Senescence and dysfunction of proximal tubular cells are associated with activated p53 expression by indoxyl sulfate

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When renal function deteriorates, uremic toxins such as indoxyl sulfate accumulate in serum and renal tubules, although indoxyl sulfate is normally excreted into the urine (17, 18, 23, 24). The administration of indoxyl sulfate and its precursor indole to 5/6-nephrectomized rats increased glomerular sclerosis in the remnant kidneys with a decline in renal function (23, 24). In addition, indoxyl sulfate induced expression of fibrosis marker genes such as transforming growth factor (TGF)-β1, tissue inhibitor of metalloproteinases-1, and type-I collagen (17, 18). The induction of fibrosis by indoxyl sulfate is mediated by organic anion transporter types 1 and 3 in the basolateral membrane of renal proximal tubular cells (9). After importation into proximal tubular cells, indoxyl sulfate induces free radical production and thus activates nuclear factor-κB, which, in turn, upregulates the expression of plasminogen activator inhibitor-1 (19). Retained indoxyl sulfate in renal failure is also associated with several detrimental effects on other organs such as altered thyroid (14), cardiac fibroblast and myocyte function (13), dysfunction of endothelial cells (6, 7, 28), the proliferation and migration of vascular smooth muscle cells (VSMCs) (21, 25, 29), calcification of VSMCs in vitro and in vivo (1, 20), and an increase in atherosclerosis (27). Current conventional hemodialysis is ineffective at removing this toxin, as 90% of indoxyl sulfate is bound to albumin, and the indoxyl sulfate-albumin complex is larger than the pores in the dialysis membrane. Indoxyl sulfate is metabolized by the liver from indole, which is generated from tryptophan in dietary proteins by intestinal flora including Escherichia coli. AST-120 is an oral adsorbent that absorbs indole in the intestine and thus decreases the serum level of indoxyl sulfate and subsequently blocks indoxyl sulfate accumulation in renal tubules and the progression of chronic renal failure (CRF) (16).

Aged kidneys are more sensitive to stress and disease, and renal aging has been associated with decreased glomerular filtration rate, glomerulosclerosis, tubular atrophy, and interstitial fibrosis (30). Senescent cells appear during the aging process, which is not only a consequence of time, but can be accelerated by many other factors, including genetic disposition, environmental factors, diet, and lifestyle (30). We previously detected cellular senescence in the kidneys of CRF rats (2). However, little is known about the molecular mechanisms of the initiation of cellular senescence and of the relationship between cellular senescence and kidney dysfunction in CRF.

Senescent cells enter irreversible growth arrest, develop a flattened, enlarged morphology, and express a different set of genes including p53, a main activator of cellular senescence and a tumor suppressor. For instance, cyclosporin A (CsA) induces the activated p53 expression in proximal tubular cells, which subsequently fall into senescence (11). In addition, p53 has six key serine residues (Ser-6, Ser-15, Ser-20, Ser-37, Ser-46, and Ser-392) that are phosphorylated as a result of various stimuli and stresses. In particular, Ser-15 phosphorylation correlates with the enhanced induction and functional activation of p53.

Here, we examined the effects of indoxyl sulfate on the proliferation, senescence, and fibrosis of proximal tubular cells, and on the kidney of CRF rats with respect to the p53 pathway.

MATERIALS AND METHODS

Reagents. Antibodies were obtained from the following suppliers: anti-β-gal, Promega (Madison, WI), anti-α-tubulin, Calbiochem (La Jolla, CA), anti-α-SMA, Sigma Chemical (St. Louis, MO), anti-p53,
anti-phospho-p53 (Ser 15), anti-rabbit IgG horseradish peroxidase (HRP)-linked antibody, and anti-mouse IgG, HRP-linked antibody, Cell Signaling Technology (Beverly, MA). Indoxyl sulfate was from Alfa Aesar (Lancashire, UK). Pifithrin-α, p-Nitro (PFTα), an inhibitor of p53, and N-acetylcysteine (NAC), an antioxidant, were from Calbiochem (La Jolla, CA). Dulbecco’s modified Eagle’s medium (DMEM)/F12 was purchased from Wako (Osaka, Japan). Trypsin-EDTA, fetal bovine serum (FBS), and insulin-transferrin-selenium (ITS) were purchased from GIBCO (Grand Island, NY). Penicillin and streptomycin were purchased from Nacalai Tesque (Kyoto, Japan).

