Differentiated Microdomains on the Luminal Surface of the Capillary Endothelium

I. Preferential Distribution of Anionic Sites

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ABSTRACT Cationized ferritin (CF), introduced systemically in vivo or by perfusion in situ, binds preferentially to certain microdomains of the luminal plasmalemma of fenestrated capillaries (mouse pancreas and jejunum). The density and affinity of binding decrease in the following order: fenestral diaphragms > coated pits > plasmalemma proper. CF binds neither to the membrane of plasmalemmal vesicles and transendothelial channels nor to the corresponding stomatal diaphragms. The distribution pattern is the same when glutaraldehyde fixation precedes the administration of the tracer by perfusion, provided fixation is followed by quenching of residual free aldehyde groups. A much smaller cationic probe (alcian blue) perfused together with the fixative reveals a similar distribution pattern. The functional implications of the association of these microdomains with structures involved in capillary permeability are discussed.

It has been generally assumed that molecular size is the parameter that controls the movement of molecules across the wall of blood capillaries in the vasculature of mammals and probably other vertebrates. The effect of molecular net charge has rarely been investigated; when done, it was found that negatively charged dextrans (Mr 60,000–70,000) permeate less readily than their neutral counterparts, the walls of rabbit ear capillaries (7). More recently, and in more extensive studies, renal glomerular capillaries were found to be less permeable to anionic macromolecules (i.e., sulfated dextrans) than to uncharged species (i.e., neutral dextran) of equal size (2, 3). In glomerular capillaries, the endothelium is provided with numerous, large, aperture-free fenestrae that allow direct access of the blood plasma to the underlying basement membrane that functions as both size and charge barrier (6). In other capillary beds, however, an endothelial layer—continuous or provided with apertured fenestrae—is interposed between the blood plasma and the basement membrane. If charge restrictions apply to these vessels, they are expected to operate at the level of the endothelium. Electrophysiological studies (10) and experiments with charged tracers indicate that the surface of endothelial cells is negatively charged in situ as well as in culture (9, 17). But there is no information concerning the distribution pattern of these charges in relation to structural elements known to be involved in capillary permeability, i.e., plasmalemmal vesicles, transendothelial channels, and fenestrae. Such information is needed to assess the possible role of molecular charge in the permeability of blood capillaries of types other than glomerular.

We demonstrate here the existence of characteristic microdomains in the distribution pattern of anionic sites on the luminal surface of the fenestrated endothelium of the capillaries of the pancreas and intestinal mucosa of the mouse.

MATERIALS AND METHODS

Animals

Male Swiss albino mice weighing 25–30 g were used in all these experiments.

Materials

Native ferritin (horse spleen), pI 4.5, twice crystallized, cadmium free (NF), and cationized ferritin (CF), pI 8.4, were obtained from Miles Laboratories (Elkhart, Ind.). Before use, ferritin solutions were dialyzed for 24–48 h at 4°C against 0.15 M NaCl. CF and NF molecules in the tracer solutions were checked by electron microscopy and found to be homogenous, monodispersed preparations. Alcian blue (color index no. 74240) from Harelco (American Hospital Supply Corp., Gibbstown, N.J.) was used as a positively charged probe of small molecular size. Minimal essential medium (MEM) amino acids (x 50 concentrate) and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.
Experimental Protocols

**In Vivo:** The animals were lightly anesthetized with ether before being injected (into the saphenous vein) with the tracer, i.e., 7 mg of CF in 0.7 ml saline/100 g of body weight, or 10-30 mg of NF in 0.7 ml saline/100 g of body weight. Fixation in situ was initiated at intervals ranging from 0 s to 20 h after administering the tracer, by injecting in the loose connective tissue of the pancreas, or in the lumen of an isolated intestinal loop, a mixture of 5% formaldehyde-3% glutaraldehyde in 0.1 M HCl-Na arsenate buffer, pH 7.4, prewarmed to 37°C. For the “0 sec” interval, the abdominal cavity was opened beforehand, and fixation in situ was started concomitantly with the i.v. injection of the tracer. For intervals >20 min, the animals were allowed to recover from anesthesia and then reanesthetized before fixation. After 10 min of fixation in situ, specimens were collected for further processing. In all cases, the animals tolerated CF and NF without overt symptoms of distress over the duration of the experiments, provided CF and NF were extensively dialyzed before use.

