

Full Paper

Exacerbation of Intracranial Aneurysm and Aortic Dissection in Hypertensive Rat Treated With the Prostaglandin F₂-Receptor Antagonist AS604872

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Abstract. Intracranial aneurysm (IA) and aortic dissection are both complications of hypertension and characterized by degeneration of the media. Given the involvement of prostaglandin F₂ α and its receptor, FP, in extracellular matrix remodeling in a mouse model of pulmonary fibrosis, here we induced hypertension and IA in rats by salt loading and hemi-lateral ligation of renal and carotid arteries and examined effects of a selective FP antagonist, AS604872, on these vascular events. AS604872 significantly accelerated degeneration of the media in both cerebral artery and aorta as evidenced by thinning of the media and disruption of the elastic lamina and promoted IA and aortic dissection. Notably, AS604872 induced expression of pro-inflammatory genes such as E-selectin in lesions and significantly enhanced macrophage infiltration. Suppression of surface expression of E-selectin with cimetidine prevented macrophage infiltration and aortic dissection. Thus, AS604872 exacerbates vascular inflammation in hypertensive rats and facilitates IA and aortic dissection. These results demonstrate that both IA and aortic dissection are caused by chronic inflammation of the arterial wall, which is worsened by AS604872, cautioning that other FP antagonists may share such deleterious actions in vascular homeostasis and suggesting that AS604872 can be used to make models of these vascular diseases with extensive degeneration. [Supplementary materials: available only at <http://dx.doi.org/10.1254/jphs.14148FP>]

Keywords: prostaglandin F₂ α receptor (FP), AS604872, intracranial aneurysm, aortic dissection, chronic inflammation

Introduction

Arterial walls are continuously exposed to blood pressure, and develop various forms of diseases such as aortic

dissection and intracranial aneurysm (IA) under excessive pressure beyond physiological condition. Aortic dissection is a life-threatening disease at a high rate of early mortality, often causing sudden death (1, 2). It is assumed that high hemodynamic stress loaded on arterial walls makes a small tear in the intima of aortic wall, which leads to aortic dissection (1). However, how the arterial wall becomes fragile to hemodynamic stress has not been fully understood. IA is a regional bulging of

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intracranial arteries mainly at bifurcation sites, and it is characterized histologically by degenerative changes of the media (3). It is a major cause of subarachnoid hemorrhage, the most severe form of cerebrovascular disease with the mortality rate of 50% (4). While IA is of high incidence in the general public, 1% to 5% in autopsy series (5, 6), and subarachnoid hemorrhage is of poor prognosis, there is currently no medical treatment available for preventing enlargement and rupture of IA except for invasive surgical procedures. Given impaired daily life ability of IA patients due to their anxiety to potential rupture (7) and social loss caused by subarachnoid hemorrhage, the development of novel therapeutic drugs to prevent rupture is strongly desired. For this purpose, mechanisms underlying the degeneration of the media and rupture of IA have to be uncovered.

Prostaglandins (PGs) consisting of PGD₂, PGE₂, PGF₂α, PGI₂, and thromboxane (TX) A₂ are arachidonic acid metabolites formed by sequential actions of cyclooxygenase (COX) and respective PG synthases. PGs exert their actions through G-protein-coupled receptors (GPCRs) specific for each PG. They are PGD receptor (DP); four subtypes of PGE receptor, EP1 to EP4; PGI receptor (IP); PGF receptor (FP); and TXA receptor (TP) (8). Because COX is the target of non-steroidal anti-inflammatory drugs (NSAIDs) that suppress symptoms of acute inflammation such as fever, swelling, and pain, PGs have been traditionally recognized as mediators of acute inflammation. However, recent studies from our laboratory have elucidated that PGs not only function in acute inflammation but also contribute to transition to and maintenance of chronic inflammation. Examples of such PG actions are seen in collagen-induced arthritis (9), experimental allergic encephalomyelitis (10, 11), and transfer colitis (12), in which PGs amplify the actions of cytokines and facilitate inflammation. Furthermore, in the rodent model of IA, PGE₂ acts on EP2 in the endothelial cells and triggers NF-κB activation, and the resultant PGE₂-EP2-NF-κB-COX-2 pathway makes the vicious cycle and amplifies the inflammatory process (13, 14). PGs are also involved in tissue remodeling associated with chronic inflammation. For example, PGI₂-IP signaling collaborates with IL-1β and amplifies expression of FGF, VEGF, and RANKL in synovial fibroblasts in collagen-induced mouse arthritis (9), and PGF₂α-FP signaling accelerates bleomycin-induced pulmonary fibrosis independently of TGF-β by inducing expression of genes involved in extracellular matrix (ECM) remodeling (15). Given that the maintenance of ECM is essential for arterial stiffness and that the decrease in ECM is a pathological hallmark of IA and aortic dissection, we have administered a FP antagonist, AS604872, to the IA model of hypertensive rats and

examined possible involvement of PGF₂α-FP signaling as a potential factor maintaining IA walls and aorta.

Materials and Methods

Human samples

Human IA samples and control arteries (middle meningeal artery or superficial temporal artery) were obtained during neck clipping of IAs or bypass surgery with written informed consent (Sample ID: H0766962, H07014337, H07035715, H07026179, 58999, 52646, 47497). The use of human IAs in research was approved by the local ethical committee at Kyoto University Graduate School of Medicine (Application Number; 276).

Animals and the hypertension and IA model

All of the following experiments including animal care and use complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Kyoto University Graduate School of Medicine.

Sprague-Dawley rats were purchased from Japan SLC (Shizuoka). The FP-deficient mouse used in the present study was previously reported (16). To induce IA, 7-week-old male rats or mice were subjected to ligation of the left carotid artery and ligation of the left renal artery under general anesthesia with the intraperitoneal injection of pentobarbital sodium (50 mg/kg). Sham operated animals were treated in the same manner without the ligation of both arteries. Animals with the ligation of the arteries were fed a special chow containing 8% sodium chloride and 0.12% 3-aminopropionitrile (#A0408; Tokyo Chemical Industry, Tokyo), an inhibitor of lysyl oxidase catalyzing cross-linking of collagen and elastin. This 'aneurysm induction' procedure is designed to increase the hemodynamic stress in intracranial artery on a contralateral side of the carotid ligation to trigger IA formation (17–19) at the right bifurcation site of the anterior cerebral artery (ACA) and olfactory artery (OA) (14, 20, 21). Systemic blood pressure was measured by the tail-cuff method (BP-98A; Softron, Tokyo), and IA induced at the right ACA-OA bifurcation was assessed at times indicated in the Results section and figure legends.

FP antagonist treatment

AS604872, (2*S*)-3-([1,1'-biphenyl]-4-ylsulfonyl)-N-[(*R*)-phenyl(2-pyridinyl)methyl]-1,3-thiazolidine-2-carboxamide, a selective FP antagonist with the *K_i* values of 158 ± 57 nM for rat FP and 323 ± 23 nM for mouse FP (22), was synthesized at KNC Laboratories Co., Ltd. (Hyogo). The purity examined by HPLC was 99.8%. AS604872 was added to the chow at 0.2% and

orally administered to rats and mice at 200 mg/kg per day for the indicated periods after the aneurysm induction. Serum concentration of AS604872 was monitored by HPLC.

