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Detection of Active Matrix Metalloproteinase-3 in Serum and Fibroblast-Like Synoviocytes of Collagen-Induced Arthritis Mice

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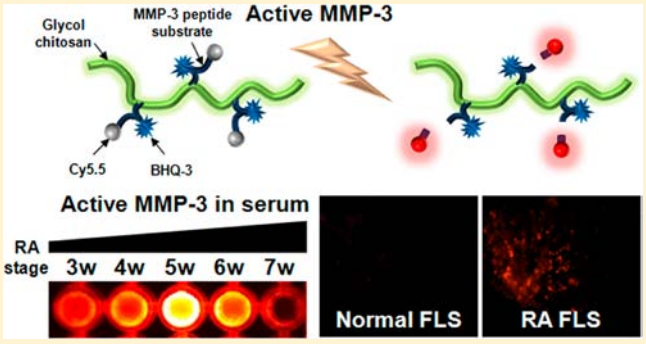
Supporting Information

ABSTRACT: The activity of rheumatoid arthritis (RA) correlates with the expression of proteases. Among several proteases, matrix metalloproteinase-3 (MMP-3) is one of the biological markers used to diagnose RA. The active form of MMP-3 is a key enzyme involved in RA-associated destruction of cartilage and bone. Thus, detection of active MMP-3 in serum or *in vivo* is very important for early diagnosis of RA. In this study, a soluble MMP-3 probe was prepared to monitor RA progression by detecting expression of active MMP-3 in collagen-induced arthritis (CIA) mice *in vivo* in both serum and fibroblast-like synoviocytes (FLSs). The MMP-3 probe exhibited strong sensitivity to MMP-3 and moderate sensitivity to MMP-7 at nanomolecular concentrations, but was not sensitive to other MMPs such as MMP-2, MMP-9, and MMP-13. In an optical imaging study, the MMP-3 probe produced early and strong NIR fluorescence signals prior to observation of erythema and swelling in CIA mice. The MMP-3 probe was able to rapidly and selectively detect and monitor active MMP-3 in diluted serum from CIA mice. Furthermore, histological data demonstrated that activated FLSs in arthritic knee joints expressed active MMP-3. Together, our results demonstrated that the MMP-3 probe may be useful for detecting active MMP-3 for diagnosis of RA. More importantly, the MMP-3 probe was able to detect active MMP-3 in diluted serum with high sensitivity. Therefore, the MMP-3 probe developed in this study may be a very promising probe, useful as a biomarker for early detection and diagnosis of RA.



INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, autoimmune inflammatory disease characterized by synovial inflammation, hyperplasia of synovial tissues, and destruction of bone and cartilage.[1] In general, erosions develop in many patients with RA during the first 2 years of the disease. Thus, early treatment based on an early diagnosis may delay progression of the disease and prevent irreversible damage.[2] However, early diagnosis of RA is often difficult because typical radiographic changes or pathognomonic signs are not observed during the initial stages of the disease.[3,4] Recently, various imaging modalities such as radiography, computed tomography (CT), ultrasonography, and magnetic resonance imaging (MRI) have been popularized as methods to detect joint space narrowing and articular bone erosion. However, CT involves ionizing radiation, ultrasonography is time-consuming and lacks a sufficient degree of standardization, and MRI is relatively expensive, time-consuming, and is limited to a single anatomic site.[5] Furthermore, the majority of contrast agents associated with CT, ultrasonography, and MRI modalities are nonspecific and cannot offer information regarding biological changes at the molecular or cellular levels involved in RA.[6]

Biological changes at the molecular or cellular level often occur long before any signs of structural, functional, or anatomic changes become evident. Thus, development of new approaches to detect specific biomarkers is required, because biomarkers of specific molecules can provide additional information about molecular and cellular events associated with a disease at an early stage, thereby facilitating earlier diagnosis of diseases. Several serological markers such as C-reactive

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protein (CRP), rheumatoid factor (RF), erythrocyte sedimentation rate (ESR), and anticyclic citrullinated peptide (Anti-CCP) appear to be prognostic with respect to the final outcome of RA and are currently used in the clinical setting.[7,8] Even though CRP and ESR are well-known as good parameters for prognostic purposes, their detection is not necessarily reflective of RA because their levels may be influenced by other stimuli of the acute phase response. Likewise, RF has limitations as a biomarker for RA diagnosis because it is nonspecific and may be present in healthy individuals or those with other autoimmune diseases. Anti-CCP exhibits high specificity for RA diagnosis, but has a relatively low sensitivity. Therefore, an accurate biomarker for the early diagnosis of RA is clearly needed by clinicians, who continue to try and develop prognostic markers that exhibit high sensitivity and specificity.

