Ultrastructure of tracheal epithelial cells migrating in an *in vivo* environment*

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Summary. The tracheal epithelium can be induced to move as a cellular sheet by heterotopic transplantation, which offers the opportunity to observe migrating cells as a group in an *in vivo* environment. We therefore investigated the ultrastructural characteristics of migrating tracheal epithelial cells with special reference to the moving front using this transplantation. The migrating epithelial cells underwent squamous metaplasia and lost their differentiated characteristics such as cilia or secretory granules. Several unique observations were made concerning the mechanism of mobility: one is that epithelial cells in the front were elongated in a direction perpendicular to the course of movement, different from previous reports *in vitro*. The second is that lamellipodia, which are regarded as the major locomotive machinery in the adult wound epithelium, did not make up the major part of the front; the major portion of the anterior fringe of the moving front was usually smooth and gently curved, and actin cables parallel to the elongated cells were observed by confocal laser microscopy, indicating that the purse-string mechanism of epithelial wound healing takes place. The third finding is that the cells in the front had irregular bleb-like structures on their antero-basal surface, which were formed even in the portion where the cells did not attach to the matrix. Few organelles were recognized in these structures. From their location, one might propose that these bleb-like structures play a role in the recognition of the substrate and thus the movement of the cell sheet.

Introduction

The cells in organisms move under various situations, e.g. during morphogenesis in embryos (Trinkaus, 1984), during the natural turnover of cells (Heath, 1996), in the defense against foreign pathogens (Hein *et al*., 1985), in the invasion of cancer cells (Strauli, 1985), and in wound healing (Woodley, 1996).

These cells move in various ways: certain kinds of cells move as independent cells, while others move as a group or as a cell sheet (Trinkaus, 1984; Woodley, 1996). For example, the cells of the immune system (Hein *et al*., 1985) and cells in embryos—such as primordial germ cells (PGCs) (Trinkaus, 1984)—move as independent cells. Epithelial cells also often lose their unity (i.e., mutual adhesiveness or collectiveness) and leave epithelial sheets as separate cells similar to mesenchymal cells in a phenomenon called the epithelial-mesenchymal transition (EMT) (Greenburg and Hay, 1982; Savagner, 2001). On the other hand, many kinds of cells move as a cell sheet; these include adult intestinal cells (Heath, 1996), epithelial cells during epiboly (Trinkaus, 1984), and epithelial cells in cutaneous wound healing (Woodley, 1996).

To investigate the movement of cell sheets in the natural environment, we developed an experimental system where epithelial cells with surrounding connective tissues were transplanted ectopically in appropriate locations (Sawada *et al*., 2004a; Yoshida *et al*., 2005). In this system, many cells may start to move as cell...
sheets under conditions similar to wound healing or regeneration (Sawada et al., 2004a; b; Yoshida et al., 2005). Among many tissues that can be regenerated, the trachea offers advantages for a study such as this. The first is the existence of cartilage in the wall which prevents tissue collapse during transplantation experiments. The second is the thinness of the epithelium (compared with the epidermis, which is very often used in regeneration studies), which may simplify the observation and interpretation. Third is the high efficiency of epithelial regeneration (Sawada et al., 2004a).

Among many reports investigating tracheal regeneration, only a few have shown the ultrastructural aspects of migrating tracheal epithelial cells (Zahm et al., 1991; Delplanque et al., 2000). Furthermore there are only a few studies that have been performed in an in vivo environment (Engelhardt et al., 1995; Baconnais-Minon et al., 1999; Delplanque et al., 2000).

In this report, we investigated the ultrastructural characteristics of migrating tracheal epithelial cells, especially in the vicinity of a moving front. Because the epithelial cells change their phenotype into squamous cells in a phenomenon called squamous metaplasia (Jetten and Harvat, 1997; Puchelle et al., 1997), various unique features of migrating cells were observed—including their relationship to extracellular matrices—which had not been observed in in vitro experiments. Their characteristics were compared with previous reports on the migrating tracheal epithelium and squamous epithelia, including epidermal cells.

