OsteoArthritis and Cartilage (2004) **12,** 239–244
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doi:10.1016/j.joca.2003.11.005

Osteoarthritis and Cartilage

ICRS International Cartilage Repair Society

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Early diagnosis of osteoarthritis using cathepsin B sensitive near-infrared fluorescent probes

Wen-Fu T. Lai†§, Chung-Hsun Chang†¶, Yi Tang†, Roederick Bronson‡ and Ching-Hsuan Tung†*

† *Center for Molecular Imaging Research, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA 02129, USA*

‡ *Tufts University School of Veterinary Medicine, Department of Biological Sciences, North Grafton, MA 01536, USA*

§ *Current Address: Institute of Medical Sciences, Taipei Medical University, Taipei, Taiwan*

¶ *Current address: Department of Orthopedic Surgery, Chang Gung Memorial Hospital, Taipei, Taiwan*

Summary

Objective: Osteoarthritis is currently diagnosed utilizing X-ray and MRI-techniques, both of which are based on the morphological changes of tissue. However, once changes are detected, the tissue has an irreversible defect. This study investigates early diagnosis of OA on a molecular basis using a recently developed cathepsin B sensitive near-infrared (NIR) fluorescent probe.

Method: Twelve male nude mice were induced osteoarthritis by intra-articular injection of collagenase (1.0%, w/v) into the right knee joint. The left knee joint served as the negative control. The cathepsin B NIR probe is activated by arthritis-associated cathepsin B, thus resulting in the emission of an intensive NIR fluorescence signal which can be detected *in vivo*. NIR fluorescence signals were acquired on an optical imaging system using an excitation wavelength of 610–650 nm and an emission wavelength of 680–720 nm.

Results: Mild to moderate degenerative cartilage was observed 1 month after collagenase injection. NIR fluorescence imaging of mice showed approximately a 3-fold difference in signal intensity between osteoarthritic and normal joints 24 h after intravenous injection of the reporter probe. Immunohistochemical evaluation also revealed cathepsin B expression in the arthritic lesion of femorotibia joints, and not in the control contra-lateral knee joints.

Conclusion: As the cathepsin B activatable NIR fluorescent imaging showed a significant difference between the osteoarthritic and normal joints, the cathepsin B activatable NIR fluorescent probe thus offers a potential new imaging technology for early OA diagnosis.
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Key words: Osteoarthritis, Cathepsin B, Near-infrared fluorescence, Collagenase, Imaging.

Introduction

Osteoarthritis (OA) is the most common form of arthritis worldwide. It is estimated that 11% of individuals 65 years of age have symptomatic knee OA. On average, 16 to 21 million Americans are attacked yearly by OA, and the number is expected to increase to 40 million by 2020 as the population ages[1]. OA is a complex, progressive disease of the joint characterized by degenerative and regenerative morphological and structural alteration. This process leads to fibrillation, erosion of articular cartilage, proliferation of normally non-dividing chondrocytes, formation of osteophytes at joint margins, and sclerosis of subchondral bone resulting in joint dysfunction at the late stage of the disease[2,3]. The etiopathogenesis of osteoarthritis is multifactorial and not completely elucidated. However, the common feature of osteoarthritis is characterized by the early inflammation followed by degeneration of chondrocytes including irreversible biodegradation of proteoglycan and type II collagen in articular cartilage[4,5].

Currently, OA is diagnosed by X-ray and MRI. The X-ray is utilized to detect hard tissue changes, while the MRI is more sensitive to detect soft tissue defects[6]. These detections are all based on morphological changes of tissue. Once the changes are seen on the X-ray or MRI, the tissue already appears an irreversible defect. Clinically, it is strongly believed that early control of the disease process is the best therapy. Hence, the accurate diagnosis of OA at its early stage is highly demanded.

High level of activity of an extracellular cysteine protease, cathepsin B, has been found around clefts and in the zones of hypercellularity in osteoarthritic cartilage[7]. High intracellular activity of cathepsin B was also found in degenerated chondrocytes[7] and synovium[8]. The particular distribution of cathepsin B suggested that it may play a critical role in cartilage catabolism in osteoarthritis[9]. Potentially this enzyme can be used as a biological marker to study OA.

