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Indoxyl sulfate, a uremic toxin, promotes cell senescence in aorta of hypertensive rats

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ABSTRACT

We demonstrated that administration of indoxyl sulfate, a uremic toxin, promotes aortic calcification in hypertensive rats. This study aimed to clarify if indoxyl sulfate could contribute to cell senescence in the aorta of hypertensive rats. The rat groups consisted of (1) Dahl salt-resistant normotensive rats (DN), (2) Dahl salt-resistant normotensive indoxyl sulfate-administered rats (DN + IS), (3) Dahl salt-sensitive hypertensive rats (DH), and (4) Dahl salt-sensitive hypertensive indoxyl sulfate-administered rats (DH + IS). After 32 weeks, their arcuate aortas were excised for histological and immunohistochemical analysis. Cell senescence was evaluated by immunohistochemistry of senescence-associated β-galactosidase (SA-β-gal), and senescence-related proteins such as $p16^{INK4a}$, $p21^{WAF1/CIP1}$, p53 and retinoblastoma protein (Rb). Both DH and DH + IS rats showed significantly higher systolic blood pressure than DN and DN + IS rats, respectively. Serum indoxyl sulfate levels were significantly higher in DN + IS and DH + IS rats than in DN and DH rats, respectively. In aorta, DH rats showed significantly increased aortic calcification and wall thickness, and increased expression of SA-β-gal, $p16^{INK4a}$, $p21^{WAF1/CIP1}$, p53 and Rb in the calcification area of arcuate aorta as compared with DN rats. More notably, DH + IS rats showed significantly increased aortic calcification and wall thickness, and significantly increased expression of SA-β-gal, $p16^{INK4a}$, $p21^{WAF1/CIP1}$, p53 and Rb in the cells embedded in the calcification area as compared with DH rats. In conclusion, indoxyl sulfate promotes cell senescence with aortic calcification and expression of senescence-related proteins in hypertensive rats.

1. Introduction

Cellular senescence is a state of permanent and irreversible cell cycle arrest with a reduced capability to respond to stress that results in insufficient regenerative capacity of organs [1,2]. Senescent cells enter an irreversible growth arrest, exhibit a flattened and enlarged morphology, and express a different set of genes such as $p16^{INK4a}$, $p21^{WAF1/CIP1}$, p53 and retinoblastoma protein (Rb). These phenotypic changes of senescent cells have been implicated in aging and age-associated diseases [3,4]. Senescence-associated β-galactosidase (SA-β-gal) is a frequently used and reliable indicator of cell senescence in both *in vitro* and *in vivo* models [5–8].

$p16^{INK4a}$ and $p21^{WAF1/CIP1}$ are cyclin-dependent kinase inhibitors (CDKI), and can inhibit the cell cycle progression and maintain the G1 phase arrest of cells [9]. p53 and Rb are the main activators of senescence. p53 can activate senescence by activating Rb through $p21^{WAF1/CIP1}$ and also independently of Rb. Rb is activated either by $p21^{WAF1/CIP1}$ or $p16^{INK4a}$ product[10]. Recent findings suggest that p53-Rb and p16-Rb pathways contribute to overall cell senescence, and the expression of $p16^{INK4a}$, $p21^{WAF1/CIP1}$, p53 and Rb are up-regulated by extrinsic and intrinsic stimuli [10,11]. In addition, common histopathologic features of renal aging and renal diseases, such as glomerulosclerosis, interstitial fibrosis, correlate with the expression of $p16^{INK4a}$, $p21^{WAF1/CIP1}$ and p53 [12–14]. $p16^{INK4a}$ induction is also found in human hearts [15], and a higher incidence of senescence-associated cellular phenotypes have been associated with hypertension and the development of atherosclerosis [16].

Less attention has been devoted to indoxyl sulfate-associated cellular senescence. We have demonstrated that indoxyl sulfate is a uremic toxin accelerating the progression of chronic kidney disease (CKD) [17–20]. Indoxyl sulfate is derived from dietary protein. A part of the protein-derived tryptophan is metabolized into indole by tryptophanase in intestinal bacteria such as *Escherichia coli*. Indole is then absorbed into the blood from the intestine, and is metabolized to indoxyl sulfate in the liver, while indoxyl sulfate is normally excreted into urine. In CKD, however, the inadequate renal clearance of indoxyl sulfate leads to an elevation of it. In fact, the serum levels of indoxyl sulfate were found to be

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markedly increased in both CKD rats and patients [20–23]. In serum, approximately 90% of indoxyl sulfate is bound to serum albumin. An oral sorbent AST-120 reduces the serum and urine levels of indoxyl sulfate in CKD rats and patients by adsorbing indole in the intestines, consequently stimulating its excretion into feces [22,23]. The administration of indoxyl sulfate to 5/6-nephrectomized rats has promoted the progression of CKD accompanied by the enhanced gene expression of transforming growth factor (TGF)-β1, tissue inhibitor of metalloproteinase (TIMP)-1 and proα1(I) collagen [24,25]. These findings support the notion that indoxyl sulfate is one of the uremic toxins stimulating the progression of CKD by increasing the renal expression of these fibrosis-related genes. The induction of nephrotoxicity by indoxyl sulfate is mediated by organic anion transporters (OATs), such as OAT types 1 and 3 in the basolateral membrane of renal proximal tubular cells [26]. Recently, we demonstrated that indoxyl sulfate contributes to the progression of aortic calcification in hypertensive rats [27]. Cellular senescence may be responsible for the development of indoxyl sulfate-related phenotypes.

In this study, we determined if indoxyl sulfate promotes cell senescence and the expression of $p16^{INK4a}$, $p21^{WAF1/CIP1}$, p53 and Rb in the aorta of hypertensive rats.

