CHOLESTEROL IN THE PLASMA MEMBRANE OF UTERINE EPITHELIAL CELLS: A FREEZE-FRACTURE CYTOCHEMICAL STUDY WITH DIGITONIN

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SUMMARY
Freeze-fracture cytochemistry with digitonin has been used to examine the cholesterol content of the plasma membrane of uterine epithelial cells during the early stages of pregnancy in the rat. Lesions caused by digitonin complexing with cholesterol were seen on both lateral and apical portions of the membrane but tight junctions and desmosomes were lesion-free. Compared with day 1 of pregnancy, lesions on the apical plasma membrane were much more extensive and some were of different morphology on day 6—the day of blastocyst implantation.

We consider mechanisms of lesion formation and interpret the results to indicate a higher content and perhaps a different organization of cholesterol in the apical plasma membrane on day 6 of pregnancy. We also suggest how this increase may occur.

INTRODUCTION
The plasma membrane of uterine epithelial cells undergoes a series of dramatic changes during the oestrous cycle and early pregnancy. The apical plasma membrane bears long, thin, regular microvilli on day 1 of pregnancy but by days 5–6, the time of blastocyst implantation, these are replaced by short, irregular, flattened microvilli and various other irregular projections (Enders & Schlafke, 1967; Tachi, Tachi & Lindner, 1970; Ljungkvist, 1972; Murphy & Rogers, 1981). The lateral plasma membrane also undergoes changes during this time, with alterations in the geometry and depth of tight junctions (Murphy, Swift, Mukherjee & Rogers, 1981, 1982b; Winterhager & Kuhnel, 1982). These changes are under the control of maternal ovarian hormones (Ljungkvist, 1972; Murphy & Rogers, 1981).

Previous work has shown that the changes in microvillar profile of the apical plasma membrane are associated with more fundamental changes in membrane structure, as seen by freeze-fracture studies on density and organization of intramembranous particles (IMPs) (Murphy, Swift, Mukherjee & Rogers, 1979, 1982a). Alterations in carbohydrates of the cell surface coat have also been reported (Hewitt, Beer & Grinnell, 1979; Murphy & Rogers, 1981).

This plasma membrane is therefore an interesting system, because changes in
profile and organization can be readily observed. Nevertheless, little attention has so far been directed towards the involvement of membrane lipids in the above-mentioned phenomena. We recently studied anionic phospholipids in the membrane by freeze-fracture cytochemistry (Murphy & Martin, 1984) and have also examined cholesterol in the large vesicles characteristic of progesterone-dominated epithelial cells (Murphy & Martin, unpublished). The purpose of the present study is, then, to extend our investigations on this plasma membrane into its lipid components, with special attention to cholesterol: this, especially, because the large vesicles that we have already shown to have cholesterol-rich membranes are thought to fuse with the apical plasma membrane (Parr, 1982; Murphy & Martin, unpublished).

MATERIALS AND METHODS

Animals

Fifteen young adult virgin female Wistar rats were used for the study: six on day 1 of pregnancy and five on day 6. Control tissue, treated as described below, was taken from these animals but two additional animals at each time were also used to provide further control tissue. The rats were maintained on tap water and rat cubes, and kept at 20°C on a 14 h light/10 h dark cycle (lights on at 0730 h). Vaginal smears were followed for several days and females showing a pro-estrous smear (Everett, 1948) were caged overnight with males of proven fertility and were separated the following morning. Insemination was determined by the presence of sperm in the vaginal smear at the time of separation, and the day on which sperm were found was designated day 1 of pregnancy.

Freeze-fracture cytochemistry

At 1100 h on days 1 and 6 of pregnancy animals were anaesthetized with sodium pentobarbitone, the abdominal cavity was opened and uteri were excised. Tissue was processed from only two animals on any one occasion, using freshly made and identically prepared batches of digitonin each time: on some occasions day 1 and day 6 tissues were processed together and on others, day 1 or day 6 tissues or tissue from control animals were processed together.

