"THE BEHAVIOR OF PLATELETS IN CAPILLARY HEMORRHAGE INDUCED BY SNAKE VENOM"

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SUMMARY

The behavior of platelets in capillary hemorrhage has been investigated by using highly purified hemorrhagic principle from a snake venom. The first capillary hemorrhage was observed through a light microscope four minutes after topical application of the hemorrhagic principle to the microcirculatory system of the rat mesentery. With time hemorrhagic spots increased in number and size. The electron microscopic observations of the affected capillaries showed a partial discontinuity of the endothelial lining. The basement membrane occasionally maintained its continuity even when the endothelial cells did not. When the endothelial gap was narrower than one micron, a single platelet which had not degranulated, was frequently seen to have a pseudopod within it. When the gap was 1-2 microns wide, platelets appeared to change their shape so as to fit the entire cell into the gap. Most of these platelets showed partial degranulation. When the gap was more than two microns wide, platelets collected and degranulated into it. In general, the wider the endothelial gap was, the more platelets collected and degranulated in it. Some degranulated platelets were found in the area enclosed by endothelial cells, a basement membrane and a pericyte. The experimental results suggest that the endothelial injury itself may provoke the reaction of platelets before the platelets make contact with extra-capillary tissue constituents.

When the blood stream of the microcirculation is disturbed somehow, the deranged hemocirculation is manifested as stasis, plasma skimming, sludge phenomenon, rolling of leukocytes, flying of thrombus, increased permeability and hemorrhage.^{1,2} The microvascular hemorrhage is one of the most serious conditions among these discomposed states. Although the thrombocyte reactions to the injured surface of large blood vessels have been studied and the capillary hemorrhage, capillarrhagia, has been investigated in a thrombocytopenia,³ an ascorbic acid deficiency^{4,5} and after the administration of a snake venom,⁶⁻¹² the

actual reactions of platelets to the injured capillary wall has been scarcely demonstrated. Because the microcirculatory system differs substantially from the large blood vessel system in terms of the vascular structure and hemodynamics, the platelet might show distinct behavior to the damaged microvascular wall.

The purpose of the present communication is to study the mode of microvascular hemorrhage by a purified snake venom and to demonstrate how platelets react to the injured microvascular wall. The used snake venom, HR 1, was purified from the venom of *Trimeresurus flavoviridis* by Omori-Sato and Osaka¹³ possessed the highest hemorrhagic activity ever purified among the hemorrhagic principles isolated from bacterial and animal origin.¹⁴ In this paper, the identification of vessel types was based on the classification by Chambers and Zwefach.¹⁵

MATERIALS AND METHODS

The purified snake venom, HR 1, was the generous donation of Drs. Omori-Sato and Ohsaka. HR 1 contained more than 80% of the hemorrhagic activity but only 0.6% of proteolytic activity present in the original venom.^{11,13} Some other properties of HR 1 are shown in Table 1.

Twenty-five adult male rats of Wistar strain, weighing about 200 g each, were divided into two groups, the experimental group of fifteen rats and the control group of ten rats. They were anesthetized intraperitoneally with sodium pentobarbital 50 mg per kg of body weight. A midline incision was made on the shaved abdomen and the ileum mesentery near the ileocecal portion was pulled out on to a glass plate. The exposed mesentery was carefully spread, stabilized

Table 1
Some Properties of HR 1

Proteolytic Activity on Casein	±
Minimum Hemorrhagic Dose on Rabbit Skin	5.8 ng
LD ₅₀ (mouse, i.v.)	4.6 µg
Sedimentation Coef. (S _{20, w})	5.8 S
M. W.	100,400
I. P.	4.3
pH-stability	pH 8.0-10.0
Heat-stability	<45°C
Inhibition by EDTA	+
Inhibition by Cysteine	+
Inhibition by Anti-hemorrhagic Factor from Snake Venom	+
Inhibition by Diisopropyl Fluorophosphate	-
Inhibition by Soybean Trypsin Inhibitor	-

at the ileum by cotton soaked in 1% gelatin-Ringer solution at a temperature of 38°C and kept humid with the same solution. In order to maintain physiological condition, the rat was placed in a chamber at 38°C. A transilluminated inverted microscope (modified Nikon inverted microscope) enabled us to observe alterations of the microcirculation which were simultaneously recorded by a 35 mm still camera (Olympus C-35), a 16 mm movie camera (Boauliau R-16) or a color TV camera (Ikegami MK-309) equipped with a video recorder (JVC CR-6300).

