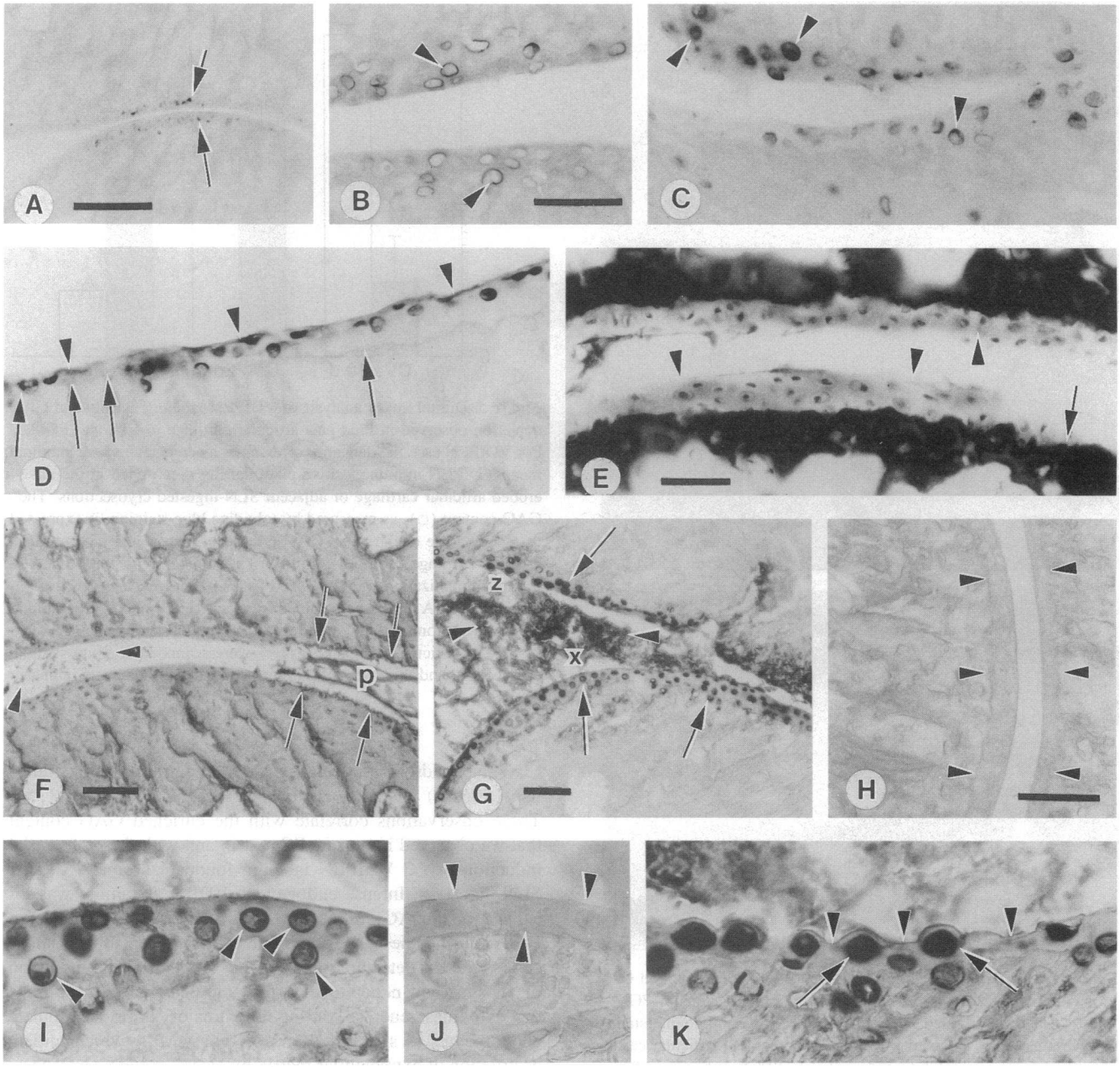

Figure 3. Distribution of endogenous VDIPEN neoepitope and GAGs in hind paw articular cartilage from CIA mice. (*A–C*) cryosections of tibiotarsal joints 10 d after immunization; (*D–K*) sections of clinical stage 2 specimens. (*A–D, G, I,* and *K*) labeled with anti-VDIPEN IgG without chondroitinase-ABC pretreatment; (*H*) stained with absorbed anti-VDIPEN IgG and (*I*) with preimmune IgG; all were counterstained with Orange-G; *E* was stained with toluidine blue, and *F* with H & E. (*A*) Articular cartilage shows focal VDIPEN induction (*arrows*). (*B*) Delicate VDIPEN staining is limited to the pericellular matrix of AC chondrocytes (*arrowheads*). (*C*) Occasional foci show intense pericellular VDIPEN labeling (*arrowheads*) with apparent chondrocyte staining (*middle arrowhead*). (*D*) Stage 2 specimen exhibits erosion of the articular surface down to the calcified layer (*arrows* at left); the eroded surface and superficial pericellular matrix show intense VDIPEN staining (*arrowheads*). (*E*) Adjacent section depicts GAG depletion from the hyaline layer (*arrowheads*) and erosion of the articular surface (*arrow*). (*F*) Metatarsal joint of stage 2 sample with inflammatory cells (*arrowheads*) and pannus (*p*) in the synovial space and extensive articular cartilage erosion (*arrows*). (*G*) Adjacent section showing VDIPEN accumulation in the articular cartilage (*arrows*) and pannus (*arrowheads*). (*H*) Metatarsal joint from a paw with stage 2 CIA stained with anti-VDIPEN IgG that was preabsorbed using 