The translaminal fibrils of the human amnion basement membrane

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Summary

The organisation of extracellular matrix beneath the human amniotic epithelium was investigated in order that the co-ordinate synthesis of basal lamina and stroma by these cells could be better understood. Transmission electron microscopy of intact tissue confirmed that stromal matrix fibrils are located between the cell surface and the basal lamina, and also penetrate the lamina. The distribution of the supralaminal fibrils and their association with the lamina was further investigated by scanning electron microscopy (SEM) after removal of the overlying epithelium. Five complementary procedures were used to remove the cells from the underlying lamina. Trypsin-EDTA treatment caused the epithelial cells to retract or detach from the lamina. SDS or ammonium hydroxide was used to extract the epithelium, which was then removed by physical shearing. Transmission electron microscopy (TEM) confirmed that the lamina densa and supralaminal fibres were present after extraction by these agents. Incubation in CHAPS, a zwitterionic detergent, did not remove the epithelium but permitted exposure of the basal lamina by mechanical scoring. Extraction with boric acid followed by osmium tetroxide produced epithelial disruption and revealed the lamina and stroma in different areas. Although the extraction pattern was different in each case, all of the five methods confirmed that individual fibrils and fibril bundles are present on the apical surface of, and enter, the lamina densa. Examination of the stromal surface of the basal lamina after fracture revealed fibrils passing from the stroma into the lamina densa. We therefore suggest that, in this tissue, newly synthesised stromal matrix components appear in an assembled fibrillar form between the basal cell surface and the basal lamina before becoming associated with the sublaminal stroma.

Key words: amnion, basal lamina, extracellular matrix fibrils, ultrastructure.

Introduction

The human amniotic epithelium is a simple monolayer of cuboidal cells that forms the interface between amniotic fluid and placental tissue. Despite this simplicity of organisation the epithelial cells have a complex basal surface from which extracellular matrix is secreted. Experiments with isolated cells and cultured tissue have shown that the epithelium has the ability to synthesise and deposit both basal laminal and stromal extracellular matrix components (Alitalo et al. 1980; Aplin et al. 1985, 1986). It therefore seems likely that the acellular 'compact layer' of amniotic stroma, which lies just beneath the epithelium (Bourne, 1960, 1962), is a product of epithelial matrix deposition. The stroma has been shown to contain collagen types I and III (Madri et al. 1983), and fibronectin (Aplin et al. 1985), and to have a perilaminal distribution of collagen types V (Modesti et al. 1984) and VII (Keene et al). The basal lamina of the amnion contains a network of type IV collagen fibrils (Yurchenko & Rubin, 1987), laminin (Aplin et al. 1985) and heparan sulphate proteoglycan (Foltz et al. 1982). The association of lamina and stroma is at least partly mediated by the presence of anchoring plaques containing type IV col-
Fig. 1. A section perpendicular to the plane of the amniotic epithelium shows the highly convoluted basal surface of these cells. The basal lamina follows the contours of the cell and is therefore present in deep infolds (arrowheads). In other regions the fibrillar matrix is not separated from the cell by lamina densa (arrow). Bar, 2 μm.

Fig. 2. A complex region of the basal surface. Fibrils of similar morphology to those in the stroma are present in a region superficial to the cell processes and basal lamina and immediately adjacent to the basal cell surface (arrowhead). Small-diameter fibrils penetrate the basal lamina (arrows). Bar, 0.3 μm.

The basal surface of the cells is covered with fine processes displaying hemidesmosomes usually toward their tips. In the infoldings between these processes the lamina appears discontinuous in restricted areas (Aplin et al. 1985) and large accumulations of matrix fibrils appear in close apposition to the basal cell surface. We therefore proposed a simple model in which the basal cell surface is composed of two functionally distinct domains: an adhesive domain of cell processes that contain the hemidesmosomes, and a secretory domain of infolds where the lamina is, in places, less well-defined and matrix fibrils are present.

