Original Article

Increased Expression of Plasminogen Activator Inhibitor-1 by Mediators of the Acute Phase Response: a Potential Progenitor of Vasculopathy in Hypertensives

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Hypertension is an important risk factor for coronary atherosclerosis, which is accelerated by inflammation and diminished fibrinolysis. We have previously shown that levels of plasminogen activator inhibitor-1 (PAI-1), the major physiologic inhibitor of fibrinolysis, are increased with atherogenic metabolic derangement. Because the liver is one of the major sources of circulating PAI-1, we here examined the effects of two proinflammatory cytokines, interleukin (IL)-1 and IL-6, on PAI-1 production in a human hepatoma cell line, HepG2. IL-1 (1 ng/ml) and IL-6 (1 ng/ml) increased the accumulation of PAI-1 in the conditioned media over 24 h (IL-1 : 2.1 ± 0.2 (mean $\pm$ SD) fold over the control; IL-6: 1.4 ± 0.2 fold; Western blot, $p < 0.05$). The increase in PAI-1 protein accumulation correlated with the increased expression of PAI-1 mRNA (Northern blot). An HMG-CoA reductase inhibitor (mevastatin, 10 μ mol/l) attenuated the PAI-1 production induced by IL-1 and IL-6. The plasma PAI-1 activity level was higher in hypertensives than in normotensives (10.0 ± 9.8 AU/ml vs. 6.2 ± 4.5 AU/ml, $p < 0.05$). The plasma PAI-1 antigen level was also higher in hypertensives than in normotensives (30.9 ± 22.4 ng/ml vs. 24.4 ± 13.3 ng/ml, $p < 0.05$). Thus, 1) IL-1 and IL-6 can increase PAI-1 production in hepatic cells and 2) mevastatin may exert anti-thrombotic effects by decreasing the PAI-1 protein production induced by these proinflammatory cytokines. These results provide further insights into how inflammation is involved in the atherothrombotic complications observed in hypertensives, which may be ameliorated by HMG-CoA reductase inhibitors. (*Hypertens Res* 2003; 26: 723–729)

Key Words: thrombosis, plasminogen activator inhibitor-1, inflammation, cytokine

Introduction

Hypertension is an important risk factor for coronary atherosclerosis, which is accelerated by inflammation and hypofibrinolysis (1). Activity of the fibrinolytic system is regulated highly at the transcriptional, translational, and post-translational levels by growth factors and cytokines (2). Increased expression of plasminogen activator inhibitor-1 (PAI-1), the

major physiologic inhibitor of fibrinolysis, may play a pivotal role in the deposition of fibrin in the affected vessels. We have previously reported that circulating PAI-1 levels were increased in an animal model of atherogenic metabolic derangement (3). Liver is one of the major sources of circulating PAI-1 (4) and insulin stimulates PAI-1 synthesis in liver cells (5), suggesting that the insulin resistance and subsequent hyperinsulinemia typically seen in hypertensives may contribute to a prothrombotic risk state.

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The secretion of cytokines specifically associated with blood vessels in hypertensives may play a role in the progression of atherosclerosis (6, 7). Several proinflammatory cytokines, including interleukin (IL)-1 β , are known to regulate the expression of the PAI-1 gene and the synthesis of PAI-1 protein in vascular cells and hematocytes (8, 9). Because cytokines may predispose to the thromboembolic events and vasculopathy associated with hypertension, we characterized the influence of IL-1 β and IL-6 on hepatic PAI-1 production *in vitro* in a cultured liver cell line and characterized the cytokine and fibrinolytic profile in hypertensives *in vivo*.

Methods

Cell Culture

HepG2 cells, a highly differentiated human hepatoma-derived cell line, were obtained from the American Type Culture Collection (Rockville, USA) and were grown to 80–90% confluence on 6-well plates or 10 cm dishes in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, USA) supplemented with 10% calf serum (Hyclone, Logan, USA) containing 100 units/ml penicillin and 100 μ g/ml streptomycin in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Then, growth medium was removed and the cells were washed twice with phosphate-buffered saline (PBS) and incubated in serum-free medium for 24 h. The medium was then replaced with fresh serum-free medium containing the indicated concentrations of recombinant human IL-1 β or IL-6 (Sigma). Mevastatin (Sigma) was dissolved in DMSO before use. Mevalonic acid lactone (Sigma) was added at a final concentration of 1 mmol/l in the indicated experiments. Media were collected and stored at -70°C until use.

