Quantification of a matrix metalloproteinase-generated aggrecan G1 fragment using monospecific anti-peptide serum

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Several members of the matrix metalloproteinase family have been reported to cleave aggrecan in the interglobular domain between Asn-341 and Phe-342. An antiserum was prepared against a peptide conjugate corresponding to the C-terminal sequence of the matrix metalloproteinase-generated aggrecan G1 fragment (Phe³³⁵-Val-Asp-Ile-Pro-Glu-Asn³⁴¹). A quantitative radioimmunoassay, with a limit of detection of about 80 pM, was developed using this antiserum. This antiserum requires the free carboxyl group of the C-terminal asparagine for optimal recognition. If the C-terminal asparagine is excised from the sequence, replaced with closely related amino acids, or extended across the matrix metalloproteinase cleavage site, there is a 40–10000-fold loss in detection. Using peptides cleaved from the N-terminus, it was determined that the antiserum requires the entire Phe-Val-Asp-Ile-Pro-Glu-Asn sequence for optimal recognition. The radioimmunoassay detects matrix metalloproteinase-

generated G1 fragments with similar sensitivity to the Phe-Val-Asp-Ile-Pro-Glu-Asn peptide, but it does not recognize intact aggrecan. Immunoreactive aggrecan G1 fragments of molecular mass 50 kDa are generated by the matrix metalloproteinases stromelysin and gelatinase A. In contrast, under identical conditions, the closely related metalloproteinases, gelatinase B and collagenase, as well as cathepsin G, cathepsin B and human leucocyte elastase, did not generate a G1 fragment recognized by the antiserum. The anti-Phe-Val-Asp-Ile-Pro-Glu-Asn serum detects stromelysin-generated aggrecan G1 fragments from mouse, guinea pig, rabbit and human, indicating that the detection is not species-specific. This antiserum and radioimmunoassay should be useful for quantifying and characterizing matrix metalloproteinase-generated aggrecan G1 fragments in articular cartilage and synovial fluids from humans and various animal models of articular-cartilage destruction.

INTRODUCTION

One of the first matrix molecules to be lost from articular cartilage in osteoarthritis and rheumatoid arthritis is the large aggregating proteoglycan, aggrecan. In addition, there is continual turnover of aggrecan in articular cartilage with age, resulting in accumulation of aggrecan G1 fragments [1]. Several members of the matrix metalloproteinase (MMP) family have been implicated in cartilage aggrecan catabolism, based, in part, on their ability to cleave aggrecan *in vitro*. For example, the MMP stromelysin-1 (EC 3.4.24.17) can degrade a number of cartilage matrix molecules including link protein [2], aggrecan [3], type-IX collagen and the telopeptide region of type-II collagen [4]. In further support of the putative role for stromelysin in cartilage matrix degradation, it has been shown that prostromelysin is increased in synovial fluids of patients with both forms of arthritis [5,6], and elevated stromelysin mRNA levels have been found in cartilage from osteoarthritis patients [7]. Animal models of accelerated matrix degradation have been established in the rat, mouse, rabbit and dog. Stromelysin has also been found in a number of these models including streptococcal cell wall arthritis [8] and collagen-II arthritis [9] in the rat and rabbit meniscectomy osteoarthritis [10] and interleukin 1 injection [11] models. Enzyme was detected in these studies by immunolocalization, *in situ* hybridization, Northern-blot analysis and e.l.i.s.a. Unfortunately, none of these techniques can differentiate pro- from active enzyme. As all MMPs, including stromelysin, are synthesized as inactive zymogens, it is important to be able

to detect increases in the activity of the enzymes and associate these increases with joint pathology. One way to begin to characterize enzyme activity is to develop techniques to localize and quantify specific proteinase-generated matrix-degradation products.

Stromelysin-1 cleaves human aggrecan in the interglobular domain (IGD) between Asn-341 and Phe-342 [3]. It has also been reported that human gelatinases A and B, interstitial collagenase, neutrophil collagenase and putative metalloproteinase 1 cleave the G1–G2 double-globe region of porcine aggrecan at the same site [12–14]. In all of these studies, the cleavage site was identified using N-terminal sequence analysis of either the C-terminal fragment (³⁴²Phe-Phe-Gly-Val) released by the proteinases [12–14] or identification of the tryptic peptide released from the C-terminus of the N-terminal hyaluronan (HA)-binding G1 fragment [3]. This G1 species was also identified as a constituent of mature human articular cartilage, demonstrating that stromelysin or stromelysin-like activity was responsible for generation of this fragment *in vivo* [3]. To extend these studies, an antibody that detects the C-terminal sequence neoepitope (Phe-Val-Asp-Ile-Pro-Glu-Asn) generated by MMP cleavage of the native aggrecan substrate was generated. The antibody was used to develop a radioimmunoassay (RIA) which allows quantitative measurement of aggrecan fragments in tissue extracts and biological fluids as a direct readout of enzyme activity. This assay does not detect intact aggrecan but quantitatively detects the G1 fragment generated by stromelysin or gelatinase A cleavage, and thereby provides a means of detecting

Abbreviations used: APMA, aminophenylmercuric acetate; GdmCl, guanidinium chloride; HA, hyaluronan; IGD, interglobular domain; MBS, *N*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; MMP, matrix metalloproteinase; RIA, radioimmunoassay; SBTI, soya-bean trypsin inhibitor; TFA, trifluoroacetic acid. Numbering of residues throughout is based on human sequence analysis [16] assuming that Val-20 of that sequence is the N-terminus of the mature protein and is therefore numbered residue 1 (Val-1).

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MMP activity in tissue extracts. Furthermore, because of the extensive sequence homology immediately surrounding the MMP cleavage site in aggrecan [15–18], this antiserum detects the stromelysin-generated G1 fragment from a number of different species.

