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Non-enzymatic harvest of renal epithelial cell sheets using temperature-responsive culture dishes.

Ai Kushida, Masayuki Yamato, Chie Konno, Akihiko Kikuchi and Teruo Okano
Institute of Biomedical Engineering, Tokyo Women's Medical University,
8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan
Fax: 81-3-3359-6046, e-mail: tokano@lab.twmu.ac.jp

We have developed a temperature-responsive culture dish grafted with poly(*N*-isopropylacrylamide) (PIPAAm). In the present study, a renal epithelial cell line, Madin-Darby canine kidney (MDCK) cell was cultured on these dishes. MDCK cells showed differentiated phenotypes such as dome formation during a long term culture, similarly on ungrafted dishes. A short time incubation at 20°C released whole confluent MDCK cells as a single contiguous cell sheet only from the polymer-grafted dishes because of the rapid hydration of PIPAAm. Since the harvest doesn't need enzymatic digestion, differentiated cell phenotypes should be retained. Immunocytochemistry with anti-β-catenin and anti-fibronectin (FN) antibodies revealed that functional cell-cell junctions were maintained even in the recovered cell sheets and the FN matrix deposited during the culture was also recovered together with cell sheets. Since these structures are highly susceptible to trypsin digestion, only the PIPAAm-grafted culture dishes enable us to recover such a cell sheet that would be promising in tissue engineering field.

Keywords: temperature-responsive culture surfaces, poly(*N*-isopropylacrylamide), MDCK cell, tissue engineering

1. INTRODUCTION

To attain the true goals promised by tissue engineering, further development of core materials and biological technologies is required. One practical shortcoming is an inability to harvest cultured cells intactly from culture dishes without deprivation of highly differentiated cell functions. Typical cell harvest techniques using digestive enzymes irreversibly damage highly differentiated cells such as hepatocytes and microglia promising in clinical use.[1,2] Another short coming is an inability to build up constructs from differentiated cells. Therefore, we have focused on novel noninvasive cell manipulation techniques using our developed temperature-responsive culture dishes.

A temperature-responsive polymer, poly(*N*-isopropylacrylamide) (PIPAAm), is grafted on culture dish surfaces by electron beam irradiation.[3] This polymer-grafted culture dish exhibits temperature-responsive surface property, i.e., the surface is hydrophobic in the culture condition at 37°C and changes to hydrophilic below the lower critical solution temperature (LCST) about 32°C. On the temperature-responsive culture dishes, various types of cells adhere, spread, and proliferate similarly to those on the ungrafted tissue culture grade polystyrene (TCPS) dishes[1,4-6] at 37°C. While adherent cells develop stress fibers and focal contacts,[7] the cells spontaneously lift up from the surfaces after only reducing culture temperature below the LCST because of rapid hydration of the grafted PIPAAm chain surfaces with no need for digestive enzymes.[1,3] When the culture temperature is reduced after cells reach confluency, cells are recovered as a single contiguous cell sheet with intact cell-cell junctions.[8]

Madin-Darby canine kidney (MDCK) cell line is a transformed renal epithelial cell line. Although MDCK cells are immortal, confluent MDCK cells show differentiated renal epithelial cell functions. Therefore confluent MDCK cells have been used frequently to examine epithelial transport system.[9] Also in tissue engineering for kidney assists and replacements, MDCK cells have been utilized,[10-12] because these unique cells have both of immortality and relatively highly differentiated functions of kidney epithelial cells. In those studies MDCK cells were harvested by trypsin-digestion, although trypsin digestion dissociates monolayer cell sheets into isolated single cells and deprives MDCK cells of its differentiated functions. With our noninvasive cell sheet harvest method, confluent monolayer cell sheets of differentiated MDCK cells are recovered from temperature-responsive surfaces simply by low temperature treatment without trypsin.

In this paper, we describe the non-enzymatic harvests of MDCK cells from temperature-responsive culture dishes and also discuss its implications for tissue engineering.

