MECHANISMS OF INVASIVENESS OF EPITHELIAL TUMOURS: ULTRASTRUCTURE OF THE INTERACTIONS OF CARCINOMA CELLS WITH EMBRYONIC MESENCHYME AND EPITHELIUM

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SUMMARY

Three different kinds of carcinoma cell, human breast tumours, mouse lung tumour and rat bladder tumours have been implanted into the developing chick wing to assay their invasive behaviour. We found that the majority of the carcinoma cells did not invade the mesenchyme but were positioned in the ectoderm. We examined the ultrastructure of the interaction of the carcinoma cells with both the ectoderm and the mesenchyme to see if there are any differences. We found no specialized adhesive junctions between carcinoma cells and mesenchyme cells. In contrast, the carcinoma cells seem to be adhesive to the ectoderm cells; desmosomes between the two have been found and also frequent parallel alignment of membranes over long distances. This difference between the interaction of the carcinoma cells with the mesenchyme and the ectoderm may explain why the carcinoma cells can penetrate into the ectoderm but rarely do so into the mesenchyme. The carcinoma cells formed a smooth border with the mesenchyme even in the absence of a basal lamina. These results are discussed in relation to the mechanisms of invasion of epithelial tumours in vitro. In particular, the relative importance of the basal lamina, and cell adhesion within the tumour, in limiting invasiveness are assessed.

INTRODUCTION

The outstanding features of malignancy are invasiveness and metastasis, the establishment of secondary tumours at sites distant to the original tumour. The cells which give rise to the secondary tumours are usually transported to new sites in the blood or lymph vessels. It has been assumed that metastasis is correlated with invasiveness. However, there are invasive tumours such as rodent ulcers of the skin which rarely metastasize (Walter & Israel, 1974). The first stages of metastasis, the entry of tumour cells into the blood or lymph vessels, may involve invasion.

One widely held view is that tumour cells invade by active cell movement (see Trinkaus, 1976; Sträuli & Weiss, 1977). We have tested whether a variety of different types of cells would move into a loose cellular meshwork. The assay site we chose was the developing chick wing bud, which at early stages of development consists of loosely associated mesenchyme cells bounded by a 2-layered ectoderm (Gould, Day & Wolpert, 1972). Besides the advantage of histological simplicity, we have found this
site to provide a good culture environment for a number of different tissues. We have found that sarcoma 180, normal and transformed fibroblasts and neuroblastoma cells all move into the chick mesenchyme. In contrast the cells from 3 different kinds of carcinomas – human breast tumours, mouse lung tumour and rat bladder tumours – all show little movement into the mesenchyme, even though clinically invasion of these tumours takes place into the underlying stromal tissues. Indeed, the unexpected finding was that the carcinoma cells showed an affinity for the wing ectoderm. The tumour cells appeared to be positioned on a common basement membrane with the neighbouring ectoderm cells (Tickle, Crawley & Goodman, 1978).

We have examined the ultrastructure of the cells in grafts of all 3 kinds of carcinoma cells and the contacts between the carcinoma and chick mesenchyme cells. We have tried to locate and examine the morphology of cells that moved into the mesenchyme, even though these cells were few and far between. It is possible that this small minority of cells are those which in vivo give rise to secondary tumours (Fidler, 1975; Fidler & Kripke, 1977).

We were interested in the propensity of the carcinoma cells to be positioned within the chick ectoderm rather than move into the loose mesenchyme. This affinity for ectoderm suggests that carcinoma cells still retain their epithelial character, and this is also reflected in the histology of many carcinomas in which infiltration is often by means of organized tongues or sheets of cells (Walter & Israel, 1974). Thus the behaviour of the carcinoma cells with the chick ectoderm may provide clues not only to the mechanisms involved in invasion but also in maintaining epithelial organization. We therefore examined the relationship of the carcinoma cells with the basal lamina of the ectoderm, and also the morphology of the contacts between the carcinoma cells and the chick ectoderm cells; both of which could play a role in the positioning of the carcinoma cells in the ectoderm.

We have concentrated on studying grafts of human breast tumours, but have also looked at the behaviour of the cells from mouse lung tumour, rat bladder tumours and, very briefly, cells from a human bladder cell line, RT4.

METHODS

Preparation of tumours for grafting

Samples of human breast tumours were obtained from the Middlesex Hospital. Four tumours were examined; 2 were benign and 2 were malignant. To obtain cellular fragments, the tumours were teased apart with forceps. Small clumps of cells were liberated and grafted. Alternatively individual cells were centrifuged at around 2000 g to form a pellet of cells, and pieces of this were used for grafting. Some of the cellular pieces obtained from the tumours were cultured overnight at 37 °C in MEM + 10 % foetal calf serum (Gibco-Biocult). Some of the cell clumps rounded up, which indicated that the cells were viable.

Rat bladder tumours were obtained which had been produced by feeding rats with repeated doses of nitrosamines (Hicks & Wakefield, 1976). Pieces of rat bladder tumour were cut out for grafting.

