Oral activated charcoal adsorbent (AST-120) ameliorates extent and instability of atherosclerosis accelerated by kidney disease in apolipoprotein E-deficient mice

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Abstract

Background. Accelerated atherosclerosis and increased cardiovascular events are not only more common in chronic kidney disease (CKD) but are more resistant to therapeutic interventions effective in the general population. The oral charcoal adsorbent, AST-120, currently used to delay start of dialysis, reduces circulating and tissue uremic toxins, which may contribute to vasculopathy, including atherosclerosis. We, therefore, investigated whether AST-120 affects CKD-induced atherosclerosis.

Methods. Apolipoprotein E-deficient mice, a model of atherosclerosis, underwent uninephrectomy, subtotal nephrectomy or sham operation at 8 weeks of age and were treated with AST-120 after renal ablation. Atherosclerosis and its characteristics were assessed at 25 weeks of age.

Results. Uninephrectomy and subtotal nephrectomised mice had significantly increased acceleration of atherosclerosis. AST-120 treatment dramatically reduced the atherosclerotic burden in mice with kidney damage, while there was no beneficial effect in sham-operated mice. The benefit was independent of blood pressure, serum total cholesterol or creatinine clearance. AST-120 significantly decreased necrotic areas and lessened aortic deposition of the uremic toxin indoxyl sulfate without affecting lesional macrophage or collagen content. Furthermore, AST-120 lessened aortic expression of monocyte chemoattractant protein-1, tumor necrosis factor-α and interleukin-1β messenger RNA.

Conclusions. AST-120 lessens the extent of atherosclerosis induced by kidney injury and alters lesion characteristics in apolipoprotein E-deficient mice, resulting in plaques with a more stable phenotype with less necrosis and reduced inflammation.

Keywords: AST-120; atherosclerosis; charcoal adsorbent; necrotic lesion; renal ablation

Introduction

Accelerated atherosclerosis and increased cardiovascular events have been extensively documented in patients across the entire spectrum of chronic kidney disease (CKD) [1, 2]. Indeed, the risk of premature cardiovascular disease (CVD) death in patients with CKD is much higher than the risk of progressing to dialysis or transplantation [3, 4]. Furthermore, the pathophysiologic mechanisms that drive CKD-related CVD are complex, as therapeutic interventions effective in the general population are inconsistently beneficial, especially as renal damage progresses. Thus, although lipid-lowering agents decrease cardiovascular events in patients with mild-to-moderate
CKD [4], in more advanced CKD or dialysis, benefits were not seen, despite decreased serum cholesterol [5]. Such findings suggest that lipid-independent risk factors for CKD-associated atherosclerotic disease may be key, especially when the underlying renal condition progresses [6].

Patients with even modestly reduced renal function may retain or produce substances as a result of altered kidney homeostasis beyond the classic ‘uremic’ substances, serum creatinine and urea. Modulation of these accumulating substances in humans with renal disease has been achieved by the oral charcoal adsorbent, AST-120 [7, 8]. This intervention reduces circulating and tissue deposition of certain uremic toxins, including indoxyl sulfate [9] and indole acetic acid. AST-120 delayed progression of CKD, at least in part due to suppression of inflammation in experimental models [10–12]. Although AST-120 has currently not been shown to decrease incidence of doubling of serum creatinine in humans [13], a Phase 3 clinical trial is underway to examine its effects on the composite end point of serum creatinine elevation or progression to transplant or dialysis [9]. Interestingly, AST-120 treatment was observed to lessen all-cause mortality after initiation of hemodialysis [14]. Furthermore, mortality in Stages 2–5 CKD patients tracked with levels of indoxyl sulfate in a recent study [15]. In the current study, we investigated whether AST-120 can also modulate acceleration in atherosclerosis observed in response to renal damage and explored the possible mechanisms. Of note, atherosclerotic plaques change composition over time, from early lipid-laden macrophage-rich lesions to necrotic, acellular, potentially unstable plaque lesions. We, therefore, also investigated whether AST-120 has differential effects on atherosclerotic lesions if given at the onset of kidney injury versus after established renal damage.

