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Induction of *Timp1* in Smooth Muscle Cells during Development of Abdominal Aortic Aneurysms

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ABSTRACT

Abdominal aortic aneurysm (AAA) is known to develop mainly by the increased diameter of aorta through metalloproteinases (MMPs). Although activities of MMPs are tightly regulated by the presence of tissue inhibitor of MMPs (TIMPs) and imbalances between MMPs and TIMPs may serve to fragility of arterial wall, little is known about TIMPs behavior in aneurysmal formation. Here, we utilized a murine experimental AAA model, and found that by immunohistochemical analysis, Timp1 as and *Timp1* mRNA levels was also revealed in aortic tissue in AAA by RT-PCR. In cultured vascular smooth muscle cells (SMCs), Tumor Necrosis Factor (TNF)-α significantly activated both *Mmp9* and *Timp1* expression, and they were blocked by Jun kinase inhibitor (SP600125) in a dose-dependent manner. Interestingly, a proteasome inhibitor (MG132), which is known as an agent for inhibition of the nuclear factor-kappa B (NF-kB), significantly inhibited the TNF-α-induced expression of *Timp1*, whereas MG132, which also works as an activator of c-Jun/AP-1 pathway, strongly increased *Mmp9*. Taken together, inflammatory cytokines, including TNF-α, may simultaneously induce MMPs and TIMPs for the remodeling of the medial layer, leading to the increased diameter of the aorta, the aneurysm.

Key words: *Abdominal aortic aneurysm, Mmp9, Timp1, TNF-α*

Abdominal aortic aneurysm (AAA) is the most common disease in human males aged over 65[5,10)] with a prevalence of 5 - 8%. AAA is characterized as a progressive dilatation of the aortic wall in the abdomen, and larger aneurysms beyond 55 mm diameter increase the risk of rupture with a high subsequent mortality[5)]. Surgical or endovascular treatments can stabilize aneurysms, however, subsequent mortality and morbidity remain high. Therefore, the establishment of medical therapies for prevention as well as intervention is an urgent requirement.

One of the crucial pathological events for aneurysm formation is the destruction of medial elastic fibers and the infiltration of lymphocytes. The initial loss of elastin in the medial layer leads to compensatory fibrosis by increased collagen deposition and the destruction of all major matrix components, which cause further distension and eventually rupture. Destruction of elastin in the medial layer is associated with the production of metalloproteinases (MMPs), particularly MMP2 and MMP9, which show a strong elastinolytic activity, have similar protein structures and the same matrix substrate preference. However, the tissues for their production, cytokines for stimuli, and activators for their conversion from pro-state to active form, are quite different. MMP2 is constantly expressed in SMCs, whereas MMP9 is only found in SMCs after inflammatory activation[9)]. On the other hand, a compensatory system to prevent the destruction of the vascular medial layer is also provided. The matrix-digesting activities of MMPs are regulated by tissue inhibitory metalloproteinase (TIMP). *Timp1*, a specific inhibitor for *MMP9*, is up-regulated in human AAA patients[16)]. Even though evidence of the involvement of MMPs and their inhibitors to aneurysm formation has accumulated, the pathogenesis of AAA is still not fully understood.

In this study, we found up-regulation of *Mmp9* and *Timp1* expression levels in the abdominal aorta of the animal model of AAA. Irregular accumula-

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tion of Mmp9 and Timp1 was detected by immunohistochemical analysis. We also found that *Mmp9* and *Timp1* are induced by TNF-α, but not by Angiotensin (Ang) II in the SMC culture system. Their activation by TNF-α was reduced by Jun kinase inhibitor in a dose dependent manner, and accelerated or reduced by adding a proteosome inhibitor. These observations suggest that the inflammatory signal could induce both Mmp9 and Timp1 to regulate the remodeling of aortic walls and development of AAA.