**Cell culture.** HK-2 cells were purchased from ATCC (Manassas, VA), and maintained in DMEM/F12 supplemented with 10% FBS, ITS, 100 U/ml penicillin and 100 µg/ml streptomycin.

**Evaluation of cell proliferation.** After culture under various conditions, HK-2 cells were harvested by trypsin digestion and counted by Trypan blue staining.

**Staining for SAβ-gal.** Serum-starved cells incubated with or without indoxyl sulfate were fixed with 0.5% glutaraldehyde, and then stained in 100 mM sodium phosphate (pH 6.0) containing 0.05% X-gal, 1 mM MgCl₂, 10 mM KCl, 3 mM K₃Fe(CN)₆, 3 mM K₄Fe(CN)₆, and 0.1% Triton X-100 at 37°C for 12 h. Senescence-activated p53 expression, which is involved in suppression of serum-induced proliferation of HK-2 cells caused by IS. A: serum-starved HK-2 cells were incubated with IS (250 µM) for 48 h and then purified mRNA was analyzed by real-time PCR using p53 primer. Amounts of p53 mRNA were evaluated as described in MATERIALS AND METHODS, and results normalized to amounts of GAPDH are expressed as ratios (fold increase) of control value. Data are shown as means ± SE of five independent experiments. *P < 0.05 vs. untreated cells. B: serum-starved HK-2 cells were incubated with IS (250 µM) for 48 h, and then cell lysates were Western blotted using anti-p53 antibody or anti-phospho-p53 (Ser-15) antibody as described in MATERIALS AND METHODS. C: intensity of p53 bands was quantified, and results normalized to amount of α-tubulin are expressed as ratios (fold increase) of control value. Data are shown as means ± SE of six independent experiments. *P < 0.05 vs. untreated cells. D: intensity of phospho-p53 bands normalized to amount of α-tubulin and expressed ratios (fold increase) of control value. Data are shown as means ± SE of four independent experiments. *P < 0.05 vs. untreated cells. E: intensity of phospho-p53 bands normalized to amount of p53 and expressed as ratios (fold increase) of control value. Data are means ± SE of four independent experiments. *P < 0.05 vs. untreated cells. F: serum-starved HK-2 cells were incubated with and without IS (250 µM) for 24 h followed by IS (250 µM) for 24 h with or without pifithrin-α (PFTα, 10 µM). After IS and PFTα (10 µM) were removed, cells were incubated with or without 10% FBS for 72 h, and then proliferation was assessed as shown in MATERIALS AND METHODS. Data are shown as means ± SE of four separate experiments. *P < 0.05 vs. FBS-treated cells. #P < 0.05 vs. IS-treated cells.
associated (SA) β-galactosidase (β-gal)-positive cells were counted in six randomly representative fields.

Quantitative real-time PCR. Total RNA was isolated using Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan). First-strand cDNAs were synthesized from template RNA (2 µg) using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, Ca). Quantitative real-time PCR proceeded using Syber Premix Ex Taq II Green (Takara Bio, Shiga, Japan) and the LightCycler PCR system (Roche, Mannheim, Germany), according to the manufacturer’s protocol with the following oligonucleotide primers: p53, 5'-CCGCAGTCAGATCCTAGCG-3' (forward) and 5'-AATCATCCATTGCTTGGGACG-3' (reverse); α-SMA, 5'-GTGTTGCCTCCCTGAGGACAT-3' (forward) and 5'-GCTGGGAACATTGAAAGTCTCA-3' (reverse); GAPDH, 5'-ATGGGGGAAGGTGAAGGTCG-3' (forward) and 5'-GGGGTGCATTAGTGCCACAAATA-3' (reverse). The expression of mRNA levels was measured as the ratio of each mRNA to GAPDH mRNA.

Immunoblotting. Immunoblotting was performed as described in the supplemental data. In brief, cell lysates were fractionated by SDS-PAGE on polyacrylamide gels, and proteins were transferred to PVDF membranes (Immobilon-P, Millipore, Bedford, MA). p53, phospho-p53 (Ser-15), and α-SMA were detected using specific antibodies and normalized to α-tubulin. The protein bands were visualized using the Chemi-Lumi One system (Nacalai Tesque, Kyoto, Japan).