Materials and methods

Experimental groups

All experiments were done using female apolipoprotein E-deficient (apoE−/−) mice on a C57BL/6 background (Jackson Laboratories, Bar Harbor, ME) maintained on normal mouse chow (R50505; PFI Feeds, St. Louis, MO) containing 18.9% protein, 11.0% fat, 0.8% calcium, 0.5% phosphorus and 3.3 IU/gm vitamin D3. Female apoE−/− mice underwent removal of 5/6th of the total renal parenchyma with ablation of the left kidney and both poles of the right kidney [subtotal nephrectomy (SNx), n = 10), uninephrectomy (UNx, n = 10) or sham operation (Sham, n = 10) at 8 weeks of age. Each of these groups were then further subdivided: SNx with no treatment (SNx), SNx with AST-120 started immediately after operation (SNx + AST-120), UNx with no treatment (UNx), UNx with AST-120 (UNx + AST-120) and sham with no treatment (Sham) or sham with AST-120 (Sham + AST-120). AST-120 was mixed in pulverized chow 5% (w/w) as previously reported [11]. Care and experimental procedures were in accordance with National Institutes of Health and Vanderbilt University Institutional Animal Care and Usage guidelines.

Systemic parameters

Systolic blood pressure (BP) was measured in conscious trained mice midway through the study and prior to sacrifice using the Bp-2000 Blood Pressure Analysis System (Visitech Systems Inc., Apex, NC) automated tail cuff as previously described [16]. The animals were acclimated to the procedure, and the mean values were based on an average of 10 stable readings. Body weight was assessed weekly. Timed urine creatinine was measured by the modified Jaffe method, and serum level of creatinine was measured by high-performance liquid chromatography (HPLC). Serum total cholesterol and triglyceride levels were determined at the end of the experiment as previously described [17, 18]. Urine indoxyl sulfate was measured by HPLC as described previously [19]. Since serum lipid measurements require fasting, indoxyl sulfate was assessed in the urine.

Assessment and characterization of atherosclerotic lesions

Mice were sacrificed under phenobarbital anesthesia and perfused with phosphate-buffered saline through the left ventricle as previously described [17, 18]. The heart, together with proximal aorta, was embedded in optimal cutting temperature compound and snap frozen in liquid nitrogen. The entire aorta, from the aortic valves to the iliac bifurcation, was dissected, and the en face preparations opened longitudinally, pinned flat and stained with Sudan IV (Sigma, St. Louis, MO). The atherosclerotic lesions were compared by computerized analysis with lesions expressed as percentage of total vascular surface [17, 18]. Ten-micrometer-thick cryosections were cut from the proximal aorta beginning at the end of the aortic sinus with modifications specific for computer analysis [17, 18]. Cryosections were stained with Oil-Red-O and Harris H&E (Sigma) to assess lipid deposition and necrosis. Bromine and collagen area were calculated from the ratio of atheroma/anuclear areas to total atherosclerotic lesion as previously described [20]. Collagen was stained by Sirius Red (Sigma). Calcium deposition was stained by von Kossa as previously described [21]. Quantitative analysis of lesion was performed using Imaging System KS300 (Release 2.0; Kontron Elektronik GmbH, Poway, CA). The operator was blinded to the group assignment.

Immunohistochemistry

Macrophage content was assessed by monoclonal rat antibody to mouse macrophages (MOMA-2; Serotec, Raleigh, NC) as previously described [17]. Indoxyl sulfate in aorta, liver and kidney was detected by antibody (Kureha Co., Tokyo, Japan) with modification of the methods previously described [12].

