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In vitro generation of three-dimensional renal structures

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ABSTRACT

End-stage renal disease is currently being treated effectively by transplantation. However, increasing demand and donor shortage make this treatment challenging. Recent advances in cell-based therapies have provided potential opportunities to alleviate the current challenges of donor shortage. In this study we developed a system to generate renal structures *in vitro* using primary kidney cells. This system involves the cultivation of expanded primary renal cells in a three-dimensional collagen-based culture system. After one week of growth, individual renal cells began to form renal structures resembling tubules and glomeruli. Histologically, these structures show phenotypic resemblance to native kidney structures. The reconstituted tubules stained positively for Tamm–Horsfall protein, which is expressed in the thick ascending limb of Henle's Loop and distal convoluted tubules. These results show that renal structures can be reconstituted in a three-dimensional culture system, which may eventually be used for renal cell therapy applications.

1. Introduction

End-stage renal disease is a devastating condition, which involves multiple organs in affected individuals. Although current treatment modalities, including dialysis and transplantation, can prolong survival for many patients, problems such as donor shortage, graft failure, and other complications remain a continued concern [1–4]. Numerous investigative efforts have been commenced in order to address these problems [5–8]. The concept of cell-based approaches using tissue engineering and regenerative medicine techniques has been proposed as a method to improve, restore or replace renal function [9–11]. Although widespread use of regenerative technology to combat kidney disease has yet to be implemented, some progress has been made [8]. An extracorporeal cell-based system, known as the renal assist device (RAD), uses human renal proximal tubular cells to improve filtration [6]. Despite demonstrations that this device can enhance the dialysis system, it is limited to *ex vivo* applications.

Another cell-based approach involves implantation of renal cells *in vivo* for renal function restoration [8]. This approach requires isolation of renal cells from donor tissue, expansion *in vitro* and reintroduction of renal cells back into the host for renal tissue regeneration [12]. The concept of renal cell therapy was demonstrated in a study in which culture expanded cells were seeded onto an artificial renal device and implanted *in vivo*. This resulted in the formation of renal structures that produce urine-like fluid [5]. In this particular study single renal cells showed the ability to reconstitute into renal tubules and glomeruli on the artificial renal device. However, the efficiency of the process of structural reconstitution could not be assessed upon implantation *in vivo*. Reconstitution of renal structure during the culture expansion stage followed by implantation would provide a more controlled assessment of renal tissue *in vivo*.

Toward this goal, we have developed a culture system that facilitates the formation of three-dimensional renal structures *in vitro*. We report a method that permits the use of primary renal cells to form tubule- and glomerulus-like renal structures *in vitro*. Such a system has the potential to eventually allow for the controlled implantation of renal structures and may ultimately serve as an effective method to maximize the outcome of cell-based therapy for renal function improvement.

2. Description of method

2.1. Isolation of primary murine renal cells

Many techniques, including flow cytometry-based cell sorting and differential sieving, have been employed to isolate and culture individual renal cell types for various experiments [13,14]. While these approaches may serve specific investigative objectives, formation of renal structures may not be adequately achieved by using single cell types, as multiple factors are believed to be involved, such as epithelial-mesenchymal cell interactions for differentiation [15]. To achieve the goal of isolating a heterogeneous population of renal cells that included those that were needed to

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generate renal tubules and glomeruli *in vitro*, a protocol involving homogenization and digestion of renal tissue was used. This was followed by filtration to obtain a non-homogenous renal cell population.

Kidneys from four-week-old C57BL/6 mice were obtained and placed in Krebs–Ringer bicarbonate buffer (Sigma, St. Louis, MO) supplemented with 1% antibiotic/antimycotic solution (Gibco Invitrogen, Carlsbad, CA). Renal capsules and adjacent connective tissues were removed using sharp scissors to prevent contamination from cells non-essential to kidney structure formation. After rinsing with sterile buffer solution, a scalpel was used to mince the tissue until a gel-like consistency was achieved. Minced tissue was placed in Krebs–Ringer bicarbonate buffer at 37 °C. Four kidneys were typically placed in 25 mL of buffer. A collagenase-based tissue dissociation reagent, Liberase Blendzyme 3 (Roche, Indianapolis, IN), was added to the solution of minced tissue and buffer at a concentration of 0.168 Wunsch units/mL. This solution was incubated with agitation in a 37 °C water bath for 30 min to facilitate tissue digestion. The solution containing digested renal tissue was filtered through a 100-μm cell strainer (BD Falcon, San Jose, CA). The filtered solution was neutralized by the addition of an equal volume of high glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco Invitrogen, Carlsbad, CA) supplemented with 1% penicillin–streptomycin (Gibco Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (FBS; Gibco Invitrogen, Carlsbad, CA). This primary renal cell-containing solution was subsequently centrifuged at 500*g* for 5 min to separate the cells from the solution. The supernatant was removed by vacuum aspiration. The resultant primary renal cells were then resuspended in growth medium. This medium is a mixture of two supplemented media types. It consists of one part DMEM containing 10% FBS and 1% penicillin–streptomycin and one part Keratinocyte Serum-Free Medium (KSFM; Gibco Invitrogen, Carlsbad, CA) supplemented with bovine pituitary extract and epidermal growth factor (Gibco Invitrogen, Carlsbad, CA); 2.5% FBS (Gibco Invitrogen, Carlsbad, CA); insulin, transferrin, and sodium selenite (ITS; Sigma-Aldrich, St. Louis, MO); and 1% penicillin–streptomycin (Gibco Invitrogen, Carlsbad, CA). The combination of supplemented DMEM and K-SFM was used to facilitate the growth of a range of cell types, including stromal and epithelial cells. The primary renal cells were plated in growth medium at a density of one 10-cm plate for each kidney used. Cells were cultured at 37 °C in 5% CO_2.