Design of animal experiments. Experimental rats were produced by Kureha (2). Seven-week-old male Sprague-Dawley rats (Clea, Tokyo, Japan) were used to produce CRF by 4/5-nephrectomy. Eleven weeks after 4/5-nephrectomy, the rats were randomized into two groups: control CRF rats (n = 6) and AST-120-treated CRF rats (n = 6). AST-120 was administered to the rats at a dose of 4 g/kg with powder chow (CE-2, Clea) for 16 wk, whereas powder chow alone was administered to control CRF rats. A control CRF rat died of uremia at the fifteenth week. Normal rats (n = 6) were used to compare the data with CRF rats. Body weight, blood pressure, and serum and urine parameters of these rats were described previously (2). These experiments were approved by Animal Care Committee of Biomedical Research Laboratories of Kureha and proceeded according to the

Fig. 3. IS promotes HK-2 cell senescence. A: serum-starved HK-2 cells were incubated with or without IS (250 µM) for indicated periods, and then cell lysates were Western blotted against anti-β-gal antibody. Representative data are from three experiments that yielded similar results. B: serum-starved HK-2 cells were incubated with IS (250 µM) for 72 h, and then SA β-gal activity was evaluated as described in MATERIALS AND METHODS. C: number of SA β-gal positive cells. Data are shown as means ± SE of three separate experiments. *P < 0.05 vs. untreated cells.

Fig. 4. IS-induced HK-2 cell senescence is inhibited by PFTα. A: serum-starved HK-2 cells were incubated with or without PFTα (10 µM) for 30 min, followed by IS (250 µM) for 72 h, and then activated SA β-gal was stained as described in MATERIALS AND METHODS. B: number of SA β-gal-positive cells. Data are shown as means ± SE of three separate experiments. *P < 0.05 vs. untreated cells. #P < 0.05 vs. indoxyl sulfate-treated cells.
Guiding Principles for the Care and Use of Laboratory Animals of the Japanese Pharmacological Society.

**Immunohistochemistry and Masson’s trichrome staining.** Paraffin-embedded sections of kidney tissues provided by Kureha from described rat models (2) were deparaffinized and hydrated. Immunostaining was performed according to the streptavidin-biotin complex method. For immunohistochemical localization of β-gal, p53, α-SMA, and p21 in kidney tissues, sections were incubated overnight with anti-β-gal antibody at 1:50 dilution, anti-p53 antibody at 1:50, anti-α-SMA antibody at 1:100, and anti-p21 antibody at 1:50. Antigen was retrieved by microwave heating twice in 0.01 M citrate buffer (pH 6.0) for 5 min at 600 W. For evaluation of fibrosis, kidney tissues were dissected, fixed in formalin, and processed for paraffin embedding, and thin sections were stained by Masson’s trichrome (MT). All of the sections were examined under light microscopy (DN100, E600, Nikon, Tokyo, Japan).

**Statistical analysis.** Results are expressed as means ± SE. To compare the values between the groups, analysis of variance and Fisher’s protected least significance difference tests were performed. Results were considered statistically significant when the P value was < 0.05.

**RESULTS**

**Indoxyl sulfate suppresses serum-induced cell proliferation by activating p53 expression.** We used an indoxyl sulfate concentration of 250 μM since our previous study revealed that this is the mean serum level of indoxyl sulfate in patients on hemodialysis (23). We initially examined whether an incubation with indoxyl sulfate for 48 h regulates HK-2 cell proliferation, because indoxyl sulfate enhances the platelet-derived growth factor (PDGF)-induced proliferation of smooth muscle cells (SMC) by promoting platelet-derived growth factor receptor (PDGFR) β expression (25). Figure 1 shows that a 48-h incubation with indoxyl sulfate suppressed serum-dependent cell proliferation, although dead cells were undetectable. We then focused on p53 expression induced by indoxyl sulfate, because p53 plays a key role in suppressing cell proliferation. Figure 2A shows that indoxyl sulfate time-dependently upregu-
lated p53 mRNA levels. In addition, Fig. 2, B–E, shows that indoxyl sulfate also increased the expression of p53 protein and of phosphorylated p53 (Ser 15) protein, of which the phosphorylation site is important for the activation of p53. To confirm that indoxyl sulfate induces activated p53 expression, we added PFTα for 24 h to cells that had been incubated with indoxyl sulfate for 24 h and then evaluated serum-induced cell proliferation. PFTα partially abrogated the indoxyl sulfate-dependent suppression of serum-induced cell proliferation (Fig. 2F). These results demonstrate that indoxyl sulfate suppresses serum-induced cell proliferation via, at least in part, by upregulating activated p53 expression.