MATERIALS AND METHODS

Materials

The materials used in this study were purchased from the following sources: biotinylated goat anti-mouse IgG (H+L), alkaline phosphatase-streptavidin and 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium from Kirkegard and Perry, Gaithersburg, MD, U.S.A.; Sepharose CL-2B from Pharmacia, Piscataway, NJ, U.S.A.; SDS/polyacrylamide gels from Integrated Separation Systems, Hyde Park, MA, U.S.A.; recombinant human stromelysin, collagenase and gelatinase A from Celltech, Slough, Bucks., U.K.; cathepsins G and B from Athens Research Tech, Athens, GA, U.S.A.; protease-free chondroitinase ABC and keratanase II from Seikagaku America, Rockville, MD, U.S.A.

Preparation of peptides

The last seven amino acids of the C-terminal sequence of the aggrecan G1 fragment generated by stromelysin are F³³⁵VDIPEN³⁴¹. This peptide was synthesized with Cys-Nle at the N-terminus to give the peptide CNleFVDIPEN. The cysteine was added as a linking amino acid to attach the peptide to the immunogenic carrier, bovine thyroglobulin, by means of the heterobifunctional cross-linker *N*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) [19]. The norleucine was added as an internal marker to quantify the number of peptide molecules cross-linked to the carrier protein. A series of substituted peptides was also prepared as specificity controls.

Peptides were synthesized via the Merrifield solid-phase technique on an Applied Biosystems 430A peptide synthesizer utilizing either tBoc or Fmoc chemistry. Using tBoc synthesis, peptides were synthesized according to the manufacturer's suggested protocols for *N*-hydroxybenzotriazole ester couplings on phenylacetamidomethyl resins for peptide acids and methylbenzhydrylamine resins for peptide amides. Peptides were simultaneously deprotected and cleaved from the resin with 90% anhydrous HF/10% anisole at 0 °C for 1 h and then extracted from the resin with 10% acetic acid and lyophilized. Using Fmoc synthesis, peptides were synthesized according to the manufacturer's suggested protocols for 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate-mediated couplings on *p*-benzyloxybenzyl alcohol resins. Peptides were simultaneously deprotected and cleaved from the resin with a solution containing 90% trifluoroacetic acid (TFA), 5% thioanisole, 3% ethanedithiol and 2% anisole at room temperature for 2 h. Crude peptides were precipitated with ethyl ether then dissolved in 10% acetic acid and lyophilized. The resulting crude peptides were purified by reversed-phase h.p.l.c. on Waters C18 Deltapak columns with a gradient of 5–50% acetonitrile in aq. 0.1% TFA. Purity of the peptides was assessed by reversed-phase h.p.l.c. on a Brownlee Spheri-5 ODS column with a gradient of 5–50% acetonitrile in aq. 0.1% TFA. All peptides were more than 95% pure. Molecular ions were obtained by electrospray ionization m.s. to confirm the structure of each peptide.

Coupling chemistry

Bovine thyroglobulin (33 nmol) was dissolved in 2.5 ml of

degassed 20 mM phosphate buffer, pH 8.0, and incubated with 10 μ mol of sulphy-MBS for 30 min at room temperature. The activated thyroglobulin-coupling mixture was applied to a PD-10 column and the activated thyroglobulin was eluted with 3.5 ml of 50 mM phosphate buffer, pH 7.0, into a vial containing 6 μ mol of lyophilized peptide. The mixture was incubated overnight at 4 °C with gentle stirring and the CNleFVDIPEN complex was separated from free peptide or reaction by-products by chromatography on a PD-10 column equilibrated with PBS. To determine the degree of coupling, 50 μ l of the sample was hydrolysed with 200 μ l of 6 M HCl containing 0.1% phenol maintained at 110 °C for 24 h. Amino acid analysis of the sample was performed using a Beckman model 6300 amino acid analyser.

Production of antiserum

New Zealand White rabbits were injected intramuscularly with 333 μ g of immunogen conjugate in 1 ml of Freund's complete adjuvant. On days 7 and 35, rabbits were again injected with 333 μ g of immunogen in Freund's incomplete adjuvant at six to ten subcutaneous sites, similarly to the approach used to generate antiserum to interleukin 1 β -converting enzyme [20]. On day 45, animals were bled, and boosted with the same amount of antigen. On day 55, animals were again boosted and 10 days later bled. This boosting and bleeding schedule was continued three to five times to obtain an adequate supply of antiserum. Serum was generated by clotting overnight and was stored at –20 °C.

Radioiodination of the probe

The synthetic probe peptide YTGEDFVDIPEN was iodinated by reaction with chloramine-T. Peptide solution (50 μ l; 220 μ g/ml) was added to 10 μ l of 0.5 M potassium phosphate buffer, pH 7.5, and then combined with 2 mCi of Na¹²⁵I and 10 μ l of freshly prepared chloramine-T (0.1 mg/ml) in water. The mixture was allowed to react for 30 s and the reaction stopped with 10 μ l of 1 mg/ml NaI plus 1 mg/ml sodium thiosulphate. The radioiodinated probe was purified by reversed-phase h.p.l.c. using a Supelco C-8 column (0.4 cm $\times$ 25 cm) with an acetonitrile gradient in 0.1% TFA to isolate the monoiodinated probe. The final probe has a specific activity of about 1500 Ci/mmol.

