Indoxyl sulfate promotes vascular smooth muscle cell senescence with upregulation of p53, p21, and prelamin A through oxidative stress

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Muteliefu G, Shimizu H, Enomoto A, Nishijima F, Takahashi M, Niwa T. Indoxyl sulfate promotes vascular smooth muscle cell senescence with upregulation of p53, p21, and prelamin A through oxidative stress. Am J Physiol Cell Physiol 303:C126–C134, 2012. First published May 2, 2012; doi:10.1152/ajpcell.00329.2011.—We previously demonstrated that indoxyl sulfate (IS), a uremic toxin, induces aortic calcification in hypertensive rats and induces oxidative stress and the expression of osteoblast-specific proteins in vascular smooth muscle cells. This study aimed to clarify whether IS stimulates senescence of cultured human aortic smooth muscle cells (HASMCs) and aorta in Dahl salt-sensitive hypertensive rats and whether AST-120, an oral sorbent, prevents senescence of aorta in subtotally nephrectomized uremic rats. IS increased the mRNA expression of p53 and p21 in HASMCs, whereas it did not change that of p16 and retinoblastoma protein (pRb). The IS-induced expression of p53 and p21 was suppressed by N-acetylcysteine, an antioxidant. IS promoted protein expression of p53, p21, and senescence-associated β-galactosidase activity in HASMCs, and N-acetylcysteine and piliflithrin-α, a p53 inhibitor, blocked these effects. IS upregulated prelamin A, a hallmark of vascular smooth muscle cell senescence, and downregulated FACE1/Zempste24 protein expression in HASMCs, and N-acetylcysteine suppressed these effects. Administration of IS to hypertensive rats increased expression of SA-β-gal, p53, p21, and oxidative stress markers such as 8-hydroxyl-2-deoxyguanosine (8-OHdG) and malondialdehyde (MDA) in the cells embedded in the calcification area of arcuate aorta. Further, the uremic rat model showed positive staining for SA-β-gal, p53, p21, prelamin A, 8-OHdG, and MDA in the cells embedded in the calcification area of arcuate aorta, whereas AST-120 reduced the expression of these biomarkers. Taken together, IS accelerates vascular smooth muscle cell senescence with upregulation of p53, p21, and prelamin A and downregulation of FACE1 through oxidative stress.

IS accelerates not only aortic calcification but also the progression of chronic kidney disease (CKD; Refs. 24, 25). A part of dietary protein-derived tryptophan is metabolized into indole in the intestine by tryptophanase of intestinal bacteria. Indole is absorbed into the blood from the intestine and is metabolized in the liver to IS, which is normally excreted into urine. In CKD, however, a decrease in the renal clearance of IS leads to its increased serum levels (24, 27). AST-120, an oral sorbent, reduces the serum and urine levels of IS in CKD rats and patients by adsorbing indole in intestines and consequently stimulating its excretion into feces (16, 26). Administration of IS and its precursor, indole, to 5/6-nephrectomized rats stimulated glomerular sclerosis in the remnant kidney accompanied by a decline in renal function (24, 25). Further, IS stimulated transcription of genes related to renal fibrosis, such as transforming growth factor-β1 (17). The induction of nephrotoxicity by IS is mediated by organic anion transporters (OATs), such as OAT1 and OAT3, which are localized in the basolateral membrane of renal proximal tubular cells (9). IS reduced superoxide scavenging activity in the kidneys of normal and uremic rats (28). Thus the nephrotoxicity of IS may be induced by stimulating ROS production and impairing the antioxidative system in the kidney. Thus IS is a nephrovascular toxin (23).

Cell senescence is a state of permanent and irreversible cell cycle arrest with a reduced capability to respond to stress, which results in insufficient regenerative capacity of organs. Senescent cells enter an irreversible growth arrest, exhibit a flattened and enlarged morphology, and express a different set of genes such as p16, p21, p53, and retinoblastoma protein (pRb). p16 and p21 are cyclin-dependent kinase inhibitors, inhibit the cell cycle progression, and maintain the G1-phase arrest of cells (30). p53 and pRb are the main activators of senescence. These phenotypic changes of senescent cells have been implicated in aging and age-associated diseases (6). 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 (7).

