Platelet Aggregation at Sites of Minimal Endothelial Injury

An Electron Microscopic Study

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THERE IS now considerable evidence to indicate that the development of a platelet thrombus at a site of mechanical injury to a blood vessel is attributable to the disruption of the endothelial lining and exposure of the underlying collagen.[1] Aggregates of intact platelets, however, are often found adjacent to unaltered endothelium after very mild injury. Although some of these changes can be shown by serial section to be continuous with altered platelets and fibrin strands accumulating at sites of demonstrable endothelial damage in another plane of section, many others show no evidence of alteration or association with fibrin and appear to be unrelated to obvious endothelial breaks.

It has recently been shown that, following depression of fibrinolytic activity in small veins with ε-aminocaproic acid (EACA), fibrin can be demonstrated under conditions in which it was not previously visible.[2] Furthermore, platelets associated with just a few fibrin strands almost invariably fail to show evidence of the alteration generally associated with exposure to thrombin or collagen. It seemed possible, therefore, that minute endothelial lesions not easily identifiable in the absence of fibrin or platelet change might be more readily recognizable after fibrinolysis was depressed and that such lesions might account for the accumulation of unchanged platelets after mild injury. Using this approach, such lesions involving only small portions of endothelial cells and closely associated with active foci of coagulation and clumps of unaltered platelets can, in fact, be demonstrated.

Methods

Male Sprague Dawley rats weighing 250–500 gm. each and fed ad libitum

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were anesthetized with intraperitoneal Nembutal. Intravenous fixative introduced distal to the undisturbed femoral vein in 5 rats provided a control preparation free of trauma. Minimal trauma was produced in 10 other rats by gentle separation of the contents of the femoral canal from surrounding structures 10 min. before the introduction of fixative. Some of the findings in these groups have been reported.[1] Three rats received EACA (Amicar, 90 mg./kg.) intravenously prior to dissection of the femoral vein.

Fixation was accomplished by intravenous perfusion of 3% glutaraldehyde in 0.2 M cacodylate buffer at pH 7.4 [3] from a point distal to the region intended for study. Additional fixative was applied externally in dissected preparations for 2 hr. The segment was then excised, sectioned transversely and postfixed in osmium tetroxide in 0.2 phosphate buffer at pH 7.4 for 2 hr. Specimens were embedded in Epon 812 after dehydration with alcohol.[4] Thin sections were stained with uranyl acetate and lead [5] and examined by electron microscopy. Representative blocks from each experimental group were sectioned serially. Single-aperture grids were used in all studies to permit an unobstructed view of large segments of the vein wall.

Results

Control Specimens

The appearance of the components of the venous wall fixed before dissection was unremarkable and has been previously described.[1] With perfusion-fixation, glutaraldehyde artifacts were rare in endothelial cells. These cells always presented a continuous layer with complex interdigitations at junctions. The endothelial cells were closely apposed, especially at the tight junctions. The subendothelial layer contained collagen and amorphous material, presumably elastin, deep to the basement membrane. A platelet clump which resembled those described below was seen only once in the course of examining many blocks from the five control experiments. The lumen contained flocculent material thought to represent plasma proteins and a few white cells and platelets were interspersed among many erythrocytes.

Minimal Trauma

Although most components of the vascular wall were unchanged when fixation followed 10 min. after dissection of the femoral sheath, moderate focal alterations were noted in some endothelial cells and in the subendothelial space in every specimen. An occasional endothelial cell displayed vacuoles, although endothelial continuity always appeared unbroken and cellular junctions intact. Platelet clusters were found associated with the surface of the endothelium, but the separation between platelet and endothelial plasma membrane was never less than 200 Å. (Fig. 1). Individual platelets retained their elliptical outlines, contained granules and mitochondria, and displayed normal microtubules arranged at the periphery. The platelets were loosely associated

and obviously extended over a large area although no platelets were seen to penetrate the endothelial layer. About 5–10 clusters, each consisting of approximately 1–4 platelets in a single plane of section, were characteristically found in a thin cross section of the vein. Most of the clumps tended to occur on the posterior wall near the area of greatest trauma during dissection. Platelets closely apposed to endothelial cells were generally found in the region of cellular junctions as opposed to those regions overlying endothelial nuclei. Structural changes in the endothelial cells were occasionally seen in these areas, but the absence of fibrin made it impossible to distinguish such lesions from artifacts induced by tissue handling.

