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In-vitro and *in-vivo* imaging of MMP activity in cartilage and joint injury

Tomoaki Fukui, Elizabeth Tenborg, Jasper H.N. Yik, Dominik R. Haudenschild*

Lawrence J. Ellison Musculoskeletal Research Center, Department of Orthopaedic Surgery, University of California Davis Medical Center, 4635 Second Avenue Suite 2000, Sacramento CA 95817, USA

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ABSTRACT

Non-destructive detection of cartilage-degrading activities represents an advance in osteoarthritis (OA) research, with implications in studies of OA pathogenesis, progression, and intervention strategies. Matrix metalloproteinases (MMPs) are principal cartilage degrading enzymes that contribute to OA pathogenesis. MMPSense750 is an in-vivo fluorimetric imaging probe with the potential to continuously and non-invasively trace real-time MMP activities, but its use in OA-related research has not been reported. Our objective is to detect and characterize the early degradation activities shortly after cartilage or joint injury with MMPSense750. We determined the appropriate concentration, assay time, and linear range using various concentrations of recombinant MMPs as standards. We then quantified MMP activity from cartilage explants subjected to either mechanical injury or inflammatory cytokine treatment in-vitro. Finally, we performed in-vivo MMP imaging of a mouse model of post-traumatic OA. Our in-vitro results showed that the optimal assay time was highly dependent on the MMP enzyme. In cartilage explant culture media, mechanical impact or cytokine treatment increased MMP activity. Injured knees of mice showed significantly higher fluorescent signal than uninjured knees. We conclude that MMPSense750 detects human MMP activities and can be used for in-vitro study with cartilage, as well as in-vivo studies of knee injury, and can offering real-time insight into the degradative processes that occurring within the joint before structural changes become evident radiographically.

1. Introduction

Osteoarthritis (OA) is a degenerative disease of the whole joint organ characterized by cartilage degradation [1], and the number of OA patients continues to increase, estimated at nearly 27 million in the United States [2] and there is no effective treatment to prevent OA or restore joints after the onset of OA. At this time, the gold standard for clinical OA diagnosis and evaluation are morphologic assessments, such as radiography [3–5], computed tomography (CT) [5,6], and magnetic resonance imaging (MRI) [3,5,6]. These imaging technologies primarily reveal the morphological changes that become evident at the later stages of OA, but do not offer insight into the process of cartilage degradation. As the field of OA research moves toward OA prevention it is becoming important to also measure the biological processes responsible for joint degradation, processes that precede the morphological and structural changes.

It is generally accepted that enzymatic activities contribute to cartilage degradation and loss in OA, and that elevated enzymatic activity precedes morphological joint space narrowing [7,8]. The ability to non-destructively image and quantify enzymatic activity would be an important tool to assess OA initiation and progression, and the efficacy of intervention strategies. While primary OA is considered idiopathic, in post-traumatic OA (PTOA) the time point of OA initiation (trauma) can be easily identified, and this is therefore an appropriate model to study the enzymatic activities during the early phases of OA.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent degradative proteinases with roles in the enzymatic cartilage degradation and OA progression. MMP-mediated degradation of type II collagen fibrils is considered one of the first irreversible steps in OA pathogenesis (reviewed in Ref. [9]), and the presence of MMPs correlates with OA symptoms, including joint effusion and pain [10,11]. Although serum level of MMP-3 is used as a biomarker for rheumatoid arthritis (RA), there is no clinically

* Corresponding author. Fax: +1 916 734 5750.
E-mail address: DRHaudenschild@ucdavis.edu (D.R. Haudenschild).

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established MMP biomarker for OA [12]. Most studies investigating MMP activity in OA rely on assays such as ELISA [13,14] or Western Blotting [14,15], and RT-PCR [14,16] to estimate protein levels and mRNA expression, respectively. However, these assays are not suitable for in-vivo use, and can only measure the amount of MMP protein but do not directly assay MMP activity. Direct measurements of MMP activity include zymography [14,16,17] and more recently fluorimetric MMP assays [14,16], but again these assays are generally not suited for *in-vivo* imaging. The development of a method to visualize MMP activities *in-vivo* hence may offer new insight into OA initiation and treatment efficacy.

An *in-vivo* fluorimetric probe was recently developed that allows non-destructive imaging of activity from a broad spectrum of MMPs (MMPSense™ 750 FAST PerkinElmer, Inc., Boston, MA). This near-infrared fluorescent probe is a peptide substrate that enables the detection of MMP activities by exhibiting fluorescent signal when cleaved by MMPs [13,15,18]. In-vivo imaging with this reagent has the potential to continuously measure real-time MMP activities non-invasively. This probe has been successfully used to detect tumor progression [13,18] or ischemia reperfusion in brain [15] with *in-vivo* mouse model, but to our best knowledge there is no previous report investigating MMPSense750 for the assessment of MMP activity in cartilage or joint injury. Moreover, although this probe is expected to be utilized for human patients in clinical setting in future, no paper has studied the kinetics of this substrate using human MMPs. The objective of the present study is to investigate the potential of MMPSense750 for detection of human MMPs and to use in an assay with cartilage explants, as well as an *in-vivo* mouse model of knee injury leading to PTOA.

