CHANGES IN THE RANDOM DISTRIBUTION OF SIALIC ACID AT THE SURFACE OF THE MYELOID SINUSOIDAL ENDOTHELIUM RESULTING FROM THE PRESENCE OF DIAPHRAGMED FENESTRAE

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ABSTRACT

Diaphragmed fenestrae (DF) are sites of increased vascular permeability. The anionic charge distribution at the luminal aspect of the DF of the endothelium of the bone marrow vessels has been studied after aldehyde fixation by means of colloidal iron (CI), native ferritin (NF), and polycationic ferritin (PCF). At pH 1.8, these cationic agents are bound by the nonmodified luminal endothelial cell surface but not at the sites of the DF. PCF was used over a pH range of 1.8-7.2 (CI is unstable at higher pH levels, whereas NF which has a pI of 4.5 is anionic above this point). PCF shows increased binding at the DF from pH 3.5 upwards. PCF binding at pH 1.8 at the nonmodified luminal cell surface is significantly diminished by neuraminidase treatment which, however, does not perceptibly reduce PCF binding at the higher pH levels. It is concluded that there are exposed sialic acid groups at the luminal cell surface which are absent or significantly fewer at the sites of the DF, whereas other anionic materials possibly with a pKa higher than that of sialic acid (pKa 2.6) are present both at the DF and at the nonmodified endothelial cell surface.

KEY WORDS vascular permeability - cell surface - diaphragmed fenestrae - endothelium - bone marrow - sialic acid - polycationic ferritin - native ferritin - colloidal iron

Fine structural studies with molecular tracers have shown that endothelial fenestrae represent one of the pathways for the transcellular permeability of vascular endothelia (review by Majno and Joris [12]). The presence of a diaphragm across these fenestrae appears to place no serious restriction on the permeability of these fenestrae, and diaphragmed fenestrae (DF) of intestinal capillaries have been found to be permeable to a variety of molecular tracers of different diameters ranging from 50 Å for horseradish peroxidase, 110 Å for ferritin (5), 75-250 Å for dextran to 300 Å for glycogen (16). Intravascular administration of carbon with particle diameters from 220 to 380 Å (mean Diam 280 Å) results in their entry into the extravascular spaces of the bone marrow and embryonic liver by permeation of the DF of the endothelial cell in the venous sinusoids of these tissues (1). There are no visible perforations in these diaphragms of a size which would permit the passage of these particulate molecular tracers. Thus, although they are discontinuous in a physi-
ological sense, they appear to be structurally continuous. Intercalated as they are in the body of the attenuated endothelial cells, they are confluent with the cell's surface and may be considered a modification of its plasmalemma. Because of the distinct functional peculiarities of these cell membrane modifications, we have examined the surface properties of the DF of the myeloid venous sinusoids by means of agents which react with cell surfaces on the basis of their Coulombic charge, viz, a positively charged iron sol (CI), native (low isoelectric point) ferritin (NF), and polycationic (high isoelectric point) ferritin (PCF). The latter two agents do not penetrate tissue sections and for these we used in this study the exposed surface of the large central venous sinusoid of the bone marrow, following procedures previously (7) developed to examine the surface properties of the endocytic sites of this vessel (see Materials and Methods).

MATERIALS AND METHODS
Male Long-Evans rats were used throughout. The details of the methods, including controls, have been recorded in an earlier study on the surface characteristics of the endocytic, large coated vesicles of the endothelium of the bone marrow (7). We present here the salient points of the procedures followed.

Sections, 30–40 μm thick, cut on a Vibratome (Oxford Laboratories Inc., Foster City, Calif.) were used for treatment with CI. Such sections cannot be used for NF or PCF because of inadequate penetration by these reagents. For these materials, we chose the large central sinusoid of the bone marrow (7). This sinusoid is provided in the same way as the smaller sinusoids and is of sufficient caliber to be opened by a scalpel when viewed under a dissecting microscope. The luminal surface of its endothelium can be exposed in fixed tissue blocks of this material for direct treatment with both NF and PCF. The bone marrow was fixed in a double aldehyde fixative by immersion as previously described (7). The Vibratome sections were immersed overnight in a dialyzed iron sol prepared according to Rinehart and Abul-Haj (14) as modified by Hardin and Spicer (9) at pH 1.8. Blocks of the large central sinusoids were treated with NF, 2× crystallized and containing 25 μg cadmium/ml (Miles Laboratories, Inc., Elkhart, Ind.) or PCF (Miles Laboratories, Inc.) in solutions ranging in pH from 1.8 to 7.2. Neuraminidase (Vibrio cholera) was obtained from Behring Diagnostics, American Hoechst Corp. (Somerville, N. J.) as a purified enzyme preparation containing 500 U/ml. Assurances of its freedom from protease activity are based on a method developed by Kuetten et al. (11) as reported by Bennett and Bondareff (3). After treatment with the surface active agent, the tissue was dehydrated, embedded in Epon 812, sectioned according to routine procedures, and stained with lead citrate.

