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Effects of Lactogenic Hormones on Morphological Development and Growth of Human Breast Epithelial Cells Cultivated in Collagen Gels

Kazuhide Takahashi,[1,3] Katsuo Suzuki,[1] Satoru Kawahara[2] and Tetsuo Ono[1]

[1]*Department of Biochemistry, Research Institute and* [2]*Department of Surgery, Kanagawa Cancer Center, 54-2 Nakao-cho, Asahi-ku, Yokohama 241*

We investigated the hormonal dependence of the growth and morphological development of human breast epithelial cells (HBEC) cultivated in collagen gels floating on serum-free medium. HBEC used had grown out from breast carcinoma tissue and from histologically non-malignant tissue fragments in the same mastectomy specimens. In the presence of all three lactogenic hormones, hydrocortisone (HC), insulin (INS), and prolactin (PRL), both carcinoma-derived and histologically non-malignant tissue-derived HBEC grew and formed cell masses with protruding tubule-like extensions that consisted of a multilayer of cells. Deletion of any one of the hormones from the medium had no effect on cell growth of HBEC derived from either tissue type. However, morphological development, especially the morphology of the tubule-like extensions, was altered in the histologically non-malignant tissue-derived HBEC by deletion of HC or INS, and by deletion of HC, but not INS, in the carcinoma-derived HBEC. The present results suggest that the carcinoma-derived HBEC have decreased responsiveness to INS as compared to that of HBEC derived from histologically non-malignant breast tissue.

Key words: Breast carcinoma — Lactogenic hormone — Morphological development — Collagen gel culture

In a pioneering study, Emerman and Pitelka[1] demonstrated that, when collagen gel was used as an extracellular matrix, mouse mammary epithelial cells grew and developed into a structure that morphologically resembled the mammary gland. Successive investigations using the collagen gel culture system showed that it facilitated the sustained growth and morphological development of mammary epithelial cells from rodents[2–11] and humans[12, 13] *in vitro*. Morphological development as well as functional differentiation of rodent mammary epithelial cells in the collagen gel culture system can be induced in response to lactogenic hormones, hydrocortisone (HC), insulin (INS), and prolactin (PRL).[2–5] In contrast to the many studies of rodent cells, there have been few reports on the hormonal inducibility of growth and morphological development of human breast epithelial cells (HBEC) *in vitro*, whether in the presence of serum[12] or in serum-free medium.[13]

In a previous study, we used collagen gel attached to a plastic culture substratum with serum-free medium as an extracellular matrix, and we found that the growth of breast carcinoma-derived HBEC required HC and INS, while that of histologically non-malignant tissue-derived HBEC required PRL along with other supplements in addition to HC and INS.[13] The morphological development of carcinoma-derived HBEC required HC, and that of non-malignant tissue-derived HBEC required INS in addition to HC. Since the morphological development of mouse mammary epithelial cells was found to be enhanced by floating the collagen gels on medium,[8–10] in the present study we used floating collagen gels to investigate the effects of the three lactogenic hormones HC, INS and PRL on the growth and morphological development of HBEC.

MATERIALS AND METHODS

Tissue collection and monolayer culture Fresh tumor tissue fragments were dissected from tumors removed at mastectomy from patients with breast carcinoma. Histologically non-malignant tissue fragments were obtained from areas away from the primary lesion in the same mastectomy specimens.[14, 15] The diagnoses were based on histological findings. The tissue fragments were minced into smaller fragments with scissors, and cultivated on plastic culture dishes in MCDB 170 serum-free medium supplemented with 10 ng/ml EGF, 5 μg/ml insulin, 5 μg/ml transferrin, 1.4 μM hydrocortisone, 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, 1 μg/ml ovine prolactin, and 25 nM prostaglandin (Sigma),[16] as previously described.[15] Cells that grew out from the tissue fragments were subcultured as monolayers in identical medium to yield a sufficient number of cells.

Collagen gel culture For standard cultures using collagen gels, a 0.5 ml base layer of type I collagen (Nitta Gelatin) gel was applied to each well of 24-well plastic

[3] To whom correspondence should be addressed.

plates at a final concentration of 0.1% collagen.[13] Monolayer cultures of the cells were harvested, and one 10-μl drop of cell suspension containing 1×10^4 viable cells was placed on each collagen layer. After incubation at 37°C for 2 h, the cells in each well were overlaid with 0.5 ml of a 0.1% collagen solution. After gelation, the wells were flooded with 1 ml of serum-free medium. Two days later, the collagen gels were detached from the plastic plate, and the media were replaced with fresh media.