Messenger RNA quantification

Total RNA extraction from the proximal descending aorta was performed using the RNeasy Mini Kit (Qiagen, Valencia, CA). Quantifications of murine monocyte chemotaxtractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α) and interleukin-1 β (IL-1β) and endogenous control Human Euk 18S ribosomal RNA (18S rRNA) levels were performed by real-time reverse transcriptase-polymerase chain reaction assay using an ABI prism 7700 sequence detection system (Applied Biosystems Inc., Foster City, CA). Probes for MCP-1 (Mm00441242_m1), TNF-α (Mm99990644_m1), IL-1β (Mm00434228_m1) and 18S rRNA were obtained from Applied Biosystems Inc.

Statistical analysis

Results are expressed as means ± SEM. Statistical difference was assessed by nonparametric Kruskal–Wallis H-test. P < 0.05 was considered to be significant.

Results

Systemic parameters

Table 1 shows systemic parameters at 25 weeks of age of sacrifice. There was no difference in body weight between any groups at any time during the study. Although BP tended to increase in mice with the most severe renal ablation, there was no significant difference among the groups as expected in these mice with C57BL/6 background, a strain resistant to development of glomerulosclerosis and hypertension following renal ablation [22]. Indeed, evaluation of the remnant kidney parenchyma of UNx and SNx revealed no significant morphological changes and no change in urine albumin excretion (data not shown). Serum total cholesterol was significantly higher in SNx than in UNx and in Sham as previously noted [23]. Treatment with AST-120 did not affect serum cholesterol or triglycerides. Creatinine clearance (Ccr) was significantly lower in SNx than in UNx and in UNx than in Sham and was not affected by AST-120. Urine indoxyl sulfate significantly increased
CKD-induced atherosclerosis is lessened by AST-120

Table 1. Systemic parameters

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>UNx + AST-120</th>
<th>UNx</th>
<th>SNx</th>
<th>SNx + AST-120</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>23.0 ± 0.522.6 ± 0.5</td>
<td>22.1 ± 0.5</td>
<td>22.6 ± 0.6</td>
<td>20.9 ± 0.4</td>
<td>20.9 ± 0.5</td>
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<tr>
<td>BP (mmHg)</td>
<td>116 ± 4</td>
<td>112 ± 5</td>
<td>112 ± 5</td>
<td>118 ± 5</td>
<td>126 ± 7</td>
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<tr>
<td>Serum cholesterol</td>
<td>251 ± 24</td>
<td>244 ± 12</td>
<td>368 ± 19</td>
<td>320 ± 17</td>
<td>386 ± 13</td>
</tr>
<tr>
<td>Serum triglycerides (mg/dL)</td>
<td>255 ± 15</td>
<td>225 ± 12</td>
<td>247 ± 8</td>
<td>236 ± 11</td>
<td>287 ± 14</td>
</tr>
<tr>
<td>Ccr (l/min per g)</td>
<td>15.3 ± 1.313.8 ± 1.6</td>
<td>10.0 ± 1.2</td>
<td>8.7 ± 0.7</td>
<td>5.6 ± 0.8</td>
<td>6.0 ± 0.7</td>
</tr>
<tr>
<td>Urine indoxyl sulfate (mg/dL)</td>
<td>296 ± 40</td>
<td>175 ± 25</td>
<td>237 ± 32</td>
<td>101 ± 13</td>
<td>436 ± 57</td>
</tr>
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</table>

*P < 0.05 versus Sham.
^P < 0.05 versus UNx.
^P < 0.05 versus SNx.

Atherosclerotic lesions

Atherosclerosis was significantly increased in mice with reduced renal mass compared to sham as assessed in en face aortas by Sudan IV staining (Figure 1). The extent of the aortic lesions was inversely proportional to the degree of reduction in renal parenchyma [Sham 3.2 ± 0.4%, UNx 7.0 ± 0.7% (P < 0.05 versus Sham) and SNx 21.0 ± 2.6% (P < 0.05 versus UNx and < 0.01 versus Sham)]. AST-120 did not affect the extent of atherosclerotic involvement in Sham animals. In contrast, AST-120 resulted in a dramatic decrease in atherosclerosis in both UNx and SNx (Figure 1).

