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Effects of Growth Factors and Hormones on Growth and Morphological Differentiation of Human Breast Epithelial Cells within Collagen Gel in Serum-free Medium

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When human epithelial cells that had grown out from either carcinoma or histologically non-malignant breast tissues were seeded within type I collagen gels in serum-free medium, they successively grew and protruded many radial duct-like extensions with lumina. Separate deletion of each of the supplements from the medium showed that growth as well as morphological differentiation of carcinoma-derived cells were prevented in the absence of epidermal growth factor (EGF) or hydrocortisone. Removal of insulin or ethanolamine plus phosphoethanolamine caused a significant inhibition of cell growth without interfering with the morphological differentiation. Contrary to the case with carcinoma-derived cells, both growth and morphological differentiation of epithelial cells derived from non-malignant breast tissues were prevented when EGF, hydrocortisone or insulin was absent. Removal of each of the other supplements (except for transferrin), including ethanolamine plus phosphoethanolamine, prolactin, or prostaglandin, caused a significant inhibition of cell growth with no apparent inhibition of morphological differentiation. The present results suggest that human epithelial cells derived from either carcinoma or histologically non-malignant breast tissues strongly depend on the presence of EGF and hydrocortisone and there is a decreased dependence on insulin in carcinoma-derived cells with respect to their growth and morphological differentiation during culture within collagen gels.

Key words: Breast cancer — Morphological differentiation — Collagen gel culture — Serum-free medium — Growth factor

Research on the mechanism of carcinogenesis of mammary epithelium would be greatly facilitated if the growth and morphogenesis of mammary epithelial cells could be studied in culture under defined conditions. For monolayer cultures of human breast epithelial cells, an excellent culture system using serum-free medium was optimized[1,2]; however, epithelial cells cultivated as a monolayer in the serum-free medium show no apparent morphological differentiation.[3] Early investigations using collagen gel as an extracellular matrix demonstrated that mammary epithelial cells themselves can form duct-like structures in the presence of sera[4] or many supplements with serum.[5–7] Under more defined conditions where serum is absent, mouse mammary epithelial cells respond to prolactin, progesterone, and adrenal corticoid to form three-dimensional architecture within a collagen gel matrix.[8] Contrary to the case with rodent cells, a combination of supplements including insulin, cholera toxin, bovine serum albumin, epidermal growth factor (EGF), and cortisol failed to induce any duct-like extensions of human breast epithelial cells grown within collagen gel.[9]

We demonstrate in the present study that human epithelial cells obtained from either carcinoma or histologically non-malignant breast tissues successfully grow and protrude many duct-like extensions within collagen gels in serum-free medium supplemented with EGF, insulin, transferrin, hydrocortisone, ethanolamine, phosphoethanolamine, prolactin and prostaglandin. By the use of this culture system, we examined the effect of each of the supplements involved on cell proliferation and morphological differentiation of human breast epithelial cells.

MATERIALS AND METHODS

Tissue collection and monolayer culture Fresh tissue fragments were dissected from the tumors at mastectomy of breast carcinoma patients. The diagnoses were based on histological findings. Histologically non-malignant tissue fragments were obtained from areas away from the primary lesion in the same mastectomy specimens.[3,9] In order to characterize the cell type and to obtain a sufficient number of epithelial cells prior to collagen gel culture, the tissue fragments were first cultivated on plastic culture dishes and cells that grew out from the tissue fragments were subcultured as described previously.[3] The serum-free medium used consisted of a basal nutrient medium, MCDB 170, supplemented with 10 ng/ml EGF (Collaborative Research), 5 μg/ml insulin, 5 μg/ml human transferrin, 1.4 μM hydrocortisone, 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, 1 μg/ml ovine prolactin, and 25 nM prostaglandin E1

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(Sigma).[1-3] Medium changes were performed every third or fourth day and cells that had grown out from the tissue fragments were harvested by successive treatments with 0.25% (w/v) trypsin-0.1% (w/v) EDTA and 0.0375% (w/v) soybean trypsin inhibitor (Sigma).

