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Sex steroids do not affect shigatoxin cytotoxicity on human renal tubular or glomerular cells

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Abstract

Background: The greater susceptibility of children to renal injury in post-diarrheal hemolytic-uremic syndrome (HUS) may be related, at least in part, to heightened renal cell sensitivity to the cytotoxic effect of Shiga toxin (Stx), the putative mediator of kidney damage in HUS. We hypothesized that sexual maturation, which coincides with a falling incidence of HUS, may induce a relatively Stx-resistant state in the renal cells.

Methods: Cultured human glomerular endothelial (HGEN), human glomerular visceral epithelial (HGEC) and human proximal tubule (HPT) cells were exposed to Stx-1 after pre-incubation with progesterone, β-estradiol or testosterone followed by determination of cytotoxicity.

Results: Under basal conditions, Stx-1 potently and dose-dependently killed HPT and HGEC, but had relatively little effect on HGEN. Pre-incubation for 1, 2 or 7 days with physiologic or pharmacologic concentrations of progesterone, β-estradiol or testosterone had no effect on Stx-1 cytotoxicity dose-response on any cell type. In addition, no steroid altered Gb3 expression (Stx receptor) by any cell type at any time point.

Conclusion: These data do not support the notion that hormonal changes associated with puberty induce an Stx-resistant state within kidney cells.

Background

The factors responsible for the age-related incidence of post-diarrheal hemolytic uremic syndrome (HUS) are unknown. Clinical data indicate that the peak incidence of HUS occurs around two years of age, but the disease clearly occurs in significant numbers of older children [1]. Population-based studies suggest that there is a decline in the incidence of HUS from a peak at age 1–2 years until a nadir at about age 11 years [1,2]. In addition, a case-control study of the Washington State outbreak of HUS revealed that the mean age of patients with HUS was 8 years, while the mean age of patients without HUS was 15 years [3]. These studies raise the possibility, therefore, that the appearance or disappearance of a factor or factors in adolescents and adults reduces susceptibility to end-organ injury in HUS.

HUS is thought to be due, at least in large part, to Shiga toxin (Stx)-mediated cell toxicity [4]. Since the kidney is a primary target of Stx in patients with HUS, it has been hypothesized that the preferential renal injury occurring in children with HUS is related to heightened renal suscepti-

bility to Stx in this age group. Such increased renal sensitivity to Stx in children, if it does indeed exist, could be due to a wide variety of factors. One immediately apparent factor that has been examined is age-related alterations in renal Stx binding and Stx receptor (galactose-α-1,4, galactose-β-1,4, glucose-ceramide (Gb3)) expression. An autopsy study determined that total kidney Gb3 levels were lower in infants than in adults (although only two individuals under 19 years of age were examined) [5]. Another study using immunostaining of biopsy samples found that glomeruli from infants (under two years of age), but not from older children or adults, bound Stx [6]. This provocative finding, albeit involving a relatively small sample size and patients who often had glomerulopathies, provided a potential explanation for augmented glomerular Stx sensitivity in young children. Confirmation of these observations is needed, however they do suggest that Gb3 levels, and in particular the pattern of renal Gb3 expression, may be a factor in the age-related incidence of renal injury in HUS.

The decreased incidence of HUS renal disease in adolescents and adults raises the possibility that factors associated with puberty may be involved. There have been no studies, to our knowledge, that have examined the effects of puberty-related hormonal alterations on renal cell sensitivity to Stx. Indeed, there is no information whatsoever on how any sex steroid affects Stx responsiveness of any cell type. It seemed reasonable, therefore, to examine whether sex steroids (estrogen, progesterone and testosterone) could modify the cytotoxic effect of Stx on renal cells. Further, since Gb3 levels may be altered with aging, the effect of sex steroids on renal cell Gb3 expression was examined.

