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Original Article

Imatinib inhibits angiotensin II-induced aortic dissection through the c-Abl signaling pathway

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Abstract: Background: Infusion of the peptide angiotensin II (AngII) has been shown to induce aortic dissection in mice, but the molecular mechanisms that underlie its effects are less well characterized. Here, we investigated whether imatinib, an inhibitor of c-Abl, would antagonize AngII development of aortic dissection in mice. Methods: Pathway signaling events were analyzed in pathological sections obtained from treated animals and confirmed in an *in vitro* model. AngII, AngII plus imatinib, and control were either delivered to old C57BL/6 male mice by mini-pumps (Alzet model 1014D) or to primary rat aortic smooth muscle cells in culture. In addition to histological examination, samples were analyzed for apoptosis by immunohistochemistry or flow cytometry, and for changes in protein and mRNA levels in c-Abl, phospho-c-Abl, and MMP2 by Western blotting and real-time PCR. Results: Imatinib prevented AngII induced aortic dissection ($P < 0.05$) and apoptosis of smooth muscle cells *in vivo* ($P < 0.05$) or *in vitro* ($P < 0.05$). Molecular analysis demonstrated that the levels of phosphorylated c-Abl and MMP2 were increased in aortas in mice treated with AngII ($P < 0.05$), and decreased in AngII plus imatinib treated mice ($P < 0.05$). Conclusions: The development of AngII-induced aortic dissection in mice was antagonized by treatment with imatinib, an inhibitor of c-Abl. These results indicate that c-Abl may be an intermediate in the AngII pathway and that imatinib might be of clinical utility for the prevention and treatment of aortic dissection in patients.

Keywords: c-abl, aortic dissection, apoptosis, angiotensin II, imatinib

Introduction

Aortic dissections and aneurysms are medical emergencies with a high mortality. They are the consequence of degeneration and damage to the vascular wall [1] which may develop in response to events that are physiologic and/or genetic in origin, such as hypertension and Marfan's Syndrome (MFS), respectively. Vascular smooth muscle cell (VSMC) integrity is a crucial factor in the maintenance of the structural properties of the vascular wall, so that changes in these cell types specifically are the focus of studies directed at understanding the development of these pathological conditions. For example, smooth muscle cell (SMC) apoptosis has been detected in abdominal aortic aneurysms (AAA) and MFS aortas [2].

Much of the emphasis of contemporary treatment for disease is to define the molecular pathways involved and to use small molecules to modulate the disease. One pathway that is involved in aortic dissection and alterations in VSMC is angiotensin II (AngII). AngII is a small peptide found in the blood plasma, which is known to have a role in regulating blood pressure. Physiologic studies of AngII have in fact produced a mouse model for AAA that recapitulates the pathologic features in humans [3, 4]. The sequence of molecular events induced by AngII in this model and in particular VSMC is of intense interest.

A number of studies implicate c-Abl as a putative intermediate in the AngII pathway in VSMC. Hypertrophy of VSMC is one example of a specific change induced by AngII. Various molecular methods have been utilized to reveal a role for c-Abl in AngII-stimulated VSMC hypertrophy [5]. In addition, exposure of cells to a small molecule inhibitor of c-Abl, imatinib, was shown to attenuate AngII-induced vascular remodeling [6]. Imatinib is a 2-phenylaminopyrimidine com-

pound that selectively inhibits the catalytic activity of the Abl, platelet-derived growth factor receptor (PDGFR), and c-Kit tyrosine kinases [7]. It is an important therapeutic for a specific type of leukemia, and it has gained importance as a tool to probe tyrosine receptor kinase pathways. We have also previously reported that blood pressure is lower in Abl-/- mice compared to wild-type mice. Furthermore, AngII induced phosphorylation of crk associated substrate (CAS) and expression of putative CAS downstream targets vinculin and paxillin was diminished in VSMC of the Abl knockout [8]. Therefore, it is possible that activation of c-Abl may mediate AngII effects on VSMC in mice and lead to aortic dissection.

