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Original Articles

Endothelial and Perivascular Anionic Sites During Immediate Transient Vascular Leakage

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Summary. To study surface charge characteristics of small blood vessels and perivascular components in vivo, rat cremaster vessels exposed to serotonin or mild thermal injury were labelled with systemically injected cationized ferritin and studied by electron microscopy. Leaky vessels showed increased density of anionic sites on the luminal endothelial plasma membrane, compared to controls. Binding of cationized ferritin and the increased density of anionic sites in leaky vessels occurred in the absence of serum factors; albumin diminished both phenomena. Anionic sites were also demonstrated a) on the surface membranes of open interendothelial junctions, b) on the attachment surface of endothelial cells, c) along the vascular basal lamina, perimysial membrane, and interstitial collagen. The biological significance of these findings is considered in relation to ligand-anionic sites interaction, inflammation, vascular permeability, and thrombosis.

Key words: Plasma membrane – Vascular permeability – Vasoactive mediators – Burn – Inflammation – Thrombosis.

Introduction

Inflammation following a variety of stimuli is commonly associated with increase in vascular permeability, which is largely due to the formation of gaps between adjacent endothelial cells (Majno and Palade 1961). Such gaps, considered to result from endothelial contraction (Majno et al. 1969), occur at specific sites along the microcirculation, depending on a variety of factors, including the nature and intensity of stimuli. For example, mild thermal injury causes a biphasic vascular response (Sevitt 1958; Allison and Lancaster 1959; Wilhelm and Mason 1960): a) *immediate* (and transient), predominantly venular leakage (Cotran and Remensnyder 1968) maximal 10–15 min after burning, and b) *de-*

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layed (and prolonged), predominantly capillary, but also venular leakage (Cotran and Remensnyder 1968; Wells 1971) occurring 15–90 min after burning and lasting for 2–4 h. The immediate, transient phase of vascular leakage can be induced in the intact animal by such agents as histamine and serotonin (Majno and Palade 1961; Shea et al. 1973), and can be suppressed by prior treatment with histamine H2-receptor antagonists (Black et al. 1972; Brimblecombe et al. 1976).

The vascular endothelial membrane is the site of a variety of biological interactions (reviewed by Pelikan et al. 1979), and contains polyanionic mucopolysaccharides (Buonassisi and Root 1975) and intrinsic anionic groups (Danon and Skutelsky 1976; Skutelsky and Danon 1976; Pelikan et al. 1979) imparting to this surface a net negative charge under physiological conditions (Danon et al. 1972). Cell surface- and tissue-associated anionic sites can be effectively labelled, in vivo, under physiological conditions, using the ultrastructural electron dense tracer cationic ferritin (CF) developed by Danon et al. (1972). The charge characteristics of the vascular membrane conceivably contribute to normal vessel functions (such as permeability and antithrombotic properties) and may play an important role in inflammatory processes. Thus, during the inflammatory reaction, a modification of cell surface anionic sites could possibly occur; also, this experimental setting would afford the opportunity to probe, in vivo, charge characteristics of open intercellular junctions, other vascular wall components, and the contiguous perivascular interstitium.

We report in this communication the observed distribution of CF-labelled anionic sites in leaking venules and perivascular tissue using serotonin and mild thermal injury to rat cremaster muscle as experimental models of immediate transient vascular leakage.

Materials and Methods

Animals

Seventeen adult male Sprague-Dawley rats (Timco Breeding Laboratories, Inc., Houston, Texas) kept under standard laboratory conditions with free access to food and water were used.

Tracer Preparation

Cationized ferritin (CF) derivatives were prepared according to Danon et al. (1972). The reagents used were: 1) native, twice crystallized, cadmium-free horse spleen ferritin, isoelectric point (pI) 4.2(4.6 (Polysciences, Inc. Warrington, PA), 2) 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (Sigma Chemical Co., St. Louis, MO), used as an activator, and 3) 1,6-hexanediamine (Eastman Kodak Co., Rochester, NY) used as a nucleophile to replace the carboxyl groups. When the cationization reaction is carried out at pH 6.5, CF derivatives with pIs ranging from 7.7 to 8.5, suitable for systemic infusion, are obtained (Kelly and Cavallo 1980). After dialysis, the final solution of CF was sterilized by filtration, suspended in 0.15 M sodium chloride, and adjusted to a protein concentration of 30 mg/ml. The CF was then examined ultrastructurally to confirm that the shape of the molecules was not altered and that the solution was monomolecularly dispersed. Further details of tracer preparation and pI estimates are as described previously (Kelly and Cavallo 1980).

