Visualization of in vivo thromboprophylactic and thrombolytic efficacy of enoxaparin in laser-induced vascular endothelial injury model using multiphoton microscopy

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Abstract: Enoxaparin is used postoperatively for the prevention of venous thromboembolism. In vitro studies and clinical trials have demonstrated the anticoagulant and antithrombotic efficacy of enoxaparin. In this study, we visualised thromboprophylactic and thrombolytic efficacy of enoxaparin in a laser-induced thrombus formation model in vivo using two-photon laser-scanning microscopy (TPLSM). Thrombus was induced by the selective irradiation of vascular endothelium in arterioles of the cecum of green fluorescent protein transgenic mice. The thromboprophylactic and thrombolytic efficacy of enoxaparin was visualised in vivo real-time using TPLSM. Platelet adhesion, aggregation, and platelet-dependent thrombus formation were observed in the laser-induced thrombus formation model with reproducibility. Laser-induced thrombus formation was significantly inhibited by enoxaparin pretreatment as the thromboprophylactic agent, as compared with control. The mean thrombus volumes were 652 microcubic meters in mice pretreated with enoxaparin and 8906 microcubic meter in control mice. Enoxaparin reduced the volume of laser-induced thrombus when using it as a thrombolytic agent. The mean rate of reduction was 59 percent. In a lipopolysaccharide-induced sepsis model, thromboprophylactic efficacy of enoxaparin was also observed in vivo in real-time. In vivo thromboprophylactic and thrombolytic efficacy of enoxaparin can be visualised at the single platelet level in the laser-induced endothelium injury model using TPLSM.

Keywords: Enoxaparin, thrombus formation, intravital imaging, multiphoton microscopy

Introduction

Venous thromboembolism (VTE), which manifests as deep-vein thrombosis or pulmonary embolism, is a common complication of cancer [1]. Cancer patients undergoing general surgery have at least twice the risk of postoperative deep-vein thrombosis and more than three times the risk of fatal pulmonary embolism [2]. The American College of Chest Physicians guidelines recommend anticoagulant thromboprophylaxis with low molecular weight heparin (LMWH) for higher-risk general surgery patients undergoing a major procedure for cancer.

Enoxaparin is one of the LMWHs that binds to and increases the activity of antithrombin III. Enoxaparin exhibits a more favourable pharmacological side effect profile compared with unfractionated heparin. Clinical trials have demonstrated the anticoagulant and antithrombotic efficacy of enoxaparin for VTE prophylaxis in cancer patients undergoing abdominal and pelvic surgery [3-5]. Therefore, enoxaparin is still the most widely used drug in the management of VTE with substantial efficacy and safety.

In vitro studies have greatly advanced our understanding of the molecular bases of haemostasis and thrombosis by identifying enzymes, cofactors, cell receptors, and associated ligands related to the haemostatic process and its regulation [6-8]. Despite these great advances, in vivo knowledge of comprehensive interactions by all components related to the haemo-
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Two-photon laser-scanning microscopy (TPLSM) has revolutionised in vivo real-time imaging with the benefits of higher resolution, increased tissue penetration, and less photodamage (or photobleaching). We previously reported a method of in vivo real-time imaging of laser-induced thrombus formation using TPLSM with an organ stabilising system, which allows high magnification (×600 or higher) and high resolution (at the single platelet level) images in living animals [9-15].

To reproduce the molecular bases of haemostasis and thrombosis in vitro, we observed the thromboprophylactic and thrombolytic effect of enoxaparin on platelet-dependent thrombus formation by laser-induced endothelium specific injury using green fluorescent protein (GFP) transgenic mice. We also observed the thromboprophylactic effect of enoxaparin in a lipopolysaccharide (LPS)-induced sepsis model.

Materials and methods

Ethics statement

This study was reviewed and approved by the Institutional Review Board and the Local Ethics Committee of the Mie University Graduate School of Medicine (No. 24-26). Written informed consent was obtained from all the patients (adults) enrolled onto the study.

The experimental protocols of in vivo studies were reviewed and approved by the Animal Care and Use Committee at the Mie University Graduate School of Medicine.

