REDISTRIBUTION OF SURFACE ANIONIC SITES ON THE LUMINAL FRONT OF BLOOD VESSEL ENDOTHELIUM AFTER INTERACTION WITH POLYCATIONIC LIGAND

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ABSTRACT

The ability of anionic groups on the luminal surface of blood vessels to redistribute by lateral migration under the influence of multivalent ligands was analyzed by electron microscopy, using cationized ferritin (CF). In vitro interaction of blood vessel segments with CF results in rapid aggregation of most anionic sites on the luminal front of the endothelium, followed by internalization or detachment of the CF patches, leaving most of the luminal surface devoid of anionic sites. Further incubation of such endothelial cells without CF results in regeneration of binding capacity for the polycationic label. Transport of CF, but not of native ferritin, across the endothelium by vesicle transport, followed by exocytosis of the interiorized CF clusters on the tissue front of the endothelium, was also observed. The possibility that such activities in the blood vessels in vivo may be associated with local changes in the normal distribution of the surface anionic sites as well as in accumulation of debris in the subendothelial layers of the vessels is suggested.

The luminal front of blood vessel endothelial cells is negatively charged (2, 12, 15). The negative surface charge is believed to contribute to the mutual repulsion between the circulating blood cells and the vessel wall, thus preventing aggregation of cells to the intima (8). It has been suggested that changes in surface charge density as a result of damage to the endothelium may initiate thrombus formation (13).

The surface charge of endothelial plasmalemma cannot be evaluated by electrophoresis, but can be determined by electron microscopical methods using polycationic labels such as colloidal iron (CI) (4) and cationized ferritin (CF) (3). Using these techniques, we have shown that the surface anionic groups on endothelial cells of the guinea pig blood vessels are randomly distributed. About 50% of the anionic groups are sialyl groups and can be removed by neuraminidase. The subendothelial components exposed after removal of the endothelium are also negatively charged (15).

Recent observations of various animal cells have indicated that plasma membrane components such as surface antigens, immune globulins, and receptors for lectins are capable of migration by diffusion in the plane of the membrane. Clustering of such receptors can be induced by bivalent or multivalent ligands such as antibodies and lectins (7, 17, 18).

Use of CF, which interacts with the negative groups of the plasmalemma of the cell at physiological pH and ionic strength and does not require prefixation (3), has made it possible to show that anionic groups, such as lectin and antibody receptors, can be redistributed on the surface of the cell (11, 14, 16).

In the present report, we describe the effect of CF on the distribution of anionic groups on the...
Interaction of Endothelial Cells with Preparation of Blood Vessels

MATERIALS AND METHODS

Preparation of Blood Vessels

Adult guinea pigs (male and female, weighing approximately 250 g) were anesthetized by inhalation of ether in a covered 1-liter beaker. The thoracic aorta just below the arch and the vena cava adjacent to the heart were dissected from the pleura. Two surgical clamps were placed at a distance of 1 cm apart, and the blood vessels were excised near the clamps, immediately removed, and placed in medium 199 (Grand Island Biological Co., Grand Island, N. Y.) with or without 100 U/ml heparin (Pularin, Evans Medical Ltd., Liverpool, England), contained in a 5-cm glass petri dish. While submerged in the medium the blood vessels were cut into 2-mm long ringlike segments with a new razor blade. The blood was washed from the vessel lumen by gentle agitation, and the segments were then transferred into the heparin-free incubation medium.

