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Impact of matrix metalloproteinases on inhibition of mineralization by fetuin

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Background and Objective: Human subjects affected by inflammatory diseases, such as periodontitis, may be at increased risk for the development of cardiovascular diseases and calcification of atheromas; however, the potential mechanisms have not been defined. Alpha-2-Heremans Schmid glycoprotein (fetuin A) is an abundant serum glycoprotein of ~49 kDa that inhibits ectopic arterial calcification. We examined whether matrix metalloproteinases (MMPs), which are increased in inflammatory diseases, including periodontitis, bind and degrade fetuin and alter its ability to inhibit calcification *in vitro*.

Material and Methods: Binding and cleavage of fetuin by MMPs were assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, *in-silico* analyses and mass spectrometry. The effects of intact and MMP-degraded human fetuin on mineralization were measured in a cell-free assay.

Results: From *in-silico* analyses and literature review, we found that only MMP-3 (stromelysin) and MMP-7 (matrilysin) were predicted to cleave human fetuin at levels that were physiologically relevant. *In-vitro* assays showed that MMP-7, and, to a lesser extent, MMP-3, degraded human fetuin in a time- and dose-dependent manner. Fetuin peptides generated by MMP-7 cleavage were identified and sequenced by mass spectrometry; novel cleavage sites were found. Hydroxyapatite mineralization *in vitro* was strongly inhibited by fetuin (> 1 μM), as was MMP-3-cleaved fetuin, while MMP-7-cleaved fetuin was threefold less effective in blocking mineralization.

Conclusion: MMP-7 and, to a lesser extent, MMP-3, affect the ability of fetuin to inhibit the formation of hydroxyapatite *in vitro*. These data suggest that the MMPs increased in inflammatory diseases, such as periodontitis, could affect regulation of mineralization and potentially enhance the risk of calcified atheroma formation.

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Calcification in the intima of blood vessels is associated with several cardiovascular diseases, including end-stage renal disease (1,2). Vascular calcification within atheromas requires the deposition of hydroxyapatite; the amount of calcification is a marker of the atherosclerotic burden and may mediate loss of arterial elasticity (2). Several cohort and cross-sectional studies have found positive associations between periodontitis and increased risk of cardiovascular diseases and vascular calcification (3–8) but the underlying mechanisms that

explain if and how periodontitis and other inflammatory diseases enhance the formation of calcifying atheromas are not defined. While there is no definitive evidence for a causal association between cardiovascular diseases and periodontitis (9), it is nevertheless important to identify potential pathophysiological mechanisms that could contribute to this link.

Vascular calcification may be initiated by several mechanisms (2,10), including loss of inhibition of calcification, induction of bone formation and cell death, which can nucleate initial calcification processes. Notably, several proteins may be involved in suppressing calcification in atheromas; these proteins include osteopontin (1,2,10,11), matrix Gla protein (12), osteocalcin and the alpha-2-Heremans Schmid glycoprotein (the human homologue of fetuin A) (13,14). Fetuin is an abundant serum sialoprotein (M_r ~49,000) (15,16) that is a member of the cystatin superfamily (17,18) and is synthesized in the liver (19). Fetuin, which is incorporated into mineralized tissues and plays an important role in bone formation and resorption (18,20,21), inhibits calcification and prevents precipitation of calcium phosphate (22,23). Fetuin may act as a crystal poison (23–25) but may also inhibit mineralization by interfering with the differentiation of cells with a mineralizing phenotype (26–29). Low levels of fetuin in serum are associated with increased levels of vascular calcification (30). In view of the relationship between low levels of intact fetuin in serum and increased risk for vascular calcification, we considered that matrix mtalloproteinases (MMPs) which are up-regulated in periodontitis could reduce the levels of fetuin in serum as a consequence of their ability to degrade this protein (31). Several enzymes that are increased in the gingival crevicular fluid of periodontitis patients, such as alkaline phosphatase (32,33), beta-glucuronidase (32,34–36), aspartate aminotransferase (37–39), lactate dehydrogenase (40,41), neutrophil elastase (42,43) and cathepsins B (40) and D (36), could potentially inhibit the function of fetuin, but none of these enzymes has been reported to degrade fetuin.

MMPs are an important family of zinc-dependent endopeptidases that are involved in the destruction of the extracellular matrix in periodontal diseases (44–46). Notably, the concentrations of MMP-3 (44–46), MMP-7 (47,48) and MMP-8 (49) are markedly increased in the gingival crevicular fluid of patients with periodontitis. MMP-9 is increased in the serum of patients with periodontitis (31), and among *Helicobacter pylori*-infected subjects, gastric cancer patients exhibited higher serum levels of MMP-3 and MMP-7 than did those with duodenal ulcer and gastritis (50). Currently there are limited data on the relationship between the concentrations in blood of other MMPs and periodontal status. As fetuin can interact with MMPs (51), we considered that the binding of MMPs to fetuin may facilitate fetuin degradation (52,53).

Previous studies have shown that complete digestion of human fetuin occurs within 12 h when it is incubated with a 10-fold molar excess of either MMP-2 or MMP-7 (52). In contrast, at equimolar ratios, neither of these enzymes can degrade fetuin, suggesting that MMP-2 and MMP-7 do not affect the regulation of mineralization by fetuin *in vivo*. While MMP-3 and MMP-7 can cleave bovine fetuin at specific sites in the C-terminus of the molecule (54), the impact of these cleavages on the regulation of mineralization has not been examined. Accordingly, we assessed how MMPs that are up-regulated in periodontitis interact with and degrade human fetuin. We also examined whether MMP-degraded fetuin loses its ability to inhibit mineralization *in vitro*.