Mice

Enhanced green fluorescent protein (EGFP)-transgenic C57/BL6-Tg (CAG-EGFP) mice were purchased from Japan SLC Inc. (Shizuoka, Japan). Ten to 12-week-old male GFP mice (20–22 g) were bred, housed in groups of six mice per cage, and fed with a pelleted basal diet (CE-7; CLEA Japan Inc., Tokyo, Japan), and had free access to drinking water. Mice were kept in the animal house facilities at Mie University School of Medicine under standard conditions of 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 the Mie University Graduate School of Medicine.

Enoxaparin

Enoxaparin sodium was purchased from Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan). The stock solution was made by dissolving it in appropriate concentrations with distilled water for the in vivo study.

LPS

LPS (E. coli, serotype 0111:B4) was purchased from Sigma-Aldrich Co., LLC. (St Louis, MO, USA). The stock solution was made by dissolving it in appropriate concentrations with distilled water for the in vivo study.

Femoral venous catheterisation

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

Organ stabilization for intravital imaging

After femoral venous catheterisation, GFP mice were anaesthetised using an anaesthetic mask.
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with 4 L/min of isoflurane (4%; Forane, Abbott, Japan). Anaesthetic maintenance was achieved using 1.5%-2% isoflurane and 4 L/min of O₂. 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 anaesthesia through laparotomy. Lower midline laparotomy was made as short as possible (< 15 mm). The cecum and terminal ileum were identified through laparotomy. After exteriorisation 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 visualise vertically all layers of the cecum by observing it through the serosa into the mucosa (serosal-approaching technique). The cecum was placed on wet gauze and kept moist during the experiments. The inflated cecum was put onto an organ stabilising system using a solder lug terminal with an instant adhesive agent (KO-10-p20, DAIso, Japan). This organ stabiliser minimised the microvibration of the observed area caused by heart beats and respiratory movements. Stabilisation and fixation of the cecum represented a critical but technically difficult part of the intravital TPLSM procedure. After the application of PBS to the observed area, a thin cover glass was placed gently on the cecum surface.

TPLSM setup

The procedures for TPLSM setup were performed as previously described [10]. Experiments were performed using an upright microscope (BX61WI; Olympus, Tokyo, Japan) and a FV1000-2P laser-scanning microscope system (FLUOVIEW FV1000MPE, Olympus, Tokyo, Japan). The use of special stage risers enabled the unit to have an exceptionally wide working distance. This permitted the stereotactically immobilised mouse to be placed on the microscope stage. The microscope was fitted with several lenses with high numeric apertures to provide the long working distances required for in vivo work, and with water-immersion optics. The excitation source in TPLSM mode was Mai Tai Ti: sapphire lasers (Spectra Physics, Mountain View, CA, USA), tuned and mode-locked at 910 nm. The Mai Tai produces light pulses of approximately 100 fs width (repetition rate of 80 MHz). Laser light reached the sample through the microscope objectives, connected to an upright microscope (BX61WI; Olympus, Tokyo, Japan). The mean laser power at the sample was between 10 and 40 mW, depending on the depth of imaging. Microscope objective lenses used in this study were the 4×UPlanSApo (numerical aperture of 0.16), the 10×UPlanSApo (numerical aperture of 0.4), and the 60×LUMPlanFI/IR (water dipping, numerical aperture of 0.9, working distance 2 mm). Data were analysed using the FV10-ASW (Olympus). TPLSM images were acquired with 512×512 pixels of spatial resolution, from a 210 μm field of view dimension, using a pixel dwelling time of 4 μs. Two-photon fluorescence signals were collected by an internal detector (non-descanned detection method) at an excitation wavelength.