The accumulation of prelamin A is a hallmark of vascular smooth muscle cell senescence (29). Lamin A and lamin C are major components of the mammalian nuclear lamina, a complex proteinaceous structure that acts as a scaffold for protein complexes that regulate nuclear structure and function. Normally, prelamin A is posttranslationally modified by farnesylation to facilitate nuclear envelope targeting and then is cleaved by the metalloproteinase FACE1/Zmpste24 to remove the farnesyl groups and produce mature lamin A, which inserts into the nuclear lamina. The accumulation of prelamin A, which might occur due to downregulation of the processing...
MATERIALS AND METHODS

Reagents. HASMCs were purchased from Cascade Biologics (Portland, OR). D-MEM, FBS, penicillin-streptomycin, and trypsin-EDTA solution were purchased from Gibco (Invitrogen, Grand Island, NY). IS, N-acetylcysteine (NAC), and Dulbecco’s PBS were purchased from Sigma-Aldrich (St. Louis, MO). Pifithrin-α, p-nitro (PFTα) was from Calbiochem (La Jolla, CA). BCA protein assay kit was purchased from Pierce (Rockford, IL). High Pure RNA isolation kit and 1st Strand cDNA synthesis kit for RT-PCR (AMV) were purchased from Roche Diagnostics (Mannheim, Germany). Syber Premix EX Taq II Green was from Takara Bio (Shiga, Japan). LightCycler-Primer Set of human GAPDH was purchased from Search-LC (Heidelberg, Germany). Human p53 and p21 primers were purchased from Nihon Gene Research Laboratories (Sendai, Japan). Antibodies used for Western blot analysis included mouse monoclonal anti-p53 antibody, rabbit monoclonal anti-p21 antibody, anti-mouse IgG, horse radish peroxidase (HRP)-linked antibody and anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology, Beverly, MA); mouse monoclonal anti-a-tubulin antibody (Calbiochem, La Jolla, CA); and goat polyclonal anti-prelamin A antibody (SC-6214,C-20), goat polyclonal anti-FACE1 antibody (SC-34777), and donkey anti-goat IgG, HRP-linked antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Chemi- Lumi one system was purchased from Nacalai Tesque (Kyoto, Japan). Antibodies used for immunohistochemistry include rabbit polyclonal anti-SA-β-gal antibody (Chemicon, Millipore, CA); rabbit polyclonal anti-p53 (FL-393), mouse monoclonal anti-p21 (F-5), and goat polyclonal anti-prelamin A (SC-6214,C-20) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); and mouse monoclonal 8-OHdG and mouse monoclonal anti-MDA antibodies (Japan Institute for the Control of Aging, Shizuoka, Japan).

Cell culture. HASMCs were maintained in DMEM containing 10% FBS supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C under 5% CO2 humidified atmosphere. The medium was replaced every 2 days until confluence. Only cells between passages 2 to 5 were used for experiments.

Total RNA isolation. HASMCs cultured in 60-mm dishes were stimulated by IS at different concentrations (100, 250, and 500 μM) for 24 h, or cells were treated with 500 μM IS for 0–24 h. Total RNA was extracted from the cultured cells using a High Pure RNA isolation kit. The purity and quantity of the RNA preparation were determined by measuring the optical densities at 260 and 280 nm.

Quantitative RT-PCR analysis. To estimate the mRNA levels of p53 and p21, quantitative RT-PCR was performed using the LightCycler system (Roche Diagnostics). Total RNA was reversely transcribed with random hexamers using 1st Strand cDNA synthesis kit for RT-PCR (AMV). The reaction was processed as follows: incubation of samples at 25°C for 10 min and then at 42°C for 60 min, followed by 99°C for 5 min and cooling to 4°C for 5 min. The quantitative PCR was performed with the LightCycler instrument using Syber Premix EX Taq II Green, LightCycler-Primer Set, human p53 primer (forward: 5’-CGGACTGACATGCTAGCG-3’; reverse: 5’-AATCATCATTGCTTGGAGG-3’), and human p21 primer (forward: 5’-GTCGACTGACTGCTCAGTCGCTCTT-3’; reverse: 5’-GCCATTGAGGATGTTAGAATT-3’). The expression of mRNA levels was measured as the ratio of each mRNA to the GAPDH mRNA.

