

Original Article

Changes in spontaneous thrombolytic activity during progression of atherosclerosis in Apo^{-/-} and LDLR^{-/-} double knockout mice

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Received December 29, 2017; Accepted February 28, 2018; Epub September 1, 2018; Published September 15, 2018

Abstract: Background: Atherosclerosis is characterized by a hypercoagulable state, during which both coagulation and thrombolytic factors are activated simultaneously. However, details regarding the thrombolytic pathway in this context remain unknown. Here we investigated the changes in spontaneous thrombolytic activity during atherosclerotic progression in Apo^{-/-}LDLR^{-/-} double-knockout mice (DKO group). Methods: We fed DKO mice and their controls (C57Bl6 mice) a high-fat diet for a total of 22 weeks and evaluated them at 14 and 22 weeks. The amount of atherosclerosis was estimated as the ratio of the atherosclerotic to total aortic intimal area. In addition, we used immunohistochemistry to analyze the expression of PAI-1, t-PA, and eNOS in atherosclerotic regions. To evaluate thrombolysis, we used a He-Ne laser to induce thrombosis in vessels of the cremaster muscle and then measured the thrombus volume over time. Results: The atherosclerotic area was increased and thrombolytic activity was decreased in DKO group compared with the control group. Furthermore, the plasma PAI-1 level was 3 times greater in the DKO group than in the control group. In support of these results, immunohistochemistry showed increased PAI-1 expression in the DKO group, whereas t-PA and eNOS expression was greater in the control group. Conclusion: Progression of atherosclerosis led to a reduction in thrombolytic activity through decreases in t-PA and eNOS levels and an increase in PAI-1 production. These findings indicate that decreases in factors that promote spontaneous thrombolytic activity may indicate increased risk for the progression of atherosclerosis.

Keywords: Thrombolysis, PAI-1, t-PA, eNOS, atherosclerosis

Introduction

Atherosclerosis, characterized by luminal thrombus formation on a ruptured atherosclerotic plaque, is a leading cause of acute coronary syndromes and cardiovascular disease in humans [1]. Pathologic alteration of the atherosclerotic plaque, such as thinning of the fibrous cap and the development of a large necrotic core, predisposes the plaque to rupture, which then triggers thrombosis [2].

Established antithrombotic treatments, such as thrombin inhibitors [3] and t-PA (tissue plasminogen activator) [4, 5], are atherosclerosis therapeutic strategies to prevent restenosis after angioplasty and endarterectomy. Conversely, retaining thrombolytic potential after fibrinolytic therapy of atherothrombosis is crucial.

Altered expression of proteases associated with thrombolysis has been implicated in the expansion of atherosclerotic plaque and hemorrhage [6]. However, the underlying mechanism is not understood in detail, although decreased fibrinolytic activity is recognized as a risk factor in ischemic cardiovascular disease [7].

Despite the need to understand fibrinolytic homeostasis in the context of ischemic diseases, few reports describe the fibrinolytic activities associated with atherosclerotic diseases [8, 9]. This deficiency is due, in part, to the lack of an appropriate experimental approach for evaluating thrombolysis. For example, whereas some studies indicate that upregulation of the

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blood PAI-1 (plasminogen activator inhibitor-1) level is closely associated with cardiovascular risk [9-11], others report the lack of direct evidence linking the blood PAI-1 level to coronary artery disease [12]. In this regard, an effective in vivo model of thrombolysis is urgently needed to investigate thrombolytic activity during the progression of atherosclerosis.

In the present study, we evaluated spontaneous thrombolytic activity during the progression of atherosclerosis by using Apo^{-/-}LDLR^{-/-} double-knockout (DKO) mice fed a high-fat diet. To this end, we followed plaque development as the ratio of the atherosclerotic area to total luminal area in the aorta. In addition, we measured plasma concentrations of PAI-1 and the PAI-1, t-PA, and eNOS (endothelial nitric oxide synthase) expression during atherosclerotic progression in DKO and control mice.