Under microscopic observation, the experimental group was treated with 50 microliter of HR 1 solution at a concentration of 300 microgram per ml of saline. The solution was applied topically to the aimed microcirculatory unit of the ileum mesentery after it was confirmed that the microcirculation was maintained normally. The alterations were recorded continuously on a video tape. A 16 mm movie film or a 35 mm film was also used at times.

Tissue was fixed for electron microscopy two minutes, four minutes, and ten minutes after the application of HR 1. The hemorrhagic lesions were fixed in situ in cold 1% osmium tetroxide in 0.1 M phosphate buffer (pH=7.4) with 4.5% sucrose. In the control group, tissue was fixed at once or ten minutes after the application of saline. Ten minutes after fixation the aimed mesentery was isolated from the other tissues and kept in the same fixative for an additional two hours.

In our preliminary study, glutaraldehyde fixation did not stop blood flow immediately as Rhodin described.^{16,17} Instead, the blood flow continued from 7 to 15 seconds after the application of the fixative. On the other hand, osmium tetroxide fixation stopped blood flow at once. To avoid alterations during fixation, only osmium tetroxide fixation was used in this study.

After dehydration through graded concentration of ethanol, the samples were embedded in flat molds in Epon 812.¹⁸ The capillaries showing hemorrhage were selected by light microscope and cut for electron microscopy on a LKB ultratome. Ultrathin sections were stained with uranyl acetate and Sato's lead solution.¹⁹ Hitachi HS-9 and JEOL JEM-100B electron microscope were used to examine ultrastructural changes.

RESULTS

Observation *in vivo*: A typical area of microcirculation could be observed in the ileum mesentery near ileocecal portion through the inverted microscope, as described by Chambers and Zweifach¹⁵ (Fig. 7a). Topical application of the hemorrhagic principle to the rat mesentery gave rise to bleeding from the capillaries in the first 4 minutes (Fig. 1b). Detailed observations at the site of hemorrhage showed that erythrocytes extravasated one by one through pin-point holes of capillary walls and that the extravasation occurred both continuously

Fig. 1 Light micrograph illustrating hemorrhagic changes in the microcirculatory unit of the rat mesentery evoked by local application of 0.05 ml of HR 1 solution at a concentration of 300 microgram per ml of saline.

(a) The microcirculatory unit before treatment. The arrow indicates the direction of blood flow. A: precapillary, Th: thoroughfare way, C: capillary, V: postcapillary. $\times 80$. One unit of the scale, $25\ \mu\text{m}$. (b) Four minutes after the application of HR 1. The arrows indicate hemorrhages at the site of capillaries. $\times 80$. (c) Seven minutes after the application of HR 1. This picture shows the same area as that framed in Fig. 1b. A new hemorrhage (arrow) $\times 160$. One unit of the scale, $12.5\ \mu\text{m}$. (d) Ten minutes after the application of HR 1. Hemorrhages are seen in a thoroughfare way and a postcapillary but not at a precapillary. $\times 80$.

and intermittently from the same hole. HR 1 did not appear to make a diffuse lesion but instead created sporadic ones along the capillary walls. With time the hemorrhagic spots increased in number and size (Fig. 1c and 1d), and often spread to thorough fare ways and to postcapillaries but not to precapillaries or arterioles. Extravasated blood cells tended to accumulate in the mesentery tissue proper and seldom in areas beyond it. The typical thrombus formation that is seen in microvascular injury caused by a puncture wound was not observed at the site of hemorrhage induced by HR 1.

Observation by electron microscopy: In the control group, capillary endothelial cells showed many cytoplasmic vesicles and a few cell junctions. They did not have fenestrations and perforations. A pericyte occasionally surrounded them. Completely continuous basement membranes were observed. The appearance of the capillaries, postcapillaries or precapillaries was not significantly different from those described as normal by previous investigators.^{15-17,20-30}

There was no remarkable changes in the microvasculature which was fixed two minutes after the application of HR 1. The capillaries affected for four minutes showed some alterations of endothelial cell shape in part. These changes were fewer but almost identical with ones observed ten minutes after the application except for extension of the changes. The capillaries affected for ten minutes revealed two kinds of alterations. First, a capillary presented very swollen and bizarr appearance at such a thick portion as its nucleus was seen (Fig. 2 and 3). Secondly, it showed gap formation in an endothelial cell lining whose wall was relatively thin. The platelets reacted to the gaps with drastic shape changes (Fig. 4-8).

