INTRAVENOUS INJECTION OF SONICATED BLOOD INDUCES PULMONARY MICROTHROMBOEMBOLISM IN RABBITS WITH LIGATION OF THE SPLENIC ARTERY

Atsushi Kisanuki, Suparp Kietthubthew, Yujiro Asada, Kousuke Marutsuka, Yoshinori Funahara, Akinobu Sumiyoshi

1First Department of Pathology, Miyazaki Medical College, Miyazaki, JAPAN, 2Department of Pathology, Faculty of Medicine, Prince of Songkla University, Hat Yai, THAILAND, 3First Department of Physiology, Faculty of Medicine, Kobe University, Kobe, JAPAN.

ABSTRACT Pulmonary thromboembolism (PTE) is found in long hospitalized patients. Chronic PTE has been reported to play an important role in cardiac failure in thalassemic patients after splenectomy. However, the mechanism of PTE in these patients remains unclear. In this study, we attempted to establish an animal model of PTE. We divided New Zealand white rabbits into three groups: Group I was injected sonicated blood, II was injected non-sonicated blood after ligation of the splenic artery, and III was injected sonicated blood after ligation of the splenic artery. After injection of the sonicated blood, we examined the platelet counts every 10 minutes until 1 hour and the rabbits were sacrificed for histological examination. Platelets significantly decreased in number immediately after the injection of sonicated blood in Groups I and III. Many pulmonary thromboemboli composed mainly of platelets were found in Group III but not in other groups. These pathological changes seem to be partly similar to those of thalassemic patients after splenectomy. This animal model is thought to be useful to study the pathogenesis of pulmonary thromboembolism, especially in thalassemic patients after splenectomy.
Pulmonary thromboembolism (PTE) is a major clinical problem, especially among hospitalized patients. In more than 95% of PTE, thrombi firstly arise in the large deep veins. PTE induces various clinical symptoms depending on the size of the occluded vessels and number of emboli. Small thromboemboli cause transient dyspnea, tachypnea and chest pain, but large ones become the cause of sudden death.

Thalassemia is a common hereditary anemic disease in southeast Asian countries including Thailand, which is characterized by imbalance of globin chain synthesis and ineffective erythropoiesis. This disease can give rise to abnormality in any organ system: hypoxemia is one of the most important features in thalassemia, particularly in those who have undergone splenectomy. Fucharoen et al. (1) demonstrated that this hypoxemic condition may be due to hyperactivity of platelets probably associated with outer membrane abnormality in thalassemic red blood cells. Evidence of pulmonary artery thrombosis had been reported to be as high as 44% in post-splenectomized β thalassemia/hemoglobin E autopsy cases (2). Recently, Sonakul et al. (3) and Sumiyoshi et al. (4) demonstrated that splenectomized thalassemic patients have a higher incidence of thromboemboli and microthromboemboli in the arteries and arterioles in the lungs than non-splenectomized ones, and these microthrombi were rich in platelets. However, the pathogenesis of PTE in splenectomized thalassemic patients is unclear. The purpose of the experiment is to establish a new animal model of PTE under conditions similar to those of thalassemic patients after splenectomy.

MATERIALS AND METHODS

Animals and experimental groups

Nine male New Zealand White rabbits (2.5-3.0 kg) were used. We divided these rabbits into three groups randomly. Blood of all rabbits was drawn before experiment. The blood with or without sonication was autologously injected into each animal. Group I was treated with sonicated blood (n=3), Group II was treated with non-sonicated blood after ligation of the splenic artery (n=3) and Group III was treated with sonicated blood after ligation of the splenic artery (n=3).

Blood drawing and sonication

One day before experiment, 20 ml of citrated blood was drawn via the ear artery from each rabbit. In Groups I and III, blood was sonicated using a sonicator (Frequency 20kHz, 7.5amps, 200watts, Heat System Ultrasonic Inc. Plainview, NY, USA). The blood was divided into 1 ml/tube and sonicated 3 times for 10 seconds each at 10 seconds interval under 50% duty cycle condition. These procedures were done under aseptic and cold (4°C) conditions. The blood drawn from Group II was not sonicated. The sonicated and non-sonicated blood was then kept at 4°C until use.

