Chapter 3

Pathogenesis of Atherothrombosis and High Blood Pressure And Their Valid Biological Risk Markers

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Introduction and Overview: Diminished Fibrinolysis Induced by Atherogenic Cytokines in Vascular Cells and its Clinical Implications for Accelerated Vasculopathy

The fibrinolytic system provides fibrin clot dissolution as a physiological defense mechanism against thrombosis (Figure 1A). This system mediates the conversion of zymogen, plasminogen, into the active protease plasmin through tissue-type plasminogen activator (t-PA) and urokinase type plasminogen activator (u-PA). The fibrinolytic system is controlled by the plasminogen activator inhibitor (PAI), which inhibits t-PA and u-PA, and by α_2-antiplasmin, which inhibits plasmin. Among PAIs, the physiologically most important is type-1 PAI (PAI-1), which is mainly synthesized by endothelial cells, smooth muscle cells, hepatocytes and adipocytes. Receptors for t-PA and plasminogen on the cell surface determine the cellular localization of plasmin-mediated proteolysis. U-PA has its own receptor, urokinase receptor (u-PAR), and determines u-PA-plasmin mediated cell surface proteolysis. Plasmin dissolves fibrin, fibronectin and laminin, activates or induces release of transforming growth factor (TGF)-β, basic fibroblast growth factor (bFGF), vascular endothelial cell growth factor (VEGF), and activates prometalloproteinases -3, -9, -12, and -13. Activated metalloproteinases can dissolve collagen, elastin and glycoproteins. As fibrinolytic components have their own receptors on the cell surface, enzymatic activities of each component are increased and the proteolytic activity on the cell surface is effectively augmented by binding to these receptors. Since the cells themselves have this fibrinolytic activity, this phenomenon is called "cellular fibrinolysis" in contrast to "blood fibrinolysis," which indicates plasmin mediated fibrin degradation within thrombus inside the vessels. By cellular fibrinolysis cells can proteolytically dissolve fibrin directly and the extracellular matrix surrounding cells indirectly by activating proteolytic enzymes. Decreased levels of circulating plasminogen activators or increased levels of PAI-1 induce diminished endogenous fibrinolysis, a failure of the defense mechanism against fibrin clot. A hypofibrinolytic state has been recognized as a marker of risk for cardiovascular disease and may play a role in thrombosis and accelerated atherosclerosis by clot derived mitogens. Diminished cell surface fibrinolysis may contribute to accumulation of the extracellular matrix and interstitial fibrosis (Figure 1B).

Recent clinical epidemiological findings suggest that insulin resistance (hyperinsulinemia, obesity, impaired glucose tolerance) is strongly related to accelerated

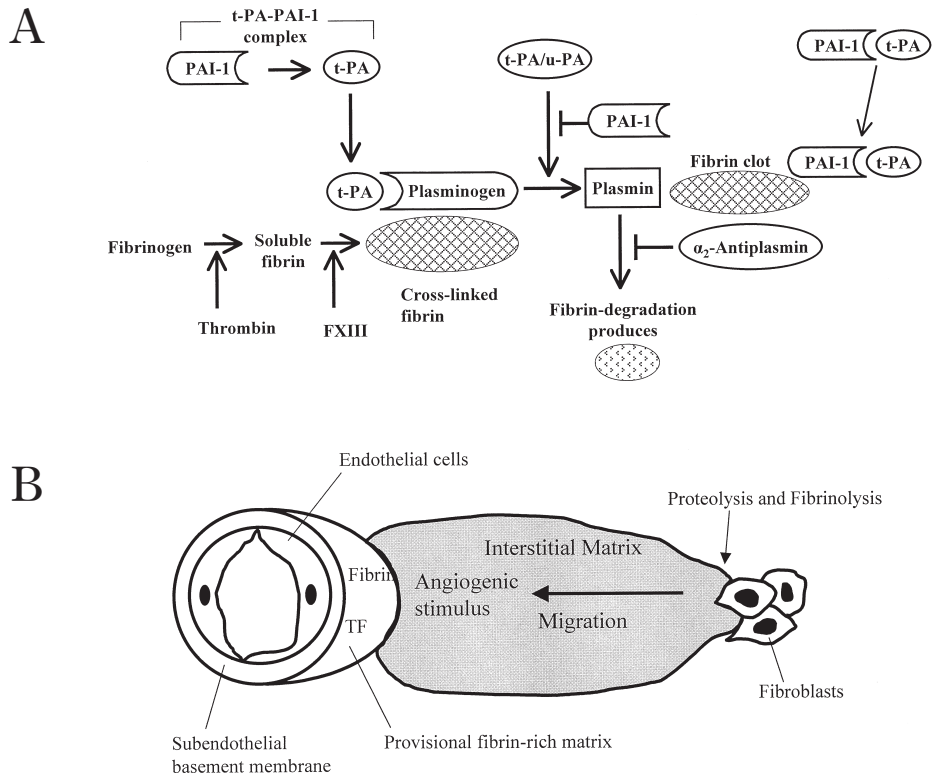

Figure 1 (A) Fibrinolytic system activation and inhibition. T-PA circulates in plasma as a complex with PAI-1 in a 1:1 ratio. The fibrin clot provides a surface on which the biding of t-PA to PAI-1 can occur. Plasminogen is activated by t-PA or u-PA. The complex of plasminogen, t-PA and fibrin promotes plasmin formation and subsequently induces lysis of cross-linked fibrin into fibrin-degradation product. PAI-1 also binds to fibrin and retains its inhibitory activity against t-PA. α_2-antiplasmin is cross-linked to fibrin by factor XIII. (B) Remodeling of coronary microvessels: a potential target for therapeutic intervention. Exudated fibrin from diseased microvessels can provide a provisional fibrin-rich matrix, to which fibroblasts can migrate by angiogenic stimulus. Proteolysis and fibrinolysis can degrade the matrix surrounding the migrating cells and promote remodeling process. Figures quoted in part from reference 93.

atherosclerosis and thrombosis. The bypass angioplasty revascularization investigation showed that in diabetic patients with multivessel coronary artery disease, the prognosis is poorer with angioplasty compared to coronary bypass surgery. The mortality from coronary heart disease in subjects with type 2 diabetes is higher compared to that in nondiabetic subjects with and without prior myocardial infarction. Elucidation of the mechanism(s) of accelerated atherosclerosis in diabetes contributes to in-depth understanding of the mechanism of atherosclerosis and thrombosis. We and others examined the role of insulin in regulating the produc-

tion of fibrinolytic proteins in cultured cells, characterized the alterations of the fibrinolytic system in diabetic animal models, and clarified the alterations of the fibrinolytic system in diabetic patients with coronary artery disease.

Cultured mouse adipocytes produce PAI-1[1]. Production of PAI-1 by these cells is increased by TNF-α, which induces insulin resistance, and by TGF-β. In vivo injection of TGF-β in mice can induce PAI-1 expression in adipose tissues. Production of PAI-1 by adipocytes in culture is increased by insulin, and insulin and TNF-α augment PAI-1 production in a synergistic manner, suggesting a potential link between vasculopathy and insulin resistance. Production of PAI-1 was increased by hydrogen peroxide simulating oxidative stress, and the increase was inhibited by antioxidants such as tetramethylthiourea. Fatty acids could increase PAI-1 production in adipocytes. These results suggest that in obesity with increased adipocyte mass, hyperinsulinemia and oxidative stress common to this condition can potentially stimulate adipocytes, produce PAI-1 and induce hypofibrinolysis by increasing circulating PAI-1 levels and the thrombotic tendency. Interleukin-1 induces PAI-1 and type-1 collagen expression in rat cardiac microvascular endothelial cells, and this induction is dependent on oxygen-centered free radicals[2]. Injection of proinsulin, a precursor of insulin, and insulin in vivo augment arterial endothelial expression of the PAI-1 gene as detected by in situ hybridization[3], suggesting that insulin and its precursor can diminish fibrinolytic activity in vivo in vascular cells. These results suggest that hyperinsulinemia can diminish fibrinolysis by increasing PAI-1 production in adipocytes and/or vascular cells.

Enhanced expression of PAI-1 in visceral fat during the development of obesity, which possibly contributed to vascular disease in obesity. In genetically obese mice from 7 weeks of age, circulating insulin, PAI-1 and adipose expression of PAI-1 mRNA are increased. In these obese mice, expression of TGF-β in adipose tissue is also elevated. In this mouse strain, diminished fibrinolysis and increased coagulation precede cardiac microvascular remodeling as shown by thickening of the arterial wall and increased perivascular fibrosis, suggesting diminished cellular fibrinolysis. In obese, hypertensive, diabetic, hyperlipidemic Otsuka Long-Evans Tokushima Fatty Rat (OLETF), the circulating plasma PAI-1 activity level is significantly increased at the early time point when high plasma glucose and hypersecretion of insulin are observed compared to that in control LETO rats.

Insulin resistance is accompanied by insulin hypersecretion at a high rate in non-diabetic normotensive obese subjects. Measurement of basal insulin levels is useful in the evaluation of the insulin response to glucose in diabetic and non-diabetic subjects. Therefore, it is reasonable to assume that insulin resistance often accompanies obesity. In obese subjects (body mass index$>$26), circulating plasma PAI-1 levels are two-fold greater compared to the value in lean subjects. The levels of t-PA antigen were increased as well, but the net fibrinolytic activity as measured by PAI-1 activity was increased in obese subjects. These results

suggest that insulin resistance can induce hypofibrinolysis and thrombotic tendency at the early stage of atherosclerosis.

As hypofibrinolysis is observed in obesity and insulin resistance, cellular fibrinolysis in the vascular wall may be altered in patients with insulin resistance or diabetes. To determine whether the expression of fibrinolytic proteins is altered in the vascular walls in diabetic patients with clinically significant coronary artery stenosis, the expression of fibrinolytic proteins in the directional coronary atherectomy specimens was investigated[4]. In coronary artery atherectomy specimens from type 2 diabetic patients compared with nondiabetic patients, PAI-1 expression was consistently increased and u-PA expression was decreased, suggesting that in diabetic atherosclerotic vascular lesions, hypofibrinolysis contributes to the induction of thrombosis or accelerated atherosclerosis.

The relationship between plasma immunoreactive insulin (IRI) levels after the oral glucose tolerance test and the extent of coronary artery disease in Japanese populations that underwent coronary angiography. Patients with significant stenosis confirmed by coronary angiography exhibited increased ΣIRI, ΣBS, ΣIRI/ΣBS, and IRI at 120 min compared to subjects without significant stenosis. IRI at 120 min was closely correlated with the severity of coronary artery disease. These results suggest that insulin resistance can diminish fibrinolysis, promote thrombotic tendency and significantly contribute to clinically apparent coronary artery disease by induction of thrombosis and coronary stenosis through reduced cellular proteolysis and subsequent extracellular matrix accumulation.

In insulin resistance observed at the early stage of atherosclerosis, insulin and TNF-α can stimulate secretion of PAI-1and induce hypofibrinolysis. As a result of hypofibrinolysis, one can speculate that small thrombus can form. Clot derived mitogens stimulate vascular cells and fibrin stroma serves as supporting matrix. These processes can induce tissue repair and ingrowth of small vessels (Figure 2A top panel). Tissue factor may contribute to this process. Hypofibrinolysis can also contribute to the expansion of thrombus formation by inhibiting clot dissolution under the presence of stress, hypoxia and plaque rupture (Figure 2A bottom panel). In diabetic coronary atherosclerotic diseases, increased expression of PAI-1 and decreased expression of u-PA can limit vascular remodeling and contribute to luminal narrowing (Figure 2B). Insulin resistance and subsequent hypofibrinolysis induced thrombotic tendency in circulation, diminished fibrinolysis in the vascular wall and contributed to the progression of atherosclerotic vascular diseases. Improvement of insulin resistance by diet and exercise may improve hypofibrinolysis and have beneficial effects on reduced hypofibrinolysis.

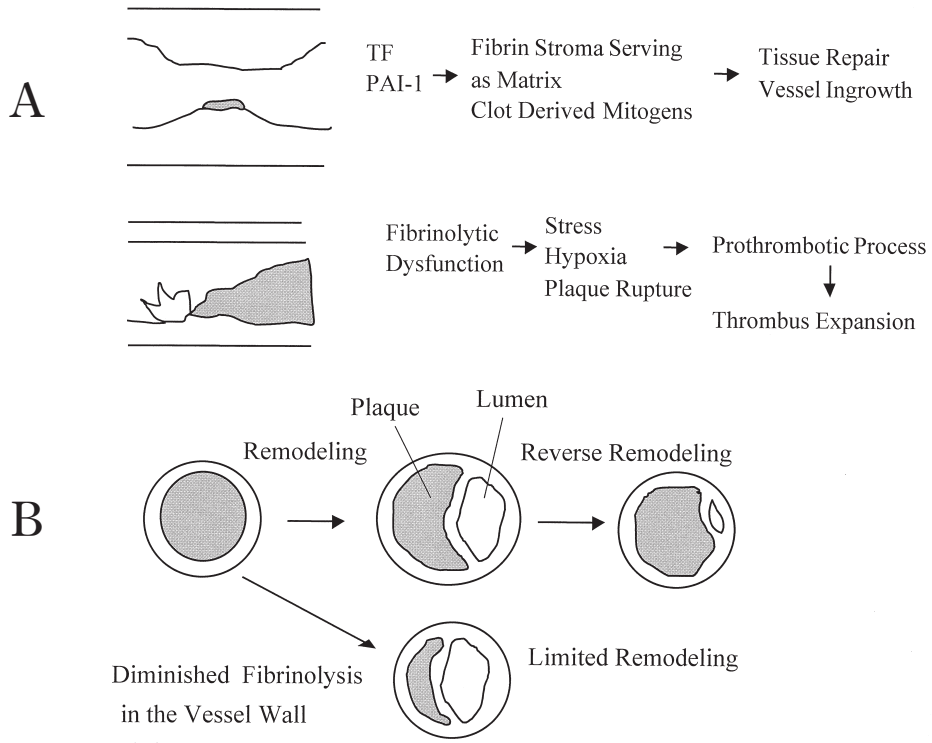

Figure 2 (A) Schema showing the potential role of fibrinolytic dysfunction and thrombus formation in atherosclerosis. (B) Schema showing the potential role of diminished intramural fibrinolysis in arterial luminal narrowing. With the increase of atherosclerotic plaque mass vessels increase in size and maintain the same lumen size (remodeling). As the plaque volume is decreased with pharmacological intervention, vessels decrease in size and maintain the same lumen size (reverse remodeling). With intramural fibrinolytic dysfunction favorable remodeling process is limited and vessels cannot maintain the same lumen size with the increase of atherosclerotic plaque mass. Figures quoted in part from reference 93.

Induction of PAI-1 in Endothelium by Interleukin-1: Implications for Impaired Coronary Microcirculation and the Role of Free Radicals

Endothelial cells are one of a major site of elaboration of fibrinolytic system proteins that affect diverse cell types, in concert with other cytokines and small molecules including t-PA, u-PA and PAI-1. Interleukin-1 (IL-1), a prototypic multifunctional cytokine elaborated by immune system cells[5], modulates expression of u-PA, PAI-1, PAI-2 and collagen in endothelial and smooth muscle cells, but the effects of IL-1 on synthesis of fibrinolytic system proteins in cardiac microvascular endothelial cells (CMEC) have not been elucidated. Because cytokines may predispose to thromboembolic phenomenon and vasculopathy associated with inflammation, the influence of IL-1 on elaboration of fibrinolytic system components in vitro in cultured rat CMEC and in rat hearts in vivo were characterized.

Proinflammatory cytokine IL-1 increased CMEC PAI-1 activity in the conditioned media (Figure 3A) in a concentration-dependent fashion. IL-1 increased PAI-1 protein accumulation in a concentration-dependent fashion (Figure 3B). Increased accumulation secondary to IL-1 (2 ng/ml) was evident by 6 hr (1.6±0.4(SD)-fold over control) and at 15 hr (2.0±0.3-fold over control) with further increases at 24 hr (4.4±0.6-fold over control). IL-1 did not augment accumulation of t-PA or u-PA. Thus, the effect of IL-1 was to increase net PAI-1 activity and accumulation. In addition, IL-1 increased accumulation of collagen elaborated by CMEC in a concentration-dependent manner with effects peaking at 2 ng/ml (3.5±0.7-fold over control at 24 hr). IL-1receptor antagonist (ra) significantly diminished but did not totally abolish accumulation of PAI-1 and collagen from CMEC exposed to IL-1. At a concentration of 200 ng/ml it suppressed the increase in PAI-1 accumulation induced by IL-1 (2 ng/ml) by 70±11% and suppressed the increase in collagen induced by IL-1 by 64±10%. Basal accumulations of PAI-1 and collagen were not affected by IL-1ra. Cycloheximide (10 μg/ml) inhibited IL-1 induced PAI-1 accumulation by 96±10% and collagen accumulation by 84±9%.

The hydroxyl radical scavenger tetramethylthiourea (TMTU, 10 mM) almost completely inhibited the accumulation of PAI-1 protein in response to IL-1 by CMEC at 24 hr (Figure 3C). Equimolar urea, used as a control, had no effect. In contrast to TMTU, dimethyl sulfoxide (DMSO) inhibited IL-1 induced PAI-1 accumulation only partially. When the cells were incubated with hydrogen peroxide (100 μM) or a superoxide generating system (xanthine oxidase 10 mU/ml plus hypoxanthine 0.6 μM), PAI-1 accumulation increased (Figure 3D). The extent to which reactive oxygen species induced PAI-1 was similar to the extent to which IL-1 induced PAI-1 (Figure 3D). TMTU inhibited accumulation of collagen in IL-1 stimulated CMEC. Equimolar urea had no effect. DMSO partially inhibited IL-1 induced accumulation of collagen. Hydrogen peroxide or xanthine oxidase plus hypoxanthine increased accumulation of collagen. The extent to which reactive oxygen species induced collagen corresponded to the IL-1 induced increase.

