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The myoepithelial cell differentiation of mucoepidermoid carcinoma in a collagen gel-based coculture model

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BACKGROUND: Mucoepidermoid carcinomas (MECs) are the most common malignant tumor of the salivary glands; however, the histogenesis of MECs has been still controversial. This study was undertaken to investigate the histogenesis of MECs by the examination of their collagen gel-based coculture tissue and transplanted tumors.

METHODS: Two cell lines from a primary and a metastatic MECs were established and characterized by the mutational analysis of the *p53* gene and *in vivo* tumorigenicity in athymic nude mice. Collagen gel-based organotypic cocultures were performed, and the ultrastructural and immunohistochemical findings were examined.

RESULTS: Two cell lines demonstrated *p53* point mutation at the same codon. A metastatic cell line of MEC showed *in vivo* tumorigenicity. Transplanted tumors and the collagen gel-based culture tissues showed poorly differentiated squamous cell carcinomas devoid of mucous cell differentiation; however, they disclosed the differentiation of myoepithelial cells.

CONCLUSIONS: MECs appear to be centered on the squamous cell differentiation, and the specific differentiation of myoepithelial or mucous cells seems to be modulated by the property of microenvironment.

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Keywords: collagen gel-based coculture; microenvironment; mucoepidermoid carcinoma; mucous cells; myoepithelial cells; squamous cells; transplantation

Introduction

The variety of histologic features in salivary gland tumors (SGTs) have enabled pathologists to investigate their histogenesis and classify them according to their presumed histogenetic backgrounds. SGTs are traditionally believed to originate from stem cells in the excretory ducts or in the acinar-intercalated duct region, the so called 'semipluripotential bicellular reserve cell theory' (1–3). According to this theory, mucoepidermoid carcinomas (MECs) have been classified as tumors originating from the excretory duct region. The main reason for segregating MECs from other SGTs is that myoepithelial cells do not participate in the formation of MECs. However, Dardick and colleagues (4–6) reported that MECs have an organized pattern, which involve luminal epithelial cells surrounded by intermediate cells, which are considered to be modified myoepithelial cells. This observation indicated that MECs share a common origin with other SGTs, and are believed to arise from acinar-intercalated duct regions. Furthermore, these investigators reported that luminal cells at all levels of the duct system, and even acinar cells, have a proliferative capacity when released following clamping of the ducts or following irradiation (7–9). For instance, a 'multicellular histogenetic theory' that ductal cells at all levels and acinar cells are candidate progenitors of SGTs has been developed.

Additionally, it is extremely hard to prove the origin of SGTs, that is, whether they are derived from acinar-intercalated or excretory duct regions, or whether they originate from stem or terminally differentiated cells. Instead, an understanding of the differentiation process might be more useful for classifying tumors of a specific organ (10). The development of *in vitro* collagen gel-based coculture model in a microenvironment that induces their differentiation might be a useful way to investigate the histogenesis of MECs. In this study, we attempted to elucidate the MEC tumorigenesis by comparing with the degree of differentiation of transplanted tumors and collagen gel-based coculture tissues with those of primary and metastatic tumors.

Materials and methods

Establishment of cell lines

We obtained the fresh tongue mass from a 39-year-old man, which was diagnosed as an MEC, high-grade malignancy. A metastatic MEC was noted in one submandibular lymph node. Fresh cancer tissue was minced and incubated for 30 min at 37°C in 0.25% trypsin. The isolated cancer cells were grown with a feeder layer of mitomycin C-treated

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National Institute of Health (NIH) 3T3 fibroblasts. The cells were fed with a mixture of Dulbecco's Modified Eagles Medium (DMEM; Gibco-BRL, USA) and Ham's nutrient mixture F12 (Gibco-BRL) at a 3 : 1 ratio supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 1×10^{-10} M cholera toxin, 0.4 mg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, and 2×10^{-11} M triiodotyronine (all purchased from Sigma, St Louis, USA). The cells were fed in an incubator at 37°C in an atmosphere containing 5% CO_2. The culture medium was changed every 2 or 3 days. The primary and metastatic cells grown by 100th passage were selected for this study. The cell line called YD-15 (Yonsei University College of Dentistry) was obtained from a primary tumor, and showed polygonal cells, while the cell line YD-15M was obtained from a metastatic lymph node and had spindle-shaped cells (data not shown).