Experimental Procedures

All animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (Nembutal, Abbot Laboratories, IL), 5.0 mg/100 g body weight. Two experimental models were used for the induction of immediate transient vascular leakage: serotonin injection into the scrotum (Majno and Palade 1961) and mild controlled thermal injury to the cremaster muscle (reviewed by Shea et al., 1973).

Induction of Vascular Changes by Serotonin. Serotonin (5-hydroxytryptamine creatinine sulfate, Sigma Chemical Co., St. Louis, MO) was injected subcutaneously into the left side of the scrotum, as described by Majno and Palade (1961). This substance readily diffuses into the underlying cremaster muscle. The serotonin salt was suspended in 0.15 M sodium chloride to yield dilutions of 1:20, 1:100, 1:1,000 and 1:10,000. A volume of 0.05 to 0.1 ml of the test material was injected. Two min after the delivery of serotonin, the CF solution was infused through the inferior vena cava using a polyethylene tube attached to an infusion pump (Harvard Apparatus Co., Millis, MA). The tracer was delivered at a constant flow of 0.1 ml/min. After 1, 3, 4, 8, and 10 min, the infusion of CF was discontinued, the cremaster muscle was exposed, and ~ 1 cm^2 area underlying the injection site was excised. The right cremaster, injected with a similar volume of saline, or not injected at all, was similarly sampled and used as control. Most observations were made on cremaster vessels removed 10 min after injection of the tracer.

Thermal Injury. The cremaster muscles were exposed and thermal injury was produced by gentle contact of a preheated disc for 20 s against the anterior surface of the muscle. The disc was kept at a constant temperature by circulating water heated to 54° C from a water bath (Neslab Instruments, Inc., Portsmouth, NH). The exposed contralateral cremaster muscle not subjected to thermal injury served as control. The skin incisions over each cremaster were then gently approximated. Two min after thermal injury, CF was infused as detailed above. After 10 min, the tracer infusion was discontinued, the scrotal incision opened, and thermally injured and contralateral cremaster muscles were sampled as in the serotonin experiments. This procedure is preferable to thermal injury to the skin, in which less consistent vascular alterations are induced (Wood and Hurley 1979).

To assess the contribution of blood factors in CF binding, the following procedures were used in separate experiments. The aorta was cannulated and vessels supplying the cremasters were washed free of blood with oxygenated Krebs-Ringer buffer (KRB) for 5–6 min. A small incision in the inferior vena cava allowed perfused fluids to drain freely. The exposed cremaster muscles were inspected to ascertain the adequacy of the perfusion procedure. Serotonin (0.1 ml, 1:1,000 dilution) was applied topically to one cremaster muscle; the contralateral cremaster served as control. Cationized ferritin (3.0 mg/ml) was then delivered in KRB alone or in a solution of KRB containing 3.0 g/dl bovine serum albumin (Sigma Chemical Co., St. Louis, MO). Tracer-containing fluids were perfused at a pressure of 90 to 110 mm Hg, and tissues were sampled 4–9 min after start of CF perfusion.

To exclude trapping of CF as the mechanism of vascular basal lamina and perivascular tissue labelling, slices of fresh cremaster muscle were incubated for 3 min with a solution of either CF or anionic ferritin (3.0 mg/ml). The specimens were washed several times to remove unbound tracer particles, and fixed as indicated below.

