Technical Bulletin #412

A Physiological and Morphological *In Vitro* Model for Normal Human Urothelium Cultured on Falcon® Cell Culture Permeable Supports

FALCON®
A Corning Brand

William I. de Boer[1], Theodorus H. van der Kwast[2], and Dominique K. Chopin[3]
[1]Department of Pulmonology, University Hospital Leiden, Leiden;
[2]Department of Pathology, ErasmusUniversity, Rotterdam, The Netherlands;
[3]Department of Urology, Hôpital Henri Mondor, Créteil, France

Introduction

Several *in vitro* models have been demonstrated for cultivation of normal human urothelium. Besides nonporous plastic substrates and collagen gels, also permeable substrates have been used like cellulose acetate, nylon, or polycarbonate. As outlined previously for the cultivation of normal mouse urothelium[1], Falcon Cell Culture Permeable Supports with polyethylene terephthalate membranes allow culture nutrients to diffuse to both the basolateral and the apical side of cells. For subsequent characterization of the cultures, Falcon Cell Culture Permeable Supports offer several advantages above the aforementioned culture substrates like its transparency for visualizing and measuring morphology resistance to solvants used in electron microscopy, rigidity during cross sectioning, and applicability for immunocytochemistry[1]. This technical bulletin summarizes advantages of these Falcon Cell Culture Permeable Supports when characterizing normal human urothelium in primary culture.

Materials and Methods

Primary Cell Cultures: Falcon Cell Culture Permeable Support membranes (0.4 µm size, Cat. No. 353090) were coated with 2.9 µg/cm^2 human collagen type IV. Other coatings (fibronectin, laminin, collagen type I) were less optimal for cellular adhesion and outgrowth. Normal human urothelium from ureters was obtained as described[2] from kidney transplantation donors or patients undergoing nephrectomy for chronic renal diseases. After dissecting the stromal layers from the urothelium, the urothelial explant with the basement membrane facing the membrane was cultured in Ham's F12 plus DMEM and 10% heat inactivated foetal calf serum[2].

Functional Assay: To test the physiological status of the cultures, dye leakage and exclusion tests were performed. After extensive rinsage with PBS, cultures were incubated with medium with phenol red in the upper compartment and medium without phenol red in the lower compartment. During 1.5 hours, medium from the lower compartment was regularly checked for phenol red leakage by spectrophotometry (wavelength at 560 nm). A similar experiment was done with trypan blue instead of phenol red. Diffusion of these dyes through a nude membrane served as a control.

Immunocytochemistry: On termination of the cultures, 40 µg/ml 5-bromo-2'-deoxyuridine (BrdU) was added during 2 hours. Cultures were rinsed with PBS, fixed with 70% (v/v) ethanol, and stored under 70% ethanol (v/v) at 4°C for immunocytochemistry. Prior to immunocytochemistry, each membrane was cut into the required number of pieces (approximately 12) with a scalpel. For revelation of BrdU incorporation, cultures were first treated with HCI and Borax buffer1,2. After preincubation with 5% (w/v) milkpowder in PBS, expression of BrdU, chain-specific cytokeratins, uroplakins, E-cadherin, or growth factors and their receptors was shown in a conjugated immunoenzyme assay with appropriate dilutions of the primary antibodies. The secondary antibodies were conjugated with either peroxidase or alkaline phosphatase. As chromogens served 3,3' diaminobenzidine (DAB) and Fast Red Violet LB with naphtol AS-MX as coupling reagent. Endogenous alkaline phosphatase activity was inhibited with 1 mM levamisole.

A double immunostaining technique was performed for BrdU and cytokeratins. Cultures were finally mounted in an aequous mounting medium.

Histology: In order to determine the number of cell layers, membranes of immunostained cultures were cut into small strips by scalpel or scissors. These pieces were dried in 1% glycerol/PBS at room temperature overnight to enforce the rigidity of the membrane and the adherence of the cells to the membrane. Finally, the pieces were embedded in paraffin for cross sectioning. Culture pieces not fixed with ethanol nor immunostained, were fixed with 1.5% glutaraldehyde (w/v) in 0.1 M cacodylate buffer pH 7.4. Transmission electron microscopy was done as described[1,2] on 1% OsO4 (w/v) and 1.5% potassium ferrocyanide (w/v) treated ultrathin Epon sections.

RNA Analysis: In order to confirm protein expression as detected by immunocytochemistry at the mRNA level, halves of confluent cultures including membranes were immersed in RNA-plus (Bioprobe, Montreuil sous Bois, France) for RNA extraction according to the manufacturer. Reverse transcription of total mRNA was carried out as described[2] and levels of Ecadherin and some fibroblast growth factor receptors were determined by semi-quantitative PCR using the transcription factor TFIID as an internal control. The procedures are described elsewhere in detail[2].