Among the family of matrix metalloproteinases (MMPs), MMP-3 is thought to play a role in the pathogenesis of RA because it is a key enzyme involved in matrix degradation in RA and is also known to be produced mainly by synoviocytes.[9] Several studies have suggested that elevated levels of MMP-3 are correlated between the serum and synovium of RA patients,[10−14] and further that serum MMP-3 is a predictor of the degree of joint destruction in RA.[15−18] Thus, some studies have suggested that MMP-3 activity may be a promising specific marker of RA-associated joint inflammation and destruction. These above-mentioned studies support the notion that MMP-3 may be a promising candidate as a new biological marker for RA diagnosis.[19]

Recently, our group developed a highly quenched MMP-3 nanoprobe to evaluate molecular events associated with the early diagnosis and monitoring of RA progression.[20] Using various optical imaging modalities, we demonstrated the ability of the MMP-3 nanoprobe to detect RA activity *in vivo* as early as 2 weeks after RA induction, whereas micro CT, histology, and clinical score are unable to detect arthritic symptoms under the same conditions. However, in our previous study, we did not demonstrate the ability of the MMP-3 probe to selectively detect the active form of MMP-3 with high sensitivity. Likewise, we did not test whether the MMP-3 probe could be applied for the detection of active MMP-3 in serum and FLSs of RA knee joints. Thus, in the present study, we prepared a soluble MMP-3 probe as a specific biomarker for RA and monitored the expression of MMP-3 in CIA mice. In addition, we investigated the ability of the MMP-3 probe to selectively detect active MMP-3 expression in diluted serum and activated FLSs from CIA mice.

MATERIALS AND METHODS

Materials. The peptide substrate for matrix metalloproteinase-3 (MMP-3) (Gly-Val-Pro-Leu-Ser(tBu)-Leu-Thr(tBu)-Met-Gly-Lys(Boc)-Gly-Gly, Ser-Leu: cleavage site) used in this study was synthesized using standard solid-phase peptide chemistry.[21] Cy5.5 mono *N*-hydroxysuccinamide ester (Cy5.5-NHS ester), a near-infrared fluorescence dye, was purchased from GE healthcare (Piscateway, NJ, USA). Blackhole quencher-3 (BHQ-3) mono *N*-hydroxysuccinamide (BHQ-3 NHS ester) was obtained from Biosearch Technologies, Inc. (Novato, CA, USA). Anhydrous dimethylformamide (DMF), *N*-methyl morpholine (NMM), 4-dimethylaminopyridine (DMAP), diethyl ether, trifluoroacetic acid (TFA), anisole, acetonitrile, dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS, pH 6.0), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), *p*-aminophenylmercuric acid (APMA), and glycol chitosan (250 kDa) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *N*-Hydroxysulfosuccinimide (Sulfo-NHS) was obtained from Pierce (Rockford, IL, USA).

Synthesis of MMP-3 Activatable Dark Quenched Fluorogenic Peptide. The MMP-3 activatable dark quenched fluorogenic Cy5.5 (NIR dye, ex/em. 675/690 nm)-MMP-3 peptide substrate-BHQ-3 (NIR dark quencher, abs. 650 nm) was prepared as described previously (see Figure S1 in Supporting Information).[20,22,23] Briefly, the N-terminus of an MMP-3 peptide substrate [Gly-Val-Pro-Leu-Ser(tBu)-Leu-Thr-(tBu)-Met-Gly-Lys(Boc)-Gly-Gly, 5.5 μmol] was chemically conjugated to Cy5.5 NHS ester (11.4 μmol; GE Healthcare) in anhydrous DMF (400 μL, Sigma) containing NMM (100 μmol; Sigma) and DMAP (5 μmol, Sigma) with shaking at room temperature for 6 h. Next, the Cy5.5-MMP-3 peptide substrate was precipitated in cold diethyl ether (Sigma) and dried under vacuum. The protected groups on the Cy5.5-MMP-3 peptide substrate were removed by TFA/distilled water/anisole (95:2.5:2.5, v/v), and the resulting peptide was further purified by semipreparative reversed-phase high liquid chromatography (RP-HPLC) and characterized by analytical RP-HPLC. The BHQ-3 NHS ester (1.5 μmol; Biosearch Technologies, Inc.) was then coupled to the primary amine of the lysine on Cy5.5-Gly-Val-Pro-Leu-Ser-Leu-Thr-Met-Gly-Lys-Gly-Gly (2.0 μmol) in anhydrous DMF (200 μL) containing NMM (15 μmol) and DMAP (3.0 μmol) with shaking at room temperature for 9 h. The resulting fluorogenic peptide was purified by reversed-phase HPLC. Specifically, the Cy5.5-MMP-3 peptide substrate-BHQ-3{Cy5.5-Gly-Val-Pro-Leu-Ser-Leu-Thr-Met-Gly-Lys(BHQ-3)-Gly-Gly} MMP-3 peptide probe fraction was collected and lyophilized. Product purity at each step (>95%) was confirmed by analytical HPLC, and the molecular weight determined by MALDI-TOF mass spectrometry was 2326 (m/z calculated: 2327) (see Figure S2 in Supporting Information).

