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Anti-receptor for advanced glycation end products therapies as novel treatment for abdominal aortic aneurysm

Fan Zhang¹, K. Craig Kent¹, Dai Yamanouchi¹, Yan Zhang², Kaori Kato¹, Shirling Tsai³, Roman Nowygrod², Ann Marie Schmidt², and Bo Liu^{1,3}

¹ Departments of Surgery, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin, WI 53705

² Columbia College of Physicians and Surgeons, New York, NY

³ Department of Surgery, Weill Medical College of Cornell University, New York, NY 10021

Abstract

Objective—Rupture of abdominal aortic aneurysms (AAA) is a devastating event potentially preventable by therapies that inhibit growth of small aneurysms. Receptor of advanced glycation end products (RAGE) has been implicated in age related diseases including atherosclerosis and Alzheimer's. Consequently, we explored whether RAGE may also contribute to the formation of AAAs.

Results—Implicating a role for RAGE in AAA, we found the expression of RAGE and its ligand AGE were highly elevated in human aneurysm specimens as compared to normal aortic tissue. In a mouse model of AAA, RAGE gene deletion (knockout) dramatically reduced the incidence of AAA to 1/3 of control (AAAs in 75.0% of controls versus 25.0% knockouts). Moreover, aortic diameter was markedly reduced in RAGE knockout animals versus controls. As to mechanism, we found that RAGE was co-expressed in AAA macrophages with MMP-9, a promoter of matrix degradation, which is known to induce AAA. In vitro, advanced glycation end products (AGE) induced the production of MMP-9 in macrophages in a dose-dependent manner while blocking RAGE signaling with a soluble AGE inhibitor prevented MMP-9 expression. In vivo, RAGE gene deficiency eliminated MMP-9 activity that was prevalent in aneurysmal wall of the wild-type mice.

Conclusions—RAGE promotes the development of AAA by inducing MMP-9 expression. Blocking RAGE in a mouse aneurysm model has a dramatic inhibitory effect on the formation of aneurysms. These data suggest that larger animal and eventually human trials should be designed to test oral RAGE inhibitors and their potential to prevent progression of small aneurysms.

INTRODUCTION

Abdominal aortic aneurysm (AAA) is a significant medical problem with a mortality rate associated with rupture as high as 90%¹. Rupture of AAAs is a devastating event that is potentially preventable by therapies that inhibit the growth of small aneurysms. Studies of the pathophysiology of aneurysms have shown that the aortic wall of aneurysms is characterized by chronic inflammation², loss of the extracellular protein elastin³, increased collagen

Reprints to K Craig Kent, 600 Highland Avenue, CSC/H4-710, Madison, WI 53792-7375, kent@surgery.wisc.edu, Tel. #: 608-265-8854, FAX #: 608-262-1842.

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metabolism, elevated activities of matrix-degrading enzymes matrix metalloproteinases (MMPs) such as MMP-9 and MMP-2⁴, and changes in cellular content, including inflammatory cell infiltration and apoptosis of vascular smooth muscle cells (SMCs)⁵. However, the precise mechanism responsible for AAA formation and progression is still under debate, and developing a better understanding of this mechanism could lead to the development of novel strategies for the prevention of this life-threatening vascular disease.

MMP-9 is considered to be an important mediator for the development of aneurysms. MMP-9 not only degrades the extracellular matrix, but it also plays an important role in many physiologic responses such as the control and regulation of the inflammation, wound healing, and remodeling relevant for atherosclerosis and restenosis⁶⁻⁸. MMPs destroy elastin and collagen, which is fundamental to the development of aneurysms². However, knowledge about the stimuli for induction of MMP-9 during the process of aneurysmal formation remains limited.

Advanced glycation end products (AGEs) are formed from proteins and peptides by non-enzymatic glycooxidation after contact with aldose sugars^{9, 10}. AGE as well as its receptor (RAGE) has been implicated in disease of the elderly age including atherosclerosis and Alzheimer's^{11, 12}. AGEs are known to accumulate in the vessel wall, where they may act to alter the extracellular matrix (ECM), cell surface receptors, or impact the function of intracellular proteins¹³. AGE/RAGE interactions elicit oxidative stress and induce proinflammatory or procoagulant cellular responses including increases in the vascular cell adhesion molecule-1 (VCAM-1) and TNF- α expression¹⁴. These events are thought to be mediated by the activation of NF- κ B by circulating AGEs. Moreover, previous studies have shown that RAGE^{-/-} mice are protected from the lethal effects of septic shock, which depend largely on the innate immune response^{15,16}. Thus, the emerging importance for AGE/RAGE in the pathophysiology of diseases of the elderly and in inflammatory processes led us to hypothesize that this ligand and receptor may contribute to the formation of AAAs.