Histological observation For histological observation, cells in collagen gels were fixed with 10% buffered formalin, dehydrated, and embedded in paraffin. Sections 4 μm in thickness were stained with hematoxylin and eosin.

Cell growth assay HBEC grown within collagen gels were washed once with phosphate-buffered saline (PBS), and the gels were then minced into small fragments with scissors and incubated with 40 U/ml collagenase (type IA-S; Sigma) for 1 h at 37°C on a reciprocating shaker. Next, the cells were washed with PBS, fixed with cold (−20°C) ethanol, and air-dried. After incubation of the cells with 3,5-diaminobenzoic acid (Aldrich) for 45 min at 60°C, DNA content was measured spectrofluorometrically (Ex at 415 nm, Em at 505 nm) by a previously described method[17] using salmon sperm DNA as a standard.

RESULTS

Growth and morphological development of HBEC derived from histologically non-malignant tissue During cultivation within collagen gels floating on serum-free medium containing HC, INS, and PRL along with other supplements, referred to hereafter as the complete medium, HBEC derived from histologically non-malignant tissue grew to form a cell mass in which the boundaries between individual cells were obscured. At 7 days after the start of culture, there were many tubule-like extensions protruding from the cell mass (Fig. 1a).

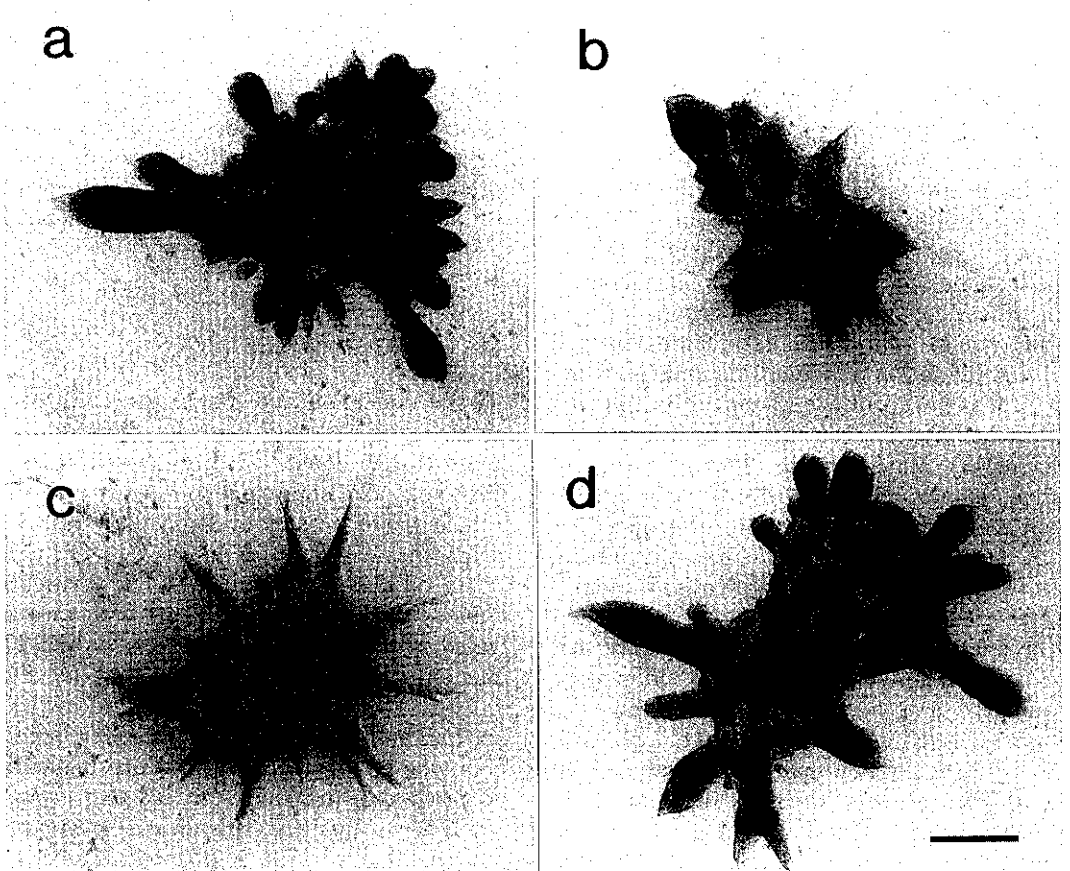

Fig. 1. Morphology of HBEC derived from histologically non-malignant breast tissues cultured for 7 days within a collagen gel floating on the complete medium (a), and on medium lacking HC (b), lacking INS (c), or lacking PRL (d). Bar=500 μm.