**In Situ:** After anesthesia and laparotomy, the abdominal aorta and vena cava caudalis were catheterized with polyethylene tubing, and the vasculature was washed free of blood by retrograde perfusion through the aorta (using a Harvard pump) with DPBS, pH 7.2-7.4, supplemented with 5% MEM amino acids, 14 mM glucose, and gassed with 95% O2-5% CO2 (12). The perfusion was carried out in an open circuit (using the cava as outlet) at a perfuse flow rate of 3 ml/min1, for a total of 4-5 min, before adding the tracer to the perfusate. CF (7 mg in 0.7 ml saline/100 g of body weight) was maintained in the vasculature for periods up to 60 min. For NF (10 mg in 0.7 ml saline/100 g of body weight) the corresponding intervals were 5-15 min. The tracers were flushed with DPBS (3 ml/min1) for 4-5 min and the vasculature was fixed by perfusion for 10 min with 2% glutaraldehyde in 0.1 M HCl-Na arsenate or HCl-Na cacodylate buffer, pH 7.2-7.4, or with a mixture of 5% formaldehyde-3% glutaraldehyde in the same buffers. In a few experiments, the 2% glutaraldehyde solution contained 0.5% alcin blue as an additive (1). All solutions used in these experiments were prewarmed to 37°C. For intervals >20 min, the animal was placed in a thermostat at 37°C.

In a few experiments, the protocol described above was modified by circulating the tracer at the concentration given in a closed system at a rate of 4-6 ml/min1 and for a period of 10-20 min, with the rest of the procedure unchanged. The closed circuit was obtained by connecting the outlet (abdominal cava) to the inlet (abdominal aorta) with a piece of tubing mounted in a LKB Perpex pump (LKB Instruments, Inc., Rockville, Md.).

In another series of experiments, the order of the last steps of the protocol was reversed and modified as follows: the vasculature was washed blood-free, fixed by perfusion as above, and then perfused with a 0.1 M solution of either glycerol or sodium borohydride for 5 min at a flow rate of 3 ml/min1 to quench free aldehyde groups generated during fixation. The quenching solution was flushed out with DPBS and then the tracer was administered as in the standard protocol.

In another set of experiments, the nature of the interactions of CF with the endothelial surface was explored by administering the tracer dissolved in 0.05 M Tris-chloride buffer, pH 7.4, containing 0.5 M KCl. The same solutions were used to flush out the tracer from the vasculature, before fixation, as above. Perfusion under these conditions did not affect significantly the morphology of the endothelium.

**Tissue Processing for Electron Microscopy:** Specimens, collected from the pancreas and the intestine after fixation either in situ (10 min) or by perfusion, were trimmed to appropriate size and further fixed by immersion for 90 min in the aldehyde solutions used in the previous steps of the protocol. They were subsequently postfixed for 90 min at 4°C in 2% OsO4 in 0.1 M HCl-Na arsenate buffer, pH 7.2, then treated in block with either 0.5% magnesium uranyl acetate in 0.15 M NaCl, or 0.5%-1% tannic acid (galloylglucoses of low molecular weight) for 30 min at room temperature (11). After dehydration in ethanol and propylene oxide treatment, the specimens were embedded in Epon. Sections, cut with a diamond knife on a MTB Ultramicrotome (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.), were stained with lead citrate and examined with a Philips 301 electron microscope operated at 80 kV.

**RESULTS**

**State of the Tracer**

**In the Injected Solution:** Both NF and CF appeared in monomolecular dispersion in the solutions used in vivo or in situ experiments as illustrated for CF in Fig. 1; aggregates were extremely rare.