Tissue Preparation

In most experiments, the specimens were rapidly excised and fixed by immersion. In a few experiments, fixation was initiated in situ by flooding the cremaster muscle with the fixative (Majno and Palade 1961). The excised specimens were gently stretched and pinned onto a sheet of dental wax, under fixative. Ten to 15 min after the initial fixation, the outer edges of the specimen were trimmed away to eliminate areas of trauma induced during sampling. All tissues were trimmed to ~ 1 mm cubes and fixed for 4 h at room temperature in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3. After washing in the same buffer, tissues were post-fixed for 1 h in 1.5% ferrocyanide-reduced osmium (Karnovsky 1971) at room temperature. This procedure enhances membrane struc-

ture and facilitates the visualization of ferritin (Karnovsky 1971). Tissues were then thoroughly rinsed with distilled water, dehydrated through an ethanol series and infiltrated and embedded in Epon 812. Ten blocks were prepared from each experiment; 1.0 μm thick sections were cut with glass knives and stained with 1% toluidine blue. Sections were examined under a light microscope to select areas exhibiting venules in which diapedesis of red blood cells could be detected. Ultrathin sections were cut with a diamond knife on an LKB Ultrotome III ultramicrotome (LKB Instruments, Inc., Bromma, Sweden). Sections of silver interference color (~60 nm thickness) were picked up on a 200 mesh copper grid, stained with lead citrate (Reynolds, 1963), and examined and photographed on a Philips EM200 or EM201 electron microscope (Philips Electronic Instruments, Mahwah, NJ). More than 1,000 vessels were studied, including approximately 3,000 interendothelial junctions.

Results

All animals tolerated the procedure well and no systemic effects were observed during the infusion of CF. The gross and microscopic changes induced in cremaster vessels by serotonin or mild thermal injury were as described in detail by Majno and Palade (1961) and Cotran and Remensnyder (1968). Briefly, with either form of injury, vessels affected were predominantly venules approximately 20 μm in diameter. In such vessels, red cells, lipid particles and leukocytes were detected in gaps produced by separation of adjacent endothelial cells. Thrombosis or necrosis of endothelial cells was not observed. No appreciable qualitative differences in ultrastructure were noted in tissues subjected to the various concentrations of serotonin used. Fixation in situ provided better morphological preservation; however, the findings were similar to those of immersion-fixed tissues. Control vessels from contralateral cremaster muscles were normal; their morphological features were as described in previous accounts (Majno and Palade 1961; Cotran 1967).

Distribution of CF in Normal and Leaky Cremaster Venules

In control venules, binding of CF to the luminal plasma membrane of endothelia occurred mostly in patches, separated by larger inter-patch areas exhibiting little or no binding of the ligand protein (Fig. 1). Often, CF molecules formed patches on the luminal aspect of closed interendothelial junctions extending over the apposing cell membranes (Fig. 1, arrow); labelling of marginal folds was similarly noted. The number of tracer molecules in the patches was variable but this increased slightly with time, averaging 10–40 molecules/patch in a section

Fig. 1. Control blood vessel to show the distribution of anionic sites in normal endothelium (*E*). CF was infused for 10 min before sampling. CF molecules are aggregated in patches (*arrow*) in a random, discontinuous, pattern labelling surface anionic sites. Endocytosis of tracer particles or shedding of CF patches is not prominent. *BL*, basal lamina; *J*, junction; *VL*, vascular lumen. ×33,000

Fig. 2. Small blood vessel exposed to serotonin. CF was infused for 1 min before sampling. Lipid particles (*L*), denoting vascular leakage, are seen in an endothelial (*E*) gap. CF molecules label few surface anionic sites along the luminal and junctional (*J*) plasma membrane (*arrows*), and basal lamina (*BL*). *RBC*, red blood cell. ×33,000