Radioimmunoassay procedure

The assay is conducted in a total volume of 300 μ l of Dulbecco's Ca²⁺/Mg²⁺-free PBS supplemented with 0.1% gelatin, 0.01% thimerosal and 1 mM EDTA. Buffer or sample (100 μ l) was mixed with 100 μ l of a 1:3000 dilution of the anti-FVDIPEN serum and incubated overnight at 4 °C. The next day, 100 μ l of the probe (about 30000 c.p.m.) was added to each of the samples. The mixture was again incubated overnight at 4 °C. The reaction was terminated by the addition of 1 ml of 0.3% dextran-coated charcoal for 10 min on ice. Under these conditions the unbound probe binds to the charcoal. The sample was clarified by centrifugation at 3000 *g* for 10 min and the supernatant decanted. The amount of radioactivity in the supernatant was determined by γ -radiation counting. Data are reported as the percentage of bound radioactivity relative to the total radioactivity bound in the absence of any competing sample.

Western-blot analysis of aggrecan fragments

Aggrecan samples were evaluated by Western blotting with or without chondroitinase ABC/keratanase II digestion. For treat-

ment with the glycosidases, samples (100 μ l) in 0.1 M sodium acetate buffer, pH 8.0, were brought to 10 mM EDTA and treated with 0.02 unit of protease-free chondroitinase ABC overnight at 37 °C. Keratanase II (0.1 unit) was then added to each sample and incubated at 37 °C for 2 h. Samples were then subjected to SDS/PAGE (4–20 % gels) under reducing conditions and transferred to nitrocellulose. To eliminate non-specific binding of antibodies to the membranes, the nitrocellulose was incubated in 5 % non-fat dry milk for 1 h at room temperature. The membranes were incubated in a 1:3000 dilution of anti-FVDIPEN serum, termed R-1776, for 1 h at room temperature. They were then washed and incubated with a 1:1000 dilution of biotinylated goat anti-rabbit IgG for 1 h at room temperature. Blots were washed and incubated with a 1:1000 dilution of alkaline phosphatase-streptavidin followed by 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium, to detect immunoreactive bands.

Preparation of aggrecan

Human aggrecan was isolated by the methods of Hascall and Kimura [21] from cartilage of patients undergoing total knee replacement surgery. Rabbit aggrecan was isolated from rabbit hind-limb stifle-joint articular cartilage from 6–8-week-old New Zealand White rabbits. Briefly, the cartilage was removed from the bone and cut into 2–4 mm² pieces in PBS containing 10 mM EDTA, 0.1 M 6-aminohexanoic acid, 50 mM benzamidinium hydrochloride, 1 mM phenylmethanesulphonyl fluoride, 50 mM *N*-ethylmaleimide and 1 mg/ml pepstatin (proteinase inhibitor cocktail). The cartilage was brought to 4 M with 8 M guanidinium chloride (GdmCl) and homogenized using a Polytron homogenizer with a 10 mm sawtooth generator. The cartilage was extracted with a 20-fold volume excess of 4 M GdmCl in proteinase inhibitors for 48 h at 4 °C. The extract was clarified by centrifugation at 3000 *g* for 30 min at 4 °C. The supernatant containing the aggrecan was brought to 50 μ g/ml with human umbilical cord HA and dialysed in a 3000-molecular-mass cut-off membrane for 24 h at 4 °C against 0.1 M sodium acetate, pH 6.0, containing the proteinase inhibitor cocktail. After dialysis, the sample was clarified by centrifugation and the aggrecan-HA-complex-containing supernatant was fractionated through an associative caesium chloride density gradient (starting density 1.5 g/ml). The bottom quarter of the gradient (A1) was harvested and brought to 4 M with solid GdmCl and fractionated on a dissociative caesium chloride density gradient (starting density 1.5 g/ml). The bottom quarter of this gradient (A1D1) was harvested and the concentration of aggrecan determined using the dimethyl Methylene Blue dye-binding assay as described previously [22]. HA (7.7 % w/w) was added to the sample which was then dialysed for 24 h at 4 °C against 0.1 M sodium acetate, pH 6.8, to generate aggrecan-HA complexes, before enzyme digestion. The integrity of these complexes was evaluated by gel-filtration chromatography on an associative Sepharose CL-2B column (1 cm $\times$ 120 cm) eluted with 0.1 M sodium acetate/0.1 % NaN₃, pH 6.8, at 4 °C.

Enzymes

Recombinant human prostromelysin (2 mM) in 25 mM Tris/HCl, pH 7.5, containing 10 mM CaCl₂, 0.05 % Brij-35 and 0.1 % NaN₃ (stromelysin buffer) and procollagenase (2 mM) in 20 mM Tris/HCl, pH 7.4, containing 5 mM CaCl₂, 150 mM NaCl and 0.1 % NaN₃ were activated with trypsin (80 nM) for 30 min at 37 °C. The samples were then incubated with soya-bean trypsin inhibitor (SBTI)-agarose for 15 min at room temperature and