Immunohistochemical characterization Cells which had grown out from tissue fragments were examined for expression of cytokeratins and human epithelial membrane antigen after the first passage.[9] Cells seeded and grown on glass cover slips were either fixed with 1% neutral formalin for the detection of the human epithelial membrane antigen or fixed and made permeable by successive treatments with methanol and acetone at −20°C for the detection of the cytokeratins.[10] The antibodies used included a polyclonal antikeratin (Dakopatts) and two monoclonal anti-cytokeratins (Labsystems, PKK3) and anti-epithelial membrane antigen (Dakopatts). The reactivities of these antibodies were visualized with an ABComplex kit (Dakopatts), utilizing an avidin-biotin amplification procedure.[11]

Collagen gel culture For standard experiments using collagen gel, 0.5 ml of the base layer of collagen was formed in each well of 24-well plastic plates by mixing in the cold 7 volumes of 0.15% acid-soluble type I collagen solution (Cellmatrix type I-A; Nitta Gelatin, Osaka), two volumes of 5-fold-concentrated MCDB 170 basal medium with supplements, and one volume of reconstitution buffer (0.2 *M* Hepes, 0.08 *M* NaOH). The final concentration of collagen gel was approximately 0.1%. Monolayer cultures of the cells were harvested as described above and the number of cells was counted with a hemocytometer. Cell density was adjusted to 1 to 2×10^6 cells/ml and a 10 μl drop was placed onto each 0.5 ml collagen gel layer. After the culture plates had been kept in a CO_2-incubator for 2 h, the cells in each well were overlaid with 0.5 ml of collagen gel and then flooded with 1 ml of serum-free medium.

Cell proliferation assay Epithelial cells within the collagen gel were washed once with phosphate-buffered saline (PBS), minced into small fragments with scissors, and transferred into 15-ml plastic test tubes. Collagenase (type IA-S; Sigma) was added to give a final concentration of 40 U/ml and the gel fragments were digested at 37°C for 1 h on a reciprocating shaker. Cells were collected and washed with PBS by centrifugation at 2,000 rpm. Cell proliferation was determined by measurement of the number of nuclei with a hemocytometer after vigorous shaking of the cells in 0.1 *M* citric acid containing 0.1% (w/v) crystal violet to dissolve the cytoplasm. Statistical analysis was performed using Student's *t* test.

Morphological and histological observation For morphological observation, cells sandwiched between collagen gels were fixed and stained with 20% Giemsa solution, or fixed with 10% buffered formalin overnight, embedded in paraffin and sectioned at 4 μm for histological observation. Sections were stained with hematoxylin and eosin, and examined histologically.

RESULTS

Growth and morphological differentiation of carcinoma-derived cells After incubation of tissue fragments for a week or so on plastic in serum-free medium, epithelial cells grew out from the tissue fragments. Under these

