Surface Charge Distribution on the Endothelial Cell of Liver Sinusoids

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ABSTRACT The topography of the charged residues on the endothelial cell surface of liver sinusoid capillaries was investigated by using electron microscopic tracers of different size and charge. The tracers used were native ferritin (pl 4.2–4.7) and its cationized (pl 8.4) and anionized (pl 3.7) derivatives, BSA coupled to colloidal gold (pl of the complex 5.1), hemeundecapeptide (pl 4.85), and alcian blue (pl >10). The tracers were either injected in vivo or perfused in situ through the portal vein of the mouse liver. In some experiments, two tracers of opposite charge were sequentially perfused with extensive washing in between. The liver was processed for electron microscopy and the binding pattern of the injected markers was recorded. The electrostatic nature of the tracer binding was assessed by perfusion with high ionic strength solutions, by aldehyde quenching of the plasma membrane basic residues, and by substituting the cell surface acidic moieties with positively charged groups. Results indicate that the endothelial cells of the liver sinusoids expose on their surface both cationic and anionic residues. The density distribution of these charged groups on the cell surface is different. While the negative charge is randomly and patchily scattered all over the membrane, the cationic residues seem to be accumulated in coated pits. The charged groups co-exist in the same coated pit and bind the opposite charged macromolecule. It appears that the fixed positive and negative charges of the coated pit glycocalyx are mainly segregated in space. The layer of basic residues is located at 20–30-nm distance of the membrane, while most of the negative charges lie close to the external leaflet of the plasmalemma.

In the last years, the study of the cell surface electrochemistry has made substantial progress by using electron dense markers of known size and electric charge. Thus, differentiated microdomains of the capillary endothelium in pancreas (18, 19), lung (16), and bone marrow (3) have been put to evidence and partially characterized. One of these microdomains, the coated pit, defined by its peculiar ultrastructure, has been extensively studied as receptor carrier for a large variety of ligands. The few data referring to the charge distribution on the coated microzone surface suggest that this is expressed differently in various cell types. Coated pits of the plasma membrane have only negative residues exposed in fibroblasts (22), and in the fenestrated capillary endothelium of the pancreas (18, 19), while in the discontinuous endothelium of myeloid sinusoids, they bind both anionic and cationic markers (2, 3).

Using different electron microscopic markers we have studied the charge distribution in the coated microdomains of the endothelial plasma membrane in liver sinusoids. Our experiments show that in these cells the coated areas concomitantly exhibit both positively and negatively charged residues which enable them to interact nonspecifically and electrostatically with a large variety of macromolecules.

MATERIALS AND METHODS

Animals

48 RAP mice, weighing 25–30 g, were fasted for 16 h and used throughout the experiments.

Materials

Native ferritin (NF), horse spleen (6 x crystallized, cadmium free), and cationized ferritin (CF) were from Miles Laboratories (Elkhart, IN USA). Anionized ferritin (AF) was prepared according to Burry and Wood (1). Alcian blue (Harco–American Hospital Supply Co., Gibbstown, NY) and hemeundecapeptide (HUP) or microperoxidase LM 11 (Sigma Chemical Co., St. Louis, MO) were used as tracers of small molecular dimensions (~2 nm). Crystallized BSA was purchased from Mann Research Laboratories, Inc. (New York) and the tetrachlauric acid was from BDH Chemical Ltd (Poole, England).

Abbreviations used in this paper: CF, cationized ferritin; e, endosomes; HUP, hemeundecapeptide; l, lumen; NF, native ferritin.
Preparation of Colloidal Gold-BSA Complex (Au-BSA)

A monodisperse solution of colloidal gold particles (particle diameter ~17 nm) was prepared according to Frens' method (5). The colloid was stabilized with BSA (40 μg/ml), washed three times in Dulbecco's phosphate buffered saline (PBS) pH 7.2, and finally resuspended in the same PBS at 4.5 μm (24 at 1:10 dilution). The Au-BSA charge was estimated by isoelectric focusing in 1% agarose isoelectric focusing containing Pharamlyte in the pH range of 3-10 (Pharmacia Fine Chemicals, Uppsala, Sweden). The pl values, measured with an antimony electrode are listed in Table I along with the molecular dimensions of the markers used.

Experimental Protocols

GENERAL EXPERIMENTAL DESIGN

In the first set of experiments we have injected the tracers either in vivo or in situ. Because of the inevitable precipitation of the cationic markers by acidic plasma proteins, the in vivo experiments were performed with anionic probes only. The sequential perfusion in situ with both anionic and cationic tracers allowed the concomitant detection in the same specimen of the relative topographical distribution of the positively and negatively charged groups of the cell surface.