Synthesis of the MMP-3 Probe. The MMP-3 probe was prepared by conjugating glycol chitosan (GC) with the activatable fluorogenic peptide (Cy5.5-Gly-Val-Pro-Leu-Ser-Leu-Thr-Met-Gly-Lys(BHQ-3)-Gly-Gly) using EDC and NHS (see Figure S3 in Supporting Information). Briefly, GC (20 mg, 0.08 μmol) was dissolved in 14 mL of PBS (pH 7.4, Sigma), to which the fluorogenic MMP-3 peptide (1.5 μmol), EDC (4 μmol, Sigma), and Sulfo-NHS (4 μmol, Pierce) were added. The mixture was then allowed to react overnight under dark conditions. Next, the resulting product was subjected to harsh dialysis against distilled water for 3 days (MWCO: 12−14 kDa, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) and lyophilized. Absorption titration was conducted using a UV−vis spectrometer to assess the amount of peptide probe bound to GC. The absorption intensity of BHQ-3 (650 nm) was calibrated and the concentration of bound peptide probe was determined from known concentrations of the standard curve.

Enzyme Selectivity and Sensitivity. Enzyme selectivity of the MMP-3 probe was evaluated in 4 mL cuvettes by coincubating 20 nM of MMP-3 probe with activated MMP-2, MMP-3, MMP-7, MMP-9, or MMP-13 human recombinant enzyme (15 nM) in TCNB reaction buffer (50 mM TRIZMA·HCl, 10 mM $CaCl_2 \cdot 2H_2O$, 0.15 M NaCl, 0.05% Brij35, pH 7.5). Each enzyme was activated with 2.5 mM of APMA in 0.1% NaOH buffer for 1 h at 37 °C. Fluorescence spectra were acquired with a fluorometer (F-7000 Fluorescence

Spectrophotometer, Hitach, Tokyo, Japan) every 10 min at 37 °C. In addition, recovery of NIR fluorescence signals was visualized with a Kodak Image Station 4000MM (Japan) equipped with a special C-mount lens and Cy5.5 bandpass emission filter (680 to 720 nm; Omega Optical, Brattleboro, VT). Using the same conditions, enzyme sensitivity of the MMP-3 probe (20 nM) was examined with various concentrations of activated MMP-3 (1.9, 3.8, 7.5, 15, and 30 nM).

To further confirm whether the MMP-3 probe was able to selectively detect pro- or active MMP-3 enzyme, the MMP-3 probe was incubated with pro-MMP-3 or APMA-activated MMP-3, and fluorescence intensity was determined using a fluorometer.

Development and Scoring of Collagen-Induced Arthritis Mice. All animal care and experimental procedures were performed according to the institutional animal care committee of the Korea Institute of Science and Technology (2011−01−017). The collagen-induced arthritis (CIA) model was established with DBA/1J mice (5-week-old, male, Orientbio, Seongnam, Korea) according to published protocols.[24] Briefly, bovine type II collagen (Sigma, St. Louis, MO) was dissolved at a concentration of 2 mg/mL in 0.1 M acetic acid by stirring for 4 h at 4 °C. An emulsion consisting of collagen and complete Freund's adjuvant (Sigma) (1:1, v/v) was prepared, followed by immunization of mice via intradermal injection of the emulsion (50 μL) into the tail. Two weeks after the primary immunization, a booster immunization was performed. The incidence of arthritis in the CIA-susceptible strain of mice was very high within six weeks of the first immunization, and 95% of the mice developed severe arthritis. The clinical severity of arthritis in each paw was quantified according to a graded scale from 0 to 4, as previously described.[24] A mean arthritis score was determined by totaling the scores of the four paws of mice and dividing the result by the total number of mice in each group.