RESULTS
The DF are irregularly dispersed in the sinusoidal wall. They may occur isolated from one another or in clusters. The latter arrangement occurs frequently in the immediate vicinity of blood cells in transmural passage (2). In the large central sinusoid, the mean of the DF distribution is 430/μm (SD 80) of cellsurface profile, as counted over 22 such distances varying from 2.7 to 3.2 μm in sections cut at 80 μm. In these sections, the DF range from 60 to 80 nm in diameter. Treatment with CI, NF, or PCF at pH 1.8 shows that all three cationic agents bind to the nonmodified endothelial cell surface but not at the sites of the DF (Figs. 1–5). The mean PCF particle density was 104/μm (SD 6) as counted over 23 distances of 0.4 μm of nonmodified cell surface profile in sections of 80 nm thickness. If this distribution would continue randomly in the same density over the DF, from six to eight particles would be expected to occur over each DF. Instead, inspection of 26 DF revealed no PCF particles over 19 DF. Four DF each one, and three DF each two electron densities which could be interpreted as PCF particles. At the nonmodified endothelial surface, virtually no interruptions in the random particle distribution occurred over distances comparable to the diameter of the DF, except at the sites of formation of large bristle-coated vesicles as has been reported earlier (7).

CI at pH 1.8 penetrated the Vibratome sections well, and iron particles were found to be present also at the abluminal surfaces of the endothelial cells (Figs. 1 and 3). There was, however, no binding of CI at the abluminal face of the DF. No NF binding occurred at either the endothelial cell surface or at the DF at pH levels above its pl (4.5), indicating the absence or effective absence (present, but possibly masked) of cationic loci at the nonmodified endothelial cell surface or at the DF. This is in essential agreement with the studies of Greer and Baker (8) on NF binding to the erythrocyte surface.

Because CI is not sufficiently stable at high pH levels and because NF is anionic above pH 4.5, PCF was used to study the anionic surface charge distribution at the endothelial cell surface over a range of pH levels. As noted above, PCF binds at
pH 1.8 consistently to the nonmodified adluminal endothelial surface but not at the sites of the DF (Figs. 5 and 6). At pH 3.5, PCF binding at the DF becomes evident and one to three PCF particles can be found at their luminal surfaces (Fig. 7). At pH 5.5 and 7.2, PCF particles are bound in increasing numbers to the adluminal face of both the endothelial cell surface and the DF (Figs. 8 and 9). At these high pH levels, there is a general increase in PCF binding at the nonmodified cell surface as compared to that occurring at pH levels below 3.5. It is to be noted that at pH 7.2 the PCF particles are aggregated in a larger mass over the DF as compared to the nonmodified endothelial cell surface (Fig. 9). At all pH levels, the PCF binding at the nonmodified endothelial cell surface as well as at the DF was, as far as could be determined at the available levels of resolution, directly to the surfaces, i.e., no particle-free zone could be observed adjacent to the cell surface.

The possibility that the absence of PCF binding at low pH at the sites of the DF resulted from the removal of some material by the acid treatment itself was considered. Blocks were exposed to 20% acetic acid for 30 min, washed in barbital buffer (pH 7.2), and then treated with PCF in the same buffer. The PCF binding in material treated this way was not different from that in material not pretreated with acid.

**Neuraminidase**

Treatment with neuraminidase followed by PCF at pH 1.8 greatly reduced the binding of PCF at the nonmodified endothelial cell surface (Fig. 10). At pH 3.5 (Fig. 11), the binding of PCF to this cell surface is still diminished as compared to the untreated control (Fig. 7). At pH 5.5, a reduction of PCF binding to the cell surface remains evident (Fig. 12). At pH 7.2, there is binding of PCF increased over that of pH 5.5 at the nonmodified endothelial cell surface as well as at the DF (Fig. 13). Also, after neuraminidase treatment, the PCF aggregates at pH 7.2 are larger at the sites of the DF than at the nonmodified cell surface (Fig. 13).

**DISCUSSION**

The three cationic agents CI, NF, and PCF applied at low pH (1.8) indicate the presence of exposed sialic acid groups at the adluminal cell surface of the endothelium of the sinusoids and the absence of exposed sialic acid groups at the sites of the DF.
Figure 2  Part of endothelial cell with two DF (arrowheads). Preparation treated as those depicted in Figs. 3–5 (pH 1.8), but without cationic surface marker. × 90,000.

Figure 3  Cl at pH 1.8. Both the adluminal and the abluminal surfaces of the endothelial cell bind Cl. At the site of DF (arrowheads) there is no Cl binding at either cell surface. × 90,000.

Figure 4  NF at pH 1.8. NF does not penetrate the tissue block and is only bound by the adluminal endothelial surface except at the DF (arrowheads). × 90,000.