Histological analysis

For histological analysis, animals were sacrificed by intraperitoneal injection of a lethal dose of pentobarbital sodium and transcardially perfused with 4% paraformaldehyde. The circle of Willis containing IA lesion was dissected out and serial frozen sections of IA walls were prepared. The aorta was dissected and frozen sections were made similarly. These sections were subjected to Elastica van Gieson staining to visualize the elastic lamina, collagen fibers, and muscle fibers in arterial walls. IA was defined as a lesion with the disruption of the internal elastic lamina in this model. Aneurysm size was calculated as a mean of the largest traverse diameter and the height of the aneurysm dome. Thickness of media was defined as the thickness of the thinnest portion of the dome relative to that of normal arterial wall distal to IA.

RNA extraction, quantitative real time (RT)-PCR, and PCR array

At the indicated times after the aneurysm induction, rats were sacrificed as described above. Total RNA was prepared from the anterior portion of the circle of Willis or the aorta using a RNeasy Fibrous Tissue Mini Kit (QIAGEN, Hilden, Germany), and transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Life Technologies Corporation, Carlsbad, CA, USA). Quantitative RT-PCR was, then, performed with SYBR Premix Ex Taq II (Takara, Shiga) and Real Time System CFX96 (Bio-rad, Hercules, CA, USA). β -Actin was used as an internal control. For quantification, the second derivative maximum method was used for crossing point determination. Conventional RT-PCR was done using a Fast Cycling PCR kit (QIAGEN) and amplified products were separated by agarose-gel electrophoresis. Primer sets used in the present study are listed in Supplementary Table 1 (available in the online version only).

PCR array analysis was done by the RT² Profiler PCR array system from QIAGEN (#PARN-013Z, Extracellular Matrix and Adhesion Molecules). The induction of each gene was calculated as a ratio of gene expressions in FP antagonist-treated rats to those in vehicle-treated control rats.

Histochemistry

Immunohistochemical analysis was performed as previously described (14). Briefly, IA and aorta of rats were sectioned at 5- μ m thickness. For human samples, paraffin-embedded slices were deparaffinized and used.

After blocking with 5% donkey or goat serum (Jackson ImmunoResearch, Baltimore, MD, USA), the sections were incubated with primary antibodies followed by incubation with secondary antibodies conjugated with fluorescent dye (Jackson ImmunoResearch). Finally, immunofluorescence images were acquired on a confocal fluorescence microscope system (CTR6500; Leica Microsystems, Tokyo or Lsm710; Carl Zeiss Microscopy GmbH, Gottingen, Germany). Primary antibodies used were mouse monoclonal anti-alpha smooth muscle actin antibody (#MS113; Thermo Scientific, Waltham, MA, USA), rat monoclonal anti-F4/80 antibody (#ab16911; Abcam, Cambridge, UK), rabbit polyclonal anti-collagen I antibody (#NB600-408; Novus Biologicals, Littleton, CO, USA), rabbit polyclonal anti-FP receptor antibody (#101802; Cayman Chemical, Ann Arbor, MI, USA), mouse monoclonal anti-AKR1C3 (PGF synthase) antibody (#A6229; Sigma-Aldrich, St. Louis, MO, USA), goat polyclonal anti-MCP-1 antibody (#sc-1785; Santa Cruz Biotechnology, Dallas, TX, USA), mouse monoclonal anti-CD68 antibody (#sc-20060; Santa Cruz Biotechnology), rabbit monoclonal anti-phospho-NF-kB p65 (s536) antibody (#3033; Cell Signaling Technology, Danvers, MA, USA), goat polyclonal anti-E-selectin antibody (#LS-C150295; Lifespan Biosciences, Seattle, WA, USA), and rat polyclonal anti-CD31 antibody (#550274; BD Biosciences, Franklin Lakes, NJ, USA). In immunohistochemistry, macrophages were defined as F4/80-staining- or CD68-staining-positive cells. Number of infiltrated macrophages was determined as the number of the above positive cells in the areas indicated in the legends of each figure or in the figures themselves.

Tunnel staining was done according to the manufacturer's instructions using ApopTag Fluorescein Direct In Situ Apoptosis Detection Kit (Millipore, Billerica, MA, USA).

In situ hybridization

Animals were sacrificed as described above, and 10- μ m frozen sections were prepared from the intracranial artery and aorta. In situ hybridization was performed using a QuantiGene ViewRNA ISH Tissue Assay Kit (Veritas, Tokyo). In this experiment, pre-designed fluorescent-labelled probes for each target gene were purchased and hybridization was performed according to the instructions. Signals were detected on a confocal fluorescence microscope system (Lsm710; Carl Zeiss Microscopy GmbH).

Electron microscopy

At 8 days after the aneurysm induction, rats were sacrificed as described above. Dissected IA preparations were fixed in 2% glutaraldehyde and 2% paraformaldehyde.

hyde in 0.1 M sodium phosphate buffer (pH 7.4) followed by the treatment with 2% OsO₄. Fixed specimens were then washed, dehydrated in a graded series of ethanol solutions, replaced in propyleneoxide, and embedded in Quetol 651 epoxy resin for 48 h. Ultra-thin sections were prepared and stained with 2% uranyl acetate followed by the incubation in modified Sato's lead solution. Images were obtained using a Jeol JEM-1200EX transmission electron microscope system. For human specimens, slices were prepared from deparaffinized paraffin-embedded samples and treated as described above and images were taken by the Hitachi H-7650 transmission electron microscope system (Hitachi High-Technologies Corporation, Tokyo).

Cimetidine treatment

Cimetidine, 2-cyano-1-methyl-3-[(5-methyl-1H-imidazol-4-yl)methylthio]ethylguanidine, (Wako Pure Chemical Industries, Osaka) was dissolved in drinking water (2.5 mg/ml) and administered to rats for 37 days immediately after the aneurysm induction operation. Effects of cimetidine on E-selectin expression were assessed by immunohistochemistry.

Statistical analyses

Data are shown as the mean $\pm$ S.E.M. Statistical comparison between two groups was made using Mann-Whitney U-test. Statistical comparisons among more than 2-groups were conducted using the Kruskal-Wallis test followed by post hoc Dunn's test. The incidence of IA across groups was statistically compared with Fisher's exact test. Survival rate was statistically analyzed by Kaplan-Meier methods with chi-square test. *P*-value smaller than 0.05 was defined as statistically significant.

Results

Treatment with a FP antagonist, AS604872, exacerbated IA formation in rats

To examine the role of PGF₂ α -FP signaling in IA formation, we first subjected mice deficient in FP (16) to the IA model. The loss of FP did not influence the systemic blood pressure after salt overloading (FP^{+/+}, 78.0 $\pm$ 8.3 mmHg, *n* = 6; FP^{+/-}, 76.7 $\pm$ 8.1 mmHg, *n* = 14, FP^{-/-}; 77.3 $\pm$ 9.8 mmHg, *n* = 9), but significantly decreased the incidence of IA formation as defined by a lesion with disrupted internal elastic lamina in a gene-dosage-dependent manner (Supplementary Fig. 1: available in the online version only). To validate the effect of the loss of FP pharmacologically, we used a specific FP antagonist, AS604872 (22). The treatment with AS604872 decreased the incidence of IA formation in wild-type mice, well mimicking the effect of FP defi-

ciency (Supplementary Fig. 1).