**In the Circulating Plasma:** In short-term experiments (20 s-2 min) and at the concentration used, CF remained in monomolecular dispersion in the circulating plasma in the majority of cases. After longer intervals or at higher concentrations, aggregates were found, presumably as a result of CF interaction with anionic plasma proteins. There was no aggregate formation with NF at the concentration and over the intervals used.

**Binding of the Tracer to the Endothelial Surface. General Remarks**

The pattern of CF binding to the endothelial surface was essentially the same in all experiments irrespective of the protocol followed, that is, tracer administered intravenously, in vivo, or tracer introduced by perfusion, in situ. At early time points, CF binding could be demonstrated more clearly in perfusion experiments primarily because of the lack of interference by plasma proteins which, upon fixation, generated a fine fibrillar or granular background. In specimens collected in vivo experiments, this background was minimal or nonexistent in vessels which, on account of their location at the periphery of tissue blocks, were drained empty of their plasma (before complete fixation) during the collection and trimming of the tissue blocks. By contrast, background interference was high in vessels that retained the full complement of their plasma, but the distribution of the tracer was the same. At late time points, the results were more informative in in vivo experiments, although they were the same in perfusion experiments, to the extent long-term observations were possible in the corresponding material.

In presenting the results, short intervals (0-20 min) and long intervals (20 min-24 h) will be considered together for the two main types of experiments performed. We will rely primarily on specimens collected from perfusion experiments for describing tracer binding over short intervals. Conversely, we will use primarily specimens collected from in vivo experiments to describe and illustrate the pattern of distribution found after long intervals. The type of specimen will be specified in the legend of each figure.

**Short Intervals (0-20 min)**

**Cationic Ferritin Binding:** In specimens fixed at “0 time” (i.e., concomitant with the administration of the tracer) or after 15 s, CF was already found in small clusters on the luminal side of the fenestral diaphragms and dispersed at low concentration over the luminal aspect of the plasmalemma proper (Fig. 3). There was no binding to the membranes of plasmalemmal vesicles and transendothelial channels and to their associated diaphragms.

After 20 s to 2 min, the large majority (>80%) of the fenestral diaphragms were marked by variably sized, but generally large, clusters of ~10-20 CF particles (per section) (Table 1) extending for ~10-40 nm above the plane of the diaphragms (Fig. 2). All coated pits opened on the luminal aspect of the plasmalemma, and most of the coated vesicles were labeled (Fig. 4). On the luminal aspect of the plasmalemma proper, labeling varied from a nearly continuous layer of single CF molecules to a scattering of individual CF molecules and small CF aggregates (Figs. 2, 4, and 5). In general, the labeling of the plasmalemma was considerably lighter than that of fenestral diaphragms. The difference was clearly seen at earlier time points (15 s) in grazing sections of the type shown in Fig. 3. The average spacing between the first row of CF particles and the underlying cell surface domains was almost the same for...
FIGURE 1 Cationic ferritin molecules in the tracer solution: the molecules, in their majority, appear randomly and individually dispersed. × 200,000.

FIGURE 2 Mouse pancreatic capillary, 30 s after in situ perfusion with CF. The ligand decorates almost continuously the plasmalemma proper (pm); it appears in characteristically high concentration on fenestral diaphragms (f), but it does not decorate the membrane or diaphragms (vd) of plasmalemma vesicles. e, endothelium; l, lumen; bm, basement membrane; pc, pericyte; and ps, pericapillary space. × 68,000.

plasmalemma and coated pits, but appeared to be smaller in the case of fenestral diaphragms (Figs. 4 and 5). CF decorated the infundibula leading to intercellular junctions but did not penetrate the junctions.

