Human Mammary Epithelial Cells Undergo Squamous Differentiation in Serum-Free Three-Dimensional Culture upon Loss of Growth Activity

Hitoshi Satoh1,2,3, Norimasa Sawada4, Yoshiki Watanabe1,2, Masaaki Satoh1, Koichi Hirata2 and Michio Mori1

1Department of Pathology and 21st Department of Surgery, Sapporo Medical University School of Medicine, South-1, West-17, Chuo-ku, Sapporo 060, Japan

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ABSTRACT. Normal human mammary epithelial cells (HMEC) isolated from surgically resected breast tissues were cultured under serum-free conditions using MCDB 170 medium. With the increase in the number of passages, in particular after the 5th passage, the number of enlarged, flattened and vacuolated cells increased while cell proliferation decreased. The senescent cells occasionally had keratohyaline granules in the cytoplasm and were positive in immunohistochemistry for keratinizing squamous epithelium-specific cytokeratin 10. When HMEC were cultured between floating double-layered collagen gels, the cells lost growth activity, showed marked stratification, and became positive for carcinoembryonic antigen (CEA). The stratified cells underwent squamous differentiation and tonofilament bundles appeared around the nuclei. The stratification and squamous differentiation of HMEC were observed within seven days after transfer to the three-dimensional culture, regardless of the number of passages. These results indicate that the HMEC in vitro ultimately differentiate into squamous epithelia and also that there is a close relationship between the squamous-type differentiation and the loss of cell proliferation.

Culture of human mammary epithelial cells (HMEC) under serum-free conditions with the use of MCDB 170 medium is well established (6), and the biological (6, 16, 17, 18) and biochemical (16) features of HMEC have been well investigated. The cells have been utilized for research on carcinogenesis (2, 16). These cells proliferate rapidly during several passages and then lose their proliferation potential (6, 16). Cells in the proliferating phase are small and polygonal, whereas those in the senescent phase are enlarged and flattened. Immunohistochemically, both the senescent and proliferating cells are positive for cytokeratins 7, 8, 14, 18 and 5 (19).

Although HMEC grow actively under serum-free conditions, monolayer cultures of the cells show no morphological differentiation (6). On the other hand, three-dimensional culture using collagen gels is an appropriate method for the study of morphogenesis and cellular differentiation, for which rodent mammary epithelial cells have been preferentially employed (3, 4, 5, 8, 20, 21). In contrast, there have been very few reports on the characteristics of human mammary epithelial cells in three-dimensional culture under serum-free conditions (17, 18, 22).

In this study, we showed that senescent cells of HMEC in serum-free culture show squamous differentiation, and also that the majority of the cells show squamous differentiation before reaching their cellular senescence in monolayer culture and become positive for carcinoembryonic antigen (CEA), when they are transferred to three-dimensional culture using collagen gels.

MATERIALS AND METHODS

Tissue collection and preparation. Normal human mammary epithelial cells were isolated from surgically resected human mammary glands according to the method of Stampfer et al. (15) with slight modification. Briefly, mammary glands were grossly separated from fatty tissues and lacerated with opposing scalpels. The tissues were then digested with 0.1% collagenase (Wako Pure Chemical) and 0.2% dispase (Godo Shusei) in Ca2+-free Hanks' balanced salt solution (HBSS) at 37°C for 60 min. The epithelial cells were collected by centrifugation at 50 x g for 5 min.

Culture procedure. Isolated epithelial cells were seeded on plastic dishes in serum-free MCDB 170 medium (Kyokuto Pharmaceutical) supplemented with 5 μg/ml insulin (Collaborative Research Inc., USA), 10 ng/ml EGF (Chemicon International Inc., USA), 5 μg/ml transferrin (Sigma Chemical Co., USA), 1.4 μM hydrocortisone (Sigma), 0.1 mM ethanolamine (Sigma), 0.1 mM phosphoethanolamine (Sigma), 1 μg
/ml prolactin (Sigma), and 25 nM prostaglandin E1 (Funakoshi). The medium was changed two or three times per week.

For routine subculture, nearly confluent cells on plastic dishes were dispersed with 0.05% trypsin plus 0.25% EDTA (Gibco Laboratories, USA) in saline, centrifuged gently, resuspended in MCDB 170 medium and seeded at a split ratio of about 1:6. Both organoids and cultures of HMEC were stored frozen in MCDB 170 with 10% dimethyl sulfoxide (DMSO; Katayama Chemical) and 20% fetal bovine serum (Filtron, Australia).

Preparation of collagen gel. Type I collagen was extracted by stirring sterile rat tail tendons for 48 hours at 4°C in acetic acid solution (3.3 mg/ml) as previously reported (13). The resulting viscous solution was centrifuged at 4,000 × g for 30 min. The supernatant was stored at 4°C until use. Collagen-mixture solution was prepared by mixing 17 volumes of collagen solution with 4 volumes of 13.3 × Ham's F-12 solution (Kyokuto) containing NaOH (13.3-fold concentrated Ham's F-12: 0.34 N NaOH = 10 : 3), and put into a CO2 incubator to be kept at 37°C to form gel.