The above results that the loss of FP and the treatment with the FP antagonist consistently suppress IA formation indicate that FP signaling promotes IA formation in mice. However, because IA progresses only limitedly and never ruptures in mice, we sought to reproduce this effect of AS604872 on IA formation in rats. In rats, IA induction causes degenerative changes of arterial walls and results in about 3% of spontaneous rupture (unpublished finding) as in humans. Therefore, IAs induced in rats mimic human IAs better than those in mice. The concentration of AS604872 in the serum of rats maintained at 200 mg/kg per day for more than one week was 1.15 $\pm$ 0.03 μ M (*n* = 3). Given the K_i value of AS604872 for rat FP, 158 $\pm$ 57 nM (22), this concentration was enough to antagonize FP signaling in rats. Similar to the findings in mice, the AS604872 treatment did not influence the systemic blood pressure: 149.0 $\pm$ 11.3 mmHg (*n* = 7) and 150.7 $\pm$ 15.5 mmHg (*n* = 6) at 1 week, 144.6 $\pm$ 8.3 mmHg (*n* = 5) and 139.0 $\pm$ 12.6 mmHg (*n* = 3) at 3 weeks, and 149.9 $\pm$ 10.1 mmHg (*n* = 5), and 170.5 $\pm$ 15.1 mmHg (*n* = 4) at 4 weeks for the vehicle-treated group and AS604872-treated group, respectively. However, on the contrary to its effects in mice, administration of AS604872 decreased neither the incidence nor the size of induced IAs (Supplementary Fig. 2: A and B, available in the online version only), but significantly accelerated the thinning of media of induced IAs compared with the vehicle-treated group as visualized by the Elastic van Gieson (EvG) staining and the immuno-staining for smooth muscle cell marker, α -smooth muscle actin (Fig. 1: A and B); the relative thickness of the thinnest portion of the media in IA walls to normal arterial walls (Fig. 1C) was significantly smaller in the AS604872-treated group than the vehicle-treated group (Fig. 1: D and E).

Degeneration of collagen fiber structure by treatment with AS604872

The finding that administration of AS604872 promoted medial thinning of arterial walls in IAs (Fig. 1) led us to suspect that degenerative changes caused by AS604872 treatment somehow impairs the arterial resistance to shear stress. Arterial stiffness largely depends on the content and structure of extracellular matrix (ECM) consisting mainly of collagen fibers in the artery. Because a previous study reported that FP stimulation promotes expression of collagens and related molecules (15), we first examined effects of AS604872 on expression of collagen genes. However, RT-PCR analysis of RNA extracted from IA walls showed that AS604872 administration influenced neither expression of genes for various forms of procollagen

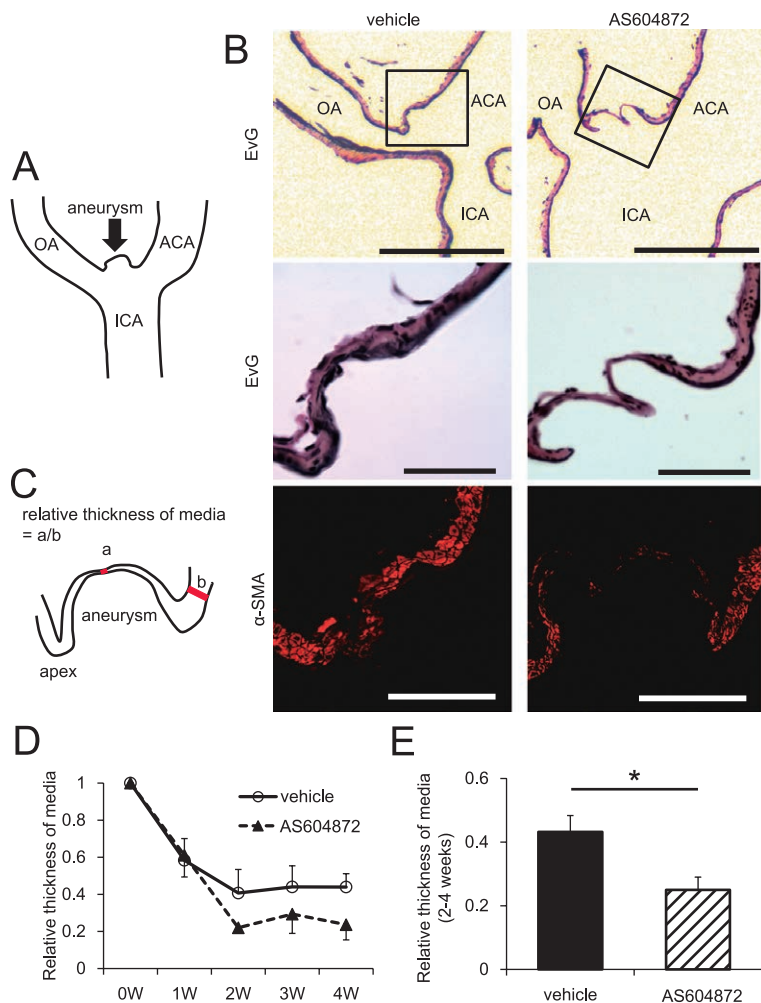


Fig. 1. Enhanced thinning of the media in intracranial aneurysm in rats treated with AS604872. A) Schematic representation of intracranial aneurysm formed in the present model. OA, olfactory artery; ACA, anterior cerebral artery; ICA, internal carotid artery. B) Intracranial aneurysm 3 weeks after the induction in rats treated with either vehicle (left panels) or AS604872 (right panels). Elastica van Gieson staining (EvG, upper and middle panels); bar, 200 μ m (upper) and 40 μ m (middle). Immunostaining for α -smooth muscle actin (α -SMA, lower panels); bar, 40 μ m. Representative image from 13 or 9 independent samples in vehicle-treated or AS604872-treated group, respectively, are shown. C) Schematic representation for calculation of thinning of the media. D, E) Time-dependent change in panel D and effects of AS604872 on panel E the thickness of the media in the aneurysm. All bars and lines indicate the mean $\pm$ S.E.M. $n = 13$ or 9 for vehicle-treated or AS604872-treated group, respectively. * $P < 0.05$.

and lysyl oxidase, an enzyme cross-linking collagen fibers (Supplementary Fig. 3A: available in the online version only), nor expression of genes for growth factors such as FGFs and PDGF- β and an embryonic type myosin heavy chain isoform (SMemb), a marker for differentiation and proliferation of vascular smooth muscle cells (23, 24) (Supplementary Fig. 3: B and C). Further, treatment with this compound did not induce apoptotic cell death in IA walls (Supplementary Fig. 3D).

We then examined the structure of IA wall of rats treated with AS604872. While treatment with this compound did not affect overall distribution of collagen type I in IA walls (Supplementary Fig. 4A: available in the online version only), electron microscopic analysis revealed that the AS604872 treatment caused marked loss of medial smooth muscle cells and induced appearance of amorphous structure, particularly in the adventitia (Fig. 2A). Higher magnified electron micrographs revealed that whereas the regular arrangement of collagen fibers remained in both the media and the

adventitia in vehicle-treated samples, the IA wall of AS604872-treated rats had collagen fibers that lost their bundled structure, becoming fused and wound in these regions, especially among smooth muscle cells of the media and within the amorphous structure in the adventitia (Fig. 2: B and C). Such disarrangement of collagen fibers is known as 'spiraled collagen' (25). We detected the spiraled collagen also in the adventitia of human IA walls but not in the wall of control superficial temporal artery (Fig. 2D), suggesting the clinical relevance of our findings on IA of AS604872-treated rats. Notably, the amorphous structures in the IA wall of AS604872-treated rats were stained positively with Alcian Blue but not with Periodic acid-Schiff, and this staining was diminished on treatment with hyaluronidase, suggesting deposition of hyaluronic acid (Supplementary Fig. 4B). Spiraled collagen and/or deposition of hyaluronic acid were previously observed in affected tissues of various inflammation-related diseases including atherosclerosis and rheumatoid arthritis (25 – 27).