In this study, we tested whether AGE/RAGE is involved in the formation of AAA by measuring the expression of AGE and RAGE in AAA tissues both in the mouse and in humans, and by testing whether gene deletion of RAGE affected the formation of aneurysms in a mouse AAA model. Moreover, we have identified a mechanism by which AGE/RAGE may promote aneurysms through the induction of MMP-9. Our results provide evidence that AGE/RAGE plays an important role in the development of aneurysms.

MATERIALS AND METHODS

Human Tissue Procurement

Human AAA specimens were obtained from patients undergoing surgical repair of AAA by the members of the Division of Vascular Surgery. As controls, normal human aorta samples were obtained from autopsy specimens (age \geq 50) through Brain and Tissue Bank for Developmental Disorders (National Institute of Child Health and Human Development). No patients were known to have connective tissue disorders. The use of human samples in this study was approved by the Institutional Review Board at Weill Cornell Medical College and Columbia Medical Center.

Animal AAA model

ApoE^{-/-} mice were obtained from The Jackson Laboratories (Bar Harbor, Maine, USA). Homozygous *RAGE*^{-/-} mice, backcrossed more than 12 generations into C57BL/6, were bred into the *apoE*^{-/-} background to obtain *apoE*^{-/-} *RAGE*^{-/-} mice. *ApoE*^{-/-} *RAGE*^{-/-} mice exhibit normal reproductive capacity, physical fitness, and overall phenotype under nominal conditions¹⁷. *ApoE*^{-/-} *RAGE*^{-/-} and *apoE*^{-/-} *RAGE*^{+/+} mice were anesthetized and AngII

(1000 ng/kg/min;Sigma, St. Louis, MO) or phosphate buffered saline (PBS) was administered by Alzet osmoticminipumps (model 2004) implanted subcutaneously, as describedpreviously¹⁸. The maximum diameter of the infrarenal aorta and the suprarenal aorta were measured 4 weeks after the pump implantation. AAA tissues were harvested after animals were anesthetized. The suprarenal aorta was removed and tissue was frozen for protein extraction or fixed with 4% PFA for cross section preparation. Tissues for cross section were then paraffin embedded for histological analysis. All procedures were approvedby the Institutional Animal Care and Use Committee at the University of Columbia.

Cell Culture

The murine macrophage cell line RAW 264.7 and rat aortic vascular smooth muscle cells were obtained from American type Culture Collection (ATCC, Manassas, VA, USA). RAW 264.7 cells and rat vascular smooth muscle cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 µg/ml streptomycin in a 5% CO₂/water-saturated incubator at 37 °C. Treatments with vehicle(PBS), AGE (Sigma), and S100b (Calbiochem) were carried out in 1% FCS.

Gelatin Zymography

For the in vitro studies, conditioned media were collected at the indicated time points and centrifuged for 5 min at 500×g to remove cells and debris. For the in vivo studies, the supra renal segments of aortas were harvested from mice 28 day after pump implantation. The tissues were homogenized in phosphate-buffered saline buffer (PBS) (pH 7.4) on ice, and homogenates were centrifuged at 10,000 rpm for 5 min, and the supernatants were collected. The total protein concentration in supernatants was determined using the Bio-Rad protein assay. Supernatants or tissue extracts containing equal amounts of protein (10 µg) were mixed with SDS gel-loading buffer and then loaded without reduction or heating onto 10 % SDS-polyacrylamidegels containing 0.1% gelatin. After electrophoresis, SDS was removed by washing with buffer (50 mM Tris/HCl, pH 7.6, 150 mM NaCl, 5 mM CaCl₂, 2 µM ZnCl₂, 0.1%, Triton X-100) for 30 min at room temperature with gentle agitation to renature enzymes. The gels were subsequently incubated in zymogen development buffer (50 mM Tris-Cl, pH 7.5; 10 mM CaCl₂; 1µM ZnCl₂; 1% Triton X-100; 0.02% NaN₃) at 37°C for 16 to 24 h . After briefly washing in water, gels were stained with Coomassie blue R-250 for 1 h. Gels were destained with 40% methanol and 5% acetic acid until clear white bands against a blue background were visible.

Western Blotting Analysis

Fifty micrograms of protein from each human aortic sample or an equal protein content of supernatants from control and AGE-treated samples were resolved on 10% SDS PAGE gels. Proteins were then transferred onto nitrocellulose membranes by electroblotting using an electroblotting apparatus. Nonspecific binding of the membranes was blocked with wash buffer containing 5% (w/v) nonfat dry milk for one hour. Membranes were washed with wash buffer three times for 10 min and incubated with appropriate dilution of RAGE, MMP-9 or MMP-2 antibodies in wash buffer overnight at 4 °C. Subsequently, the membranes were washed with wash buffer and incubated with an appropriate secondary antibody (horseradish peroxidase-conjugated goat antimouse or antirabbit IgG) for 1 h. After washing the membrane three times for 10 min, band detection was revealed by enhanced chemiluminescence using ECL Western blotting detection reagents and exposed to ECL hyperfilm.

