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Abstract. Activity of blood cells, erythrocytes, leucocytes, and platelets, in microcirculation was observed by using an intravital microscope and confocal laser scanning microscope connected with an image processing system including fluorescence and phosphorescence emission methods. Dynamic functions of the blood flow were mainly observed in mesentery, brain, and liver tissues of rats. The results are summarized as follows: Deformability of diabetic erythrocytes was significantly lower than that of healthy controls, particularly at high shear rate. The spring constant and Young’s modulus of diabetic erythrocytes obviously stiffened, making them hard to deform in the capillary. During hemorrhagic shock and thrombosis, flow velocity and oxygen partial pressure of blood decreased in the brain and liver tissues that can be visualized by using FITC stained erythrocytes and Pd-porphyrin derivative as a pO₂ probe. Platelet adhesion and thrombus formation in the micro-vessels accelerated under the photodynamic reaction; diabetic platelets showed augmented adhesion and aggregation on the vessel wall which was followed by acute thromboembolism. Active oxygen radicals take part in thrombus formation, accompanied with adhesion of the activated leucocytes. Fluorescent dye probes, rhodamine G and acridine orange, are quite useful for visualization of the flow behavior of platelets and leucocytes, respectively.

Keywords: microcirculation, erythrocyte deformability, hemostasis, active oxygen, image analysis

Introduction

Microcirculation is the generic name for the finest level of the circulatory system consisting of arterioles, capillaries, and venules whose diameters range from 5 to 50 μm. They play very important roles to maintain homeostasis and tissue metabolism as well as being involved in many types of disease. In order to elucidate mal-function of tissue metabolism, measurement of blood flow velocity or volume and oxygen tension is essential for studies on the microcirculation. Target locations for observing the microcirculation with a microscope are limited to tissues that are close to the body surface or can be exteriorized and extended under the microscope. In case of targeting thin and transparent tissues, like a mesentery, we can observe not only a vascular bed but also the dynamic behavior of each blood cell clearly. Blood flow in a parenchymatous organ microcirculation with some thickness, however, can be visualized using a fluorescent microscope by labeling blood cells with fluorescent dye. A fundamental process of the oxygen supply to tissue is the unloading of oxygen molecules from erythrocytes in the capillary network. Under the healthy condition, erythrocytes can deform, enabling them to flow smoothly through the capillary with low shear stress, so organ tissues receive efficient oxygen and the inner surface wall of capillary

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is less injured by the flow. Decrease in the deformability of erythrocyte, however, leads to high shear stress and significant increase in flow resistance of the blood circulation that may be followed by a low oxygen supply. Since the hardness of erythrocytes may affect the growth of microangiopathy, it is very important to investigate the relationship between deformability of erythrocytes and hardness of the cell membrane under various flow shear rates (1). As for measuring deformability of erythrocytes as well as the adhesive characteristic of leucocytes and platelets, in vitro methods using membrane filter (2), micro-pipette (3), or micro-array channel silicon device (4, 5) have been used; however, hemo-dynamical properties of each cells were not sufficiently investigated. As well known, leucocytes and platelets play important roles to activate immune function and thrombus formation. Inherent and healthy tissue metabolism also depends on the adhesive properties of both cells related with abundant oxygen uptake from the blood flow in the microcirculation system. In order to measure blood oxygen tension (pO$_2$) in vivo, the oxygen electrode method has mostly been used; however, if electrodes are used to measure micro-vessels, their edges will cause vascular wall injury and blood flow stasis, as is essentially inevitable with physically invasive techniques. Moreover, it is difficult to fix the measurement point, and the spatial resolution is not sufficient for observing micro-vessels. There have been some studies to quantify oxygen tension in micro-vessels optically using phosphorescent intensity and lifetime (6, 7). Since the blood flow distribution and pO$_2$ are closely related in the microcirculation system, a practical system to measure both data simultaneously is very much required. In our study, a new system to measure blood flow velocity, vessel diameter, and blood pO$_2$ simultaneously and continuously even in a thick parenchymatous organ such as the pial, kidney, and liver was realized. Animal experiments showed the effectiveness of the system for measurements of each parameter during asphyxia and acute hemorrhage shock models.

