Active matrix metalloproteases (MMPs) play a significant role in the pathogenesis of many diseases including osteoarthritis (OA), which involves progressive proteolytic degradation of cartilage. Clinical success of OA interventions that target MMPs has been limited by a lack of information about the presence and activity of specific disease-related proteases. We therefore developed a chemoproteomics approach based on MS to characterize the release and activity of MMPs in an in vitro model of the early inflammatory phase of posttraumatic OA (PTOA). We designed and synthesized chemical activity-based probes (ABPs) to identify active MMPs in bovine cartilage explants cultured for 30 days with the proinflammatory cytokine, interleukin-1α. Using these probes in an activity-based protein profiling-multidimensional identification technology (ABPP-MudPIT) approach, we identified active MMP-1, -2, -3, -7, -9, -12, and -13 in the medium after 10 days of culture, the time at which irreversible proteolysis of the collagen network in the explant was detected using proteolytic activation of FRET-quenched MMP substrates. Total MMP levels were quantified by shotgun proteomics, which, taken with ABPP-MudPIT data, indicated the presence of predominantly inactive MMPs in the culture medium. The selectivity of the ABPP-MudPIT approach was further validated by detection of specific endogenous MMPs activated de novo with 4-aminophenylmercuric acetate. The utility of the new ABPP-MudPIT approach for detecting molecular biomarkers of PTOA disease initiation and potential targets for therapeutics motivates possible application in other diseases involving MMP activity.

Osteoarthritis (OA)² is a common disease that develops with age or after traumatic injury and involves a debilitating, progressive destruction of joint tissues. Matrix-degrading enzymes produced by chondrocytes and synovial fibroblasts mediate cartilage matrix degradation in OA (1–3). Recent efforts to develop new OA therapies have focused on modulation of the activation of one such class of enzymes, the matrix metalloproteinases (MMPs) (4–6). MMPs are zinc-dependent endopeptidases, and their activity, activation, and localization are tightly regulated by interactions with other proteins, proteoglycan core proteins, and other matrix molecules (7). MMP activity is integral to normal extracellular matrix (ECM) remodeling processes such as the cartilage-to-bone transition during ossification, wound healing, and embryonic development, whereas dysregulation of MMP activity has been implicated in many pathophysiological processes including rheumatoid arthritis, periodontitis, and tumor cell invasion and metastasis (8). Thus, OA therapeutics would need to inhibit pathological MMP activity while preserving activity required for healthy tissue remodeling. Unfortunately, the clinical efficacy of protease modulation therapies has been disappointing across various disease states (9), primarily due to the highly complex and recursive nature of the MMP protease network and its reciprocal regulation by intracellular kinase signaling networks (10, 11). Computational systems biology approaches have begun to reveal how inhibition of one pathway in the complex MMP network can enhance other pathways in nonintuitive and deleterious ways and have provided some insights into the clinical failure of MMP inhibitors. These approaches have also motivated development of new experimental tools to measure active MMPs in a multiplex fashion to reveal network activity and yield a more complete understanding of both the mechanism and function of the specific MMPs that are active during disease progression.

In the last few decades, researchers have applied an enormous diversity of molecular, biochemical, cell biological, pro-

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2 The abbreviations used are: OA, osteoarthritis; MMP, matrix metalloproteinase; GAG, glycosaminoglycan; ABPP-MudPIT, activity-based protein profiling-multidimensional protein identification technology; ABP, activity-based probe; PTOA, post-traumatic osteoarthritis; TIMP, tissue inhibitor of metalloproteinase; ECM, extracellular matrix; APMA, 4-aminophenyl mercuric acetate; IAA, iodoacetamide; IL, interleukin; TCEP, tris(2-carboxyethyl)phosphine hydrochloride.
teomic, and high-throughput technologies to characterize MMP activity (12–14). However, extensive protease posttranslational modification and the presence of endogenous inhibitors have made unbiased robust characterization of active proteases challenging, especially in the context of complicated disease models. For example, zymography, a common technique to monitor active MMPs, fails to account for inhibition by tissue inhibitors of metalloproteases (TIMPs), and cannot readily detect MMPs that do not cleave known matrix proteins (15, 16). Fluorescence-based monitoring of synthetic polypeptide protease substrate cleavage has been used extensively to monitor real-time average MMP activity, but the potential of target peptides to be cleaved by multiple closely related proteases has limited the use of this technique to characterize specific active MMPs beyond well-controlled in vitro systems (17–20).

Recently, the Cravatt group (21, 22) addressed these limitations by developing activity-based probes (ABPs), tight-binding protease inhibitors designed to be photocross-linked specifically to active metalloproteases. These probes include a click chemistry handle for pulldown of active proteases from complex mixtures, which are then identified by traditional MS (21, 22). In a related advancement, immobilized reversible inhibitors have been used as affinity ligands for selective retention and detection of active MMPs (23). Despite the promise of ABP techniques for active protease detection, their limited sensitivity for detecting low concentrations of labeled active proteases has been a barrier to broad implementation.