Material and methods

Experimental animals

Double-homozygous Apo^{-/-} deficient and LDLR^{-/-} deficient mice (DKO mice, 129 × C57BL/6J background) were obtained originally from the Jackson Laboratory (Bar Harbor, Maine, USA). Subsequently, the animals were bred by sibling mating. For the control group, C57BL/6 mice (age: 10 to 13 weeks) were obtained from SLC (Hamamatsu, Japan). All animals were maintained at Kobe Gakuin University in air-conditioned rooms (22.5 ± 0.5°C; humidity, 50% ± 5%) on a 12-12-h light-dark photo cycle. Male mice were used in the present study. Animals had free access to diet and drinking water. Experimental high-fat diet (Fatty energy proportion: 20%; CMF, Oriental Yeast Co., Tokyo, Japan) were provided for a total of 22 weeks, and mice were evaluated after 14 and 22 weeks of feeding. All procedures were conducted in compliance with the *Guiding Principles for the Care and Use of Animals in the Field of Physiological Science* of the Physiological Society of Japan [13].

Measurement of atherosclerosis

We assessed the progression of atherosclerosis by estimating the area of atherosclerotic regions as a percentage of the entire surface area of the aorta, as previously described [14]. Briefly, hearts were exposed through abdomi-

nal incision, and PBS (pH 7.4) followed by 10% neutral-buffered formalin (Nacalai tesque, Tokyo, Japan) was infused through an indwelling 20-gauge butterfly needle (Top Kasei, Tokyo, Japan). In addition, the major blood vessels were washed with PBS and fixed with 10% neutral-buffered formalin solution by reflux through a femoral artery. Connective tissue and minor branching blood vessels were removed from the aortic arch. The extracted vessels were kept in 10% neutral-buffered formalin until processed, when they were incised along the longitudinal plane and pinned flat. The tissue was washed with distilled water for 30 seconds, treated with 60% isopropyl alcohol for 1 minute, and stained with Oil Red O (to identify atherosclerotic plaques) at 37°C for 15 minutes. Finally, the tissue was washed with 60% isopropyl alcohol and distilled water.

Stained specimens were photographed (Pentax K-7, Ricoh, Tokyo, Japan); images were transferred to a personal computer and analyzed by using image analysis software (Image-Pro Plus, Media Cybernetics, Rockville, Maryland, USA). The whole area (W) of the dissected aorta and the portion that stained positively with Oil Red O (R) were calculated; the resulting ratio [(R ÷ W) × 100%] was used as an index of atherosclerotic progression.

Experimental thrombolysis model

Our model for evaluating thrombolysis in vivo was based on a previously described model [15]. Briefly, mice were anesthetized with sodium pentobarbital (60 mg/kg IM), and femoral blood vessels were exposed through a median incision. Cannulae (PE 50, Becton Dickinson, Franklin Hills, New Jersey, USA) were introduced into a femoral vein and artery for administering Evans blue dye and monitoring blood pressure, respectively. The blood vessel on the cremaster muscle was isolated, placed on a Perspex plate, and an O-ring was placed around the tissue to prevent vessel movement [16]. The Perspex plate then was attached to the stage of a microscope (BX51, Olympus, Tokyo, Japan) and the preparation viewed by using a long working-distance objective. Arterioles (diameter, 30 to 35 µm) were selected for irradiation. The He-Ne laser beam was introduced into the microscope by using a dichroic mirror and focused on the center of the selected ves-

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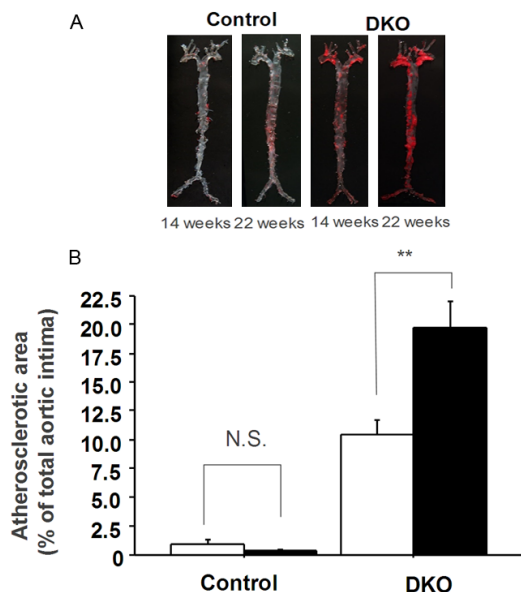


Figure 1. Progression of atherosclerosis after 14 and 22 weeks of high-fat feeding in control and DKO mice. A. Typical images of atherosclerotic regions identified by Oil Red O staining of the entire aortic tree in each group. B. Comparison of atherosclerotic area (mean \pm SEM) according to weeks of high-fat feeding. \square : 14 weeks; n = 9/group; \blacksquare : 22 weeks; n = 11/group, **: P<0.01; N.S.: not significant.

sels. The diameter of the laser spot on the focal plane was 15 μ m with a power of 25 mW. Evans blue (1.6 mg/kg; E. Merck, Darmstadt, Germany) was injected through the cannula; 5-second irradiation was repeated every 30 seconds until a mural thrombus that occluded 90% of the vessel lumen was formed. After stabilization for 10 min, images of the formed thrombus were captured for 60 minutes and stored in the computer.