2. MATERIALS AND METHODS

2.1 Reagents

N-Isopropylacrylamide (IPAAm) was kindly provided from Kohjin (Tokyo, Japan). The following materials were purchased from respective companies. Trypsin-ethylenediaminetetraacetic acid (EDTA) solution, streptomycin, and penicillin were from Gibco BRL Life Technologies (Grand Island, NY); bovine serum albumin fraction V (BSA) was from Sigma; Dulbecco's modified Eagle's Medium (DMEM) was from IWAKI glass (Chiba, Japan); fetal bovine serum (FBS) was from PAA Laboratories (Exton, PA); rabbit anti-bovine FN polyclonal antibody was from Biogenesis (UK); mouse anti-β-catenin monoclonal antibody (clone 14) was from Transduction

Laboratories (Lexington, KY); fluorescein isothiocyanate (FITC)-conjugated goat antibody against rabbit immunogloblins (Ig) and FITC-conjugated goat antibody against mouse IgG were from ICN Pharmaceuticals, Inc. Cappel Products (Aurora, OH); and rhodamine-conjugated phalloidin was from Molecular Probes (Eugene, OR).

2.2 Preparation of cell culture surfaces grafted with PIPAAm

Specific procedures for the preparation of PIPAAm-grafted cell culture dishes are described elsewhere.[3, 13] Briefly, IPAAm monomer in 2-propanol solution was spread onto polystyrene culture dishes (Falcon 3001). Then, the dishes were subjected to electron beam irradiation with an Area Beam Electron Processing System (Nisshin High Voltage, Kyoto, Japan). The PIPAAm-grafted dishes were rinsed with cold distilled water to remove non-grafted IPAAm. Untreated Falcon 3001 dishes were used as control surfaces. Presence of PIPAAm on the culture dish surfaces was confirmed by electron spectroscopy for chemical analysis (ESCA750, Shimadzu, Kyoto, Japan). The amount of grafted PIPAAm was determined by attenuated total reflection Fourier transform infrared spectrophotometry (ATR-FTIR). The grafted amount of PIPAAm used in the present study was estimated as 2 $\mu g/cm^2$ by ATR-FTIR.

2.3 Cells and cell culture

MDCK cells (passage 57) were provided by Health Science Research Resources Bank (JCRB 9029, Osaka, Japan). MDCK cells were maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO_2. Cells were used between passage 60 and 65 during all experiments. MDCK cells were harvested with 0.25% trypsin-2.65 mM EDTA in PBS, and subcultured at a 1:4 split ratio. Cell morphology was monitored under a phase contrast microscope (ET300, Nikon, Tokyo, Japan).

2.4 Recovery of cells from temperature-responsive culture dishes by low-temperature treatment

MDCK cells were plated on PIPAAm-grafted culture dishes at the cell density of $5X10^5$ cells/35 mm dish (about one quarter of the confluent cell density) and cultured at 37°C. TCPS dishes were also used as a control to confirm that the observed cell detachment depends on PIPAAm hydration. These dishes were transferred to another incubator set at 20°C and cell detachment was monitored under a phase contrast microscope.

2.5 Fluorescence microscopy

Cultured MDCK cells were fixed at 37°C with prewarmed 4% paraformaldehyde in Dulbecco's phosphate buffered saline (PBS) for 20 min in order to avoid the hydration of PIPAAm below the LCST. In some case, cells were fixed after a short time incubation at 20°C. They were washed with PBS and permeabilized with 0.5% Triton X-100 in PBS for 2 min. Then, they were blocked with 0.1% BSA in PBS for 90 min, and reacted with a primary antibody at an appropriate concentration over night at 4°C. Following three washes with 0.1% BSA in PBS, they were incubated for 1 h with a 1: 1000 dilution of FITC-conjugated goat anti-rabbit or mouse Ig antibody and again washed three times. To examine F-actin, the cells were stained with a 1:100 dilution of rhodamine-conjugated phalloidin instead of antibodies. The stained dishes were observed under a microscope with fluorescence equipment (ET300, Nikon, Tokyo, Japan). Fixed cell sheets were stained without the primary or secondary antibodies as negative control. In the negative controls, no fluorescence was detected under the condition used in the study.

3. RESULTS

MDCK cells were plated on temperature-responsive culture dishes grafted with PIPAAm. MDCK cells adhered, spread and proliferated on PIPAAm-grafted dishes as observed on ungrafted TCPS dishes. When plated at about one quarter of the confluent cell density, MDCK cells reached confluency on the sixth day after plating. Even after reached confluency, MDCK cells kept on slowed proliferation and developed cell domes, fluid-filled blister-like structures composed of scores to hundreds of cells.[14,15] (Fig. 1) These cell domes reflect renal epithelial cell differentiated phenotypes such as fluid transport. MDCK cells comprise a single contiguous monolayer cell sheet even in cell domes.