Imaging methods using TPLSM

The surface of the cecum was initially screened at lower magnifications by setting out the X/Y plane and adjusting the Z axis manually to detect the optimal observation area. Each area of interest was subsequently scanned at a higher magnification (water-immersion objective 60× with or without 2× zoom) by manually setting the X/Y plane and adjusting the Z axis (either automatically or manually) to obtain...
high-resolution, clear TPLSM images. The scanning areas were 200×200 μm (600×) or 100×100 μm (600× with 2× zoom). The imaging depth or imaging stack was determined arbitrarily to allow real-time three-dimensional visualisation in vivo. The laser power was adjusted according to the imaging depth. When imaging at larger depths, we increased the laser power level (up to 100%) manually using the laser power level controller.

Our organ stabilising technique enables imaging of tissue microvasculature at high magnification (×600 or higher) and high resolution (at the single platelet level) in vivo in real-time using TPLSM. By using our system, we can perform laser irradiation for the vascular endothelial layer of cecal arterioles selectively and reproducibly under in vivo real-time imaging of the cecal arterioles at high magnification and high resolution [10].

Laser-induced thrombus was formed in serosal arterioles of the cecum of 30 to 100 μm in diameter according to the method of Nishimura et al [16]. The vascular endothelium was visualised and identified as a thin layer of the luminal surface of the top of the vessel, which is different from vascular smooth muscle. Laser irradiation at the two-photon wavelength of 910 nm (a Mai Tai Ti:sapphire laser light applied through a ×60 water-immersion microscope objective) was performed for the selected endothelial region for 15 to 40 sec. Laser pulses (910 nm; joules, maximum up to 192 mW) were delivered along the transverse axis of the target endothelium (via the 60× microscope objective with
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Irradiation intensity (W/cm²) was set depending on the vessel diameter. After identification of photobleaching of vascular endothelium, the laser power was reduced for in vivo imaging of thrombus formation to avoid additional photobleaching, extravasation, and undesirable vessel rupture.

The sophisticated and selective irradiation of the endothelial layer by using digital zoom (total magnification was approximately \( \times3000-6000 \)) enabled reduction of undesirable target tissue damage [17].

**In vivo thromboprophylactic effect of enoxaparin**

Enoxaparin at a dose of 1 mg/kg or PBS (the same volume as enoxaparin) was administered intravenously via the femoral venous catheter. Thirty minutes later, laser-induced endothelium injury was achieved for real-time imaging of the thromboprophylactic effect of enoxaparin. Thrombus formation was imaged in both enoxaparin-pretreated mice and control mice.

We also imaged the thromboprophylactic effect of enoxaparin under the LPS-induced sepsis model in vivo.

**In vivo thrombolytic effect of enoxaparin**

Laser-induced thrombus was performed by irradiating endothelia of the cecal arterioles. One hour later, platelet-dependent thrombus formation was observed. Enoxaparin at a dose of 1 mg/kg or 5 mg/kg was then administered intravenously via the femoral venous catheter for real-time imaging of its thrombolytic effect.

**Results**

**In vivo real-time imaging of blood flow in arterioles of murine cecum**

Within the vessels of GFP mice, leukocytes were recognised as large, round cells and platelets were recognised as small cells. In contrast, erythrocytes were not identified [18]. Under normal blood flow of the arterioles, leukocytes or platelets seldom adhered to the endothelium (Figure 1 and Supplementary movie 1).

**Platelet-dependent thrombus formation by laser-induced endothelium injury**

The endothelium of cecal arterioles was identified and then injured by a laser of 192-mW of power for 15-40 sec at 900 nm as mentioned above (Materials and Methods).

Figure 4. Thrombolytic effect of enoxaparin in the laser-induced thrombus model in vivo. The left panel (A) indicates an almost complete obstruction 60 min after laser-induced thrombus formation, resulting in a near complete cessation of blood flow. Supplementary movie 7 shows a process of thrombus development with the adhesion of rolling platelets on the surface of the growing thrombus. The right panel (B) shows in vivo thrombolytic efficacy of enoxaparin for laser-induced thrombus. Supplementary movie 8 shows a process of thrombolysis with the release of aggregated platelets on the surface of the thrombus at the single platelet level.
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After irradiation, photobleaching was observed on the luminal surface of the top of the vessel wall (Figure 2 and Supplementary movie 2). The mean length of such photobleaching was 20.7 ± 1.78 μm (mean ± standard deviation). There was no significant difference in the length of photobleaching between enoxaparin-pretreated mice and control mice, suggesting an achievement of a similar degree of laser-induced endothelium injury for each mouse.

