Oxidative stress modulates vascular smooth muscle cell phenotype via CTGF in thoracic aortic aneurysm

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Aims

Dissection and rupture of the ascending aorta are life-threatening conditions resulting in 80% mortality. Ascending aortic replacement in patients presenting with thoracic aortic aneurysm (TAA) is determined by metric measurement. However, a significant number of dissections occur outside of the parameters suggested by the current guidelines. We investigate the correlation among altered haemodynamic condition, oxidative stress, and vascular smooth muscle cell (VSMC) phenotype in controlling tissue homoeostasis.

Methods and results

We demonstrate using finite element analysis (FEA) based on computed tomography geometries that TAA patients have higher wall stress in the ascending aorta than non-dilated patients. We also show that altered haemodynamic conditions are associated with increased levels of reactive oxygen species (ROS), direct regulators of the VSMC phenotype in the microregional area of the ascending aorta. Using in vitro and ex vivo studies on human tissues, we show that ROS accumulation correlates with media layer degeneration and increased connective tissue growth factor (CTGF) expression, which modulate the synthetic VSMC phenotype. Results were validated by a murine model of TAA (C57BL/6J) based on Angiotensin II infusion showing that medial thickening and luminal expansion of the proximal aorta is associated with the VSMC synthetic phenotype as seen in human specimens.

Conclusions

Increased peak wall stress correlates with change in VSMC towards a synthetic phenotype mediated by ROS accumulation via CTGF. Understanding the molecular mechanisms that regulate VSMC towards a synthetic phenotype could unveil new regulatory pathways of aortic homoeostasis and impact the risk-stratification tool for patients at risk of aortic dissection and rupture.

Keywords

Thoracic aortic aneurysm • ROS • CTGF • VSMC phenotype

1. Introduction

Dissection of the ascending aorta is associated with excessive mortality.1–3 Aortic dissection and thoracic aortic aneurysm (TAA) are highly interrelated, as the only preventive treatment for aortic dissection is elective aortic replacement in patients with dilated ascending aortas.4,5 The current American College of Cardiology (ACC)/American Heart Association (AHA) guidelines recommend replacing the ascending aorta at the time of aortic valve replacement if the ascending aorta is > 5.5 cm.1,2,6,5 However, the optimal timing of aortic surgery in TAA patients remains uncertain.1–7,10,11 In a recent study, we reported that > 60% of patients with acute type A aortic dissection presented with aortic diameters < 5.5 cm.2,4–7 Therefore, the current surgical guidelines for ascending aortic aneurysm repair would fail to prevent the majority of acute aortic dissections seen in our cohort of patients. Although metric measurements are the current indications for elective surgical intervention, they are imperfect predictors of aortic dissection and rupture1,2,6,9,12,13 as they do not consider the status of cellular and extracellular components of the aortic tissues.
Biomechanical studies including our recent work have shown that aortic dissection or rupture occur when aortic wall stress exceeds aortic wall strength. We recently characterized the role that geometric indices and aortic wall stress distribution might play in the pathogenesis of thoracic aortopathies. In that prior work, fine element analysis (FEA) was applied to normal human thoracic aortic resection from electrocardiogram-gated computed tomography angiography (CTA) scans. We demonstrated the presence of increased wall stress in the human thoracic aorta above the sinotubular junction and distal to the origin of the left subclavian artery (the two locations where the tears resulting in aortic dissections typically occur). Although factors that increase aortic wall stress—such as aortic diameter and hypertension—or decrease wall strength—have been identified as risk factors for aortic dissection and rupture, there remains neither a clear understanding of the cellular pathophysiological mechanisms of aortic dilatation nor molecular mechanisms linking aortic wall stress with altered tissue homoeostasis. Therefore, we undertook to correlate computationally predicted wall stresses with changes in the local arterial wall homoeostasis.

Recent studies show that reactive oxygen species (ROS) are modulated by altered haemodynamic conditions and directly regulate cellular and extracellular components of the aortic wall. Vascular smooth muscle cells (VSMCs) are the main source of extracellular matrix (ECM) proteins in the aortic media, and the interplay between aortic wall stress, VSMCs, and ECM proteins is critical for the structural and functional integrity of the proximal aorta. At homeostasis, mature VSMCs maintain a quiescent differentiated state directed towards contractile function. In vascular pathologies contractile, VSMCs can change dramatically to a synthetic, fibroblast-like cell expressing abundant ECM proteins, and proteases. Therefore, VSMC phenotypic modulation in response to arterial stress potentiates the metabolic deregulation of ECM proteins. Here, we demonstrate that ROS, modulated by altered haemodynamic conditions, directly regulate the VSMC phenotype via connective tissue growth factor (CTGF). CTGF is a matricellular protein belonging to the transforming growth factor beta superfamily involved in ECM biosynthesis and VSMC proliferation and apoptosis. Since matricellular proteins are a class of non-structural and secreted proteins that regulate the bi-directional interaction between VSMCs and ECM proteins and act as mediators of biomechanical forces, we investigated CTGF role with respect to development of TAA in human surgically resected specimens. We therefore aimed to test the hypothesis that human ascending aortic aneurysms have higher wall stress that correlates with increased ROS-injury and change in the VSMC phenotype driven by CTGF.

