Antithrombin III reduces collagen-stimulated granule secretion of PDGF-AB and the release of soluble CD40 ligand from human platelets

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Abstract. Although antithrombin-III (AT-III), an anti-coagulant, has been shown to affect human platelet functions, the direct effect of AT-III on platelets is still unknown. We recently reported that the collagen-induced phosphorylation of the heat shock protein 27 (HSP27) via the p44/p42 mitogen-activated protein (MAP) kinase is sufficient for granule secretion and the release of soluble CD40 ligand (sCD40L) from platelets but not platelet aggregation. In the present study, we investigated whether AT-III affects the collagen-induced secretion of the platelet-derived growth factor (PDGF)-AB and sCD40L release. AT-III inhibited collagen-stimulated platelet aggregation. The collagen-induced secretion of PDGF-AB was significantly suppressed by AT-III. AT-III also reduced sCD40L release. AT-III markedly attenuated the collagen-induced phosphorylated levels of p44/p42 MAP kinase. In addition, AT-III suppressed collagen-induced HSP27 phosphorylation. These results strongly suggest that AT-III reduced collagen-stimulated platelet granule secretion due to the inhibition of HSP27 phosphorylation via p44/p42 MAP kinase.

Introduction

It is generally recognized that antithrombin III (AT-III), a member of the serpin family, is one of the most important physiological inhibitors of coagulation such as thrombin (1,2). AT-III itself slowly prevents thrombin-activity, but when heparin and heparan sulfate proteoglycans exist on microvascular and macrovascular endothelial cells, the inactivating ability for thrombin is further enhanced. AT-III is clinically used as a therapeutic for sepsis or septic shock with disseminated intravascular coagulation and plays a pivotal role in coagulation abnormality (3). In contrast to heparin, which prevents coagulopathy, but does not reduce overall mortality, AT-III significantly increases the survival of endotoxin-induced shock without improving coagulopathy (4,5). Accumulating evidence suggests that AT-III demonstrates not only an anticoagulant effect but also an anti-inflammatory effect. It has recently been shown that AT-III directly affects platelets, resulting in the elicitation of the anti-inflammatory effect (6). However, the exact mechanism of AT-III in platelets remains to be clarified.

It is well recognized that collagen is a pivotal activator for human platelets, and its receptors on platelet membranes are mainly classified into glycoprotein VI (GPVI) and integrin α2ß1 (7). The firm adhesion of activated integrin α2ß1 by binding to collagen and strengthening of GPVI-collagen interactions leads to integrated signaling and the further up-regulation of integrin activity, resulting in the enhancement of granule secretion, and the development of coagulant activity (8-10). Heat shock proteins (HSPs) are expressed in a variety of cells when exposed to environmental stress such as heat and chemicals (11). HSPs are classified into high-molecular-weight HSPs such as HSP90 and HSP70, and low-molecular-weight HSPs such as HSP27 and αB-crystallin, based on their apparent molecular sizes. Low-molecular-weight HSPs with molecular masses from 10 to 30 kDa have high homology in their amino acid sequences (12). Low-molecular-weight HSPs may have chaperoning functions similar to the high-molecular-weight HSPs (12).

It is recognized that HSP27 activity is regulated by post-translational modifications such as phosphorylation (12,13).

HSP27 becomes rapidly phosphorylated in response to various stresses, as well as exposure to cytokines and mitogens (14,15). Human HSP27 is phosphorylated at three serine residues (Ser-15, Ser-78 and Ser-82). While HSP27 exists in an aggregated form under unstimulated conditions, it is rapidly dissociated as a result of stimulation-responsive phosphorylation. It has been shown that HSP27 phosphorylation is catalyzed by the MAP kinase superfamily such as p38 MAP kinase, the stress-activated protein kinase/c-Jun *N*-terminal kinase (SAPK/JNK) and p44/p42 MAP kinase (16,17). It has been reported that collagen regulates the phosphorylation of HSP27, which is most likely required for human platelet activation (18). We recently showed that in human platelets the collagen-induced phosphorylation of HSP27 via p44/p42 MAP kinase is correlated with platelet granule secretion such as the platelet-derived growth factor (PDGF)-AB and the release of soluble CD40 ligand (sCD40L), but not for platelet aggregation (19). However, the exact mechanism of collagen in platelet activation is not precisely clarified.