Western blot analysis. Serum-starved HASMCs were incubated with 500 μM IS for 1 to 3 days, or cells were preincubated with 2.5 mM NAC and 10 μM PFTα for 30 min and then stimulated with IS for 48 h. Cells were lysed in lysis buffer (1% NP-40,150 mM NaCl, 50 mM Tris-HCl, complete inhibitor, and 10% glycerol) and were fractionated by SDS-PAGE on polyacrylamide gels. Then, proteins were transferred to PVDF membranes (Immobilon-P; Millipore, Bedford, MA). Some membranes were blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween (TBS-T) for 1 h at room temperature. After blocking, the membranes were incubated for 2 h with primary antibodies and then washed and incubated with secondary antibodies. The membranes were developed with enhanced chemiluminescence (Pierce, Rockford, IL) and X-ray film (Fuji, Tokyo, Japan). A representative Western blot showing the expression of the indicated proteins is shown in Fig. 1. The gray densities of the bands on X-ray film were measured using Quantity One software (Bio-Rad, Hercules, CA). The protein bands were quantitated by scanning densitometry using the NIH Image program (software). The results obtained in three independent experiments were expressed as the mean ± SE. Significant differences from control (set at 100%) were calculated using the Student’s t-test. p<0.05, **p<0.01, ***p<0.001 vs. 0 h. B and D: effect of IS at various concentrations on p53 and p21 expressions after incubation for 24 h. Expressions of mRNA level for p53 and p21 were normalized to GAPDH. Results are means ± SE; n = 4. *P < 0.05, **P < 0.01 vs. 0 μM IS.

enzyme FACE1, induces DNA damage and mitotic dysfunction in vascular smooth muscle cells, accompanied by limited reparative capacity, and consequently plaque instability and rupture.

This study aimed to clarify whether administration of IS stimulates vascular smooth muscle cell senescence by regulating the expression of p53, p21, prelamin A, and FACE1 through oxidative stress in HASMCs and aorta of hypertensive rats and whether AST-120 prevents accumulation of SA-β-gal, p53, p21, prelamin A, 8-hydroxy-2′-deoxyguanosine (8-OHdG) and malondialdehyde (MDA) in the aorta of subtotally nephrectomized uremic rats.

Fig. 1. Effects of indoxyl sulfate (IS) on mRNA expression of p53 and p21 in human aortic smooth muscle cells (HASMCs). A and C: time course of the effect of IS (500 μM) on p53 and p21 expressions. *p < 0.05, **p < 0.001 vs. 0 h. B and D: effect of IS at various concentrations on p53 and p21 expressions after incubation for 24 h. Expressions of mRNA level for p53 and p21 were normalized to GAPDH. Results are means ± SE; n = 4. *P < 0.05, **P < 0.01 vs. 0 μM IS.
temperature. After being washed with TBS-T, the membranes were incubated with mouse monoclonal anti-human p53 antibody (1:1,000) and rabbit monoclonal anti-human p21 antibody (1:1,000), respectively. Some membranes were blocked with 5% BSA in TBS-T for 1 h at room temperature and then incubated with goat polyclonal anti-human prelamin A antibody (1:1,000), goat polyclonal anti-human FACE1 antibody (1:1,000), and mouse monoclonal anti-human α-tubulin antibody (1:5,000), respectively. Then, the membranes were further incubated with HRP-linked second antibody (1:5,000) for 1 h at room temperature. After being washed with TBS-T three times, the protein bands were visualized using Chemi-Lumi one 5,000) for 1 h at room temperature. After being washed with TBS-T, the membranes were further incubated with HRP-linked second antibody (1:5,000) for 1 h at room temperature. After being washed with TBS-T, the membranes were further incubated with HRP-linked second antibody (1:5,000) for 1 h at room temperature.

SA-β-gal staining. Serum-starved cells incubated with or without IS 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 MgCl2, 3 mM K3Fe(CN)6, 3 mM K4Fe(CN)6, and 0.1% Triton X-100 at 37°C for 12 h. SA-β-gal-positive cells were counted in six randomly representative fields.

