Ultrastructural Changes of Endothelium Associated With Thrombocytopenia

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In a study of the relationship between thrombocytopenia and increased vascular fragility, changes in the endothelium of capillaries and postcapillary venules of the tongue were examined by electron microscopy. Adult male albino rabbits (4 kg) were maintained thrombocytopenic (platelets <20,000/cu mm) up to 24 hr by one to three injections of guinea pig anti-rabbit platelet serum. Within 6 hr the normal projections and folds of the lumenal surface of the endothelial surface were largely effaced. In addition, the endothelium became thinner. In places, pores and membranous diaphragms were observed. Endothelial junctions appeared normal. Identical findings were observed if rabbits were made thrombocytopenic by administration of intraperitoneal busulfan. Intravenously administered Thorotrast was observed in endothelial cells and in the extravascular spaces within 3 min after injection into thrombocytopenic animals, while it was seen only intravascularly in control rabbits. With the spontaneous restoration of circulating platelets, the endothelium reverted to normal.

Severe thrombocytopenia is associated with petechiae and purpura. In experimental thrombocytopenia, abnormally large numbers of red blood cells (RBCs) are present in thoracic duct lymph, and blood vessels have increased permeability to plasma proteins. Restoration of normal vascular competence quickly follows platelet replacement. It would appear, therefore, the platelets are needed to maintain endothelium.

This paper reports the results of an electron microscopic study of the structure and permeability of the endothelium of lingual capillaries and postcapillary venules of the rabbit following platelet depletion by an antiplatelet antibody and by busulfan. We have observed remarkable attenuation and fenestration of the endothelium associated with platelet depletion and increased permeability to colloids, as revealed by the transmural passage of Thorotrast.

MATERIALS AND METHODS

Healthy male albino New Zealand rabbits weighing 4-4½ kg were used in these experiments. The tongue was studied because it has a rich vasculature, is easily accessible, and is suited for repeated biopsy. The vessels examined were capillaries as defined by Majno (vessels of a single endothelial cell thickness with a diameter of ≤ 10 μ) and therefore may include smaller postcapillary venules. We limited our observations to the capillaries supplying muscle. Animals were anesthetized with intravenous pentobarbital and biopsied along the lateral edge of the tongue with a corneoscleral punch (Storz, St. Louis, Mo.). Bleeding was controlled with 6-0 silk sutures.

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platelet antiserum (PAS) prepared by the method of Mattison. A dose of 1 cc usually reduced the platelet count from normal (400,000–500,000/cu mm) to severely depressed levels (0–30,000/cu mm) within 15 min. Platelets were counted by the method of Bull et al. The platelet count remained low for about 9 hr before it began to rise spontaneously. Therefore, injections were given every 8 hr when it was desired to maintain thrombocytopenia. When the platelet count was ≤ 30,000/cu mm, minimal trauma produced petechiae. As a control, rabbits were given a similar dose of normal guinea pig serum. No changes in the platelet count were observed in this group.

Thrombocytopenia was also produced by the intraperitoneal injection of busulfan (Myleran, Burroughs-Wellcome, Research Triangle, N.C.) according to the method of Evensen et al. Severe thrombocytopenia and granulocytopenia developed 14 days after the first injection of busulfan, and biopsies were done at that time.

To study the permeability of the endothelium to particulate matter, two rabbits made thrombocytopenic by PAS injection were each given 2 cc of a suspension of thorium dioxide stabilized in dextran (Thorotrast, Fellows-Testagar, Detroit, Mich.) intravenously. Tongue biopsies were made 1, 3, and 5 min after injection. A normal rabbit was given a similar dose of Thorotrast.

All biopsied material was immediately placed in cold 3% glutaraldehyde in phosphate buffer at pH 7.4 and sliced into cubes no larger than 1 cu mm. After 1 hr fixation in glutaraldehyde, the tissue was rinsed and then fixed in 1% OsO4 in phosphate buffer at pH 7.4. The tissue was dehydrated in ethanol, cleared with propylene oxide, and embedded in araldite. Thick sections (0.5 μ) were cut with glass knives, stained with toluidine blue, and examined by light microscopy.

