Regulation of Cytoskeletal Structure in Human Mammary Carcinoma Cells MCF-7 by Culture Substrata

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ABSTRACT. Three-dimensional cellular structures formed by MCF-7 human mammary carcinoma cells within collagen gels were isolated with collagenase and cultivated on plastic substratum to examine whether the cytoskeleton specific for cells forming cellular structures (S-type) changes to that specific for cells grown as monolayers (M-type). The cytoskeleton isolated as 0.05% Triton-insoluble fraction from the cellular structures after culture for 1 day on plastic was exclusively S-type. However, both types of cytoskeletons were observed in the cellular structures cultivated for 7 days on plastic as well as in the cells grown as monolayers for 2 days after dissociation of the cellular structures with trypsin. By use of an antibody raised against a 65-kD polypeptide that was specific for the M-type cytoskeleton, the presence of the polypeptide was found to be restricted to the cells grown out as monolayers from the edge of the cellular structures. In the cells grown for 2 days as monolayers, a mixture of cells both having and lacking the polypeptide was observed. After a 7-day culture of the dissociated cells as monolayers on plastic, however, most of the cells had M-type cytoskeletons. The present results show that the apparent change in the cytoskeleton of MCF-7 cells from S-type to M-type does not occur in cells involved in the three-dimensional cellular structures even in the absence of collagen gels, but that it occurs in cells which are grown as monolayers for at least 7 days on plastic substratum.

The mammary gland and mammary epithelial cells have been well investigated as a useful model to analyze the mechanism involved in the morphogenesis of epithelial cells. The early pioneering work of Emerman and Pitelka (3) has demonstrated that it is possible to form three-dimensional cellular structures resembling the mammary gland in situ by the use of collagen gels as an extracellular matrix in place of fat tissues. Subsequent investigations using collagen gels succeeded in inducing the morphological development in mammary epithelial cells of mouse (13, 16), rat (9), and human (11, 15). However, little is known about the cellular factors associated with its morphological development.

Human mammary carcinoma cell line MCF-7 cells have been previously shown to form three-dimensional cellular structures within collagen gels (12), yielding a model system that permits the biochemical analysis of the morphological development in human mammary epithelial cells. By the use of this culture system, it has been demonstrated that the cytoskeleton changes from that specific for cells in monolayers (M-type) to that for cells forming three-dimensional cellular structures (S-type) within collagen gels (12).

In the present study, to examine the stability of the cytoskeleton in the cellular structures once formed, we isolated the cytoskeletal structures from collagen gels with collagenase followed by cultivation on plastic substratum with or without trypsinization. By the use of an antibody against a polypeptide that was specific for the M-type cytoskeleton, we demonstrated here that the isolated cellular structures on plastic substratum retain their S-type cytoskeletons for at least 7 days of cultivation and that M-type cytoskeleton appeared in cells grown out as monolayers from the cellular structures or in cells grown on plastic for 7 days after being dissociated by treatment with trypsin.

MATERIALS AND METHODS

Collagen gel culture. For standard culture using collagen gels, a layer (0.5 ml) of 0.21% type I collagen was made in each well of 24-well plastic plates as described previously (11, 12). After gelation of collagen layers, MCF-7 cells that had been maintained as monolayers in RPMI1640 medium supplemented with 10% fetal bovine serum and 10⁻⁸ M 17β-estradiol were harvested by treatment with 0.25% trypsin and 0.1% EDTA followed by seeding onto each collagen gel layer as 10 µl drops of cell suspension containing 10⁴ viable cells. The

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cells were then incubated for 2 h at 37°C in a CO₂-incubator and flooded with 1 ml of medium. Each collagen gel layer was detached from the culture plates after cultivation for 3 days.

Isolation of cellular structures from collagen gels. The collagen gels involving three-dimensional cellular structures such as cylindrical or domed structures formed by MCF-7 cells were washed twice with phosphate-buffered saline (PBS) and minced into small fragments by a pair of scalpels. The gel fragments were incubated with 40 U/ml of type IA collagenase for 1 h at 37°C in a reciprocating shaker. The cellular structures were collected, washed twice with PBS by centrifugation and placed onto plastic dishes containing a growth medium. In

Fig. 1. Phase-contrast micrograph of three-dimensional cellular structure of MCF-7 cells that was isolated from collagen gels with collagenase and cultivated on plastic culture dish for 1 day (A, B, C) or 7 days (D, E). The cellular structure of MCF-7 cells in A and B adhered onto the surface of the plastic dish by means of the cells that grew out from the edge of the cellular structures. In C, an enlargement of monolayers of cells in B, surrounding the spherical cell mass. Bar, 200 μm.
some experiments, the isolated cellular structures that had been cultivated on plastic substratum for 5 days were dissociated by treatment with 0.25% trypsin and 0.05% EDTA followed by seeding onto plastic culture dishes as monolayers.