Animal experimental design 1: hypertensive rat model. Experimental rats were produced by Kureha (Tokyo, Japan) as previously described (2). Briefly, the rat groups consisted of 1) Dahl salt-resistant normotensive rats (DN; n = 8), 2) Dahl salt-resistant normotensive IS-administered rats (DN + IS; n = 8; 200 mg/kg of IS in drinking water), 3) Dahl salt-sensitive hypertensive rats (DH; n = 8), and 4) Dahl salt-sensitive hypertensive IS-administered rats (DH + IS; n = 7; 200 mg/kg of IS in drinking water). After administration of IS for 32 wk, the rats were anesthetized and the aortas were excised for immunohistochemical analysis. Body weight, blood pressure, and serum and urine parameters of these rats were described previously (2). These experiments were approved by the Biomedical Research Laboratories Institutional Animal Care Committee.

Animal experimental design 2: subtotally nephrectomized uremic rat model. Experimental rats were produced by Kureha as previously described (3). Briefly, 7-wk-old male Sprague-Dawley rats (Clea, Tokyo, Japan) were used to produce uremic rats by 4/5-nephrectomy. Eleven weeks after 4/5-nephrectomy, the rats were randomized into the following two groups: uremic rats (n = 5) and AST-120-treated uremic rats (n = 5). AST-120 was administered to the rats at a dose of 4 g/kg with powder chow (CE-2, Clea), whereas powder chow alone was administered to uremic rats. Normal rats (n = 3) were used to compare the data with uremic rats. After administration of AST-120 for 20 wk, the rats were anesthetized and the aortas were excised for immunohistochemical analysis. All the experiments were approved by the Biomedical Research Laboratories Institutional Animal Care Committee.

Immunohistochemistry. Immunohistochemistry was processed according to the streptavidin-biotin complex (SABC) method. Paraffin-embedded fixed tissue sections were deparaffinized with xylene and rehydrated with ethanol. Antigen was retrieved with 0.01 M citrate buffer (pH 6.0) twice for a 5-min microwave treatment. Endogenous peroxidase was inhibited with 0.3% H2O2 in methanol for 10 min, followed by a rinse in PBS. Sections were blocked by appropriate 10% serum (Nichirei, Tokyo, Japan) at room temperature for 30 min. Sections were incubated with the primary antibody at 4°C overnight. Rabbit polyclonal anti-SA-β-gal (1:500), rabbit polyclonal anti-p53 (1:50), mouse monoclonal anti-p21 (1:50), goat polyclonal anti-prelamin A (1:100), mouse monoclonal anti-8-OHdG (1:20), and mouse monoclonal anti-MDA (1:100) antibodies were used as the primary antibodies. Sections were further incubated with the second antibody (Nichirei) at room temperature for 30 min and then with peroxidase-conjugated streptavidin (Nichirei) at 37°C for 30 min. The localization of SA-β-gal, p53, p21, prelamin A, 8-OHdG, and MDA was visualized using 3,3′-diaminobenzidine tetrahydrochloride (DAB tablet; Merck, Darmstadt, Germany) at a concentration of 30 mg/ml, containing 0.03% H2O2. Finally, sections were counterstained by 1% methylene green, dehydrated, and mounted. All sections were examined under light microscopy (DN100, E6000; Nikon, Tokyo, Japan).

Statistical analysis. Results are expressed as means ± SE. To compare values among the groups of three or more, ANOVA and Fisher’s protected least significance different test were performed. Results were considered statistically significant when P value was <0.05.

RESULTS

IS upregulated mRNA expression of p53 and p21 through ROS in HASMCs. Senescent cells display high level expression of the cyclin-dependent kinase inhibitors p21 and p16 and tumor suppressor proteins p53 and pRb. Cell cyclin arrest mediated via p53 and pRb is important for senescence of vascular smooth muscle cells. We determined by using RT-PCR if IS induces mRNA expression of p53, pRb, p21, and p16 in HASMCs. The results of RT-PCR analysis showed that IS treatment enhanced the mRNA expression of p53 and p21 in time- and dose-dependent manners (Fig. 1, A–D), whereas the mRNA expression of p16 and pRb showed no significant change (data not shown).