Since the first atherosclerotic lesions in the apoE⁻/⁻ model develop in the proximal aorta, we also performed cross-sectional analysis of the aortic sinus (Supplementary Figure 1). Lesions in this area were very extensive in all groups. Paralleling results observed by the en face assessments, treatment with AST-120 significantly lessened the extent of sinus lesions in SNx mice (436 138 ± 13 594 μm² in SNx versus 348 827 ± 34 777 in SNx + AST-120, P < 0.05). AST-120 also numerically decreased sinus lesions of mice with a lesser degree of renal ablation [UNx (478 876 ± 44 843 versus 397 366 ± 12 174 μm² in UNx + AST-120, NS) and Sham (434 285 ± 20 063 versus 472 337 ± 37 421 μm² in Sham + AST-120, NS)].

Characteristics of atherosclerotic lesions

Several key characteristics are known to influence plaque stability (Figure 2). The lesion area positive for macrophage, as assessed by MOMA-2 staining, was not different among the groups and was not affected by AST-120 (Figure 2A). Collagen content, assessed by Sirius Red staining, was not different among groups (Figure 2B). Calcium deposition assessed by von Kossa staining showed occasional staining of the aortic valve and only slight deposition in the vascular intima in all of the experimental groups, with no effect by AST-120 (data not shown). By contrast, necrotic areas were significantly increased in UNx and further increased in SNx (3.4 ± 0.7% in Sham, 7.6 ± 1.2% in UNx and 9.5 ± 2.0% in SNx; Figure 2C and D). Furthermore, AST-120 lessened the necrotic areas in both UNx and SNx (Figure 2C and D). These findings indicate that the extent of necrotic areas is inversely proportional to the extent of renal ablation and that AST-120 can lessen these potentially destabilizing areas of the plaque.

To further define the mechanisms for the beneficial effects of AST-120, we also determined expression of several inflammatory factors likely involved in the necrotic processes within the atherosclerotic lesions. In SNx mice, treatment with AST-120 significantly decreased aortic messenger RNA (mRNA) expression of MCP-1 (SNx,
AST-120 caused a directionally similar pattern of lessening inflammation (UNx versus UNx + AST-120 for TNF-α, 2.67 ± 0.90 versus UNx + AST-120, 0.61 ± 0.19, P < 0.05, MCP-1: 1.65 ± 0.32 versus 1.07 ± 0.12, NS, IL-1β: 4.07 ± 1.37 versus 1.56 ± 0.48, NS). Treatment with AST-120 has previously been shown to modulate deposition of indoxyl sulfate within the remnant renal tissue [12]. Levels of indoxyl sulfate in atherosclerotic lesions were reduced by AST-120 (SNx, 47.4 ± 2.8% versus SNx + AST-120, 34.9 ± 2.7%, P < 0.05; Figure 4). Similar to results in SNx, AST-120 decreased the deposition of indoxyl sulfate in mice with UNx (UNx, 37.2 ± 3.2% versus UNx + AST-120, 12.4 ± 4.1%, P < 0.05). AST-120 also decreased indoxyl sulfate in liver (SNx, 57.0 ± 2.8% versus SNx + AST-120, 34.5 ± 1.3%, P < 0.05) and kidney (SNx, 34.1 ± 2.8% versus SNx + AST-120, 19.1 ± 3.6%, P < 0.05).

Discussion

The present study makes the novel observation that AST-120 lessens the accelerated atherosclerosis that develops in models of renal ablation. This benefit was not dependent on reduction in systemic BP, serum lipid levels or improvement in renal function. Instead, amelioration in atherosclerosis by AST-120 was associated with compositional changes in the plaque, with reduction in the necrotic area and inflammation.