In vivo *tumorigenicity*

To investigate the *in vivo* tumorigenicity of the MEC cell lines, 3×10^6 to 5×10^6 cells, mixed in DMEM, were injected subcutaneously into 4–6-week-old male athymic nude mice (nu/nu; BALB/C, Japan SLC). Tumor formation was examined everyday, and when the mass had grown to more than 1 cm, it was surgically removed and fixed in a 10% formalin solution, and paraffin-embedded for histologic evaluation.

DNA extraction, polymerase chain reaction (PCR), and DNA sequencing

DNA was extracted from harvested culture cells using the phenol–chloroform method. The *p53* exons 5–9 were each amplified with 100 ng of genomic DNA, 20 pmol of each primer, 2 μmol dNTP (dATP, dCTP, dTTP and dGTP), 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, and 0.2 units of Taq polymerase (Perkin-Elmer, USA). The conditions used for PCR were: 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 60°C, and extension for 1 min at 72°C in an automated thermal cycler (Perkin-Elmer). The primer sequences and PCR fragment sizes were as follows: exon 5 (245 bp) 5′-TTCCTCTTCCTGCAGTACTC-3′ (sense), 5′-ACCTGGGCAACCAGCCCTGT-3′ (anti-sense); exon 6 (175 bp) 5′-ACAGGGCTGGTTGCCCAGGGT-3′ (sense), 5′-AGTTGCAAACCAGACCTCAG-3′ (anti-sense); exon 7 (190 bp) 5′-GTGTTGTCTCCTAGGTTGGC-3′ (sense), 5′-GTCAGAGGCAAGCAGAGGCT-3′ (anti-sense); exon 8 (212 bp) 5′-TATCCTGAGTAGTGGTAATC-3′ (sense), 5′-AAGTGAATCTGAGGCATAAC-3′ (anti-sense); exon 9 (138 bp) 5′-GCAGTTATGCCTCAGATTCAC-3′ (sense), 5′-AAGACTTAGTACCTGAAGGGT-3′ (anti-sense).

The PCR products were cloned using the pGEM T-easy vector system (Promega, USA), and then sequenced using an automated DNA sequencer (Autoassembler 1.0, Perkin-Elmer). HT3 (codon 245 of exon 7: GGC → GTC) and Caski cell lines (wild-type *p53* gene) were used as positive and negative controls, respectively.

Collagen gel-based organotypic coculture

The two types of collagen gel were prepared, as described by Asselineau & Prunieras (11). The one type included NIH 3T3 fibroblasts, which were obtained from the ATCC. To prepare the collagen gel embedded with fibroblasts, eight volumes of ice-cooled type I collagen (Nitta Gelatin, Japan) was mixed with one volume of reconstitution buffer (200 mM HEPES, 0.05N NaOH), one volume of 10× culture media and NIH 3T3 fibroblasts (1.2×10^5/ml). The other type was established by the same components merely devoid of NIH 3T3 fibroblasts. The type I collagen mixture was then dispensed into a 12 mm Millicell (Millipore Co., USA), which was placed in a six-well plate. The mixture was then polymerized overnight at 37°C. MEC cells (3×10^5/ml) were then seeded onto the collagen gel and submerged in the culture media for 5 days. The cells were then exposed to the air by feeding culture media below the epithelial layer. The cells were cultured in an incubator at 37°C with an atmosphere containing 5% CO_2. The medium was changed every other day, and the culture tissues were maintained for 2 weeks. Subsequently, they were fixed in 10% formalin, paraffin-embedded, and processed for histologic examination.