In the present study, we examined the effect of AT-III on collagen-induced platelet granule secretion and its exact mechanism. We show that AT-III suppresses the collagen-induced secretion of PDGF-AB from the human platelet granule and the release of sCD40L due to the inhibition of HSP27 phosphorylation via p44/p42 MAP kinase.

Materials and methods

Materials. Collagen was purchased from Nycomed Pharma GmbH (Munich, Germany). AT-III was a generous gift from CSL Behring (Tokyo, Japan). AT-III was extracted from human blood and its purity was >97.9% (analyzed by High Performance Liquid Chromatography) or >98.2% [analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE)]. AT-III was stored as a freeze-dried agent and dissolved with distilled water. PDGF-AB enzyme-linked immunosorbent assay (ELISA) kits and sCD40L ELISA kits were purchased from R&D System, Inc. (Minneapolis, MN). Phospho-specific p44/p42 MAP kinase and p44/p42 MAP kinase antibodies were obtained from Cell Signaling, Inc. (Beverly, MA). Phospho-specific HSP27 (Ser-15) and phospho-specific HSP27 antibodies (Ser-78) were from Stressgen Biotechnologies (Victoria, BC, Canada). Phospho-specific HSP27 antibodies (Ser-82) were from Biomol Research Laboratories (Plymouth Meeting, PA). The other materials and chemicals were obtained from commercial sources.

Preparation of platelets. Human blood was donated from healthy volunteers into a 1/10 volume of 3.8% sodium citrate. Platelet-rich plasma (PRP) was obtained from blood samples by centrifugation at 155 x g for 12 min at room temperature. Platelet-poor plasma was prepared from residual blood by centrifugation at 2,500 x g for 5 min. All participants signed an informed consent after receiving a detailed explanation and the study was approved by the Committee of Ethics of the Gifu University Graduate School of Medicine.

Measurement of platelet aggregation induced by collagen. Platelet aggregation using citrated PRP was carried out in a PA-200 aggregometer (Kowa Co. Ltd., Tokyo, Japan), which can determine the size of platelet aggregates based upon particle counting using laser scattering methods (small size, 9-25 µm; medium size, 25-50 µm; large size, 50-70 µm) (20), at 37˚C with a stirring speed of 800 rpm. The platelets were preincubated for 1 min, and then platelet aggregation was monitored for 4 min. The percentage of transmittance of the isolated platelets was recorded as 0%, and that of the appropriate platelet-poor plasma (blank) was recorded as 100%. When indicated, PRP was pretreated with AT-III for 15 min.

Protein preparation after collagen stimulation. After the stimulation with collagen, platelet aggregation was terminated by the addition of ice-cold EDTA (10 mM) solution. The mixture was centrifuged at 10,000 x g at 4˚C for 2 min. To measure PDGF-AB and sCD40L as described below, the supernatant was isolated and stored at -20˚C for subsequent ELISA. For Western blot analysis, the pellet was washed twice with phosphate-buffered saline and then lysed and immediately boiled in a lysis buffer containing 62.5 mM Tris/Cl, pH 6.8, 2% SDS, 50 mM dithiothreitol, and 10% glycerol.

Measurement of PDGF-AB and sCD40L levels. The PDGF-AB and sCD40L levels in samples were determined using each kit following the manufacturer's instructions.

