

## UREMIC TOXICITY OF INDOXYL SULFATE

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### ABSTRACT

Indoxyl sulfate, a uremic toxin, is accumulated in the serum of chronic kidney disease (CKD) patients. A part of the dietary protein-derived tryptophan is metabolized into indole by tryptophanase in intestinal bacteria. Indole is absorbed into the blood from the intestine, and is metabolized to indoxyl sulfate in the liver. Indoxyl sulfate is normally excreted into urine. In CKD, however, an inadequate renal clearance of indoxyl sulfate leads to its elevated serum levels. The oral adsorbent AST-120 reduces the serum levels of indoxyl sulfate by adsorbing indole in the intestines and stimulating its excretion into feces. I have proposed a protein metabolite theory by which endogenous protein metabolites such as indoxyl sulfate play a significant role in the progression of CKD. A progressive decline in the glomerular filtration rate leads to increased serum levels of endogenous protein metabolites such as indoxyl sulfate, and to the adverse effects of their overload on the remnant nephrons. Indoxyl sulfate stimulates progressive both tubulointerstitial fibrosis and glomerular sclerosis by increasing the expression of transforming growth factor- $\beta$ 1, a tissue inhibitor of metalloproteinase-1 and pro $\alpha$ 1(I) collagen, leading to a further loss of nephrons. AST-120 delays the progression of CKD by removing serum indoxyl sulfate. Moreover, indoxyl sulfate induces oxidative stress in tubular cells, mesangial cells, vascular smooth muscle cells, endothelial cells and osteoblasts as well as stimulating aortic calcification in hypertensive rats, it is also involved in the progression of CKD, cardiovascular disease (CVD) and osteodystrophy. Thus, the removal of indoxyl sulfate by AST-120 ameliorates the progression of not only CKD, but also of CVD and osteodystrophy.

Key Words: Indoxyl sulfate, Chronic kidney disease, Protein metabolite theory, Oxidative stress, Oral adsorbent AST-120

### INTRODUCTION

The uremic syndrome is considered to be caused by an accumulation of uremic toxins due to kidney dysfunction. Ninety compounds have been identified as uremic toxins.<sup>1,2)</sup> Sixty-eight have a molecular weight of less than 500 Da, 12 exceed 12,000 Da, and 10 have a molecular weight between 500 and 12,000 Da, 25 solutes are protein-bound. Uremic toxins include low-molecular-weight compounds (e.g. indoxyl sulfate, p-cresyl sulfate, 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid, asymmetric dimethylarginine), middle-molecular-weight peptides, and proteins modified by advanced glycation and oxidation. These uremic toxins are considered to be involved in a variety of symptoms which may appear in patients with stage 5 chronic kidney disease (CKD). This review focuses on the role of indoxyl sulfate, a uremic toxin, in the progression of both CKD and cardiovascular disease (CVD).

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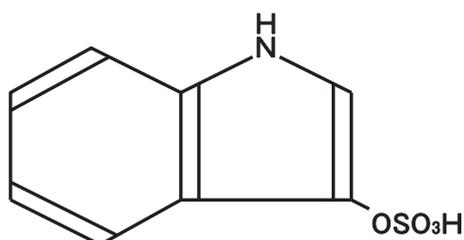
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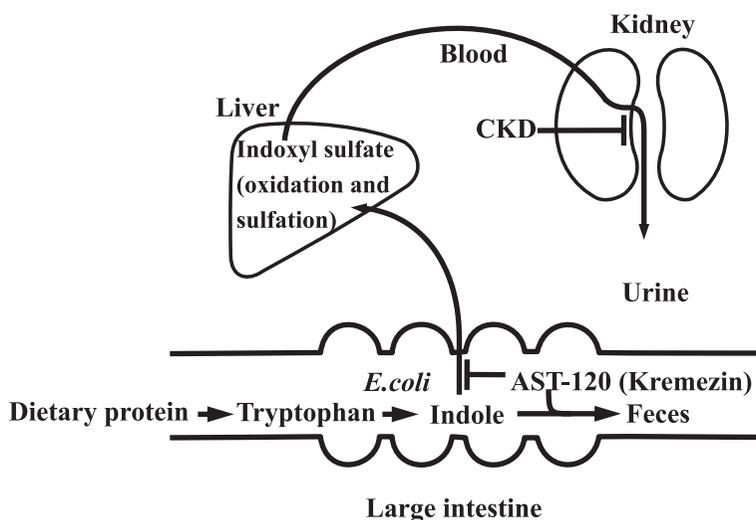
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### *Indoxyl sulfate is a uremic toxin*