The tyrosine kinase c-Abl is a substrate of the Src family kinases [9]. c-Abl is expressed in various cell types, including VSMC, endothelial cells, and fibroblasts [5], and it localizes to both the nucleus and cytoplasm in cell [10]. Previous studies have reported that activation of c-Abl tyrosine kinase can lead to cell death [11, 12]. However, even though activation of c-Abl is known to occur in response to a variety of signals, it has not been investigated in VSMC upon exposure to AngII.

Mechanisms that lead to AAA may in part be due to molecules induced by AngII in VSMC. One candidate gene is matrix metalloproteinase (MMP)2. MMP2 is one of the principal matrix-degrading proteases and is known to play a particularly important role in the development of AAA. Several studies have shown that MMP2 expression is increased in the extracellular matrix (ECM) in the walls of AAA [13, 14]. Degradation of the aortic wall may result and thus, contribute to loss and cell death of VSMC.

In the present study, we utilized *in vivo* and *in vitro* models to determine whether activation of c-Abl in VSMC may mediate Ang II-induced aortic dissection.

Materials and methods

Ethics statement

Animal experiments were approved by the Institution Animal Care and Use Committee at Tongji Medical College, Huazhong University of Science and Technology, China. All mice were obtained from and housed in the Animal Center of Tongji Medical College.

Animals and treatment

C57BL/6 male mice from 12 to 30 weeks of age and weighing 25 to 35 g were used [15]. All mice were housed in a pathogen-free room at 25°C with free access to a normal diet and water. Mini-pumps (Alzet osmotic pump model 1014D; DURECT Corporation, Cupertino, CA, USA) were primed at 37°C in normal saline for 6 h. After mice were anesthetized with 3% chloral hydrate, mini-pumps were implanted subcutaneously in the midscapular region to achieve a delivery rate of 3 mg/kg per day over the course of two weeks. AngII and imatinib mesylate, the inhibitor of c-Abl, were dissolved in 0.9% NaCl (Sigma, St. Louis, MO, USA). All mice (n=45) were randomly divided into three study groups based on bodyweight: (1) AngII, 3 mg/kg per day; (2) AngII plus irrigation with imatinib mesylate, 60 mg/kg per day; and (3) sham-treated animals implanted subcutaneously with mini-pumps containing only 0.9% NaCl. Mice were euthanized at four weeks after implantation of minipumps, and aortic tissue was immediately removed. Autopsies were performed in addition on animals which died prior to this time point.

Histology and immunohistochemistry

Aortic tissue was placed into 4% paraformaldehyde for fixation, embedded in paraffin, and subsequently sectioned at 4 μm. Three different sections of each aorta were histologically examined after hematoxylin/eosin staining. Aortic dissection was defined as a splitting of the middle layer (media) from the outer layer of the aorta. Cross sections of affected areas and corresponding control regions of the ascending aorta were sectioned, and histological examination and immunohistochemical staining were performed. Blinded analysis of the study groups was performed.

Evaluation of apoptosis

Terminal transferase dUTP nick end labeling (TUNEL) was carried out to detect apoptotic VSMC in deparaffinized 4 μm sections using the ApopTag Peroxidase In Situ Cell Apoptosis Detection Kit I according to the manufacturer's instructions (EMD Millipore Corporation, Billerica, MA, USA). Three sections were selected from areas of the descending aorta, and three fields per section were examined at a

magnification of 400 ×. Two investigators counted TUNEL-positive VSMC, and their observations were averaged. The apoptotic index was calculated using the following formula: 1000 × (number of TUNEL-positive nuclei per field/total number of nuclei per field).

