Development of a Primary Culture System of Rat Kidney Proximal Tubule Cells for Transport Studies

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Abstract: Primary culture of proximal tubule cells have provided useful model for the study of proximal tubule cell function, especially transport studies. Kidney is excised from the anesthetized rat, cortex is separated and proximal tubule cells are isolated by using DNAse-collagenase and separated by using different mesh size sieves and collagen coated surfaces of flasks containing 15% Dulbecco's Modified Eagle's medium (DME) to exclusively avoid other cells or cell debris. The cells are then seeded onto rat-tail collagen- coated flasks containing 15 % DME. After one day, the above medium is changed and the cells are suspended with new 15% DME medium. After two days, the above medium is replaced with routine serum free culture medium containing growth factors, antibiotics and cholera toxin in different proportions. The medium is replaced once in 2 days. The cells are confluent in 6-8 days. The cells are then sub cultured in rat-tail collagen coated 24 well plates. These cells are confluent and ready for doing transport experiments after 2 or 3 days. Our histochemical methods reveal that the cells are positive for gamma glutamyl transpeptidase and glucose-6-phosphatase and hence they are of proximal tubule origin. Fluoroskan methods show cellular absorption of fluorescein methotrexate is concentration dependent and hence these cells exhibit original transport properties of the intact proximal tubule. Hence, the above methods are well suited to grow good quality proximal tubule cells and to assess its property for transport studies.

Keywords: Fluorescein methotrexate, histochemistry, primary culture, rat proximal tubule cells, transport.

INTRODUCTION

The kidneys act as organs of excretion, transport and metabolism. The kidney is a complicated organ, comprising several different cell types and having a sophisticated three dimensional organization [1]. Because of this complexity, the intact kidney is difficult to employ for adequate study of the many biochemical, pharmacological and physiological processes. Cell cultures, either primary cells or established cell lines [2], have provided useful model systems for the study of renal cell functions, especially transport studies. Established cell lines have several limitations, including resembling specific kidney cell types to a limited extent and being of poorly defined origin [3]. Primary cultures that are derived from known segments of the nephron minimize these problems [4].

Primary cultures of the renal proximal tubule have become important tools for examination of the mechanisms and control of transport processes [5]. The proximal tubule is a frequent target for nephrotoxic compounds, because of to its ability to transport and accumulate xenobiotics and their metabolites and of the presence of an organ-selective set of biotransformation enzymes [6]. This study presents a method for culturing rat proximal tubule cells for long-term use in transport or toxicity studies, based on critical modifications of a previous protocol [7].

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Enzyme histochemistry plays an important role in renal cell culture to define the site of origin and to reveal functional properties of the cells [8]. Though several techniques are available for the demonstration of enzymes in tissue sections, techniques for the direct demonstration of enzymes in cultured renal cells are very limited. This study developed histochemical methods to directly demonstrate γ-glutamyl-transpeptidase (GGT) and glucose-6-phosphatase (G-6-P) activities in cultured cells of proximal tubule origin, since both enzymes are sensitive enzyme markers of proximal tubules [9]. The transport properties of these cultured cells were also assessed using the model compound fluorescein-methotrexate (FL-MTX). Renal proximal tubule cells are known to transport folate compounds [10] and since FL-MTX is a folate analogue, transport in this study could be followed using fluorescence techniques. We have been doing folate transport studies in our laboratory and hence we have chosen FL-MTX as a model compound in this study.

METHODS

Animals and Basic Procedures

The animal protocols were approved by the Institutional Animal Care Committee (Louisiana State University Health Sciences Center-Shreveport) and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (150 – 175 g; Harlon, Houston, TX) were allowed access to pelleted rat food (Teklab Rodent Chow 8640) and water ad libitum. Prior to the experiment, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 g/kg body weight). All glassware and surgical tools were sterilized in an autoclave. The kid-

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neys were excised through an abdominal incision and immediately placed in cold Hank's balanced salt solution (HBSS) in 50 ml centrifuge tubes. The renal capsules were removed slowly and the kidneys were washed with cold HBSS in 50 ml centrifuge tubes for three or four times to remove blood and other remaining contents.