RT-PCR

Total RNA from macrophages culture treated with AGE and S100b was isolated using the RNeasy[®] Mini kit following the instructions of the manufacturer (Qiagen). Messenger RNA of MMP-9 and GAPDH expression in each sample was determined by reverse transcription-

polymerase chain reaction (RT-PCR) using GeneAmp RNA PCR Core Kit (PerkinElmer Life Sciences).

ELISA assay of AGE

Aortic tissues lysates were prepared using cell lysis buffer for determining protein concentrations and measure AGE product. The competitive AGE-ELISA procedure was performed as described previously¹⁹.

Immunohistochemistry

Immunostaining for AGE and RAGE using monoclonal anti-AGE and anti-RAGE antibody (a gift from A. M. Schmidt) were performed as described previously²⁰. Slides were visualized with a Nikon Eclipse E800 upright microscope. Digital images were acquired using a RetigaEXi CCD digital camera and processed and analyzed using IPLab software.

Statistical analysis

Data are expressed as mean \pm SE. Unpaired Student's *t* test was used to evaluate the statistical differences between normal and AAA patient groups. Values of $p < 0.05$ were considered significant.

Results

AGE and RAGE are highly expressed in human AAA

Previous reports have implicated AGE to be involved in the pathogenesis of vascular inflammation as well as a number of diseases of the elderly. Since AAA is a disease of the elderly, we began our studies by determining whether the expression of AGE is altered in AAA samples from humans. Immunostaining revealed a markedly increased accumulation of AGE in AAA tissues compared to normal aortic tissue (Fig. 1A and B). The expression of AGE was then quantified in human AAA tissues and normal human aortic tissues by ELISA. As shown in Fig. 1C, the accumulation of AGEs in AAA tissues was 2.7 fold higher than that found in normal aortic tissues. Since the expression of RAGE is often upregulated in parallel with its ligands, we next evaluated RAGE expression in human AAA tissues by immunostaining and Western blotting. As shown in Fig. 2A–C, human AAA tissue contained significantly higher levels of RAGE expression compared with tissue from normal aorta. Increased staining for RAGE was predominantly observed in CD68-positive macrophage cells and to a lesser extent in smooth muscle cell specific α -actin (SMA)-positive SMC (Fig. 2D–I).

MMP-9 expression is associated with AGE/RAGE signaling in AAA tissues

MMP-9 has been reported as an important factor responsible for the development of AAA^{2, 21, 22}. Consistent with previous reports, we found significantly higher MMP-9 activity in AAA tissues compared to normal aortic tissues, measured by gelatin zymography (Fig. 2J). Immunostaining of AAA tissue showed that MMP-9 was mostly expressed in inflammatory cells, but not in SMCs nor in fibroblasts (Fig. 2K).

RAGE is up-regulated in mouse AAA model

To further explore the potential involvement of AGE/RAGE signaling pathway in AAA development, we examined RAGE expression in a mouse model of AAA. The mouse model was created as described in methods by continuous infusion of Ang II into apoE null mice. Consistent with previous reports, Ang II produced an AAA-like dilation involving the suprarenal aorta in approximately 75 % of the treated mice. Immunostaining revealed a marked upregulation of RAGE expression in the suprarenal aorta of AngII-infused mice as compared to the aortic tissue of mice infused with saline (As shown in Fig. 3A–B). Similar to what was

we observed in human AAA specimens, the highest expression of RAGE was found in CD45 labeled cells, a marker for leukocytes (Fig. 3D). RAGE was infrequently colocalized with cells positive for the SMC marker α -actin suggesting that RAGE may also emanate from vascular SMCs (Fig. 3C).

RAGE is necessary for AAA development

To further test the role of RAGE in the development of AAA, we generated a strain of mice that are deficient in both RAGE and apoE (RAGE^{-/-} apoE^{-/-}). Ang II was administered to double “knock-out” mice (RAGE knockout) or single “knock-out” (control or RAGE wild-type) mice for 28 days. As shown in Fig. 4, six out of the eight or 75% of the control mice developed aneurysms, defined as a 50% or larger increase in suprarenal aorta compared to infra renal aorta (the aneurysm model). In contrast, only two out of the eight, or 25%, of the RAGE knock-out mice developed AAA (Fig. 4A&B). Consistently, the maximum diameter of the suprarenal abdominal aorta in the RAGE knock-out mice was significantly diminished compared to the control mice (Fig. 4C). Furthermore, two of the control mice died prior prematurely due to ruptured aorta. No premature death occurred in RAGE knock-out mice.