Cell culture

Mouse aortic smooth muscle cells (RASMC) were cultured from thoracic aortas of young male mice. Animals were killed by cervical dislocation and soaked in 70% alcohol for 5 min. Endothelium and adventitia was stripped from aortic tissues and placed in 6-well plates. The aortic tissue was cut into 2 × 3 mm^2 pieces, placed in 50 cm^2 culture flasks without medium to prevent floating, and incubated at 37°C in a humidified, 5% CO_2 cell culture chamber for one hour. Cultures were grown in Dulbecco's modified Eagle medium (DMEM) containing 20% fetal bovine serum (FBS) (HyClone, Logan, UT, USA), 100 units/mL penicillin, and 100 mg/mL streptomycin (Gibco, Life Technologies, Grand Island, NY, USA) [16]. Tissues were incubated at 37°C in a humidified, 5% CO_2 atmosphere for 5 to 9 days, or until outgrowth of spindle-shaped SMC was observed extending from the tissue. After removing the explant, cells were dispersed by treatment with 0.25% trypsin (Gibco, Life Technologies), centrifuged, resuspended in high glucose DMEM with 10% FBS, and placed into 50 cm^2 culture flasks. Post-confluent cultures assumed a hill and valley topography characteristic of SMC grown *in vitro*. Greater than 99% of cells were confirmed to be RASMC by staining with a polyclonal antibody against SMC-specific α-actin (Boster, Pleasanton, CA, USA). All experiments were performed with approval on laboratory equipment from the Histology and Embryology Laboratory of Tongji Medical College, Huazhong University of Science and Technology.

Cell treatment

AngII (Sigma, Spain) and imatinib mesylate were resolubilized in 0.9% NaCl (Enzo, Farmingdale, NY, USA) [7].

Confluent monolayers of RASMC were used at passages three through five. Cells were plated at 50,000 cells/well onto 6-well plates and allowed to attach for 24 h. Cells were then starved for 24 h in 0.2% FBS containing medium in order to synchronize them in the G0/G1 phase of the cell cycle. They were then incubated for 24 h with drugs in culture medium containing 0.2% FBS and divided into three study groups: (1) AngII alone (1 μM); (2) AngII (1 μM) plus imatinib mesylate (0.5 μM); (3) Control (0.9% NaCl). The concentration of 1 μM AngII was chosen as this dose induced apoptosis in mice VSMC in culture [17].

Flow cytometric analysis of apoptosis

An annexin V-fluorescein isothiocyanate kit (KeyGen Biotech. Co. Ltd., Nanjing, China) was used to detect phosphatidylserine externalization as a marker of apoptosis. After drug stimulation, RASMC were dispersed by treatment with trypsin without EDTA (Life Technologies), centrifuged (2000 rpm, 5 min), and washed in cold phosphate buffered saline (PBS) twice. Approximately 1-5 × 10^5 cells were collected, resuspended in binding buffer, and incubated with FITC-labeled annexin V and the DNA-intercalating agent, propidium iodide. After 15 min incubation in the dark at room temperature, stained cells were immediately analyzed by flow cytometry (Becton Dickinson, San Jose, CA, USA). Data were analyzed on biparametric FL1 (Green)/FL2 (Red) dot plots using quadrant statistics.

Real-time PCR

Levels of MMP2 and control mRNAs were determined using semi-quantitative real-time PCR. After treatment, total cellular RNA was extracted from RASMC with TRIzol reagent (15596-026; Gibco, Life Technologies), and the concentration and purity were evaluated on a spectrophotometer. RNA was reverse transcribed with the RevertAid Reverse Transcriptase (EP0442; Fermentas, Thermo Scientific, Waltham, MA, USA), dNTP (R0191; Fermentas), RiboLock RNase Inhibitor (E00381; Fermentas). Real-time PCR was carried out using the Real-time PCR Detection System (SLAN, Hongshi, China) with SYBR qPCR mix (QPS-201; TOYOBO, Osaka, Japan), according to the manufacturer's instructions. Cycling was performed as follows: 95°C for 1 min, followed by 40 cycles of 95°C 15 s, 58°C 15 s, 72°C 1 min, and a final incubation at 72°C 45 s. Primers for amplification were created using Primer Premier Software (Premier Biosoft International, Palo Alto, CA, USA) based

