ORIGINAL PAPER

Human umbilical vein endothelial cells accelerate oxalate-induced apoptosis of human renal proximal tubule epithelial cells in co-culture system which is prevented by pyrrolidine dithiocarbamate

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Abstract Oxalate is the most common component of kidney stones and elevated urinary levels induce renal tubular cell toxicity and death which is essential for crystal attachment. Endothelial cells, in some studies have been shown to regulate certain functions of renal proximal tubule cells. The aim of this study was to evaluate the effect of endothelial cells on tubular cell apoptosis in a co-culture system mimicking the in vivo renal physiological settings. The human umbilical vein endothelial cells (HUVEC) and human renal proximal tubule epithelial cells (RPTEC) were exposed to increasing concentrations (0–1.0 mM) of oxalate with or without 10 μM PDTC pretreatment for 24 h. In HUVEC, RPTEC and HUVEC–RPTEC co-cultures, the cell viability was measured using the WST-1 assay and cell death with the TUNEL analysis using the flow cytometry. The treatment of RPTECs with oxalate lead to 8.9–26.2% cell death which was reduced to 0–1.6% with the PDTC pretreatment. The death rate of RPTECs was significantly increased by 15–19% at different oxalate concentrations when co-cultured with HUVECs. In contrast, cell viability was not substantially altered in PDTC pretreated RPTECs that were co-cultured with HUVECs. Apoptosis was the way of cell death as similar rate of apoptosis was observed in cell culture systems. Although cell viability of RPTECs was further reduced when co-cultured with HUVECs, it was restored with the pretreatment of PDTC. This is the first study focusing on the role of endothelial cells on RPTEC apoptosis following hyperoxaluria.

Keywords Human renal proximal tubular epithelial cells · Apoptosis · Endothelial cells · Pyrrolidine dithiocarbamate

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Introduction

Hyperoxaluria is a major risk factor for calcium oxalate (CaOx) urolithiasis by causing crystal formation and deposition in the kidneys. Although supersaturation is the main driving force for crystallization, stone formation does not occur unless crystals adhere to renal tubular epithelium. Studies on crystal–cell interaction revealed that proximal tubule is the primary site of events and tubular cell injury is the end result of this interaction [1]. There is clear evidence that calcium oxalate crystals preferentially adhere to injured renal epithelial cells rather than to healthy cells [2]. Epithelial cell injury causes exposure of the basal lamina, which becomes a site of crystal attachment. Some investigators reported that oxalate and/or CaOx crystals have direct toxic effects on renal tubular cells and yet others suggested

the crystal–cell interaction process as the responsible mechanism for renal tubular epithelial cell injury [1, 3]. Additional studies have disclosed that oxalate-induced toxicity is related to increased production of free radicals [4]. The peroxidation of membrane phospholipids has been considered as the mechanism for oxalate-induced epithelial cell injury [5]. However, none of the above hypotheses alone was able to clarify the concerns about the underlying mechanisms of renal tubular epithelial cell injury.

Recent studies have led the physicians to focus on the role of renal vascular endothelium. Proximal tubular epithelial cells are in close contact with renal circulation and endothelial cells have been shown to regulate proximal tubular epithelial cell ion transport [6]. In our recent study, we have shown that hyperoxaluria induces the expression of asymmetrical dimethylarginine (ADMA) which is a marker of endothelial dysfunction in renal tissue of ethylene glycol administered rats [7]. This finding indicated the role of endothelium on CaOx stone formation for the first time. Since renal epithelial cell injury is accepted to be the key and initial element for stone formation, the possible role of vascular endothelium on renal tubular epithelial cell death was of great interest. This study was conducted to investigate whether renal tubular epithelial cell death could be further enhanced by endothelial cell dysfunction induced by oxalate and could be prevented by restoration of endothelial function.

Materials and methods

Cell lines

Primary human umbilical vein endothelial cells (HUVECs) (American Type Culture Collection via LGC Standards, Teddington, UK) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Primary human renal proximal tubule epithelial cells (RPTECs) were purchased from the American Type Culture Collection via LGC Standards (Teddington, UK) and maintained in renal epithelial cell basal medium supplemented with renal epithelial cell growth kit (LGC Standards, Teddington, UK). All cells were cultured at 37°C and in a humidified atmosphere of 5% CO_2 in air.

