Figure 6. Analysis of VWF multimers of patients with sepsis-induced DIC. VWF multimers in the plasma of patients with sepsis-induced DIC with ADAMTS13 activity levels lower than 20% were analyzed by SDS-agarose gel electrophoresis, as described in "Patients, materials, and methods." VWF multimer patterns of patients and healthy subjects (N) were analyzed simultaneously. *Representative unusually large multimers of VWF found in the plasma of patients with ADAMTS13 activity levels lower than 20%.

unusually large VWF multimers in humans. TTP is a fatal thrombotic microangiopathic disease if patients are not treated appropriately, but the incidence of TTP is low.[8,22] While searching for the role of ADAMTS13 in common thromboembolic diseases, we found severe secondary ADAMTS13 deficiency in patients with sepsis-induced DIC and showed its clinical correlation to the development of renal failure in this study.

DIC is associated with a variety of disease states such as sepsis, advanced malignancy, severe tissue damage, and pregnancy-related complications. Sepsis may be the most common pathogenic disease that leads to the development of DIC, and the endotoxemia and high cytokine levels in the circulation are thought to induce tissue factor expression that in turn initiates fibrin thrombus formation in the circulation. Microthrombi formed in the circulation cause ischemia of and damage to a variety of organs. Lines of evidence have suggested that proteases released from white blood cells may also be involved in the development of organ injuries. This study showed that patients with sepsis-induced DIC frequently exhibited decreased antigen and activity levels of ADAMTS13 and that severe ADAMTS13 deficiency was found in these patients at high incidence. Many patients in this study had undergone transfusion with ADAMTS13-containing blood products, such as fresh frozen plasma and platelet concentrates, soon before blood sample collection for the determination of ADAMTS13 levels, suggesting that the levels of ADAMTS13 in the plasma samples of these patients might not reflect the severity of ADAMTS13 deficiency before blood transfusion. Thus, severe secondary ADAMTS13 deficiency in sepsis-induced DIC might be more common. Clinical manifestations and laboratory data of these patients with sepsis and secondary severe ADAMTS13 deficiency were nearly indistinguishable from those of patients with TTP, though the former had evidence of infection (Table 2), indicating that there exists a subset of patients who have secondary severe ADAMTS13 deficiency caused by sepsis and in whom the disease course is clinically similar to that of TTP. In addition, they might also have the same ADAMTS13 deficiency pathophysiology for the development of TMA seen in patients with idiopathic TTP.

Organ failure might be caused by tissue factor–dependent fibrin thrombus formation and platelet aggregation because of severe ADAMTS13 deficiency in the patients with sepsis-induced DIC with ADAMTS13 activity levels lower than 20%. This notion was supported by the correlation between severe secondary ADAMTS13 deficiency and renal failure in patients with sepsis-induced DIC with ADAMTS13 activity levels lower than 20%. We could not find any significant difference in the ADAMTS13-specific activity levels between these 2 groups (not shown). One possibility is that small molecular forms of ADAMTS13 could be lost in urine because of renal injuries. However, we could not determine whether this was the case because no urine samples were available for study.

Table 4. Correlation between presence of unusually large multimers of VWF and serum creatinine levels of patients with sepsis-induced DIC and ADAMTS13 activity levels lower than 20%

	Presence, n = 26	Absence, n = 25	P
Creatinine, mg/dL	2.39 ± 2.24	1.34 ± 1.35	< .05*
ADAMTS13 activity, %	6.6 ± 6.8	8.9 ± 6.0	NS

Values are mean ± SD.

To convert creatinine from milligrams per deciliter to micromoles per liter, multiply milligrams per deciliter by 88.4.

Presence indicates unusually large VWF multimers present in the plasma of patients; absence, unusually large VWF multimers absent in the plasma of patients. NS, not significant.

*Statistically significant (Welch *t* test).

In a previous report by Reife et al,[25] patients with TMA who did not have DIC were analyzed for the correlation between ADAMTS13 activity levels and serum creatinine levels without distinguishing TTP from HUS. They found that creatinine levels in patients with severely decreased ADAMTS13 activity levels were significantly lower than those in patients without severely decreased ADAMTS13 activity levels. These data are contrary to our findings that patients with severe ADAMTS13 deficiency (ADAMTS13 activity less than 20%) had significantly higher serum creatinine levels than did patients with the ADAMTS13 activity levels higher than 20%. Given that patients with HUS were not distinguished from patients with TTP in the report by Reife et al,[25] it is possible that the patients without severe ADAMTS13 deficiency in that study included patients with HUS. We studied patients with sepsis-induced DIC, and this difference in patient groups explains the opposing findings. There was no apparent difference between the platelet counts of patients with ADAMTS13 activity levels less than 20% and those of patients with ADAMTS13 activity levels greater than 20%. The combination of underlying DIC and platelet transfusion in these patients may account for the data.

The presence of the unusually large multimers of VWF in the plasma of patients with severe secondary ADAMTS13 deficiency and its correlation with serum creatinine levels supports the notion that severe secondary ADAMTS13 deficiency may correlate with the development of renal failure in sepsis-induced DIC. There was no significant correlation between the unusually large multimers of VWF and ADAMTS13 activity levels, possibly because of technical difficulties in determining the unusually large VWF multimers and the differences in endothelial cell damage among these patients.

Decreased specific activity of ADAMTS13, presumably caused by its cleavage by proteases, was a mechanism for severe secondary ADAMTS13 deficiency in patients with sepsis-induced DIC. Various proteases have been shown to degrade ADAMTS13 in vitro.[21] Thrombin and plasmin are generated in DIC, and these enzymes may cleave ADAMTS13, resulting in the inactivation of ADAMTS13. Our data suggest that granulocyte elastase may be one of the proteases that cleave ADAMTS13, together with thrombin and plasmin, under in vivo pathologic conditions. In this regard, the case report by Galbusera et al[26] of chronically relapsing

TTP—which showed that α1-antitrypsin (the physiologic granulocyte elastase inhibitor) therapy was effective at preventing the appearance of unusually large VWF multimers in the circulation but not at preventing TTP relapse—was interesting and suggested the link between granulocyte elastase and cleavage of ADAMTS13. Correlation between ADAMTS13 activity and antigen levels and E-XDP levels, not only in patients with TTP but also in patients with pathogenic *E coli* infection–related HUS, would be a further study to investigate the role of granulocyte elastase in TMA development. Specific inhibitors of these proteases are present at high concentrations in blood, indicating that cleavage of ADAMTS13 by these proteases may depend on the kinetic balance between ADAMTS13, the proteases, and their inhibitors. Thus, cleavage of ADAMTS13 by these proteases may not proceed completely in vivo. It is possible that other proteases could also digest ADAMTS13 in the disease state. This possibility should be investigated in a future study.

Because serum albumin levels decreased in most patients, liver injuries associated with the underlying disease might be an additional mechanism for decreasing ADAMTS13 antigen levels given that this enzyme is synthesized in the liver. Mutations or polymorphisms of the *ADAMTS13* gene are another possible cause of a decrease or an increase of ADAMTS13-specific activity. These possibilities should also be explored in future studies.

In conclusion, the precise analysis of ADAMTS13 antigen and activity levels in disease states offers insight into the roles of ADAMTS13 in thromboembolic diseases. Severe ADAMTS13 deficiency takes place secondarily in disease states such as sepsis-induced DIC, and it may not be specific for idiopathic TTP and may not have a solo diagnostic value for idiopathic TTP. Although the mechanisms of severe ADAMTS13 deficiency in sepsis are different from those of idiopathic TTP, the clinical features of patients with sepsis-induced DIC and severe ADAMTS13 deficiency are similar to those of patients with idiopathic TTP. Sepsis may have the same pathophysiology of severe ADAMTS13 deficiency for TMA development as idiopathic TTP, raising the possibility of novel supportive therapies for patients with sepsis and severe ADAMTS13 deficiency, such as ADAMTS13 supplementation, α1-antitrypsin administration, and use of synthetic granulocyte elastase inhibitors. Given that severe secondary ADAMTS13 deficiency might correlate with the development of organ injury in patients with sepsis-induced DIC, determining the ADAMTS13 levels of patients in severe condition at the time of hospital admission would provide better understanding of the extent of disease. Current analyses of ADAMTS13 levels in disease states are retrospective; thus, prospective study is needed for the timely execution of ADAMTS13 supplementation for patients not only with TTP but also with secondary ADAMTS13 deficiency.