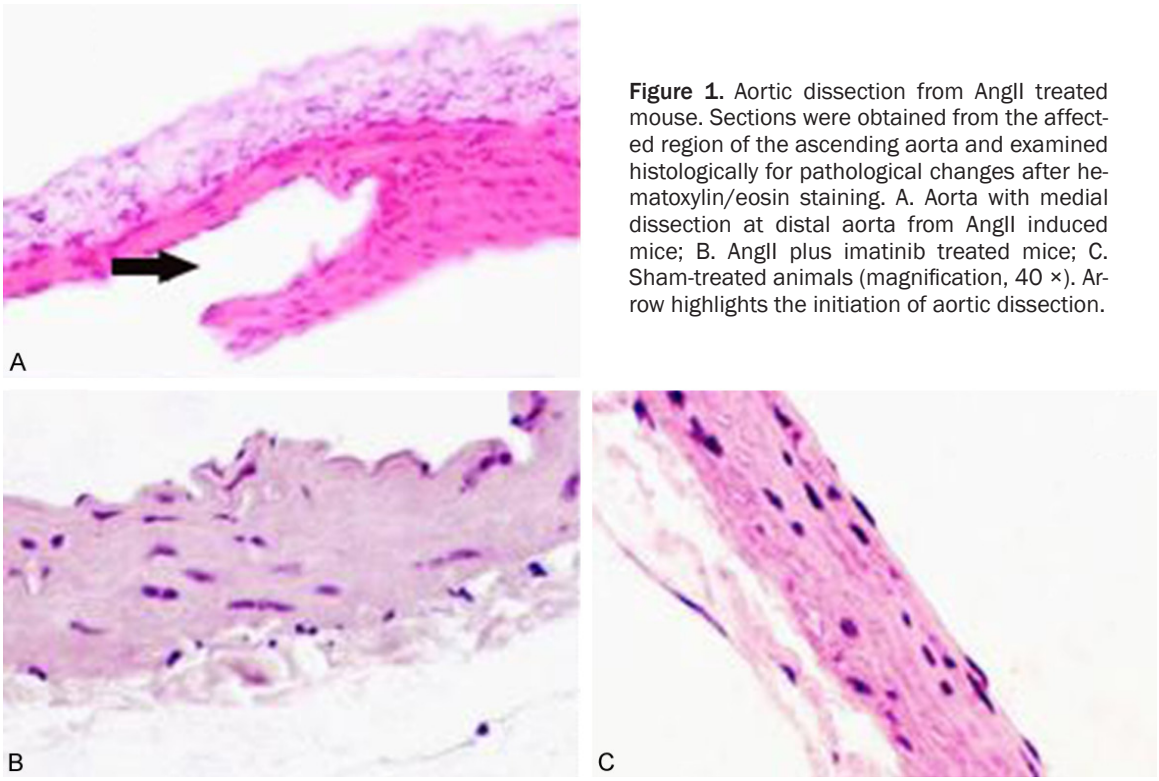

Figure 1. Aortic dissection from AngII treated mouse. Sections were obtained from the affected region of the ascending aorta and examined histologically for pathological changes after hematoxylin/eosin staining. A. Aorta with medial dissection at distal aorta from AngII induced mice; B. AngII plus imatinib treated mice; C. Sham-treated animals (magnification, 40 ×). Arrow highlights the initiation of aortic dissection.

on primary RNA sequences from GenBank. The sequences used are as follows:

MMP2 sense, 5'- CAAGGATCGGTTTATTTGGCG-3; MMP2 antisense, 5'- GCCTCGTACACG GCATCAATC -3'; β-actin sense, 5'- CGTTGACATCCGTAAAGACCTC -3'; β-actin antisense, 5'- TAGGAGCCAGGGCAGTAATCT -3'.

Western blotting

Protein was extracted from aortic samples (n=5 per group/time point) and treated RASMC with 0.1% sodium dodecyl sulfate (SDS). Total cellular protein concentration was measured by the bicinchoninic acid protein assay (BCA; Pierce, Rockford, IL, USA). Total protein was electrophoretically separated on an SDS-PAGE and blotted onto nitrocellulose membranes. Nonspecific binding was blocked by incubation of the membrane for 2 h in 5% nonfat milk. The membranes were washed in PBS 30 min and incubated with appropriate primary antibodies overnight at 4°C. The primary antibodies used were as follows: MMP2 (rat; 1:1000, Abcam, Cambridge, England); c-Abl (rat, 1:1000; Cell Signaling, Danvers, MA, USA); phospho-c-Abl (rat, 1:1000; Cell Signaling). Specific binding was detected with HRP-conjugated secondary antibodies and an ECL Western Blotting Kit (Thermo Fisher Scientific, Rockford, IL, USA). The blots were quantified on a Bio-Rad Gel Documentation System (Hercules, CA, USA).