Previously, we have demonstrated that IS induced ROS generation in HASMCs (19). Then, we checked the effects of NAC, an antioxidant, on mRNA expression of p53 and p21 in HASMCs. As shown in Fig. 2, A and B, the IS-induced mRNA expression of p53 and p21 was suppressed by the addition of NAC.
2.5 mM NAC in HASMCs. Thus IS induced mRNA expression of p53 and p21 through ROS production in HASMCs.

**IS upregulated protein expression of p53 through ROS in HASMCs.** We detected the effect of IS on protein expression of p53 in HASMCs. Serum-starved HASMCs were pretreated with 2.5 mM NAC for 30 min and then stimulated with 500 μM IS for 48 h. Expression of protein levels for p53 was normalized to α-tubulin. Results are means ± SE; n = 4. *P < 0.05, **P < 0.01 vs. control; #P < 0.05 vs. IS.

**Fig. 3.** IS upregulated protein expression of p53 by reactive oxygen species (ROS) production in HASMCs. A: time course of the effect of IS on protein expression of p53 in HASMCs. B: NAC suppressed IS-induced protein expression of p53 in HASMCs. Serum-starved HASMCs were pretreated with 2.5 mM NAC for 30 min and then stimulated with 500 μM IS for 48 h. Expression of protein levels for p53 was normalized to α-tubulin. Results are means ± SE; n = 4. *P < 0.05, **P < 0.01 vs. control; #P < 0.05 vs. IS.

**Fig. 4.** IS upregulated protein expression of p21 by ROS and p53 in HASMCs. A: time course of the effect of IS on protein expression of p21 in HASMCs. B: NAC, an antioxidant, and pifithrin-α, p-nitro (PFTα), a p53 inhibitor, inhibited IS-induced protein expression of p21 in HASMCs. Serum-starved HASMCs were pretreated with 2.5 mM NAC and 10 μM PFTα for 30 min, and then stimulated with 500 μM IS for 48 h. Expression of protein levels for p21 was normalized to α-tubulin. Results are means ± SE; n = 4. **P < 0.01, ***P < 0.001 vs. control; #P < 0.05 vs. IS.
protein expression of p21 time dependently in HASMCs, whereas NAC and PFTα inhibited the IS-induced protein expression of p21. These results indicate that IS upregulated protein expression of p21 through ROS and p53 in HASMCs.

IS induced SA-β-gal activity through ROS and p53 in HASMCs. The activity of SA-β-gal has been described as a marker of senescent cells in vivo and in vitro (7). SA-β-gal staining was found in intimal vascular smooth muscle cells of advanced human atherosclerotic plaques. The results of SA-β-gal staining showed that IS significantly increased the SA-β-gal activity in a time-dependent manner in HASMCs (Fig. 5A), and the increase in the IS-induced SA-β-gal activity was significantly suppressed by the addition of NAC and PFTα (Fig. 5B). These results suggest that IS accelerated cellular senescence through ROS and p53 in HASMCs.

IS upregulated protein expression of prelamin A and downregulated protein expression of FACE1 through ROS in HASMCs. Recent evidence demonstrated that prelamin A is a senescent maker in vascular smooth muscle cells. Then, we examined if IS affects protein expression of prelamin A and FACE1 in cultured HASMCs. The results showed that IS upregulated prelamin A (69 kDa) protein expression and downregulated FACE1 (54.8 kDa) protein expression in HASMCs, whereas NAC blocked these effects (Fig. 6, A and B). Thus IS induced upregulation of prelamin A and downregulation of FACE1 through ROS in HASMCs.

Immunostaining of SA-β-gal, p53, p21, prelamin A, 8-OHdG, and MDA in the arcuate aorta of hypertensive rat model. To confirm the in vitro findings, we examined the localization of senescent biomarkers such as SA-β-gal, p53, p21, and prelamin A and oxidative stress biomarkers such as 8-OHdG and MDA in the calcification area of arcuate aorta by immunohistochemistry. DH rats showed increased expression of SA-β-gal, p53, p21, prelamin A, 8-OHdG, and MDA in the cells embedded in the calcification area of arcuate aorta compared with DN rats (Fig. 7). More notably, DH + IS rats showed markedly increased expression of SA-β-gal, p53, p21, prelamin A, 8-OHdG, and MDA in the cells embedded in the calcification area of arcuate aorta compared with DH rats (Fig. 7). Thus both hypertension and IS promoted vascular smooth muscle cell senescence through oxidative stress in rats.