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Review

Aging and plasminogen activator inhibitor-1 (PAI-1) regulation: implication in the pathogenesis of thrombotic disorders in the elderly

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Abstract

Thrombotic cardiovascular diseases increase in incidence in the elderly, a tendency dependent on the age-related changes in vascular and hemostatic systems that include platelets, coagulation, and fibrinolytic factors as well as in the endothelium. The hypercoagulability of and advanced sclerotic changes in the vascular wall may contribute to the increased incidence of thrombosis in the elderly. One of the important key genes for aging-associated thrombosis is plasminogen activator inhibitor-1 (PAI-1), a principal inhibitor of fibrinolysis. The expression of PAI-1 is not only elevated in the elderly but also significantly induced in a variety of pathologies associated with the process of aging. These conditions include obesity, insulin resistance, emotional stress, immune responses, and vascular sclerosis/remodeling. Several cytokines and hormones, including tumor necrosis factor-α, transforming growth factor-β, angiotensin II, and insulin, positively regulate the gene expression of PAI-1. The recent epidemic in obesity with aging in the industrialized society may heighten the risk for thrombotic cardiovascular disease because adipose tissue is a primary source of PAI-1 and cytokines. Emotional or psychosocial stress and inflammation also cause the elevated expression of PAI-1 in an age-specific pattern. Thus, PAI-1 could play a key role in the progression of cardiovascular aging by promoting thrombosis and vascular (athero)sclerosis. Further studies on the genetic mechanism of aging-associated PAI-1 induction will be necessary to define the basis for cardiovascular aging in relation to thrombosis.

Keywords: PAI-1; Obesity; Stress; Immune response; Vascular remodeling

1. Introduction

The incidence of thrombotic cardiovascular disease increases with age [1], and recent studies have begun to address the important clinical problem of "aging and thrombosis" [2]. Age-related changes may occur in the vascular and hemostatic systems, which include platelets, coagulation, and fibrinolytic factors as well as in the endothelium. Aging-associated sclerotic changes in the vascular wall may also contribute to the increased incidence of thrombosis in the elderly [3]. The hypercoagulability of the blood in the elderly may be yet another cause of the increased thrombotic tendency. For example, platelet activity is enhanced with advancing age, and aging is associated with increased plasma levels of several blood coagulation factors (e.g., factor VII, factor VIII, and fibrinogen) [4], all of which have been shown to be risk factors for thrombotic diseases [5]. On the other hand, a proportional increase in natural anticoagulant factors (e.g., protein C, protein S, antithrombin, tissue factor pathway inhibitor, etc.) has not been observed in the elderly [6]. The fibrinolytic system is impaired by aging since a progressive prolongation of the euglobulin lysis time [7] and an increase in plasminogen activator inhibitor-1 (PAI-1), a principal

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regulator of fibrinolysis [8], have been observed with aging [9]. Thus, the inappropriate expression of procoagulant/antifibrinolytic genes may underlie the occurrence of thrombotic events, which are frequently observed in the elderly. However, the molecular link between aging and prothrombotic states due to aberrant expressions of procoagulant/antifibrinolytic genes remains to be elucidated. One aim of this review is to describe the pathological significance of PAI-1 in cardiovascular aging in relation to thrombosis based upon clinical observations and animal studies.

2. PAI-1 and its regulation in various clinical states associated with aging

PAI-1 is a rapid and specific inhibitor of both tissue-type and urokinase-type plasminogen activators (t-PA and u-PA) and may be the primary regulator of plasminogen activation in vivo [8]. The synthesis of PAI-1 is increased in activated or injured endothelial cells and smooth muscle cells, and abundant PAI-1 is also secreted by activated platelets. The increased expression of this potent inhibitor in vivo will suppress the normal fibrinolytic system and create a prothrombotic state, resulting in pathological fibrin deposition followed by tissue damage. Increased expression of PAI-1 in vivo is related to the development of tissue pathologies [10] such as thrombosis, fibrosis, and cardiovascular disease [11]. Factors inducing PAI-1 expression in vitro and pathologies associated with elevated PAI-1 in vivo are listed in Table 1.

2.1. Myocardial infarction

A rise in the circulating level of PAI-1 has been shown to precede the occurrence of myocardial infarction [12]. Survivors of myocardial infarction had impaired fibrinolytic activity due to elevated levels of plasma PAI-1 [13], which is also associated with early recurrence of myocardial infarction [14]. Acute increases in plasma PAI-1 levels in patients with acute ST-elevated myocardial infarction are strongly associated with the risk of mortality during a 1-month period [15]. Thus, PAI-1 seems to be a risk factor for the development and recurrence of thrombotic cardiovascular diseases. It is also known that the renin–angiotensin system is activated after acute myocardial infarction [16]. A strong relationship has been shown between the activation of the renin–angiotensin system and plasma PAI-1 [17], and it is known that angiotensin II can induce the expression of PAI-1 [18]. The plasma level of another fibrinolytic inhibitor, thrombin-activatable fibrinolysis inhibitor (TAFI), is also associated with increased risk for cardiovascular diseases [19,20]. The activity of TAFI in young patients with myocardial infarction was found to be significantly higher and has been correlated positively with the PAI-1 level [21], suggesting that a hypofibrinolytic state largely contributes to the occurrence of cardiovascular events.

Table 1
Stimulating factors of PAI-1 synthesis and clinical conditions associated with increased PAI-1 expression

Factors inducing PAI-1 synthesis	Clinical conditions with increased PAI-1
Endotoxin	Sepsis
Thrombin	Coronary heart disease
TNF-α	Atherosclerosis
TGF-β	SLE/lupus nephritis
Interleukin-1	Antiphospholipid syndrome
Insulin	Obesity
Dexamethasone	Insulin resistance
PDGF	Lung fibrosis
Basic FGF	Hyperoxic lung injury
Lipoprotein (a)	Preeclampsia
Angiotensin II	Malignancy/tumor metastasis

PDGF—platelet derived growth factor; FGF—fibroblast growth factor; SLE—systemic lupus erythematosus.

2.2. Obesity and insulin resistance

Clinically, thrombotic cardiovascular diseases occurring in aged subjects are often associated with obesity. Obesity is an independent risk factor for the development of thrombotic cardiovascular disease [22]. In a large community-based sample, an increased body-mass index has been associated with increased risk of heart failure [23]. The increased incidence of cardiovascular disease may be associated with impaired fibrinolysis, which has been shown to be present in obese patients [24]. For example, increased plasma PAI-1 levels have been correlated with the amount of visceral fat in obese humans [25], and PAI-1 is commonly and predictably elevated in individuals with insulin resistance and type II diabetes [26]. Vascular dysfunction caused by insulin resistance is associated with the activation of the renin–angiotensin system [27]. Taken together, obesity, insulin resistance, and hypertension are closely related in terms of PAI-1 induction, resulting in the development of thrombotic cardiovascular disease. In this context, we have speculated on the potential benefit of therapies that might prevent an acute increase in plasma PAI-1. These potentially include angiotensin-converting enzyme inhibitors [28], insulin-synthesizing [29] or-sensitizing agents [30], and other agents that improve endothelial function and nitric oxide production systematically.

2.3. Atherosclerosis

By limiting extracellular proteolysis in developing atherosclerotic lesions, PAI-1 may play a significant role not only in the organization of mural thrombi within the plaque but also in the neointimal proliferation of smooth muscle cells and in the neovascularization of the plaque. High plasma levels of PAI-1 may be associated with the development of atherosclerosis. Investigations of PAI-1 expression in the arteries of atherosclerotic subjects have

revealed significantly increased levels of PAI-1 mRNA in severely atherosclerotic vessels, including the abdominal aorta, iliac artery, and femoral artery, as compared with those in normal or mildly affected arteries [31]. In situ hybridization analysis revealed an abundance of cells (e.g., endothelial cells, smooth muscle cells, and macrophages) positive for PAI-1 mRNA within the thickened intima of atherosclerotic arteries, mainly around the base of the plaque [31,32]. Fibrin, which is a consistent component of atherosclerotic plaques, may contribute to plaque growth through the stimulation of smooth muscle cell proliferation [33,34] and through the binding and accumulating of low-density lipoprotein [35]. Intravascular or mural thrombosis is a frequent histological feature of atherosclerotic lesions and appears to play a role in the intimal thickening and fibrosis characteristic of advanced lesions. Thus, localized alterations in fibrinolytic activity due to the increased expression of PAI-1 in blood vessels may contribute to the progression of atherosclerotic process by promoting fibrin deposition and extracellular matrix accumulation in the lesions [36].