Statistical analysis

The results are presented as mean ± SEM. Differences between various treatment conditions were evaluated by one-way ANOVA, followed by Levene's test using statistical software IBM SPSS Statistics for Windows, Version 18.0 (Amonk, NY, USA). Differences were considered statistically significant when $P < 0.05$.

Results

Imatinib prevented AngII induced aortic dissection

Spontaneous aortic dissection or aneurysm has been shown to occur in C57BL/6 male mice infused with AngII [9]. To recapitulate this model, we treated C57BL/6 male mice with AngII over a four-week period. Aortic dissections or aneurysm were found in 4/15 AngII-treated C57BL/6 mice. Spontaneous dissection was observed from the descending aorta to in AngII-treated C57BL/6 mice. Control ani-

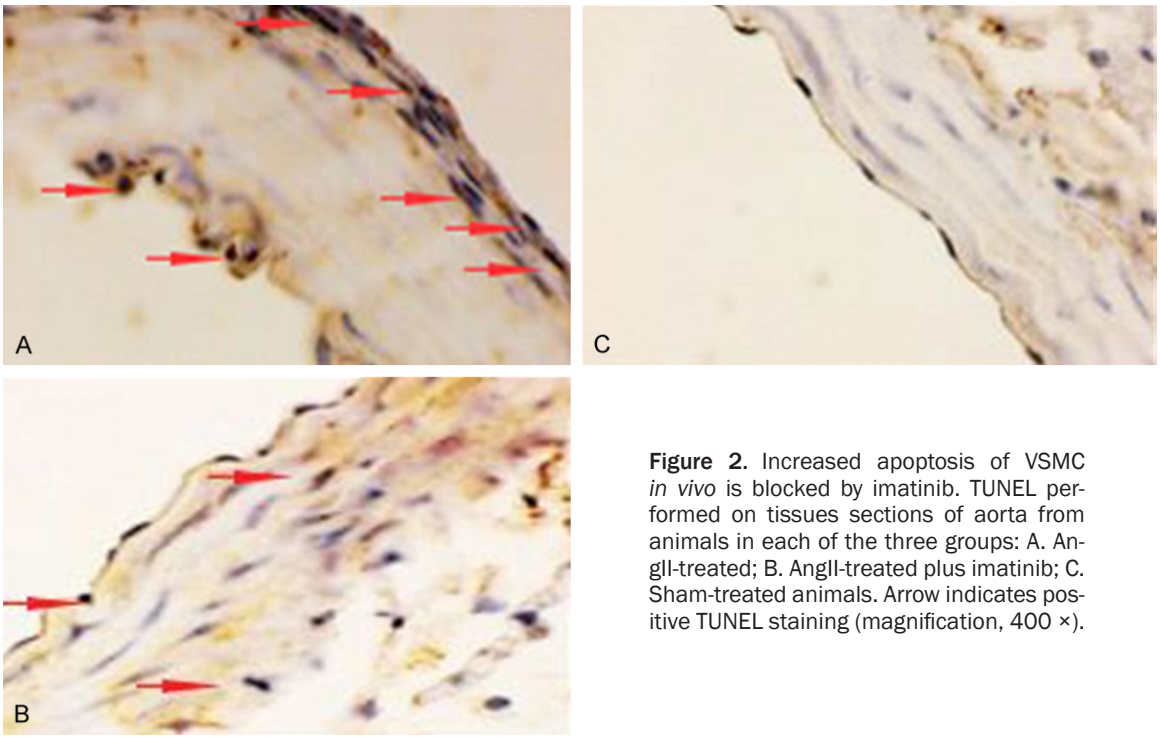

Figure 2. Increased apoptosis of VSMC *in vivo* is blocked by imatinib. TUNEL performed on tissues sections of aorta from animals in each of the three groups: A. AngII-treated; B. AngII-treated plus imatinib; C. Sham-treated animals. Arrow indicates positive TUNEL staining (magnification, 400 ×).