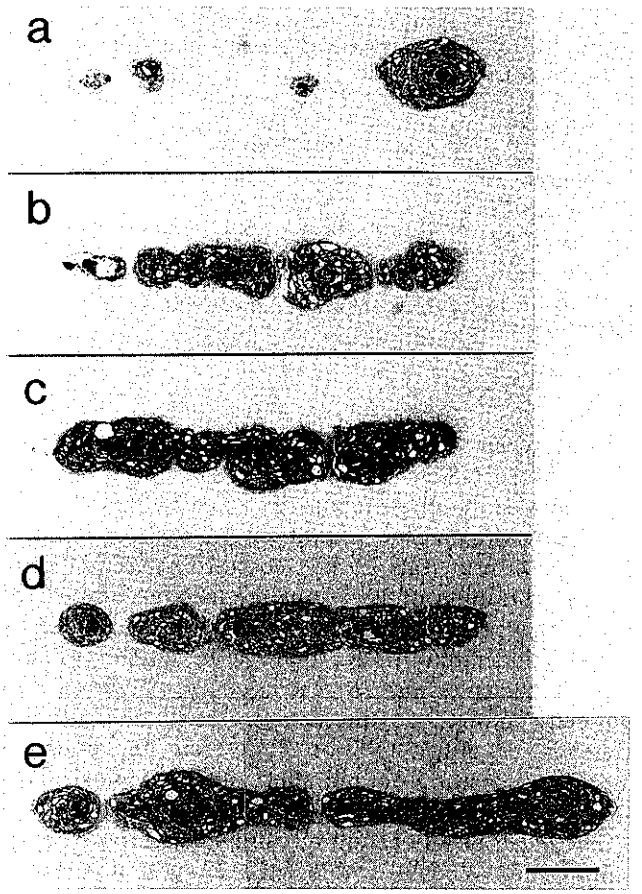

Fig. 2. Serial cross section of a cell mass with protruding tubule-like extensions of HBEC derived from histologically non-malignant tissue grown within floating collagen gel for 7 days in the complete medium. H-E staining. Bar=200 μm.

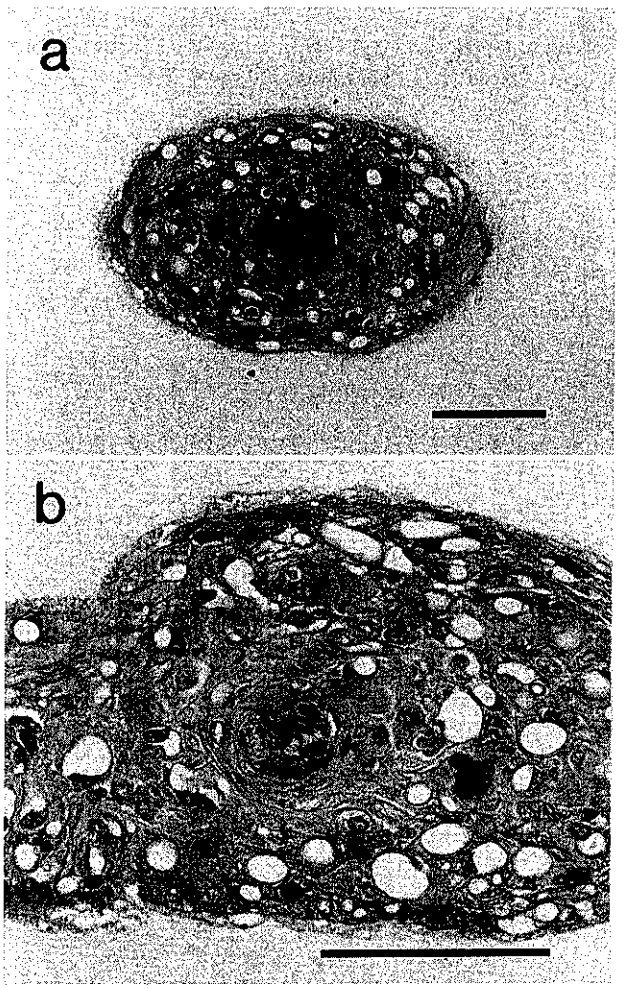

Fig. 3. Histological section of a tubule-like extension protruding from the cell mass (a) and the cell mass (b). Note that the central regions of the tubule-like extension and the cell mass are strongly stained with eosin. Bar=100 μm.