Conventional ultrastructural examination of this complex cell–matrix interface in thin sections gives a very restricted view of such a tortuous two-dimensional surface. Consequently, we felt that a much better understanding of the cell–matrix interface would be achieved if large areas of the lamina and its associated fibrils could be examined by scanning electron microscopy (SEM). However, it seemed inevitable that removal of the epithelial lagen, which are attached to the lamina by anchoring fibrils containing collagen type VII (Keene et al. 1987).
cells would produce unwanted disruption of some structures and distortions of others. In order to overcome the possibility of misinterpreting such artefacts we have used a variety of procedures that remove the epithelial cells by different mechanisms. Other workers have extracted human amniotic epithelium with ammonia (Mignatti et al. 1986) or detergent followed by scraping (Liotta et al. 1980; Erickson, 1980), in order to provide a basement membrane substratum for experimental studies of metastasis (Liotta et al. 1980), endothelial growth (Madri et al. 1983), neutrophil invasion (Azzarelli and Lafuze, 1987), and neural crest cell migration (Erickson, 1987). At least one of these treatments appears to reveal supralaminar fibrils in SEM micrographs, although it received no comment by the authors (Mignatti et al. 1986). We now report the results of five cell-removal procedures and reveal how the translaminar fibrils of the amnion basement fibrils can be better visualised by SEM.

Materials and methods

Normal term placentas were obtained immediately after delivery by Caesarean section. The membranes were removed and washed extensively in phosphate-buffered saline without divalent cations and the amnion then separated from the chorion. When appropriate the tissue was fixed in 2.5% glutaraldehyde in Sorensen’s buffer, post-fixed in 1% osmium tetroxide and routinely embedded and sectioned for transmission electron microscopy (TEM).

Semi-thin cryosections were also prepared after the method of Tokuyasu (1973) (Griffiths et al. 1984), so that the morphology of the basal lamina and associated fibrils could be examined without solvent dehydration and resin embedding. The tissue was fixed in 4% paraformaldehyde–0.1% glutaraldehyde in a phosphate buffer for 1 h at room temperature, embedded in 2–3 M-sucrose in 0.1 M-phosphate buffer, dissected into small cubes, and placed on a cryopin (Ultracut E, Reichart). Dry sections were cut and floated onto sucrose, then mounted on glow-discharged Formvar-coated nickel grids. They were stained with 3% uranyl oxalate at neutral pH, washed, then air dried in a loop of 1–5% Methocyl (Fluka), 0.3% uranyl acetate in a desiccator and examined on a Philips 400 TEM.

When the basement membrane was to be exposed for scanning electron microscopy (SEM) the epithelium was removed in one of five ways. Whole-cell detachment was achieved with a trypsin (0.25%)/EDTA (10 mM) mixture at 37°C by a procedure designed to produce viable cell suspensions (Aplin et al. 1985). The detachment process, which was monitored on an inverted bright-field microscope, resulted in the removal of single cells and cell clumps, whilst leaving other regions with rounded up cells and more recently exposed areas of subjacent basement membrane. Chemical extraction of the epithelial cells was carried out with a variety of agents with different modes of action. The end points of the extraction processes were determined morphologically rather than biochemically by examination of the epithelial surface with an

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Fig. 4. SEM of an amnion incubated in trypsin-EDTA for 60 min. A cell in the uppermost part of the figure in the process of detachment has retracted from the lamina leaving behind a smooth surface that is severely damaged by the presence of holes through which the meshwork of stromal fibrils (x) may be seen. Bar, 1 μm.

Fig. 5. Trypsin-EDTA treatment for 40 min. Fibrils of various diameters are found on the upper surface of the lamina. In some places they occur in bundles and have amorphous material associated with them (arrow). Bar, 1 μm.