**Deformability and elasticity of erythrocytes**

The diameter of the human erythrocyte is about 7 $\mu$m, while the inner diameter of capillary is less than 7 $\mu$m. When the erythrocyte goes through a capillary, it deforms to a parachute-like shape easily even with little force and reforms its original shape quickly when the force is taken away. Deformability of erythrocytes is a crucial factor for maintaining smooth blood flow in the microcirculation supplying oxygen and nutrients to organic tissues. Decrease in the deformability may depend on the elasticity of the erythrocyte, especially on the cell membrane structure that leads to high shear stress on the blood vessel wall and significant increase in flow resistance. Since high stiffness of erythrocytes may affect on the growth of microangiopathy, it is very important to investigate elasticity of the cell in relation to the deformability. By in vivo measurement, we can observe the deformation of erythrocytes under an intra-vital microscope, but it is troublesome to analyze the relationship between the deformability and flow shear rate. From this point of view, a micro-channel flow system made of a crystal glass plate was developed and used for analysis of the erythrocyte deformability as a capillary model (1, 8). Flow channels of fifty narrow ditches were straight cut on the glass plate by means of silicon micro-machining technology, as shown in Fig. 1. The width of each ditch is 9.1 $\mu$m, its depth is about 10 $\mu$m, and the length is 130 $\mu$m. Concentration of erythrocyte suspension was adjusted with Krebs-Ringer solution that was poured into the micro-channel flow system. Hydrostatic pressure of 10 – 30 cmH$_2$O was applied to enable erythrocytes to flow smoothly through the channels. Local area images of erythrocyte flow were observed under an intra-vital microscope and recorded by using a high-speed video camera of 1000 frames/s. Deformation of erythrocytes was analyzed by a computer-based image processor, and the degree of deformability, deformation index ($DI$), was obtained from the lengthwise to crosswise ratio of the parachute-like formation of an individual erythrocyte (Fig. 2). Flow velocity of the erythrocytes was simultaneously measured by applying the image correlation method (9) on the recorded images, and flow shear rate was also obtained. Deformability of erythrocytes ($DI$) increases non-linearly with increase in the flow velocity and it reaches maximum deformation at high shear rate (Fig. 3). The relationship between deformability and flow velocity of erythrocytes through the micro-channel was empirically represented by an exponential function as

![Fig. 1. Transparent micro-channel flow system on crystal glass substrate made by micro-machining technology.](image-url)
Image Analysis of Microcirculation

\[ \text{Deformation Index} = \frac{L}{D} \]

\( DI (v) = C + a [1 - \exp(-v/b)] \]

, where \( v \) is flow velocity, and the other parameters, \( C \), \( a \), and \( b \), are coefficients dependent on the sample and channel geometrical factors. As can be recognized, deformability of diabetic erythrocytes was much lower than that of controls, especially at higher shear rate. At \( v = 4 \text{ mm/s} \), average \( DI \) was about 1.6 in the healthy controls and it was 1.05 in diabetes mellitus (DM) patients \( (P<0.01) \). At \( 2 \text{ mm/s} \), average \( DI \) was about 1.3 in the healthy controls and it was 0.95 in DM patients \( (P<0.01) \). Inverse relationship between deformability and HbA1c of erythrocytes, a clinical index of blood glycosylation, was obtained from the diabetic samples: \( DI \) decreased when HbA1c increased. The \( DI \) was 1.05 with 6.4% HbA1c, while it was 0.95 with 12.1% HbA1c at 5 mm/s of the flow velocity \( (P<0.01) \).

To verify the change of deformability of erythrocyte in diabetes, the spring constant and the Young’s modulus of the erythrocyte were measured by using an atomic force microscope (AFM, NVB100; Olympus, Tokyo) in the contact mode. By applying Hooke’s law and the Hertz model on the force curve, respectively, both elastic constants of the sample can be obtained. Cantilever probe having the spring constant of 0.02 N/m was used. In all experiments, blood samples were drawn with heparin from healthy adult volunteers and diabetic patients whose informed consents were accepted. The blood samples were diluted in the phosphate-buffered saline solution and set on a poly-l-lysine coated dish vessel. As shown in Fig. 4, the spring constant and the Young’s modulus of diabetic erythrocytes were much higher than those of healthy controls. It means that it becomes too difficult for the diabetic erythrocyte to deform itself as the result of glycosylation. In healthy young subjects group \( (\text{HbA1c} <5\%) \), average data of the spring constant of erythrocytes was 3.64 mN/m and the Young’s modulus was 0.03 MPa, while the DM patients \( (\text{HbA1c} >6.4\%) \) had a spring constant of 9.46 mN/m and a Young’s modulus of 0.12 MPa. All of the data showed statistically significant difference with \( P<0.01 \). These characteristics are well coincident with the deformability of erythrocytes in various conditions.