Recently, a series of protease sensitive near-infrared fluorescence (NIRF) probes for *in vivo* imaging of disease-associated proteases were developed in this laboratory [10–13]. The probes were designed to have a low fluorescence signal in their initial state, due to serious

*Address to correspondence to: Ching H. Tung, Ph.D., Center for Molecular Imaging Research, Massachusetts General Hospital, 149 13th St., Rm. 5406, Charlestown, MA 02129, USA. Tel: +1 617 726-5779; Fax: +1 617 726-5708; E-mail: tung@helix.mgh.harvard.edu

Received 7 July 2003; revision accepted 2 November 2003.

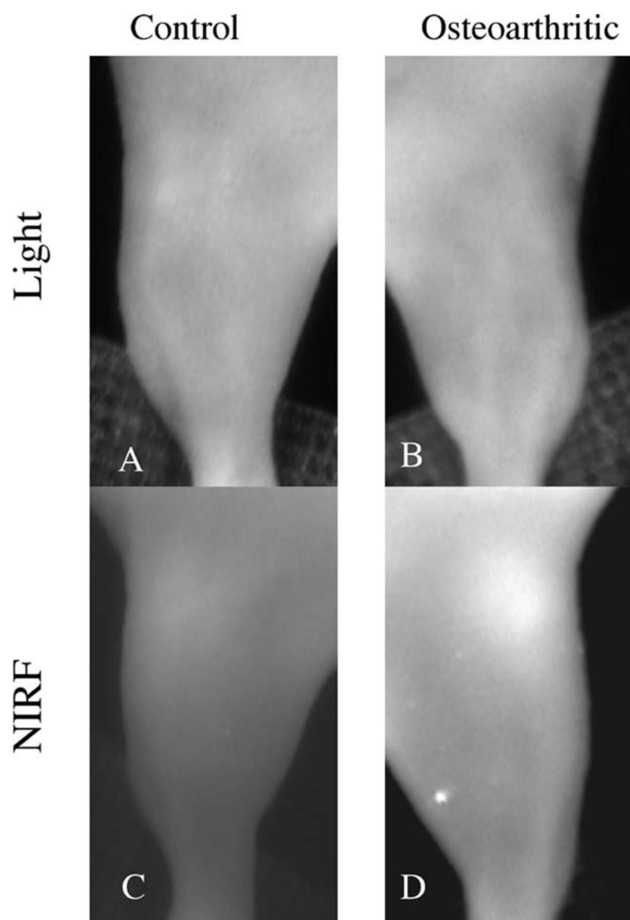

Fig. 1. Near infrared fluorescence reflectance imaging 24 h after IV injection of the cathepsin B sensitive autoquenched probe in a representative animal; (A and B) white light images, (C and D) NIRF images. Note that the arthritic knee joints reveal significantly higher joint tissue fluorescence values compared to contralateral joints.

self-quenching between multiple near-infrared (NIR) fluorochromes. The NIRF signal is not released until the probe is recognized and activated (digested) by the targeted protease. The cathepsin B selective probe has previously been applied to tumor detection, tumor characterization, and atherosclerotic plaque localization *in vivo*[10,14–17]. With these findings, we thus hypothesize that cathepsin B probe may be used in early OA diagnosis. Using an OA mouse model, we are able to demonstrate that the cathepsin B probe can be activated in the osteoarthritic joints at an early stage of the disease.

Materials and methods

GENERAL

Bacterial collagenase and most histological reagents were purchased from Sigma (St Louis, MO), unless specifically mentioned. Cathepsin B sensitive probe consisting Cy5.5 fluorochromes was synthesized as previously published[10]. Briefly, a synthetic graft copolymer consisting of poly-L-lysine (35 kDa) sterically protected by multiple methoxypolyethylene glycol (5 kDa) side chains was used as a delivery vehicle of quenched fluorochromes. About 30% of the lysine residues were PEG-ylated, 15% were reacted with Cy5.5 and the rest 55% were remained free. The overall molecular weight of the probe is about 500 kDa. The free lysine residues on the backbone serve as sites for cleavage by cathepsin B[10].