Examination of the histology of aortic sections revealed substantial expansion and cellularity of the tunic adventitia in the control mice that were treated with angiotensin II, a characteristic of AAA associated inflammation (Fig. 5A&B). This adventitial response was markedly diminished in RAGE knockout mice (Fig. 5C). To determine whether MMP-9 is involved in the development of aneurysms in the mouse AAA model, we examined levels of MMP-9 activity in aortic tissue from control and RAGE knock-out mice. In this particular experiment, we compared four samples from each. Three out of the four control mice developed AAA-like dilation in comparison to zero of four RAGE knockout mice. Elevated MMP-9 activity was identified in the three control mice with aortic dilatation, whereas MMP-9 was barely detectable in the control mouse without aortic dilatation as well as all of the aortic specimens from RAGE knock-out mice (Fig. 5D).

AGE induces MMP-9 in Macrophages

To further evaluate the relationship between AGE/RAGE and MMP-9 production, we stimulated rat vascular SMCs or a macrophage cell line (RAW 264.7) with increasing amounts of the RAGE ligand, AGE or S100b. S100b, a member of the S100 family of proteins containing two EF-hand-type calcium-binding domains, is another ligand of RAGE²³. As shown in Fig. 6A and B, both AGE and S100b increased MMP-9 activity (gelatinolytic activity at 92-kDa) in macrophages in a concentration dependent manner without significantly affecting the activity of MMP-2, another MMP that has been associated with the development of aneurysms. In contrast, the SMCs treated with AGE did not evidence increased enzymatic activity of either MMP-9 or MMP-2 (Fig. 6C). The stimulatory effects of AGE and S100b on MMP-9 were confirmed by Western blotting (Fig. 6D). To test whether AGE stimulates MMP-9 at a transcriptional level, we analyzed MMP-9 mRNA levels by RT-PCR. Compared to control cells, macrophages treated with AGE or S100b demonstrated significantly enhanced levels of MMP-9 mRNA (Fig. 6E). To determine whether the observed effect on MMP-9 is indeed mediated by AGE/RAGE signaling, we pre-treated macrophages with a RAGE inhibitor: soluble RAGE (sRAGE), a truncated form of RAGE that is composed of only the extracellular ligand-binding domain and lacking the cytosolic and transmembrane domains²⁴. As shown in Fig. 6F, inhibition of RAGE signaling with sRAGE blocked MMP-9 induction in response to AGE.

Discussion

In this study, we clearly demonstrate a significant association between AGE/RAGE and aneurysm formation in humans as demonstrated by the presence of these two proteins in human aneurysmal tissues. To determine cause and effect, we employed a mouse model of AAA produced by the infusion of angiotensin II in ApoE deficient mice. We were able to demonstrate a markedly reduced incidence of AAA in mice that were lacking the gene for RAGE. To our knowledge, this is the first study to report an association between AGE/RAGE and aneurysms. Our findings suggest a novel association between AGE and RAGE and the pathogenesis of this important and lethal condition.

It is well known that the degradation of AGE is diminished with aging which in turn results in its tissue accumulation²⁵. Indeed, several studies have reported that AGE and RAGE are involved in age related diseases such as Alzheimer's, diabetes and atherosclerosis^{26, 27}. AGE has a number of actions that contribute to its provocative effects on disease including effects on extracellular matrix as well as its ability to induce inflammation and the generation of reactive oxygen species.

Likely the most prevalent risk factor for aneurysms is age. Aneurysms become increasingly prevalent with age with the incidence in the 9th decade being almost 10 times that in the 6th decade. Thus, it is a plausible hypothesis that AGE and RAGE might play a role in the development of AAA. Previous investigators have reported that RAGE and AGE are involved in other vascular diseases specifically restenosis and atherosclerosis. In fact our laboratory has demonstrated an important role for RAGE in restenosis^{17, 28, 29}. However, over the past several years it has become clear that aneurysms once thought to be a form of atherosclerosis, have a pathogenesis that is distinct from that of occlusive vascular disease.

We chose in our initial studies of RAGE to employ a model that uses angiotensin-II to induce the development of aneurysms. This is a unique model that is characterized by the initial formation of dissection followed by dilatation of the supra renal aorta. Interestingly, this model may more closely parallel in humans, aortic dissection which produces aneurysms secondary to a weakened dissected wall; a process that may differ from the one that predisposes to the traditional infrarenal aneurysm in humans. There are two additional animal models of AAA, one resulting from the external application of calcium chloride and a second that is produced by the intraluminal infusion of elastase. These models are similar in that in all three, inflammation is essential. However, the differences are sufficiently great that the role of RAGE in the formation of AAA should be confirmed using conditions that more precisely model those that produce the traditional infrarenal aneurysm.