IL-1 and LPS increased PAI-1 protein in rat hearts in vivo with a maximum

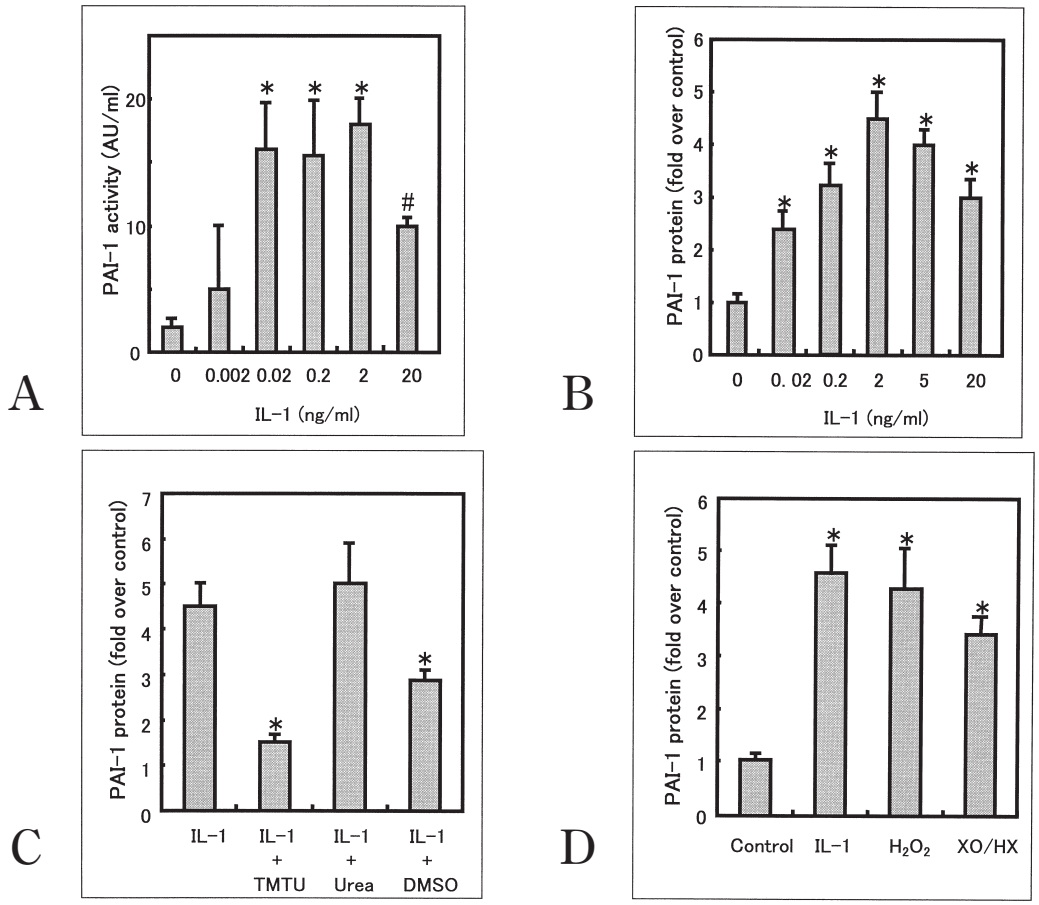

Figure 3 **A**: Effects of IL-1 on activity of PAI-1 in conditioned medium of CMEC. Confluent cells were serum starved overnight and then incubated with fresh serum free media containing IL-1 (0 to 20 ng/ml) for 24 hr. Conditioned media were harvested and activity of PAI-1 was assayed. # $P<0.05$, *$P<0.01$ compared with values in untreated control cells. **B**: Effects of IL-1 on the concentration of PAI-1 in conditioned medium of CMEC. Confluent cells were serum starved overnight and then incubated with fresh serum free media containing IL-1 (0 to 20 ng/ml) for 24 hr. Conditioned media were harvested, and concentrations of PAI-1 assayed by Western blot. Values are fold increase over control without IL-1. *$P<0.01$ compared with untreated, control cells. **C**: Effects of the oxygen centered free radical scavenger, tetramethylthiourea (TMTU), urea (chemical control), and dimethyl sulfoxide (DMSO) (another scavenger) on elaboration by CMEC of PAI-1 induced by IL-1 (2 ng/ml). PAI-1 was assayed by Western blotting. Values are fold increase over control without IL-1. *$P<0.01$ compared with results in IL-1 treated cells without TMTU or DMSO. **D**: Effects of hydrogen peroxide (H_2O_2), and xanthine oxidase plus hypoxanthine (XO/HX) (both free radical generating systems) on the elaboration of PAI-1 in CMEC. PAI-1 was assayed by Western blotting. Values are fold increase over control without IL-1. Figures quoted in part from reference 2.

induction of 2.7±0.3-fold and 3.4±0.7-fold at 24 hr compared with that in animals infused with saline. Immunohistochemical analysis indicated a consistent, relatively strong signal for PAI-1 antigen in the endothelial layer within the small arteries of the hearts from rats injected with IL-1 (Color Fig. 10). PAI-1 antigen was evident also in microvessels in the myocardium. Endothelial cells of large epicardial vessels showed only a weak signal after stimulation with IL-1. This general pattern of PAI-1 antigen expression was relatively uniform throughout the vasculature in all animals studied. A similar pattern of PAI-1 antigen distribution was observed in rat heart tissues of animals injected with LPS. In contrast, the arteries from hearts of animals injected with saline showed little or no staining.

IL-1 produced by immune system cells influence production of (proteo)fibrinolytic system proteins in CMEC. Furthermore, IL-1 increased matrix protein type-1 collagen. Increased PAI-1 and collagen induced by IL-1 was attenuated by cycloheximide, suggesting that PAI-1 and collagen expression induced by IL-1 requires new protein synthesis. IL-1ra diminished accumulation of PAI-1 and collagen from CMEC exposed to IL-1. This verified, at least in part, the specificity of the IL-1 response. Thus, the effects appear to have been mediated through the IL-1 receptor. Because basal accumulations of PAI-1 and collagen were not affected by the IL-1ra, constitutive synthesis appears to be independent of IL-1.

Reactive oxygen species are commonly produced by inflammatory cells during the course of inflammatory processes. In glomerulonephritis glomerular cells can generate reactive oxygen intermediates, independently of infiltrating cells, that may play an autacoid role in glomerular injury. IL-1 can induce production of both superoxide and hydrogen peroxide. To determine whether reactive oxygen species were involved in the increased expression of PAI-1 induced by IL-1, the effects of radical scavengers TMTU and other antioxidants were compared. The hydroxyl radical scavenger TMTU, and to a lesser extent DMSO, inhibited IL-1 induced PAI-1 expression. To determine whether reactive oxygen species could directly induce PAI-1 expression in CMEC, cells were incubated with either hydrogen peroxide or a superoxide generating system. Both increased PAI-1 accumulation.

In many cell types NADPH oxidase is involved in the generation of reactive oxygen species. Endothelial cells contain membrane-bound oxidase(s) that use NADH and NADPH as substrates for electron transfer to molecular oxygen, and can produce reactive oxygen species. NADPH oxidase generated reactive oxygen may mediate production of PAI-1 by CMEC. Since IL-1 and reactive oxygen species had similar effects on accumulation of collagen from CMEC, a common mechanism may be operating in the signal transduction pathways leading to induction of collagen synthesis by IL-1 in CMEC. Oxidative stress is suggested to play a role in the development of perivascular fibrosis, and fibrolytic responses may modulate the interstitial and perivascular fibrosis of intramyocardial coronary arteries[6]. Type-1 collagen, the predominant matrix protein deposited in myocardial disease in humans, may contribute. Furthermore, impairment of the microcir-

culation may induce myocytolytic necrosis and reperfusion injury. Because oxidant stress and cytokines appear to be involved in myocardial ischemia, reperfusion injury and thrombosis[7)-9)], the results are consistent with the possibility that IL-1 may influence cardiac remodeling of the extracellular matrix by inducing collagen synthesis and perivascular fibrosis and that it may alter coronary microcirculation dynamics by altering microvascular fibrinolysis associated with activation of immunocompetent cells.

CMEC were used to determine whether altered elaboration of specific fibrinolytic system proteins is likely to occur in cardiac microvasculature under conditions of ischemia. CMEC have distinctive characteristics compared with endothelial cells isolated from large vessels. When IL-1 was included in the medium, the concentrations of matrix protein collagen increased, suggesting that CMEC are capable of producing extracellular matrix proteins.

IL-1 is a prototypic multifunctional cytokine. Unlike lymphocyte and colony-stimulating growth factors, IL-1 affects diverse cell types, often in concert with other cytokines or small mediator molecules. Both IL-1 and LPS, a potent inducer of cytokines elaborated by immune system cells, administered in vivo increased PAI-1 in rat heart as judged from analysis of Western blots. PAI-1 immunostaining was evident in microvessels, showing that PAI-1 synthesis in coronary microvessels can occur in vivo and potentially contribute to high concentrations of PAI-1 in the coronary circulation.

Activation of blood coagulation and inadequate activation of endogenous fibrinolysis may contribute to a “no reflow” phenomenon by inducing fibrin deposition and formation of microthrombi[10)]. Thus, the poor prognosis associated with TIMI 2 vs TIMI 3 may be a reflection in part of microvascular damage and is, therefore, a predictor of a poor outcome in patients with myocardial infarction[11)-12)]. The findings suggest a potential efficacy for antioxidants in ameliorating microvascular fibrinolytic system dysfunction related to inflammatory disease or ischemia and reperfusion injury. Antioxidants may not only inhibit direct effects of cytokines but suppress elaboration of PAI-1 and collagen and their potentially adverse consequences.

Insulin and Proinsulin Regulate PAI-1 and Type-1 Collagen in Rat Cardiac Microvascular Endothelial Cells: Potential Role in Metabolic Syndrome

Hyperinsulinemia, hypertension, hyperlipidemia, and obesity are concomitant disorders of many individuals. This cluster of metabolic abnormalities has been called the insulin resistant syndrome, because insulin resistance and hyperinsulinemia have been postulated to be the underlying features. PAI-1 may predict risk of thrombosis and myocardial infarction. The role of PAI-1 in the insulin resistance syndrome is therefore interesting. Increased PAI-1 levels have been associated with cardiovascular risk factors such as obesity, insulin resistance, increased levels of glucose, insulin, and proinsulin[13].

The primary role of endothelial cells is to serve as a barrier between the blood and surrounding tissues. Insulin has been shown to modulate expression of PAI-1 in hepatoma cell line but not in endothelial cells from vessels with large caliber[14]. In contrast to endothelial cells isolated from large vessels, the roles played by the endothelial cells of microvasculature are not as well understood. Endothelial cells of microvessels have been proposed to function in angiogenesis. Endothelial cells are facing blood circulation so that after vascular injury and clot formation they have access to plasma plasminogen activators involved in fibrinolysis, and may participate in insulin-associated intravascular processes that are characterized by fibrin degradation. PAI-1 production in brain microvascular endothelial cells can be regulated by t-PA[15]. But effects on synthesis of proteins related to fibrinolysis by CMEC have not been characterized. It is hypothesized that one important function of CMEC might be the regulation of intravascular fibrinolysis via production of PAI-1. Because hyperinsulinemia may predispose to thromboembolic phenomenon and vasculopathy associated with diabetes, the influence of insulin on elaboration of fibrinolytic system components and extracellular matrix protein collagen type I in vitro in cultured rat CMEC and on coronary microvessels in diabetic rat hearts in vivo were characterized.

Insulin increased CMEC PAI-1 activity in the conditioned media (Figure 4A) in a dose dependent fashion. Proinsulin had similar effects (Figure 4B). Insulin and proinsulin also increased PAI-1 protein accumulation (Figure 5A). Cycloheximide (10 μg/ml) used to inhibit protein synthesis lead to a marked reduction in baseline PAI-1 protein accumulation and insulin or proinsulin induced PAI-1 accumulation. Insulin and proinsulin did not augment accumulation of t-PA or u-PA by zymography. Thus, the net effect of insulin and proinsulin was to increase PAI-1 activity and accumulation. Insulin and proinsulin also increased accumulation of collagen from CMEC (Figure 5B). Cycloheximide (10 μg/ml) lead to a marked reduction in baseline collagen accumulation and insulin or proinsulin induced collagen accumulation. Cycloheximide did not affect cell viability as judged from persistent exclusion of trypan blue.

Coronary microvessels ($<$100 μm) of rats rendered diabetic exhibited markedly increased tortuosity. Hearts obtained 12 weeks after injection of saline exhibited

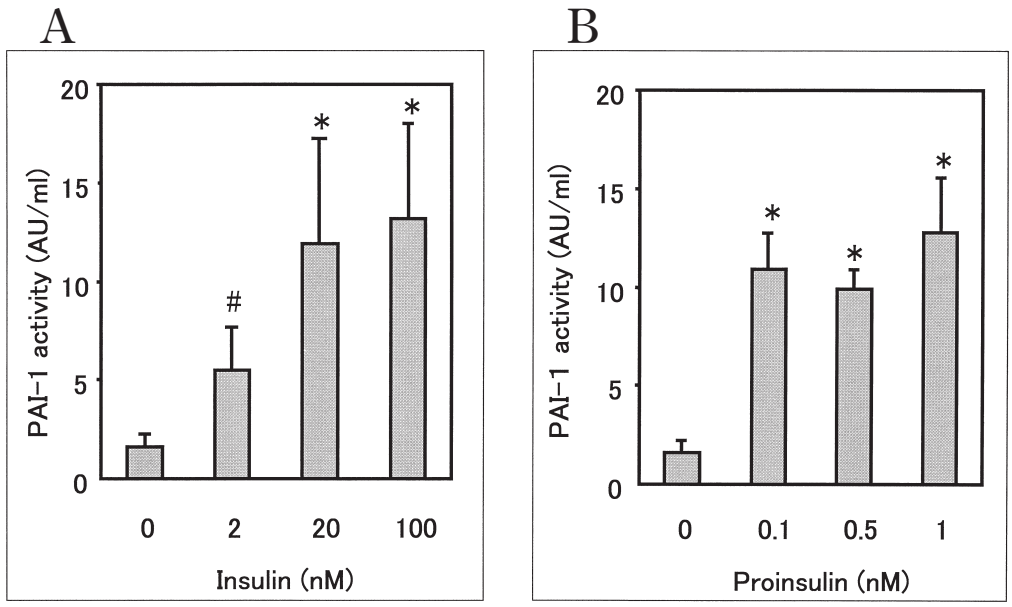

Figure 4 A: Effects of insulin on activity of PAI-1 in conditioned media of CMEC. Confluent cells were serum starved for overnight and then incubated with fresh serum free media containing insulin (0-100 nM) for 24 hours. Conditioned media were harvested and activity of PAI-1 was assayed. #P<0.05, *P<0.01 as compared to untreated control cells. B: Effects of proinsulin on activity of PAI-1 in conditioned media of CMEC. Confluent cells were serum starved for overnight and then incubated with fresh serum free media containing proinsulin (0 to 1 nM) for 24 hours. Conditioned media were harvested, and activity of PAI-1 was assayed. *P<0.01 as compared to untreated, control cells. Figures quoted in part from reference 94.

no such changes in microvessels in the myocardium. The total capillary density was significantly decreased in diabetic group compared with controls. In the diabetic group the arteriolar portion and intermediate portion were significantly increased while the venular portion was significantly decreased, suggesting that diabetic state may induce arteriolization of venules.

Hyperinsulinemia is associated with increase in coronary risk factors. Insulin can stimulate production of PAI-1 in CMEC, thereby leading to diminished fibrinolysis. Without agonist stimulation CMEC expressed PAI-1 functionally and antigenically. At plasma concentrations of insulin there was a 3 fold increase in PAI-1 activity. The increase in activity was accompanied by increase in protein. Although zymography is semiquantitative in nature, insulin and proinsulin did not augment accumulation of t-PA or u-PA as determined by this method, suggesting that the net effect of insulin and proinsulin is to attenuate fibrinolysis. The concentrations of insulin used in the present study were selected to be consistent with the concentrations of insulin seen in normal subjects and in patients with non-insulin dependent diabetes[16]. Although typical concentrations of proinsulin in

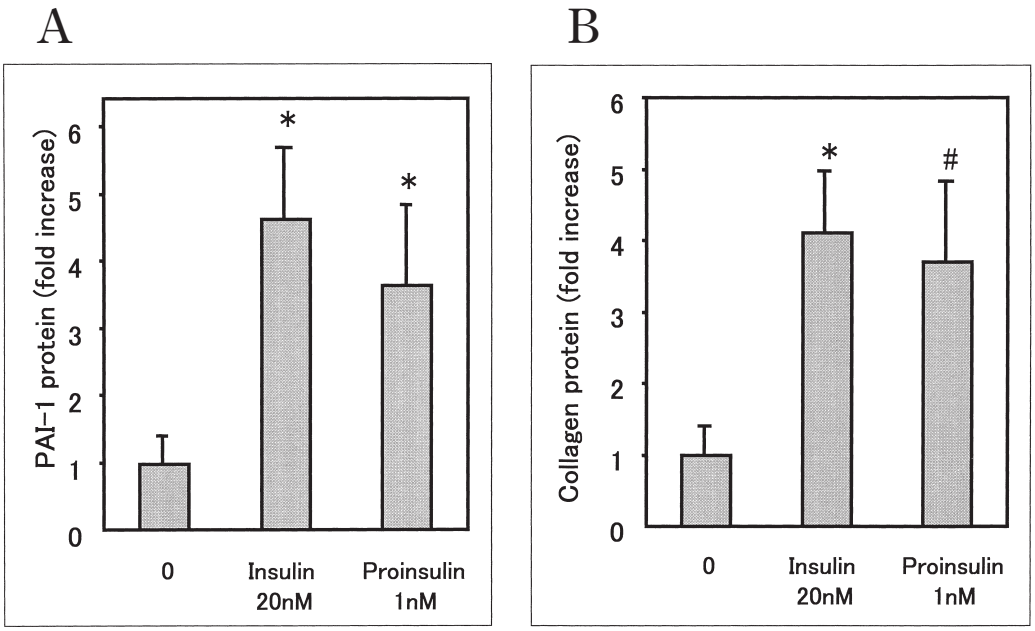

Figure 5 A: Effects of insulin (20 nM) and proinsulin (1 nM) on concentrations of PAI-1 in conditioned media of CMEC. Confluent cells were serum starved for overnight and then incubated with fresh serum free media containing insulin (20 nM) or proinsulin (1nM) with or without cycloheximide (10 μg/ml) for 24 hours. Conditioned media were harvested, and concentrations of PAI-1 were assayed by Western blot. Values are fold increase over control. *P$<$0.01 as compared to untreated, control cells. B: Effects of insulin (20 nM) and proinsulin (1 nM) on concentrations of collagen in conditioned media of CMEC. Confluent cells were serum starved for overnight and then incubated with fresh serum free media containing insulin or proinsulin with or without cycloheximide (10 μg/ml) for 24 hours. Conditioned media were harvested and concentrations of collagen were assayed by Western blot. Values are fold increase over control. #P$<$ 0.05, *P$<$0.01 as compared to untreated, control cells. Figures quoted in part from reference 94.

plasma range from 0.002 to 0.01 nM in normal subjects, in obese diabetic subjects precursors of insulin may account for much higher proportion of immunoreactive insulin.

The second messengers involved in the signal transduction pathways leading to induction of PAI-1 and collagen by insulin in CMEC remain incompletely understood. Because insulin and proinsulin had similar effects on collagen accumulation by CMEC, common mechanism may be operating in the signal transduction pathways leading to collagen induction in CMEC. Because insulin and proinsulin appear to be involved in vasculopathy and thrombosis, our results also suggest that insulin may influence cardiac remodeling of extracellular matrix by inducing collagen synthesis and perivascular fibrosis, and may alter coronary microcirculation by altering microvascular fibrinolysis associated with hyperinsulinemic states.

Pathogenesis of microvasculopathy in diabetes has been attributed to hyperglycemia per se[17]. Because elevated insulin levels are recently implicated in the pathogenetic factor in the development of brain small-vessel disease, and fibrinolysis is implicated in atherosclerosis, we hypothesized that insulin may play a direct role. To test this hypothesis in vivo the effects of insulin deficiency induced by streptozotocin treatment on coronary microvessels was examined. PAI-1 immunostaining has been previously shown in microvessels[18], showing that PAI-1 synthesis in coronary microvessels can occur in vivo. Remodeling of capillary network can occur in vasopressin administration and coronary artery occlusion. Streptozotocin administered in vivo induced marked tortusity of microvessels, increase in arteriolar portion and intermediate portions in capillary density in rat heart tissues. Thus, it is likely that after insulin deficiency due to streptozotocin PAI-1 expression in CMEC is decreased, leading to increased perivascular proteolysis and decreased maintenance of integrity in microvessels. These combined data suggest that subsequent to changes in circulating insulin levels CMEC could play a pivotal role in regulating the fibrinolytic response in microvessels.

The events leading to altered fibrinolysis in insulin resistant syndrome in humans may be complex and regulated by multiple interactions between insulin, glucose, lipids, and other cytokines and growth factors. However, the ability of CMEC to express PAI-1 will play a critical role in controlling perivascular proteolysis and maintenance of integrity of the microvessels in diabetic microvasculopathy and potentially in microvascular angina. The findings suggest that insulin and proinsulin induce PAI-1 and collagen in CMEC, and insulin deficiency impairs microvascular integrity in vivo and contribute directly to coronary microvasculopathy.

Induction of PAI-1 in Endothelium by bFGF and its Modulation by Fibric Acid: a New Face of the Pleiotropic Actions of Fibrates

bFGF, a growth factor elaborated by vascular cells, modulates expression of plasminogen activators in endothelial cells (ECs) and smooth muscle cells[19)-20)]. Excessive plasmin generation and proteolysis induced by bFGF mediated overproduction of plasminogen activators may interfere cell adhesion or atherosclerotic plaque development. However, the influence, if any, of bFGF on synthesis of PAI-1 in ECs has not been elucidated. The potential influence may well contribute to physiologic or pathophysiologic consequences of elaboration of bFGF. Because local synthesis of PAI-1 and the balance between plasminogen activation and inhibition determine generation of plasmin which in turn influences regional fibrinolytic balance, thus modulating atherothrombotic[21)] and angiogenic[22)] properties of ECs. A lipid-lowering fibric acid derivative, which activates peroxisome proliferator activated receptor-α (PPARα), reduces PAI-1 expression in human hepatoma cells in vitro[23)]. The influence of bFGF and fibric acid on elaboration of PAI-1 in vitro by cultured ECs were characterized, and to delineate potential mechanisms that may underlie increased PAI-1 expression in vivo was sought.