Table I

NIRF signal intensity of osteoarthritic joints compared to control joints. Near infrared fluorescence signal intensity (680–720 nm) 24 h after IV injection of the cathepsin B sensitive probe in 12 animals: $P<0.0001$

Mouse	OA joint	Control joint	OA/Control
#1	481.0	160.0	3.0
#2	404.0	79.8	5.1
#3	230.2	107.8	2.1
#4	548.3	106.3	5.2
#5	251.4	84.4	3.0
#6	245.7	93.0	2.6
#7	299.2	148.8	2.0
#8	337.6	146.5	2.3
#9	305.9	160.6	1.9
#10	412.8	171.6	2.4
#11	392.5	136.3	2.9
#12	218.2	87.6	2.5
Mean	343.9	123.6	2.8
SD	105.2	33.8	

INDUCTION OF OSTEOARTHRITIS

Twelve 10-week-old, male nude mice were used in this study (Charles River Lab, Wilmington, MA). The animals were housed in cages with a sawdust bottom in an air-conditioned room at constant temperature and were fed a standard laboratory diet. All animal studies were approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital. Ketamine (80 mg/kg of body weight) and Xylazine (12 mg/kg) delivered subcutaneously were used as anesthetics, and for euthanasia, pentobartobital (100 mg/kg) was administered intraperitoneally.

The collagenase-induced osteoarthritis in mouse was adapted from a previously described model[18,19]. The bacterial collagenase 1.0% (w/v) solution was prepared and filtered through a 0.2 μm bacteria filter. The right knee joint of mouse was injected once (22s needle, Hamilton, Reno, Nevada), intra-articularly through the patellar ligament, with 10 μl of the collagenase solution. The left control knee was injected with the same volume of normal saline.

IN VIVO IMAGING

One month after the induction of OA, each mouse was injected systemically with 2 nmole of cathepsin B probe. Twenty-four hours later, the animals were anesthetized and whole body optical imaging using a laboratory-built optical imaging system was performed[20]. The system contained a 150 W halogen lamp with an excitation and an emission bandpass filter of 610–650 nm and 680–720 nm, respectively (Omega Optical, Brattleboro, VT). Both white light and NIRF images were detected by a 12-bit CCD camera (Kodak, Rochester, NY). Whole-body NIRF images were

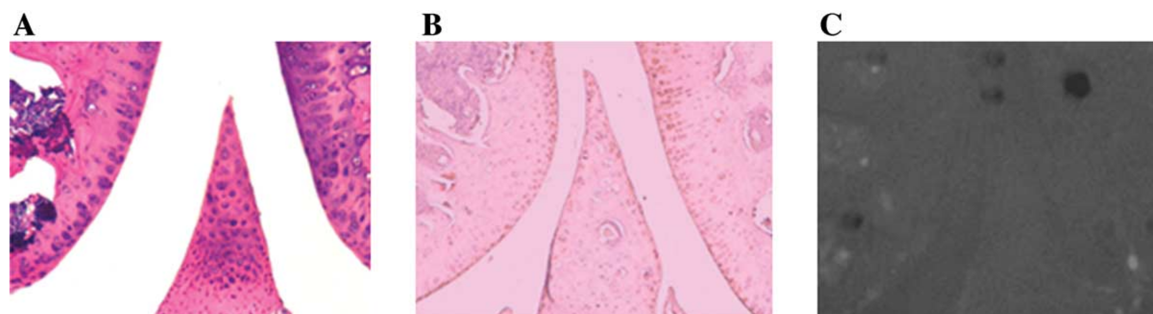

Fig. 2. Histology, immunohistochemistry, and microscopic fluorescence of the control joint. (20×) (A) H/E staining indicated that the epiphysis of the distal femur comprised cancellous bone covered by a thin layer of compact bone. The outer surface on the epiphysis appears smooth and was covered with hyaline cartilage. The meniscal disc appeared normal. Chondrocytes and disc cells were found within the disc. (B) Immunohistochemical staining for cathepsin B is not found in the normal knee joint. (C) NIRF signal was not detectable in the control joints.

obtained with acquisition times of 2 min, and the white light images were with 0.075 s.

IMMUNOHISTOCHEMISTRY

After imaging, animals were euthanasized and knee joint tissues were sagittally excised *en bloc*. Specimens were fixed in formalin for 5 days, and subsequently decalcified in 5% formic acid for 4 days. Standard processing of the tissue processing apparatus was followed by embedding the knee joints in paraffin and serial sectioned (10 μm). Tissue sections were stained with hematoxylin/eosin and the arthritic areas were evaluated using a cartilage and synovium score ranging from 0 (no alterations), + (mild pathological alterations), ++ (moderate pathological alterations), to +++ (markedly pathological alterations).