Three-dimensional culture using collagen gels (floating sandwich method). The collagen mixture was dispensed into a plastic dish and allowed to stand at 37°C for about 5 min to form gel. After rinsing the gel with the culture medium, the cells were seeded onto the gel and cultured. When the cells formed a subconfluent monolayer, they were covered with another layer of collagen gel. After being cultured for 24 hrs, the double-layered gels were allowed to float from the dish into the culture medium.

Immunohistochemistry and histological observations. For immunohistochemical analysis of cytokeratin, cells were seeded onto clean glass coverslips and fixed with acetone for 30 min at −20°C when they reached subconfluence. The coverslips were rinsed with phosphate-buffered saline (PBS), air-dried and stored at −20°C until use.

The expression of cytokeratin was examined with polyclonal anti-keratin (pan-keratin) antibody (DAKO), monoclonal anti-cytokeratin 8 (M 20: ICN Immunobiological, USA) and anti-cytokeratin 10 (RKSE 60: ICN) antibodies. These antibodies were applied to the cultured cells by an indirect immunofluorescence method.

For histological and immunohistochemical examinations of the cells cultured by the floating sandwich method, they were fixed with 1% glutaraldehyde, dehydrated, and embedded in paraffin. Sections about 4 μm in thickness were stained with hematoxylin and eosin or treated with polyclonal anti-CEA antibody (DAKO) by the avidin-biotin complex method.

Ultrastructural study. After being cultured by the floating sandwich method for one week, the cells were fixed in 1% glutaraldehyde-4% paraformaldehyde adjusted to pH 7.4 with 0.1 M cacodylate buffer. They were then postfixed with 1% osmium, dehydrated and embedded in Epon 812.

Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a JEOL 1200-EX transmission electron microscope.

BrdU staining. HMEC cultured on a plastic dish were incubated with bromodeoxyuridine (BrdU: DAKO) for 24 hrs and fixed in cold 70% ethanol. HMEC cultured in floating collagen gels were also incubated with BrdU for 24 hrs and fixed with 1% glutaraldehyde. BrdU labeling was examined by using anti-BrdU mouse antibody (Amersham, United Kingdom).

RESULTS

Normal human mammary epithelial cells cultured on plastic dishes. As reported previously (6, 16), small epithelial cells were derived from isolated and fragmented mammary ducts and actively proliferated during 3-5 serial passages. They maintained a cobblestone epithelial morphology until senescence (Fig. 1a). At senescence, the cells were heterogeneous in shape and consisted of large, flattened, vacuolated cells and cobblestone-like cells (Fig. 1b). However, all of these cells were immunohistochemically positive for pan-keratin (Fig. 1c) showing that the cells were epithelial in nature. Some of the senescent cells were positive for keratin 8 (Fig. 1d), in accordance with the observations reported by Taylor-Papadimitriou et al. for cultured human mammary epithelial cells (19). Furthermore, a few senescent cells were positive for keratin 10 (Fig. 1d), which is specific for keratinizing squamous epithelium (10). On the other hand, no CEA-positive cells were detected immunohistochemically under these conditions (data not shown). Since the cells peeled off the culture dishes easily as they underwent senescence, the following culture method between double-layers of floating collagen gels was employed to investigate the properties of the senescent cells.

Normal human mammary epithelial cells cultured between double-layers of floating collagen gels. After 5 passages on plastic dishes, HMEC were transferred into the floating gels, and then maintained for 7 days. As shown in Fig. 2a, HMEC become stratified in the floating double-layered collagen gels, and the results of H and E staining suggested that the stratified cells undergo squamous differentiation under such conditions. On the other hand, the cells distant from collagen gels were immunohistochemically positive for CEA (Fig. 2b). Furthermore, after 2 passages, HMEC proliferated actively on plastic dishes, and also showed marked stratification in the floating gels (data not shown). At the 4th day after transfer of the cells to the three-dimensional culture, 50-60% of the cells released from double layered collagen gels by collagenase digestion was keratin 10-positive (data not shown). This observation shows that HMEC have a potential to differentiate keratinizing squamous epithelium.

Growth potential of cultured mammary epithelial cells. BrdU labeling of the cells cultured on plastic
Fig. 1. Phase-contrast photomicrographs and immunocytochemical demonstration of human mammary epithelial cells (HMEC) cultured on plastic dishes. a: Phase-contrast photomicrograph of HMEC in primary culture showing a cobblestone epithelial morphology (×85). b: Phase-contrast photomicrograph of HMEC cultured through 5 passages showing a heterogeneous cell population, with large, flattened and vacuolated cells (×85). c: All HMEC were positive for pan-keratin (×170). d: Some large cells were positive for keratin 8 (×170). e: A few of these cells were positive for keratinizing-specific keratin 10 (×170).
Fig. 2. Photomicrographs of HMEC cultured in floating collagen gels. a: HMEC stratified and showed squamous differentiation (H & E staining, ×85). b: HMEC showing squamous differentiation were positive for CEA (×85).

dishes showed that the majority of the cells, which were small and polygonal in shape, proliferated actively, while large and vacuolated cells did not show growth activity (Fig. 3a). On the other hand, the cells cultured between the collagen gels hardly showed growth potential (Fig. 3b), but showed squamous differentiation and became positive for CEA regardless of the number of passages.