Co-culture experiment

For co-culture system, RPTECs were seeded on the bottom of the 96-well plates or 24-well plates at 1.5×10^4 cells/cm^2 and HUVECs were plated into Nunc tissue culture inserts with 0.2-μm pore size (1.5×10^4 cells/cm^2) which

were then placed above the underlying RPTECs. The pore size of the insert membrane would not allow the migration of HUVECs but permit the diffusion of secreted soluble factors of $<2 \times 10^6$ Da. Sodium oxalate was added to co-culture system in increasing concentrations (0–1 mM) and incubated for 24 h. In PDTC experiment, cells were pretreated with 10 μM pyrrolidine dithiocarbamate (PTDC) for 24 h and then exposed to increasing concentrations of sodium oxalate for further 24 h. Cell viability and apoptosis assay details were explained below.

Cell viability assay

Cellular viability and proliferation was assessed by WST-1 assay (Roche Applied Science, Mannheim, Germany) which is based on the reduction of tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1.6-benzene disulfonate) to a soluble formazan-class by mitochondrial succinate tetrazolium reductase in metabolically active cells. HUVECs and RPTECs were plated at 5,000 cells/well and allowed to attach to the 96-well plate overnight. Cells were pretreated with 10 μM pyrrolidine dithiocarbamate (PTDC) for 24 h and then exposed to increasing concentrations (0–1 mM) of sodium oxalate for 24 h. To quantify cell viability, cells were incubated with WST-1 reagent (5/50 μl DMEM) for 1 h and the absorbance was measured using a plate reader at 450 nm (reference 650 nm). The cell viability was calculated as the mean percentage of control (untreated) cell viability, which represents 100%.
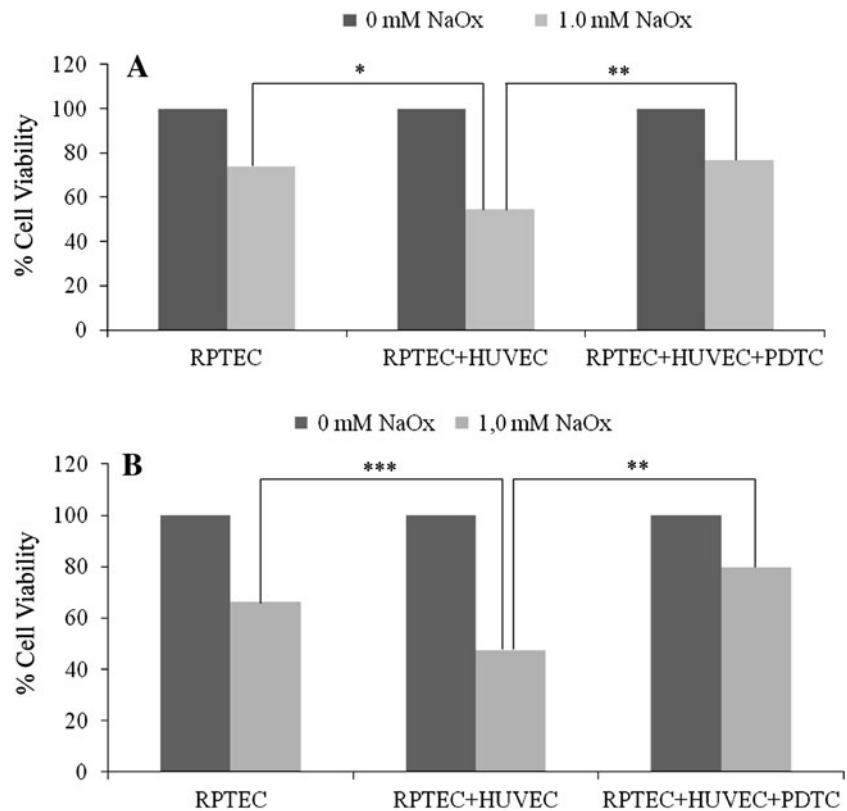
Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

Following the exposure of RPTECs to 0.5, 0.75 and 1 mM of sodium oxalate with or without the PTDC pretreatment, all cells were collected and washed twice with PBS. After fixation with 4% (w/v) paraformaldehyde in PBS, cells were permeabilized using 0.1% (v/v) Triton X-100 in 0.1% (w/v) sodium citrate buffer for 2 min at room temperature. Cells were then labeled with terminal deoxynucleotidyl transferase (TdT) and FITC-dUTP, using the In situ *Cell Death Detection Kit* (Roche Diagnostics) according to the manufacturer's instructions. Cells were then washed twice in PBS and the fluorescence intensity was measured by flow cytometry using a BD FACSCalibur (BD Biosciences). Minimum detection rate of apoptosis was fixed to 0.1% after exclusion of background value.