Immunohistochemistry

Paraffin-embedded tissues from both surgical specimens and the culture tissues were used for immunohistochemical staining. After deparaffinization and rehydration, endogenous peroxidase was blocked with 3% H_2O_2 in methanol, and sections were incubated with normal horse serum at room temperature. Subsequently, primary antibodies, anti-keratin antibody (AE1/3) (DAKO, Denmark), cytokeratin 10/13 (DAKO), and anti-α-smooth muscle actin (DAKO) were applied overnight at room temperature to detect cytokeratins and myoepithelial cell differentiation. Slides were then incubated with biotin-labeled horse anti-mouse/anti-rabbit IgG (Vector Laboratory, USA) at room temperature for 30 min, and with horseradish peroxidase streptavidin at room temperature for 30 min. The visualization was performed by 3,3′-diaminobenzidine tetrachloride, and the slides were counterstained with Meyer's hematoxylin.

Ultrastructural study

The culture tissue was fixed immediately in 2.5% glutaraldehyde for 24 h, and post-fixed in 1% osmium tetroxide for 2 h at room temperature. The fixed tissue was then embedded in Epon812 resin and ultramicrotome (Ultracut Reichert Jung, Germany) sections were stained with uranyl acetate and lead citrate, and examined under a Philips CM-10 transmission electron microscope.

Results

Histologic findings

Primary and metastatic tumors

The primary tumor obtained from a tongue mass was mainly composed of a poorly differentiated squamous cell carcinoma. Clusters of mucous cells were scattered and showed a mucicarmine-positive reaction (Fig. 1). Histologically, glandular differentiation and mucous cells were found in a metastatic lymph node (Fig. 2).

Transplanted tumors

When MEC cells were subcutaneously injected into the nude mice, only the metastatic cell line (YD-15M) developed

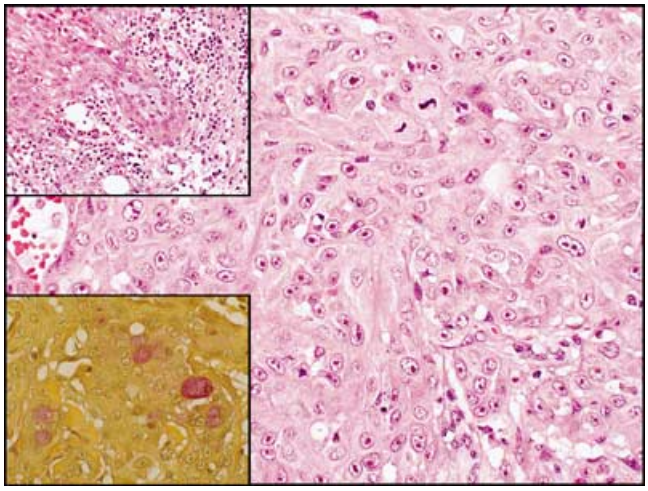

Figure 1 Mucoepidermoid carcinoma, high-grade malignancy showing frequent mitoses and pleomorphism. Inset: upper left; a cluster of mucous cells is found. Lower left; mucicarmine-positive mucous cells.

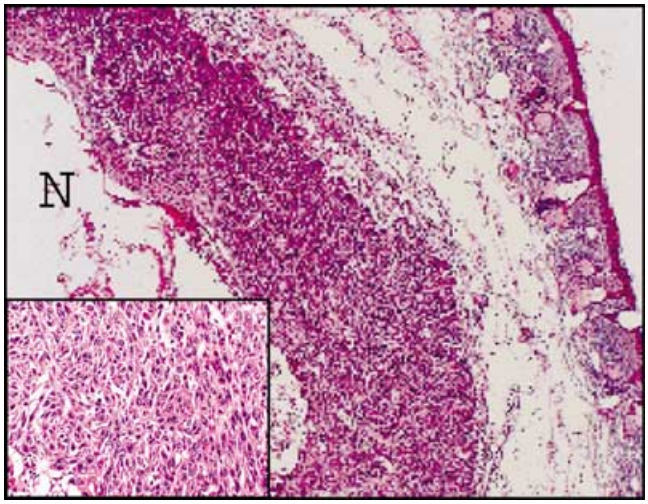

Figure 3 A transplanted tumor derived from YD-15M cell line is well circumscribed with necrotic change (N). Inset: a poorly differentiated squamous cell carcinoma devoid of mucous cells is found.

tumors, which occurred in two out of five nude mice. The tumors were well circumscribed by surrounding fibrous tissue, and no invasive growth was evident. The central portion of the transplanted tumors underwent severe ischemic necrosis. Transplanted tumors showed a poorly differentiated squamous cell carcinoma devoid of mucous cells or glandular differentiation (Fig. 3).

p53 *mutation*

Exon 7 showed the same point mutation in the YD-15 and YD-15M cell lines at codon 258. Specifically, the GAA sequence was changed to GCA, which resulted in a Glu to Ala amino acid mutation (data not shown).

