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Research Paper

Fragmentation of kidney epithelial cell primary cilia occurs by cisplatin and these cilia fragments are excreted into the urine

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ABSTRACT

The primary cilium, which protrudes from the cell surface, is associated with the pathogenesis of various diseases, including acute kidney injury (AKI). Primary cilium length dynamically changes during the progression of diseases. However, its relevance in disease and the underlying mechanism are largely unknown. In this study, we investigated the role of primary cilia in AKI induced by cisplatin, an effective anticancer drug, and the underlying mechanisms. In addition, we evaluated the usefulness of length alteration and deciliation of primary cilia into the urine for the diagnosis of AKI. Cisplatin induced shortening, elongation, and normalization of the primary cilia in kidney epithelial cells over time. During shortening, primary cilia fragments and ciliary proteins were excreted into the urine. During deciliation, cell proliferation and the expression of cyclin-dependent kinase inhibitor and proliferating cell nuclear antigen were not significantly changed. Shortening and deciliation of primary cilia were observed before significant increases in plasma creatinine and blood urea nitrogen concentration occurred. Pretreatment with Mito-Tempo, a mitochondria-targeted antioxidant, prevented cisplatin-induced primary cilium shortening and inhibited the increases in superoxide formation, lipid peroxidation, blood urea nitrogen, and tissue damage. In contrast, isocitrate dehydrogenase 2 *(Idh2)* gene deletion, which results in defect of the NADPH-associated mitochondrial antioxidant system, exacerbated cisplatin-induced changes in mice. Taken together, our findings demonstrate that cisplatin induces deciliation into the urine and antioxidant treatment prevents this deciliation, renal dysfunction, and tissue damage after cisplatin injection. These results suggest that cisplatin-induced AKI is associated with primary cilia and urine primary cilia proteins might be a non-invasive biomarker of kidney injury.

1. Introduction

The primary cilium, a cellular organelle which is anchored on the basal body and protrudes from the cell surface, acts as a sensor and signal transducer in the cell. The length of the primary cilium is dynamically altered under physiological and pathological conditions. This alteration is strongly linked to the assembly and disassembly of the microtubule that forms the core of the primary cilium and consists of tubulins [1]. In the kidneys, primary cilia reside on the apical surface of epithelial cells, including the parietal cells of Bowman's capsule and tubular epithelial cells, and they detect changes in renal fluid flow and the composition of kidney ultrafiltrate, and activate intracellular signaling to respond to those changes [2]. Perturbations in the primary cilium structur and function, including changes in length, are not only causally but also consequentially associated with various genetic or non-genetic human diseases, including polycystic diseases of the kidney. Changes in length can occur through injury and repair. Recent studies have demonstrated that epithelial cells in the kidney can be injured by various stresses, including ischemia/reperfusion (I/R), which can lead to shortening of the primary cilium through resorption and/or disruption (deciliation) [3–5]. Such length changes are affected by multiple factors, including cell cycle, cytokines, and reactive oxygen species (ROS) [3–6]. Furthermore, several studies have reported that primary cilium length influences cell susceptibility to stress [7,8], although the exact molecular mechanisms remain to be elucidated.

Cisplatin (cis-diamminedichloridoplatinum II) is one of the most

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widely used chemotherapeutic agents. Approximately one third of patients who receive cisplatin therapy develop acute kidney injury (AKI) [9,10]. Cisplatin is mainly removed from the body through excretion via the kidneys. However, during this process, cisplatin accumulates in the kidney tubular epithelial cells and injuries the cells through various pathways [11–14]. Among which oxidative stress is a major factor. Cisplatin metabolites form a complex with glutathione (GSH) the major antioxidant in the mitochondria [15,16], which leads to depletion of GSH in the mitochondria as the cisplatin-GSH complex can easily pass through the mitochondrial membrane [4,6]. Consequently, the mitochondrial redox balance is disrupted, resulting in oxidative damage of mitochondria and cells. Recently, we found that primary cilium length is influenced by ROS and oxidative stress [4,6,17]. Lavagnino et al. reported that hypoxia-inducible factor, which is linked to oxidative stress, is also associated with cilium length [18]. Furthermore, one major anti-tumor effect of cisplatin is the blockage of tubulin assembly into microtubules, resulting in cell death [19]. Recently, Wang et al. observed the shortening of primary cilia and loss of kidney tubular cells in cisplatin-injected mice, and they suggested that these changes increase kidney cell susceptibility to cisplatin. Based on these lines of evidence, we investigated why primary cilia are shortened in cisplatin-induced AKI, and whether these changes can serve as a diagnostic marker. In the present study, we found that cisplatin shortens primary cilia via deciliation rather than resorption into the cell body, and this shortening is caused by increased ROS and oxidative stress. In addition, we found that deciliated primary cilia are excreted into the urine. These findings indicate that cisplatin-nephrotoxicity is associated with primary cilia, and urine primary cilium proteins can be a useful non-invasive indicator for the diagnosis of kidney injury.