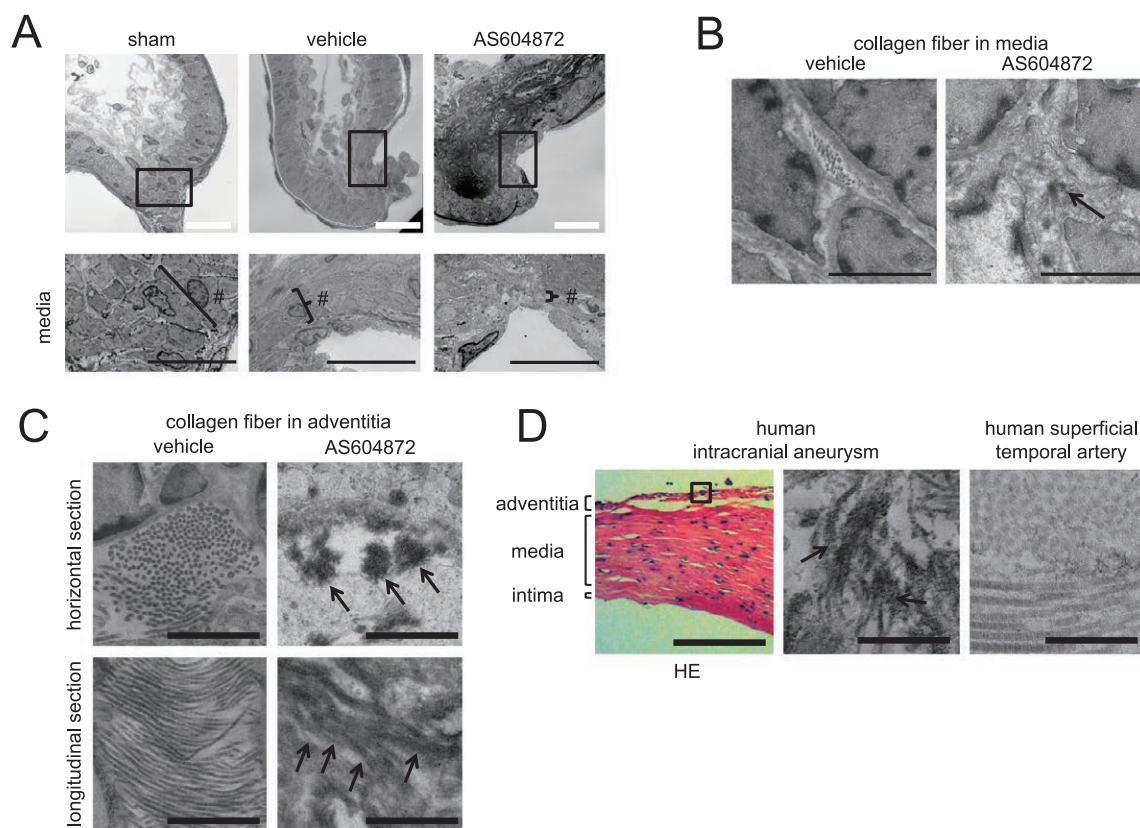


Fig. 2. Degeneration of collagen fiber structures in IA of rats treated with AS604872. A) Transmission electron microscopy (TEM) images of IA walls. Sham-operated rats or rats with the aneurysm induction treated for 8 days with either vehicle or AS604872 were subjected to the analysis; bar, 25 μm . Areas indicated by squares are magnified in lower panels. # indicates the medial smooth muscle cell layer; bar, 12.5 μm . Representative images from 5 independent specimens in each group are shown. B, C) TEM images of collagen fibers in the media (B) and the adventitia (C) of IA walls of rats treated with vehicle (left panel) or AS604872 (right panel). Note the absence of regularly arranged collagen fibers in the AS604872-treated preparation. Arrows indicate “spiraled collagen”; bar, 1 μm . Representative images from 5 independent specimens in each group are shown. D) TEM images of collagen fibers in the adventitia of human IA walls (middle) and the wall of control human superficial temporal artery (right). Arrows indicate the spiraled collagen; bar, 500 nm. Hematoxylin-eosin (HE) staining of human IA wall is shown in the left panel with the adventitia being indicated by a square; bar, 200 μm . Representative images from 3 independent specimens in each group are shown.

AS604872 administration accelerated aortic dissection in hypertensive rats

Chronic systemic hypertension imposed on rats for the aneurysm induction leads to premature death of animals; the death rate was accelerated after 100 days of hypertension and all died by around 200 days of hypertension (Fig. 3A). One presumed cause was heart failure because significant increase in the wet weight of the heart and the size of the left ventricle was seen already at 4 weeks after the induction of hypertension (Supplementary Fig. 5: A – D, available in the online version only). The other cause for premature death appeared to be aortic dissection. About 40% of the animals died of massive hemorrhage in thoracic cavity due to aortic dissection in thoracic aorta histologically defined by the presence

of pseudo-lumen. Notably, the death of AS604872-treated rats occurred significantly earlier than the vehicle-treated group (Fig. 3A, $n = 8$ for each, $P = 0.005$, by the Kaplan-Meier method); the mean survival period was 114.3 ± 3.5 days in the vehicle-treated group ($n = 8$) and 33.4 ± 4.0 days in the AS604872-treated group ($n = 8$, $P = 0.014$). The rats treated with AS604872 showed 100% incidence of aortic dissection and all died by 50 days after induction of hypertension (Fig. 3: A and B, $n = 8$ for each, $P = 0.026$). Many of them exhibited massive hemorrhage in the thoracic cavity through the dissection (Supplementary Fig. 5: E and F, available in the online version only). In the aorta of AS604872-treated rats, the elastic lamina of aortic arch was severely disrupted and fragmented before rupture at prospective

