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Abstract

Background: The uremic syndrome is characterized by a deterioration of kidney function due to the accumulation of uremic toxins. Currently, 100 different uremic toxins have been identified. Uremic toxins are particularly difficult to remove by conventional dialysis treatments and are the major causes of mortality in patients with chronic kidney disease (CKD). p-Cresol is a well-known uremic toxin which accumulates in uremic serum. Our aim was to evaluate the in vitro effect of p-cresol on apoptosis and necrosis in renal tubular cells (RTCs) to better understand the pathophysiological effect of this toxin on the kidney. Methods: We studied apoptosis and necrosis in RTCs, which were incubated for 24 h with increasing concentrations of p-cresol. A DNA ladder was noted in treated cells as a qualitative marker of the apoptotic process. Furthermore, we performed quantitative analysis of cell viability using a flow cytometer and assessed caspase-3 activity. Results: Incubation with p-cresol for 24 h resulted in a significant reduction in RTC viability. DNA isolated from RTCs incubated with increasing p-cresol concentrations for 24 h showed a ‘ladder' pattern of apoptosis at p-cresol concentrations of 10, 5 and 2.5 mg/l. However, we did not observe any significant changes in apoptosis levels detected by annexin V and caspase-3 compared with untreated cells. Cytofluorimetric analysis of necrosis highlighted significantly higher cell death rates in RTCs incubated with the higher p-cresol concentrations (range 40-10 mg/l) compared with other concentrations (5-2.5 mg/l) and untreated cells ($p < 0.05$). Necrosis induction was stronger at higher p-cresol concentrations. Conclusion: It is necessary to develop new therapeutic and dialytic strategies to manage p-cresol concentrations in CKD.

Introduction

The retention of compounds in the body that are normally secreted into the urine by healthy kidneys gives rise to a progressive deterioration in physiologic function and the chemical milieu. These compounds are called uremic toxins [1]. Currently, 100 different uremic toxins have been identified. On the basis of their hydrophobicity, they exist in two forms: a free water-soluble form and a bound form which is reversible to serum protein. The latter can alter protein function. Protein-bound solutes and peptides are particularly

difficult to remove by conventional dialysis treatment [2,3,4]. The identification and characterization of uremic toxins are fundamental for critical evaluation and treatment of patients with chronic kidney disease (CKD). Uremic patients show states of immunodeficiency and infection susceptibility, which are the major causes of mortality in CKD patients [5]. In uremic patients, p-cresol is a uremic toxin which is associated with immunodeficiency. p-Cresol, a 108.1-Da volatile low-molecular-weight compound, is a member of the phenol family, which possesses a partially lipophilic moiety which strongly binds to plasma protein under normal conditions [6]. p-Cresol is excreted into the urine of healthy persons [7] and is retained in patients with CKD [8,9]. It is produced by intestinal bacteria that metabolize tyrosine and phenylalanine [9]. In uremia, modifications in the intestinal flora result in the overgrowth of bacteria that are specific p-cresol producers [10]. p-Cresol is metabolized through conjugation, mainly sulfation and glucuronization [9,11], but the unconjugated p-cresol is removed, at least in part, via the urine [12]. Studies showed a strong correlation between plasma concentrations of p-cresol and hospitalization frequency [13], clinical symptoms of the uremic syndrome [14], cardiovascular events [15] and mortality [16]. Furthermore, increase plasma p-cresol concentrations lead to the development of CKD [16]. Free plasma p-cresol might be pathophysiologically important, since the free fraction probably exerts biological activity, i.e. toxicity, in analogy with many drugs [17]. Its concentration can be expected to be markedly lower than total concentrations in view of the important protein binding [18]. In 1997, using HPLC analysis, De Smet et al. [18] found that total p-cresol increased progressively during the development of CKD, whereas protein binding decreased. In healthy controls, virtually no free p-cresol could be found; in contrast, in hemodialysis (HD) patients, protein binding was only ±90%.

The pathophysiological effects of uremic toxins and the role of p-cresol were studied in different in vitro models [4,19,20]. It is well known that p-cresol decreased the functional capacity of phagocytes [21,22,23,24] and inhibited the release of platelet-activating factor by macrophages [19]. A leukocyte cell line has been used to highlight cellular dysfunction caused by uremia [4]. Many studies revealed dual effects of uremic retention solutes on leukocyte function: blunting upon stimulation and basal activation linked to microinflammation [25], malnutrition and atherosclerosis [26]. Vanholder et al. [24] demonstrated that granulocyte function was depressed after prolonged incubation with p-cresol.