Figure 5  PCF at pH 1.8. Note the absence of PCF binding at the site of the DF (arrowheads). LCV, large bristle-coated vesicle. In agreement with earlier observations (7), there is at low pH no binding of cationic marker at the large bristle-coated vesicle, the bristle coating of which is often poorly preserved at acidic pH levels (7). × 90,000.
FIGURES 6–9  PCF at pH levels ranging from pH 1.8 to 7.2. Fig. 6 shows the binding of PCF at the endothelial surface except at the DF (arrowheads). At pH 3.5 (Fig. 7) there is some binding of PCF at the DF (arrowheads). The binding at the DF (arrowheads) as well as at the endothelial cell surface increases progressively at pH 5.5 (Fig. 8) and pH 7.2 (Fig. 9). × 90,000.

FIGURES 10–13  PCF binding at various pH levels after treatment with neuraminidase. After neuraminidase treatment there is a considerable reduction in the PCF binding at pH 1.8 at the endothelial cell surface (Fig. 10). There is no binding at this pH at the DF (arrowheads). At pH 3.5 (Fig. 11) the binding of PCF after neuraminidase is still significantly reduced. There is some PCF binding at the DF (arrowheads). At pH 5.5 (Fig. 12) and pH 7.2 (Fig. 13), PCF binding after neuraminidase treatment progressively increases at both the endothelial cell surface and the DF (arrowheads). × 90,000.
The reliability of these agents for the presence of sialic acid is shown by the sensitivity of their binding to the cell surface after neuraminidase treatment. PCF binding at higher pH levels depends on anionic charges derived from other than sialic acid groups. We have suggested (7) that this binding of PCF at higher levels (from ~4.5 upwards and including physiological pH levels) may be the consequence of the presence of anionic materials with pKₐ values higher than that of sialic acid (pKₐ 2.6).

Cl, which in contrast to NF and PCF is capable of penetrating the Vibratome sections, shows that there are anionic charges on account of exposed sialic acid groups at the abluminal endothelial cell surface and the absence of such charges at both the adluminal and the abluminal site of the DF.

Various speculations can be adduced for the absence of sialic acid at the DF (enzymatic removal, shedding, masking). Adopting the view that the peptide residues of the glycoproteins are integral membrane components while the sialic acid-carrying carbohydrate moieties are exposed at the surface, the concept of the fluid mosaic model of the plasma membrane (17), which allows for a rapid translational movement of membrane components, provides at this time an attractive explanation for the present observations. The localized absence of sialic acid reported here for the DF is similar to that reported for the endocytic, large coated vesicles at this location (7) and at the oocyte surface of Xenopus laevis as reported by Brummett and Dumont (4). It is difficult to relate the absence of sialic acid to the specific functional processes at these sites, viz., permeability and endocytosis. Though the DF and the large coated vesicles are cell membrane modifications with diverse functional properties, a common factor in these two different functional qualities is possibly the arrest and capture of the particulate which is to be transported through the vascular interface or to be endocytosed. It is possible that the absence of sialic acid and/or perhaps the localized increase at these sites of high pKₐ, nonsialated materials have a role in this. Such factors, together with the presence of diaphragms spanning the fenestrae, are likely to have, as yet unknown, modifying effects on particulate permeability at these sites.

A common factor present at the sites of the two functionally different membrane specializations (permeability and endocytosis) is that there is, in each case, a change in the normal cell membrane stability at these places. Based on the effect of poly-L-lysine and other cationic polymers, Quinton and Philpott (13) have postulated that the anionic sites may be of importance in the stabilization of the epithelial cell membrane. Katsuyama and Spicer (10) hold it to be likely that such charges have a role in maintaining the structural stability of the membranes of different components of the exocrine pancreas. On the basis of the present experiments, one might wish to implicate in the membrane stabilization function more specifically certain sialic acid-containing membrane components rather than the negative surface charges in general, as PCF at physiological pH shows that negative surface charges from other sources than sialic acid are maintained or possibly even increased at both the DF as well as at the endocytic, large bristle-coated vesicles (7). The in vivo experiments of Simionescu and Simionescu (15) also show that the DF exhibit neuraminidase refractory anionic sites. It would thus appear that the absence of exposed sialic acid groups at the endothelial cell surface is associated with the appearance of specific localized functional changes, i.e., permeability and endocytosis, which in these cases are accompanied with definite structural modifications of the cell membrane, viz., the formation of, respectively, DF and large bristle-coated vesicles. Furthermore, the present work indicates that, in addition to sialoglycoproteins, non-neuraminidase-sensitive, high pKₐ materials, possibly high pKₐ carboxyl groups of amino acids, are an important measure responsible for the anionic nature of the luminal surface of the sinusoidal endothelial cells of the bone marrow.

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REFERENCES


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