Interaction of Endothelial Cells with Multivalent Ligands

Blood vessel segments were incubated in 2 ml of medium 199 containing 0.5 mg/ml CF (Miles-Yeda, Rehovot, Israel), warmed to 37°C. Incubation was carried out in a water bath, with shaking, at 37°C, for time periods varying from 30 s to 60 min. When blood vessel segments were prepared in the presence or in the absence of heparin, no noticeable differences were observed in the density or distribution of CF on endothelial cell surfaces. Thereafter, the blood vessel segments were thoroughly washed in CF-free medium 199 by quick aspiration and addition of fresh medium. The vessel segments were then reincubated in tissue culture medium for the desired periods of time, or immediately fixed by addition of Karnovsky fixative (6), as described. In several experiments, 10% bovine serum albumin (BSA, Armour Pharmaceutical Co., Chicago, Ill.) or 20% fetal calf serum (Grand Island Biological Co.) were added to the ferritin-free incubation medium. In a group of experiments, incubation of vessel segments with the CF was terminated by addition of Karnovsky fixative (6), as described. In several experiments, 10% bovine serum albumin (BSA, Armour Pharmaceutical Co., Chicago, Ill.) or 20% fetal calf serum (Grand Island Biological Co.) were added to the ferritin-free incubation medium. In a group of experiments, incubation of vessel segments with the CF was terminated by addition of Karnovsky fixative (6), as described. In several experiments, 10% bovine serum albumin (BSA, Armour Pharmaceutical Co., Chicago, Ill.) or 20% fetal calf serum (Grand Island Biological Co.) were added to the ferritin-free incubation medium. In a group of experiments, incubation of vessel segments with the CF was terminated by addition of Karnovsky fixative (6), as described. In several experiments, 10% bovine serum albumin (BSA, Armour Pharmaceutical Co., Chicago, Ill.) or 20% fetal calf serum (Grand Island Biological Co.) were added to the ferritin-free incubation medium. In a group of experiments, incubation of vessel segments with the CF was terminated by addition of Karnovsky fixative (6), as described. In several experiments, 10% bovine serum albumin (BSA, Armour Pharmaceutical Co., Chicago, Ill.) or 20% fetal calf serum (Grand Island Biological Co.) were added to the ferritin-free incubation medium. In a group of experiments, incubation of vessel segments with the CF was terminated by addition of Karnovsky fixative (6), as described. In several experiments, 10% bovine serum albumin (BSA, Armour Pharmaceutical Co., Chicago, Ill.) or 20% fetal calf serum (Grand Island Biological Co.) were added to the ferritin-free incubation medium.

Aldehyde Fixation

An equal volume of Karnovsky fixative containing 1% formaldehyde and 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, was added to the medium containing blood vessel segments. After fixation for 1 h at room temperature (about 25°C), the tissue segments were washed three times with saline buffered with Veronal-acetate at pH 7.4 (VBS). In one series of experiments, 0.1 M glycine was added to the washing medium to quench free aldehyde groups and thus prevent non-specific adsorption of ferritin to the surface of the membranes. No difference was noted whether glycine was used or not.

Labeling with Colloidal Iron

Positively charged CI was prepared according to the procedure of Gasic et al. (4) and used undialyzed at pH 1.8. Aldehyde-fixed tissue segments or erythrocytes were washed twice with distilled water, incubated with CI for 5 min at room temperature, washed with 12% acetic acid, and then washed again with distilled water. The labeled tissue and erythrocytes were then transferred to VBS for further processing for electron microscopy.

Processing for Electron Microscopy

Aldehyde-fixed tissue segments in VBS were cut into 2 x 1 mm strips along the longitudinal axis of the blood vessel, postfixed for 1 h at 4°C in 1% OsO4, and washed twice in VBS. In some experiments the tissue blocks were labeled after aldehyde fixation and washing with 0.5 mg/ml CF in VBS, then washed twice in VBS, postfixed in OsO4, and washed in VBS. Before labeling with CF, a control sample was incubated in 10 μg/ml poly-L-lysine (Miles-Yeda, Rehovot, Israel) in VBS for 10 min at room temperature, followed by two washes with VBS. The blocks were dehydrated in graded ethanol, stained with 50% saturated uranyl acetate in 50% ethanol, and embedded in Epon (9) flat in the mold. ~600-Å thick slices (gray interference color) were obtained with an MT-2 Sorvall microtome (DuPont Instruments, Sorvall Operations, Newtown, Conn.), mounted on naked 400-mesh copper grids, and coated with carbon without additional straining. A Jeol 100-A electron microscope was used at 80 kV.

Analysis of Labeled Charge Density on Cell Surfaces

Ferritin particles on micrographs of tangential sections of endothelial cell membranes facing the blood vessel...
FIGURE 1 (a) Endothelial cell of guinea pig aorta, fixed with Karnovsky fixative and stained with CF. The ferritin particles are evenly distributed over the luminal front of the endothelial cell. × 60,000. (b) Endothelial cell of guinea pig aorta, fixed with Karnovsky fixative and stained with CI. The CI particles appear in small clusters randomly distributed over the entire luminal surface of the endothelial cell. × 60,000.

