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Ratiometric Optical Imaging of Subclinical Inflammation with a Thrombin-cleavable Probe: A Future Tool for the In Vivo Visualization of Clinically Silent Synovitis?

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It is universally accepted that effective treatment of rheumatoid arthritis (RA) requires initiation of therapy at the early phase of articular inflammation. In addition to clinical and serological assessments, imaging of joint tissues provides useful information for the early diagnosis of RA (1). Although radiographic (X-ray) imaging can be used to detect bone erosions and to monitor disease progression in terms of bone involvement, X-ray does not detect early synovitis. More advanced musculoskeletal imaging modalities, including magnetic resonance imaging (MRI) and ultrasound (US), have been developed to visualize early inflammatory changes in the synovial tissue of affected joints. MRI and US are now widely used to help diagnose RA in its pre-radiographic stage as well as identify joints with low-grade inflammation in patients during clinical remission of RA (1,2). Optical imaging, employing intravenous fluorescent dyes or fluorescence-tagged probes, has the potential to enable rapid visualization of inflammatory foci within the joints (3). The near-infrared (NIR) fluorescent dye indocyanine green (ICG) has shown promise as a safe and inexpensive agent for the detection of early inflammatory changes in an animal model of arthritis (4) and in patients with various forms of articular inflammation including RA (5,6). In the human studies, performed on the hands, there was good agreement between the results of optical imaging and those of MRI or power Doppler US (5,6). However, ICG is a non-specific probe and its accumulation in the joints largely depends on enhanced vascular permeability within the inflammatory foci. Therefore, there is increasing interest in the development of functional imaging probes that target arthritis-associated molecules within the affected tissues in a disease process-specific manner (3).

In this issue, Friedman and colleagues (7) report successful non-invasive in vivo imaging of established and subclinical synovitis in mouse models of inflammatory arthritis using a thrombin-cleavable dual-fluorescence ratiometric probe. This ratiometric activatable cell-penetrating peptide (RACPP) probe, recently developed by this group (8), takes advantage of fluorescence resonance energy transfer (FRET) and thrombin cleavage. In brief, this 'smart' probe has three components: the NIR fluorophore Cy7 attached to a polyanionic peptide domain, the far-red fluorophore Cy5 attached to a polycationic peptide domain, and a short linker peptide between the two domains having a modified amino acid sequence

(NleTPRSFL) from the cleavage site of thrombin-specific protease-activated receptor (PAR)1 (8). The intact (uncleaved) probe naturally forms a hairpin structure, in which the proximity of fluorophores results in quenching of Cy5 fluorescence (with an emission peak in the far-red spectrum) by Cy7 (with an emission peak in the NIR spectrum) via FRET. Upon thrombin cleavage, the distance between the fluorophores increases and FRET is canceled, resulting in the restoration (increase) of Cy5 fluorescence intensity, a decrease in Cy7 emission intensity, and also in preferential retention of the Cy5-containing polycationic fragment within the tissue. The ratio of Cy5 and Cy7 fluorescence emission serves as the readout of thrombin activity. This ratiometric probe was originally designed to monitor blood coagulation (i.e., thrombus formation) in vivo in real-time at high spatial and temporal resolution (8). The involvement of active thrombin in inflammation, most notably in inflammatory arthritis (9,10), has obviously prompted Friedman and colleagues to utilize the above-described RACPP_{NleTPRSFL} probe for the ratiometric detection of joint inflammation in two animal models of RA. Indeed, the presence of thrombin in rheumatoid joints has been reported before (9). Thrombin, a downstream participant of the coagulation cascade, is not only involved in the proteolytic generation of fibrin (which is also deposited in RA joints) from fibrinogen, but it also can generate the anaphylatoxin C5a from complement component C5. C5a has been shown to attract and activate neutrophils and has been found indispensable for the spontaneous development of arthritis in K/BxN transgenic (Tg) mice (10).