We have demonstrated that indoxyl sulfate (Fig. 1) is a uremic toxin accelerating the progression of CKD.<sup>3-6)</sup> Fig. 2 shows the metabolism of indoxyl sulfate and the effect of an oral sorbent (AST-120: Kremezin). 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 uremia, 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 markedly increased in both uremic rats



**Fig. 1** Chemical structure of indoxyl sulfate (molecular weight: 213). Indoxyl sulfate is a uremic toxin derived from dietary protein, and is synthesized from indole, a metabolite of tryptophan. Its serum levels are elevated in chronic kidney disease (CKD) patients. It is an accelerating factor of CKD, and a small hydrophobic anion.



**Fig. 2** Metabolism of indoxyl sulfate and effect of AST-120 (Kremezin). Indoxyl sulfate is derived from dietary protein. A part of protein-derived tryptophan is metabolized into indole by tryptophanase in intestinal bacteria such as *Escherichia coli*. Indole is absorbed into the blood from the intestine, and is metabolized to indoxyl sulfate in the liver, it is normally excreted in urine. In uremia, however, reduced renal clearance of indoxyl sulfate leads to elevated serum levels of indoxyl sulfate. AST-120 reduces the serum and urine levels of indoxyl sulfate in chronic kidney disease (CKD) patients by adsorbing indole in the intestines, and consequently stimulating its excretion in feces.

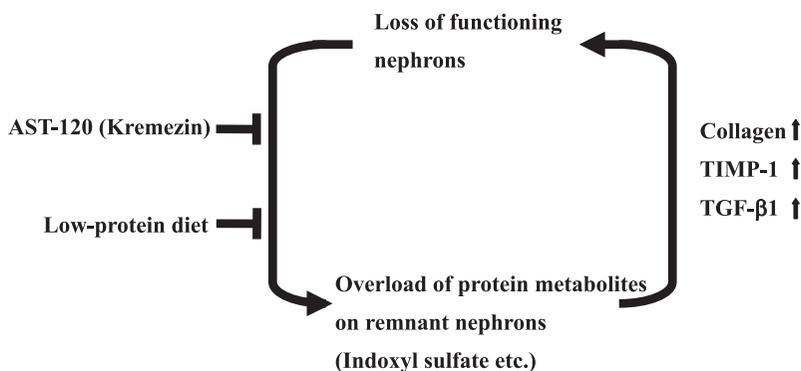
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and patients.<sup>4,7,8)</sup> In serum, approximately 90% of indoxyl sulfate is bound to serum albumin. AST-120 reduces the serum and urine levels of indoxyl sulfate in uremic rats and patients by adsorbing indole in the intestines, consequently stimulating its excretion into feces.<sup>4,7-13)</sup> 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)- $\beta$ 1, tissue inhibitor of metalloproteinase (TIMP)-1 and pro $\alpha$ 1(I) collagen.<sup>6,14)</sup> 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.

*Protein metabolite theory*

I have proposed a protein metabolite theory by which endogenous protein metabolites such as indoxyl sulfate play a significant role in the progression of CKD (Fig. 3).<sup>12,15,16)</sup> The initial insult leads to a loss of functioning nephrons via a disease-specific pathophysiological process. A progressive decline in the glomerular filtration rate leads to increased circulating levels of endogenous protein metabolites such as indoxyl sulfate, and to the adverse effects of their overload on the remnant nephrons, especially proximal tubular cells. Indoxyl sulfate is normally excreted into urine mainly via active secretion by the proximal tubular cells. Indoxyl sulfate, for example, stimulates progressive tubulointerstitial fibrosis, glomerular sclerosis and the progression of CKD by increasing the gene expressions of TGF- $\beta$ 1, TIMP-1, and pro $\alpha$ 1(I) collagen, leading to a further loss of nephrons,<sup>6,14)</sup> completing the vicious circle of progressive renal injury.

