Possible continuity of subplasmalemmal cytoplasmic network with basement membrane cord network: ultrastructural study

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SUMMARY

The ultrastructure of the subplasmalemmal cytoplasm of the cell and the associated basement membrane as well as the area of the cell-basement membrane border were observed with high resolution electron microscopy after preparation of the tissues with cryofixation or glutaraldehyde fixation followed by freeze substitution. The subplasmalemmal cytoplasm of the smooth muscle cells of rat epididymal tubules and the podocyte processes of the mouse glomerular visceral epithelium were found to be composed of a fine network of irregular anastomosing strands. This network closely resembled the previously characterized cord network of the basement membrane. The cords are known to be composed of a 1.5 to 3 nm thick core filament made up of type IV collagen which is surrounded by an irregular ‘sheath’ of other components. The strands in the subplasmalemmal network showed ultrastructural features similar to those of the cord network. Ribbon-like, 4.5 nm wide heparan sulfate proteoglycan ‘double tracks’ were previously reported to be associated with the cord network. Structures similar in size and appearance to the double tracks were also found in the subplasmalemmal network. At the cell-basement membrane border, the lamina densa of the basement membrane was in contact with the cell without the intervening space of a lamina lucida which was recently found to be an artefact caused by conventional tissue processing. Furthermore, the subplasmalemmal network appeared to be continuous, through the plasma membrane, with the cord network of the basement membrane. These results raise the possibility that the subplasmalemmal region of the cell and the associated basement membrane is occupied by a seemingly ultrastructurally unified network in spite of the fact that two such areas have been known to be composed of biochemically distinct components.

Key words: glomerular basement membrane, smooth muscle cell, cryofixation, freeze substitution

INTRODUCTION

The basement membrane separates the connective tissue from the parenchymal cells of epithelia, endothelia, muscle fibers and others. These sheet-like layers of extracellular material are known, after processing the tissues with conventional methods of preparation, to be layered structures composed of a lamina lucida, a lucent layer next to the plasma membrane of the cell, followed by a lamina densa, the main component of the basement membrane, and finally, by the pars fibroreticularis which is an area of transition between the basement membrane and the connective tissue (Kefalides et al., 1979; Inoue, 1989). The lamina densa is mainly composed of a three-dimensional network of irregular strands referred to as ‘cords’ (Inoue and Leblond, 1988; Inoue et al., 1983). The lamina lucida is crossed by a few cords which extend from the lamina densa to the plasma membrane of the cell.

However, in recent studies with the application of new advanced methods of tissue preservation such as cryofixation and freeze substitution, the possibility that the lamina lucida was an artefact was raised (Goldberg and Escaig-Haye, 1986; Reale and Luciano, 1990). In order to further examine this problem, a systematic study of basement membranes from a number of different tissues was done in this laboratory with the methods of cryofixation or aldehyde fixation followed by freeze substitution. It was concluded, from the results of these studies, that the lamina lucida of basement membranes was an artefact caused mainly by the process of conventional tissue processing (Chan et al., 1993; Chan and Inoue, 1994).

As a result of these findings, it now seems likely that the lamina densa of the basement membrane is therefore in direct contact with the surface of the associated cell in its original state in the living condition without the intervening layer of a lamina lucida. While the lamina densa is known to be composed of a network of cords, as described above, the subplasmalemmal cytoplasm of the cell is also reported to be filled with a network structure. By use of the high voltage electron microscope, the cytoplasm was observed to be filled with the network structure referred to as the microtrabecular lattice (Wolosewick and Porter, 1979). This filamentous network was reported to be denser and more compact in peripheral areas of the cytoplasm where few organelles were present and little particle motion was observed (Luby-Phelps et al., 1988). In the present study, high resolution electron microscope observations were made in the area of the cell-basement membrane border in tissues prepared with freeze substitution following
cryofixation or aldehyde fixation. The aim of these observations was to elucidate the detailed ultrastructure of this border area, and also to examine any possible relationship between the network of the subplasmalemmal cytoplasm and the network of cords of the associated basement membrane.

**MATERIALS AND METHODS**

The rat and mouse tissues were prepared for electron microscopy with methods described previously (Chan et al., 1993) with cryofixation by slam freezing, or glutaraldehyde fixation, each of which was followed by freeze substitution.