Several renal cell types have been demonstrated to respond to Stx. Based on histologic evidence, glomerular endothelial cells have been invoked as being primarily involved in HUS renal injury [7]. In vitro studies have demonstrated that human glomerular endothelial cells (HGEN) bind Stx-1 and are modestly sensitive to the toxin's cytotoxic effect [8]. Recent work indicates that other renal cell types may be Stx targets in HUS: cultured human proximal tubule (HPT) [9] and human visceral glomerular epithelial (HGEC) [10] cells express abundant amounts of Gb3 and are highly sensitive to the cytotoxic effect of Stx-1. Consequently, the current study examined the effect of sex steroids on Stx-1 cytotoxicity on, and Gb3 expression by, HGEN, HGEC and HPT.

Methods

Cell culture

Primary cultures of HPT were obtained from Clonetics (San Diego, CA) and studied at the third passage. The identity and purity of these cultures was established by immunofluorescent staining as previously described [9] (in addition to the extensive characterization by Clonetics, we have determined that these cells lack von Willebrand factor and platelet endothelial cell adhesion molecules (PECAM), but are positive for cytokeratin, immunofluorescence and alkaline phosphatase activity). HPT were maintained in 1:1 Dulbecco's Modified Eagle Media:Ham's F12 containing 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, 250 μg/L amphotericin B, 1 μg/ml hydrocortisone, 10 μg/ml insulin, 5.5 mg/ml transferrin, 6.7 μg/ml selenium, 0.2 gm/L ethanolamine, 6.5 ng/ml L-thyroxine, 10 ng/ml epidermal growth factor, and 10% fetal bovine serum (FBS). Hydrocortisone was present in the media since HPT do not grow well in the absence of corticosteroids (personal observation).

Primary cultures of HGEN were obtained from Cell Systems (Kirkland, WA) and studied at the third passage. These cells have been extensively characterized by Cell Systems; in addition, we determined that these cells have von Willebrand factor and PECAM, but lack cytokeratin, immunofluorescence. HGEN were maintained in EGM2-MV media (Clonetics) which contains epidermal growth factor, hydrocortisone, vascular endothelial cell growth factor, basic fibroblast growth factor, insulin-like growth factor-1, ascorbic acid, heparin and FBS.

Primary cultures of HGEC were obtained from nephrectomies as previously described [10] and studied at the third passage. Briefly, glomeruli were obtained by sieving renal cortex, collagenase digested, and cultured in GEC media (Media 199 + 20% FBS + 100 μg/ml Endothelial Growth Supplement (PerImmune, Rockville, MD) + 100 U/ml penicillin/streptomycin). Primary cultures were passaged when 40% confluent and maintained in GEC media thereafter. Cell identity and purity was determined as previously described [10]: all cells stained (by immunofluorescence) negative for von Willebrand factor, PECAM and anti-myosin, but were positive for cytokeratin.

Stx-1 cytotoxicity and Gb3 expression

Confluent cells in 96 well plates were placed in their maintenance media without serum for 24 hr and exposed to either high or low concentrations (see Results) of progesterone, β-estradiol or testosterone (all from Sigma Chemical Co., St. Louis, MO) for 1, 2 or 7 days in serum-free media. Subsequently, Stx-1 (purified in our laboratory [9]) was added for 72 hr at concentrations of 10^{-5} – 10^{-11} grams/liter. The neutral red cytotoxicity assay was performed [9]. Briefly, 50 μg/ml neutral red in Media 199 containing 5% serum was added for 2–3 hours at 37°C. Cells were washed with 1% $CaCl_2$ + 1% formaldehyde and solubilized in 1% acetic acid in 50% ethanol. Absorption was read at 450 nm.