A 2.1 fold increase in PAI-1 mRNA expression induced by bFGF was seen with human umbilical vein ECs (HUVECs). The baseline concentration of PAI-1 in the conditioned media of HUVECs was 181.7±32.7 ng/ml. bFGF increased PAI-1 protein accumulation in the media in a concentration-dependent fashion. Peak effects were seen with 10 ng/ml (1.8±0.3 fold over control). Baseline activity of PAI-1 in the conditioned media was 12.0±0.6 AU/ml at 4 hour. bFGF increased PAI-1 activity at a concentration of 0.1 ng/ml (1.7±0.4 fold over control) and 1 ng/ml (2.3±1.1 fold over control). At 10 and 100 ng/ml PAI-1 activity somewhat diminished (1.4±0.4 fold over control and 1.5±0.8 fold over control, respectively). The baseline concentration of t-PA in the conditioned media of HUVECs was 22.1±2.2 ng/ml. bFGF increased t-PA protein accumulation in the media (24.9±2.7 at 1 ng/ml and 24.0±2.8 at 10 ng/ml, $p<0.05$).

An increase in PAI-1 mRNA expression was seen in ECV cells. The expression of PAI-1 mRNA by bFGF was increased in a concentration-dependent fashion with 1.4 fold increase over control at 10 ng/ml and 2.0 fold at 100 ng/ml. The baseline concentration of PAI-1 in the conditioned media of ECV cells was 254±28 ng/ml. bFGF increased PAI-1 protein accumulation in the media in a concentration-dependent fashion. Peak effects were seen with 10 ng/ml bFGF (1.9±0.2 fold over control). The response was diminished somewhat with concentrations of 100 ng/ml (1.4±0.2 fold over control). The baseline concentration of t-PA in the conditioned media of ECV cells was 0.71±0.05 ng/ml. bFGF increased t-PA protein accumulation in the media (0.88±0.05 at 1 ng/ml and 0.93±0.02 at 10 ng/ml, $p<0.01$). Total protein content in the conditioned media was not altered by bFGF in HUVECs and EVC cells.

To investigate the intracellular mechanisms involved in the induction of PAI-1

mRNA ECV cells were treated for 1 hr with each of the various inhibitors of intracellular signaling pathway before addition of bFGF (Figure 6A). Inhibition of ERK kinase with PD98059 blocked basal and bFGF stimulated PAI-1 mRNA levels. In contrast, GF109203X, an inhibitor of protein kinase C pathway, and genistein, an inhibitor of tyrosine kinase, had no significant effect. β-actin mRNA levels did not change in any experimental conditions. To determine whether bFGF influences PAI-1 mRNA half-life ECV cells were stimulated with bFGF for 2 hr and medium was changed and actinomycin D (5 mg/ml) was added with or without bFGF and cells were incubated for up to 6 hr (Figure 6B). The rate of PAI-1 mRNA decrease was not changed by bFGF, suggesting that PAI-1 mRNA increase by bFGF was due to an increase of transcription from the PAI-1 promoter.

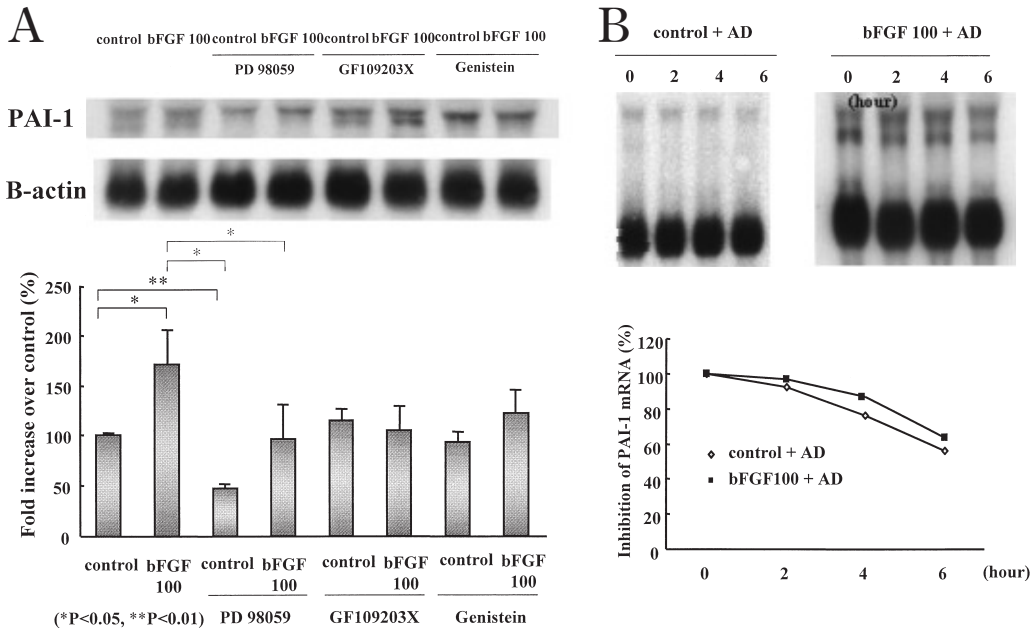

Figure 6 Effects of PD98059 and actinomycin D on PAI-1 mRNA. (A) Effects of PD98059 (30 μM), GF109203X (2 μM) and genistein (100 μM) on PAI-1 mRNA levels. ECV cells were incubated with or without indicated inhibitors for 1 hr and then bFGF was added. Cells were incubated for 4 hr. Total RNA was isolated and analyzed by Northern blot. PAI-1 mRNA levels were analyzed by densitometry and normalized to β-actin signals. The upper panel shows a representative autoradiograph from three separate experiments. *$p<0.05$ and **$p<0.01$ compared to control. (B) Effects of actinomycin D (AD) on PAI-1 mRNA levels. ECV cells were pretreated with bFGF (100 ng/ml) for 2 hr. Each medium was changed and actinomycin D (5 μg/ml) was added with or without bFGF. The incubation continued for up to 6 hr. Total RNA was isolated and analyzed by Northern blot. PAI-1 mRNA levels were analyzed by densitometry and normalized to β-actin signals. The upper panel shows a representative autoradiograph from three separate experiments. Figures quoted in part from reference 37.

To identify the 5' flanking region of the PAI-1 gene responsible for the effects of bFGF, transient transfections with several PAI-1 promoter-luciferase reporter constructs were performed in ECV cells. Higher basal promoter activity was observed in 1F and 2F, suggesting that the region between -747bp and -553bp may contain a repressor element. bFGF increased promoter driven luciferase activity by 67±16 %. Relative to the largest promoter fragment tested, bFGF effect was reduced with deletion of the region at -747 to -553bp and -553 to -313 bp. Deletion of the region at -313 to -260 bp completely abolished the bFGF effect. Deletion of the region at -260 to -205 bp resulted in no further reduction. These data indicate that the major sequence determinant of responsiveness resides between -313 to -260 bp. To determine whether a protein which can bind to Ets-1 like site was responsible for bFGF effect EMSA was performed using oligonucleotides corresponding to the PAI-1 Ets-1 like site. The oligonucleotide bound, besides a constitutive protein complex, a bFGF-inducible nuclear protein complex, suggesting that bFGF induced nuclear translocation and DNA binding of the Ets-1 like transcription factor to the PAI-1 promoter (Figure 7A). Addition of a 10-100-fold excess of cold probe inhibited DNA-protein complex formation in a dose-dependent manner. The addition of Ets-1/Ets-2 antibody inhibited DNA-protein complex formation. To further characterize the responsible region a mutant construct containing 2-nucleotide substitu-

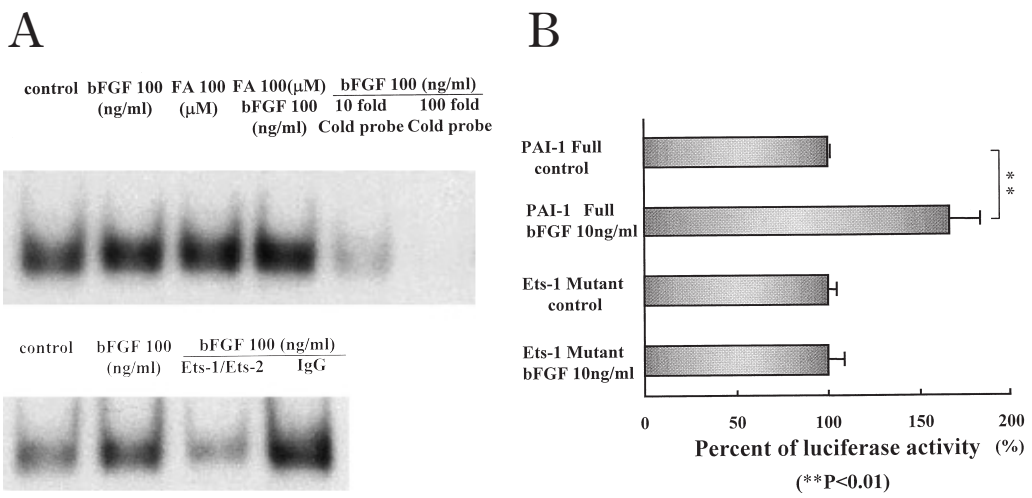

Figure 7 EMSA and mutational analysis of PAI-1 promoter. (A) EMSA of ECV cells stimulated with bFGF for 2 hr. Some cells were pretreated with fenofibric acid for 24 hr before bFGF stimulation. EMSA was performed using probes from Ets-1 like site of PAI-1 promoter and anti-human Ets-1/Ets-2 IgG or control rabbit IgG. The panel shows a representative autoradiograph from three separate experiments. (B) Effects of two point mutation in Ets-1 like site on PAI-1 promoter activity. Promoter activity was determined and values were reported as relative to the value for the PAI-1 full or Ets-1 mutant plasmid without bFGF stimulation. **$p<0.01$ compared to control. Figures quoted in part from reference 37.

tion in the Ets-1 like site was generated. Compared with the bFGF induced increased promoter activity in the wild-type substitution in the Ets-1 like site reduced bFGF stimulation (Figure 7B). These results suggest that Ets-1 like site is critical for bFGF effect.

Fenofibric acid diminished PAI-1 mRNA expression in ECV cells in a dose dependent manner as assessed by Northern blotting. Fenofibric acid markedly diminished but did not totally abolish accumulation of PAI-1 protein elaboration from ECV cells exposed to bFGF (Figure 8 right). When fenofibric acid was present in media of ECV cells transfected with the PAI-1 promoter-luciferase reporter construct, basal PAI-1 promoter activity and bFGF stimulated PAI-1 promoter activity were diminished (Figure 8 left). Thus, fenofibric acid inhibited PAI-1 expression at least partly at the level of transcription. Fenofibric acid did not inhibit bFGF induced nuclear translocation or DNA binding of Ets-1 like transcription factor to the PAI-1 promoter. Fenofbric acid decreased the baseline concentration of t-PA in the media by 84±08% and suppressed the increased induced by bFGF by 82±10%. Cell viability and total protein in the conditioned

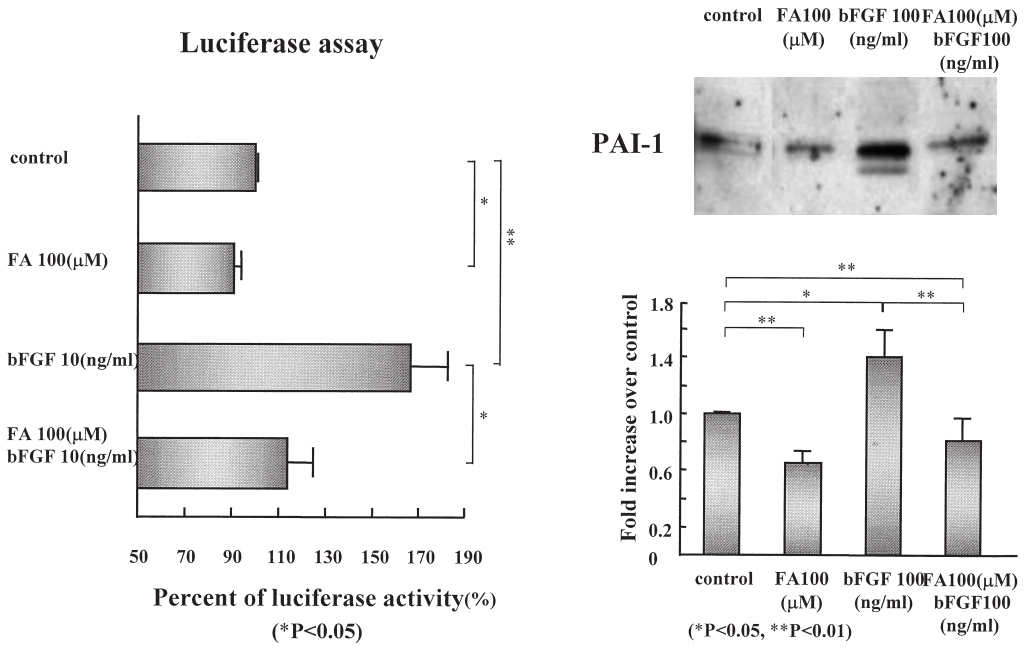

Figure 8 Effects of fenofibric acid on PAI-1 expression. (Right) Effects of fenofibric acid on the concentration of PAI-1 in conditioned medium of ECV cells. Confluent cells were serum starved for 24 hr, incubated with fresh serum free media containing fenofibric acid (0, 100 μM) for 24 hr, and exposed to bFGF (0, 100 ng/ml) for 24 hr. Conditioned media were harvested, and concentrations of PAI-1 were assayed by Western blot. Values are fold increase over control without bFGF. (Left) Effects of fenofibric acid (100 μM) on basal and bFGF inducible PAI-1 transcriptional activity. *p<0.05 and **p<0.01 compared to control. Figures quoted in part from reference 37.

media were unaffected by fenofibric acid at the concentrations used.

bFGF was shown to influence production of PAI-1 in ECs. A paradoxical decrease of PAI-1 activity with high concentrations of bFGF in media of HUVEC may reflect co-induction of plasminogen activators by bFGF. Increased PAI-1 protein correlated with increased mRNA and activity of the PAI-1 promoter. The induction of PAI-1 was inhibited by ERK kinase inhibitor, suggesting that ERK kinase dependent pathway is involved in the regulation of endothelial PAI-1 gene expression by bFGF. The results in this study extend our previous observation that ERK kinase pathway plays a critical role in mediating the response of PAI-1 to angiotensin II in smooth muscle cells[24] and offer a novel insight into the mechanism of PAI-1 gene response in two distinct vascular cell components. The concentrations of the inhibitors used were relevant in ECV cells[25]. Search for the putative regulatory sequence in the promoter revealed sterol regulatory element binding protein like site (-569 to -558 bp); activator protein (AP)-1 like site (-334 to -324 bp); Ets-1 like site (-296 to - 284 bp); and CCAAT/enhancer binding protein (C/EBP) like site (-224 to -210 bp). Deletion analysis of PAI-1 promoter suggested that a region between -313 bp and -260 bp was primarily mediating the bFGF response, showing that the Ets-1 like site may be involved in the bFGF inducible PAI-1 promoter activity. EMSA and mutational analysis of Ets-1 like site further suggested that this Ets-1 like sequence is responsible for the bFGF induced PAI-1 expression. Although antibody specific for Ets-1 did not inhibit DNA-protein complex formation, the addition of Ets-1/Ets-2 antibody raised against carboxy terminal domain highly conserved among Ets gene family and essential for DNA binding inhibited DNA-protein complex formation. These results suggest that other members of the Ets gene family may be at least partly responsible for mediating bFGF effects.

Control of proteolysis is important for angiogenesis. PAI-1 may diversely modulate angiogenesis. Low PAI-1 levels may result in excessive proteolysis, failure of cell adhesion, prevention of coordinated EC sprouting and inhibition of angiogenesis. Tumor cells fail to invade in PAI-1 knockout mice because of a lack of vascularization[26]. PAI-1 promoted angiogenesis by inhibition of proteolysis[27], suggesting that inhibition of excessive proteolysis by PAI-1 may contribute to neovascularization. Alternatively, inhibition of proteolysis can also inhibit angiogenesis due to the inhibition of fibrinolysis in the provisional matrix[28], which might affect EC migration. Ets-1 modulates angiogenic properties of ECs and growth factors that promote angiogenesis may also contribute to atherosclerotic plaque development[29]. Thus, proteolysis mediated by plasmin requires tight control during angiogenic processes and induction of PAI-1 by bFGF as shown in this study may contribute to this fine control of neovascularization.

Fenofibric acid diminished the increased accumulation of PAI-1 induced by exposure of cells to bFGF. Because basal accumulation of PAI-1 was affected also by fenofibric acid, constitutive synthesis was modified as well. Fenofibric acid

binds and activates PPARα and activation of PPARα negatively influences AP-1 dependent pathway. In this study deletion of the promoter region containing AP-1 like site inhibited bFGF effect and EMSA suggested that fenofibric acid did not inhibit nuclear translocation or DNA binding of Ets-1 like transcription factor to the PAI-1 promoter. Thus, inhibition of PAI-1 synthesis by fenofibric acid may involve AP-1 activation pathway.

The potential consequences of fenofibric acid action are of particular clinical interest. Use of fibric acid derivatives decreases the incidence of cardiac events in patients with coronary artery disease and low LDL levels despite inducing only a modest increase in HDL[30]. Although increased local fibrinolysis may induce degradation of extracellular matrix and destabilization of advanced plaques, reduced PAI-1 expression by ECs may shift the local balance to increased fibrinolytic capacity in blood that could limit the extent of acute thrombosis following plaque rupture. A shift in the local fibrinolytic balance toward increased fibrinolysis may decrease thrombotic risk, thereby contributing to clinical benefit seen with fibrates independent of their salutary effects on lipids.

Increased Expression of PAI-1 by Mediators of the Acute Phase Response: a Potential Progenitor of Vasculopathy in Hypertensives and the Promising Role of Statins

Hypertension is an important risk factor for coronary atherosclerosis, which is accelerated by inflammation and hypofibrinolysis. Circulating PAI-1 levels were increased in an animal model of atherogenic metabolic derangement[31]. Liver is one of the major sources of circulating PAI-1[32] and insulin stimulates PAI-1 synthesis in liver cells[16], suggesting that the insulin resistance and subsequent hyperinsulinemia typically seen in hypertensives may contribute to a prothrombotic risk state.

The secretion of cytokines specifically associated with blood vessels in hypertensives may play a role in the progression of atherosclerosis. Several proinflammatory cytokines including IL-1β are known to regulate the expression of the PAI-1 gene and the synthesis of PAI-1 protein in vascular cells and hematocytes. Because cytokines may predispose to the thromboembolic events and vasculopathy associated with hypertension, the influence of IL-1β and IL-6 on hepatic PAI-1 production *in vitro* in a cultured liver cell line and the cytokine and fibrinolytic profile in hypertensives *in vivo* were characterized.

IL-1β increased PAI-1 protein accumulation in a concentration-dependent fashion at 24 h. Peak effects were seen at an IL-1β concentration of 1 ng/ml (2.1 ± 0.2 fold over the control). Increased accumulation secondary to IL-1β was evident at 0.01 ng/ml, and induction was somewhat diminished at 3 ng/ml. In contrast to IL-1β, IL-6 increased PAI-1 protein accumulation only modestly. Peak effects were seen at a concentration of 1 ng/ml (1.4 ± 0.2 fold over the control). Increased accumulation secondary to IL-6 was evident at 0.1 ng/ml, and induction was somewhat decreased at 3 ng/ml. Combined treatment with IL-1β (1 ng/ml) and IL-6 (1 ng/ml) increased PAI-1 protein accumulation by 2.4 ± 0.2 fold over the control (Figure 9). Total protein content in the conditioned media was not altered by IL-1β or IL-6.

Mevastatin (10 μM) significantly decreased the IL-1β-, IL-6-, and IL-1β+IL-6-induced accumulation of PAI-1 protein into the conditioned media (Figure 9). Addition of mevalonic acid lactone (1 mM) was able to reverse the mevastatin-mediated reduction of PAI-1 accumulation. Mevalonic acid lactone alone did not noticeably modify PAI-1 accumulation.

An increase in PAI-1 mRNA expression was seen in HepG2 cells treated with IL-1β (1 ng/ml) and IL-6 (1ng/ml) as assessed by Northern blotting. Both the 3.2kb and 2.2kb products were increased. The combination of IL-1β (1 ng/ml) and IL-6 (1 ng/ml) also increased PAI-1 mRNA. Mevastatin (10 μM) decreased the PAI-1 mRNA expression induced by IL-1β, IL-6, and IL-1β+IL-6 significantly. Both the 3.2kb and 2.2kb products were decreased.

