Colloidal Silica-Coated Tissue Culture Dishes for Primary Cell Cultures : Growth of Rabbit Renal Proximal Tubule Cells

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ABSTRACT

The use of colloidal silica as a substratum for primary cultures of differentiated cells has significant advantages over classic tissue culture polystyrene. In this report, the growth and the level of expression of differentiated function of primary rabbit renal proximal tuble(RPT) cell cultures on colloidal silica is examined, using hormonally defined serum-free medium. Primary RPT cells grew to confluence more rapidly on collidal silica than on tissue culture polystyrene(TC*). Moreover, following three passages, the RPT cells increased in number threefold more than parallel cultures on TC*. The morphology of primary RPT cells on colloidal silica were found by means of transmission electron microscopy to possess a polarized morphology with a brush border, and differentiated markers were retained even after passaging, including the Na+/glucose cotransport system and Glut 7.

INTRODUCTION

The tissue culture substratum has remained one of the primary factors that sets limits to the successful culturing of anchorage-dependent animal cells(2, 11). In vivo, anchorage-dependent amimal cells adhere in a very precise manner to an extracelluar matrix(ECM), so as to maintain normal tissue integrity(3, 9). In vitro, anchorage-dependent animal cells must similarly interact with a substratum to grow and function. However, in the in vitro situation, animal cells and their associated ECM proteins generally interact with a synthetic substratum rathe than with an intact ECM. Soda lime silicate glass was the primary substratum used for this purpose up until the 1970's, when injection-molded polystyrene was introduced as a tissue culture material. The poystyrene surfaces that were used initially did not permit appropriate cell attachment and spreading and only achieved generalized use following a glow-discharge treatment(1).

Glow-discharge-treated polystyrene [now generally called tissue culture polystyrene(TC*)] very likely is a more favorable surface for cell adhesion due to its increased wettability and higher oxygen content relative to untreated polystyrene(4). Presumably, the increased wettability and surface oxidation of the TC* surface is directly responsible for the increased adsorption of such ECM proteins as ibronectin and vitronectin onto the surface, particularly in the case in which serum-supplemented medium is utilized. Consequently, improved cell attachment and spreading is obtained. In the case in which serum-free medium is utilized, the polystyrene surface must in many cases be coated with purified vitronectin, fibronectin or other cell attachment factors, so as to permit effective culturing (2,11). However, procedures for coating dishes with such attachment factors are labor-intensive and expensive. Moreover, such coated dishes are only effective for a limited number of cell types.

When considering primary tissue culture preparations in particular, the initial cell attachment to the culture dish is often minimal, and consequently, confluent monolayers are not obtained, Thus many primary culture systems are still not effective experimental tools, even when using serum-free medium. Here, we describe the use of colloidal silica as an alterative substratum for

culturing primary and short-term cultures of rabbit renal proximal tubule (RPT) cells. We show that the use of colloidal silica is significantly advantageous for culturing RPT cells as compared with TC*.

MATERIALS AND METHODS

Materials

Colloidal silica-coated culture dishes were prepared by Corning Costar (Cambridge, MA, USA) (13) using Ludox HS-40 (NEN Life Science Products, Boston, MA, USA). Ludox HS-40 is a 40% wt/vol solution of colloidal silica consisting of negatively charged silica particles with an average particle diameter of 12 nm, a specific surface area of 220 m^2/g and pH 9.7. Corning Costar TC* dishes (35 mm) were spincoated with 0.4 mL of a 10% (wt/vol) solution of Ludox HS-40 using a Benchtop Photo-Resist Spinner (Headway Research, Garland, TX, USA). The dishes were dried in a forced-air oven for 60 min at 50℃~70℃, soaked in a 1% (vol/vol) sulfuric acid solution for 60min, rinsed 3× in deionized water, flushed with flowing deionized water for 30 min and once again dried. Finally, the dishes were sterilized by gamma irradiation (2.5 Mrad).

The basal medium consisted of a 50:50 mixture of Dulbecco's modified Eagles medium (DMEM) and Ham's nutrient mixture F-12 containing 15 mM HEPES, pH 7.4, 20 mM sodium bicarbonate, 0.5 mM sodium pyruvate and 17.5 mM glucose (DMEM/F12) (5). At the time of use, the basal medium was further supplemented with 5μg/mL bovine insulin, 5μg/mL human transferrin, 5×10^{-8} M hydrocortisone and 92 IU/mL penicillin (Medium RK-1) (5). The water utilized was purified through a MilliQ® Deionization System (Millipore, Bedford, MA, USA). The powdered culture medium utilized in these studies was from Life Technologies (Gaithersburg, MD, USA). Hormones and other chemicals for tissue culture were obtained from Sigma Chemical(St. Louis, MO, USA).