Here, we describe how modifications of ABP chemical structures, sample preparation methods, and detection methods together provide sufficient improvements in detection limits such that ABPs can be deployed to illuminate protease activity changes in biological samples. Specifically, we demonstrate the application of a functional proteomic approach based on MS–activity-based protein profiling-multidimensional identification technology (ABPP-MudPIT), to characterize active MMPs in an in vitro model of early-stage cartilage degradation in post-traumatic osteoarthritis (PTOA). Early after joint injury, an up-regulation of inflammatory cytokines in the synovial fluid promotes cartilage degradation (24, 25). Our in vitro model consists of cultured bovine cartilage explants treated with the pro-inflammatory factor IL-1α; the culture medium is a surrogate for the synovial fluid aspirates obtained clinically from patients with PTOA. We designed and synthesized next generation ABPs coupled to a pulldown handle for affinity capture of specific active MMPs and incorporated a cleavable linker to decrease probe selectivity. We optimized tissue sample preparation and analysis with the ABPP-MudPIT approach to quantify specific active MMPs in pooled conditioned culture medium with sufficient sensitivity. We also conducted an extensive validation of our ABPs for measuring active metalloproteases in the PTOA model. Our findings suggest that the ABPP-MudPIT approach could be adapted for active protease measurement in other complex disease models. Furthermore, our analysis of MMP activities in a model of PTOA may lead to the development of synovial fluid biomarkers for earlier clinical diagnosis and more effective treatments to slow tissue degradation in PTOA.

Results

Synthesis and validation of cleavable ABPs

Due to large variations in MMP active site structure and universal applicability, we synthesized three distinct ABPs (21, 22, 26) (supporting Schemes I–III). All three probes contain active-site targeting warheads based on the potent MMP inhibitor, marimastat, and were previously found to bind MMPs (21, 22). Probes with warheads similar to that of Probe 1 in this study have been shown to broadly target MMPs (21, 26), and previous studies suggest that Probes 2 and 3 likely target a wide variety of metalloproteases including ADAMTS family members (Probe 3) (22). To promote efficient enrichment and identification of active MMPs, we incorporated a selectively cleavable linker into each ABPs, consisting of a unique disulfide bridge protected by vicinal dimethyl groups (27). To validate the utility of the linkers, we monitored the stability of the three ABPs at various dithiothreitol (DTT) concentrations, temperatures, and levels of UV irradiation, each representing reductive conditions employed during labeling of active MMPs and avidin bead capture of tagged target molecules during the ABPP-MudPIT protocol. A time course characterization of the solution phase probes via LC/MS confirmed the stability of all three probes under all tested conditions. Furthermore, an optimized cleavage condition, 10 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) in phosphate buffer (pH 7.7), 0.5% diethanolamine, and 20% methanol at 37 °C, was found to be optimal for release of captured proteins from avidin beads. We further validated the linker modification in Probe 1 (Fig. 1A), which has the most well-established warhead (21). Probe 1 was incubated with human endometriotic epithelial (12Z) cell lysate (10 μg) spiked with various concentrations of MMP-2, and then photocross-linked to the bound proteins. Dose-dependent ABP labeling of recombinant MMP-2 was observed by biotin immunoblot, suggesting that the probe linker modification did not interfere with active protein labeling by the ABP, and was effective in the presence of complex background proteins in the cell lysates (Fig. 1B). An additional study was performed to check the sensitivity limits and selective release of active proteases from solid support. In these screens, active MMP-2 from 30 ng of recombinant protein was confidently identified in the background of cell lysate using ABPP-MudPIT (Fig. 1B). The detection sensitivity in biological samples is expected to be even greater as, in this experimental system, activity of the commercially available MMP-2 catalytic domain is known to be very low, and TIMPs present in the cell lysate (28) are expected to further decrease the effective concentration of active MMP-2.

Monitoring average MMP activity in IL-1α–treated bovine cartilage explant culture medium

Bovine calf cartilage explants (Fig. 2A) were cultured for 30 days in the presence or absence of IL-1α (10 ng/ml) (29). Conditioned medium was collected every 2–3 days and pooled into three 10-day averaged samples, concentrated, and normalized to total protein (Fig. 2A). Consistent with earlier reports of MMP activity (30), and supporting the histology data (Fig. 2B), all untreated control samples and the day 10 IL-1α–treated
sample (pooled medium from days 1 to 10) showed minimal MMP activity, whereas days 20 and 30 IL-1α-treated samples (pooled medium from days 11–20 and 21–30, respectively) showed robust MMP activity, measured as proteolytic activation of commercially-available quenched-FRET MMP substrates (17, 18) (Fig. 2C). The specific peptide probe chosen for this screen was efficiently cleaved by a variety of MMPs, especially MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-12, and MMP-13, and reliably represents average MMP activity in a given sample (18). Protease activity was suppressed by the addition of a mixture of the three newly designed ABPs (Fig. 2C), demonstrating their potent inhibitory efficiency via MMP active site binding.

To visualize the labeling pattern of ABPs, the explant culture medium samples were incubated with ABPs, photocross-linked, separated on SDS-PAGE, and transferred to polyvinylidene difluoride membrane, where biotin-labeled proteins were visualized using streptavidin-horseradish peroxidase (Fig. 2D). As expected, greater protease activity correlated with greater ABP-mediated biotin labeling of active proteases (22). Biotin-labeled proteins in control samples were below the detection limit, indicating minimal association of the ABPs with metalloprotease active sites, whereas the IL-1α–treated explant culture medium contained an increasing amount of biotin-labeled active proteases over time. Notably, active protease concentrations detected by biotin immunoblot correlated with the protease activity detected in the quenched-FRET substrate cleavage assay (Fig. 2C).

Using ABPP-MudPIT to identify specific active MMPs in IL-1α–treated bovine cartilage explant culture medium

We used our new ABPs in an ABPP-MudPIT approach to identify specific active proteases within the treated and untreated pooled explant medium samples. Concentrated samples were first covalently labeled with the ABPs; after pulldown with avidin beads and TCEP-mediated cleavage, the released proteases were digested and tryptic peptides were subjected to multidimensional LC-tandem MS (LC-MS/MS) analysis using spectral counting. ABPP-MudPIT detection identified active metalloproteases in all three of the IL-1α–treated samples but not in untreated samples (Fig. 3, A and B). MMP-3 was the only active enzyme identified in the day 10 IL-1α–treated sample. Day 20 and 30 IL-1α–treated samples contained many more active MMPs including MMP-1, MMP-2, MMP-3, MMP-9, and MMP-12. MMP-13 was also detected in day 20 IL-1α–treated samples.