A low-protein diet delays the progression of CKD by suppressing renal TGF- $\beta$ 1 expression in uremic animals in which it also reduces the serum levels of indoxyl sulfate.<sup>4,11)</sup> The administration of AST-120 decreases the serum and urine levels of indoxyl sulfate, and delays the progression of CKD by reducing the gene expressions of TGF- $\beta$ 1, TIMP-1, and pro $\alpha$ 1(I) collagen.<sup>13,17-24)</sup>



**Fig. 3** The protein metabolite theory, a mechanism for the progression of chronic kidney disease (CKD). Endogenous protein metabolites such as indoxyl sulfate play a significant role in the progression of CKD. The initial insult leads to a loss of functioning nephrons via a disease-specific pathophysiological process. A progressive decline in the glomerular filtration rate leads to increased circulating levels of endogenous protein metabolites such as indoxyl sulfate, and to the adverse effects of their overload on the remnant nephrons, especially proximal tubular cells. Indoxyl sulfate stimulates progressive tubulointerstitial fibrosis, glomerular sclerosis, and the progression of CKD by increasing the gene expression of transforming growth factor (TGF)- $\beta$ 1, tissue inhibitor of metalloproteinase (TIMP)-1 and pro $\alpha$ 1(I) collagen, leading to a further loss of nephrons and completing the vicious circle of progressive renal injury. If the overload of indoxyl sulfate, for example, is alleviated by a low-protein diet or by the administration of AST-120, the chain of events leading to the further progression of renal damage might be interrupted.

AST-120 is widely used not only in Japan but also in Korea and Taiwan for the treatment of CKD patients to delay the progression of CKD. If the overload of indoxyl sulfate is alleviated, for example, by a low-protein diet or by the administration of AST-120, the chain of events leading to a progression of renal damage might be interrupted. In fact, many clinical and experimental studies have demonstrated that both dietary protein restriction and oral sorbent AST-120 can suppress the progression of CKD.

#### *Induction of free radicals by indoxyl sulfate*

Indoxyl sulfate induces free radical production not only in renal tubular cells<sup>21)</sup> but also in glomerular mesangial cells.<sup>26)</sup> Free radicals induced by indoxyl sulfate in renal tubular cells activate the nuclear factor (NF)- $\kappa$ B which, in turn, up-regulates the expression of plasminogen activator inhibitor (PAI)-1.<sup>25)</sup> Free radicals induced by indoxyl sulfate in mesangial cells change their redox status, which then activates mitogen-activated protein kinases and cell proliferation.<sup>26)</sup> Thus, indoxyl sulfate induces the generation of free radicals in the kidneys. Administration of indoxyl sulfate have reduced the superoxide scavenging activity in the kidneys of uremic rats. Therefore, the nephrotoxicity of IS may be induced by impairing the kidney's anti-oxidative systems.<sup>27)</sup>

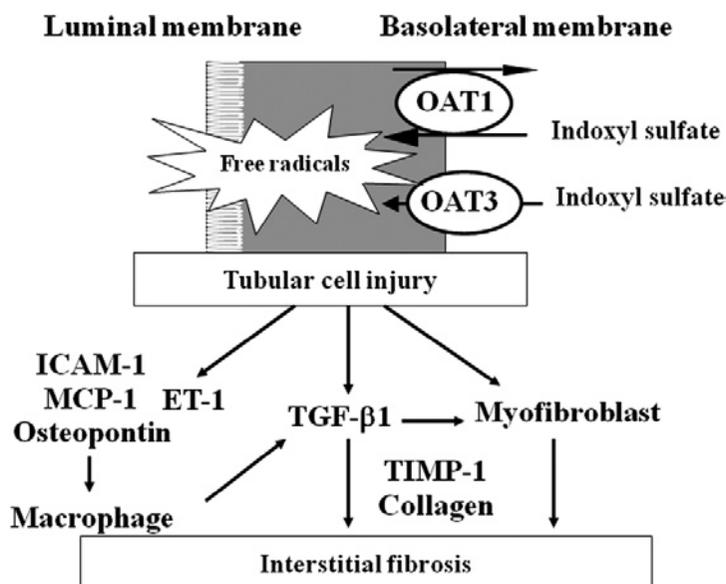
AST-120 alleviates oxidative stress in the kidney of uremic rats by reducing serum levels of indoxyl sulfate,<sup>28)</sup> and reduces the urine level of acrolein, a lipid peroxidation end product. Acrolein is considered to play an important role as a mediator of oxidative damage in a variety of human diseases. Thus, AST-120 alleviates oxidative stress in the kidneys by reducing serum levels of indoxyl sulfate.

