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Glomerular filtration drug injury: In vitro evaluation of functional and morphological podocyte perturbations

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ABSTRACT

The kidney is an organ that plays a major role in the excretion of numerous compounds such as drugs and chemicals. However, a great number of pharmacological molecules are nephrotoxic, affecting the efficiency of the treatment and increasing morbidity or mortality. Focusing on glomerular filtration, we propose in this study a simple and reproducible in vitro human model that is able to bring to light a functional podocyte injury, correlated with morphologic/phenotypic changes after drug exposure. This model was used for the evaluation of paracellular permeability of FITC-dextran molecules as well as FITC-BSA after different treatments. Puromycin aminonucleoside and adriamycin, compounds known to induce proteinuria in vivo and that serve here as positive nephrotoxic drug controls, were able to induce an important increase in fluorescent probe passage through the cell monolayer. Different molecules were then evaluated for their potential effect on podocyte filtration. Our results demonstrated that a drug effect could be time dependent, stable or scalable and relatively specific. Immunofluorescence studies indicated that these functional perturbations were due to cytoskeletal perturbations, monolayer disassembly or could be correlated with a decrease in nephrin expression and/or ZO-1 relocation. Taken together, our results demonstrated that this in vitro human model represents an interesting tool for the screening of the renal toxicity of drugs.

1. Introduction

The kidney represents an important target for drug-induced toxicity since it is responsible for the excretion of numerous xenobiotics and is continuously exposed to drugs. The filtering unit of the kidney, the nephron, contains capillaries whose walls function as a filtration barrier. In addition to the glomerular endothelial cells, the filtration process implies specialised cells, the podocytes that cover the capillary. The structural organisation of the podocyte that displays an interdigitating structure (foot process) assures a selective filtration of molecules depending on their size [1]. Considering that the cut-off is around 70 kDa, the finding of albumin in urine is the first symptom of glomerular filtration dysfunction that could be observed in numerous pathologies [2]. Disorganisation of the podocyte architecture using nephrotoxic drugs, such as puromycin aminonucleoside (PAN) or adriamycin (ADR), results in an increase of protein barrier crossing, confirming the involvement of this cell as a major actor of glomerular filtration [3,4]. There is a need of simple relevant models to evaluate the effects of pharmacological drugs on glomerular filtration, to screen new compounds and to elucidate cellular events involved in functional perturbations. Numerous rodent models [5,6] have been developed to study kidney physiology and functionality, but recently research is focusing on the podocyte considering that this cell is the “weak link” in the filtration process [7]. In vitro approaches have been studied using rodent podocytes, but the establishment of a human differentiation inducible cell line by M. Saleem offers new perspectives for the study of the human podocyte [8]. This cell line has been already used for fundamental research [9] as well as for the study of cellular events involved in kidney pathologies [10].

Considering that such a model could be used for the screening of numerous compounds, we chose a simple, standardisable approach focusing on the podocyte to evaluate the effects of pharmacological

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compounds on glomerular filtration. The goal of our study was to set up an in vitro human podocyte model in order to be able to predict the deleterious effect of drugs on glomerular filtration. Selective glomerular filtration was determined by measuring the apparent permeability of 70 kDa dextran molecules or BSA, before and after drug exposure. The analysis of morphological modifications was done to correlate functional perturbations to phenotypic/morphological changes.

2. Materials and methods

2.1. Cell culture

Except when specified, all the reagents were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Podocytes were routinely cultured in RPMI 1640 medium containing 10% foetal bovine serum (FBS), 1% insulin-transferrin-selenium-A supplement, and 1% penicillin/streptomycin solution. The confluent monolayer was regularly disrupted by using a trypsin-EDTA solution.

The immortalised human podocytes were routinely cultured at 33 °C with 95% air and 5% CO_2 in 75 cm^2 flasks (BD Falcon, Le Pont de Claix, France). To obtain fully differentiated podocytes, the cells were switched to a 37 °C incubator (5% CO_2) and cultured in the same medium for 12–14 d.

For cultures on permeable supports, undifferentiated cells (grown at 33 °C) were seeded at a density of 2.5 × 10^5 cell per filter (BD-Falcon cell culture inserts, 24-well size, 3 μm pore size), cultured 24 h at 33 °C for cell adhesion and then switched to 37 °C for 12–14 d. Medium was changed twice a week.

Caco-2 cells, approved by the FDA for in vitro transport studies, were chosen as a positive control for epithelium tightness since these cells display a significant transepithelial electrical resistance (TEER) when cultured on permeable support. These cells, grown on DMEM high glucose medium supplemented with FBS (10%) and 1% penicillin/streptomycin solution, were seeded at a density of 10^5 cells per filter (3 μm pore size BD Falcon inserts, 24-well plate format). These cells were used when the TEER (measured with an EVOM resistance meter; World Precision Instrument, Sarasota, USA) reached a value in the range of 400 Ω cm^2.