Results and Discussion

Within 20 days, culture areas were fully covered with cells[2]. The cells showed a cuboidal morphology in multiple cell layers (up to 5 layers) as seen by cross sectioning and transmission electron microscopy (**Figures 1 and 2**). Immunocytochemical characterization demonstrated the expression of cytokeratin 18 preferentially in superficial umbrella cells, cytokeratin 19 and E-cadherin in all cells, and several uroplakin-positive umbrella cells (**Figure 2**). In addition, epidermal growth factor receptor expression and BrdU incorporation was seen only in basal and intermediate layers. No background staining was visible during immunocytochemistry, while the data also show that antibodies have access to all cell layers. The expression data correspond with *in vivo* expression patterns and support the morphological resemblance of the cultures with human urothelium *in vivo*. The dye leakage tests pointed out that there was no leakage of phenol red or trypan blue into the lower compartment whereas nude membranes leaked both dyes within several minutes reaching maximal absorption within 30 minutes. This confirms the functional integrity of the cultures since no active or passive transport of the dye occurred. Finally, we demonstrated that mRNA can be extracted from whole cultures while adherent to the membrane and specific mRNAs can be detected by RTPCR techniques. As an example, in **Figure 3** the presence of two different mRNAs in urothelial cells cultured on membranes and urothelial cells scraped from the ureter wall are compared indicating similar profiles.

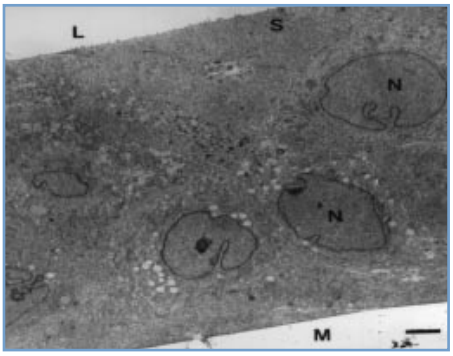

Figure 1: Transmission electron micrograph of a primary urothelial culture (M=membrane; L=luminal surface, S=superficial cell; N=nucleus; arrow=desmosome). Original magnification=3,000x. Scale bar=2.5 µm.

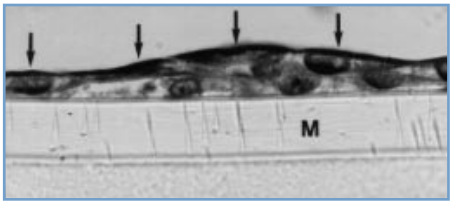

Figure 2: Cross section of a confluent urothelial culture previously immunostained with a chain-specific antibody against cytokeratin 18 (M=membrane; arrow=cytokeratin 18 expressing superficial cell). Original magnification=1,000x

In our first report[1], we showed that Falcon® Cell Culture Permeable Supports with PET membranes were suitable for cultivation of primary cultures of normal mouse urothelium. Using different techniques on the same membrane piece we could assess proliferation by incorporation of both [3H]-thymidine and BrdU, differentiation by cytokeratin expression and morphology on cross sections, and migration by morphometry during culture. The techniques included morphometry, immunocytochemistry with dyes or fluorescence, determination of incorporated radioactivity, and transmission electron microscopy[1]. On primary cultures of normal human urothelium, we were able to assess the same parameters as well as performing a functional dye transport assay. Recently, we also assessed migration of human urothelial cells on these membranes by morphometry during culture, and by localization in a thick gel of Corning® Matrigel® Matrix using confocal laser scanning microscopy[3]. Our data point out that using Falcon Polyethylene Terephthalate Cell Culture Permeable Supports we were able to obtain an *in vitro* model resembling normal human urothelium *in vivo* both morphologically and physiologically.

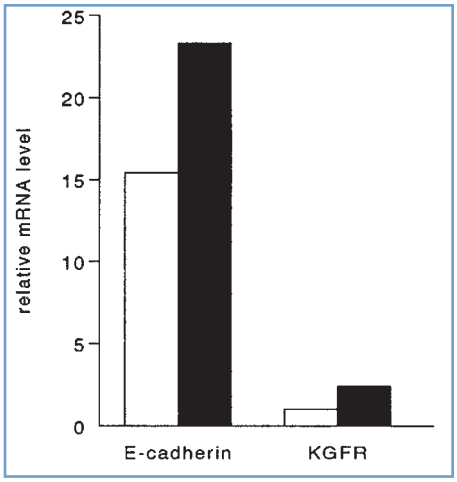

Figure 3: Relative mRNA levels for E-cadherin and keratinocyte growth factor receptor (KGFR) in primary cultures (open bars) or urothelium isolated freshly from the ureter wall (solid bars) were expressed as (specific signal - background)/ (TFIID signal - background).

References

1. de Boer, W.I., Rebel, J.M.J., Vermey, M., Thijssen, C.D.E.M., and van der Kwast, T.H. Multiparameter analysis of primary cultures grown on Cyclopore membranes. J. Histochem. Cytochem. 42:277 (1994).
2. de Boer, W.I., Vermeij, M., Gil Diez de Medina, S., Bindels, E., Radvanyi, F. van der Kwast, T., and Chopin, D. Functions of fibroblast and transforming growth factors in primary organoid-like cultures of normal human urothelium. Lab. Invest. 75:147 (1996).
3. de Boer, W.I., Houtsmuller, A.B., Izadifar, V., Muscatelli-Groux, B., van der Kwast, T.H., and Chopin, D.K. Expression and functions of EGF, TGF, and TGF-b-growth-factor family members and their receptors in invasive human transitional-cell-carcinoma cells. Int. J. Cancer 71:284 (1997).

Corning acquired the Falcon® and Matrigel® brands. For information, visit www.corning.com/discoverylabware.

CORNING

Corning Incorporated
Life Sciences
836 North St.
Building 300, Suite 3401
Tewksbury, MA 01876
t 800.492.1110
t 978.442.2200
f 978.442.2476
www.corning.com/lifesciences