Collagen gel-based coculture tissue

Both MEC cell lines were reconstructed by collagen gel-based coculture with or without fibroblasts. The hematoxylin–eosin sections mimicked squamous cell carcinomas showing nuclear atypia and pleomorphism in both types of collagen gel (Fig. 4a,b). However, no mucous cell or glandular differentiation was observed in the culture environment. Interestingly, the invasive growth was prominent only in the culture tissue grown by the collagen gel embedded with fibroblasts (Fig. 4a).

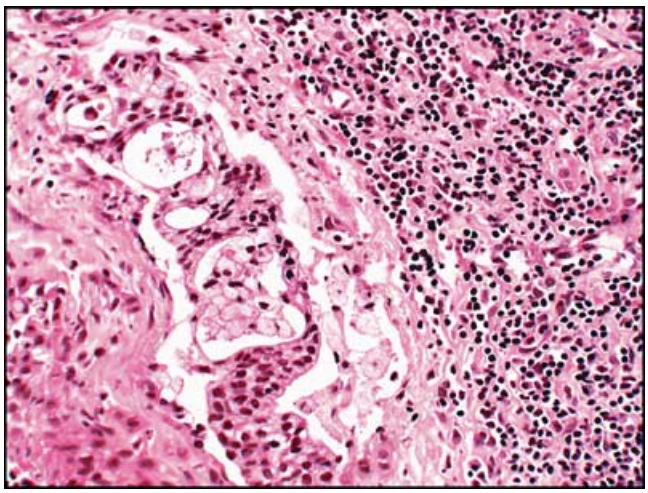

Figure 2 A metastatic MEC in a lymph node showing a focus of glandular formation and mucous cells.

Immunohistochemical study

To detect cytokeratin expression in the primary and metastatic tumors, and in the culture tissue and the transplanted tumors in the nude mice, we performed immunohistochemical staining for AE1/3 and cytokeratin 10/13. AE1/3 antibodies react with both acidic and basic keratin groups recognizing epidermal differentiation (12). Cytokeratin 10/13 shows the specific distribution in the suprabasal cells of the skin and oral mucosa (13). AE1/3 and cytokeratin 10/13 were demonstrated in the surgical specimens from both the patient and the nude mice and in the cultured tissues, suggesting that the culture conditions successfully induced squamous cell differentiation (Fig. 5a,b). No positive reaction for α-smooth muscle actin was found in the primary or metastatic MEC (data not shown). In contrast, transplanted tumors showed a few positive cells of α-smooth muscle actin (Fig. 5c). The collagen gel-based culture tissues showed a diffuse positive reaction for α-smooth muscle actin in the cytoplasm of stratified cancer cells (Fig. 5c). There is no difference in expression pattern between the culture tissues grown by collagen gel with and without fibroblasts.

Ultrastructural findings

The ultrastructure of the three-dimensional culture tissue was examined, and it was found to be mainly composed of squamous cells that showed a perinuclear distribution of tonofilaments, interconnected to neighboring cells by well-developed desmosomes (Fig. 6). Some cells displayed numerous rough endoplasmic reticulum (RER), ribosomes, and desmosomes, suggesting that they were epithelial cells, but no evidence of specific differentiation, such as the presence of tonofilaments, secretory granules, or