Statistical analysis

Results are expressed as means ± standard deviation. Experiments were repeated three times and the mean was

Fig. 1 The effects of sodium oxalate on cell viability and apoptosis both alone and co-culture experiment with or without pyrrolidine dithiocarbamate. **a** Sodium oxalate caused a decrease in RPTEC viability. Co-incubation with HUVECs further increased death of RPTECs. Pretreatment of co-cultured cells with PDTC provided better survival rate of RPTECs compared to non-PDTC experiment. $*p = 0.004$, $**p = 0.003$. **b** Sodium oxalate caused a higher rate of apoptosis of RPTECs. Co-incubation of RPTECs with HUVECs in the presence of NaOx further increased the rate of apoptosis. But, pretreatment with PDTC preserved cell viability. $***p = 0.005$

Results

The effect of sodium oxalate on cell viability

The dose-dependent effect of NaOx on RPTEC and HUVEC viability was tested after incubation of cells with NaOx for 24 h. The NaOx concentration of 5.0 mM significantly reduced cell viability below 75% and minimal lethal concentration was achieved at 1.0 mM for both types of cells. The study was conducted on NaOx concentrations below 1.0 mM. At concentrations of 0.5, 0.75 and 1.0 mM of NaOx, viability of RPTECs was 93.09 ± 1.5, 83.04 ± 0.9, 72.29 ± 1.5 and 95.8 ± 1.1, 79.9 ± 2.5 and $80.3 \pm 1.6\%$ for HUVECs, respectively ($p < 0.05$).

The effect of endothelial cells on proximal tubule epithelial cell viability

The co-incubation of RPTECs with HUVECs alone did not cause any change in cell viability. However, the addition of NaOx on co-incubation medium caused a significant death of RPTECs. The rate of cell viability of RPTECs was $74.7 \pm 5.8\%$ at 0.5 mM ($p = 0.0002$), $67.2 \pm 3.2\%$ at 0.75 mM ($p = 0.0001$) and $54.5 \pm 4.0\%$ at 1.0 mM concentration of NaOx ($p < 0.0001$). Compared to the effect of NaOx on RPTECs alone, co-incubation with HUVECs increased the lethal effect of NaOx on RPTECs. Viable cell population was further decreased by 17.3% at 0.5 mM ($p = 0.02$), 16.2% at 0.75 mM ($p = 0.006$) and 15.2% at 1.0 mM concentration of NaOx ($p = 0.01$) in co-incubated cells compared to RPTECs alone (Fig. 1a).

The role of restoration of endothelial function with pyrrolidine dithiocarbamate on proximal tubule epithelial cell viability

Pyrrolidine dithiocarbamate is a potent antioxidant with the ability of suppressing NF-κB which is an important mediator of oxidant cell injury. Pretreatment of RPTECs with 10 μM concentration of PDTC resulted in proliferation of both types of cells at basal state and this protection was also observed when cells were further incubated with NaOx at different concentrations (Fig. 1a).

As mentioned before co-incubation of RPTECs with HUVECs caused a decrease in viability of RPTECs in the presence of NaOx. When co-incubated cells were pretreated with 10 μM concentration of PDTC for 24 h before the addition of NaOx, addition of PDTC saved 28.6% of cells from lethal effect of NaOx at concentration of

presented here. Comparisons between two groups was made by the unpaired, Student's t test. Statistical significance was set at $p < 0.05$.

0.5 mM ($p = 0.01$) and 27.6% at 0.75 mM ($p = 0.001$) and 21.9% at 1.0 mM concentration ($p = 0.01$) (Fig. 1). Pretreatment with PDTC provided a cell viability similar to the effect of NaOx on RPTECs alone except at concentration of 1.0 mM (Fig. 1a).