A second group of experiments was designed to demonstrate whether the interaction of the markers used with the cell surface was electrostatic in nature. As generally accepted (8, 18), perfusion with buffers of high ionic strength was considered a mean to detach the bound tracers. Additionally, the binding pattern of the markers was investigated after the cell surface charged groups were chemically modified. The ionization state of the plasma membrane basic groups was diminished by reaction with aldehydes, and the charge of the acidic residues was reversed by their substitution with an amine.

CELL SURFACE LABELING

IN VIVO: The anesthetized animals were injected via the iliac vein with 0.1 ml of NF (10 mg), or Au-BSA. After 1-2 min, the portal vein was perfused with 10 ml of PBS, pH 7.2, 37°C, at 3 ml/min, followed by 8 ml of fixative mixture (0.5% paraformaldehyde, 1.5% glutaraldehyde, 2.5 mM CaCl₂ in 0.1 M HCl-Na cacodylate buffer, pH 7.4) (9).

IN SITU: After anesthesia and laparotomy, a catheter was placed into the portal vein and the liver was washed free of blood by perfusing 14-20 ml PBS (3 ml/min, 37°C). One of the following tracers, in 0.3 ml PBS, were injected: NF (10 mg), anionized ferritin (10 mg), HUP (0.5 mg), CF (1.5 mg). Au-BSA, undiluted. At time intervals ranging from 30 s to 3 min, the liver vasculature was washed with 10 ml PBS and perfused with the same fixative mixture. Alcian blue (0.1%) was injected in 5 ml of aldehyde mixture followed by 5 ml of fixative without dye.

DOUBLE LABELING EXPERIMENTS: We followed the same steps as in the preceding section, except that all perfusates were kept at 4°C. 0.3 ml of undiluted Au-BSA were injected via the portal vein; after 1 min the unbound tracer was removed with 3 ml PBS, and 1.5 ml of cationized ferritin was infused for another minute. The final wash of the liver vasculature was done with 10 ml cold PBS, and the organ was fixed by perfusion. Alternatively, a short fixation step with 1% formaldehyde in between the two tracers was used.

Some Physical Characteristics of the Tracers Used

| Tracer         | M₀ (Da) | Molecular diameter (nm) | pl   |
|----------------|---------|-------------------------|------|
| Anionic        |         |                         |      |
| Native ferritin| 480,000*| 11                      | 4.2-4.7 |
| Anionized ferritin | 480,000 | 11                      | 3.7  |
| Au-BSA complex |         | 17                      | 5.1  |
| Hemeundecapeptide | 2,000 | 2                      | 4.85 |
| Cationic       |         |                         |      |
| Cationized ferritin | 480,000 | 11                      | 8.4  |
| Alcian blue    | 1,600   | 2                      | 10.0 |

* Molecular weight of the apoprotein only.

Experiments to Test the Electrostatic Nature of the Binding

DETACHMENT OF THE BOUND LIGAND BY AN INCREASED IONIC STRENGTH: Since the liver is drastically affected by a direct perfusion with buffered 0.3 M NaCl, the organ was lightly fixed with 6 ml 1% formaldehyde (3 ml/min flow rate) after in situ injection of a tracer and then washed with 10 ml PBS, pH 7.2 containing 0.3 M NaCl. The fixation was then completed with the procedure mentioned above.

MODIFICATION OF THE IONIZATION STATE OF PLASMA MEMBRANE CATIONIC GROUPS: Before injecting the anionic markers, the liver, washed free of blood, was perfused for 3 min with 10 ml 1% formaldehyde, or acetaldelyde in 0.1 M HCl-Na cacodylate buffer, pH 7.2, containing 2.5 mM CaCl₂.

CATIONIZATION OF PLASMA MEMBRANE: A perfusion circuit via vena porta, thoracic vena cava, was established and the liver was fixed in situ for 30 min with the aldehyde mixture, then extensively washed with PBS. The perfused fluid was switched to 10 ml of 1 M Na-dimethyltrimethylenediamine (Merck-Suchard, Hohenbrum, Federal Republic of Germany) pH 5.0, to which 400 mg of 1-ethyl-3-(3-dimethylaminopropy)carbodiimide HCl (Sigma Chemical Co.) was gradually added. This solution was recycled at 2 ml/min for 1–2 h, while maintaining the pH value at 5.0 with 0.1 M HCl. The liver vasculature was then washed with PBS until the pH of the effluent was raised to 7.2. CF or NF alone, or CF followed by Au-BSA with 10-ml PBS washing in between were injected and the unbound tracer was removed with PBS. For the control, the carbodiimide was omitted.