Ten μm thick tissue sections were selected from formalin-fixed paraffin-blocks, deparaffinized with xylene and rinsed three times with 100% ethanol and once with distilled water. The deparaffinized sections were pretreated with hyaluronidase 1 mg/ml and collagenase 1 mg/ml in phosphate-buffered saline for 1 h at 37°C and the staining procedure was performed with a modification of the avidin–biotin–peroxidase complex (ABC) technique[21,22]. The slides were incubated with the primary antibody against cathepsin B (1:40 dilution, sc-6493, Santa Cruz Biotechnology, Santa Cruz, CA) for 18 h and with the secondary biotinylated donkey antibody against goat-IgG (1:200 dilution, sc-2402, Santa Cruz biotechnology) for 2 h at room temperature in a humidified chamber. Thereafter, slides were rinsed with PBS and visualized with a chromogen of oxidized diaminobenzidine-H_2O_2 reaction. Positive immunoreactions appeared as dark brown staining on a blue background; counterstaining was done with hematoxylin. All processes were conducted at room temperature. Tissue was viewed using a Nikon Eclipse 800 microscope and images were digitally captured using a CCD-SPOT RT digital camera and compiled using Adobe Photoshop™ software (v5.5).

STATISTICAL ANALYSIS

To quantitate NIRF signal, a circular region-of-interest (ROI) was manually defined around the joint, and the average signal within this ROI was obtained. Data are presented mean±SD. Statistical analysis of different groups was conducted using a two-tailed student *t*-test. A *P*-value <0.05 was considered to be significant.

Results

According to the published protocol, mice will have mild to moderate OA symptom 3 to 4 weeks after the intra-articular injection of collagenase[18,19]. In our preparation, the animals appeared normal as described except one showed mild deformity on osteoarthritic joints at 1 month. The observed osteoarthritic alterations in the collagenase model closely resembled previously published studies.

To check if OA could be detected by NIRF imaging, the cathepsin B probe was administered i.v. to the arthritic mouse. A clearly detectable NIRF signal was observed in the arthritic joint 2–3 h later, but the highest target to background signal was observed at 24-h time point. As seen on Fig. 1, a significantly higher NIRF signal was found in the arthritic joints. The average signal intensity was 2.8-fold higher in the osteoarthritic joints compared to control joints 24 h after probe injection (343±105 and 123±33 $P<0.0001$, Table I). More than 5-fold of signal intensity difference between arthritic and control

Table II
*Collagen-induced arthritis. Histologic evaluation of the osteoarthritic alteration in the collagenase injected (1%) knee joints of individual mice (n=12)**

Pathological alteration	0	+	++	+++
Cartilage englargement	0	2	7	3
Cartilage fibrillation	0	0	9	3
Erosion of non-calcified cartilage	0	2	8	2
Erosion of calcified cartilage	4	5	3	0
Chondrocyte loss	0	5	7	0
Chondrocyte cluster	0	8	4	0
Synovial infiltration	0	8	4	0
Synovial hyperplasia	0	0	9	3
Erosion of bone	12	0	0	0

*Key: 0, no alterations; + mild pathological alterations; ++ moderate pathological alterations; +++ markedly pathological alterations.