MATERIALS AND METHODS

Generation of a mouse model for AAAs

AAA was induced by peri-aortic application of 0.5 M $CaCl_2$ in wild-type mice of the C57BL/6J strain (CLEA Japan, Inc.), under phenobarbital anesthesia, at 7 weeks of age. For the control (Sham group), saline was substituted for $CaCl_2$. At 6 weeks post-induction, the mice were sacrificed by an overdose of phenobarbital, and after perfusion-fixation and washing by 4% formaldehyde or PBS at physiological perfusion pressure, the abdominal aorta was excised for histological examination. All aortic morphometric procedures were performed by an investigator blinded to the experimental groups. Experiment protocols were approved by the Committee of Animal Experimentation at Hiroshima University.

Immunohistochemistry

Aortic tissues were embedded with Paraplast (McCormick Inc.) and sectioned at 6 μm thick serially. To examine the localization of Mmp9 and Timp1 in AAA tissue, sections were incubated with anti-mouse MMP9 goat antibody (R&D Systems; 1:50 dilution) or anti-Timp1 goat polyclonal antibody (LSBio; 1:50 dilution), and anti-goat IgG-Rhodamine conjugated antibody (Molecular Probe Inc.; 1:500 dilution). Signals were detected by fluorescence microscope (DMI 4000; Leica microsystems).

Cell culture

Mouse aortic SMCs were isolated from 5 weeks old male wild type mice as previously described[13] and maintained in DMEM with 10-20%FBS. Growth of cells at 70-80% confluence was arrested by incubation with serum free medium for 48 hr before stimulating cells with combinations of recombinant protein and chemicals, including TNF-α (Peprotech), AngII (Sigma Aldrich), MG-132 (Calbiochem), and SP600125 (Wako).

RT-PCR detection

Total RNA was isolated from the aortic samples restricted to the region of AAA by using a RNeasy Fibrous Tissue Mini Kit (Qiagen). Reverse transcription was performed using a ReverTra Ace qPCR RT Kit (TOYOBO). Real-time PCR was conducted using SYBR Premix Ex Taq II (Takara Bio Inc.) and the following *Mmp9* primers: forward 5'-GCCCTGG AACTCACACGACA-3' and reverse 5'-TTGGAAA CTCACACGCCAGAAG-3', and *Timp1* primers: forward 5'-TGAGCCCTGCTCAG CAAAGA-3' and reverse 5'-GAGGACCTGATCCGTCCACAA-3'. Amplification conditions were 5 s at 95℃, 20 s at 60℃, and 15 s at 72℃ for 49 cycles. G3PDH was used as an internal control. Band density was analyzed by scanning densitometry and measured using Opticon (MJ Research).

RESULTS

Induction of Mmp9 and Timp1 by AAAs

Mmp9 accumulation in the medial layer of AAA has been reported[18]. First, we tested Mmp9 expression at 6 weeks after AAA application. Mmp9 was found weakly in the overall medial layer of both Sham and AAA application, and strongly in the exposed portion of the operation side (Fig. 1A-1F). RT-PCR also showed a noticeable tendency of increased *Mmp9* accumulation in the aorta of both Sham and AAA application (Fig. 1G), suggesting that up-regulation of *Mmp9* might occur weakly in response to the operation and strongly in reaction to $CaCl_2$.

In addition to Mmp9, we tested Timp1 distribution after AAA application, because up-regulation of *MMP9* and *TIMP1* in human AAA patients has been reported[16]. At 6 weeks after AAA application, accumulation of Timp1 in the medial layer of aorta, significantly on the operation side, was detected after AAA application by antibody staining (Fig. 1H-1M). RT-PCR also revealed a significant up-regulation of *Timp1* at 6 weeks after AAA application compared to the sham operation (Fig. 1N). These data demonstrated that Timp1 was induced by AAA application in our AAA mouse model.

TNF-α up-regulates Mmp9 and Timp1 via different pathways

Since up-regulation of Mmp9 and Timp1 was found in the aorta after AAA application, we hypothesized that AngII, a vasopressor, could induce *Mmp9* and/or *Timp1* expression in the SMCs culture system. Although we found a tendency of *Mmp9* up-regulation at 6 hr after stimulation (Fig. 2A), as in a previous report[15], we could not detect the statistical significance of either the induction of *Mmp9* or *Timp1* by Ang II in our results (Fig. 2A and 2B).