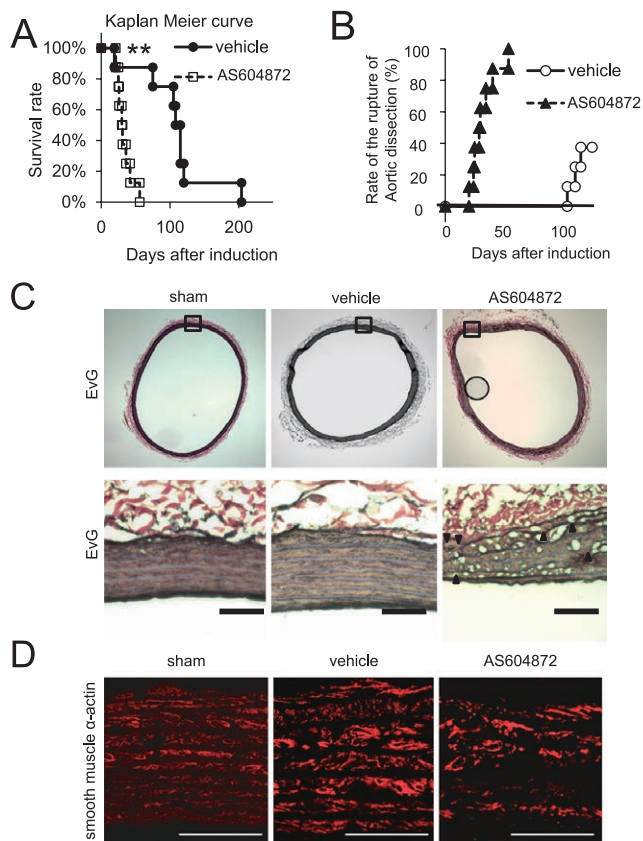


Fig. 3. Facilitation of aortic dissection in rats by treatment with AS604872. A) Kaplan Meier curve for survival rate of rats treated with vehicle (n = 8) or AS604872 (n = 8) after the IA induction. ** $P < 0.01$. B) Incidence rate of the rupture of aortic dissection in rats treated with vehicle or AS604872 after the IA induction. n = 8 for each group. C) EvG staining of the unruptured aorta from rats sham-operated or operated for IA induction and treated with vehicle or AS604872. Areas shown by the square in upper panels are magnified in the lower panels. Arrow heads indicate disruption of the internal elastic lamina; bar, 50 μ m. Representative images from 5 independent sections in each group are shown. D) Disarrangement and loss of smooth muscle cells in the media of rat IA walls. Immunohistochemistry for α -SMA in aortic walls of sham-operated rats (left) or rats treated with vehicle (middle) or AS604872 (right) two weeks after the IA induction. Representative images from 5 independent sections are shown. Bar, 50 μ m.

sites of the entry of aortic dissection (Fig. 3C), and extensive loss and disarrangement of the medial smooth muscle cells were also noted (Fig. 3D).

Administration of AS604872 exacerbated inflammation in IA walls and aorta

Vascular inflammation plays the crucial role in IA formation (13, 28, 29) and is characterized by macrophage infiltration, NF- κ B activation, and expression of inflammation-related genes such as MCP-1, COX-2, and TNF- α (13, 28, 30, 31). We wondered whether AS604872

treatment accelerated this process. Indeed, H&E staining revealed enhanced cell infiltration in the wall, especially in the adventitia, of not only IA (Supplementary Fig. 6A: available in the online version only) but also the aorta (Fig. 4A) of AS604872-treated animals compared to control rats. Immunostaining showed that most of them were positive for CD68 or F4/80, markers of macrophages (Fig. 4B, Supplementary Fig. 6A). The number of infiltrated macrophages in the adventitia of arterial walls defined as such was significantly higher in the wall of both aorta (Fig. 4C) and IA (Supplementary Fig. 6B) of AS607348-treated rats than that in control animals. We also noted that immunostaining signals for activated NF- κ B, pSer⁵³⁶-p65, were remarkably enhanced in both IA walls and aorta; in the aorta (Fig. 4D), they overlapped with CD68 signals and those for α -SMA, a marker for smooth muscle cells, in the media, and in the IAs (Supplementary Fig. 6C), the signals were found in endothelial cells and CD68-positive macrophages. Consistent with NF- κ B activation, RT-PCR analysis of RNA extracted from these tissues revealed up-regulation of expression of pro-inflammatory NF- κ B target genes such as TNF- α , COX-2, and IL-1 β in thoracic aorta (Fig. 4E) and IA walls (Supplementary Fig. 6D) of rats treated with AS604872. These results suggest that administration of AS604872 facilitates vascular inflammation and exacerbates IA formation and aortic dissection.

Enhanced induction of MCP-1 and E-selectin in aorta and IA by AS604872 treatment

Our previous findings revealed that macrophages are the major population of inflammatory cells recruited to IA walls through an action of MCP-1 and plays a crucial role in inflammation there for IA induction (30 – 32). Since the AS604872 treatment accelerated the macrophage infiltration both in IA and aorta in hypertensive rats (Fig. 4, Supplementary Fig. 6), we examined whether the AS604872 treatment enhanced the expression of MCP-1 in both tissues. We found that MCP-1 was induced more extensively both in aorta and IA wall of AS604872-treated rats than vehicle-treated control rats as expected (Fig. 5: A and B, Supplementary Fig. 7: available in the online version only). In the aorta, the MCP-1 signals overlapped with those for markers of macrophages and smooth muscle cells (Fig. 5B), while the signal was detected in endothelial cells and macrophages, the later mainly present in the adventitia, in the IA, as previously demonstrated (30) (Supplementary Fig. 7).

Because inflammatory cell infiltration is determined partly by chemotactic substances such as MCP-1 and partly by molecules mediating cell-cell or cell-matrix adhesion, we sought for additional factor(s) mediating