250 ng/ml of the peptide YTGEDFVDIPEN. Layers of articular cartilage (*arrowheads*) and the subchondral bone are completely unlabeled. (*I*) Higher magnification of area (×) in *G* shows intense VDIPEN labeling of the articular cartilage PC matrix (*arrowheads*), the articular surface, and moderate staining of AC interterritorial zones. (*J*) VDIPEN staining is absent from articular cartilage (*arrowheads*) of an adjacent section treated with preimmune IgG. (*K*) Higher magnification of region (*z*) in *G* exhibits erosion of the articular surface (*arrowheads*) and intense VDIPEN staining of chondrocytes and associated pericellular matrix (*arrows*). Bar for *A*, 200 μm; bar for *B–D* and *I–K*, 50 μm; bar for *E*, 25 μm; bars for *F* and *H*, 100 μm; bar for *G*, 50 μm.

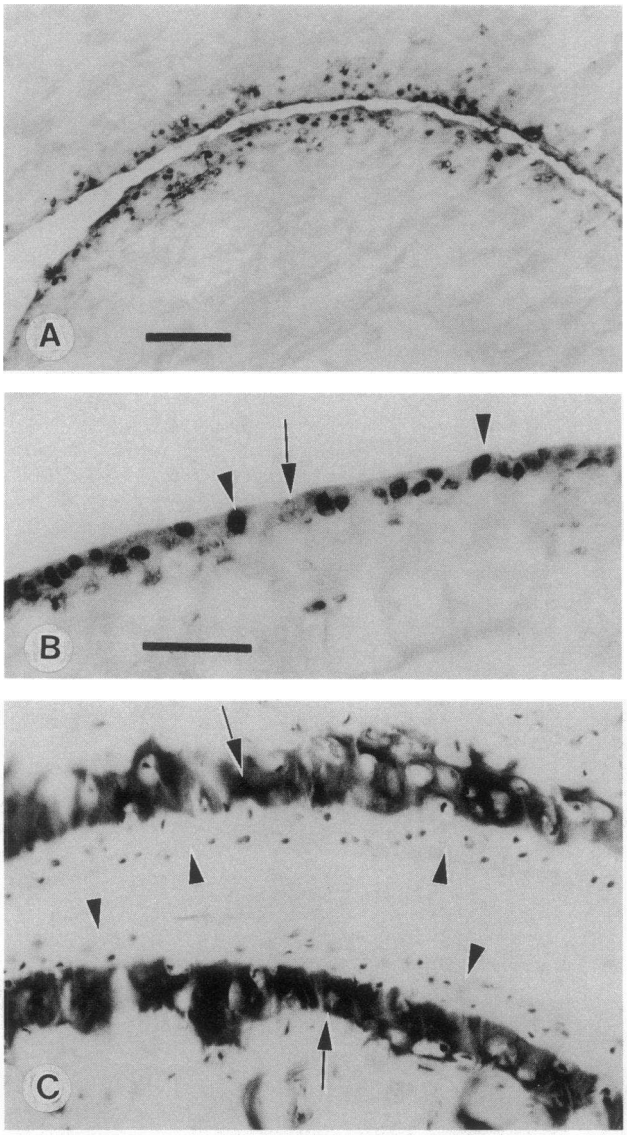

Figure 4. Distribution of endogenous VDIPEN neoepitope and GAGs in paw articular cartilage of mice exhibiting clinical signs of PGIA for 1 mo. (*A* and *B*) Labeled with anti-VDIPEN IgG and counterstained with Orange-G; (*C*) stained with toluidine blue. (*A*) Articular cartilage of a tibiotarsal joint exhibits widespread VDIPEN induction. (*B*) VDIPEN staining is concentrated in the chrondrocytes and adjacent pericellular matrix of the articular cartilage (*arrowheads*), whereas the interterritorial matrix (*arrow*) is moderately labeled. (*C*) Articular cartilage ECM (*arrowheads*) shows marked depletion of GAGs, whereas the calcified layer (*arrows*) exhibits a normal level of GAG staining. Bar for *A*, 100 μm; bar for *B* and *C*, 50 μm.