We next tested if TNF-α, a mediator for inflammation[2], could affect the expression of *Mmp9* and *Timp1*, and found a significant up-regulation of both *Mmp9* and *Timp1* at 6 hr after stimulation (Fig. 2C and 2D). This showed similarities to a previous report[6]. TNF-α, in addition to activating the NF-kB-mediated signaling pathway[12], has been reported to activate c-Jun/AP-1 pathway[14].

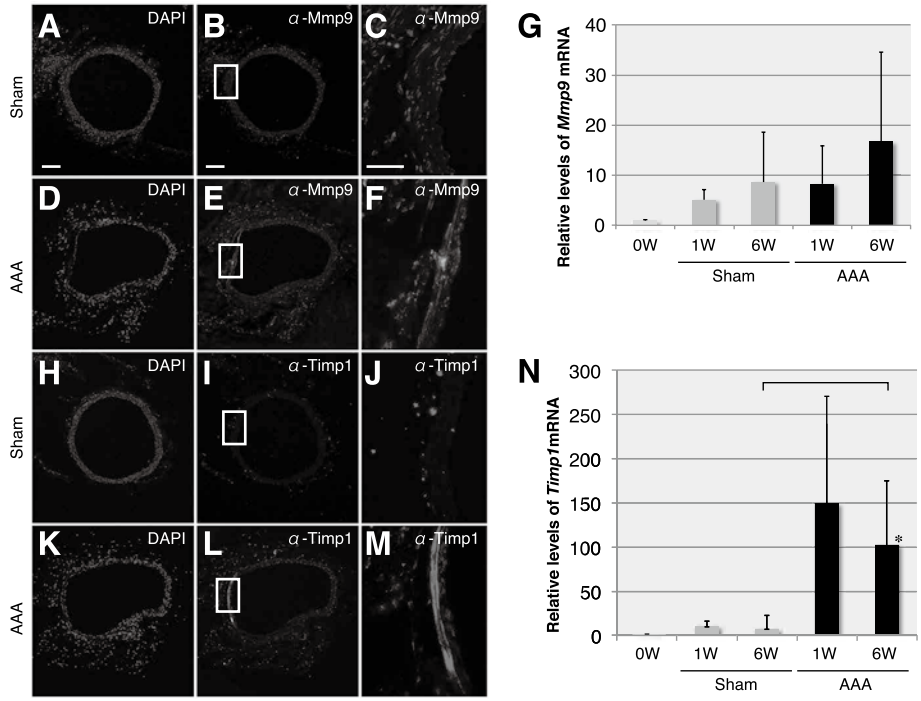

Fig. 1. MMP9 and Timp1 protein accumulation in the murine AAA experimental model.

Serial sections of aorta 6 weeks after Sham (A-C, H-J) or AAA operation (D-F, K-M) are shown. Representative immunofluorescence staining images are shown for DAPI (A, D, H, K), MMP9 (B, C, E, F) and Timp1 (I, J, L, M). Scale bars are represented to 100 um (A, B, D, E, H, I, K, L) and 50 um (C, F, J, M), respectively. The mRNA expression levels of *Mmp9* (G) or *Timp1* (N) in the aortic tissue, which was determined by RT-PCR, are shown. Data are presented as mean ± SD (n = 4-5). Statistical analysis used the Student's t-test, and asterisks indicate the difference from control (*$p < 0.05$).

Then, to ascertain which pathway was required for *Mmp9* induction, we used a proteasome inhibitor, MG132, which can inhibit the TNF-α-induced degradation of I-kBα and nuclear translocation of the p65/NF-kB subunit. Interestingly, MG132 activates the c-Jun/AP-1 pathway[7,17]. We also used a SP600125, which is a specific inhibitor of Jun Kinase[1]. Our results showed that, in addition to TNF-α-induced activation, the expression of *Mmp9* was significantly activated in MG132 treatment (Fig. 2E) and remarkably repressed by SP600125 treatment (Fig. 2G), suggesting that, rather than the NF-kB, the c-Jun signaling pathway is dominant for the *Mmp9* activation by TNF-α, as previously reported[18]. On the other hand, TNF-α-induced activation of *Timp1* was conspicuously repressed by MG132 (Fig. 2F) as well as SP600125 treatment (Fig. 2H), demonstrating that the *Timp1* would be induced through the NF-kB as well as the c-Jun pathway by TNF-α induction. It is noteworthy that AS-601245, a Jun Kinase inhibitor, repressed both *Mmp9* and *Timp1* expression, while BAY 11-7082, an inhibitor for NF-kB pathway, showed a suppressive effect restricted for *Timp1* transcription (data not shown). These observations indicate that Mmp9 and its endogenous inhibitor Timp1 are induced by the same stimuli through different signaling in addition to the common pathway, c-Jun.