Preparation of isolated cytoskeletons. The three-dimensional cellular structures or cells grown as monolayers on plastic were harvested with a rubber policeman in stabilizing buffer (6), designated as SB, which was slightly modified so as to consist of 0.15 M NaCl, 1 mM MgCl₂, 1 mM EGTA, 2 mM ATP, 20 mM HEPES (pH 6.9), and 0.5 mM phenylmethylsulfonyl fluoride (12). Next, the cells were homogenized in 5 volumes of SB containing 0.05% Triton X-100 and 30% glycerin in a Dounce homogenizer with a tight fitting pestle by 5 strokes at room temperature. The cell homogenate layered onto a discontinuous sucrose gradient that was composed of 1 ml each of 40, 50, 60, 70, and 80% sucrose in SB was spun at 150,400 × g (40,000 rpm) for 1 h at 20°C in a centrifuge equipped with an RPS50-2 rotor (Hitachi Koki Co., Tokyo). Isolated cytoskeleton was identified as a white flocculent layer in the sucrose gradient.

Two-dimensional gel electrophoresis. The isolated cytoskeleton was dissolved by two-dimensional gel electrophoresis (8) using a carrier ampholite having a pH range of 3–10 (Bio-Rad Laboratories, Richmond, CA). Slab gels as the second dimension of electrophoresis were stained with Coomassie blue.

Preparation of monoclonal antibody against the 65-kD polypeptide. The 65-kD polypeptide separated in SDS-PAGE as the second dimension of electrophoresis was excised and eluted electrophoretically from the gels. The monoclonal antibodies were obtained by a routine procedure (4) with BALB/c mice injected with electrophoresed 65-kD polypeptide as immunogens with Freund's complete adjuvant. Spleen cells from immunized mice were fused with NS-1 myeloma cells and hybridoma cells were screened by immunoblot analysis. Cells showing positive reactions were subcloned by limiting dilution.

Immunoblots. Polypeptides separated in SDS-PAGE were electrophoretically transferred (14) onto a polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, MA) (7) as previously described (12). The reactivity of the antibody against the 65-kD polypeptide was visualized with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions.

Indirect immunofluorescent microscopy. The three-dimensional cellular structures or cells grown on glass slides of Lab-Tek chambers (Nunc Inc., Naperville, IL) were washed once with SB and then treated with 0.05% Triton X-100 and 30% glycerin in SB for 10 min at room temperature. The slides were then fixed with chilled (−20°C) methanol for 5 min and air-dried. The slides were incubated with the antibody for 60 min at room temperature followed by FITC-conjugated antibodies against the mouse immunoglobulins (IgG + IgM + IgA) for another 1 h at room temperature. Anti-actin antibody was purchased from Amersham International plc.

**RESULTS**

Morphological change. Most of the three-dimensional cellular structures isolated from collagen gels by treatment with collagenase adhered onto the surface of plastic dishes after culture for 1 day. The cellular structures derived from the cylindrical structures adhered by means of the cells that grew out as monolayers (Fig. 1A), while those derived from the domed structures appeared as a spherical cell mass (Fig. 1B) adhering by means of the monolayered cells that surrounded the cell mass (Fig. 1C). After culture on plastic for 7 days, the cellular structures derived from the domed structure became flattened, remaining in the form of many cellular folds on the cellular sheet (Fig. 1D), and some other cellular structures remained as distinct cell masses at the central region of the cellular sheets (Fig. 1E). Such morphological changes in the cellular structure and outgrowths of cells as monolayers from the edge of the cell mass could be observed until around 7 days of culture, after which point no additional changes in its morphology or outgrowth of cells were observed even when the culture was continued for up to 2 weeks with several medium changes.

Isolated cytoskeleton. The 0.05% Triton-insoluble cytoskeleton isolated from the cellular structures grown for 1 day on plastic sedimented as a single flocculent layer at the boundary between the 60% and 70% sucrose layers after ultracentrifugation (Fig. 2, tube 1), corresponding to S-type cytoskeleton (12). At 7 days of culture, the cytoskeleton of the cellular structures was...
K. Takahashi and T. Ono

separated into two discrete layers with a different buoyant density from each other in a sucrose gradient (Fig. 2, tube 2). The cytoskeleton with a smaller buoyant density that sedimented at the boundary between the 50% and 60% sucrose layers is the M-type cytoskeleton, and the other at the boundary between the 60% and 70% sucrose layers is the S-type. At 5 days of culture on plastic substratum, the three-dimensional cellular structures were dissociated into single cells by treatment with trypsin followed by cultivation as monolayers on plastic substratum. Two days later, the cytoskeleton isolated from the cells separated into two cytoskeleton with different buoyant densities, consisting of S- and M-type cytoskeletons (Fig. 2, tube 3). After culture of the dissociated cells as monolayers for 7 days on plastic, M-type cytoskeleton with a smaller buoyant density became predominant and no apparent S-type cytoskeletons sedimented at the boundary between the 60% and 70% sucrose layers (Fig. 2, tube 4).