2. Methods

2.1 Patients population and tissue collection
From January 2010 to February 2011, 106 patients were enrolled according to the approved IRB protocol #809349. This study conforms to the principles outlined in the Declaration of Helsinki. A detailed description of the subjects enrolled, the patients’ demographics, and the exclusion criteria are summarized in Supplementary material online, Table S1 and S2.

2.2 Finite element analysis
Wall stress was calculated using the finite element method, where complex geometry is parsed into small elements upon which numerical simulations are more readily carried out. A uniform pressure of 120 mmHg was applied to the luminal surface and a non-linear large deformation model was used. The aortic wall was modelled with first-order triangular shell elements. A uniform wall thickness of 1.7 mm was used based on echocardiography data. The arterial wall was assumed to be hyperelastic, homogeneous, and isotropic. A detailed description of the method is summarized in the Supplementary material online.

2.3 H2O2 and CTGF treatment of VSMC
A detailed description of the method is summarized in the Supplementary material online.

2.4 Statistical analysis
The data were analysed using the SPSS software (version 15; SPSS). Continuous variables were expressed as means ± standard deviation. Comparisons of continuous variables between groups were performed with Student’s t test or non-parametric (Mann–Whitney U test) tests as appropriate, depending upon normal distribution. Statistical differences between non-dilated and TAA patients and between treated cells compared with untreated, were analysed by Student’s t-test. Differences were considered statistically significant at values of $P < 0.05$.

3. Results

3.1 Altered wall stress correlates with accumulation of ROS and dysfunctional structure of the proximal aorta
A subset of 16 patients who underwent chest CT was retrospectively identified and analysed for image segmentation and aneurysm reconstruction followed by FEA. Figure 1A and B show that the average peak wall stress for patients with an aneurysmal ascending aorta was significantly higher than in patients with a normal ascending aorta (384 ± 34 vs. 236 ± 27 kPa, $P < 0.001$).

In the same patient population oxidative stress was analysed by staining for nitrotyrosine. Our results show intense staining in the medial layer of the TAA aorta (Figure 1F). Furthermore, antioxidant enzymes superoxide dismutases 1 and 2 (SOD1–2) gene expression were reduced in aneurysmal tissues by 2.2 ± 0.3 and 1.9 ± 0.5 fold, respectively, when compared with non-dilated (Figure 1D) ($P < 0.05$). Movat Pentachrome staining was then used to analyse the distribution and integrity of collagen fibres and proteoglycans. Verhoeff–Van Gieson staining was used to detect elastic organization. Staining on three sections/group was quantified using a score from 0 to 3. The aorta of TAA subjects shows increased proteoglycan and collagen deposition (blue and yellow staining, respectively, score = 2.5 ± 0.8) compared with non-dilated aortas (score 0.3 ± 0.5), together with dramatic loss of elastin fibres (black staining) and fragmentation (score = 2.3 ± 0.6 vs. score = 0.3 ± 0.3) (Figure 1C).