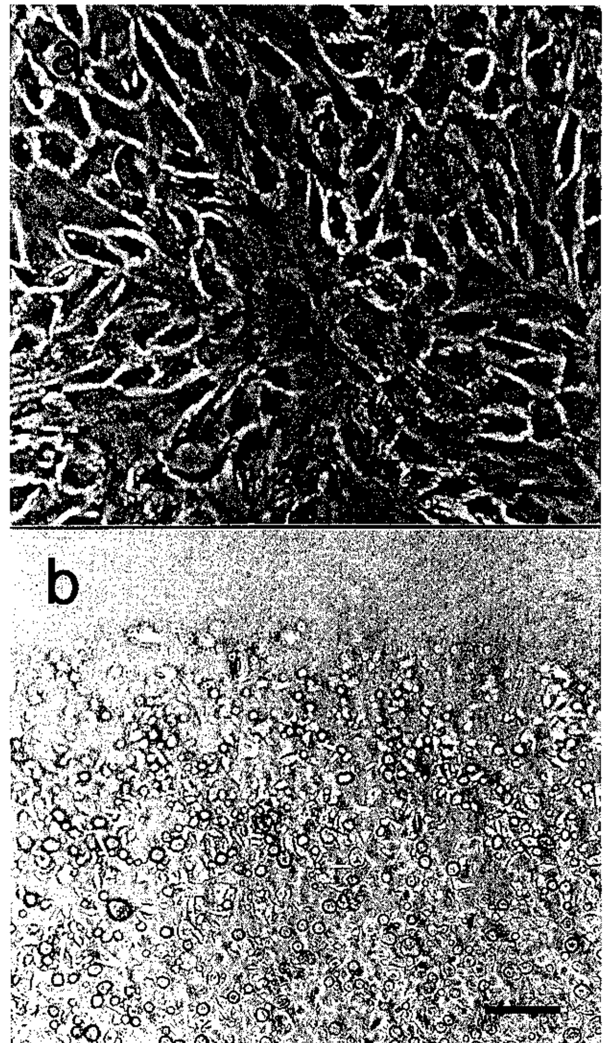

Fig. 1. Phase-contrast micrographs of human epithelial cells derived from breast carcinoma. (a) Monolayer culture on a plastic culture dish after the first passage of cells that had grown out from the tissue fragments. Bar=50 μm. (b) At the second passage, the cells grown as monolayers were harvested and seeded as a micromass (1×10^4 cells/10 μl) between two layers of type I collagen gel. Bar=200 μm.

culture conditions, epithelial cells were predominant, but few if any of the fibroblasts present disappeared during successive cultivation. After the first passage, the predominant cells showed polygonal morphology (Fig. 1a) and immunohistochemical characterization revealed that these cells uniformly displayed the presence of cytokeratins and a low level of expression of epithelial membrane antigen (data not shown), which are characteristics of cultured epithelial cells.[9]

Table I. Effect of Supplement Deletion on Growth of Human Epithelial Cells Derived from Breast Carcinoma

Medium	Mean number of nuclei±SD ($\times 10^3$)[a]	%	P[b]
Control	13.3±2.5	100	
Minus EGF	7.3±1.4	55	<0.01
Minus insulin	9.1±0.6	68	<0.05
Minus transferrin	12.9±0.3	97	
Minus hydrocortisone	7.8±0.2	59	<0.05
Minus ethanolamine and phosphoethanolamine	7.7±0.6	58	<0.05
Minus prolactin	11.7±0.2	88	
Minus prostaglandin	9.6±0.5	72	

a) Triplicate cultures after cultivation for 7 days were scored and the mean±SD is given.
b) Compared with control by Student's *t* test.