As recommended by Elias, Goerke & Friend (1978), Robinson & Karnovsky (1980) and Severs & Robenek (1983) tissue was fixed before exposure to the sterol-binding cytochemical: 2 mm transverse slices of uterus were placed in 2-0% glutaraldehyde in 0-1 M-cacodylate buffer (pH 7-4) for 60 min and to ensure that the epithelial cells were freely exposed to the cytochemical agent, the slices of uterus were cut in half at right angles to the transverse cut. Tissue was then transferred to fresh fixative as above containing 2 mg/ml digitonin (Sigma; 80%) and incubated for 120 min. Control tissue was treated similarly but without digitonin in the second solution.

Following three washes in fresh buffer, the tissue was transferred to 30% glycerol in buffer. Samples of 1 mm³ were frozen in freshly thawed Freon-22 cooled with liquid N, transferred to liquid nitrogen and then fractured without etching at −115°C in a Balzers Apparatus (model BAF 300). After platinum shadowing with an electron gun and carbon deposition, the replicas were separated from tissue in sodium hypochlorite, washed in water, placed on copper grids and examined in Siemens Elmiskop 102 or JEOL 100CX electron microscopes operating at 80 kV.

Freeze-fracture 'runs' were repeated until successful replicas were obtained from at least two samples of uterus from each of the 15 rats. The terminology of Branton et al. (1975) is used in referring to membrane fracture faces.

Quantification of membrane area occupied by lesions

Although not without difficulty, owing to the variability in lesion size and morphology, we attempted to estimate the percentage of apical plasma membrane occupied by lesions as follows.

A transparent grid with 1 cm × 1 cm squares drawn on it was placed over pictures enlarged to ×75 000 and the numbers of such squares that fell over lesions were expressed as a percentage of
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the total number of squares that fell over fractured membrane. From two to five pictures were measured for each animal and at least 1000 squares were counted for each group of animals.

RESULTS

Incubation of tissues in digitonin produced lesions in the plasma membrane of uterine epithelial cells that were similar in appearance and dimensions to those reported for other cell membranes (Elias et al. 1978; Meyer, 1981; Severs & Robenek, 1983, for a review). In general, digitonin lesions seen in this study were the typical tubular scallops, with dimensions of 50–70 nm wide and 200–400 nm long, but scale-like plates with irregular dimensions were also seen. The form and distribution of digitonin-induced lesions were different, however, depending on the day of pregnancy on which cells were examined.

On day 1 of pregnancy, the apical plasma membrane showed only a few tubular lesions (Fig. 1), which were sparse and usually occurred in groups of 2–4, but occasionally singly as well. There was no obvious clustering of IMPs at this time and the membrane in general was not extensively disrupted. The apical plasma membrane of all the cells we saw had a similar appearance, with no obvious 'regionalization' of lesions. Quantification showed that only 6.5% of the apical membrane was occupied by lesions. In contrast, the lateral plasma membrane on day 1 of pregnancy was sharply divided into regions of differing digitonin lesion concentration. As can be seen in Fig. 2, the tight junction region of interlocking strands shows few, if any, lesions. Below the tight junction, however, which at this time extends about 0.4 μm down the lateral membrane (see also Murphy et al. 1982a), the lateral membrane is extensively disrupted by tubular digitonin lesions, except for the immediate area of desmosomes. Other than in these specialized regions, we could see no difference in lesion distribution either at different levels down the same lateral membrane or between lateral membranes of different cells. Fig. 3 shows lesions on the lateral plasma membrane deep below the tight-junction region and this appears to be similar to the lower portions of Fig. 2.