Material and methods

Reagents

We obtained heparin–agarose beads and 4-amino phenylmercuric acetate (APMA) from Sigma-Aldrich (Oakville, ON, Canada). Human fetuin was from MyBiosource (San Diego, CA, USA) and antibody to human fetuin was from Alpha Diagnostics (San Antonio, TX, USA). Human recombinant pro-MMP-3, human recombinant MMP-7, manufacturer-activated human recombinant MMP-7 (active), antibody to human MMP-7 and MMP Inhibitor II (*N*-hydroxy-1,3-di-(4-methoxybenzenesulphonyl)-5,5-dimethyl-[1,3]-piperazine-2-carboxamide) (55) were from Calbiochem (San Diego, CA, USA).

In-silico analysis

We searched online databases (ExPASy PeptideCutter, http://expasy.org/tools/peptidecutter; MEROPS Peptidase Database, http://merops.sanger.ac.uk; and CutDB, http://cutdb.burnham.org) for both predicted and reported cleavages of human fetuin by enzymes that are reported to be increased in periodontitis, including alkaline phosphatase (32,33), beta-glucuronidase (32,34–36), aspartate aminotransferase (37–39), lactate dehydrogenase (40,41), neutrophil elastase (42,43), cathepsins B (40) and D (34,36,44–46), MMP-3 (44–46), MMP-7 (47,48) and MMP-8 (49). There is no predicted cleavage of human fetuin by most of these enzymes; however, human fetuin is reported to be degraded after overnight incubation with MMP-7 at a very high enzyme : substrate ratio (52). Therefore, we focused subsequent analyses on *in-vitro* experiments.

MMP assay

A fluorimetric MMP assay kit (SensoLyte®) was obtained from Anaspec (Fremont, CA, USA). This assay detects MMP-7 and MMP-3 activities in biological samples using a 7-dimethylaminocoumarin-4-acetate)/nitro-2-1,3-benzoxadiazol-4-yl fluorescence resonance energy transfer peptide. When the fluorescence resonance energy transfer peptide is not cleaved, the fluorescence of 7-dimethylaminocoumarin-4-acetate is quenched by nitro-2-1,3-benzoxadiazol-4-yl. After cleavage of the peptide by MMP-7 or MMP-3, the fluorescence of dimethylaminocoumarin-4-acetate is restored

and can be measured at excitation/emission wavelengths of 370 nm/460 nm in a fluorescence plate reader.

Fetuin cleavage by MMPs

Fetuin was incubated in TNCB buffer (50 mM Tris, pH 7.5, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% BRIJ 35) with MMP-7 or MMP-3 for 0, 1, 2, 4, 8 or 24 h, and at various ratios of fetuin : MMP, as indicated in the Results. Dithiothreitol was not included in the buffer as we did not intend to reduce the disulfide bridges in fetuin (56) for degradation assays.

We used MMP-7 and MMP-3 that were either unactivated or supplier pre-activated, or that we activated *in vitro* with APMA (1 mM in TNCB buffer for 1 h at room temperature, as described previously (57)). In some experiments, the reversible, binding-site-targeting MMP inhibitor II (58) was co-incubated with fetuin and enzymes in digestion experiments. This inhibitor has an half maximal inhibitory concentration (IC_{50}) of 18.4 nM for MMP-3 and an IC_{50} of 30 nM for MMP-7 (55,58).

Proteins obtained following the digestion of fetuin were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12% gels) and stained with Coomassie Blue. Individual protein bands were excised and destained with ammonium bicarbonate. Gels were shrunk and alkylated, and the peptides were subjected to trypsin digestion before peptide extraction. Samples were evaporated to dryness and analyzed by tandem mass spectrometry (Protein Technology Service, Hospital for Sick Children, Toronto, ON, Canada). For prediction of cleavage sites, SCAFFOLD 3 (Proteome Software, Portland, OR, USA) was used with a Web-based database for the amino-acid sequence of human fetuin (Swissprot database-http://web.expasy.org) and applied to the analysis of the peptides produced from the digestions.

Fetuin binding to MMPs

We assessed whether intact fetuin was bound by MMP-7 before potential digestion of fetuin by MMP-7. Purified MMP-7 (or bovine serum albumin as a control) was attached to heparin–agarose beads and incubated with fetuin for 20 min. MMP-7 that had been bound to beads previously was incubated with fetuin at specific dilutions. Quantification of the bound whole fetuin vs. free fetuin was estimated by dot-blotting proteins onto nitrocellulose and immunodetection with antibody to fetuin. Appropriate dilutions of human fetuin standards were dot-blotted in parallel. Quantification of dot densities were used to estimate the kDa and maximum binding (Bmax) values for MMP-7–fetuin binding by Scatchard plots. In separate experiments, the efficacy of MMP-7-mediated digestion of fetuin when the MMP-7 was attached to agarose was assessed by separating fetuin-degradation products by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Mineralization assay

The assay for measurement of hydroxyapatite crystals formed *in vitro* was based on an original paper by Chen and co-workers (59) and modified by Dr G. Hunter (University of Western Ontario, London, ON, Canada). The following solutions: 10 μL of phosphate solution (10 mM KH_2PO_4, 20 mM Na_2HPO_4, and 150 mM NaCl, pH 7.4), 10 μL of calcium solution (50 mM $CaCl_2$, 50 mM Hepes and 150 mM NaCl, pH 7.4) and 80 μL of test agents in buffer solution (50 mM Hepes and 150 mM NaCl, pH 7.4), which contained 38 μM fetuin and equal volumes of either vehicle (TNCB buffer) or TNCB buffer containing MMP-3 or MMP-7, were added sequentially to wells of 96-well plates (to give a total volume of 100 μL per well). After incubation (up to 4 h at 23°C), the supernatant was removed, leaving the nascent hydroxyapatite crystals bound to the bottom of the well. Electron microscopy (see below) was used to confirm that the crystals did indeed attach to the bottom of the wells.