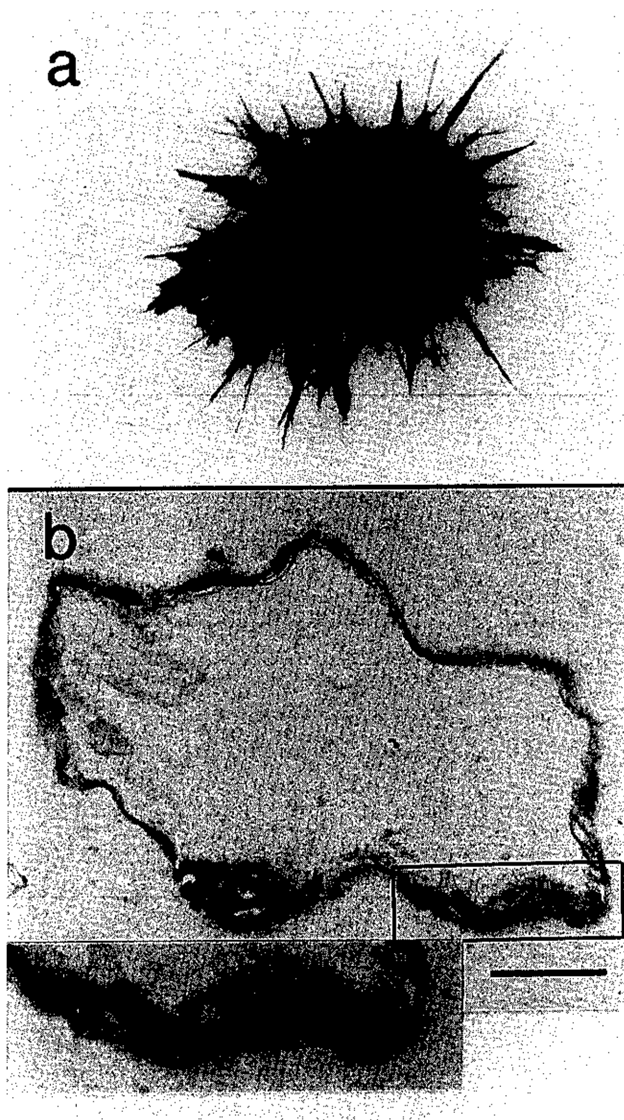

Fig. 2. (a) Bulk morphology of the cell mass which consisted of human epithelial cells derived from breast carcinoma and cultivated between two collagen gels for 7 days in the serum-free medium. The cell mass was stained with Giemsa solution. ×17. (b) Representative histological section of a duct-like extension that protruded from the periphery of the cell mass. H-E staining. Bar=50 μm. The inset is an enlargement of the lower portion of Fig. 2b, as indicated by a rectangle.

To assess the growth and morphogenetic potentials of the epithelial cells, we harvested the cells grown as monolayers and seeded them as a micromass between two layers of collagen gel, followed by feeding with serum-free medium (Fig. 1b). Under these conditions, the cell mass grew slowly and extended many radial duct-like extensions from its periphery within a week (Fig. 2a). These duct-like extensions were shown to contain a lumen surrounded for the most part by a single layer of elongated epithelial cells (Fig. 2b).

Since the serum-free medium used here has been optimized for monolayer culture of human breast epithelial cells,[1,2] deletion of supplements from the medium was carried out to examine the possible contribution of the supplements to cell proliferation and morphological differentiation. After a 7-day culture, carcinoma-derived cells sandwiched between two collagen layers in the control serum-free medium grew slowly; that is, the number of cells increased from 1.0×10^4 to 1.3×10^4 (Table I). Deletion of either EGF, insulin, hydrocortisone, or ethanolamine plus phosphoethanolamine from the control medium caused a significant inhibition of cell proliferation, and the resulting cell numbers after cultivation for 7 days were 55% ($P<0.01$, *t* test), 68% ($P<0.05$), 59% ($P<0.05$), or 58% ($P<0.05$), respectively, of those in the control cultures (Table I). Removal of transferrin, prolactin, and prostaglandin did not result in a significant reduction in cell number.

Contrary to the case of cell proliferation, the number of radial extentions from the carcinoma-derived cell mass was reduced and some short extensions were observed when either EGF (Fig. 3b) or hydrocortisone (Fig. 3e)