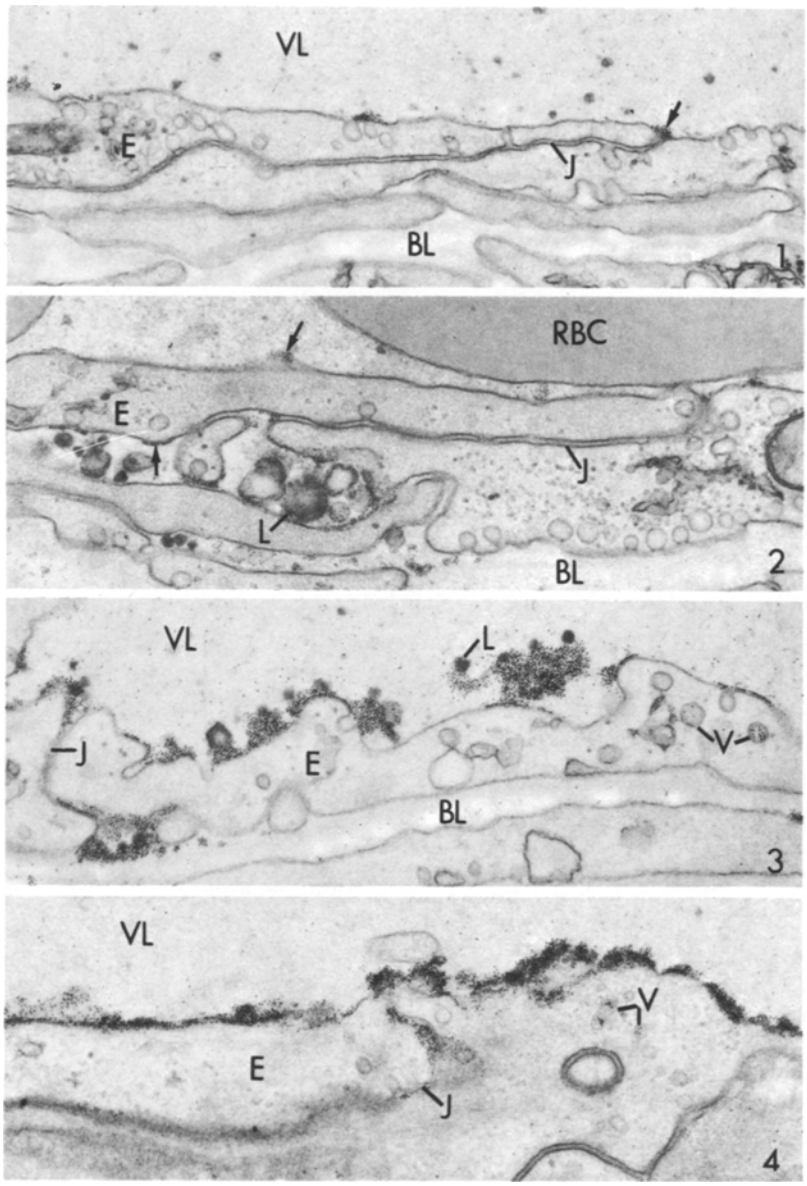

Fig. 3. Small blood vessel exposed to serotonin. CF was infused for 10 min before sampling. Numerous, large, patches and short rows of CF molecules label the irregular profile of the endothelial (*E*) luminal plasma membrane. Large CF patches, presumably in the process of being shed, are associated with lipid (*L*) particles. Segments of a junction (*J*) show surface membrane labelling with CF. Endocytic vacuoles (*V*) containing CF are evident. *BL*, basal lamina; *VL*, vascular lumen. ×33,000

Fig. 4. Small blood vessel subjected to thermal injury. CF infusion was started immediately after burning and the specimen was sampled 10 min thereafter. The luminal endothelial (*E*) plasma membrane is extensively labelled with CF. Large patches of CF are confluent in segments of the membrane. A cell junction (*J*) is partly labelled with CF. A few CF-containing vacuoles (*V*) are seen. *VL*, vascular lumen. ×33,000

of tissue obtained 10 min after exposure to the tracer. Cationized ferritin particles were present in small numbers at the mouth of vesicles at the luminal front or in cytoplasmic vesicles. In the vascular lumen, CF molecules were seen: a) as isolated particles, b) forming discrete aggregates, interpreted as patches shed from the endothelial surface (Skutelsky and Danon 1976; Pelikan et al. 1979), and c) occasionally bound to lipid particles. Although at earlier time intervals there were fewer CF molecules seen in all such locations, the overall distribution of the tracer did not change appreciably during the time intervals studied.