We confirmed ABPP-MudPIT identification of active MMP-2 by immunoblot. After ABP labeling of day 20 untreated and day 20 IL-1α–treated pooled medium, pulled down proteins were cleaved from the agarose beads and detected with an MMP-2 antibody. Consistent with the ABPP-MudPIT analysis, MMP-2 was observed only in the IL-1α–treated samples (Fig. S3, lane 2 versus lane 1). The enrichment of the lower molecular weight form of MMP-2 by ABP pulldown, rather than the higher molecular weight form of MMP-2 prominent in the unprocessed IL-1α–treated sample (Fig. S3, lane 2 versus lane 3), verified that the ABPs targeted the active form of MMP-2. The enrichment of MMP-2 from the explant sample demonstrates the feasibility of the ABPP-MudPIT-labeled protein analysis approach to identify specific proteases in complex samples, but also introduce the potential for using ABP labeling and selective release to recover whole labeled proteins for additional further characterization, such as glycosylation patterns and other post-translational processing that may affect apparent activity.

Quantifying total secreted MMPs in bovine cartilage explant culture medium with shotgun proteomics

We next performed a shotgun proteomic analysis based on LC-MS/MS for global characterization of the proteases present...
in the explant culture medium. Proteases were first digested and then separated by off-gel electrophoresis. The pre-separated fractions were run on LC-MS/MS to determine the total amount of MMPs in the medium. Comparing spectral counts for each MMP in the IL-1α–treated and untreated culture medium samples, we found that total MMP-1, MMP-3, MMP-9, MMP-12, and MMP-13 released into explant culture medium was higher in IL-1α–treated samples, with MMP-3 and MMP-9 demonstrating marked decreases and increases, respectively, during the 30-day culture period (Fig. 4). In contrast, MMP-2 total protein levels remained relatively constant across the untreated and IL-1α–treated samples.
ABBP-MudPIT detects chemically activated proteases

Comparing active protease profiles from ABPP-MudPIT with the total protease profiles quantified by shotgun proteomics suggested that a significant fraction of the total secreted proteases is inactive, either as zymogens or endogenously inhibited by TIMPs. This excess of inactive proteases presented an opportunity to definitively validate the specificity and sensitivity of the modified ABPP-MudPIT approach to detect active metalloproteases. We incubated the untreated and IL-1\textsuperscript{β}/H9251–treated culture medium samples with the chemical activator 4-aminophenyl mercuric acetate (APMA), along with our multifunctional ABPs (31). APMA activation disrupts the interaction between the MMP propeptide domain and the active site zinc atom, allowing autolytic cleavage activation of the enzyme (32). Chemical activation of MMPs with APMA, followed by profiling of specific MMP activity using ABPP-MudPIT, detected all MMPs in both untreated and IL-1\textsubscript{β}–treated culture medium samples (Fig. 5 and Fig. S2). In samples without chemical activation, the ABPP-MudPIT approach detected active MMPs only in samples collected later in the treatment period (Fig. 3B). Consider specifically ABPP-MudPIT detection of MMP-1, MMP-2, MMP-3, MMP-9, MMP-12, and MMP-13 activity in IL-1\textsubscript{β}–treated day 20 samples. Without chemical activation, this sample exhibited a low concentration of active MMPs (except for MMP-1 and 13) (Figs. 3B and 5 and Fig. S2), but a high concentration of zymogens (Figs. 4 and 5), indicating that most of the proteases in the sample are inactive. After APMA activation, ABPP-MudPIT detected robust MMP-2 and MMP-3 activity, thus verifying the ability of our ABPs to detect specific MMP activity in complex samples and supporting the minimal background inherent in active protease detection using ABPP-MudPIT probes with cleavable linkers. The observation that the spectral count of total protein (from shotgun proteomics) does not match MMP activity measurements after APMA activation reflects the dynamic and complex environment of active MMPs and MMPs associated with endogenous TIMPs (33).

**Discussion**

Previously developed ABPs have generally included both a targeting moiety (known as a warhead, allowing specific ABP binding to the active target protein), and an affinity handle (allowing selective identification or enrichment of the labeled species). This work introduces three ABPs designed based on the literature (21, 22). However, each ABP incorporates an additional feature, an optimized, selectively cleavable linker, which has a unique disulfide bridge protected by vicinal dimethyl groups (27). Previous studies of ABP recombinant protease labeling were limited to confirming that the newly incorporated bulky dimethyl protected disulfide and PEG linker does not dramatically hinder probe binding (Fig. 1B). Similar reducible disulfide-containing linkers have been previously used in conjunction with biotin for target purification; however, without additional stabilization due to their labile nature, particularly with respect to the potential for sulfur scrambling with free cysteines, these reagents are far too reactive for use in quantitative proteomics applications. The vicinal dimethyl groups introduced here stabilize the disulfide linker, making it inert to cross-reactivity with free cysteine thiols and even DTT (34), whereas allowing targeted reduction-mediated release from avidin capture beads. The linker allows a second significant advancement. Established
ABPP-MudPIT approaches that require on-bead tryptic digest prior to MS (21, 22) introduce additional background signal from tryptic peptides of proteins adsorbed nonspecifically to agarose capture beads. ABPP-MudPIT probes with protected disulfide-mediated cleavage were expected to limit background signal and increase overall sensitivity by allowing selective release of bead-captured ABP-labeled proteins prior to tryptic digest.