DISCUSSION

In this study, we demonstrated for the first time the up-regulation of Timp1 in addition to that of Mmp9 in the medial layer of the mouse model for AAAs. ECM remodeling is critical to aneurysm formation[10]. ECM is largely synthesized in SMCs and functions to resist mechanical stress in their medial layer[4]. However, by responding to many stimuli, such as cytokine, growth factors and others, SMCs simultaneously produce a proteolytic enzyme, MMPs, which are essential for the extracellular matrix turnover associated with physiological and pathological tissue remodeling. These MMPs may further act on cytokines, chemokines and protein mediators to regulate various aspects of inflammation[11]. MMP activity is suppressed by TIMPs, which are also produced by SMCs. Since *Timp1*-deficient mice showed enhanced AAA[3], and genetic polymorphism of the *Timp1* gene was also found in human acute aortic dissection[8], remodeling of the extracellular matrix of the vascular wall should be tightly regulated by a quantitative balance of MMPs and TIMPs. These findings suggest that *Timp1* prevents disruption of aortic tissue in our experimental AAA model.

We also found the *Timp1* and *Mmp9* expression induced by TNF-α, but not by AngII, in the SMC

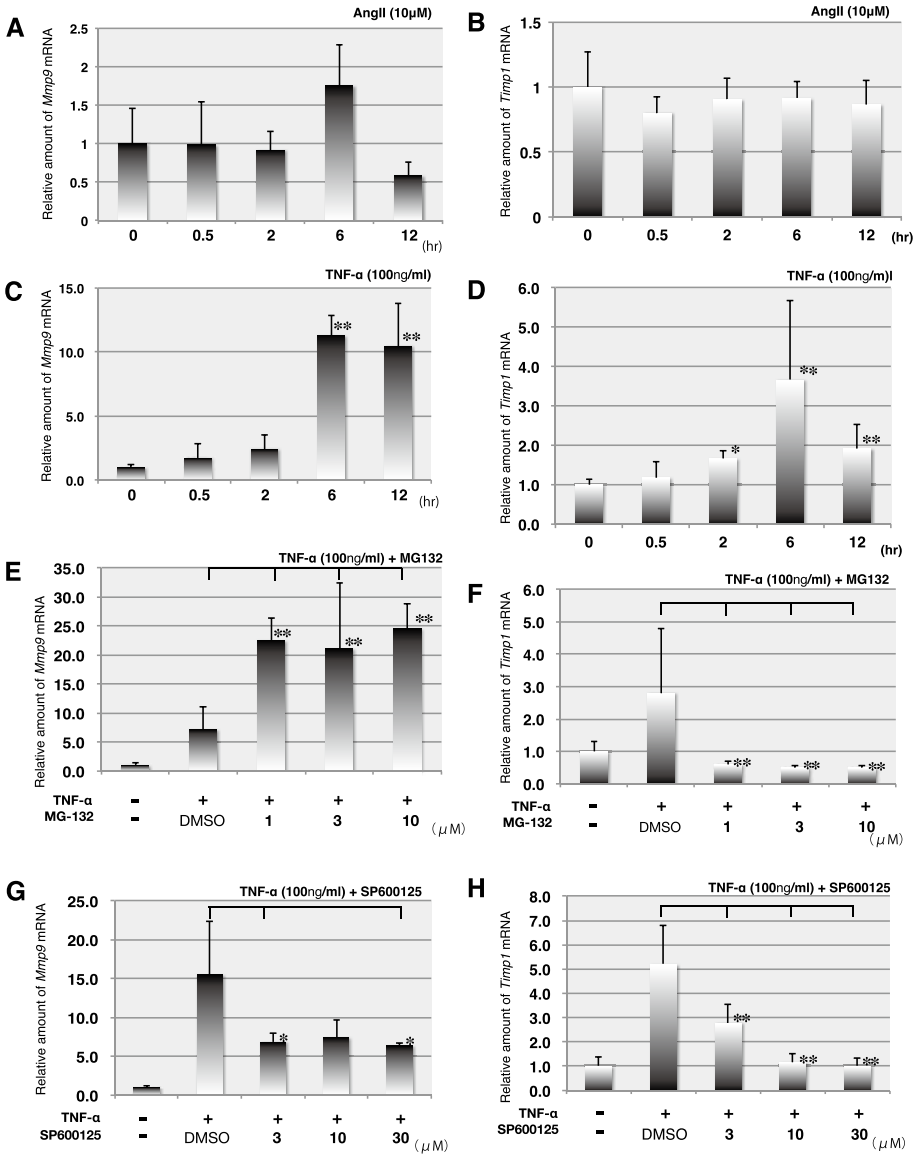

Fig. 2. Effects of AngII and TNF-α on the expression of *Mmp9* and *Timp1* genes in mouse aortic SMCs.

The *Mmp9* (A, C) or *Timp1* (B, D) mRNA expression level was determined by RT-PCR after stimulation with 10 μM of AngII (A, B) or 100 ng/ml of TNF-α (C, D) for the indicated periods. TNF-α, but not AngII, increased the *Mmp9* and *Timp1* mRNA level at 6 hrs after stimulation. The effect of proteasome, MG132 (E, F), or Jun kinase, SP600125 (G, H), inhibitor was tested in various concentrations (1-10 μM) or (3-30 μM), treated 2 hr or 1 hr before the 6 hr continuous stimulation of TNF-α (100 ng/ml), respectively. The mRNA level of *MMP9* (E, G) or *Timp1* (F, H) was determined by RT-PCR. Data are presented as mean ± SD (n = 3). Statistical analysis used Student's t-test, and asterisks indicate the difference from control (*$p < 0.05$; **$p < 0.01$).

