S100A12 Mediates Aortic Wall Remodeling and Aortic Aneurysm

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Rationale: S100A12 is a small calcium binding protein that is a ligand of RAGE (receptor for advanced glycation end products). RAGE has been extensively implicated in inflammatory states such as atherosclerosis, but the role of S100A12 as its ligand is less clear.

Objective: To test the role of S100A12 in vascular inflammation, we generated and analyzed mice expressing human S100A12 in vascular smooth muscle under control of the smooth muscle 22α promoter because S100A12 is not present in mice.

Methods and Results: Transgenic mice displayed pathological vascular remodeling with aberrant thickening of the aortic media, disarray of elastic fibers, and increased collagen deposition, together with increased latent matrix metalloproteinase-2 protein and reduction in smooth muscle stress fibers leading to a progressive dilatation of the aorta. In primary aortic smooth muscle cell cultures, we found that S100A12 mediates increased interleukin-6 production, activation of transforming growth factor β pathways and increased metabolic activity with enhanced oxidative stress. To correlate our findings to human aortic aneurysmal disease, we examined S100A12 expression in aortic tissue from patients with thoracic aortic aneurysm and found increased S100A12 expression in vascular smooth muscle cells.

Conclusions: S100A12 expression is sufficient to activate pathogenic pathways through the modulation of oxidative stress, inflammation and vascular remodeling in vivo. (Circ Res. 2010;106:145-154.)

Key Words: S100A12 • calgranulins • smooth muscle cell differentiation • RAGE • aortic aneurysms

Members of the S100/calgranulin family such as S100A8 (myeloid related protein-8; calgranulin A), S100A9 (myeloid related protein-14; calgranulin B), and S100A12 (EN-RAGE; calgranulin C), are members of the calcium binding proteins implicated in the regulation of a variety of intracellular and extracellular activities. They are endogenously expressed in cells intimately linked to vascular disease such as granulocytes and myeloid cells. Calgranulins are associated with disease activity in chronic inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, asthma, and Kawasaki vasculitis. In a number of clinical settings, serum concentrations correlate with disease activity and, therefore, may serve as biomarkers of cell stress.

Calgranulins promote inflammation and vascular perturbation by activating RAGE (receptor for advanced glycation end products) and toll-like receptor-4; both receptors are strongly linked to vascular dysfunction and atherosclerosis. The importance of RAGE as a mediator of vascular stress and inflammation in atherosclerosis was recently shown in apolipoprotein E–null mice that also lacked RAGE.

In the absence of RAGE, apolipoprotein E–null mice developed significantly less atherosclerosis in euglycemic and hyperglycemic conditions. Less is known about the RAGE ligands, the S100/calgranulins. S100/calgranulins are not detectable in normal vascular smooth muscle cells (VSMCs) but are induced in VSMCs after endothelial cell wire injury, and in neovascular SMCs in the atherosclerotic arterial wall. Burke et al found strong expression of S100A12 in human coronary artery SMCs associated with plaque rupture and sudden cardiac death, suggesting that S100A12 is either a marker or mediator of VSMC dysfunction.

To study this issue, we exploited the fact that S100A12 is not present in mice and generated transgenic (TG) mice expressing human S100A12 in VSMCs driven by the smooth muscle (SM)22α promoter. We found aneurysmal dilatation of the aorta with disarray of elastic fibers, increased fibrosis, increased matrix metalloproteinase (MMP)-2 protein, and reduction in VSMC contractile elements. Furthermore, cultured aortic VSMCs from TG mice had increased production of cytokines and elevated measures of oxidative stress. We
next examined human aortic tissue obtained from patients with thoracic aortic aneurysms (TAA)s and found strong expression of S100A12 in the VSMCs near the site of cystic media necrosis. Together these data demonstrate that expression of S100A12 in VSMCs is sufficient to modulate aortic wall remodeling and suggest that overexpression of S100A12 in human aortic aneurysms is pathogenic.

**Methods**
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org and contains details on generation of TG (TG) mice expressing human S100A12 in VSMCs driven by the SM22-α. Hemizygous TG mice were mated with C57BL6/J (The Jackson Laboratory). TG and wild-type (WT) littermates not expressing the transgene were used for all experiments. All procedures were carried out with the approval of the Institutional Animal Care and Use Committee of The University of Chicago. Human vascular tissue was obtained with informed consent and approved by the University of Chicago Institutional Review Board.

**Results**

**Generation of TG Mice Expressing Human S100A12 in Aortic Smooth Muscle**
Human S100A12 was expressed in TG mice under control of the SM22α promoter (Figure 1A). Of the 9 S100A12 founder animals, 3 lines were propagated based on a variable copy number. TG mice developed normally and were fertile and healthy. There were no differences in body weight, plasma cholesterol and blood pressure between TG and WT littermate mice (Online Table 1). Mating of hemizygous TG mice with C57BL/6J WT mice produced ~50% TG offspring with an equal gender distribution. TG and WT mice, age 12 to 15 months, had no differences in mortality (5% versus 7%, n=20 for each group; P=NS).