FIGURE 2 Endothelial cell of guinea pig aorta, incubated for 1 min with CF, washed, and fixed with aldehydes. The ferritin particles are seen in clusters, separated by unlabeled gaps. Some ferritin is seen in a phagocytic vacuole near the surface of the cell. × 80,000.

FIGURE 3 (a) Endothelial cells of guinea pig aorta, incubated for 1 min with CF, washed, and fixed with aldehydes. A large CF patch is seen on a tangentially sectioned membrane (center). Note the paracrystalline organization of the ferritin within the cluster. × 100,000. (b) Endothelial cell of guinea pig aorta, fixed with aldehydes and labeled with CF. The even distribution of the CF particles is seen on the tangentially sectioned membrane (center). × 100,000.

FIGURE 4 Endothelial cell from guinea pig aorta, incubated for 5 min with CF followed by washing, additional incubation for 5 min in CF-free tissue culture medium, and fixation with aldehydes. The ferritin patches are separated by wide unlabeled gaps. Most of the CF patches are located in a "micro cap" arrangement, on top of cytoplasmic protrusions. Open plasmalemmal pit and ferritin-free vesicles are seen near the cell surface. × 80,000.
RESULTS

Distribution of CF on Aldehyde-Fixed Endothelial Cells

Cationized ferritin was randomly distributed over the luminal surface of the endothelial cell membranes (Fig. 1a). There was no CF detected on the tissue front of the endothelium or within the junctional complexes, or on endothelial cells that were pretreated with poly-L-lysine. There was an average of 2,500 particles (SD ± 180) per $\mu m^2$ of membrane area (Fig. 3b). No significant difference in particle density was found between endothelial cells of the aorta and vena cava. Random particle distribution was also observed on endothelial cells labeled with positively charged colloidal iron (Fig. 1b). Incubation of aldehyde-fixed blood vessel segments with native ferritin did not show any binding of ferritin to the endothelial cell surfaces.

Redistribution of Anionic Groups after Interaction with Polycations

Incubation of unfixed blood vessel segments in the presence of CF for 1 min at 37°C, followed by washing twice with VBS and fixation, showed clustering of most anionic sites on the luminal surface of the endothelial cells. The ferritin clusters were separated by narrow gaps of unlabeled membrane (Fig. 2). Similar unlabeled gaps were observed when the tissue segments were fixed without intermediate washing with ferritin-free medium. In perpendicular view, the clustered ferritin appeared in most instances as multilayered aggregates (Fig. 2). In some specimens, the clustered ferritin was in the form of a monolayer (Fig. 4). In the latter, a paracrystalline arrangement was commonly found with practically no distance between the particles (Fig. 3a). The density of ferritin particles in such areas was as high as 4,400 per $\mu m^2$ (SD ± 150). No morphological changes could be detected in the trilaminar structure of the plasma membrane underneath the ferritin clusters (Figs. 2 and 3a). Some ferritin was already seen within phagocytic vesicles near the surface of the cell (Fig. 2). 5-min incubation in CF, followed by washing and an additional 5-min incubation in ferritin-free medium resulted in greater distances between ferritin clusters and larger areas of unlabeled membrane (Fig. 4). Clustered ferritin patches were often seen in regions rich in cytoplasmic protrusions, sometimes assuming a caplike form. Vesicles generally free of ferritin were often seen in these areas, as well as fusion of vesicles with the plasmalemma (Fig. 4).

After 10 to 20 min incubation in a CF-free incubation medium after the initial interaction with CF, many clusters of ferritin were observed detached or nearly detached from the membrane (Fig. 5a–c). In most of the cells, vacuoles containing ferritin particles were also seen (Fig. 5a). In order to determine whether the lack of ferritin labeling on large areas of the luminal membrane at this stage was due to lack of CF by overconsumption of the ferritin from the incubation medium, we incubated another aldehyde-fixed blood vessel segment in the remaining incubation medium and found a normal labeling pattern as with fresh medium. The presence of 10% BSA or 20% calf serum in the incubation medium drastically increased the aggregation and detachment of the ferritin clusters from the cell surfaces.