Ultrastructures of the epithelial cells in the collagen gels. As shown in Fig. 4a, the cells cultured between the collagen gels were observed electron microscopically to be a mixture of cells with glandular features and those with squamous differentiation. These two types of cells were often observed to be attached to each other by desmosomes. Some of the cells with features of squamous differentiation possessed microvilli on their surface (Fig. 4b). In Fig. 4c, typical cells showing squamous differentiation can be seen. The cells have abun-

Fig. 3. Immunocytochemical demonstration of growth activity of HMEC (passage 3) using BrdU counterstained by hematoxylin. a: In HMEC cultured on plastic dishes small, polygonal cells proliferated but large, vacuolated cells lost growth activity (×85). b: HMEC cultured in floating collagen gels for 3 days completely lost growth activity (×170).
Fig. 4. Transmission electron micrographs of HMEC cultured in floating collagen gels. a: The cells with glandular features and with squamous differentiation co-existed. b: Abortive junctional complexes and glandular structures with microvilli were observed between the cells showing squamous differentiation. c: Squamous differentiation such as abundant tonofilament bundles around the nuclei, well-developed desmosomes and interdigitations on the cell surface were observed. Bar=2 μm.
Cells undergo squamous differentiation rather than glandular differentiation. The incidence of cells with such squamous differentiation increased with time in culture. At the 7th day of culture between collagen gels, more than 80% of the cells showed squamous differentiation, regardless of the number of passages.

**DISCUSSION**

The results of the present study showed that mammary epithelial cells undergo squamous differentiation under certain conditions of cell culture. Such squamous differentiation of mammary epithelial cells was shown to be closely related to the loss of growth activity.

Squamous differentiation was suggested in histological examination by the finding of an eosinophilic homogeneous layer of stratification. This was confirmed electron microscopically by the finding of abundant tonofilament bundles in the cytoplasm of epithelial cells, and further supported by immunohistochemistry in that some of the cells were positive for cytokeratin 10, which is known to be a marker for keratinizing squamous epithelial cells. Such squamous differentiation was frequently observed in the mammary epithelial cells cultured between floating collagen gels after five passages in culture on plastic dishes. However, squamous differentiation was observed even in the early passages of cultivation, during which the cells still actively proliferated in monolayers on plastic dishes if they were transferred to the floating gel culture. Since culturing HMEC between floating collagen gels strongly suppresses the growth activity of the cells, such squamous differentiation is considered to be related to the suppression of proliferation rather than a result of cell senescence.

Squamous differentiation of mammary epithelial cells has rarely been observed in physiological and pathological conditions. However, since mammary epithelial cells are derived from the epidermis during embryonal development, it is conceivable that suppression of growth activity in human mammary epithelium ultimately induces squamous differentiation. Schaefer et al. (14) reported evidence that an increased level of cyclic adenosine monophosphate induces squamous differentiation of human mammary epithelial cells *in vitro*. The factor most likely responsible for the induction of squamous differentiation of HMEC in the present study was the extracellular matrix. It is known that differentiated functions and morphology of epithelial cells are induced and that the morphology differs when the cells are cultured between such matrices. However, the present study did not clarify why the mammary epithelial cells undergo squamous differentiation rather than glandular differentiation in our experimental conditions. One possible explanation for the induction of squamous differentiation could be the serum free conditions employed in this study. Yang et al. (22) reported that the presence of serum reduced the squamous differentiation of cells cultured in collagen gels. Since serum contains fibronectin, which reacts with cell surface receptors and thus modulates cell phenotypes (12), it is conceivable that the extracellular matrix component and serum factors influence the phenotype of human mammary epithelial cells *in vitro*. Finally, the expression of CEA observed in the mammary epithelial cells in association with squamous differentiation must be discussed briefly. CEA is now known to be an intercellular adhesion molecule, and is a generally accepted marker for human colon adenocarcinoma, but is also found in certain types of human breast cancers, such as intraductal and infiltrating duct carcinomas (9). CEA has also been observed in the lung and bronchial mucosa in association with squamous metaplasia (7, 11). Benchimol et al. (1) suggested that the production of CEA promotes cellular movement and the formation of a multilayered cell array. Thus, the appearance of CEA in the mammary epithelial cells in the present study seems to be related to cellular stratification and squamous differentiation rather than neoplastic transformation.

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