Culture Medium

The cell culture basal medium was a 50:50 mixture of Dulbecco's Modified Eagle's (DME) medium (with high glucose and HEPES) and Ham's F-12 medium. The culture medium was supplemented with 0.05% of bovine serum albumin (Armour Pharmaceutical Co., Kankakee, IL) and with transferrin (10 μg/ml), selenium (10 μg/ml), insulin (10 μg/ml), hydrocortisone (50 ng/ml), triiodothyronine (8 pg/ml), epidermal growth factor (20 ng/ml), glutamine (4 mM) and cholera toxin (10 ng/ml). Transferrin, selenium, insulin, hydrocorticosone, and epidermal growth factor were purchased from Collaborative Biomedical Research (Bedford, MA). Triiodothyronine and glutamine were purchased from Sigma (St Louis, MO) and GIBCO Life Technologies (Grand Island, NY) respectively. The above medium also contains 1% antibiotics (Penicillin and fungizone from GIBCO).

Isolation of Rat Proximal Tubule Cells

All procedures were carried out in a biological safety cabinet. The cortex was trimmed from the medulla in petri dish containing cold HBSS and minced into very small pieces (about 1 mm^3). HBSS was aspirated and the cortex pieces were washed in cold PBS carefully. These minced cortical pieces were then transferred into trypsinizing flask (Bellco, NJ) that was kept in a water bath at 37°C. After addition of a DNAse (1 mg/ml) and collagenase (2 mg/ml) Type I (Sigma) solution, the cortical pieces were digested by stirring for 30 minutes. After digestion, the solution was passed through an 80-mesh sieve (Fisher Scientific, Pittsburgh, PA) to remove cell debris and glomeruli. Again, the suspension was passed through a 170-mesh size sieve to remove distal tubules. Proximal tubule cells remained on the sieve filter and were collected by washing the sieve filter with HBSS. The proximal tubule cell suspension was transferred into 50 ml centrifuge tubes containing 10 ml of cold HBSS and was centrifuged for 10 minutes at 1000 rpm at 4° C. The proximal tubule cell pellet was collected. Cell count and cell viability were measured by mixing 0.1 ml of cells of the final cell suspension with 0.4 ml of 0.4 % trypan blue and counting the cells on a hemacytometer.

Primary Culture

The tissue culture flasks (75 cm^2, Costar, Cambridge, MA) were coated with rat-tail collagen (Sigma) and then treated with fetal calf serum (FCS) overnight at 4° C. The FCS was removed and the flasks were rinsed with PBS before use. The final proximal tubule cell pellet was resuspended with 6 ml of 15 % DME (15 ml of FCS per 100 ml DME) and dispensed into a collagen-coated flask (flask-1) containing an additional 10 ml of 15 % DME. After 3 hours, the suspension was transferred to a second collagen-coated flask (flask-2). The original flask (flask-1) was discarded to remove the cell debris and other cells that had adhered to the surface during the 3 hours. The cells in the new flask (flask-2) were incubated at 37° C for two days, and then the media was changed to 15 ml of the serum-free growth medium described above. Cells reached confluency in about 6-8 days.

Subculture

When the proximal tubule cells were confluent in the flasks, the cells were subcultured for various assays. The cells were washed with PBS and then loosened from the substratum using trypsin (0.5 mg/ml, Sigma) for an incubation period of 7 minutes at 37° C. EDTA (GIBCO) was then added to the suspension and the detached cells were centrifuged for 10 minutes. To the final pellet, the serum-free growth medium was added in order to seed onto new collagen-coated flasks or plates.

Enzyme Histochemistry

For GGT, the cells in 24 well plates were washed with 1 ml PBS and incubated with 1 ml incubation buffer for 40 minutes at 37° C. Incubation buffer comprised 0.5 ml of 20 mM of the substrate L-glutamic acid γ (4 – methoxy – β naphthylamide) in acetone, 0.5 ml of 25 mg /ml glycylglycine in 100 mM Tris assay buffer at pH 7.2 and 1 ml of a stock solution containing 15 mg/ml of the stable diazonium salt of o-aminoazotoluene (Fast Garnet GBC Base). Controls were performed by incubation in the same buffer without substrate. Cells were fixed by incubating with a 1% formaldehyde solution for 20 minutes at 4°C and washed with PBS several times before viewing under light microscope.

For G-6-P, cells in 24 well plates were incubated with 1 ml of test solution comprising 5 mM glucose 6-phosphate, 2 mM lead nitrate, 0.40 M sucrose and 0.08 mM sodium cacodylate (pH 6.7) at 37°C. for 80 minutes. Controls were performed by incubation in the same solution without substrate. The test solution was removed and the cells were washed once with PBS. The cells were then incubated with 1 ml of a 1% ammonium sulfide solution for 5 minutes. The ammonium sulfide solution was removed and the cells were washed three times with PBS. After removing PBS, the cells were viewed under light microscope. All the chemicals used in both assays were purchased from Sigma.

