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Cytoskeleton and surface structures of cells directly attached to the tooth in the rat junctional epithelium

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Objective: It is still an open question whether cells directly attached to the tooth (DAT) cells are migratory or non-migratory cells. The purpose of this study was to examine cytoskeletal and surface structures of DAT cells that might be involved in migration.

Methods: We investigated the distribution of stress fibers composed of actin filaments in DAT cells using phallacidin fluorescent dye methods in a confocal laser scanning microscope. To observe the three-dimensional structure of the DAT cell surface, the osmium maceration scanning electron microscope (SEM) method, which removes various soluble materials between DAT cells and the enamel, was employed.

Results: Stress fibers were found in the most apically located DAT cells, and were arranged in parallel to the presumable cervical-line, whereas some of the fibers ran parallel to the tooth axis in the more coronally located DAT cells. The parallel arrangement to the tooth axis of the fibers may be involved with migration for turnover, and the parallel accumulation to the presumable cervical-line may be concerned with the cervical contraction of DAT cells. Osmium maceration SEM images at high magnification revealed the existence of microvilli-like structures on the enamel surfaces (facing to the tooth surface) of DAT cells after removal of the soluble matrices. The thicknesses of the microvilli-like structures on the enamel surfaces and cell processes of intercellular bridges were significantly different.

Conclusion: DAT cells possess stress fibers arranged in parallel to the tooth axis and to the presumable cervical-line in the cytoplasm, and microvilli-like structures on their enamel surfaces. These results suggest that these structures contribute to DAT cell migration.

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The junctional epithelium is located at the dento-epithelial area in the floor of the gingival sulcus, between the cervical enamel surface facing the cemento-enamel junction and the gingival sulcular epithelium continuing from the gingival oral epithelium, which forms the dento-epithelial junction. Ultrastructurally, the junctional epithelium composed of poorly developed non-keratinized squamous epithelium is characterized by wide intercellular spaces, thin cytoplasmic processes or concavities and fewer tonofilaments. It is believed that the junctional epithelium plays a significant role in homeostasis and in the maintenance of healthy gingiva to protect from occlusal forces or bacterial infection by adhering and sealing the oral epithelium and the tooth (1, 2).

Salonen *et al.* proposed that cells aligned in the most superficial layer facing the enamel in the junctional epithelium have been termed as 'cells directly attached to the tooth' (DAT cells) (3). These cells possess firm adhesive structures that bind to the tooth through integrin $\alpha_6\beta_4$ and laminin-5 in the internal basal lamina (4–8). Therefore, it has been believed that DAT cells might be non-migratory cells, namely, independent cells that are attached to the tooth but do not participate in the turnover of the junctional epithelium (3).

More recently, however, the proliferative activities of DAT cells have been demonstrated (9, 10). Besides, investigations examining transferrin receptors in DAT cells suggest the active metabolism and high turnover of these cells (11–13). Pöllänen *et al.* reported that DAT cells have a more important role in tissue dynamics (14) and in the reparative capacity of the junctional epithelium than has previously been thought (15).

The mechanism of DAT cell turnover is not fully understood, although possible mechanisms have been proposed (14). To elucidate the mechanism of DAT cell migration, we examined the cytoskeletal and surface structures of DAT cells in this study. To observe the cytoskeleton, we used a confocal laser scanning microscope (CLSM) to identify DAT cells. We observed the cell surface of DAT cells by employing the osmium maceration scanning electron microscope (SEM) method, which removes various soluble materials that cover the surfaces of DAT cells facing to the tooth surface (16–19).

Materials and methods

Animals and tissue preparation

Twenty-four 9-week-old male Sprague-Dawley rats, weighing about 250 g each were used in this study. The animals were deeply anesthetized with an intraperitoneal injection of sodium thiopental and perfusion fixation was carried out. This study was conducted in accordance with the Guidelines for the Treatment of Experimental Animals at the Tokyo Dental College.

Observation of the cytoskeleton in DAT cells

The animals were transcardially perfusion-fixed with 10% neutral buffered formalin for 2 h at room temperature. After the fixation, maxillary jawbones were removed and decalcified with 10% EDTA for 24 h at room temperature. The gingival tissues including the junctional epithelium were dissected by a fine surgical knife, and the tissues from M1 and M2 molar areas of the maxilla including the periosteum were removed carefully by a fine dental excavator. Whole mount specimens were incubated with BODIPY FL conjugated phallacidin (6.0 U/ml; Molecular Probes, Inc., Oregon, USA) for cytoskeletal F-actin, and with TO-PRO-3 iodide (2.0 μM/ml; Molecular Probes, Inc.) for nuclear counter staining, in 0.1 M cacodylate buffer for 2 h at room temperature. After washing with 0.1 M cacodylate buffer, specimens were mounted on glass-bottom culture dishes with ProLong® Antifade Kit (Molecular Probes, Inc.) and were examined using a CLSM (MRC-1024UV: Bio-Rad Laboratories Ltd, Hertfordshire, UK). The enamel surfaces of DAT cells were reconstructed and integrated using *z*-axis serial sections of about 0.4 μm in thickness. The pictures were photographed under 60 and 100 times by an oil emulsions objective lens, after confirmation of the cells in the CLSM.

Transmission electron microscopic observations

The animals were perfusion-fixed transcardially with modified Karnovsky's fixative solution containing 2.0% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 for 60 min at room temperature. After the fixation, maxillary jawbones were removed and the enamel was decalcified with 10% EDTA at room temperature. The gingival tissues at the M1 and M2 areas were carefully removed from the tooth surface, and were embedded in EPON 812. After ultra-thin sections were cut, specimens including the enamel surfaces of DAT cells were stained with uranyl acetate and lead citrate. They were observed and photographed using a transmission electron microscope (TEM) (H-7100: Hitachi, Co., Tokyo, Japan). To verify the SEM images with TEM observations, the spattered samples were embedded in EPON 812. Ultra-thin sections were cut, stained, and observed as same manner as the mentioned above.