Integrating a quenched-FRET substrate-based screen of active protease inhibition within the workflow of the ABPP-MudPIT technology is an additional important system optimization introduced in this work. These complementary screens allow a baseline approximation for identifying an appropriate probe concentration that limits nonspecific protein labeling due to excess probe, whereas promoting unbiased and complete labeling of all metalloproteases in a given sample. For the ABPP-MudPIT sample preparation, the concentration of incubating probes was chosen as the concentration needed to fully inhibit quenched substrate cleavage by the most active sample. Truly quantitative results would require further validation by titrated ABPP-MudPIT labeling studies of active and inactive proteases, as protease inhibition does not necessarily guarantee protease labeling or capture.

ABPP-MudPIT approaches extend the information gained from the quenched substrate cleavage assay by allowing rapid identification of specific ABP-labeled active proteases in a complex mixture. ABPP-MudPIT identified active MMPs in IL-1α-treated day 20 and 30 explant culture medium samples (Fig. 3, A and B), but not in the untreated control medium. This profile of specific MMP activity matches the average MMP activities suggested by earlier published literature (30) and quenched FRET-based assessments (Fig. 2C). Similarly, the relative total MMP protein abundance across samples as suggested by shotgun spectral counts generally confirms previous reports of increased MMP and ADAMTS expression and production in response to cytokine treatment and injurious compression (35, 36). The observed increase in the release of MMP-1, -2, -3, -9, -12, and -13 with IL-1α treatment compared with untreated controls (Fig. 4) is consistent with a previous report of increased MMP-1, -3, -9, and -13 gene expression 24 h after mechanical injury to bovine calf cartilage (35). Other explant model systems have also shown increased expression of MMP-1, -3, -8, -9, and -13 following IL-1β treatment (36, 37).

Comparing the total protein levels (Fig. 4) to active protein levels as identified by ABPP-MudPIT (Fig. 3, A and B) suggests

![Figure 5. Comparison of spectral counts of MMP-1, -2, -3, -9, -12, and -13 with ABPP-MudPIT and shotgun methods. Shown are APMA-activated MMPs in day 20 IL-1α-treated explant culture medium. The complex mixtures of proteases were treated with APMA along with ABPs and the activity of the MMPs was profiled using ABPP-MudPIT. The graph presents mean values of two biological replicates and standard error are plotted to add visualization of the data and comparison across conditions.](image-url)
the broader potential and clinical relevance of parallel, proteomic-mediated specific active and total protease quantification. Specific observations include that culture medium from IL-1α-treated samples contained comparable total MMP-1 and MMP-13 proteins on day 10 (pooled days 1–10) and day 30 (pooled days 21–30), but only active protease on day 30. Also, in treated culture medium samples, the level of MMP-3 protein decreased over time, whereas MMP-3 activity remained relatively constant. Finally, whereas similar levels of MMP-2 total secreted protein were observed in treated and untreated samples across the treatment period, active MMP-2 was only identified in IL-1α-treated culture medium on days 20 (pooled days 11–20) and 30. Taken together with the histology findings (Fig. 2B), such studies reveal the temporal and spatial complexities of inflammation-mediated cartilage degradation by MMPs.

We hypothesize that the low levels of total and active proteases observed in the untreated and IL-1α-treated explant culture medium on day 10 is the result of limited diffusion within the dense avascular ECM of cartilage. In a recent publication describing IL-1α treatment of similar bovine cartilage explants (25), the dominant process occurring during the first 10 days is the aggrecanase-mediated degradation and loss of the densely packed, space-filling aggrecan from the ECM (almost 80% loss by day 10). The same study showed that loss of collagen (presumably due to MMP-1 and/or MMP-13) does not start until most of the aggrecan is lost; a similar finding was previously reported in other cytokine-stimulated explant proteolysis studies (25, 38, 39). As we recently documented for age-matched normal bovine cartilage tissue (38), IL-1α treatment results in extensive proteolytic degradation of cartilage matrix. Eighty percent or more of the sulfated glycosaminoglycans (sGAT, predominantly chondroitin sulfate GAGs covalently attached to the core protein of the proteoglycan aggrecan) in cartilage are lost by 10–14 days of IL-1α treatment. In the present study, histological staining for sGAG revealed a similar dramatic loss of aggrecan-GAG (Fig. 2B); this aggrecan loss proceeded with increasing culture duration from the outer surfaces of the explant inward. The delay in the release of collagen (and other matrix macromolecules) is likely due, in part, to the densely packed space-filling nature of aggrecan within native cartilage tissue, such that transport of macromolecules out of the cartilage is delayed until the aggrecan is lost. Although the loss of matrix during explant culture may originate from proteolysis of pre-existing matrix or newly synthesized matrix, or even passive loss of matrix components from degraded tissue, matrix loss under IL-1α treatment conditions is predominantly due to proteolysis of pre-existing matrix associated with newly activated matrix proteases (25, 40).

In the present study, the observation that active MMPs (with the exception of MMP-3) did not appear in the medium until culture days 20 or 30 is consistent with the inhibition of macromolecule transport through the aggrecan-dense ECM. However, MMP-2 readily diffused from the explant regardless of cartilage degradation or activation state, and high concentrations of MMP-3 diffused from the treated samples as early as day 10. It is known that collagenases (e.g., MMP-13) can be activated by MMP-2, MMP-3, and MMP-9, and it has been speculated (39) that these MMPs (along with MMP-12) may be involved in proteolytic events that occur after aggrecanolysis and prior to collagenolysis. This is consistent with their appearance earlier than MMP-1 and MMP-13 in the present study. Although previous literature has identified gene expression of MMP-12 in cartilage-bone samples, to our knowledge, this is the first observation of active MMP-12 protein in cytokine-stimulated intact cartilage tissue. Although beyond the scope of this work, extending the current ABPP-MudPIT approach to characterize the temporal and spatial changes in active proteases within the cultured explant is expected to enable a more complete understanding of the mechanism of aberrant protease activation and define strategies for improved clinical interventions.