Primary Kidney Cell Cultures

Each experimental study was conducted with independently derived primary cultures by a modification of the method of Chung et al. (5,8). To summarize, a kidney from a male New Zealand white rabbit (2-2.5 kg) was perfused by the renal artery, first with phosphate-buffered saline (PBS) and then with a 0.5% (wt/vol) solution of iron oxide (6). The renal cortex was removed, and the tissue was subjected to 4 strokes in a Model 1984 Sterile Dounce Homogenizer (type A pestle, 15 mL; Bellco Glass, Vineland, NJ, USA). The homogenate was washed through a series of 2 meshes (253 μm and then 83 μm). The material that remained over the 83-μm mesh (proximal tubules and glomeruli) was transferred into basal medium, and glomeruli (containing iron oxide) were removed with a stir bar. The remaining RPTs were then incubated for 2 min at 23℃ in basal medium containing 0.125 mg/mℓ collagenase (class IV; Worthington Biochemical, Freehold, NJ, USA) and 0.025% soybean trypsin inhibitor, washed twice by centrifugation at 500×g, resuspended in Medium RK-1 and finally inoculated into 35-mm culture dishes at 0.5 mg protein/dish (5). The cultures were maintained in a humidified, 5%, CO_2/95% air environment at 37℃. Medium was changed one day after plating and every two days thereafter.

Passaged Kidney Cell Cultures

Primary and passaged RPT cells were subcultured by removing the medium, washing the monolayers twice with PBS and subsequently detaching the cells from the culture dishes using a 0.05% EDTA/0.5 mM trypsin solution in PBS (EDTA/trypsin). Trypsin action was stopped using a 0.1% solution of soybean trypsin inhibitor in PBS. The cells were washed by centrifugation at 500× g, resuspended in basal medium and inoculated into culture dishes containing Medium RK-1, further supplemented with 10 mg/mL epidermal growth

factor (EGF) and 30 μg/mL bovine pituitary extract (Collaborative Research, Bedford, MA, USA).

Cell Growth Studies

To determine the cell number present in primary and passaged kidney cell cultures, the cells in representative culture dishes were detached utilizing EDTA/trypsin, and the cell number was determined utilizing a Model Zf Particle Counter (Beckman Coulter, Hialeah, FL, USA). Values are the averages of triplicate determinations [± standard deviation (SD)]. The growth rate was determined from the slope of a plot of the $\log_2$ (cell number) vs. culture time.

Electron Microscopy

Confluent monolayers were fixed with 2% (vol/vol) glutaraldehyde in 0.05 M sodium cacodylate (pH 7.4) and postfixed, first in 0.5% osmium tetroxide/0.8% $K_3Fe(CN)_6$/0.05 M cacodylate (pH 7.4), then in 0.15% tannic acid/0.05 M cacodylate (pH 7.4) and finally in 2% (wt/vol) aqueous uranyl acetate. For transmission electron microscopy (TEM), the culture dishes were placed on a 21-㎟ histomold (Peel-A-Way-Products®) containing Epona-raldite (Electron Microscopy Sciences, Fort Washington, PA, USA). Semi-thin, 0.5-μm and thin, 90-nm sections were cut at 90° relative to the plane of the coverslip and photographed with a JEOL 100CXII Electron Microscope(JEOL USA, Peabody, MA, USA).

Western Analysis

RPT cell cultures were washed twice with PBS, removed into sample buffer for sodium dodecyl sulfate(SDS) gel electrophoresis, sonicated and cleared by centrifugation in a Model 5415 Microcentrifuge (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) for 5 min at 14000 rpm. The cell lysates were equalized with respect to protein content, as assessed by the DC Protein Assay (Bio-Rad, Hercules, CA, USA), electrophoresed through a 7.5% SDS polyacrylamide gel and transferred to nitrocellulose membranes using a Trans-Blot® System (Bio-Rad) (12). The nitrocellulose blots were blocked for 1 h in Tris-buffered saline (TBS) containing 3% bovine serum albumin (BSA), incubated for 3 h in Western Wash Buffer containing 3% BSA and primary rabbit antiserum to either the Na+/glucose cotransport system or Glut 7 (Chemicon International, Temecula, CA, USA). The blots were then washed 5× with Western Wash Buffer, incubated for 1 h with a goat anti-rabbit IgG/alkaline phosphatase conjugate (Bio-Rad), washed 3× with Western Wash Buffer and 2× with TBS. Signals were visualized by incubation in a reaction mixture containing 0.33mg/mL nitro blue tetrazolium (NBT) and 0.17 mg/mL 5-bromo- 4-chloro-3- indolyl phosphate (BCIP) in 0.1 M Tris, pH 9.5, containing 0.1 M NaCl and 0.05 M $MgCl_2$. The Western Wash Buffer used above was prepared by the addition of 0.292% NaCl and 0.3% Nonidet® P-40 (NP40) to TBS.