Immunostaining of SA-β-gal, p53, p21, prelamin A, 8-OHdG, and MDA in the arcuate aorta of uremic rat model. AST-120 reduces serum levels of IS in uremic rats (16). To further study...

Fig. 5. IS induced senescence-associated β-galactosidase (SA-β-gal) activity by ROS and p53 in HASMCs. A: time course of the effect of IS on SA-β-gal activity in HASMCs. B: NAC, an antioxidant, and PFTα, a p53 inhibitor, suppressed IS-induced SA-β-gal activity in HASMCs. Serum-starved HASMCs were pretreated with 2.5 mM NAC and 10 μM PFTα for 30 min and then stimulated with 500 μM IS for 48 h. Results are means ± SE; n = 4. *P < 0.05, **P < 0.001 vs. control (0 day); ***P < 0.001 vs. IS.
whether AST-120 prevents cellular senescence induced by IS, we also examined the localization of SA-β-gal, p53, p21, prelamin A, 8-OHdG, and MDA in the arcuate aorta of uremic rats by immunohistochemistry. Expression of SA-β-gal, p53, p21, and prelamin A was detected only in the calcification area of arcuate aorta in uremic rats but not in normal rats or AST-120-treated uremic rats (Fig. 8). Expression of 8-OHdG and MDA was increased in the calcification area and the media of arcuate aorta in uremic rats compared with normal rats, whereas AST-120 reduced the expression of 8-OHdG and MDA (Fig. 8). Thus AST-120 might be effective in preventing vascular smooth muscle cell senescence in uremic rats.

**DISCUSSION**

The novel findings in the present study are 1) IS induced cultured vascular smooth muscle cell senescence with upregulation of p53, p21, and prelamin A through oxidative stress; 2) IS induced accumulation of prelamin A, 8-OHdG, and MDA in the cells embedded in calcification area in the aorta of hypertensive rats; and 3) AST-120 reduced the accumulation of SA-β-gal, p53, p21, prelamin A, 8-OHdG, and MDA in the cells embedded in calcification area in the aorta of uremic rats.

Figure 9 shows schematic effects of IS on HASMCs. IS accelerates vascular smooth muscle cell senescence by upregulating the expressions of p53, p21, and prelamin A through ROS production. p53 induces senescence by activating p21 or independently of p21. These effects of IS were observed even at a concentration of IS comparable to its serum levels in hemodialysis patients (mean serum level: 249 μM, maximum serum level: 557 μM; Ref. 24).

Our recent study (1) demonstrated that IS promoted aortic calcification in hypertensive rats with expression of osteoblast-specific proteins in the cells embedded in the aortic calcification area. IS induced ROS such as superoxide by upregulating NADPH oxidase Nox4 and the expression of the osteoblast-specific proteins in cultured vascular smooth muscle cells (19). Further, IS promotes cell senescence with aortic calcification in aorta of hypertensive rats (2). In the present study, we further extended our research demonstrating that IS induced cell senescence of cultured vascular smooth muscle cells and the cells embedded in the calcification area were positive for SA-β-gal, p53, p21, prelamin A, 8-OHdG, and MDA in hypertensive rats. Our findings are consistent with the report by Nakano-Kurimoto et al. (22) that senescence of vascular smooth muscle cells enhances the calcification through initiating the osteoblastic transition (22). Taken together, our research revealed that IS induces senescence and osteoblastic differentiation of vascular smooth muscle cells through oxidative stress, accelerating aortic calcification.

Senescent cells enter irreversible growth arrest, develop a flattened, enlarged morphology, and express a different set of genes including p53, p21, p16, and pRb. The expression of these cell cycle regulatory proteins can be upregulated by some stressors, such as oxidative stress and DNA damage (6, 7, 34). IS induced expression of p53 and p21 in HASMCs, which was inhibited by NAC. However, we could not observe any effect of IS on the expression of p16 and pRb in HASMCs. IS-induced p21 was also inhibited by PFTα, a p53 inhibitor. Thus IS-induced expression of p53 and p21 through oxidative stress and induced expression of p21, at least partly, through activation of p53. p53 controls p21 expression through direct DNA binding to the p21 promoter and transactivation, and the p53-p21 axis regulates cell proliferation and senescence (8, 12, 15).