Fig. 1. Normal capillary. Collagen (C) is abundant and is seen in cross section and longitudinally (arrow) showing its periodicity. Part of a pericyte (P) is present and is enveloped by the basement membrane (BM). Vesicles (V) are numerous and measure about 700A. The thickness of the endothelium averages 2000 A. The winding course of an intracellular junction (J) is seen. A Golgi body (G) is in the area of the nucleus (N). × 22,500.
Thin (silver to gray) sections were cut with a diamond knife (DuPont Instruments, Wilmington, Del.) on a Porter Blum ultramicrotome (Sorvall, Norwalk, Conn.) and stained in aqueous uranyl acetate for 30 min at 60°C, followed by 30-60 sec of staining with lead citrate. These sections were then examined in a Siemens Elmskop IA.

RESULTS

Normal Animals

Capillaries in the tongue muscle had a structure like that described by Majno, Bruns and Palade, and Luft. Briefly, this consisted of a single layer of endothelium measuring 0.15–0.30 μ (1500–3000 Å) in thickness in areas away from the nucleus (Figs. 1 and 2). Occasionally the luminal surface was thrown up into folds and small projections. The endothelium was of the continuous variety (see below) having no fenestrations. It contained many vesicles about 700 Å in diameter. In no place did the vesicles singly or in groups bridge the thickness of the endothelium. There were small numbers of mitochondria, ribosomes, lysosomes, and scanty endoplasmic reticulum. A basement membrane (BM) having a thickness of 1000–1500 Å was present. Pericytes were common. They were within the BM. Outside the BM, loose connective tissue and collagen were encountered. No extravascular RBCs or white blood cells were seen. Control animals injected with guinea pig serum had normal vessels.

PAS-thrombocytopenic Animals

Animals were biopsied 1, 9, and 24 hr after the induction of thrombocytopenia. The changes to be described were observed as early as 1 hr but were more commonly present at 9 and 24 hr. Thrombocytopenic vessels showed a comparative lack of luminal projections, thereby leaving a smooth, effaced surface (Fig. 3A). Extravascular RBCs were frequently seen but were only very rarely observed in the wall of a vessel. The altered endothelium was thinner than normal, often only by 500 Å. Vesicles bridged its full thickness. Fenestrations in the wall were common (Fig. 3B). They typically were covered by a thin diaphragm only about 70 Å thick. Endothelial vesicles remained, however, in contrast to normal fenestrated endothelium (see below). These changes were seen in about half of the capillaries examined and were interspersed with areas of normal-appearing endothelium. One PAS-treated animal was allowed to correct spontaneously its platelet deficiency. The observed endothelial changes were not found 24 hr after a platelet count of >100,000/cu mm.

Busulfan-thrombocytopenic Animals

Busulfan-treated animals having thrombocytopenia longer than 12 hr showed endothelial changes identical to PAS animals (Fig. 3C).

Thorotrast Administration

After 9 hr of PAS-induced thrombocytopenia, Thorotrast was injected intravenously into two animals. Biopsies were taken at 1, 3, and 5 min. In a control rabbit injected with Thorotrast, the normal endothelial structure was maintained, and Thorotrast was observed only in the vascular lumen (Fig. 4). In the thrombocytopenic animals, the endothelial changes of thrombocytopenia were
Fig. 2. (A) Normal endothelium. It is of a fairly uniform thickness of about 1500–2500 Å. The basement membrane (BM) is seen. The lumen (L) contains precipitated plasma proteins. x 45,000. (B) Normal capillary adjacent to a muscle cell (MC). The endothelium shows an intercellular junction having four darkened areas (arrows) where gap junctions are formed. x 45,000.