**Cryofixation-freeze substitution**

The epididymis was isolated from Sherman rats under anesthesia and the epididymal tubules were dissected out from the caput and corpus regions. The tissue was cut into small pieces (1-2 mm³) and subjected to ‘slam freezing’ in a CF-100 cryofixation system (LifeCell Corporation, Woodlands, Texas) by slamming the tissue onto the surface of a cryogenic copper block maintained at −180°C. The cryofixed tissue was kept in liquid nitrogen. For freeze substitution, the tissue was transferred into absolute acetone containing 1% osmium tetroxide and was kept in liquid nitrogen. For freeze substitution, the tissue was cryofixed and freeze substituted. The lamina densa material of the basement membrane (BM) of two parallel smooth muscle cells (SMC) are fused into a single layer which fills the entire space between the endothelial cells (En). U, urinary sinus. (B) Rat epididymal tubule cryofixed and freeze substituted. The lamina densa material of the basement membrane (BM) of the mouse kidney after fixation with glutaraldehyde followed by freeze substitution. The lamina densa material of the basement membrane fills the entire space between the base of podocyte processes of the visceral epithelium (Ep) and endothelial cells (En). U, urinary sinus. (B) Rat epididymal tubule cryofixed and freeze substituted. The lamina densa material of the basement membrane (BM) of two parallel smooth muscle cells (SMC) are fused into a single layer which fills the entire space between the cells, except where a group of collagen fibrils are present. Bars, 100 nm.

**RESULTS**

The peripheral cytoplasm of the podocyte processes and the glomerular basement membrane of the rat kidney (Fig. 1A), and smooth muscle cells with the associated basement membrane of the rat epididymis (Fig. 1B) were examined at higher magnification. In each specimen, the peripheral subplasmalemmal cytoplasm of the cell was composed of a network of irregular anastomosing strands which showed a strong resemblance to the network of ‘cords’ previously observed as the main component of basement membranes (Inoue and Leblond, 1988). An example is shown in Fig. 2A,B in the case of the smooth muscle cells of the rat epididymis. The network of the subplasmalemmal cytoplasm of the smooth muscle cell (Fig. 2A) resembles the cord network of the associated basement membrane (shown in Fig. 2B at the same magnification for comparison). The irregular strands composing the cytoplasmic network are similar in appearance and thickness to the cords making up the network in the basement membrane, and the size and shape of the openings of these networks were similar. Thus, the thickness of the strands of the subplasmalemmal network is 4.3±1.4 nm (n=50 in this and all other measurements) as compared to 4.4±1.3 nm of the thickness of the cords in associated basement membrane (Table 1). Similarly, in the glomerulus the mean thickness of strands in peripheral cytoplasm of podocyte processes is almost identical to that of the cords in the glomerular basement membrane (4.0 nm). The size of the openings of the network of either strands or cords was expressed in terms of the interstrand space diameter index (ISSDI) or intercordal space diameter index (ICSDI), as has been done previously (Inoue and Leblond, 1988) since the openings seen in the micrographs in fact resulted from the projection of a three-dimensional network onto a two-dimensional plane and, therefore, some of them are not necessarily true openings. The ISSDI of both the podocyte processes and smooth muscle cells, as well as the ICSDI of the smooth muscle basement membrane, were very similar (about 17 nm) while the ICSDI of the glomerular basement membrane was a little smaller (14 nm) (Table 1).

The close similarity of the networks observed in the subplasmalemmal cytoplasm and the basement membrane was even more pronounced when the network of the peripheral

**Fig. 1.** Two types of basement membranes prepared with the technique of freeze substitution. (A) Glomerular basement membrane (BM) of the mouse kidney after fixation with glutaraldehyde followed by freeze substitution. The lamina densa material of the basement membrane fills the entire space between the base of podocyte processes of the visceral epithelium (Ep) and endothelial cells (En). U, urinary sinus. (B) Rat epididymal tubule cryofixed and freeze substituted. The lamina densa material of the basement membrane (BM) of two parallel smooth muscle cells (SMC) are fused into a single layer which fills the entire space between the cells, except where a group of collagen fibrils are present. Bars, 100 nm.
cytoplasm in both tissues was observed at high magnification. Fig. 2C shows a high magnification view of the peripheral cytoplasm of a podocyte process from a glomerulus of the mouse kidney. The strands (thick arrows) composing the network are irregular in thickness and the density along their lengths and they are frequently anastomosing, features very similar to those observed in cords in basement membranes in this and in previous studies (Inoue and Leblond, 1988; Inoue et al., 1983; Laurie et al., 1984). Ribbon-like 4.5 nm wide double tracked structures were also associated with the strands (Fig. 2C, paired arrows), and they closely resembled the ‘double tracks’ previously observed in the basement membrane and were found to be composed of heparan sulfate proteoglycan (HSPG) (Inoue et al., 1989). In limited areas, the network of strands was replaced by a network of filaments of about 1.5 nm in thickness (Fig. 2C, single thin arrows). These filaments resembled in appearance the core filaments of the cords (Inoue and Leblond, 1988).