2.4. Stress

Hypercoagulability and thrombotic diseases appear to be induced also by mental [37] and psychosocial stress [38]. Because aged subjects may have lower tolerance to stress, they are susceptible to thrombosis caused by a variety of stress factors [39]. Chronic stress, defined as feelings of fatigue, lack of energy, increased irritability, and demoralization, has also been associated with elevated plasma PAI-1 antigen in middle-aged men [40]. The stress-mediated activation of the sympathetic nervous system, whose activity is heightened in older subjects [41], may contribute to the induction of PAI-1 [42]. Oxidative stress, one of the characteristics of diabetes, boosts PAI-1 expression by activating the PAI-1 promoter through an AP-1 response element [43]. Thus, the stress-induced PAI-1 may be responsible for the onset of thrombotic disease associated with a variety of stress factors, especially in the elderly.

2.5. Endotoxemia

PAI-1 is an acute-phase reactant linked to inflammatory and prothrombotic markers because it is induced by a variety of cytokines [e.g., tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), interleukin-1 and -6], but most strongly by the endotoxin of Gram-negative bacteria [44,45]. Endotoxin (lipopolysaccharide, LPS) profoundly alters the fibrinolytic system [46], frequently leading to prothrombotic states. Recently, PAI-1 has been regarded as a prognostic marker of sepsis caused by Gram-negative bacteria [47], which is often observed in hospitalized elderly patients. Septic patients with high plasma PAI-1 levels have a poor prognosis because of progressive multiple organ failure due to microvascular fibrin deposition and subsequent cell damage [48,49]. After endotoxin administration, elderly individuals are more susceptible to endotoxin-induced effects than the young, showing severe abnormalities in the cardiorespiratory system, such as hypotension, increased heart rate, and increased respiratory rate [50]. Overall, PAI-1 is regarded as a key molecule in the development of septic organ damage because this protein is strongly induced by inflammatory mediators and promotes microvascular and extravascular fibrin deposition.

2.6. Malignancy

A couple of reports have stated that basal plasma PAI-1 levels were found to be significantly elevated in patients with malignant conditions [51], which are sometimes observed in elderly subjects. Deep-vein thrombosis is sometimes observed in patients with malignancy due, not only to the increased activation of coagulation, but also to impaired fibrinolysis. An increasing number of studies demonstrate that high PAI-1 levels indicate a poor prognosis for the survival of patients with a variety of cancers, including breast [52], lung [53], and gastric [54] cancer. PAI-1 may play a critical role in tumor-cell invasion, and the possible mechanism is that PAI-1 blocks the interaction of integrins with vitronectin, thereby loosening the cells from their substratum and promoting cell migration [55].

2.7. Genomics on the PAI-1 up-regulation in relation to thrombosis

The genomics of PAI-1 is relevant to the PAI-1 regulation in association with thrombotic/bleeding phenotype as follows. There have been several reports describing elevated plasma PAI-1 levels in familial or sporadic venous thrombophilia [56]. On the other hand, several individuals have been identified with little or no detectable functional PAI-1 in their plasma due to the mutation in the PAI-1 gene [57], and all have had lifelong bleeding problems [58]. Moreover, disruption of the PAI-1 gene in mice was associated with a mild hyperfibrinolytic state and increased resistance to thrombosis [59]. Transgenic mice overexpressing the human PAI-1 gene developed thrombotic problems in the extremities [60], and an excess of PAI-1 can promote coronary arterial thrombosis in these mice [61]. The coronary thrombi developed in an age-dependent manner in the transgenic mice, and 90% of the mice older than 6 months had spontaneous thrombotic occlusions of the coronary arteries [61].

An association between one of the DNA sequence variations of the human PAI-1 gene, the 4G/5G polymorphism, and plasma PAI-1 levels has been suggested, with the 4G homozygotes having the highest PAI-1 levels and the 5G homozygotes having the lowest [62]. For example, in young myocardial infarction patients, the prevalence of the unfavorable 4G allele was higher than in healthy controls [62]. Furthermore, the 4G/4G genotype has

been shown to be significantly associated with a history of coronary artery disease in patients diagnosed by coronary angiography [63] and also in patients with noninsulin-dependent diabetes mellitus [64,65]. However, it is still controversial whether the 4G/5G polymorphism increases the risk for myocardial infarction and thromboembolism [66].

3. PAI-1 induction in animal models of aging and prothrombotic states

Experimental studies on animals have also demonstrated a link between increased expression of PAI-1 and thrombotic events. In the following, we describe the induction of the PAI-1 gene in a variety of mouse models of aging and prothrombotic states.

3.1. PAI-1 expression in a mouse model of premature aging, "klotho"

A mouse model of premature aging, named the "*klotho* (*kl/kl*) mouse", was generated through the insertional mutation of a transgene disrupting a newly found gene locus named "*klotho*" [67]. The *kl/kl* mouse exhibits a syndrome resembling human aging, including a short life span, growth retardation, osteoporosis, arteriosclerosis, obstructive pulmonary disease, and atrophy of the skin. Higher levels of renal PAI-1 mRNA expression and active PAI-1 antigen in the plasma were found in *kl/kl* mice in comparison with wild-type mice [68], suggesting impaired fibrinolysis in this mouse model of aging. The kidneys of *kl/kl* mice showed severe sclerotic changes, with calcification and spontaneous glomerular fibrin deposition. These observations suggest that the aging-associated induction of PAI-1 contributes to the development of renal sclerotic changes and thrombosis. Interestingly, in the heart of *kl/kl* mice, the cardiomyocytes and the cells in the myxomatous-degenerated mitral valve with calcification also expressed abundant PAI-1 mRNA [68]. The induction of PAI-1 gene expression in cardiomyocytes may contribute to microvascular injury and cardiac muscle degeneration in the hearts of *kl/kl* mice.

3.2. PAI-1 induction in an experimental model of vascular remodeling

One candidate for the paracrine factor involved in vascular remodeling would be the metalloproteinases (MMPs), of which activity is increased in the arteries of aged animals [69]. The plasminnogen activator/plasmin system is an important regulatory system in the onset of cardiac wound healing and arterial remodeling [70] because plasmin can modulate the activity of MMPs by activating proMMPs to MMPs [71]. Age-dependent induction of PAI-1 would enhance the accumulation of ECM components in a variety of tissues, including cardiac and vascular tissues. It has been reported that adenoviral PAI-1 overexpression resulted in the prevention of cardiac rupture after myocardial infarction through the inhibition of local proteolysis [72]. Moreover, PAI-1-deficient mice were found to be resistant to the progression of coronary perivascular fibrous change in a model of long-term nitric oxide (NO) synthase inhibition [73]. Mice deficient in PAI-1 showed less development of cardiac fibrosis after infarction than wild-type mice [74], suggesting that PAI-1 deficiency may prevent the increase of collagen deposition by accelerating matrix degradation. Thus, PAI-1 could regulate the activation of MMPs and has indeed been implicated as an important modulator during the process of cardiac repair and vascular remodeling.

3.3. PAI-1 induction in a mouse model of obesity

High expression levels of PAI-1 mRNA have been detected in murine adipose tissue [75]. This observation suggests that adipose tissue is the primary source of PAI-1 in the obese condition. Adipose-derived PAI-1 expression is dramatically up-regulated and significantly increased as a function of age in genetically obese mice, whose adipocytes express PAI-1 mRNA abundantly [76]. PAI-1 expression in cultured adipocytes has been strongly induced by insulin [76] and glucose [77], suggesting that PAI-1 expression in adipocytes may be strongly associated with insulin resistance [78]. Interestingly, insulin-resistant adipocytes can still respond to insulin stimuli in terms of the induction of the PAI-1 gene [79], suggesting that the expression of PAI-1 is up-regulated by insulin signal independently of insulin sensitivity.