Osmium maceration scanning electron microscope method

The animals were perfusion-fixed transcardially with fixative containing 1.0% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 for 20 min at room temperature. After the fixation, maxillary jawbones were removed and the enamel was decalcified with 10% EDTA. The gingival soft tissues at the M1 and M2 areas were carefully separated from the tooth surfaces and transferred to 1% OsO_4 in 1.25% potassium-ferrocyanide for post-fixation at room temperature for 2 h. After post-fixation and rinsing, the gingival tissues were macerated in 0.1% OsO_4 in phosphate-buffered saline for 60 h at room temperature with shaking in a rotating agitator, according to the maceration methods described by Segawa and by Riva *et al.* (16–19). After rinsing, the specimens were dehydrated in an acetone series and by critical point drying, then were mounted on sample stages with the enamel face up, and spattered with platinum-palladium. DAT cells of the junctional epithelium were examined by high-resolution field emission SEM (JSM-6340F: JEOL, Co., Akishima, Japan).

Statistical analysis of microvilli-like structures and intercellular bridges

For histomorphometry, osmium maceration SEM images of DAT cells and suprabasal cells were photographed randomly, and were printed at a final magnification of ×15,000. The thicknesses of the microvilli-like structures and cell processes forming intercellular bridges of DAT cells or suprabasal cells were measured. Three

areas were measured: (i) surfaces of DAT cells facing to enamel, (ii) surface of DAT cells facing to intercellular space and (iii) intercellular bridges of suprabasal cells. The data obtained from each area were statistically analyzed using Excel software (Microsoft Excel X for Mac; Microsoft, Co., Washington, USA).

Data for each group were compared using the Kruskal–Wallis *H*-test (SNK: Student–Newman–Keuls test) ($p < 0.01$). Total estimated numbers of cell processes were 500 at the enamel surface, 150 at the lateral surface, and 60 at the intercellular bridges.

Results

Arrangement of the cytoskeleton in DAT cells

The intracellular arrangement of actin filaments in DAT cells adjacent to the enamel was discernible by phallacidin fluorescent dye staining using a CLSM. The actin filaments were detected clearly as bundles of green-colored fluorescence and bordered on the plasma membranes of DAT cells. The bundles were found in the perinuclear cytoplasm at the coronal side of the junctional epithelium (Fig. 1a).

In the most apically located DAT cells, bundles of actin filaments were observed in the peripheral cytoplasm (Fig. 1b), and formed stress fibers, which ran in various directions in the cytoplasm facing to enamel. These fibers were arranged in parallel to the presumable cervical-line and were surrounded by the cervical area. The stress fibers in the more coronally located DAT cells were arranged in parallel to the tooth axis (Fig. 1c).

On the other hand, a few bundles of actin filaments could be seen at the most coronal side of DAT cells (Fig. 1a). Amorphous structures

