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Effects of shiga toxin 2 on cellular regeneration mechanisms in primary and three-dimensional cultures of human renal tubular epithelial cells

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ABSTRACT

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) causes post-diarrheal Hemolytic Uremic Syndrome (HUS), which is one of the most common causes of acute renal failure in children in Argentine. The aim of the present work was to study the effects of Shiga toxin type 2 (Stx2) on regenerative mechanisms of primary cultures of human cortical renal tubular epithelial cells (HRTEC) and three-dimensional (3D) cultures of HRTEC. Primary cultures of HRTEC were able to develop tubular structures when grown in matrigel, which showed epithelial cells surrounding a central lumen resembling the original renal tubules. Exposure to Stx2 inhibited tubulogenesis in 3D-HRTEC cultures. Moreover, a significant increase in apoptosis, and decrease in cell proliferation was observed in tubular structures of 3D-HRTEC exposed to Stx2. A significant reduction in cell migration and vimentin expression levels was observed in HRTEC primary cultures exposed to Stx2, demonstrating that the holotoxin affected HRTEC dedifferentiation. Furthermore, a decreased number of cells expressing CD133 progenitor marker was found in HRTEC cultures treated with Stx2. The CD133 positive cells also expressed the Stx receptor globotriaosylceramide, which may explain their sensitivity to Stx2. In conclusion, Stx2 affects the regenerative processes of human renal tubular epithelial cells *in vitro*, by inhibiting cell dedifferentiation mechanisms, as well as tubules restoration. The development of 3D-HRTEC cultures that resemble original human renal proximal tubules is a novel *in vitro* model to study renal epithelial repair mechanisms after injury.

1. Introduction

Post-diarrheal hemolytic uremic syndrome (HUS) [1,2], due to Shiga toxin (Stx)-producing *Escherichia coli* (STEC), is a common cause of acute renal failure in children younger than 5 years [2]. *E coli* O157:H7 and other STEC strains express Shiga toxins type 1 and/or 2 (Stx1, Stx2), which are associated with HUS [3]. Stx2 is more virulent than Stx1, and STEC strains expressing Stx2 are highly prevalent in Argentine [4,5]. Stxs are AB5 holotoxins, consisting of an A subunit monomer non-covalently linked to a pentamer of B subunits [6]. The pentamer binds to the globotriaosylceramide (Gb3) receptor on the surface of several eukaryotic cells, followed by holotoxin internalization and translocation to the endoplasmic reticulum by a retrograde pathway [7,8]. The Stx A subunit exhibits RNA N-glycohydrolase activity on the 28S ribosomal subunit, which produces the inhibition of protein synthesis [7,9].

The human kidney expresses high levels of Gb3 [10], being a major target organ for Stx in HUS. Acute tubular damage was reported in kidneys of patients with HUS [11,12] and the proximal tubule seems to be an important early target of Stx action in the kidney [11]. We have previously shown that Stx2 inhibited protein synthesis [13], and decreased cell viability and proliferation, as well as stimulated apoptosis in human renal tubular epithelial cells

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(HRTEC) primary cultures [13,14]. The treatment with C-9, a specific inhibitor of glucosylceramide synthase, decreased Gb3 expression levels and prevented the cytotoxic effects of Stx2 on HRTEC [15], and on renal proximal tubules of an experimental rat model of HUS [16].

It is known that after acute renal injury tubular cells have the ability to regenerate and restore the renal tubular epithelium. The process of regeneration involves the dedifferentiation of surviving tubular cells, which migrate and proliferate to cover the injured area [17]. Subsequently, cells redifferentiate to restore the normal functional epithelium [18,19]. Several groups have demonstrated the presence of a subpopulation of human renal proximal tubular cells, characterized by the expression of the progenitor markers CD133 and CD24 [20–22] as well as vimentin [23–25]. These cells have been proposed to be involved in the regeneration of tubular cells, particularly after acute kidney injury [23,25,26]. Vimentin, the major intermediate filament protein of mesenchymal cells, has been shown to participate in critical functions, such as cell adhesion, migration, and cell signaling, therefore, it is frequently used as a marker of cell development and dedifferentiation [27].

The aim of the present work was to study the cytotoxic action of Stx2 on the regenerative process of HRTEC primary cultures. For this purpose, a novel model of three-dimensional (3D) culture of human renal tubular epithelial cells (3D-HRTEC) was developed and characterized, and tubulogenesis was evaluated in the absence and presence of Stx2. Furthermore, mechanisms and markers involved in cell dedifferentiation were analyzed in HRTEC, such as cell migration, proliferation, and vimentin expression. The progenitor cell marker CD133 was also evaluated. Three-dimensional cultures of HRTEC were used to study the effects of Stx2, in order to evaluate whether these human renal cultures have the ability to recover from Stx2 effects. Our results show that 3D-HRTEC cultures constitute a novel and accurate model for studying regeneration mechanisms in human renal cells after injury.