Fig. 6. The same treatment as in Fig. 5, viewed at higher magnification. Fibrils are observed on the surface of the lamina where they bend with a range of curvatures, and criss-cross each other in an apparently random pattern before disappearing into the stroma (arrowheads). Bar, 2 μm.
inverted microscope. The end point was regarded as the situation where no detectable cell debris could be observed on large patches of the membrane. Ammonium hydroxide (0-2 M, pH 11.5), which has been used to expose basal laminal matrix deposited on collagen gels (Schor et al. 1985), was found to act more slowly on tissue than on cultured cells (Jones and Scott-Burden, 1979), and therefore proved to be a useful solubilising agent. Amnion was extracted with ammonium hydroxide for times ranging from 30 min to 2 h at ambient temperature (Mignatti et al. 1986). In order to preserve the morphology of the extracted matrix (Allen et al. 1988), scraping of the cell layer was avoided. Instead, small pieces of tissue (about 4 cm²) were placed in a Petri dish and vigorously sheared across the epithelial surface with a Pasteur pipette. The extracting solution was changed once or twice during the course of solubilisation. SDS was chosen as the other cell-solubilising agent because of its limited effectiveness in solubilising basement membranes. (In the absence of reducing agents SDS will not solubilise collagen type IV from rat glomerular basement membrane (Taylor & Price, 1984) or produce bulk extraction of the GBM, lens capsule or Decemet's membrane (Hudson & Spiro, 1976; Kefalides, 1973.) SDS was used at 0.2 or 1.0% (w/v) for times ranging from 30 min to 2 h and the tissue was sheared with a pipette. Owing to its tendency to foam, the SDS solution was changed up to five times. Variations in the incubation times with these agents gave no significant change in the outcome. Mechanical removal of the epithelium was facilitated by mild treatment of epithelium and basal lamina and was therefore inappropriate to this investigation.

Trypsinised tissue was fractured in a way that exposed the distal (or stromal) surface of the basal lamina (Allen et al. 1988). After critical-point drying the tissue was mounted on a specimen stub (Cambridge Instruments) with a silver colloid adhesive (EcoBond, Hitech Ltd, Scunthorpe). A second stub covered with an adhesive tab (Agar Aids, Essex) was pressed firmly against the surface of the tissue. The stubs were then pulled apart without lateral shearing.

The extracted tissues were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M-sodium cacodylate (pH 7.2), and post-fixed in 1% (w/v) osmium tetroxide for 1 h, except tissues that had been previously exposed to osmium, which were thus given no additional fixation. The tissue was then dehydrated in a graded series of ethanol solutions, transferred to Arkalone (ICI), and critical-point dried from carbon dioxide. All prepared surfaces were sputter coated with gold (5 nm) before examination on the high-resolution mode of the ISI SS40 scanning microscope.

Results

Transmission electron microscopy of intact amnion

The amniotic epithelial cell has a convoluted basal surface (Fig. 1), which was difficult to visualise in three dimensions when examined in thin section. The lamina densa does not form a flat well-defined structure such as that found in the cornea (Hay & Revel, 1969; Hay, 1980), but follows the basal cell surface, although in some regions it was found to be either absent or indistinct. Another unusual feature of human (Aplin et al. 1985) and monkey (King, 1980) amnion is the presence of matrix fibrils between the cell surface and basal lamina (Figs 1, 2). These supralaminal fibrils were morphologically indistinguishable from those within the densely fibrillar region of the superficial stroma (Fig. 2). Some of them clearly penetrated the lamina and perhaps passed through it (Figs 2, 3). However, the complex topology and variable appearance of the lamina render this surface and the relationship of the fibrils to it difficult to interpret.

Scanning electron microscopy of amnion after epithelial removal

Epithelial detachment by trypsin-EDTA treatment. Trypsin-EDTA was used to cause cell rounding up and detachment and thus permit exposure of the basement membrane. After 60 min of digestion, areas of matrix were observed beneath the detaching cells (Figs 4, 5).

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The major component of this subcellular matrix was an amorphous smooth surface folded into an anastomosing system of ridges and blebs. This surface was thought to be the residual electron-opaque lamina densa because of its immediately subcellular location. Holes of various sizes were observed in the lamina. The larger of these gaps were much bigger than the discontinuities observed in TEM and were therefore considered to be an artefact that had arisen as a direct consequence of proteolysis and fragmentation during cell detachment. Stromal matrix fibrils, which were visible through the holes, formed a densely interwoven meshwork in close proximity to the basal lamina. Fibrils also extended from the surface of detaching cells through the large gaps down into the stromal layer or attached directly to the lamina at points where it remained intact.