Western blot analysis. Western blot analysis was performed as described previously (21). Briefly, SDS-PAGE was performed by the method of Laemmli (22) in a 10% or 12% polyacrylamide gel. The proteins in the gel were transferred onto a polyvinylidine fluoride (PVDF) membrane. The membranes were then blocked with 5% fat-free dry milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T, 20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween) for 2 h before incubation with the indicated primary antibodies. The antibodies used in these studies were phospho-specific p44/p42 MAP kinase, p44/p42 MAP kinase, phospho-specific HSP27 (Ser-15), phospho-specific HSP27 (Ser-78), phospho-specific HSP27 (Ser-82), and HSP27 antibodies or GAPDH, respectively. Peroxidase-labeled anti-mouse IgG or anti-rabbit IgG antibodies were used as secondary antibodies. The first and second antibodies were diluted for optimum concentration respectively, with 5% fat-free dry milk in TBS-T. Peroxidase activity on PVDF membranes was visualized on X-ray film by means of an ECL Western blotting detection system following the manufacturer's protocol.

Statistical analysis. The data were analyzed by the Student's t-test, and a p<0.05 was considered to be significant. All data are presented as the mean ± SEM.

Results

Effect of AT-III on platelet aggregation by collagen stimulation. We first examined the effect of AT-III on platelet aggregation stimulated by collagen using a platelet aggregometer. AT-III markedly reduced the collagen-stimulated platelet aggregation in a dose-dependent manner
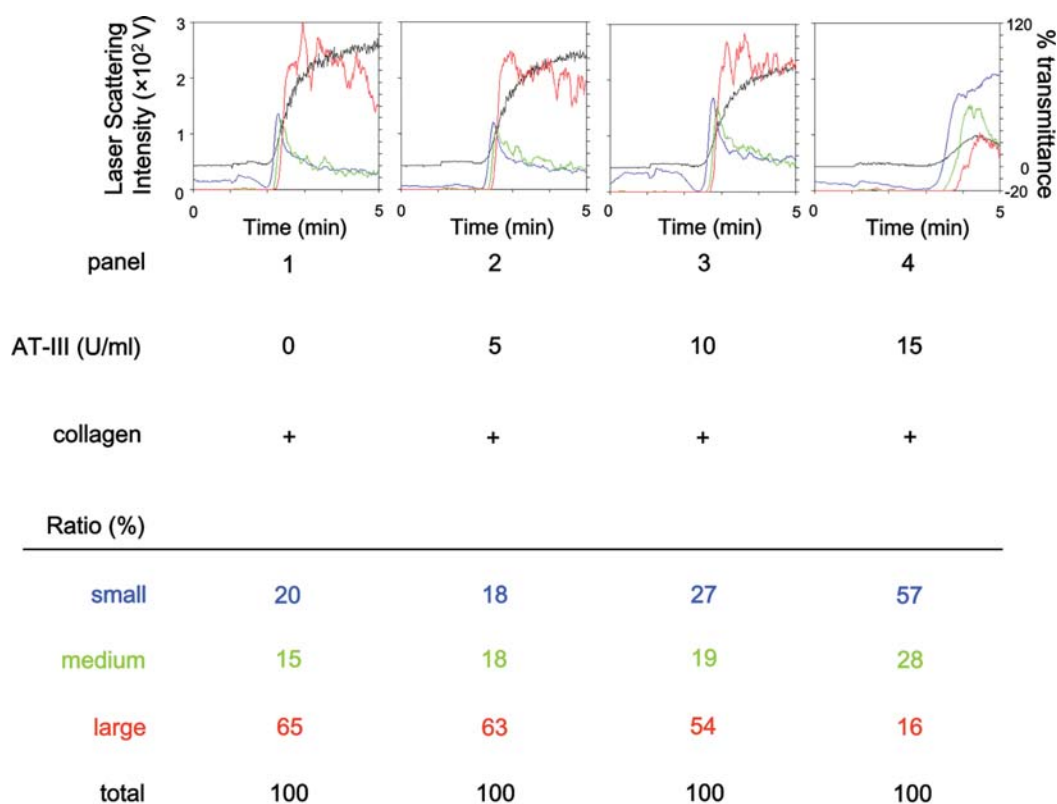
Figure 1. Effect of AT-III on the collagen-induced platelet aggregation. PRP was pretreated with various doses of AT-III at 37˚C for 15 min, and stimulated by 1.0 mg/ml collagen for 5 min. The reaction was terminated by the addition of ice-cold EDTA (10 mM) solution. Black line indicates the percentage of transmittance of each sample (the isolated platelets were recorded as 0%, and platelet-free plasma was recorded as 100%). Blue line indicates small aggregates (9-25 μm); green line, medium aggregates (25-50 μm); red line, large aggregates (50-70 μm). The distributions (%) of aggregated particle sizes were measured using laser scattering methods. Representative results obtained from five healthy donors are indicated.
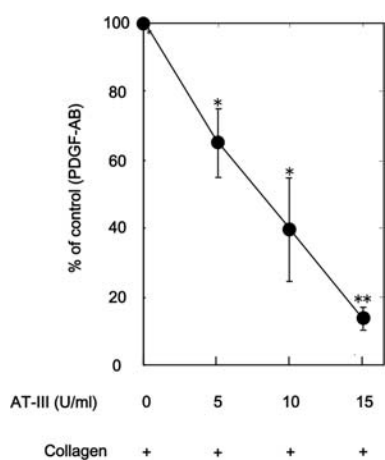