**Indoxyl sulfate promotes cellular senescence by activating p53 expression.** We further examined the promotion of β-gal expression and SA β-gal activity by indoxyl sulfate, because SA β-gal is a marker of cellular senescence (5) and of p53 expression activated by CsA-induced senescence in HK-2 cells (11). Figure 3 shows that indoxyl sulfate time-dependently induced β-gal expression (A) and significantly increased SA β-gal activity (B and C). On the other hand, prior incubation with PFTα significantly suppressed indoxyl sulfate-induced SA β-gal activity (Fig. 4, A and B). These results demonstrate that the induction of β-gal expression correlates with increasing SA β-gal activity and that indoxyl sulfate evokes cellular senescence by activating p53 expression.

**NAC inhibited indoxyl sulfate-induced activated p53 expression.** We examined the relationship between reactive oxygen species (ROS) production and activated p53 expression using NAC, because indoxyl sulfate induces ROS production in HK-2 cells (19). Figure 5 shows that NAC inhibited indoxyl sulfate-induced p53 mRNA expression (A) and suppressed indoxyl sulfate-induced p53 protein, phosphorylated p53 protein expression and the phosphorylation levels of p53 (B–E). These results indicate that indoxyl sulfate-induced ROS increases the levels of p53 protein, phosphorylated p53 protein, and p53 phosphorylation.

**NAC and PFTα inhibited indoxyl sulfate-induced α-smooth muscle actin expression.** We previously detected a fibrosis marker gene in CRF rats (17, 18). Here, we examined the expression of α-smooth muscle actin (α-SMA), which is a marker of fibrosis. Figure 6 shows that indoxyl sulfate time-dependently upregulated α-SMA mRNA (A), whereas NAC and PFTα inhibited the indoxyl sulfate-induced expression of α-SMA mRNA and protein (B–D). These results suggest that indoxyl sulfate promotes α-SMA expression through ROS-induced activated p53 expression.

**β-Gal, p53, and α-SMA are expressed and colocalized in renal tubules of CRF rats.** We immunostained sections of the renal cortex with anti-β-gal, anti-p53, and anti-α-SMA antibodies to confirm the findings in vitro. We previously found that AST-120 suppresses indoxyl sulfate staining intensity in renal tubules and indoxyl sulfate concentrations in the serum and urine of CRF rats (16). Therefore, serum indoxyl sulfate levels decrease in AST-120-administered CRF rats. In fact, AST-120 suppresses ROS production and the expression of fibrosis marker genes in the renal tubules of CRF rats (16, 22, 26). We also recognized increased β-gal expression in the renal tubules of CRF rats compared with normal rats and AST-120-treated CRF rats (2).
The present study examined renal cortex sections from CRF rats in which β-gal expression had previously been detected in renal tubules (2), to determine whether p53 and α-SMA colocalize with β-gal at the same sites in these rats.

Figure 7 shows more intense immunoreactivity of p53, α-SMA, and β-gal proteins in the renal tubules of CRF rats than in control and AST-120-treated CRF rats. MT staining shows that the extent of interstitial fibrosis was more prominent in CRF rats than in control and AST-120-treated CRF rats (Fig. 7). These results demonstrate that β-gal, p53, and α-SMA protein levels increase in vivo as well as in vitro, and that p53 and α-SMA are colocalized with β-gal in the renal tubules of CRF rats. Furthermore, interstitial fibrosis was induced in CRF rats, and AST-120 suppressed it.

**DISCUSSION**

The present findings suggest that indoxyl sulfate positively regulates histological and functional aggravation of CRF perhaps via the following mechanisms (Fig. 8). First, indoxyl sulfate promotes activated p53 expression through ROS. The indoxyl sulfate-induced activated p53 expression sequentially suppresses serum-induced cell proliferation, elicits cellular senescence, and facilitates fibrosis. Together with the findings indicating that AST-120 inhibits expression of β-gal, p53, and α-SMA in the renal tubules of CRF rats, the present findings support the notion that indoxyl sulfate-induced activated p53 expression is implicated in one of the molecular mechanisms connecting cellular senescence with functional CRF aggravation.