Furthermore, AST-120 increases NO synthesis in the kidneys of CKD rats by increasing the renal expressions of endothelial nitric oxide synthase and neuronal nitric oxide synthase through the alleviation of indoxyl sulfate overload on the kidney.<sup>29)</sup>

#### *Nephrotoxicity of indoxyl sulfate*

Fig. 4 shows the exact mechanism underlying the nephrotoxicity of indoxyl sulfate. Approximately 90% of indoxyl sulfate accumulated in the blood of uremic patients is bound to serum albumin. Thus, the urinary excretion of indoxyl sulfate is considered to occur mainly by tubular secretion and only secondarily by glomerular filtration. Organic anion transporters (OAT1 and OAT3) play an important role in the transcellular transport of indoxyl sulfate in the tubular cells and in the induction of its nephrotoxicity.<sup>30,31)</sup> Indoxyl sulfate in the blood is taken up by OAT1 and OAT3 at the basolateral membrane of tubular cells (OAT1: proximal, OAT3: proximal and distal), and is accumulated in the tubular cells at high concentrations in uremic patients.<sup>32)</sup> The accumulation of indoxyl sulfate generates free radicals, reduces superoxide scavenging activity, and consequently causes tubular cell injury by impairing the kidney's anti-oxidative systems.<sup>27)</sup>

The damaged tubular cells produce TGF- $\beta$ 1 as well as chemokines such as intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1), osteopontin and endothelin-1 (ET-1). These chemokines promote the infiltration of macrophages which produce TGF- $\beta$ 1. The secreted TGF- $\beta$ 1 stimulates the production of TIMP-1 and collagen. The damaged tubular cells are transformed into myofibroblasts through an epithelial-to-mesenchymal transition induced by TGF- $\beta$ 1, these changes facilitate interstitial fibrosis. The indoxyl sulfate accumulated in uremic serum accelerates tubular cell injury and induces subsequent interstitial fibrosis, thus acting as a nephrotoxin.



**Fig. 4** Nephrotoxicity of indoxyl sulfate.

Indoxyl sulfate in the blood is taken up by organic anion transporters (OAT1 and OAT3) at the basolateral membrane of renal tubular cells in which it is accumulated. Indoxyl sulfate generates free radicals, reduces superoxide scavenging activity, and consequently causes tubular cell injury by impairing the kidney's anti-oxidative systems. The damaged tubular cells produce transforming growth factor (TGF)- $\beta$ 1 as well as chemokines such as intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1), osteopontin and endothelin-1 (ET-1). These chemokines promote the infiltration of macrophages which produce TGF- $\beta$ 1. The secreted TGF- $\beta$ 1 stimulates production of tissue inhibitor of metalloproteinase (TIMP)-1 and collagen. The injured tubular cells are transformed into myofibroblasts through an epithelial-to-mesenchymal transition induced by TGF- $\beta$ 1. These changes facilitate interstitial fibrosis. Thus, indoxyl sulfate accumulated in chronic kidney disease (CKD) serum accelerates tubular cell injury and subsequent interstitial fibrosis.

#### *Cardiovascular disease in CKD patients*

Cardiovascular disease accounts for 40 to 50% of deaths among dialysis populations, and its mortality among hemodialysis and peritoneal dialysis patients is much higher, especially in younger age-categories, than age- and sex-matched controls without CKD.<sup>33,34</sup> Vascular calcification plays a vital role in the development of cardiovascular morbidity and its resultant increased mortality, while vascular calcification affects both vascular intima and media layers, its underlying mechanism remains poorly understood. Hyperphosphatemia, calcium overload, increased oxidized low-density lipoprotein cholesterol, uremic toxins, increased oxidative stress, hyperhomocysteinemia, hemodynamic overload and dialysate-related factors as well as traditional cardiovascular risk factors may play a role in CKD-related cardiovascular disease.<sup>35</sup>