The mouse lung tumour, CMT 64 (Franks, Carbonell, Hemmings & Riddle, 1976) was grafted in 2 forms: as a solid tumour and as a cell line that had been growing in tissue culture. The cells were removed from the tissue culture vessel by a standard treatment with 0.1 % trypsin (Difco 1:250) and 0.901 M EDTA in calcium- and magnesium-free saline. When the cells started to detach from the surface of the dish, chicken serum was added to give a final
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concentration of 10% serum. The cells were washed with tissue culture medium (Basal medium Eagle's + 10% chicken serum (Gibco-Biocult) with HEPES buffer) by centrifugation at least twice. The lung tumour cells were then mixed with quail wing mesenchyme cells (Tickle et al. 1978) and a composite pellet made by centrifuging at 2000 g for 10 min. The quail wing mesenchyme cells served to bind the lung tumour cells together.

We also carried out a few experiments with a human bladder tumour cell line, RT4 (Rigby & Franks, 1970). Pellets of these tumour cells were prepared in the same way as the mouse lung tumour cell pellets but the cells were not mixed with quail cells.

In all cases pieces of adjacent tumour or pellet to that grafted were fixed and sectioned, to examine the morphology of the grafted tissue.

Grafting technique

The chick embryos which were hosts for the grafts were stage 20/21 (Hamburger & Hamilton stages). A hole, of side 200 μm, was cut into the dorsal surface of the wing bud. Between a third to one half of the depth of the wing mesenchyme was removed together with the overlying ectoderm and a piece of tumour or cell pellet was implanted. In some cases the graft was held in place by a pin of platinum wire, 25 μm thick. This pin was removed safely after 4 h. The host chick embryos were re-incubated after grafting, for a further day or 2 days before the wing bearing the graft was removed for histology.

Histology and electron microscopy

The wing was fixed in half-strength Karnovsky fixative (cacodylate-buffered formalin/glutaraldehyde mixture, Karnovsky, 1965) at 4 °C overnight. After fixation the tissue was rinsed in 0.1 M cacodylate buffer and then postfixed for 1 h at 4 °C in 1% osmium tetroxide, dehydrated in a graded series of ethanols, cleared in propylene oxide and finally embedded in Araldite. Thick (1 μm) and ultrathin sections were prepared using a Cambridge Huxley ultramicrotome (Mark 1). The thick sections were stained with 1% toluidine blue (in 1% borax) (Trump, Smuckler & Benditt, 1961). Serial ultrathin sections were cut at this level before taking off a thick section to check the position of the grafted cells. This procedure was continued throughout the graft. The thin sections were stained with lead citrate (Reynolds, 1963) and examined in a Philips EM 300 microscope.

RESULTS

In many cases the grafts were forced out of the mesenchyme during healing and protruded from the wing. The ectoderm came to lie, over some extent of the graft, between the implanted cells and the mesenchyme. The interaction between the grafted cells and adjacent tissue was examined in both situations; where the graft abutted mesenchyme and also where the tumour cells were next to ectoderm.

Malignant breast tumours

Morphology of tumour cells. Lumina were often found within the graft, with cells arranged around the edge (Figs. 1, 2). These tubules may have been isolated intact from the tumour (Goldenberg, Goldenberg & Sommers, 1969), or may result from the reorganization of individual tumour cells within the cell pellet. We have found such a reorganization of tubules in pellets of disaggregated cells from embryonic mesonephros.

The cells surrounding lumina were polarized. The apical surfaces of the cells were studded with microvilli. Just below the apices of the cells there were lateral junctional complexes (Fig. 3). Some of the cells were very electron-dense while others were less so
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(Fig. 1). Similar dark and light cells have been described in other epithelia and may well be characteristic of all epithelia. The reasons and significance of why some cells are more electron-opaque than others is not known. It has been suggested that the electron opacity reflects the stages of the cell cycle (Tannebaum, Weiss & Marx, 1969). Some of the cells contained glycogen bodies.

There were extensive cell–cell contacts in both malignant tumours. Typically the cells were joined by numerous desmosomes. We often saw several desmosomes around the perimeter of a cell in a single section. In addition many cells possessed fingerlike processes which interdigitated with similar projections from adjacent cells (Fig. 3). These projections have also been seen in other kinds of epithelia such as gall bladder (Kaye, Wheeler, Whitlock & Lane, 1966) and ureter (Hicks, 1965). We have used the presence of these projections as a criterion to identify isolated epithelial cells (see later). Another type of contact, which occurred frequently, was the parallel alignment of the membranes of 2 adjacent cells over considerable distances, up to 3.5 μm.

There is the general problem in obtaining tumour cells from pieces of malignant tissue, which is the possibility that some of the cells are not in fact from the tumour but are from adjacent normal epithelia. We were interested to see if we could find any of the features that have been variously suggested to be typical of malignant breast cells. The nuclei of carcinoma cells have been said to be irregular in outline (Murad & Scarpelli, 1967; Murad, 1971). While the nuclei of some of the cells were very indented and irregular in outline (Fig. 2) this was by no means true of all the cells in the graft (see also comments of Tannebaum et al. 1969). Another morphological feature found to be associated with malignancy is the presence of intracellular ducts lined with microvilli (Sykes, Recher, Jernstrom & Whitescarver, 1968; Goldenberg et al. 1969). We found one example of an intracellular duct in each of the malignant tumours (Fig. 4). McNutt (1977) has suggested that the presence of microfilaments might be a sensitive indicator of malignancy. The cells in the graft contained filaments of diameter $8.4 \pm 1.0 \text{ nm} (n = 8)$.