Expression of S100A12 protein within the vasculature was examined by immunofluorescence microscopy in 10-week old mice (Figure 1D). S100A12 was detected in the VSMC-rich medial layer of the aorta from TG but not in WT mice. Furthermore, primary aortic VSMCs from TG mice, but not from WT littermates, expressed S100A12 when cultured for up to 8 passages (Figure 1E). Expression of S100A12 protein was detected in tissue extracts rich in vascular smooth muscle cells, including the aorta and lung (Figure 1F and Online Table 1). No expression was detected in the esophagus, stomach, urinary bladder, kidney, liver, and brain (data not shown).

To compare S100A12 protein expression between TG mice and humans with aortic disease, we performed semiquantitative immunoblotting using 10 µg of protein extract from the ascending aorta of patients undergoing aortic root repair for TAAas. S100A12 expression in TG animals was comparable to protein levels observed in patients with aneurysmal aortic disease (Figure 1G). VSMCs were cultured from those surgical samples and increased expression of S100A12 was observed in TAA-SMCs but not in control SMCs (Figure 1H). This increase is not explained by contaminating leukocytes in the primary VSMC culture, because immunoblotting and quantitative RT-PCR for CD68 and CD45 was negative (data not shown). Stimulation with lipopolysaccharide (LPS) (1 µg/mL) revealed a strong upregulation of S100A12 in both, control and TAA-SMCs and is comparable to the expression level achieved in cultured SMCs from TG aorta (Figure 1H).

**S100A12 TG Mice Have Pathological Aortic Remodeling**
The aorta was examined in 10- and 16-week-old TG and WT littermate mice. TG mice displayed disrupted aortic wall architecture which was seen at age 10 weeks and was more pronounced at 16 weeks of age (Figure 2). Histological grading of median elastin degradation revealed a significant increase in elastin fiber disruption in TG mice (grade 2 for 10-week-old TG mice and grade 2 to 3 for 16-week-old TG mice compared to no elastic fiber disruption in WT mice; Figure 2Q). Furthermore, we observed absent wall thickening in TG mice. Quantification of the aortic wall thickness from histology sections of the aortic arch revealed significant increase in wall thickness at 10 and 16 weeks of age (Figure 2R). In parallel, the TG mice showed loss of VSMCs and overt fibrosis within the medial layer of the aorta as demonstrated by Masson trichrome staining (Figure 2J and 2L). Quantitative analysis of the aortic arch revealed increased fibrosis in the medial layer in TG mice at 10 weeks (12±4% versus 2±0.4%) and at 16 weeks (22±3% versus 3±0.3%) compared to WT littermates (Figure 2S). This phenotype was also present in other TG lines (Online Figure I).

S100A12 protein is a monocytes chemotaxant, resulting in neutrophil and monocyte recruitment in vivo. We therefore examined aortic tissue to evaluate if infiltration of inflammatory cells precedes the pathological remodeling observed in 10-week-old mice. At 4 and 6 weeks of age, TG aorta showed normal architecture with normal wall thickness, absent fibrosis, and no inflammatory cells within the aortic wall, either in the vasa vasaor or intima or media (data not shown). Because detection of infiltrating leukocytes by microscopy has limited sensitivity, we assayed aortic tissue for myeloperoxidase (MPO), a heme protein derived from leukocytes. Expression of S100A12 in smooth muscle resulted in a significant increase in MPO activity in TG aorta. This was observed at all ages studied, but most profoundly in TG mice with established aortic remodeling at age 10 and 16.
weeks (Figure 2T). Enhanced MPO activity is linked to generation of oxidative stress, and therefore may initiate or exacerbate dysfunction of VSMCs in the TG mice. In aortic tissue of 8-week-old mice, we found a reduction of vinculin, smooth muscle myosin heavy chain, and smooth muscle α-actin. We noted an increase of VCAM-1 in the aortic tissue of TG mice (Figure 3A). Furthermore, we found increased MMP-2 protein in the aortic tissue of TG mice compared to WT mice (6.2 fold; P<0.001; Figure 3B). To elucidate whether this increase depends on infiltrating leukocytes, we assayed cultured aortic SMCs from TG aorta and WT aorta. TG-SMCs showed a significant increase of MMP-2 protein expression in TG but not in WT mice. G, Immunoblotting with α-S100A12 IgG of lung and aortic tissue extracts reveals S100A12 protein expression in TG but not in WT mice. I, Immunoblotting with α-S100A12 IgG and β-actin IgG of whole cell lysates from cultured VSMCs shows no S100A12 expression in WT-VSMCs at passage 3 to 8 (lanes 4 to 6). J, Immunoblotting with α-S100A12 IgG of lung and aortic tissue extracts reveals S100A12 protein expression in TG-VSMCs at passage 3 to 8 (lanes 4 to 6). K, Immunoblotting with α-S100A12 IgG and β-actin IgG of whole cell lysates of cultured aortic SMCs, SMCs from human thoracic aneurysm at baseline (lane 1) and after LPS 1 μg/mL for 24 hours (lane 2), SMCs from human control aorta at baseline (lane 3), and after 1 μg/mL LPS for 24 hours (lane 4), SMCs from TG aorta (lane 5), and SMCs from WT aorta (lane 6).  