IS promoted the expression of SA-β-gal activity in HASMCs. Acid β-galactosidase is a lysosomal hydrolase (18). Since senescence may induce this enzyme, or lysosomal activity may...
increase in senescent cells, lysosomal β-galactosidase, or the spliced, short form of this enzyme may increase (7, 18). The present quantitative analysis of senescent cells in HASMCs showed increased production of SA-β-gal, revealing that vascular smooth muscle cells are at a risk of IS-induced senescence-related changes.

IS upregulated prelamin A expression in HASMCs, whereas it downregulated FACE1 expression. A hallmark of vascular smooth muscle cell aging, in vitro and in vivo, is the accumulation of prelamin A, which is due to oxidative stress-induced downregulation of the processing enzyme FACE1 (29). Prelamin A-induced DNA damage and mitotic dysfunction act in concert to accelerate vascular smooth muscle cell senescence. These events severely limit vascular smooth muscle cell reparative capacity, resulting in plaque instability and rupture.

Recent histological examination of human atherosclerotic lesions has revealed that vascular smooth muscle cells and

Fig. 7. Immunostaining of SA-β-gal, p53, p21, prelamin A, 8-hydroxyl-2′-deoxyguanosine (8-OHdG), and malondialdehyde (MDA) in the arcuate aorta of hypertensive rat model. DN, Dahl salt-resistant normotensive rats; DN + IS, Dahl salt-resistant normotensive IS-administered rats; DH, Dahl salt-resistant hypertensive rats; DH + IS, Dahl salt-resistant hypertensive IS-administered rats.

Fig. 8. Immunostaining of SA-β-gal, p53, p21, prelamin A, 8-OHdG, and MDA in the arcuate aorta of uremic rat model. Normal, normal rats; uremic, uremic rats; uremic + AST-120, AST-120-treated uremic rats.

Fig. 9. Schematic representation of IS functions in HASMCs. IS accelerated cellular senescence by upregulating the expressions of p53, p21, and prelamin A through ROS production. p53 could activate senescence by activating p21 or independently of p21.
endothelial cells exhibit morphological features of cell senescence (4, 5). More notably, SA-β-gal-positive vascular cells have been demonstrated in human atherosclerotic plaques of coronary arteries obtained from patients with ischemic heart disease (14). Angiotensin II and aldosterone induce vascular smooth muscle cell senescence in concert through oxidative stress (13). The present study demonstrates that IS might be one of risk factors inducing vascular cell senescence in atherosclerotic lesions in CKD patients.

Our findings are important not just because of the understanding of pathogenesis of vascular calcification in CKD patients but also for exploring potential therapeutic targets. IS cannot be removed efficiently by hemodialysis due to its albumin binding. However, AST-120, an oral sorbent, can reduce serum levels of IS in CKD patients by adsorbing indole, a precursor of IS, in the intestine (16, 24, 26). AST-120 suppresses ROS production as well as the senescence and fibrosis of renal tubules in the kidney (3, 16, 20, 31–33). AST-120 prevents progression of CKD (16, 20) and suppresses oxidative stress and the progression of cardiac damage in rat model of CKD (11). The present study revealed that the cells embedded in the calcification area in uremic rat aorta were positive for SA-β-gal, p53, p21, prelamin A, 8-OHdG, and MDA, whereas AST-120 reduced the expression of these biomarkers. A clinical study demonstrated that AST-120 reduces arterial stiffness (pulse wave velocity) and intima media thickness in nondiabetic CKD patients before dialysis (21). Thus AST-120 might be effective in preventing senescence and calcification in aorta of CKD stage 5 patients. Further study will be required to completely elucidate the vascular toxicity of IS and the therapeutic effects of its removal on the progression of cardiovascular disease in CKD patients.

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DISCLOSURES

F. Nishijima is employed by Kureha Corporation. The other authors declare no competing interests.

AUTHOR CONTRIBUTIONS

Author contributions: G.M. and F.N. performed experiments; G.M., H.S., F. Nishijima is employed by Kureha Corporation. The other authors declare no competing interests.

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