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Fig. 1. General view of cells of a malignant breast tumour around a lumen (l). Note the regular outline of the nuclei. Scale bar, 5 μm.

Fig. 2. Another part of organized epithelium from a malignant breast tumour. The nuclei are indented and irregular in outline. Scale bar 2 μm.

Fig. 3. Outlined region in Fig. 2 showing sub-apical junctional complex, interdigitating arms and desmosome. Scale bar, 250 nm.

Fig. 4. Intracellular duct (i) in a cell from a malignant tumour. Note abundant filaments (arrowed) and bleb (b). Adjacent tumour cells (t) are darkly stained and 2 neighbouring cells are held together with desmosome (double arrowed). On one side the tumour cell abuts the chick mesenchyme (m) with extensive cell contact along the surface of the bleb; and the chick mesenchyme cells appear to be deformed. Scale bar, 2 μm.

Fig. 5. Two Donné cells in the lumen of a breast tubule. Note the apical complexes at a where the cells in the epithelium seal off the lumen, and interlocking projections between the 2 Donné cells (arrowed). Scale bar, 1 μm.

Fig. 6. Border where cells from malignant breast tumour abut chick mesenchyme cells (m). Scale bar, 5 μm.
In addition to these epithelial cells, the pellets and grafts of the malignant tumours contained other cell types. We have identified macrophages in one malignant tumour, and in both tumours we have found what have been called corpuscles of Donné or foam cells.

We have found Donné cells amongst the cells in the graft and also in the lumina of tubules (Fig. 5). These cells showed a range of morphology with varying degrees of degeneration. Some cells which were highly vacuolated were found within the lumina of tubules – a characteristic position for Donné cells (Hollman, 1974). Even these cells show features which we think indicate an epithelial origin. The cells have fingerlike projections from the cell surface. Where 2 such cells occur adjacent to each other in the same lumen the projections from each cell interlock in a way similar to those from neighbouring cells in intact epithelia (Fig. 5).

**Interaction of graft cells with mesenchyme.** In 3 out of 6 grafts one cell was found away from the graft in the wing mesenchyme. We recognized these cells because they were very electron-dense and rounded in shape, with small projections from the cell surface. In 1 case the cell also contained a number of inclusions and extensive Golgi apparatus, which suggested that it was a Donné cell.

In places where the graft abutted the mesenchyme there was often a smooth border between the carcinoma cells and the chick mesenchyme cells (Fig. 6) even though no basal lamina was present. Occasionally a rounded bulge or bleb from a carcinoma cell pressed against mesenchyme cells (Fig. 4) and there was extensive contact between the 2 types of cell. In regions where the apical surfaces of the tumour cells came to lie close to the host mesenchyme there was a space between the grafted cells and the chick cells.

**Interaction with the chick ectoderm cells.** Desmosomes were found between the tumour cells and the chick ectoderm cells (Figs. 7, 8) but were infrequent. In contrast, many desmosomes were observed between the carcinoma cells. However, when we looked for desmosomes between the chick ectoderm cells we found they were scarce. It is
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possible that the number of desmosomes between the chick ectoderm cells and the breast carcinoma cells is limited by the ability of the chick ectoderm cells to form desmosomes.

The chick ectoderm cells did not form processes to interlock with the short projections from the adjacent tumour cells. There were, however, stretches where the membranes of adjacent chick and tumour cells ran parallel to each other (Figs. 7, 9, 10). The distance between the parallel membranes was measured in 2 cases and was 23 and 27 nm, around the same distance that was noted between neighbouring tumour cell membranes (approximately 25 nm). In 2 cases the separation between the membranes of a tumour cell body and its own fingerlike projections was 20 and 23 nm.

There appeared to be 2 main types of orientation of the tumour cells within the ectoderm. They either penetrated into the ectoderm as far as the basal lamina (Fig. 10) or they were elongated along the surface of the ectoderm to form what looked like a periderm layer (Fig. 7). The carcinoma cells interacted with the basal lamina by putting out long processes which were closely applied to the basal lamina. Sometimes these processes appeared to run beneath the ectoderm cells and thus separate them from the basal lamina (Fig. 7). In 1 case the processes of a tumour cell buckled the basal lamina, but there was nevertheless a continuous basal lamina (Fig. 11). This cell had small pinocytotic vacuoles and some hints of hemidesmosomes; both are characteristic of myoepithelial cells.

We have also seen Donné cells that have penetrated the ectoderm in thick sections. In one case it looked as though a Donné cell (recognized by vacuolar cytoplasm) might be breaking through into the mesenchyme (Fig. 12).

Fig. 13. General view of a piece of a graft of benign breast tumour. Note the variation in electron density of the cells within the epithelium. Scale bar, 5 μm.