**Figure 1.** Generation of S100A12 TG mice. A, Human cDNA S100A12 was expressed under control of the smooth muscle promoter SM22α. B, PCR showing TG + mice. C, Southern blotting revealed S100A12 incorporation. D, Immunofluorescence microscopy of aortic frozen sections from WT mice (top) and TG mice (bottom) were stained with α-S100A12 IgG (red), α-smooth muscle actin IgG (SMA) (green) and DAPI (blue). E, Immunofluorescence microscopy of cultured primary VSMCs isolated from WT mice (left) and TG mice (right) were stained with α-S100A12 IgG (red) and DAPI (blue). F, Immunoblotting with α-S100A12 IgG of 10 μg whole cell lysates from cultured VSMCs shows no S100A12 expression in WT-VSMCs (lanes 1 to 3) and strong S100A12 expression in TG-VSMCs at passage 3 to 8 (lanes 4 to 6). H, Immunoblotting with α-S100A12 IgG and β-actin IgG of whole cell lysates of cultured aortic SMCs, SMCs from human thoracic aneurysms at baseline (lane 1) and after LPS 1 μg/mL for 24 hours (lane 2), SMCs from human control aorta at baseline (lane 3), and after 1 μg/mL LPS for 24 hours (lane 4), SMCs from WT aorta (lane 5), and SMCs from WT aorta (lane 6).

TG mice developed aortic aneurysms. We examined the aortic root and the midaortic arch using serial in vivo echocardiography at age 10 weeks, 16 weeks and 22 weeks (n=4 for each group; Figure 4). At 10 weeks of age, no difference between TG and WT mice was observed in the aortic root size (Figure 4) and in the size of the aortic arch (data not shown). At age 16 and 22 weeks, the aortic root and arch were consistently dilated in the TG mice compared to WT littermates. In TG male mice, the aortic root increased significantly at age 16 weeks by ≈23% (1.72±0.19 mm; P=0.01) and only ≈5% in WT mice (1.45±0.17 mm; P=NS), and remained dilated without further changes at age 22 weeks (1.75±0.15 and 1.46±0.11 mm). In TG female mice, the aortic root increased significantly at age 16 weeks by ≈20% (1.59±0.17 mm; P=0.01) and only ≈5% in WT mice (1.33±0.15 mm; P=NS) and remained dilated without further changes at age 22 weeks (1.61±0.12 and 1.32±0.17 mm). Similarly, measurements for the aortic arch dimensions were equal at the 10-week baseline measurement, and TG mice had an ≈20% increase at age 16 weeks without further progression at 22 weeks (both sexes; data not shown). We did not observe spontaneous rupture of the aorta, and there was no difference in mortality between TG and WT littermates observed in mice aged up to 15 months.

**S100A12 Augments Cytokine Production in Vascular Smooth Muscle Cells**

Previously it was shown that S100A12 induces upregulation of proinflammatory cytokines, most notably induction of
interleukin (IL)-6 in RAGE-bearing human monocytes and in human cord blood–derived mast cells lacking RAGE expression. We therefore tested the hypothesis that S100A12 augments IL-6 production in cultured primary aortic VSMCs harvested from TG mice and WT littermate controls. IL-6 was increased in the cell culture supernatant from S100A12 expressing VSMCs compared to WT-VSMCs (417 ± 35 versus 160 ± 20 ng/mL; P = 0.001; Figure 5A). This difference did not derive from variable leukocyte contamination in the cultured primary aortic VSMCs as assessed by immunoblot using anti-murine CD45 IgG and by RT-PCR using specific primers (data not shown). In VSMCs, IL-6 is induced on stimulation with LPS. At a dose not associated with cytotoxic effects (data not shown), there was a significant increase in IL-6 production in both TG-VSMCs and WT-VSMCs (2130 ± 160 and 415 ± 54 ng/mL, respectively;
Figure 3. Abnormal aortic tissue composition. A, Immunoblot from aortic tissues from TG and WT (n=3 for each group). *P<0.05. B, Representative gelatin zymogram from aortic tissue lysates from WT and TG mice (n=4 for each group for quantitation). *P<0.01. C, Gelatin zymogram from concentrated supernatant of cultured aortic SMCs harvested from WT and TG aorta. The insert shows an immunoblot of lysed SMCs using anti-MMP-2 IgG.

Figure 4. In vivo ultrasound of the aortic root of TG mice and WT littermate mice. A through C, Baseline ultrasound measurements were performed at 10 weeks of age (A), followed by repeat ultrasound at 16 weeks (B) and 22 weeks (C) of age. Ao indicates proximal aorta; PA, pulmonary artery; BC, brachiocephalic trunk. Aortic root dimensions were measured 0.5 mm proximal of the aortic valve. Scale bar=1 mm. D and E, Male (D) and female (E) TG mice (n=4) showed significant dilatation of the aortic root at age 16 and 22 weeks compared to their respective gender-matched littermates (n=4 for each group). *P<0.01.

P=0.001). However, the increase was 5 fold higher in the S100A12 expressing VSMCs (P<0.01; Figure 5A). Pretreatment of the VSMCs with 5 and 10 µg/mL of soluble RAGE attenuated the LPS induced IL-6 secretion by 40% in the TG-VSMCs (1278±65 and 1345±69 ng/mL, respectively; P<0.05) but not in WT-VSMCs (390±61 and 410±56 ng/mL, respectively; P=NS). Soluble RAGE had no effect on baseline IL-6 secretion in TG- or WT-VSMCs.

