Microvascular Reviews and Communications

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Vol.2 No.1

Three-dimensional imaging of growing thrombus *in vivo*

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Abstract

Although several methods have been developed to investigate thrombus or hemostatic plug formation in microvessels *in vivo*, our knowledge on platelet behavior during such a process is still limited. We, therefore, developed new instrumentation which enables us to induce interactions of individual platelets and to detect their behavior with endothelium in and around the growing thrombus. To evoke a platelet reaction, focal hemorrhage was induced with a nitrogen-dye laser focused through the microscope optics using mesenteric microcirculation of the rat. Platelet deposition at the site of injury was then observed during thrombus formation in real-time. The system was able to provide images with high spatial resolution at video-frame rate that could be used to reconstruct three-dimensional model of a thrombus. (Abstract movie) [MVRC 2(1): 8-12,2008]

Key Words: thrombus formation, three-dimensional reconstruction, confocal, real-time, intravital microscopy

Received 2007/7/8, Accepted 2007/8/7

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Introduction

Process of thrombus or normal hemostatic plug formation upon vascular injury is initiated by the activation of circulating platelets. Biological and rheological properties of platelets have been extensively studied in the experimental systems *in vitro* such as in a parallel-plate flow chamber. Because platelet responses are affected by many factors in the normal environment, such as endothelium, other circulating cells, and blood flow *per se*, it is inevitable to examine such interactions *in vivo*. In this regard, several methods have been developed to evoke platelet activation and to observe thromboembolic process *in vivo*¹⁻³⁾. Among these, the most recent technology was developed by Falti *et al.*³⁾ with which vascular injury is induced by laser ablation technology and high-speed, near simultaneous acquisition of images of multiple fluorescent probes and the three-dimensional reconstruction of thrombus were made possible. Here we describe a new intravital imaging system that is essentially a modified technique of Falti *et al.*³⁾. Because the system utilizes an analog 3CCD camera without using binning function, it was possible to acquire the image in real-time, i.e. $3 \times 640 \times 480$ pixels format, 30 frames s^{-1} which enabled us to evaluate a single platelet behavior *in situ*.

Materials and Methods

Preparation of rats for intravital microscopy

Experiments were approved by the local ethical committee on the use of laboratory animals. Male Wistar rats (250-280 g; Clea Japan, Tokyo) were anesthetized with an intramuscular injection of sodium pentobarbital at 50 mg kg^{-1} , and the femoral vein was cannulated with a polyethylene catheter. Platelets circulating *in vivo* were labeled with an intravenous injection of carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, Morikawa et al. Molecular Probes, Inc., Eugene, OR, USA) as described previously^{4,5)}. This compound is membrane-permeable and after it has entered cells it is hydrolyzed by esterase which predominantly occurs in platelets and leukocytes. After the dye infusion, the abdomen was opened and the ileocecal portion of the mesentery was arranged on the surface of the glass-coverslip for intravital observation. The mesentery was superfused continuously with the Krebs-Henseleit buffer saturated with 95% $\text{N}_2/5\% \text{ CO}_2$ at 2.0 ml min^{-1} at 37°C .

Thrombus generation by laser ablation of microvascular walls

Either an arteriole or a venule was chosen and endothelial injury was induced by a pulsed nitrogen-dye laser at 440 nm applied through the microscope objective using the MicroPoint laser system. Arterioles with a diameter of 15-30 μm or venules with that of 20-35 μm were targets for the injury.

Intravital imaging of thrombus formation

The imaging system constructed in this study is a

modified method of Falati *et al.*³⁾. The system was developed around an Olympus BX51W1 up-right microscope with a trinocular head (Olympus, Inc., Tokyo, Japan). Applications of the intravital microscopy in these experiments require either a 40 x (LUMPlanFI/IR, NA 0.8) or a 60 x (LUMFL, NA 1.1) water immersion lens. For confocal microscopy, we used a Yokogawa CSU21 confocal scanner (Yokogawa, Inc., Saitama, Japan) based on Nipkow disk technology. This confocal scanner uses the same pinholes for entrance and exit light beams but is also equipped with a second rotating disk that contains approximately 20,000 pinholes, each with a microlens. The disks rotate together at 1,800 rpm so that the light beams raster-scan the specimen. This technology allows the system to capture up to 360 flashed-frames s^{-1} . An argon-krypton two-line laser (Melles Griot, Carlsbad, CA, USA) provides the fluorescent light for confocal microscopy with excitation at 488 nm and 568 nm. The objective lens was mounted on a piezoelectric driver (Physik Instrumente-PZ73E, PI-Polytec CO., Ltd, Tokyo, Japan) which was controlled by a function generator (SG-4105, Iwatsu, Tokyo, Japan). A schematic representation of the instrumentation is shown in Figure 1.