3.2 Synthetic VSMC phenotype and thoracic aortic aneurysm
To determine the impact of altered wall stress and tissue microstructure on cellular components, we analysed differences in the VSMC population of the aortic media in the region at high (concave) and low (convex) wall stress by the expression of a spectrum of markers: those of the contractile phenotype included SMA, myocardin, and smoothelin B. The synthetic phenotype was characterized by the decrease of contractile markers and up-regulated expression of osteopontin (OPN), MMP-9, ELK-1, and vimentin. We show that
human, surgically resected specimens from dilated and non-dilated aortas show a differential expression of SMA, vimentin and smoothelin B by western blotting and immunohistochemistry (Figure 2A–C). Smoothelin B and SMA are expressed abundantly in non-dilated aortas, both at the convex and concave side, whereas vimentin is minimally detectable. In contrast in the dilated aorta, smoothelin B and SMA levels are diminished in particular in the concave side of the ascending aorta (high wall stress), whereas vimentin is up-regulated. The expression of these markers was quantified by densitometry and the difference between their expression at the concave and convex side of the ascending aorta was plotted against CT scan measures of the ascending aorta diameter. As shown in Figure 2B, we observed a linear correlation between ascending aorta diameter and the expression of VSMC markers of phenotype. To validate the results, we performed real-time PCR using total mRNA extracted from surgically resected specimens. This analysis confirmed that in the dilated aorta, there is a decrease in contractile markers, such as SMA (−2.2 ± 0.4), and accumulation of markers of the synthetic phenotype such as OPN (2.04 ± 0.3) and MMP-9 1.7 ± 0.4. The myocardin-serum response factor (SRF)–Elk-1 complex has been shown to regulate the expression of SMC plasticity at the transcription level. SRF activates genes involved in smooth muscle differentiation and proliferation by recruiting muscle-restricted myocardin, as well as ternary complex factors of the ETS-domain family. Several growth signals have been shown to repress smooth muscle genes by triggering the displacement of myocardin from SRF by Elk-1. The opposing influences of myocardin and Elk-1 on smooth muscle gene expression are mediated by structurally related SRF-binding motifs that compete for a common binding site on SRF. In the tissues analysed ELK-1 was undetectable in control non-dilated aorta, but is significantly induced in TAA specimens (Figure 2E and F), whereas myocardin expression was not changed. ELK-1 and SRF expression were also assayed by immunofluorescence on primary human VSMCs harvested from non-dilated and aneurysmal patients. Once again, ELK-1 expression was higher in TAA isolated VSMCs when compared with controls and co-localized in the nuclei of the cells with SRF (Figure 2G).

3.3 Overexpression of connective tissue growth factor is associated with thoracic aortic aneurysm

We then investigated possible downstream effectors of ROS-mediated VSMC regulations. RT² PCR Array for 84 matricellular and cellular adhesion protein genes was performed in a total of 16 patients followed by validation in a total of 20 patients (Figure 3A). A complete list of tested molecules is provided in Supplementary material online, Table S3. Among these genes, we found CTGF to be associated with TAA in human specimens. CTGF up-regulation was then validated.
by immunohistochemistry, real-time PCR and western blotting (Figure 3B–E). As shown in Figure 3E both nitrotyrosine and CTGF are up-regulated in aneurismal tissues with no significant difference in the concave and convex side of the ascending aorta. CTGF and nitrotyrosine quantification revealed that there is a linear correlation between ascending aorta diameter and the expression of these markers (Figure 3F).

3.4 CTGF modulates VSMC synthetic phenotype in vitro

To test the effect of CTGF as a direct modulator of the VSMC phenotype and ECM biosynthesis primary human VSMCs were treated with hrCTGF in the presence or absence of a CTGF neutralizing antibody. Figure 4A shows that CTGF induces vimentin expression, which was blocked by CTGF neutralizing antibody. Increasing concentration of hrCTGF (from 20 to 80 ng/mL), doses previously reported to modulate collagen synthesis in vitro,18, 19, 28 was used to test a dose-dependent response of vimentin under CTGF treatment by real-time PCR. As shown in Figure 4B, CTGF induces an up-regulation of the synthetic marker vimentin, 15- and 25-fold, respectively. Notably, CTGF did not significantly alter smoothelin B expression. Also, consistent with a phenotypic transition towards a synthetic phenotype we observed a significant up-regulation of the COL1, FN, ITGα5, and ITGβ1, MMP-2, -9, -14 (P < 0.05) in response to CTGF treatment (Figure 4C and D). Then, we isolated aortic VSMCs from dilated and non-dilated ascending aorta according to previously described protocol.4, 20 To test the ability of human isolated VSMCs to modulate their phenotype in response to CTGF stimulation, we treated the cells with hrCTGF and assayed smoothelin B and vimentin expression. In response to CTGF,
VSMCs up-regulated the expression of synthetic markers of phenotype (Figure 4E).

3.5 Oxidative stress mediates the expression of synthetic VSMC markers via CTGF

To test whether the accumulation of synthetic VSMCs is mediated by ROS via CTGF, human patient-derived VSMCs were treated with H₂O₂ in the presence or absence of 30 μM SOD mimetic MnTMPyP and then CTGF expression was quantified (Figure 5A). We observed that MnTMPyP blocks H₂O₂-induced CTGF expression in VSMCs. To confirm the modulation of VSMC phenotype towards a synthetic phenotype we assayed the expression of vimentin (+3.91), MMP-2 (+1.61), MMP-9 (+7.14), and CTGF (+2.96) in patient-derived VSMC treated with 100 or 200 μM H₂O₂ (P < 0.05) (Figure 5B and C). We then tested whether CTGF neutralizing antibody was able to modulate vimentin expression under H₂O₂ treatment. Figure 5D shows a reduction of vimentin when VSMCs were pre-treated with CTGF neutralizing antibody (P < 0.05).