For determination of Gb3 content, cells grown in 6-well plates were placed in serum-free media for 24 hr followed by addition of the steroids for 1, 2 or 7 days. Gb3 was quantitated as previously described [9]. Briefly, cells were scraped off the plates, centrifuged, the pellet extracted in chloroform:methanol:water and separated on high performance thin layer chromatography-silica plates (Mallinckrodt Baker Inc., Paris, KY) by ascending chromatography. The plates were dried, immersed in 0.5% polyisobutylmethacrylate in acetone, and sequentially incubated with Stx-1, anti-Stx-1 monoclonal antibody (purified from a hybridoma cell line, 13C4 (ATCC, Rockville, MD), as previously described [9]), and ^{125}I-goat anti-mouse IgG (DuPont NEN, Boston, MA). Gb3 concentrations were calculated by densitometry (Eagle Eye II, Stratagene, LaJolla, CA) and standardized to total protein. Prior to centrifugation, a cell aliquot was solubilized in 0.1 N NaOH, mixed with Bradford reagent (Bio-Rad, Richmond, CA) and protein concentration determined by measuring absorbance at 590 nm.

Statistics

All data were analyzed by one way analysis of variance. Individual conditions were compared using Student's t-test after the Bonferroni correction. *P* values < 0.05 were taken as significant.

Results

Stx-1 (72 hr exposure to toxin) dose-dependently killed HGEN, HGEC and HPT (Figure 1). In HGEN, Stx-1 had an LD50 of about 10^{-5} g/L (140 pM), while in HGEC the LD_{50} for Stx- was about ^{10}-8 g/L (140 fM) and in HPT the LD_{50} for Stx was about $10^{-9.5}$ g/L (4.4 fM).

Exposure for 1, 2 or 7 days to high (1 μM) or physiologic concentrations (reference range of physiologic steroid concentrations based on Barnes Hospital Laboratory Manual [11]) of progesterone (30 nM), β-estradiol (100 pM), or testosterone (30 nM) had no effect on HGEN (Table 1), HGEC (Table 2) or HPT (Table 3) responsiveness to Stx-1. For the sake of brevity, the tables illustrate the effect of 10^{-5}, 10^{-8}, and 10^{-11} g/L Stx-1, however a full dose-response from 10^{-5} – 10^{-11} g/L Stx-1, in ten-fold concentration increments, was done for each condition: there was also no significant effect of these other concentrations of sex steroids on the cytotoxic effects of Stx-1 on any of the renal cell types. In addition, physiologic or pharmacologic concentrations of progesterone, estradiol or testosterone had no effect on baseline (without exposure to Stx-1) HGEN, HGEC or HPT survival.

The effect of 1, 2 or 7 days of exposure to physiologic or pharmacologic concentrations of progesterone, estradiol or testosterone on Gb3 expression by HGEN, HGEC or HPT was also examined. A representative blot of Gb3 content is shown in Figure 2 (figure illustrates 2 day steroid exposure in HPT) to demonstrate that Gb3 isolated from cells migrated on the gel at the same position as purified Gb3. As for Stx-cytotoxicity, none of the steroids, at any dose or exposure time, modified Gb3 content in any cell type (n = 3 each condition, data not shown).

Immunoreactive steroid concentration was determined in media samples at the start and conclusion of the 7 day experiment (analyzed in clinical laboratory at Associated Regional University Pathologists, Salt Lake City, UT) in order to determine the stability of the added steroids. Estradiol and testosterone concentrations did not vary over 1, 2 or 7 days in culture media. Progesterone levels also did not fall after 1–2 days in culture media, however after 7 days in media the progesterone concentration significantly dropped (80% decrease). Consequently, fresh progesterone was added to the 7 day experiments every two days.

The failure of steroids to affect Stx cytotoxicity was not due to inactivity of the steroid preparations. Similar preparations and concentrations of steroids from the same supplier (Sigma) were tested by Dr. Wayne Meikle in the Division of Endocrinology at the University of Utah using an in-house estrogen and progesterone binding assay and a testosterone-dependent stimulation of prostate cancer cell apoptosis assay [12]; all the steroids retained biologic activity (data not shown).