Close observation, at high magnification, of the area at the border of the peripheral cytoplasm and the basement membrane showed that fine filaments similar to those observed in the network of the cytoplasm were seen passing through the plasma membrane of the cell, as shown in Fig. 3A,B. The segments of the filaments which passed through and were included within the plasma membrane were more positively identified when the plasma membrane was sectioned either obliquely (Fig. 3C) or tangentially (Fig. 3D).

Table 1. Network in subplasmalemmal cytoplasm of the cell and basement membrane

<table>
<thead>
<tr>
<th>Tissue (species)</th>
<th>Network</th>
<th>Thickness of strands/cords nm±s.d.</th>
<th>ISSDI/ICSDI* nm±s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerulus (rat)</td>
<td>Subplasmalemmal cytoplasm network of strands</td>
<td>4.0±1.0</td>
<td>17.3±3.4</td>
</tr>
<tr>
<td></td>
<td>Basement membrane cord network</td>
<td>4.0±0.9</td>
<td>14.0±2.9</td>
</tr>
<tr>
<td>Smooth muscle of epididymis (mouse)</td>
<td>Subplasmalemmal cytoplasm network of strands</td>
<td>4.3±1.4</td>
<td>17.0±4.8</td>
</tr>
<tr>
<td></td>
<td>Basement membrane cord network</td>
<td>4.4±1.3</td>
<td>17.6±3.8</td>
</tr>
</tbody>
</table>

*The size of the openings of the network are defined as the interstrand space diameter index (ISSDI) or the intercordal space diameter index (ICSDI).

Fig. 2. High magnification view of the network observed in the subplasmalemmal cytoplasm of a smooth muscle cell (A) and in its associated basement membrane (B) of the rat epididymal tubule. PM, plasma membrane of the smooth muscle cell. Arrows indicate a strand (A) and cord (B). (C) Subplasmalemmal cytoplasmic network of a podocyte process in the mouse glomerulus observed at very high magnification. Irregular flimsy strands (thick arrows) forming a network are often associated with 4.5 nm wide double tracked structures (paired arrows). In limited areas, the network of strands is replaced by a network of fine filaments about 1.5 nm in width (thin arrows). Bars: A, B, 100 nm; C, 10 nm.
As previously reported, fine filaments made up of type IV collagen were usually associated with a ‘sheath’ composed of other components such as laminin to form the cords in basement membranes, and occasionally, cords were found to be ‘denuded’, that is, without the association of this sheath material (Inoue and Leblond, 1988). Similarly, in the peripheral cytoplasm of the smooth muscle cell, both irregular strands with an appearance similar to that of cords and the denuded strands made up of only fine filaments were observed (Fig. 4A). In areas where the network passed through the plasma membrane, the network seemed to always be composed of only fine filaments and not of cords or strands, with the possible exception of HSPG ‘double tracks’ because the double tracks in the basement membrane were occasionally seen passing through the plasma membrane into the space of the subplasmalemmal cytoplasm. Incidentally, the ‘darkness’, or density, of the cell cytoplasm or basement membrane, noticeable particularly at low magnification, appears, with high resolution observation, to be related to the amount of this ‘sheath’ material that is associated with the fine filaments of the network in both the cytoplasm or basement membrane.

The periphery of the cell which was not in association with the basement membrane, but was apposed by an ‘open’ space, was also observed at high magnification. Fig. 5A shows a podocyte process of the mouse kidney in which a few strands, extending from the network in the subplasmalemmal cytoplasm, stick out 15-25 nm through the plasma membrane into the space of urinary sinus in the form of either fine filaments (Fig. 5A, arrows) or strands (arrow with asterisk). Another example is the surface of a capillary endothelial cell (Fig. 5B) of the rat ciliary body which faces the lumen of the capillary. Strands continuous from those of the subplasmalemmal network were seen sticking out into the space of the lumen mostly in the form of strands, rather than fine filaments, with an average length of about 20 nm but extending occasionally to more than 70 nm (Fig. 5b, arrows with asterisk).
DISCUSSION

After preparation of the tissues in this study with the freeze substitution technique, the lamina lucida of the basement membranes was no longer seen and the lamina densa was observed to be in direct contact with the surface of the cells in agreement with previous reports in which the lamina lucida was shown to be an artefact (Goldberg and Escaig-Haye, 1986; Reale and Luciano, 1990; Chan et al., 1993; Chan and Inoue, 1994).

The slow, gentle dehydration of the tissues at low temperature also permitted good preservation of the network structure in the subplasmalemmal region of the cell cytoplasm. It has generally been accepted that the cytoplasm contains a lattice-work made up of filamentous components (Luby-Phelps et al., 1988) referred to as the microtubular lattice (Wolosewick and Porter, 1979; Porter, 1984). As well, the cytoplasmic lattice-work, or network, was reported to be more dense in the subplasmalemmal regions (Buckley and Porter, 1967; Spooner et al., 1971; Yamada et al., 1971). Such subplasmalemmal structures have been observed in various cultured cells and nerve cells (Buckley, 1974, 1975; Buckley and Porter, 1967; Yamada et al., 1971) as three-dimensional networks of filaments 4-6 nm in thickness. These filaments were described as being inserted into the plasma membrane on the cytoplasmic side.