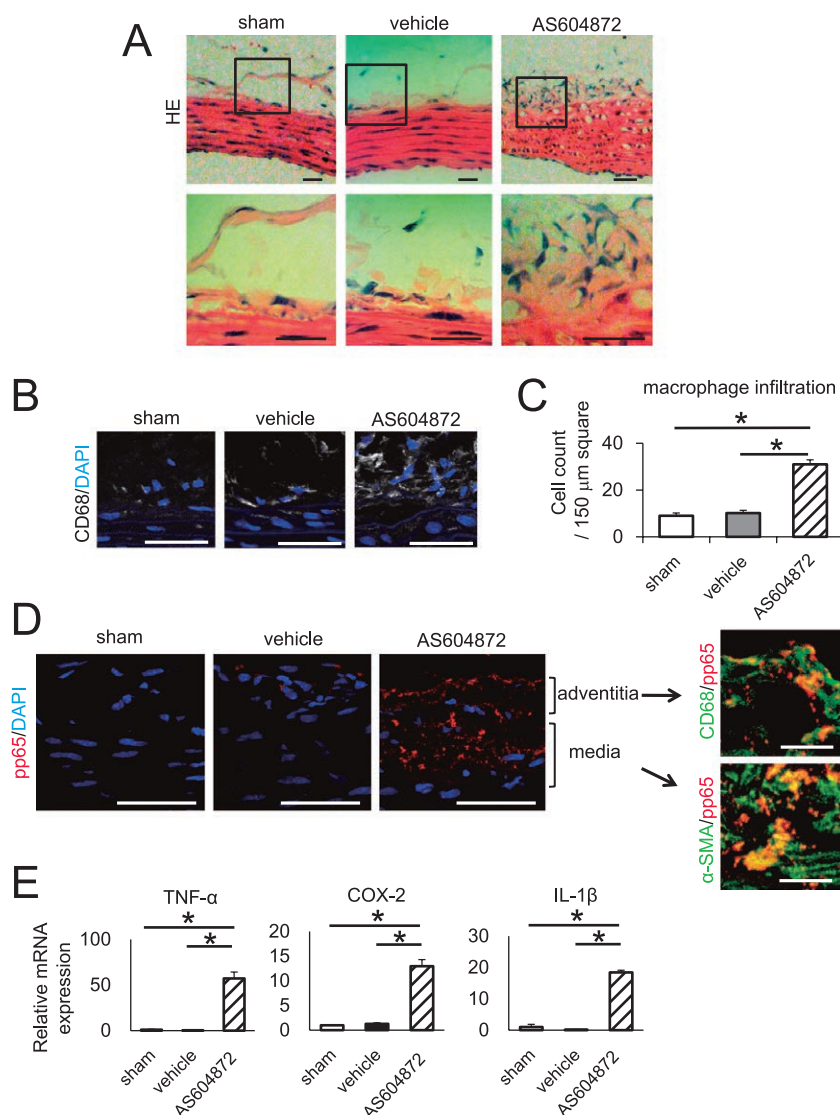


Fig. 4. Enhanced inflammation in aorta after the IA induction by treatment with AS604872. A) Inflammatory cell infiltration in the aorta after the IA induction. HE staining of the aorta from sham-operated rats or rats operated for the IA induction and treated with vehicle or AS604872 is shown. Areas in the adventitia shown in squares in upper panels are magnified in lower panels. Note the marked increase in infiltrating cells in the aorta of AS604872-treated rat. Bar, 50 μ m. B, C) Immunohistochemistry for CD68 (B) and quantification of infiltrating CD68-positive cells in the aorta (C). Sham-operated rats ($n = 4$) and rats operated for IA induction and treated with vehicle ($n = 6$) or AS604872 ($n = 6$) were sacrificed 2 weeks after the IA induction for immunohistochemistry. Gray and blue in panel B indicate the signals for CD68 and the nuclear staining by DAPI, respectively. Bar, 50 μ m. The number of CD68-positive cells in the 150- μ m square in adventitia of the aorta was determined and are shown as the mean $\pm$ S.E.M. in panel C. * $P < 0.05$. D) Immunohistochemistry for the Ser536-phosphorylated form of NF- κ B p65 subunit (pp65) in the aorta. Rats were treated as described in (B) and used for analysis. Red and blue indicate the signals for pp65 and DAPI, respectively. Bar, 50 μ m. Representative images from 4 independent experiments are shown. Merged images with CD68 or α SMA are shown in the right. Bar, 10 μ m. Representative images from 5 independent sections in each group are shown. E) Enhanced expression of inflammation-related genes in the aorta by AS604872 treatment. Rats were treated as described in panels B and C and total RNA was extracted from the aorta for quantitative RT-PCR. $n = 4$ for each group. All bars indicate the mean $\pm$ S.E.M. * $P < 0.05$.

macrophage infiltration in the aorta of AS604872-treated rats. We performed the PCR array analysis on RNA from aorta and found that E-selectin expression was most highly induced (Supplementary Fig. 8; available in the online version only). The PCR array analysis also reflected macrophage infiltration as shown by expression of integrin, α M and β 2, and further showed up-regulation of genes for matrix remodeling such as various matrix metalloproteinases, laminins, tenascin, and fibronectin and CD44, a receptor for hyaluronic acid. Among them, we focused on E-selectin because it regulates recruitment of macrophages in various inflammatory diseases (33). We confirmed the up-regulation of E-selectin gene expression in the aorta from AS604872-treated rats by RT-PCR analysis (Fig. 5C). Immunohistochemistry showed that the signals for E-selectin first appeared in endothelial cells at the early stage (Supplementary

Fig. 9: available in the online version only) and then spread to macrophages and smooth muscle cells in the whole wall of aorta of AS604872-treated rats (Fig. 5D). The most numerous and strongest signals were found in macrophages in the adventitia (Fig. 5D). The initial E-selectin signal in endothelial cells well overlapped with that for p-p65, a NF- κ B activation marker, suggesting the induction of E-selectin under NF- κ B activation (Supplementary Fig. 9). Notably, treatment with AS604872 did not appear to enhance E-selectin expression in endothelial cells at the early stage (Supplementary Fig. 9). We then examined the significance of E-selectin expression in the AS604872-induced exacerbation of inflammation in aorta and IA by administering cimetidine to rats in combination with AS604872. Cimetidine, an H_2 -blocker, is also known to down-regulate cell-surface expression of E-selectin (34, 35). Cimetidine co-administered with

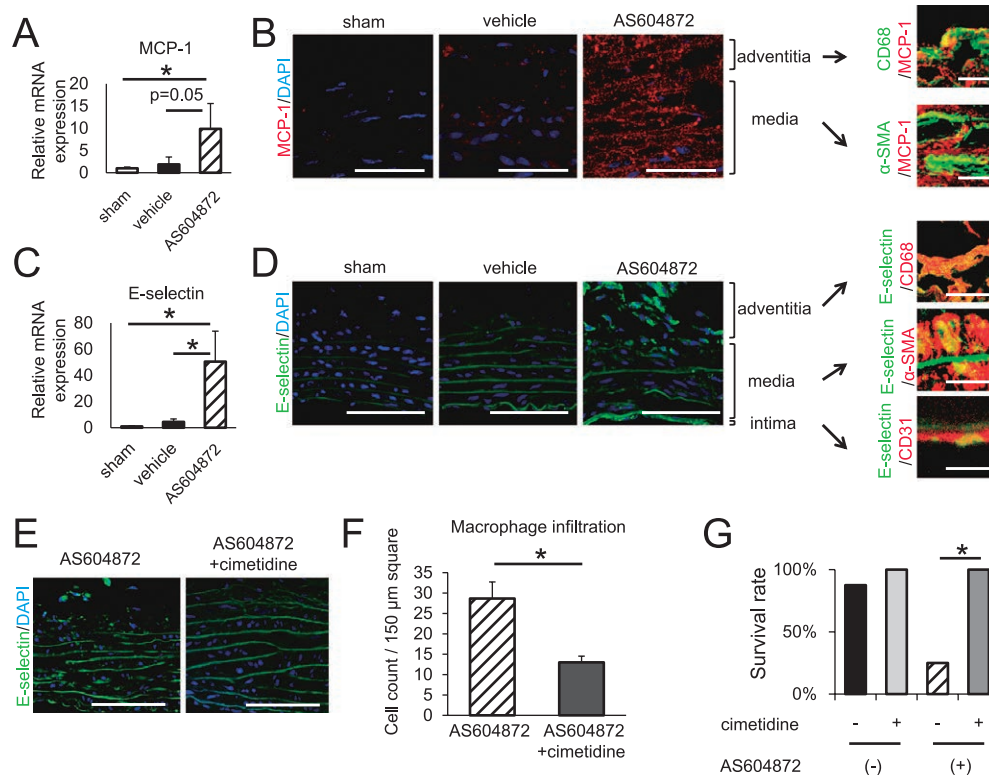


Fig. 5. Enhanced expression of MCP-1 and E-selectin in the aorta by AS604872 treatment. A, B) Quantitative RT-PCR analysis (A) and immunohistochemistry (B) for MCP-1 expression in the aorta. Rats were treated as described in Fig. 4B, and subjected to the analysis. The fold changes in MCP-1 mRNA were calculated as a ratio to the expression in the sham-operated group and are shown as the mean $\pm$ S.E.M. $n = 4$ for each group. $*P < 0.05$. In panel B, red and blue indicate the signals for MCP-1 and the nuclear staining by DAPI, respectively. Bar, 50 μ m. Merged images of MCP-1 (red) with CD68 or α SMA (green) are shown in right panels. Bar, 10 μ m. Representative images from 4 independent sections in each group are shown. C, D) Quantitative RT-PCR analysis (C) and immunohistochemistry (D) for E-selectin expression in the aorta. Rats were treated as described in Fig. 4B and subjected to the analysis. The fold changes in E-selectin mRNA were calculated as in panel C. $n = 3, 5$, or 4 for sham-operated, vehicle-treated, or AS604872-treated group, respectively. $*P < 0.05$. In panel D, green and blue indicate the signals for E-selectin and DAPI, respectively. Bar, 75 μ m. Merged images of E-selectin (green) with α SMA, CD68, or CD31 (red) are shown in the right panels. Bar, 20 μ m. Representative images from 4 independent sections in each group are shown. E, F, G) Effects of cimetidine on E-selectin protein expression (E) and macrophage infiltration (F) in the aorta and survival rate (G) of rats treated with AS604872. Cimetidine was administered in drinking water for 37 days after the IA induction to rats treated with either vehicle ($n = 8$) or AS604872 ($n = 6$), and the survival of treated mice was monitored. The aorta was dissected from mice for immunohistochemistry for E-selectin and CD68. Green and blue (E) indicate the signals for E-selectin and DAPI, respectively. Bar, 75 μ m. Representative images from 4 independent sections in each group are shown. Macrophage infiltration was examined as described in Fig. 4C and is shown as the mean $\pm$ S.E.M. (F). $n = 3$ for each group. $*P < 0.05$. Survival rate is compared among rats treated with or without cimetidine in combined administration with vehicle or AS604872 at 37 days after the IA induction (G). $*P < 0.05$.