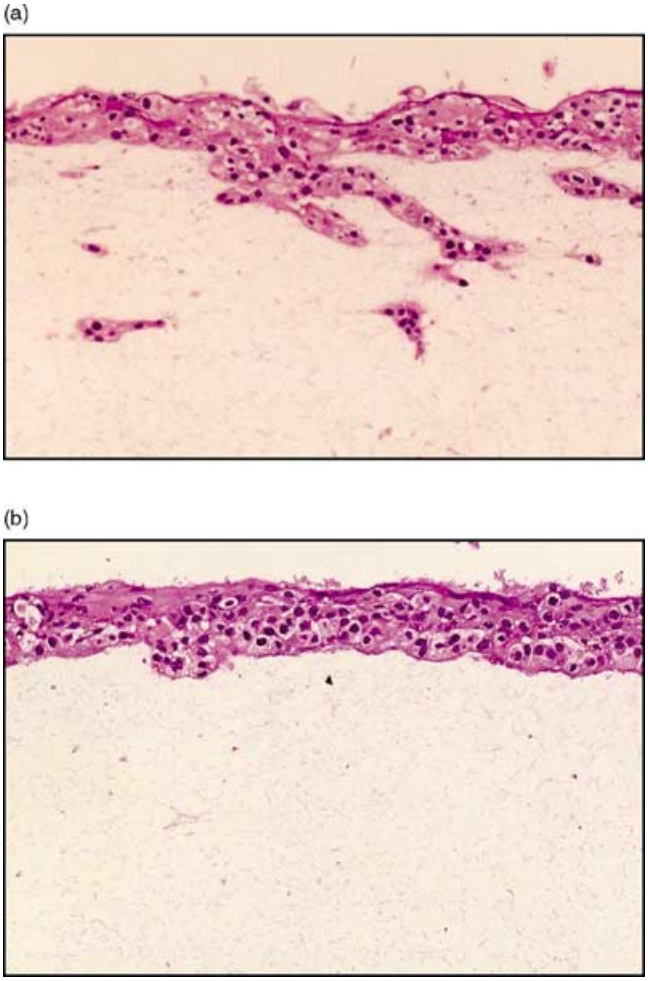

Figure 4 (a) Collagen gel-based culture tissue of YD-15 cell line shows invasive growth into a dermal equivalent with fibroblasts. (b) Collagen gel-based culture tissue of YD-15M cell line grown by a collagen gel devoid of fibroblasts shows stratified pleomorphic cancer cells.

myofilaments, was found (data not shown). Interestingly, some cells demonstrated pinocytotic vesicles and thin filament bundles, with focal densities simulating myoepithelial cells, but these cells showed no basal lamina (Fig. 7). Prominent microvillus projections were also noticed in culture cells. No mucous cells or glandular differentiation was found.

Discussion

The *p53* tumor suppressor gene is most commonly associated with the cause of human cancer. Mutations to the *p53* gene are very common in head and neck squamous cell carcinomas (14–17), and are also frequent events in the MECs of salivary glands (18, 19). The present case also showed a point mutation in codon 258 of exon 7. Both the primary and metastatic tumors showed the same mutation.

Collagen gel-based organotypic cocultures have been developed to provide the *in vitro* stromal microenvironment for the reconstruction of *in vivo* conditions (20). The present study attempted to reconstruct MECs by collagen gel. Regardless of the presence or the absence of fibroblasts in the collagen gel, this system replicated squamous cell