**Blood flow and oxygen tension in microcirculation**

Blood flow in organ microcirculation can be visualized by perfusing fluorescent isothiocyanate (FITC) labeled erythrocytes. Before the experiment, blood was withdrawn from a donor rat, and the erythrocytes were centrifuged, washed, and labeled by FITC. A 0.1-ml suspension of erythrocytes was injected into a cannulated femoral vein of each rat. Since the peaks of absorption and emission spectra of FITC are 450 and 520 nm, respectively, it was excited by irradiation of a mercury lamp through a band pass filter \( (450 - 490 \text{ nm}) \). Microscopic images of the FITC labeled blood flow were recorded by a high-speed video camera with an image intensifier \( (\text{Ektapro CR Imager 2000; Kodak, Tokyo}) \). After recording the high-speed images, they are replayed.

**Fig. 2.** Parachute-like deformation of red blood cells in the micro-channel capillary flow and the deformability defined by the lengthwise to crosswise ratio of erythrocyte deformation.

**Fig. 3.** Erythrocyte deformability of diabetic patients compared with those of healthy controls. Relation between the deformation index and flow velocity of erythrocytes can be represented by exponential function. Redrawn from Ref. 1.
at the ordinary video rate of 30 frames/s, and blood flow velocity of the FITC-labeled erythrocytes was analyzed by a computer-based image processor (9). On the other hand, blood \( pO_2 \) can be measured by the luminescent quenching method using a phosphorescent molecular probe, Pd-meso-tetra-(4-carboxyphenyl)-porphyrin (Pd-TCPP), which is chemically stable and nontoxic. Pd-TCPP previously dissolved in dimethyl sulfoxide solution with NaCl and bovine serum albumin (30 mg/kg body wt, 15 mg/ml) was administered to an objective rat via a femoral vein and was excited with the second harmonic of a Q-switched Nd-YAG pulse laser (532 nm in wavelength, 6 ns in pulse width, 1 Hz in pulse recurrent frequency, 10 mJ/cm\(^2\)/pulse in irradiation energy). Phosphorescence emission was detected by a photo-multiplier tube through a long pass filter (>620 nm). From the phosphorescence lifetime of the emission decay curve, \( pO_2 \) was obtained by using Stern-Volmer equation (10).

In the experiment, Wistar male rats (300 g body weight) were anesthetized with an intraperitoneal injection of \( \alpha \)-chloralose and urethane and tracheostomized and mechanically ventilated with room air. For the liver and kidney observation, a rat received an abdominal midline incision and longitudinal dissection; the targeted organ was put on an acrylic plate like a spoon. During the experiment, the organ was fixed loosely with saline-soaked cotton to reduce the influence of respiratory movement. For the observation of cerebral pial microcirculation, the head of the rat was fixed in a stereotaxic frame, and craniotomy was performed in the left parietal bone exposed by longitudinal midline skin incision. Both the dura matter and arachnoid membranes were incised together, and thereafter, the window was covered with a cover glass by means of the cranial window method. Artificial cerebrospinal fluid (at body temperature and pH 7.4 for maintaining the physiological condition) was perfused over the exposed pial surface at a speed of 0.1 ml/min. Flow velocity of FITC-labeled erythrocytes and \( pO_2 \) were alternately measured at a given position of micro-vessels by changing two light sources at 1-Hz interval using an electromechanical shutter installed in the microscope. Figure 5 shows a flow image of the fluorescence labeled erythrocytes in a cerebral pial microcirculation. Under the normal, physiological condition, RBC velocity and \( pO_2 \) in the pial venule (inner diameter was about 55 \( \mu \)m) were maintained at about 0.5 mm/s and 40 mmHg, respectively. Both of them, however, decreased drastically during acute hemorrhagic shock, and they recovered after reperfusion of the blood; in particular, it must be noted that the flow velocity and oxygen tension showed excessive elevation over the pre-shock value just after

**Fig. 4.** The spring constant and the Young’s modulus of diabetic erythrocytes compared with those of healthy controls. Elastic property of diabetic erythrocyte shows significantly high stiffness compared with that of a healthy erythrocyte (*\( p < 0.01 \)). Redrawn from H. Minamitani et al. Trans IEJ of Japan. 2002;122-C:1664–1671.