3.4. Stress-induced PAI-1 and thrombosis in association with aging

A dramatic induction of PAI-1 gene has been observed in a mouse restraint-stress model [80], indicating that PAI-1 is a major stress-induced gene. The specific localization of the increased PAI-1 mRNA in epithelial cells, vascular smooth muscle cells, cardiovascular endothelial cells, adrenomedullar chromaffin cells, and neural cells of the para-aortic sympathetic ganglion has been demonstrated in restraint-stressed aged mice [80]. Restraint stress activates the hypothalamic–pituitary–adrenal axis and the sympathetic nervous system, leading to the increased secretion of glucocorticoid hormone and adrenaline, both of which induce PAI-1 expression in vivo [42,81]. The magnitude of PAI-1 mRNA induction due to restraint stress is the highest in the adipose tissue among the tissues examined, and the adipocytes are responsible for this induction [80]. Thus, adipose tissue/adipocytes may be one of the principal sources of PAI-1 expression in response to stress.

More importantly, stress-induced PAI-1 expression has been dramatically enhanced in aged mice [80], indicating an increased ability of aged animals to mount a PAI-1 response to stress. The mRNA induction of a procoagulant gene,

tissue factor (TF), in several tissues due to restraint stress is also higher in aged mice than in young mice [82]. These responses may elevate the procoagulant/antifibrinolytic potential, contributing to the increased incidence of stress-associated thrombotic events in the elderly. Indeed, stress-induced renal glomerular thrombosis is more pronounced in aged mice compared with young mice [80]. This difference in the thrombosis phenotype between young and aged mice may result from a much greater induction of the PAI-1 gene at the systemic and regional levels in aged mice. Thus, an age-related increase in the PAI-1 response to stress may exacerbate vascular injury and subsequent tissue damage as aging progresses.

3.5. Increased microthrombosis with PAI-1 induction in LPS-treated aged animals

Aged rats have shown increased susceptibility to hemorrhaging and intravascular hypercoagulation following endotoxin administration, resulting in a higher mortality of aged rats as compared to young rats [83]. In these studies, a greater increase in PAI-1 activity and a more significant decrease in total PA activity have been demonstrated in the plasma of aged rats treated with endotoxin in comparison with young rats [84]. Interestingly, renal glomerular fibrin deposition and renal PAI-1 gene expression were markedly induced and sustained in LPS-treated aged mice, as compared with young mice [85]. This increased response of the aged mice to LPS in PAI-1 induction, together with the observation that little fibrin was detected in LPS-treated PAI-1 deficient mice, suggests that PAI-1 contributes to an enhanced thrombotic tendency in aged mice suffering from endotoxemia. Thus, aged animals may tend to develop thrombosis due to the high antifibrinolytic potential in endotoxemia and inflammatory processes.

3.6. Enhanced immune response with cytokine induction in aged animals

The expression of CD14, which is a major receptor for LPS on the cell surface triggering a signaling cascade leading to cytokine production [86], has been induced by LPS in a variety of tissues [87]. The expression of CD14 in rat cardiac tissues was found to be more increased in aged animals after LPS treatment, suggesting that innate immune response is augmented with aging [88]. The magnitude of the induction in tissues of CD14 and Toll-like receptor 4 (TLR4), which is identified as another signaling receptor for LPS [89], was found to be greater in LPS-treated aged mice than in young mice [85], suggesting that LPS binding and signaling inside cells is augmented in aged mice. Indeed, higher levels of TNF-α have been detected in the plasma of LPS-treated aged mice in comparison with those of young mice [85], and this response of TNF-α may result in the dramatic induction of PAI-1 in aged mice. Overall, the greater magnitude of the induction of CD14 and TLR4 gene in LPS-treated aged mice may cause a larger increase in PAI-1 expression, leading to enhanced tissue microthrombosis.

Obesity could be considered a low-grade inflammatory state [90]. Several observations indicate that interleukin-6 and TNF-α are elevated in obesity [91], the latter contributing to the insulin-resistant state [92]. Interleukin-6 probably plays an important pathogenic role in a variety of disorders associated with chronic stress and physiological aging [93], such as the induction of PAI-1 [94]. Obese mice treated with neutralizing antibodies to TNF-α not only acquire increased insulin sensitivity but also significantly reduced levels of plasma PAI-1 antigen and adipose-tissue PAI-1 and TGF-β mRNAs [95]. These observations provide direct evidence that TNF-α is a common link between obesity, insulin resistance/hyperinsulinemia, PAI-1, and TGF-β, the last of which is also elevated in obese mice [96]. This establishes a central role for TNF-α in a number of the metabolic disorders associated with obesity. Similar striking elevation of TNF-α was observed in restraint-stressed aged mice [80], suggesting that the induction of cytokines in response to stress is augmented in aged individuals.

4. Procoagulant proteins/molecular markers and platelet function in the elderly

The levels of fibrinogen and factor VIII, both of which are acute-phase reactants, are significantly increased in the elderly [97]. Elevated levels of fibrinogen and factor VIII have been correlated with increased risk of venous thrombosis and cardiovascular events [98,99]. In contrast, factor VII is not an acute-phase reactant and has been identified as an independent risk factor for cardiovascular events [100]. Importantly, factor VIIa is also increased in centenarians [101], suggesting that the coagulation response, initiated by the binding of factor VIIa to TF, is accelerated in the elderly.

Molecular markers of thrombin generation also increase with age [102]. For example, elevated levels of the prothrombin fragment 1+2 (F1+2) have been observed in the elderly, suggesting the presence of excessive plasma factor Xa activity [103]. Other molecules of prothrombotic markers (e.g., fibrinopeptide A and B, factor X-activation peptide, factor IX-activation peptide, and the thrombin–antithrombin complex) have been observed to increase with age [101]. Centenarians have been found to have higher levels of the plasmin–antiplasmin complex and D-dimer compared with younger controls, suggesting a hypercoagulable state with reactive hyperfibrinolysis [101].

Although the proximate cause of elevated coagulation factor levels with aging may be multifactorial, recent studies have demonstrated that certain genomic elements regulate the age dependency of expression. Two genetic elements, AE5′ and AE3′, which contribute to the age-related increase

in factor IX levels, have been discovered in the human factor IX gene [104]. AE5′, which is present in the 5′ untranslated region and a consensus motif for the transcriptional factor, is necessary for the liver-specific expression of the human factor IX gene and for its stable transcription as the individual ages. AE3′, which is an element in the 3′ untranslated region, would increase human factor IX mRNA stability with age. The elements that control age-related gene expression were also discovered in the gene of anticoagulant protein C [105]. However, in general, it appears that the elevation in the anticoagulant proteins levels with aging does not keep pace with that of coagulant protein levels, thus contributing to a prothrombotic state in the elderly [106].

Platelet function is a critical determinant of the propensity to thrombosis, because activated platelets greatly accelerate thrombin generation. Markers of platelet activation, β-thromboglobulin and platelet factor 4, are significantly elevated with age [107]. Platelets from elderly patients may be less susceptible to inhibition by prostacyclin because the density of both high- and low-affinity receptors for prostacyclin decreases with aging [108]. The increase platelet activity with aging is correlated with a larger content of platelet phospholipids, suggesting an age-related increase in platelet transmembrane signaling or second messenger accumulation [109]. Von Willebrand factor, which enhances platelet interaction with the damaged endothelium or subendothelium and which is associated with atherosclerosis, also increases with age [110].

5. Alterations in the vascular wall with aging

Structural changes in the vascular wall at the level of the extracellular matrix, vascular smooth muscle, and endothelium could contribute to the increased risk for thrombosis in the elderly. Advanced age is accompanied by stiffness and dilation of the arteries due to the degeneration of elastic fibers and the increase in collagen content [111]. Gene polymorphisms of elastin and angiotensin II type-I receptor may predispose the elderly to a highly significant age-dependent stiffening and loss of vessel distensibility [112,113]. Aging is also associated with reduced endothelium-dependent dilation [114]. The aged blood vessels express less endothelial nitric oxide (NO) synthase [115], resulting in less NO production [116]. Decreased NO production may contribute to increased platelet activation and arterial thrombosis [117] as well as enhanced atherogenesis [118]. Also, the angiotensin II pathways may play a role in age-related endothelial dysfunction. The expression of angiotensin II is increased in the arterial intima with advancing age [119], and the cardiac expression of receptors for angiotensin II is significantly increased [120]. These observations suggest that age-associated arterial remodeling and the development of atherosclerosis are partially mediated by the increased angiotensin II signaling.