Figure 2. Effect of AT-III on the collagen-induced PDGF-AB secretion in human platelets. PRP was pretreated with various doses of AT-III at 37˚C for 15 min, and stimulated by 1.0 mg/ml collagen for 30 min. The reaction was terminated by the addition of ice-cold EDTA (10 mM) solution. The mixture was centrifuged at 10,000 x g at 4˚C and the supernatants were then subjected to ELISA for PDGF-AB. The net increased levels of collagen alone are represented as 100%. Representative results from five independent experiments are shown. Each value represents the mean ± SEM. *p<0.05, compared with the value of the control.

in the range between 5 and 15 U/ml (Fig. 1) as previously shown (6). AT-III dose-dependently decreased large aggregates (50-70 μm) according to the analysis of the size of platelet aggregates (Fig. 1). On the contrary, small aggregates (9-25 μm) and medium aggregates (25-50 μm) were markedly increased by AT-III (Fig. 1).

Effect of AT-III on the collagen-induced platelet granule secretion. In order to investigate whether AT-III affects collagen-induced human platelet granule secretion, we next examined the effect of AT-III on the collagen-induced secretion of PDGF-AB from platelets. We found that collagen stimulated the secretion of PDGF-AB (255±20 pg/ml for control; 8990±1050 pg/ml for 1.0 mg/ml collagen, as measured during the stimulation for 30 min). AT-III (15 U/ml) significantly suppressed the collagen-induced PDGF-AB secretion from human platelets in a dose-dependent manner (Fig. 2). AT-III markedly inhibited the PDGF-AB secretion and 15 U/ml of AT-III caused an ~80% reduction in the collagen effect (Fig. 2).