After less-prolonged treatment (40 min digestion), areas were found in which supralaminal fibrils were present in much greater numbers on the upper surface of the residual lamina densa (Fig. 6). This suggests that the fibrils, or their anchorage points, are trypsin-sensitive. When present, they extended for long distances over the lamina (up to 7-5 µm) and frequently formed bundles as suggested by TEM of intact tissue (Figs 1, 2). These bundles became incorporated either into the stromal meshwork where large gaps were present or into ridges or blebs on the surface of the lamina.

When the distal (‘under’) surface of the lamina was visualised by the tape-stripping method, single fibrils and bundles of fibrils were found closely associated with the stromal surface of the lamina densa. Examination at high magnification showed fibrils running obliquely into the lamina densa from beneath, and blunt-ended terminations of fibrils that may have broken during stripping (Fig. 7).

Epithelial extraction by SDS or NH₄OH. Extensive shearing was required to remove the partially solubilised epithelial cells during these extractions. This inevitably gave rise to some heterogeneity over the exposed surface, so that in some areas cells or fragments of cells remained attached. The results shown are typical of areas in which the cells were successfully removed. After SDS extraction of the epithelium much of the surface was occupied by an amorphous sheet (Fig. 8), which was similar in appearance to the lamina after trypsin treatment. However, the upper surface was less smooth, having more frequent small blebs. Holes ranging from 0-1 µm to 1 µm were present within the lamina. On the superficial (epithelial) side of the lamina there was an abundant network of fibrils occurring singly and in bundles of different widths. The largest bundles observed after extraction contained 20–30 fibrils, a range that is consistent with the sizes of bundles observed in TEM of intact tissue (Figs 1, 2). Fibrils could be readily seen to traverse...
the lamina before either becoming associated with the stroma visible at gaps in the lamina densa, or simply disappearing into the lamina (Fig. 8).

When the same type of preparation was examined by TEM the epithelial cells were found to separate from the underlying matrix by cleavage through the plane of the lamina lucida (Figs 9, 10). Intermediate filaments were abundant in the cytoplasm of the extracted, detaching cells (Fig. 9), although the internal membranes were disrupted. Discontinuities in the lamina densa were obvious as well as the peaks and troughs corresponding to the folding seen in intact tissue (Fig. 1). Bundles of fibrils were retained on the epithelial side of the lamina after extraction (Fig. 10). The fibril diameters corresponded exactly to those of the most abundant fibril type found in the stromal layer (18 nm) immediately below the basal lamina.

Very similar features were evident in tissue extracted with ammonia (Fig. 11). The extensive fibril network above the lamina had a similar distribution to SDS-extracted and trypsin/EDTA-treated tissue. However, the rougher appearance of both fibrils and lamina suggested a less-efficient extraction of amorphous components. As it was difficult to correlate the presence of amorphous patches of matrix in TEM of unextracted tissue with the fibril- and lamina-associated material seen by SEM after extraction, the nature of this material remains undefined.

**Epithelial disruption by boric acid–osmium tetroxide.** This procedure exposed both lamina and stroma in different areas. In some places remnants of cells sat upon patches of basal lamina (Fig. 12), although in other adjoining regions cells and lamina were removed, revealing the underlying stroma. At low magnification the laminal region was covered in ridges and blebs. The ridge pattern seemed complementary to the pattern of indentations observed by SEM on the basal cell surface (Allen et al. 1988). In places, ridges also seemed to reveal the outline of the cell boundaries. The ridges and blebs were very heterogeneous in shape and size and were therefore thought to be a mixture of cell fragments and genuine undulations in the surface of the basal lamina. At higher magnification (Fig. 13) some of these ridges were observed to be composed of fibril bundles that were partially coated with amorphous material. Single supralaminal fibrils were also observed (Fig. 14), although present in much reduced numbers in comparison with other preparations. In regions of the tissue where large patches of lamina remained, the small holes present in the other preparations were conspicuously absent.