Apoptosis is the way of oxalate-induced death of renal proximal tubular epithelial cells

Apoptosis was tested on RPTECs either alone, co-incubated with HUVECs and PDTC pretreated culture media with TUNEL assay. Exposure of RPTECs alone to NaOx was associated with increased apoptosis in a dose-dependent manner from 9.8 to 33.6%. But pretreatment with PDTC almost totally prevented apoptosis of RPTECs. Maximum rate of apoptosis was 0.9% and observed at 1.0 mM concentration of NaOx (Fig. 1b).

Compared to RPTECs-alone experiment, co-incubation of RPTECs with HUVECs resulted in a dramatic increase in apoptotic cell rate in parallel with NaOx concentration. Except at high concentration (1.0 mM) of NaOx, addition of PDTC prevented apoptosis of RPTECs. Even at higher concentrations of NaOx, the rate of apoptosis was half of non-PDTC pretreated group ($p = 0.003$) (Fig. 1b).

Discussion

The present study provides evidence that the death of renal tubular epithelial cells can be enhanced when co-incubated with vascular endothelial cells in the presence of oxalate. Induction of apoptosis was the way of cell death. Both apoptosis and cell death could be prevented by pretreatment with PDTC, a potent antioxidant that antagonizes endothelial nitric oxide synthesis and endothelial dysfunction.

The pathophysiological mechanisms of calcium oxalate stone formation are still in dispute. The process begins with the attachment of formed crystals to renal tubular epithelial cells [2]. In individuals with hyperoxaluria, attachment of crystals is facilitated by preexisting renal epithelial cell injury which is under the effect of various factors [2]. In addition to the direct toxic effect of oxalate, renal tubular epithelial cell injury is thought to be mediated by increased free radical production [4]. As a novel result of this study, for the first time, it has been shown that renal epithelial cell injury is also under the control of endothelial cells.

There are many hypotheses on stone formation. Of these, as a least popular one is intravascular phenomenon has been proposed by Low and Stoller [8]. They proposed that stones form within the vasa recta of renal papillae. Injury to vasculature result in atherosclerotic-like lesion and the eventual calcification erodes the papillary duct and

serves as a nidus. Although following studies could not verify them [9], our results partly support this hypothesis where the correct order of events might be oxalate elevation in intravascular area, resulting in endothelial dysfunction first and eventually renal epithelial cell injury and death upon which facilitated crystal attachment occurs.

In our previous study, we had shown that hyperoxaluria induces asymmetrical dimethyl arginine (ADMA) expression which is a marker of endothelial dysfunction in renal tissue of ethylene glycol administered rats [7]. The present study was planned to demonstrate the interaction in a direct manner. Decreased cell viability after co-incubation of RPTECs with HUVECs and prevention of cell death with pretreatment with PDTC which antagonizes ADMA and restores endothelial cell function confirm the results of our first study. Both of these studies put forward a new understanding, "endothelial cell hypothesis" in stone formation.

In their recent study, Ilbey and his co-workers [10] showed that administration of PDTC to hyperoxaluria-induced rats prevented crystallization in renal tubules by decreasing oxidative stress in renal tissue. Although they claimed the findings to be the result of direct action of PDTC on renal tubular oxidant stress, their findings are actually in parallel and supportive to our current study. Ethylene glycol administration stimulated iNOS and NF-κB/P65 expression together with increased levels of malondialdehyde and nitric oxide in renal tissue and PDTC did well inhibit them. Protective role of PDTC was attributed to the prevention of hyperoxaluria-induced peroxidative damage to renal tubular cells but did not demonstrate tubular cell death in their study. Since iNOS requires to be induced by certain cytokines; the induction of iNOS expression in renal tissue is supposed to be by p38-MAPK whose expression was increased with ethylene glycol administration. But it is not obvious whether p38-MAPK activation was a direct result of hyperoxaluria or another mediator exists in this process. Since p38-MAPK activity was shown to be stimulated by vascular endothelial growth factor receptor-2 activation [11] and vascular endothelial growth factor gene polymorphism was associated with calcium oxalate stone disease [12]; it can be postulated here that ethylene glycol-induced elevation of plasma oxalate levels initially affects the vascular endothelium and activates VEGF which eventually induces p38-MAPK and then iNOS expression and cause NO production.