Fig. 3. (A) Capillary from an animal made thrombocytopenic by PAS. The endothelium is attenuated to a thickness of about 700 Å thereby nearly allowing a vesicle (arrow) to bridge its thickness. The process of a pericyte (P) is present. x 45,000. (B) Capillary from a PAS animal. The endothelium is bridged by a vesicle (double arrow), and at least two fenestrations with diaphragms (arrows) are observed. A slightly thinned but otherwise normal basement membrane (BM) is present. x 45,000. (C) Capillary from busulfan-treated animal. Changes identical to PAS-treated animals are present. Two fenestrations (arrows) are present. x 45,000.
Fig. 3. See legend on facing page.
Fig. 4. (A) Normal capillary 3 min after Thorotrast. The lumen (L) contains a red cell (RBC) and Thorotrast (T). Many fewer radiodense glycogen granules (GG) are seen in the endothelial cytoplasm. The endothelium is normal and displays a mitochondrion (M). The basement membrane (BM) is clearly seen. × 45,000. (B) Normal capillary 5 min after Thorotrast. A red cell (RBC) is in the lumen (L). Thorotrast (T) is abundant. A junction (J) is present. Collagen (C) is in the subendothelium. × 45,000.
Fig. 5. (A) Thrombocytopenic vessel 1 min after Thorotrast injection. The changes associated with thrombocytopenia, i.e., attenuation and three fenestrations (arrows), are present. Thorotrast is near the upper fenestration but is contained within the lumen. \( \times 45,000 \). (B) Thrombocytopenic vessel 3 min after Thorotrast injection. Thorotrast particles (T) are seen extravascularly. Note the complete bridging (arrow) of the endothelium by a vesicle apparently covered by two diaphragms. \( \times 45,000 \).
Fig. 6. (A) Thrombocytopenic vessel 5 min after Thorotrast injection. More extravascular Thorotrast is observed (T). Note that the basement membrane, collagen, and connective tissue do not act as barriers to the Thorotrast. × 45,000. (B) Intracellular junction in a thrombocytopenic animal. The junctions were normal and did not participate in the leakage of Thorotrast (T) from the lumen (L). This long junction was found in a thick perinuclear area. × 72,000.
present. At 1 min (Fig. 5A), Thorotrast was only intravascular. At 3 min (Fig. 5B) and 5 min (Fig. 6A), it was seen in the extravascular space also. Thorotrast was not concentrated at areas near fenestrations as has been reported in fenestrated intestinal endothelium of normal animals using smaller molecules (horseradish peroxidase and ferritin) and a much longer period of observation. We did not observe Thorotrast in intracellular junctions or fenestrations (Fig. 6B). It appears that Thorotrast traverses the endothelium via vesicles (Fig. 7) in thrombocytopenic animals. Similar transport was not seen within 5 min in normal animals.

**DISCUSSION**

Electron microscopic studies have shown that capillary structure is variable with dissimilar types having distinct functions present in different organs. Majno has divided endothelium into three types. Type 1 is the continuous endothelium seen in muscle, lung, and central nervous system. This endothelium is at once permeable enough to allow passage of materials and nutrients, yet impermeable to the cellular elements of the blood and the bulk of plasma and its proteins. Type 2 is fenestrated endothelium as present in the renal glomerulus and tubules, intestinal mucosa, choroid plexus, and endocrine glands.

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**Fig. 7.** The passage of Thorotrast across the endothelium in thrombocytopenia. Each figure is x 45,000. The lumen (L) is oriented toward the top of each figure. (A) Thorotrast is seen in an outpouching of the endothelium. (B) A vesicle contains a Thorotrast particle. (C) A vesicle has apparently opened at the ablumenal side and released a particle of Thorotrast. (D) Thorotrast is present between the endothelial cell and pericytes.
These tissues are characterized by fluid production or absorption. The fenestrated endothelium would appear to afford increased permeability to fluid but resistance to cellular passage. Fenestrated endothelium only rarely has vesicles. Type 3 is the endothelium found in the sinusoids of the bone marrow, liver, and spleen where cellular passage is permitted.

The above reviews\textsuperscript{3,9,10} did not show thinned areas or fenestrations in continuous endothelium, although Palade and Bruns\textsuperscript{12} did find occasional fenestrations in the lamina propria of the lingual mucosa. We found no fenestrations in normal tissue. In thrombocytopenia, the endothelium became fenestrated but, unlike normal fenestrated endothelium, many vesicles were still present.\textsuperscript{13} The observation that RBCs escape in thrombocytopenia indicates that in this respect the endothelium functionally resembles type 3. With the large number of extravascular RBCs and the very rare observation of RBCs in the vascular wall, it must be surmised that the actual passage through the endothelium is very rapid. Using capillary microscopy in thrombocytopenic humans, Humble\textsuperscript{14} observed that RBCs were rapidly “hurled from the vessel” and came to rest several cell diameters away from the capillary. Van Horn and Johnson\textsuperscript{15} demonstrated that RBCs go through the endothelial cells rather than between them.