AS604872 to rats indeed suppressed the expression of E-selectin in the aorta (Fig. 5E), decreased the number of infiltrating macrophages (Fig. 5F), and significantly improved the survival rate of AS604872-treated rats by suppressing the occurrence of the rupture of aortic dissection in thoracic aorta (Fig. 5G). Consistent with the inhibition of the rupture of aortic dissection, the cimetidine treatment remarkably suppressed the inflammatory responses, NF- κ B activation and MCP-1 expression, in the aorta (Supplementary Fig. 10: available in

the online version only). These results combined together suggest that the exacerbation of inflammatory responses in arterial walls by treatment with AS604872 is at least partially mediated by E-selectin induction.

E-selectin was also induced in the IA lesion. However, although treatment with cimetidine significantly inhibited E-selectin protein expression, it failed to suppress the development of IA (Supplementary Fig. 11: available in the online version only), suggesting that macrophage infiltration and the development of IA was mainly regu-

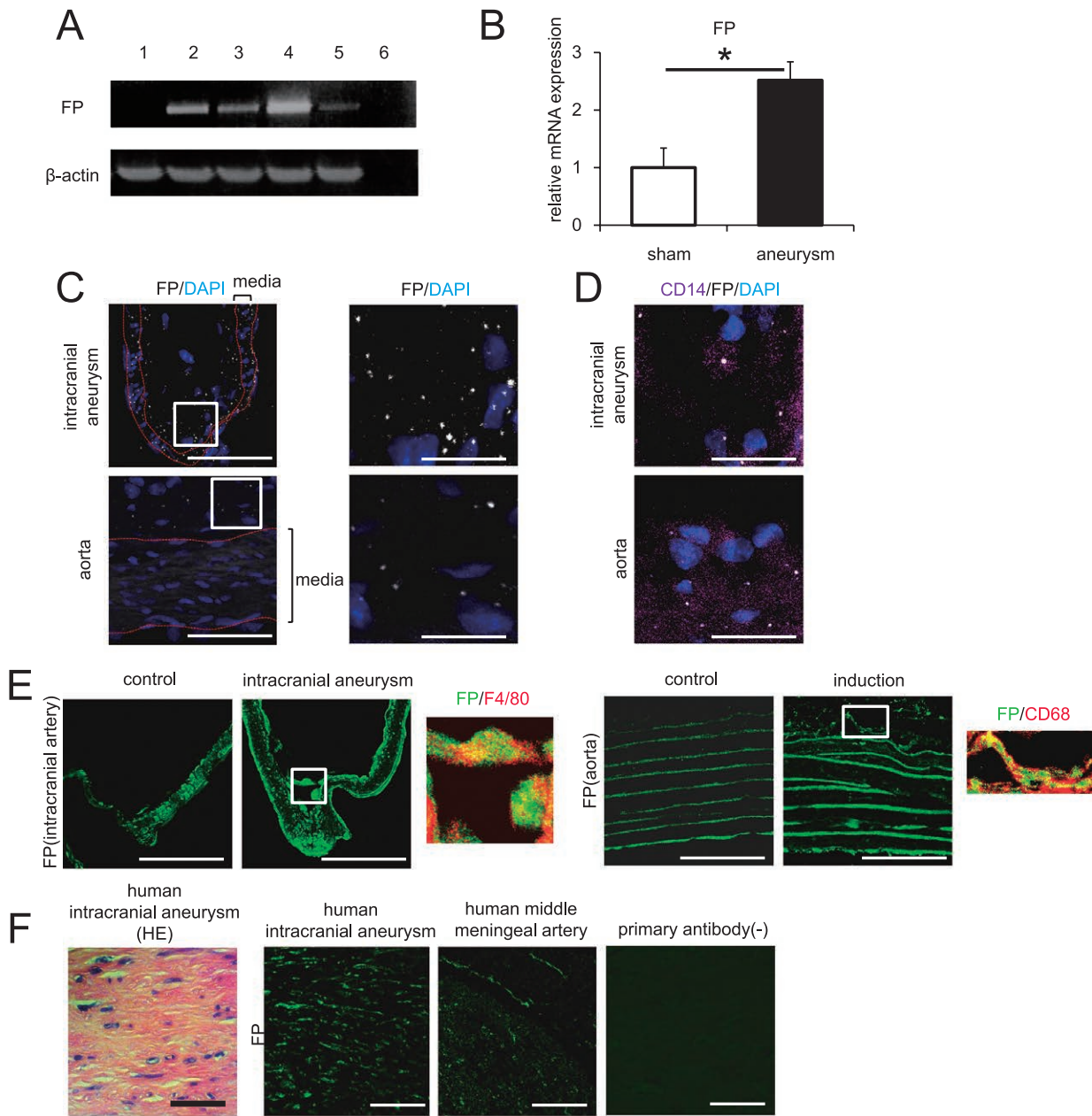


Fig. 6. FP expression in IA walls and aorta. A) RT-PCR analysis for FP gene expression in various arteries from rats. β -Actin served as the internal control. 1, aorta; 2, renal artery; 3, femoral artery; 4, carotid artery; 5, intracranial artery; 6, negative control (PCR reaction without primers). Representative images from 3 independent experiments are shown. B) Quantitative RT-PCR for FP mRNA in intracranial arteries from sham-operated rats (sham, $n = 3$) and rats 2 weeks after the IA induction (aneurysm, $n = 4$). All bars indicate the mean $\pm$ S.E.M. $*P < 0.05$. C) In situ hybridization for FP mRNA in IA walls (upper panels) and aorta (lower panels) from rats collected 2 weeks after the IA induction. Bar, 50 μ m. Regions in the square in the adventitia in the left panels are magnified in the right panels. Bar, 10 μ m. Gray and blue indicate the signals for FP and the nuclear staining by DAPI, respectively. The red line shows the media of arterial walls. Representative images from 3 independent sections in each group are shown. D) Double in situ hybridization for FP and CD14 mRNA in the IA wall (upper) and the aorta (lower) of rats collected 2 weeks after the IA induction. Gray, magenta and blue indicate the signals for FP, CD14 and DAPI, respectively. Bar, 20 μ m. Representative images from 3 independent sections in each group are shown. E) Immunohistochemistry for FP in the IA wall and the aorta of rats collected 2 weeks after the IA induction. Double immunostaining for FP and either F4/80 or CD68 in adventitia indicated by white squares are shown in the right. Green and red indicate the signals for FP and those for the macrophage markers, respectively. Bar, 50 μ m. Representative images from 4 independent sections in each group are shown. F) Immunohistochemistry for FP (green) in human IA wall and control middle meningeal artery. Immunostaining without the primary antibody is shown in the right, and HE staining of a serial section is shown in the left. Bar, 50 μ m. Representative images from 3 independent sections in each group are shown.

lated by MCP-1 and not by E-selectin, which is consistent with previous reports (30, 31).