Morphological study of sonicated blood

Morphology of the sonicated red blood cells (RBCs) was evaluated by a scanning electron microscopy. Some blood samples after sonication were fixed with a mixture of 4% neutralized formaldehyde and 1% glutaraldehyde in a 0.1 M phosphate buffer, pH 7.2 for several hours. After post-fixation with 2% OsO₄ in 0.1 M phosphate buffer for 90 minutes, they were dehydrated in a graded series of t-butyl alcohol and dried in a critical point dryer (ID-2, EIKO, Mito, JAPAN). They were coated with platinum-palladium and observed with a S-800 microscope (Hitachi, Tokyo, JAPAN).
Splenic artery ligation and blood reinjection

Animals were anesthetized by intravenous injection of sodium pentobarbital (25 mg/ml/kg). We opened the midline of rabbit abdominal wall in 2 cm length, then exposed the splenic arteries and ligated them with suture materials in Groups II and III. After closing the abdomen, we inserted and placed a polyethylene tube into the femoral artery for blood sampling from each rabbit throughout the experiment. Stored blood was warmed to 37°C in a water bath before the injection. The volume of 20 ml of sonicated or non-sonicated blood from each rabbit was injected autologously to each rabbit via the ear vein using a vein infusion set at the rate of 2 ml/min. One ml of citrated blood was collected in heparinized tubes for blood cell counting in the following time sequences; before and immediately after blood injection (0 minute), and at 10, 20, 30, 40, 50 and 60 minutes after blood injection.

Tissue sampling and morphological studies

Rabbits were sacrificed by an overdose injection of sodium pentobarbital (50 mg/ml/kg i.v.) after collecting blood at 60 minutes and then perfused with 0.1 M phosphate buffered saline. The lungs, liver, kidneys and spleen were removed for morphological evaluation. For histological examination, tissues were fixed in 4% paraformaldehyde for 12 hours at 4°C, dehydrated in a graded series of ethanol and embedded in paraffin. Hematoxylin-eosin (HE), phosphotungstic acid hematoxylin (PTAH), elastica von Gieson (EVG) stains were used for morphological examinations. Some sections were immunohistochemically examined using monoclonal anti-fibrin antibody (a kind gift from Dr. Tomofumi Kurokawa, Takeda Chemical Industries, Ltd., Osaka, JAPAN). We used streptavidin-biotin immunoperoxidase method (Histofine SAB-PO kit, Nichirei, Tokyo, JAPAN). For ultrastructural evaluation, tissues were fixed with a mixture of 4% neutralized formaldehyde and 1% glutaraldehyde in a 0.1 M phosphate buffer, pH 7.2, for 12 hours at 4°C. They were postfixed with 2% OsO₄ in 0.1 M phosphate buffer for 90 minutes at room temperature, dehydrated in a graded series of ethanol and embedded in Epon 812 as usual. The semithin sections (1 μm thick) were stained with toluidine blue, and ultrathin sections were cut and stained with uranyl acetate and lead citrate. Sections were examined with a JEOL 1200EX (Nihon Denshi, Tokyo, JAPAN).

The investigation conforms with the Guide for the care and use of laboratory animals published by the US National Institutes of Health (NIH publication No.85-23, revised 1985) and it was carried out at the Experimental Animal Center in Miyazaki Medical College.

RESULTS

Blood counting and morphological changes

The injection of the sonicated blood immediately decreased the platelet count in Groups I and III (Fig. 1). The most significant decrease was observed within 10 minutes and the reduction of platelet counts was prolonged until 60 minutes in both groups, while no change of platelet count was observed in Group II. The sonicated RBCs were distorted and fragmented compared with normal RBCs (Fig. 2).

Histological findings

Histological changes were observed mainly in the lungs. All rabbits of Group III showed various stages of thromboemboli from platelet aggregates (Figs. 3 & 6), which are considered to be “early stage” of thrombosis and some of which may disaggregate with time (4), to frank thrombi with fibrin formation (Fig. 4) in the capillaries and arterioles. Furthermore large thrombi
The number of platelets before and after injection of the sonicated or non-sonicated blood. The curve of Group II was significantly different from those of Groups I and III (two way analysis of variance).

Scanning electron micrographs of rabbit red blood cells. Sonicated red blood cells (Groups I and III) are distorted and fragmented. Inset shows normal red blood cells. Bar=10μ.
The lung in Group III. Many platelet-rich thromboemboli (arrow) are present in the arterioles, and in the capillaries of the alveoli. (HE, x80).

The lung in Group III. Thromboemboli are also present in the medium-sized arteries, which is composed of platelets with some fibrin (arrow). (A : HE, x50. B : anti-fibrin antibody, x50).
FIG. 5.

Histological change of the lung in Group III. A saddle thrombus composed of platelets and fibrin is noted in the large pulmonary artery. (HE, x40).

FIG. 6

Electron micrograph of the alveolar wall in Group III. The capillary lumen is obstructed by densely packed platelets. Some platelets show shape change with disappearance of dense granules and dilatation of the open canalicular system. (x 12,000).
composed of platelets and some fibrin were occasionally noted in the medium- and rather large-sized arteries (Fig. 5). No thromboembolism could be found in the lungs of Groups I and II. The spleen of Group I without ligation of the splenic artery showed pooling of packed platelets without fibrin in the sinuses. No pathological changes including platelet aggregation and thrombus formation could be detected in other organs in all groups.