Hydroxyapatite crystals were stained with 75 μL of Alizarin Red S (0.5% Alizarin Red S, pH 4.2; 5 min of staining). Then, the Alizarin Red solution was removed by pipetting. To dissolve the crystal-bound Alizarin Red, 100 μL of cetylpyridinium chloride solution (100 mM) was added to each well and incubated at 23°C for 30 min. The relative abundance of hydroxyapatite crystals formed in each well was estimated from the amount of Alizarin Red S stain remaining in the cetylpyridinium chloride solution, which was determined by measuring the absorbance at 540 nm using a spectrophotometer.

The authenticity of the hydroxyapatite crystals formed was assessed by embedding crystals in electron microscopy embedding resin, thin sectioning and examination of unstained sections by transmission electron microscopy. Briefly, the dried mineral was scraped from the bottom of the culture well, dropped into molds and embedded in Quetol-Spurr resin (Electron Microscopy Sciences, Hatfield, PA, USA). Sections (of 100 nm thickness) were cut on an RMC MT6000 ultramicrotome (Thermo Fisher, Toronto, ON, Canada), placed on formvar coated-grids and viewed on an FEI Tecnai 20 transmission electron microscope (FEI, Hillsboro, OR, USA). The samples were not fixed or stained. Electron diffraction analysis of crystals (60) was conducted (Mount Sinai Hospital, Department of Pathology, Toronto, ON, Canada) using comparisons with hydroxyapatite standards.

Statistical analyses

For all data shown, experiments were repeated at least three times and were conducted on different days. For each individual experiment, at least three replicates were analyzed. For continuous variables, means and standard deviations were computed. For the binding experiments, means, standard errors and r^2 were computed. Between-group comparisons were assessed using analysis of variance, and individual group differences were analyzed *post hoc* with Tukey's test. The type I error threshold for estimation of statistical significance was set at $p < 0.05$.

Results

We quantified the enzyme activity of three different MMP-7 formulations [unactivated, manufacturer-activated and APMA-activated (1 mM APMA in TNCB buffer for 1 h at room temperature, as described previously) (56)]. Enzyme activity was estimated using a fluorimetric MMP assay that provides increased fluorescence after MMP-3- or MMP-7-induced cleavage of the MMP substrate. In time-course experiments that evaluated substrate degradation by the MMP-7 formulations, there was a plateau of enzyme activity approaching 1 h (Fig. 1), which was considered to be the optimal time for APMA activation. While the APMA-activated MMP-7 exhibited the highest activity of the three formulations throughout the time course, the activity levels were not markedly different (< 5% difference between groups at 1 h). For all subsequent experiments, we used APMA-activated MMP-7 and APMA-activated MMP-3.

Time-course studies were conducted (at 37°C) using MMP-3 and MMP-7 incubated with fetuin at an enzyme/substrate ratio of 1 : 60, and analysis of fetuin degradation was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. MMP-3 and MMP-7 were not detected in the polyacrylamide gels because these proteins were present at much lower molar concentrations than fetuin. MMP-3 produced limited cleavage of human fetuin that was detectable only after 24 h of incubation, whereas marked degradation of fetuin was readily observed after 1 h of incubation with MMP-7 (Fig. 2A). By 24 h there was no intact fetuin remaining after incubation with MMP-7. The prominent fetuin-degradation fragments found after treatment with MMP-7 for 2 h were ~40 and ~18 kDa; after longer periods of incubation the 18-kDa fragment predominated. Notably, based on densitometry of intact fetuin, MMP-7-mediated degradation of fetuin was inhibited by > 90% with the MMP II inhibitor [1 μM; Fig. 2B; IC_{50} = 30 nM; (55)], suggesting that the cleavage was attributable to the catalytic activity of MMP-7 and not to nonspecific degradation by undefined mechanisms in the assay.

To complement the time-course experiments, digestions of human fetuin by MMP-3 and MMP-7 were conducted by incubation for 4 h with various enzyme/substrate ratios, and the relative amounts of full-length fetuin were evaluated by densitometry of the stained gels. For MMP-7, 24% of the fetuin was degraded at an enzyme/substrate ratio of 1 : 60, 71% at an enzyme/substrate ratio of 1 : 30 and 100% at an enzyme/substrate ratio of 1 : 15. For MMP-3, even at an enzyme/substrate ratio of 1 : 10, only 6% of the fetuin was degraded. As the relative amount of MMP-7 was increased relative to the amount of substrate (i.e. an elevated ratio of enzyme/substrate), MMP-7 caused more extensive fetuin degradation and the relative amount of intact fetuin was decreased. Therefore, MMP-7, but not MMP-3, appears to be relevant for studying pathophysiological fetuin degradation.