#### *Vascular toxicity of indoxyl sulfate*

Indoxyl sulfate has been shown to inhibit endothelial proliferation and wound repair,<sup>36</sup> and to stimulate the proliferation of rat vascular smooth muscle cells<sup>37</sup> and human aortic smooth muscle cells.<sup>38</sup> Such *in vitro* experiments suggest that indoxyl sulfate may play a role in the dysfunction of endothelial and vascular smooth muscle cells in CKD patients. Moreover, the serum level of indoxyl sulfate has been associated with pentosidine and HDL-cholesterol, the

risk factors of atherosclerosis in hemodialysis patients.<sup>39)</sup> Thus, indoxyl sulfate may be involved in the pathogenesis of atherosclerosis.

We have recently demonstrated that indoxyl sulfate promotes aortic calcification and aortic wall thickening in hypertensive rats.<sup>40)</sup> Osteoblast-specific proteins such as osteopontin, core binding factor 1 (Cbfa1), alkaline phosphatase (ALP), and osteocalcin are expressed in the cells embedded in the aortic calcification area. Thus, indoxyl sulfate is a vascular toxin that may be responsible, at least in part, for the progression of atherosclerosis in CKD patients.

We have further demonstrated *in vitro* that indoxyl sulfate stimulates the generation of free radicals such as superoxide by up-regulating NADPH oxidase Nox4, and induces the expressions of osteoblast-specific proteins such as Cbfa1, ALP, and osteopontin in human aortic smooth muscle cells.<sup>41)</sup> Free radicals derived from NADPH oxidase Nox4 are important in inducing the transdifferentiation of human aortic smooth muscle cells into cells with a more osteoblastic phenotype. These effects of indoxyl sulfate have been observed even at a concentration of indoxyl sulfate found in hemodialysis patients.<sup>4)</sup>

Endothelial dysfunction, which is a critical precursor of CVD, is the most important cause of morbidity and mortality in CKD patients. NO is a crucial factor in endothelial function. In CKD patients, the overall production of NO is reduced. There are several mechanisms for reduced NO bioavailability, one of which is increased oxidative stress, which has emerged as a constant feature of CKD caused by an imbalance between its oxidative and anti-oxidative systems. Accumulated uremic toxins contribute to the enhanced generation of free radicals.

We have demonstrated that indoxyl sulfate inhibits NO production and cell viability by inducing free radicals such as superoxide through the induction of NADPH oxidase Nox4 in human vascular endothelial cells.<sup>42)</sup> Indoxyl sulfate has been shown to induce the expression of Nox4 mRNA and the production of superoxide and peroxynitrite in human vascular endothelial cells.

A recent clinical study has demonstrated that indoxyl sulfate may play a significant role in vascular disease and its higher rate of mortality observed in CKD patients.<sup>43)</sup> Indoxyl sulfate levels exhibited an inverse relationship to renal function and a direct relationship to aortic calcification and pulse-wave velocity. The highest indoxyl sulfate tertile has proved to be a powerful predictor of overall and cardiovascular mortality in CKD patients.

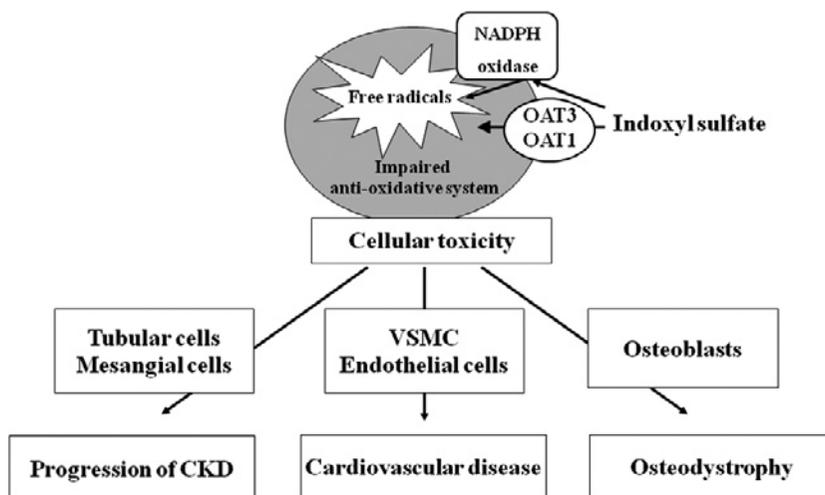
#### *Osteoblast cytotoxicity of indoxyl sulfate*