3.6 Angiotensin II infusion provokes media thickness, aortic aneurysm development, nitrotyrosine, and vimentin accumulation

Finally, to examine the role of ROS on CTGF and VSMC phenotype in vivo, we used an Angiotensin II (Ang II) infusion model in wild-type mice (C57BL/6J) under a hypercholesterolaemic diet. Seven-week-old male mice were fed with a hypercholesterolaemic diet and infused with saline (Controls), or Ang II (1000 ng/kg/min) using osmotic pumps, for 28 days, as previously described. Twenty-eight treated and 12 untreated mice were used. Aortic aneurysms were observed in 50% of the Ang II-treated group compared with the control (saline) group, none of which developed aortic pathology. Eight per cent of AngII-treated mice died from aortic dissection or rupture before the 28th day of treatment. During the course of Ang II infusion luminal expansion was observed throughout the entire ascending aorta that progressed during prolonged treatment. Animals subjected to chronic Ang II infusion show increased luminal diameter 1.12 ± 0.02 vs. 1.81 ± 0.06 mm (P < 0.01) (Figure 6A–C) and increase in systolic blood pressure (201 ± 13 vs. 127 ± 10 mmHg). Pathological medial changes were present.
**Figure 4** CTGF modulates VSMC synthetic phenotype in vitro. (A) vimentin and smoothelin B expression in VSMCs treated with hrCTGF in the presence or absence of CTGF neutralizing antibody and relative densitometry. (B) vimentin and smoothelin B expression assayed by qPCR (*p < 0.05). (C and D) In vitro hrCTGF treatment of VSMCs on ECM components analysed by qPCR (*p < 0.05). (E) Smoothelin B and vimentin expression in VSMC isolated from non-dilated and dilated tissues treated with 80 ng/mL of hrCTGF. GAPDH as a loading control. Data are means ± SD.

**Figure 5** Oxidative stress mediates the expression of synthetic VSCM markers via CTGF. (A) Western blotting and densitometry showing CTGF expression in VSMC treated with H₂O₂ in the presence or absence of MnTMPyP. GAPDH was used as a loading control. (B and C) CTGF, vimentin, MMP-2, and MMP-9 expression in VSMC treated with 100 or 200 μM H₂O₂ assayed by qPCR. Fold change were normalized against actinB (*p < 0.05). (D) Western blotting and densitometry of VSMCs treated with 100 μM H₂O₂ assayed for vimentin expression in the presence or absence of CTGF neutralizing antibody. Data are means ± SD.
concentrically and include wall thickening (6.2 vs. 14.1 mm, \( P < 0.01 \)) and extensive elastin fragmentation (Figure 6C). Concomitantly with luminal expansion, we observed nitrotyrosine accumulation and up-regulation of both CTGF and vimentin further corroborating the observation in aneurysmal human tissues (Figure 6D and E). To evaluate whether CTGF and vimentin up-regulation were indeed related to ascending aorta dilatation real-time PCR was performed using RNA extracted from both the ascending and descending aorta of saline and AngII-treated mice (Figure 6D).

4. Discussion

The current indications for surgical intervention of a dilated ascending aorta are imperfect predictors of adverse aortic events, as metric measurements do not consider the cellular and molecular characteristic of the aortic tissues. The data reported in this paper addresses directly the problem of aortic tissue ‘quality’ in patients with dilatation of the proximal aorta linking wall stress to oxidative injury and VSMC activation. This work provides several new insights into the pathophysiology of thoracic aortic aneurysm and dissection:

- The function of the thoracic aorta is the result of a dynamic, regulated and highly preserved micro-structural organization, which correlates with both haemodynamic conditions and the cellular and extracellular components of the aortic wall. Growing evidence suggests that altered wall stress distribution and predisposing genetic background participate in the overall risk of thoracic aortopathies.1,2

Figure 6 Angiotensin II infusion provokes media thickness, formation of aortic aneurysms, and vimentin accumulation. (A) Ang II-induced ascending aortic aneurysm (B) AA expansion (in mm) in mice with Ang II-induced TAA compared with saline-infused controls measured by TEE. (C) Histology of the ascending aorta showing medial thickness and measure of luminal diameter (mm). (D) CTGF and vimentin quantification by qPCR performed using RNA obtained from the ascending (AA) and descending (DA) aorta. (E and F) Immunohistochemistry showing CTGF, nitrotyrosine and vimentin expression in saline vs. ANGII-induced aneurysmal aorta. Magnification \( \times 10–40 \). Data are means ± SD.*\( P < 0.05 \).