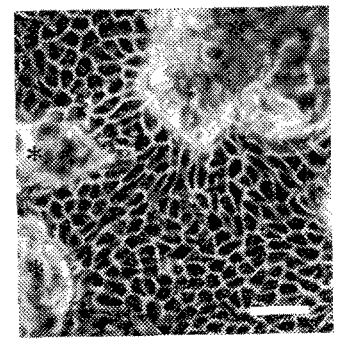

Fig. 1 Phase contrast microscopy of MDCK cells on PIPAAm-grafted culture dishes. MDCK cells were plated on culture dishes grafted with a temperature-responsive polymer, PIPAAm, and cultured at 37°C for 6 weeks. MDCK cells developed cell domes (*), fluid-filled bliter-like structures. Bar = 50 μm.

After cells reached confluency, the culture dishes were transferred into another CO_2 incubator set at 20°C, below the LCST. MDCK cells were detached as a single sheet from the periphery of culture dishes grafted with PIPAAm. Similar observations were previously obtained with other cell types including primary hepatocytes, retinal pigmented epithelial cells, and bovine aortic endothelial cells (BAECs).[8] Figure 2a shows a six-week cultured MDCK cell sheet which was completely detached and floated up in culture medium after reducing temperature. As soon as cells were detached from PIPAAm-grafted surfaces, MDCK cell sheets contracted to decrease in cell sheet diameter. Sometimes, detached cell sheets were folded and wrinkled.

MDCK cell sheets were double-stained with rhodamine-conjugated phalloidin for F-actin and anti-β-catenin polyclonal antibody in order to examine cell-cell junctions (Fig. 2). In epithelial cells, E-cadherin is utilized as the essential homotypic cell

adhesion molecule in the establishment and stabilization of cell-cell junctions.[16] E-cadherin cytoplasmic domain is complexed with β-catenin, and β-catenin is in contact with actin filaments via α-catenin.[17] Before reducing temperature, MDCK cells showed the essentially identical distribution of F-actin and β-catenin on PIPAAm-grafted culture dishes (Figs. 2b and c, respectively) as well as on ungrafted TCPSdishes. Actin filaments were organized along cell-cell junctions to develop peripheral bands. β-Catenin was co-localized with F-actin in peripheral bands. as observed before reducing temperature. Even in detached and undulating MDCK cell sheets (Figs. 2d and e), F-actin and β-catenin were co-localized at cell-cell junctions as observed before reducing temperature. This finding strongly suggests that cell-cell junctions were functionally maintained even in detached and undulateing cell sheets.

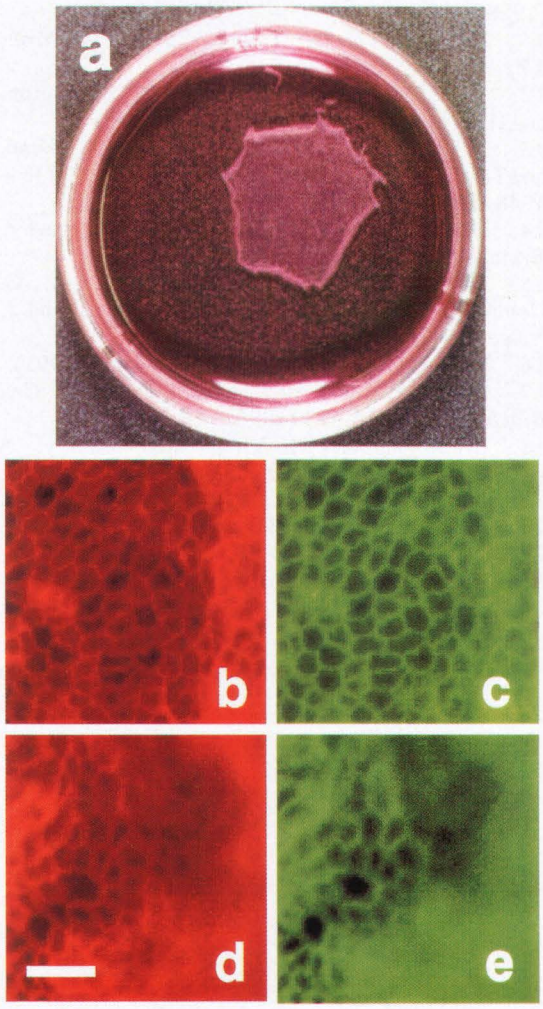

Fig. 2 Recovery of intact MDCK cell sheets from temperature-responsive culture dishes. After 5-week culture at 37°C, the culture temperature was decreased to 20°C. Then, MDCK cells were detached from the dish surfaces and floated up in culture medium (a). Before (b and c) or after (d and e) reducing temperature, MDCK cell sheets were fixed, double stained with rhodamine-conjugated phalloidin for F-actin (b and d) and anti-β-catenin antibody (c and e), and observed under a fluorescence microscope.