Materials and Methods

Animals and surgery

Adult male DA rats were used both as donors and recipients in this study since they are syngeneic and their organs are easily transplantable. The animals were allowed to move freely until dissection and during experimentation under light cycles of 12 h light-12 h dark in the animal facility at Yokohama City University. The experiments were performed under analgesia and anesthesia with carprofen (32 mg/kg body weight) followed by pentobarbital (32 mg/kg body weight). Six rats were used as recipients. The experimental protocol was approved by the Animal Ethics Committee of Yokohama City University.

Donor rats were killed by an overdose of pentobarbital anesthesia, and tracheae were excised and cut to 8 mm lengths. The membranous part (fibrous wall) of the trachea was removed before transplantation.

Sagittal incisions (about 6 cm for 4 transplants) were made on the back skin of the recipient rats, open spaces were made between the dermis and the fascia of the back muscle, and the donor trachea was transplanted between the skin and the fascia with the concave surface towards the back muscles and fixed at the four corners with #7-0 surgical thread (Fig. 1). Four tissues were transplanted on the back of each recipient.

The epithelial cells started moving three to four days after surgery and covered the surface of the fascia, forming a cyst-like structure in five days (Fig. 1, 2).

Light microscopy

Four days post operatively the trachea was excised from the donor along with the surrounding tissues, and fixed with periodate-lysine-paraformaldehyde (PLP) fixative for 2 h. The tissue blocks were immersed in 30% sucrose in a 0.1 M phosphate buffer, pH 7.4, and frozen in isopentane cooled with liquid nitrogen.

Ten μm thick sections were cut from the frozen tissue with a Leica CM1800 Cryostat (Nussloch, Germany), stained with hematoxylin-eosin or with Masson's trichrome stain, and observed under an Olympus BH-2 microscope (Tokyo).

Scanning electron microscopy (SEM)

The excised trachea was fixed with 2.5% glutaraldehyde and 1.25% formaldehyde in a 0.1 M phosphate buffer, pH 7.4, overnight or for several days. Specimens were then postfixed in cold 1% osmium tetroxide in a phosphate buffer for 2 h, rinsed with distilled water, dehydrated in an ascending series of ethanol, substituted in isoamylacetate, dried in a Hitachi HCP-2 critical point dryer (Hitachi Co. Ltd., Tokyo), coated with gold in a Hitachi E101 Ion Sputter, and observed and photographed under a Hitachi S700 scanning electron microscope (SEM) operated at 25 kV.

The area occupied by ciliated cells was measured on photographs taken at x200, and calculated using Image J software. Three each of the donor trachea and extended portion were used for the measurement.

Transmission electron microscopy (TEM)

After post-fixation with osmium tetroxide, the tissues were stained en bloc with 0.5% uranyl acetate, dehydrated with a graded series of ethanol, substituted with propylene oxide, and embedded in Epon 812. Semithin sections were obtained and stained with toluidine blue. Ultrathin sections were obtained using an Ultracut-N...
Confocal laser scanning microscopy (CLSM)

The specimens were treated with 0.5% Triton X-100 in PBS for 30 min and subsequently with Alexa 546-phalloidin (1:100) (Molecular Probes Inc., Eugene, OR, USA) and SYBR Green I (1:100) (Molecular Probes Inc.) in PBS for 2 h at room temperature. Then the migrating epithelium with granulation tissues underneath was removed from other parts of the specimen, mounted on a slideglass, and observed and photographed under a Zeiss LSM510 Confocal Laser Scanning Microscope (Carl Zeiss, Oberkochen, Germany).

Results

Light microscopy showed the epithelial cells starting to move along the surface of the back muscle fasciae around
Fig. 3. Scanning electron microscopy of the migrating tracheal epithelium 4 days after surgery. Open arrows indicate the direction of epithelial movement. a: A low power scanning electron micrograph of the migrating tracheal epithelium. The donor portion containing cartilage has been removed. The epithelia move towards the center of the view from both sides. Arrowheads indicate the moving front. The dotted line indicates the approximate section shown in the reembedded Epon section in Figure 7. Bar=1 mm. b: An area in the rectangle in a. Most of the migrating cells show a relatively flat surface, except for several ciliated cells (white arrowheads). Bar=100 μm. c and d: A higher magnification of the area shown in rectangles (C) and (D) in b. The contour of the cells can often be surmised from the difference in surface texture. Note that the cells of the moving front bulge over another area of the epithelium. Each swelling very possibly corresponds to each cell, and the cells seem to be elongated in a direction perpendicular to the direction of movement. There are no apparent lamellipodial protrusions. E: erythrocytes, M: extracellular matrix fibrils. Bar=50 μm
Ultrastructure of migrating tracheal epithelium