2. Methods

2.1. Experimental design

Five-week-old Dahl salt-resistant rats (Dahl-Iwai, $n = 16$) and Dahl salt-sensitive rats (Dahl-Iwai, $n = 16$) were purchased from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan), and were fed powder rat chow (CE-2, Clea, Tokyo, Japan) and water for 2 weeks. Then, the rats were fed the chow (CE-2) with salt (1.5% NaCl) intake in water. At 16 weeks of age, Dahl salt-sensitive rats spontaneously developed hypertension with systolic blood pressure (BP) more than 140 mm Hg, and the rats were divided into two groups: Dahl salt-resistant normotensive rats (DN, $n = 16$) and Dahl salt-sensitive hypertensive rats (DH, $n = 16$), then the rats randomized into control rats and indoxyl sulfate-administered rats (200 mg/kg of indoxyl sulfate in water). Thus, the rat groups consisted of (1) Dahl salt-resistant normotensive rats (DN, $n = 8$), (2) Dahl salt-resistant normotensive indoxyl sulfate-administered rats (DN + IS, $n = 8$), (3) Dahl salt-sensitive hypertensive rats (DH, $n = 8$), and (4) Dahl salt-sensitive hypertensive indoxyl sulfate-administered rats (DH + IS, $n = 8$). At 48 weeks of age (32nd week of the study), the rats were anesthetized, and their blood samples were collected from the aorta after a 24-h urine sample had been collected by using a metabolic cage. BP was measured using the tails of the rats with a pneumatic cuff and a sphygmomanometer for small animals (UR-5000, Ueda Avancer Co., Tokyo, Japan). Serum levels of indoxyl sulfate were measured by using the method reported in our previous paper [27]. Aortas were excised for histological and immunohistochemical analysis. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). All animal studies were performed under the Institutional Guide for Care and Use of Laboratory Animals (Biomedical Research Laboratories, Kureha, Tokyo, Japan).

2.2. Histopathology of aorta

Tissues were removed, weighed and fixed with 10% formalin, dehydrated at room temperature through ethanol series and embedded in paraffin. Then, they were cut into 3-μm sections. Aorta tissues were stained with hematoxylin and eosin (HE), and von Kossa to detect calcification. The calcification area and the wall thickness of aorta were evaluated with von Kossa staining as described before [27].

2.3. Immunohistochemistry

Immunostaining for SA-β-gal, $p16^{INK4a}$, $p21^{WAF1/CIP1}$, p53 and Rb in tissues were performed in paraffin-embedded fixed tissues as described previously [27]. Briefly, sections were deparaffinized and hydrated. Endogenous peroxidase was inhibited with 0.3% H_2O_2 in methanol for 10 min, followed by a rinse in phosphate-buffered saline. Sections were blocked by 10% serum (Nichirei Co., Tokyo, Japan) at room temperature for 30 min. Immunostaining was processed according to the streptavidin–biotin complex (SABC) method.

For immunohistochemical localization of SA-β-gal in aorta tissues, sections were incubated overnight with anti-β-galactosidase antibody (Promega Corp. Madison, WI, USA) at 1:20 dilution as reported by Gruber et al. [28]. Antigen retrieval was performed by microwave in 0.01 mol/l citrate buffer (pH 6.0) for 5 min at 600 W twice. For immunohistochemical localization of $p16^{INK4a}$, $p21^{WAF1/CIP1}$, p53 and Rb in aorta, mouse monoclonal anti-$p16^{INK4}$ antibody (Clone F-12, Santa Cruz Biotechnolgies, Santa Cruz, CA, USA) at 1:100 dilution, mouse monoclonal anti-$p21^{WAF1/CIP1}$ antibody (Clone F-5, Santa Cruz Biotechnolgies, Santa Cruz, CA, USA) at 1:50 dilution, rabbit polyclonal anti-p53 antibody (Clone FL-393 Santa Cruz Biotechnolgies, Santa Cruz, CA, USA) at 1:100 dilution, and rabbit polyclonal anti-Rb (Clone C-15, Santa Cruz Biotechnolgies, Santa Cruz, CA, USA) at 1:50 dilution were used. Antigen retrieval was performed by microwave in 0.01 mol/l citrate buffer (pH 6.0) for 5 min at 600 W twice.

All the sections were examined under light microscopy (DN100, E600, Nikon, Tokyo, Japan). Positive areas were determined using Adobe Photoshop® by a blinded observer, and measured using NIH Image 1.62. The localization of the markers was analyzed from singly stained consecutive sections. In aorta, positive areas for SA-β-gal, $p16^{INK4a}$, $p21^{WAF1/CIP1}$, p53 and Rb were measured.

2.4. Statistical analysis

Results are expressed as mean ± SE. The differences in time course (the 4th week to the 32nd week) of systolic BP and serum indoxyl sulfate were analyzed by repeated measure two-way analysis of variance (ANOVA). The differences in histological and immunohistochemical values at the 32nd week were analyzed by Fisher's least significant difference (LSD) test of ANOVA. *P*-value less than 0.05 was considered significant.

3. Results

3.1. Laboratory parameters

Fig. 1 shows the time course of systolic BP and serum indoxyl sulfate in rats. Both DH and DH + IS rats showed significantly higher systolic BP than DN and DN + IS rats, respectively, although there were no significant differences in systolic BP between DH and DH + IS rats. Serum indoxyl sulfate levels were significantly higher in DN + IS and DH + IS rats than in DN and DH rats, respectively. However, the serum levels of indoxyl sulfate in DH + IS rats were significantly higher than in DN + IS rats.

3.2. Histological analysis in aorta

Table 1 shows aortic calcification and wall thickness in aorta of rats. In arcuate aorta, DH rats showed significantly increased calci-