In order to examine extracellular matrix (ECM), detached MDCK cell sheets were fixed and double-stained with rhodamine-conjugated phalloidin and anti-FN antibody (Fig. 3). In the presence of serum, FN is one of the major ECM components to cell adhesion onto dish surfaces. The area from which the cells had detached was not stained either with anti-FN antibody or rhodaimine-conjugated phalloidin (right side in Figs. 3a and b). No difference in fluorescence intensity of FN was observed between the detached domain and the other area where cells still adhered (data not shown). These results show that the great majority of FN matrix was detached from temperature-responsive culture dishes together with MDCK cell sheets.

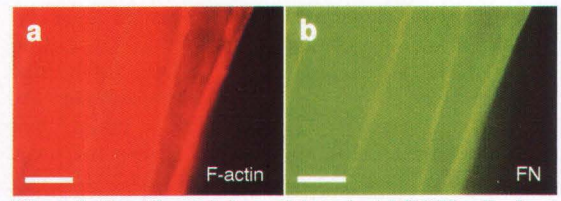

Fig. 3 Double-staining of detached MDCK cell sheet with rhodamine-conjugated phalloidin and anti-FN antibody. MDCK cells were cultured on PIPAAm-grafted dishes. After 5-week culture, the temperature was decreased to 20°C. Detached cell sheets were fixed, double-stained with rhodamine-conjugated phalloidin for F-actin (a) and anti-FN antibody (b). Bar = 200 μm.

4. DISCUSSION

MDCK cells are an immortal renal epithelial cell line and retain differentiated cell functions. Accordingly, MDCK cells have been utilized in tissue engineering for kidney replacements.[10-12] Long term culture is required for the recovery of differentiated phenotypes, because MDCK cells develop its apical-basal cell polarity, that is essential to functions, only after making a confluent monolayer cell sheet.[18] However, by trypsin digestion, few differentiated confluent MDCK cells were harvested from culture dishes and this enzymatic digestion dissociates monolayer cell sheets into isolated single cells and deprives MDCK cells of its differentiation functions. With our noninvasive cell harvest method, cell-cell junctions, which are highly susceptible to depletion of divalent cations, were intactly maintained, because neither digestive enzyme nor EDTA was required. So confluent monolayer cell sheets of differentiated MDCK cells can be recovered from temperature-responsive surfaces simply by low temperature treatment.

Recovered cell sheets can be utilized with two- and three-dimensional manipulation techniques and represent a significant advance with notable potential. In natural organs, the parenchyma comprises intimately associated cell sheets. For example, kidney glomeruli comprises sheets of podocytes and fenestrated endothelial cells that are interconnected via basement membrane to form a continuous three-dimensional tissue lattice. Similar bilayers are also observed in renal tubules, in which epithelial cells and peritubular capillary endothelial cells make separate monolayer cell sheets respectively and the cell sheets are combined via basement membrane, although cell sheets form tubular

structures. In order to not only investigate molecular mechanisms of tissue functions but also develop a novel hybrid artificial organ with a three-dimensional structure similar to normal tissues, we have focused on the re-construction of cell sheets, not isolated co-cultured single cells, into tissue-like structures as a breakthrough technology. To accomplish this, temperature-responsive culture dishes has a great potency. Using temperature-responsive culture dishes, FN matrix deposited during culture was also recovered with cell sheets. FN matrix adhering to the basal side of cell sheets can function as a glue to attach cell sheets onto other surface or cell sheets. In fact, cell sheets recovered from the temperature-responsive surfaces easily adhere onto other surfaces.[13]

Using the above-mentioned cell sheet recovery-transfer technique, we have succeeded in fabricating stratified cell sheets to reconstruct liver lobule-like structure from two cell sheet types of hepatocytes and endothelial cells.[19] In addition, many new experiments useful for fundamental studies of basic cell biology, cell-cell interactions, and structural matrix are now possible with the recovered cell sheets.

5. ACKNOWLEDGMENTS

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