Analysis of thrombus volume

Changes in thrombus volume were analyzed by using image analysis software (Image-Pro Plus, Media Cybernetics) as previously described [14]. Briefly, 2D images of thrombi were captured *in situ* at 5-minute intervals. Subsequently, 3D images were constructed by establishing optical density values relative to that of an area of the blood vessel lumen not involved in thrombus formation. Integrative optical density (IOD) values corresponding to thrombus volume were computed. Changes in thrombus volume were calculated according to the following formula: Relative thrombus volume = $\text{IOD}_n \div \text{IOD}_0$ (IOD_n , IOD at a particular time interval during thrombolysis; IOD_0 , IOD immediately after the stabilization of thrombus). The extent

of thrombolysis was expressed as a percentage of the initial thrombus volume (that is, IOD₀).

Determination of fibrinolytic factors in plasma

After 14 and 22 weeks of feeding the high-fat diet, blood was drawn from the abdominal aorta of mice into 3.14% sodium citrate by using a 23-gauge needle. All samples (citrate plasma) were stored at -80°C until analysis. Plasma PAI-1 levels were determined by using a commercial ELISA kit (IMPAIKT-TOT, Innovative Research, Novi, Michigan, USA).

Immunohistochemistry

The heart was exposed, and a butterfly catheter was inserted into the left ventricle. The heart was flushed with 10 mmol/L PBS (pH 7.4) for about 3 minutes, to remove all blood. Blood vessels then were fixed by perfusion with 4% paraformaldehyde in PBS (Wako Pure Chemical Industries, Osaka, Japan).

The aortic root, aorta, and brachiocephalic artery were analyzed by immunohistochemistry and histology, and data from the brachiocephalic artery are shown. To this end, the blood vessels of interest were fixed in OCT compound (Tissue-Tek and Sakura, Japan) and frozen by using a dry ice-acetone mixture or liquid nitrogen. Frozen OCT-embedded tissue blocks were cut into 6- μ m sections, which were placed on poly-L-lysine-coated microscope slides (Muto Pure Chemicals, Tokyo, Japan). These slides were examined after immunoperoxidase staining with anti-PAI-1 antibody (dilution, 1:100; PAI-1 Rabbit PAb, LabVision, Fremont, California, USA), anti-t-PA antibody (1:100; t-PA Rabbit PAb, LabVision), and anti-eNOS antibody (1:100; eNOS Rabbit PAb, LabVision). Epitopes recognized by the primary antibody were visualized by labeling with streptavidin and biotinylated horseradish peroxidase (LSAB2 kit, Dako, Kyoto, Japan) as described previously [17]. The slides then were counterstained with Mayer's hematoxylin (TA-125-MH, LabVision). During histology, cross-sections were obtained from at least three sections (luminal side, intraplaque, and intimal side) of each plaque.

Statistical analysis

Results were expressed as mean \pm SEM. Comparisons among groups were made by using factorial ANOVA. Differences between groups of each determined parameters were analyzed

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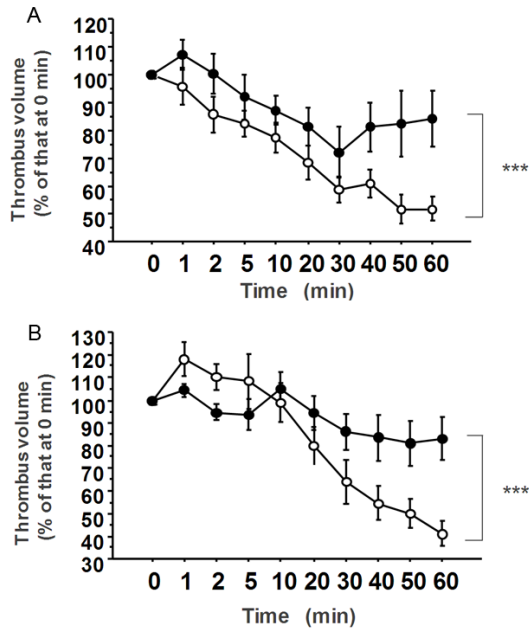


Figure 2. Relative thrombus volume (mean \pm SEM) over time in control (\circ) and DKO (\bullet) mice fed a high-fat diet for (A) 14 weeks and (B) 20 weeks. (n = 8 per group; ***: P<0.001).