We examined whether S100A12 was released into the cell supernatant where it could activate VSMC surface RAGE. S100A12 was not detected in either cell supernatant from TG-VSMCs or WT-VSMCs under basal conditions, but was detected in the supernatant of TG-VSMCs on stimulation with low dose LPS (1 µg/mL; Figure 5B), suggesting that S100A12 may amplify LPS induced IL-6 production in a RAGE-dependent manner. IL-6 was measured in the supernatant of freshly harvested aortic rings (n=4 mice per group).

At baseline, there was no difference in the IL-6 production, but in response to stimulation with 1 µg/mL LPS, the aortic ring tissue from TG mice produced 2.2 fold more IL-6 than the aortic tissue from WT mice (980±175 and 450±51 ng/mL, respectively; P=0.03; Figure 5C). We next examined serum IL-6 levels in TG and WT littermate mice housed in a barrier facility (Figure 5D). There was no difference in serum IL-6 at 4 weeks (41±5 and 50±8 ng/mL; P=NS) and at 8 weeks (45±7 and 43±8 ng/mL; P=NS). As the mice aged, we found increased serum IL-6 in TG mice at 12 weeks (95±18 and 55±10 ng/mL; P=0.04) and at 16 weeks (135±15 and 55±11 ng/mL; P=0.01, n=6 in each group and time point).

The increased collagen deposition in the aortic medial layer and the histological changes in S100A12 TG mice are similar to the aortic remodeling observed in mice that model Marfan syndrome.22,23 Fibrillin-1 regulates transforming growth factor (TGF)-β sequestration and in the presence of fibrillin-1 mutations, there is an increase in TGF-β availability and increased Smad2 phosphorylation and nuclear localization.23 Similar to Marfan syndrome, aortic VSMCs from S100A12 TG mice displayed increased phosphorylation and nuclear translocation of Smad2 (Figure 5E). Furthermore, nuclear translocation of pSmad2 depends on the availability of TGF-β, because treatment of TG-VSMCs with neutralizing TGF-β antibody prevented nuclear translocation of pSMad2 (Figure 5E).

S100A12 Expression Increases Oxidative Stress and Metabolic Activity in Vascular Smooth Muscle Cells

We investigated the effect of S100A12 on VSMC proliferation because some members of the S100 protein family have been implicated in VSMC migration, proliferation, and cy-
BrdUrd-positive nuclei, respectively; 

Reduced proliferation, TG-VSMCs bioreduced MTS into formazan 1.9- to 2.7-fold more than WT-VSMCs (Figure 6B). Reduced proliferation, 

Figure 5. S100A12 induces IL-6 and nuclear translocation of pSmad2. A, ELISA measuring IL-6 from cell culture supernatant from TG-VSMC and WT-VSMC cultures stimulated with 1 µg/mL LPS and soluble RAGE as indicated, \( P < 0.001 \) compared to baseline condition; \( P < 0.05 \) compared to LPS-stimulated condition. B, Immunoblotting with α-S100A12 IgG using culture supernatant from TG-VSMCs (lanes 1 and 2) and WT-VSMCs (lanes 3 and 4) revealed S100A12 protein release into the cell culture supernatant only from TG VSMCs after treatment with LPS. C, IL-6 was also produced from aortic ring tissues (3 mm) after LPS exposure. \( P = 0.03 \). D, IL-6 ELISA from mouse serum. \( P < 0.05 \). E, Immunofluorescence microscopy of cultured primary VSMCs isolated from WT mice (top) and TG mice (middle and bottom) were stained with α-pSmad2 IgG and DAPI. Scale bar=20 µm. Pretreatment with neutralizing TGF-β (0.5 µg/mL) is shown (bottom).

S100A12 is expressed in vascular smooth muscle cells in human thoracic aneurysms

To evaluate weather increased S100A12 occurs in human aortic diseases, we studied a 34 year old male with familial thoracic aneurysm syndrome. The patient (II.1 in Figure 7A) developed a thoracic aneurysm affecting the proximal aorta, just above the sinus of Valsalva at the age of 24, and, at age 34, he developed marked dilatation above the previous repair site, necessitating repair. His brother displayed a similar course at age 33, and both were non-Marfanoid. The patient underwent clinical genetic evaluation for fibrillin-1, TGF-β receptor-1, TGF-β receptor-2, ACT2, and MYH11. A novel mutation was identified in MYH 11 c3757-3759delAAG, leading to the deletion of amino acid 1253, a lysine. The histopathology of the resected proximal aorta showed disruption of the tunica media with accumulation of myxoid material, degeneration and loss of VSMCs, as well as disruption of the elastic fibers, consistent with cystic medial necrosis (Figure 7). Strong expression of S100A12 protein was seen in the medial layer,
particularly in areas of degeneration and dissection. S100A12 was not detected in control aortic tissue. The S100A12 staining colocalized with smooth muscle actin positive staining (Figure 7E).