On day 6 of pregnancy the distribution and appearance of digitonin lesions was different from that described above. Fig. 4 show apical plasma membrane of an epithelial cell from a rat on day 6. This membrane no longer bears the numerous circular profiles of cross-fractured microvilli (see Murphy et al. 1982a, for more details) but is also extensively disrupted by digitonin–cholesterol complexes. Individual tubular scallops are still seen in some regions, but, more particularly, nearly the entire membrane is thrown into a series of scale-like corrugations that sometimes interleave with regions of cross-fractured cytoplasm. The corrugations are irregular in size and are often devoid of IMPs, but have IMPs around the edges. IMPs in general are displaced into various irregular aggregations by the treatment. Measurements showed that 97.6% of apical membrane was affected by lesions on day 6. The lateral plasma membrane (Fig. 5) on day 6 is more similar to that described for day 1. The tight junction region now extends about 1.3 μm down the lateral membrane (see also Murphy et al. 1982b), but is still more or less free from lesions. Lateral
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Figs 1–2. For legend see p.168
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Fig. 1. Freeze-fractured apical plasma membrane (P face) of a uterine epithelial cell from a rat on day 1 of pregnancy. A few tubular lesions are visible (some are arrowed) but they are sparsely distributed. IMPs are randomly dispersed. $m$, cross-fractured microvillus. Shadowing direction is indicated by an arrowhead in the bottom right-hand corner in all figures. $\times50,000$.

Fig. 2. Lateral plasma membrane (P face) from an epithelial cell on day 1 of pregnancy. The tight-junction region (t) shows very few, if any, lesions but below this lesions become more frequent and disrupt most of the lateral membrane except for desmosomes ($d$). $l$, uterine lumen. $\times50,000$.

Fig. 3. Similar to Fig. 2 (day 1) but deep below the tight junction. The entire lateral membrane shows lesions except for desmosomes ($d$). As in Fig. 2, only tubular type lesions are seen. $\times50,000$.

Fig. 4. Apical plasma membrane (P face) of a uterine epithelial cell from a rat on day 6 of pregnancy. The entire fracture face is disrupted and many scale-like lesions are seen. Some tubular lesions are also seen (arrows). IMPs are aggregated into various arrays. $\times50,000$.

Fig. 5. Lateral plasma membrane (P face) from a cell on day 6 of pregnancy. The fracture plane exposes only the deeper part of the tight junction (t) in this micrograph but it is free from lesions. Below the junction tubular lesions are again seen on the membrane. $l$, uterine lumen. $\times50,000$. 


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membrane deep below the tight junction (Fig. 5) again shows a high concentration of the tubular scallops but the scale-like corrugations seen on the apical membrane at this time were not in evidence. The general appearance of both apical and lateral plasma membrane as described above for day 6 was consistent between cells and we did not see any marked deviations.

Control cells incubated without digitonin showed no complexes of any kind and were identical to those seen in our previous studies (Murphy et al. 1982a,b).

DISCUSSION

A positive response to digitonin, as seen here in freeze-fracture cytochemistry, is generally held to indicate the presence of cholesterol. Experiments with artificial lipid vesicles have shown that digitonin produces typical lesions only when the medium in which the vesicles were formed contained cholesterol (Elias et al. 1978). Moreover, in those instances in which correlations have been performed there seems to be general agreement on cholesterol distribution as determined biochemically and cytochemically (see Severs & Robenek, 1983, for a review). Lastly, digitonin and other saponins do not appear to be as susceptible to false negatives as filipin (Fujimoto & Ogawa, 1983; Severs & Simons, 1983). This latter factor, and the partly comparative nature of our study, prompted us to start our examination of cholesterol in this plasma membrane using digitonin.

The tubular lesions we observed in the plasma membrane of uterine epithelial cells are quite similar to those noted in both liposomes (Elias et al. 1978) and several studies on various tissues (Elias et al. 1978; Fischbeck, Bonilla & Schotland, 1982; Severs, Warren & Barnes, 1981; Severs & Robenek, 1983). These structures are usually considered to be complexes of digitonin with cholesterol. The scale-like lesions we saw in day 6 cells have also been reported previously in erythrocyte membranes by Elias et al. (1978) and Meyer (1981). The mechanism of formation of lesions is, however, the subject of considerable debate. In the interaction between digitonin and sterols the bulky sugar moiety of the former is considered to play an important part (Akiyama et al. 1980) and as mentioned elsewhere (Murphy & Martin, 1984) most models of lesion formation in freeze-fracture cytochemistry imply some kind of incorporation of the chemical into the lipid bilayer (see Severs & Robenek, 1983, for a review). The idea of incorporation is supported by Miller (1984), who concluded from a study on artificial lipid bilayers that tubular lesion formation depended on the formation of bilayers with more digitonin-cholesterol complexes in the outer than in the inner monolayer. Curvature or buckling of the bilayer, as a consequence of this induced asymmetry could then cause the lesions characteristic of digitonin.