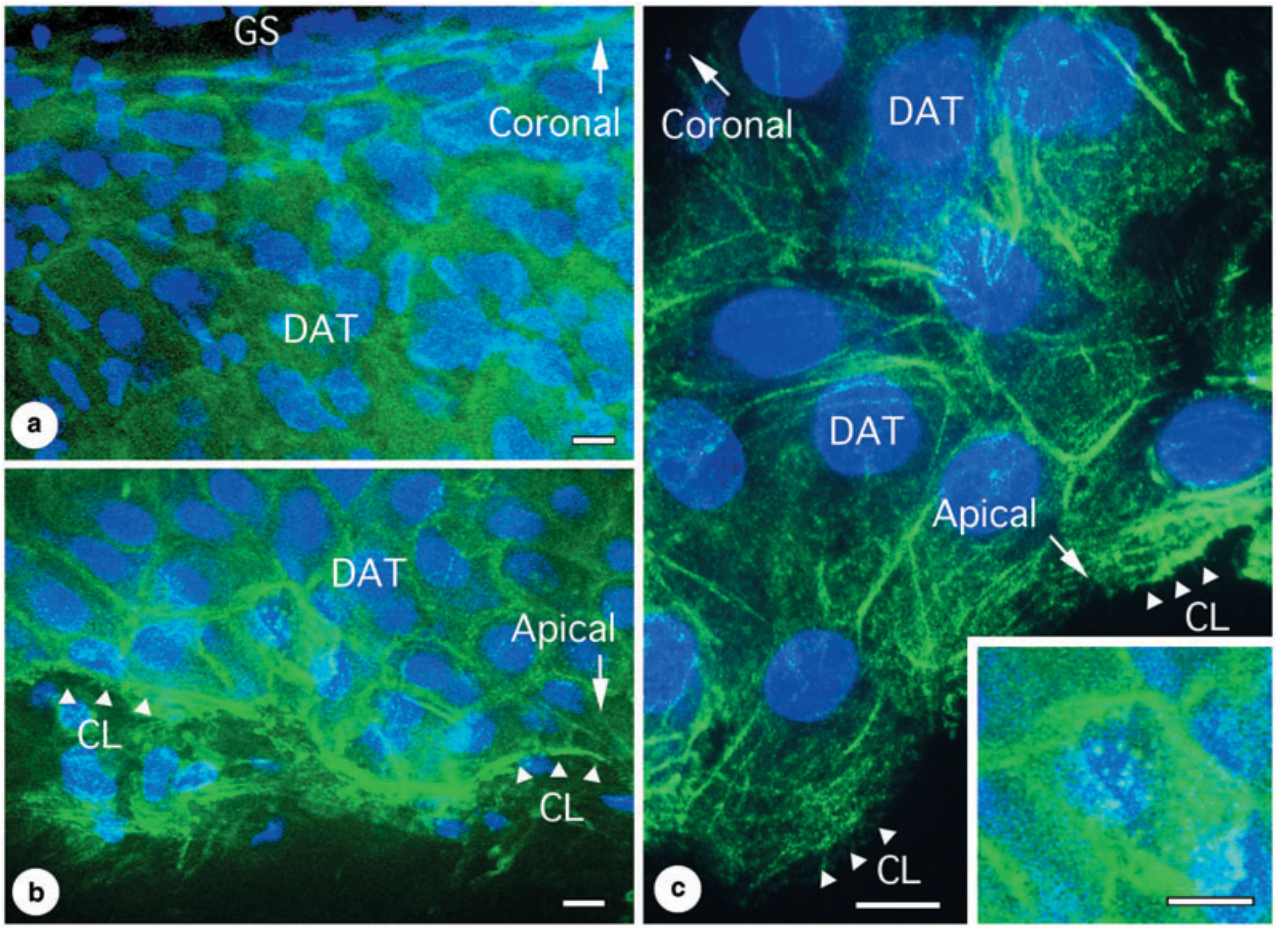

Fig. 1. Confocal laser scanning microscope. Distribution of actin filaments in directly attached to the tooth (DAT) cells. Actin filaments can be detected clearly as bundles of green-colored fluorescence and border on the plasma membrane of the cells. The bundles are found in the perinuclear cytoplasm at the coronal part of the junctional epithelium (a). In the apical part of DAT cells, the bundles of filaments are distinct in the peripheral cytoplasm (b). The bundles forming stress fibers at the most apically located DAT cells are arranged in parallel to the presumable cervical-line and are surrounded by the cervical area. Stress fibers in the more coronally located DAT cells run parallel to the tooth axis from the apical to the coronal directions (c). Focal adhesions, which are concentrated actin filaments, are distinct in the apically located DAT cells at high magnification images of the cells (c, inset). BODIPY FL phallacidin fluorescent staining/green color. Original magnification: (a) and (b), ×550; (c), ×1200; inset, ×1150. Bar = 10 μm. Arrow indicates coronal or apical direction of tooth axis; CL, presumable cervical-line; GS, gingival sulcus.