To detect established arthritis with the ratiometric RACPP_{NleTPRSFL} probe, Friedman et al used the spontaneous autoimmune K/BxN Tg model (11) of RA. The passive version of this model, K/BxN serum transfer-induced arthritis (STIA) (11) was utilized for the detection of subclinical inflammation. The authors successfully mapped high Cy5/Cy7 emission ratios to regions of active inflammation in the arthritic (swollen) hind paws of K/BxN Tg mice. The correct anatomical sites of ratiometric signal detection were confirmed by localization of high Cy5 fluorescence intensity within the inflamed synovium in tissue sections prepared from the arthritic joints. Cy5/Cy7 emission ratios also positively correlated with the physical parameters of arthritis such as visually observable swelling and measurable joint thickness. Intriguingly, using this probe in the STIA model, the authors found increased Cy5/Cy7 emission ratios in the yet asymptomatic joints of normal mice 1 day after the transfer of K/BxN serum, suggesting that the probe could be used to detect subclinical synovitis. The localization of the thrombin-cleaved probe predicted the onset of overt arthritis in the STIA model in most, albeit not all, of the joints imaged, but the probe was more effective in predicting full-blown arthritis in the ankle than in the interphalangeal joints. As demonstrated by immunohistochemistry, high Cy5 fluorescence in 1-day STIA co-localized with neutrophils, mast cells, and macrophages infiltrating the synovium as well as with citrullinated histone 3, a marker of neutrophil extracellular traps (NETs) released from neutrophils activated by, at least in part, locally produced C5a fragments. The specificity of thrombin cleavage in generating high Cy5/Cy7 emission ratios was verified by ex vivo treatment of arthritic joint tissue sections with the RACPP_{NleTPRSFL} probe in the presence of a thrombin inhibitor (which abolished the high Cy5 fluorescence signal), and by treatment of the tissue sections with an analog probe having a linker not cleavable by thrombin, which failed to disrupt FRET between Cy7 and Cy5. On the basis of their findings, the authors

hypothesize that reciprocal regulation between thrombin activation and inflammation exists in arthritis, i.e., inflammatory tissue damage can induce tissue factor release with subsequent activation of proteases, including thrombin, in the coagulation cascade, while thrombin can be involved in the recruitment and activation of inflammatory cells upon generation of C5a fragments. Additionally, NETs, produced by activated neutrophils, contain multiple citrullinated antigens that may be recognized by anti-citrullinated protein antibodies (ACPA), potentially leading to immune complex deposition and further recruitment of inflammatory cells in the joints of ACPA-positive RA patients.

The study by Friedman and colleagues adds two pieces of important information to existing knowledge. First, the authors demonstrate that the ratiometric thrombin-cleavable optical probe is capable of detecting clinically silent synovitis in a mouse model of inflammatory arthritis. Second, they outline a molecular mechanism that may drive the vicious cycle between inflammation and the activation of the coagulation system in the arthritic joints. The limitations of the study, acknowledged by the authors, are that imaging of the joints in 1-day STIA was performed at a single time point, and that images were captured only at the medial and plantar aspects of the hind paws, resulting in negative findings for some joints that subsequently became swollen.

While the etiology and time course of inflammatory arthritis are different in mice and humans (12), it is very likely that there are similarities between the two species in the pathology of early synovitis. Moreover, the presence or activity of thrombin has been reported in the inflamed joints of both humans and mice (9,10). The question arises whether the thrombin-cleavable imaging agent described by Friedman et al in the mouse STIA model is suitable for the detection of subclinical arthritis in humans (e.g., in seropositive, but asymptomatic siblings of RA patients) before the onset of RA symptoms, or during clinical remission of established disease in RA patients. Since tissue penetration by far-red fluorescence (emitted by Cy5) is limited compared to NIR fluorescence (emitted by Cy7 or ICG), the ratiometric probe relying on enhancement of fluorescence emission in the far-red spectrum may not be able to perform well in human joints that lie much deeper below the surface than those in mice. In humans, even the NIR fluorescent ICG, with deep tissue penetrance, has been found useful for imaging inflammatory foci only in the small joints of the hand (5,6,13), but not in larger joints. Potential toxicity or other adverse effect of the imaging probe described in the study of Friedman et al is much less of a concern, as an analogous imaging agent with a peptide linker cleavable by tumor cell-released proteases, was approved by the FDA for use in a first-in-human phase I clinical trial (14). The imaging agent (named AVB-620) used in the clinical study was well tolerated upon infusion into patients before they underwent breast cancer surgery, and it helped the surgeons identify residual tumor tissue in the breast area as well as metastatic foci in sentinel lymph nodes intraoperatively (14).

In summary, while the ratiometric $\text{RACPP}_{\text{NleTPRSFL}}$ probe holds promise as a safe and potentially powerful imaging tool capable of detecting silent synovitis in clinical settings, the optical properties of the probe, i.e., the composition or chemistry of fluorophores, may need to be improved to enable deep tissue penetration and effective in vivo visualization of early inflammatory processes in human joints.

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