Minimal Injury after EACA

Although the general appearance of the endothelium and of the subendothelial space in these preparations could not be distinguished from that seen after mild injury alone, many groups of platelets near the endothelial surface were now intimately associated with fibrin (Fig. 2). In spite of this, there was no discernible alteration in the shape or granule content of the platelets, and microtubules were seen in characteristic peripheral distribution. Serial sections at the sites of occasional platelet groups, however, revealed minute structural alterations of the endothelium closely associated with the fibrin (Fig. 3). These endothelial flaws occurred near complex endothelial junctions and fibrin strands were often associated with them in spite of the fact that these junctions appeared to remain closely apposed. Additional aggregates, however, remained unassociated with either demonstrable endothelial alterations or fibrin.

Discussion

These experiments provide evidence that at least some of the platelet clumps that tend to accumulate near the endothelial surface following minimal venous injury are associated with minute lesions of the endothelial cells. Such lesions are difficult to appreciate by routine electron microscopy following standard methods of tissue preparation since the absence of fibrin and platelet change make them impossible to distinguish from artifacts induced by tissue handling subsequent to sacrifice of the animal. When fibrinolytic activity is inhibited by EACA, however, minute foci of fibrin formation can be identified and some are clearly associated with such lesions. Furthermore, the fibrin observed must have been formed prior to the infusion of fixative which abruptly terminates the coagulation process.

Most of the lesions associated with traces of fibrin under these conditions involve small portions of the endothelial cells close to the complex

cellular junctions. Although interruptions in the endothelial cell membrane appear to be present in some instances, no clearly demonstrable break is evident in the majority. In addition, platelets aggregating in or near these areas show no evidence of change and are not seen beneath the endothelium in contact with collagen, as is the case with more severely traumatized areas. It seems unlikely, therefore, that the platelet aggregation is attributable to direct contact with collagen. A more likely possibility is that injury permits access of the plasma proteins to the subendothelial tissues with resultant activation of procoagulant factors and local evolution of thrombin. In the presence of a normally active fibrinolytic system, fibrin would not become visible unless the rate of fibrin formation were sufficiently great or, as in these experiments, the fibrinolytic activity were depressed. Under these conditions, platelet alteration would not necessarily occur. The coagulation mechanism may also be triggered by breaks in the plasma membrane with exposure of the cytoplasmic contents of the endothelial cells to the plasma, or by separations at the endothelial cell junctions induced by injury.[6] On the other hand, even severe injury to the cell does not appear to attract platelets if the plasma membrane remains intact.[1]

The frequency with which these minute lesions occur near cell junctions may well account for such earlier observations as those of Samuels and Webster.[7] These investigators, using *en face* silver preparations, demonstrated that platelets tended to adhere at the silver lines between apparently intact endothelial cells following minimal injury. More recent electron microscopic observations on such preparations by Florey, Poole, and Meek [8] have shown, however, that silver tends to deposit to some extent on all endothelial cell surfaces and that the silver lines correspond only roughly to the cell junctions. Mild injury producing cellular disruptions close to the relatively fixed junctions could readily produce the effect noted by Samuels and Webster.[7]

A considerable number of platelet aggregates lying close to the endothelial surface fail even on serial section to show evidence of associated endothelial abnormality or fibrin following the inhibition of fibrinolysis by EACA. Since such clumps are also seen in similar preparations obtained from animals treated with heparin, their presence suggests that the release of thrombin is not a necessary prerequisite for their occurrence in all instances.

Summary

Routine electron microscopic examination of rat veins 10 min. after minimal mechanical injury reveals platelet aggregates adjacent to apparently intact endothelium. When fibrinolysis is depressed by ε-amino-

caproic acid (EACA) and sections are studied serially, many platelet aggregates are found to be intimately associated with fibrin and to be adjacent to minute lesions of the endothelial cells. These lesions characteristically tend to occur near cellular junctions, a phenomenon that may explain the tendency of platelets to deposit along the silver lines in *en face* preparations following mild injury.