We did not determine whether typical molecules that lie downstream of p53 are expressed in response to indoxyl sulfate. We examined p21 expression induced by indoxyl sulfate (see supplemental Fig. 1 at the AJP-Cell Physiology website) because p53 controls p21 expression through direct DNA binding to the p21 promoter and transactivation (8), and the p53-p21 axis regulates cell proliferation and senescence (8, 10).
independent pathway also contributes to the induction of p21 (Supplemental Fig. 1A). These results indicate that a p53-dependent suppression of serum-induced cell proliferation. In addition, p21 mRNA expression induced by indoxyl sulfate began to increase before p53 mRNA expression (Fig. 2A and Supplemental Fig. 1A). These results indicate that a p53-independent pathway also contributes to the induction of p21 expression and cellular effects through ROS production induced by indoxyl sulfate, because p21 expression is regulated by p53-dependent and independent pathways (10). We previously demonstrated that the kidneys of CRF rats are dysfunctional, fibrotic, and senescent, although which indoxyl sulfate-induced factor(s) is implicated in these effects remains unknown (17, 18). The present study demonstrates that indoxyl sulfate-induced activated p53 expression regulates not only the serum-induced proliferation and activation of SA β-gal but also α-SMA expression. Although many groups have already investigated the mechanism of suppressed cell proliferation and senescence induced by p53 in detail, the molecular mechanism of α-SMA expression induced by p53 in the kidneys of CRF rats remains unclear. The present results indicate that PFTα suppresses indoxyl sulfate-induced α-SMA expression, but we did not examine whether indoxyl sulfate-activated p53 directly binds to the α-SMA promoter region and regulate α-SMA expression in the human osteogenic sarcoma cell line Saos-2. We consider that indoxyl sulfate-induced activated p53 also binds to the promoter region of α-SMA, thus inducing α-SMA expression in HK-2 cells. Therefore, activated p53 expression provoked by indoxyl sulfate might be closely related to dysfunction, senescence, and fibrosis in the kidneys of CRF rats.

AST-120 suppresses fibrosis and dysfunction in the kidney by removing the indoxyl sulfate precursor indole from the intestine and thus decreases indoxyl sulfate accumulation in the serum and renal tubules (16). AST-120 also attenuates ROS production in the renal tubules of CRF rats (22, 26). Our immunohistochemical analyses revealed that expression of β-gal, p53, and α-SMA in CRF rats are localized in the renal tubules, whereas these proteins are barely detectable in normal rats and in AST-120-treated CRF rats (Fig. 7). In addition, p21 expression in CRF rats is localized with both β-gal and p53 (Supplemental Fig. 3). These results indicate that indoxyl sulfate, ROS, β-gal, p53, α-SMA, and p21 are expressed and colocalized in the renal tubules of CRF rats, and that these effects are suppressed when AST-120 decreases the indoxyl sulfate concentrations in the serum and renal tubules. Therefore, indoxyl sulfate-induced kidney dysfunction, fibrosis, and senescence might be accelerated by ROS-induced activated p53 expression in vivo as well as in vitro.

The present study is limited to in vitro and animal experiments. Clinical study is required to prove that p53-induced cell senescence is involved in the progression of renal fibrosis in CRF patients. Furthermore, the clinical effects of AST-120 on the p53-induced cell senescence in the kidneys should also be studied.

The present study shows that indoxyl sulfate-induced activated p53 expression is a critical event in the suppression of cell proliferation and in the initiation of fibrosis and senescence in proximal tubular cells. In addition to reducing indoxyl sulfate accumulation in the serum of CRF rats using AST-120, expression levels of β-gal, p53, α-SMA, and p21 were decreased in renal tubules. Therefore, understanding the process of indoxyl sulfate-dependent activated p53 expression is essential to elucidate the molecular mechanisms of indoxyl sulfate actions not only in proximal tubular cells but also in other cells such as VSMCs and ECs.

![Progression of CRF](image_url)

**Fig. 8.** Schematic representation of IS functions in proximal tubular cells. IS induces activated p53 expression through ROS production. ROS-induced activated p53 by IS facilitates induction of α-SMA and SA β-gal, thereby promoting fibrosis and cellular senescence, respectively. Cell proliferation is also suppressed by IS-induced activated p53. Thus IS-induced activated p53 expression is a central role of aggravation of renal function in CRF.
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DISCLOSURES

F. Nishijima is employed by Kureha Corporation. The other authors declared no competing interests. No conflicts of interest, financial or otherwise, are declared by the author(s).

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