Of particular note, only a few aggrecanases (ADAMTS-4) were identified in the medium of IL-1α–treated explants in the present study (Fig. 4) and no ADAMTS proteases were detected by ABPP-MudPIT. This result was expected as it has been shown in our previous studies (25) and those of others (34) that aggrecanase-mediated aggrecan degradation is an early event during cytokine stimulation of bovine cartilage ex vivo, and high levels of ADAMTS-4 have been identified in the bovine cartilage model (41). Difficulties identifying ADAMTS may be attributed to their likely association with cells and matrix inside the explants (40) or their overall low concentration (42). Active aggrecanase detection may also be hindered by suboptical probe design. It has been shown that the similar kind of warhead as Probe 3 can target ADAMTS-4 (22) although targeting of ADAMTS-5 was not demonstrated. However, as even ADAMTS-4 was not detected there is a possibility that the chemical modification on these multifunctional probes may hinder the reactivity toward these proteases or new ABPs may need to be designed that better detect ADAMTS family members using ABPP-MudPIT.

In conclusion, we have developed new multifunctional photoaffinity ABPs with cleavable linkers that are able to selectively enrich and pulldown active MMPs in an in vitro bovine explant model of PTOA. Selective pulldown and release of these MMPs minimizes background and enhances active protease detection. Use of these probes with the ABPP-MudPIT approach enabled detection of active MMPs in IL-1α–treated explant culture medium samples, but not in untreated control samples. Shotgun proteomics suggested that a large proportion of the total MMPs in all samples are inactive. The specificity of ABPP-MudPIT approach to detect active MMPs was confirmed with chemical activation of MMPs. Taken together, using the ABPP-MudPIT approach, we revealed a time-resolved profile of active MMPs release from cartilage in a clinically relevant disease model of early-phase inflammation-induced cartilage degradation. This approach may lead to the identification of critically important molecular biomarkers of PTOA disease initiation and potential therapeutic targets.

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3 K. C. Ravindra, C. C. Ahrens, Y. Wang, J. Y. Ramseier, J. S. Wishnok, L. G. Griffith, A. J. Grodzinsky, and S. R. Tannenbaum, unpublished data.

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Profiling MMP activity in an in vitro model of osteoarthritis
Profiling MMP activity in an in vitro model of osteoarthritis

Experimental procedures

Bovine cartilage harvest and culture

Cartilage disks were harvested from the femoropatellar grooves of 1- to 2-week-old calves (obtained from Research'87, Boylston, MA). Briefly, full thickness cartilage cylinders were cored perpendicular to the surface using a 6-mm thermal punch. After a level surface was obtained by removing the most superficial layer (~500–700 μm), two sequential 1-mm slices of middle zone cartilage were cut from each cylinder (~30–35 mg each). The disks were equilibrated in serum-free medium (low-glucose Dulbecco’s modified Eagle’s medium; 1 g/liter) supplemented with 10 mM HEPES buffer, 0.1 mM nonessential amino acids, 0.4 mM proline, 20 μg/ml of ascorbic acid, 100 units/ml of penicillin G, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B for 2 days (5% CO2; 37 °C). After equilibration, cartilage explants were untreated (control) or treated with the 10 ng/ml of IL-1α (R&D Systems). For both treatment conditions, each cartilage explant was cultured in a single well with 1 ml of medium, and medium from 4 wells was collected every 2–3 days and combined (i.e. 4 ml of conditioned medium collected). The collected medium was pooled into day 10 (days 1–10), day 20 (days 11–20), and day 30 (days 21–30) time points for analysis, resulting in samples of ~16 ml of total medium per time point for each treatment group.

Histology

Bovine explant samples from the various treatments were fixed in 4% formaldehyde (Sigma). Samples were paraffin-embedded, sectioned (5 μm thickness), and stained with 0.1% toluidine blue in deionized water to visualize GAG retained in the cartilage.

Culture medium sample preparation

The medium samples were pooled into 3 time points: day 10 (medium collected between start of experiment to day 10), day 20 (medium from day 11 to 20), and day 30 (medium from day 21 to 30). The 16 ml of pooled medium for the 3 time points, treated or untreated, was concentrated to 1 ml using pooled medium samples were aliquoted for each planned experiment to analysis. For activity-based labeling, the samples were processed for mass spectrometry analysis.

Quenched-FRET MMP substrate assay

Reactions for quenched-FRET substrate assays were composed of a 1:1 mixture of pooled explant culture medium containing 10 μg/ml of total protein and 5 μM quenched-FRET substrate (Dabcyl-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(5-FAM)-NH2) (Biozyme Inc., Apex, NC) diluted from 5 mM DMSO stock into PBS. For FRET–substrate assays run with a mixture of the three ABPs, reactions contained a 2:1:1 mixture of pooled explant culture medium (10 μg/ml of total protein), 0.2 μM ABP mixture (0.066 μM of each ABP diluted from 10 mM stock into Tris buffer), and 5 μM FRET-quenched substrate. Experiments were performed at 37 °C and fluorescence readings were measured every 20 min for 8 h. (The average change in fluorescence units with respect to time was observed for the linear range of the resulting fluorescence saturation curve.)

