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A Branched Fluorescent Peptide Probe for Imaging of Activated Platelets

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Abstract: Novel fluorescent probes for thrombi and activated-platelet detection were developed that were based on the glycoprotein IIb/IIIa (GP-IIb/IIIa) binding sequence, Pro-Ser-Pro-Gly-Asp-Trp. Linear, Pro-Ser-Pro-Gly-Asp-Trp-Aha-Gly-Cys(Cy5.5)-NH_2 (1PF), and branched, (Pro-Ser-Pro-Gly-Asp-Trp-Aha)$_2$-Lys-Gly-Cys(Cy5.5)-NH_2 (2PF), fluorescent-labeled peptide probes were synthesized. A third probe, also branched, (Pro-Ser-Pro-Gly-Glu-Trp-Aha)$_2$-Lys-Gly-Cys(Cy5.5)-NH_2 (2CF), was synthesized as control. The platelet-binding activity of the probes was tested in clots generated from human platelet-rich plasma. Fluorescence reflectance imaging results showed that 2PF has a 16-fold increase in fluorescence intensity compared to the autofluorescence of clots. The linear conjugate, 1PF, and free dye did not show appreciable fluorescence enhancement. 2PF fluorescence was also found 5.5-fold higher than that of the control probe, 2CF. Overall, our results suggest that 2PF binds tightly to GP-IIb/IIIa and potentially can be used for in vivo imaging of thrombosis.

Keywords: Activated platelets; thrombi detection; fluorescence; IIb/IIIa; receptor imaging

Vascular thrombosis is the pathological hallmark of clinical syndromes such as myocardial infarction and stroke. These common and life-threatening events are the leading cause of death and disability in developed countries.[1,2] Platelets, along with fibrin, are the major components of vascular thrombi.[3] Platelets originate in bone marrow from the cytoplasm of megakaryocytes and circulate in the vascular system to survey its integrity, discriminating between normal endothelial cell lining and areas with lesions.[4] Platelets circulate in a resting form, and they are activated by a number of stimuli in vascular lesions. Once activated, they adhere to the exposed matrix and trigger a series of signal transduction and activation events.

Glycoprotein IIb/IIIa (GP-IIb/IIIa) is a surface receptor and a major player in the regulation of platelet adhesion and aggregation during thrombus formation.[5] In resting platelets, GP-IIb/IIIa does not bind to soluble ligands. Upon platelet activation by an agonist, a signaling process is initiated, which causes conformational changes within GP-IIb/IIIa. These conformational changes increase the affinity of the receptor for its primary ligand, fibrinogen. Bound fibrinogen then acts as bridging molecule facilitating the interaction of adjacent platelets. Due to its critical role in mediating platelet aggregate formation, GP-IIb/IIIa is a primary target of antithrombotic agents.[6,7]

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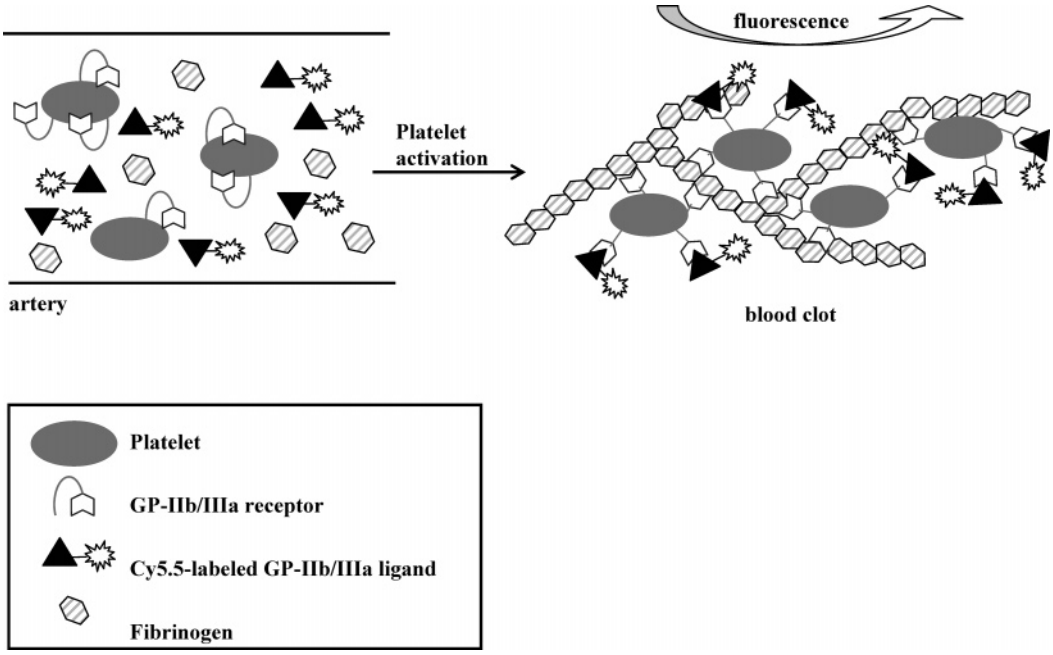

Figure 1. A schematic representation of blood-clot detection by the fluorescence-labeled GP-IIb/IIIa binding probe.