Localization characteristics of CF were comparable between thermal- and serotonin-induced injury (Figs. 2–4). At 1 min after CF infusion, the luminal endothelial plasma membranes demonstrated a few CF patches (Fig. 2) which increased in number and size at later time intervals (Figs. 3 and 4). CF patches in experimental tissues were larger and more frequent, compared to those in control tissues (Fig. 1). Such patches were seen often, but not always, on projections of the endothelial surface (Figs. 3 and 5). Cationized ferritin patches often consisted of several rows of tracer molecules having a pyramidal configuration, but in some instances the labelled segment exhibited a single or double row of particles (Fig. 3). On the average, cross sections of CF patches in experimental tissues included 80–100 particles, compared to 10–40 particles in controls. Since binding of CF in altered junctions and vascular wall components was more readily demonstrated 10 min after the injection of CF, all subsequent observations were made on tissues obtained at this time interval. In endothelial junctions exhibiting variable degrees of intercellular separation (Figs. 5–7), the tracer formed one or two rows of orderly arranged molecules on the apposing plasma membranes. Often this labelling was continuous but limited to a segment of the junction (Figs. 3–7); CF patches were seen rarely in interendothelial gaps or cell junction infundibula (Fig. 8). In a few open junctions, endocytosis of CF particles was seen. The kinetics of membrane labelling with CF, lateral movement of CF patches (Skutelsky and Danon 1976; Pelikan et al. 1979), and interiorization or shedding from labelled segments were not studied in detail in our experiments. However, the number of shed patches and pinocytotic vesicles containing CF particles was greater in experimental vessels at the time intervals studied, compared to control vessels.

Labelling of the basal surface of the endothelial membrane was noted only in vessels in which extensive leakage of CF had occurred (Figs. 9 and 10). Such labelling took the form of aggregates of CF particles numbering 5–10 molecules regularly spaced along the membrane surface (Fig. 10). Although in favorable

Figs. 5–7. Detail of endothelial (*E*) cell junctions (*J*) from small blood vessels exposed to serotonin. CF was infused for 10 min before sampling. Nearly continuous labelling of junctions of cell membranes is demonstrated. Leaked lipid particles (Fig. 6) and CF with labelling of portions of the basal lamina (BL) are seen (Figs. 6 and 7). *VL*, vascular lumen. Figs. 5 and 6: ×33,000; Fig. 7: ×43,500

Fig. 8. Detail of an endothelial (*E*) cell junction (*J*) from a small blood vessel exposed to serotonin. CF was infused for 10 min before sampling. Note labelling with CF in a short linear and patch configuration over part of the junction. *RBC*, red blood cell. ×22,800