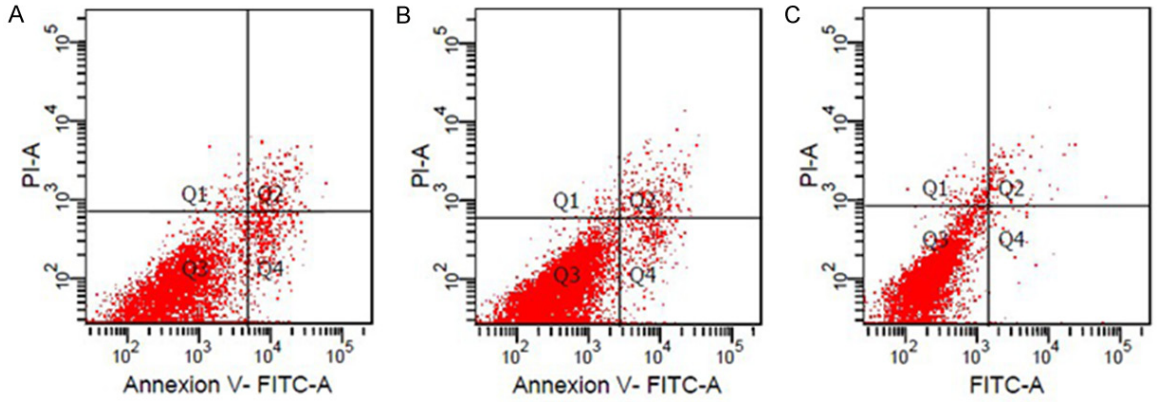

Figure 3. Increased apoptosis of AngII treated VSMC is blocked by imatinib *in vitro*. Cells were treated and the percentage of apoptotic cells was determined by flow cytometry. A. AngII (1 μM), percentage of apoptotic cells is 12.0 ± 1.67%; B. AngII (1 μM) plus imatinib mesylate (0.5 μM) of the apoptosis cells is 5.6 ± 0.76%; C. Control (0.9% NaCl), percentage of the apoptotic cells is 1.83 ± 0.71% ($P < 0.05$).

mals displayed no abnormal aortic pathologies. In contrast, only 2/15 AngII plus imatinib treated mice displayed aortic dissections or aneurysm. Interestingly, although imatinib decreased the frequency of aortic dissection or aneurysm when compared to AngII-treated mice (13.3% versus 26.7%, $P < 0.05$), these mice did not revert to a normal pathology. Pathological differences were still evident in the media of the descending aorta from AngII plus imatinib treated mice when compared with the medial layer of controls (**Figure 1**).

Imatinib blocks AngII induced VSMC apoptosis

Deterioration of the aortic wall in aortic dissection is thought to be in part due to apoptosis of VSMC. To determine whether apoptosis occurs in VSMC in our model and whether this might be mediated by c-Abl, we looked for apoptotic VSMC *in vivo*. We performed an in situ assay (TUNEL) to identify apoptotic cells. We found a significant increase of apoptosis in VSMC in AngII-treated mice when compared to that observed in vehicle-treated controls ($P < 0.05$;