In Vivo Molecular Imaging of MMP-3 Activity in CIA Mice. CIA Mice were intravenously injected with the MMP-3 probe (10 μg/100 μL in PBS pH 7.4) between 3 and 7 weeks after the first immunization (n = 14). At 1 h postinjection of the MMP-3 probe, CIA (n = 14) and normal (n = 5) mice were anaesthetized with isoflurane (1%, w/v in 2 L oxygen) and near-infrared optical images of the hind limbs were acquired using eXplore Optix (ART Advanced Research Technologies Inc., Montreal, Canada). Laser power and count time settings were optimized at 10 μW and 0.3 s per point. Additionally, we checked the residual fluorescence signal at CIA joints before the probe injection every week (see Figure S4 in Supporting Information). The residual fluorescence signal is normal range before the probe injection.

Detection of Active MMP-3 in CIA Mice Serum. A total of 200 μL of peripheral blood from CIA mice (n = 7) was collected from the tail vein between 3 and 7 weeks after RA induction. Blood was clotted for 1 h and centrifuged for 10 min at 13,000 rpm. Serum was stored at −80 °C until assayed. To generate the MMP-3 kit for detection of active MMP-3, 100 μL of 10 μM MMP-3 probe was dissolved in PBS/DMSO (7:3, v/v), added to each well of maleic anhydride-activated 96-well plates (Pierce), and incubated for 24 h at room temperature. Next, 100 μL of 5% bovine serum albumin (BSA) was added to each well for 1 h to block nonspecific binding, after which plates were washed with TCNB buffer to remove excess BSA. To determine the level of active MMP-3 in serum, collected serum from CIA mice was diluted 10-fold in PBS (pH 7.4), and 100 μL of the diluted serum was added and further incubated for 1 h at 37 °C in the MMP-3 probe kit. After incubation, the MMP-3 probe kit was imaged using a 12-bit CCD camera (Kodak Image Station 4000MM) with a special C-mount lens and a Cy5.5 band-pass emission filter (680 to 720 nm; Omega Optical). MMP-3 fluorescence intensity was measured in quadruplicate and reported as the mean ± SD.

Determination of Pro and Active MMP-3 Levels in CIA Mice Serum by Western Blotting. Western blotting was used to determine the relative levels of pro and active MMP-3 in CIA mice serum. At a predetermined time, serum was obtained from CIA mice in each group (n = 3/group). The protein concentration of serum was assayed using a NanoDrop3300 (Thermo Scientific), and 10 μg of protein for each sample was mixed with 5× sample buffer and heated to 100 °C for 10 min and subjected to 7.5% SDS-PAGE. Gels were then transferred to nitrocellulose membrane (Invitrogen, NY) and blocked with 5% skim milk in 1× Tris-buffered saline containing Tween 20 (TBST, 50 mM Tris−HCl, pH 7.5, 150 mM NaCl, supplemented with 0.2% Tween 20). The membrane was then probed with primary antibody (anti-MMP-3 at 1:1000 or β-actin at 1:5000) diluted in 5% skim milk. After an overnight incubation at 4 °C, the membrane was washed three times with 1× TBST and incubated with a goat anti-mouse peroxidase (HRP)-linked immunoglobulin G (IgG) antibody (1:5000 diluted in 5% skim milk) at room temperature for 1 h. Bands were visualized with an ECL detection system (enhanced chemiluminescence, GE) and developed with X-ray film. The film was scanned by a HP Scanjet G3010 (City, Country).

Detection of MMP-3 Levels in CIA Mice Serum using MMP-3 ELISA Kit. MMP-3 protein levels were detected with an MMP-3 ELISA kit according to the manufacturer's instructions (Uscn Life Science Inc., USCNK). The sensitivity of the MMP-3 ELISA kit for MMP-3 was 0.08 ng/mL.

MMP-3 Activity and Immunofluorescence Study in FLS. After sacrifice of CIA mice at 7 weeks, excised knee joint tissues (n = 5) were cryo-sectioned at a thickness of 5 μm. For NIR fluorescence imaging to detect MMP-3 activity in FLS, knee tissue sections were viewed by fluorescence microscopy (ex: 650 nm, em: 700 nm, Olympus IX81, Meta Morph v 7.5.3.0, New York, USA). For immunofluorescence evaluation of FLS in knee tissue, immunofluorescence anti-CD13 was used as a specific FLS marker to stain FLS in knee joint tissue as follows: tissue sections were washed with 0.01 M PBS (pH 7.4) and blocked in 3% serum albumin containing 0.1% Triton X-100 for 30 min. The sections were then incubated in a cocktail of mouse anti-CD13 (1:1000; abcam) for 1 h at room temperature. After washing with 0.01 M PBS, the sections were further incubated for 30 min with a mixture of FITC-conjugated goat anti-mouse IgG (1:2000; Santa Cruz Biotechnology Inc., Santa Cruz) and fluorescence images were subsequently acquired.