Tissue Processing for Electron Microscopy

After 5-min fixation in situ, blocks of ~1 mm² were immersed in the same fixative for 1 h, postfixed in 1% OsO₄ in 0.1 M HCl-acetate-veronal buffer, pH 7.6 (12), and stained in block with 0.5% uranyl acetate (10). The peroxidatic reaction was performed according to Graham-Karnovsky method (6) as modified by Simionescu et al. (17).

For an unambiguous identification of the endothelial cells of liver sinusoids, their peculiar feature of forming sieving plates was considered (24).

RESULTS

Pattern of Cell Surface Labeling

ANIONIC MARKERS: 30 s to 3 min after in vivo or in situ injection of NF, the endothelial cells of the mouse liver sinusoids bound this marker only in the coated microdomains of their plasma membrane (Fig. 1). NF was also contained in coated vesicles and in some small uncoated vesicles (~200-nm-diam). The coated pits bound NF as distinct particles in single rows at a relatively large distance (20–30 nm) from the outer layer of the plasma membrane (Fig. 2 a). All the coated pits existing at a given time on the plasma membrane were labeled with NF. At 1 min after injection NF appeared concentrated in large vesicles or vacuoles (800–1,500-nm-diam) (Figs. 1 and 2 c), which were never seen in connection with plasma membrane. The number of these large vesicles containing NF and the density of their load was significantly increased in time, suggesting that they function as intracellular reservoirs in which other organelles specialized in binding and transporting the ligands, discharge their content. The dynamics of the NF endocytosis in liver sinusoidal endothelium seemed to be that depicted in Fig. 2: the marker was bound only by coated pits (Fig. 2 a), which by invagination formed coated vesicles (Fig. 2 b). These vesicles either lost their coat or fused directly with large storage vesicles that concentrated the internalized particles (Fig. 2 c).

The Kupffer cells bound NF not only on their coated pits, but also to their microvillar projections, in a typical pattern for a macrophage. The hepatocytes, as well as the fat-storing cells were completely devoid of NF.

The binding pattern of anionized ferritin or Au-BSA was...
similar; they decorated in a single row, at 20–30 nm from the outer leaflet of the membrane, only the coated microdomains of the plasma membrane (Figs. 3 and 4). HUP labeled the entire luminal surface of the endothelial cells and accumulated in coated pits and coated vesicles (Fig. 5).

**CATIONIC MARKERS**: CF injected in situ decorated in random patches the whole surface of the endothelial plasma membrane, on both fronts (Fig. 6 a). CF bound in single or multiple rows at a short distance (≈5 nm) of the membrane (Fig. 6, b and c). All the coated pits existing at a time were labeled with CF. The marker was also bound to the microvilli of the hepatocytes, and was internalized by the coated vesicles of these cells.

Alcian blue uniformly decorated the whole surface of the endothelial plasma membrane (Fig. 7).

**DOUBLE LABELING**: When two markers of opposite charge were sequentially injected—Au-BSA complex followed by CF (with or without light fixation in between)—each of them preserved its distinctive pattern of binding. Accordingly, coated pits were decorated by both tracers (Fig. 8).

**Evidence for the Electrostatic Nature of the Binding**

**EFFECTS OF HIGH IONIC STRENGTH**: Perfusion with PBS containing an increased salt concentration removed all the bound NF or Au-BSA complex from the coated pits (Fig. 9), in spite of the preceding formaldehyde light fixation. Similarly, CF was displaced from the whole membrane surface. The return of the perfusate salt content to the isotonic conditions restored the CF normal binding pattern. Additionally, the protein content of the effluent collected in aliquots and, determined by the amido-black method (14) did not increase as a result of the ionic strength rise. This suggested that no extraction of the extrinsic membrane protein occurred throughout the procedure. When instead of formaldehyde the liver was shortly fixed with glutaraldehyde before the high salt concentration perfusion, both anionic and cationic markers remained bound in coated pits, but at a significantly lower density; CF was completely removed from the plasma membrane proper, but was still attached to the coated pits at a distance from the membrane similar to that found for NF (Fig. 10).

**PREVENTION BY ALDEHYDES OF ANIONIC MARKER BINDING**: When the tissue was reacted with formaldehyde or acetaldehyde before injecting the anionic markers, no such ligand was bound by the coated pits of the endothelial cells. The binding pattern of cationic probes was not altered by this treatment. If the formaldehyde was perfused after NF injection, the marker remained bound by the coated microdomains.