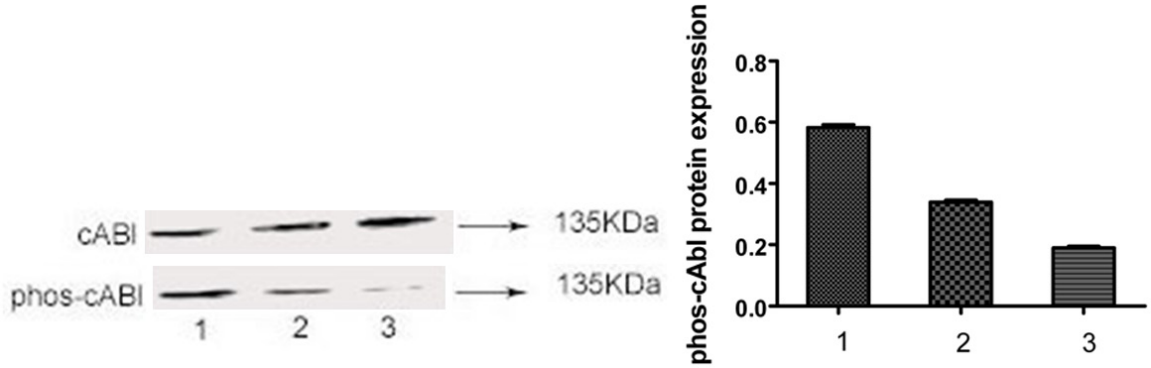

Figure 4. Phosphorylation of c-Abl protein is increased in AngII-treated mouse aorta *in vivo*. Protein lysates were prepared from aortic tissue treated with (1) AngII; (2) AngII plus imatinib; (3) Vehicle control (0.9% NaCl). Western blots are shown in the first panel and quantification in the second.

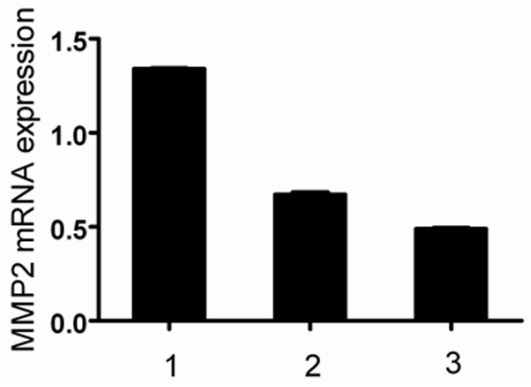

Figure 5. MMP2 mRNA and protein expression is increased in AngII-treated VSMC *in vitro*. mRNA and protein were isolated from cells treated with (1) AngII (1 μM); (2) AngII (1 μM) plus imatinib mesylate (0.5 μM); (3) Vehicle control (0.9% NaCl).

Figure 2). However, treatment with imatinib significantly reduced the number of apoptotic VSMC per field in AngII-treated mice ($P < 0.05$; **Figure 2**).

To confirm these results, we performed analysis of apoptosis on RASMC *in vitro*. Imatinib was found to significantly inhibit RASMC apoptosis stimulated by AngII *in vitro*, as evaluated by flow cytometry (12.0 ± 1.67% versus 5.6 ± 0.76%; **Figure 3**). Therefore, results obtained *in vitro* corroborate findings from tissue specimens and implicate c-Abl as a potential mediator of the AngII induced pathologies.

Activation of c-Abl occurs in response to AngII treatment

Phosphorylation of c-Abl is a signal that the protein is activated. Western blotting was performed on protein lysates prepared from treated tissue and RASMC samples to determine whether c-Abl became phosphorylated in response to AngII in our model systems. Importantly, protein levels of c-Abl remained unaltered in mice aorta and in RASMC *in vitro* regardless of treatment (**Figure 4**). However, levels of phospho-c-Abl did increase proportionally in samples treated with AngII (**Figure 4**). To determine whether AngII induced molecular events that might be potentially downstream of c-Abl, MMP2 protein levels were examined. MMP2 protein was also found to be significantly upregulated in the aortas of AngII-treated mice and in Ang II-treated RASMC *in vitro*. Imatinib influenced both c-Abl phosphorylation and MMP2 protein levels so that aortas from AngII treated animals and RASMC displayed decreased c-Abl phosphorylation and MMP2 levels relative to AngII treated animals (**Figure 4**).

In order to determine the mechanism for the increase in MMP2 protein, real-time PCR was performed on RNA extracted from RASMC treated *in vitro*. Real-time PCR demonstrated that MMP2 mRNA expression was significantly increased in AngII-treated RASMC. However, increases in MMP2 mRNA were inhibited in AngII-treated RASMC plus imatinib ($P < 0.05$; **Figure 5**). These results demonstrated that the mechanism for increased MMP2 protein was at the transcriptional level.

Discussion

AngII perfusion in apoE-deficient and wild-type C57BL/6 mice has been shown to promote the formation of aortic dissection and aneurysm

[15, 18]. These model systems [15] are invaluable for delineating the molecular mechanisms that underlie this pathological process and potentially the development of new therapies. Two results led us to test more directly the role of c-Abl in aortic dissection and thus the utility of imatinib in the treatment of it. The first is that imatinib was shown to interfere with AngII induced vascular remodeling [6]. The second was from our own work where we found that the systolic blood pressure was reduced in knockout Abl mice [8].