3–4 days after the transplantation (Fig. 2a). Beneath the migrating epithelial cells, there was typically an immature granulation tissue containing many fibroblastic cells, but it was not fully invaded by the microvasculature (Fig. 2b). The migrating epithelial cells did not usually have cilia or mucous secretory granules but displayed a stratified appearance. The moving front epithelium often thickened to variable thicknesses occasionally reaching 50 μm, and was usually thicker than that of the epithelium behind. It often tipped upward (Fig. 2b). If the donor trachea was removed carefully, the migrating epithelial cells were easily recognized by SEM (Fig. 3a). The migrating cells constituted a continuous sheet on which only a limited number of ciliated cells were observed (Fig. 3b). The density of the ciliated cells in the migrating epithelium was always much lower than that in the donor trachea. The average area occupied by ciliated cells in the donor portion was 24.8% (average of 1.54 mm² from 3 specimens) whereas that in the extended portion was only 0.3% (average of 1.24 mm² from 3 specimens). There were no gaps between the migrating cells (Fig. 3b). The

Fig. 4. In a few areas, the moving front shows protrusions similar to lamellipodia (arrows), to which extracellular fibrils converge. M: extracellular matrix fibrils. Bar=10 μm

Fig. 5. The donor portion. a: A low magnification view. A rise in the surface due to the cartilage underneath can be observed. Bar=500 μm. b: A higher magnification of the area in the rectangle in a. Note that many cells with cilia can be observed. c: A higher magnification of the rectangle in b to show ciliated cells. Bar=10 μm
Fig. 6. Legend on the opposite page.
Ultrastructure of migrating tracheal epithelium 229

Fig. 6. Transmission electron micrographs of several representative migrating epithelial fronts (a, c–f, g, i), their vicinity 4 days after surgery (h, j–l), and a normal trachea (b). a–f are taken at the same magnification. The migrating cells appear stratified. Open arrows indicate the direction of epithelial movement. Bar=10 μm. a: At the front, the cells are often obliquely elongated showing a tapering appearance, and the upper layer cells appeared to be overriding the lower cells (see also Fig.6c, f). Slightly behind the moving front, a distinction between upper layers and lower layers is apparent. The upper 2-3 layers (U) are composed of flat cells, whereas the lower layer (L) is composed of rather cuboidal cells. There are distinct spaces between the cells, which have many lateral protrusions that touch each other in these spaces. E: erythrocytes, F: fibrin. Bar=10 μm. b: For comparison, a normal rat trachea is shown at the same magnification as a and c–f. c: Another moving front. Characteristics similar to those in a are observed. On the uppermost cells, many short microvilli are observed. F: fibrin. The same magnification as a. d: Another moving front. In this front, the thickness of the epithelial front is much greater than that in a and c. L: lipid droplets, D: a densely stained possibly dead epithelial cell, F: fibrin. The same magnification as a. e: Another moving front in which the cells in the front have almost engulfed another epithelial cell (Ep). F: fibrin. The same magnification as a. f: Another moving front. In this photograph, c and e, the epithelial front is lifted from and not attached to the underlying fibrin matrix (F). On the uppermost cells, short microvilli are observed. D: a lightly stained possibly dead epithelial cell. The same magnification as a. g–i: Higher magnifications of the antero-basal surface of moving front cells shown in Figures 6a and c. The moving front shows irregular blebs (arrows) that, while variable in thickness and size, are usually thicker than the lateral protrusions (P in g) and contain few organelles. In g and i, many fibrin fibrils are seen and often fit in the basal invagination of the cells. These fibrils show c. 20 nm periodicity (h). Many of the bleb-like structures are not attached to fibrin fibrils, except for the basal-most structures. Bars=5 μm (g, i), 100nm (h). j: Cells in mitosis (M) in the upper layer of migrating cells. Bar=10 μm. k: Cells in the upper layer containing phagosomes (V) and internalized material similar to erythrocytes (E). Bar=10 μm. l: A high magnification of desmosomes (arrowheads) in the migrating epithelium. Bar=1 μm
the base were rather cuboidal in shape, and the upper cells had a darker appearance (Fig. 6a). On the apical surface of the uppermost cells, many small microvilli were observed (Fig. 6c, f, k). The lateral surface of the cells exhibited many protrusions (Fig. 6g, l) and formed many desmosomes between the cells (Fig. 6l). The cells contained bundles of tonofilaments but were not as abundant as keratinocytes in the skin. The desmosomes became less conspicuous in the cells closer to the front (Fig. 6g).