Following MMP-7 degradation (24 h), fragments were cut out of the polyacrylamide gels at sites in the gels that were expected to contain fetuin and fetuin fragments (Fig. 3A), and these fragments were analyzed by tandem mass spectrometry using Scaffold 3 analysis of the Swiss-Prot protein sequence database. From a series of analyses, continuous amino-acid sequences (i.e. without interruption) of fetuin digestions by MMP-7 were compared with the reported cleavage sites of bovine fetuin cleaved by MMP-7 (54). Based on these comparisons and our examination of predicted sequences obtained from all of the peptides identified in the various analyses, we identified a high-probability (> 95%) cleavage site in human fetuin at R317-H318. We also identified novel predicted cleavage sites (A167-L168 and P192-L193), which were based on examination of fetuin fragments of < 20 kDa (Fig. 3B) that included assembly and contiguity analyses of the sequences of the various tryptic peptides that were detected in all samples. In contrast to our analyses, based on the online databases ExPASy PeptideCutter, MEROPS Peptidase Database, CutDB and Swissprot, MMP-7 was predicted to cleave fetuin at the sequence YDLR-HTFM (amino-acid positions 312–313 at the cleavage site).

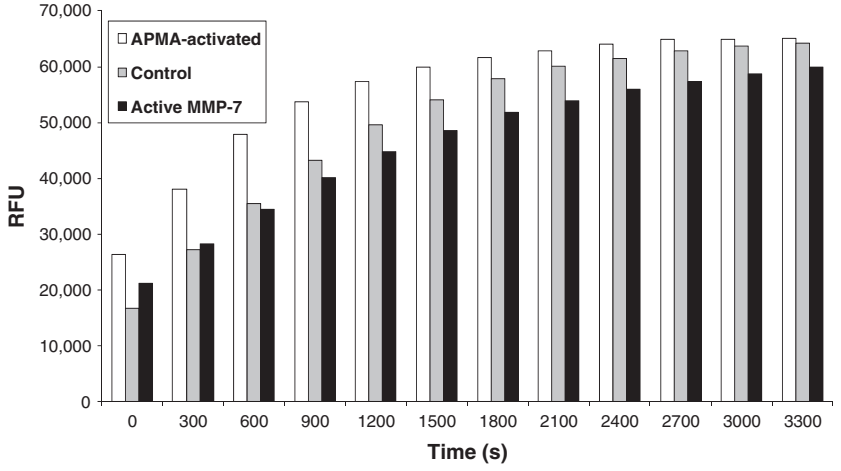

Fig. 1. Matrix metalloproteinase (MMP) catalytic activities of recombinant MMP-7 preparations were measured using a dimethylaminocoumarin-4-acetate/nitro-2-1,3-benzoxadiazol-4-yl fluorescence resonance energy transfer peptide. After cleavage by MMP-7, the fluorescence of dimethylaminocoumarin-4-acetate is restored. In this assay the fluorescence was measured at excitation/emission wavelengths of 370 nm/460 nm in a fluorescence plate reader. The three different MMP-7 preparations evaluated were: 4-amino phenylmercuric acetate (APMA)-activated; unactivated (control); and manufacturer-activated (active MMP-7). Data are expressed as relative fluorescence units (RFU) and were measured after the indicated incubation times using a fluorescence peptide.