Statistical Analysis. Statistical analyses were performed using ANOVA. We compared serum MMP-3 levels each week following RA induction. P-values <0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Matrix metalloproteinases (MMPs) are overexpressed in serum and synovial fluid of RA patients because MMPs are correlated with articular cartilage degradation and persistent synovial

inflammation. Among several members of the MMP family, MMP-3 is considered a particularly important marker of joint inflammation and destruction in RA. Thus, a technique for detection of active MMPs in serum is required for early diagnosis of RA disease progression and activity. In the present study, we prepared a MMP-3 specific probe consisting of GC and a dark-quenched fluorogenic peptide [Cy5.5 (NIR dye)-MMP-3 peptide substrate-BHQ-3(NIR dark quencher)] and evaluated whether it could be useful for the early detection of RA disease. Specifically, we analyzed whether the probe could detect MMP-3 levels in serum and FLSs using the CIA mouse model. The principle of the MMP sensor is based mainly on the recovery of quenched fluorescence following cleavage of a specific peptide substrate; when the probe encounters the specific MMP of interest, the dark-quenched fluorogenic peptide is cleaved due to specific recognition of the substrate by the MMP, producing a pronounced NIR fluorescence signal recovery due to dequenching of the dye (Figure 1).

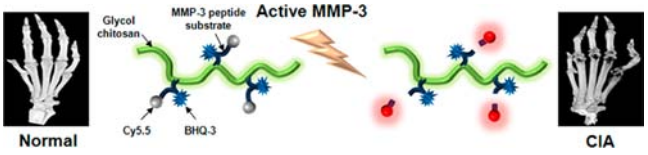

Figure 1. Schematic diagram of the MMP-3 probe. When MMP-3 encounters the specific substrate recognition site of the probe at the disease site, the fluorogenic peptide probe is cleaved by MMP-3, resulting in a pronounced NIR fluorescence signal recovery.

Characterization of the MMP-3 Probe. The specific MMP-3 probe was prepared by coupling GC with a dark-quenched fluorogenic peptide [Cy5.5-GVPLSLTMGK(BHQ-3)GG, purity: >95%, *m/z*: 2326]. Conjugation of the dark-quenched fluorogenic peptide to GC was confirmed by measuring the absorption intensity of BHQ-3 (650 nm) using a UV−vis spectrometer. By measuring the absorption intensity produced from the MMP-3 probe, we estimated that 117.9 molecules of the fluorogenic MMP-3 peptide were bound to each molecule of GC, and the molecular weight of the MMP-3 probe is 524,235 Da.

Enzyme Selectivity. The enzyme selectivity of the MMP-3 probe was evaluated by incubating a fixed concentration of the MMP-3 probe (20 nM) and activated MMP-2, MMP-3, MMP-7, MMP-9, or MMP-13 (15 nM) in reaction buffer at 37 °C for 1 h. NIR fluorescence signal data clearly demonstrated that significant time-dependent recovery of NIR fluorescence intensity occurred with MMP-3 and MMP-7 (37.1- and 15-fold for MMP-3 and -7, respectively) (Figure 2A). Importantly, there was a proportional recovery of NIR fluorescence intensity depending on MMP-3 concentration (1.9, 3.8, 7.5, 15, and 30 nM) (r^2 = 0.97) (Figure 2B); however, there was no recovery of NIR fluorescence signals against MMP-2, MMP-9, and MMP-13. Together, these results indicated that the probe was able to detect active MMP-3 with a high degree of sensitivity.

In Vivo Monitoring of MMP-3 Activity in CIA. The RA mouse model was developed by injecting a Type II collagen and CFA emulsion in tails, followed by detection of MMP-3 expression *in vivo*. After the first immunization, swelling in limbs was observed and gradually increased as shown in Figure 3A,B. At 7 weeks after RA induction, the clinical RA scores reached their highest level. Additionally, to evaluate whether the MMP-3 probe could be used to determine levels of MMP-3