The results of the present study confirm the presence of a similar fine network in the subplasmalemmal region and also show it to be ultrastructurally similar to the cord network of the basement membrane (Inoue and Leblond, 1988; Inoue et al., 1983). This similarity was further highlighted through measurement of the thickness of the cord-like strands and the diameter of the inter-strand spaces (openings of the network of strands) of the subplasmalemmal cytoplasmic network. They were found to be almost identical, respectively, to the thickness of the cords, and the width of the intercordal spaces of the basement membrane cord network. An exception was in the size of the intercordal spaces of the mouse glomerular basement membrane (14 nm) which was smaller than that of most of the other strand and cord networks (17 nm). This difference appears to be mainly due to the highly specialized function (filtration) of this particular basement membrane (Farquhar, 1981).

In addition to the close morphological similarity between the network of the subplasmalemmal cytoplasm and of its associated basement membrane, ultrastructural evidence was obtained in this study suggesting the possibility that the networks in these two distinct locations are continuous. Close observations of the border between these two networks revealed that the network of core filaments of the cords, reported to be composed of type IV collagen, was continuous, through the plasma membrane of the cell, with the network composed of filaments of similar appearance occupying the subplasmalemmal cytoplasm of the cell. Incidentally, where cells were not associated with the basement membrane, strands of the network in the peripheral cytoplasm were seen protruding through the plasma membrane out into the extracellular ‘open’ space.

The biochemical nature of the cord-like strands, fine filaments and double tracked structures in the subplasmalemmal cytoplasmic network, although ultrastructurally resembling basement membrane cords, type IV collagen filaments and HSPG double tracks, respectively, is not clear at present from the results of this ultrastructural study. It is known that basement membrane components such as laminin and type IV collagen are mediators of cell-basement membrane interactions (Paulsson, 1992). These components bind to integrins, transmembrane receptors, at the cell surface and, on the cytoplasmic side, the internal portions of the integrins are linked to the cytoskeleton through the actin-associated proteins, talin, vinculin and α-actinin (Burridge et al., 1988; Albelda and Buck, 1990). Another cell surface protein in epithelia in mature tissues is syndecan, a heparan/chondroitin sulfate containing proteoglycan having well conserved transmembrane and cytoplasmic domains (Saunders et al., 1989; Mali et al., 1990). It was proposed that this proteoglycan mediates a linkage between the cytoskeleton and the extracellular matrix (Bernfield et al., 1985).
intramembrane and cytoplasmic components remain to be done with other techniques such as, for example, immunolabeling, it is of interest to note that some components of the transmembrane complex have been shown to take the form of thin filaments when observed in the electron microscope. An integrin, for example, is known to contain, in addition to its globular domains, two tails 2 nm in thickness and 10-20 nm in length. They extend through the cell membrane to reach the peripheral cytoplasm (Nermut et al., 1988). The thickness of the tails is comparable to that of the filaments observed in this study traversing the cell membrane. Within the cytoplasm, the thickness of the actin filaments of the cytoskeleton is in the same order of magnitude as that of the thin intracellular filaments. Therefore, the various ultrastructural components observed in this study within the cell membrane and the subplasmalemmal cytoplasm are most probably components of the cell-basement membrane adhesion complex.

In any case, whatever the biochemical nature of these ultrastructural cytoplasmic and transmembrane components, it is again intriguing that both the basement membrane and the subplasmalemmal cytoplasm show very similar ultrastructural features, as revealed in this study.

In summary, the results of the ultrastructural study reported here show a striking similarity between the cord network of the basement membrane and the network in the subplasmalemmal cytoplasm, and also indicate the probable continuity of these networks. The presence of such a seemingly unified network would be functionally advantageous in maintaining the structural integrity of the area of the border between the subplasmalemmal region of the cells and the basement membrane, the connective tissue structure that is present in the immediate vicinity of the cells. It is possible that such an arrangement may be vital for the tissue in order to carry out fundamentally important biological processes such as growth and differentiation.

This work was supported by the Medical Research Council of Canada, grant MRC MA 12279, and also by an MRC Grant to Dr C. P. Leblond to whom I am greatly indebted. I thank Dr F. L. Chan for his help during the preparation of tissues and Ms Cindy Gates for her excellent technical assistance.

**REFERENCES**


(Received 5 October 1994 - Accepted 26 January 1995)