Pulldown protocol for MMPs

After labeling 100 μg of proteases with a mixture of three ABPs, the reaction mixture was exposed to UV irradiation for 5 min at 250 milliwatt/cm². The proteins were denatured by adding 20 μl of 2.5% (w/v) SDS to the reaction mixture, vortexing slightly, and then heating at 90 °C for 5 min. After cooling, the proteins were precipitated using the chloroform/methanol method. The precipitated protein pellets were air-dried for 5 min at room temperature. The protein pellet was then rehydrated in 100 μl of 8 M urea (8 M urea, 50 mM NH4HCO3 (ABC)) for 15–20 min at room temperature. After obtaining a clear solution, cysteine bridges were reduced by adding 10 μl of freshly prepared 38 mM DTT (DL-DTT) (in ABC) to the 100-μl sample and incubating this mixture for 30 min at 37 °C. Alkylation was carried out by adding 15 μl of 200 mM iodoacetamide (IAA) solution (freshly prepared in 50 mM ABC) to the 110-μl sample and incubating it for 1 h at room temperature in the dark. Chloroform/methanol precipitation was repeated to remove the excess DTT and IAA. The protein pellet was re-solubilized by stepwise dilution with 2.5% (w/v) SDS solution.

After solubilizing the protein, the SDS was further diluted to 0.1% with pulldown buffer (50 mM ABC, 150 mM NaCl) before adding to streptavidin-agarose beads. Prior to adding the protein mixture, the streptavidin column was prepared using pre-washed immobilized streptavidin-agarose beads (Thermo Scientific, Rockford, IL) packed into a column (dimensions inner diameter = 9 mm, outer diameter = 7 mm, height = 60 mm), with a final column volume of 2 ml. Pre-prepared protein samples were added directly to the streptavidin column and incubated overnight at 4 °C. To remove nonbiotinylated proteins, the column was washed with 10 column volumes of 50 mM ABC with 0.1% SDS, 5 column volumes of 50 mM ABC, and 5 column volumes of deionized water. Disulfides were selectively cleaved by treatment with 10 mM TCEP in phosphate buffer (pH 7.7), 0.5% diethanolamine, and 20% methanol at 37 °C for 3 h. Immediately after releasing the proteases from the beads, the pH was adjusted to 7.8. The cleaved proteins were trypsinized at 37 °C for 3 h with sequencing grade trypsin and surfactant Protease-MAX™ (Promega, Madison, WI). Peptides were desalted with OMIN C18 pipette tips (Agilent Technologies, Chicago, IL) and processed for mass spectrometry analysis.

Immunoblotting

Ten μg of total protein was treated with 500 nM of the probe mixture and incubated at 37 °C for 30 min. After incubation, the samples were UV-irradiated for 5 min on ice. Immediately after irradiation, 5× loading dye was added to the mixtures, followed by boiling for 5 min at 90 °C. The denatured samples were run on TGX™-SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes. The membranes were
blocked with 5% BSA in 1× TBS containing Tween 20 (TBST, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, supplemented with 0.2% Tween 20) for ~1 h at room temperature, followed by washing with TBST, 3 × 5 ml. The membrane was then incubated with streptavidin-horseradish peroxidase antibody at room temperature for 1 h before washing with TBST. Biotinylated MMPS were visualized with an ECL detection system (Enhanced Chemiluminescence, GE Healthcare). Initial validation of modified probe activity was conducted using lysates of the 12-Z endometriotic epithelial cell line, obtained as a gift from Steve Palmer (EMD-Serono) with permission from the originator, Anna Starzinski-Powitz (43). Cells were cultured and lysed as previously described (10).

**Nano-Chip LC-MS/MS**

Analysis of tryptic peptides of enriched endogenous active MMPS was performed using a Q-TOF 6510 instrument (Agilent Technologies) equipped with a nano-LC-chip cube. The HPLC system consisted of a nano flow analytical pump and a capillary loading pump (Agilent 1200 series). The solvents were double-distilled water (buffer A) and acetonitrile (buffer B) with 0.1% formic acid. Parameters for the analytical (nano) pump were at a flow rate of 400 nl/min, and elution gradient as follows: 1% buffer B at 0 min, 1% buffer B at 1 min, 45% buffer B at 101 min, 75% buffer B at 109 min, 98% buffer B at 116 min, 1% buffer B at 120 min, and 10 min post-time. Parameters for loading the capillary pump were as follows: 2% acetonitrile, 98% HPLC grade water, 0.1% formic acid. The loading pump was operated in isocratic mode. The QTOF instrument was externally calibrated. Peptides were enriched and separated via nano-LC (0.150 × 75 mm, packed with Zorbax 300SB-C18, 5-mm material, 300-Å pore size) integrated in the HPLC Chip (G4240-62001). A full scan was acquired over a range of m/z 100–2500 at six spectra/s, and MS/MS scans were acquired over m/z 50–2500 at three spectra/s with a maximum of five precursors per cycle. Fragmentation energy was applied at a slope of 3.0 volts/100 Da with a 2.8 offset.

Protein expression values (spectrum counts) were calculated in Scaffold software with the imported peptide hits from Spectrum Mill. The threshold for including a protein was a minimum of two distinct peptides with 95% confidence.

**References**

1. Okada, Y., Shinmei, M., Tanaka, O., Naka, K., Kimura, A., Nakanishi, I., Bayliss, M. T., Iwata, K., and Nagase, H. (1992) Localization of matrix metalloproteinase 3 (stromelysin) in osteoarthritic cartilage and synovium. *Lab. Invest.* 66, 680–690 Medline
2. Chin, J. R., Murphy, G., and Werb, Z. (1985) Stromelysin, a connective tissue-degrading metalloendopeptidase secreted by stimulated rabbit synovial fibroblasts in parallel with collagenase: biosynthesis, isolation, characterization, and substrates. *J. Biol. Chem.* 260, 12367–12376 Medline
3. MacNauls, K. L., Chartrain, N., Lark, M., Tocci, M. J., and Hutchinson, N. L. (1990) Discoordinate expression of stromelysin, collagenase, and tissue inhibitor of metalloproteinases-1 in rheumatoid human synovial fibroblasts. *J. Biol. Chem.* 265, 17238–17245 Medline
4. Treeberg, L., and Nagase, H. (2012) Proteases involved in cartilage matrix degradation in osteoarthritis. *Biochim. Biophys. Acta* 1824, 133–145 CrossRef
5. Vincenti, M. P., Clark, I. M., and Brinckerhoff, C. E. (1994) Using inhibitors of metalloproteinases to treat arthritis: easier said than done? *Arthritis Rheum.* 37, 1115–1126 CrossRef Medline
6. Levin, J. I. (2004) The design and synthesis of aryl hydroxamic acid inhibitors of MMPS and TACE. *Curr. Top. Med. Chem.* 4, 1289–1310 CrossRef Medline