Serial cross-sections of the cell mass, which presented radiating tubule-like extensions, revealed that both the extensions and the cell mass consisted of a multilayer of cells (Fig. 2). There were many cells with vacuoles, and the central region of the extensions was occasionally strongly stained with eosin (Fig. 3).

Deletion of HC from the medium altered the morphological development, including that of the tubule-like extensions from the cell mass, of HBEC derived from histologically non-malignant tissue (Fig. 1b), with the number of extensions being reduced in comparison with that in HBEC cultured in the complete medium. When HBEC were cultivated for 7 days within collagen gels floating on INS-deleted medium, the tubule-like extensions formed were much finer than those formed in the complete medium (Fig. 1c). Deletion of PRL from the medium did not affect the morphological development of the cells (Fig. 1d).

The effect of deletion of each of the three lactogenic hormones from the medium on cell growth was determined by measurement of DNA content after cultivation of the cells from histologically non-malignant tissue within collagen gels for 7 days. The mean DNA content of cultures of HBEC on the complete medium was 1.03 μg (Table I). Deletion of HC from the medium slightly increased the DNA content, but this increase was not statistically significant. When INS or PRL was deleted from the medium, the mean DNA content was reduced to 79% and 84%, respectively, of that of cultures on the

Table I. DNA Content of Human Breast Epithelial Cells Derived from Histologically Non-malignant Tissue Cultured within Collagen Gels Floating on Different Media

Media	DNA content (μg/culture)[a]	%
Complete	1.03±0.17	100
Minus HC	1.30±0.16	126
Minus INS	0.81±0.10	79
Minus PRL	0.86±0.27	84

a) Mean±SD for three cultures after cultivation for 7 days.

Table II. DNA Content of Cultures of Carcinoma-derived Human Breast Epithelial Cells Cultured within Collagen Gels Floating on Different Media

Media	DNA content (μg/culture)[a]	%
Complete	1.08±0.36	100
Minus HC	0.97±0.39	90
Minus INS	1.22±0.68	113
Minus PRL	1.72±0.30	159

a) Mean±SD for three cultures after cultivation for 7 days.

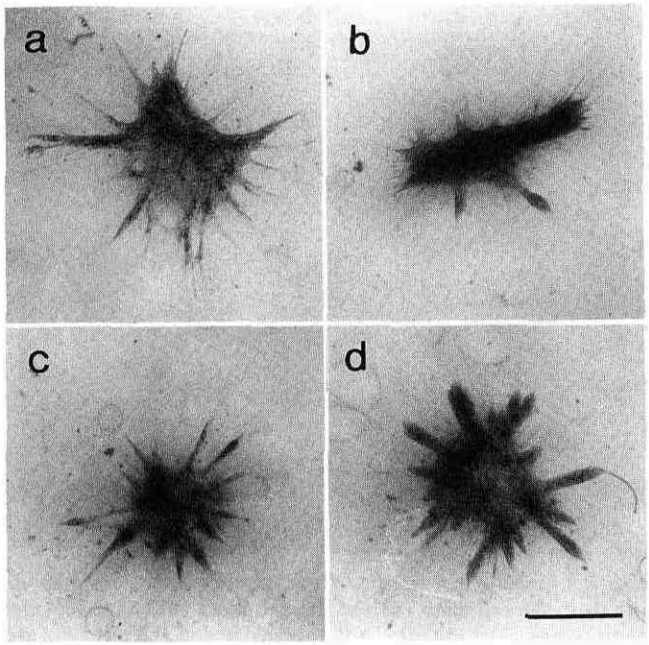

Fig. 4. Photomicrographs of the overall morphology of the cell mass formed by carcinoma-derived HBEC cultured for 7 days within collagen gels floating on complete medium (a), and on medium lacking HC (b), lacking INS (c), or lacking PRL (d). Bar=1 mm.

complete medium; these changes had no statistical significance.