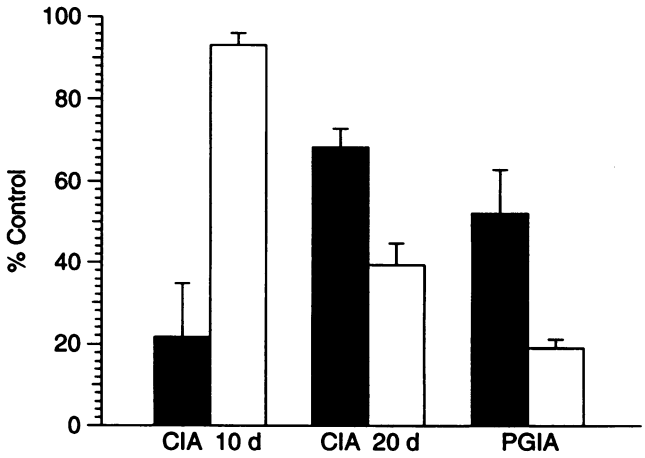

Figure 5. Digital image analysis of VDIPEN-labeling density and GAG depletion observed in hind paw articular cartilage in CIA and PGIA. For VDIPEN (■), the data are expressed as a percentage of the maximal mean VDIPEN immunopositive pixel density determined in the uneroded articular cartilage of adjacent SLN-digested cryosections. The GAG content (□), as measured by toluidine blue staining, is expressed as a percentage of the mean GAG gray-level pixel density in normal articular cartilage. Both hind paws of CIA animals killed at 10 and 20 d after immunization were analyzed (n = 4 and 7 mice, respectively). Similarly, PGIA data were obtained from a pool of three mice exhibiting clinical symptoms for 1 mo and from one animal symptomatic for 5 mo. 10–12 articular cartilage surfaces were scanned per 5 μm sagittal cryosection, and three sections were made per paw; brackets indicate the SEM.

to localize them and to determine the time course during which the enzymes they represent may play a role in aggrecan cleavage. In any case, our observation of VDIPEN induction is the first to localize any specific aggrecan degradation product in an arthritis model and is the most direct evidence that MMP-mediated aggrecan degradation does take place in situ in CIA and PGIA. At minimum, our observation of VDIPEN induction is the most specific evidence for the involvement of MMP activity in animal models of arthritis available to date.

The chondrocyte PC matrix is rich in aggrecan and hyaluronan (50, 63) and also exhibits elevated PG synthesis (35). These observations correlate with the enriched GAG content that we observed in murine PC matrix and with the preferential induction of PC VDIPEN labeling found in normal AC after SLN digestion. Intense endogenous VDIPEN staining was localized in the AC chrondrocyte PC matrix in both CIA and PGIA, during the early phases of disease when inflammatory cells were not detectable in the synovial cavity. The induction of an aggrecan neoepitope at this strategic location suggests that cytokines generated during inflammatory arthritis stimulate chondrocytes to synthesize and secrete MMPs, which initiate destruction of extracellular matrix by cleaving aggrecan. Therefore, the early appearance of VDIPEN labeling in PC matrix is a highly sensitive indicator of the commencement of joint pathology initiated by chondrocytes. Additional chondrocyte-generated proteases not detected by our VDIPEN antibody also participate in aggrecan and CII degradation within the pericellular matrix (52, 60, 62). The later appearance of VDIPEN labeling at the articular surface further suggests that MMPs originating from the synovial cavity and pannus may also participate in destruction of articular cartilage. Other ECM-degrading proteases secreted by inflammatory cells could also increase accessibility of the superficial AC to MMPs, thus enhancing further VDIPEN labeling at the articular surface. The recent localization of VDIPEN staining in damaged regions of human RA and OA cartilage also indicates that the VDIPEN neoepitope is a relevant marker of MMP-mediated aggrecan catabolism in human disease (64). Analogous observations have also been reported for degradation of CII in organ culture and arthritic

human cartilage (52). Further studies of animal and arthritic patient cartilage using immunocytochemical methods to detect aggrecan neoepitopes generated by active MMPs and other proteases as described here should clarify further the role of these enzymes in the chondrodestructive process.

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