The accumulation of natural RAGE ligands, such as AGE as well as the HMGB-1 and S100A12 polypeptides, has been associated with the activation of inflammatory cells and the production of proinflammatory mediators³⁰. Amongst these ligand-RAGE effects are the cellular activation of signal transduction cascades that generate reactive oxygen species which in turn trigger inflammation³¹. Multiple intracellular pathways including the MAP kinases and PI3 kinase have been reported to be activated by RAGE³². Activation of these signal transduction pathways in turn turns on the redox-sensitive transcription nuclear factor $\text{NF-}\kappa\text{B}$ ³¹, which in turn leads to gene expression of pro-inflammatory cytokines and leukocyte adherence molecules³³.

Extracellular matrix (ECM) proteins, such as collagens, elastin and proteoglycans, are important structural components in arteries³⁴. Degradation of these structural components by matrix metalloproteinases (MMPs), such as MMP-2 and MMP-9, has been reported as important contributor to the pathogenesis of AAA in various animal models of AAA^{35, 36} and in humans^{2, 37}. Moreover, systemic administration of MMP antagonists can decrease

aneurysmal dilatation in the elastase-induced rodent model of AAA³⁸. There is preliminary data in humans that treatment of patients with small aneurysms with the MMP inhibitor, doxycycline, may decrease the growth rate of aneurysms³⁹. In the current study, we demonstrate that AGE is a potent inducer of MMP-9 production in macrophages in vitro. Moreover, the formation of aneurysms in the angiotensin model is associated with enhanced expression of MMP-9 and this enhanced expression is eliminated in the RAGE knockout model. It is likely that AGE/RAGE at least in part affects the formation of aneurysms via the induction of MMP-9. As a possible mechanism for AGEs' effect on MMP-9, it has been demonstrated that AGE/RAGE induces NF- κ B activation through a variety of signal transduction pathways^{40–42}. The NF- κ B in turn, through activation of a protein 1 (AP-1) binding site in the MMP-9 promoter, is essential for activation of MMP-9 gene⁴³. Nevertheless, the precise mechanism of AGE/RAGE induced MMP-9 needs to be further studied.

The soluble form of RAGE (sRAGE) can potentially bind to an AGE ligand thereby acting as a decoy, preventing the AGE–RAGE interaction and RAGE activation⁴⁴. Administration of a recombinant soluble form of RAGE (sRAGE) consisting of the extracellular ligand-binding domain has been shown not only to suppress the development of atherosclerosis, but also to stabilize established atherosclerosis in diabetic apolipoprotein E null mice^{45, 46}. Inhibition of the AGE/RAGE interaction opens new possibilities for the treatment of small aneurysms. An unanswered question is the timing of therapy. Animal models allow institution of preventative therapies at the inception of the processes that lead to aortic dilatation. However, most aneurysms are discovered after aortic dilatation has already occurred and presumably many years after the inciting events. Whether RAGE inhibitors will be useful at the later stages of AAA development remains to be determined. Encouraging is the fact that increased levels of RAGE remain present in the tissue of the relatively large aneurysms sampled in this study and inflammation also appears to play an important role in aneurysms of all sizes.

In summary, our results suggest that AGE and RAGE are involved in the development of AAAs. These findings contribute to a better understanding of the mechanisms underlying the pathogenesis of AAAs. Manipulation of the interaction between AGE and RAGE may ultimately lead to novel therapies to treat and prevent AAA progression.

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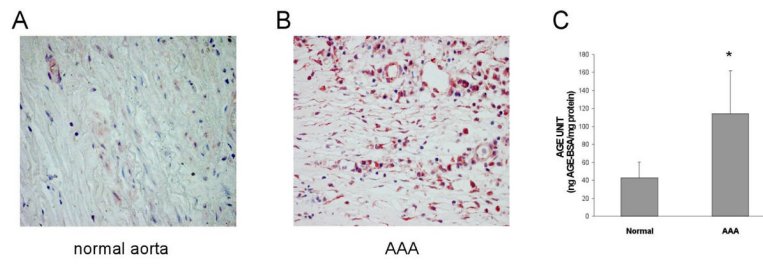


Fig. 1. AGE expression in human AAAs

(A) Normal aortic tissue from cadaveric specimens and (B) human AAA tissue collected during surgical repair. Samples were stained with an antibody specific for AGE (magnification: x200). (C) Tissue samples were lysed and subjected to an antibody specific for AGE and quantified by ELISA.