The moving front of the epithelium is thicker than the epithelium behind it (Fig. 6a, c). Occasionally it formed a very thick structure composed of several layers of the cells (Fig. 6d). The cells in the front were usually obliquely elongated (Fig. 6a, c, e), and the upper layer cells gave an appearance of overriding the lower layer cells as they moved beyond the latter. At the antero-basal surface of the cells in the front, irregular bleb-like structures different from either apical microvilli or lateral protrusions were formed. These bleb-like structures were not uniform in size or shape and did not seem to contain specific structures other than ribosomes, flocculate or fine granular materials, and occasional empty spaces (Fig. 6g, i). They were formed even in the portion where the cells did not attach to the matrix.

Beneath the basal surface of migrating epithelial cells, there were abundant fibrils coursing randomly (Fig. 6a, c–g, i). These fibrils had a periodicity of about 20 nm (Fig. 6h), characteristic of fibrin. Very often the fibrin fibrils extended into the invaginations of the basal plasmalemma of the cells (Fig. 6d, g). Distinct hemidesmosomes were not observed. The basement membrane was not seen in the vicinity of the moving fronts.

Many cells in the vicinity of the moving front contained lipid droplets. There were also phagocytic vacuoles containing leukocytes, erythrocytes, and epithelial cells themselves (Figs. 6e, k). Occasionally, mitotic figures with condensed chromosomes were observed within the migrating epithelium (Fig. 6j). Dead cells with either dense (Fig. 6d) or empty (Fig. 6f) appearances were occasionally observed.

By correlational observations under SEM and TEM, the structure of the moving front without lamellipodia-like structures became more apparent. In most cases, the moving front showed a roundish cross-sectioned contour (Fig. 7, see also Fig. 6f). The cells demonstrated bleb-like structures on the antero-basal surfaces.

By CLSM, thick actin cables were seen along the moving front, in a direction perpendicular to the course of the movement (Fig. 8). The cables extended from one cell to another. Although occasional lamellipodia-
like structures were observed, a configuration similar to that shown in Figure 8 occupied most of the front fringe. From the actin along the lateral border of the cells, it can be surmised that the cells in the front were elongated in a direction perpendicular to the course of movement.

**Discussion**

Epithelial cells were induced to move but became metaplastic

In the present experiments, tracheal epithelial cells were induced to move by heterotopic transplantation in an *in vivo* environment, as was shown in our previous reports on other transplantations (Sawada et al., 2004a). In addition, we demonstrated that the tracheal epithelium changed in morphology to squamous epithelium. Similar squamous metaplasias have been very often encountered during recovery from stress or injury to the tracheal epithelium (Wilhelm, 1953; Keenan et al., 1983; Inayama et al., 1988; Shimizu et al., 1992; Puchelle and Zahm, 1996; Puchelle et al., 1997), or in vitamin A deficiency (Jetten et al., 1987); in these experiments, the ciliated epithelial cells and secretory cells were lost, and poorly differentiated cells appeared (Inayama et al., 1988; Shimizu et al., 1992). Differentiated tracheal cells such as ciliated cells were, however, sometimes observed even in the newly extended portion of the epithelium, suggesting that ciliated cells can migrate in their differentiated form as reported by Baeza-Squiban et al. (1991).