In CKD patients, renal endothelial cells are chronically exposed to uremic toxins. p-Cresol inhibits cytokine-induced expression of endothelial adhesion molecules and stimulates monocyte adhesion to endothelial cells [21]. In particular, at concentrations commonly found in uremia, two solutes, p-cresol and indoxyl sulfate, induce a dose-dependent inhibition of endothelial proliferation [27]. p-Cresol strongly increases endothelial monolayer permeability [28], and it is able to inhibit proliferation, invariably decreases cell migration and tube formation, and arrests the cell cycle of late endothelial progenitor cells at G2/M phase [20]. Whereas the effects of p-cresol in monocytes and endothelial cells are well known, it remains to be determined what happens in renal epithelial cells, considering that the kidney is the principal organ affected by the uremic syndrome. In vivo, renal tubular cells (RTCs) are exposed to glomerular filtrate which contains all the materials present in the blood except for erythrocytes, blood cells and many proteins - which are too large to cross the basement membrane of the glomerulus. Middle and small water-soluble uremic toxins are present in glomerular filtrate. The protein binding of p-cresol is close to 90%. [26]. The unbound fraction is filtered by glomerula and removed through renal tubes and RTCs.

The major goal of this study is to investigate the effect of p-cresol on renal epithelial cells in terms of apoptosis and necrosis to better understand the pathophysiological role and impact of this toxin in the kidney.

Patients and Methods

p-Cresol Stock Solution

In 2007, Cohen et al. [29] recommended the use of the highest reported concentration in uremic plasma/serum (Cmax) as a starting point for testing the in vitro effect of the substances. In 2010, a uremic Cmax of 40.7 mg/l was reported in uremic patients for p-cresol [30]. We dissolved the powder (Sigma, St. Louis, Mo., USA) in complete cell medium [RPMI 1640 with stable L-glutamine (International PBI Italy, Milan, Italy) and 10% fetal bovine serum (Sigma)] to obtain a solution of ×100. Dilutions were made with the same medium.

RTC Culture

The human RTC cell line was obtained from renal epithelial cells derived from nephrectomy tissue and immortalize by infection with an SV40 virus. RTCs were grown in complete liquid phase medium (RPMI 1640 with stable L-glutamine) supplemented with 10% heat-inactivated (30 min at 56°C) fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma). RTCs were maintained in an incubator at controlled atmosphere (5% CO2) at 37°C and passaged every 2nd or 3rd day.

Trypan Blue Exclusion Cell Viability Assay

The number of viable cells was determined by trypan blue (Sigma); 50 µl of cells were stained with 50 µl of trypan blue. Cell viability is calculated by the number of viable cells (unstained) divided by the number of total cells in percent at ×20 magnification.

Induction of Cytotoxicity with p-Cresol

RTCs were plated at 3 × 105 cells per well in 6-well plates and incubated with increasing concentrations of p-cresol for 24 h in complete RPMI 1640 under standard conditions (37°C in 5% CO2 for 24 h). p-cresol concentrations were scalar: 40, 20, 10, 5 and 2.5 mg/l for 24 h. We used untreated RTCs as a negative control. Each concentration was tested five times.

Detection of DNA Fragmentation

Apoptosis is characterized by DNA fragmentation which shows a ladder-like pattern and nuclear fragmentation in several smaller fragments.

Untreated and p-cresol-treated RTCs (2 × 106 cells) were harvested and washed with Dulbecco's PBS. The DNA fragmentation assay was performed using an apoptotic DNA ladder extraction kit (BioVision, Milpitas, Calif., USA) according to the manufacturer's protocol.

DNA ladder fragmentation was detected by electrophoresis on 1.2% agarose gels staining with Syber Safe (Life Technologies, Monza, Italy); the bands were visualized under ultraviolet light and photographed.

Annexin V and Propidium Iodide Detection Assay

Before incubation, all cells and cellular debris were collected and washed in Dulbecco's PBS (without calcium and magnesium) at pH 7.4.