By contrast with the structures mentioned above, plasmalemmal vesicles, transendothelial channels and their associated diaphragms remained unlabeled (Figs. 2, 4, and 5). In those cases in which the decoration of the luminal aspect of the plasmalemma was almost continuous, the transition from labeled plasmalemma to unlabeled vesicles, channels, and their diaphragms was generally abrupt. Moreover, when a vesicle was preceded by an infundibulum (defined by a first angular change in the plasmalemma profile), the latter was also unlabeled (Fig. 5). A feltwork of fibrillar material appeared to be concentrated in the cytoplasmic matrix immediately under the plasmalemma proper. This feltwork was absent or less well
FIGURE 3 Mouse pancreatic capillary 15 s after an in vivo CF injection. In this oblique section through the endothelium, some fenestral diaphragms are fully included in the thickness of the section and appear heavily and nearly homogeneously labeled (fd1). Other fenestral diaphragms are only partly included; hence, the corresponding fenestrae appear labeled only in part (fd2). For still other fenestrae, the section misses the fenestral diaphragms entirely, passing on either their luminal (f1) or abluminal (f2) side. CF also decorates the plasmalemma proper (arrowheads), but in much lower concentration. x 100,000. Inset: mouse pancreatic capillary fixed at time 0. CF binds immediately and almost exclusively to fenestral diaphragms (f). x 82,000.

TABLE I

Average Number of CF Bound to Different Domains of the Luminal Front of the Endothelial Cell (Capillaries of Mouse Pancreas 1 min after CF Administration in Situ)

| Structural Elements       | Number of Particles per Reference Surface Unit (2.5 x 10^3 nm²) | Aggregate Area Examined (μm²)* |
|---------------------------|---------------------------------------------------------------|--------------------------------|
| Plasma membrane           | 3.6                                                           | 3.2                            |
| Coated pit                | 8.5                                                           | 1.1                            |
| Vesicle diaphragm         | 0.2                                                           | 1.25                           |
| Channel diaphragm         | 0.1                                                           | 0.25                           |
| Fenestral diaphragm       | 16.3                                                          | 1.25                           |

The number of structural elements examined was 100 for coated pits, 500 for vesicle diaphragms, 100 for channel diaphragms, and 500 for fenestral diaphragms. The surface areas for a coated pit and a fenestral diaphragm were estimated at 1.0 x 10⁶ nm² and 2.5 x 10⁶ nm², respectively. The latter figure was used as unit of reference to calculate the CF density on all other structures investigated.

* For the plasma membrane, the average thickness of a section was assumed to be ~50 nm, and the average cell surface surveyed was estimated at ~0.05 μm²/μm length of endothelial cell profile.

No CF binding to any structure was found when a high salt solution (0.5 M KCl), buffered to pH 7.4, was used both to administer the tracer and then flush it out from the microvasculature. No CF was found in the pericapillary spaces in any of these short-term experiments.

ALCIAN BLUE BINDING: In alcian blue-treated specimens, there was heavy, quasicontinuous labeling of a layer of 20–30 nm on the luminal side of the plasmalemma and heavy labeling of fenestral diaphragms and coated pits. By comparison, the plasmalemma vesicles were either not labeled or only lightly labeled by discontinuous deposits, and the same applied to the diaphragms of plasmalemma vesicles. The tracer reached the pericapillary spaces, where it produced a characteristic staining of basement membranes and a much lighter staining of the tissue aspect of the plasmalemma (Fig. 7).

NATIVE FERRITIN: Native ferritin did not bind to any endothelial structures, had access to plasmalemma vesicles, permeated a small fraction of fenestral diaphragms at the time points investigated, and was found in detectable amounts in pericapillary spaces past a 10-min interval.

Long Intervals (20 min–20 h)

CATIONIC FERRITIN BINDING: After intervals of 20 min or longer, the labeling of the plasmalemma decreased, whereas that of the fenestral diaphragms and coated pits remained at the same high level as before. 60 min after CF administration, the plasmalemmal labeling was light and spotty, whereas ~80% of the fenestral apertures and ~50% of the coated pits and vesicles were still labeled.

Beginning with 20 min, some of the multivesicular bodies of the endothelium were marked by CF which was present only within the light matrix (not in the vesicles) of these bodies (Figs. 8 and 9); the number of the labeled multivesicular bodies developed around plasmalemma vesicles (Figs. 4 and 6).