**Fig. 5.** Flow image of FITC-labeled erythrocytes in cerebral pial venule (inner diameter is about 55 \( \mu \)m) obtained by using fluorescent imaging method and high speed video camera.
the reperfusion (Fig. 6). On the other hand, in arterioles, drastic decrease of erythrocyte velocity and $pO_2$ was not observed during acute hemorrhagic shock. These results suggest that the developed system is quite useful for measuring blood flow and oxygen delivery in various organ microcirculatory systems.

**Adhesive activity of platelets and leucocytes induced by photodynamic reaction**

DM brings forth some risk factors for major cardiovascular events. The risk of stroke, myocardial infarction, peripheral vascular disease, and cerebral and coronary ischemia are increased twofold to fourfold in most populations of diabetic subjects. The so-called macroangiopathy and microangiopathy are complicated in diabetic individuals, and they have been investigated extensively for possible involvement in accelerated atherosclerosis and increased tendency to thrombosis. Platelets and leucocytes are important participants in the response of the body to small diameter blood vessels, and platelet and leucocyte adhesion, platelet aggregation, and endothelium injury are accelerated in DM that have been observed through in vivo and in vitro experiments. In this study, cell adhesion and platelet aggregation were induced by filtered light following administration of a photosensitizer in arterioles and venules of rat mesentery in vivo. An additional in vitro experiment concerning the cell adhesion with endothelial cells was also carried out. Photochemical reaction is a useful technique for inducing thrombus formation and acute thromboembolism realized by administration of a

![Fig. 6. Time course changes of erythrocyte flow velocity and oxygen tension $pO_2$ in cerebral pial venule. Both parameters decreased drastically during acute hemorrhagic shock and recovered after reperfusion of the blood.](image)

![Fig. 7. Photochemical reaction induces platelet adhesion and acute thromboembolism in organ microcirculation as the result of production of reactive oxygen species. a: normal state before light irradiation, b: platelet adhesion and thrombus formation at 3 min after light irradiation, c: thromboembolism at 5 min after light irradiation, and d: complete vascular shut down at 11 min after light irradiation.](image)
photosensitizing agent, followed by activation of the agent using specific light radiation (Fig. 7). This treatment results in a sequence of photochemical processes and cytotoxic reactive oxygen species (ROS) that causes irreversible photo-damage to tissue cells. However, it can also be used for a positive application like photodynamic therapy for cancer treatment and atherosclerosis exclusion.

In the experiment, male Wistar rats weighing 180–200 g (7 weeks after birth) were used. To induce the diabetic condition, streptozotocin (55 mg/kg bw) was injected through the external jugular vein and after 3 weeks, they were used as DM rats. Normal male Wistar rats of the same age were also used as a control. The animals were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg bw). Platelet aggregation and leucocyte adhesion were observed in rat mesenteric arterioles and venules (vessel diameter 30–60 μm) under intravital and fluorescent microscopes. The microscopic video image was obtained by using a high-speed video camera and analyzed to measure blood velocity in the vessels using the image correlation method (9). In order to induce acute thromboembolism by the photochemical reaction, filtered light from a mercury lamp (540 nm in wavelength and 25 mW/mm²) was irradiated on selected mesenteric micro-vessels following intravascular administration of Photofrin (5 mg/kg bw) or Zn coproporphyrin III (3 mg/kg bw) as a photosensitizer agent. Change of blood flow in the irradiated area was observed, and outset time of platelet adhesion To and complete vessel occlusion (vascular shut down, VSD) time Tv were examined with image analysis. The process of thrombus formation was assessed by two parameter, To and Tv – To. In mesenteric venules of control animals, To = 0.5–4 min, Tv = 1–20 min, and Tv – To = 1–15 min, while in arterioles, outset time and VSD time are elongated by 20–40% because of high blood flow velocity. In the state of DM, however, To = 20 s–2 min, Tv = 1.5–9 min, and Tv – To = 1–7 min that means thrombus is readily formed in the DM microcirculation. Even in diabetic arterioles, as was expected, faster thrombus formation was also observed in comparison with the control state. This experimental procedure is quite useful to assess pharmaceutical effectiveness of thrombus inhibitors for diabetes mellitus.