Discussion

S100A12 is constitutively expressed in neutrophilic granulocytes where it accounts for up to 5% of the cytoplasmic protein. As it relates to chronic vascular perturbation, S100A8/9 are present in foam cells and endothelial cells in the atherosclerotic aortic wall and elevated plasma levels of the S100A8/9 heterodimer are associated with increased risk of cardiovascular events. Furthermore, transcriptional profiling of platelets identified S100A8/9 as a novel regulator of atherothrombosis and S100A12 was identified in SMCs in ruptured coronary artery plaques in patients with sudden cardiac death. Together, these data suggest a possible role of S100A12 in mediating VSMC dysfunction. Beyond serving as a marker for atherothrombotic risk, it is still unclear whether S100A12 indeed regulates the development of vascular disease. To determine whether S100A12 expression is sufficient to mediate vascular disease, we expressed human S100A12 in murine vascular smooth muscle cells. S100A12 expression was sufficient to change the phenotype of VSMCs with increased IL-6 production, enhanced TGF-β signaling pathways, increased oxidative stress, and increased MMP2 protein. Whereas our in vitro studies using cultured VSMCs from WT and TG aorta demonstrated a direct effect of S100A12 on VSMCs, the biology leading to aortic remodeling appears likely to be more complex in vivo. We found increased MPO activity in aortic tissue as evidence for infiltrating leukocytes, although inflammatory cells were not yet seen on histology. We speculate that enhanced MPO activity triggers oxidative stress, thereby providing at least in part an environment capable of augmenting VSMC dysfunction, leading to remodeling of the aortic wall with aneurysm formation. In vivo, we found markedly reduced expression of contractile SMC fibers and increased collagen accumulation, together with increased MMP-2 levels and increased elastin fiber breakdown. It is interesting, that S100A12 augments the breakdown. It is interesting, that S100A12 augments the levels of latent MMP-2 but had only minimal effect on the active form of MMP-2 when assessed by standard gelatin zymography. Accumulation of extracellular matrix proteins and increased elastin fiber breakdown. It is interesting, that S100A12 augments the MMP synthesis and increase ECM degradation.
We found S100A12 increases LPS induced IL-6 production in VSMCs. Some S100A12 may be released and mediate effects through cell surface receptors. However, the observation that soluble RAGE could only partially block LPS induced IL-6 production argues for the importance of intracellular S100A12. We speculate that S100A12 may serve as an amplifier of LPS signaling. To explain the pathology in S100A12 TG mice, it is possible that normal amounts of LPS present in a pathogen free mouse barrier facility may propagate enough IL-6 in the VSMCs of S100A12 TG mice to sustain vascular inflammation and remodeling. TG mice have normal vascular morphology by histology in the first weeks of life but develop morphological remodeling by the age of 8 to 10 weeks, followed by functional impairment, leading to aneurysmal dilation. This time course suggests that environmental factors may trigger or augment SMC dysfunction in S100A12 TG mice, and exposure to pathogens may be among those triggers. Based on our in vitro results (Figure 5), we hypothesize that a more proinflammatory environment would alter the severity of vascular remodeling and possibly lead to rupture of the aneurysmal remodeled aorta. However, under the conditions described here in a pathogen-free barrier facility, we did not observe impaired survival or rupture of the aortae. Therefore, S100A12 may accelerate vascular disease via increased IL-6 production under conditions associated with increased endotoxin levels. In humans, increased endotoxin levels have been found in periodontal disease, chronic inflammatory bowel diseases, and cigarette smoking, and this may all be sufficient to increase S100A12 levels and amplify IL-6 production.

Furthermore, we demonstrated increased metabolic activity and mitochondrial ROS production in cultured TG-VSMCs. S100 proteins have been previously shown to increase cytosolic H$_2$O$_2$ production via the NADPH oxidase system in VSMCs. Activation of RAGE by advanced glycation end products is associated with mitochondrial superoxide generation in rat mesangial cells when cultured in high glucose medium. Together, these data suggest a role for AGE/RAGE mediated mitochondrial dysfunction. Our studies here demonstrate that intracellular expression of S100A12 can serve as a potent inducer of mitochondrial ROS. Of interest, S100A12 and its homolog S100A8/9 are associated with strong antimicrobial activity and have cytostatic effects when added to mammalian cell lines. In agreement with those findings, we found reduced proliferation of VSMCs from S100A12 TG mice using BrdUrd incorporation as the measurement for cell proliferation. Of interest, other cell proliferation assays using bioreduction of MTS to formazan showed increased activity reflecting an increase in metabolic activity.

Figure 7. S100A12 is expressed in VSMCs in human aortic tissue with cystic media necrosis. A, Pedigree of a family with familial thoracic aneurysms with the MYH11 mutation. Affected family members with aortic dissection and aortic valve replacement are indicated by . B and C, Three-dimensional reconstruction of the computed tomography scans of the proximal aorta shows a 5.2-cm aneurysm distal the area of prior aortic repair. D, Resected TAA aorta and control aortic tissue were stained using Verhoeff–van Giessen (VVG), $\alpha$-smooth muscle actin (SMA), and $\alpha$-S100A12 antibodies. The tunica interna, media (MEDIA), and adventitia (AD) are shown. E, Colocalization of SMA and S100A12 is shown by immunofluorescence microscopy.
Lastly, we identified strong expression of S100A12 in aortic SMCs with a novel MYH11 mutation. It was recently discovered that mutations in VSMC-specific β-myosin (MYH11) and α-actin (ACTA2) can also cause TAAs, focusing attention on the importance of the maintenance of SMC contractile function in preserving aortic structure and preventing TAAs. The mechanisms leading to the expression of S100A12 in VSMCs in aneurysmal aortic vasculature are not well understood, but our data show that at least 1 mechanism leading to S100A12 expression in VSMCs can be mediated by LPS stimulation. S100A4, another member of the S100 family is endogenously expressed in VSMCs and promotes VSMC dedifferentiation. Furthermore, S100A4 levels are increased in TGF-receptor 2 mutant smooth muscle cells isolated from TAA patients, suggesting a potential role of S100 proteins in mediating VSMC dysfunction and vascular disease. In summary, our data demonstrate that targeted expression of human S100A12 in murine VSMCs is sufficient to modulate VSMC function and promote vascular remodeling and identify S100A12 expression as a potential therapeutic target.