In specimens fixed by perfusion with aldehydes and exposed to the tracer without quenching, CF decorated the entire luminal surface of the endothelium including apparently the stomatal diaphragms of plasmalemma vesicles; the inner aspect of the membrane of the plasmalemmal vesicle was not labeled. But in specimens in which a quenching step was introduced between fixation and exposure to the tracer, the pattern of CF distribution was entirely similar to that described above (Fig. 6).

TABLE 3

Plasma membrane Coated pit Vesicle diaphragm Channel diaphragm Fenestral diaphragm

Number of particles 3.6 8.5 0.2 0.1 16.3

Aggregate area examined (μm²)*

3.2 1.1 1.25 0.25 1.25

The number of structural elements examined was 100 for coated pits, 500 for vesicle diaphragms, 100 for channel diaphragms, and 500 for fenestral diaphragms. The surface areas for a coated pit and a fenestral diaphragm were estimated at 1.0 x 10⁶ nm² and 2.5 x 10⁶ nm², respectively. The latter figure was used as unit of reference to calculate the CF density on all other structures investigated.

* For the plasma membrane, the average thickness of a section was assumed to be ~50 nm, and the average cell surface surveyed was estimated at ~0.05 μm²/μm length of endothelial cell profile.
FIGURE 4  Mouse pancreatic capillary 1 min after in situ perfusion with CF. The ligand is densely bound to an f, and, to a lesser extent, and with some discontinuities, to the pm; it is absent from the membranes of plasmalemma vesicles and their associated diaphragms (v). A grazing section through a labeled coated vesicle or pit appears at cv. Note the accumulation of fibrillar material beneath the regions of the plasmalemma to which CF is bound (arrowheads). × 100,000. Right inset: this micrograph illustrates the striking difference between the intense labeling by CF of a coated pit (cp) (in this case, in its course of internalization) and the absence of CF decoration from the membranes and the diaphragms of two adjacent plasmalemma vesicles (v). × 130,000. Left insets: details of CF decoration in a mouse pancreatic capillary 1 min after a CF injection in vivo. The membranes and diaphragms of transendothelial channels (c) are not labeled by CF, whereas the pm is labeled. × 130,000.

increased continuously at subsequent times (Fig. 11). Over all these intervals, there was no labeling of plasmalemma vesicles and no detectable transport of CF across the endothelium.

After 4–7 h, CF was cleared from the circulation as well as from the endothelial surface with the sole exception of 60–80% of fenestral diaphragms which still displayed one to three rows of CF particles. At these late time points, some (not all) endothelial cells had large vacuoles (200–400 nm in diameter) that appeared to open on the tissue front. Many of these vacuoles contained clusters of CF frequently packed in regular arrays. Similar clusters were found between the tissue front of the endothelium and the basement membrane (Fig. 10).

20 h after CF administration, the only endothelial structures still marked by CF were multivesicular bodies (≥35%) and some large vacuoles (Fig. 10).

DISCUSSION

Our findings establish the existence of differentiated microdomains on the blood front of the fenestrated endothelium in pancreatic and jejunal capillaries. These domains are the result of an unequal distribution of anionic sites detected by probe molecules of large (CF, Mr 480,000; molecular diameter, 11 nm) as well as small size (alcian blue, Mr ~1,300; estimated molecular diameter, ≤2 nm). The fenestral diaphragms are the domains that appear to have the highest concentration of high affinity (low pK) anionic sites. They are the first to be labeled, and the last to lose their label in vivo experiments. They are also the first to bind the cationic probes in perfusion experiments in situ. The coated pits are the microdomains with the next highest concentration of anionic sites. They are followed by the plasmalemma proper whose labeling is often discontinuous. The domains that do not bind the cationic probes are the plasmalemma vesicles open to the blood front of endothelium, the transendothelial channels and the stomatal diaphragms of both structures. The lack of labeling of the membranes of plasmalemma vesicles and channels cannot be ascribed to the impermeability of the diaphragms for the tracers, because infundibula leading to plasmalemma vesicles are not labeled either, and because alcian blue (molecular diameter, ≤2 nm) does not label (or labels sparingly) these structures. Moreover, large molecules (lectins) appear to have access, across stomatal
FIGURE 8. Muscular venule (mouse pancreas) 60 min after a CF injection in vivo. The micrograph illustrates the labeling of a multivesicular body (mv), a particularly frequent occurrence in the endothelium of arterioles and venules. sm, smooth muscle; and me, myoendothelial junction. × 62,000.