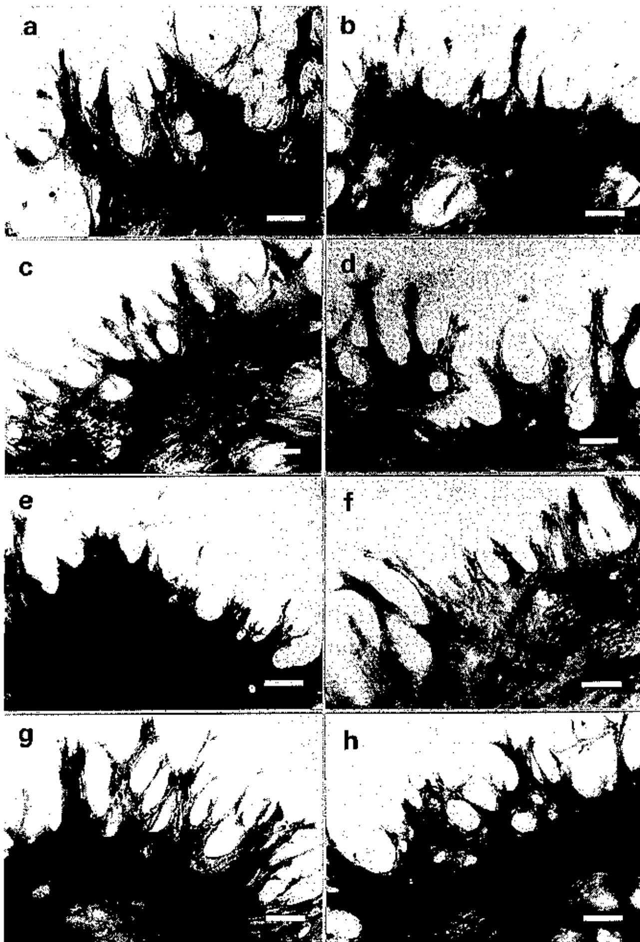

Fig. 3. Effect of deletion of each supplement on morphological differentiation of human epithelial cells derived from breast carcinoma. Cells seeded between two collagen gels were cultivated for 7 days in the serum-free medium (a), or in that lacking EGF (b), insulin (c), transferrin (d), hydrocortisone (e), ethanolamine plus phosphoethanolamine (f), prolactin (g), or prostaglandin (h). Cell masses were stained with Giemsa solution. Bar=200 μm.

was deleted. Removal of each of the other supplements, including insulin, transferrin, ethanolamine plus phosphoethanolamine, prolactin, and prostaglandin, failed to interfere with the morphological development of the cells (Fig. 3).

Growth and morphological differentiation of cells derived from histologically non-malignant tissues As compared to the carcinoma-derived cells, the growth potential of epithelial cells derived from histologically non-malignant breast tissues was significantly higher within collagen gels in the serum-free medium. The number of cells increased from 2.0×10^4 to 7.2×10^4 during 7 days of cultivation

Table II. Effect of Supplement Deletion on Growth of Human Epithelial Cells Derived from Histologically Non-malignant Breast Tissues

Medium	Mean number of nuclei±SD ($\times10^4$)[a]	%	P[b]
Control	7.2±1.6	100	
Minus EGF	2.4±0.2	33	<0.01
Minus insulin	1.7±0.3	24	<0.01
Minus transferrin	5.1±0.1	71	
Minus hydrocortisone	1.6±0.3	22	<0.01
Minus ethanolamine and phosphoethanolamine	3.7±0.5	51	<0.05
Minus prolactin	4.2±1.1	58	<0.05
Minus prostaglandin	3.7±0.6	51	<0.05

a) Triplicate cultures after cultivation for 7 days were scored and the mean±SD is given.
b) Compared with control by Student's *t* test.

(Table II). Deletion experiments showed that removal of each of the supplements, except for transferrin, significantly inhibited the cell proliferation (Table II). Growth dependence of cells from non-malignant tissues on EGF, insulin or hydrocortisone was especially strong.

Epithelial cells from non-malignant tissues also grew and developed many radial duct-like extentions within collagen gels in the serum-free medium (Fig. 4a), as the carcinoma-derived cells did. The number of these radial extensions was reduced and some short extensions were observed when either EGF (Fig. 4b), insulin (Fig. 4c) or hydrocortisone (Fig. 4e) was absent from the medium. In contrast to this, removal of transferrin, ethanolamine plus phosphoethanolamine, prolactin or prostaglandin caused no apparent prevention of morphological differentiation in the cells.

DISCUSSION

Prior to seeding human breast cells between collagen gels, cultivation of the cells which had grown out from tissue fragments as a monolayer on plastic permitted us to observe cell morphology and characterize cell type, allowing the collection of a pure population of epithelial cells. Thus, collected human epithelial cells from either carcinoma or histologicaly non-malignant breast tissues were found to have a potential to grow and to develop many duct-like extensions within collagen gels in serum-free medium supplemented with EGF, insulin, transferrin, hydrocortisone, ethanolamine, phosphoethanolamine, prolactin, and prostaglandin. This result suggests that formation of the duct-like extensions by the human breast epithelial cells may depend on a high cell density

