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Serum-free culture of rat proximal tubule cells with enhanced function on chitosan

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ABSTRACT

The proximal tubule performs a variety of important renal functions and is the major site for nutrient reabsorption. The purpose of this study is to culture rat renal proximal tubule cells (PTCs) on chitosan without serum to maintain a transcellular pathway to transport water and ions effectively without loss of highly differentiated cell function. The effect of chitosan, which is structurally similar to glycosaminoglycans, in the absence of serum on the primary cultured PTCs was compared that of collagen with or without serum. Two days after seeding, more tubule fragments and higher PTC viability were observed on chitosan than on collagen with or without serum. Proliferation marker Ki-67 immunostaining and phosphorylated extracellular-regulated kinase (ERK) expression results displayed similar proliferation capability of PTCs established on chitosan without serum and collagen with 2% fetal bovine serum after 4 days of incubation. When grown to confluence, PTCs formed a monolayer with well-organized tight junctions and formation of domes on chitosan without serum. Moreover, evaluation of the transepithelial electrical resistance showed that both chitosan and serum were involved in the modification of water and ion transport in confluent cells. By showing the direct suppression of PTC growth and dome formation treated with heparinase, we demonstrated that the interaction between cell surface heparin sulfate proteoglycan and chitosan played an important role in PTC proliferation and differentiation. A successful primary culture of PTCs has now been produced on chitosan in serum-free culture condition, which offers potential applications for chitosan in renal tissue engineering.

1. Introduction

The proximal tubule performs a variety of important renal functions and is the major site for reabsorption of most nutrients. Primary cultures of renal proximal tubule cells (PTCs) provide a useful tool for studying metabolic functions, transport processes, and a variety of cytotoxic or ischemic effects [1–5]. The differentiated PTCs display apical–basal polarity and vectorial liquid transport. Domes develop when these PTCs form a monolayer on non-permeable substrates and are considered as differentiated characteristic of transporting epithelia in vitro [6,7].

The success of renal epithelial tissue engineering is strongly dependent on the development of cell culture techniques to maintain a normally functional and differentiated state in primary cultured PTCs. However, the innate properties of PTCs limit the growth that is possible if the unique functions of PTCs in vitro are to be preserved. For example, Miller reported that the use of primary cells from rat renal cortex with serum-supplemented media has been largely unsuccessful due to fibroblast overgrowth [8]. Chung et al. also reported that serum-containing medium enhanced the growth of fibroblasts and this disturbed or inhibited the isolation and culture of rabbit PTCs [9]. Therefore, proximal tubules have been grown in primary culture by several laboratories using serum-free, hormonally defined media [10–15].

Extracellular matrix (ECM) plays an important role in regulating epithelial cell behavior such as survival, adhesion, proliferation and differentiation [16,17]. ECM remodeling and changes in the composition of the proximal tubule and the surrounding interstitium are associated with inflammatory and chronic renal disease [18–20]. The development of tissue-engineered renal proximal tubule needs a biocompatible material as a synthetic ECM to support the growth of PTCs to form a well-defined paracellular barrier and transcellular pathway to transport ions, water and other molecules in the proper manner. Chitosan is a widely used biomaterial that possesses good biocompatibility, biodegradability, low allergenicity and nontoxicity [21,22]. Chitosan is a deacetylated product of chitin and is structurally analogous to glycosaminoglycans (GAGs). Being structurally similar to ECM components, chitosan-based biomaterial is able to stimulate the attachment, proliferation and function of various cells [23–27]. However, little is known about the utility of chitosan in culturing PTCs. Therefore, we tested

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whether chitosan could be used to support the survival, proliferation and differentiation of PTCs in serum-free conditions. Previously, collagen has been routinely used for culturing PTCs, and therefore the behavior of PTCs on a collagen-coated surface in the absence of serum or with low serum concentration to restrict fibroblast growth [14] was compared in this study. This study suggest that chitosan itself is potentially useful as a tissue-engineering scaffold for culturing rat PTCs with well-defined functions.

2. Materials and methods

2.1. Preparation of chitosan-coated tissue culture plates

Briefly, 1 ml 0.5% (w/w) chitosan solution (degree of deacetylation $\geqslant$ 80%; Sigma–Aldrich) dissolved in 3% (w/v) acetic acid was added into each well of 6-well tissue culture plates (Corning Costar) and dried at 60 °C to form a thin film with a uniform thickness of about 5–8 μm by using a scanning electron microscope (data not shown). Subsequently, the plates were neutralized by 0.5 N NaOH aqueous solution for 1 h, washed thoroughly with Milli-Q water and exposed to ultraviolet light overnight. For cell culture, the chitosan-coated film was transparent without any visible defects.