6. Summary

Hypercoagulability and advanced vascular sclerotic changes may contribute to the increased incidence of thrombosis in the elderly. One of the important key genes for the age-associated prothrombotic state is PAI-1 (Fig. 1). The expression of PAI-1 is not only elevated in the elderly but also significantly induced in a variety of pathologies associated with the process of aging. These conditions include obesity, insulin resistance, psychosocial stress, immune responses, and vascular sclerosis/remodeling, all of which accompany aging. Indeed, the expression level of

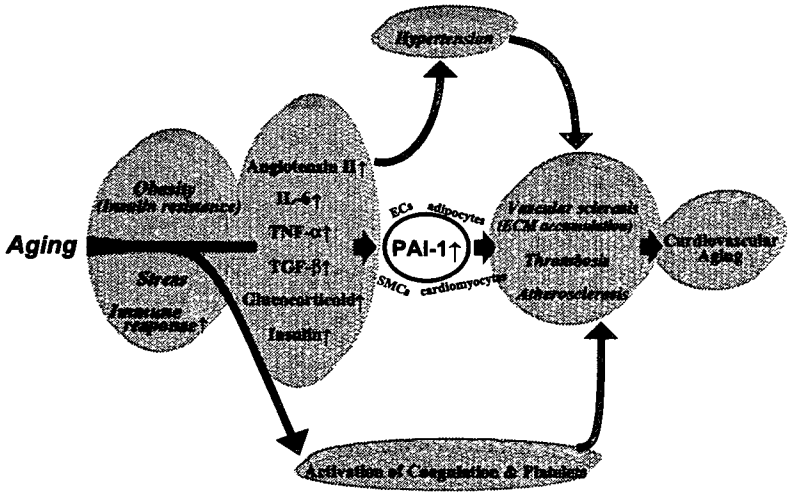

Fig. 1. A key role of PAI-1 in cardiovascular aging. A variety of pathologies associated with aging process cause the PAI-1 induction. This response is enhanced by specific function of several cytokines and hormones. PAI-1 could play a key role in the progression of cardiovascular aging by promoting thrombosis and vascular (athero)sclerosis. ECs—endothelial cells; SMCs—smooth muscle cells.

PAI-1 has been regarded as an important marker for cardiovascular risk. Several cytokines and hormones, including TNF-α, TGF-β, angiotensin II, and insulin, positively regulate the gene expression of PAI-1. These components are primarily synthesized or affected by adipocytes/adipose tissue, which is highlighted because of its relevance to the increased risk for atherosclerosis and cardiovascular events. Thus, PAI-1 could play a key role in the progression of cardiovascular aging and must be considered the most crucial gene for thrombosis and vascular (athero)sclerosis in current developed societies, where the elderly, the obese, and individuals exposed to stress are increasing in number. Further studies on the mechanism of age-regulated expression of PAI-1 are necessary in order to define the basis for cardiovascular aging in relation to thrombosis. It is also important for future clinical research to establish the most promising strategies for controlling PAI-1 expression so that cardiovascular diseases associated with aging can be prevented.

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ORIGINAL ARTICLE

Obesity enhances the induction of plasminogen activator inhibitor-1 by restraint stress: a possible mechanism of stress-induced renal fibrin deposition in obese mice

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Summary. *Background and objectives:* Cardiovascular/thrombotic diseases are frequently induced by a variety of stressors. Obese patients are susceptible to thrombotic diseases associated with stress, but the underlying mechanism is still unknown. We have begun to investigate the expression of a primary inhibitor of fibrinolysis, plasminogen activator inhibitor-1 (PAI-1), in association with tissue thrombosis, using restraint-stressed obese mice. *Methods and results:* We analyzed the expression of PAI-1 after restraint (immobilization) stress in genetically obese mice in comparison with their lean counterparts. Dramatic increases in PAI-1 antigen in plasma and in tissue extracts were observed in the obese mice exposed to restraint stress. The induction of PAI-1 mRNA by stress in the tissues was also pronounced in the stressed obese mice as compared with the lean mice, especially in the hearts and adipose tissues. *In situ* hybridization analysis revealed that strong signals for PAI-1 mRNA were localized in the adipocytes, cardiovascular endothelial cells, and renal glomerular cells of the stressed obese mice. Histological examination revealed that renal glomerular fibrin deposition was detected only in the obese mice after 2 h of restraint stress. *Conclusions:* Obesity enhances the stress-mediated PAI-1 induction in the blood and tissues. This phenomenon may be associated with the increased risk of stress-induced renal fibrin deposition in obese subjects.

Keywords: adipose, fibrin deposition, obesity, PAI-1, stress.

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Introduction

Obesity is an independent risk factor for the development of cardiovascular/thrombotic disease [1,2]. The increased incidence of cardiovascular disease may be associated with elevated levels of coagulation factors (e.g. factor VII, fibrinogen) and plasminogen activator inhibitor-1 (PAI-1) in plasma, which have been observed in obese patients [3,4]. PAI-1 is the primary inhibitor of plasminogen activation *in vivo*, and increased PAI-1 in plasma compromises the normal fibrin clearance mechanisms, promoting thrombosis [5], which can take the form of coronary artery disease [6]. In obese humans, increased plasma PAI-1 levels have been correlated with the amount of visceral fat [7], suggesting that adipose tissue is the primary source of PAI-1 in this condition. For example, relatively high levels of PAI-1 mRNA have been detected in the human [8] and murine adipose tissue [9], and adipose-derived PAI-1 expression has been found to be dramatically increased in genetically obese mice [10]. PAI-1 expression in cultured adipocytes is strongly upregulated by glucocorticoids [11], insulin [12], tumor necrosis factor-α [9], and transforming growth factor-β [12], all of which have been found to be elevated in obese subjects [13].

Mental and physical stressors decrease fibrinolytic activity [14,15] and contribute to the occurrence of thrombotic complications. For example, PAI-1 has been strongly induced by acute inflammatory stress [16] and hypoxic stress [17] *in vivo*. Chronic stress, defined as feelings of fatigue, increased irritability, and demoralization, has also been associated with elevated plasma PAI-1 antigen levels in humans [18]. Recently, the presence of psychosocial stressors, including financial stress, stress at the workplace and home, and major life events during the past year, has been associated with increased risk of acute myocardial infarction [19]. We have also reported that PAI-1 expression is dramatically induced by restraint (immobilization) stress, a typical physicopsychological stress [20], with maximal induction in the adipose tissue *in vivo*, a change

contributing to the development of tissue thrombosis [21]. In this context, obese individuals are susceptible to stress-mediated pathological changes, including thrombotic complications [22], possibly because of the stress-induced imbalance of the coagulation and fibrinolytic systems, and thus, obese animals may have lower tolerance to stress insults [23]. Taken together, these observations have led us to hypothesize that obesity may enhance the stress-mediated induction of PAI-1, thus causing thrombosis in obese patients.

In the present study, the effect of obesity on stress-induced PAI-1 expression and subsequent tissue thrombosis was investigated using genetically obese mice. A greater induction of the PAI-1 gene in response to restraint stress was observed in obese mice as compared with their lean counterparts, and renal glomerular fibrin deposition was induced only in the stressed obese mice. These observations suggest a possible mechanism of stress-induced fibrin deposition in the tissue of obese subjects.

Materials and methods

Restraint stress and tissue preparation

Male obese mice (C57BL/6J ob/ob) of 6 weeks old and their lean counterparts (C57BL/6J +/?) were obtained from The Jackson Laboratories (Bar Harbor, ME, USA). The mice were placed into conical centrifuge tubes fitted with multiple punctures so as to allow ventilation. We used 50 mL-tubes (20 mm in diameter) for lean mice and 100 mL-tubes (30 mm in diameter) for obese mice to subject them to the same level of restraint stress. The tubes were placed in horizontal holders and the animals thus maintained for a continuous period of restraint [20]. During this time, the animals were provided with water only. After 2 or 20 h of restraint, the mice ($n = 8$ in each time point) were sacrificed by overdose inhalation anesthesia with methoxyflurane (Pitman-Moore, Mundelein, MD, USA), which did not influence the PAI-1 expression (not shown). This experimental protocol was approved by the Animal Care and Use Committee of Nagoya University. Tissues were rapidly removed by standard dissection techniques, and either minced and immediately frozen in liquid nitrogen for preparation of total RNA or protein extraction. Other portions of tissues were fixed in chilled 4% paraformaldehyde and embedded in paraffin for *in situ* hybridization or for fibrin immunohistochemistry.