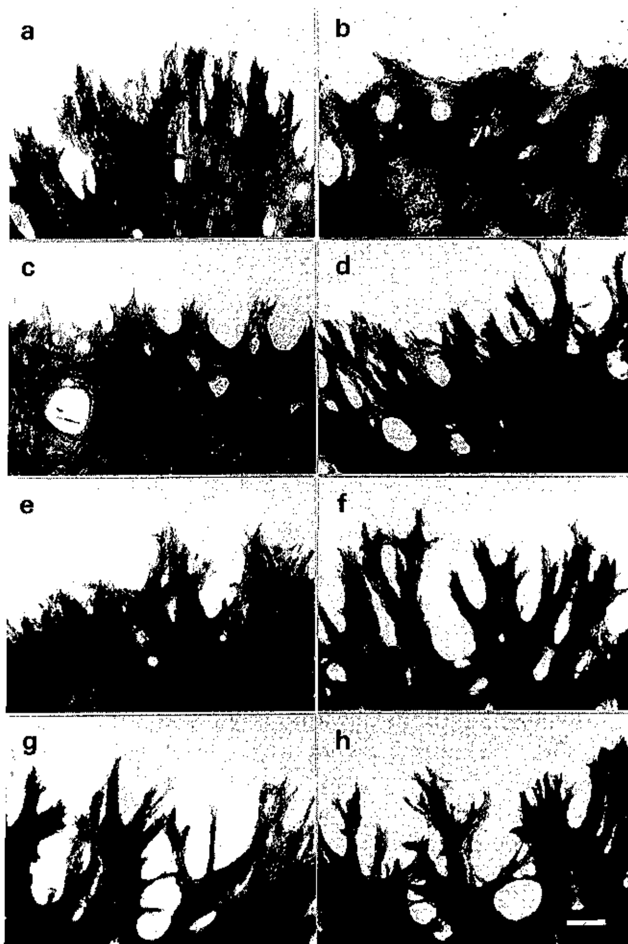

Fig. 4. Effect of deletion of each supplement on morphological differentiation of human epithelial cells derived from histologically non-malignant breast tissues. Cells seeded as a cell mass between two collagen gels were cultivated for 7 days in the serum-free medium (a), or in that lacking EGF (b), insulin (c), transferrin (d), hydrocortisone (e), ethanolamine plus phosphoethanolamine (f), prolactin (g), or prostaglandin (h). Cell masses were stained with Giemsa solution. Bar=200 μm.

such as 10^4 cells/10 μl at seeding within collagen gels where serum is absent. Since cells grow very slowly within collagen gels, cultivation of the cells as a micromass, which minics to some degree the organ culture system, may promote morphological differentiation of the cells. This possibility is supported by a recent study showing that human breast epithelial cells embedded in collagen gels at a low cell density such as 1 to 5×10^4 cells/0.5 ml failed to form any duct-like extentions in serum-free medium that contained EGF, cortisol, and insulin in addition to other supplements.[9]

Deletion experiments showed that EGF and hydrocortisone were essential to proliferation as well as morphological differentiation of both epithelial cells derived from carcinoma and non-malignant breast tissues. A significant suppression of cell proliferation without interfering with the morphological differentiation by the removal of ethanolamine plus phosphoethanolamine, observed in either cell type, suggests that the predominant activities of these factors are mitogenic and that the stimulation of cell proliferation itself is insufficient for the cells to differentiate morphologically. While prolactin and prostaglandin can be classified as factors similar to ethanolamine or phosphoethanolamine for the cells from non-malignant tissues, growth dependence on each of them was not apparent in carcinoma-derived cells. Growth of the cells derived from non-malignant tissues depends on a wider spectrum of growth factors or hormones as compared to that of the carcinoma-derived cells.

Growth dependence of breast epithelial cells on EGF, hydrocortisone and insulin in addition to other supplements has been suggested previously in a monolayer culture system[1, 12, 13] and in a collagen gel culture system.[9] However, the present results show that there is a differential dependence on insulin between epithelial cells derived from carcinoma and non-malignant tissues with respect to the morphological differentiation. This suggests that insulin has two biological activities, promotion of growth and promotion of morphological differentiation, that are separable from each other. In the carcinoma-derived cells, morphological differentiation alone was independent of the presence of insulin. The mechanism involved in morphological differentiation of mammary epithelial cells is not understood yet; however, the carcinoma-derived cells may not require insulin when other growth factors or hormones such as EGF or hydrocortisone are present. Alternatively, since many human breast carcinoma cell lines have been found to produce insulin-like growth factor-I-related protein,[14] the carcinoma-derived cells themselves may possibly produce insulin-like factor, thereby leading to their independence of exogenous insulin.

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