Cell viability, apoptosis and necrosis were assessed using the annexin V-FITC kit (Beckman Coulter, Brea, Calif., USA) according to the manufacturer's protocol. This kit is based on the binding properties of annexin V to phosphatidylserine and on DNA-intercalating capabilities of propidium iodide (PI). Analysis was performed using a Navios flow cytometer (Beckman Coulter). Bi-parametric analysis revealed three distinct populations: viable cells with low FITC and low PI signals; apoptotic cells with high FITC and low PI signals, and necrotic cells with high FITC and high PI signals. We used negative controls (untreated cells): quadrants encompassed unstained cells, cells stained with annexin V-FITC alone (for FL-1 fluorescence) and cells stained with PI alone (detected in FL-4). A minimum of 15,000 events were collected for each sample.

Determination of Caspase-3 Activity

RTCs were assayed for activation of caspase-3, an effector caspase able to cleave various cytoplasmic or nuclear substrates, which leads to many morphological features of apoptotic cell death. Caspase-3 concentration was measured using the human caspase-3 instant ELISA kit (eBioscience, San Diego, Calif., USA) with a fluorometric assay.

RTCs incubated with p-cresol for 24 h and controls were processed according to the manufacturer's instructions and finally caspase-3 levels were measured in cell lysates at 450 nm in the VICTOR4 Multilabel Plate Reader (PerkinElmer Life Sciences, Waltham, Mass., USA). The amount of caspase-3 (ng/ml) was calculated from the standard curve according to the manufacturer's protocol. Each experiment was performed in triplicate.

Statistical Analysis

Statistical analysis was performed using the SPSS 15 software package. Results are presented as percentages, or medians and interquartile ranges (nonparametric variables). The Kruskal-Wallis test for multiple comparisons was applied to compare the different p-cresol concentrations. In case of a significant difference between the groups, the Mann-Whitney test was applied in order to detect which group or groups were different from the others. A value of $p < 0.05$ was considered statistically significant.

Results

In our experiment, RTC viability was 90%, which was assessed by trypan blue exclusion. The cytotoxic effect of p-cresol was studied in an RTC cell line incubated for 24 h with or without increasing concentrations of this uremic toxin. After 24 h, cell apoptosis and necrosis were assessed using different methods.

DNA isolated from RTCs incubated with increasing p-cresol concentrations for 24 h showed a ‘ladder' pattern of apoptosis at p-cresol concentrations of 10 and 5 mg/l. The results showed that at 2.5 mg/l p-cresol, DNA ladder formation was similar to that in untreated cells, suggesting the presence of physiological apoptotic events. At 2.5 mg/l p-cresol, physiological apoptosis expression was not modified. At higher concentrations (20-40 mg/l), DNA did not show the apoptotic ladder fragmentations typical of an apoptosis-inducing effect (fig. 1).

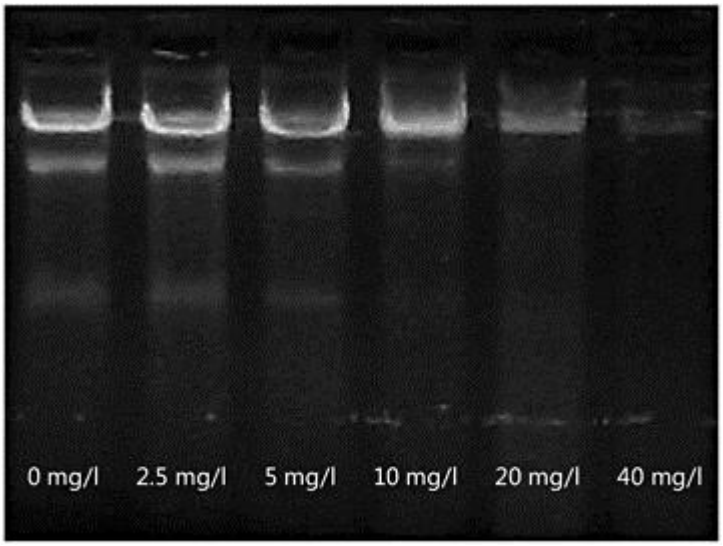

Fig. 1. Qualitative analysis of apoptosis by DNA ladder detection.

Quantitative analyses of injured, necrotic and apoptotic cells are reported in table 1.