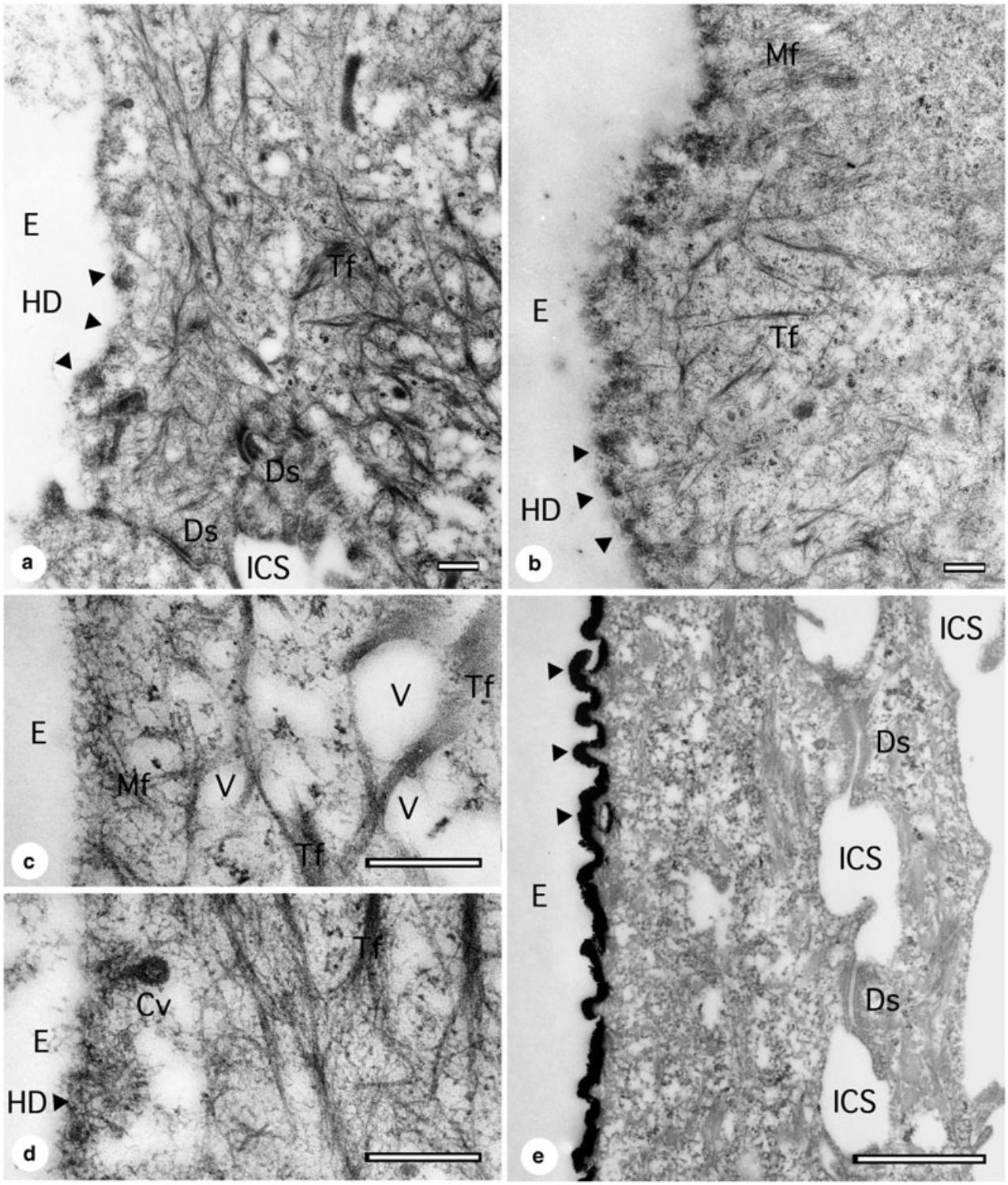

Fig. 2. Transmission electron microscope. Ultrastructure of directly attached to the tooth (DAT) cells at the enamel surface. Lamina densa and lucida of the internal basal lamina are not precisely defined in ultra-thin sections (a). The internal basal lamina is detected as an amorphous structure with a filamentous meshwork (b). At the surfaces of DAT cells, hemi-desmosomes (arrowheads) are recognized as high-density plaques associated with the microfilament meshwork (a, b, c). Some vesicles (Cv and V) are seen in the peripheral cytoplasm, and concavities of the plasma membrane are observed at the peripheral cytoplasm at the enamel side (d). In thin sections of the osmium maceration SEM samples, numerous cell processes (arrowheads) showing microvilli-like structures 100–180 nm in thickness can be found on the plasma membrane of DAT cells facing to the enamel (e). Original magnification: (a), ×7000; (b), ×7000; (c), ×19,000; (d), ×19,000; (e), ×22000. Bar = 1.0 μm. Cv, coated vesicle; Ds, desmosome; E, enamel space; HD, hemi-desmosome; ICS, intercellular space; Mf, microfilament; Tf, tonofilament; V, vesicle.

reacting weakly for phallacidin could be detected in the cytoplasm. Focal adhesions, which are concentrated actin filaments, could be observed in high magnification images of DAT cells at the apical parts (Fig. 1c, inset).

Ultrastructure of DAT cells

Lamina densa and lucida of the internal basal lamina close to DAT cells were not clearly defined in ultra-thin sections (Fig. 2a). The internal basal lamina was discerned as an amorphous structure with a filamentous meshwork (Fig. 2b). At the surfaces of DAT cells, hemi-desmosomes were recognized as high-density plaques associated with the microfilament meshwork, which were connected to bundles of tonofilaments (Figs 2a, b and d).

A few clathrin-coated vesicles were detected in the peripheral cytoplasm, and some of these continued directly to the plasma membrane facing the enamel. Concavities of the plasma membrane and small vesicles fringed by microfilaments were observed in the peripheral cytoplasm on the enamel side (Fig. 2d). Neighboring suprabasal cells were connected to each other by thick cell processes associated with desmosomes and gap junctions. Microvilli-like structures were occasionally found in the intercellular spaces.

Images of thin sections of the osmium maceration SEM samples were characterized by the presence of electron dense materials on the surface of the plasma membrane facing the enamel. Numerous cell processes showing microvilli-like structures 100–180 nm in thickness could be found on the surface. These processes, which were relatively short and bent in places with thick spattered materials, measured approximately 200 nm in thickness and 230–320 nm in height (Fig. 2e).

Scanning electron microscope images of DAT cells following osmium maceration

After the osmium maceration, the surfaces of the decalcified DAT cells facing to enamel were well preserved and were recognized as smooth, continuous, and flat faces. The faces revealed sheets associated with numerous and irregular cracks revealing the intercellular spaces. At lower magnification, the cracks were larger and polygonal fragments could be detected at the more coronal sides of the DAT cells. Polygonal fragments, which corresponded to flattened cells, were observed at the bottom of the gingival sulcus. The number of such fragments increased at the transition areas of the junctional epithelium and the sulcular epithelium, indicating cell