2. Materials and methods

2.1. Cell culture

HRTEC primary cultures were isolated from kidneys removed from pediatric patients undergoing nephrectomies, indicated for the correction of urological problems by the pediatric surgical section at the Hospital Nacional Prof. A. Posadas, Buenos Aires, Argentina. The Ethics Committee of the Hospital Nacional Prof. A. Posadas approved the use of human renal tissues for research purposes. The primary cultures were performed according to the methods described previously [14]. To amplify the primary cultures, cells were grown in flasks to confluence in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine and 100 U/ml penicillin/streptomycin, and incubated in 5% CO_2 atmosphere at 37 °C. Aliquots of cells were resuspended in 10% DMSO, stored in liquid nitrogen, and used between 3 and 5 passages.

2.2. Three-dimensional cultures of HRTEC (3D-HRTEC). Tubulogenesis assay

To develop 3D-HRTEC cultures, HRTEC primary cultures were treated with trypsin, and cells were washed and quantified. Cells (~4 × 10^4 cells/well) were then seeded on an extracellular matrix (Matrigel, BD Biosciences, 150 μl/cm2), and allowed to grow in the same culture medium used for primary cultures. Tubulogenesis was then monitored under an inverted optical microscope (Nikon Eclipse TS100), and micrographs were obtained using a digital camera (Nikon Coolpix S10). Isolated 3D structures were fixed with 4% paraformaldehyde in phosphate buffer 0.1 M (pH 7.4), dehydrated and embedded in paraffin. Sections of 5 μm were stained with hematoxylin-eosin. For some experiments, HRTEC were incubated with 10 ng/ml or 10 pg/ml of pure Stx2 (Phoenix Laboratory, Tufts Medical Center, Boston, MA, USA), for different periods of time. The toxin was then removed and the cells were trypsinized, and seeded on matrigel to evaluate tubulogenesis. Lipopolysaccharide (LPS) contamination, analyzed by Limulus amoebocyte lysate assay, demonstrated that Stx2 contained <10 pg LPS/ng of pure Stx2.

2.3. Apoptosis on 3D-HRTEC cultures

3D-HRTEC cultures were developed for 2 days, and tubular structures were treated or not with 10 ng/ml Stx2 for 30 min, washed, and incubated for 24 h in Stx2-free culture medium. The percentage of apoptotic cells was established morphologically by fluorescence microscopy after staining with acridine orange/ethidium bromide (AO/EB, 1:1, v/v) in a final concentration of 100 mg/ml. Each experiment was performed in duplicate, counting 15–20 tubules per 3D culture. Apoptotic cells were defined on the basis of nuclear morphological changes such as chromatin condensation and staining, as described previously [14].

2.4. Cell proliferation on 3D-HRTEC cultures

Cell proliferation rate was measured by incorporation of 5-Bromo-2-DeoxyUridine (BrdU) into the DNA of cells in S-phase of the cell cycle. The 3D-HRTEC cultures were developed for 2 days, and tubular structures were treated or not with 10 ng/ml Stx2 for 30 min. Tubular structures were then pulse-labeled with 100 μM BrdU, and incubated for 24 h in Stx2-free culture medium. For BrdU detection, indirect immunofluorescence was performed using a primary antibody against BrdU (Sigma-Aldrich, MO, USA) and Alexa fluor 488 goat anti-mouse IgG as secondary antibody. Total number of cells was calculated by staining cell nuclei with Hoechst (1 μg/ml). Each experiment was performed in duplicate, counting 15–20 tubules per 3D culture.

2.5. Cell migration in 3D-HRTEC cultures

Seventy percent confluent HRTEC cultures were pulse-labeled with 10 μM BrdU for 24hs, and then, treated or not with 10 ng/ml Stx2 for another 24hs. Cells were trypsinized and seeded (1 × 10^4 cells per well of a 96 well plate) onto non-labeled 3D-tubular structures that were already developed in matrigel for three days. BrdU positive nuclei were detected by indirect immunofluorescence, as described previously [14]. Tubular structures were mechanically detached from matrigel, mounted on slides, and nuclei were stained with Hoechst (1 μg/ml) to detect all cells. The fluorescence was observed using a Nikon Eclipse E−2000 fluorescence microscope. For non-fluorescence observations, migration and aggregation was observed under an inverted optical microscope (Nikon Eclipse TS100), and micrographs were obtained using a digital camera (Nikon Coolpix S10).

2.6. Wound healing assay

HRTEC were grown to confluence and growth-arrested in serum-free medium for 24 h, followed by the incubation with Stx2 (10 ng/ml) for 30 min. Then, confluent cultures were scraped with a sterile 200-μl pipette tip to produce a linear wound, washed, and incubated in RPMI supplemented with 5% FBS. Healing was monitored after wounding, using a Nikon Eclipse TS100 inverted microscope. Digital images were obtained at 0 h and 24 h (three