2. Materials and methods

2.1. Animal preparation

All experiments were conducted with 8-week-old male C57B/6, *Idh2* gene-deleted (*Idh2*$^{-/-}$), and wild-type (*Idh2*$^{+/+}$) littermates [20]. C57B/6 mice were purchased from Samtaco (Seoul, Korea). The mice were allowed free access to water and standard mouse chow. The animal study was approved by the Institutional Animal Care and Use Committee of Kyungpook National University. Mice were intraperitoneally injected with either cisplatin (10 mg/kg or 20 mg/kg body weight; Sigma, St. Louis, MO, USA) or 0.9% saline (vehicle). Some mice were injected daily with (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl) triphenylphosphonium chloride monohydrate (Mito-Tempo, a mitochondria-targeted antioxidant; 0.7 mg/kg body weight; Sigma) one week before cisplatin injection. To collect urine, mice were individually placed in metabolic cages for 6 h. At the end of experiments, kidneys were snap-frozen in liquid nitrogen or perfusion-fixed with PLP (4% paraformaldehyde, 75 mM L-lysine, 10 mM sodium periodate; Sigma) solution. Frozen tissues were stored at −80 °C until use.

2.2. Measurement of functional parameters

Concentrations of blood urea nitrogen (BUN) and plasma creatinine (PCr) were measured using a Vitros 250 (Johnson & Johnson, New Brunswick, NJ, USA).

2.3. Histology

Paraffin-embedded tissues were cut into 3-μm sections using a microtome (Leica, Bensheim, Germany). To determine histological damage, kidney sections were stained with periodic acid-Schiff (PAS) according to the manufacturer's instruction. Images were captured using i-Solution software (IMT, Vancouver, Canada). Kidney tubular damage was scored as follow: 0, no damage; 1, mild damage with rounded epithelial cells and dilated tubular lumen; 2, moderate damage with flattened epithelial cells, dilated lumen, and congestion of lumen; and 3, severe damage with flat epithelial cells lacking nuclear staining and congestion of the lumen. At least 50 tubules per kidney were analyzed.

2.4. Measurement of superoxide levels in kidney tissue

Superoxide levels were measured using dihydroethidium (DHE; Sigma) as described previously [21]. Briefly, kidney lysates were transferred into wells of a 96 plate and 10 μM DHE reagent was added. The absorbance was read using an emission/excitation filter of 530 nm/620 nm at a temperature of 37 °C. Superoxide levels were expressed as a value per milligram protein of the kidney lysates.

2.5. BrdU-incorporation assay

To determine cell proliferations, 5-bromo-2′-deoxyuridine (BrdU, 50 mg/kg body weight; Sigma) incorporation assay was performed. BrdU, as a thymidine analog, is incorporated into newly synthesized DNA during the S phase of cell replication [22]. BrdU was administered to mice beginning on 1 day before cisplatin injection, every other day until sacrifice. Kidney sections were subjected to immunohistochemical staining using anti-BrdU (Serotec, Oxford, UK) antibody. The cortical and outer medullary regions were observed under a Leica microscope (DM2500, Wetzlar, Germany).

2.6. Western blot analysis

Western blot analysis was performed using anti-4-hydroxynonenal (4-HNE; Abcam, Cambridge, MA, USA), anti-acetylated-α-tubulin (ac-α-tubulin, Sigma), anti-Arl13b (Proteintech, Chicago, IL, USA), anti-proliferating cell nuclear antigen (PCNA; DAKO, Carpinteria, CA, USA), anti-p21 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; NOVUS, Littleton, CO, USA) antibodies.

2.7. Immunofluorescence

Kidney sections were deparaffinized and rehydrated, and then washed with phosphate-buffered saline (PBS) for 5 min each. The sections were incubated in PBS containing 0.1% sodium dodecyl sulfate (SDS; Sigma) for 1 min and washed in PBS for 10 min. To expose the antigen epitope, the sections were boiled in 10 mM sodium citrate buffer (pH 6.0) for 10 min, cooled for 20 min, and then washed three times with PBS for 5 min. The sections were blocked with 1% bovine serum albumin in PBS (blocking buffer) for 30 min and then incubated with anti-ac-α-tubulin, anti-AQP-1 (Alomone Labs, Jerusalem, Israel), and anti-AQP-2 (Alomone Labs) antibodies diluted in blocking buffer overnight at 4 °C. After washing, the sections were incubated with FITC-conjugated goat anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) or goat anti-rabbit IgG (Vector Laboratories) for 60 min, and then washed three times with PBS for 5 min. To stain cell nuclei, 4′-6-diamidino-2-phenylindole (DAPI; Sigma) was placed on the sections for 1 min.

To detect fragments of primary cilia in the urines, slide glasses were smeared urines, fixed, immunostained using anti-ac-α-tubulin and -Arl13b antibodies, and then observed under a Leica microscope.

2.8. Measurement of primary cilium length

Primary cilia lengths were measured as previously described [4]. Kidney sections were processed for immunofluorescence staining with anti-ac-α-tubulin antibody for primary cilia, and nuclei were counterstained with DAPI. Images were captured using a Leica microscope. Five to ten fields in the outer medulla of kidneys were randomly captured (magnification, 400 ×) and primary cilia length were measured