The osteoblast cytotoxicity of indoxyl sulfate has recently been reported. It induces oxidative stress in cultured osteoblasts to impair osteoblast function and down-regulates parathyroid hormone (PTH) receptor expression.<sup>44)</sup> Its accumulation in blood due to renal dysfunction is at least one of the factors, inducing skeletal resistance to PTH, and consequently leads to osteodystrophy such as low-turnover bone. Administration of AST-120 decreases the osteoblast cytotoxicity of indoxyl sulfate and suppresses the progression of low-turnover bone in CKD rats.<sup>45)</sup>

#### *Uremic toxicity of indoxyl sulfate*

Fig. 5 shows the overall uremic toxicity of indoxyl sulfate. It induces the cellular production of free radicals such as superoxide by activating NADPH oxidase, especially Nox4, and/or by its uptake through OAT1 and OAT3, and consequently impairing the cellular anti-oxidative system. It induces free radicals in renal tubular cells and glomerular mesangial cells, and stimulates the progression of CKD. It also induces free radicals in vascular smooth muscle cells and vascular endothelial cells, and aggravates CVD, as well as inducing free radicals in osteoblasts, and causing osteodystrophy.

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**Fig. 5** Uremic toxicity of indoxyl sulfate.

Indoxyl sulfate induces the cellular production of free radicals such as superoxide by activating NADPH oxidase, especially Nox4, and/or by its uptake through organic anion transporters (OAT1 and OAT3), consequently impairing cellular anti-oxidative system. It also induces free radicals in renal tubular cells and glomerular mesangial cells, and stimulates the progression of chronic kidney disease (CKD), as well as inducing free radicals in vascular smooth muscle cells (VSMC) and vascular endothelial cells, and aggravating cardiovascular disease. Moreover, indoxyl sulfate induces free radicals in osteoblasts and causes osteodystrophy.

#### *Clinical effects of AST-120*

We determined whether AST-120 could reduce the serum and urine levels of indoxyl sulfate and suppress the progression of CKD in undialyzed CKD patients.<sup>12)</sup> Administration of AST-120 significantly decreased the serum and urine levels of indoxyl sulfate. Among the patients in whom the urinary excretion of indoxyl sulfate was reduced by AST-120, it also significantly improved the slope of the  $1/\text{SCr}$ -time plot. The change in the slope of that plot showed a significant negative correlation to changes in the urine level of indoxyl sulfate. Thus, patients who showed a greater decrease in urinary indoxyl sulfate also exhibited a more marked suppression in the advance of CKD. These results support the notion that indoxyl sulfate, a protein metabolite, is involved in the progression of CKD, and that AST-120 delays that progression, at least in part, by reducing the serum and urine levels of indoxyl sulfate.

A multicenter, randomized, double-blind, placebo-controlled, dose-ranging study of a US population was designed to examine the nephroprotective effects of 3 doses of AST-120 versus a placebo in patients with moderate to severe CKD and elevated serum indoxyl sulfate levels who were also following an adequate protein-intake diet.<sup>46)</sup> Patients were randomly assigned to 1 of 3 doses of AST-120 (0.9, 2.1, or 3.0 g) or a placebo 3 times daily for 12 weeks. AST-120 significantly decreased serum indoxyl sulfate levels in a dose-dependent fashion. Furthermore, significant improvements in malaise were observed in a dose-dependent fashion. These results suggest that a dose of 9 g/day is an optimal dose for that population, and that it may prove useful in the treatment of patients with CKD.

A significant increase in plasma TGF- $\beta$ 1 has been observed in CKD patients. The progression of CRF may be caused by the persistent renal production of TGF- $\beta$ 1. Administration of

AST-120 (6 g/day) was shown to significantly reduce the plasma levels of indoxyl sulfate and TGF- $\beta$ 1 and to improve the slope of the reciprocal of serum creatinine.<sup>47)</sup> These results support the notion that indoxyl sulfate and TGF- $\beta$ 1 are involved in the progression of CRF, and that the oral adsorbent AST-120 impedes that progression, at least in part, by reducing the overproduction of TGF- $\beta$ 1.