FIGURE 9. Mouse pancreatic capillary 20 min after a CF injection in vivo. The polycation is found only at the level of f and in multivesicular bodies mv. Neither the plasmalemma proper pm nor the coated pits cp or coated vesicles cv are decorated by CF at this time point. × 110,000. In Fig. 6 and especially in Fig. 7, some ferritin molecules seem to be present within the cavity of the small vesicles contained in multivesicular bodies. This is an imaging artifact: the average thickness of the sections (~60 nm) being greater than the average diameter of the vesicles (~45 nm), the image of some ferritin molecules located above or below the vesicles is superimposed on the image of the vesicles.

FIGURE 6. Mouse pancreatic capillary from a perfusion experiment in which the endothelium was fixed for 5 min with 2% glutaraldehyde, followed by quenching (of free aldehyde groups) with 0.1 M glycine. CF was then injected and allowed to remain in the vasculature for 1 min before the specimen was refixed by perfusion. The pattern of CF binding to the endothelial cell surface is similar to that obtained in specimens exposed to CF without prior fixation. × 96,000.

FIGURE 7. Mouse pancreatic capillary 1 min after perfusion in situ with glutaraldehyde containing alcian blue. The latter forms a quasicontinuous dense layer on, or slightly above, the pm. It does stain f, but does not bind to either the membrane or diaphragm (vd) of plasmalemma vesicles. Alcian blue stains locally the basement membrane to which it gained access probably through the fenestrae of the endothelium (arrowheads). × 90,000.
the plasma, and that the latter is continuously removed from the circulation to some tissue sinks. The final result will be the progressive loss and eventually the complete removal of bound CF from the endothelium. So far, accumulation of CF was detected in the liver, spleen (macrophages), and kidney (interstitia).

Because the plasmalemma, coated pits, and plasmalemma vesicles are expected to have a common fluid bilayer, it is of interest to identify the means by which the endothelial cell maintains the existence of these domains and prevents the randomization of their anionic sites. In the case of coated vesicles, the geodetic cages presumably formed by clathrin may function as stabilizing structures of the specificity of the corresponding microdomains. The plasmalemma vesicles do not have detectable infrastructures, whereas the plasmalemma proper appears to be backed by a poorly organized (or poorly preserved) fibrillar feltwork. That feltwork may represent a stabilizing infrastructure which maintains local differences between the plasmalemma proper and the membrane of plasmalemma vesicles, and defines the sites of opening or formation of such vesicles. Other stabilizing interactions within the membrane itself cannot be excluded.

Because the differentiated microdomains correspond to structures involved in the control of endothelial permeability, it is expected that these structures will select permeant molecules according to charge, in addition to size. Fenestral diaphragms are expected to discriminate against anionic molecules, whereas stomatal diaphragms, plasmalemma vesicles, and transendothelial channels may favor the penetration of anionic molecules for diffusion, filtration, or vesicular transport. It is noteworthy that the majority of plasma proteins are anionic, but the size range at which charge discrimination becomes effective remains to be established for each specific structure.

Preliminary perfusion experiments using specific hydrolases indicate that the high affinity sites on fenestral diaphragms are sulfated glycosaminoglycans, primarily heparan sulfate, presumably present as part of proteoglycans (15).

The striking difference in detectable anionic sites between
 FIGURE 12 Diagrammatic representation of the time course of CF binding to, and detachment from, the luminal front of the fenestrated endothelium of murine pancreatic capillaries.

was effected by large vacuoles (~120–250 nm), which occurred in high number only in the endothelial cells of these vessels. The CF clusters were not attached to the membrane of the vacuoles and appeared to be retained in the subendothelial space by the capillary basement membrane. These appearances were reminiscent of those described in the aortic endothelium by Skutelsky and Danon (18).

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