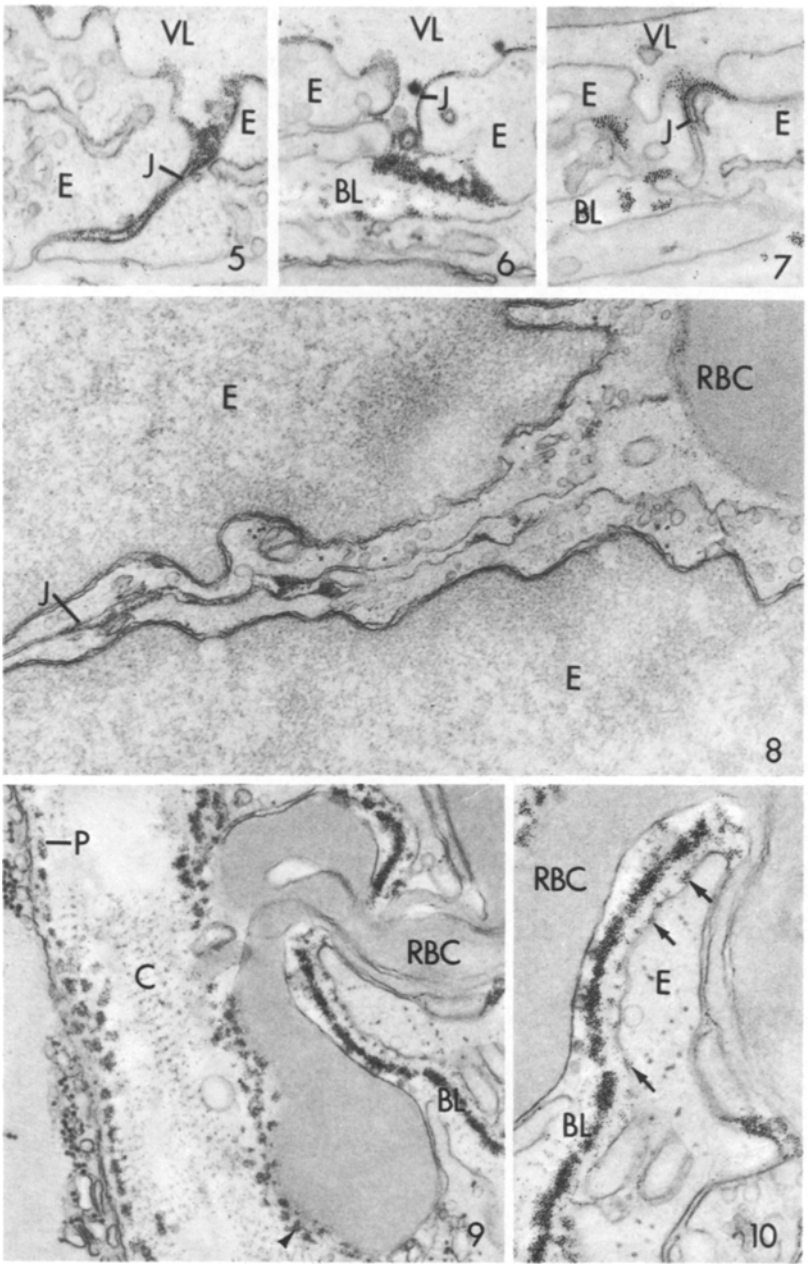

Fig. 9. Small blood vessel exposed to serotonin. CF was infused for 10 min before sampling. A red blood cell (*RBC*) is emigrating through an interendothelial gap. The surface membrane of the endothelial junction is virtually unlabelled with CF whereas at the leading edge of the RBC there are large accumulations of the tracer (*arrow head*). Note CF labelling along the basal lamina (*BL*), interstitial collagen (*C*) and perimysial membrane (*P*). ×26,400

Fig. 10. Detail of Fig. 9 to demonstrate regular cluster of CF molecules (*arrows*) along the attachment surface of the endothelial (*E*) membrane. The basal lamina (*BL*) is heavily labeled with CF. *RBC*, red blood cell. ×39,600

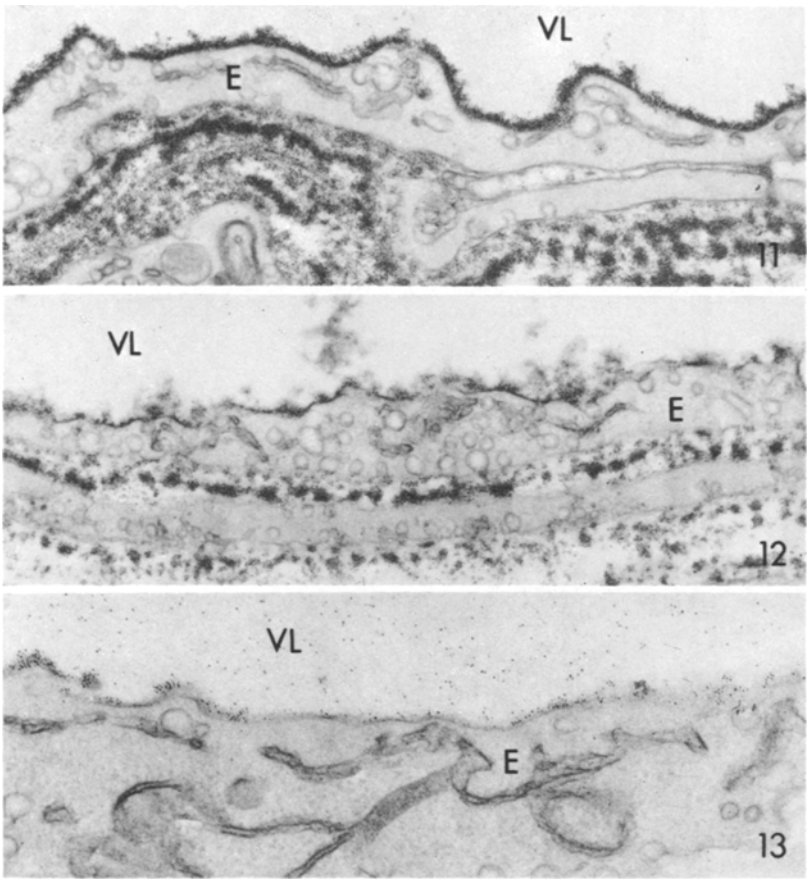

Fig. 11. Vessel perfused with KRB to wash out blood, followed by exposure to serotonin and perfusion with CF (3.0 mg/ml) in KRB. Nearly continuous binding of CF to the luminal surface of the endothelial cell (*E*) is evident. *VL*, vascular lumen. ×33,000