Discussion and Conclusion

The reasons why HUS primarily affects children are unknown. It is evident that the peak incidence of HUS occurs in very young children (1–2 years of age). The factors responsible for this apparent heightened sensitivity to infection with Stx-expressing bacteria have not been extensively investigated and were not examined in the current study. Indeed, it is conceivable that not all of the factors involved in HUS occurrence in young children are identical to those involved in the diminished HUS incidence in adolescents and adults. Numerous factors could be involved in age-related Stx sensitivity, including differences in the degree of enteric invasion by Stx-producing *E. coli*; in Stx transport across the gut, in circulating red or white blood cell toxin binding and delivery to the kidney, in the immune response to Stx, in renal Stx binding, and/or in intrinsic or cofactor-regulated (e.g. inflammatory cytokines) renal Stx responsiveness. The current study evaluated one of these possibilities, namely that renal cell Stx-1 responsiveness decreases with age due, at least partially, to hormonal changes associated with puberty. As stated earlier, there is no precedent in the literature for examination of the effect of sex steroids on Stx responsiveness, however, the coincidental fall in clinically apparent HUS in adolescents and adults [1] raises the possibility that

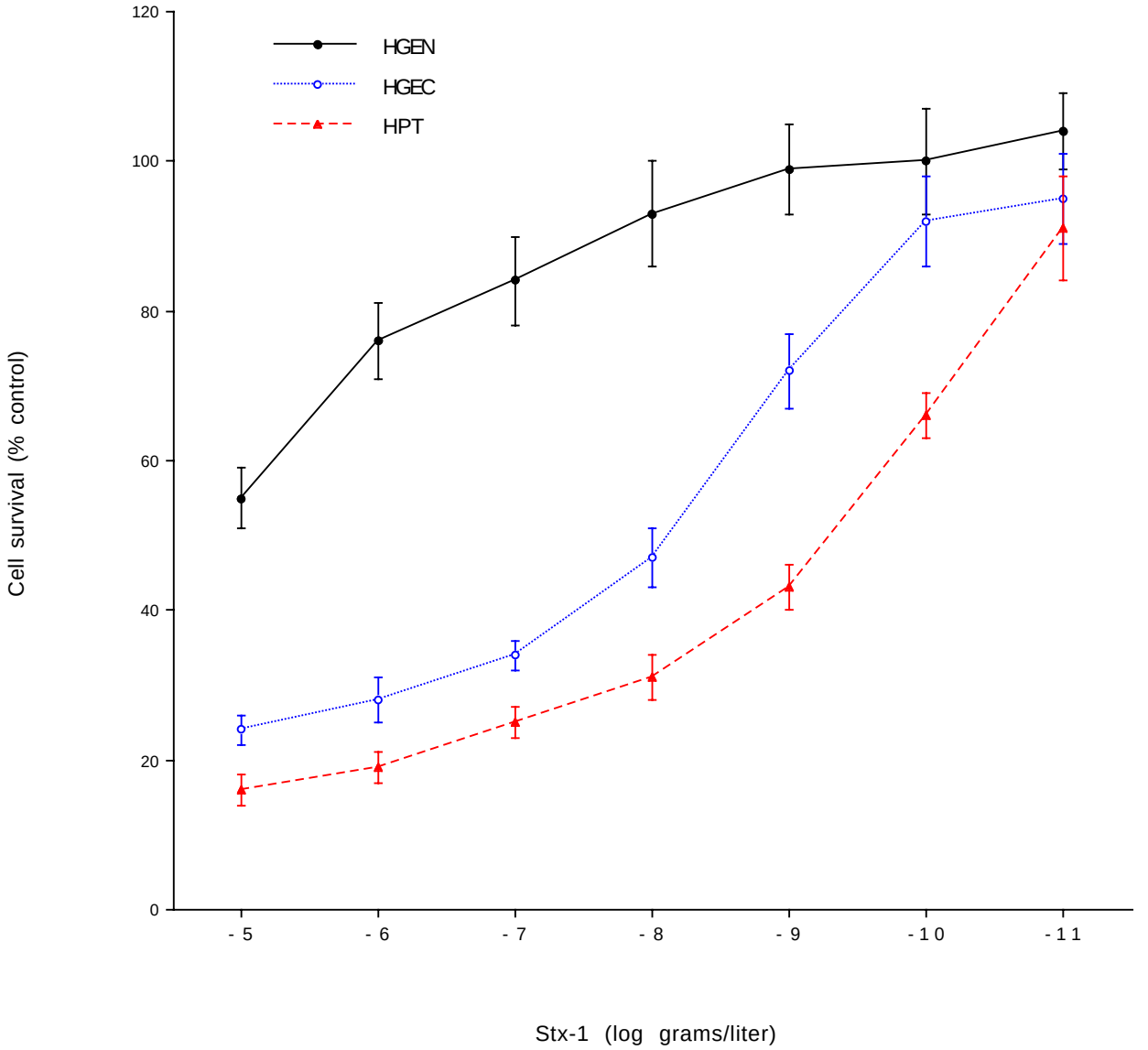

Figure 1
Effect of Stx-1 (72 hr exposure) on cell survival in cultured human glomerular endothelial cells (HGEN), human glomerular epithelial cells (HGEC) or human proximal tubule cells (HPT). N = 9 for each data point. % control refers to control cells not exposed to toxin.

hormonal changes associated with puberty induce a relatively Stx-resistant state in the kidney.