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Disclosures

None.

References


Supplemental Material

Material and Methods

**Generation of TG mice.** Human full length S100A12 cDNA (Origene, Rockville, MD) was amplified by PCR using primers with added restriction site for Hind III (5’) and BamH I (3’) and ligated into the Bluescript II SK (Stratagene, La Jolla, CA) vector containing the smooth muscle cell promoter SM22. Verification of the construct was obtained by restriction analysis and by sequencing. The transgene was excised from the vector prior pronuclear injection into fertilized C57BL/6J mouse oocytes performed at the University of Chicago transgenic core facility. The genotype was determined by PCR analysis of genomic DNA extracted from tail biopsies using the following primers: 5’-CCGACAGACTGCTCCAACTTGGTGTCTTTC-3’ and 5’-GATATGAATTCTTGAAAGTCGACCTGTTCA-3’. PCR products with a predicted size of 530 bp were analyzed by agarose (1.8%wt/vol) gel electrophoresis in 0.5x TBE buffer and confirmed by Southern blotting. Briefly, 10 µg genomic DNA was digested with KpN I and Pst I, separated on a 1% agarose gel and transferred to a nylon membrane. The blot was then hybridized with a radiolabelled DNA probe using ExpressHyb hybridization solution (Clontech). Hemizygous TG mice were mated with wild type C57BL6/J (Jackson Laboratory). TG hemizygous and littermates not expressing the transgene were used for all experiments. Mice were housed at all times in a pathogen free barrier facility and maintained on normal rodent chow with free access to food and water.

**Primary Aortic Smooth Muscle cell culture (murine).** Primary mouse aortic vascular smooth muscle cells were isolated under sterile conditions following a modified protocol described by Ray. Briefly, working under a dissecting microscope, the aorta was dissected from the proximal aorta to the iliac bifurcation and the adventitia was removed leaving the aorta as a smooth tube. Next, the proximal aorta and the aortic arch were cut longitudinally and than cut into 2x2mm tissue fragments, washed three times in PBS, and digested with 7.5 mg/ml collagenase type II for 1 hours at 37°C. The tissue fragments were placed and gently attached to the cell culture dish bottom containing 100% FBS. After 2-3 days, cells were growing out of the aortic tissue and DMEM medium containing 1x antibiotics was added to the cell culture dish. Subsequently, cells were cultured in DMEM containing 10 % FBS and antibiotics. Cells of passage three were tested for contaminating leukocytes by immunoblotting with α CD45.2 antibody (Pharmingen), α CD68 (Pharmingen) and by qRT-PCR. Only cell cultures testing negative for CD45 and CD 68 were propagated, and VSCM from passage 4-8 were used for experiments. These primary cells were characterized as smooth muscle cells by immunofluorescence staining using α-SMA IgG (Sigma). 24 hours prior to all experiments, SMC were placed in DMEM containing 0.5% FBS and 1x antibiotics.

**Primary Aortic Smooth Muscle cell culture (human).** Aortic tissue resected from patients undergoing surgery for thoracic aneurysms (TAA) or control aortic tissue from patients undergoing surgery for aortic valve replacement or heart donors were dissected under a microscope and the muscular layer was minced under steril conditions. Next, the tissue fragments were washed in PBS three times followed by digestion with collagenase type II (7.5 mg/ml for 1 hour at 37°C). The tissue fragments were processed as described above for murine aortic SMC.
Histopathology. The murine aorta was perfused in situ with PBS (2 min) followed by 4% paraformaldehyde (5 min) under physiologic pressure (∼45 cm water) by canulating the left ventricle. After dissection, the aorta was fixed in 10% formalin for 12 hours prior to tissue processing. Seven µm sections obtained from the proximal aortic arch were stained with H&E, Masson Trichrome or Verhoff-vanGiessen stain (VVG). The microscopic images of the MT stained slides were then acquired and stored on the Chromavision Automated Cellular Imaging System (ACIS, Clarient, Aliso Viejo, CA), a computer program that allows quantification of chromogenic activity. Analysis on all visible vascular areas was performed, thereby improving analytical accuracy and resolution over manual slide analysis. Histological grading of medial elastin degradation was performed on VVG stained slides of the aortic arch using the following grading system: grade 1: no disruption; grade 2: disruption of one elastic fiber with intact neighboring fibers; grade 3: disruption of two or more fibers in direct contact, grade 4: disruption of all elastic fibers from the internal elastic lamina to the external elastic lamina. Histological measurement of the aortic wall thickness was measured on VVG stained slides of longitudinally cut sections of the aortic arch from the internal elastic membrane to the external elastic membrane. For all experiments, at least 4 animals were used per group and graded in a blinded manner. For immunofluorescence microscopy, frozen aortic sections were incubated with S100A12 IgG (Abcam 37657), smooth muscle α-actin (Sigma, 42547). Rabbit IgG (Zymed; Invitrogen) or omission of the primary antibody was used as a negative control.