the SBTI-agarose-bound trypsin was removed from the sample by centrifugation. Recombinant human gelatinase A (1.4 mM) in 20 mM Tris/HCl, pH 7.5, containing 5 mM CaCl₂, 150 mM NaCl, 0.01 % Brij-35 and 0.1 % NaN₃ was activated with 1.1 mM aminophenylmercuric acetate (APMA) for 2 h at 25 °C. Human gelatinase B (1.1 mM) (kindly supplied by Dr. G. Goldberg, Washington University, St. Louis, MO, U.S.A.) was activated with 2 mM APMA for 4 h at 37 °C. Both gelatinases were dialysed against 20 mM Tris/HCl, pH 7.5, containing 10 mM CaCl₂, 150 mM NaCl and 0.05 % Brij-35 to remove any residual APMA. All enzymes were evaluated on SDS/PAGE to confirm that there had been a conversion in molecular mass into the activated species with APMA treatment. To confirm stromelysin activity, second-order rate constants were determined as described previously [23] using the peptide substrates Mca [(7-methoxycoumarin-4-yl)acetyl]-Pro-Leu-Ala-Nva-Dpa [*N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH₂ (cleavage at the Ala-Nva bond). Similar activity measurements for the gelatinases and collagenase were performed using the peptide substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ [24] (cleavage at the Gly-Leu bond). The $k_{\text{cat}}/K_{\text{m}}$ values were determined in a semicontinuous h.p.l.c.-based assay as previously described [24,25]. Briefly, the fluorescent peak area corresponding to the hydrolysis product, Mca-Pro-Leu-Gly-Leu, was quantified and plotted as a function of incubation time. The resulting curve was fitted to a first-order rate equation to determine k_{obs} , the first-order rate constant corresponding to appearance of product. As these experiments were performed at $[S] < K_{\text{m}}$, division of k_{obs} by the enzyme concentration gives the kinetic constant $k_{\text{cat}}/K_{\text{m}}$. Two criteria were used to judge that the progress curves were indeed first-order: (i) the substrate concentration used in the experiments was much less than K_{m} ($1 \mu\text{M} < K_{\text{m}} = 70 \mu\text{M}$), and (ii) the rate constant was identical at a substrate concentration 5-fold higher. Previous active-site titrations of both gelatinases using recombinant human tissue inhibitor of metalloproteinase-1 demonstrated that the enzyme concentration was at least 90 % of the predetermined protein concentration. Each of the enzymes was added to the aggrecan-HA aggregates in 100 mM sodium acetate/10 mM CaCl₂, pH 6.8, at a molar ratio of aggrecan to enzyme of 10:1. Enzyme activity was inhibited by bringing each of the samples to 10 mM with EDTA.

Human leucocyte elastase (isoenzyme 3) (kindly supplied by B. Green, Merck Research Laboratories) in 50 mM Tes/500 mM NaCl, pH 7.5, human cathepsin G in 100 mM sodium acetate, pH 6.8, and human cathepsin B in 100 mM sodium acetate/1 mM EDTA/10 mM dithiothreitol were added to aggrecan in 100 mM sodium acetate, pH 6.8, at the same molar ratios as described for the metalloproteinases. Elastase and cathepsin G activities were inhibited with 10 mM phenylmethanesulphonyl fluoride. Cathepsin B activity was inhibited with 10 mM iodoacetic acid.

Digestion of guinea-pig and mouse aggrecan

For the guinea-pig sample, a 4 M GdmCl/proteinase-inhibitor extract of fetal guinea-pig stifle-joint cartilage was dialysed against water, lyophilized and digested with human stromelysin in stromelysin buffer for 18 h at 37 °C. Enzyme activity was then inhibited with 10 mM EDTA. For the mouse sample, mouse stifle joints were opened and incubated with 100 μ g/ml stromelysin in stromelysin buffer for 18 h at 37 °C. The supernatant was removed and brought to 10 mM with EDTA. An aliquot of each sample with or without enzyme treatment was evaluated for the G1 fragment terminating in F³³⁸VDPEN³⁴¹ by Western blotting.

Figure 1 Anti-FVDIPEN RIA displacement curve using anti-FVDIPEN serum R-1776

In a competitive binding RIA format, as described in the Materials and methods section, increasing amounts of unlabelled YTGEDFVDIPEN peptide were added to a mixture of the antiserum R-1776 and ^{125}I -YTGEDFVDIPEN. Data are reported as percentage of peptide bound to the antibody/background antibody control plotted against concentration.

Table 1 Inhibition of YTGEDFVDIPEN binding in RIA

Competing peptide	IC ₅₀ (pM)
YTGEDFVDIPEN	2×10^2
FVDIPEN	1×10^2
YTGEDFVDIPE	$> 2 \times 10^6$
YTGEDFVDIP	$> 2 \times 10^6$
YTGEDFVDI	$> 2 \times 10^6$
YTGEDFVD	$> 2 \times 10^6$
YTGEDFVDIPENF	1×10^6
YTGEDFVDIPENFF	5×10^5
YTGEDFVDIPENFFG	$> 2 \times 10^6$
YTGEDFVDIPENFFGV	$> 2 \times 10^6$
VDIPEN	1×10^3
DIPEN	4×10^3
IPEN	9×10^5
PEN	3×10^7
AcFVDIPED	7×10^4
AcFVDIPED-NH ₂	7×10^4
AcFVDIPEQ	1×10^5
AcFVDIPEdN	8×10^5
AcFVDIPEN-NH ₂	5×10^3
AcFVDIPEA	1×10^6
AcFVDIPAN	2.8×10^4
AcFVDIAEN	8.2×10^3
AcFVDAPEN	8.2×10^3

RESULTS

Development of an RIA for aggrecan G1 terminating in FVDIPEN³⁴¹

A number of MMPs have been reported to cleave aggrecan between Asn-341 and Phe-342 to generate a G1 fragment terminating in F³³⁵VDIPEN³⁴¹ [3,12–14]. The goal of this study was to establish a direct RIA to measure the MMP-generated aggrecan G1 fragment. It was important that the antiserum should not recognize either intact aggrecan or G1 fragments generated by proteinases (cathepsin G, human leucocyte elastase, cathepsin B) known to cleave in the IGD of aggrecan at sites other than Asn³⁴¹–Phe³⁴². In order to achieve such specificity, the

