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Dynamic pathology for leukocyte–platelet formation in sepsis model

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ABSTRACT

Background: This study aimed to evaluate the dynamic pathology for *in vivo* real-time leukocyte–endothelium–platelet aggregation in a mouse model of sepsis.

Materials and methods: A lipopolysaccharide-induced model of sepsis was analyzed in green fluorescent protein transgenic mice using two-photon laser-scanning microscopy (TPLSM). The real-time process of leukocyte–endothelium–platelet complex (LEPC) formation was assessed *in vivo* using blood flow dynamics.

Results: TPLSM allowed direct visualization of LEPC formation at the single-platelet level. Leukocytes rolling number and speed, blood flow velocity, and shear rate gradually decreased with time during the acute phase of sepsis compared with those in the control groups. The number of adherent leukocytes and platelet counts gradually increased over time in the septic group. In the septic group, microcirculatory dysfunction was seen in the postcapillary venules before the capillaries.

Conclusions: In vivo real-time imaging and analysis of LEPC formation can be achieved with little inter-experimental variation using TPLSM. In the acute phase of sepsis, new treatment strategies should target the postcapillary venules because their LEPC formation and blood flow dynamics start to change before those in the capillaries.

1. Introduction

Microvessel occlusion, followed by microvascular thrombus formation and hypoperfusion, is a major contributor to organ dysfunction during sepsis [1]. It is well recognized that sepsis involves a complex interaction between the inflammatory and coagulation systems [2]. During inflammation, leukocyte–endothelium–platelet complex (LEPC) is observed in *in vitro* and *in vivo* models of sepsis.

Leukocyte–endothelium interaction is a prerequisite for the recruitment of leukocytes during acute inflammation [3,4]. This process can be resolved into successive steps of leukocyte margination, rolling, firm adhesion, and transendothelial and extravascular migration [4]. Although basic aspects of leukocyte adhesion and interaction with platelets were studied intensively in recent decades, the intravascular leukocyte movement with platelets *in vivo* was poorly investigated. *In vitro* thrombus formation was extensively studied using

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techniques common to the study of biochemical processes, cell biology, and molecular biology. Given the large number of variables in this complex process, it is necessary to study thrombosis in an intact living animal in the presence of an intact blood vessel wall, circulating blood, and all the blood components. In recent years, the progress in digital technologies has brought strong innovative impulses for the improvement of standard methods in biological imaging. In our previous study, we reported that combination with two-photon laser-scanning microscopy (TPLSM) and organ-stabilizing systems could identify early thrombus formation at a single-platelet level *in vivo* [5]. Thus, it is now possible to study thrombus formation precisely (at the single-platelet level) in live mice. This technique includes a continuous video documentation of leukocyte movement with optional time compression. It also allows the monitoring of every single leukocyte and platelet and offers dramatic improvements to study leukocyte and platelet locomotion with blood flow dynamics in the intravascular space. The results of the study showed that almost all circulating leukocytes attached to the endothelium do not adhere for a long time. Rather, they move inside the blood vessel, detach in most cases from the endothelium, or transmigrate through the endothelial wall after proinflammatory stimulation. The phenomenon of "intravascular crawling" reflects the migratory activity of circulating leukocytes and depends on the expression of adhesion molecules.

Although there have been recent studies of thrombus research using TPLSM [6–9], detailed thrombus formation and dynamic blood flow analysis under septic condition remain obscure. Even though recent studies developed some excellent thrombus visualizations using single-photon microscopy and other systems, the advantages of TPLSM for thrombus investigation in septic conditions have not previously been reported. Thus, in deep tissues such as the microvessels of the intestinal mucosal layer, long-term monitoring of *in vivo* real-time thrombus formation and blood flow analysis under septic conditions remain a technical challenge.

In this study, we demonstrated high-resolution and high-contrast *in vivo* real-time TPLSM imaging of LEPC formation and analysis of blood flow dynamics in postcapillary venules and capillaries in an acute-phase septic model. This model could be used for initial screening or validation of novel antiseptic agents.

2. Materials and methods

2.1. Transgenic mice

Enhanced green fluorescent protein-transgenic C57/BL6-Tg (CAG-EGFP) mice were purchased from Japan SLC Inc (Shizuoka, Japan). Male green fluorescent protein (GFP) mice aged 10–12 wk (20–22 g) were bred, housed in groups of six mice per cage, fed a pelleted basal diet (CRF-1; Oriental Yeast Co Ltd, Tokyo, Japan), and provided with free access to drinking water. Mice were kept in the animal house facilities at Mie University School of Medicine under standard conditions of relative humidity (50 ± 10%), temperature (23 ± 2°C), and light (12/12 h light–dark cycle) according to the Institutional Animal Care Guidelines. The experimental protocols were reviewed and approved by the Animal Care and Use Committee at Mie University Graduate School of Medicine.

2.2. Lipopolysaccharide

Lipopolysaccharide (LPS) from *Escherichia coli* serotype 0128:B12 was purchased from Sigma–Aldrich (St Louis, MO). The stock solution was made by dissolving it in appropriate concentrations with distilled water for the *in vivo* study. LPS (10 mg/kg) was administered to the acute septic model mice, and 0.9% NaCl equivalent to the LPS final solution volume was administered to the control group.

2.3. Femoral venous catheterization

To administer accurate doses of LPS reliably, a catheter (M-FAC/FVC; Neuroscience, Tokyo, Japan) was placed in the right femoral vein of anesthetized GFP mice under surgical microscopy.

2.4. Organ stabilization for intravital imaging

After femoral venous catheterization, GFP mice were anesthetized using an anesthetic mask with 4 L/min isoflurane (4%; Forane; Abbott, Tokyo, Japan). Anesthetic maintenance was achieved using 1.5%–2% isoflurane. Body temperature was kept at 37°C throughout the experiments using a heating pad. Normal saline (200 μL) was administered at 1–2-h intervals for hydration during anesthesia through laparotomy. Lower midline laparotomy was made as short as possible (<15 mm). The cecum and terminal ileum were identified through laparotomy. After exteriorization of the cecum, air was introduced through the tip of the collapsed cecum using a syringe with a small needle. Optimal inflation of the cecum enabled us to visualize vertically all layers of the cecum by observing it through the serosa into the mucosa (serosal-approaching technique). The cecum was placed on a wet gauze and kept moist during the experiments. The inflated cecum was put onto an organ-stabilizing system using a solder lug terminal with an instant adhesive agent (KO-10-p20; DAISO, Higashihiroshima, Japan). This organ stabilizer minimized the microvibration of the observed area caused by heart beats and respiratory movements. Stabilization and fixation of the cecum represented a critical but technically difficult part of the intravital TPLSM procedure. After the application of phosphate-buffered saline to the observed area, a thin cover glass was placed gently on the cecum surface.

2.5. TPLSM setup

The procedures for TPLSM setup were performed as previously described [5]. Experiments were performed using an upright microscope (BX61WI; Olympus, Tokyo, Japan) and an FV1000-2P laser-scanning microscope system (FluoView FV1000MPE; Olympus). The use of special stage risers enabled the unit to have an exceptionally wide working distance. This permitted the stereotactically immobilized mice to be placed on the microscope stage. The microscope was fitted with several lenses with high numeric apertures to provide the long