To generate a laser-induced thrombus, the system was fitted with a nitrogen ablation laser (MicroPoint, Photonic Instruments, St. Charles, IL, USA) which was introduced via the epi-illumination port and was focused on the specimen through the microscope objectives. The output of the laser was at 337 nm but was subsequently tuned through a dye cell. We used coumarin as dye, which emits at 440nm. The laser delivers 4-nsec energy pulses, at a frequency between 3 and 10 Hz, over a surface approximately 1- μm in diameter; the energy of the pulses can be controlled by the operator. In order to conduct consistent ablation, the objective lens was kept at a stationary position until we became certain about a

Fig.1 Schematic representation of the experimental setup. See text in Materials and Methods for details.

successful ablation. The ablation was considered successful if erythrocytes left from the vessel, indicating that all components of the vessel layers were damaged. We then started to operate a piezoelectric driver which allows very fine movement in the vertical direction of the sample.

Video acquisition and three-dimensional reconstruction of developing thrombus

The central part of the trinocular stand was connected to an analog color CCD camera (JK-TU52H, Toshiba, Tokyo) that allowed us to record color images with acceptable spatial and temporal resolutions (640 x 480 pixels format, 30 frames s⁻¹). Analog signals were digitized via a frame grabber (Meteor-II, Matrox Electronic Systems Ltd, Tokyo). The computer had a 3 GHz processor, 1 GB of RAM, and three SCSI hard drives including one 160GB-RAID-0. This computer system was capable to acquire real-time color images for the duration of approximately 90 min. Using a piezo-electric driver on an objective lens, optical slices are obtained. Imaging software (StreamPix ver. 3.7.0, Norpix Inc., Montreal, Quebec, Canada) was used to control the hardware components during image acquisition. Finally acquired images were stacked to generate a three-dimensional reconstruction of a developing thrombus by three kinds of imaging software; StreamPix, IP Lab (ver. 3.6.4, Scanalytics, Inc., Fairfax, VA, USA) and Vox Blast (ver. 3.0, VayTek, Inc., Fairfield, Iowa, USA).

Results

Tissue depth of the formed images

Figure 2 shows a representative image of a three-dimensional reconstruction. Here we first positioned the microscope lens at the center of the venule, namely at the point where the largest diameter was seen. The position of the lens was then moved upwards by 22.5 μm and images composed of 640 x 480 pixels with 1.5- μm steps for the total depth of 45 μm were collected. The 45- μm height of the images was intentionally greater than the diameter of the venule in order to compensate for inexact positioning of the lens before the start of the experiment. As seen in Panel A of the x-z section which transverses to the microvessels on this mesentery, only the top half of the circumference of this venule, but not the bottom half of it was visualized. When the dissecting plane was moved 18.7- μm more deeply (Panel B), the shape of the platelet thrombus on this venular wall became apparent. However, when a further 18.3- μm deeper section was made (Panel C), the system failed to form a sharp image. The result suggests a limitation of this optic system as a whole on the tissue depth of image acquisition which was approximately a 30- μm depth from the tissue surface. Therefore microvessels and thrombi residing in the superficial layer lying no more than 30 μm from the surface were subjected to this study.

Thrombus formation upon laser ablation

Upon the injury by laser ablation, a thrombus started to form typically over a 20 second to 1 min period. Thereafter either did it remain rather constant in size

Fig.2 Three-dimensional images of CFSE-associated fluorescence at 10 min after the initial ablation of the venule. At each x-y confocal plane, a 2D image was obtained and these images were stacked in sequential order with an imaging-software to yield a 3D images of the rat mesentery. The image seen in panel A was reconstituted from twenty-eight different x-y planes covered 45- μm depth of the tissue, and each images are 1.5- μm apart. The x-y dissecting plane was moved 18.7- μm (panel B), and further 18.3- μm more deeply (panel C). Scale bar=30 μm . a, arteriole; v, venule.

with producing small emboli or it embolized as a whole cluster and started to form another thrombus. A small population of platelets tethering at the growing thrombus appeared to change their shape from normal discoid to flattened morphology. Figure 3 (Movie 1) shows such a process between 20 seconds and 40 seconds after the start of laser ablation. Spatial distribution of platelets in and around the thrombus was evaluated by three-dimensional reconstruction.