A capillary presented a very thick appearance when an endothelial cell laid its nucleus in the cytoplasm. In such an endothelial wall, the spaces were newly formed between an endothelial cell and basement membrane (Fig. 2 and 3). A part of endothelial cytoplasm was sometimes noticed left over in the space (Fig. 2) or along the basement membrane (Fig. 3). There were observed fibrins (Fig. 2) and amorphous substance (Fig. 3) in the spaces which were thought to be brought in from plasm. The necrotic or degenerative changes of endothelial cells was not apparent. It was probably too soon for these alterations to become visible as morphologic changes because tissues were fixed by ten minutes after exposure of the toxin.

Platelets were seen to collect toward the endothelial gaps when capillary walls were damaged. The number of collecting platelets seemed to depend on the size of the gap. It could be said in general that the wider the gap was, the more platelets were observed. In thin wall of capillaries, there were many gaps which varied in diameter and increased in number with time. A pinpoint gap (less than one micrometer) was often filled with a strikingly deformed platelet. A

Fig. 2 Electron micrograph of an affected capillary fixed ten minutes after application of HR 1. Although two endothelial junctions (J) seemed to be almost intact, narrow space (S1) and wide space (S2) were formed in the capillary wall. The narrow space (S1) is located between two endothelial cells (E), and the wide space (S2) between an endothelial cell and basement membrane. In S2, such a variety of substances as fibrin strands (f), vacuoles and an endothelial debris (E') were observed. $\times 18,700$. Bar, $0.5 \mu\text{m}$.

Fig. 3 Electron micrograph of an affected capillary wall. Wide and round spaces (Sa and Sb) are found in the capillary wall and amorphous material (arrow) is seen in the space of Sa. The endothelial cell junction (J) is almost intact here. $\times 24,000$. Bar, $0.5 \mu\text{m}$.

Fig. 4 Electron micrograph of a capillary with an endothelial gap in its lining wall. Note that a platelet (white capital P) has a pseudopod (arrow) lodged within the gap. The platelet granules remain in the center of the cytoplasm. Other platelets in the capillary lumen (L) appear to be close to other damaged portion of the capillary wall. At this stage, the platelets have no contact with outside collagen fibers (C). E: capillary endothelial cell. $\times 28,000$. Bar, $0.5 \mu\text{m}$.

Fig. 5 Electron micrograph of platelets (P) lodged in and passing through narrow gaps ($1-2\mu\text{m}$) of the capillary wall. These platelets show marked changes in shape and some of them have already begun to release their granules (arrow). R: erythrocyte. $\times 24,000$. Bar, $0.5\mu\text{m}$.

Fig. 6 Electron micrograph of a capillary with a 1-2 μm gap in its wall. In the capillary lumen (L), two platelets (P) have changed shape with pseudopod formation and with release of intracytoplasmic granules toward the injured portion. Outside the capillary wall, empty platelets (P') are observed, which maintain a round shape inspite of complete release of their granules. Note the striking contrast in the morphology of the platelets before and after passage through the capillary wall. A few collagen fibers are observed near the injured capillary wall. B: basement membrane, E: endothelial cell, L: capillary lumen. $\times 31,000$. Bar, 0.5 μm .

Fig. 7 Electron micrograph of a severely injured capillary wall with a $6.2\text{ }\mu\text{m}$ gap. Many platelets have aggregated at the gap and appeared to begin forming a hemostatic plug. This is a very rare observation. E: endothelial cell, L: capillary lumen. $\times 10,600$. Bar, $1\text{ }\mu\text{m}$.

single platelet was seen to have a pseudopod lodged within it (Fig. 4). This reaction of the platelet to the gap did not appear to be associated with the existence of a basement membrane or collagen fibers outside of the capillary. In fact, it occurred most often when the platelet had no contact with them. Platelet granules lay in the central region of the cell and degranulation had not yet occurred at this stage. The larger gaps were also replenished with many platelets which appeared to change their shape to fit the entire cell into the gap space (Fig. 5-7). Most of these platelets showed partial degranulation and some released granules could be seen between accumulated platelets. Typical platelet metamorphoses were seen in Fig. 6. The platelets in the capillary lumen and apart from the endothelial gap maintained their original shape. They had varying numbers of granules and amount of cytoplasm. On the other hand, the two platelets close to an endothelial gap formed pseudopodia and the filamentous structures in their cytoplasm were more evident than those of other platelets. Part of the plasma membrane of a platelet facing an endothelial gap seemed to

Fig. 8 Electron micrograph of an affected postcapillary. Several gaps were found in an endothelial lining of the postcapillary. The basement membrane (B) still looked intact. A space (S) is observed between the basement membrane and a pericyte. $\times 13,000$. Bar, $1\ \mu\text{m}$.

disappear and amorphous material around the platelet was observed. Just outside the capillary wall vacant platelets lay along the wall without any granules or cytoplasm. A striking contrast was seen among these three types of platelets, normal in the vessel lumen, deglanulating towards gaps and vacant outside of the capillary. In wide gaps of endothelial lining, thrombus formation was seen in progress (Fig. 7).