*J. Biol. Chem.* (2018) 293(29) 11459–11469
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7. Hadler-Olsen, E., Fadnes, B., Syte, I., Uhlin-Hansen, L., and Winberg, J.-O. (2011) Regulation of matrix metalloproteinase activity in health and disease. FEBS J. 278, 28–45 CrossRef MedLine

8. Woessner, J. F. (1991) Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J. 5, 2145–2154 CrossRef MedLine

9. Vandenbroucke, R. E., and Libert, C. (2014) Is there now hope for therapeutically matrix metalloproteinase inhibition? Nat. Rev. Drug Discov. 13, 904–927 CrossRef MedLine

10. Miller, M. A., Meyer, A. S., Beste, M. T., Lasisi, Z., Reddy, S., Jeng, K. W., Chen, H., Lee, T., Madsen, M. C., Le, H., Levine, A. L., and Buckwalter, J. A. (2011) Post-traumatic osteoarthritis: improved understanding and opportunities for early intervention. J. Orthop. Res. 29, 802–809 CrossRef MedLine

11. Miller, M. A., Oudin, M. J., Sullivan, R. J., Wang, S. J., Meyer, A. S., Im, H., Frederick, D. T., Tadros, J., Griffith, L. G., Lee, H., Weissleder, R., Flaherty, K. T., Gertler, F. B., and Lauffenburger, D. A. (2016) Reduced proteolytic shedding of receptor tyrosine kinases is a post-translational mechanism of kinase inhibitor resistance. Cancer Discov. 6, 382–399 CrossRef MedLine

12. Cravatt, B. F., Wright, A. T., and Kozaarich, J. W. (2008) Activity-based protein profiling: from enzyme chemistry to proteomic chemistry. Annu. Rev. Biochem. 77, 383–414 CrossRef MedLine

13. Niphakis, M. J., and Cravatt, B. F. (2014) Enzyme inhibitor discovery by activity-based protein profiling. Annu. Rev. Biochem. 83, 341–377 CrossRef MedLine

14. Berger, A. B., Vitorino, P. M., and Bogov, M. (2004) Activity-based protein profiling. Am. J. Pharmacogenomics. 4, 371–381 CrossRef MedLine

15. Mungal, B. A., and Pollitt, C. C. (2001) In situ zymography: topographical considerations. J. Biomed. Biophys. Methods 47, 169–176 CrossRef MedLine

16. Yan, S. J., and Blomme, E. A. (2003) In situ zymography: a molecular pathology technique to localize endogenous protease activity in tissue sections. Vet. Pathol. 40, 227–236 CrossRef MedLine

17. Moghissi, S. K., and Rasmussen, F. H. (2007) Fluorescent substrates for the proteasinas ADAM17, ADAM10, ADAM8, and ADAM12 useful for high-throughput inhibitor screening. Anal. Biochem. 366, 144–148 CrossRef MedLine

18. Miller, M. A., Barkal, L., Jeng, K., Herrlich, A., Moss, M., Griffith, L. G., and Lauffenburger, D. (2011) A proteolytic activity matrix analysis (PrAMA) for simultaneous determination of multiple protease activities. Integr. Biol. (Camb), 3, 422–438 CrossRef MedLine

19. Nagase, H., and Fields, G. B. (1996) Human matrix metalloproteinase specificity studies using collagen sequence-based synthetic peptides. Biopolymers 40, 399–416 CrossRef MedLine

20. Caes, C. J., Jeschke, G. R., and Turk, B. E. (2010) Active site determinants of substrate recognition by the metalloproteinase TACE and ADAM10. Biochem. J. 424, 79–88 MedLine

21. Saghatelian, A., Jessani, N., Joseph, A., Humphrey, M., and Cravatt, B. F. (2004) Activity-based probes for the proteomic profiling of metalloproteases. Proc. Natl. Acad. Sci. U.S.A. 101, 10000–10005 CrossRef MedLine

22. Sieber, S. A., Niessen, S., Hoover, H. S., and Cravatt, B. F. (2006) Proteomic profiling of metalloproteinase activities with cocktails of active-site probes. Nat. Chem. Biol. 2, 274–281 CrossRef MedLine

23. Freije, R. J., and Bischoff, R. (2003) Activity-based enrichment of matrix metalloproteinases using reversible inhibitors as affinity ligands. J. Chromatogr. A1009, 159–169 CrossRef MedLine

24. Anderson, D. D., Chubinskaya, S., Guiñak, F., Martin, J. A., Oegema, T. R., Olson, S. A., and Buckwalter, J. A. (2011) Post-traumatic osteoarthritis: improved understanding and opportunities for early intervention. J. Orthop. Res. 29, 802–809 CrossRef MedLine

25. Li, Y., Wang, Y., Chubinskaya, S., Schoerbel, B., Florine, E., Kopesky, P., and Grodzinsky, A. J. (2015) Effects of insulin-like growth factor-1 and dexamethasone on cytokine-challenged cartilage: relevance to post-traumatic osteoarthritis. Osteoarthritis Cartilage 23, 266–274 CrossRef MedLine