Fig. 14. Projection from a myoepithelial cell into the stroma. Note basal lamina around projection and additional basement membrane material giving multilayering effect. Scale bar, 0.5 μm.

Fig. 15. Region of Fig. 13 at higher power showing the apical surfaces of the cells covered with microvilli and pinocytotic vesicles in the sub-apical cytoplasm. Also note cell contacts, many desmosomes, interlocking projections (arrowed) from adjacent cells and sub-apical junctional complexes. The cells also contain abundant filaments. Scale bar, 1 μm.

Fig. 16. Inset. Two cells away from a graft (g) of a benign tumour. The cell containing the vacuoles (single arrow) is a Donné cell and the other (double arrow) is a breast epithelial cell. Scale bar, 25 μm.

Fig. 16. Breast epithelial cell shown in a thin section. Note the broad protrusion (p) at one end of the cell, also the finger-like processes which interdigitate with each other (arrowed). At one end of the cell are many pinocytotic vesicles (pv). There is also an extensive Golgi apparatus. Scale bar, 1 μm.

Fig. 17. Graft of benign tumour cells in the chick wing: the interaction between the apical surfaces of the epithelial cells and the chick mesenchyme cells. Note the space around the tumour cells which corresponds to the length of the microvilli, also the apical junctional complexes (arrowed). Scale bar, 2 μm.

Fig. 18. Benign breast tumour epithelium fusing with the chick ectoderm (e), and taking up the position of the periderm. Scale bar, 25 μm.
Benign tumours

Morphology of tumour cells. The epithelial cells sat on a basal lamina, thus separated from the underlying stroma. Within the epithelial layer we could distinguish both epithelial cells and myoepithelial cells. The myoepithelial cells were readily recognized because of their position at the base of the epithelium, and by the hemidesmosomes they formed with the underlying basal lamina (Tannebaum et al. 1969). The epithelial cells of the breast do not form hemidesmosomes. The electron density of the cells within the epithelium (Fig. 13) varied. The basal lamina was well defined and continuous round the projections from the myoepithelial cells into the stroma and in some cases appeared to be multi-layered (Fig. 14). Pinocytotic vacuoles were found at the apex of the cells beneath the microvilli (Fig. 15). Some cells contained glycogen bodies.

The cell–cell contacts were very similar to those in the malignant tumours: desmosomes, interlocking processes and parallel alignment of adjacent cell membranes were all found (Fig. 15). We found a cell in which a desmosome was formed across a small invagination of the cell surface. Similar desmosomes have been seen in disaggregated epithelial cells (Overton, 1974) and in some tumours and regenerating epithelia (Caputo & Prandi, 1972).

We looked for the characteristics we have already mentioned as being possible indicators of malignant cells. We found some cells in a graft of one benign tumour with very indented nuclei but the nuclei of cells in the other benign tumour had very regular outlines (Fig. 13). However, in this tumour we found one case where a cell appeared to have an intracellular duct. The cells in both benign tumours contained filaments. These were $9.3 \pm 2.0$ nm ($n = 10$) in cells of one tumour and $6 \pm 0.8$ nm ($n = 6$) in cells of the other tumour.

We found some Donné cells in both benign tumours.

Grafts of benign tumours

Interactions with the mesenchyme. In 1 case the graft buckled out of the limb so that the apical surfaces of the carcinoma cells were facing the outside of the limb. This kind of orientation of apical surfaces was also seen in grafts of embryonic pigmented retina (Tickle et al. 1978). In the other benign tumour, the apical surfaces of the cells were facing into a cyst within the limb. In both cases the apical surfaces were not in contact with the chick limb mesenchyme.

In 1 graft 3 cells were seen away from the graft in the chick mesenchyme. Two of these cells occurred in the same thick section (Fig. 16, inset) and were found in the adjacent thin sections. One of these cells was an epithelial cell from the tumour, while the other was probably a corpuscle of Donné. The epithelial tumour cell (Fig. 16) was more electron dense than the chick cells, and had small fingerlike projections of the cell surface. These processes were aligned parallel to each other and interdigitated with each other, none being available from adjacent cells (Fig. 16). The cell was very rounded, with a broad protrusion bearing the fingerlike processes at one end, the original base of the cell within the epithelium. The other end of the cell opposite
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to the protrusion contained small vacuoles, typical of the apical regions of cells (Fig. 15).
The other cell away from the graft in the same region and which was also found in thin sections was very elongated and contained large inclusions which could be easily seen in the thick section (Fig. 16, inset). In thin sections we could find no clear fingerlike projections from the surface and there were no pinocytotic vacuoles. The cell did contain lysosomes which may be typical of Donné cells. The nucleus was very indented and the cell also contained filaments.

As in grafts of the malignant breast tumours, we noticed that the apical surfaces of the breast epithelial cells were non-adhesive to the surrounding tissues. If these surfaces were adjacent to the chick wing mesenchyme there was a well defined space of varying width. In some cases the width of the space corresponded with the length of the microvilli, the tips of which appeared to contact the adjacent tissue (Fig. 17).

Interaction with the chick ectoderm. Only one of the grafts of the benign tumours was adjacent to the chick ectoderm. The breast epithelium fused with the chick wing ectoderm. Two desmosomes were found between a tumour cell and a chick wing ectoderm cell. The membranes were not parallel over long distances (see malignant tumour).