Although hemorrhages in venule or arteriole were rarely happened under intravital microscopic observation, the endothelial gap or subendothelial space formation of venular wall was noticed (Fig. 8). The endothelial gap was usually not so large that a platelet or two were able to fill it up. Polymorphonuclear leukocytes were sometimes seen sliding in the subendothelial spaces.

DISCUSSION

The extravasation of blood cells is believed to occur very quickly in capillary hemorrhage. Sunada *et al.*³¹ observed in an animal with a low blood calcium concentration that it took about 60 milliseconds for an erythrocyte to extravasate across the venular wall. Rhodin¹⁶ described that a two per cent solution of buffered glutaraldehyde did not stop the capillary blood flow instantaneously, but buffered

one per cent osmium tetroxide did. In the present study, we made a similar observation so that osmium tetroxide was used for optimal fixation.

In order to complete the hemorrhagic process, blood cells must pass through two main components for a capillary wall, an endothelial lining and basement membrane. As far as an endothelial lining is concerned, two kinds of extravasating routes are possible. One route is through an endothelial cell cytoplasm and the other through an endothelial junction. Using serial sections of capillaries of thrombocytopenic guinea pigs, van Horn and Johnson³² maintained that erythrocytes penetrate weakened endothelial cytoplasm rather than an endothelial junction. McKay *et al.*⁶ also described that injection with the hemorrhagin derived from *Vipera palestinae* venom appeared to produce gaps in the endothelial cell rather than between endothelial cells. On the other hand, Gore *et al.*³ considered that the thrombocytopenic animals displayed widened intercellular junctional spaces in the endothelial lining through which erythrocytes extravasated. As these three studies were carried out under different experimental conditions, and their findings were inconsistent, the precise mechanism of capillary hemorrhage is still not understood.

HR 1 is known to induce the release of such mediators as histamine or serotonin from various isolated organs and tissues.³³ These mediators cause the opening of endothelial junctions.^{29,34} *In vitro* experiments have shown that HR 1 disrupts isolated glomerular basement membranes with release of proteins and carbohydrates.³⁵ Therefore, it was proposed that HR 1 acts by opening endothelial cell junctions and that the adjacent basement membrane is disrupted enzymatically to permit the extravasation of blood cells. This point of view is supported by the fact that there is a time lag of four minutes before capillary hemorrhage is observed. In the present study, however, it is also considered that the endothelial gaps could be made both in an endothelial cell and between two endothelial cells, because the number of gaps were sometimes seen more than the number of cell junctions which is usually noticed in blood vessels of this size (Fig. 8).

Platelet-collagen adhesion is generally believed to be the primary and critically important step in the formation of a hemostatic plug.^{36,37} This theory may be applicable in the hemostatic process of large or middle-sized blood vessels. Tranzer and Baumgartner,³⁸ however, showed that platelets could adhere to the basement membrane of a capillary when its wall was denuded of endothelial cells. In addition, Johnson³⁹ demonstrated by autoradiography that platelet material entered the endothelial cells in the thrombocytopenic animals. She believed that platelets would adhere and aggregate on injured endothelial cells and on all types of connective tissue in the vessel wall. In the present study, platelets were seen to have a pseudopod or the whole cell body lodged within an endothelial gap as a plug. These findings suggested that the endothelial injury itself can provoke

the reaction of platelets in this type of hemorrhage before they contact the basement membrane or extra-capillary collagen fibers.

Recently, a prostaglandins and their derivatives come to appear before the footlights in terms of correlation between endothelial cells and platelets.^{40,41} The phospholipase A₂ released from endothelial cells can provoke platelets to cleave free arachidonic acid from phosphatidyl choline or phosphatidyl inositol. Arachidonic acid, then, converts to prostaglandin (PG) G₂ and PG H₂. Thromboxane A₂ which is formed from PG H₂ induces the platelet release reaction. On the other hand, prostacyclin (PG I₂), which is produced from PG H₂ only in endothelial cells, inhibits platelet adhesion to endothelial cells. The prostaglandin derivatives undoubtedly play a major role in the maintenance of blood circulation. These recent evidences support Johnson's idea^{39,42} that platelets react not only at hemorrhage but also always sustain endothelial cells. The present study demonstrated that platelets could begin to take a necessary step of reaction to the injured microvessels even before the contact with the extra-capillary tissue constituents. The damaged endothelial cells might induce platelet reaction by failing to produce prostacycline.

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