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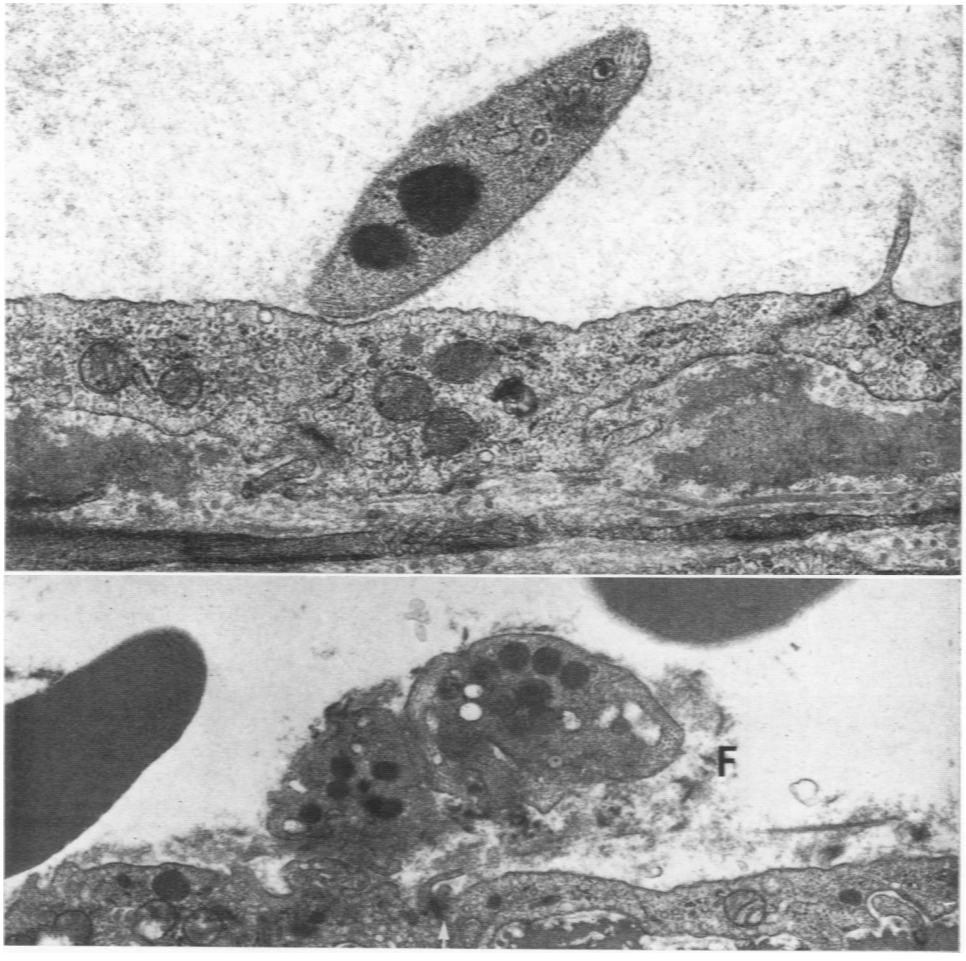
[*Illustrations follow*]

Legends for Figures

Fig. 1. Unaltered platelet adjacent to apparently intact endothelial cell seen in routine preparation after minimal injury of rat vein. Platelet retains normal shape and contains granules and peripheral microtubules. It is separated from endothelial cell by 200 Å. Serial sections reveal other platelets forming a loose aggregate. No fibrin is seen. × 17,000.

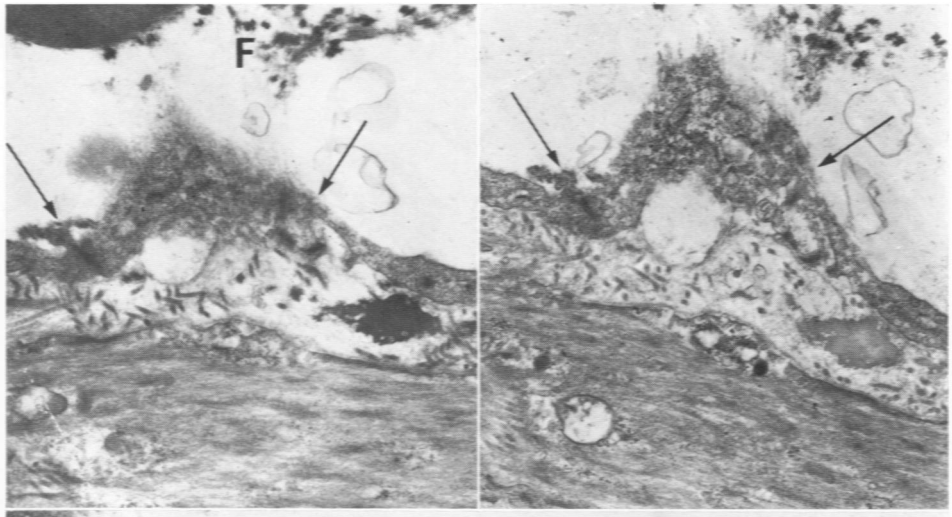
Fig. 2. Unaltered platelets adjacent to intact endothelium of mildly injured rat vein after EACA. Fibrin *(F)* is seen intimately associated with platelets which contain granules and peripheral microtubules. Platelets overlie endothelial junction *(arrow)*. × 17,000.