The dynamic process of laser-induced thrombus formation has been described previously [10]. In brief, individual platelets were translocated on the surface of the laser-injured endothelium. Platelets then attached and adhered to the endothelium downstream of the laser-injured region. Gradually, they adhered to the laser-injured endothelium in a linear fashion (straight line formation). Circulating platelets adhered to the surface of the linear platelet adhesion. Platelet-dependent thrombus was formed forward to the upstream of the laser-injured endothelium with cycles of attachment and detachment of platelet aggregates. The thrombus developed in size with the adhesion of rolling platelets on the surface of the growing thrombus.

Approximately 60 min after laser-induced endothelial injury, platelet-dependent thrombus was formed, with a mean size of 8906 ± 1469 μm³ (n=5 arterioles from three mice).

**Thromboprophylactic effect of enoxaparin in the laser-induced thrombus model in vivo**

Initially, enoxaparin at a dose of 5 mg/kg was used to visualise in vivo antithrombotic efficacy in the laser-induced endothelium injury model.

Figure 3 (Supplementary movies 3-6) shows the time course of laser-induced thrombus formation and in vivo thromboprophylactic efficacy of enoxaparin in the laser-induced endothelium injury model. No thrombus was formed up to 60 min following irradiation using enoxaparin at a dose of 5 mg/kg. To compare laser-induced thrombus size between enoxaparin-pretreated mice and control mice, enoxaparin at a dose of 1 mg/kg was adopted. The mean volumes of laser-induced thrombus 60 min after endothelial injury were 652 ± 432 μm³ in enoxaparin-pretreated mice (n=5 arterioles from three mice) and 8906 ± 1469 μm³ in control mice (n=5). Intravenous administration of enoxaparin at a dose of 1 mg/kg significantly inhibited laser-induced thrombus formation (p < 0.01),
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as compared with control (no-exoxaparin pretreatment).

Thrombolytic effect of enoxaparin in the laser-induced thrombus model in vivo

To visualise the in vivo thrombolytic effect, enoxaparin at a dose of 1 mg/kg was administered intravenously 60 min after irradiation of the vascular endothelium. The mean thrombus volume 60 min after laser-induced endothelial injury was 8366 ± 2037 μm³ (n=3 arterioles from three mice). The mean thrombus volume 60 min after enoxaparin administration was 3643 ± 3384 μm³ (n=3 arterioles from three mice).

The rates of reduction in thrombus volume by enoxaparin administration were 16.1%, 66.6%, and 94.3% in each mouse. The mean rate of reduction was 59% ± 39.7%. Intravenous administration of enoxaparin at a dose of 5 mg/kg significantly decreased the size of laser-induced thrombus, as compared with control (no-exoxaparin treatment).

Figure 4 shows an almost complete obstruction 60 min after laser-induced thrombus formation, resulting in a near complete cessation of blood flow. Supplementary movie 7 shows a process of thrombus development with the adhesion of rolling platelets on the surface of the growing thrombus.

Figure 4B shows in vivo thrombolytic efficacy of enoxaparin for laser-induced thrombus. Supplementary movie 8 shows a process of thrombolysis with the release of aggregated platelets on the surface of the thrombus at the single platelet level.

Thromboprophylactic efficacy of enoxaparin in the LPS-induced sepsis model in vivo

Postoperative surgical site infection is one of the major concerns among patients undergoing major gastrointestinal surgery because it may develop into lethal sepsis. In general, sepsis causes haemostatic abnormalities and coagulation disorders, resulting in disseminated intravascular coagulation or multiple organ failure/injury. Therefore, we also sought to examine the thromboprophylactic effect of enoxaparin in the LPS-induced sepsis model.

We found that the time to maximum thrombus volume (approximately 8000 μm³) after laser irradiation was shorter in LPS-administered mice (approximately 30 min after laser-induced endothelium injury) than in control mice (approximately 60 min after injury), indicating hypercoagulation due to LPS.