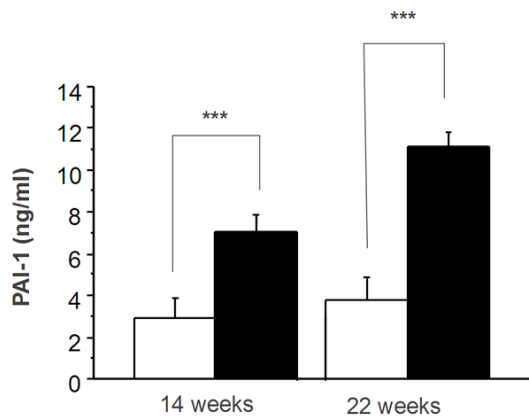


Figure 3. Plasma PAI-1 concentrations of control (\square) and DKO (\blacksquare) mice fed a high-fat diet for 14 or 22 weeks (n = 19-21 per group; ***: P<0.001).

using Student's t-test. P value of <0.05 was defined as statistically significant. No adjustments were made. All analyses were accomplished by the statistical package JMP (JMP 13 SAS Institute Japan).

Results

Body weight

After 14 weeks of feeding the high-fat diet, body weight (mean \pm SEM) was 27.8 ± 0.5 g (n

= 24) in the control mice and 33.8 ± 0.6 g (n = 40) in the DKO group. After 22 weeks of feeding, the control group weighed 34.2 ± 1.0 g (n = 30) and the DKO mice weighed 35.5 ± 0.5 g (n = 43). Body weight did not differ significantly between the control and DKO groups.

Development of atherosclerosis

Typical images of the dissected blood vessels and aortic root in each group are shown in **Figure 1A**. In both groups, we evaluated the extent of atherosclerosis after 14 and 22 weeks of high-fat feeding (**Figure 1B**). The atherosclerotic area in the aorta at 14 weeks was $0.9\% \pm 0.4\%$ in the control group (n = 9) compared with $10.4\% \pm 1.4\%$ (n = 9) in the DKO group, and at 22 weeks was $0.3\% \pm 0.9\%$ (n = 11) in controls compared with $19.7\% \pm 2.3\%$ in DKO mice. The difference between time points was significant (P<0.01) in the DKO mice only.

Spontaneous thrombolytic activity

Spontaneous thrombolytic activity, evaluated as change in thrombus volume over time, in both groups is shown in **Figure 2**. The thrombus volume at 60 minutes relative to that immediately after thrombus stabilization (that is, time 0) was $51.8\% \pm 4.4\%$ at 14 weeks and $41.0\% \pm 5.5\%$ at 22 weeks in the control group compared with $84.1\% \pm 10.0\%$ at 14 weeks and $83.2\% \pm 9.5\%$ at 22 weeks in the DKO group (n = 8 per group and time point). At both time points, relative thrombus volume was greater (P<0.001) in DKO mice than in controls.

Plasma PAI-1 level

The plasma PAI-1 concentration in the control group was 2.9 ± 1.0 ng/ml after 14 weeks of high-fat feeding and 3.7 ± 1.1 ng/ml after 22 weeks (**Figure 3**). These values did not differ significantly. In contrast, the PAI-1 level of DKO mice was 7.0 ± 0.8 ng/ml at 14 weeks compared with 11.1 ± 0.7 ng/ml at 22 weeks (P<0.001).

Immunohistochemistry

We used immunohistochemistry to assess the production of PAI-1, t-PA, and eNOS in the vascular endothelium of cross-sections of the left brachiocephalic trunk. The DKO group-but not control mice-showed endothelium-specific expression of PAI-1 (**Figure 4A, 4B**). In contrast,