As mentioned, various ROS, O₂⁻, H₂O₂, ·OH and ·O₂, take part in the thrombus formation induced by activation of the photosensitizing agent in blood plasma and accompanied with adhesion of the activated platelets and leucocytes. Increase in the dose of photosensitizing agent and light energy promotes shortening of To and Tv – To, while high flow shear rate leads to a low possibility of platelet adhesion. Interaction between these blood cells and endothelium depends on the production rate of ROS in the area of photochemical reaction. For visualization of flow behavior of the platelets and leucocytes, fluorescent dye probes, rhodamine G and acridine orange, were used, respectively, and increase in area of the platelet aggregation and number of adherent leucocytes in the states of rolling and sticking were evaluated by the microscopic observation. In the experiments, to elucidate the contribution of ROS to VSD, we administered some quenchers to eliminate the ROS: SOD for O₂⁻ quencher, DMSO for ·OH quencher, catalase for H₂O₂ quencher, and L-histidine for ·O₂ quencher. Administration of SOD and L-histidine resulted in significant elongation of the outset and VSD time compared with the non-administered control (Fig. 8). On the other hand, administration of DMSO and catalase showed little elongation of the outset time of platelet adhesion, while causing significant elongation of VSD time. From these results, it might be considered that ·O₂ and O₂⁻ strongly activate adhesive function of platelets and leucocytes and also induce endothelial cell retraction, which promotes acute thromboembolism in micro-vessels.

![Figure 8. Effect of quenchers of reactive oxygen species on the outset time of platelet adhesion (the upper figure) and complete vascular shut down time (the lower figure). ·O₂ and O₂⁻ significantly activate adhesive function of platelets and leucocytes and also induce endothelial cell retraction, which promotes acute thromboembolism in micro-vessels.](image-url)
release adhesion molecules that are followed by the strong cell interaction indispensable for platelet aggregation and thrombus formation.

**Conclusion**

In this paper, we presented some examples of the bio-imaging method and functional analysis of blood flow and blood cell activity in organic microcirculation. As shown, the transparent micro-channel flow system made by crystal glass plate is quite useful for direct observation of the flow velocity and deformation of individual erythrocyte in the flow passage. AFM is also usable to measure the microscopic elasticity of the cell membrane. Our results suggest that in DM, the erythrocytes become significantly glycosylated, making it hard for them to deform themselves. The hardness of the erythrocytes may affect the growth of microangiopathy. Although it is not yet clear how the glycosylated erythrocytes relate to microangiopathy, DM patients have higher risk factors for malfunction of the microcirculation system compared with healthy people. The macroangiopathy and microangiopathy are complicated in diabetic individuals, and they are closely connected with accelerated atherosclerosis and increased tendency for thrombosis. Platelets and leucocytes are also important participants in the response in small diameter blood vessels; and acceleration of platelet and leucocyte adhesion, platelet aggregation, and endothelium injury were observed in the diabetic microcirculation in this study. Photochemical reaction was a useful treatment to induce thrombus formation and acute thromboembolism realized by administration of a photosensitizing agent, followed by activation of the agent using specific light radiation. This treatment results in a sequence of photochemical processes and cytotoxic ROS that causes irreversible photo-damage to tissue cells. It might be considered that $^1O_2$ and $^3O_2$ strongly activate the adhesive function of platelets and leucocytes and also induce endothelial cell retraction, resulting in an increase in the sub-endothelial area. Activation of both platelets and endothelial cells also released adhesion molecules that are followed by the strong cell interaction indispensable for platelet aggregation and thrombus formation. Through animal experiments, simultaneous measurement of blood flow velocity and oxygen tension is required to examine the functional behavior of the microcirculation without a physically invasive technique. In our study, a new system to measure both data continuously even in a thick parenchymatous organ such as the pial, kidney, and liver was realized by using fluorescent and phosphorescent molecular probes. As mentioned, the developed system is quite useful for measuring blood flow distribution and oxygen delivery in organ microcirculatory systems. It can be used for analyzing various clinical pathophysiological conditions of blood flow change during asphyxia and acute hemorrhagic shock.

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