Determination of PAI antigen in mouse plasma and tissue extracts

The blood was collected into 20 mM EDTA (final concentration), centrifuged at 3000 *g* for 5 min, and then the plasma was removed and stored at −70 °C. Extraction of tissues was performed as described [24], and the protein concentration of the supernatant was determined by bicinchoninic acid (BCA) assay. The tissue extracts were also stored at −70 °C. PAI-1 antigen in the plasma and in the tissue extracts was determined by employing enzyme-linked immunosorbent assay (ELISA) specific for murine PAI-1, which was established in our laboratory [25]. The results are expressed as nanogram of PAI-1 per milliliter of plasma or picogram of PAI-1 per milligram of tissue.

Quantitative reverse transcription-polymerase chain reaction

We have developed a quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay to determine the concentration of PAI-1 mRNA in murine tissues as described in previous studies [16]. Briefly, total RNA was prepared from unfixed tissues using the Ultraspec™ RNA Isolation System (Biotecx Laboratories, Inc., Houston, TX, USA), and then quantified by measuring absorption at 260 nm. The complementary RNA (cRNA) standard was then *in vitro* transcribed using the Riboprobe Gemini II (Promega, Madison, WI, USA). Thereafter, 1 μg of total tissue RNA and the cRNA standard to be used as a competitor for the target mRNA were combined and reverse transcribed using a Gene Amp RNA PCR kit (Perkin-Elmer/Cetus, Norwalk, CT, USA). Serial twofold dilutions of the RT mixture were amplified using specific primers for PAI-1 or β-actin in the presence of ^{32}P-end-labeled sense primer (5×10^5 cpm). After PCR amplification for 30–35 cycles (95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min), 20-μL of aliquots of the PCR products were electrophoresed on a 2% agarose gel. The appropriate bands corresponding to the standard cRNA product and the target mRNA product were excised from the gel and the incorporated radioactivity in each was determined using a scintillation counter. The number of molecules of the target mRNA were determined by extrapolation using the cRNA standard curve. The concentration of PAI-1 mRNA was calculated and expressed as picogram mRNA per microgram total tissue RNA. Variations in sample loading were assessed by measuring β-actin mRNA.

Statistical analysis

All statistical analyses were performed with STATA ver.7 software (STATA Corp., College Station, TX, USA). Comparison of all quantitative RT-PCR results between two age groups (obese vs. lean) was performed with the two-sample *t*-test. Welch's method was applied when variance between two-group was unequal. The *P*-value < 0.05 was considered statistically significant.

In situ *hybridization*

In situ hybridizations for PAI-1 mRNA were performed using riboprobes as described previously [16,26]. After hybridization, the slides were dehydrated by immersion in a graded alcohol series containing 0.3 M NH_4Ac, and dried. The slides were then coated with NTB2 emulsion (Kodak, Rochester, NY, USA; 1:2 in water), and exposed in the dark at 4 °C for 8–12 weeks. The slides were developed for 2 min in D19 developer (Kodak), fixed, washed in water and counterstained with hematoxylin

and eosin. No specific hybridization signal could be detected in parallel sections using ^{35}S-labeled sense probes in each experiment (not shown).

Fibrin immunohistochemistry

Immunohistochemical staining was performed using the Histostain-SP Kit (Zymed Labs., South San Francisco, CA, USA), as described previously [16,26]. Briefly, the tissue sections were deparaffinized, treated with 2% hydrogen peroxide, and incubated with 10% normal goat serum for 30 min. The slides were then incubated with 10 μg mL^{-1} of rabbit anti-mouse fibrinogen/fibrin antibody (a kind gift of Dr E. Plow, Cleveland Clinic), containing 0.1% goat serum at 4 °C overnight, followed by incubation for 1 h at 25 °C. In control experiments, tissues were incubated with preimmune (normal) rabbit IgG instead of the primary antibody. The slides were then washed and treated sequentially with biotinylated goat anti-rabbit IgG (Zymed Labs.), streptavidin-peroxidase conjugate (Zymed Labs.) and aminoethylcarbazole chromogen containing 0.03% hydrogen peroxide (Zymed Labs.). After rinsing in distilled water, the slides were counter-stained with Gill's modified hematoxylin. The specificity of the antibody for fibrin in the extensively perfused tissues was indicated by the absence of staining in tissues from control mice (not shown). Quantitative evaluation of fibrin was achieved by counting the number of glomeruli positive for fibrin in each kidney section (magnification, ×400) in a blinded fashion.

Results

Induction of PAI-1 expression by restraint stress in obese and lean mice

We performed restraint experiments using genetically obese mice and their lean counterparts, and then analyzed PAI-1 expression in their blood and tissues (Fig. 1). Basal (no stress) levels of PAI-1 antigen in plasma and PAI-1 mRNA expression in the adipose tissue were significantly elevated in the obese mice as compared with the lean mice. Interestingly, the induction of PAI-1 antigen in plasma and PAI-1 mRNA expression in the tissues after restraint stress was pronounced in the obese mice. Moreover, in contrast to the lean mice, plasma PAI-1 antigen and adipose PAI-1 expression in the obese mice was dramatically induced by short-duration (2 h) stress. Most importantly, PAI-1 mRNA expression in the hearts was unchanged after restraint stress in the lean mice, but was significantly (threefold) increased in the obese mice. Finally, only short-duration stress induced renal PAI-1 mRNA in the lean mice, while both short- and long-duration stress

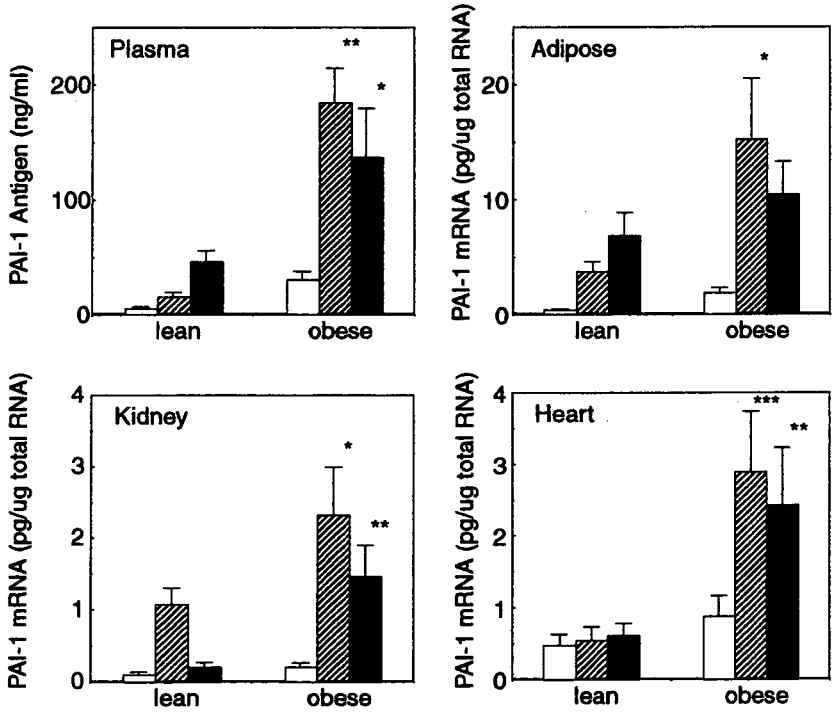

Fig. 1. PAI-1 antigen in plasma and mRNA in tissues after restraint stress in obese and lean mice. Six-week-old C57BL/6J ob/ob mice and their lean counterparts, C57BL/6J +/? mice, were placed into restraint tubes for 2 or 20 h, and then, the blood and tissues were removed. PAI-1 antigen levels in plasma (ng mL^{-1}) were measured by ELISA assay as described in Materials and methods. Total tissue RNA was prepared and analyzed for PAI-1 mRNA expression level by quantitative RT-PCR assay as described in Methods. Open bars, no stress; hatched bars, 2 h-restraint stress; closed bars, 20 h-restraint stress. The data are represented as the mean and SD (n = 8) in each category, and the error bars represent inter-animal variation. *P < 0.05 in obese vs. lean; **P < 0.02 in obese vs. lean; ***P < 0.01 in obese vs. lean.