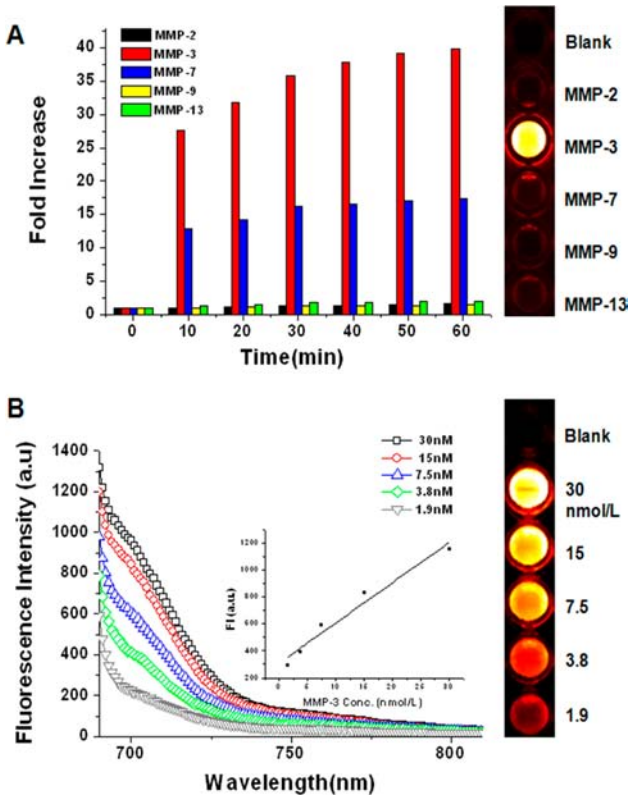

Figure 2. A. MMP-3 probe in the presence of various recombinant MMPs (MMP-2, -3, -7, -9, -13) as a function of time following incubation for 60 min at 37 °C. B. Fluorescence intensities of the MMP-3 probe (20 nM) in the presence of various concentrations of MMP-3 (30, 15, 7.5, 3.8, 1.6 nM) for 30 min at 37 °C.

during progression of RA in mice, fluorescence images and intensities were observed between weeks 3 and 7 after CIA induction. In control (normal) mice treated with the MMP-3 probe (10 μg/100 μL/mouse), NIR fluorescence intensity was not substantially increased. However, the MMP-3 probe in CIA mice produced strong NIR fluorescence signals up to 5 weeks after the first immunization, but decreased gradually thereafter (Figure 3A). Changes in NIR fluorescence signals by MMP-3 were not correlated with the RA scores because MMP-3 levels are increased at an early stage during the progression of RA, before any erythema or swelling can be observed with the naked eye and prior to the start of erosion.[15,25,26]

Detection of Active MMP-3 in Serum from CIA Mice. MMP-3 is used as a biologic marker of RA because of the increasing evidence that elevated MMP activity, most notably MMP-3 activity, is involved in the development of RA.[15,22,25] Therefore, detection of active MMP-3 levels in serum of RA patients is very important for early stage RA diagnosis. We evaluated the presence of MMP-3 enzyme in serum from CIA mice by Western blotting to confirm the presence of both pro and active MMP-3. Specifically, the two MMP-3 forms in serum were increased up to 5 weeks after the first immunization, but decreased gradually thereafter (Figure 4A). Interestingly, we found that the level of active MMP-3 was significantly lower than pro MMP-3.

To evaluate which kinds of MMP-3 are capable of specifically producing the strong NIR fluorescence signal, the MMP-3 probe was incubated in pro MMP-3 or active MMP-3 and the recovery of NIR fluorescence signal was determined. A strong

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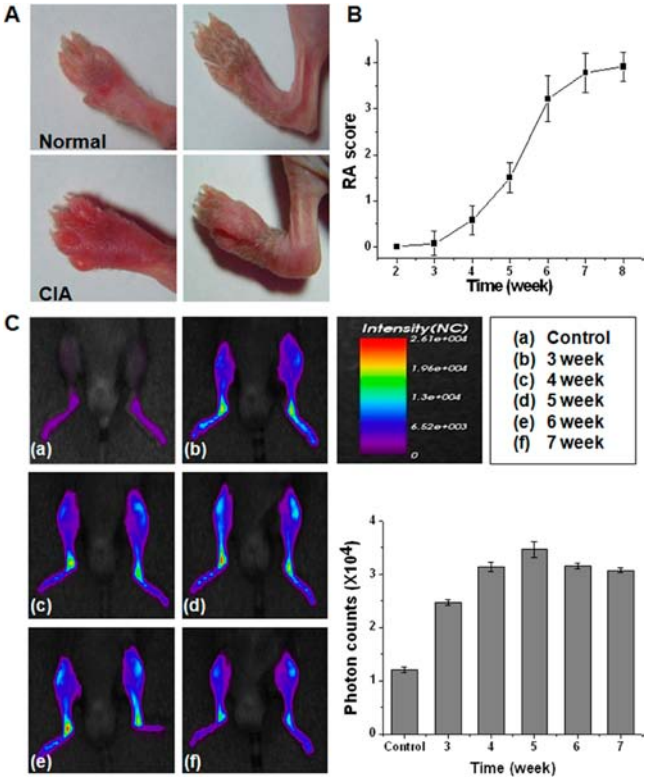