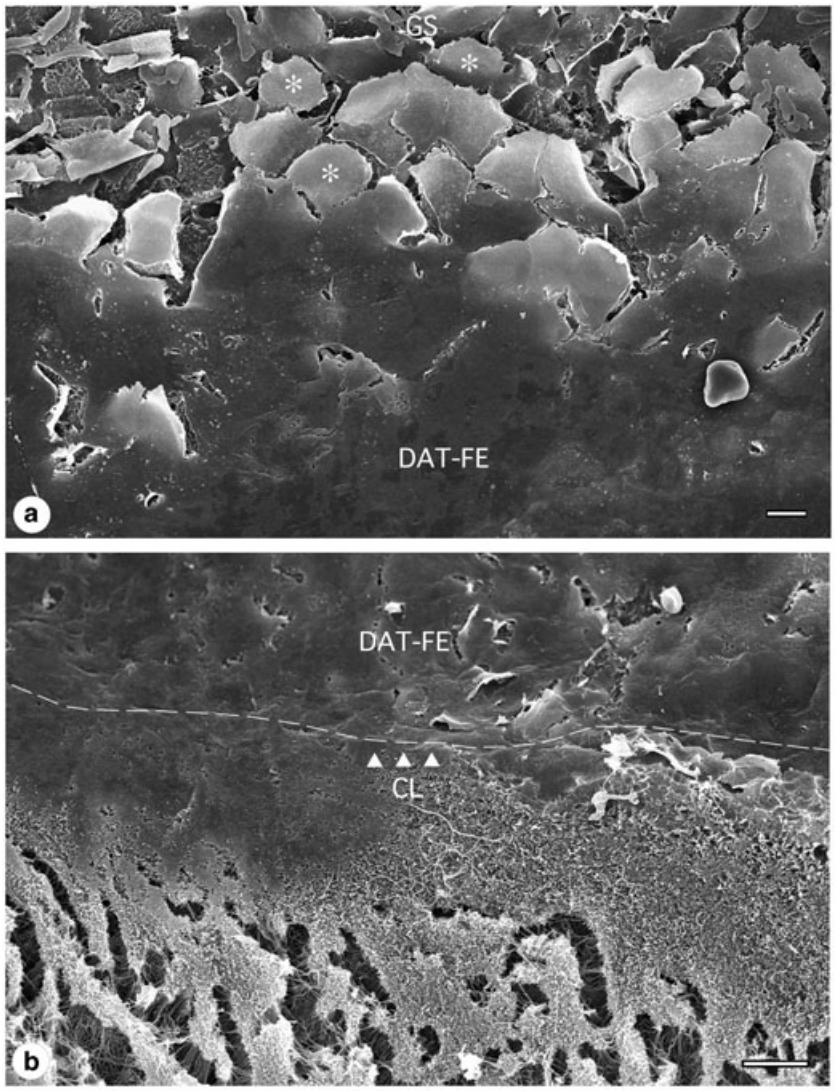

Fig. 3. Scanning electron microscopy images of directly attached to the tooth (DAT) cells following osmium maceration. (a) Coronal side near the gingival sulcus bottom. (b) Apical side near the cemento-enamel junction. At lower magnification, DAT cells can be recognized as smooth, continuous and flat faces associated with numerous and irregular cracks. The cracks become larger and polygonal fragments are distinct at the more coronal side. Polygonal fragments corresponding to flattened cells are observed at the bottom of the gingival sulcus (asterisks). The number of fragments increases at the transition of the junctional epithelium and the sulcular epithelium indicating cell desquamation (a). In the area of the presumable cervical-line, the surfaces of DAT cells are flat and smooth. The presumable cervical-line is not always obviously distinct, but the cementum is recognized as a rough filamentous surface and gingival connective tissue is seen as bundles of thick collagen fibrils (b). Original magnification: (a), ×700; (b), ×1300. Bar = 10.0 μm. CL, presumable cervical-line; DAT-FE, DAT cell facing to enamel; GS, gingival sulcus.

desquamation. Between these fragments, irregular intercellular spaces were evident (Fig. 3a).

In the area of the cemento-enamel junction, the cell surfaces of DAT cells were flat and smooth, and irregular cracks were distinct in places. Although the cemento-enamel junction was not always clearly defined, the cementum was discernible as a rough filamentous surface and the gingival connective tissue could be recognized by the presence of bundles of thick collagen fibrils (Fig. 3b).

When the maceration was greater, individual DAT cells were distinctly observed by the existence of large cracks indicating intercellular spaces (Fig. 4a). Just inside the surfaces of DAT cells, cellular processes of suprabasal cells and migrating cells could be detected. The migrating cells, which had smooth surfaces, were frequently found just inside the cell surfaces of DAT cells through dilated intercellular spaces (Fig. 4b).

Thin intercellular bridges were detected on the lateral surfaces of the DAT cells. The cell processes revealed numerous and long microvilli-like structures (Fig. 5a). In contrast, the bridges between the suprabasal cells just underneath the DAT cells were thick and sparse (Fig. 5b). Those thick processes 200–300 nm in thickness contacted processes of the neighboring cells, and some of these were attached directly to components of the internal basal lamina. Processes without any contacts were also seen.

Dental pellicle and extracellular matrices constituting the internal basal lamina were removed in various grades by the osmium maceration. At high magnification, short and thin microvilli-like structures 180–260 nm in thickness were detected and were arranged diffusely on the cell surfaces of DAT cells in the apical and middle parts of the junctional epithelium. Round- or flat-headed tips of microvilli-like structures were frequently observed (Figs 6a and b).

To analyze the dimensions of the microvilli-like structures and intercellular bridges, the thickness of each cell process was measured histomorphometrically. The thicknesses of these cell processes were 0.23 ± 0.02 μm ($n = 500$) at the enamel surfaces of DAT cells, 0.26 ± 0.03 μm ($n = 150$) at the lateral surfaces of DAT cells, and 0.42 ± 0.06 μm ($n = 60$) between suprabasal cells. These thicknesses are significantly different by the Kruskal–Wallis *H*-test (SNK: Student–Newman–Keuls test) ($p < 0.01$) (Fig. 7).