To allow for attachment to the tissue culture plates, cells were left undisturbed for the first 48 h after isolation. After this, the medium was removed from the cells and they were rinsed with warm Dulbecco's phosphate buffered saline (PBS; Gibco Invitrogen, Carlsbad, CA) to remove dead cells and debris. Fresh, pre-warmed growth medium was subsequently added to the culture plates, which were then placed back in the incubator. Cells were subcultured when they reached approximately 90–95% confluence. To passage cells, the growth medium was removed and cells were rinsed with PBS. Pre-warmed PBS supplemented with 0.5 mM ethylenediaminetetraacetic acid (EDTA; Gibco Invitrogen, Carlsbad, CA) was added to the plates and they were incubated at 37 °C for 5 min. This additional rinse with EDTA–PBS enhances efficacy of the subsequent cell detachment from the culture plate. After the 5-min treatment, the PBS/EDTA solution was removed from the plates and replaced with warm 0.05% Trypsin–EDTA (Gibco Invitrogen, Carlsbad, CA). Cells were again incubated at 37 °C for 5 min. The trypsin was neutralized with DMEM containing 10% FBS. The detached cells were collected and centrifuged at 500*g* for 5 min. The supernatant containing the neutralized trypsin solution was aspirated and the cells were resuspended in growth medium. Cells were subcultured so that one plate gave rise to five plates.

2.2. Characterization of primary murine renal cells

Cultivation of a heterogeneous population of renal cells requires identification of key cell types that would contribute to the formation of renal structures. The expression of several key proteins of tubular and glomerular cells was confirmed by immunofluorescence microscopy. Cells cultured were isolated as described above and allowed to expand to 90% confluence prior to passage, which took 5–7 days. Protein markers including aquaporins 1, 2, and 3, and Tamm–Horsfall protein (THP; [16–18]) were used to identify cells composing tubules. Cells derived from glomeruli were identified with antibodies specific to synaptopodin and von Willebrand factor [19,20].

Cells were processed for immunofluorescence microscopy as follows. Renal cells were plated onto 6-well tissue culture dishes. At 50–70% confluence, cell-containing dishes were placed on ice for media removal and three rinses with cold PBS. Plates were then removed from the ice and fixed with 4% paraformaldehyde (Polysciences, Inc., Warrington, PA) in PBS at room temperature for 10 min. The fixative was removed and cells were rinsed three times with PBS. Cells were permeabilized with 0.1% Triton-X 100 (Sigma, St. Louis, MO) in PBS for three minutes at room temperature. After three rinses with PBS, cells were blocked in a solution of 10% serum of the species from which the secondary antibodies were generated in PBS at room temperature for 30–45 min. Blocking was followed by incubation at room temperature for one hour with the primary antibodies diluted in 3% serum of the species from which the secondary antibodies were generated in PBS. Primary antibodies used included those directed against aquaporin-1 (Alpha Diagnostic International, San Antonio, TX), Tamm–Horsfall protein (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), synaptopodin (Research Diagnostics, Inc., Concord, MA), and von Willebrand factor protein (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For each antibody, a sample in which the primary antibody was omitted served as an internal control. After primary antibody treatment, three 5-min rinses with PBS were used to remove unbound primary antibody. Cells were then treated with the secondary antibodies diluted in 3% serum in PBS for 30 min. Secondary antibodies used included fluorescein-conjugated goat anti-rabbit IgG, fluorescein-conjugated horse anti-mouse IgG, and fluorescein-conjugated rabbit anti-goat IgG (Vector Laboratories, Burlingame, CA). After three 5-min PBS rinses, cells were mounted in VECTASHIELD HardSet mounting medium with DAPI (Vector Laboratories, Burlingame, CA).

Analysis of cells by immunofluorescence microscopy demonstrated that the isolated primary murine renal cells express protein markers characteristics of cells within renal proximal tubules (Fig. 1a; aquaporin-1; [17]); collecting ducts (Fig. 1b; aquaporin-2; Fig. 1c; aquaporin-3; [16]); and the thick ascending limb of Henle's Loop and distal convoluted tubules (Fig. 1d; Tamm–Horsfall protein; [18]). Glomerular cells were also present, indicated by positive staining for the visceral glomerular epithelial cell protein synaptodpodin (Fig. 1e; [19]) and von Willebrand factor protein, a protein expressed in glomerular endothelial cells (Fig. 1f; [20]). These results indicate that primary murine renal cell cultures comprise a heterogeneous cell population that contains components essential to the generation tubule and glomerular structures.

2.3. In vitro generation of three-dimensional renal structures from primary murine renal cells

We have developed a simple and reproducible technique that uses primary cells to form three-dimensional renal structures *in vitro*. This system utilizes rat tail collagen type I to suspend the primary renal cells in a three-dimensional matrix. Growth medium is added above the cell-containing collagen gel matrix to