Endothelial changes associated with thrombocytopenia have not been described. In the valuable studies of Johnson and colleagues,\textsuperscript{16} thrombocytopenic endothelium was characterized as unchanged from normal, yet examination of their published micrographs clearly shows endothelial thinning and fenestrations (see Fig. 2, reference 16). After we observed these changes in association with thrombocytopenia, we confirmed the observation in busulfan-induced thrombocytopenia to be certain that they were not due to antiserum against endothelium or to other effects of heterologous serum infusion. Since busulfan produces thrombocytopenia by interfering with platelet production, as contrasted with peripheral destruction due to PAS, we also excluded the possibility that the endothelial changes observed were secondary to the release of some platelet factor. Normal guinea pig serum infusion did not result in endothelial changes. All the observed alterations disappeared when the platelet count returned to normal.

Recently, endothelial changes of swelling, vacuolation, and desquamation have been described following endotoxin\textsuperscript{17} and rattlesnake venom\textsuperscript{18} administration. In these two studies, neither thrombocytopenia nor the changes we have observed in association with thrombocytopenia were observed. Gimbrone et al.\textsuperscript{19} perfused isolated dog thyroid glands with heparinized dog plasma either with or without platelets. Vascular integrity was better maintained with the platelet-containing medium. After 5 hr of infusion with the platelet-poor medium, endothelial disruption and extravascular hemorrhage occurred.

Thorotrast (200 Å) crossed the endothelium within 5 min in thrombocytopenia but not control animals. Smaller molecules such as horseradish peroxidase (50 Å) and ferritin (110 Å) are able to traverse normal fenestrated intestinal endothelium via the fenestrations and not by the vesicles. This movement requires 4 hr for ferritin,\textsuperscript{11} with horseradish peroxidase taking somewhat less time.\textsuperscript{11} In nonfenestrated endothelium, Bruns and Palade\textsuperscript{20} demonstrated that ferritin crossed the endothelium via the vesicles, reaching a maximal extravascular concentration in about 24 hr. Others\textsuperscript{16} studying thrombocytopenic animals were unable to demonstrate extravasation of Thorotrast but did ob-
serve that the passage of carbon particles across the endothelium was dependent on thrombocytopenia. Intracellular junctions were intact. We found that Thorotrast gains access to the extravascular spaces via vesicles. How thrombocytopenia enhances vesicular transport is unclear. Vesicular formation, energy requirements, and rate of transendothelial migration have been studied but offer no clues to the role platelets may or may not play. Perhaps a thinner endothelium would simply take less time to traverse at a given rate of migration.

It is still unclear how platelet levels affect endothelial structure and function. Platelets do repair lesions in the endothelium. This function has been studied and is closely related to aggregation. It is thought that this function is reflected clinically by the bleeding time. The maintenance of a normally thick non-fenestrated endothelium supplying muscle tissue would appear to be a separate function of platelets. Spontaneous extravasation of RBCs and plasma and their appearance in lymph do not require vascular trauma or endothelial tears. Roy and Djerassi differentiated these two functions in the following experiment. Using irradiated thrombocytopenic dogs, they found that numbers of infused platelets, too small to result in detectable increases in the peripheral platelet count, decreased the number of RBCs in thoracic duct lymph by 50% with no effect on the bleeding time. Only with much larger platelet infusions did the peripheral platelet count increase and the bleeding time decrease. Likewise, Aursnes observed that RBCs in ear lymphatics often decreased dramatically 1 day prior to an increase in the platelet count during the spontaneous recovery of postirradiation thrombocytopenia. These observations are consistent with the concept that only a small number of platelets is needed to maintain vascular integrity. Of clinical interest is the observation that patients with immune thrombocytopenic purpura (ITP) bleed less than patients with aplastic anemia having the same platelet count. Perhaps the constant turnover of platelets in ITP affords greater vascular integrity, although other explanations based on platelet size and efficiency have been proposed.

Johnson and colleagues proposed that platelets are incorporated into endothelial cells and thereby affect endothelial support. Presumably, the lack of platelets would therefore preclude this support and lead to dysfunctional endothelium. They supported their position by micrographs interpreted as showing platelets in the process of being assimilated into endothelium. Cronkite et al demonstrated endothelial incorporation of labeled sulfur from tagged platelets after their infusion into thrombocytopenic animals. Similar results were found using tritiated DFP-labeled platelets.

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REFERENCES


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