Staining with CF or CI of blood vessel segments fixed with aldehydes after they had been incubated with CF for 5 min, and then incubated in CF-free medium for 20 min, did not reveal any additional labeling with either of the particles, on the membrane surfaces that had been free of the label (Figs. 6 and 7). It is noteworthy that clustered CF was rarely seen on the membrane after exposing the endothelial cells to CI at pH 1.8 (Fig. 7).

When further CF was added to the medium, after initial incubation for 5 min with CF and subsequent incubation for 30 min at 37°C without CF, large areas of the luminal face were again covered with a layer of cationized ferritin, leaving a smaller proportion of the membrane free of label (Fig. 8). After subsequent 60-min incubation without CF, the entire luminal surface of endothelial cells was apparently regenerated and when CF was added no label-free surfaces could be detected (Fig. 9).

Endocytosis of CF by endothelial cells was limited after incubation periods of 1 and 5 min at 37°C. Small phagocytic vesicles containing a few ferritin particles were located at the luminal front of the cells (Fig. 2). Longer periods of incubation in the presence of cationized ferritin resulted in more phagocytosis, with large phagocytic vacuoles.
FIGURE 5 (a) Endothelial cell of guinea pig aorta, incubated for 1 min in CF, followed by washing, additional incubation in ferritin-free tissue culture medium for 10 min, and fixation in aldehydes. CF patches, separated by narrow unlabeled gaps, are partially detached from the cell surface. Vacuoles containing internalized ferritin are located near the surface of the cell. × 60,000. (b) Endothelial cell of guinea pig aorta, incubated for 5 min with CF, followed by 10 min of incubation in ferritin-free tissue culture medium and fixation with aldehydes. Partially detached ferritin aggregates are localized mainly on cytoplasmic protrusions. × 60,000. (c) Endothelial cell of guinea pig aorta, incubated for 5 min with CF, followed by incubation for 15 min in ferritin-free tissue culture medium and aldehyde fixation. Few CF patches are still located on the cell surface. Large ferritin clusters, believed to represent CF patches that have been detached from endothelial cell surfaces, are seen in the external medium. × 60,000.
appearing in the entire cytoplasm (Fig. 10). After incubation for 30 min, ferritin clusters were also found outside the endothelial cells, along the cell membranes facing the elastic laminae (Figs. 10 and 11). No CF was seen between cells beyond tight junctions (see Figs. 9 and 10).

No endocytosis or transfer of ferritin across the endothelium was observed when unfixed vessel

![Figure 10](image10.png)  ![Figure 11](image11.png)

**Figure 10** Endothelium of guinea pig aorta, incubated for 30 min with CF. Interiorized ferritin is seen in several phagocytic vacuoles within the cell cytoplasm. Clustered ferritin is also seen along the cell membrane facing the elastic laminae. Binding capacity for CF on the luminal front of the cells is completely regenerated. × 40,000.

**Figure 11** Endothelial cell of guinea pig aorta, incubated for 30 min with CF. Interiorized ferritin is seen within phagocytic vesicles in the cytoplasm. Exocytosed CF clusters are seen along the cell membrane facing the connective tissue elements of the elastic laminae. × 80,000.
segments were incubated with native ferritin under the same conditions and time periods.