HGEN, HGEC and HPT were chosen for examination in this study because these cell types, at least *in vitro*, have been shown to be injured by Stx-1 [6–8]. Mesangial cells were not examined since Stx-1 has not been demonstrated to affect their viability [13]. Previous studies have separately examined the cytotoxic effect of Stx-1 on HGEN [8], HGEC [10], and HPT [9], however this is the first study to directly compare Stx-1 sensitivity of these renal cell types. These data indicate that, as suggested from the previous studies, within the limits of the cell culture system, HPT are the most sensitive to Stx-1 cytotoxicity of the three cell types, HGEC are slightly less sensitive, and HGEN are at least three orders of magnitude less sensitive than either

Table 1: Effect of sex steroids on Stx-1 cytotoxicity in human glomerular endothelial cells (HGEN). Cells were exposed to high and low concentrations of steroids for 1, 2 or 7 days followed by addition of varying concentration of Stx-1 for 72 hr. Cell survival was determined at the end of the experiment. % survival refers to percent of cells surviving after exposure to Stx-1 ± steroids. N = 9 each data point. Results are expressed as mean ± SEM.

HGEN	**Dose**	**[Stx-1]** gm/L	**Stx alone** (% survival)	**Day 1** (% survival)	**Day 2** (% survival)	**Day 7** (% survival)
Estradiol	1 μM	-5	54 ± 4	49 ± 3	49 ± 6	48 ± 6
	1 μM	-8	90 ± 7	86 ± 6	85 ± 8	95 ± 6
	1 μM	-11	102 ± 8	102 ± 10	105 ± 4	91 ± 8
	100 pM	-5	54 ± 4	51 ± 3	55 ± 3	58 ± 4
	100 pM	-8	90 ± 7	87 ± 6	96 ± 9	85 ± 9
	100 pM	-11	102 ± 8	96 ± 6	103 ± 11	115 ± 11
Progesterone	1 μM	-5	54 ± 4	51 ± 4	50 ± 4	45 ± 6
	1 μM	-8	90 ± 7	84 ± 9	85 ± 8	95 ± 7
	1 μM	-11	102 ± 8	100 ± 4	103 ± 6	95 ± 7
	30 nM	-5	54 ± 4	53 ± 3	53 ± 4	52 ± 5
	30 nM	-8	90 ± 7	91 ± 9	92 ± 7	94 ± 9
	30 nM	-11	102 ± 8	102 ± 7	104 ± 7	100 ± 7
Testosterone	1 μM	-5	54 ± 4	58 ± 5	55 ± 2	56 ± 3
	1 μM	-8	90 ± 7	91 ± 8	92 ± 9	94 ± 10
	1 μM	-11	102 ± 8	103 ± 12	95 ± 8	102 ± 7
	30 nM	-5	54 ± 4	52 ± 4	54 ± 4	55 ± 3
	30 nM	-8	90 ± 7	86 ± 6	90 ± 6	94 ± 8
	30 nM	-11	102 ± 8	96 ± 6	100 ± 5	104 ± 8