**Mechanical removal of the epithelium following partial solubilisation by CHAPS.** Treatment with this zwitter-ionic detergent caused extraction of the epithelial surface with loss of the microvillous projections, but did not remove the cells. Scores in the epithelium were made as
described in Materials and methods. At the edge of the scored regions the plane of exposure passed steeply from the cell surface through a zone of hollow nuclei to the subnucleus cytoplasm (Fig. 15). Large areas of subnuclear cytoplasm with clearly delineated cell peripheries and junctional regions were present within the scores (not shown). In patches where the scoring had exposed the basement membrane, supralaminal fibrils were found in abundance (Fig. 16). Removal of the epithelium in this way probably caused disruption in the organisation of the fibrils, for some were displaced and lay over regions of adjoining basal cytoplasm. At higher magnification (Figs 17, 18) the fibrils had the same smooth appearance, although the basal lamina was covered in small blebs of various sizes. Despite the unusual appearance of the lamina in this preparation, fibrils were seen to penetrate it in the same way as before.

Discussion

This study has confirmed the existence of supralaminal fibrils in the human amnion and established that they can extend for distances of several microns across the epithelial side of the lamina densa, form bundles, and penetrate the lamina on the superficial side before emerging into the stroma. Although their composition is at present unknown, their morphology suggests strongly that they belong to a major class of collagenous fibrils present in the sublaminal stroma.

The compact layer of acellular stroma beneath the basal lamina varies from 5 to 50 μm in thickness but is of uniform width in a particular individual (Bourne, 1962). We believe that its principal function is to provide mechanical support for the laterally growing epithelium. Its cellular origin is a concern of the present study. In previous studies we (Aplin et al. 1985, 1986) and others (Alitalo et al. 1980) showed that cultured term amnion epithelial cells produce a mixture of interstitial and basal laminal matrix components, and the results suggest that the latter is quantitatively dominant. In this tissue no stromal cell approaches the basal lamina, so it is reasonable to suppose that the epithelial cells synthesize and secrete components of both the basal lamina and its subjacent stroma. On the basis of the present results, we suggest that the supralaminal fibrils observed in SEM after removal of epithelial cells are newly deposited collagenous components destined for incorporation into the underlying stroma. The observation that these fibrils cross the lamina densa supports this idea, although the precise sequence of morphogenetic events remains unclear.

The complexity of this cell–matrix interface does not arise of necessity from the requirement of the epithelial cells to produce both basal lamina and stroma. The avian corneal epithelium, which synthesises both stromal and laminal components in early embryogenesis, possesses a continuous flat basal lamina (Hay & Revel, 1969; Meier & Drake, 1984) without supralaminal fibrils. When the corneal basement membrane was examined by SEM after
Fig. 12. Boric acid-osmium tetroxide disruption of the epithelium showing large fragments of epithelial cells (c) attached to the lamina densa, which is elevated into ridges (arrowheads) and blebs. The cell outlines are marked by prominent ridges. Bar, 10 μm.

Fig. 13. The same preparation at higher magnification showing a portion of basal lamina lying above the stromal matrix (left). A fibrillar bundle (arrow) that traverses the lamina densa is covered in places by amorphous material. Bar, 1 μm.

Fig. 14. Although individual supralaminal fibrils are largely extracted by boric acid-osmium tetroxide, occasional fibrils are seen on the upper surface of the lamina. Bar, 1 μm.

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Fig. 15. The edge of a score in the epithelium after partial extraction with CHAPS. The extracted cell surface (cs) lies above the hollow extracted nuclei (en) and basal cytoplasm (bc). Bar, 10 µm.

Fig. 16. Areas of basal cytoplasm (bc) surround a patch of basal lamina covered by fibrils. Bar, 1 µm.