Here, we report for the first time an analysis of peak wall stress of non-dilated compared with dilated ascending aortas. Our results show that aneurysmal aortas have higher peak wall stress compared with non-aneurysmal. Aortic wall stress can predict aortic dilation,34 and by extension rupture and dissection. In addition, hydrostatic pressure-induced wall stress influences VSMC physiological responses in the aortic wall further supporting this putative relationship.

- Despite its clinical importance, the cellular and molecular determinants of aortic wall structure are relatively unexplored in human pathological specimens. Our data and recent studies show that ROS are induced by altered haemodynamic conditions and directly regulate the cellular and extracellular components of the aortic wall.3,4 The higher level of nitrotyrosine in the TAA aorta is also in accordance with the marked inflammation previously observed in these types of tissues.5,6 Furthermore, both our human and murine studies revealed that dilatation of the proximal aorta is associated with induction of the VSMC synthetic phenotype markers including vimentin, MMPs and ELK-1/ESR pathways along with disorganization of the ECM structure. According to previous reports the dysfunctional organization of the media, along with increased oxidative stress, could be responsible for the increased propensity to dissect and rupture in patients with dilated aortas. Notably, chronic infusion of Ang II was previously used in ApoE−/−mice to generate both thoracic and abdominal aortic aneurysm.7,8 Growing evidence indicate that Ang II induces its pleiotropic vascular
effects through NADPH-driven generation of ROS and it has been reported that NADPH activity shifts VSMCs towards a synthetic phenotype and increases collagen I production in prosthetic vascular graft. Our results show that Ang II infusion in mice induces medial thickening and luminal expansion of the proximal aorta together with accumulation of synthetic VSMC as seen in human TAA specimens. In addition, our data reveals a specific dilatation of the ascending thoracic aorta using Ang II chronic infusion on wild-type hypercholesterolemic mice (CS7BL/6j).

- We reported that CTGF expression is up-regulated in human and murine thoracic aneurysmal aorta, it is induced by ROS, and directly regulates VSMC synthetic markers. In response to vascular injury VSMC become synthetic and increase their ability to produce ECM playing in this way a critical role in vascular repair. However, the negative effect of VSMC plasticity is that it can predispose to an abnormal response that ultimately contributes to the progression of vascular disease. Here, we demonstrate that CTGF induces the expression of VSMC synthetic markers both directly and indirectly mediating ROS effect. As reported in Figure 1, the presence of dilatation of the ascending aorta is associated with reduced SOD 1 and 2 expressions. We, therefore, tested in vitro whether the exogenous delivery of SOD mimetics could revert the phenotypic differentiation of primary isolated H2O2-treated VSMC. We decided to use MnTMPyP for its stability over the use of SOD adenosine.

There are several limitations associated with this study. First, FEA techniques do not include the effects of blood flow. However, the effects of wall shear stress have been shown to be several orders of magnitude lower than static stresses calculated using pressure-deformation analyses. Secondly, static boundary conditions were assumed, so the systolic motion of the heart was not captured in this model. Studying the effects on ascending aortic wall stress through the cardiac cycle represents an area of future research. Thirdly, material properties derived from the strain testing of excised abdominal aortas were used in this work; studies have shown that the arterial wall in the ascending aorta has twice the breaking strength of AAA specimens, but detailed elastic properties of the ascending aorta are unavailable. Finally, a uniform wall thickness was assumed in these models, even though pathological specimens show significant variation in aortic thickness. Unfortunately, no validated techniques for extraction of local wall thickness from imaging data are available, particularly in the proximal ascending aorta where systolic motion artefact confounds any attempts to accurately determine aortic thickness. Further multi-scale studies linking micro- and macro-structure of the aortic wall with the mechanical forces are needed to fully evaluate the mechanism regulating the VSMC-matrix complex and, overall, the aortic tissue homoeostasis. However, the delineation of the cellular and extracellular regulators of aortic dilation, such as CTGF and its molecular pathway could help in the surveillance of patients with aortic wall pathologies and in optimizing medical therapy as well as the timing of surgical intervention.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Conflict of interest: none declared.

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