Transport Studies

For the transport studies with fluorescent substrates, the cells in the 24 well plates were washed twice with room temperature pH 7.4 inucbation buffer (20 mM HEPES, pH 7.4, containing NaCl, KCl, $CaCl_2$, $MgCl_2$, D-glucose, and $NaHCO_3$) [10] and then treated with 1.5 ml of the above buffer containing 1, 2, 4, 8 and 16 μM FL-MTX for 1 hour**.** The cells were washed with the incubation buffer and the fluorescence intensity was read in a micro plate reader (Biorad model 3550) with the filter pair 485 nm (excitation) and 538 nm (emission). FL-MTX was purchased from Molecular Probes (Eugene, OR) and all other chemicals in the incubation buffer were purchased from Sigma.

RESULTS

Cell Culture Medium and Growth of Proximal Tubule Cells in Culture

The cell culture medium is similar to that of a previous protocol [7], but with higher amounts of certain growth factors. Hydrocorticosone was increased 1.5 fold, while triiodo-

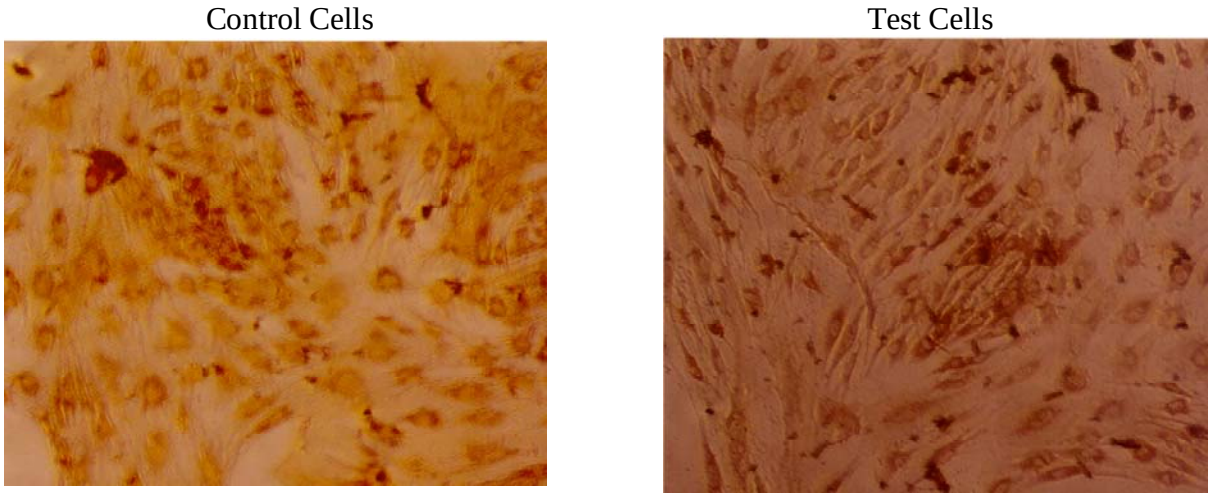

Fig. (1). Histochemical localization of γ-glutamyl-transpeptidase (GGT) activity in the cultured rat proximal tubule (RPT) cells. Cells were grown to confluency in 24 well plates at passage number 7. GGT-positive cells were reddish brown. Magnification (100 X) for both control and test cells.

thyronine, epidermal growth factor and glutamine were increased 2 fold compared with the previous concentrations of these growth factors [7]. In addition, 10 ng/ml of cholera toxin was added to the culture medium [11]. These variations were necessary so that the cells in both flasks and multi-well plates grew enough to become fully confluent.

With this procedure, a large number of homogeneous, viable proximal tubule cells were obtained. By increasing the duration of the digestion with the DNAse – Collagenase mixture to 30 minutes, a higher yield of single, viable proximal tubule cells resulted. The cell yield was ~ 35×10^6 cells per rat and cell viability was routinely > 95 %. The proximal tubule cells obtained from one kidney were sufficient to seed one 75 cm^2 flask (seeding density of 150-200 µg cellular protein/cm^2). By using the sieving method, the other cells and debris that remained in the final cell suspension was < 3 %. After seeding the final cell suspension into a flask for 3 hrs, the entire suspension was transferred to a new flask. Cell debris and other tissue remained attached to the collagen-coated surface of the first flask. In this way, the occurrence of other cells or cellular contents in the proximal tubule cell suspension was greatly diminished. These changes in cell preparations and in the growth medium enhanced the growth of the cells such that cells could be readily subcultured. Cells have been passaged to 16 passages with a similar growth rate and viability. Cells retained a cuboid cobblestone appearance up to about 6-7 passages, then became elongated in appearance.