Systolic blood pressure levels of hypertensives involved and normotensives studied were 139 ± 20 mmHg and 118 ± 13 mmHg, respectively ($p<0.01$), and dias-

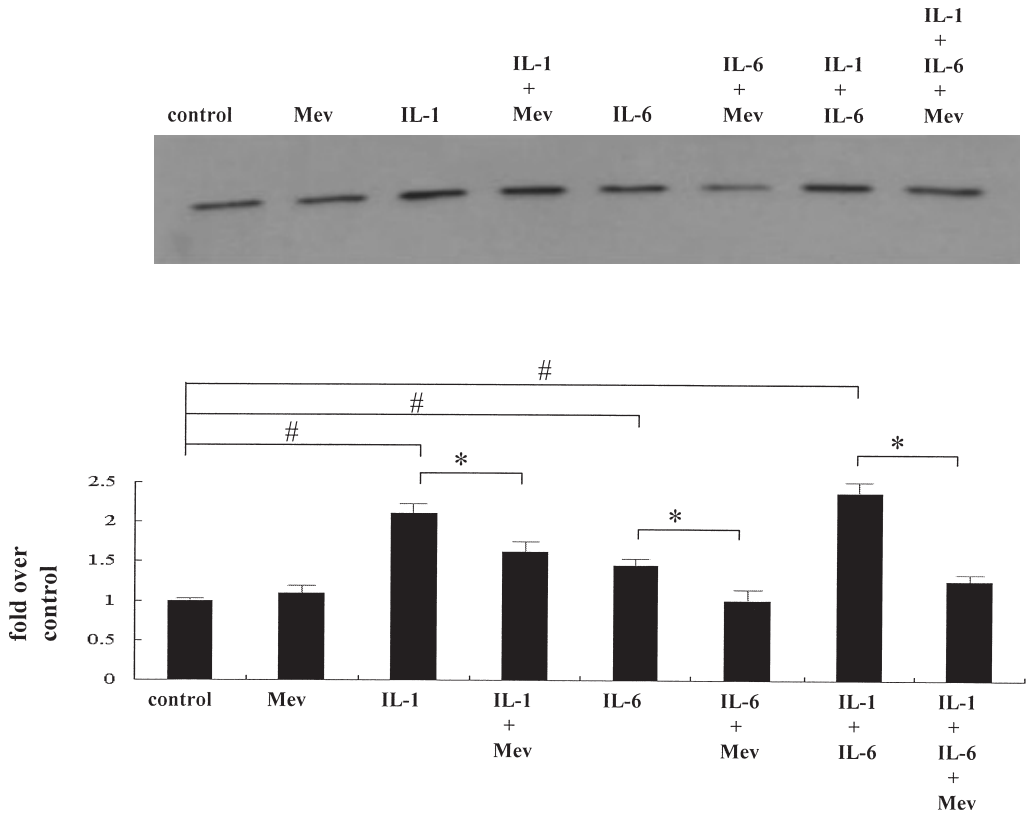

Figure 9 Effects of combination treatment with IL-1β, IL-6, and mevastatin on accumulation of PAI-1 in the conditioned medium of HepG2 cells. When cells became 80-90% confluent, they were serum starved for 24 h and then incubated with fresh serum-free media containing IL-1β (1 ng/ml), IL-6 (1 ng/ml), and mevastatin (Mev, 10 μM) for 24 h. Conditioned media were harvested, and accumulation of PAI-1 protein was assayed by Western blotting as described in the Methods section. Values are fold increase over the controls without agents. *$p<0.05$ between the two groups indicated; # $p<0.01$ compared with the values in untreated control cells. A representative blot from four separate experiments is shown in the upper panel. Figures quoted in part from reference 89.

tolic blood pressure levels of hypertensives and normotensives were 80 ± 14 mmHg and 71 ± 11 mmHg, respectively ($p<0.01$). The plasma PAI-1 activity level was higher in hypertensives than in normotensives (10.0 ± 9.8 AU/ml vs. 6.2 ± 4.5, $p<0.05$). The plasma PAI-1 antigen level was higher in hypertensives than in normotensives (30.9 ± 22.4 ng/ml vs. 24.4 ± 13.3, $p<0.05$). The plasma t-PA antigen level was also higher in hypertensives than in normotensives (10.4 ± 3.9 ng/ml vs. 8.7 ± 4.1, $p<0.05$), indicating that the endothelial damage continued despite the blood pressure reduction. The plasma IL-6 level was not significantly higher in hypertensives than in normotensives (1.7 ± 1.2 pg/ml vs. 1.5 ± 1.2). The plasma insulin level was higher in hypertensives than in normotensives (6.1 ± 2.9 mU/ml vs. 4.6 ± 1.7, $p<0.05$).

Both IL-1β and IL-6 contributed to the regulation of PAI-1 expression.

Although IL-6 alone had only a modest effect on PAI-1 production, in combination with IL-1β it caused a significant induction of PAI-1 protein production and mRNA expression. On the other hand, mevastatin significantly diminished the effects of IL-1β and IL-6. Supplementation with mevalonic acid lactone as exogenous mevalonic acid reversed the mevastatin-mediated reduction of PAI-1, suggesting that a metabolite(s) along the mevalonate pathway of the cholesterol synthesis was involved in the regulation of PAI-1.

HepG2 cells were used to determine whether changes in the level of PAI-1 are likely to occur in the liver under conditions of inflammatory response. The acute-phase response activated by intravascular or extravascular inflammation plays an important role in atherothrombosis, and the liver is a major source of acute-phase protein production[33]. The changes in the concentrations of acute-phase proteins, including PAI-1, are due largely to changes in their production in hepatocytes. IL-6 is the chief stimulator of production of most acute-phase proteins[34], but other other cytokines such as IL-1 also contribute to the regulation. PAI-1 production was increased by the proinflammatory cytokines IL-1β and IL-6 in HepG2 cells; this finding was consistent with those of previous studies.

HMG-CoA reductase inhibitors (statins) exert various beneficial clinical effects on coronary diseases beyond their effects on serum cholesterol levels[35]. Recent studies have focused on the pleiotropic effects of statins, and especially on inflammation and the fibrinolytic system[36]. Although the effects of statins on PAI-1 in vascular cells and hematocytes have been studied previously, it remains unclear whether statins affect the hepatic PAI-1 expression under stimulation by proinflammatory cytokines. Mevastatin decreased the PAI-1 production induced by IL-1β and IL-6 in HepG2 cells. The decrease in PAI-1 antigen was correlated with a reduction in PAI-1 mRNA levels. Mevastatin may exert anti-thrombotic effects by decreasing PAI-1 expression in the liver under proinflammatory conditions. Because basal accumulations of PAI-1 were not affected by mevastatin, constitutive synthesis appears to be regulated independently. It is known that statins exert therapeutic effects beyond that of simply lowering plasma cholesterol. PAI-1 may become a major target of statin therapy.

Previousy IL-1 was shown to up-regulate PAI-1 expression via an 805 bp promoter, protein binding to the promoter element was not induced by IL-1 and the identity of the protein was not fully confirmed. Unlike lymphocyte and colony-stimulating growth factors, IL-1β affects diverse cell types often in concert with other cytokines or small mediator molecules. Peripheral blood monocytes, one of the major sources of circulating cytokines, may be preactivated in hypertensives. PAI-1 levels are increased in an animal model of atherogenic metabolic derangement[31]. Thus, it is likely that PAI-1 synthesis in the liver can occur in vivo in the presence of high blood pressure, and can potentially contribute to the high concentrations of PAI-1 in circulation.

The level of t-PA antigen, a sensitive index of endothelial damage, was in-

creased in our cohort of Japanese hypertensives, reflecting endothelial damage in our study population. The plasma levels of both PAI-1 activity and PAI-1 antigen were also elevated in our hypertensive patients. PAI-1 levels may be regulated by a diverse range of mechanisms[37]. High prevalence and incidence of diabetes is readily recognized in Japan, and hyperinsulinemia and diabetes are predictors of the development of hypertension. Because insulin can stimulate PAI-1 synthesis in the liver[16], the increased insulin levels observed in hypertensives may have contributed to the increase in plasma PAI-1. Conversely, because the angiotensin converting enzyme inhibitor can attenuate plasma PAI-1 levels, the use of the angiotensin converting enzyme inhibitor in our study may have attenuated the increase in PAI-1 levels in hypertensives. Angiotensin and statin can modulate inflammation[38]. This may have been the reason for the only modest increase of plasma IL-6 levels in hypertensives receiving the angiotensin converting enzyme inhibitor and statin in our study.

There is a close link between diminished fibrinolysis and atherogenic metabolic derangement[39]. Inadequate activation of endogenous fibrinolysis may contribute to microcirculatory dysfunction by inducing fibrin deposition and formation of microthrombi in coronary circulation. Thus, the poor cardiovascular prognosis associated with hypertension may be a reflection, in part, of microvascular damage. Statins may be effective for ameliorating hypofibrinolysis related to high blood pressure and moderate inflammation, and that statins may suppress the enhancement of PAI-1 and its potentially adverse consequences.

Regulation of PAI-1 in Adipocytes: In Search of Missing Link Between Obesity and Thrombosis and the Role of Adipocytokine

Adipocytes abundantly produce tumor necrosis factor (TNF)-α, which is considered to produce insulin resistance. Elevated concentrations of PAI-1 are seen with insulin resistant states including type 2 diabetes and obesity. They are associated with an increased risk of thromboembolic events. Adipocytes secrete PAI-1 and appear to contribute to the elevated PAI-1 in blood seen under these conditions[1), 40)-43)]. Therefore, the effect of TNF on adipocyte PAI-1 production was investigated.

Proinflammatory cytokine TNF increased PAI-1 activity in the conditioned media as previously described[1)]. TNF also increased PAI-1 protein accumulation (Figure 10). Accumulation was evident in 6 hours with further increases at 24 hour. TNF did not alter accumulation of t-PA or u-PA. Thus, the net effect of TNF was to increase PAI-1 activity and accumulation. Total protein content was not altered by TNF. Cycloheximide (25 μg/ml) inhibited TNF induced PAI-1 accumulation by 96$\pm$10%.

Hydroxyl radical scavenger TMTU (20 mM) almost completely inhibited the increase in PAI-1 activity and the PAI-1 protein accumulation in TNF stimulated cells at 24h (Figure 10). Equimolar urea as a control had no effect on changes in PAI-1 activity or protein accumulation induced by TNF. In contrast to TMTU, DMSO only partially inhibited TNF induced increase in PAI-1 activity or PAI-1 accumulation. When the cells were incubated with hydrogen peroxide (100 μM) or a superoxide generating system by xanthine oxidase (10 mU/ml) plus hypoxanthine (0.6 mM), PAI-1 activity increased and PAI-1 accumulation also increased (Figure 11). Chronic exposure of adipocytes to low concentrations of TNF (1 ng/ml) over 5 days under the presence of insulin (20 nM) also increased PAI-1 production (2.1$\pm$0.5 fold). No increase in PAI-1 activity was observed, presumably due to spontaneous inactivation of PAI-1 in prolonged culture.

TNF modulated production of proteins related to fibrinolysis in adipocytes as with endothelial cells, smooth muscle cells, HepG2 hepatoma cells and human mesangial cells. This effect was in contrast to the effect of TNF on PAI-1 expression in chondrocytes and synovial fibroblasts, in which TNF increased fibrinolytic capacity by decreasing PAI-1 expression. TNF induced PAI-1 expression was inhibited by cycloheximide, suggesting that PAI-1 increase induced by TNF requires protein synthesis.

The regulatory effects of TNF on PAI-1 protein were both time and dose dependent. Maximum effect was achieved at 5 ng/ml. The time course of TNF mediated changes in PAI-1 was slow. A large lag time of several hours after the start of TNF stimulation was observed before stimulation of PAI-1 proteins became apparent, suggesting the role of autocrine factor, which may induce gene expression.

The second messengers involved in the signal transduction pathways leading to

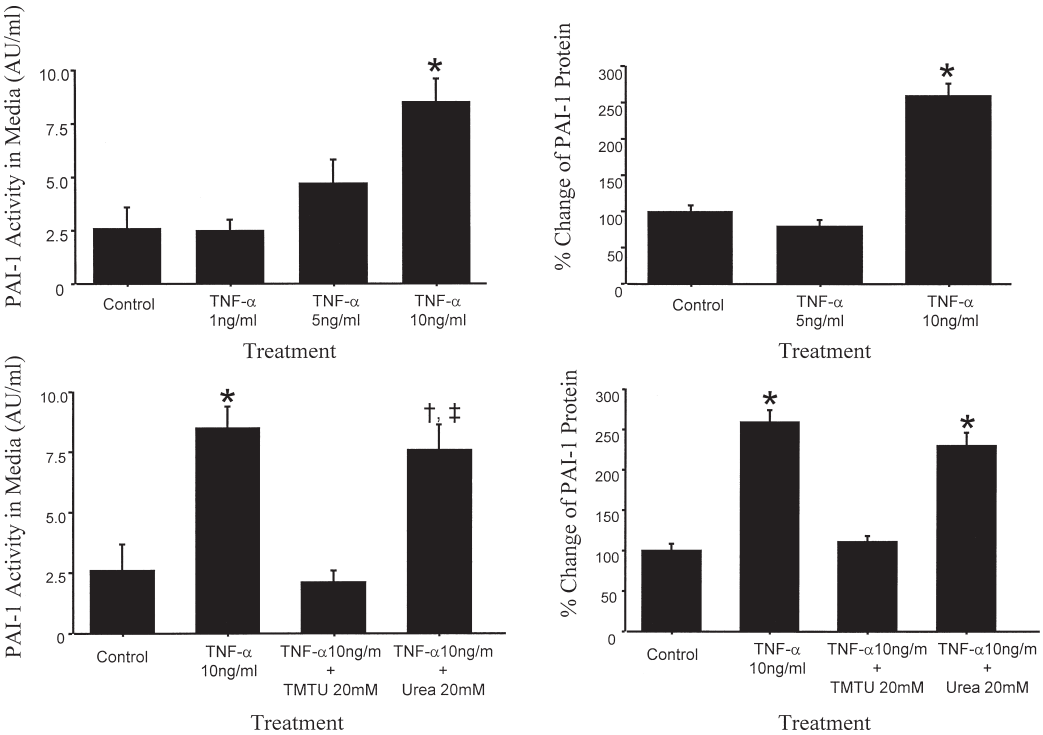

Figure 10 Upper left: Effects of TNF on activity of PAI-1 in conditioned media. Confluent cells were incubated in fresh media for overnight and then incubated with fresh media containing TNF (0 to 10 ng/ml) for 24 hours. Conditioned media were harvested and activity of PAI-1 assayed. Upper right: Accumulation of PAI-1 protein by adipose cells. Confluent cells were incubated in fresh media for overnight and then incubated with fresh media containing TNF (0 to 10 ng/ml) for 24 hours. Conditioned media were harvested, and concentrations of PAI-1 assayed by Western blot. Values are fold increase over control without TNF. Lower left: Effects of TMTU and urea on activity of PAI-1 in conditioned media of adipose cells induced by TNF (10 ng/ml). Confluent cells were incubated in fresh media for overnight and then incubated with fresh media containing TNF for 24 hours. Conditioned media were harvested and activity of PAI-1 assayed. Lower right: Effects of TMTU and urea on concentration of PAI-1 induced by TNF (10 ng/ml). Confluent cells were incubated in fresh media for overnight and then incubated with fresh media containing TNF for 24 hours. Conditioned media were harvested and concentrations of PAI-1 assayed by Western blot are expressed as fold increase over control without TNF. *$p<0.01$ compared with control. †$p<0.05$ compared with control. ‡$p<0.01$ compared with 10 ng/ml TNF + 20 mM TMTU. Figures quoted in part from reference 47.

induction of PAI-1 remain incompletely understood. Reactive oxygen intermediates are agents commonly produced by inflammatory cells during inflammatory process. In glomerulonephritis glomerular cells can generate reactive oxygen intermediates, independently of infiltrating cells, which may serve as an autacoid

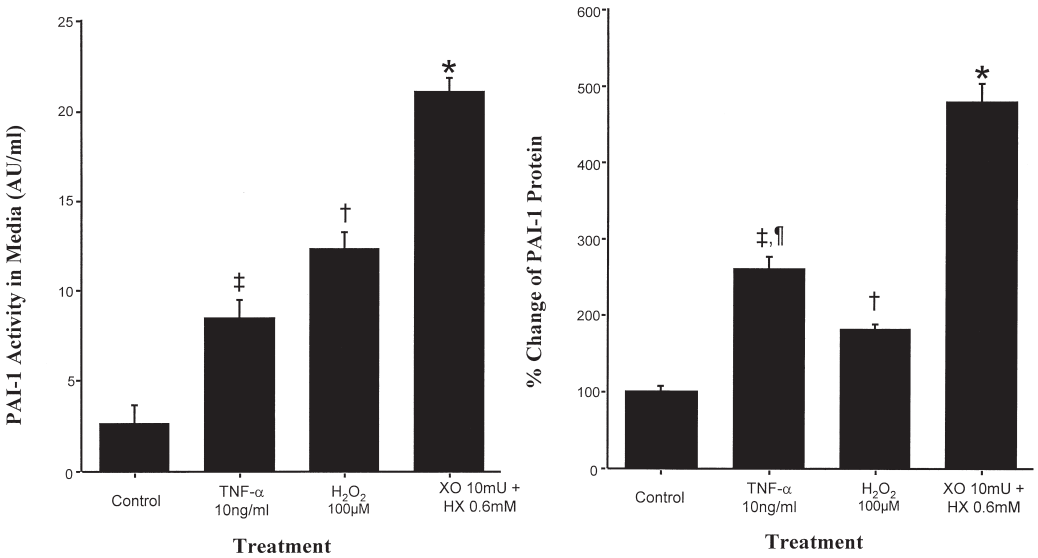

Figure 11 Left: Effects of hydrogen peroxide (H_2O_2), and xanthine oxidase plus hypoxatnine (XO/HX) on activities of PAI-1. Right: Effects of hydrogen peroxide (H_2O_2), and xantine oxidase plus hypoxanthine (XO/HX) on concentrations of PAI-1. Values are fold increase over control without TNF. *$p<0.001$ compared with control, 10ng/ml TNF or 100 μM H_2O_2. †$p<0.05$ compared with control. ‡$p<0.01$ compared with control. ¶ $p<0.05$ compared with 100 μMH_2O_2. Figures quoted in part from reference 47.

role in glomerular injury. TNF can induce both superoxide and hydrogen peroxide production. To determine whether reactive oxygen intermediates are involved in the expression of PAI-1 induced by TNF, the effects of radical scavengers TMTU and other antioxidants were compared. The hydroxyl radical scavenger TMTU, and to a lesser extent DMSO, inhibited the TNF induced PAI-1 expression. To determine whether reactive oxygen intermediates could directly induce PAI-1 expression, cells were incubated with hydrogen peroxide or a superoxide generating system. In many cell types NADPH oxidase is involved in the generation of reactive oxygen species. However, endothelial cells and fibroblasts lack NADPH oxidase system, but can still produce reactive oxygen species on exposure to proinflammatory cytokines. The expression of additional non-NADPH oxidase dependent reactive oxygen intermediates production by adipocytes. TNF may influence the alterations in fibrinolysis associated with activation of immunocompetent cells, inflammation, or both.

Fibrinolytic potential of adipocytes can be regulated by TNF, which may be increased in human obesity and insulin resistance. Plasma PAI-1 levels are increased in these populations. Elevated levels of PAI-1 expression suggest limited plasminogen activator activity. This in turn may promote or stabilize fibrin deposits upon vascular injury or in the area of vasculitis.

Adipocyte was used to establish a model to study comparative regulation of

fibrinolytic proteins. However, there are certain limitations that have to be considered when using in vitro model to gain insight into very complicated physiologic system in vivo. One of the limitations of this model is that cells are constantly exposed to high levels of cytokines during the experiments. Adipocytes are a useful tool to study the cytokine regulating of the production of fibrinolytic proteins. Detailed studies have to be performed to establish the specific cytokine effects on the underlying processes, such as transcription, translation, cellular processing and transport and secretion. The potential efficacy of antioxidants in ameliorating inflammatory diseases not only inhibit their direct effects but also aid the suppression of PAI-1 when desirable such as vasculopathy.