Figure 3. A. Optical images of normal and CIA mice limbs. B. Arthritic scores were measured by examining the paws of each mouse between 2 and 8 weeks after the first immunization. C. *In vivo* NIRF images of CIA mice models after intravenous injection of the MMP-3 probe (n = 14) at different stages of RA development, specifically between 3 and 7 weeks after the first immunization.

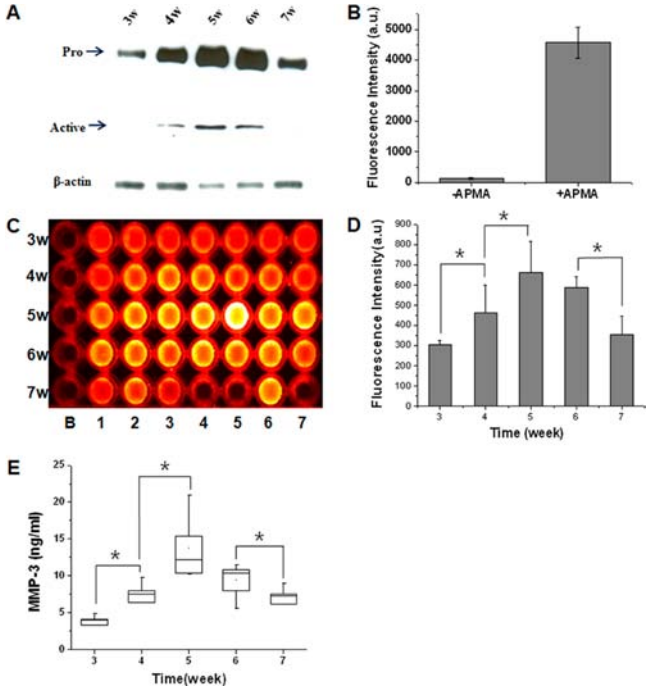

Figure 4. A. Confirmation of MMP-3 expression at different stages of arthritis development by Western blot. MMP-3 is known to appear as 57 kDa (pro) and 28 kDa (active) bands. β-actin was used as an internal control. B. Graph of NIR fluorescent signal recovery by pro and active MMP-3. C. NIR fluorescent image of CIA mice serum analyzed by the MMP-3 kit. D. NIR fluorescent intensity of CIA mice serum analyzed with the immobilized MMP-3 kit. E. Serum concentrations and median values of MMP-3 in CIA model pathogenesis. Values are the mean ± SEM * $P < 0.05$ by ANOVA.

NIR fluorescence signal was induced by active MMP-3, indicating that the MMP-3 probe specifically detected active MMP-3 and was able to differentiate active MMP-3 from pro MMP-3.

We also determined the level of active MMP-3 expression in serum from CIA mice at different stages of induced RA using a MMP-3 diagnostic kit prepared by immobilizing the MMP-3 probe onto wells of maleic anhydride-activated 96-well plates as previously described.[27] 10-fold diluted serum from mice with different stages of RA was incubated with the MMP-3 diagnostic kit, and the NIR fluorescence signal was visualized and quantified with a Kodak Image Station 4000MM. As shown in Figure 4C,D, the NIR fluorescence signal in serum was substantially increased up to 5 weeks after the first immunization and decreased thereafter. In addition, levels of total MMP-3 detected by ELISA showed a similar pattern (Figure 4E). Based on Western blot, ELISA, and NIR fluorescence data obtained with the MMP-3 diagnostic kit, all three approaches were able to detect MMP-3 levels in serum of CIA mice with different stages of RA. However, the Western blotting and ELISA assays exhibited limitations with respect to detection of MMP-3 levels in serum. Specifically, while the Western blot method was able to determine levels of both pro- and active MMP-3 (Figure 4A), the analysis range was limited to several micrograms. Likewise, while the ELISA kit could analyze total MMP-3 levels in serum down to several nanomoles with high sensitivity (Figure 4E), it detected only total levels of MMP-3, and thus could not specifically differentiate between pro- and active forms of MMP-3. Surprisingly, the MMP-3 probe used in the present study could detect both pro- and active MMP-3 levels with 10-fold diluted serum with high sensitivity at nanomolar concentrations. As recently reported, MMP-3 tended to be more elevated in the progressive group and MMP-3 levels in serum of RA patients were 103 ± 71.0 ng/mL for nonprogressive group and 180.7 ± 179.4 ng/mL for progressive group.[28] Thus, the MMP-3 probe and diagnostic kit may be very useful for the detection of active MMP-3 enzyme in RA models.