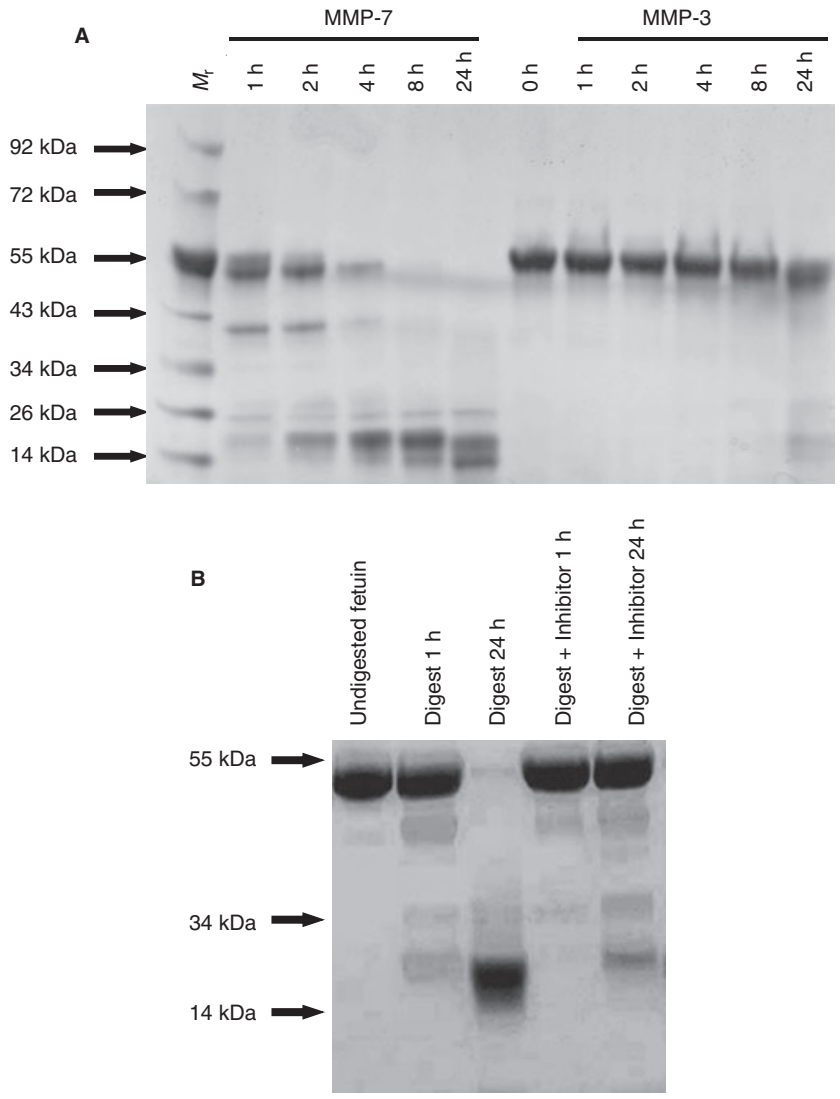

Fig. 2. (A) Digestion of human fetuin by matrix metalloproteinase (MMP)-7 and MMP-3. Fetuin was incubated with purified enzymes at an enzyme/substrate ratio of 1 : 60 and for the time-periods indicated. The degradation fragments were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then stained with Coomassie Blue. (B) Inhibition of MMP-7-mediated degradation of fetuin with MMP Inhibitor II ($C_{21}H_{27}N_3O_8S_2$; 1 μM). MMP-7 and human fetuin were co-incubated in the presence and in the absence of the MMP inhibitor, for 1 h or 24 h, as indicated.

As MMP-7 evidently cleaves human fetuin (Fig. 2), we examined the initial interaction of MMP-7 with fetuin by *in-vitro* binding studies with MMP-7 bound to heparin–agarose beads. We first measured the amount of MMP-7 that bound to beads by dot-blot analysis and compared this with known amounts of MMP-7 as standards (Fig. 4A). For assessment of the binding of fetuin to MMP-7, fetuin at various concentrations (1.1–111 μM) was incubated with MMP-7 bound to heparin–agarose beads and the relative amounts of bound and free fetuin after 20 min of binding were quantified by immunodetection and densitometry of dot-blots (Fig. 4B). The time of 20 min for binding was chosen because we anticipated that there would be very limited fetuin degradation by MMP-7 at this time. Measurements of the free vs. the bound amounts of fetuin allowed computation of Bmax (0.87 ± 0.17) and kDa ($2.96 \pm 1.92 \times 10^{-9}$ M; $r^2 = 0.87$), indicating high-affinity binding of fetuin to MMP-7 (Fig. 4B).

As MMP-7 binds tightly to heparin (59), we attached MMP-7 to heparin–agarose in binding buffer and the catalytic efficacy of the bound enzyme was assessed. The binding of MMP-7 to heparin–agarose did not affect catalytic activity, and bead-bound MMP-7 efficiently degraded fetuin (after 24 h of digestion; Fig. 4C). Therefore, the heparin that was used to attach MMP-7 for the binding assays (Fig. 4B) evidently did not occlude functionally the catalytic site of MMP-7.

For assessing the effect of hydroxyapatite mineralization *in vitro* by fetuin, we first examined the authenticity of the hydroxyapatite crystals that were formed in the *in-vitro* mineralization assays. Transmission electron microscopy showed small, crystalline structures that formed at the bottom of the dishes (Fig. 5A). Electron diffraction analysis of the mineralized material showed that the d-spacings and intensities of the pattern of the hydroxyapatite formed in the *in-vitro* assay matched the published standards for hydroxyapatite (Table 1).

In preliminary trials that examined mineralization in time-course experiments, spectrophotometric absorbance (540 nm) measurements of mineral-bound Alizarin Red showed a time-dependent increase of absorbance (from 0.5 to 2.2 absorbance units) which peaked at 3.5 h (Fig. 5B). During the experiments in which we optimized the conditions for the mineralization assay, we found that freshly prepared Alizarin Red staining solutions produced somewhat (~30%) lower absorbance measurements than did Alizarin Red preparations that were made at least 1 wk in advance of the assay. Furthermore, we found that assays conducted with the aged Alizarin Red preparations were more sensitive in detecting fetuin-induced inhibition of mineralized material than were assays conducted with freshly made Alizarin Red. Accordingly, when the mineralization assays were conducted for 3.5 h with 1-wk-aged Alizarin Red solutions, the absorbance attributable to Alizarin Red was ~3 units (Fig. 5C). In assays that used 2 μM human fetuin in the mineralization medium, there was a