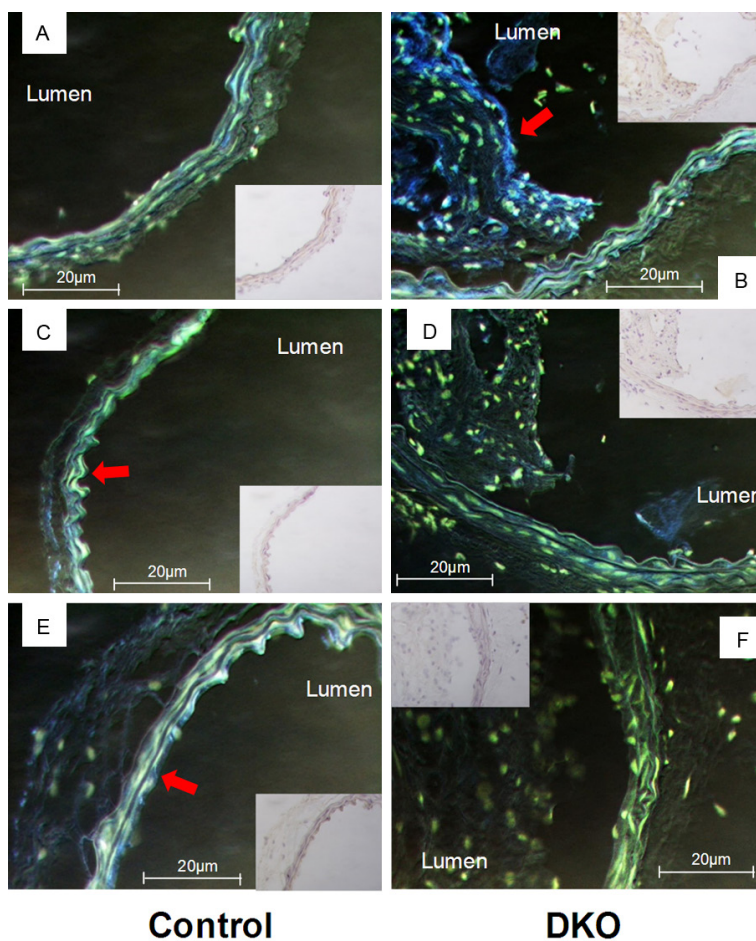


Figure 4. Immunofluorescence and pseudo-color treatment of the brachiocephalic artery in (A, C, E) control and (B, D, F) DKO mice after 22 weeks of high-fat feeding (A, B): Anti-PAI-1, (C, D): Anti-t-PA, (E, F): Anti-eNOS. Red arrows indicate positive areas. Magnification: 200 ×.

control mice-but not the DKO group-demonstrated endothelial expression of t-PA (**Figure 4C, 4D**) and eNOS (**Figure 4E, 4F**).

Discussion

Unstable, vulnerable, atherosclerotic plaques frequently rupture and therefore are strongly associated with acute cardiovascular events [18]. Unstable plaque is characterized by having a thin fibrous cap, lipid-rich core, intensive inflammatory cell infiltrate, and insufficient or abnormal elastin and collagen protein [19]. Plaque fissure or rupture initiates the thrombotic process by exposing platelets to the aforementioned thrombogenic cellular and acellular components of plaque. These triggers lead to thrombin formation, with subsequent thrombus development and lumen occlusion [20].

In these situations, the pharmacokinetics of thrombolytic activity are highly important, but how changes in thrombolytic activity affect atherosclerotic progression is unknown. In the current study, we established a murine model of thrombolysis by using DKO mice that lack both Apo and LDL-R so that we might investigate the pathology of, and changes in, thrombolytic activity during atherosclerotic progression. Specifically, we assessed plasma PAI-1 concentration as a pathologic marker and endothelial cell production of PAI-1, t-PA, and eNOS as indicators of spontaneous thrombolytic activity. Furthermore, we used the Entire Aorta method to follow atherosclerotic progression in our control and DKO mice [14]. This procedure enabled us to investigate not only atherosclerotic progression but also the site of plaque development. According to our results, the predilection sites of atherosclerosis were the aorta, carotid artery, abdominal aorta- the same as those in which arteriosclerosis develops preferentially in humans [21, 22].

DKO mice had more aortic atherosclerotic plaque after 22 weeks of high-fat feeding than after 14 weeks. In addition, relative residual thrombus volume was greater in DKO mice than controls at both 14 and 22 weeks. These findings indicate that spontaneous thrombolytic activity during atherosclerotic progression was decreased significantly in the DKO group.