In another study, AST-120 (6 g/day) was administered to CKD patients. When comparing the  $\Delta$ GFR in the observation and intervention periods for each group, the rate of decline in GFR was found to be significantly retarded in the AST-120 group while no significant difference was observed in the control group.<sup>48)</sup> Thus, AST-120 treatment slowed the decline in renal function in CKD patients with moderately decreased renal function.

A low-protein diet and treatment with renin-angiotensin system (RAS) blockers can delay the progression of CKD. The influence of AST-120 on the preservation of renal function was studied in CKD patients. Adding AST-120 (6 g/day) to a low-protein diet together with RAS blocker therapy delayed the deterioration of CKD, especially in patients with early or rapid progression.<sup>49)</sup>

The effects of AST-120 on early stage overt diabetic nephropathy have been determined in a prospective, randomized, controlled study for patients with type 2 diabetes.<sup>50)</sup> A significant reduction in urinary indoxyl sulfate was observed at month 12 in the AST-120 group but not in the control group. A significant difference was observed in changes in the mean levels of serum creatinine versus time between the two groups. The primary end points (defined as exceeding 2 mg/dl of serum creatinine) were counted in 70% of the control subjects, but in only 17% of the AST-120 group, and the Kaplan-Meier analysis was found to be statistically significant. Thus, the administration of AST-120 initiated at an early stage overt of diabetic nephropathy stunts the progression of renal dysfunction.

The CAP-KD study (Carbonaceous oral Adsorbent's effectiveness on Progression of chronic Kidney Disease) has recently demonstrated that AST-120 treatment (6 g/day) for 1 year significantly slowed the decline of eGFR (estimated glomerular filtration rate) in Japanese CKD patients as compared with conventional therapy, although there was no significant improvement in the composite primary endpoint.<sup>51)</sup>

The effect of AST-120 in CKD patients has been evaluated retrospectively by the 24-month dialysis-free rate and 50% dialysis-free period.<sup>52)</sup> The latter was significantly prolonged in the AST-120 group compared to the non-AST-120 group. When AST-120 treatment was started at a serum creatinine level below 3 mg/dL, the dialysis-free period lasted more than 24 months in the AST-120 group, compared with only 16.2 months in the non-AST-120 group. The risk of dialysis initiation was increased 3.48-fold in patients who were not administered AST-120. Thus, AST-120 was found to delay the initiation of dialysis in CKD patients.

The intima media thickness (IMT) and stiffness of the carotid arteries is related to coronary artery disease, and CKD patients are at high risk for such diseases. AST-120 treatment (6 g/day) for 2 years was found to significantly reduce arterial stiffness (pulse wave velocity) and carotid IMT in non-diabetic CKD patients before dialysis.<sup>53)</sup> In addition, the slope of the reciprocal serum creatinine concentration over time became significantly less steep in the AST-120 group than in the non-AST-120 group. Thus, AST-120 slowed the progression not only of CKD but also of CVD.

AST-120 has been shown to retard the deterioration of renal function in patients with CKD by decreasing serum nephrotoxic substances such as indoxyl sulfate. A high level of serum indoxyl sulfate may be one of the mechanisms underlying the progression of atherosclerotic lesions, which are the leading cause of cardiovascular events or deaths in dialysis patients. A retrospective study has examined whether AST-120 given to CKD patients in the pre-dialysis period influences

their prognosis after the initiation of dialysis. AST-120 given prior to the initiation of dialysis improved the prognosis of CKD patients under dialysis.<sup>54)</sup>

## CONCLUSION

Indoxyl sulfate, a uremic toxin, is markedly accumulated in the serum of CKD patients, and induces oxidative stress in a variety of cells such as renal tubular cells, glomerular mesangial cells, vascular smooth muscle cells, vascular endothelial cells and osteoblasts. Indoxyl sulfate stimulates the progression of CKD, CVD and osteodystrophy. Thus, the removal of indoxyl sulfate by the oral sorbent AST-120 ameliorates the progression of not only CKD, but also of CVD and osteodystrophy.

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