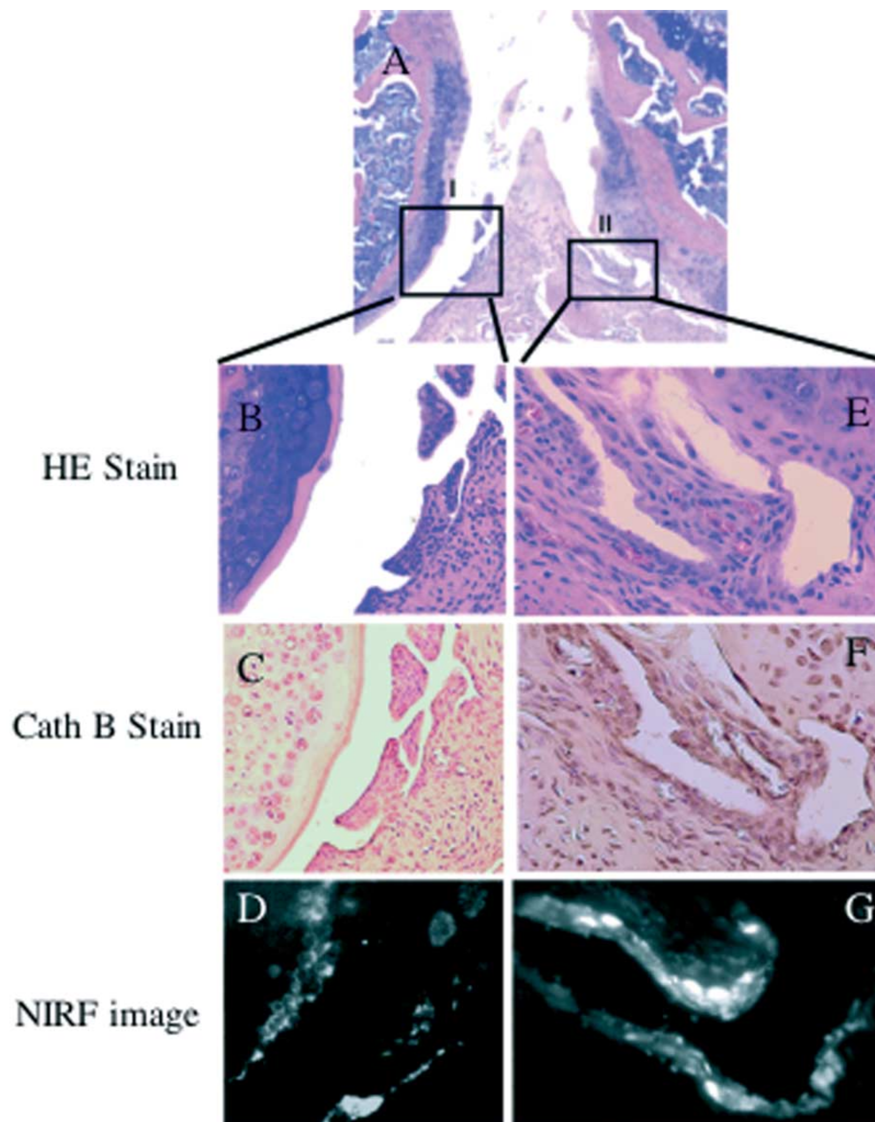

Fig. 3. Histology, immunohistochemistry, and microscopic fluorescence of the arthritic joint. (A) Cartilage of the femorotibial joints shows mild to moderate signs of degeneration. Fibrillation, clefting, erosion, and eburnation are noticed in this area. (10×) (B) Milder degeneration was characterized by the degenerated chondrocytes and synovial villi formation (40×). (E) Synovial hyperplasia is found on the edges of the meniscus. (C) Cathepsin B expression is found in the intra- and peri-cellular areas of degenerated chondrocytes (40×), and (F) in hyperplasic synovium (40×). Near infrared fluorescence microscopy shows a significant fluorescence signal correlatively to the areas of cathepsin B expression (D and G, 40×).

joints was observed after the removal of skin (data not shown).

The histology sections of the contralateral control joints appeared normal. The epiphyses were composed of cancellous bone covered by a thin layer of compact bone, and the outer surface on the epiphysis was covered with hyaline cartilage (Fig. 2A). In contrast, osteoarthritic joints showed mild to moderate deformity. An overview of the osteoarthritic alterations is listed in Table II. The osteoarthritic lesions in the knee joint were moderate, but none of animals have bone erosion at this time point. The cartilage on the anterior aspect of the femorotibial joints showed signs of degeneration, including fibrillation, cleft formation and erosion (Fig. 3A). Moderate to marked synovial proliferation was noted in the condylar process of tibial and femoral bone. Granulation tissue and vascular formation were found in the surface layer. Synovial hyperplasia was also found on the edges of menisci (Fig. 3B and E).

To check if cathepsin-B expression correlated with the cathepsin-B NIRF signal, immunohistochemistry was performed with anti-cathepsin-B antibody. Cathepsin B expression was noted in the arthritic lesion of femorotibia joints. The majority of cathepsin B was distributed in panni, hyperplasic synovium and in intracellular and pericellular areas of degenerated chondrocytes (Fig. 3C and F). Cathepsin-B expression was not detected in the contralateral normal knee joints (Fig. 2B).

The NIRF signal distribution is histological sections correlated with that of cathepsin B, which is locally distributed in the degenerated cartilage (Fig. 3C and D) and hyperplastic synovium (Fig. 3F and G). As expected, the cathepsin B

(Fig. 2B) and NIRF signal (Fig. 2C) in contralateral joints were minimally detectable.