	0 mg/l	2.5 mg/l	5 mg/l	10 mg/l	20 mg/l	40 mg/l
Caspase-3, ng/ml	1.914±0.188	2.353±0.482	2.558±0.538	2.425±0.428	2.306±0.307	1.988±0.293
Annexin V, %	0.3 (0.14–14.18)	5.81 (0.01–21.77)	3.88 (0.32–30.31)	2.515 (0–28.33)	0.095 (0.01–1.61)	0.19 (0.03–0.3)
PI, %	13.78±7.43	15.6±6.75	18.18±8.86	40.16±18.62	66.15±11.09	82.25±2.52

Table 1. Caspase-3 and rate of cellular positivity for annexin V and PI in RTCs after 24-hour incubation with increasing concentrations of p-cresol (means ± SD or medians with interquartile ranges in parentheses)

RTCs incubated for 24 h with p-cresol demonstrated a significant reduction in viability. The effect of p-cresol on RTCs is expressed as percent cell necrosis. Cytofluorimetric analysis of necrosis using the annexin V/PI assay highlighted significantly higher cell death rates in RTCs incubated with the maximum concentration of p-cresol compared with other concentrations and untreated cells ($p < 0.05$).

Our results revealed that p-cresol at 40 mg/l strongly induced necrosis in RTCs. Compared with controls, RTCs incubated with 20 mg/l p-cresol had a significantly higher level of necrosis [71.22 (51.34-76.08) vs. 13.78% (7.09-18.47) $p < 0.05$]. At 10 mg/l p-cresol, necrosis was significantly higher compared with untreated cells. p-Cresol concentrations of 5 and 2.5 mg/l induced necrosis in 15.54 (9.17-29.07) and 17.77% (5.74-23.4) of cells, respectively. However, no significant difference was found between necrosis induced by 5 and 2.5 mg/l of p-cresol and that in controls. Necrosis induction was more marked at higher p-cresol concentrations (fig. 2).

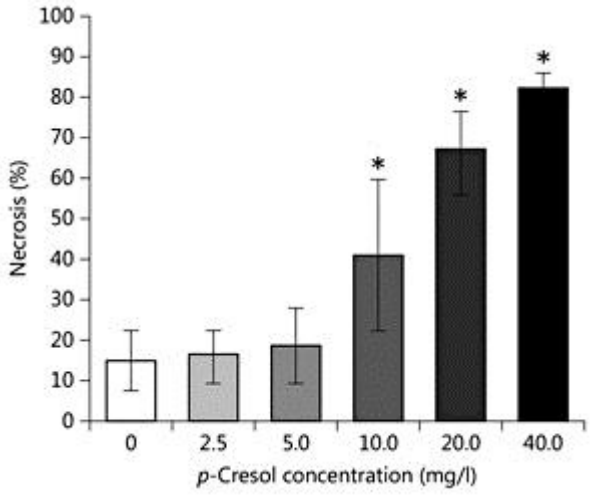

Fig. 2. Percentage of cellular necrosis after a 24-hour incubation with increasing p-cresol concentration. * $p < 0.05$.

There was no significant difference in apoptosis levels detected by annexin V compared with untreated cells. p-Cresol had the same effect on RTC apoptosis at maximum, mean and normal uremic concentrations. Similar results were obtained and confirmed by caspase-3 analysis. With respect to apoptosis, we did not detect any effect of p-cresol on RTCs compared with the control.

Discussion

p-Cresol is a protein-bound uremic toxin accumulating in the body of patients with compromised renal function. Due to its cytotoxic effects, CKD and HD patients are at increased risk of vascular damage,

morbidity and mortality [31]. The cytotoxic effects of p-cresol on monocytes has been described in many studies [4,24].

In the present report, we performed an in vitro study to clarify the effect of p-cresol on RTCs. We studied the cytotoxic effects of p-cresol on apoptosis and necrosis after a 24-hour incubation of these cells with increasing concentrations of this uremic toxin. The maximum concentration tested was 40 mg/l, which is the highest concentration observed in uremic patients. Our in vitro data show that p-cresol induces necrosis at higher concentrations, but we did not observe any significant pro-apoptotic effects of increasing concentrations of p-cresol in RTCs.

Cohen et al. [29] defined the guidelines to work with uremic toxins in in vitro studies. In case of protein-bound uremic toxins, it is recommended to add albumin together with the highest total concentration of the substances to ensure that the concentration of the free toxin reaches the desired value. We did not add albumin because cells in renal glomerula are exposed only to the free fraction of p-cresol. Moreover, the free fraction is the only fraction that has a cytotoxic effect, as shown in different studies in literature [31].

In this study, free p-cresol causes a toxic reaction in RTCs, which leads to cell death especially at high concentrations. Cell death can occur in two different pathways: apoptosis and necrosis. We detected apoptotic and necrotic pathways at different p-cresol concentrations.