Detection of Active MMP-3 in FLSs from CIA Mice. Up-regulation of MMP-3 activity can be a specific marker of inflammation and destruction of joints in RA, because MMP-3 is known to be exclusively generated from inflamed synovial tissue. Indeed, MMP-3 has been shown to localize to both FLSs and cartilage in RA. Thus, to investigate the correlation between activation of FLSs and MMP-3 specific probe accumulation, we performed a histological study. Specifically, tissue sections of arthritic and nonarthritic knee joints were examined using CD13, a FLS-specific cell surface marker. Histological data showed that the NIR fluorescence signal of the MMP-3 probe generated by active MMP-3 and the FLS specific anti-CD13 stain were much stronger in arthritic knee joints than nonarthritic knee joints (Figure 5) and that the two signals colocalized well. Together, these results suggested that active MMP-3 expression was closely correlated with FLS activation at RA joints and was related with RA progression.

Compared with conventional methods (i.e., Western blotting and ELISA), the MMP-3 probe described in this study has several promising advantages. Specifically, the MMP-3 probe

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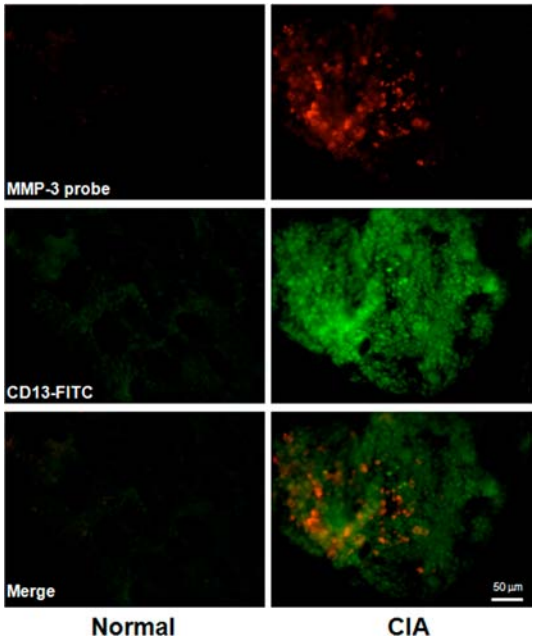

Figure 5. Co-localization of MMP-3 probe signal and FLS positive anti- CD13-FITC signal in the arthritic joints of normal (n = 5) and CIA mice (n = 5). Red fluorescence indicates a site of MMP-3 probe accumulation while green fluorescence indicates a FLS positive site. Yellow signal indicates a merged red and green signal.

can be used to detect MMP-3 expression *in vivo*, and thus may be useful for the early diagnosis of RA and similar diseases. More importantly, the MMP-3 probe was readily immobilized onto 96-well plates and used successfully as a specific MMP-3 diagnostic assay kit. Such MMP-3 diagnostic kits can quickly and selectively detect active MMP-3 levels in diluted serum at nanomolar levels, and is superior to Western blot and ELISA, which are time-consuming, relatively labor-intensive, and in the case of ELISA unable to distinguish between the pro- and active forms of MMP3. Therefore, we believe that the MMP-3 probe used in this study is a very powerful and promising probe and diagnostic assay kit for the detection of active MMP-3 in serum and FLSs for early detection of RA.

CONCLUSIONS

In the present study, we describe an MMP-3 probe capable of monitoring MMP-3 expression in a model of RA progression. More importantly, as a MMP-3 diagnostic assay kit, the MMP-3 probe was able to selectively detect active MMP-3 expression with high sensitivity in diluted serum of CIA mice. Furthermore, the probe was able to detect the MMP-3 generated by activated FLSs in arthritic knee joints. Thus, the approach described in this study may help with decisions for early diagnosis, monitoring disease progression, and providing personalized medication solutions for the treatment of RA.

ASSOCIATED CONTENT

Supporting Information

Details of synthetic scheme of the dark-quenched fluorogenic MMP-3 peptide and chemical structures of Cy5.5-NHS ester and black hole quencher-3; MALDI-TOF data of the dark-quenched fluorogenic MMP-3 peptide; Synthesis of the MMP-3 probe; *In vivo* fluorescence images of normal and CIA mice limbs after probe injection. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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