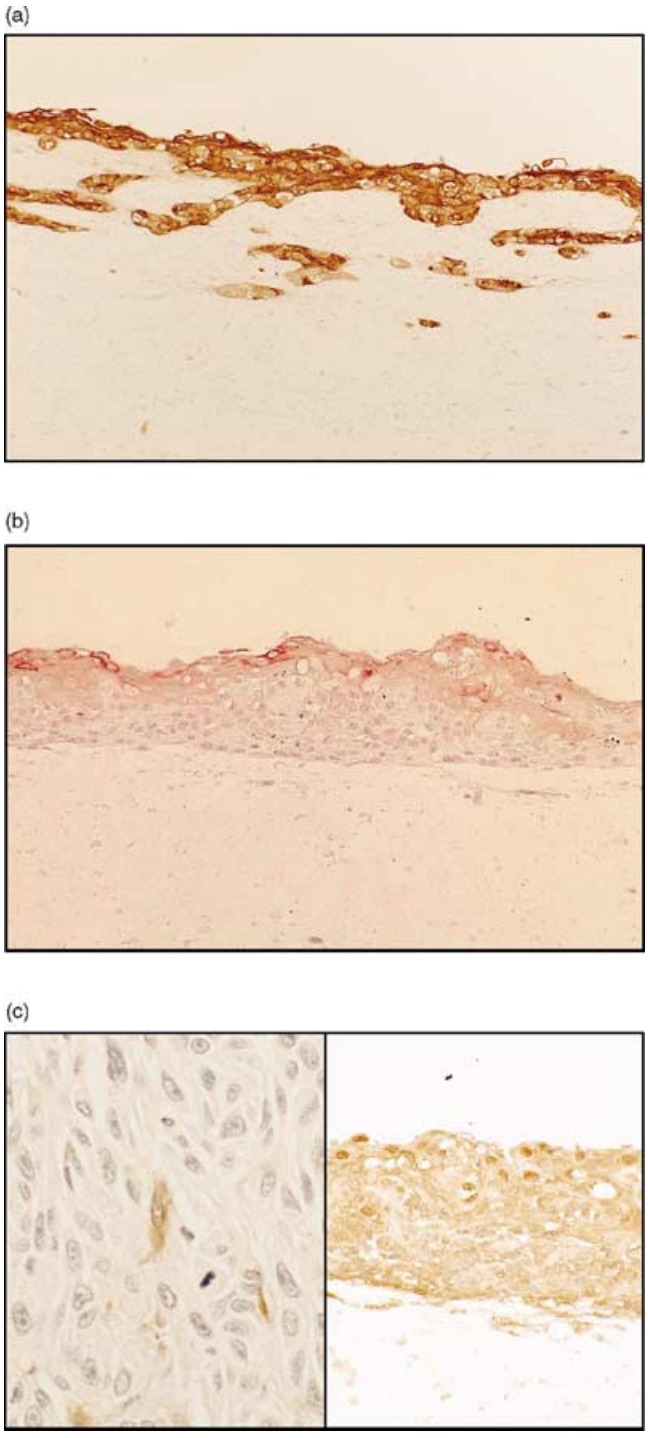

Figure 5 (a) Cytokeratin AE1/3 expression of the culture tissue. (b) Cytokeratin 10/13 expression of the culture tissue. (c) Left: a transplanted tumor shows a few positive cells for α-smooth muscle actin. Right: three-dimensional culture tissue shows a diffuse positive reaction for α-smooth muscle actin in the cytoplasm of cancer cells.

carcinomas; however, no glandular or mucous cell differentiation was found. Transplanted tumors showed poorly differentiated squamous cell carcinomas devoid of mucous cell differentiation as well. Recent studies have been focused on the interrelationship between tumor mesenchyme and

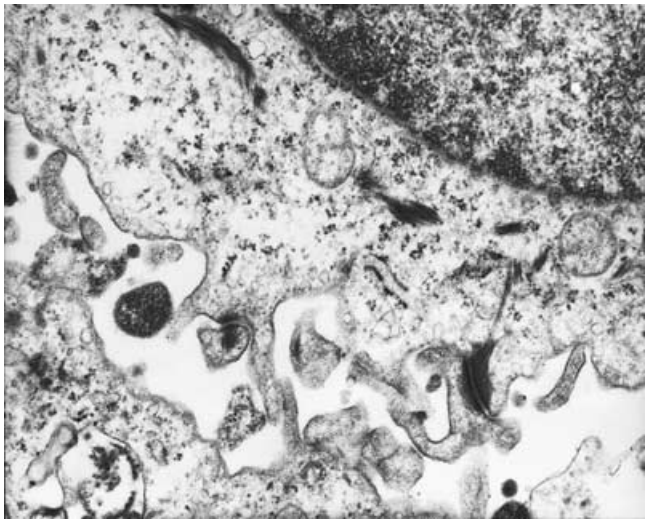

Figure 6 A squamous cell showing well-developed desmosomes and tonofilaments.

parenchymal cancer cells (21). The microenvironment of the local host tissue can be an active participant in the tumorigenesis, and the cancer-associated stromal cells can modulate cancer progression (22). Further, primary prostate stromal cells can modulate the morphogenesis of prostate epithelial cells (23). Hence, the differentiation process of MECs might be influenced by their mesenchymal components. Furthermore, the fact that the invasive growth was prominent in the culture tissue by the collagen gel with fibroblasts supports that the invasiveness may be influenced by the mesenchymal components (21, 22, 24).