Table 1 Plasminogen activator inhibitor-1 (PAI-1) antigen levels in tissue extracts of obese and lean mice after restraint stress (all data are presented as picogram of PAI-1 per milligram of tissue, mean ± SE. $n = 8$)

	Before stress		2 h-stress		20 h-stress	
	Obese	Lean	Obese	Lean	Obese	Lean
Adipose	44 ± 8.6	18 ± 3.8	436 ± 83	94 ± 17	409 ± 71	165 ± 31
Kidney	28 ± 2.7	23 ± 2.5	237 ± 33	149 ± 16	168 ± 19	35 ± 2.9
Heart	78 ± 14	69 ± 9.1	252 ± 55	74 ± 15	211 ± 46	77 ± 20

remarkably did so in the obese mice. The magnitudes of the stress-induced PAI-1 mRNA expression in other tissues, including the liver, lung and aorta, were similar in the lean and the obese mice (data not shown).

We examined the PAI-1 expression at antigen level as well in each tissue extract of obese and lean mice after restraint stress (Table 1). In general, large increases in PAI-1 antigen were observed in tissue extracts of the stressed obese mice, which showed similar kinetics with the mRNA level. A larger induction of PAI-1 antigen was shown in the kidneys, hearts, and adipose tissues of obese mice as compared with that in the lean mice. Especially, the adipose-derived PAI-1 antigen induced by stress was about 10-fold higher than basal level, mostly contributing to a marked increase in plasma PAI-1 in the stressed obese mice.

Cellular localization of PAI-1 mRNA in the tissues of stressed obese mice

In control (i.e. before stress) epididymal fat tissues, a few adipocytes were slightly positive for PAI-1 mRNA in both the obese and lean mice (Fig. 2A,E). After a 2-h period of restraint stress, the adipocytes specifically expressing considerable amounts of PAI-1 mRNA increased in both the obese and the lean mice (Fig. 2B,F). The adipocyte-specific signals for PAI-1 mRNA were stronger in the obese mice than those in the lean mice (compare Fig. 2D,H). In the restraint-stressed obese mice, the adipocytes of larger size specifically produced abundant PAI-1 mRNA, and the signals were still considerably strong after a 20-h period of stress (Fig. 2G). In contrast, the signals for PAI-1 mRNA in the epididymal fat tissues of lean mice exposed to 20 h of stress had increased in comparison with those in mice exposed to 2 h of stress (compare Fig. 2B,C).

In the hearts, only focal signals for PAI-1 mRNA were localized in the cardiovascular cells in unstressed obese mice, and few signals were detected in unstressed lean mice (Fig. 3, left panels). However, increased signals for PAI-1 mRNA were observed in some cells (which seemed to be the endothelia of the microvessels lying between the cardiomyocytes) of obese mice exposed to 2 or 20 h of restraint stress, but not in the hearts of stressed lean mice (Fig. 3, middle and right panels).

There was no detectable signal for PAI-1 mRNA in the kidneys of unstressed mice (Fig. 4, left panels). However, some glomerular cells of stressed obese mice expressed considerable amounts of PAI-1 mRNA, while only weak signals for PAI-1 mRNA were detected in the kidneys of stressed lean mice (Fig. 4, right panels). These results are consistent with the data obtained by quantitative RT-PCR assay (see Fig. 1).

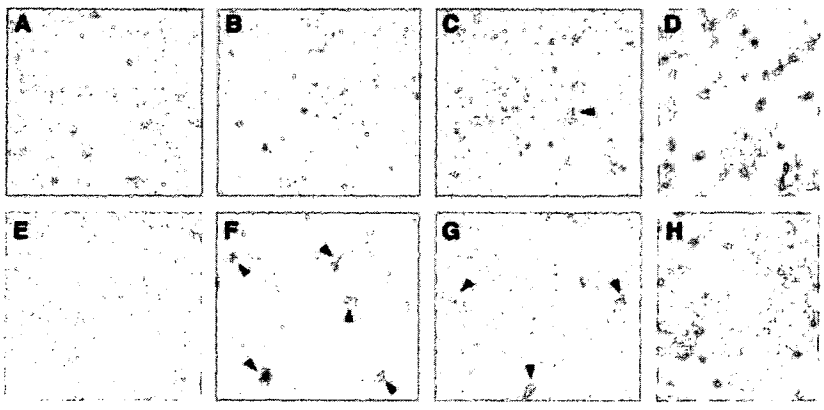

Fig. 2. *In situ* hybridization analysis of PAI-1 mRNA in adipose tissues of the stressed obese and lean mice. Epididymal fat tissues were harvested from 6-week-old obese and lean mice before and after 2 or 20 h-restraint stress and analyzed by *in situ* hybridization using ^{35}S-labeled cRNA probes as described in Materials and methods. The hybridization signal for PAI-1 mRNA corresponds to black dots in panels A–C, E–G (high magnification, ×400) and to light blue dots in panels D, H (low magnification, ×200). Panels A–D: adipose tissues of lean mice (A, no stress; B and D, 2 h-stress; C, 20 h-stress). Panels E–H: Adipose tissues of obese mice (E, no stress; F and H, 2 h-stress; G, 20 h-stress). Arrowheads indicate strongly positive cells for PAI-1 mRNA. All slides were exposed for 8 weeks at 4 °C.

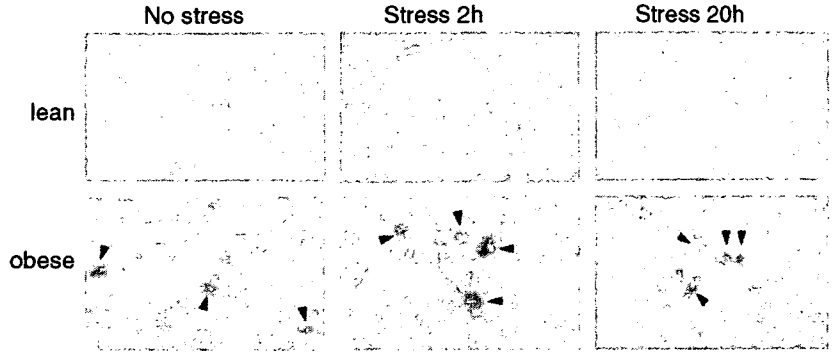

Fig. 3. *In situ* hybridization analysis of PAI-1 mRNA in hearts of the stressed obese and lean mice. Cardiac tissues were harvested from 6-week-old obese and lean mice before and after 2 or 20 h-restraint stress and analyzed by *in situ* hybridization using ^{35}S-labeled cRNA probes as described in Methods. The hybridization signal for PAI-1 mRNA corresponds to black dots in all panels. Arrowheads indicate strongly positive cells for PAI-1 mRNA. All slides were exposed for 12 weeks at 4 °C. Magnification, ×400.

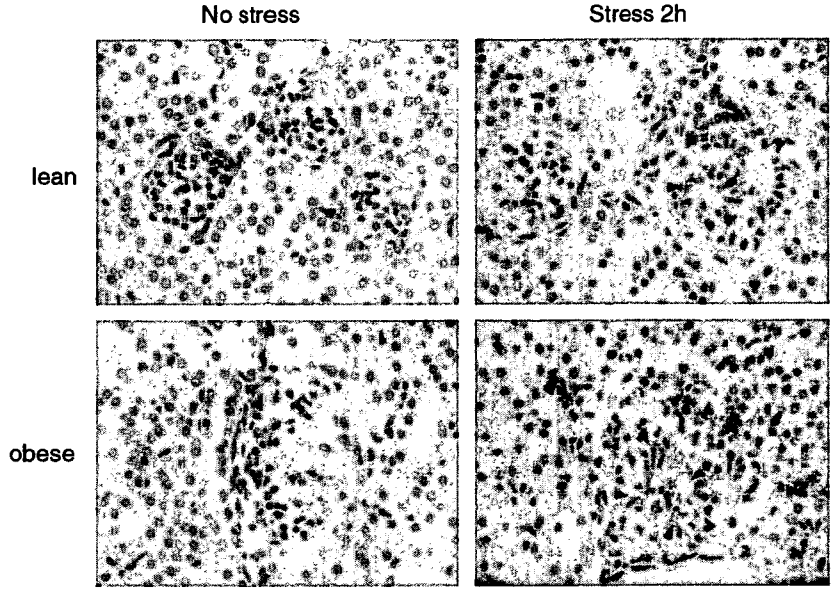

Fig. 4. *In situ* hybridization analysis of PAI-1 mRNA in kidneys of the stressed obese and lean mice. Renal tissues were harvested from 6-week-old obese and lean mice before and after 2 h-restraint stress and analyzed by *in situ* hybridization using ^{35}S-labeled cRNA probes as described in Materials and methods. The hybridization signal for PAI-1 mRNA corresponds to blue dots in all panels. Arrowheads indicate strongly positive cells for PAI-1 mRNA. All slides were exposed for 12 weeks at 4 °C. Magnification, ×400.