Effect of AT-III on the collagen-stimulated release of sCD40L from human platelets. We next examined the effect of AT-III on the collagen-stimulated sCD40L release from platelets. We found that collagen stimulated the release of sCD40L (63±10 pg/ml for control; 4573±250 pg/ml for 1.0 mg/ml collagen, as measured during the stimulation for 30 min). AT-III significantly inhibited the collagen-stimulated release of sCD40L (Fig. 3). The suppressive effect of AT-III was dose-dependent and 15 U/ml of AT-III caused an ~80% reduction in the collagen effect (Fig. 3).
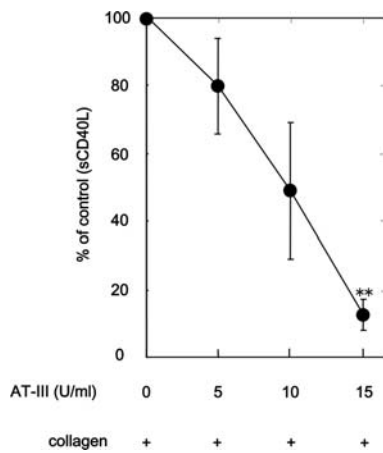
Figure 3. Effect of AT-III on the collagen-induced sCD40L release in human platelets. PRP was pretreated with various doses of AT-III at 37°C for 15 min, and stimulated by 1.0 mg/ml collagen for 30 min. The reaction was terminated by the addition of ice-cold EDTA (10 mM) solution. The mixture was centrifuged at 10,000 x g at 4°C, and the supernatants were then subjected to ELISA for sCD40L. The net increased levels of collagen alone are represented as 100%. Representative results from five independent experiments are shown. Each value represents the mean ± SEM. *p<0.05, compared with the value of the control.
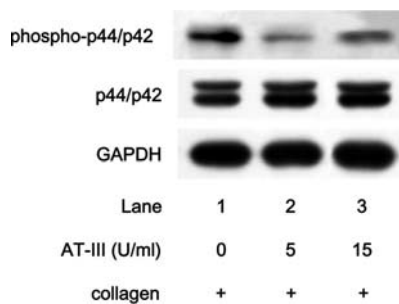

Figure 4. Effect of AT-III on the collagen-induced phosphorylation of p44/p42 MAP kinase in human platelets. PRP was pretreated with the indicated doses of AT-III at 37°C for 15 min, and stimulated by 1.0 mg/ml collagen for 5 min. The reaction was terminated by the addition of ice-cold EDTA (10 mM) solution. Lysed platelets were subjected to Western blot analysis using antibodies against phospho-specific p44/p42 MAP kinase, p44/p42 MAP kinase or GAPDH. Representative results from at least three independent experiments are shown.

Effect of AT-III on the collagen-induced phosphorylation of p44/p42 MAP kinase or HSP27 in human platelets. We previously showed that collagen stimulates the phosphorylation of HSP27 via p44/p42 MAP kinase in human platelets, and subsequently induces the secretion of PDGF-AB and the release of sCD40L (19). Therefore, we next examined the effect of AT-III on the collagen-induced phosphorylation of p44/p42 MAP kinase in human platelets. AT-III markedly reduced the collagen-induced phosphorylation levels of p44/p42 MAP kinase (Fig. 4).

In our previous study (19), we demonstrated that collagen elicits HSP27 phosphorylation at three serine residues, Ser-15, Ser-78 and Ser-82, in human platelets. Furthermore, the effect of AT-III on the collagen-induced phosphorylation of HSP27 (Ser-15, Ser-78 and Ser-82) was examined. The
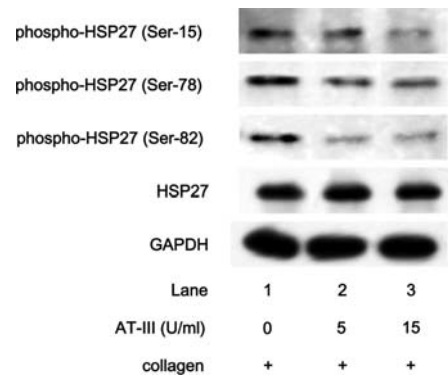

Figue 5. Effect of AT-III on the collagen-induced phosphorylation of HSP27 in human platelets. PRP were pretreated with the indicated doses of AT-III at 37°C for 15 min, and then stimulated by 1.0 mg/ml collagen for 5 min. The reaction was terminated by the addition of ice-cold EDTA (10 mM) solution. Lysed platelets were subjected to Western blot analysis using antibodies against total HSP27, phospho-specific HSP27 (Ser-15, Ser-78 and Ser-82) or GAPDH. Representative results from at least three independent experiments are shown.

phosphorylation levels of HSP27 (Ser-15, Ser-78 and Ser-82) induced by collagen were markedly attenuated by AT-III (Fig. 5).