**Solubility of cytoskeletal components.** We have shown previously that the M-type cytoskeleton consisted of two specific polypeptide components with apparent molecular sizes of 65-kD and 80-kD (12). These two polypeptides as well as 4 other prominent cytoskeletal polypeptides were insoluble in 0.05% Triton (Fig. 3A), while the cytoskeleton isolated from monolayered cells as 0.5% detergent-insoluble fraction sedimented on a 80% sucrose layer (Fig. 3B) and lost the two polypeptides that were specific for the M-type cytoskeleton (Fig. 3C). This indicated that the two polypeptides are soluble in 0.5% Triton. In contrast, the actin as well as 3 other prominent polypeptides was present in both types of cytoskeleton (12) and was insoluble in 0.5% Triton.

![Fig. 3. Solubility of cytoskeletal component in Triton X-100.](image)

**A.** Two-dimensional gel electrophoresis of the 0.05% Triton-insoluble cytoskeleton isolated from the cells grown as monolayers (M-type cytoskeleton). Arrow indicates the 65-kD polypeptide which is one of two polypeptide components specific for M-type cytoskeleton. The actin is indicated as A.

**B.** Sedimentation of the 0.5% Triton-insoluble cytoskeleton from the monolayered culture of MCF-7 cells.

**C.** Two-dimensional gel electrophoresis of the 0.5% Triton-insoluble cytoskeleton isolated as shown in B. Note that the M-type cytoskeleton-specific 65-kD polypeptide is lost, while the actin remains.

![Fig. 4. Reactivity of an antibody raised against the 65-kD polypeptide.](image)

**A.** Polypeptides of M-type cytoskeleton separated on two-dimensional gel electrophoresis were transferred onto a PVDF membrane and reacted with the anti-65-kD polypeptide. Reactivity was visualized according to the method described in Materials and Methods. B. The Coomassie blue-stained polypeptides which were transferred onto the PVDF membrane.
Regulation of Cytoskeleton by Culture Substrata

Triton. To examine whether the two cytoskeletons co-exist in the cellular structures or in the monolayered cells (Fig. 2), we chose the 65-kD polypeptide (pI = 5.5) as the M-type cytoskeleton-specific component and prepared an antibody against it (Fig. 4).

**Immunofluorescent microscopy.** Immunofluorescent microscopy using the antibody prepared against the 65-kD polypeptide revealed that the 65-kD polypeptide was distributed along thick filaments that run for long distances and in a complex network surrounding nuclei (Figs. 5A and B). The actin, a common component in the two types of cytoskeletons, was distributed in a fine cytoskeletal network spreading over the cells (Fig. 5C). Treatment of the cells with 0.5% Triton solubilized nearly all the 65-kD polypeptide (Fig. 5D), while the actin remained unchanged after the same treatment (Fig. 5E).

When the cellular structures after culture for 7 days on plastic were treated with 0.05% Triton and reacted with the antibody against the 65-kD polypeptide, the presence of the polypeptide was found to be restricted to the edge of the cellular sheet (Fig. 6A). The cellular sheet consisting of multilayers of cells and the cell mass at the central region of the cellular sheet failed to react with the antibody. Contrary to this, the actin was distributed equally in the cellular sheet (Fig. 6B). In the 0.05% Triton-treated monolayer cultures of MCF-7 cells grown for 2 days after treatment with trypsin, a mixture of cells having or lacking the 65-kD polypeptide were present (Figs. 6C and D). In contrast, the actin was found in every cell grown as monolayer and no apparent difference among cells in its reactivity to the antibody could be seen (Fig. 6E). After treatment of the cells with 0.5% Triton, no positively stained cells could be detected by the anti-65-kD polypeptide antibody, while the reactivity of cells to the anti-actin antibody

Fig. 5. Intracellular distribution of the 65-kD polypeptide and the actin. Cells grown on glass coverslips were subjected to 0.05% (A, B, C) or 0.5% Triton X-100 (D, E) prior to reaction with the antibody against the 65-kD polypeptide (A, B, D) or the actin (C, E).
Fig. 6. Indirect immunofluorescent micrograph of the cellular structures cultivated on plastic for 7 days (A, B) or the cells grown as monolayers plastic for 2 days after being dissociated with trypsin (C, D, E). The 0.05% Triton-treated cellular structures or cells in monolayers were reacted with the antibody against the 65-kD polypeptide (A, C, D) or the actin (B, E).

was not affected (data not shown).