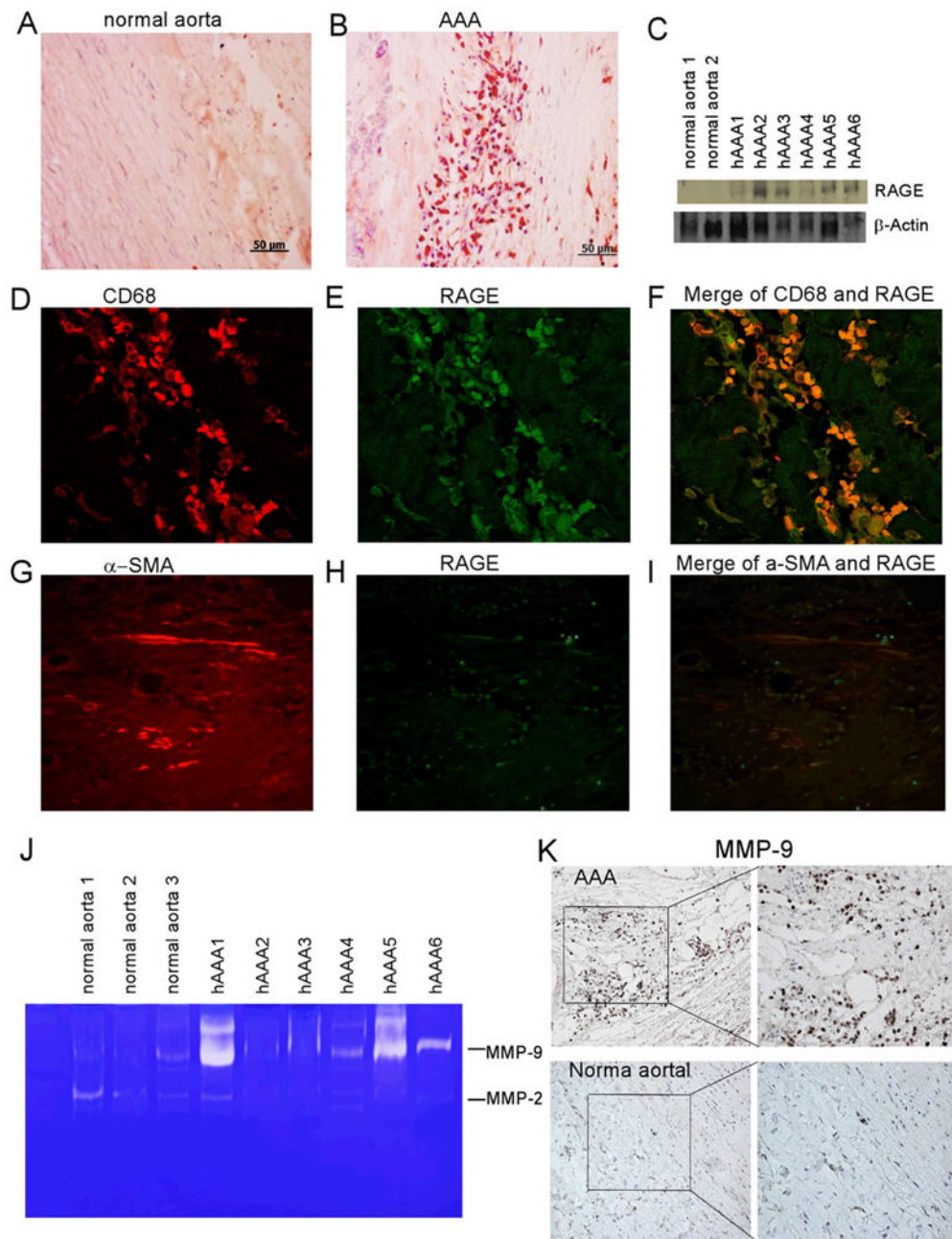


Fig. 2. RAGE expression in macrophages and SMCs of tissue from human AAA
 (A) Immunohistochemical staining of RAGE in normal aorta or (B) tissue from human aneurysms (magnification: x200). (C) Western blot showing RAGE expression in human AAA samples (n = 6) and no expression in normal human aorta tissue. Equal protein loading was confirmed by re-probing with β-actin. (D–F) Double-immunofluorescent staining for RAGE and CD68, a marker for macrophages. (G–I) Double-immunofluorescent staining for RAGE and α-SMA, a marker for SMC (magnification: x200). (J) Gelatin zymography for MMP-2 and MMP-9 in protein extracts from normal human aorta and AAA tissue. (K) Immunohistochemistry for MMP-9 in human normal and AAA tissues (magnification: left x100; right x200).