Fig. 12. Vessel perfused with KRB to wash out blood, followed by exposure to serotonin and perfusion with a KRB solution of bovine serum albumin (3.0 g/dl) and CF (3.0 mg/ml). The quantity of CF perfused was the same as in the experiment illustrated in Fig. 11. Binding of CF to the luminal endothelial (*E*) surface is patchy and diminished relative to findings illustrated in Fig. 11. *VL*, vascular lumen. ×33,000

Fig. 13. Control vessel, unexposed to serotonin, from the same experiment illustrated in Fig. 12. Few patches of CF are seen on the luminal endothelial (*E*) surface. *VL*, vascular lumen. ×43,500

sections heavier accumulations of CF molecules were often present at the vicinity of the interstitial aspect of a junction (Figs. 3 and 6), labelling of large segments of the basal lamina (Figs. 9 and 10), interstitial collagen and perimysium (Fig. 9) was also evident. The labelling of basal lamina and collagen fibers by CF particles was uniform and regular and in the case of collagen (Fig. 9) conformed

with binding along the known periodicity of the fibers. Interendothelial gaps exhibiting diapedesis of blood cells showed substantial (Fig. 9) or virtually complete loss of the junction-associated CF molecules. Particles of lipid material were more abundant and of larger size in leaking vessels (Figs. 2, 3 and 6), when compared with controls (Fig. 1).

Binding of CF was increased and exhibited a nearly continuous distribution on endothelial surfaces of serotonin-treated vessels from which blood had been washed previously (Fig. 11), compared to binding in vessels subjected to systemic CF infusion (Figs. 3 and 4). Binding of CF in patches resulted from the addition of bovine serum albumin to the CF-KRB perfusate in serotonin exposed vessels (Fig. 12); these patches of bound CF were diminished relative to experiments illustrated in Figure 11. Control vessels perfused with bovine serum albumin and CF demonstrated few patches of the tracer (Fig. 13), resembling findings obtained when CF was infused systemically (cf. Fig. 1).

Cremaster muscle incubated with CF demonstrated clustering of large numbers of molecules along the adventitial side of the vascular basal lamina of small vessels and to collagen and perimysial membrane, as in the serotonin experiments. By contrast, in cremaster muscle incubated with anionic ferritin tracer molecules were few in number and exhibited no distinct association with such tissue components.

Discussion

Our studies show that cell membranes of normal, continuous endothelium of small blood vessels exhibit sparse, randomly distributed surface anionic sites, as demonstrated by labelling with CF. Such distribution of surface negative sites has also been reported by other investigators in unfixed endothelia of vascular explants of aorta and vena cava of guinea pigs (Danon and Skutelsky 1976; Skutelsky and Danon 1976) and human endothelial cells in culture (Pelikan et al. 1979). Formation of CF clusters over the endothelial luminal membrane in vivo may result from interaction of the ligand with membrane-associated negative sites, followed by aggregation and lateral migration to form CF patches of variable size (Skutelsky and Danon 1976; Pelikan et al. 1979). In cell junctions of normal vessels, clustering of CF particles was limited to the luminal end (Fig. 1), although often a marginal fold was also labelled. Similar findings have been reported in cell junctions of vascular explants in culture systems (Danon and Skutelsky 1976). Interiorization and shedding of CF patches was relatively unimpressive in control vessels. Except for the presence of CF at various sites, control vessels studied were morphologically normal (Majno and Palade 1961; Cotran 1967).