Growth and morphological development of carcinoma-derived HBEC Carcinoma-derived HBEC within collagen gels floating on complete medium grew and formed a cell mass with protruding tubule-like extensions after 7 days of culture (Fig. 4a). However, the radial extensions were finer than those formed by histologically non-malignant tissue-derived HBEC cultured on complete medium, with the overall morphology of the three-dimensional structure being similar to that of the structure formed when non-malignant tissue-derived cells were cultured on INS-deleted medium. Histological observation revealed that these tubule-like extensions and the cell mass consisted of a multilayer of cells containing eosinophilic materials in their central regions (data not shown). Deletion of HC from the medium altered the morphological development of the cell mass formed by carcinoma-derived HBEC, that is, the number of protruding extensions was reduced as compared with that of the cells cultured on the complete medium (Fig. 4b). Deletion of either INS or PRL from the complete medium apparently did not affect the morphological development (Figs. 4c and d).

The mean DNA content of carcinoma-derived HBEC cultured for 7 days within collagen gels floating on complete medium was 1.08 μg (Table II). Deletion of either HC or INS from the medium did not significantly affect the DNA content of the cells. In the absence of PRL, however, mean DNA content was 1.72 μg, being 159% of mean DNA content of cells within collagen gels floating on complete medium (Table II). However, this difference between the mean DNA contents of the two culture media was not statistically significant.

DISCUSSION

HBEC derived from either carcinoma or histologically non-malignant breast tissue grew within collagen gels floating on serum-free medium containing the three lactogenic hormones HC, INS and PRL to form an initially spherical cell mass in which the boundaries between the individual cells were obscure. During subsequent cultivation for 7 days, the cell mass formed protruding tubule-like extensions (Fig. 1). Formation of this three-dimensional structure was shown to be facilitated by floating the gels on medium as opposed to attaching the gels directly onto the plastic substrata.[13] This result accords with previous findings for mouse mammary epithelial cell cultures.[8-10] However, the lumen was infrequently surrounded by polarized epithelial cells, de-

spite the fact that there were many eosinophilic and necrotic cells in the central regions of both the tubule-like extensions and the cell mass. Deletion of either HC or INS from the medium altered the morphology of the protruding tubule-like extensions from the cell mass in cultures of HBEC derived from histologically non-malignant tissues. However, the morphological development of carcinoma-derived HBEC cultures was affected by deletion of HC, but not by deletion of INS. The overall morphology of the three-dimensional structure formed by the carcinoma-derived HBEC on the complete medium was similar to that formed by HBEC derived from histologically non-malignant tissue on medium lacking INS. With respect to morphological development, it is suggested that carcinoma-derived HBEC may not respond to INS and may form a three-dimensional structure with a morphology similar to that formed by histologically non-malignant tissue-derived cells in INS-depleted medium. For both carcinoma-derived HBEC and HBEC derived from histologically non-malignant tissue, these hormonal responses are essentially the same as those found when collagen gels are attached directly onto plastic plates.[13]

Contrary to these effects on morphological development, deletion of any of the three lactogenic hormones did not affect the growth of HBEC derived from either carcinoma or histologically non-malignant breast tissues within collagen gels floating on serum-free medium. These results are quite different from those obtained in previous research using collagen gels attached directly to plastic substrata, which showed that the growth of histologically non-malignant tissue-derived HBEC depended on the presence of all three hormones, and that that of carcinoma-derived HBEC depended on HC and INS.[13] That the growth of carcinoma-derived HBEC and HBEC derived from histologically non-malignant tissue within floating collagen gels is not dependent on any of the three hormones suggests that floating the gels on the medium alters in some unknown manner the growth dependence of the cells on these hormones.

The most characteristic difference between the cells within floating gels and those within attached gels is their shape. While the cells in attached gels are elongated,[13] the cells in floating gels are round. Rounding of the cell is affected by contraction of the gel, induced by the presence of the cells in it, and is enhanced by floating the gel on the medium.[18] In the human breast carcinoma cell line MCF-7, a change in the culture substrate is accompanied with a change in cytoskeletal structure.[19] The loss of growth dependence of HBEC on the lactogenic hormones within floating collagen gel is suggested to be related to this change in cell shape and cytoskeletal structure.

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