Figure 4 (Movie 2) and Figure 5 (Movie 3) are the representative three-dimensional images where the scan was made on the longitudinal or radial axis of the venule, respectively. As seen, the optical resolution of the system was sufficient to visualize the interaction between the growing thrombus and circulating platelets on this

Fig.3(Movie 1) Developing thrombus upon laser ablation on a venule. Two dimensional images were acquired between 20 seconds and 40 seconds after the start of laser ablation. Towards the end of this movie, the whole thrombus embolized.

Fig.4(Movie 2) Three-dimensional reconstruction of a thrombus induced by laser ablation on a venule. Spatial distribution of platelets in the thrombus was evaluated by this scanning. Scans were made on the x-z plane (vertical) traversing the longitudinal axis of the microvessels. Note that single platelets in the thrombus were visualized.

Fig.5(Movie 3) Three-dimensional reconstruction of a thrombus induced by laser ablation on a venule. Spatial distribution of platelets in the thrombus was evaluated by this scanning. Scans were made radially on the x-y plane (horizontal) from the top surface to the approximately the centerline of this venule.

venule.

Figure 6 shows temporal changes in thrombus shape. At 0 second, the initial image showed minimal fluorescence. At 20 seconds, platelets started to accumulate on the point of injury on the vessel wall. At 26 seconds, the platelet thrombus expanded further.

Discussion

In the present study, we established an intravital imaging system with which the platelet interaction can be visualized in real-time during thrombus formation on the mesenteric microcirculation. The optical-and the time-resolution of the system were sufficient to visualize the interaction between the growing thrombus and circulating platelets under the current condition. Utilizing an analog camera system offers the advantage that thrombus formation is visualized on-line with high resolution. This approach therefore enabled us to obtain three-dimensional geometry of the growing thrombus and to study the kinetics of thrombus growth *in vivo*.

To examine the process of thrombus formation, assessment must be ideally performed vertically as well as horizontally through the whole volume of a thrombus. The current system enables to rapidly acquire the information for a thrombus residing in the superficial layer lying no more than 30 μm from the mesenteric surface. It could be speculated that due to the light scattering properties of the imaged tissue, confocal images often suffer from significant intensity attenuation in deeper parts of the specimen^{6,7}. The reason could be that both the laser as well as the emitted light are

Fig.6 Three-dimensional reconstruction of a growing thrombus showing temporal changes in shape and volume. At each x-y confocal plane, a 2D image was obtained and these images were stacked in sequential order with an imaging-software to yield a 3D image. Representative images of different x-y planes covered 28- μm depth of the tissue, and each images were 1- μm apart. Bar=10 μm .

absorbed and scattered more in deeper parts of the specimen than in parts near the objective lens. This could have caused a greater rejection of the light from the sample to form the image through an array of microlens of Nipkow spinning disk.

Despite the non-ideality of this system discussed above, the system is able to acquire high-spatial resolution images with video-frame rate that can provide us important information to analyze platelet behavior *in vivo*. As seen in Figure 3 (Movie 1), the system is capable of acquiring very sharp images of individual platelets at the site of growing thrombus. Although circulating free flow platelets were rapidly moving, time resolution appears to be appropriate to detect the behavior of tethering and/or adherent platelets of which are the majority in and around a thrombus without severe blur along the longitudinal axis of the vessel (Fig. 4 and 5 (Movie 2 and 3)).

With the laser-induced injury, we observed that as soon as the endothelium was damaged by the laser, platelets rapidly started to accumulate to form a hemostatic plug which was confined at the injured site (Fig. 6). Our methodology allowed us to examine process and content of growing thrombus at the sites of injured vasculature. At present, our system is equipped to capture two different fluorophores that can be excited by 488 nm and 568 nm without crossover of fluorescence. To gain the insight for the kinetics of growing thrombus, its laser source and filter systems should be developed further to visualize the components of growing thrombus other than platelets, e.g. fibrin and tissue factors, which was accomplished by the system of Falati *et al*³). In conclusion, the current instrumentation enables us to unravel the biological and rheological properties of single platelet behavior in and around a developing thrombus *in vivo*.

Acknowledgements

This work was supported by Health Labour Sciences Research Grant, Research on Advanced Medical Technology from the Ministry of Health Labour and Welfare (to M.K.), and Grant-in-Aid for Creative Scientific Research, Leading Project for Biosimulation, the 21st Century Center-of-Excellence Program from the Ministry of Education, Culture, Sports, Science and Technology in Japan (to M.S.). Our gratitude goes to Mr. Toshihiro Ochiai who immensely helped us to make this system work.

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