**EFFECTS OF PLASMA MEMBRANE CATIONIZATION**: Charge reversal of the plasma membrane by substituting its carboxyl groups with an amine resulted in the reversal of the binding patterns of the markers: CF did not bind any longer, but Au-BSA complex or NF decorated instead, indiscriminately the whole endothelial surface (Fig. 11). Moreover, NF was retained in the space of Disse, it labeled the hepatocyte membrane and became closely attached to the membrane, as CF did in normal liver (Fig. 11 b). The perfusion with diamine only, did not alter CF binding.

**DISCUSSION**

The cell surface labeling pattern by electron microscopic markers such as those used by us, is generally considered to be determined by electrostatic interactions between the tracers and the corresponding cell surface binding sites. The coated microdomains we have focused on, have a well developed...
FIGURE 2 Native ferritin, perfused in situ: the ligand is bound on the top of long glycosaly thread of coated membrane domains occurring as either coated pits (a) or coated vesicle (b). The marker is finally concentrated in large, smooth membrane vesicles (c). Sp, sieving plate; SD, space of Disse. Bar, 0.1 μm. × 144,000 (a); × 123,000 (b); × 103,000 (c).

FIGURE 3 Anionized ferritin is bound only to the membrane of the coated pits and vesicles (arrowheads). Bar, 0.2 μm. × 85,000.
glycocalyx, which could nonspecifically trap the tracers of large dimensions. Consequently, it was necessary to prove that the decoration of the coated pits and vesicles as well as the rest of the cell surface does reveal the distribution of the plasma membrane exposed charged residues, accessible to probes of appropriate size and charge.

Liver perfusion with buffered solutions containing high salt concentrations (0.3 M NaCl) reversibly removed all markers from their binding sites, irrespective of their charge. The short formaldehyde fixation following the tracer perfusion did not impair the high salt detachment of the probes. Substitution of formaldehyde with glutaraldehyde, more effective in promoting cross-linkage, resulted in the preservation of some of the bound anionic or cationic tracers, but only on the coated pits. Considerations on this observation will be made later.

Aldehyde treatment of cells before marker injection abolished the attachment of the anionic ligands to the plasma membrane, but leaving the CF binding pattern unchanged. Formaldehyde was known to displace the alkaline segment of the protein titration curve by 3 Units toward the lower values of pH, while leaving unmodified the acidic segments (4). Therefore, the formaldehyde treatment at pH 7.2 would significantly reduce the ionization of the basic, mainly amino residues, quenching the cell surface binding sites for anions. The same anionic marker persisted on the plasma membrane when injected before the aldehyde. As formaldehyde reacts with free uncharged amino groups only (4), binding of the anionic marker to the coated pit basic residues could keep the
FIGURE 6 Cationized ferritin is bound in random, discontinuous zones all over the endothelial plasma membrane and is internalized by coated pits and vesicles (a) (arrowheads). The cationic marker attaches very closely to the membrane of the coated microdomains (b and c). Bar, 0.1 μm × 60,000 (a); × 230,000 (b); and × 254,000 (c).

FIGURE 7 Alcian blue labels the entire cell surface, coated pits included. Bar, 0.2 μm × 79,600.

latter in an ionized state, preventing a reaction with aldehydes and the detachment of the bound particle.

In situ substitution of the carboxyl groups of the plasma membrane by a diamine abolished the CF binding. Such a modified cell surface bound particulate anionic markers in the pattern a normal cell was decorated by the cationic ferritin. As the possible resulting compounds of such cationization are either terminal amines or a transitory ω-acylsourea (7), both positively charged, the cell surface acquired net cationic charge, located on the sites of the original carboxyl groups. Therefore, the observed binding patterns were exclusively determined by the electrostatic interactions of the tracers with the fixed charges of the membrane. Chemical modifications of these charged groups produced alterations of the binding patterns compatible and predictable by the accepted theoretical considerations.

Coated vesicles were reported to be involved in endocytosis of native ferritin in a variety of cell types: spinal ganglion cells (13), peritoneal macrophage (11), and myeloid sinusoidal endothelium (2) for example. Data referring to the anionic material uptake in the liver are contradictory. While the native ferritin uptake was attributed to the parenchimal cells only (21), the sinusoidal macrophages (Kupffer cells) were reported to be responsible for the ingestion of negatively charged colloidal carbon particles (15). At least for the time intervals used in our experiments, the hepatocytes did not bind or internalize any of the anionic markers. The luminal front of the hepatocyte plasma membrane appeared to expose only negatively charged sites that bound cationized ferritin which is internalized via coated vesicles.