Figure 2 C-terminal truncations and extensions

Peptides with (a) C-terminal truncations or (b) C-terminal extensions were evaluated for their ability to displace the radiolabelled probe peptide described in the legend to Figure 1. (a) Displacement of the probe peptide by the control peptide YTGEDFVDIPEN (●) was evaluated and compared with displacement by peptide YTGEDFVDIPE, which lacks the C-terminal Asn (■), peptide YTGEDFVDIP, which lacks the C-terminal Glu-Asn (◆), peptide YTGEDFVDI, which lacks the C-terminal Pro-Glu-Asn (○), and peptide YTGEDFVD, which lacks the C-terminal Ile-Pro-Glu-Asn (□). (b) The peptide was extended by one amino acid to YTGEDFVDIPENF (■), by two amino acids to YTGEDFVDIPENFF (◆), by three amino acids to YTGEDFVDIPENFFG (○) or by four amino acids to YTGEDFVDIPENFFGV (□) and compared with the control peptide YTGEDFVDIPEN (●).

antiserum should recognize the free carboxyl group of Asn-341 which is normally in amide linkage with Phe-342. In an attempt to raise such an antiserum, CNleFVDIPEN was conjugated to thyroglobulin using the cross-linking reagent sulpho-MBS, which specifically orients the carboxyl group of Asn-341 away from the thyroglobulin. This conjugate was then injected into rabbits. The degree of peptide substitution on thyroglobulin was found to be approx. 25 mol of peptide/mol of thyroglobulin. Typically, it was found necessary to incorporate at least 20 mol of a short peptide on to a carrier protein to elicit a significant antibody response in either rabbits or guinea pigs.

A number of rabbit antisera were generated and an RIA was established using an antiserum, termed R-1776, at a final dilution of 1:9000. This RIA detects the peptide antigen Y³³⁰TGEDFVDIPEN³⁴¹ with a log-linear range of detection of 30–800 pM (Figure 1) with an interassay variation of less than 5% and an intra-assay variation of less than 10%. The anti-FVDIPEN serum, R-1776, possessed remarkable specificity for the free carboxyl group on Asn-341. When the C-terminal asparagine was excised from the sequence to generate the peptide Y³³⁰TGEDFVDIPE³⁴⁰, there was a more than 10000-fold loss in recognition by the antiserum (Table 1, Figure 2a). Additional C-terminal truncation peptides were prepared and none

Figure 3 N-terminal truncations

Peptides excised from the N-terminus of the FVDIPEN sequence were evaluated for displacement of the probe peptide described in the legend to Figure 1. The peptide VDIPEN lacking the N-terminal Phe (◆), DIPEN lacking the N-terminal Phe-Val (○), IPEN lacking the N-terminal Phe-Val-Asp (□) and PEN lacking the N-terminal Phe-Val-Asp-Ile (◇) were compared with the immunogen peptide sequence FVDIPEN (■) and the control peptide YTGEDFVDIPEN (●).

were recognized by the antiserum at concentrations up to 1 μ M, confirming the requirement for the C-terminal asparagine (Table 1, Figure 2a). A series of peptides was also prepared that extend across the MMP cleavage site to determine whether aggrecan G1 fragments that were generated by cleavage distal to this C-terminal site would be recognized by the anti-FVDIPEN serum. There is a more than 2500-fold loss in recognition by these spanning peptides (Table 1, Figure 2b). Cathepsin B has been shown to hydrolyse the 'double-globe' fragment of aggrecan at Phe³⁴³-Gly³⁴⁴ [26]. Interestingly, the sequence Y³³⁰TGEDFVDIPENFF³⁴³ is not recognized by R-1776, indicating that cathepsin-B-generated aggrecan G1 fragments should not be recognized by the antiserum. The truncation and extension peptide-mapping data suggest that protease cleaving N- or C-terminal to the peptide bond Asn³⁴¹-Phe³⁴² will generate fragments that are recognized at least 1000-fold less well than those terminating in Asn-341.

Additional peptides were synthesized to characterize the length of the peptide required for maximal recognition by the antiserum. When the sequence F³³⁵VDIPEN³⁴¹ was shortened by one, two or three amino acids from the N-terminus, there was a 10–30000-fold loss in recognition (Table 1, Figure 3). However, the peptides F³³⁵VDIPEN³⁴¹ and Y³³⁰TGEDFVDIPEN³⁴¹ were recognized equally well. Therefore FVDIPEN was determined to be the minimum sequence required for optimal recognition by the antiserum.

Using the minimal sequence optimally recognized by the antiserum, peptides were prepared in which the C-terminal asparagine was replaced with an Asp, Asp-NH₂, Gln, D-Asn, or Asn-NH₂. The antiserum again recognized these peptides with reduced sensitivity (50–1000-fold) (Table 1). Together, these data indicate that the free COO⁻ on the C-terminal asparagine, as well as the appropriately spaced side-chain amide, are required for optimal recognition by the antiserum.

Specificity of the antiserum was further examined by sequentially substituting an alanine residue for the four C-terminal amino acids within the native epitope (Table 1). Replacement of Asn-341, Glu-340, Pro-339 or Ile-338 with alanine resulted in a greater than 500-fold loss in detection. This alanine scan shows that each of these residues contributes to epitope recognition but that the residues proximal to the C-terminus contribute most

Figure 4 Detection of stromelysin-cleaved human aggrecan

A limit digest of human aggrecan was prepared with stromelysin. Intact (■) and stromelysin-digested (●) aggrecan were quantified in the competitive binding RIA using the probe peptide described in the legend to Figure 1.

significantly. Together the peptide-mapping data indicate that the optimal length of peptide recognized by the antiserum is the seven amino acid sequence F³³⁵VDIPEN³⁴¹ and that, although specificity is directed primarily against the free C-terminal asparagine, other residues contribute significantly to the binding of the antiserum.