Where the two different epithelia fused together there was a common basement membrane continuous along the base of the cells. The fusion of these 2 epithelia is interesting because they do not have the same basic structure. It appeared that the breast tumour cells extended along the chick ectoderm in the region where the 2 epithelia were fusing and so took up the position of the periderm (Fig. 18).

Mouse lung tumour

Morphology of the cells. The cells have many features in common with the cells from the human breast tumours. In the solid tumour the cells were arranged in an epithelium separated from the stroma by a basal lamina. The cells within the epithelium showed a range of electron density. The cells which had been growing in culture also showed a similar range of electron density (Fig. 19). Cells from the solid tumour and those which had been growing in culture contained large lipid inclusions (Fig. 19). In this respect the cells are reminiscent of the alveolar B cells of the lung (see also Franks et al. 1976). The apical surfaces of the cells within the solid tumour were studded with short microvilli.

A frequent type of cell-cell contact was the interdigitation of small projections from adjacent tumour cells both in grafts of the solid tumour and cell pellet. In the solid tumour there were sub-apical junctional complexes. Desmosomes were also present but were not as well defined as those between cells from the breast tumours. Unlike the contacts between breast tumour cells, we did not find long stretches where the membranes of adjacent tumour cells ran parallel to each other.

As with breast tumours, the nuclei of many cells were indented in outline, although others were regular (Fig. 19). We did not find any intracellular ducts. In one cell within the solid tumour we measured filaments which were \(9.4 \pm 1.1\) nm \(n = 8\) in diameter.
Interaction of graft cells with mesenchyme. There was usually a smooth border where a group of cells in the graft abutted mesenchyme. For instance, cultured cells became associated into loose tubules and had a smooth edge bordering on to the mesenchyme.

Fig. 19. Mouse lung tumour cells positioned in the ectoderm and in register with chick ectoderm cell (e). Note quail cell (q) in mesenchyme. Scale bar, 5 μm.

Fig. 20. Mouse lung tumour cells were mixed with quail cells and implanted into the chick wing for 2 days. The lung tumour cells are surrounded by mesenchyme cells and have become loosely arranged into 2 tubules. Scale bar, 50 μm.
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We do not know whether in this particular case a basal lamina was present around the group of tumour cells. We have observed instances where tumour cells form a straight border with the mesenchyme and there is no basal lamina present (see also breast tumour cells). In other instances in the pellet of tumour cells, there were spaces between the tumour cells and adjacent mesenchyme cells. These regions of non-adherence were present where tumour cells had processes from the surface (see also breast tumour cells).

Interaction with chick ectoderm. Tumour cells were found within the ectoderm positioned on a common basal lamina with the neighbouring chick cells (Fig. 19). In cases such as this one, where the lung tumour cells were grafted in a mixed pellet with quail wing mesenchyme cells, there was a striking difference in the behaviour of the 2 types of cells of the graft. The lung tumour cells became associated with the ectoderm while the quail cells remained within the mesenchyme (Fig. 19).

The lung tumour cells were often closely apposed to the neighbouring ectoderm cells, which in some cases appeared to be deformed by the tumour cells. No special junctions between the tumour cells and the ectoderm cells were found. Although adjacent tumour cells had projections which interlocked we never observed this kind of interaction between tumour cells and host ectoderm cells.

The lung tumour cells within the ectoderm are aligned on the basal lamina. Short processes from the lung tumour cells are closely applied to the basal lamina and in some places appear to run between ectoderm cells and the basal lamina (see breast tumour).

Rat bladder tumours

Morphology of the cells. The cells were very similar in morphology to those from the human breast tumours and the mouse lung tumour. Most of the cells were electron dense. A striking feature was that the cells contained prominent swathes of filaments, which were arranged in crescent-shaped bands. There was a well defined basal lamina beneath the epithelial tumour cells and well developed hemidesmosomes were present. In most cases, the base of the tumour cells was straight. In a few cases there were long processes in the stroma, but these were bounded by an intact basal lamina with hemidesmosomes (see benign tumours and Fig. 14).

The cell-to-cell contacts within the tumour were extensive and of the same types as in the human breast tumours and mouse lung tumour.

The morphology of the nuclei of the bladder tumour cells was variable; some nuclei were very indented. We have measured some of the filaments in the tumour cells and these were 6.8 ± 0.9 nm (n = 10) in diameter, and therefore within the size range of microfilaments. We did not observe any intracellular ducts.

Interaction with the mesenchyme. Bladder tumour cells usually formed a smooth border with the chick mesenchyme even without a basal lamina (Fig. 21). The apical surfaces of the cells were non-adhesive as were those of the breast tumour cells. These surfaces were studded with sparse microvilli. Where the tumour mass protruded out of the limb the apical surfaces of the cells faced the outside and subapical junctions sealed together the outer layer of cells (see also benign tumour grafts).
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Interaction with the ectoderm. The bladder tumour cells were found in the ectoderm aligned on a common basement membrane with the chick ectoderm cells (Fig. 22). Although many desmosomes were found between tumour cells we did not find any well formed desmosomes between tumour and ectoderm cells. However, we did find some darkening of membranes in places where tumour cells came close to chick cells (Fig. 23). There did not seem to be stretches where the membranes of adjacent chick and tumour cells ran parallel to each other. Although the tumour cells possessed many small projections from the lateral surfaces there were none on adjacent chick ectoderm cells.