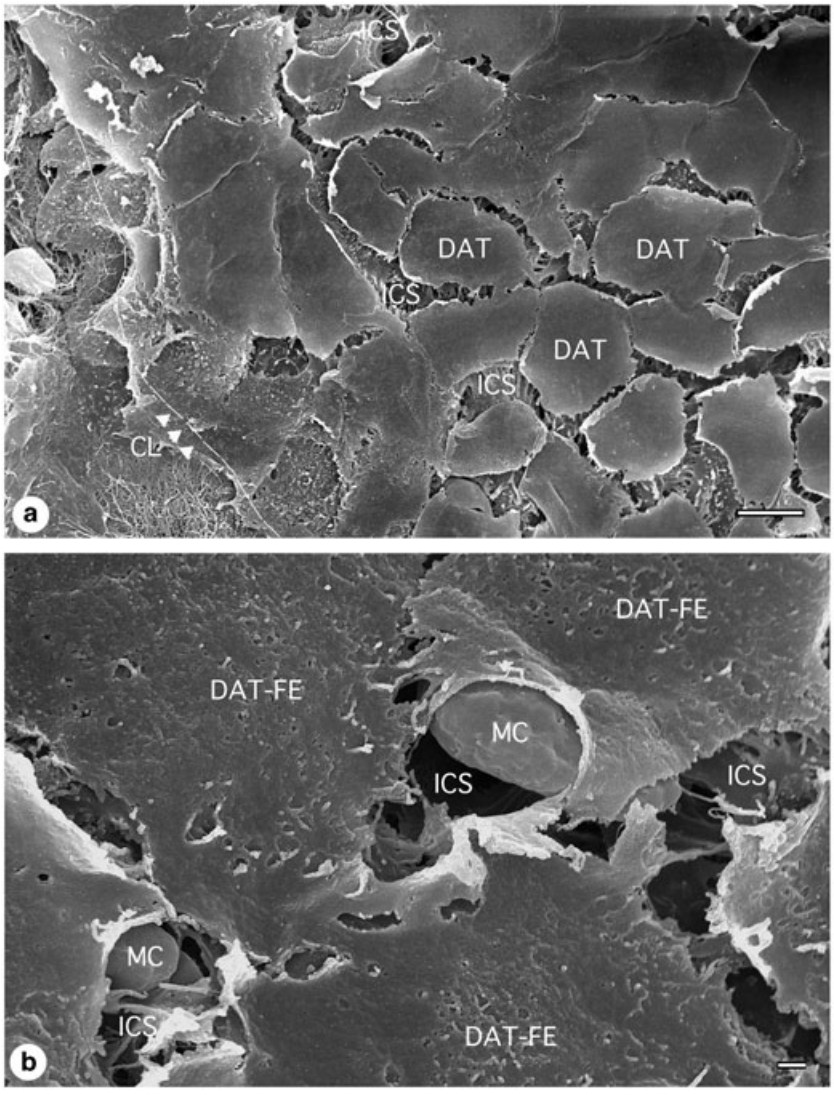

Fig. 4. Scanning electron microscopy images of directly attached to the tooth (DAT) cells following osmium maceration. In specimens of advanced osmium maceration, individual DAT cells are seen by the existence of large cracks indicating the intercellular spaces (a). Just inside the surface, cellular processes of cells inside the junctional epithelium and migrating cells (MC) are detectable (b). Original magnification: (a), ×1300; (b), ×5700. Bar = 10.0 μm (a), = 1.0 μm (b). CL, presumable cervical-line; DAT-FE, DAT cell face to enamel; ICS, intercellular space.

Discussion

It is well known that the turnover rate of the junctional epithelium is exceptionally rapid. Calculation of the mitotic index per 1000 cells of the total cell population demonstrated that the turnover time is 4–6 days in monkeys. Taking the unit length along the surface to the distance along the basement membrane of both the oral epithelium and the junctional epithelium into account, the turnover time estimated is 50–100 times faster for the junctional epithelium than for the oral epithelium surface (1).

It was previously believed that only epithelial cells facing the internal basal

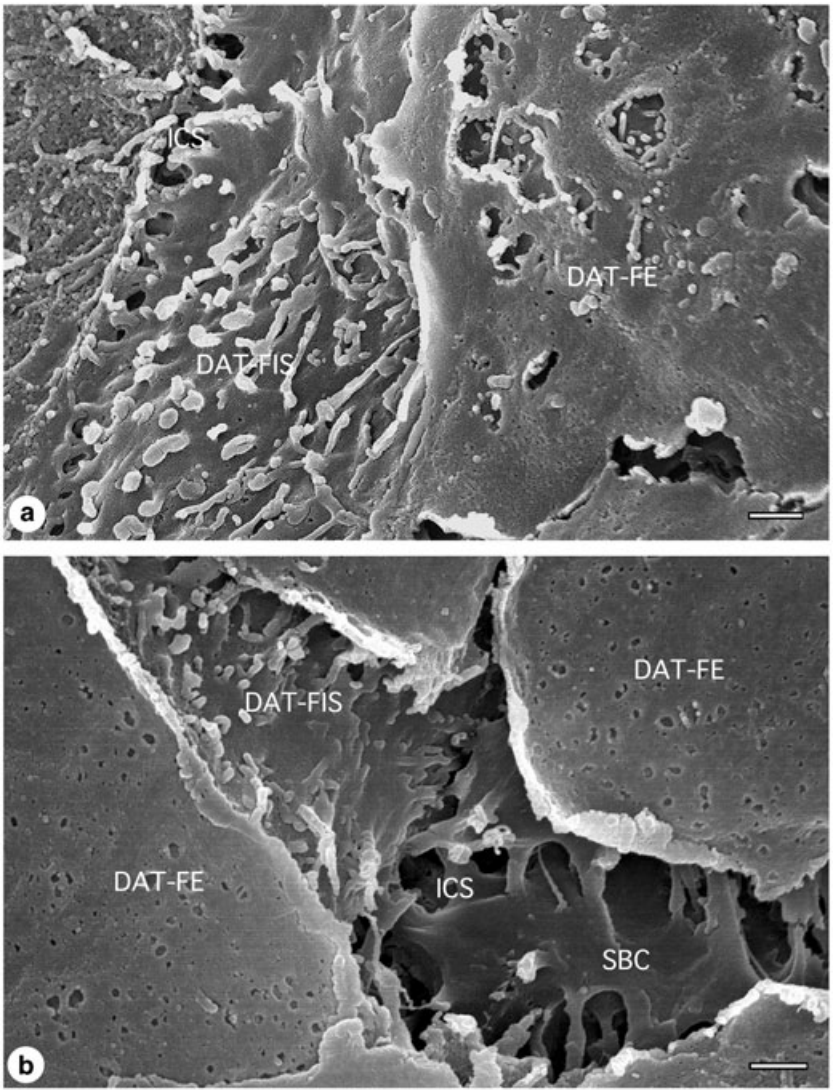

Fig. 5. Scanning electron microscopy images of directly attached to the tooth (DAT) cells following osmium maceration. On the DAT cell facing to intercellular space (DAT-FIS), thin intercellular bridges can be observed. Numerous and long microvilli-like structures are distinct (a). Bridges between the suprabasal cells just underneath the DAT cells are thicker and fewer than those at the lateral side (b). Original magnification: (a), ×9500; (b), ×10,500. Bar = 1.0 μm. DAT-FE, DAT cell face to enamel; ICS, intercellular space; SBC, suprabasal cell.

lamina are rapidly dividing, but not the DAT cells. However, recent investigations using autoradiography demonstrated that a significant number of DAT cells are capable of synthesizing DNA, as do basal cells along the connective tissue (9, 10). The results of our previous studies using argyrophilic nucleolar organizer region (AgNORs) staining also support the idea that DAT cells have proliferative activity (20, 21).