Fig. 17. A region where supralaminal fibrils have been exposed after scoring. The lamina does not have a smooth appearance after this treatment and many amorphous blebs are present. Bar, 0.5 µm.

removal of the epithelium by detergent extraction, a smooth surface exhibiting immunoreactivity to type IV collagen antibodies was exposed (Meier & Drake, 1984). Indeed, after removal of the amniotic epithelium by blotting, there remains a matrix containing collagen type IV, laminin and perilamin collagen type V, as well as the major stromal collagen types I and III (Madri et al. 1983). We therefore suppose that the smooth subcellular sheet revealed in all treatments, except mechanical scoring, comprises a network of collagen type IV (Yurchenko and Rubin, 1987) with associated amorphous components as observed by conventional TEM (Laurie et al. 1982; Inoue et al. 1983). Its smooth appearance at this level of analysis may, however, be due to the dehydration, critical-point drying, and coating processes used to prepare the tissue. Under our conditions of extraction it seems likely that the amorphous components identified in TEM remain insoluble, so that individual fibrils of type IV collagen are not resolved by SEM (Yurchenko and Rubin, 1987). However, the finest fibrils visible within the basal lamina by TEM of intact tissue probably do correspond to the type IV collagen network observed.
Fig. 18. Fibrils disappear into the subjacent matrix within the scored regions. Bar, 0.5 μm.

in metal replicas after high salt extraction (Yurchenko and Rubin, 1987).

The existence of abundant translaminal fibrils has been convincingly demonstrated by TEM in amphibian epidermis (Ellison and Garrod, 1984), although their biochemical composition remains undefined. These structures are probably involved in cell adhesion to the matrix, for they emerge from the hemidesmosomal membrane, cross the lamina lucida and penetrate the lamina densa. The translaminal fibrils that we have described here are dissimilar, for they are not associated with hemidesmosomes and appear more randomly oriented with respect to the lamina. We therefore consider their existence in amnion to be a consequence of the pattern of matrix synthesis, rather than acting as structural elements directly involved in cell adhesion to the lamina. Nevertheless, anchoring fibrils are present in amnion, although at lower frequency than in skin or cornea (Keene et al. 1987).

Removal of epithelial cells from other tissues including glomerulus (Carlson et al. 1986) and intestine (McCluggage and Low, 1984; Komuro, 1985; Warfel and Hull, 1988) with either detergent, boric acid or boric acid–osmium has revealed a smooth subcellular lamina on examination by SEM. Inspection of the extracted human colon by TEM after boric acid extraction (Warfel and Hull, 1988) has demonstrated that the smooth surface corresponds to the lamina densa or its unextracted residue. In these tissues the laminal surface lacks the fibrils and rugosities that are associated with matrix morphogenesis in amnion. Both human and rat intestinal basal lamina contained holes after extraction. In the case of the rat these holes were sometimes filled by processes of stromal cells. Despite the apparent discontinuities of the amniotic lamina detected by TEM of intact tissue, we have not observed epithelial cell processes penetrating to the stroma. Trypsin–EDTA treatment produced large holes that were clearly artefacts of proteolysis or fragmentation at the time of epithelial detachment (Allen et al. 1988). The existence of discontinuities in the lamina densa could therefore not be unequivocally established in SEM.

The methodology described here should be useful in the morphological examination of other tissues. It is important, however, to stress the need for a multiplicity of complementary experimental approaches to combat or circumvent artefacts that may arise in any new method of visualisation of inner layers of the tissue. Immunofluorescence analysis of cell-free areas of exposed perilaminar matrix should give further insight into the composition of the fibrils.

The observations of the present study substantiate our previous account of amnion morphology (Aplin et al. 1985), in which we noted that the presence of basal cell invaginations gives rise to a large (about 10-fold) increase in the basal cell surface compared to the area covered. The increased area of cell–substratum contact allows the cells to adhere to the lamina via hemidesmosomes while depositing large quantities of stromal extracellular matrix in the acellular zone beneath the lamina. Despite the clear topographical description of matrix organisation reproduced here, we still cannot provide an analysis of the temporal sequence of events that leads to the incorporation of newly secreted supralaminar fibrils into the sublaminal stroma.

Nevertheless, we feel sure that the development of the densely fibrillar acellular zone beneath the epithelium must toughen the surface region of the tissue and so help to contain the growing and highly motile foetus.
References


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