**Comparison with observations of other experimental systems**

There have been a few reports concerning the tracheal cells during wound healing *in vitro* (Zahm et al., 1991; McGuire and Parks, 2003), while many reports have been published on the ultrastructure of keratinocytes during wound healing both *in vivo* and *in vitro*, which is a typical stratified epithelium (Odland and Ross, 1968; Krawczyk, 1971; Radice, 1980; Woodley, 1996; Singer and Clark, 1999). In this experiment, we made a similar observation, such as the appearance of cortical microfilament bundles, a decrease in tonofilaments and desmosomes, the elevation of phagocytic activities, and the loss of apical-basal polarity in the regenerating epithelium. However, we also observed significant differences in structure which may be related to the migratory mechanism of the cells as described below.

**Moving machinery and the mechanism of movement of the epithelium**

There are two hypotheses; for the molecular mechanism for closing the wound opening of epithelial sheets: one is the purse-string mechanism in which actin cables in the leading edge cells are linked by adherens junctions and their contraction makes the edges of leading front cells smaller, and the other is the lamellipodial crawling mechanism in which cytoplasmic protrusions emanate from the leading edge of the cells and drag the cells forward (Jackinto et al., 2001). The embryonic epithelium uses the former mechanism for wound healing, and the adult epithelium is postulated to use the latter. An *in*
vitro scratch assay of tracheal cells showed conspicuous lamellipodia (Zahn et al., 1991; 1997). Lamellipodia and related structures were also observed in moving front keratinocytes (Odland and Ross, 1968; Krawczyk, 1971; Carlson, 2007) and the tracheal epithelium in other experimental systems (McGuire et al., 2003). Although we used adult animals in the present observation, the major portion of the moving front seemed to lack lamellipodia, and the cells in the front were shown by CLSM to contain thick actin cables that extended from one cell to another along the border of the moving fronts. The lack of lamellipodia in a moving epithelium has been recently reported although the mode of movement seems to differ from ours (Ewald et al., 2008). These findings suggest the participation of the purse-string mechanism, at least in part. The observation that the cells in the front were elongated in a direction perpendicular to the movement and formed a continuous sheet also differs from findings by many previous studies using various systems in which the cells were elongated radially (Repesh and Oberpriller, 1978; Gipson and Keezer, 1982; Yoshii et al., 2005; Carlson, 2007) and showed gaps between them (Orthonne et al., 1981; Clark, 1985). However, because of the occasional presence of lamellipodia-like structures, the two mechanisms described above are not mutually exclusive (Danjo and Gipson, 1998; Jackinto et al., 2001). Poujade et al. (2007) observed collectively moving MDCK cells in culture, and showed that the migrating cells were not homogeneous: leading cells at the tops of outgrowths have lamellipodia, and following cells along the sides have pluricellular subcortical actin belts suggesting the purse string mechanism. In our system a similar mechanism may be operating, although we did not observe distinct leading cells with well-developed lamellipodia.

Under the moving front, there are many small bleb-like structures even where the cells were not attached to the fibrin matrix. These structures were not observed under the SEM, probably because of the overriding upper layer cells. These bleb-like structures also have structural similarities to the lamellipodia observed in moving front keratinocytes and the tracheal epithelium in other experimental systems (Odland and Ross, 1968; Krawczyk, 1971; Zahn et al., 1991; McGuire et al., 2003). However, our bleb-like structures seemed to be much smaller than those lamellipodia. Judging from their location, they might play a role in the recognition of the substrate and thus the movement of the cell sheet.

Regarding the mechanism of the movement of stratified squamous cells, there are also two hypotheses: the leap-frog model (Laplante et al., 2001; Danjo and Gipson, 2002), in which individual cells at the tip of the regenerating epithelium crawl over each other, and the sliding model in which all the cells slide simultaneously towards the wound center (Zhao et al., 2003). In our observation, the upper cell layer and lower cell layer showed distinct structural differences, and apical cells were very often moved over the lower front cells. Laplante et al. (2001) made a similar observation with keratinocytes in vitro and argued for the leap-frog mechanism for their migration.

In conclusion, the present system provides a new method of investigating the movement of epithelial cell sheets under a condition similar to that in vivo. The cells shared many characteristics reported for migrating epidermal cells and tracheal cells in vitro, but from these observations there are still crucial differences relating to the mechanisms. Further studies are needed on this intriguing regeneration system.

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