CKD dramatically increases atherosclerosis and cardiovascular events [1, 2]. This detrimental connection is especially prominent in patients with advanced CKD, who are
also the most resistant to conventional lipid-lowering therapies [5]. Recent interest in lipid-independent risk predictors has identified inflammation as an important contributor to CVD in the general population [24, 25]. This mechanism may be an especially relevant predictor of cardiovascular mortality at every stage of CKD [26, 27]. In this connection, AST-120, an oral charcoal adsorbent that reduces circulating and tissue uremic toxins, delays progression of experimental and human CKD [8, 10–12] at least in part, by suppressing inflammation [10, 11, 28]. Therefore, we investigated whether AST-120 can modulate acceleration of atherosclerosis induced by reduction in renal mass. The present study finds that AST-120 lessens development of renal injury-induced acceleration of atherosclerosis. This effect is especially marked in mice with the most extensive renal ablation (Figure 1). Since disease development in this model proceeds from the proximal aortic root down the length of the vessel, it is possible that the therapeutic effects of AST-120 are most apparent in the most recently developed lesions captured by en face measurements. The salutary effects were independent of BP or serum lipids, findings that parallel divergence between beneficial effects on lipid deposition and effects on systemic pressure and serum lipid concentrations following treatment with an angiotensin II receptor blocker (ARB) [29, 30]. Furthermore, AST-120 did not change renal function over the duration of the study, indicating that the vascular benefit was not linked to improvement in renal function as assessed by creatinine clearance.

Lack of salutary effects of AST-120 on atherosclerosis in mice with intact kidneys while benefiting mice with reduced renal parenchyma seems related to factors specifically accumulating in the latter, including indoxyl sulfate. Along the same lines, in the absence of such renal injury-related factors, AST-120 was not beneficial in Sham animals with intact kidneys. Thus, AST-120 is postulated to adsorb toxins related to renal ablation, although exactly which toxins are removed remains uncertain. Notably, the benefit of AST-120 on atherosclerosis was not reflected in changes in Ccr. Instead, AST-120 may have adsorbed other retained substances produced by the remnant renal tissue. In this connection, AST-120 has been shown to decrease serum and kidney levels of indoxyl sulfate in clinical [9] and experimental CKD [11, 12]. Indoxyl sulfate has also been associated with increased vascular stiffness, calcifications and mortality in Stages 2–5 CKD [15]. In vitro, indoxyl sulfate was recently shown to be associated with upregulation in oxidative stress-mediated increased mRNA expression of intercellular adhesion molecule-1 (ICAM-1) and MCP-1 [31]. In the current study, reduction in atherosclerosis in response to AST-120 was accompanied by reduction in urinary indoxyl sulfate as well as in aorta, liver and kidney. These findings suggest the possibility that deposition of uremic toxins could contribute to disease progression. AST-120 may modulate renal injury-induced atherosclerosis in part by decreasing this tissue deposition. This reduction of indoxyl sulfate, in turn, is expected to lessen inflammation. Indeed, a reduction in markers on inflammation was documented in aortae of AST-120-treated mice. Our observations complement recent in vitro and in vivo findings that exogenous indoxyl sulfate activates leukocyte–endothelial cell interactions [32] as well as...
clinical findings showing that serum indoxyl sulfate is correlated with cardiovascular mortality in CKD patients [15] and that CKD patients treated with AST-120 before initiation of dialysis have significantly better 5-year survival after dialysis was begun compared with patients who did not receive this treatment before dialysis [14]. Taken together, the observations raise the possibility that AST-120 can modulate underlying vasculopathy in CKD patients.