Based on the fact that DAT cells typically express a high density of transferrin receptors at the coronal part of the junctional epithelium, it was suggested that DAT cells have an active metabolism and a high turnover (12, 13). Recently, Pöllänen *et al.* showed that DAT cells play a more important role in tissue dynamics (14) and in the reparative capacity of the junctional epithelium than previously thought (15). Thus, it is natural to think that proliferating DAT cells should move in the junctional epithelium tissue during the turnover process. Few studies on the cytoskeleton of DAT cells have been reported, although it is reasonable to investigate the cytoskeleton of DAT cells with respect to their mobility.

We clearly observed bundles of actin filaments in the cytoplasm of DAT cells using phallacidin fluorescent dye staining and CLSM. At the most apically located DAT cells, the bundles formed stress fibers arranged in parallel to the presumable cervical-line as encircled by the gingival cervix. At the more coronally located DAT cells, the stress fibers were arranged in parallel to the tooth axis from the apical to the coronal direction as shown in Fig. 1. Bundles of stress fibers may be involved in cellular constriction of DAT cells at the gingival cervix, while on the coronal side, they may participate in the migration of DAT cells from the apical to the coronal direction (22, 23).

We also detected focal adhesions in the apical and middle portions of DAT cells in higher magnification images of the CLSM shown in an inset of Fig. 1(c). These focal adhesions may be concerned with cell movement.

In ultrathin sections of DAT cells, numerous concavities and clathrin-coated vesicles were found in the cytoplasm, as reported previously by Sawada and Inoue (24). These concavities and vesicles may possibly be involved in the exchange of materials at the interface between the enamel and the DAT cells.

Although a large number of studies have reported the ultrastructure and components of the internal basal lamina in both the mature and the developing junctional epithelium (2, 4–7, 10, 20, 25–30), the structures of the inner surfaces of DAT cells have not yet been fully clarified in relation to the function(s) of these cells.

The surfaces of newly erupted teeth are covered with a dental cuticle in close association with the internal basal lamina of the junctional epithelium. After tooth eruption, the enamel surface is covered with an acquired enamel pellicle (31, 32). Through these components and the internal basal lamina, DAT cells in the junctional epithelium are attached and migrate on to the enamel surface under physiological conditions. For these reasons, it is

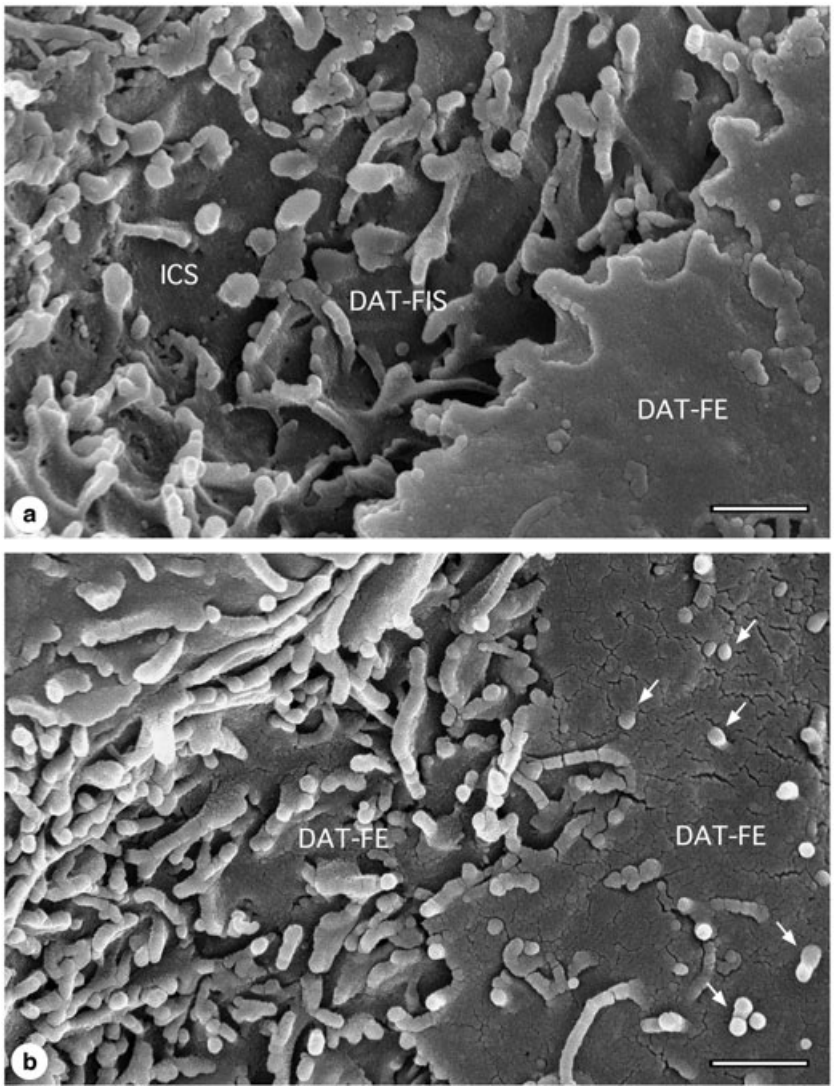

Fig. 6. Scanning electron microscopy images of directly attached to the tooth (DAT) cells following osmium maceration. In specimens after removal of the dental pellicle and extracellular matrices by osmium maceration, short and thin microvilli-like structures are detected at higher magnification (a). The microvilli-like structures are arranged diffusely on the DAT cell facing to enamel (DAT-FE). Round- or flat-headed tips of microvilli-like structures (arrows) are frequently observed (a, b). Original magnification: (a), ×18,500; (b), ×18,500. Bar = 1.0 μm. DAT-FIS, DAT cell face to intercellular space; ICS, intercellular space.

hard to directly observe the inner surface structures of DAT cells, even after decalcification of the enamel tissue by demineralization in substrates such as EDTA (10, 33).