A series of peptidic antagonists against GP-IIb/IIIa have been developed using systemic library screening approaches and shown to be potent inhibitors of human platelet aggregation.[8] For example, the hexapeptide Pro-Ser-Pro-Gly-Asp-Trp has been found to strongly bind to GP-IIb/IIIa (IC_{50} = 0.017 μM), but not to $\alpha_v\beta_3$ integrin (IC_{50} > 100 μM). We reasoned that this peptide sequence, tethered with an appropriate reporter molecule, could be used as an in vivo imaging probe to detect activated platelets and thrombus formation. A schematic representation of our detection mechanism is shown in Figure 1.

Recently, we developed two novel fluorescent imaging probes that shed some light on the critical process of thrombosis: a protease-sensitive probe has been designed to report thrombin activity, and a peptide fragment derived from α2-antiplasmin has been synthesized to target the blood coagulation activated factor XIII (FXIIIa).[9,10] Applying these two probes, we were able to locate thrombi and detect regional enzyme activity in living animals using intravital fluorescence imaging.[11−13] In this paper, we report the progress in targeting another critical component of thrombosis, activated platelets.

Linear, Pro-Ser-Pro-Gly-Asp-Trp-Aha-Gly-Cys(Cy5.5)-NH_2 (1PF), and a branched, (Pro-Ser-Pro-Gly-Asp-Trp-Aha)$_2$-Lys-Gly-Cys(Cy5.5)-NH_2 (2PF), fluorescent-labeled GP-IIb/IIIa peptide probes were prepared that were based on the reported binding sequence, Pro-Ser-Pro-Gly-Asp-Trp (Figure 2). A third probe, also branched, (Pro-Ser-Pro-Gly-Glu-Trp-Aha)$_2$-Lys-Gly-Cys(Cy5.5)-NH_2 (2CF), was synthesized as control by substituting the Asp residues with Glu residues. All peptides were synthesized on an automatic solid-phase peptide synthesizer (Applied Biosystems, 433A), using Rink Amide resin with N-α-FMOC-protected amino acids and standard HBTU coupling chemistry. A Fmoc-Lys(Fmoc) residue was used to generate the branching point for the two-arm peptide syntheses, and 6-aminohexanoic acid (Aha) was added as a spacer. The peptides were cleaved under standard conditions and purified by reverse phase HPLC. Mass spectrometry data were obtained with MALDI-TOF: 2P, M + H = 1811 *m*/*z* (calculated 1811); 2C, M + H = 1841 *m*/*z* (calculated 1839); 1P, M + H = 931 *m*/*z* (calculated 930).

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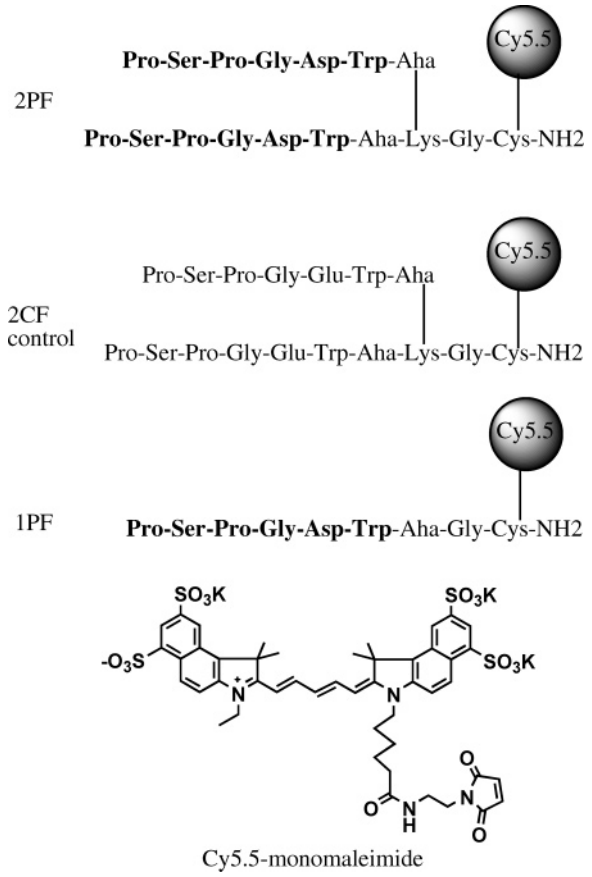

Figure 2. Chemical structures of the synthesized Cy5.5-labeled probes. The GP-IIb/IIIa specific binding sequence is shown in bold. Aha: 6-aminohexanoic acid.

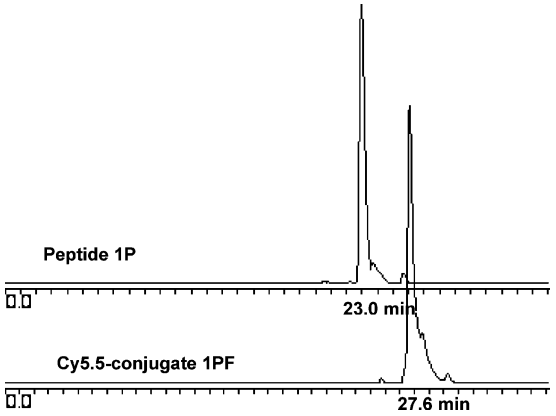

Figure 3. HPLC chromatograms of the peptide 1P and its Cy5.5 conjugate 1PF.