Enzyme Histochemistry

Under light microscopic examination, GGT–positive cells were reddish brown (Fig. **1**). G-6-P activity was seen as black granular deposits of lead surface on the cells (Fig. **2**). GGT and G-6-P activity in these cells revealed that the cells were of proximal tubule origin, since these activities are mainly confined to proximal tubules of the nephron [12].

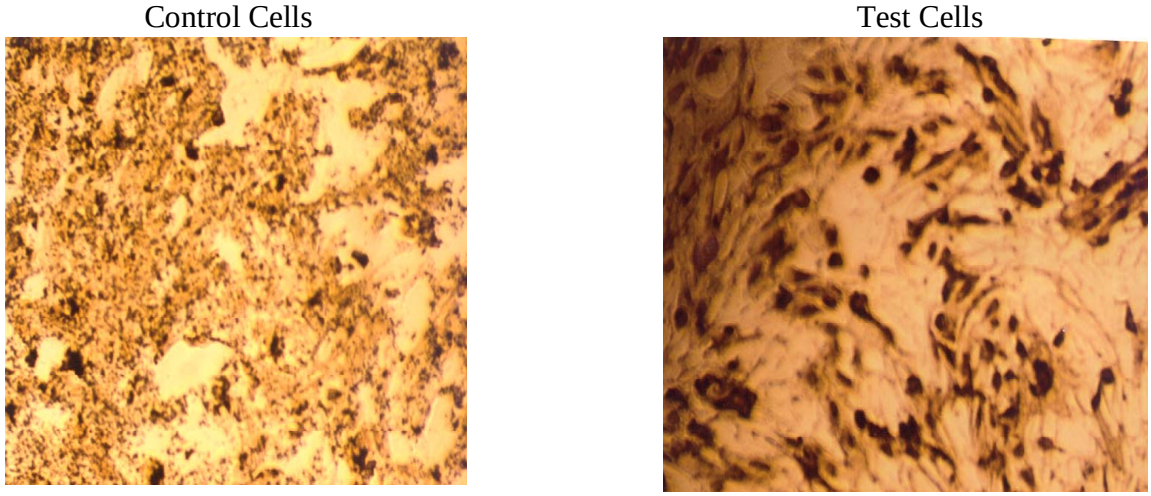

Fig. (2). Histochemical localization of glucose-6-phosphatase (G-6-P) activity in the cultured rat proximal tubule (RPT) cells. Cells were grown to confluency in 24 well plates at passage number 8. G-6-P activity was seen as black granular deposits of lead sulfide concentrated on the individual cells, whereas there was an amorphous distribution of lead nitrate in control cell populations. Magnification (100 X) for both control and test cells.

3.3. Transport studies FL-MTX, being a reduced folate derivative, should be transported by the reduced folate carrier protein [13]. Previous studies have shown that proximal tubule cells derived from human tissue retain significant amounts of folate transport by the reduced folate carrier [14]. In these rat proximal tubule cells, transport of the folate analogue, FL-MTX was concentration dependent (Fig. **3**) and hence the cells exhibited reliable transport properties.

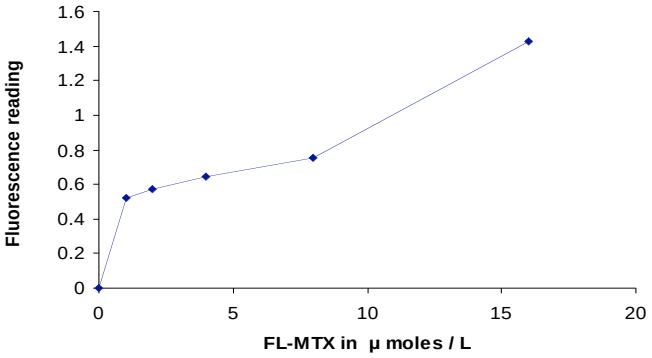

Fig. (3). Transport of FL-MTX by cultured rat proximal tubule (RPT) cells. Cells were grown in 24 well plates and then exposed to FL-MTX (1, 2, 4, 8 and 16 µM) for 1 hour ; after the cells were washed to remove unattached substrate, transport was determined by the increase in fluorescence (microplate reader). The data represent the mean of five experiments ± SEM with cells at passages 1, 3,5,7,9.

DISCUSSION

In this study, we developed a protocol for culturing pure quality proximal tubule cells for transport studies. Unlike other studies, for transport studies, the cells of proximal tubule origin should exhibit original characters of proximal tubule such as reabsorptive and secretory functions. In this study, we carried out some modifications and in addition, we developed histochemical and fluorescent methods to assess directly the quality of the growing cultured cells.