26. Levy, D. E., Lapierre, F., Liang, W., Ye, W., Lange, C. W., Li, X., Grobelny, D., Casabonne, M., Tyrrell, D., Holme, K., Nadzan, A., and Galardy, R. E. (1998) Matrix metalloproteinase inhibitors: a structure activity study. J. Med. Chem. 41, 199–223 CrossRef MedLine

27. Gartner, C. A., Elias, J. E., Bakalarski, C. E., and Gygi, S. P. (2007) Catch-and-release reagents for broadscale quantitative proteomics analyses. J. Proteome Res. 6, 1482–1491 CrossRef MedLine

28. Chen, C.-H., Miller, M. A., Sarkar, A., Best, M. T., Isaacson, K. B., Lauffenburger Da Griffith, L. G., and Han, J. (2013) Multiplexed protease activity assay for low-volume clinical samples using droplet-based microfluidics and its application to endometriosis. J. Am. Chem. Soc. 135, 1645–1648 CrossRef MedLine

29. Stevens, A. L., Wishnok, J. S., White, F. M., Grodzinsky, A. J., and Tannenbaum, S. R. (2009) Mechanical injury and cytokines cause loss of cartilage integrity and upregulate proteins associated with catabolism, immunity, inflammation, and repair. Mol. Cell. Proteomics 8, 1475–1489 CrossRef MedLine

30. Dickinson, S. C., Vankemmelbeke, M. N., Buttle, D. J., Rosenberg, K., Heinegård, D., and Hollander, A. P. (2003) Cleavage of cartilage oligomeric matrix protein (thrombospondin-5) by matrix metalloproteinases and a disintegrin and metalloproteinase with thrombospondin motifs. Matrix Biol. 22, 267–278 CrossRef MedLine

31. Keow, J. Y., Pond, E. D., Gisar, J. S., Cravatt, B. F., and Crawford, B. D. (2012) Activity-based labeling of matrix metalloproteinases in living vertebrate embryos. PLoS ONE 10.1371/journal.pone.0043434

32. Springman, E. B., Angleton, E. L., Birkedal-Hansen, H., and Van Wart, H. E. (1990) Multiple modes of activation of latent human fibroblast collagenase: evidence for the role of a Cyst73 active-site zinc complex in latency and a “cysteine switch” mechanism for activation. Proc. Natl. Acad. Sci. U.S.A. 87, 364–368 CrossRef MedLine

33. Olson, M. W., Gervasi, D. C., Mobashery, S., and Fridman, R. (1997) Kinetic analysis of the binding of human matrix metalloproteinase-2 and -9 to tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2. J. Biol. Chem. 272, 29975–29983 CrossRef MedLine

34. Kellogg, B. A., Garrett, L. K., Anderton, A. J., Cawston, T. G., and Brown, A. (2001) Shed factor VIII is a potent activator of ADAM17/TEM8, a shed factor XI inhibitor. J. Biochem. Biophys. Methods 42, 266–274 CrossRef MedLine

35. Keow, J. Y., Pond, E. D., Gisar, J. S., Cravatt, B. F., and Crawford, B. D. (2012) Activity-based labeling of matrix metalloproteinases in living vertebrate embryos. PLoS ONE 10.1371/journal.pone.0043434

36. Koshy, P. J., Lundy, C. J., Rowan, A. D., Porter, S., Edwards, D. R., Hogan, A., Clark, I. M., and Cawston, T. E. (2002) The modulation of matrix metalloproteinase and ADAM gene expression in human chondrocytes by interleukin-1 and oncostatin M: a time-course study using real-time quantitative reverse transcription-polymerase chain reaction. Arthritis Rheum. 46, 961–967 CrossRef MedLine

37. Dozin, B., Malpel, M., Camardella, L., Cances, R., and Pietrangelo, A. (2002) Response of young, aged and osteoarthritic human articular chondrocytes to inflammatory cytokines: molecular and cellular aspects. Matrix Biol. 21, 449–459 CrossRef MedLine

38. Pratta, M. A., Yao, W., Decicco, C., Tortorella, M. D., Liu, R. Q., Copeland, R. A., Magolda, R., Newton, R. C., Trzaskos, J. M., and Arner, E. C. (2003) Aggrecan proteolytic cartilage collagen from proteolytic cleavage. J. Biol. Chem. 278, 45539–45545 CrossRef MedLine

39. Nilsson, C., Belloni, D. A., and Bay-Jensen, A. C. (2013) The development and characterisation of a competitive ELISA for measuring active ADAMTS-4 in a bovine cartilage ex vivo model. Matrix Biol. 32, 143–151 CrossRef MedLine
42. Glasson, S. S., Askew, R., Sheppard, B., Carito, B., Blanchet, T., Ma, H.-L., Flannery, C. R., Peluso, D., Kanki, K., Yang, Z., Majumdar, M. K., and Morris, E. A. (2005) Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis. *Nature* **434**, 644–648 CrossRef Medline

43. Zeitvogel, A., Baumann, R., and Starzinski-Powitz, A. (2001) Identification of an invasive, N-cadherin-expressing epithelial cell type in endometriosis using a new cell culture model. *Am. J. Pathol.* **159**, 1839–1852 CrossRef Medline

44. Ravindra, K. C., Ho, W. E., Cheng, C., Godoy, L. C., Wishnok, J. S., Ong, C. N., Wong, W. S., Wogan, G. N., and Tannenbaum, S. R. (2015) Untargeted proteomics and systems-based mechanistic investigation of artesunate in human bronchial epithelial cells. *Chem. Res. Toxicol.* **28**, 1903–1913 CrossRef Medline