FP expression in IA walls and aorta

We next examined expression of FP in IA walls and the aorta to identify the type of cells in which FP possibly functions. We used RT-PCR and in situ hybridization for mRNA expression and immunohistochemistry. RT-PCR analysis of various arteries under the basal conditions revealed that FP is not expressed in the aorta as reported previously (36) but is expressed in middle-sized arteries including renal, femoral, carotid, and intracranial arteries (Fig. 6A). Upon induction of hypertension, and consequently, IA formation, expression of FP mRNA was significantly increased in the intracranial artery of rats (Fig. 6B). In situ hybridization analysis with a cyclic amplification technique of these experimental animals detected appearance of punctate signals in the adventitia of both IA walls and the aorta (Fig. 6C). Double in situ hybridization analysis revealed that the signals for FP mRNA were well co-localized with those for CD14, a marker for macrophages (Fig. 6D), suggesting the presence of FP in macrophages infiltrating in IA and aorta. The in situ hybridization results were confirmed also by immunohistochemistry, in which positive signals for FP were seen in macrophages present in the adventitia (Fig. 6E). In addition, FP protein expression was also induced in endothelial cells of both IAs and aorta in the rats (Fig. 6E). The signal specificity of immunohistochemistry was confirmed using samples from FP-deficient mice (Supplementary Fig. 12: available in the online version only). Notably, Immunohistochemical analysis revealed that FP expression was also up-regulated in the adventitia of human IA walls compared with that in control middle meningeal artery (Fig. 6F). These results suggest that FP in macrophages may be a target for AS604872 in its enhancing effects on vascular inflammation. Finally, we examined expression of PGF synthase, an enzyme responsible for PGF₂ α production, by immunohistochemistry. Signals for PGF synthase were detected mainly in medial smooth muscle cells of both aorta and intracranial artery and increased in intensity after induction of hypertension in rats, suggesting increased production of PGF₂ α in situ (Supplementary Fig. 13: available in the online version only).

Discussion

In this work, we have administered a FP antagonist, AS604872, to mice and rats subjected to the IA model and examined its effects on development of IA in these animals. We have obtained apparently opposite results

in mice and rats (Fig. 1, Supplementary Fig. 1). While administration of AS604872 decreased incidence of IA formation in mice (Supplementary Fig. 1), rats administered with AS604872 developed IA at a similar incidence (above 80%) and much exacerbated pathology compared to control vehicle-treated rats (Fig. 1, Supplementary Fig. 2). Decreased IA incidence was also seen in mice deficient in FP, suggesting that AS607842 induced a phenotype similar to FP deficiency in mice (Supplementary Fig. 1). These opposite effects may be due to differences between the two types of animals, including the following possibilities: 1) Such difference in the outcome between mouse and rat could be explained by the stages each species animal reach in this disease model. The mouse develops IA only to the early stage, while the rat has its elastic lamina at bifurcations of intracranial arteries already disrupted in about two thirds of animals before aneurysm induction and develops it to the stage of degenerative changes once it begins. 2) The PGF₂ α -FP signaling may function differently in the vasculature between these animals. Indeed, in some genetically engineered animals, phenotype is quite different between rats and mice (37). 3) such a difference may just reflect the strain difference between the mouse C57BL/6 strain and the rat SD strain. 4) AS604872 may act on an additional target to FP to accelerate the degenerative changes in the rat, which should be tested in rats with genetic deletion of FP (see below). Nonetheless, these findings caution us not to simply extrapolate the findings obtained in mice to other animals. This caution is extremely important in the current situations where many experiments are being carried out in transgenic mice. Results obtained by gene-engineered mice should be reevaluated in other animal species using pharmacological reagents before drawing any definite conclusion.

The most prominent feature of IA caused by AS604872 administration in rats was strong degenerative changes such as formation of spiraled collagen and deposition of hyaluronic acid in the media and consequent marked thinning of the artery (Figs. 1 and 2, Supplementary Fig. 4). AS604872 administration also induced aortic dissection (Fig. 3, Supplementary Fig. 5: E and F). Aortic dissection, like IA, is one of the complications associated with hypertension, and occurs spontaneously in this model of rat. However, AS604872 administration much accelerated the progression of this disease, almost all rats treated with this compound exhibiting rupture within 50 days (Fig. 3). Here again, degeneration worsened; the elastic lamina of aortic arch was severely disrupted and fragmented, apparently making the aorta much more fragile to hypertensive stress, in AS604872-administered animals. Such degenerative changes were not associated

with down-regulation of collagen synthesis (Supplementary Fig. 3), but accompanied by enhanced infiltration of macrophages, activation of NF- κ B, and expression of pro-inflammatory genes such as TNF- α , IL-1 β , and COX-2 (Fig. 4). PCR array analysis of affected aorta from AS604872-treated rats confirmed recruitment of macrophages as suggested by high expression of integrin α M β 2 and high expression of various types of matrix metalloproteinases possibly derived from macrophages (Supplementary Fig. 8). Interestingly, expression of various ECM proteins and connective tissue growth factor was also enhanced, indicating extensive remodeling of ECM. Such macrophage recruitment appears to be amplified by a positive feedback loop as indicated by high expression of a macrophage chemoattractant, MCP-1, and an adhesion molecule, E-selectin (Fig. 5). Indeed, cimetidine treatment that suppressed surface expression of E-selectin (34, 35), suppressed macrophage infiltration and prevented aortic dissection with significant increase in the survival rate (Fig. 5: F and G).

The findings discussed above suggest that both IA and aortic dissection are diseases of macrophage-dominant chronic inflammation of arterial wall that is triggered by hypertensive stress and ends in degeneration of the media. This view is also supported by examination of clinical samples (30, 38–42). Our results indicate that AS604872 acts on some steps in this process and markedly facilitates inflammation. We suspect that triggering events in aortic dissection occur in the endothelium, which is directly exposed to stress. Indeed, E-selectin expression appeared first in the endothelium of the aorta in association of activation of NF- κ B and then spread to other layers (Fig. 5D, Supplementary Fig. 9). We also detected NF- κ B activation first in the endothelium in IA (28). However, AS604872 appears not to affect these triggering events because E-selectin expression was not critical in the pathology of IA that was also enhanced by administration of this compound (Supplementary Fig. 9). Our initial hypothesis that FP signaling acts in ECM remodeling associated with inflammation was not supported by the RT-PCR analysis that showed no change in expression of ECM-related genes (Supplementary Fig. 3). Our *in situ* hybridization and immunohistochemical studies showed expression of FP mRNA and protein in macrophages infiltrating the artery (Fig. 6). It is therefore plausible that AS604872 acts on FP in macrophages to shut off endogenous PGF $_2\alpha$ action and facilitates their infiltration and activation. We have tested this possibility by examining effects of latanoprost, a FP-specific agonist, on adhesion, migration, and activation of macrophages and its antagonism by AS604872, but could not obtain supporting evidence (M. Fukuda and T. Aoki, unpublished observations).

We have therefore not verified that the findings obtained by the use of AS604872 represent the actions of this compound on FP. This issue should be examined by the use of other FP antagonists and/or by generating and examining the phenotype of FP-deleted rats.

In conclusion, we have found that administration of AS604872 induces extensive degeneration of the arterial wall and facilitates formation of IA and aortic dissection in rats exposed to hypertensive stress. Our findings support a view that both IA and aortic dissection result from chronic inflammation of the arterial wall. Establishment of models for IA and aortic dissection with extensive degeneration using AS604872 will help investigate the underlying molecular mechanisms in future studies.

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