RESULTS AND DISCUSSION

Growth of Primary RPT Cells on Colloidal Silica

The ability of primary RPT cells to grow on a colloidal silica and TC* surface was examined over time (Figure 1) After an initial lag period, the cells on the colloidal silica surface grew at a rate of 1 doubling/day and ultimately achieved confluence. Although the primary cells also grew logarithmically on TC*(Figure 1), fewer cells were present on TC* as compared with colloidal silica. Consistently, in four additional culture sets, significantly more primary kidney cells were obtained on colloidal silica as compared with TC*(Table 1).

As in primary cultures, the growth rates were very similar on colloidal silica and TC*. The advantage of colloidal silica in the primary cultures

Table 1. Growth of Primary Proximal Tubule Cells on Colloidal Silica

Experiment	Days in Culture	Cell No:TC*	Cell No: Colloidal Silica
Experiment 1	4	54240±8500 (100%±12%)	280540±77920 (517%±143%)
Experiment 2	4	80920±17240 (100%±21%)	294217±25625 (364%±32%)
Experiment 3	6	125280±4040 (100%±3%)	413920±82340 (330%±7%)
Experiment 4	7	299540±70620 (100%±24%)	581560±26840 (194%±9%)

Primary rabbit kidney proximal tubule cell cultures were initiated on either the TC* or the colloidal silica substratum. After the indicated number of days, the cell number was determined. Values are the averages(±SD) of triplicate determinations

was very likely derived from more efficient adhesion of the plated material, RPTs, on colloidal silica. Increased adhesion of the RPTs on colloidal silica can be explained, at least in part, by the physical properties of this particular surface.

The colloidal silica, being composed of a colloidal dispersion of very small negatively charged particles, has an increased surface area as compared with TC*, Thus, the colloidal silica surface provides an increased number of productive binding sites, both for the tubular epithelial cells and their associated ECM proteins (10).

TEM of Primary Cultures

When examining the primary renal cell cultures on colloidal silica by means of TEM, the cultures were found to be composed of polarized epithelial cells with microvilli on the apical surface(Figure 2). Cell projections were present basolaterally and were in direct contact with colloidal silica. Unlike the case with either the original colloidal silica surface or with TC* (data not included), extracellular, particulate material was dispersed over the colloidal silica. Possibly, the particulate material might consist of silica that has been released from the silica film by dissolution. However, an analysis of this material by means of an EDAX-designed X-ray microanalysis system (EDAX International, Mahwah, NJ, USA), which uses TEM, indicates that no silica was present in the particulate material (unpublished observation), Thus, the particulate material might possibly be secreted basement-membrane proteins.

Table 2. Growth of Renal Proximal Tubule Cells through Three Passages

Passage No.	Colloidal Silica		TC* Surface	
	Fold Increase in Cell No.	Doublings	Fold Increase in Cell No.	Doublings
P1	6.5±2.1	2.7±0.4	7.4±0.0	2.9±0.1
P2	2.1±0.2	1.1±0.2	1.1±0.0	0.1±0.0
P3	1.8±0.1	0.9±0.1	0±0	0±0
P1+P2+P3:		4.7±0.7		3.0±0.1

Primary cultures of rabbit renal proximal tubule cells were initiated on either the TC* or the colloidal silica surface. When the cells were confluent, they were counted utilizing a Coulter counter and passaged (P1) at 23000 cells per dish. Ten days later, the cells were counted and passaged again (P2) at 29900 cells per dish. After another ten days, the cells were counted and passaged (P3) at 6400 cells per dish. Values were averaged(±SD) from duplicate determination. The fold increase in cell number as compared with the initial inoculum, and the number of doublings that occurred following passaging was then calculated.