In addition to lessening the extent of the atherosclerotic lesions, the current study also reveals that AST-120 modulates the characteristics of these lesions. Assessments of necrotic areas have been applied to characterize advanced lesions in mice with intact kidneys [20, 33–35]. This assessment provides significant additional insight into the evaluation of the plaque burden that has furthered our understanding that atherosclerotic plaques are not static but undergo dynamic remodeling that influences plaque stability. Previously, we showed that changes in the lipid content and composition, macrophage infiltration, collagen content and elastin integrity were affected by the stage of the lesion and treatment interventions [29, 30, 36]. Thus, whereas necrotic areas within the atherosclerotic lesions were significantly increased in both UNx and SNx, AST-120 decreased these areas in both groups of renal-ablation mice when started at the onset of renal damage (Figure 2C and D). Plaque composition is a crucial component of risk for acute coronary events, as vulnerable plaques have increased inflammatory cytokines, expanded necrotic areas, induced a focal decrease in collagen deposition and are prone to rupture and thrombosis [37]. Indeed, apoE−/− mice with selective deficiency of macrophage p38 MAPK had increased necrotic and apoptotic lesions, although no change in proximal atherosclerotic lesions [20]. Increased necrosis and apoptosis linked to reduced effectorcytosis were also noted in atherosclerotic lesions of mice reconstituted with macrophages defective in the tyrosine kinase Merkt receptor [33]. That study suggested that less effectorcytosis and more apoptosis predicts greater inflammation in the microenvironment that would in turn promote more necrotic lesions. Contrary to the beneficial effects of lessening collagen deposition in progressive renal injury, maintenance of collagen in atherosclerotic lesions is viewed as a beneficial stabilizing effect on the atherosclerotic plaque [29, 30]. Our result that AST-120 prevented a significant decrease in collagen content, together with the effect to decrease necrotic lesions, supports the idea that AST-120 treatment promotes more stable atherosclerotic lesions.

AST-120 significantly dampened gene expression of several markers of inflammation. It is interesting that in addition to AST-120, other interventions have been shown to benefit renal injury-associated atherosclerosis, including ARBs [29] and inhibitor of oxidant stress and inflammation [38]. Each of these interventions benefits atherogenesis independently of effects on systemic BP, circulating lipid levels or kidney function [29, 38]. Instead, benefits have been associated with a reduction in the inflammatory component of atherosclerosis [29, 38]. ARBs have also been shown to modulate macrophage infiltration and lipid homeostasis in arterial foam cells [29, 36]. We recently showed that reduction in renal mass by UNx not only increases atherosclerosis but also activates macrophage NFkB, a nuclear factor central in the inflammatory pathway [36]. Notably, treatment with an ARB lessened NFkB activation and decreased lesion size [36]. The common thread for these beneficial therapies seems to involve interference with inflammation, which may be direct, or as in the case of AST-120, indirect, through decrease in indoxyl sulfate levels. Antioxidant N-acetylcysteine also inhibits renal injury-induced acceleration of atherosclerosis with lessening the inflammatory component of atherosclerosis [38]. By contrast, we observed only slight and sporadic calcium deposition in some aortic valve leaflets with little positive staining in the vascular intima or media in any experimental group with/without AST-120. Differences between these results and previous reports describing vascular calcification in nephrectomized mice [39–41] may reflect more severe kidney dysfunction, differences relating to the method of renal ablation and/or difference in diet composition, which could affect levels of vitamin D, calcium and phosphate, which would favor tissue calcium deposition.

In summary, our study shows AST-120 lessens atherosclerosis development and decreases unstable plaque characteristics in apoE−/− mice with renal dysfunction in association with reduced vascular inflammation. These results provide further support for the use of AST-120 to decrease cardiovascular events in patients over a spectrum of CKD. Since ARB therapy is a mainstay of CVD therapy in the general population and reduces atherosclerosis in experimental CKD as well as cardiovascular morbidity and mortality in hemodialysis patients [42, 43], it is possible that the combination of an ARB and AST-120 may be of particular benefit in reducing CVD in CKD.

Supplementary data
Supplementary data is available online at http://ndt.oxfordjournals.org.

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Conflict of interest statement. None declared.

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