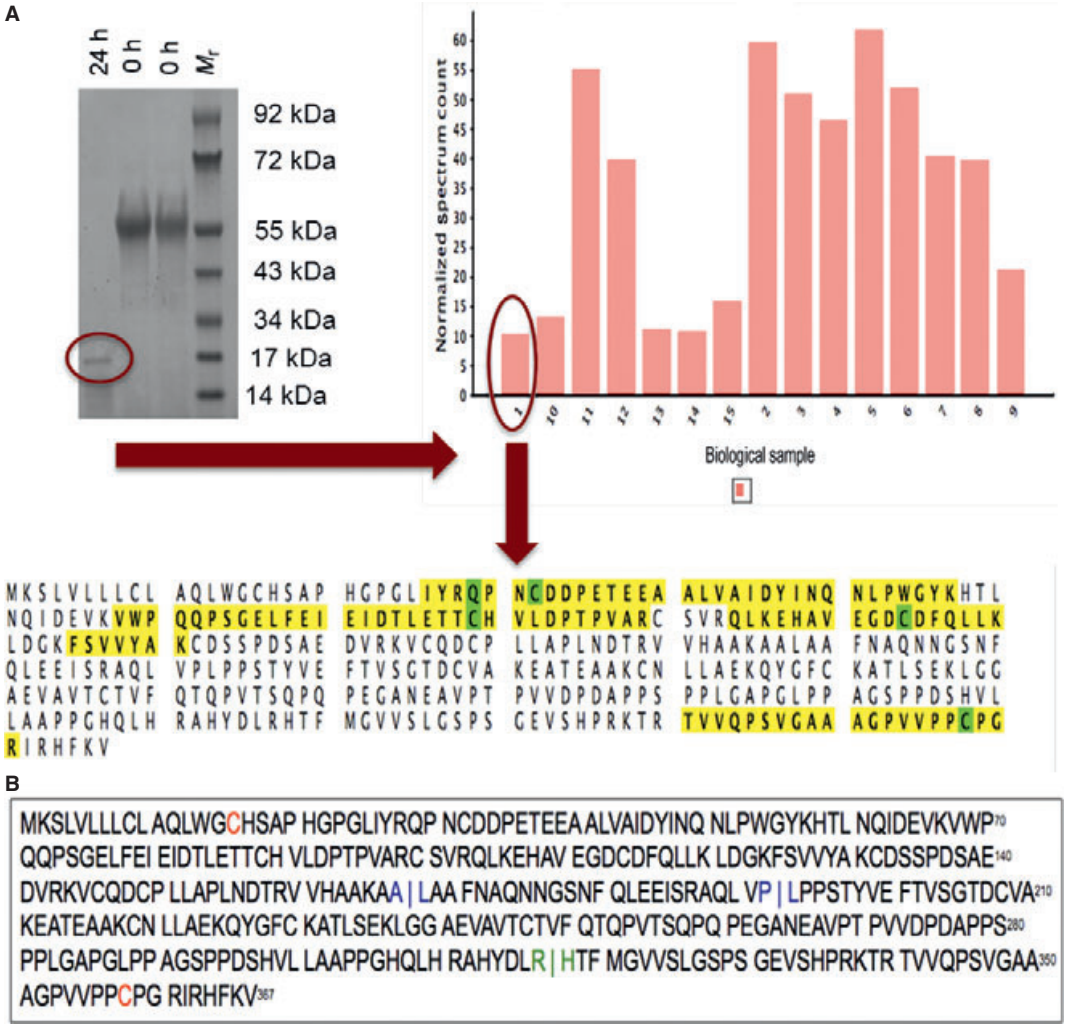

Fig. 3. (A) Typical sodium dodecyl sulfate–polyacrylamide gel of fetuin after no treatment (0 h) or after degradation by matrix metalloproteinase (MMP)-7 (at an enzyme/substrate ratio of 1 : 60) for 24 h that was used for mass spectrometry analysis of fetuin degradation. In this example, a fragment of ~18 kDa (circled area) was cut out of the gel, subjected to tryptic digestion and analyzed by mass spectrometry. Data from a single sample are shown here. Four uninterrupted, matched (> 95% probability) tryptic peptides from the cut-out fragment are highlighted in yellow and are overlaid over the whole known amino-acid sequence for human fetuin. Amino acids with post-translational modifications detected by mass spectrometry are highlighted in green. (B) The full amino-acid sequence of human fetuin, indicating the location of a disulfide bond (C16-C358; red letters), predicted the MMP-7 cleavage site based on reported data of bovine fetuin (the position changed in human fetuin to R317-H318; green letters), and predicted novel cleavage sites based on data from mass spectrometry of cleavage fragments formed after MMP-7 digestion of fetuin for 24 h (A167-L168 and P192-L193; blue letters).

10-fold reduction of Alizarin Red absorbance compared with controls ($p < 0.001$; Fig. 5C).

We next conducted mineralization assays with MMP-3- and MMP-7-degraded human fetuin. In preliminary trials we found that fetuin-mediated inhibition of mineralization was not affected when the fetuin was degraded by MMP-7 for < 4 h. Accordingly, we conducted longer fetuin digestions (24 h at an enzyme/substrate ratio of 1 : 60) in which we anticipated that there would be degradation of fetuin into fragments of < 20 kDa (Fig. 3A). For MMP-7-degraded fetuin there was threefold more mineralization than with intact fetuin (2 μM fetuin; $p < 0.001$; Fig. 5C). In assays that examined MMP-3-degraded fetuin under the same molar ratios and incubation times, there was a small, but nonsignificant, change of Alizarin Red absorbance ($p > 0.2$).