The bladder tumour cells were aligned with the chick ectoderm cells (Fig. 23), the common basal lamina usually extending uninterrupted under the composite ectoderm. There were, however, what appeared to be occasional breaks in the basal lamina (Fig. 23). Elsewhere the bladder tumour cells appeared to be tightly adherent to the basal lamina and hemidesmosomes were formed.

Human bladder tumour cell line, RT4. Preliminary investigations into the behaviour of these bladder tumour cells when implanted into the chick wing bud showed no invasion into the mesenchyme, but bladder cells in the ectoderm (Fig. 24).

DISCUSSION

The majority of the carcinoma cells did not move into the mesenchyme but were positioned in the ectoderm. It could be argued that we did not allow sufficient time for the carcinoma cells to invade into the mesenchyme. However, we purposely chose to allow one or two days to keep any contribution to invasion, from cell division within the graft, to a minimum. We have looked at 1 experiment where we left a graft of mouse lung tumour cells for 5 days, and here there was no invasion either. We have also found that other cells such as trophoblast, sarcoma 180, various fibroblasts and neuroblastoma had moved into the mesenchyme after one day (Tickle et al. 1978). We also recognize that our experiments might be criticized in that we test the invasion of carcinoma cells into a foreign mesenchyme. However, the virtue of our assay is that it has enabled us to compare the behaviour of a number of different types of cell under

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Fig. 21. Bladder tumour cells adjacent to the chick mesenchyme. Note basal lamina (arrow) at one edge of the graft where it abuts chick mesenchyme; note also the rounding up of the graft where the cells are not in contact with the mesenchyme. These are the apical surfaces (double arrow); note sparse microvilli. Scale bar, 1 μm.

Fig. 22. Bladder tumour cells (electron-dense) positioned in the chick ectoderm on common basal lamina (arrows). Scale bar, 5 μm.

Fig. 23. Bases of rat bladder tumour cells positioned in ectoderm showing an apparently discontinuous basal lamina and a small focal junction (double arrow) between a chick ectoderm process and adjacent bladder tumour cell. Note also hemidesmosomes on tumour cell process and at base of tumour cell (arrows). Scale bar, 1 μm.

Fig. 24. Cell of human bladder tumour cell line, RT4, positioned in chick ectoderm (e). Scale bar, 25 μm.
the same conditions. Cells of the 3 different kinds of epithelial tumours showed very similar behaviour. We think therefore that these results may reflect general features of carcinoma cell behaviour which may be relevant to invasiveness in vivo.

Non-invasion into mesenchyme

We have found a smooth border even in the absence of a basal lamina (see later discussion of the role of basal lamina) and no invasion into the adjacent mesenchyme. The tumour cells do not appear to put out long processes into the mesenchyme, but occasionally rounded bulges of a cell deform adjacent mesenchyme cells. We have found no distinct junctions between carcinoma and mesenchyme cells, although the 2 types of cells may be close together over extensive distances especially along the surface of a bulge. We presume in these cases that it is the basal or lateral surfaces of the tumour cells that face the mesenchyme. When the apical surfaces of the tumour cells lie next to the mesenchyme cells there is often a space, and the tumour cells show no contact with the mesenchyme cells. Occasionally, however, the tips of microvilli on apical surfaces touch adjacent mesenchyme cells.

This lack of contact suggests that the apical surfaces of the carcinoma cells are non-adhesive. It has been noticed that normal epithelial surfaces in organ culture are non-adhesive (De Ridder, Mareel & Vakaet, 1975). In addition there have been several reports that the upper surfaces of epithelial cell sheets in culture, which appear from ultrastructural studies to be equivalent to the apical surfaces of epithelia in vivo (Middleton, 1973; Pickett et al. 1975), do not permit the spreading of individual cells dropped on to them (Di Pasquale & Bell, 1974; Elsdale & Bard, 1974). Elsdale & Bard (1974) have suggested that this property of epithelial cell surfaces may control morphogenesis of structures, such as tubules (see also Bultjens & Edwards, 1977).

It is not clear whether the non-adhesiveness of the apical surfaces is due to the presence of microvilli. There have been some suggestions that the disappearance of microvilli that occurs during attachment of blastocysts may increase the adhesiveness of the surface of the uterine epithelium (Nilsson, 1966; Potts, 1969). On the other hand it has been suggested that cells may initially form contacts readily at the tips of projections (Pethica, 1961). However, these point contacts have to broaden to give a stable adhesion (Armstrong, 1970; Garrod & Born, 1971; Middleton, 1976; Jones, Gillett & Partridge, 1976; Partridge & Jones, 1977). This lateral spreading of an adhesion may not occur if structured microvilli are the contacting projections. Another possibility is that the tips of the microvilli are non-adhesive.