Intracellular Signal Transduction Modulating Expression of PAI-1 in Adipocytes

Adipocytes stimulated with TGF-β elaborate PAI-1[32]. Small GTP-binding and GTPase proteins such as Rho family members have been shown to down-regulate TGF-β expression in chicken embryonic heart cells. This pathway is involved in diverse processes in a myriad of cell types[44] including cytoskeletal reorganization and transcriptional activation. Its potential influence on PAI-1 expression is controversial. Rho-mediated responses depend on the availability of geranylgeranylated Rho and its activation by GTP loading. In cultured heart cells HMGCoA reductase inhibitors up-regulate TGF-β signaling and PAI-1 expression[45]. Small G protein Rho-associated coiled-coil forming protein kinase (rho-kinase), an effector of activated Rho, inhibits myosin light chain phosphatase, and alters the actin cytoskeleton[46]. Because of its effects on TGF-β signaling in embryonic chicken heart cells, we studied effects of inhibition of rho-kinase on expression of PAI-1 in adipocytes and characterized potential molecular mechanisms involved[47].

Inhibition of rho-kinase by Y-27632 increased PAI-1 mRNA in a concentration-dependent manner (113$\pm$28% over control at 1 μM, 124$\pm$29% at 10 μM and 190$\pm$35% at 30 μM, respectively). By contrast, the PAI-1 signal at baseline was faint in preadipocytes, and Y-27632 (30 μM) did not affect PAI-1 mRNA expression in these cells.

The mechanism responsible for the increase in PAI-1 expression induced by Y-27632 was evaluated. Pretreatment of adipocytes with a tyrosine kinase inhibitor (genistein, 100 μM) attenuated the increase of PAI-1 mRNA expression induced by 30 μM of Y-27632 (72$\pm$26% of control) (Figure 12). By contrast, the protein kinase C inhibitor (GF109203X, 2 μM) and the MAP kinase kinase inhibitor (PD98059, 30 μM) did not attenuate the increased PAI-1 expression induced by inhibition of rho-kinase by Y-27632 (30 μM). These inhibitors had no effect on basal PAI-1 expression. Thus genistein appeared to inhibit signaling pathway unmasked by effects of inhibition of rho-kinase. The p38 MAP kinase inhibitor (SB202190, 4 μM) exerted no effect on the increased PAI-1 mRNA expression induced by inhibition of rho-kinase with Y-27632 (30 μM).

Because genistein is not only a tyrosine kinase inhibitor but also a flavonoid[48], the effects of other tyrosine kinase inhibitors and those of a flavonoid were investigated. Use of other tyrosine kinase inhibitors, herbimycin A (1 μM) and tyrphostin 23 (100 μM), both non-flavonoid tyrosine kinase inhibitors, did not attenuate PAI-1 induction induced by Y-27632 (30 μM), suggesting that genistein exerted the inhibitory effect through a non-tyrosine kinase mechanism. In keeping with this possibility daidzein (100 μM), another flavonoid with only weak tyrosine kinase inhibition, decreased basal PAI-1 expression and attenuated PAI-1 expression induced by Y-27632 (66$\pm$43% and 88$\pm$25% compared with control, respectively). Accumulation of PAI-1 protein in conditioned media of adipocytes was increased by Y-27632 (30 μM) as assessed by Western blotting (258$\pm$86% over control at 30 μM).

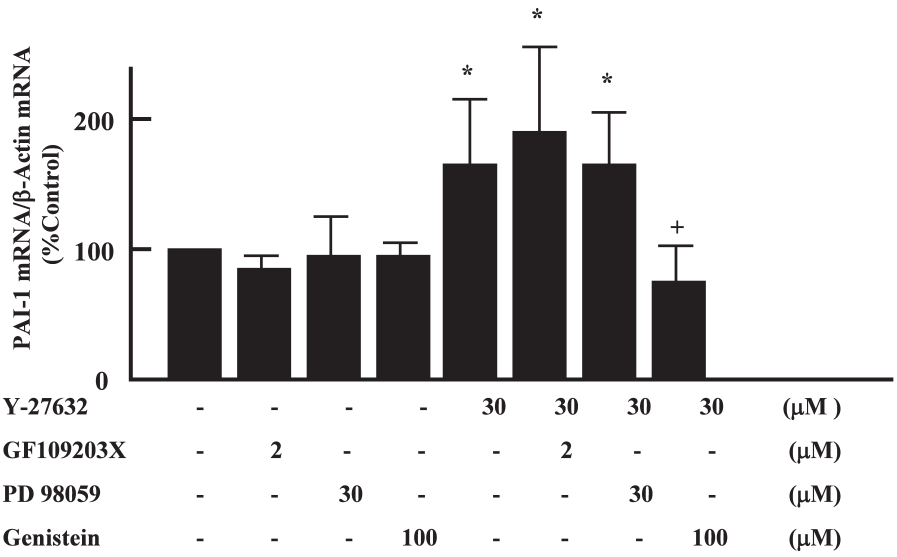

Figure 12 A bar graph showing that pretreatment of adipocytes with 100 μM genistein attenuated the increased PAI-1 mRNA expression induced by inhibition of rho-kinase with 30 μM of Y-27632 (72±26% of control). By contrast, 2 μM GF109203X and 30 μM PD98059 did not decrease PAI-1 expression induced by inhibition of rho-kinase with 30 μM of Y-27632. The data are % stimulation in three independently performed experiments. *$P<0.05$ compared with control; $^{+}P<0.05$ compared with Y27632 30 μM. Figure quoted in part from reference 86.

Genistein (100 μM) completely inhibited the increase of PAI-1 induced by Y-27632 (109±37% of control).

Adipose tissue is a probable source of PAI-1 in blood of patients with insulin resistant states including type 2 diabetes and obesity. In this study PAI-1 expression was increased in adipocytes compared with that in preadipocytes. The results are compatible with the concept that adipocytes are one of the major cell types responsible for PAI-1 secretion into blood. Inhibition of rho-kinase by Y-27632 increased PAI-1 mRNA expression in a dose-dependent manner. In human fat tissue preadipocytes and stromal cells may also contribute to PAI-1 production[49].

TGF-β is a potent inducer of PAI-1 in human adipocytes[50] and cultured heart cells. HMGCoA reductase inhibitors up-regulate both TGF-β signaling and PAI-1 expression through inhibition of geranylgeranylation of a rho-family member, RhoA GTPase[45]. Thus, it is likely that Y-27632 could increase PAI-1 expression by up-regulating TGF-β signaling in adipocytes, or more specifically de-repressing rho pathway-dependent suppression of TGF-β expression although adipocyte TGF-β expression was not determined in this study. Because angiotensin II-induced PAI-1 gene expression is inhibited by Y-27632 in heart in vivo and in cultured smooth muscle cells in vitro and because rho-kinase inhibitors suppress PAI-1 synthesis in

monocytes, the effects of Y-27632 may be cell or organ specific and dependent upon the agonists used[51].

The tyrosine kinases, protein kinase C and MAP kinase may influence PAI-1 expression in mesangial cells and HepG2 cells. In adipocytes, pretreatment with genistein attenuated the increase of PAI-1 mRNA expression induced by Y-27632. By contrast, the protein kinase C inhibitor (GF109203X), the mitogen-activated protein kinase kinase inhibitor (PD98059), and the p38 mitogen-activated protein kinase inhibitor (SB202190) exerted no effects on the increased PAI-1 mRNA expression induced by inhibition of rho-kinase. Therefore, it is likely that a genistein sensitive mechanism we identified is involved in the cell signaling after inhibition of rho-kinase with Y-27632. Inhibitors were used at the concentrations previously used in adipocytes. However, higher concentrations may result in different responses.

Genistein reduces induction of PAI-1 by TNF-α, bFGF, insulin and IGF-1[51]. Yet other tyrosine kinase inhibitors, herbimycinA and tyrphostin 23, did not reduce induction of PAI-1 in the present study. These results are consistent with the likelihood that genistein was not acting as a tyrosine kinase inhibitor but rather exerted its effects via a different mechanism. Genistein is not only a tyrosine kinase inhibitor but also a flavonoid. It exerts diverse effects in adipocytes such as inhibition of mitotic clonal expansion, triglyceride accumulation and peroxisome proliferators-activated receptor-γ. Because we found that another flavonoid also decreases PAI-1 expression, it appears that flavonoids themselves can inhibit PAI-1 expression in adipocytes. Flavonoids are polyphenolic compounds that exist widely in plants, inhibit the proliferation of tumor and nontumor cells in culture, induce apoptosis, exert estrogenic and antiestrogenic effects and function as antioxidants. Flavonoids alter the activity of a number of intracellular enzymes, including tyrosine kinases. Primarily because of their antiproliferative effects, flavonoids have been a focus of active exploration seeking to identify anti-cancer agents. Genistein, a soy isoflavone, inhibits proliferation of a number of cancer cell lines and commonly induces differentiation. In this regard it is of interest that flavonoids can function as antioxidants and that oxidative stress can induce PAI-1 in adipocytes as we previously reported[47]. It is not clear at the mechanistic level how the flavonoids regulate Rho/rho-kinase system. It is of interest that activation of a rho-like small G protein is related to oxygen deprivation tolerance in plants[52].

Flavonoids may provide a means for down-regulating PAI-1 expression thereby diminishing cardiovascular risk in conditions such as obesity and insulin resistant states in general. The effect they exert on expression of PAI-1 in adipocytes appears to reflect an impact on the rho-kinase pathway, perhaps through activating the pathway and thereby suppressing expression of TGF-β and its induction of PAI-1. Modulation of activity of the Rho/rho-kinase pathway and its effect on PAI-1 gene expression by a genistein-sensitive mechanism in adipocytes suggests

that pharmacological interventions with flavonoids may be beneficial in decreasing PAI-1 expression and reducing thrombotic events in patients with diverse insulin resistant states including type 2 diabetes mellitus and obesity.

Angiotensin Blockade Improves Hypofibrinolysis and Cardiac Complications in Genetically Obese Mice: Clinical Implications for Diabetic Cardiovascular Diseases

Accelerated atherosclerosis in coronary arteries is a major determinant of prognosis in diabetic patients[53]. Pathogenesis of atherosclerosis in these patients involves multiple risk factors such as impaired glucose tolerance, hyperlipidemia, obesity and hypertension. In addition impairment of coronary microcirculation is frequently accompanied by insulin resistance. Retinopathy may predict impairment of coronary microcirculation. Histologically perivascular fibrosis around arterioles is frequently found in myocardial biopsy specimens in patients who exhibit chest pain, ischemic changes in electrocardiogram and nuclear imaging on exercise, and no significant stenosis or spasm in coronary angiogram[54]. These results suggest that in non-insulin dependent diabetic patients development of insulin resistance may be temporally and pathogenically closely correlated with abnormalities of coronary microcirculation and development of diabetic cardiovascular diseases.

Patients with diabetes mellitus exhibit abnormalities of hemostatic system. Diabetic patients exhibit high PAI-1[55] and tissue factor (TF), an important initiator of extrinsic coagulation system. In addition to regulation of hemostasis in circulation, coagulation and fibrinolytic system can play a critical role in vascular remodeling. Vascular remodeling is a complex process involving both the migration of vascular cells and matrix accumulation, and fibrin provides a provisional scaffold for cell migration. The interaction of cell surface proteolytic activity mediated by plasmin generation and extracellular matrix regulates cell migration and matrix turnover[37]. These results suggest that coagulation and fibrinolysis can play a major role in coronary micorvascular remodeling in diabetic cardiovascular diseases.

The alterations of fibrinolysis and coagulation were characfgerized and their relations to coronary microvascular remodeling of arterioles in genetically obese mice (ob/ob) which develop insulin resistance and mild non-insulin dependent diabetes mellitus[56] were elucidated. Tissue angiotensin modulates production of PAI-1. In HEART study angiotensin converting enzyme inhibitor (ACEI) decreased plasma PAI-1 antigen and activity in patients with acute myocardial infarction[57]. Therefore, it was also sought to determine whether ACEI has potential beneficial effects on hemostatic system and coronary microvascular remodeling in this obese animal model.

At 10 weeks of age obese mice exhibited more than 2 fold increase in body weight. This difference persisted at 20 weeks. Body weight of obese mice treated with ACEI, temocapril, was not significantly different from untreated obese mice. Significant increase in heart weight in obese mice was observed at 10 weeks and at 20 weeks. Left ventricular weight was also increased significantly in obese mice at 10 weeks and at 20 weeks. Heart weight and left ventricular weight of obese mice

treated with temocapril from 10 to 20 weeks were significantly decreased as compared to untreated obese mice.

Increase in plasma glucose level was detected in obese mice at 10 weeks (231±15 mg/dl vs 103±7 in control, $p<0.01$) and at 20 weeks (242±16 vs 116±9, $p<0.01$). Immunoreactive insulin level was also elevated in obese mice at 10 weeks (2.98±0.49 ng/ml vs 0.56±0.19, $p<0.01$) and at 20 weeks (3.03±0.36 vs 1.02±0.35, $p<0.01$). Temocapril did not affect the plasma glucose or insulin concentrations in obese mice. PAI-1 activity measured at 10 weeks was higher in plasma from obese mice (5.5±3.2 AU/ml) than control (2.0±0.9, $p<0.05$). This difference persisted further at 20 weeks (3.5±1.9 vs 2.0±0.3, $p<0.05$), suggesting that obesity increases plasma PAI-1 activity. Temocapril significantly prevented the increase in PAI-1 activity in obese mice at 20 weeks (1.2±0.6, $p<0.05$).

On Elastica-Masson's trichrome staining strong fibrosis was noted around coronary arterioles and small arteries in obese mice at 10 and 30 weeks (Color Fig. 11). Obese mice exhibited significant increase in fibrosis to lumen ratio and fibrosis to wall ratio at 10 weeks and at 20 weeks compared to control groups. The fibrosis to lumen ratio and fibrosis to wall ratio of obese mice treated with temocapril from 10 to 20 weeks were significantly smaller than those of untreated obese mice and were similar to those of control mice.

Although only faint immunoreactivity for PAI-1 and TF were detected in microvessels of control mice, strong immunoreactivity for PAI-1 and TF was revealed in endothelium in obese mice. No immunoreactivity was detected in specimens using control isotype IgG. Immunoreactivity for PAI-1 and TF was reduced in the specimens from obese mice treated with temocapril.

Expressions of PAI-1 and TF mRNA were examined in heart tissues from obese and lean mice. At 10 weeks expression of PAI-1 mRNA was markedly increased in obese mice. Expression of TF mRNA was moderately increased as well in obese mice. Expressions of PAI-1 and TF mRNA became more intensified at 20 weeks from obese mice than that in lean counterpart. Treatment with temocapril significantly prevented the increase in PAI-1 mRNA expression in obese mice. Temocapril moderately reduced TF expression in obese mice as well. Expressions of β-actin mRNA was not altered. On in situ hybridization PAI-1 mRNA signals were noted in the medial cells (Color Fig. 12).

Genetically obese mice due to defects in leptin develop obesity, insulin resistance, hyperinsulinemia, high glucose and mild non-insulin dependent diabetes mellitus[56]. In obese mice plasma PAI-activity was increased as compared to control mice consistent with previous observations. On immunohistochemical staining strong immunoreactivity for PAI-1 and TF was observed in heart tissues of obese mice compared to control lean mice. On RT-PCR mRNA levels of PAI-1 and TF in heart tissues were increased in obese mice. These results suggest that diminished fibrinolysis and increased coagulation are present in heart tissues at early stages in obesity. Work done in several laboratories implicates close associ-

ation between diabetes and alterations of hemostatic system. In diabetic patients fibrinolytic activity is diminished[39] and hyperinsulinemia increases plasma activity of PAI-1 in vivo[3), 58]. Oxidative stress is increased in diabetes, and oxygen-centered free radicals increase PAI-1 production in coronary microvascular endothelial cells[2]. TNF abundantly present in adipose tissue in diabetes increases PAI-1 production in adipose cells[47]. Taken together, these results imply that fundamental abnormalities in hemostatic system may exist in cardiac tissues during obesity and insulin resistance before the development of overt diabetes mellitus.

Obese mice exhibited increased perivascular fibrosis. Alterations in fibrinolysis and coagulation played a role during this process. In these mice increased expression of PAI-1 may diminish plasmin generation, reduce proteolysis and contribute to accumulation of extracellular matrix. In addition increased TF expression may induce procoagulant activity and provide fibrin as provisional matrix for cell migration. It has been reported that diabetic patients develop less coronary collateral circulation, and angiogenesis in diabetes may not be beneficial in this population[59]. In a preliminary study we investigated the capillary profiles in these obese mice using alkaline phosphates staining of arteriolar capillaries[60] and endothelium staining using lycopersicon esculentum (tomato) lectin. There was only a mild increase in arteriolar capillaries and total capillary density in obese mice, but no effect was seen with temocapril.

Angiotensin regulates fibrinolytic activity[57]. Angiotensin II inhibits insulin signals. In this study increase in cardiac PAI-1 and TF expression, and microvascular remodeling observed in the insulin resistance and early diabetes was prevented by inhibition of ACE activity, suggesting that modulation of angiotensin system may be beneficial in preventing the microvascular remodeling in obesity. Angiotensin II and its hexapeptide angiotensin IV stimulate PAI-1 synthesis in endothelial and smooth muscle cells. Agiotensin II induces TGF-β[61], which induces perivsacular fibrosis. Angiotensin II and TGF-β stimulate TF expression. Therefore, it is likely that inhibition of ACE activity may improve perivascular fibrosis in this obese mouse through reduced expression of PAI-1 and TF. Decreased perivascular fibrosis in obese mice by ACEI may be beneficial for coronary microcirculation.

Obese mice exhibit coronary microvascular remodeling as well as alterations in cardiac PAI-1 and TF expression. Inhibition of ACE activity prevented these alterations. These observations may have important implications for prevention and therapy of diabetic cardiovascular diseases.

Coronary Capillary Remodeling in Non-insulin-dependent Diabetic Rats: Amelioration by Inhibition of Angiotensin Converting Enzyme and Future Promises

The prevalence of hypertension is higher in patients with diabetes compared with nondiabetic patients. Non-insulin-dependent (type II) diabetes mellitus (NIDDM) and hypertension are closely associated. The coexistence of hypertension and diabetes are clinically important because they constitute multiple risk factors for macrovascular and microvascular diseases. Diabetes and insulin resistance are major risk factors in the pathogenesis of atherosclerotic cardiovascular diseases, and clinical and experimental investigations suggest that renin angiotensin system is important for the development of various cardiovascular diseases including hypertension. Diabetes is independently associated with left ventricular mass and angiotensin II and insulin have been suggested to be additive stimuli to left ventricular hypertrophy. ACEI can significantly retard the progression of diabetic renal disease, but the effects of ACEI on diabetic cardiovascular complications are not fully clarified. OLETF rats are an established model of human NIDDM[62)-63)], which exhibit hypertension, obesity, hyperglycemia and hyperlipidemia. Cardiac lesions in OLETF rats with advanced age have been previously characterized, indicating that OLETF rats are a useful model to study the pathogenesis of cardiac complications in NIDDM. The effects of an ACEI on cardiac complications of early stages of NIDDM using OLETF rats was investigated.

Body weight of vehicle treated OLETF rats at 20 weeks was significantly greater than that in the LETO rats of the same age (557 ± 36 g vs 432 ± 30, $p<0.05$). Body weight of ACEI, cilazapril, treated group (468 ± 13) was not significantly different from that of LETO rats. Heart weight of OLETF rats was greater than that of LETO rats (1284 ± 78 mg vs 1083 ± 261), and was significantly decreased by treatment with cilazapril (1006 ± 18, $p<0.05$). As body weight of OLETF rats was substantially greater than that of LETO rats, heart weight to body weight ratio of OLETF rats tended to be lower than that of LETO rats (0.23 ± 0.01 % vs 0.28 ± 0.06) and was significantly decreased by treatment with cilazapril (0.22 ± 0.01, $p<0.05$ vs LETO). Blood pressure of OLETF rats was significantly higher than that of LETO rats (95 ± 4 mmHg vs 81 ± 2, $p<0.05$). Cilazapril lowered blood pressure of OLETF rats to a significant extent (59 ± 7, $p<0.05$).