Discussion

In the present study, we focused on the effects of AT-III on collagen-induced human platelet functions. First, we found that AT-III significantly suppressed collagen-induced platelet aggregation and affected the distribution of aggregated particle sizes measured in laser scattering methods. We next investigated the effect of AT-III on collagen-induced granule secretion and showed that collagen-induced granule secretion of PDGF-AB was significantly inhibited by the pretreatment with AT-III. In addition, sCD40L release was also attenuated when the platelets were pretreated with AT-III. Since we recently reported that the collagen-induced phosphorylation of HSP27 via p44/p42 MAP kinase is positively correlated with platelet granule secretion such as PDGF-AB and the sCD40L release from platelets (19), we next examined the effects of AT-III on these phosphorylations. AT-III markedly suppressed the collagen-induced phosphorylation of p44/p42 MAP kinase and HSP27 at three serine residues, Ser-15, Ser-78 and Ser-82. Therefore, our results suggest that AT-III inhibits both granule secretion and sCD40L release by inhibiting HSP27 phosphorylation via p44/p42 MAP kinase in human platelets.

Platelets rapidly respond to a variety of stimuli and release the materials stored mainly in two specific granules, dense-granules and α-granules, although platelets are anucleate cells that lack genomic DNA (23). These specific granule populations store different types of constituents. Between them, α-granules contain large adhesive and healing proteins such as PDGF-AB and the von Willebrand factor (24). It is well recognized that PDGF-AB secreted from platelet α-granules is a potent growth factor, which mainly acts on connective tissue (25). PDGF-AB induces the proliferation of vascular smooth muscle cells and plays a key

the development of arteriosclerosis (25). In addition, platelets also release the inflammatory mediators of atherosclerosis, such as sCD40L. CD40L, a member of the tumor necrosis factor-α superfamily, is stored in the cytoplasm of resting platelets and is immediately translocated on the platelet membrane after activation by agonists such as ADP (26,27). sCD40L is subsequently cleaved from the cell surface-expressed CD40L as a functional soluble fragment. It is generally known that the platelet-released sCD40L induces inflammatory responses via the CD40 expressed on vascular endothelial cells that produce inflammatory substances (23,28). It has been shown that platelet-derived sCD40L becomes mobilized in acute coronary thrombotic indications (29). The elevation of plasma sCD40L is reportedly associated with an increased risk of cardiovascular events in patients with unstable coronary artery disease (30). In addition, it has been shown that sCD40L enhances the stimulation-induced platelet release of inflammatory substance from dense-granules (31).

In the present study, we demonstrated that AT-III markedly suppressed the collagen-induced secretion of PDGF-AB, and the release of sCD40L from human platelets. Therefore, it is possible that inhibition by AT-III of sCD40L release from platelets plays a role in the immunosuppressive effect of this agent. Based on our findings as a whole, it is most likely that AT-III acts not only as an anticoagulant agent but also as an anti-inflammatory or anti-atherogenic agent through the reduction in the granule secretion of PDGF-AB and the release of sCD40L from platelets. AT-III is clinically used for the patient in critical state, such as severe sepsis or severe vascular disease (3). Taking our present findings into account, it is likely that AT-III could be effective to these patients not only as an anticoagulant agent but also as an anti-inflammatory agent. It has been recently reported that AT-III acts on platelet-membrane receptors directly, such as syndecan-4 (6). This report led us to speculate that AT-III acts on a certain platelet-membrane receptor, and subsequently suppresses the collagen signaling via inhibiting the phosphorylation of p44/p42 MAP kinase. Further investigation is required to clarify the exact mechanism underlying the AT-III-effects on human platelets.

In conclusion, our results strongly suggest that AT-III suppresses collagen-induced PDGF-AB secretion and the release of sCD40L due to the inhibition of HSP27 phosphorylation via p44/p42 MAP kinase in platelets.

Acknowledgements

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