In places where the apical surfaces of the carcinoma cells abut mesenchyme it is not surprising that there is no invasion because there is no contact between the tumour and chick cells. What then prevents invasion of carcinoma cells where these come to lie in close contact with the mesenchyme cells? In the absence of a basal lamina it seems likely that it is the contacts between the carcinoma cells that prevent invasion by these cells into the mesenchyme (see later).
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Behaviour of a small minority of cells

There were a few cells that did invade the mesenchyme. We have speculated that these cells may be those that are important in establishing metastases, in line with the suggestions of Fidler (1975). However, some of these cells in human breast tumours were Donné cells. They appeared to be able to invade through the ectoderm, presumably with accompanying breakdown of the basal lamina. They resemble mouse peritoneal macrophages which also move into the wing mesenchyme. Donné cells are found in large numbers in involuting mammary glands. The nature of these cells in man and other mammals remains, apparently, a mystery. There seem to be 2 possibilities: either they are wandering histiocytes, or degenerating epithelial cells. The characteristics that have been cited in favour of the idea that these cells are histiocytes are that they do not divide and are phagocytic (Taylor-Papadimitriou, Shearer & Tilly, 1977). However, these features are by no means definitive. Hollman (1974) recently concluded that the weight of evidence was that Donné cells of mice were degenerating epithelial cells (Sekhri, Pitelka & De Ome, 1967), whereas in humans they were probably histiocytes. We do not know what role, if any, these cells play in the development and invasiveness of human breast tumours in vivo.

However, if they are able to migrate across epithelia they may produce local gaps in the basal lamina.

Carcinoma cell interaction with ectoderm

The carcinoma cells of all types were found positioned in the ectoderm on a common basal lamina with the neighbouring ectoderm cells. It is not clear in these experiments whether the basal lamina orients the carcinoma cells within the ectoderm. In one case we observed bladder tumour cells apparently aligned along the inner face of the basement membrane (Tickle et al. 1978). The tumour cells within the ectoderm also show a strong affinity for the basal lamina and put out long processes which appear to separate the chick ectoderm cells from the basal lamina. A similar affinity for the basement membrane has been previously reported in ascites tumour cells (Birbeck & Wheatley, 1965).

When the carcinoma cells are positioned within the ectoderm they are separated from the wing mesenchyme cells in most cases by an intact basal lamina. It is not clear whether invasion invariably involves breakdown of the basal lamina (Tarin, 1972); and the histology of many epithelial tumours shows that invading tongues of carcinoma are often bounded by a well formed basal lamina. Indeed the presence of a basement membrane cannot be used as a criterion for non-invasive carcinoma; in, for example, carcinoma of the cervix (Luibel, Sanders & Ashworth, 1960) or in breast carcinoma (Erlandson & Carstens, 1972). Gaps in the basal lamina, if present, may be very small, in, for example, breast carcinoma (Ozzello & Sanpitak, 1970) and bladder tumours (Hicks & Chowaneck, 1977). Also, as we have found in some cases here, multiple layers of basal lamina material are present in many tumours (Tarin, 1969; Tandler, 1971; Hicks & Chowaneck, 1977). Finally, there are situations where the basal lamina is missing between epithelium and mesenchyme and yet no invasion has taken place. In regenerating amphibian limbs, for example, although there
is no basal lamina between the ectoderm cells and the redifferentiating mesenchyme (Salpeter & Singer, 1960), the ectoderm does not invade. Also basal lamina breakdown often occurs in the pre-invasive stages of tumour development and cannot therefore be linked directly with invasion (McNutt, 1977). An alternative view is that gaps in the basal lamina in carcinomas might result not from breakdown by tumour cells, but from synthesis not keeping pace with the expansion of the epithelium (Frei, 1962). This interpretation may fit other cases where the basal lamina has been found to be incomplete – for example, at the branching tips of developing salivary glands (Bluemink, Van Maurik & Lawson, 1976) where a local increase in cell proliferation accompanies lobule formation (Bernfield, Banerjee & Cohn, 1972). However, no evidence for localized cell division could be found during bud formation in the developing lung (Wessells, 1970); in this case expansion must occur by redeployment of cells.

The affinity of the carcinoma cells for basal lamina might be one mechanism that controls the positioning of these cells within other epithelia, both here and for instance in metastases of mammary cells in the lung (Brooks, 1970). Another mechanism which could lead to positioning of carcinoma cells is the formation of contacts between carcinoma cells and epithelial cells. We have found desmosomes between grafted carcinoma cells and wing ectoderm cells. In addition, we have noticed other interactions, such as the frequent parallel alignment of membranes of adjacent tumour and chick cells separated by about 20 nm over long distances.

Hybrid desmosomes between different types of epithelial cells in mixed aggregates have been reported (Overton, 1974; Overton & Kapmarski, 1975). The frequency of desmosome formation between different types of cells seems to depend on the number of desmosomes found within each type of tissue. Thus our speculation that the number of desmosomes that we found between the carcinoma cells and the ectoderm cells was limited by the small number of desmosomes between ectoderm cells, seems reasonable.