In our animal model presented here, we found that imatinib reduced the incidence of aortic dissection in AngII treated animals (**Figure 1**). This result is consistent with our hypothesis that AngII activates c-Abl. In addition, we found that the apoptosis of VSMC in AngII-treated mice was decreased after the intervention with imatinib, and we confirmed that imatinib can reduce the apoptosis of AngII-treated VSMC *in vitro* (**Figures 2** and **3**). Studies from other laboratories indicate though that imatinib attenuated AngII-induced vascular remodeling through the PDGF-signaling pathway [6]. This result does not necessarily contradict our finding that imatinib inhibits aortic dissection. It is possible that AngII elicits variable responses that are dependent upon diverse signaling pathways.

AngII has emerged as a critical peptide hormone that affects the function of vasculature, including induction of VSMC apoptosis. Therefore, AngII warrants investigation as a primary player in the pathogenesis of aortic dissection. Previous studies have shown that apoptosis of SMC occurs in AAA and may contribute to the disease process [2]. In addition, the most common form of arterial aneurysm in humans is characterized by a loss of VSMC from the vessel media, leading to progressive dilatation and eventually rupture [19]. Loss of SMC might, therefore, be the basis for aneurysmal disease and aortic dissection. The mechanism of imatinib inhibition of aortic dissection in mice may be the prevention of apoptosis of VSMC. We cannot conclude definitively that imatinib inhibits apoptosis by blocking c-Abl, as the molecule targets additional tyrosine receptor kinases, such as the PDGF receptor. However, imatinib has not been reported to inhibit apoptosis through the PDGF signaling pathway.

The level of phosphorylation of c-Abl in the aorta of AngII treated-mice was significantly higher than that of AngII plus imatinib treated-mice. This finding was confirmed *in vitro* (**Figure 4**). The significance of phosphorylation in our model systems remains unclear although we attempted to link phospho-c-Abl to apoptosis of VSMC. Results from other studies support our observations. Firstly, AngII was found to activate c-Abl in murine VSMC, and it is important for reactive oxygen species (ROS-), cSrc-, NOX-, and CAV1-dependent growth-related angiotensin II receptor, type 1 (AGT1R) signal transduction [5]. Secondly, AngII-stimulated VSMC apoptosis has also been shown to be mediated via ROS synthesis [20] which is increased in AAA. The increased oxidative stress could promote apoptosis of SMC [21]. Moreover, AngII-induced c-Abl activation mediated by ROS production has been observed [5]. Finally, it has been confirmed that c-Abl mediates apoptosis of cell types other than VSMC [12, 13].

Although we have focused on the role of c-Abl in apoptosis of AngII-treated VSMC and aortic dissection, the possibility exists that AngII activates multiple intracellular signaling pathways with diverse cellular outcomes. A previous study has shown that low dose of AngII induced hypertrophy of VSMC via c-Abl *in vivo* and [5], it may contradict the observation that c-Abl mediates VSMC apoptosis induced by high doses of AngII. However, the dose of AngII may lead to two different outcomes. Regardless, apoptosis and hypertrophy were both mediated via ROS synthesis in AngII-stimulated VSMC. Whether apoptosis and hypertrophy also co-exist in VSMC, requires further investigation.

MMP2 presents an important molecular mechanism for the degeneration of the vascular wall and subsequent aortic dissection in AngII treated animals. MMP2 is known to play a critical role in the remodeling of the ECM of arterial vessels, and it is expressed at a higher level in smooth muscle cells from the walls of AAA than in cells from controls [21]. Inhibition of MMP2 has been already suggested as a potential strategy for the prevention of the development of AAA and aortic dissection in animal models [22]. These results together with our findings indicate that imatinib could become an important component of treatment. Nevertheless, the relationship of imatinib and the regulation

of MMP2 to the c-Abl signaling pathway and pathological outcome requires further study [23].

Conclusions

We demonstrate for the first time that c-Abl is involved in the pathogenesis of aortic dissection in Ang II-treated mice. The results of the present study may provide a new therapeutic approach for patients suffering from aortic dissection.

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Disclosure of conflict of interest

None.

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