In the early phase of laser-induced thrombus formation, platelets attached and adhered to the endothelium downstream of the laser-injured region (straight line formation) (Figure 5A and Supplementary movie 9). We also observed that enoxaparin pretreatment disturbed straight line formation of platelets in LPS-administered mice, confirming in vivo thromboprophylactic efficacy of enoxaparin with LPS-induced sepsis (Figure 5B and Supplementary movie 10).

Discussion

The mechanisms underlying platelet aggregation and thrombus formation have been extensively investigated in in vitro studies [19, 20]. Animal models (in vivo studies) for vascular thrombosis have facilitated our comprehensive understanding of physiological and pathophysiological thrombus formation [21, 22]. Furthermore, intravital imaging of thrombus formation in living animals provides a direct visualisation of the dynamic process of platelet adhesion, aggregation, and thrombus formation in real-time [17, 23, 24].

Previously, we reported a method of in vivo real-time imaging of laser-induced thrombus formation using TPLSM with an organ stabilising system, and demonstrated platelet-dependent thrombus development, including platelet translocation, adhesion, aggregation in a linear fashion, and thrombus growth with the adhesion of rolling platelets on the surface of developing thrombus after laser-induced endothelium specific injury [10].

Strengths of our study protocol include clear visualisation of the vascular wall layer at higher magnification and higher resolution because of intravital TPLSM with an organ stabilising system, and sophisticated and reproducible laser irradiation of vascular endothelium owing to clear and nearly static visualisation of vascular layers. In addition, intravascular events, such as platelet-platelet interactions and platelet-endothelial interactions, may be more physiological (or pathophysiological) because there is
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no need to label an antibody for platelet visualisation in cellular interactions in vivo. Therefore, we believe that our results of thromboprophylactic and thrombolytic efficacy of enoxaparin in an in vivo laser-induced vascular endothelial injury model are more reliable and reproducible, compared with those of previously reported procedures [17, 25, 26].

Enoxaparin, one of the LMWHs, has been widely used as an anticoagulant and antithrombotic drug with similar features to heparin in vitro and in vivo, based on its antithrombin III-activating properties [27, 28].

In this study, we visualised in vivo thromboprophylactic and thrombolytic efficacy of enoxaparin at the single platelet level. The thromboprophylactic effect of enoxaparin was strong and constant even at a dose of 1 mg/kg. The thrombolytic effect of enoxaparin was also strong based on the rate of reduction in thrombus size (> 50%). The thromboprophylactic effect of enoxaparin in the LPS-induced sepsis model was also observed. These results complement and support the data regarding thromboprophylactic and thrombolytic effects of enoxaparin previously demonstrated by in vitro and in vivo studies, and clinical trials.

Several limitations of our study protocol should be taken into account when interpreting our results. Species differences in the efficacy of enoxaparin on thrombogenesis and thrombosis between humans and mice should be considered. The methods and dosages of enoxaparin administration between our experimental model (intravenous) and clinical use (subcutaneous) are different.

However, intravital TPLSM imaging of thrombus formation at high resolution (at the single platelet level) and high magnification (×600 or higher) is required for further understanding of physiological haemostasis and thrombosis. Our methods may help screen and evaluate new anticoagulant or antiplatelet drugs in preclinical murine thrombosis models.

In conclusion, in vivo thromboprophylactic and thrombolytic efficacy of enoxaparin can be visualised at the single platelet level in the laser-induced endothelium injury model using TPLSM. Our methods may help screen and evaluate new anticoagulant or antiplatelet drugs in preclinical murine thrombosis models.

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Disclosure of conflict of interest

K.T., A.M. and M.K. designed and performed experiments and analyzed and interpreted data and wrote the manuscript. K.T., Y.K., K.M., and M.O. performed experiments and analyzed data. Y.T., M.K., S.S., Y.O., Y.I., K.U., T.A., and Y.M. designed experiments and analyzed and interpreted data. A.M. and M.K. designed experiments and interpreted data.

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