Comparison of interaction of carcinoma cells with ectoderm and mesenchyme

We have not found any special adhesions between carcinoma cells and mesenchyme cells, either where the graft borders on to the mesenchyme or where the odd individual carcinoma cell is surrounded by mesenchyme, although often the cells come close together. However, we have found evidence of adhesive interactions between carcinoma and ectoderm cells, both in the form of special junctions (desmosomes) and also in the frequent parallel alignment of membranes. It seems possible therefore that the carcinoma cells associate with the ectoderm because they can form adhesive junctions with these cells, whereas they remain separate from the mesenchyme to which they are not so adhesive. The reason that carcinoma cells do not move into the mesenchyme would be simply that they are more tightly held together within the graft.

Cell contacts within tumours

It has been suggested for a long time that reduced cell adhesiveness may account for the invasiveness of tumour cells (Coman, 1944). Surprisingly, there have been few
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Recent attempts to compare the adhesiveness of normal and tumour cells. One of the problems is how to measure adhesiveness (see Curtis, 1967). Even so, it is by no means clear that reduced adhesiveness is a consistent feature of tumour cells. For instance, observations on a variety of cervical carcinoma cultures showed that adhesiveness was not always decreased, and was very variable (Auersperg & Worth, 1966).

Desmosomes are common between epithelial cells; fibroblasts do not seem to form them. There have been several investigations to see if the number of desmosomes is reduced in carcinomas (McNutt & Weinstein, 1969; Fulker, Cooper & Tanaka, 1971). The results tend to support the idea that loss of desmosomes is associated with invasiveness. However, in some cases it appears that the number of desmosomes is decreased in tumours in pre-invasive stages, which may not progress to invasion for some time. Hence, in these cases, the loss of desmosomes does not immediately lead to invasion (see Weinstein, Merk & Alroy, 1976). In addition, there are several types of adhesive interaction between carcinoma cells. Desmosomes are probably not the only adhesive junctions between the cells. The parallel alignment of adjacent membranes separated by a gap of around 20 nm probably represents a region where the cells are stuck together (Johnson, 1972). It is interesting that the short lateral projections from the tumour cells come to lie at this same distance from the tumour cell body, suggesting that this distance may be energetically favourable (Curtis, 1967). Interlocking cell processes also may serve to hold the cells together. Unless the contributions from all these types of interaction are known, we cannot estimate how strongly the cells in a carcinoma are held together. From these considerations, we suggest that the main barrier to the invasion of single cells from an epithelial tumour into the underlying stroma may be the adhesion between the cells within the tumour.

Role of cell movement in invasiveness

It is not clear from our experiments whether the tumour cells actively insinuated themselves into the ectoderm. However, human breast carcinoma cells have been shown to penetrate into the intact epithelium of the chick chorioallantoic membrane (Ambrose & Easty, 1976). It seems likely that the carcinoma cells move short distances or at least undergo dramatic shape changes to take up positions within epithelia although they show little propensity to move into the mesenchyme. Penetration of an epithelium also may involve displacement of the normal epithelial cells. This has been observed during implantation of tumour cells in the peritoneum and involves the rounding up of the peritoneal epithelial cells to expose bare basal lamina (Birbeck & Wheatley, 1965; Buck, 1973). Carcinoma cells show a strong affinity for the basal lamina compared to that of the normal ectoderm cells. This difference may reflect the greater strength of carcinoma cell attachment to basal lamina or may be due to the weakening of the normal cell attachment by, for instance, the secretion of proteolytic enzymes by the neighbouring tumour cells (Reich, 1973). The production of enzymes may well cause the rounding up of the peritoneal epithelial cells.

It has been suggested that the appearance of microfilaments might be involved in the onset of invasion by tumour cells (Franks, Riddle & Seal, 1969; Malech & Lentz, 1974). McNutt (1977) has recently suggested, based on observations of skin tumours,
that microfilaments might be a sensitive indicator of invasive carcinoma. One obvious problem with features that can only be distinguished at the ultrastructural level is that they must occur widely throughout the tumour. This is because only a small population of the tumour cells can be sampled for electron-microscopy. This may be the reason that we did not identify microfilaments in samples from all the carcinomas we studied. It is of interest that microfilaments are present in epithelial cells during the development of glands such as the salivary gland (Spooner & Wessells, 1970) in which branchings and invaginations of a growing epithelium take place. As there is no movement of cells as individuals, the role of the microfilaments seems to be to enable the cells to undergo the shape changes involved in cleft formation.

We have suggested that the movement of cells, as individuals, is not of major importance in the invasiveness of carcinomas into an underlying stroma (Tickle et al. 1978). This conclusion is supported by the only recent study in tissue culture which showed that individual carcinoma cells did not invade into populations of fibroblasts (Wilbanks & Richart, 1966). In mixed populations of fibroblasts and epithelial cells in culture, islands of epithelial cells are formed, which shows the affinity between epithelial cells (Steinberg & Garrod, 1975; Pitts & Burk, 1976; Fentiman, Taylor-Papadimitriou & Stoker, 1976). In this respect carcinoma cells appear to be like normal epithelial cells; a difference may exist in that carcinoma cells are able to invade into disparate epithelia.

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