Apoptosis is an active programed process of autonomous cellular disassembling which elicits no inflammatory response. It is characterized by a variety of cellular changes including loss of membrane phospholipid asymmetry, chromatin condensation, DNA cleavage and cellular contraction [30]. Caspases are a family of aspartate-specific cysteine proteases that act in a kinase-like stepwise signaling manner and cause apoptosis. Caspases are present in all cells and are activated by autoproteolytic cleavage. Active caspases proteolyze additional caspases generating a caspase cascade to cleave proteins critical for cell survival. The final outcome of this signaling pathway was a form of controlled cell death termed apoptosis. The subgroup of caspases involved in apoptosis are initiators or effectors, such as caspase-3 [32].

DNA ladders are produced by cleavage of genomic DNA between nucleosomes to generate fragments with lengths corresponding to multiple integers of approximately 180 bp [33]. These morphologic changes represent the classic hallmarks of apoptosis that are distinct from cell necrosis. Necrosis has been characterized as passive, accidental cell death resulting from environmental perturbations with uncontrolled release of inflammatory cellular contents [34].

At the maximum concentration (40 mg/l) of p-cresol observed in uremic patients, p-cresol had a strong effect on epithelial cells, resulting in generalized necrosis. In spite of the absence of a pro-apoptotic effect of p-cresol in our in vitro model, we rejected the hypothesis that apoptosis was involved in the pathogenic

mechanism of this toxin. de Carvalho et al. [35] studied apoptotic activation in polymorphonuclear leukocytes (PMN) from healthy subjects incubated with p-cresol. They did not detect necrosis and did not observe any changes in apoptosis, viability or expression of caspase-3 in these cells.

Our in vitro data highlight that necrosis is the principal mechanism of death in RTCs incubated with increasing concentrations of p-cresol.

p-Cresol is a phenol molecule. On the basis of their chemical properties, phenols were used as organic disinfectant: the molecular structure is optimal to permeate plasma membranes and alter cellular permeability. In this study, we hypothesized that the cell dose-dependent necrosis in RTCs is caused by gradual loss of cell permeability at increasing p-cresol concentrations, but apoptotic signals may not be involved in RTC death.

In vivo, under normal conditions, the glomerular filter clears molecules with a molecular weight of up to 58 kDa, therefore the small percentage of total free p-cresol (108.1 kDa) is filtered [8]. In CKD patients, some substances, which can be cleaned physiologically, are retained. In this context, the total volume of the glomerular filtrate is diminished and the relative p-cresol concentration increases.

This condition determines serious effects on renal tubular epithelial cells. In addition, many clinical adverse effects were reported to be correlated with high concentrations of free p-cresol [16,31]. Epithelial cell death may have strong pathophysiological consequences on renal function, which is already compromised in CKD patients.

Cendoroglo et al. [33] reported an increased apoptosis level in PMN isolated from healthy controls and treated with uremic plasma. This is in agreement with the study by Sardenberg et al. [36], who showed that in PMN from HD and uremic patients apoptosis was increased compared to PMN from healthy subjects. In addition, Ferrante [22] observed that PMN isolated from uremic patients had impaired function and showed a lower migration capacity [37].

Furthermore, toxicity is not a single monofactorial process whereby only one or a few toxins affect many different metabolic processes at a time. A recent survey of the literature revealed the retention of at least 90 compounds in uremia, and it is very likely that it is only the tip of the iceberg.

To our knowledge, this is the first study to explore the cytotoxicity of p-cresol in RTCs. Nevertheless, we acknowledge the study limitations, e.g. the lack of a pool of uremic toxins that mimic the uremic composition in the blood; these preliminary results can be considered as hypothesis generating and results need to be validated by further in vitro studies in the future.

In conclusion, our data indicate that the uremic toxin p-cresol has a harmful effect on RTC viability and causes RTC injury and necrosis. We observed an increase in necrosis rates by p-cresol in the renal epithelium; the cytotoxic effect of p-cresol results in generalized cellular damage and death. It was suggested that the activation of cellular necrosis plays a critical role in the p-cresol-induced mechanism in RTCs. These results were consistent with clinical studies showing a link between high concentrations of plasma free p-cresol and HD patient outcome, which is reflected by hospitalization rates for inflammatory disease and cardiovascular events [13,36].

Our results highlight the necessity of developing new therapeutic and dialytic strategies to increase p-cresol removal in CKD and HD patients.

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Disclosure Statement

The authors report no conflicts of interest.

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