Both anionic and cationic markers labeled the microvillar projections of the Kupffer cells and seemed to be actively taken up by numerous smooth membrane vesicles, "worm-like structures" (25), and coated vesicles.

In contradistinction, the endothelial plasma membrane appeared to be heterogeneous in terms of the distribution of the positively charged moieties. The large anionic tracers used (11–17 nm) were bound exclusively to the coated microdomains. Small anionic molecules such as HUP (2 nm) labeled the entire endothelial surface. The discrepancy between the binding pattern of the two size classes of anionic markers could be tentatively explained either by different access to the exposed cationic residues of the plasma membrane, and/or by different local charge densities on the cell surface required for binding. NF was eluted from a DEAE cellulose column, equilibrated with 10 mM potassium phosphate buffer pH 7.2, at lower salt concentrations than HUP (data not shown), suggesting that the density of its exposed negative charges was comparatively smaller. Consequently, the plasma membrane
Au-BSA and cationized ferritin, sequentially perfused, decorate the same coated pits (a and b) and vesicles (c). Both tracers appear to follow the same intracellular route to endosomes (c). FSC, fat storing cell; SD, space of Disse. Bar, 0.1 μm. × 140,000 (a); × 126,000 (b); × 110,000 (c).

Native ferritin is completely displaced from the original binding sites (coated pits) by perfusion with buffered 0.3 M NaCl. SD, space of Disse. Bar, 0.2 μm. × 55,000.

should expose an increased local concentration of positive residues to bind NF or Au-BSA complex. The assertion that the coated pits and vesicles would contain higher densities of accessible basic sites than the rest of the plasmalemma was supported by the increased accumulation of HUP reaction product in these microdomains. Also, a short glutaraldehyde fixation prevented the detachment of both CF and NF by high salt treatment, in coated pits only, probably by cross-linking the tracer particles with the amino-rich glyocalyx of the coated microzones.

In contrast with the exposed membrane basic charges, the negative sites accessible to large markers on the endothelial
cell surface were not restricted to coated pits but were present as discrete patches all over the plasmalemma.

All the coated pits existing at a time were labeled by the injected tracer, either anionic or cationic. The pattern of labeling obtained when tracers of opposite charge were sequentially perfused, indicated that the same coated pit bound both kinds of markers. Therefore, these coated microdomains simultaneously exposed on their surface both basic and acidic residues.

A close examination of the localization of the markers in coated pits and vesicles revealed that the large anionic particulate markers were bound to sites constantly located in planes differently spaced from the membrane. Thus, while the anionic ferritins and Au-BSA complex were attached at the top of the glycocoalyx, 20-30 nm away from the outer leaflet of the membrane, the cationized ferritin was bound very close to the phospholipid bilayer (~5 nm). It appeared that a main stratification of the positive and negative charges exposed on the coated pits exists (Fig. 12).

The CF particles retained by short glutaraldehyde fixation on the coated pits, after perfusion with high salt concentration, were not any longer located close to the membrane, but rather at the distance where NF was normally displayed. If cross-linkage between the marker and the amino-rich segments would have explained this selective retention, then it appeared that the predominantly basic zone of the coated pits was 20-30 nm away from the membrane.

Transformation of the cell surface carboxyl groups in amino residues by cationization, determined the NF particles to bind near the plane of the membrane, where CF used to be normally attached.

The physiological significance of this active, nonspecific uptake of both cationic and anionic probes exhibited by the liver endothelial cells is still unclear. We could not find if the cationic probes were bound and internalized by these endothelial cells in vivo. It is also intriguing that, in spite of a predictable strong competition by the plasma acidic proteins, NF or Au-BSA particles are recognized and bound by the endothelial coated pits in vivo as well as in situ. It seems unlikely that the sinusoidal lining cells of the liver are continually internalizing plasma proteins. Rather a certain "preference" toward larger molecules or aggregates could drive this nonspecific kind of endocytosis. In this case, the affinity of
the coated pits to molecules of different size and charge
densities remains to be comparatively studied.

We would like to thank Ioana Andreescu and Carmen Barboni for
providing excellent technical assistance, Manda Misici and Margareta
Mitroaica for electron microscopy sectioning, and Victor Ionescu for
photographic work. We are especially grateful to Drs. Maya and
Nicolae Simionescu for their generous help and criticism.

This project was supported by The Ministry of Education, Rom-
ania, and by National Institutes of Health Grant HL-26343,
awarded to Nicolae Simionescu and Maya Simionescu.

Received for publication 14 October 1983, and in revised form 24
April 1984.

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