Similar data were reported by Huang et al. [13] where they were able to show that excessive CaOx crystal accumulation could enhance endothelial NOS (eNOS), iNOS, and NAD(P)H oxidase protein expression in the kidney. Increased superoxide formation was thought to be derived from NAD(P)H oxidase and uncoupled eNOS, and increased nitrotyrosine formation from iNOS and

ED1-positive cells that gathered around the CaOx crystals. Co-treatment with L-NAME reduced renal oxidative nitrosative stress and tubular damage. This study again confirms that CaOx stone formation is a cumulative result of endothelial dysfunction. They stated that increased ROS production was a result of NAD(P)H oxidase activation by calcium oxalate crystals and resulting eNOS uncoupling further amplifies oxidative stress in kidneys. Increased ROS production enhances macrophage/monocyte infiltration and release of inflammatory cytokines stimulates iNOS expression in renal tubules resulting in further damage. The results of our present study are in accordance with these findings and prove that hyperoxaluria-induced endothelial cell dysfunction plays a crucial role in renal tubular cell death.

Endothelial cells not only play role in pathological processes, but also regulate some physiological mechanisms. Proximal tubule epithelial cells are in close contact with the renal capillaries. Vascular endothelial cells has been shown to regulate proximal tubule epithelial cell function especially sodium transport through NOS-dependent up-regulation of cGMP in these cells [6]. Additionally, the endothelium of the peritubular capillaries have been shown to affect proximal tubule acidification, probably via stimulation of Na^+/H^+ exchanger and endothelium-derived nitric oxide (EDNO) which has been thought to play important role in this function [14]. Confirmatory results were reported by Wang et al. [15] who found that nitric oxide regulates $HCO3^-$ and Na^+ transport by a cGMP-mediated mechanism in the kidney proximal tubule. If physiological transport mechanisms are under the control of vascular endothelial cells, any pathological condition affecting endothelial function should have direct effects on renal tubule function. The above-mentioned hyperoxaluria-induced changes do further support this critical relationship.

Recent studies relate urolithiasis with some systemic disorders such as obesity, hypertension, hyperlipidemia and metabolic syndrome all of which are characterized by systemic endothelial dysfunction [16–19]. Most of these studies demonstrate increased ROS in these conditions and disturbed endothelial function is supposed to be the result of oxidative stress induced by high lipids, blood pressure or glucose. In our previous study, we have shown that hyperoxaluria causes systemic endothelial dysfunction as ADMA levels were found to be increased in hyperoxaluric rats [7]. Since hyperoxaluria is the result of excess oxalate in the blood stream, initial event could be systemic endothelial dysfunction in this cascade. All of these studies indicate well that urolithiasis is not a disease restricted only to kidneys but a part of a systemic disorder or may be a separate syndrome.

Oxalate-induced death of renal tubular epithelial cells exhibits predominantly the features of apoptosis [20]. Oxalate-induced free radical production is the offender of this process. Increase in the rate of apoptosis with co-incubation of RPTECs with HUVECs indicates that apoptosis of renal tubular epithelial cells are mediated by endothelial cells. Our results did clearly show that pretreatment with PDTC will preserve endothelial cell function and prevent tubular apoptotic changes indicating the direct role of endothelial cells in stone formation.

There are some limitations in this study. First of all, we were not able to study possible mediators such as nitric oxide, ADMA, p38-MAPK, eNOS or iNOS. Secondly, cell culture studies help to explain pathogenetic mechanisms in a direct manner but may not reflect the natural physiological human conditions. Last but not the least, use of only oxalate reflects only hyperoxaluric part of the concept, but actually the condition is the result of calcium oxalate crystals.

Conclusion

This is the first study demonstrating the role of endothelial cells on renal tubular cell injury and apoptosis induced by oxalate and its prevention with PDTC, an antagonist of ADMA and endothelial cell function restorer. Our results indicate a new pathogenetic mechanism "endothelial cell hypothesis" in CaOx stone formation and help to better understand systemic underlying causes of calcium oxalate stone disease where treatment strategies targeting endothelial cell preservation may help in the prevention and/or management of stone disease.

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