In vivo ultrasound. A 40MHz high frequency Visual Sonics Vevo 660 ultrasound machine was used to measure the diameter of the ascending aorta at the sinus of Valsalva at various time points. The mice were anesthetized, shaved and in a parasternal long axis view the ascending aorta, aortic arch and the neck vessels were visualized in one plane. In all studies, at least 4 animals per group were used.

Immunoblot. Aortic tissue samples were prepared using T-PER-tissue lysis buffer (Pierce). The following primary antibodies were used: S100A12 (Abcam 37657), mS100A8 (R&D), mCD45.2 (BD Pharmingen), vinculin (Sigma-Aldrich), SM-MHC (Santa Cruz), SM-α-actin (Sigma-Aldrich), VCAM-1 (Santa Cruz), β-actin (Sigma), GAPDH (Sigma), MMP-2 (Millipore). In all Western blot studies, at least 3 animals per group were used; results of representative experiments are shown.

Zymography. Mouse aortas were homogenized and 10µg whole tissue lysates were loaded onto zymogram gelatin gels (Invitrogen) for electrophoresis. For cell culture experiments, VSMC were grown for 3 days in DMEM supplemented with 0.5%FBS and cell culture supernatant was concentrated using a centrifugal filter device (Millipore) prior to loading onto zymogram gelatin gels (Invitrogen). After the required developing time, gels were stained with blue code (Pierce) and images obtained with an alpha imager.

Myeloperoxidase activity. Mouse aortas were perfused in situ with PBS for 2 minutes and rapidly retrieved from the heart to the iliac bifurcation at sacrifice, followed by mechanical disruption in hexadecyltrimethylammonium bromide using several freeze/thaw cycles to relases myeloperoxidase from leukocyte granules. The homogenate was centrifuged and stored at -80 °C until enzymatic activity of myeloperoxidase was measured using a colorometric assay at 460nm as previously described. Briefly, the sample was mixed with o-dianisidine (Sigma) and 0.0005% hydrogen peroxide, and absorbance change was measured at 460 nm.

Cholesterol Assay. Levels of total cholesterol were determined in plasma of fasted mice (4 hours) using chromogenic assays (Fisher Scientific).
Blood pressure measurement. Arterial blood pressure was recorded non-invasively using a tail cuff with volume pressure recording (CODA Standard, Kent Scientific Corporation). Blood pressure was recorded for 10 min in awake mice and the average systolic and diastolic pressure obtained in the last 3 min of recording is reported.

ELISA. Concentrations of mouse IL-6 were determined in plasma or in culture supernatant harvested from VSMC or 3 mm aortic ring tissue by Quantikine ELISA (R&D Systems Inc.). For cytokine measurement from cell culture supernatant, VSMC were cultured in 24 well plates in DMEM containing 10%FBS and antibiotics. Once cells were 95% confluent, the medium was changed to DMEM with 0.5% FBS for 24 hours. Next, the cells were washed with PBS followed by stimulation with 1 µg/ml LPS in serum free DMEM for 24 hours. Cell culture supernatant was harvested after 24 hours, centrifuged to remove all cell debris and stored at -80 for IL-6 ELISA (R&D). To determine whether the concentration of LPS used in the experiments affected cell viability, cells were assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Cell viability assay, Promega). Cell viability in the presence of up to 20 µg/ml LPS was indistinguishable from viability in the absence of LPS. If indicated, cells were pretreated with recombinant soluble RAGE (R&D Systems Inc.). For IL-6 released directly from the aorta, 3 mm aortic ring tissues were placed in 24 well plates containing serum free DMEM and if indicated containing 1 µg/ml LPS. Tissue supernatants were harvested after 24 hours, centrifuged to remove all cell debris and analyzed by IL-6 ELISA (R&D).

Cell proliferation assay. Primary aortic VSMC (3000 cells/ml) were seeded into a 96 well plate. At indicated time points (24, 48, 72 and 96 h), MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was added according to the manufacture’s protocol (Promega, non-radioactive cell proliferation assay) and cells were incubated for 45 min. The reduction of MTS by the cells into a formazan product was measured directly at 490 nm using an Elisa plate reader (Fluostar, BMG labtech). For BrdU incorporation, cells (3000 cells/ml) were seeded onto glass cover slips and grown for 24-96 hours. At indicated time points, cells were pulsed with 10µM BrdU (Sigma Aldrich) for 3 hours followed by detection of BrdU incorporation using anti BrdU monoclonal antibody (BD Biosciences) according to the manufactures. Nuclei were stained with DAPI. All nuclei and BrdU positive nuclei were counted on five high power fields by a blinded investigator using a fluorescence microscope.

Immunofluorescence microscopy. VSMC grown on glass coverslips were fixed with 20% ice cold methanol for 5 min, washed in PBS, and blocked for 1 hour with 5%FBS prior to incubation with primary antibody (1:1000 for rabbit anti S100A12 (Abcam 37657), 1:2000 for rabbit anti pSmad2 (Cell Signaling 3122), 1:2000 for mouse anti 8-oxo-dG (Trevigen 4354), 1:3000 for rabbit anti BrdU (BD Pharmingen), Cells were washed with PBS and incubated with secondary antibodies (AlexaFluor488, AlexaCy3). If indicated, cells were pretreated with neutralizing TGF-β antibody (Cell Signaling #3711). For experiments measuring mitochondrial ROS production, MitoSox Green (Invitrogen, 500nM) was added for 1 hour to the cell culture before cells were fixed with 10% ice cold methanol. Cells were imaged by confocal lasermicroscopy (Zeiss 500 Meta) and fluorescent intensity was measured in 200 cells (n=100 for TG-SMC and n=100 for WT-SMC).