DISCUSSION

In the present study we have established that anionic groups on the luminal surface of blood vessels, like those on macrophages (14), lymphocytes (11), and fibroblasts (16), are capable of lateral migration and redistribution under the influence of multivalent ligands, in a manner similar to that of receptor sites for antibodies (17) and lectins (7, 18) on other cells. The clustering of anionic sites is probably due to cross-linking of the CF by negatively charged molecules; the fact that surface aggregation is more rapid in the presence of serum albumin supports this hypothesis. The presence of similar gaps in the specimens that were fixed without washing with ligand-free medium before fixation, i.e., in presence of the CF in the external medium, rules out the possibility that washing may have contributed to the formation of unlabeled gaps. It further indicates that glutaraldehyde did not cross-link the CF particles to the membrane that had been apparently depleted of its anionic sites. The appearance of ferritin aggregated on top of cytoplasmic protrusions may be associated with induction of protrusion formation by the aggregates of CF. In addition, there is a morphological similarity between "capping" and the cap form on the protrusions of the endothelium, suggesting that the latter phenomenon may be due to a similar mechanism which induces cap formation in other cells (18). Endothelial cells have been shown to be contractile, capable of reducing their size when stimulated with histamine-like mediators (10). Such activity should be associated with changes in membranes configuration which will result in protrusions and invaginations. The internalization of vesicles calls for membrane regeneration (14). The presence of vesicles in proximity to, and often in the process of fusion with, the plasma membrane suggests that fusion of vesicles with the plasmalemma provides the replacement membrane in this process. The absence of CF in the open pits which occur along the cell surface (Fig. 4), suggest that the pits represent fusion of vesicles with the plasmalemma rather than endocytosis. Alternatively, if what we see are endocytotic vesicles, the existence of a "diaphragm" which may prevent the penetration of CF into the vesicle should be considered. However, there was no morphological evidence for the presence of such a structure.

Further incubation of CF-interacted cells in tissue culture medium without CF resulted in almost complete disappearance of the clustered ferritin from the cell surface. This takes place by two mechanisms: (a) detachment of the clustered ferritin from the surface and release into the medium. The observation that, upon and after detachment, the CF particles remain clustered suggests that the surface anionic groups are detached with the CF clusters. Whether this includes only the oligosaccharide chains containing the anionic groups, or the entire glycoproteins or glycolipids of the plasmalemma, is still unknown. The possibility that the aggregates are attached to a pseudopod of another plane of section was ruled out by observation of serial sections; (b) internalization of the attached ferritin. The latter is evidenced by the presence of CF in phagocytic vacuoles within the cytoplasm of the endothelial cells. The internalization of the CF, accumulation of CF in vesicles in the cytoplasm facing the luminal front of the endothelium, the subsequent increase in the number of such vesicles in the tissue front after further incubation periods, and finally the presence of vesicles opening to the tissue front (Fig. 11) suggest that CF may be transported by vesicles and vacuoles across the endothelium. It is noteworthy that native ferritin was not pinocytized and not transported across the endothelium of either the vena cava or the aorta under these experimental conditions, i.e., in vitro incubation for 1 h at 37°C. When native ferritin was injected in vivo into mice, as previously described (1), pinocytosis was observed in the capillary endothelium but not in the aorta or vena cava. It is assumed that the positively charged particles serve as a trigger for the endocytosis. The endocytosis of CF that has been observed in other cell types such as Ehrlich ascites tumor cells (16) and mouse peritoneal macrophages (14) supports this assumption.

Since the CF, upon its internalization, is attached to the anionic groups on the membrane surface, the presence of clustered CF outside the cells on the tissue front of the endothelium (Figs. 10 and 11) suggests that a mechanism exists whereby ferritin can be released from the vacuole membrane upon exocytosis. This mechanism might be similar to that which operates in the release of the clustered ferritin from the cell surface. The mode of action is still unknown. However, this mechanism of transport across the endothelium of large vessels is relevant to some observations of Joris and Majno (5) regarding accumu-
lation of debris in the intima and media of coronary arteries. Although Joris and Majno do not suggest this hypothesis, we think that some of the cell debris may be transported in a way similar to that of CF. However, it should be kept in mind that the present in vitro experimental conditions may not reflect perfectly the in vivo hemodynamics.

If such a mechanism of endocytosis and exocytosis does exist in vivo as well as in vitro, it must be associated with short periods during which parts of the membrane of the endothelium are devoid of charge, and other periods (and places) during which newly formed membrane patches face the lumen. The regeneration of the binding capacity for CF in the newly formed membrane may be due to the replacement of the specific anionic constituents rather than the insertion by fusion of complete membrane patches that carry already the negatively charged groups. Whatever the mechanism might be, the characteristics of the newly inserted surface components are as yet unknown. The interaction of the charge-depleted membranes and the regenerated surface constituents with blood platelets and other cellular elements of blood, as well as lipoproteins and their eventual role in the thrombogenesis and atherogenesis, should be a subject for study.

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