Recognition of stromelysin-digested aggrecan

To determine whether the anti-FVDIPEN serum could recognize the stromelysin-generated aggrecan G1 fragment, human aggrecan was digested with recombinant human stromelysin, and the digest evaluated by both RIA and Western-blot analysis. The F³³⁵VDIPEN³⁴¹ sequence in intact aggrecan was not recognized by RIA (Figure 4) whereas the stromelysin-generated G1 fragment terminating in F³³⁵VDIPEN³⁴¹ was (Figure 4). Assuming that aggrecan has a molecular mass of 2000 kDa, the log-linear range of detection for digested aggrecan is similar to the standard peptide ranging from about 30 to 900 pM. These data indicate that the aggrecan fragment is recognized similarly to the peptide and that quantification of the stromelysin-generated aggrecan G1 fragment would be reasonably accurate when standardized with the YTGEDFVDIPEN peptide. When 1.5 μ M aggrecan was digested with 150 nM stromelysin, there was a time-dependent generation of a G1 product which was recognized by the anti-FVDIPEN serum by both RIA and Western-blot analysis (Figure 5). Under these conditions, there was almost complete digestion of the aggrecan, generating FVDIPEN-positive G1 fragment, within 5 h. By 24 h, virtually all the aggrecan had been digested. No FVDIPEN-positive fragment was generated up to 24 h without the addition of stromelysin, indicating that generation of the G1 fragment terminating in F³³⁵VDIPEN³⁴¹ was entirely dependent on specific cleavage by the proteinase. As expected, the fragment from the stromelysin-digested sample migrated as a polydisperse 50 kDa band (Figure 5, inset), when evaluated by Western blotting. Together, these results support previously published sequencing data [3,12–14] indicating that stromelysin cleaves the aggrecan molecule between Asn-341 and Phe-342. Recognition by the antiserum on Western blots was completely blocked by preincubation of a 1:3000 dilution of the antiserum with 10 μ g/ml F³³⁵VDIPEN³⁴¹ peptide but was not affected by preincubation of the serum with the same concentration of spanning peptide F³³⁵VDIPENFFGVG³³⁶ (results not shown), confirming that the recognition of the G1 fragment on immunoblots is specific for the FVDIPEN sequence. Furthermore, the stromelysin-

Figure 5 Detection of stromelysin-generated aggrecan G1 fragment over time

Human aggrecan was incubated with (○) or without (■) stromelysin at a molar ratio substrate/enzyme of 10:1 over a 24 h time period. Samples from each time point were evaluated in the competitive binding RIA described in the legend to Figure 1. Samples from the 0 h time point without enzyme (lane 1), or with enzyme for 0 h (lane 2), 5 min (lane 3), 15 min (lane 4), 30 min (lane 5), 1 h (lane 6), 2 h (lane 7), 4 h (lane 8), 6 h (lane 9) and 24 h (lane 10) were also evaluated by Western blotting (inset) without chondroitinase ABC/keratanase II treatment.

Figure 6 Generation of anti-FVDIPEN-positive aggrecan G1 fragments with multiple enzymes

Aggrecan was digested for 2 h at 37 °C with stromelysin (lane 4), collagenase (lane 5), gelatinase A (lane 6), gelatinase B (lane 7), human leucocyte elastase (lane 8), cathepsin G (lane 9) or cathepsin B (lane 10) and compared with intact aggrecan (lanes 3 and 11) and stromelysin-digested aggrecan as a control (6 h digest; lane 2). Molecular-mass markers are shown in lane 1. Samples were treated with chondroitinase ABC and keratanase II and then evaluated by Western blotting using the anti-FVDIPEN serum.

generated G1 fragment maintains its ability to bind to HA as assessed by gel-filtration chromatography on a Sepharose CL-2B column under associative conditions (results not shown). Stromelysin, activated with either trypsin or APMA, generates the FVDIPEN-positive G1 fragment and this fragment is generated by stromelysin digestion at either pH 5.5 or 7.5 (results not shown).

Reactivity of the anti-FVDIPEN serum against G1 fragments generated by digestion of aggrecan with other proteinases

To determine whether other enzymes could cleave aggrecan at the Asn³⁴¹-Phe³⁴² bond and generate a G1 fragment terminating in F³³⁵VDIPEN³⁴¹, human aggrecan was digested with stromely-

Figure 7 Generation of anti-FVDIPEN-positive fragments in different species

Cartilage samples from rabbits (lane 1), mice (lane 2) and guinea pigs (lanes 3) were evaluated by Western blotting for aggrecan G1 fragment terminating in FVDIPEN. All cartilage samples were treated with human stromelysin followed by chondroitinase ABC and keratanase II before evaluation on blots stained with anti-FVDIPEN serum.

sin, collagenase, gelatinases A and B, human leucocyte elastase and cathepsins B and G. All the samples were treated with chondroitinase ABC, followed by keratanase II before electrophoresis. Even at a ratio of 1 mol of proteinase to 10 mol of aggrecan, the only enzymes that generated the FVDIPEN-positive band were stromelysin and gelatinase A (Figure 6). Human leucocyte elastase, stromelysin, gelatinase A and cathepsin G generated a band that migrated with molecular masses ranging from approx. 50 to 60 kDa on reducing SDS/PAGE, detected by Coomassie Blue staining (results not shown); however, only the 50 kDa bands generated by stromelysin and gelatinase A were recognized by the antiserum. Under the same conditions, cathepsin B, collagenase and gelatinase B generated minimal amounts of 50–60 kDa aggrecan fragments as detected by reducing SDS/PAGE. Furthermore, no immunoreactive band was detected. Thus it appears that stromelysin and gelatinase A are considerably more effective at cleaving aggrecan in the IGD and generating the G1 fragment terminating in F³³⁵VDIPEN³⁴¹ than the related MMPs gelatinase B or collagenase. It is interesting to note that gelatinase A had a k_{cat}/K_m of 190 000 M⁻¹·s⁻¹ compared with gelatinase B which had a k_{cat}/K_m of 905 000 M⁻¹·s⁻¹, using the same peptide substrate, even though gelatinase A appears significantly more active at cleaving the IGD of aggrecan. Also, it appears that, after glycosidase treatment, the FVDIPEN-positive fragment migrates as a more homogeneous band (compare Figures 5 and 6), suggesting that this fragment contains keratan sulphate.