Discussion

We examined the cleavage of fetuin by two specific MMPs – MMP-3 (44–46) and MMP-7 (47,48) – that are known to be increased in the gingival crevicular fluid of patients with periodontitis. The rationale for focusing on MMP-3 and MMP-7 was based on the initial *in-silico* analyses indicating that of those enzymes known to be increased in tissues affected by

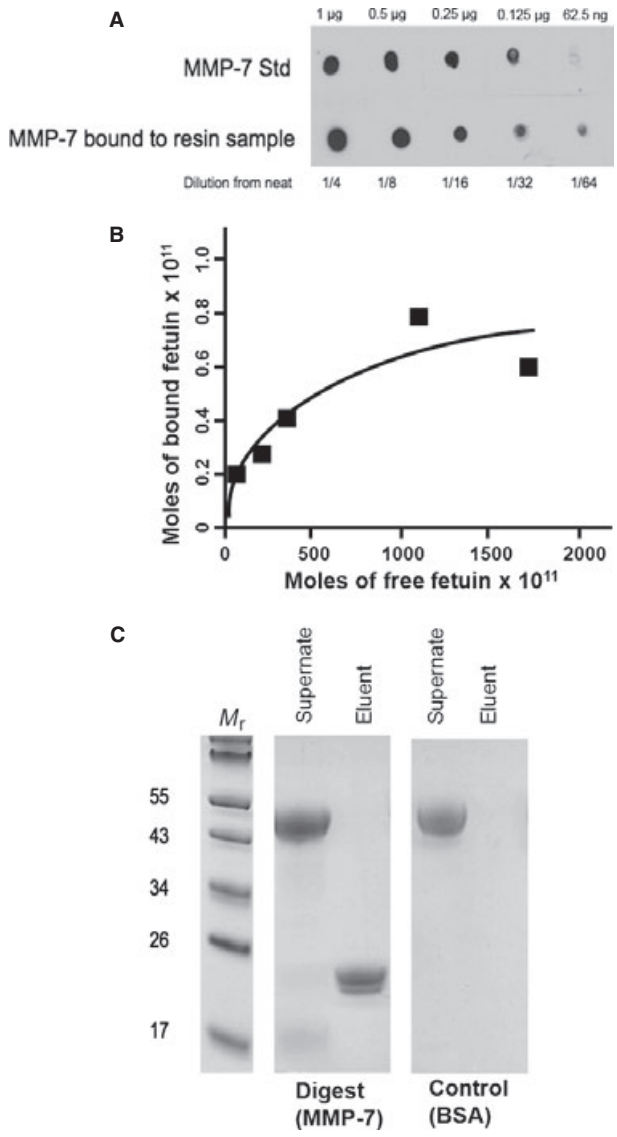

Fig. 4. (A) Dot-blot assay to assess the amount of matrix metalloproteinase (MMP)-7 bound to heparin–agarose beads. MMP-7 was eluted from a fixed volume of pelleted beads, dotted onto the membrane and then stained with antibody to MMP-7. MMP-7 standards (MMP-7 Std) were included to estimate the amount of MMP-7 bound to the beads. (B) Estimates of binding affinity based on measurements of free fetuin vs. bound fetuin with MMP-7 attached to heparin–agarose beads. Computations of Bmax and kDa were performed by Scatchard analysis. (C) MMP-7 or bovine serum albumin (BSA) was bound to heparin–agarose beads and incubated with human fetuin for 24 h. Supernates or materials eluted from beads were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 12% gels and stained with Coomassie Blue. For MMP-7-bound beads, the bead eluent protein migrates at ~20 kDa, which is the approximate size of the fetuin digestion products identified in Fig. 2. For beads with bound BSA, fetuin is found only in the supernate fraction and no fetuin or digestion products are attached to the beads.

periodontitis, cleavage of fetuin was predicted for only these two MMPs. Despite the predicted cleavage of fetuin by MMP-3 and MMP-7 (54), we found that only MMP-7 degraded human fetuin efficiently. Notably, the digestion of human fetuin by MMP-7 was strongly reduced by an MMP inhibitor (55) (IC_{50} = 30 nM), suggesting that the digestion was MMP-specific. Even when incubated at high enzyme/substrate ratios, human fetuin was not efficiently degraded by MMP-3. Based on these findings we focused subsequent studies on the interaction between purified MMP-7 and fetuin. These experiments indicated very tight binding of these two proteins, consistent with our data showing that MMP-7-mediated degradation of fetuin increased proportionately when the enzyme/substrate ratio was increased.

Bovine fetuin and human fetuin exhibit ~70% amino acid identity (61) and the similarity of the arrangement of cysteine residues and of disulfide loops has led to the proposal that bovine and human fetuin proteins are highly similar (61). We found that MMP-7 digestion fragments of human fetuin exhibited novel and unexpected cleavage sites that were different from the predicted cleavage sites for bovine fetuin (54). These variations between predicted and actual cleavages by MMP-7 indicate that small, species-dependent differences of fetuin structure could explain the discrepancies of the observed cleavage sites vs. the predicted cleavage sites.

We found that human fetuin inhibited mineralization in an *in-vitro* model of hydroxyapatite formation. The inhibitory effect of fetuin on this mineralization assay was reduced after digestion by MMP-7 but was not substantially altered by MMP-3 under the digestion conditions used here. Furthermore, it would appear that the fetuin fragment of ~18 kDa is not as effective as the full-length molecule for the inhibition of mineralization. These data indicate that MMP-7 may be important for the control of biological mineralization *in vivo*.