In this protocol, proximal tubule cells were collected by sieving technique. We used 80 mesh sieves to remove cell debris and glomeruli and 170 mesh size sieves to remove distal tubules from the proximal tubule cell suspension. Liu and Preisig [15] used 80 and 170 mesh size sieves to collect proximal tubules. We transferred the final pellet into 15% DME in collagen coated flasks and then kept for three hours to remove debris and also other proteinous substances, in case if they were found in the proximal tubule suspension. Actually debris and other proteinous substances will easily precipitate and deposit on the collagen coated surface. The proximal tubules would remain in the DME suspension. The above suspension was then transferred to a new collagen coated flask. In this way, we can eliminate debris and other substances present in the proximal tubule suspension.

The cells were grown very well in rat tail collagen coated flaks and also well plates. Normally, collagen coated surface will improve morphological and biochemical properties of proximal tubule epithelial cells in culture [16]. We also observed that the cells became detached or grew very slow in uncoated flasks or plates. Cholera toxin was added to the medium in addition to different proportions of growth factors. In this condition, cells became confluent very soon. Cholera toxin increases growth [17] and transport properties of rat renal proximal epithelial cells in culture [18]. We have focused on increasing attachment, improvement of growth rate and retention of proximal tubule function by using this medium.

Though the principles of the histochemical techniques are the same for tissue sections, we adapted our methods in such a way so as to preserve enzymes and cells and also to avoid diffusion of enzymes and / or reaction products. Normally, histochemical demonstration of the enzymes of the cells under culture will be carried out by the way of scraping the cells in the well plates and freezing and then subjected to sectioning and staining [19]. Freeze drying is not suitable for the demonstration of all enzymes since it destoroys proteins [20]. The methods we developed were comparatively simple, rapid and sensitive for the direct histochemical demonstration of GGT and G-6-P on renal cells (primary or cell lines) in 24 well plates or other well plates. For GGT, we followed the method of Rutenberg *et al.* [21] with many modifications (cells were incubated for 40 minutes in the test solution and fixed with formalin for 20 minutes) suitable for well plates and for G-6-P, we followed the method of Wachstein and Meisel [22] for tissue sections but many modifications (cells were incubated for 80 minutes in the test solution and then incubated with ammonium sulfide solution for 5 minutes) suitable for 24 well plates. The incubation periods, and other procedures were entirely different from those that used for the tissues. Since it is known that formalin will destroy G-6-P [23], in this study, formalin was used as a fixative for the demonstration of GGT and not for G-6-P. All the cells we cultured using our protocol were positive for GGT and G-6-P histochemistry.

Since we worked with folate to assess the mechanism of its transport, we developed a method by using confocal microscope and fluroskan micro plate reader to evaluate the effect of ethanol on endothelin mediated folate transport by using endothelin –B receptor antagonist (RES-701-1) and protein kinase-c selective inhibitor(BIM). We observed that ethanol inhibited FL-MTX uptake was reversed by the addition of the (RES-701-1) or (BIM) (unpublished data). We used the Fluorescent (fluroskan) method to evaluate the transport property of these cells. FL-MTX, a folic acid analogue, was used in this study as a transport substance. The transport of FL-MTX is mediated by the reduced folate transporter [13]. From this, we can conclude the cells exhibit functional carrier proteins which is a measure of the quality of the proximal tubule cells for transport properties.

Several studies show that the transport property can be manipulated by culture conditions such as cell density, where transport increases with density. GGT activity is also associated with cell density [24]. From this, we can also conclude that GGT activity is a measure of transport properties. Normally GGT is expressed in cells having secretory or absorptive function [25]. GGT activity is also a measure of cell growth [26] and renal epithelial cellular glutathione transport [27]. Methotrexate transport appeared to be correlated with GGT activity [28]. G-6-P is a marker enzyme for gluconeogenesis [29] and glucose 6-phosphate transport is required for the functioning of the G-6-P [30]. Increased trans-

port activities are consistent with increased rates of intermediary and oxidative metabolism in the cells [4].

For transport studies, especially to evaluate the molecular mechanism, the cells in the culture exhibit all functional properties of the *in vivo* proximal tubule. After characterizing these cells, we revealed that the cells were of proximal tubule origin and exhibiting good transport properties. From this, we can conclude that our proximal tubule isolation method is reliable and the cells in the culture are appropriate for transport and also for other *in vitro* studies. We have sub cultured these cells easily for about 16 passages and the cells also grew very well and exhibited reliable transport properties at the same rate in all the passages. The methods (histochemical and florescent) we adapted will be useful to assess directly the quality of renal or other primary cells and cell lines in cultures.

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