**Ultrastructural findings**

Platelet thrombi in the alveolar capillaries were observed in Group III (Fig. 6). The densely packed platelets occluded the dilated capillary lumen showed shape change with loss of dense granules. In Group I, the splenic histiocytes phagocytized platelets and RBC fragments probably derived from sonicated ones. In the liver of Groups I and III (sonicated blood injection groups), some Kupffer's cells phagocytized platelets and RBC fragments. No thrombi could be observed in the glomeruli in any group.

**DISCUSSION**

We established a rabbit model of PTE by autologous injection of sonicated blood after splenic artery ligation. We think that our model may reproduce the condition of PTE seen in thalassemic patients after splenectomy. In this model, sonicated abnormal RBCs circulate without trapping by the spleen because of ligation of the splenic artery. This pathophysiological state seems to be similar to that of post-splenectomized thalassemic patients in which abnormal RBCs circulate without trapping by the spleen. Some thrombosis animal models have been reported previously (6-8), but they are fatal and their morphological changes are very different from those of our model. Mosers et al. (9) reported a dog model of chronic PTE treated with tranexamic acid. They could make pulmonary hypertension and organized and recanalized thrombi after forming venous thrombi in the femoral veins. The emboli were derived from thrombi of deep vein and the spleen function was preserved. Because PTE in thalassemic patients is mainly seen after splenectomy, our model may be more appropriate for studying PTE in thalassemic patients.

The platelet count was significantly decreased after the injection of the sonicated blood. This means that sonicated blood activated platelets and induced platelet aggregation in the circulation. Setiabudy-Dharma et al. (10) reported that no platelets in citrated plasma are aggregated. Although we considered that the hemoglobin from destructed RBCs may induce the platelet aggregation in the vascular systems, the platelet aggregation site was not specified except for the spleen in Group I in this study. We observed that the splenic histiocytes and Kupffer's cells phagocytized platelets and RBC fragments in the platelet decreased groups (Groups I and III). No platelet thrombi were found in the lung except for the sonicated blood injection with splenic artery ligation group (Group III). It is considered that the decrease of platelet in Group I may be due to increased phagocytosis of platelets by the reticuloendothelial system which was activated by sonicated RBC fragments.

The pathogenesis of pulmonary thromboemboli of this model is unclear. It is known that thrombocytosis is a common finding after splenectomy, but thromboembolism is merely happened except thalassemia, myeloproliferative disease, paroxysmal nocturnal hemoglobinuria and hereditary non-spherocytic hemolytic anemia (11-13). Sumiyoshi et al. (4) and Fucharoen et al. (1) have proposed a vicious circle in the lung of thalassemic patients, especially after splenectomy. In such a clinical setting, hyperactive platelets are present together with abnormal RBCs and dysfunctional endothelium under hypoxemic conditions, which in turn may develop spontaneous platelet aggregation and finally may result in the formation of pulmonary microthromboemboli. Opartkiattikul et al. (14) reported high availability of the platelet factor 3 (PF3) in β-thalassemia
/HbE splenectomized patients. They suggested that abnormal erythrocytes might remain in the circulation longer and might cause platelet activation that triggers thrombosis. In our model, no thromboembolism was detected in the normal blood injection group even after splenic artery ligation. Therefore, it is considered that circulating sonicated abnormal RBCs, which should be trapped by the splenic reticulo-endothelial system, may activate platelets in the pulmonary vascular system.

Thromboembolic events in thalassemic patients after splenectomy and restricted only in the lung (3, 4). On the other hand, thrombi in thrombotic thrombocytopenic purpura are only rarely or never seen in the lung (15). The reason why such difference occurs is unknown, although platelet activation must be involved in the pathogenesis of both diseases.

In summary, we established a rabbit model of PTE with ligation of the splenic artery and intravenous injection of sonicated blood. Many platelet-rich thrombi were noted in the pulmonary circulation in this model. These conditions and findings are similar to those of thalassemic patients after splenectomy. We suggest that this model is useful not only for studying the pathogenesis of PTE occurring in thalassemic patients after splenectomy but also for evaluating the therapeutic efficacy of drugs.

Acknowledgements

Ms. Suparp Kiethubthew visited the Department of Pathology, Miyazaki Medical College and did this work as an exchange scientist under the Japanese Society for the Promotion of Science (JSPS) and National Research Council of Thailand (NRCT) Cooperation Programs supporting Southeast Asian Countries. The authors also thank Ms. Ritsuko Sotomura and Ms. Masuko Inomata for their technical assistance.

REFERENCES

8. BEVIGLIA, L., POGGI, A., ROSSI, C., MCLANE, M.A., CALABRESE, R.,