Plasma glucose level of vehicle treated OLETF rats was significantly greater than that in the LETO rats of the same age (152 ± 4 mg/dl vs 126 ± 6, $p<0.05$), and was not significantly different from that of the cilazapril treated OLETF rats (146 ± 10). Plasma insulin level of OLETF rats was greater than that of LETO rats (1.37 ± 0.38 ng/ml vs 1.26 ± 0.25) and was significantly decreased by treatment with cilazapril (0.87 ± 0.22, $p<0.05$). HOMA equivalent values were significantly increased in OLETF rats compared to the values in LETO rats (213 ± 63 vs 162 ± 37, $p<0.01$) and was significantly decreased by treatment with cilazapril (132 ± 46, $p<0.01$). Plasma cholesterol level of OLETF rats was significantly higher than that of

LETO rats (84±9 mg/dl vs 66±11, $p<0.05$). Cilazapril treatment did not lower cholesterol of OLETF rats (94±12). Plasma triglyceride of 20 week OLETF rats was significantly higher than that in LETO rats (146±34 mg/dl vs 36±6, $p<0.05$). Cilazapril significantly lowered triglyceride level of OLETF rats (44±7, $p<0.05$).

Using double staining technique remodeling of capillary network was quantitated at 20 weeks. Arteriolar capillary portion containing AP was stained blue and venular capillary portion containing DPPIV was stained red. Intermediate capillary portion was stained violet. The total capillary density was significantly increased in OLETF rats compared with control LETO rats (Figure 13). In OLETF rats the number of venular capillary portion shown as red was significantly decreased compared with the value in LETO rats. In contrast the number of intermediate capillary portion shown as violet was significantly increased and arteriolar capillary portion shown as blue was also significantly increased. Treatment with cilazapril in OLETF rats significantly prevented the increase in total capillary density, and ameliorated the decrease in venular capillary proportion and increase in intermediate capillary proportion and arteriolar capillary proportion. Capillary domain area was significantly decreased in OLETF rats compared to the values in LETO rats (374±22 μm^2 vs 490±65, $p<0.01$). Treatment with cilazapril ameliorated the decrease of capillary domain area in OLETF rats (430±42). There was no difference in the C:M ratio between OLETF and LETO rats (1.08±0.13 vs 1.18±0.12). Cilazapril did not alter the C:M ratio of OLETF rats (1.00±0.07).

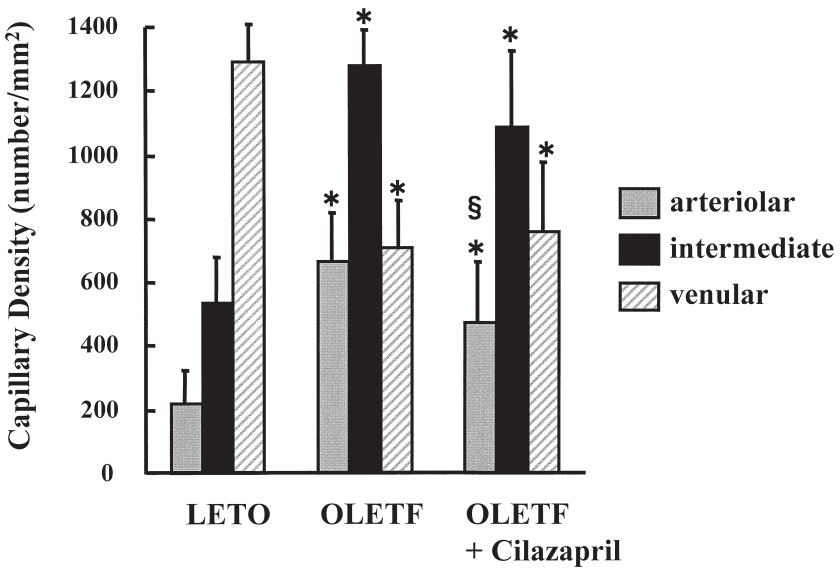

Figure 13 Capillary density (number/mm^2) for arteriolar, intermediate and venular capillary portions in subendocardium of LETO and OLETF rats at 20 weeks of age, and OLETF rats treated with cilazapril from 5 to 20 weeks of age. * $p<0.05$ compared to LETO rats. § $p<0.05$ compared to OLETF rats. Figure quoted in part from reference 95.

Collagen/non collagen protein ratio in heart tissue of vehicle treated OLETF rats was significantly greater than that in the LETO rats of the same age (21.4 ± 0.8 μg collagen/mg protein vs 7.4 ± 1.9, $p < 0.05$). The increase was significantly prevented in the cilazapril treated OLETF group (8.2 ± 3.8, $p < 0.05$). On Elastica-Masson's trichrome staining perivascular fibrosis and increase in vessel wall thickness previously reported in aged OLETF rats were not noted around arterioles less than 100 μm diameter in OLETF and LETO groups at 20 weeks, suggesting perivascular fibrosis and increase in the vessel wall thickness were not apparently present at early stage of NIDDM.

Renin angiotensin system participates in the pathogenesis of cardiac hypertrophy and fibrosis induced by hypertension and myocardial infarction. Cardiac renin angiotensin system may be activated in diabetes induced by streptozotocin. However, whether the renin angiotensin system is involved in the pathogenesis of cardiac complications in NIDDM is not clear. OLETF rats characterized by early increase in serum insulin, late onset of hyperglycemia and mild course of diabetes mellitus[62] also exhibit hypertension. Thus, they are suitable for elucidating the role of renin angiotensin system in cardiac complications observed in NIDDM. Although increase in cardiac expression of TGF-β, a potent growth factor involved in fibrosis, and perivascular fibrosis in aged OLETF rats are reported[63], the early changes in cardiac tissues in OLETF rats have not been previously characterized. Therefore, whether the alterations of coronary microvascular remodeling and extracellular matrix components in young OLETF rats before the onset of overt diabetes is present were investigated.

In rats with streptozotocin induced insulin dependent diabetes mellitus (IDDM) captopril partially prevented diabetic cardiomegaly, suggesting that ACE inhibitors may be useful for cardiomyopathy in IDDM. Cilazapril prevented increase in heart weight and collagen protein content in OLETF rats, suggesting that inhibition of renin angiotensin system may be beneficial on cardiomyopathy in NIDDM as well as in IDDM. Because ACEI leads to inhibition of bradykinin degradation and bradykinin inhibits cardiac remodeling, the effect of cilazapril may be at least partly through bradykinin. Furthermore, angiotensin type 2-receptor exerts an antiproliferative action on endothelial and smooth muscle cells. Further investigations to determine the potential involvement of bradykinin and angiotensin type-2 receptor for protection of cardiovascular complications in OLETF rats are necessary.

Significant capillary network remodeling can occur in rats subjected to coronary artery occlusion or nitric oxide depletion[60), 64), 65)], suggesting that capillary network can undergo substantial adaptation under various pathological conditions. Indeed, in OLETF rats the number of venular capillary portion was significantly decreased compared with the value in LETO rats. In contrast the number of intermediate capillary portion was significantly increased and arteriolar capillary portion was also significantly increased. These results suggest that in early stages

of NIDDM arterialization of venular capillary portion and intermediate capillary portion may be induced. In young OLETF rats capillary domain area, defined as the area to which one capillary provides oxygen[60], was decreased as compared to LETO rats, suggesting an adaptation process in response to metabolic alterations, microcirculatory dysfunction or myocyte atrophy to effectively deliver oxygen. This capillary network remodeling was prevented by cilazapril. Because angiotensin II can potentiate angiogenic activity in microcapillary endothelial cells and ACE inhibitor may suppress tumor angiogenesis, inhibition of capillary network remodeling by cilazapril is caused at least partly by the direct action in addition to its hypotensive action. Indeed, angiotensin II can upregulate vascular endothelial growth factor, a potent mitogen for endothelial cells, in rat heart endothelial cells. In IDDM model rats treated with streptozotocin there was a reduction in vascular surface area, and reduction in length and diameter of capillaries and post capillary venules. The structural changes may underlie the reduced function of the diabetic heart and limited angiogenesis in diabetes. Because the blood pressure was measured through femoral artery under anesthesia, the blood pressure values were generally lower than the values previously reported. Whether hypotensive action provided by other antihypertensive drugs can also alter coronary capillary remodeling in OLETF rats needs further investigation.

Although perivascular fibrosis was not observed in young OLETF rats, collagen/non collagen protein ratio in heart tissue was increased suggesting an extracellular matrix remodeling. In streptozotocin treated rats increase in extracellular spaces between cardiac myocytes and deposition of collagen fibrils and amorphous components of the ground substances are reported. The extracellular matrix remodeling could increase the distance between capillary and myocyte and stiffness of the ventricular wall, and reduce both oxygen delivery and left ventricular contractility. In young OLETF rats activation of the renin angiotensin aldosterone system may include a progressive remodeling of the heart mediated, in part, by the induction of various cytokines and growth factors as in patients with heart failure. Cardiac aldosterone may also be increased in heart and coronary blood vessels, exerting autocrine or paracrine action[66].

Although cilazapril did not significantly affect plasma glucose or cholesterol, plasma insulin, HOMA equivalent values, body weight and triglyceride levels were significantly reduced, suggesting that cilazapril had minor but beneficial effects on diabetes itself. ACEI had improved glucose metabolism and insulin resistance in fructose fed rats. Thus, it can not be completely ruled out that the effects of cilazapril may be at least partly mediated through the improvement of insulin resistance.

Angiotensin II appears to be involved in the development of cardiovascular complications observed in early stages of NIDDM in OLETF rats, and inhibition of ACE ameliorated myocardial hypertrophy, coronary capillary network remodeling, and increase in collagen content. The results support the concept that ACE inhibi-

tion may be useful not only in the treatment but also in the prevention of cardiac complications in patients with NIDDM. Amelioration of coronary capillary network remodeling may at least partly explain the favorable anti-ischemic effects of ACEI in high-risk patients[67].

Allograft Inflammatory Factor-1 Augments Macrophage Phagocytotic Activity: a New Inflammatory Modulator of Atherosclerosis

Allograft inflammatory factor (AIF)-1 was originally identified as a gene product expressed in infiltrating macrophages in heterotopic cardiac allografts of a rat model[68]. The expression of AIF-1 was mostly limited to cells of a monocyte/macrophage lineage and augmented by interferon (IFN)-γ, suggesting a novel molecular involvement in allogeneic responses. AIF-1 is also expressed in macrophages and microglial cells of experimental autoimmune diseases, in devascularized rat skeletal muscles[69] and in human cerebral infarctions[70]. Upon injury with balloon angioplasty vascular smooth muscle cells also expressed AIF-1[71]. Thus, AIF-1 may play a pivotal role not only in immune responses to alloantigens but also in various host responses to inflammatory stimuli. However, little is known concerning the functions of AIF-1 in these lesions.

AIF-1 or AIF-1-like genes have been cloned from a wide range of organisms. The similarity of AIF-1 sequences reaches to 70%, which suggests that AIF-1 is an essential molecular component functioning across the species barriers. We have previously cloned mouse AIF-1 cDNA, established monoclonal antibodies against recombinant mouse AIF-1 proteins, and analyzed the effects of the AIF-1 overexpression on cytokine productions of a mouse macrophage cell line upon stimulation with bacterial lipopolysaccharide (LPS)[72]. The effects of AIF-1 overexpression on phagocytotic activity and the incorporation of acetyl-low density lipoprotein (LDL) were investigated and potential pathophysiological roles of AIF-1 in macrophages in the development of atheroscerosis were examined.

Iba-1, a homologue of AIF-1, was shown to be co-localized with F-actin in phagocytotic cups formed during phagocytosis of zymosan particles and the transfectants that overexpressed mutant Iba-1 exhibited suppressed phagocytosis. Thus, the overexpression of functional AIF-1 may enhance the phagocytosis. To examine this possibility, RAW 264.7 cells were transfected with expression vectors, pRc/CMV alone (mock transfectants) or pRc/CMV-mAIF-1 (AIF-1 transfectants). And the phagocytotic activity was tested by incubating the cells with FITC-labeled latex beads. The amount of AIF-1 protein expressed in the transfectants was determined as in an increasing order with wild type RAW 264.7 cells＝mock transfectants＜＃182＜＃24＝＃203 as previously reported[72]. The fraction of cells that incorporated FITC-beads were demonstrated in FL-1 histogram as the sharp peaks with a factor of integer multiplication. The M1 region was considered as the cells that ingested more than one bead per cell. AIF-1 transfectants incorporated FITC-microbeads more than 3 times compared with control cells as shown in Figure 14A. This ratio was in proportion to the expression level of AIF-1 (RAW 264.7 cells＝mock transfectants＜＃182＜＃24＝＃203).

As the phagocytosis of latex beads was affected by an overexpression of AIF-1, whether the overexpression of AIF-1 also induced an enhanced incorporation of modified LDL in RAW264.7, mock transfectants, and AIF-1 transfectants as well

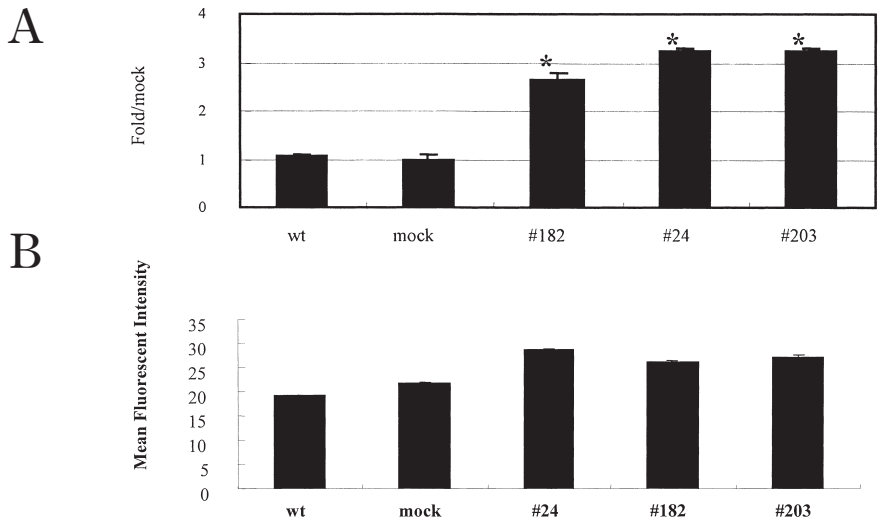

Figure 14 (A) Bar graph showing the phagocytotic activity [phagocytosed cell fractions (wt, transfectants) /phagocytosed cell fractions in mock transfectants] of each cell line expressed as fold over mock group. Significant difference versus mock *$p<0.01$. (B) Influence of AIF-1 overexpression on incorporation of acetyl-LDL. RAW 264.7 (wild-type, wt), a vector control (mock) and three AIF-1 transfectants (#24, #182, #203) were incubated with DiI-labeled acetyl-LDL *in vitro* for 30 min in triplicate. The incorporation of acetyl-LDL was quantitated with flow cytometry as described in Materials and Methods. Multiple comparisons were performed with Scheffe's method and only the significant differences between AIF-1 and mock transfectants were demonstrated in the graph.

was determined. As shown in Figure 14B, the incorporation of acetyl-LDL was significantly enhanced in AIF-1 transfectants when compared with the control cells. The DiI-labeled acetyl-LDL was incorporated as droplets in cytosol of incorporating cells as observed with fluorescent microscope. When the time course was plotted with the mock transfectants and AIF-1 transfectants, significant difference in the incorporation was impressively demonstrated in early phase at 30 min. The difference was less at 1 hr or 2 hr of incubation probably due to the saturation in uptake.

AIF-1 expression in monocytes may modulate the development of atherosclerosis by enhancing incorporation of degenerated LDL. However, transgenic mice that overexpress AIF-1 in macrophage-specific manner are necessary to directly examine the effect of AIF-1 expression on the development of atherosclerosis. Instead the expression of AIF-1 in the atherosclerotic lesions and also the cell populations that express AIF-1 was examined with an immunohistochemical analysis in the apoE$^{-/-}$ mice. As shown in Color Fig. 13A, AIF-1 was expressed by cells that constitute plaques. Some of these cells were positive for MOMA-2, suggesting

that these cells were macrophages (Color Fig. 13B) and the others were positive for α-actin, suggesting that those were vascular smooth muscle cells (Color Fig. 13D). The AIF-1 expression was weak in control mice (Color Fig. 13C). Meanwhile, no significant signals were detected when control non-immune IgG was used, or the staining without the primary monoclonal antibody. The levels of AIF-1 expression were similar between control and apoE$^{-/-}$ mice at 8 weeks, but the level of expression was shown to be increased in apoE$^{-/-}$ mice as compared to that of control mice at 28 weeks by immunoblot analysis.

AIF-1 was originally identified in rat cardiac allografts with chronic rejection. Studies of endomyocardial biopsy specimens from human heart transplants and allogeneic grafts in two marine sponge species also showed increased AIF-1 expression in allografts, suggesting that AIF-1 expression was induced in response to allogeneic antigens. The expression of AIF-1 was also increased in various host responses to inflammatory stimuli[69), 71)]. We have previously identified AIF-1 gene and product in a mouse system[72)]. The mouse system has an advantage of availability of a number of genetically manipulated stocks. Recombinant AIF-1 proteins abrogate proliferation and differentiation of cultured satellite cells of skeletal muscles, suggesting that AIF-1 proteins are secreted to extra-cellular compartments. Human vascular smooth muscle cells transfected with AIF-1 cDNA showed enhanced proliferation and the amount of AIF-1 expression in cardiac allografts correlates with the severity of rejection[73)].

Resident peritoneal cells, most of which are macrophages, expressed only a little amount of AIF-1, indicating that AIF-1 expression is confined to a subpopulation of macrophages in a resting condition. Thus, precise histological analysis of AIF-1 expression in vasculopathy and examination of the effect of AIF-1 overexpression on cellular function were carried out to determine the exact cell subpopulation(s) expressing AIF-1 in vascular beds and to pursue functional roles of AIF-1. Immunohistological analysis revealed that AIF-1 positive cells were observed among mononuclear cells resided in plaques as well as among smooth muscle cells. These results were consistent with a previous report on human AIF-1[73)].

To analyze functions of AIF-1 in cells of a monocyte/macrophage lineage, we generated transfectants that constitutively express AIF-1 proteins. The parental cell line RAW 264.7 expressed little AIF-1. We have previously reported that, although no detrimental influences of AIF-1 overexpression on RAW 264.7 cells were noted, these AIF-1 transfectants showed significant morphological changes especially upon stimulation with LPS[72)]. In addition, we showed that the LPS-stimulated transfectants produced significantly larger amounts of IL-6, -10 and -12p40 than those by control cells[72)].

The AIF-1 transfectants incorporated FITC-microbeads more than 3 times compared with control cells. This ratio was in proportion to the expression level of AIF-1. The AIF-1 transfectants also showed significantly enhanced incorporation of acetyl-LDL. These results suggest that AIF-1 functions in the development

of atherosclerotic vasculopathy, because macrophage phagocytotic activity is one of the major determinants for susceptibility of atherosclerosis as we previously reported[74]. It seems necessary to determine whether the characteristic pattern of phagocytosis observed in AIF-1 transfectant represents the immune or inflammatory responses in vivo and results in alteration of atherosclerotic changes. To address this issue studies employing transgenic mice that overexpress AIF-1 with a macrophage-specific promoter are being undertaken in our laboratories.

The present finding that AIF-1 overexpression resulted in the enhanced phagocytosis may have potential important clinical implications not only in atherosclerosis but also in engraftment of allogeneic transplants. When the transplantation of allograft is clinically taken into consideration, regulation of AIF-1 expression appears to be important for prolongation of the graft survival. It seems important to study how AIF-1 expression is regulated not only for the better understanding of AIF-1 biology but also for the development of new treatments that improve atherosclerotic vasculopathy[74]. AIF-1 is a new inflammatory modulator of vasculopathy.

Cardiovascular Risk Factors and Endothelial Dysfunction in Japanese Hypertensive Patients: a Valid Biological Risk Marker of Early Atherosclerosis

Hypertension, hyperlipidemia, diabetes and smoking are risk factors of atherosclerosis in Caucasians. Their relative contributions to early atherosclerosis among Japanese are not thoroughly investigated[75]. Endothelial cells maintain vascular tone and structure. In response to shear stress stimuli derived from flowing blood endothelium releases nitric oxide, which causes smooth muscle cell relaxation. Flow mediated vasodilation (FMD) of brachial arteries, a useful noninvasive method to determine peripheral endothelial functions, is reduced in patients with hypertension and cardiovascular risk factors.