Stress-induced fibrin deposition in the kidneys of obese mice

Immunohistochemical analysis of fibrin deposition in renal tissue sections revealed that glomerular fibrin deposition developed after 2 h of restraint stress in obese mice (Fig. 5B), but that no significant glomerular fibrin was detected in stressed lean mice (Fig. 5A). Quantitative analysis of stress-induced fibrin deposition was performed by counting the glomeruli positive for fibrin. Although glomerular fibrin deposition was detected in < 5% of the glomeruli and only in two of eight restraint-stressed lean mice, all of the stressed obese mice (n = 8) showed glomerular fibrin deposition, and the percentages of the glomeruli positive for fibrin in these stressed obese mice were 6–15% (Fig. 5C). Meanwhile, only slight fibrin deposition was observed within the vasculature of the adipose tissue of two of eight stressed obese mice, while none of the stressed lean mice showed fibrin deposition in their adipose tissue (data not shown). No fibrin deposition was detected in other tissues, including the livers, hearts, lungs, and intestines of the obese mice after 2 h-restraint (data not shown).

Discussion

Although obesity and physicopsychological stress are associated with an increased incidence of cardiovascular/thrombotic diseases, little information is available about the underlying

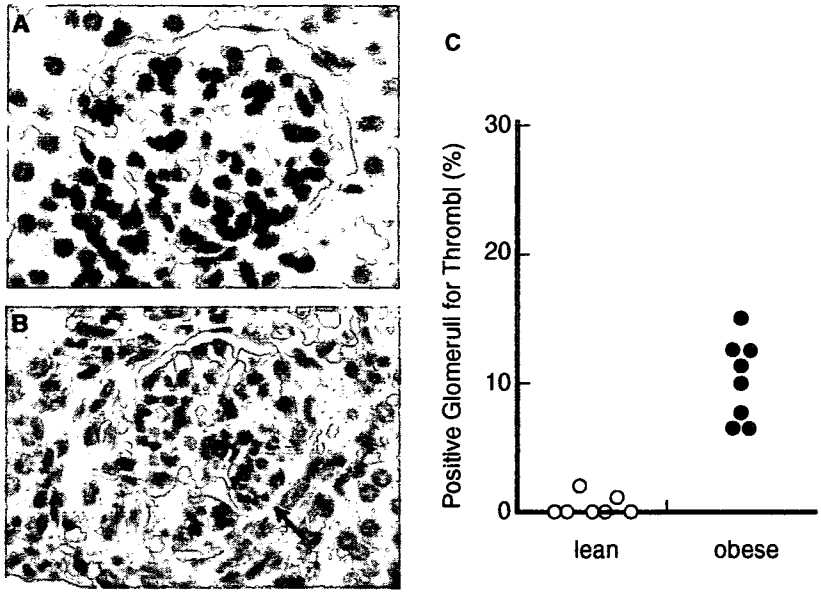

Fig. 5. Stress-induced glomerular fibrin deposition in obese and lean mice. Renal tissues were removed from 6-week-old obese and lean mice after 2 h-restraint stress ($n = 8$), and then, analyzed by immunohistochemistry for fibrin as described in Materials and methods. Panels A and B: renal glomerulus of lean (A) and obese (B) mice after 2 h-restraint stress. An arrow denotes glomerular fibrin deposition in the stressed obese mouse (B). Magnification, ×400. Quantitative data were obtained by counting the number of glomeruli positive for fibrin out of at least 100 glomeruli in each kidney section and the percentage of the glomeruli positive for fibrin in each mouse was shown in (C).

mechanisms. We have previously studied the expression of PAI-1 in a mouse restraint-stress model, and revealed that stress-induced PAI-1 contributes to tissue thrombosis *in vivo* [21]. In this study, we investigated the effect of obesity on stress-induced PAI-1 expression and observed that PAI-1 expression was significantly increased by restraint stress in obese mice (Fig. 1–4; Table 1). Even restraint stress of short duration caused a dramatic induction of PAI-1 in the obese mice, which stood in marked contrast to the state of the lean mice (Fig. 1). Importantly, the adipose tissue of obese mice remarkably responded to stress in the synthesis of PAI-1 (Fig. 2; Table 1), resulting in a systemic prothrombotic state in the obese mice, as shown by the high levels of plasma PAI-1 [8]. Adipocytes, and the adipose tissue in general, are the principal site of PAI-1 production in obesity [7,10,26], and thus, obese animals may possess a large potential to synthesize PAI-1 in response to stress because of their abundant adipose mass, leading to a marked increase in systemic and regional anti-fibrinolytic activity.

More interestingly, the synthesis of PAI-1 in cardiac tissues was significantly increased by stress only in the obese mice (Fig. 3). This implies that PAI-1 response to stress in cardiac tissues may be modulated by obesity-linked hormonal abnormalities. *In situ* hybridization analysis showed that the increased signals for PAI-1 mRNA were localized in cardiovascular endothelial cells in the stressed obese mice (Fig. 3), i.e. in those cells in which inflammatory mediators could induce PAI-1 gene expression [12,16]. Cardiomyocytes are another source of heart PAI-1 *in vivo*, as shown in our previous study [27,28]. Taken together, these results suggest that the induction of cardiac PAI-1 may result in a decreased fibrinolytic potential in the heart of obese mice.

We have previously shown that acute inflammatory stress immediately caused PAI-1 mRNA induction in the kidney, resulting in the development of glomerular fibrin deposition [16]. We have demonstrated here the marked induction of PAI-1 mRNA in the kidneys of obese mice after short-duration restraint stress (Fig. 1). *In situ* hybridization analysis revealed that renal glomerular cells in obese mice specifically produced PAI-1 mRNA in response to stress (Fig. 4). Renal glomerular fibrin deposition was markedly induced by stress in obese mice (Fig. 5), suggesting that stress-mediated PAI-1 induction contributes to the suppression of systemic or regional fibrinolytic activity and to the development of tissue thrombosis. Increased dehydration by restraint stress did not seem to enhance fibrin deposition in obese mice because the elevation of hematocrit was < 5% after 2 h-restraint and not different between the obese and lean mice (data not shown).

Indeed, renal thrombosis was detected in morbid obesity in humans [29], and pathological changes such as glomerular capillary occlusion and fibrin cap are observed in diabetic nephropathy, which is often accompanied by obesity. However, our observations do not necessarily match the clinical situation in obese patients because stress-induced (arterial) thrombosis is frequently encountered in lung, heart, and brain. This discrepancy may be because of differences between species because murine models of hypercoagulability developed thrombi at unexpected sites where thrombosis rarely occurs in humans [30,31]. The development of thrombosis would be dependent upon the vascular-bed specific hemostatic balance

[32], which may explain why some organs do not develop thrombosis in this model. In any case, several reports describing a pathological role of PAI-1 for cardiovascular diseases in obese subjects [33] may support our finding that the systemic PAI-1 induction by stress can lead to tissue thrombosis in obese animals.

In conclusion, we have demonstrated here that obesity enhances the stress-mediated induction of the PAI-1 gene, which may contribute to renal fibrin deposition in stressed obese mice. The induction of cardiac PAI-1 expression in the stressed obese mice may be extrapolated to the high incidence of stress-associated cardiovascular disease in obese subjects. This study presents a possible molecular mechanism of stress-induced fibrin deposition in the tissue in obese conditions.

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