Few studies have examined the fine three-dimensional structure of the surfaces of DAT cells facing to enamel. In this study, we used the osmium maceration method to remove the dental cuticle, the enamel pellicle, and components of the internal basal lamina, to expose the surface of junctional epithelium facing to enamel and to observe surface structures by SEM. By employing this method, soluble materials were removed and the cell membranes were preserved quite well (16–19).

In osmium maceration SEM images, cell membranes of surfaces of DAT cells facing the enamel were not flattened, but short and thin microvilli-like structures were diffusely arranged. These structures in SEM samples were confirmed as short cell processes showing microvilli-like structures 100–180 nm in thickness in TEM specimens. Although the outline of these structures is obscure in conventional TEM images because of the existence of the extracellular matrices of junctional epithelium cells, we could detect distinctly the cell processes of the cells facing the enamel.

In general, microvillous structures are found in the intestinal epithelium for absorption (microvillus) and in fibroblasts or keratinocytes for migration (lamellipodia) (33–37). Although the microvilli-like structures in the DAT cells were morphologically similar to microvilli observed in absorbing epithelia, the structures in DAT cells were fewer and shorter than those in the absorbing cells, suggesting that the microvilli-like structures are not counterparts for absorbing function. On the other hand, it has been reported that keratinocytes can migrate using the organization of actin filaments and the lamellipodia, which is the prerequisite for cell spreading and migration and involves vectorial growth bursts of actin filament bundles and arrays (33, 37). Therefore, we are tempted to suggest that those structures are concerned with migration of DAT cells.

Round- or flat-headed tips of microvilli-like structures were frequently observed in osmium maceration specimens shown in Fig. 6(a and b). From these morphological characteristics, these structures are probably attached directly to the enamel surface.

The mechanism of turnover of proliferating DAT cells is not fully understood, although three possible mechanisms have been proposed; (i) daughter cells produced by dividing DAT cells replace degenerating cells on the tooth surface, (ii) daughter cells enter the exfoliation pathway and gradually migrate coronally between the basal cells and the DAT cells to eventually break off into the sulcus, or (iii) epithelial cells move/migrate in the coronal direction along the tooth surface and are replaced by basal cells migrating around the apical termination of the junctional epithelium (14). From our results on the distribution of the cytoskeleton and the existence of microvilli-like structures in DAT cells,

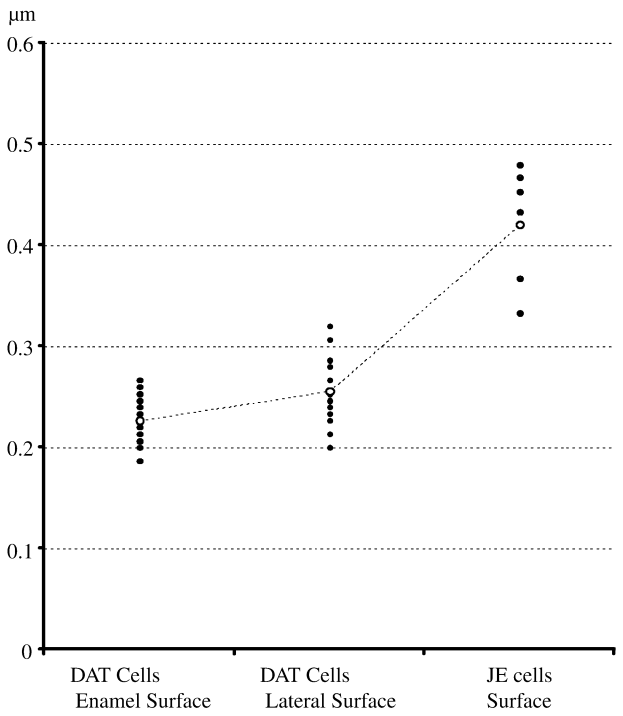

Fig. 7. Thickness of the microvilli-like structures of directly attached to the tooth (DAT) cells and cell processes forming intercellular bridge of junctional epithelium (JE) cells (μm).

we surmise that DAT cells migrate in the coronal direction along the enamel surface, the third possibility originally suggested by Pöllänen *et al.* (14), since we found no evidence of degenerating DAT cells or of migration between basal cells and DAT cells.

In fact, it is hard to consider that DAT cells might enter the exfoliation pathway breaking away from the surface of the internal basal lamina. It is well known that the internal basal lamina differs from other basement membranes such as the external basal lamina, and is a very specialized structure responsible for anchoring, binding and mediating secretion or transport of many kinds of substances (2, 4–7, 26–30). More recently, Sawada and Inoue (24) proposed yet another new function of the dento-gingival junction in the internal basal lamina. They demonstrated the presence of mineral deposits and a delicate mutual basement membrane–mineral interaction in the dento-gingival junction of monkeys, where the network arrangement of mineral crystals in the internal basal lamina resembles the pattern of the cord network in the basement membrane. This may imply that the adhesion of DAT cells to the enamel through the internal basal lamina is exceedingly firm. Taking the adhesion structures of DAT cells into account, the migration speed of DAT cells may be lower than that of other junctional epithelium cells such as suprabasal cells, participating in the regular turnover of this tissue.

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