Inflammation is implicated in vasculopathy. The plasma levels of TNF-α and IL-6 are elevated in atherosclerotic cardiovascular diseases. Plasma level of t-PA, which plays a crucial role in modulating thrombosis and thrombolysis[76], is a sensitive index of endothelial damage and is associated with risk of accelerated atherosclerosis.

Endothelial dysfunction may be reversed by antihypertensive therapy in experimental hypertension and in humans. To evaluate the relative contribution of hypertension to early atherogenesis, FMD, plasma levels of t-PA, a sensitive index of endothelial damage, and TNF-α and IL-6, established markers of inflammation, were determined in Japanese normotensive and hypertensive patients under treatment.

Patients were recruited from the Hokkaido University Hospital (n=119). Written informed consent was obtained from all patients in accordance with the institutional ethical guidelines. Patients with a history of essential hypertension (systolic blood pressure$\geqq$140 mmHg, diastolic blood pressure$\geqq$90 mmHg measured before any treatment) that resulted in antihypertensive treatment (calcium blocker n=47, β blocker n=17, angiotensin converting enzyme inhibitor n=17) were included as the hypertension group (n=67). Control subjects were matched to hypertensive subjects according to age and gender (n=52). Blood pressure was expressed as the mean of 3 different sphyngomanometric measurements, each performed on 3 separate days. The possibility of secondary hypertension was excluded by clinical and laboratory tests. Exclusion criteria also included heart failure, valvular heart disease, congenital heart disease, acute coronary syndrome and liver disorders. Clinical data, including risk factor assessment for coronary heart disease, biochemical data, and results of investigations were recorded. A known history of smoking (current smokers included individuals who stopped smoking$<$5 years before the enrollment) was recorded. Body mass index (BMI) was calculated as the ratio of weight (kg) to the square of height (m^2). Routine lipid estimation was performed including total cholesterol, triglyceride (TG) and high-density lipoprotein (HDL) levels. Subjects with an elevated serum total cholesterol ($>$220 mg/dl) or subjects with a history of elevated serum total cholesterol that resulted in cholesterol lowering treatment were considered to have a positive history of hyperlipidemia.

No subject had documented familial hyperlipidemia. Subjects with an elevated fasting plasma glucose (>126 mg/dl) or subjects with a history of elevated fasting plasma glucose that resulted in anti-diabetes treatment were considered to have a positive history of diabetes mellitus.

The blood pressure was in good control among hypertensives. No difference in brachial artery diameter was noted between the two groups. FMD was significantly lower in hypertensives (9.9±5.8%) than in normotensives (14.6±7.6%, $p<0.01$). Endothelium independent vasodilation was not significantly lower in hypertensives (16.0±6.3%) than in normotensives (16.7±5.8%).

Plasma t-PA antigen level was higher in hypertensives than in normotensives (10.4±4.2 ng/ml vs 8.4±4.2, $p<0.05$), indicating continued endothelial damage despite blood pressure reduction (Figure 15). Plasma TNF-α level was higher in hypertensives than in normotensives (2.6±1.5 pg/ml vs 2.1±1.4, $p<0.05$), indicating persistent inflammation (Figure 15). Plasma IL-6 level was not higher in hypertensives than in normotensives (1.8±1.2 pg/ml vs 1.5±1.2).

The hypertensives were divided into the following two subgroups according to the control of blood pressure. High hypertensives (H-HT) had insufficient control of blood pressure (systolic blood pressure>130 mmHg and/or diastolic blood pressure>85 mmHg). Low hypertensives (L-HT) had good control of blood pressure (systolic blood pressure≦130 mmHg and diastolic blood pressure≦85 mmHg). The clinical characteristics in terms of age, gender, cholesterol, TG, HDL, BMI,

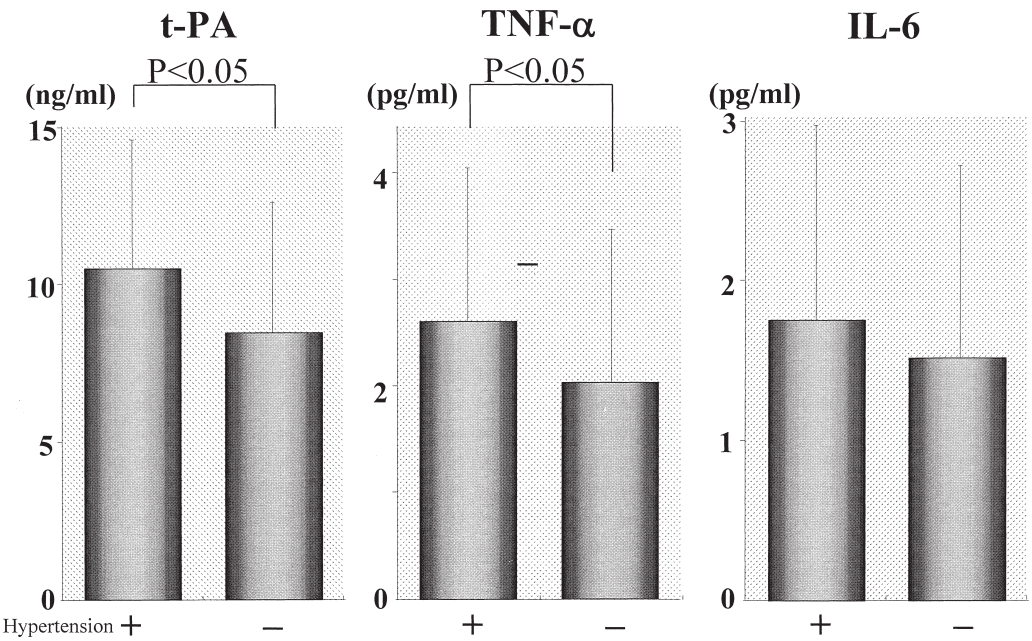

Figure 15 Comparison of plasma levels of t-PA, TNF-α and IL-6 levels in normotensives (N, n=52) and hypertensives (HT, n=67). Figures quoted in part from reference 80.

HOMA, brachial artery diameter and prevalence of diabetes and smoking did not differ between H-HT and L-HT. Calcium blocker was used in 67% in H-HT and 71% in L-HT. Angioensin converting enzyme inhibitor was used in 28% in H-HT and 19% in L-HT and b blocker was used in 22% in H-HT and 33% in L-HT. The blood pressure of L-HT (119±8 / 70±8 mmHg) was not significantly higher than the values in normotensives (118±13 / 71±11 mmHg). The blood pressure of H-HT was significantly higher than the values in normotensives and L-HT (149±17 / 85±14, $p<0.01$). Despite good control of blood pressure FMD in L-HT was significantly lower than the value in normotensives. Plasma level of TNF-α in L-HT was also significantly higher than the value in normotensives.

FMD was significantly reduced as the total number of risk factors increased (Figure 16). Furthermore, hypertenisves without hyperlipidemia, diabetes or smoking exhibited significantly lower FMD compared to normotensives (Figure 17). Among hypertensives addition of one or two other risk factors did not further reduce FMD (Figure 17). There was no difference in endothelium independent relaxation between normotensives and hypertensives regardless of the number of risk factors. Among low-risk hypertension patients, in whom hypertension was the only risk factor or hypertension was accompanied by only one other risk factor, plasma levels of t-PA, TNF-α and IL-6 were moderately elevated in hypertensives as compared to normotensives.

FMD was lower in hypertensives despite good control of blood pressure,

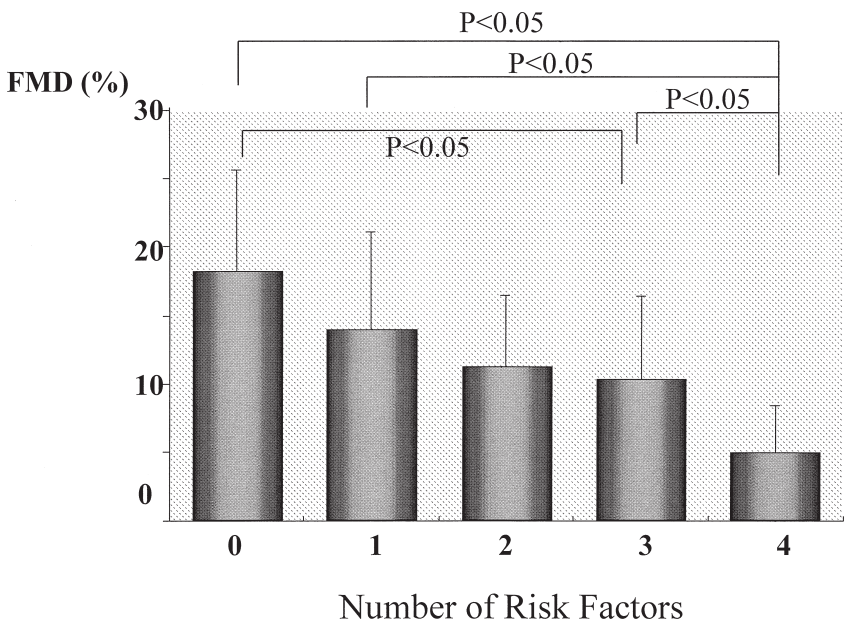

Figure 16 Relationship between FMD and total number of risk factors. Risk factors include hypertension, hyperlipidemia, diabetes mellitus and smoking. Values are expressed as mean±SD. Figure quoted in part from reference 80.

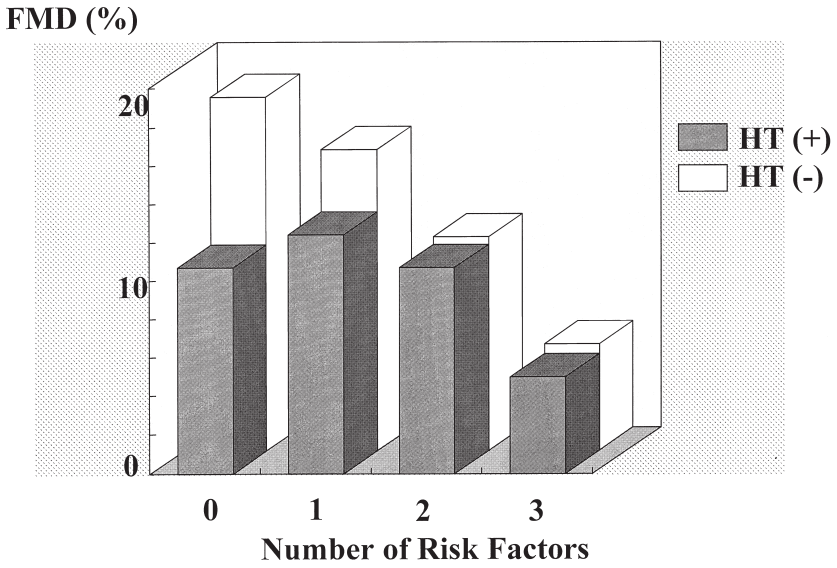

Figure 17 Relationship between FMD and total number of risk factors (hyperlipidemia, diabetes and smoking) in subjects with or without hypertension. Values are expressed as mean±SD. Figure quoted in part from reference 80.

suggesting that reduction of shear stress as a result of blood pressure decrease may not be sufficient to fully restore endothelial function. There was no difference in endothelium independent vasodilation in hypertensives compared with normotensives, suggesting that there may be no disruption of endothelium independent vasodilation.

In addition to decreased FMD hypertensives exhibited higher plasma TNF-α levels than normotensives. Secretion of proinflammatory cytokines is upregulated in monocytes from hypertensives[77]. Although TNF-α and IL-6 are the major determinants of acute phase protein production[78], their roles have not been thoroughly investigated in hypertensives. Inflammatory cytokines impair endothelium-dependent dilatation in human veins in vivo and vascular inflammatory responses are implicated in hypertension induced end-organ damage[79]. Thus, mild inflammatory reaction in hypertensives might be an informative index to disclose impairment in vascular reactivity.

Level of t-PA antigen was increased in hypertensives reflecting endothelial damage in our study population[80]. TNF-α modulates fibrinolytic system in endothelium and IL-6 induced by TNF-α has procoagulant properties. A close link between fibrinolysis and atherogenic metabolic derangement is suggested[81]. Increased t-PA level may reflect chronic endothelial stimulation by thrombin and endothelial damage during vasculopathy. Recently upregulation of basal t-PA release and subsequent depletion of endothelial t-PA stores are implicated in coronary atherosclerosis[81].

Even a mild inflammatory reaction coupled with an alteration of fibrinolysis may disturb endothelial regulation of vascular tone in the arterial circulation in essential hypertension. Despite medical treatment and similar blood pressure levels L-HT group had lower FMD than the normotensive subjects, suggesting that FMD might have not been fully improved by medication. TNF-α is linked to hypertension and angiotensin II is implicated in TNF-α regulation. Furthermore, use of angiotensin converting enzyme inhibitor is reported to improve FMD. Although statistically not significant frequent use of angiotensin converting enzyme inhibitor in H-HT group may partly explain the tendency of higher FMD and lower plasma TNF-α levels in H-HT group as compared to L-HT group. Alterations in endothelial function may provide a link between systemic inflammation and hypertension.

The presence of hypertension alone without hyperlipidemia, diabetes or smoking was associated with reduced FMD, suggesting that hypertension per se is a very important risk factor among Japanese. Furthermore, addition of other risk factors only moderately reduced FMD in hypertensives, suggesting that future treatment of hypertension may need to include anti-inflammatory therapeutic strategies directing at reducing inflammation[82]. Because the cross-sectional nature of the present study does not permit causal inferences, further studies on fibrinolytic factors and inflammatory reactions could clarify the pathogenesis of early atherosclerosis in hypertension and provide different preventive measures against clinical cardiovascular events[83)-90)]. Accurate noninvasive evaluation of peripheral vascular function and morphology may become useful for predicting future cardiovascular events in Japanese hypertensives[91)-92)]. Impairment of FMD reflects risk factor burden and hypertension alone indicates a high-risk status for early atherosclerosis. Persistent endothelial damage and moderate inflammation may increase the risk of early atheroscleosis synergistically under the presence of hypertension in Japanese.

Conclusion

Collectively, in patients prone to cardiovascular diseases cytokines can be derived from atherome, aneurysm or vessels with damages of hypertension (Figure 18). Cytokines can be from adipose tissue and chronic infections such as mild respiratory diseases or gingival infection. These cytokines can stimulate liver, adipose tissue and endothelial cells. The products from these tissues can serve as high-risk biomarkers in circulation and they can also have various biological active properties. In high-risk patients the factors of metabolic abnormalities can directly or indirectly stimulate vascular, liver and endothelial cells and induce PAI-1 transcription and secretion. Thus, PAI-1 can serve as an important cardiovascular risk marker and also regulate atherothrombosis and vessel remodeling (Figure 19).

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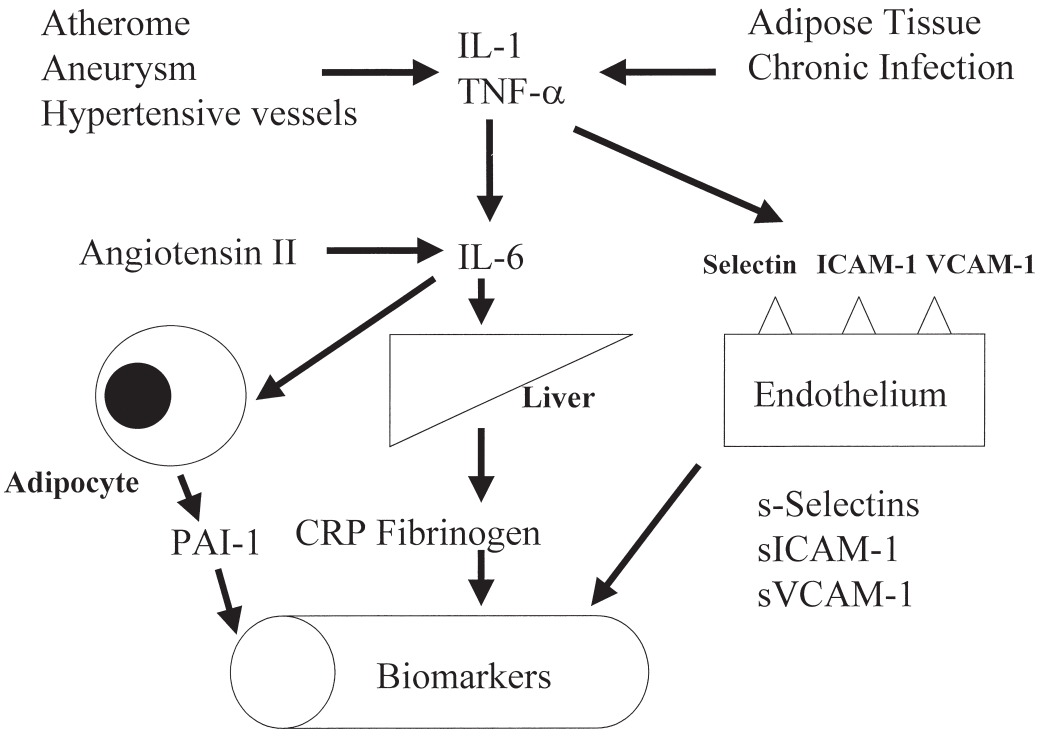

Figure 18 In patients prone to cardiovascular diseases cytokines can be derived from atherome, aneurysm or vessels with damages of hypertension. Cytokines can be from adipose tissue and chronic infections such as mild respiratory diseases or gingival infection. These cytokines can stimulate liver, adipose tissue and endothelial cells. The products from these tissues can serve as high-risk biomarkers in circulation and they can also have various biological active properties.

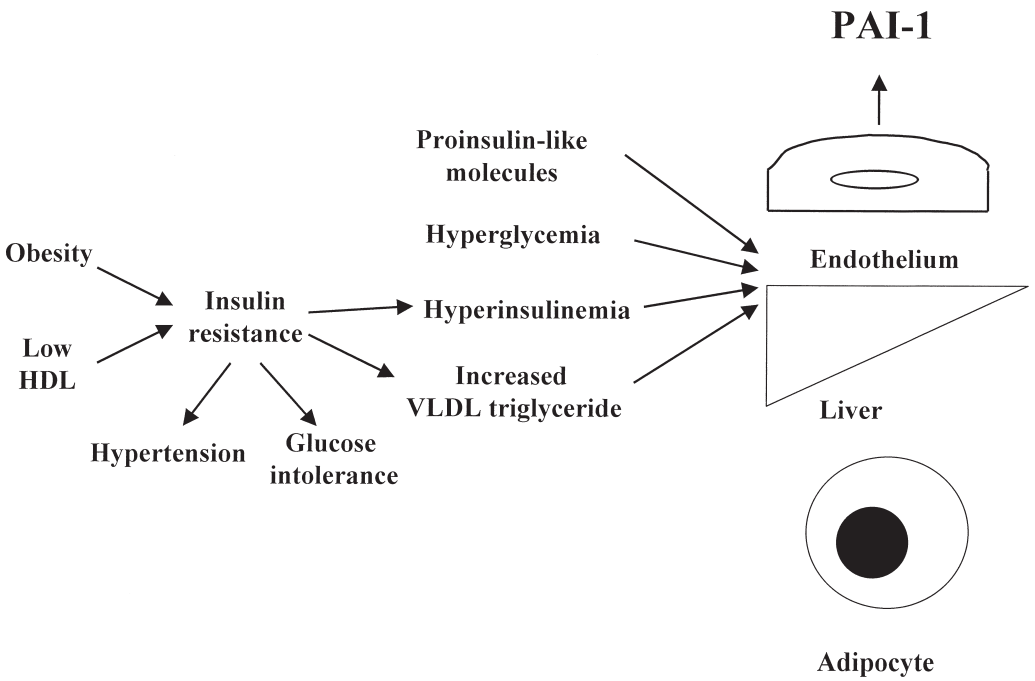

Figure 19 In high-risk patients the factors of metabolic abnormalities such as proinsulin, insulin, hyperglycemia and VLDL as well as inflammatory cytokines can directly or indirectly stimulate vascular, liver and endothelial cells and induce PAI-1 transcription and secretion. Thus, PAI-1 can serve as an important cardiovascular risk marker and also regulate atherothrombosis and vessel remodeling.