DISCUSSION

The morphological development of the mammary gland during embryogenesis requires the presence of stroma (5, 10). It has been shown that the role of stroma in the morphogenesis of the mammary epithelial cells as the extracellular matrix could be replaced in vitro by collagen gel (2, 3, 9, 11, 13, 15). By the use of the collagen gel culture system, changes in cell shape were observed to occur during the morphological development (2), and it has been suggested that the contraction of collagen gel is important in these changes (1). Our previous study showed that MCF-7 human mammary carcinoma cells form three-dimensional cellular structures within floating collagen gels, accompanied by alteration of cytoskeletal structure with significant decreases in the amounts of two polypeptide components (12). The present results show that the three-dimensional cellular structures isolated from collagen gels had exclusively S-type cytoskeleton after culture for 1 day on plastic substratum but changed to predominantly M-type cytoskeleton, in addition to the S-type one, after 7-day culture. There are two possible explanations for the existence of the two types of cytoskeletons in the cellular structure; either cell possesses both types of cytoskeletons, or cells having one of the two types of cytoskeletons coexist. Immunofluorescent microscopic observations support the latter possibility and suggest that the presence of M-type cytoskeleton may be restricted in cells which are located in the outermost region of the cellular sheet, corresponding to the region where newly dividing cells grew out as monolayers on plastic. In contrast, the three-dimensional cellular structures once formed were stable for up to 2 weeks on plastic substratum, suggesting that they retain their S-type cytoskeletons.

After cultivation of the dissociated cells as monolayers on plastic substratum for 2 days, they became to have M-type cytoskeleton in addition to the S-type one. The present result suggests that cells having M-type or S-type cytoskeleton are present in mixed population. The cells which have M-type cytoskeleton may be derived from cells that grew out as monolayers from the cellular structures and the cells which have S-type cytoskeleton may be derived from the cells that formed the three-dimensional cellular structures before being tryp-
sinized. If this assumption is correct, the cultivation of the dissociated cells as monolayers for 2 days on plastic is insufficient for the cells to have the M-type cytoskeleton in place of the S-type one. After cultivation of the dissociated cells as monolayers on plastic for 7 days, however, the cytoskeleton in most cells changed to the M-type. When the three-dimensional cellular structures that had been formed within collagen gels were treated successively with collagenase and trypsin, followed by cultivation of the dissociated cells on plastic substratum for 7 days, the 0.05% Triton-insoluble cytoskeleton also changed to M-type (data not shown). One possible explanation for the change in cytoskeleton of monolayered cells is that the cells which have M-type cytoskeleton divide successively to overwhelm the nondividing cells with S-type cytoskeleton. Alternatively, it is also possible that the MCF-7 cells which previously had S-type cytoskeletons change to the M-type one during culture for 7 days on plastic.

It was suggested that the major difference between S- and M-type cytoskeletons with respect of their buoyant density was due to the presence or absence of the 65-kD and 80-kD polypeptides as the cytoskeletal components (12). The present results show that after exposure of the monolayered MCF-7 cells to 0.5% Triton the isolated cytoskeleton not only loses the two polypeptides but also sediments on the 80% sucrose layer. However, the 0.05% detergent-insoluble S-type cytoskeleton, which sediments at the boundary between the 60% and 70% sucrose layers, was virtually lacking in the 65- and 80-kD polypeptides (12). The present results suggest that the 4 other basal polypeptides can form the 0.5% detergent-labile cytoskeletal network, such as that found in the three-dimensional cellular structures, even in the absence of the two polypeptides specific to the M-type cytoskeleton. The latter two polypeptides are speculated to interact with the cytoskeletal network that consist of the 4 basal polypeptides to form a fine cytoskeletal network with a small buoyant density.

Cultivation of the three-dimensional cellular structures on plastic substratum somewhat resembles the primary culture of tissue fragments from the mammary gland on plastic. In the latter case, many cells that grow out from the tissue fragments as monolayers and the cells are trypsinized on successive subcultures. The present results suggest that the monolayer cultures of mammary epithelial cells may affect the cellular properties, including the cytoskeletal structure, characteristic of the mammary epithelium in vivo. It was demonstrated that mouse mammary epithelial cells maintained on floating collagen gels retained many characteristics of the mammary epithelial morphology as well as the ability to produce milk protein casein (2, 3). However, the same cells cultivated on plastic substratum lost these differentiated characteristics, being accompanied by drastic alterations of morphology.

REFERENCES

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