Flow cytometry. Cells were detached with trypsin and resuspended in PBS. The cell suspension was then labeled with the mitochondrial fluorescence probe MitoTracker Green
(Invitrogen) at 500 nM for 60 min to quantify mitochondrial content or with the mitochondrial superoxide fluorescence probe MitoSOX Red (Invitrogen) at 5 micromolar for 20 min to quantify mitochondrial reactive oxygen species. Cells were then washed with PBS and fluorescence was quantified as relative fluorescence units on a FACS Calibur Instrument (Becton Dickinson). Values are reported as mean fluorescence units. Where indicated, cells were treated with 10µg/ml bovine serum albumine (Sigma) or 10µg/ml recombinant S100A12 (Abnova) prior to harvesting.

**Quantitative RT PCR.** Total RNA was isolated from the indicated VSMCs with the QiagenRNA mini kit including a DNase step. cDNA was synthesized from 1 µg RNA using Superscript first strand synthesis system (Invitrogen) followed by PCR amplification using SYBR GreenER (Invitrogen) with a IQ5 cycler (BioRAD). The following primers were used: for β-Actin F: 5-TGTGATGGGGAATGGGTAGAA and R: 5-TGTGGTGCCAGATCTTCTCCATGT; for GAPDH F: 5-TCAACAGCAACTCCACTTTCCA and R: 5-ACCCGTGTCTGCAGGCGATC; and for mCD45 F:5-CCAGTGATGGGTGTATCCAC and R: 5-GGGGTATCAACAGGAAAGGC

The ΔC_{T} value was used to describe the difference between the TG and WT normalized to the house keeping genes, GAPDH and β-actin.

Relative mRNA expression= 2exp (ΔC_{T} target gene- ΔC_{T} housekeeping gene).

**Computed tomography.** The CT was performed on a multi-slice CT scanner with 64 detector rows at a collimation width of 0.625 mm (Philips, Cleveland, OH). Imaging parameters for this scan included a gantry rotation time of 420ms, pitch of 0.2, table increment of 0.33 mm, tube voltage of 120 kV, and tube current set at 800 mA. The patient received 120 cc of low osmolar contrast (Omnipaque 350) at 5cc/s through an antecubital vein, followed by 20 cc of a 70%-30% saline contrast mix also at 5cc/s. Axial slice data were reconstructed using retrospective gating at 75% phase of the cardiac cycle, which corresponds to mid diastole. The displayed image is a volume rendering of the axial slices depicting the contrast filled lumen of the heart, aorta and major branches.

**Statistics.** All continuous data are reported as mean ± SD and discrete variables were summarized by percentage. All experiments were performed in at least 3 replicates per group and all cell culture experiments were repeated twice. Independent sample t-test and one way analysis of variance were used for mean comparison between two or multiple groups, respectively. For repeated measurements of the aortic root dimensions we performed a two tailed paired two sample t-test, and differences were considered significant at a level of p<0.05. The Spearman rank correlation, χ² test and Fisher exact test were also performed to describe association between different outcome variables. The Bonferroni correction was used to adjust for multiple comparisons. Two tailed probability values of p less than 0.01 were considered statistically significant for each test to ensure an overall study significance level of p less than 0.05.

References:


Online Table I

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<th>Age (weeks)</th>
<th>WT-littermate mice</th>
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<tr>
<td>Weight (g)</td>
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<tr>
<td></td>
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Online Figure I

**Aortic remodeling in transgenic mice line 61.** (A-L) Representative histology of aortic sections obtained from the proximal ascending aorta from 16 weeks old WT mice (left column) and from 16 weeks old TG mice line 61 (middle column) showing the proximal ascending aorta and pulmonary artery (PA). The right column shows the mid aortic arch from 16 weeks old TG mice line 61 (N-vagal nerve, Br-bronchus, E-esophagus, scale bar is 100µm in the 25x magnification A-C, and 10 µm in the 400x magnification D-L). The lower three panel shows serial sections stained for H&E, Masson’s Trichrome (MT) and Verhoeff-van Giessen (VVG) in a 400x magnification (scale bar =10µm) from the field marked in A-C. (M-N) In vivo ultrasound of the aortic root showed significant dilatation of the aortic root in TG mice line 61 at age 16, 22 and 56 weeks compared to their respective sex-matched littermates (n=4 for each group, * p<0.01).

Online Figure II

**Increased metabolic activity in VSMC transfected with S100A12.** Rat VSMC (A7r5 cells, ATCC) were stably transfected with S100A12 driven by the CMV promoter (pcDNA3.1) or mock transfected. Three thousand cells/ml were grown in 96 well plates and treated with MTS at indicated time points and conversion to formazan was measured at 490nm (* p < 0.05).
Online Figure II

MTS/Formazan (OD 490nm)

p < 0.01

S100A12

vector

time (h)

24 48 72 96