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References

1. Lundgren CL, Brown SL, Nordt TK, et al. Elaboration of type-1 plasminogen activator inhibitor from adipocytes: a potential pathogenetic link between obesity and cardiovascular disease. *Circulation* 1996; 93: 106–110
2. Okada H, Woodcock-Mitchell J, Mitchell J, et al. Induction of plasminogen activator inhibitor typpe-1 and type-1 collagen expression in rat cardiac microvascular endothelial cells by interleukin-1 and its dependence on oxygen centered free radicals. *Circulation* 1998; 97: 2175–2182
3. Nordt T, Sawa H, Fujii S, Sobel BE. Augmentation of arterial endothelial expression of the plasminogen activator inhibitor type-1 (PAI-1) gene by proinsulin and insulin in vivo. *J Moll Cell Cardiol* 1998; 30: 1535–1543
4. Sobel BE, Woodcock-Mitchell J, Schneidr DJ, et al. Increased plasminogen activator inhibitor type-1 in coronary artery atherectomy specimens from type 2 diabetic compared with

nondiabetic patients. *Circulation* 1998; 97: 2213–2221
5. Dinarello CA. Biologic basis for interleukin-1 in disease. *Blood* 1996; 87: 2095–2147
6. Brilla CG, Matsubara L, Weber KT. Advanced hypertensive heart disease in spontaneously hypertensive rats. Lisinopril-mediated regression of myocardial fibrosis. *Hypertension* 1996; 28: 269–275
7. Grech ED, Dodd NJ, Jackson MJ, et al. Evidence for free radical generation after primary percutaneous transluminal coronary angioplasty recanalization in acute myocardial infarction. *American Journal of Cardiology* 1996; 77: 122–7
8. Stephens NG, Parsons A, Schofield PM, et al. Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). *The Lancet* 1996; 347: 781–6
9. Habib FM, Springall DR, Davies GJ, et al. Tumour necrosis factor and inducible nitric oxide synthase in dilated cardiomyopathy. *The Lancet* 1996; 347: 1151–1155
10. Bottiger BW, Motsch J, Bohrer H, et al. Activation of blood coagulation after cardiac arrest is not balanced adequately by activation of endogenous fibrinolysis. *Circulation* 1995; 92: 2572–2578
11. Ito H, Maruyama A, Iwakura K, et al. Clinical implications of the "no reflow" phenomenon. A predictor of complications and left ventricular remodeling in reperfused anterior wall myocardia infarction. *Circulation* 1996; 93: 223–228
12. Ito H, Okamura A, Iwakura K, et al. Myocardial perfusion patterns related to thrombolysis in myocardial infarction perfusion grades after coronary angioplasty in patients with acute anterior wall myocardial infarction. *Circulation* 1996; 93: 1993–99
13. Nordt TK, Schneider DJ, Sobel BE. Augmentation of the synthesis of plasminogen activator inhibitor type-1 by precursors of insulin: a potential risk factor for vascular disease. *Circulation* 1994; 89: 321–330
14. Klassen KJ, Nordt TK, Schneider DJ, Sobel BE. Constitutive biosynthesis of plasminogen activator inhibitor type-1 by cultured human aortic endothelial cells independent of insulin. *Coronary Artery Disease* 1993; 4: 713–719
15. Shatos MA, Doherty JM, Penar PL, Sobel BE. Suppression of plasminogen activator inhibitor-1 release from human cerebral endothelium by plasminogen activators: a factor potentially predisposing to intracranial bleeding. *Circulation* 1996; 94: 636–642
16. Schneider DJ, Sobel BE. Augmentation of synthesis of plasminogen activator inhibitor type 1 by insulin and insulin-like growth factor type I: implications for vascular disease in hyperinsulinemic states. *Proc Natl Acad Sci USA* 1991; 88: 9959–9963
17. Nordt TK, Klassen KJ, Schneider DJ, Sobel BE. Augmentation of synthesis of plasminogen activator inhibitor type-1 in arterial endothelial cells by glucose and its implications for local fibrinolysis. *Arterioscler Thromb* 1993; 13: 1822–1828
18. Fujii S, Sawa H, Saffitz JE, et al. Induction of endothelial cell gene expression of plasminogen activator inhibitor type-1 (PAI-1) by thrombosis in vivo. *Circulation* 1992; 86: 2000–2010
19. Bochaton-Piallat ML, Gabbiani G, Pepper MS. Plasminogen activator expression in rat arterial smooth muscle cells depends on their phenotype and is modulated by cytokines. *Circ Res* 1998; 82: 1086–93
20. Wang W, Chen HJ, Schwartz A, et al. T cell lymphokines modulate bFGF-induced smooth muscle cell fibrinolysis and migration. *Am J Physiol* 1997; 272: C392–8
21. Eitzman DT, Westrick RJ, Xu Z, et al. Plasminogen activator inhibitor-1 deficiency protects

against atherosclerosis progression in the mouse carotid artery. *Blood* 2000; 96: 4212–5
22. Mandriota SJ, Pepper MS. Vascular endothelial growth factor-induced in vitro angiogenesis and plasminogen activator expression are dependent on endogenous basic fibroblast growth factor. *J Cell Sci* 1997; 110: 2293–302
23. Nordt TK, Kornas K, Peter K, et al. Attenuation by gemfibrozil of expression of plasminogen activator inhibitor type 1 induced by insulin and its precursors. *Circulation* 1997; 95: 677–83
24. Takeda K, Ichiki T, Tokunou T, et al. Critical role of Rho kinase and MEK/ERK pathways for angiotensin II-induced plasminogen activator inhibitor-1 gene expression. *Arterioscler Throm Vasc Biol* 2001; 21: 868–873
25. Liu J, Gao B, Mirshahi F, et al. Functional CB1 cannabinoid receptors in human vascular endothelial cells. *Biochem J* 2000; 346: 835–40
26. Bajou K, Noel A, Gerard RD, et al. Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. *Nat Med* 1998; 4: 923–8
27. Bajou K, Masson V, Gerard RD, et al. The Plasminogen Activator Inhibitor PAI-1 Controls In Vivo Tumor Vascularization by Interaction with Proteases, Not Vitronectin. Implications for antiangiogenic strategies. *J Cell Biol* 2001; 152: 777–84
28. Stefansson S, Petitclerc E, Wong MK, et al. Inhibition of Angiogenesis in vivo by Plasminogen Activator Inhibitor-1. *J Biol Chem* 2001; 276: 8135–41
29. Celletti FL, Waugh JM, Amabile PG, et al. Vascular endothelial growth factor enhances atherosclerotic plaque progression. *Nat Med* 2001; 7: 425–9
30. Rubins HB, Robins SJ, Collins D, et al. Gemfibrozil for the secondary prevention of coronary heart disease in men with low levels of high-density lipoprotein cholesterol. Veterans Affairs High-Density Lipoprotein Cholesterol Intervention Trial Study Group. *N Engl J Med* 1999; 341: 410–8
31. Zaman AK, Fujii S, Sawa H, et al. Angiotensin-converting enzyme inhibition attenuates hypofibrinolysis and reduces cardiac perivascular fibrosis in genetically obese diabetic mice. *Circulation* 2001; 103: 3123–3128
32. Brown SL, Sobel BE, Fujii S. Attenuation of the synthesis of plasminogen activator inhibitor type 1 by niacin. A potential link between lipid lowering and fibrinolysis. *Circulation* 1995; 92: 767–772
33. Munford RS. Statins and the acute-phase response. *N Engl J Med* 2001; 344: 2016–2018
34. Blake GJ, Ridker PM. Inflammatory bio-markers and cardiovascular risk prediction. *J Intern Med* 2002; 252: 283–294
35. Sposito AC, Chapman MJ. Statin therapy in acute coronary syndromes: mechanistic insight into clinical benefit. *Arterioscler Thromb Vasc Biol* 2002; 22: 1524–534
36. Kwak B, Mulhaupt F, Myit S, Mach F. Statins as a newly recognized type of immunomodulator. *Nat Med* 2000; 6: 1399–1402
37. Kaneko T, Fujii S, Matsumoto A, et al. Induction of plasminogen activator inhibitor-1 in endothelial cells by basic fibroblast growth factor and its modulation by fibric acid. *Arterioscler Thromb Vasc Biol* 2002; 22: 855–860
38. Brasier AR, Recinos A, Eledrisi MS. Vascular inflammation and the renin-angiotensin system. *Arterioscler Thromb Vasc Biol* 2002; 22: 1257–1266
39. Sobel BE. Increased plasminogen activator inhibitor-1 and vasculopathy. A reconcilable paradox. *Circulation* 1999; 99: 2496–2498
40. Loskutoff DJ, Samad F. The adipocyte and hemostatic balance in obesity: studies of PAI-1.

Arterioscler Thromb Vasc Biol 1998; 18: 1–6
41. Mavri A, Stegnar M, Krebs M, et al. Impact of adipose tissue on plasma plasminogen activator inhibitor-1 in dieting obese women. *Arterioscler Thromb Vasc Biol* 1999; 19: 1582–7
42. Morange PE, Alessi MC, Verdier M, et al. PAI-1 produced ex vivo by human adipose tissue is relevant to PAI-1 blood level. *Arterioscler Thromb Vasc Biol* 1999; 19: 1361–5
43. Crandall DL, Busler DE, McHendry-Rinde B, et al. Autocrine regulation of human preadipocyte migration by plasminogen activator inhibitor-1. *J Clin Endocrinol Metab* 2000; 85: 2609–14
44. Takai Y, Sasaki T, Matozaki T. Small GTP-binding proteins. *Physiol Rev* 2001; 81: 153–208
45. Park HJ, Galper JB. 3-Hydroxy-3-methylglutaryl CoA reductase inhibitors up-regulate transforming growth factor-beta signaling in cultured heart cells via inhibition of geranylgeranylation of RhoA GTPase. *Proc. Natl. Acad. Sci. U S A* 1999; 96: 11525–30
46. Maekawa M, Ishizaki T, Boku S, et al. Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. *Science* 1999; 285: 895–8
47. Sakamoto T, Woodcock-Mitchell J, Marutsuka K, et al. TNF-alpha and insulin, alone and synergistically, induce plasminogen activator inhibitor-1 expression in adipocytes. *Am J Physiol* 1999; 276: C1391–7
48. Harmon AW, Harp JB. Differential effects of flavonoids on 3T3-L1 adipogenesis and lipolysis. *Am J Physiol* 2001; 280: C807–13
49. Bastelica D, Morange P, Berthet B, et al. Stromal cells are the main plasminogen activator inhibitor-1-producing cells in human fat: evidence of differences between visceral and subcutaneous deposits. *Arterioscler Thromb Vasc Biol* 2002; 22: 173–8
50. Birgel M, Gottschling-Zeller H, Rohrig K, Hauner H. Role of cytokines in the regulation of plasminogen activator inhibitor-1 expression and secretion in newly differentiated subcutaneous human adipocytes. *Arterioscler Thromb Vasc Biol* 2000; 20: 1682–7
51. Schneider DJ, Nordt TK, Sobel BE. Stimulation by proinsulin of expression of plasminogen activator in hibitor type-I in endothelial cells. *Diabetes* 1992; 41: 890–5
52. Baxter-Burrell A, Yang Z, Springer PS, Bailey-Serres J. RopGAP4-dependent Rop GTPase rheostat control of Arabidopsis oxygen deprivation tolerance. *Science* 2002; 296: 2026–8
53. Haffner SM, Lehto S, Rönnemaa T, et al. Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. *N Engl J Med* 1998; 339: 229–234
54. Murakami H, Urabe K, Nishimura M. Inappropriate microvascular constriction produced transient ST-segment elevation in patients with syndrome X. *J Am Coll Cardiol* 1998; 32: 1287–94
55. McGill JB, Schneider DJ, Arfken CL, et al. Factors responsible for impaired fibrinolysis in obese subjects and NIDDM patients. *Diabetes* 1994; 43: 104–109
56. Samad F, Loskutoff DJ. The fat mouse: a powerful genetic model to study elevated plasminogen activator inhibitor 1 in obesity/NIDDM. *Thromb Haemost* 1997; 78: 652–655
57. Vaughan DE, Rouleau J-L, Ridker PM, et al. The HEART study investigators. Effects of ramipril on plasma fibrinolytic balance in patients with acute anterior myocardial infarction. *Circulation* 1997; 96: 442–447
58. Nordt TK, Sawa H, Fujii S, Sobel BE. Hyperinsulinemia increases plasma activity of PAI-1 in vivo independently of an acute phase reaction. *Fibrinolysis and Proteolysis* 1997; 11 (suppl 1): 51–54
59. Schaper W, Buschmann I. Collateral circulation and diabetes. *Circulation* 1999; 99: 2224–

2226
60. Koyama T, Xie Z, Gao M, et al. Adaptive changes in the capillary network in the left ventricle of rat heart. *Jpn J Physiol* 1998; 48: 229–241
61. Moriguchi Y, Matsubara H, Mori Y, et al. Angiotensin II-induced transactivation of epidermal growth factor receptor regulates fibronectin and transforming growth factor-β synthesis via transcriptional and posttranscriptional mechanisms. *Circ Res* 1999; 84: 1073–1084
62. Kawano K, Hirashima T, Mori S, et al. Spontaneous long-term hyperglycemic rat with diabetic complications: Otsuka Long-Evans Tokushima Fatty (OLETF) strain. *Diabetes* 1992; 41: 1422–1428
63. Yagi K, Kim S, Wanibuchi H, et al. Characteristics of diabetes, blood pressure, and cardiac and renal complications in Otsuka Long-Evans Tokushima Fatty rats. *Hypertension* 1997; 29: 728–735
64. Xie Z, Gao M, Batra S, Koyama T. The capillarity of left ventricular tissue of rats subjected to coronary artery occlusion. *Cardiovasc Res* 1997; 33: 671–676
65. Goto D, Fujii S, Tarikuz Zaman AKM, et al. Long-term blockade of nitric oxide synthesis in rats modulates coronary capillary network remodeling. *Angiogenesis* 1999; 3: 137–146
66. Silvestre J-S, Robert V, Heymes C, et al. Myocardial production of aldosterone and corticosterone in the rat: physiological regulation. *J Biol Chem* 1998; 273: 4883–4891
67. Yusuf S, Sleight P, Pogue J, et al. Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. *N Engl J Med* 2000; 342: 145–153
68. Utans U, Liang P, Wyner LR, et al. Chronic cardiac rejection: identification of five upregulated genes in transplanted hearts by differential mRNA display. *Proc Natl Acad Sci USA* 1994; 91: 6463–6467
69. Kuschel R, Deininger MH, Meyermann R, et al. Allograft inflammatory factor-1 is expressed by macrophages in injured skeletal muscle and abrogates proliferation and differentiation of satellite cells. *J Neuropathol Exp Neurol* 2000; 59: 323–332
70. Postler E, Rimner A, Beschorner R, et al. Allograft-inflammatory-factor-1 is upregulated in microglial cells in human cerebral infarctions. *J Neuroimmunol* 2000; 104: 85–91
71. Autieri MV, Carbone C, Mu A. Expression of allograft inflammatory factor-1 is a marker of activated human vascular smooth muscle cells and arterial injury. *Arterioscler Thromb Vasc Biol* 2000; 20: 1737–1744
72. Watano K, Iwabuchi K, Fujii S, et al. Allograft inflammatory factor-1 augments productions of interleukin-6, -10 and -12 by a mouse macrophage cell line. *Immunology* 2001; 104: 307–316
73. Autieri MV, Kelemen S, Thomas BA, et al. Allograft inflammatory factor-1 expression correlates with cardiac rejection and development of cardiac allograft vasculopathy. *Circulation* 2002; 106: 2218–2223
74. Ishimori N, Iwabuchi K, Fujii S, et al. Mixed allogeneic chimerism with wild type strains ameliorates atherosclerosis in apolipoprotein E-deficient mice. *J Leukoc Biol* 2001; 69: 732–740
75. Muratani H, Kimura Y, Fukiyama K, et al. Control of blood pressure and lifestyle-related risk factors in elderly Japanese hypertensive subjects. *Hypertens Res* 2000; 23: 441–449
76. Sobel BE. Insulin resistance and thrombosis: a cardiologist's view. *Am J Cardiol* 1999; 84: 37J–41J
77. Dörffel Y, Lätsch C, Stuhlmüller B, et al. Preactivated peripheral blood monocytes in patients with essential hypertension. *Hypertension* 1999; 34: 113–117
78. Anderson JL, Muhlestein JB, Carlquist J, et al. Randomized secondary prevention trial of

azithromycin in patients with coronary artery disease and serological evidence for Chlamydia pneumoniae infection: The Azithromycin in Coronary Artery Disease: Elimination of Myocardial Infection with Chlamydia (ACADEMIC) study. *Circulation* 1999; 99: 1540–1547
79. Muller DN, Mervaala EMA, Schmidt F, et al. Effect of bosentan on NF-kB, inflammation, and tissue factor in angiotensin II-induced end-organ damage. *Hypertension* 2000; 36: 282–290
80. Furumoto T, Saito N, Dong J, et al. Association of cardiovascular risk factors and endothelial dysfunction in Japanese hypertensive patients: implications for early atherosclerosis. *Hypertens Res* 2002; 25: 475–480
81. Newby DE, McLeod AL, Uren NG, et al. Impaired coronary tissue plasminogen activator release is associated with coronary atherosclerosis and cigarette smoking: direct link between endothelial dysfunction and atherothrombosis. *Circulation* 2001; 103: 1936–1941
82. Peeters AC, Netea MG, Kullberg BJ, et al. The effect of renin-angiotensin system inhibitors on pro- and anti-inflammatory cytokine production. *Immunology* 1998; 94: 376–379
83. Furumoto T, Fujii S, Saito N, et al. Relationships between brachial artery flow mediated dilation and carotid artery intima-media thickness in patients with suspected coronary artery disease. *Jpn Heart J* 2002; 43: 117–125
84. Yamashita T, Ito F, Iwakiri N, et al. Prevalence and predictors of renal artery stenosis in patients undergoing cardiac catheterization. *Hypertens Res* 2002; 25: 553–559
85. Sugawara T, Fujii S, Zaman AKM T, et al. Coronary capillary network remodeling and hypofibrinolysis in aged obese diabetic rats: Implications for increased myocardial vulnerability to ischemia. *Mol Cell Biochem* 2003; 248: 165–170
86. Goto D, Fujii S, Kaneko T, et al. Intracellular signal transduction modulating expression of plasminogen activator inhibitor-1 in adipocytes. *Biochem Pharmacol* 2003; 65: 1907–1914
87. Oyama N, Oyama N, Komatsu H, et al. Left ventricular asynchrony caused by an intramuscular lipoma: computed tomographic and magnetic resonance detection. *Circulation* 2003; 107: e200–e201
88. Goto D, Fujii S, Kitabatake A. Rho/Rho-Kinase as a novel therapeutic target in treatment of cardiovascular diseases. *Drugs of the Future* 2003; 28: 267–271
89. Dong J, Fujii S, Goto D, et al. Increased expression of plasminogen activator inhibitor-1 by mediators of the acute phase response: a potential progenitor of vasculopathy in hypertensives. *Hypertens Res* 2003; 26: 723–729
90. Kaneko T, Fujii S, Matsumoto A, et al. Induction of tissue factor expression in endothelial cells by basic fibroblast growth factor and its modulation by fenofibric acid. *Thrombosis Journal* 2003; 1: 6
91. Onozuka H, Fujii S, Mikami T, et al. In vivo echocardiographic detection of cardiovascular lesions in apolipoprotein E-knockout mice using a novel high-frequency high-speed echocardiography. *Circ J* 2002; 66: 272–276
92. Yamada S, Mikami T, Nishihara K, et al. Measuring medium-sized muscular arteries using a novel broadband 15-MHz linear array probe. *J Med Ultrasonics* 2003; 30: 177–185
93. Fujii S, Goto D, Zaman T, et al. Diminished fibrinolysis and thrombosis: clinical implications for accelerated atherosclerosis. *J Atheroscle Thromb* 1998; 5: 76–81
94. Okada H, Fujii S, Sobel BE, et al. Insulin and proinsulin regulate type-1 plasminogen activator inhibitor and type-1 collagen expression in rat cardiac microvascular endothelial cells: potential role in development of coronary microvasculopathy. *Pathogenesis* 1999; 1: 179–188
95. Sugawara T, Fujii S, Zaman AKM T, et al. Coronary capillary remodeling in non-insulin-dependent diabetic rats: amelioration by inhibition of angiotensin converting enzyme and its potential clinical implications. *Hypertens Res* 2001; 24: 75–81