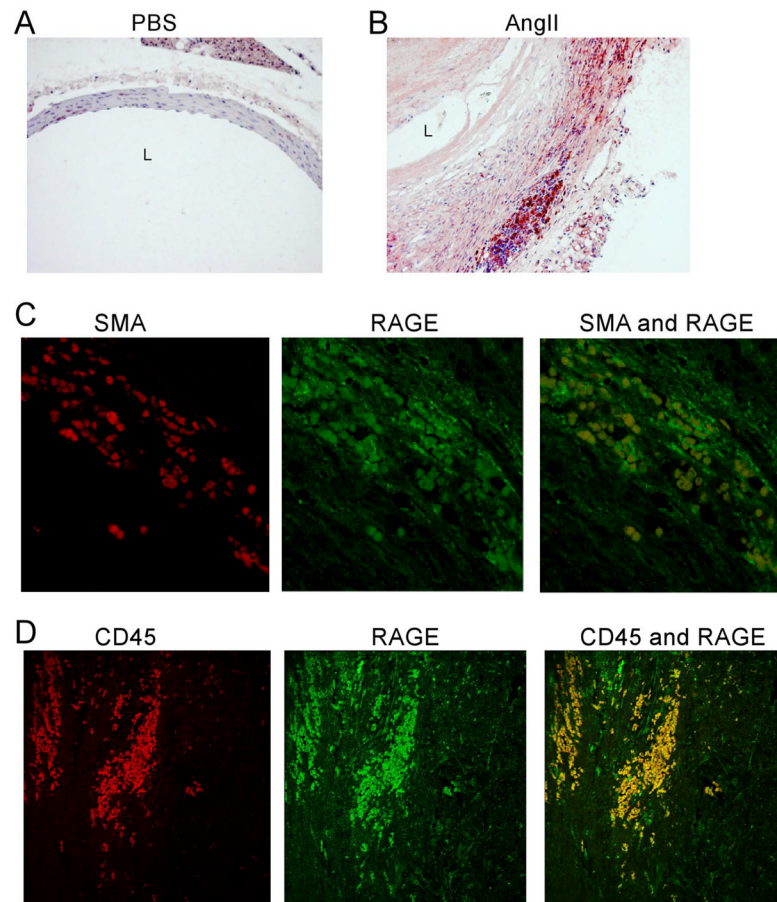


Fig. 3. RAGE expression in SMC and inflammatory cells in an Angiotensin mouse model of AAA (A–B) Immunohistochemistry for RAGE expression in ApoE^{-/-} mice infused with PBS (A) or Angiotensin II (B) (black L indicates lumen). (C–D) Double immunofluorescence staining for RAGE with α -SMA (C) or CD45 (D).

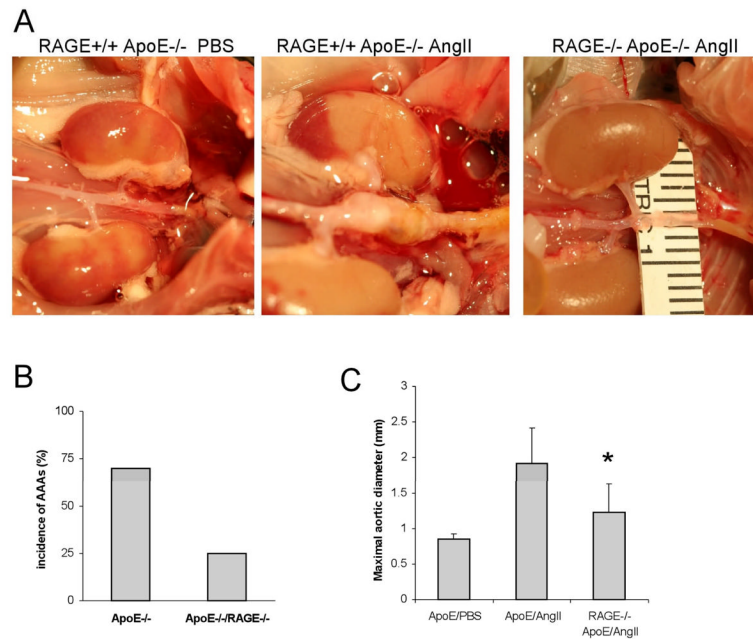


Fig. 4. Effect of RAGE deletion in the angiotensin mouse AAA model
 RAGE^{+/+}ApoE^{-/-} (control) or RAGE^{-/-} ApoE^{-/-} (RAGE knockout) mice were treated with PBS vehicle or Ang II for 4 weeks, at which time aortas were dissected and removed. (A) Representative examples of abdominal aortas from control mice treated with PBS (left), Ang II (middle) and RAGE knockout mice treated with AngII (right). (B) Aneurysm incidence (mice with aneurysms/total mice examined) in control and RAGE knockout mice; suprarenal aortas with > 1.5-fold the diameter of the infrarenal aorta were considered aneurysms. (C) Maximum suprarenal/infrarenal diameter for each group; *p< 0.05.