2.2. Apparent permeability measurements

The evaluation of selective filtration was done with four FITC-dextrans possessing different molecular weights (4, 20, 70 and 150 kDa). Apparent permeability was calculated with the following formula:

$$P_{app} = \frac{Vr}{C_0} \times \frac{1}{S} \times \frac{C_2}{t}$$

Where P_{app} is the apparent permeability, Vr is the volume of medium solution in the receiving chamber, C_0 is the initial concentration of drug in the basal compartment, S is the area of the monolayer, C_2 is the concentration of drug found in the apical compartment after an incubation of 4 h, and t is the incubation time.

The experiments were performed with undifferentiated cells, differentiated podocytes or Caco-2 cells (control of epithelial tightness). Podocyte filtration functionality was determined in the basal to apical direction. Cells were first incubated for 1 h at 37 °C in serum-free RPMI medium (SF-RPMI) and then incubated with FITC-dextran or FITC-BSA at a concentration of 0.5 g L^{-1} in the basal compartment (1 mL). After an incubation of 4 h at 37 °C, 100 μL were taken from the apical compartment, transferred into a 96-well plate and fluorescence was measured using a fluorimeter (Exc = 485 nm, Em = 538 nm; Fluoroskan Ascent, ThermoFisher Scientific, Dardilly, France). All the assays were performed in quadruplicates (n = 4).

2.3. Modulation of podocyte transport by drugs

Drug effect on podocyte permeability was evaluated by using FITC-BSA, the gold standard protein for determination of glomerular filtration efficiency. The two known nephrotoxic drugs PAN and adriamycin ADR were used as positive controls for podocyte injury. Different compounds including risedronate (Rise), gentamycin (Gent), vancomycin (Vanco), ketoprofen (Keto), heparin were evaluated for their capacity to modulate podocyte filtration function.

Podocyte filtration was determined in the basal to apical direction with the same procedure as described above. The epithelial crossing of FITC-BSA was then performed as described above.

In addition, a high and a low concentration were tested during several days (3, 7 or 10 d). The apparent permeability of the podocytes was evaluated at the end of the incubation time. The two concentrations were chosen in agreement with the literature [11–13] and after preliminary assays were done to evaluate the highest nontoxic dose for each compound in our podocyte model. Untreated and PAN-treated cells served as negative and positive control, respectively, for podocyte dysfunction. Each experiment was done in quadruplicate (n = 4).

2.4. Cytotoxicity studies

Cell toxicity was evaluated by using the lactate dehydrogenase (LDH) release test that was purchased from Promega Corporation (Madison, USA). The experiments were performed according to the manufacturer's instructions.

2.5. Immunofluorescence studies

Immunofluorescence experiments were performed with Phalloidin-iFluor 555 (Abcam, Cambridge, UK) and 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI; Life Technologies, Carlsbad, USA) to visualise cell cytoskeleton and nucleus, respectively. Mouse anti-ZO-1 antibody (clone 1) and mouse anti-nephrin antibody (clone 174CT2.1.1), used to visualise cell-cell contact complexes, were purchased from BD Bioscience (Le Pont de Claix, France) and from Sigma-Aldrich, respectively. Anti-mouse Alexa Fluor 488 conjugated secondary antibodies, used to detect primary antibodies, were obtained from Life Technologies.

Differentiated podocytes grown on glass coverslips were fixed with paraformaldehyde (3.7% in PBS) and permeabilised with Triton X-100 (0.5%). Then the cells were incubated with primary antibodies directed against nephrin (1/50) or ZO-1 (1/50) for 1 h at room temperature, washed and incubated with the anti-mouse Alexa Fluor 488 conjugated secondary antibodies (1/200) in the presence of Phalloidin-iFluor 555 and DAPI to visualise F-actin and cell nucleus, respectively. The images were acquired using an epifluorescence inverted microscope (IX81, Olympus, Tokyo, Japan) equipped with a cell imaging software (Soft Imaging System GmbH, Munster, Germany).

3. Results

3.1. Setup of the podocyte in vitro model: morphological aspects

Fig. 1 illustrates the cell line cultured at 33 °C (undifferentiated cell, left panel) or at 37 °C (differentiated cells, right panel). Undifferentiated cells formed a monolayer of relatively small cells with an intracytoplasmic peripheral pattern of actin staining. In contrast, differentiated podocytes appeared as large spreading cells that possess an important and multidirectional actin staining with small cytoplasmic extensions between cells resembling the specific foot process structure that is observed in vivo.