Table 2: Effect of sex steroids on Stx-1 cytotoxicity in human glomerular epithelial cells (HGEC). Cells were exposed to high and low concentrations of steroids for 1, 2 or 7 days followed by addition of varying concentration of Stx-1 for 72 hr. Cell survival was determined at the end of the experiment. % survival refers to percent of cells surviving after exposure to Stx-1 ± steroids. N = 9 each data point. Results are expressed as mean ± SEM.

HGEC	**Dose**	**[Stx-1]** gm/L	**Stx alone** (% survival)	**Day 1** (% survival)	**Day 2** (% survival)	**Day 7** (% survival)
Estradiol	1 μM	-5	54 ± 4	51 ± 4	51 ± 3	48 ± 5
	1 μM	-8	90 ± 7	79 ± 10	94 ± 8	86 ± 5
	1 μM	-11	102 ± 8	96 ± 10	107 ± 5	95 ± 8
	100 pM	-5	54 ± 4	53 ± 4	52 ± 3	50 ± 4
	100 pM	-8	90 ± 7	88 ± 8	89 ± 6	83 ± 7
	100 pM	-11	102 ± 8	106 ± 7	96 ± 9	98 ± 9
Progesterone	1 μM	-5	54 ± 4	54 ± 49	56 ± 5	54 ± 2
	1 μM	-8	90 ± 7	83 ± 9	90 ± 9	90 ± 5
	1 μM	-11	102 ± 8	101 ± 3	101 ± 6	103 ± 7
	30 nM	-5	54 ± 4	50 ± 6	58 ± 4	50 ± 5
	30 nM	-8	90 ± 7	86 ± 4	92 ± 6	78 ± 9
	30 nM	-11	102 ± 8	97 ± 7	105 ± 7	93 ± 7
Testosterone	1 μM	-5	54 ± 4	53 ± 4	55 ± 4	50 ± 4
	1 μM	-8	90 ± 7	90 ± 11	90 ± 7	84 ± 6
	1 μM	-11	102 ± 8	103 ± 9	100 ± 7	105 ± 5
	30 nM	-5	54 ± 4	59 ± 6	59 ± 4	54 ± 9
	30 nM	-8	90 ± 7	84 ± 8	89 ± 5	82 ± 7
	30 nM	-11	102 ± 8	98 ± 6	106 ± 8	97 ± 6

Table 3: Effect of sex steroids on Stx-1 cytotoxicity in human proximal tubule cells (HPT). Cells were exposed to high and low concentrations of steroids for 1, 2 or 7 days followed by addition of varying concentration of Stx-1 for 72 hr. Cell survival was determined at the end of the experiment. % survival refers to percent of cells surviving after exposure to Stx-1 ± steroids. N = 9 each data point. Results are expressed as mean ± SEM.

HPT	Dose	[Stx-1] gm/L	Stx alone (% survival)	Day 1 (% survival)	Day 2 (% survival)	Day 7 (% survival)
Estradiol	1 μM	-5	54 ± 4	49 ± 4	50 ± 4	46 ± 5
	1 μM	-8	90 ± 7	81 ± 7	83 ± 7	84 ± 9
	1 μM	-11	102 ± 8	97 ± 5	99 ± 7	107 ± 7
	100 pM	-5	54 ± 4	49 ± 4	60 ± 4	49 ± 6
	100 pM	-8	90 ± 7	95 ± 8	90 ± 7	87 ± 8
	100 pM	-11	102 ± 8	97 ± 6	103 ± 5	92 ± 9
Progesterone	1 μM	-5	54 ± 4	56 ± 4	52 ± 3	60 ± 5
	1 μM	-8	90 ± 7	99 ± 9	81 ± 7	91 ± 9
	1 μM	-11	102 ± 8	107 ± 8	101 ± 5	106 ± 8
	30 nM	-5	54 ± 4	50 ± 6	52 ± 3	54 ± 4
	30 nM	-8	90 ± 7	86 ± 4	83 ± 5	90 ± 5
	30 nM	-11	102 ± 8	97 ± 7	105 ± 5	92 ± 7
Testosterone	1 μM	-5	54 ± 4	57 ± 3	49 ± 4	58 ± 5
	1 μM	-8	90 ± 7	92 ± 7	89 ± 5	91 ± 8
	1 μM	-11	102 ± 8	94 ± 5	97 ± 6	100 ± 6
	30 nM	-5	54 ± 4	51 ± 4	64 ± 7	57 ± 3
	30 nM	-8	90 ± 7	98 ± 8	97 ± 7	85 ± 5
	30 nM	-11	102 ± 8	109 ± 10	102 ± 6	103 ± 7