At the C-terminus of the peptide, a thiol-reactive fluorophore, Cy5.5 monomaleimide, was anchored onto the side chain of the cysteine residue in aqueous buffer. The Cy5.5-labeled conjugates were purified by reverse phase HPLC. As shown in Figure 3, the Cy5.5 conjugate 1PF and the free peptide 1P were easily separated by HPLC, eluting at 27.6 and 23.0 min, respectively. All purified conjugates were characterized by MALDI-TOF: 2PF, M + H = 2852 *m*/*z* (calculated 2850); 2CF, M + H = 2879 *m*/*z* (calculated 2878); 1PF, M + H = 1970 *m*/*z* (calculated 1969). The

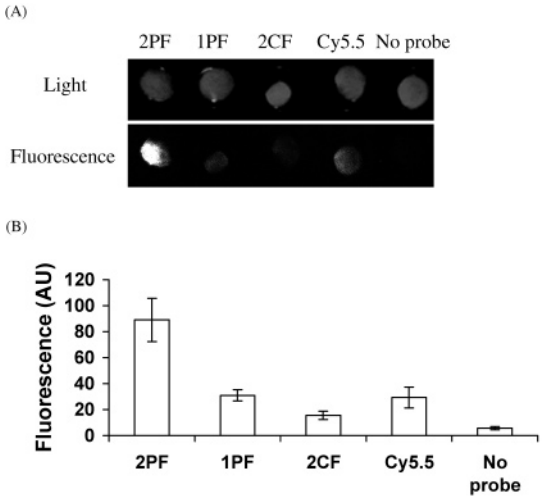

Figure 4. In vitro imaging of platelet binding: (A) light (top raw) and fluorescence (bottom raw) images of blood clot with 5 μM 2PF, 1PF, 2CF, Cy5.5 or saline; (B) quantitated fluorescence intensity of the clots. Experiments were performed in triplicate, mean ± SD.

synthesized conjugates had all the same excitation and emission maxima, at 675 and 694 nm, respectively.

The platelet-binding activity of the synthesized probes was tested with human platelet rich plasma (PRP) obtained by centrifugation of human whole blood of a healthy volunteer (800 rpm, 15 min, room temperature). To generate blood clots, 90 μL of PRP were initially mixed with 2PF, 1PF, 2CF, free Cy 5.5, or normal saline, with a probe final concentration of 5 μM. In a 96-well plate, the PRP/probe solution was then mixed with 5 μL of thrombin (0.5 unit) and 5 μL of 0.4 M $CaCl_2$. Clots were incubated at 37 °C for 90 min and then washed in Tris-buffered saline (TBS), spun in situ in their well plates, and resuspended in TBS. The clots were then imaged using a fluorescence reflectance imaging system with excitation at 630 ± 15 nm and emission at 700 ± 20 nm.[14] All experiments were performed in triplicate.

The imaging results showed that 2PF has a 16-fold increase in fluorescence intensity compared to the autofluorescence of clots. The fluorescence signal of 2PF was also 5.5-fold higher than that of the control probe, 2CF (Figure 4), although the sequence difference between these two probes (Figure 2) was minimal. Structurally, the only difference is that the Asp residues in 2PF were replaced by Glu residues in the control probe 2CF. Both amino acids are negatively charged, but Glu is one methylene longer at the side chain. This result suggests that 2PF binds to a specific and tight pocket of GP-IIb/IIIa which cannot accommodate small structural changes. Such peptide-sequence specificity in GP-IIb/IIIa binding was also reported in the original study by Hayashi et al.[8] In comparison, Cy5.5 by itself seemed to be only nonspecifically trapped inside of the clot, resulting in

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a slight fluorescence signal enhancement. Interestingly, the single arm conjugate, 1PF, was found much less effective than the branched conjugate, 2PF (Figure 4), giving about the same fluorescence as free Cy5.5. The enhanced targeting nature of 2PF suggests the importance of using a branched structure.

An additional advantage of the 2PF branched probe in blood-clot imaging is its low molecular weight (2850 Da).[15,16] Although in the past much effort has been concentrated on producing thrombus-targeted imaging agents using high-molecular-weight antibodies which bind specifically to clot components, the success of these imaging agents has been limited by their long clearance time.[17] In fact, the long residency of unbound agents in the blood usually results in a high background signal, which makes clot detection difficult.[18] Our prior experience with the peptide-based factor XIIIa probe has shown that thrombi in animals can be clearly visualized as early as 30 min after probe injection.[12] We expect that 2PF will have similar pharmacokinetics. Our initial in vivo animal experiments have revealed the potential of 2PF for clot imaging (data not shown). We are undertaking further studies to demonstrate its clinical applicability.

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