1

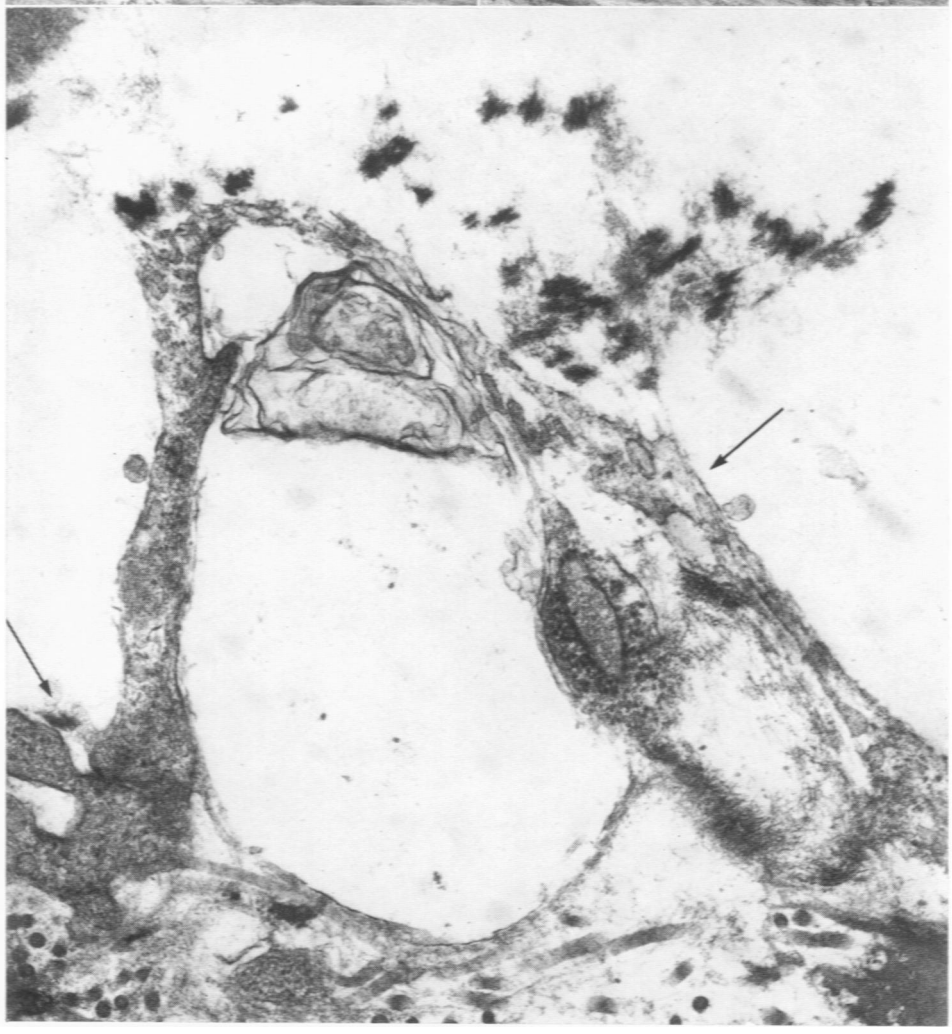
2

Fig. 3. Selection from serial sections of an endothelial cell 10 min. following minimal trauma after EACA. Arrows indicate sites of junctions with adjacent cells. **A.** Central endothelial cell is obliquely sectioned but intact. Fibrin *(F)* appears remote from cell. × 12,000. **B.** Central endothelial cell still appears intact although more distorted. Fibrin is seen closer to cell. × 12,000. **C.** Central cell is disrupted with myelin figure and loss of cellular organization. Fibrin is now intimately associated with the cell. Junction on the right cannot be seen in this picture, but its probable location is determined from adjacent sections. × 27,000.



3A

3B



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