Ability of RPT Cells to be Passaged on the Colloidal Silica Surface

The successful passaging of the RPT cells is dependent upon the tissue culture surface and the medium components. To compare the ability of RPT cells to be passaged on colloidal silica and TC*, primary cultures were passaged three times at equivalent densities, both onto the colloidal silica and the TC* surface. A portion of the passaged cultures were utilized for determinations of cell number. Table

2 shows the results of this study. After the first passage, the growth obtained on colloidal silica and TC[+] was equivalent. However, after the second and third passages, the RPT cells continued to divide on colloidal silica, unlike the case with TC[+].

The increased growth, which was obtained as the RPT cells were passaged on colloidal silica, cannot only be explained by improved adhesion, but also by an increased growth rate on colloidal silica. If however, inappropriate adhesion did occur following the second subculturing on polystyrene, the result would be as observed, i.e., no significant cell division. The initial adhesion of the RPT cells to the culture dish following subculturing might in part be influenced by the basement-membrane proteins that are synthesized by the newly plated cells and deposited onto the culture dish. For this reason, the ability of the culture dish to adsorb such newly synthesized basement-membrane proteins might influence the process of cell adhesion and is a possible mechanism by which the colloidal silica surface promotes increased growth(3,7,9).

Expression of Differentiated Function on Colloidal Silica

The cultures that were passaged on colloidal silica and TC[+] were observed by means of light microscopy to retain an epithelial morphology (data not included). The state of differentiation of the renal cells was also assessed by analyzing the levels of two transport systems, the Na^+/glucose cotransport system and Glut 7, by means of Western analysis. Both the Na^+/glucose cotransport system, a distinctive marker of the renal proximal tubule (5), and Glut 7, a facilitated diffusion system for glucose, are present in RPT cells. The results of the Western analysis indicated that the level of expression of the Na^+/glucose cotransport system was retained following two passages (Figure 3A), and the level of expression of Glut 7 was found to be retained following three passages on colloidal silica (Figure 3B). Thus, the use of a colloidal silica surface did not promote the growth of undifferentiated cells.

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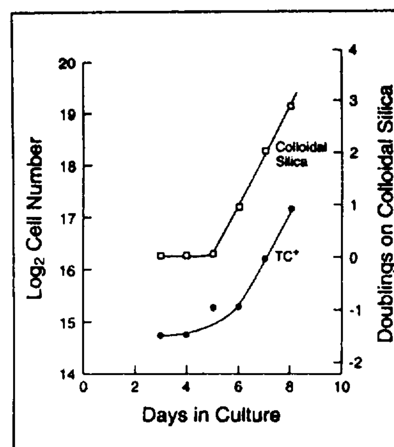

Figure 1. Growth of primary RPT cells. Primary RPT cells were grown either on colloidal silica or the TC[+] surface. Periodically, cultures were removed, the cell number determined and the average value calculated. The $\log_2$ (cell number) was plotted as a function of culture time.

Figure 2. TEM of primary RPT cells on colloidal silica. Primary cells on colloidal silica were photographed at 2000× magnification

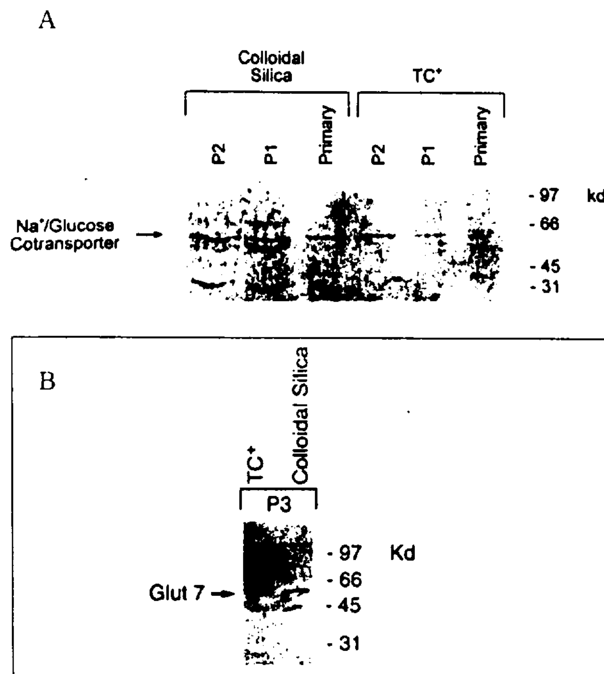

Figure 3. Expression of the Na⁺/glucose cotransport system and Glut 7. Primary RPT cells were initiated on either colloidal silica or the TC⁺ surface and passaged as described in Table 2. The passaged cultures were examined with regards to the level of expression of (A) the Na⁺/glucose cotransport system and (B) Clut 7 by Western analysis.

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