Utility of anti-FVDIPEN serum across species

The sequence surrounding the stromelysin-cleavage site in aggrecan is extremely conserved among species [15–18]. In mouse [15], human [16], rat [17] and rabbit [18] aggrecan the sequence around the cleavage site is DIPEN³⁴¹FFGVG. As a number of arthritis models have been developed in these species, as well as in the guinea pig, cartilage-derived samples (see the Materials and methods section) from each of these species, except rat, were digested with stromelysin and evaluated by Western blotting (Figure 7). On digestion of the mouse, rabbit and guinea-pig samples, a FVDIPEN-positive band with a molecular mass of

50 kDa was generated with human stromelysin. These data indicate that the same serum can be used to recognize stromelysin-generated G1 fragments from a variety of animal species, as well as from humans.

DISCUSSION

An anti-peptide serum was prepared against the C-terminal sequence, F³³⁵VDIPEN³⁴¹, of the MMP-generated aggrecan G1 fragment, and used for development of an RIA and for Western blotting. Using this antiserum, it was shown that stromelysin-1 degrades aggrecan within the IGD releasing a G1 fragment with the C-terminus F³³⁵VDIPEN³⁴¹. The proteinase does this in a time-dependent manner, generating a fragment that is recognized by both RIA and Western blotting. This fragment retains its ability to bind to HA and therefore should remain bound within the cartilage matrix *in vivo*. Gelatinase A also has the capacity to generate significant levels of the fragment recognized by the antiserum. It has been reported that stromelysin, collagenase, gelatinase A and gelatinase B can all cleave aggrecan at Asn³⁴¹-Phe³⁴² [3,12–14]. In this study, we have shown that collagenase, gelatinase B, cathepsin B, cathepsin G and human leucocyte elastase do not generate the aggrecan G1 fragment terminating in F³³⁵VDIPEN³⁴¹ at the same concentrations under which stromelysin and gelatinase A are effective. This appears to be the result of a combination of factors. First, collagenase, cathepsin B and gelatinase B appear to be relatively poor at cleaving aggrecan in the IGD, generating few G1 fragments. Second, human leucocyte elastase [27] and cathepsin G appear to cleave aggrecan in the IGD, but at sites other than the MMP-cleavage site. Lack of recognition of these G1 fragments by the antiserum would be expected on the basis of the peptide-mapping results which indicate that peptides truncated on the C-terminus or extended across the MMP-cleavage site are not recognized by the antiserum. Thus G1 fragments with C-terminal sequences that extend across the MMP-cleavage site, such as those generated by elastase (cleavage at Val³⁷⁸-Ile³⁷⁹) or cathepsin B (cleavage at Gly³⁴⁴-Val³⁴⁵), would not be recognized. A careful kinetic analysis, in which the enzyme preparations are active-site titrated, must be completed to determine conclusively which MMP has the most activity against the aggrecan substrate. The RIA described in this study will be useful for such analyses, as it can easily quantify the MMP-generated G1 fragment terminating in F³³⁵VDIPEN³⁴¹. It is interesting to note, however, that gelatinase A, which has approximately one-fifth the activity of gelatinase B against a peptide substrate, can generate the FVDIPEN-positive G1 fragment under conditions in which gelatinase B cannot. These data are consistent with those characterizing relative proteoglycan cleavage by these enzymes which show that stromelysin and gelatinase A at pH 7.5 have similar activity against proteoglycan/acrylamide bead cleavage whereas gelatinase B was 2–3-fold less active [28].

After chondroitinase/keratanase digestion of the samples, the anti-FVDIPEN-positive fragments migrate as more homogeneous bands on the gels. This result suggests that there may be keratan sulphate N-terminal to the F³³⁵VDIPEN³⁴¹ sequence. It has been reported that Thr-352 and Thr-357, which are C-terminal to the stromelysin-cleavage site, are substituted with keratan sulphate [29]. Further analysis of the MMP-generated aggrecan G1 fragment will be required to confirm that there are keratan sulphate chains attached to this molecule as well as the specific location of these glycosaminoglycan chains.

One of the significant advantages of this antiserum is that it does not recognize intact aggrecan or aggrecan fragments generated by proteinases that cleave at sites other than the MMP

site, but does recognize the G1 fragment after MMP cleavage. The antiserum does not recognize peptides in which this asparagine has been substituted, or peptides extending across the cleavage site. This is to be expected as the peptide immunogen was linked to the thyroglobulin carrier through the N-terminal phenylalanine leaving the C-terminal asparagine exposed. Similar approaches have been used in the past for both link protein [30] and protein kinase C [31] to generate specific anti-peptide antibodies that recognize cleaved protein fragments but do not recognize the intact parent molecules. The other significant advantage of this anti-FVDIPEN serum is that it can detect stromelysin-generated fragments from many species. We have shown that stromelysin-generated fragments from humans, rabbits, mice and guinea pigs can be detected using this serum. This may not be so surprising as the D³³⁷IPEN³⁴¹ sequence is identical in rat [17], human [16], rabbit [18] and mouse [15] aggrecan. In all these systems, it will be interesting to determine whether there is a correlation between enzyme, or enzyme-inhibitor complex levels, and the levels of aggrecan-degradation product. It has been suggested that MMPs, including stromelysin, may play a role in cartilage degeneration in animal models in a number of these species [7–11]. We have reported that stromelysin is elevated in synovial fluid from patients with traumatic joint injury, rheumatoid arthritis and osteoarthritis [5–7] as well as in a model in the rabbit in which interleukin 1 is injected intra-articularly [11]. We cannot differentiate active stromelysin from proenzyme in any of these systems. The use of antibodies that detect fragments generated by the enzymes will help resolve the issue of whether these MMPs play a role in cartilage matrix degradation in these models, as well as human joint diseases. The antiserum described in this study will be useful for such experiments as it can detect aggrecan fragments from human cartilage as well as fragments from a number of other species.