The only lesions seen on lateral plasma membranes at both the times of pregnancy that we studied were of the tubular form and we could see no difference in their density between the two days. Interestingly, despite the fact that on day 6 the tight junction is three times deeper than previously, the junctional membrane is still free from lesions. Presumably, this deeper and still lesion-free junctional region either occupies membrane that on day 1 displayed lesions below the much shallower junction then
present, or represents a newly organized junctional membrane region (see Murphy et al. 1981, 1982b, for quantification and further data). The finding that tight-junctional membrane does not display lesions with sterol-binding agents is in agreement with findings by others (Elias et al. 1978, 1979; Robenek, Jung & Gebhardt, 1982; Matsuda, Fujita & Ishimura, 1983). No data are available on the cholesterol content of tight junctional regions and it is possible that the lack of lesions in these areas may reflect an inability of digitonin to penetrate junctional membrane, rather than a paucity of cholesterol. Some authors, however, have argued that junctional membrane may be a specialized region of lower cholesterol content (Robenek et al. 1982) and if the absence of lesions does reflect a real paucity of cholesterol then the rapid changes in depth of tight junctions and their associated membrane in uterine epithelial cells may be useful in studying cholesterol dynamics.

In contrast to the lateral plasma membrane, the types of lesions we saw on the apical plasma membrane were neither of the same density nor of the same form on days 1 and 6 of pregnancy. As discussed above (and see Murphy & Rogers, 1981; Murphy et al. 1982a) the apical plasma membrane undergoes a remarkable transformation in outline between these times and loses its regular microvilli. Changes in microfilament organization are almost certainly involved in this phenomenon (Ljungkvist, 1972; Lunam & Murphy, 1983), but in view of the present findings with digitonin it is tempting to speculate that cholesterol is somehow involved as well. The finding of only a few lesions on day 1, compared with the extensive disruption on day 6, suggests that the apical membrane contains more cholesterol on day 6— the time when blastocysts are able to implant (Tachi et al. 1970). Notwithstanding the greater extent of lesioning, perhaps of more interest is the different lesion morphology on day 6. This may reflect only a higher content of cholesterol or may be influenced by the higher density of intramembrane particles on day 6 (Murphy et al. 1982a). On the other hand, different lesions may reflect different arrangements of cholesterol and, or, other lipids as well.

Most authors seem to agree that the effect of cholesterol on biological membranes at physiological conditions is to condense the acyl chains, giving rise to a partially ordered state (Shinitzky & Henkart, 1980; Severs et al. 1981; Robertson, 1983). On day 6 the apical plasma membrane with its higher cholesterol content would therefore be less fluid and this may be important for attachment of the blastocyst, although this aspect awaits further study. If the reproductive function of a higher cholesterol content on day 6 remains obscure, however, the way in which it is brought about may be less so: large apical vesicles begin to appear in uterine epithelial cells on day 4 of pregnancy and we have already shown that these vesicles have cholesterol-rich membranes (Murphy & Martin, unpublished). Since the vesicles are thought to fuse with the apical plasma membrane, by day 6 (Parr, 1982) this may be a vehicle for increasing cholesterol content.

Notwithstanding the false negatives known to be associated with filipin as a cytochemical probe (Severs & Robenek, 1983; Severs & Simons, 1983), we have begun a short study on the apical plasma membrane of uterine epithelial cells using this reagent (Murphy & Dwarte, unpublished data). In agreement with the results
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reported here, we find many more filipin-induced lesions on day 6 than on day 1, so in the case of this plasma membrane at least, digitonin and filipin seem to give the same result.

C.R.M. was a Nuffield Dominions Demonstrator in the Department of Human Anatomy at Oxford while part of this work was done and the support of the Nuffield Dominions Trust is gratefully acknowledged. We are also grateful to Drs D. M. Shotton and S. Bradbury, both of Oxford, for their support of this work and to Miss Anne Woods for typing the manuscript.

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(Received 5 February 1985 – Accepted 6 May 1985)