2. Methods

2.1. Assessment of optimal MMPSense750 concentration

We first wanted to determine the appropriate MMPSense750 concentration for *in-vitro* studies using purified recombinant human MMPs. Human MMP-3, -9, and -13 were chosen based on their established importance in OA [11,19,20]. To achieve comparable results between the different enzymes, the amount of active enzyme in each assay was normalized using the specific activity (Units of enzyme activity per weight) provided by the manufacturer (Supplementary information 1). MMPSense750 was added to media containing the active proteases, the reactions were incubated at 37 °C, and the resulting fluorescent signal was measured at different time points as described in detail below.

Recombinant human MMP-3 (Enzo Life Sciences, Farmingdale, USA) was reconstituted to various concentrations in assay buffer consisting of 50 mM sodium acetate, 10 mM $CaCl_2$, 150 mM NaCl and 0.05% Brij-35 at pH 6.0 [9]. Recombinant human MMP-9 and MMP-13 (Enzo Life Sciences) were reconstituted in the assay buffer consisting of 50 mM HEPES, 10 mM $CaCl_2$ and 0.05% Brij-35 at pH 7.5 [21].

MMPSense750 (24 nmol per vial) was reconstituted in 1200 μl sterile phosphate-buffered saline (Invitrogen) as recommended by the manufacturer, then added into the MMP solutions at 0.2, 0.7 and 2.0 μM final concentration. Imaging was performed on an IVIS-Spectrum imaging system at multiple time points for up to 72 h after adding MMPSense750.

2.2. Cartilage explants

Cartilage explants were harvested from the weight-bearing area of the femoral articular surfaces of bovine stifle knee joints purchased from a local slaughterhouse (Petaluma, CA). A 6 mm dermal biopsy punch was used to isolate cartilage cylinders, which were then cut to 2 mm height from the articular surface using a custom jig. Explants were cultured for 3 days in DMEM with 10% FBS and 1% penicillin-streptomycin (all from Invitrogen, Carlsbad, CA) at 37 °C and 5% CO_2. Six joints were used, and 1 or 2 explants from each joint was randomly assigned to one of three treatment groups; IL-1β, mechanical injury, or control. There was no significant difference among the cartilage weights of each group. The IL-1β group was treated with 10 ng/ml IL-1β (R&D Systems, Minneapolis, MN). The explants in the mechanical injury group were mechanically compressed with an Instron 8511.20 digital servo-hydraulic mechanical testing device using displacement control. A compressive preload of ~0.5 N was applied, and then the explant was loaded to 30% strain at a strain rate of 100%/s, held at 30% strain for 100 ms, then unloaded. Following compression, all loaded explants were transferred to fresh culture medium and returned to an incubator at 37 °C and 5% CO_2 until the termination of the experiment. In the control group, the explants were given a preload of ~0.5 N and then returned to the culture media. The culture media were replenished at day 3. The media were collected at 3 and 6 days after IL-1β stimulation or mechanical injuries. MMPSense750 was added to a final concentration of 0.7 μM, and the fluorescence measured at 60 min and 24 h after adding MMPSense.

2.3. Animal model of joint injury

Eight adult male BALB/cByJ mice (9-week-old at time of injury) were obtained from Jackson Laboratory (Bar Harbor, Maine). All animals were maintained and used in accordance with National Institutes of Health guidelines on the care and use of laboratory animals. This study was approved by our Institutional Animal Care and Use Committee (IACUC). The right knees of the mice were injured with a single mechanical compression as previously described in our PTOA model [22]. Briefly, the tibial compression system consists of two custom-built loading platens; the bottom platen that holds the knee flexed, and the top platen that holds the heel. The platens were aligned vertically and positioned within an electromagnetic materials testing machine (Bose ElectroForce 3200) (Eden Prairie, MN). Mice were anesthetized using isoflurane inhalation, then the right leg of each mouse was subjected to a single dynamic axial compression (1 mm/s loading rate) to a target load of 12 N. This causes a transient anterior subluxation of the tibia, which injures the anterior cruciate ligament and leads to PTOA within 8 weeks. The contralateral uninjured knees served controls, and were used to normalize the data within each animal.

All mice received an injection of 2 nmol of MMPSense750 via the orbital sinus at 24-h post-injury, and IVIS imaging was performed 24 h after the injection (48-h post-injury). Mice were euthanized immediately after the imaging and both knees were dissected for isolation of total RNA and analysis of mRNA expression.

2.4. Quantitative real-time RT-PCR

Total RNA was extracted from injured and uninjured knees using the miRNeasy Mini Kit (Qiagen Valencia, CA) and reverse transcribed by the QuantiTect Reverse Transcription Kit (Qiagen). 2 μl of cDNA was used for quantitative RT-PCR (in a final volume of 10 μl) performed in triplicate in a 7900HT RT-PCR system with gene-specific probes according to the manufacturer's conditions. Results were normalized to the 18s rRNA and calculated as fold-change in mRNA expression relative to the untreated control, using the $2^{-\Delta\Delta CT}$ method. The probes used are shown in Supplementary information 2.

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