Discussion

In this study we have used cathepsin B sensitive probes with collagenase-induced osteoarthritic joint model to relate NIRF signal with tissue parameters. One of the major problems in OA is the low rate of matrix synthesis and the inability of the chondrocytes to exceed the rate of matrix degradation. These combined factors lead to the overall destruction of the cartilage as seen in OA. Cartilage degradation is mediated by elevated proteolytic activity of enzymes and cathepsin B and several other proteases may potentially participate in matrix degradation[23]. In experimental osteoarthritis, cathepsin B is up-regulated in synovial tissue during the early degenerative phase. Furthermore, progression of experimental osteoarthritis is accompanied by up-regulation of cathepsin-B in cartilage[8]. Results in this research showed that the collagen-induced osteoarthritic joints demonstrate pathological alterations including erosion and fibrillation of cartilage, and synovial hyperplasia. In histological sections, the microscopic NIRF imaging also correlated with histopathological and immunohistochemical findings. The immunohistochemical reaction of cathepsin B was found in the inflamed joint tissues such as degenerated chondrocytes and hyperplastic synovial lining, but not in the normal joints. These results agree with previous studies that cathepsin B participates in articular cartilage catabolism in osteoarthritic joints[24].

Chondrocytes from normal cartilage contained very few lysosomes and only a minor cell population was cathepsin B positive. A high proportion of chondrocytes from active OA cartilage contained a large number of lysosomes and an excess of cathepsin B in intracellular organelles; the enzyme was also stored in an active form[25]. In this study, minimal to no cathepsin B activity could be detected by immunohistochemistry in either chondrocytes or matrix of normal cartilage. Thus the cathepsin B may act as a plausible marker to differentiate osteoarthritic joints from normal joints. It has been reported that cathepsin B reaches the highest level when macrophages were present in the joint space 20 days after the induction of arthritis[26]. This suggests that the macrophage infiltrate may have stimulated proteinase production in chondrocytes through cytokine release. The profile of proteinase expression also suggests their involvement in the breakdown of cartilage and bone in the arthritic joint[26].

In humans, OA evolves slowly, and its diagnosis is delayed because its initial phase activity is clinically silent. In addition, current non-invasive imaging modality such as radiography, computerized tomography (CT), magnetic resonance imaging, and conventional ultrasonography (5–10 MHz) only allow detection of permanent tissue change. It has been suggested that MRI seems to be a more sensitive way of detecting inflammation, and visualizing destructive bone changes than the conventional radiography in arthritis[27]. However, MRI is still not sensitive enough in OA diagnosis. For example, recurrence of synovitis presented in most knees can only be detected after 2 months.

Improvement in qualitative imaging of OA requires the introduction of high resolution and reliable modalities that allow assessment of the early changes of osteoarthritic joints at the molecular level. Imaging protease activity using optical probes provides morphologic details at the molecular basis. Our data showed the NIRF imaging on the hyperplastic synovial lining and degenerated chondrocytes from experimental osteoarthritis knees within a month. The detectability of the NIRF signal at the early arthritic stage shows that this non-invasive optical imaging technology can potentially be used for early OA diagnosis.

In this study, cathepsin B acts as a biological marker for OA. In addition to cathepsin B, there are other proteases that may be also involved in the pathogenesis of OA, and can serve as biomarkers. For example, metalloproteinases, including stromelysin and collagenase, are a big family of enzymes known to play key roles in cartilage degradation[23]. Cathepsin K, a major osteoclastic peptidase, is known to be involved in bone resorption[24,28]. These proteases are also potential targets for OA diagnosis.

In additional to target selection and probe optimization, imaging systems play important roles in advancing this technique into clinics. NIRF tomographic systems[17] and arthroscopes have to be developed for orthopedic applications. The potential disadvantage of using this optical technology is still the fundamental limitation of light, diffusion. Photons will be scattered in a complex environment including tissue, cartilage, fluid, and bone. High resolution image in patient joints could be a challenge.

In summary, cathepsin B was found up-regulated in osteoarthritic chondrocytes as well as hyperplastic synovium at an early stage of osteoarthritis. Such early physiological changes can be detected using the developed cathepsin B sensitive NIRF probe. The imaging technology may provide a new strategy for early detection of osteoarthritis at the molecular level.

Acknowledgements

Part of this research was supported by NIH R33-CA88365.

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