2.2. Isolation of rat renal proximal tubules

Isolation of rat renal proximal tubules was performed by modification of previously published methods [28]. Male Wistar rat aged 8 weeks were anesthetized with isoflurane (1.5% in mixture of 70% N_2O and 30% O_2). After the abdominal aorta were cannulated, the kidneys were first flushed with 50 ml Hank's Balanced Salt Solution (HBSS) without calcium and magnesium (GIBCO) and then perfused with 30 ml HBSS with calcium and magnesium (GIBCO) containing 60 mg collagenase (type II, Sigma–Aldrich). After digestion, the kidneys were transferred to a sterile Petri dish, washed with HBSS and decapsulated. Subsequently, renal cortices were dissected, chopped, placed into HBSS containing 30 mg collagenase and incubated at 37 °C for 5 min. The fragments were filtered through 125 m sieve, washed three times with fresh HBSS, centrifuged at 0.8*g* for 5 min and resuspended in Dulbecco's Modified Eagle Medium (DMEM)/F12 medium (GIBCO) with 3% bovine serum albumin (BSA, Sigma–Aldrich) at 4 °C for 15 min. Proximal tubules were purified by further centrifugation in 45% Percoll solution (Pharamacia Biothech) at 20,000*g* for 30 min at 4 °C, washed three times with HBSS and resuspended in culture medium which consists of equal parts of DMEM/F12 (GIBCO) and keratinocyte serum-free medium (KSFM, GIBCO) mixture supplemented with 50 g ml^{-1} bovine pituitary extract (GIBCO) and 10 ng ml^{-1} epidermal growth factor (GIBCO), 5 μg ml^{-1} insulin, 5 μg ml^{-1} transferrin, 5 ng ml^{-1} selenium (I-T-S, GIBCO), 10^{-3} M hydrocortisone, 0.5% dimethyl sulfoxide (DMSO, Sigma–Aldrich), 30 ng ml^{-1} cholera toxin (Sigma–Aldrich) and 1% antibiotics (GIBCO) with or without the addition of 2% fetal bovine serum (FBS, Biological Industries). For comparison, type I collagen (BD Biosciences)-coated TCPS with the concentration of 20 g ml^{-1} and chitosan-coated TCPS were used in this study. Proximal tubules were seeded at a density of 1.7 mg ml^{-1} and incubated at 37 °C in a humidified atmosphere of 5% CO_2/95% air for 8 days and the culture medium was changed every day. Cell morphology was observed with an inverse phase-contrast microscope (Olympus IX 71).

2.3. Cell viability assay

Cell viability was determined using Alamar blue (Invitrogen) [29] and calcein AM (Live/Dead Assay, Invitrogen) [30] assays. Alamar blue solution (5%) was added directly to culture medium and incubated at 37 °C for 5 h at indicated time points. Fluorescence in each well was measured quantitatively on a microplate reader (ELx800; BIOTEK, Saga, Japan) with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Cell viability was normalized to the seeded quantity of tubules and expressed as the percentage of the absorbance recorded for chitosan.

The viability of cells in the dome was determined by staining with 5 mM calcein AM to label live cells and with 5 mM ethidium homodimer to label dead cells. Cells were incubated in culture medium containing calcein AM and ethidium homodimer at 37 °C for 60 min and then washed twice with phosphate-buffered saline (PBS) for microscopic observation (Leica DMI 600).

2.4. Immunocytochemical characterization

For immunocytochemical characterization, cultured PTCs were fixed with 4% paraformaldehyde in PBS for 20 min, washed three times with PBS, and then permeabilized with 0.1% Triton X-100 in PBS for 5 min at the time points indicated. After blocking with 10% BSA in PBS for 1 h, PTCs were incubated at 4 °C overnight with mouse monoclonal antibodies against E-cadherin (BD Biosciences, 1:200), Ki-67 (Invitrogen, 1:100), Zonula Occludens-1 (ZO-1) (Invitrogen, 1:50), and Na^+-K^+ ATPase α-1 subunit (Abcam, 1:200). FITC-conjugated secondary antibodies (Invitrogen, 1:100) were used to visualize the signal by reacting with PTCs for 1 h at room temperature. PTCs were also counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma–Aldrich). Fluorescence images were visualized by fluorescence microscopy (Leica DMI 600) or confocal microscopy (Zeiss LSM510).

2.5. Western blot analysis

Cell lysates were prepared in ice-cold lysis buffer (Millipore) with protease inhibitor cocktail (Roche) for 30 min and then were centrifuged at 16,000*g* for 5 min at 4 °C. The supernatant was added to 10% sodium dodecylsulfate (SDS) and heated at 95 °C for 5 min. Protein extracts were separated by SDS on 10% polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking with I-block (Millipore), membranes were incubated with the mouse monoclonal antibody against extracellular-regulated kinase (ERK), phosphorylated ERK (Abcam, 1: 2000), ZO-1 (Invitrogen, 1:500), and Na^+-K^+ ATPase α-1 subunit (Abcam, 1:1000) overnight at 4 °C. After washing with Tris-buffered saline and Tween 20 (TBST), the blots were incubated with peroxidase-labeled secondary antibody for 2 h at room temperature and detected by chemiluminescence.

2.6. Alkaline phosphatase (ALP) assay

The expression of the PTC brush-border enzyme ALP was assessed using a kit from Abcam following the manufacturer's instructions. After 8 days of culture, ALP activity was measured spectrophotometrically at 410 nm according to the release of *p*-nitrophenol from *p*-nitrophenylphosphate substrate, and results were normalized to protein concentrations measured by a bicinchoninic acid (BCA) kit (Sigma–Aldrich).

2.7. Quantitation of domes

Domes were counted by randomly choosing 3–5 microscopic fields per well every 2 days. Results were expressed as the average dome number per microscopic field of 1 mm^2 area.