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REFERENCES

- 1 Bayliss, M. T., Holmes, M. W. A. and Muir, H. (1989) *Trans. Orthop. Res. Soc.* **14**, 32
- 2 Quang, N., Murphy, G., Roughley, P. J. and Mort, J. S. (1989) *Biochem. J.* **259**, 61–67
- 3 Flannery, C. R., Lark, M. W. and Sandy, J. D. (1992) *J. Biol. Chem.* **267**, 1008–1014
- 4 Wu, J.-J., Lark, M. W., Chun, M. W. and Eyre, D. E. (1991) *J. Biol. Chem.* **266**, 5525–5528
- 5 Walakovits, L. A., Moore, V. L., Bhardwaj, N., Gallick, G. S. and Lark, M. W. (1992) *Arthritis Rheum.* **35**, 35–42
- 6 Lohmander, L. S., Hoerner, L. A. and Lark, M. W. (1993) *Arthritis Rheum.* **36**, 181–189
- 7 Wolfe, G. C., MacNaul, K. L., Buechel, F. F. et al. (1993) *Arthritis Rheum.* **36**, 1540–1547
- 8 Case, J. P., Sano, H., Lafyatis, R., Remmers, E. F., Kunkumian, G. K. and Wilder, R. L. (1989) *J. Clin. Invest.* **84**, 1731–1740
- 9 Hasty, K. A., Reife, R. A., Kang, A. H. and Stuart, J. M. (1990) *Arthritis Rheum.* **33**, 388–397
- 10 Mehraban, F., Riera, H., Kuo, S. Y., Chang, C. and Moskowitz, R. W. (1993) *Trans. Orthop. Res. Soc.* **18**, 682
- 11 McDonnell, J., Walakovits, L. A., Lark, M. W. et al. (1992) *Arthritis Rheum.* **35**, 799–805
- 12 Fosang, A. J., Neame, P. J., Hardingham, T. E., Murphy, G. and Hamilton, J. A. (1991) *J. Biol. Chem.* **266**, 15579–15582
- 13 Fosang, A. J., Neame, P. J., Hardingham, T. E., Murphy, G. and Hamilton, J. A. (1993) *Trans. Orthop. Res. Soc.* **17**, 83
- 14 Fosang, A., Last, K., Knaeuper, V. et al. (1993) *Biochem. J.* **259**, 273–276
- 15 Walcz, E., Deak, F., Horvath, P., Fulop, C., Doege, K. J. and Glant, T. T. (1993) *Trans. Orthop. Res. Soc.* **18**, 656
- 16 Doege, K. J., Sasaki, M., Kimura, J. and Yamada, U. (1991) *J. Biol. Chem.* **266**, 894–902

-
- 17 Doege, K., Sasaki, M., Horigan, E., Hassell, J. R. and Yamada, Y. (1987) *J. Biol. Chem.* **262**, 17757–17767
- 18 Bayne, E., MacNaul, K., Donatelli, S. et al. (1994) *Trans. Orthop. Res. Soc.* **19**, 308
- 19 Kitagawa, T. and Aikawa, T. (1976) *J. Biochem. (Tokyo)* **79**, 233–236
- 20 Miller, D. K., Ayala, J. M., Egger, L. A. et al. (1993) *J. Biol. Chem.* **268**, 18062–18069
- 21 Hascall, V. C. and Kimura, J. H. (1982) *Methods Enzymol.* **82**, 769–800
- 22 Farndale, R. W., Buttle, D. J. and Barrett, A. J. (1986) *Biochim. Biophys. Acta* **883**, 173–177
- 23 Teahan, J., Harrison, R., Izquierdo, M. and Stein, R. L. (1989) *Biochemistry* **28**, 8497–8501
- 24 Knight, C. G., Willenbrock, F. and Murphy, G. (1992) *FEBS Lett.* **296**, 263–266
- 25 Harrison, R. K., Teahan, J. and Stein, R. L. (1989) *Anal. Biochem.* **180**, 110–113
- 26 Fosang, A. J., Neame, P. J., Last, K., Hardingham, T. E., Murphy, G. and Hamilton, J. A. (1992) *J. Biol. Chem.* **267**, 19470–19474
- 27 Mok, M. T., Ilic, M. Z., Handley, C. J. and Robinson, H. C. (1992) *Arch. Biochem. Biophys.* **292**, 442–447
- 28 Murphy, G., Cockett, M. I., Ward, R. W. and Docherty, A. J. P. (1991) *Biochem. J.* **277**, 277–279
- 29 Barry, F. P., Gaw, J. U., Young, C. N. and Neame, P. J. (1992) *Biochem. J.* **286**, 761–769
- 30 Hughes, C. E., Caterson, B., White, R. J., Roughley, P. J. and Mort, J. S. (1992) *J. Biol. Chem.* **267**, 16011–16014
- 31 Kikuchi, H., Imajoh-Ohmi, S. and Kanegasaki, S. (1993) *Biochim. Biophys. Acta* **1162**, 171–176
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