We found that the inhibition of mineralization by fetuin seems to be reliant on an intact fetuin structure and not on sequestration of fetuin by MMP-7 and MMP-3. Under conditions in which fetuin was not substantially degraded by MMP-7 (20-min co-incubations), there was little effect on the ability of fetuin to regulate mineralization (data not shown). As fetuin is an abundant serum protein and evidently binds MMP-7 with high affinity, it may inhibit the catalytic

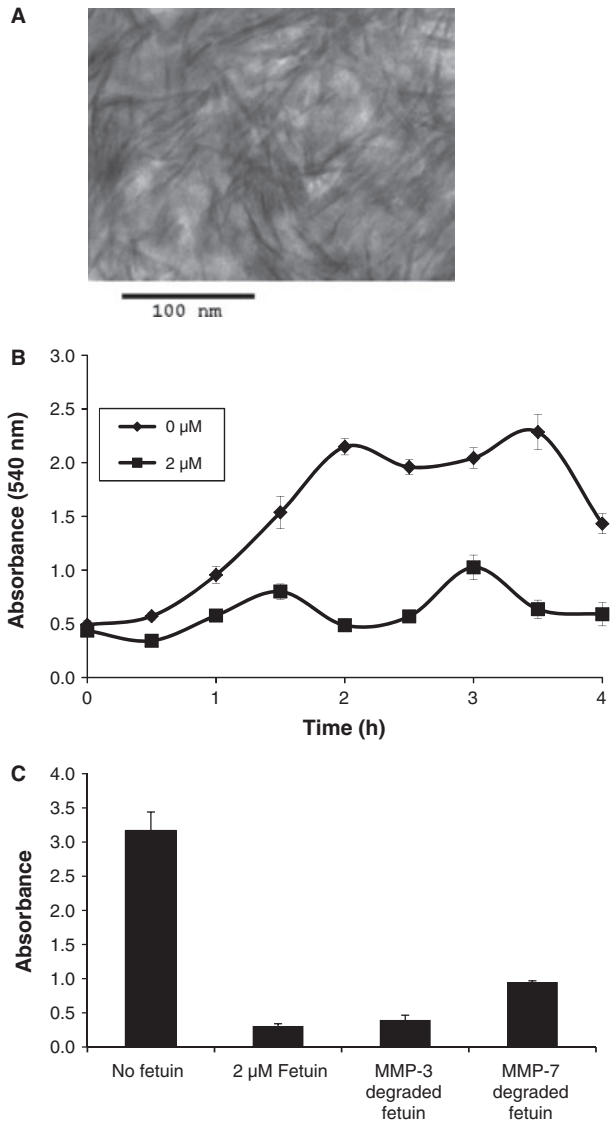

Fig. 5. (A) Transmission electron micrograph of a thin section of unstained hydroxyapatite crystals formed at the bottom of a culture dish. The scale bar indicates the magnification. (B) Measurements of Alizarin Red absorbance in hydroxyapatite mineralization assays over 0–4 h and with 0 or 2 μM fetuin, as indicated. Data are given as mean ± standard deviation of the absorbance at 540 nm. (C) Mineralization assays were conducted, as described in the Methods, with TNCB buffer (50 mM Tris, pH 7.5, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% BRIJ 35) containing no fetuin, 2 μM human fetuin, or 2 μM human fetuin that had been predigested with matrix metalloproteinase (MMP)-3 or MMP-7 for 24 h at an enzyme/substrate ratio of 1 : 60. Data are given as mean ± standard deviation of the absorbance at 540 nm.

Table 1. Electron diffraction analysis of hydroxyapatite mineralization

Hydroxyapatite: JCPDS d-spacing (Å)	Hydroxyapatite: JCPDS intensity (%)	Sample: calculated d-spacing (Å)	Sample: visual intensity (%)
3.44	40	3.39	40
3.08	19	3.03	20
2.81	100	2.76	100

Data were derived from electron diffraction analysis and were compared with published standards. Comparison of crystals formed *in vitro* with standards show close agreement, indicating that with the assay conditions used here, calcium hydroxyapatite crystals formed were authentic. Joint Committee on Powder Diffraction Standards (JCPDS).

activity of MMPs, analogous to tissue inhibitors of matrix metalloproteinases (62,63), but this possibility has not been examined here.

There is considerable interest in the potential association between inflammatory periodontal diseases and the development of cardiovascular diseases in general (64) and the formation of calcified atheromas in particular (8). However, apart from suggestions that periodontal pathogens may play a role in this association (65), little is known about other putative mechanisms, based on biochemical processes that could explain the statistical evidence for this relationship. Conceivably, linkages may exist between different inflammatory diseases and cardiovascular diseases (66), which could be mediated by reductions in the levels of intact fetuin in serum. In this context, we found a 50% reduction in the level of fetuin in patients with severe osteoarthritis (67). Although not investigated in patients with osteoarthritis, MMPs released into the bloodstream because of severe inflammatory joint disease may also contribute to fetuin degradation.

MMP-7 has been implicated in the pathogenesis of atheroma formation (68). If MMP-7 that is produced in periodontitis is important for the regulation of calcification of atheromas, then the concentration of MMP-7 in the blood would need to be increased. Notably, the concentration of MMP-9 is increased in the blood of patients with periodontitis (31), and the concentrations of MMP-3 and MMP-7 are increased in the serum of patients with gastric cancer (50). Therefore, to evaluate the potential biological impact of the degradation of fetuin by MMP-7 in periodontitis, it will be important to determine whether the concentrations of MMP-7 are increased in the blood of patients with periodontitis. We conclude that, based on the data described above, MMP-7 may be able to cleave serum fetuin, potentially increasing the risk of vascular calcification. If this notion is correct, MMP-7 levels in the blood may be instructive for assessing the risk of vascular calcification in inflammatory diseases, such as periodontitis.

The definition of such a mechanism, if demonstrated *in vivo*, could explain why there may be a relationship between inflammatory periodontal diseases and calcification in cardiovascular diseases.

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