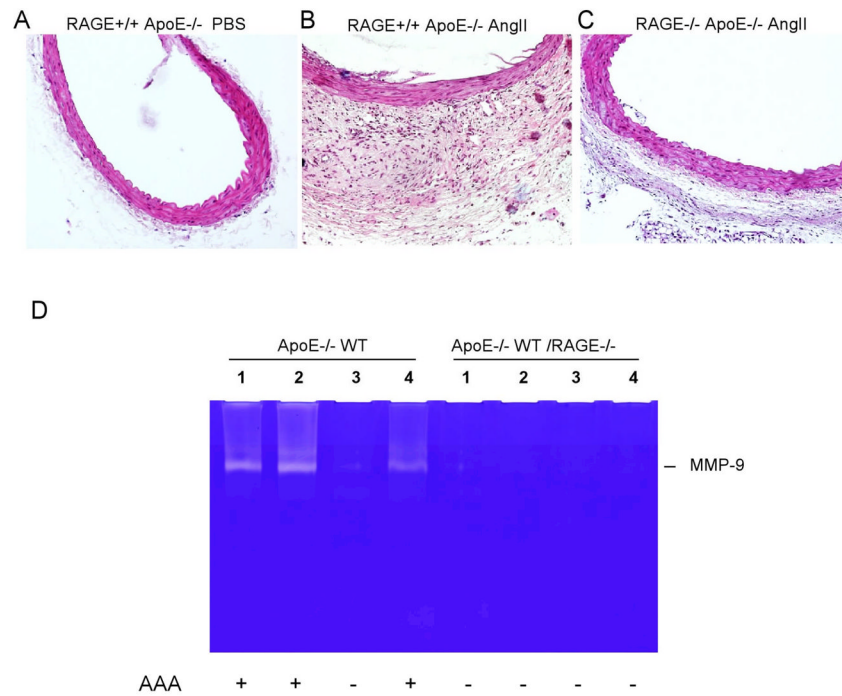


Fig. 5. Analysis of MMP-2 and 9 in mouse aortic tissues

(A–C) Histological sections of the mouse aorta were stained with hematoxylin/eosin. Apo E^{-/-} mice treated with PBS (A), ApoE^{-/-} mice treated with AngII (B), and RAGE^{-/-} ApoE^{-/-} mice treated with AngII (C). (D) Gelatin zymography for MMP-9 of protein extracts from suprarenal aorta tissue in angiotensin treated ApoE^{-/-} and ApoE^{-/-} RAGE^{-/-} mice.

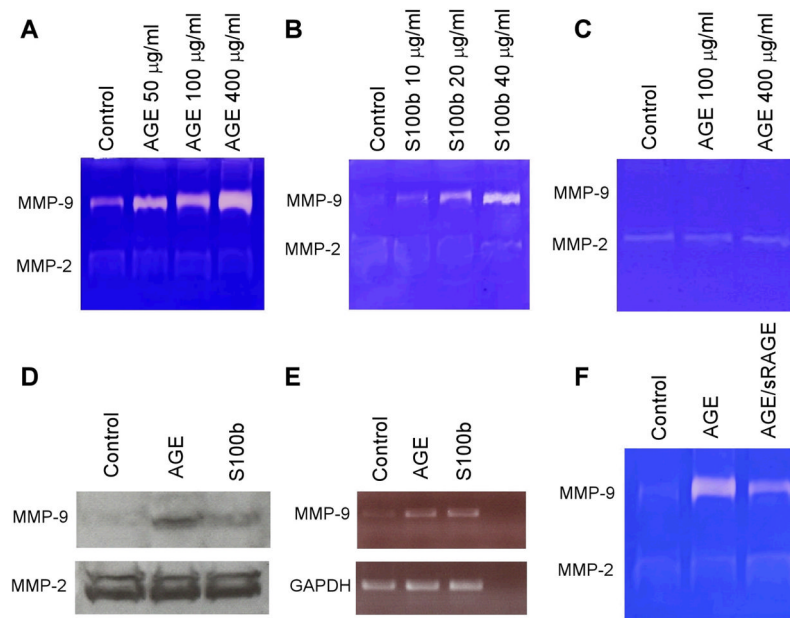


Fig. 6. In vitro measurement of MMP-2 and 9 in macrophages and SMCs following stimulation with AGE

(A) RAW 264.7 cells (macrophage cell line) were treated with 50, 100 and 400 μ g/mL of AGE for 24 h, then assessed for MMP-9 and MMP-2 activity by gelatin zymography. (B) RAW 264.7 cells were treated with 10 to 40 μ g/mL of S100b for 24 h. Supernatant was collected and assessed for MMP-9 and MMP-2 activity by gelatin zymography. (C) Rat SMCs cells were treated with 100 or 400 μ g/mL of AGE for 24 h, then assessed for MMP-9 and MMP-2 activity by gelatin zymography. (D-E) In RAW 264.7 cells, protein levels of MMP-9 and MMP-2 in response to AGE and S100b were confirmed by Western blotting (D) or mRNA expression of MMP-9 was measured by RT-PCR (E). (F) RAW 264.7 cells were treated with AGE (200 μ g/ml) and 100 μ M sRAGE or solvent for 24 h then assessed for MMP-9 and MMP-2 activity by gelatin zymography.