By comparison, the number and size of CF patches was substantially increased in vessels subjected to serotonin or thermal injury (Figs. 3 and 4). It is unlikely that this increased binding of CF resulted from a decrease in shedding or interiorization of CF for, compared to controls, both luminal shedding and endocytosis of CF appeared slightly increased in such vessels. Because distinct differences in CF binding were seen in leaking vessels when compared

to controls studied at the same time interval (cf. Figs. 1, 3 and 4), it appears likely that increase in CF labelling of surface endothelium in our experimental animals represents an actual increase in number of anionic sites. The following possibilities could be considered to explain such a phenomenon. First, the increase in density of anionic sites could be due to interaction of plasma membrane and serum factors. In this regard, experiments in which blood was washed from the cremaster vessels demontrated that a) serum factors are not a prerequisite for CF binding, b) increase in density of anionic sites in leaky vessels occurs in the absence of serum factors, and c) albumin decreases the labelling of anionic sites in vivo. Second, the interaction of CF and surface anionic sites could expose additional anionic groups by inducing conformational changes on membrane constituents (Burry and Wood 1979) or by lateral migration and/or detachment of CF patches (Pelikan et al. 1979). Whereas these latter mechanisms might have contributed to overall CF binding density, quantitative differences in CF binding between leaky and control vessels cannot be explained satisfactorily by these mechanisms alone. Third, under the action of histamine-like mediators, endothelial cells undergo contraction (Majno et al. 1969), which results in alterations of cell membrane profiles. It is conceivable that under such conditions additional anionic sites are exposed, which could account for increased CF density in leaky endothelium.

Binding and clustering of CF particles were also noted along the cell surfaces of interendothelial junctions in experimental vessels. Such findings were taken to indicate that open junctions exhibit a highly anionic surface. Similarly, regular clustering of CF was noted along the adventitial aspect of the endothelial plasma membrane in vessel segments in which substantial leak of tracer occurred (e.g., Fig. 9). This observation confirms, in vivo, that the attachment surface of endothelial cells exhibits anionic sites. The distribution of such sites was random in our experiments but continuous in the experiments of Pelikan et al. (1979). This discrepancy in results could be due to a) prefixation of endothelial cells prior to interaction with CF [glutaraldehyde fixation greatly changes surface membrane charges (Burry and Wood 1979)], as in the experiments of Pelikan et al. (1979), or b) clustering of anionic sites by CF, as in our experiments, or c) the fact that such anionic sites have a discontinuous distribution in vivo.

The vascular basal lamina has been considered a mechanical barrier which functions as a coarse filter preventing the passage of larger macromolecular and aggregated materials (reviewed by Cotran 1967). Our studies show that this component of the vascular wall is heavily labelled with CF in leaking vessels (Fig. 9). Because of a filtration function, it could be argued that localization of CF in this component of the vascular wall could be due to trapping of tracer particles, also. Whereas filtration might be a contributing mechanism, incubation of cremaster vessels with CF, but not anionic ferritin, demonstrated labelling of the vascular basal lamina; this labelling could not be removed with repeated washings of the tissue. Additionally, perivascular interstitial collagen and perimysial membrane also showed labelling with CF, whether tissues were incubated with the tracer or the tracer leaked through endothelial gaps (Fig. 9). In all, such findings were taken to represent binding of the tracer (Danon and Skutelsky 1976), rather than random localization of particles, as

might be expected with anionic ferritin (Danon and Skutelsky 1976; Johansson 1978). Acidic groups derived from aspartic and glutamic acids (Kefalides 1978), phosphates and sulfates (Danon and Skutelsky 1976) in collagen-related materials and, on cell surfaces, sialic acid (Danon and Skutelsky 1976) and potentially carboxyl groups of proteins and phosphate groups of phospholipids (Burry and Wood 1979), presumably account for most of the anionic sites disclosed in our experiments.

We can only speculate about the significance of our findings. An increase in endothelial charge density during inflammation could, by electrostatic repulsion, minimize excessive loss of plasma proteins. Attachment and aggregation of cells to the intima, which is influenced by surface charge density (Sawyer and Srinivasan 1972), could be similarly decreased. Thus, increase in endothelial charge density could modulate inflammation, hemostasis, or both. Finally, charge alone cannot account for endothelial nonthrombogenicity for the thrombogenic subendothelial area and perivascular tissues are also negatively charged. Whether alterations in endothelial surface charges can influence other vascular processes remains to be investigated.

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