Because we hypothesized that it was not easy to promote thrombolysis of the formed thrombus after atherosclerotic progression, we compared the plasma PAI-1 concentration between the control and DKO groups. The plasma PAI-1 level at 14 weeks did not differ between groups. In contrast, the plasma PAI-1 level at 22 weeks was significantly higher in DKO mice than control mice. We considered that this finding reflects a decrease in the overall thrombolytic

activity during atherosclerotic progression and that this decrease is due to enhanced inhibitory activity in the fibrinolytic system. The high lipoprotein levels in DKO mice may contribute to this effect, given that lipoprotein upregulates the production of PAI-1 in vascular endothelial cells [23]. In addition, high dietary fat promotes both atherosclerosis and gains in adipose tissue, which in turn might hamper spontaneous thrombolytic activity because adipose tissue upregulates the production of PAI-1 [24]. We considered it necessary to examine the PAI-1 expression in adipose tissue, atherosclerotic plaque regions, and vascular endothelium. The increased PAI-1 level during atherosclerotic progression supported the results from our spontaneous thrombolysis model. A thrombotic tendency and unstable plaque are known to accompany increased PAI-1 levels and decreased fibrinolytic activity in atherosclerotic progression [25, 26]. Furthermore, hypercholesterolemia, hypertension, diabetes mellitus, and smoking are all associated with endothelial cell damage and are all known risk factors of atherosclerosis [27]. Together these data imply the importance of PAI-1 in thrombolysis.

Like PAI-1, t-PA is an important contributor to fibrinolytic activity [28]. Little t-PA was expressed on the endothelial cell surface in both control and DKO mice, and the amount of t-PA produced did not differ between groups. Together, these findings indicate that, rather than the activation of plasminogen, endothelial dysfunction may be responsible for the decrease in t-PA expression during atherosclerotic progression. In contrast to t-PA expression, PAI-1 production was increased significantly in regions of endothelium associated with plaque. These data may indicate a critical role for inhibition of PAI-1-mediated plasmin activation during atherosclerotic progression.

In addition, NO-derived eNOS is another important factor in thrombolysis [29, 30]. In the current study, eNOS expression was apparent in vascular endothelial cells in our control mice but not in the DKO mice. In addition, eNOS expression was very low in plaque-associated regions of endothelium. The negligible expression of eNOS in DKO mice suggests that NO production was diminished in the atherosclerotic regions. Atherosclerosis promotes dysfunction of endothelium-dependent relaxation [31], suggesting that the decrease in eNOS

associated with atherosclerotic progression in our mice reduced NO bioavailability. Furthermore, the atherosclerosis-associated reduction in eNOS production that occurred in our DKO mice corresponds to the decreased levels of NO in atherosclerotic human coronary arteries [32].

Because of NO's important role in homeostasis of the microcirculation [33], atherosclerosis-associated decreases in vasodilation likely accelerate concurrent reductions in spontaneous thrombolysis. In addition, activation of iNOS due to vascular endothelial cell injury may exceed that of eNOS, in proportion to atherosclerotic development [32]. Furthermore, the generation of superoxides, leading to the production of peroxynitrite and hydrogen peroxide, would have an even greater effect on the progression of atherosclerosis than would the synthesis of NO [34, 35]. Therefore, iNOS expression should be assessed in future experiments.

Thrombi contain large amounts of PAI-1 because they consist primarily of platelets, which contain 90% or more of the total PAI-1 in the body [36, 37]. In addition, the PAI-1 level in platelets may increase during atherosclerotic progression because lipoprotein and adipose tissue levels increase as well, thus making a plaque-associated thrombus particularly difficult to dissolve. This result confirms reports that the PAI-1 level increases during myocardial infarction [38] and cerebral stroke [39]. In addition, during activation, platelets secrete not only PAI-1, but also another serpin, named protease nexin-I (PN-I), which also inhibits t-PA. Recently, it has been established that, besides platelet PAI-1, platelet PN-I also has relevant antifibrinolytic properties in both human and mice [40]. Furthermore, inhibitors such as thrombin-activatable fibrinolysis inhibitor (TAFI) [41] and alfa2 antiplasmin [42] besides PAI-1 regulate fibrinolysis, albeit through different mechanisms. Therefore, further investigations about these inhibitors are necessary. These observations suggest that, like the situation that occurs during atherosclerotic progression, the increased concentration of PAI-1 during myocardial infarction and cerebral stroke upsets the dynamic balance of fibrinolysis because of excessive plasmin inhibition through the inhibition of t-PA.

Conclusions

All of the findings described might contribute to a combined mechanism by which spontaneous thrombolytic activity is decreased during atherosclerosis progression. Our current findings indicate that the progression of atherosclerosis decreases spontaneous thrombolytic activity via PAI-1-associated inhibition of t-PA, which increases the conversion of plasminogen to plasmin.

Disclosure of conflict of interest

None.

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