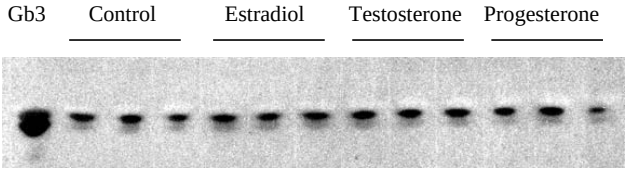

Figure 2
Representative blot of Gb3 expression by human proximal tubule cells after 48 hr exposure to media alone (control), 1 μM β-estradiol, 1 μM progesterone or 1 μM testosterone. A Gb3 standard is shown for reference. Identical results were obtained at 1 and 7 days of steroid exposure.

HGEC or HPT. Such differential sensitivity is quite interesting, particularly considering that glomerular endothelial cell damage is a prominent histologic feature in biopsies obtained from patients with clinically well established HUS [7]. The significance of these observations remains, therefore, to be determined. Although speculative, it is tempting to propose that HPT and HGEC may be early targets of Stx-1 and that this could play a role in the pathophysiologic processes that characterize renal injury in HUS.

Estrogen, progesterone or testosterone did not affect Stx-1 mediated renal cell cytotoxicity or Gb3 expression. Cells were exposed to the steroids for up to one week in order to allow time for steroid-regulated gene transcription and protein expression. In addition, both physiologic and pharmacologic concentrations of steroids were utilized. Despite these efforts to be insure that the system was optimized in order to detect a steroid-mediated alteration of Stx responsiveness, the concern still exists that this system may not really reflect the influences of sex steroids in vivo. Indeed, one might argue that an animal model, in which the testes or ovaries are removed and the animals are given Stx in the presence and absence of steroid replacement, would be better. Such studies would, however, be problematic. Traditional animal models, such as mice, rats, rabbits or pigs do not develop typical renal disease when given either Stx or *E. coli* 0157:H7 [14]. Further, animal models that most closely reflect human HUS are only in the early stages of development [14]. Hence, the cell culture system remains the most reasonable means to assess an effect of sex steroids on renal cell Stx-1 responsiveness; such systems have clearly been demonstrated to be responsive to sex steroids (e.g. testosterone-regulated KAP promoter activity in proximal tubule cells [15] as well as estrogen-regulated endothelial cell function [16]). Given these limitations, our data suggest that sex steroids do not alter renal cell sensitivity to the cytotoxic effects of this Stx-1. Consequently, the reasons why clinically apparent HUS primarily occurs in children remains to be determined.

List of Abbreviations

HUS hemolytic-uremic syndrome

HGEN human glomerular endothelial cells

HGEC human glomerular visceral epithelial cells

HPT human proximal tubule cells

Stx-1 Shiga toxin-1

Gb3 galactose-α-1,4, galactose-β-1,4, glucose-ceramide

Competing interests
None declared.

Authors' Contributions
Author 1 performed the analysis of cytotoxicity, maintained cell culture and was involved in experimental design. Author 2 performed Gb3 analysis and assisted with cell culture. Author 3 designed, interpreted and supervised the experiments.

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