culture system. We expected that up-regulation of *Timp1* and *Mmp9* could be induced by a vasoconstrictor, AngII, and a mediator of inflammation, TNF-α, although both of them were reacted only by TNF-α, suggesting that inflammation would be a key factor for their induction. Because AAA always begins with atherosclerosis, which is exaggerated by inflammation, it is reasonable that an inflammation signal is involved in the regulation of *Timp1* and *Mmp9*. In addition, our results indicated that TNF-α induction of *Timp1* and *Mmp9* might be involved in at least two distinct pathways for each induction. In addition to usage of the common pathway for Jun kinase, NF-kB for *Timp1* and an unidentified pathway for *Mmp9* may also participate. In fact, both inhibitors, NF-kB and Jun kinase, were valid for *Timp1* repression, whereas Jun kinase, but not NF-kB, was partially effective for *Mmp9* suppression. Since systemic inflammation is a critical reaction for biophylaxis, many signaling pathways known to be simultaneously activated should be systematically regulated for its completion. Therefore, both proteolytic enzymes and their inhibitors are coincidently induced by inflammation of the Jun kinase pathway, whereas usage of additional signaling pathways to regulate them allows well-balanced enzymatic activity: proteolytic in the acute inflammation phase or inhibitory in the termination phase. In the medial

layer of the inflammatory state, MMPs and TIMPs are simultaneously induced. As long as MMPs and TIMPs maintain an adequate balance for remodeling of the aortic tissue, it would result in mild to moderate formation of AAA. Once an imbalance between MMPs and TIMPs issues from a partial activation and/or inhibition through an additional signaling pathway, it may cause severe AAA formation. Thus, protection from the disruption of aortic tissue by TIMPs in the usage of chemical substances would be useful for the prevention of AAA *in vivo*.

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