Salivary glands arise as a focal thickening of the oral epithelium into the underlying mesenchymal tissue (25). In salivary gland development, the mesenchymal tissue, namely, type I and type III collagen and basement membrane proteoglycans are known to play a significant role in branching morphogenesis. The cytodifferentiation of secretory cell types appears to be initiated by direct contact between epithelial and specific mesenchymal components (26). Mucous cells, a highly differentiated cell type, need a specific microenvironment for terminal differentiation induction. Even though the carcinogenesis of SGTs is not identical to the development of salivary glands, an appropriate mesenchymal interaction is at least partly required for mucous cell differentiation.

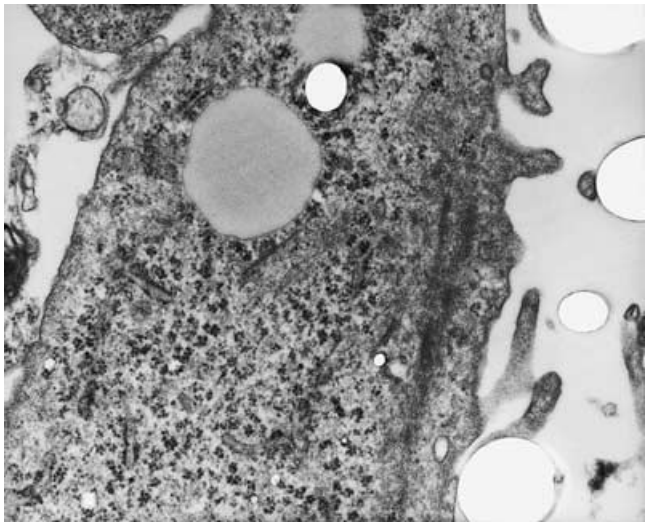

Figure 7 An epithelial cell showing pinocytotic vesicles and thin filaments with focal densities. No basal lamina is found.

Glandular differentiation has been induced in a collagen gel culture system in various cancer cell lines (27–30). In pancreatic or endometrial adenocarcinomas, collagen gel or Matrigel reproduced glandular differentiation in well to moderately differentiated adenocarcinomas (27–29), but not in poorly differentiated adenocarcinomas, illustrating that gland-forming ability is an intrinsic property of cancer cells (29). Consequently, the failure of mucous cell differentiation in the present study appears to result from the lack of a differentiation capability of the cancer cells as well as the absence of an appropriate mesenchymal interaction in a collagen gel-based coculture model or host mesenchymal tissue of the nude mice.

The participation of myoepithelial cells in MEC is still controversial. Some investigators have demonstrated immunohistochemical and ultrastructural evidences for the presence of myoepithelial cells (6, 31, 32). Conversely, other investigators found no immunologic markers for myoepithelial cell differentiation in MECs (33–35). According to the present study, the culture tissue showed that some cells had thin filaments with focal densities in their cytoplasm, and found that this corresponded to the immunohistochemical results shown by a diffuse positive reaction for α-smooth muscle actin in organotypic culture tissue. A few α-smooth muscle actin-positive cells were also found in the transplanted tumor. Interestingly, the α-actin and myofilament bundles appeared in many *in vitro* epithelial cells to be an artifact of culturing (36). The process of growing cells *in vitro* can be regarded as a trauma introducing them into a new physical and biochemical environment. We speculated that these *in vitro* microenvironments might stimulate myoepithelial cell differentiation in MECs.

Based on the present study, MECs appear to be centered on the differentiation to squamous cells, and the specific differentiation of myoepithelial or mucous cells seems to be modulated by the property of microenvironment. Further, the analysis of the differentiation and invasion process in concert with the mesenchymal components will contribute to the elucidation of the histogenesis of MECs, and clarify the classification of SGTs.

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