Western Blotting

PAI-1 protein was assayed by Western blotting as previously described (10). In brief, equivalent amounts of conditioned medium were loaded on 8% polyacrylamide gel. Proteins were electrophoresed and transferred to polyvinylidene difluoride membranes, which were then blocked with Tween-Tris buffered salt solution (TTBS) containing 5% skim milk. The membranes were washed several times with TTBS containing 0.5% skim milk and incubated overnight at 4°C with mouse anti-human PAI-1 monoclonal antibody (American Diagnostica, Greenwich, USA; 1:500 dilution in TTBS). After being washed with TTBS containing 0.5% skim milk, the membranes were incubated with rabbit anti-mouse immunoglobulin G (IgG) antibody (Sigma; 1:10,000 dilution in TTBS) for 1 h at room temperature. Immunological detection was carried out with a ProtoBlot Western blot AP System (Promega, Madison, USA). Blots were analyzed by the method of Huang and Amero (11). Total protein in the

conditioned media was assayed using a BCA protein assay kit (Pierce, Rockford, USA).

Northern Blotting

Total cellular RNA was extracted by the acid guanidium thiocyanate–phenol–chloroform method. Northern blot analysis was performed as described in our previous study (12). RNA was electrophoresed (10 μ g per lane) in 1% agarose gels containing 0.66 mol/l formaldehyde and transferred onto Hybond-N⁺ nylon membranes by capillary blotting. The membranes were prehybridized for 3 h at 50°C with 0.5 ml salmon testes DNA (200 μ g/ml) and sequentially hybridized for 24 h at 50°C with human PAI-1 and human β -actin cDNA probes as previously described (12). The probes were labeled by random priming with the 30 μ Ci [α -³²P]dCTP. The membranes were exposed for autoradiography at -80°C.

Study Population

Patients were recruited from the Hokkaido University Hospital. 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 140 mmHg, diastolic blood pressure 90 mmHg measured before any treatment) for which they were receiving treatment (calcium blocker 79%; β -blocker 26%; angiotensin converting enzyme inhibitor 32%) were admitted into the hypertension group ($n = 97$; 63.6 ± 10.9 years; male 63%). Thirty-nine percent of patients in the hypertension group were also administered statins for concomitant hyperlipidemia. Control subjects were matched to hypertensive subjects according to age and gender ($n = 67$; 59.4 ± 14.8 years; male 73%). Blood pressure was expressed as the mean of three different sphygmomanometric measurements, each performed on three 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, liver diseases, and inflammatory disorders.

Blood Sampling and Laboratory Assay

Morning blood samples were taken after an overnight fast. The samples were drawn into plastic tubes containing 0.103 mol/l trisodium citrate at a volume of 1:9 and centrifuged at $3,000 \times g$ for 15 min. Aliquots of plasma were stored at -80°C until analysis. Personnel blinded to the clinical data performed the laboratory measurements. Plasma levels of PAI-1 activity were measured by spectrophotometric assay as previously described (5). Plasma levels of PAI-1 antigen and tissue-type plasminogen activator (t-PA) antigen were measured by enzyme-linked immunosorbent assay (ELISA) (Biopool, Umeå, Sweden). The plasma concentra-

Fig. 1. Effects of IL-1 β on accumulation of PAI-1 in the conditioned medium of HepG2 cells. Upon reaching 80–90% confluence, cells were serum starved for 24 h and then incubated with fresh serum-free media containing IL-1 β (0 to 10 ng/ml) 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 the means $\pm$ SD of the fold increase over the controls without IL-1 β (n = 6). * p < 0.05 compared with the values in untreated control cells. A representative blot from six separate experiments is shown in the upper panel.

Fig. 2. Effects of IL-6 on accumulation of PAI-1 in the conditioned medium of HepG2 cells. Upon reaching 80–90% confluence, cells were serum starved for 24 h and then incubated with fresh serum-free media containing IL-6 (0 to 10 ng/ml) 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 the means $\pm$ SD of the fold increase over the controls without IL-6 (n = 6). * p < 0.05 compared with the values in untreated control cells. A representative blot from six separate experiments is shown in the upper panel.

tion of IL-6 was measured by enzyme-linked immunoassay (R & D Systems, Minneapolis, USA; lower detection limit, 0.094 pg/ml) (13). Levels of PAI-1, t-PA, and IL-6 were within the detection limit of the assays. The plasma im-

Fig. 3. Effects of combination treatment with IL-1 β , IL-6, and mevastatin (Mev) 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-1 β (1 ng/ml), IL-6 (1 ng/ml), and Mev (10 μ mol/l) 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 the means $\pm$ SD of the fold increase over the controls without agents (n = 4). * 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.

munoreactive insulin level was measured by ELISA (13). All assays were performed in duplicate and the intra- and interassay variabilities were less than 10%.

Statistical Analysis

Data are the means $\pm$ SD. Differences were assessed by analysis of variance with Bonferroni's least significant *post hoc* test used for comparisons within multiple groups. Values of p < 0.05 were considered to indicate statistical significance.

Results

Effects of IL-1 and IL-6 on Accumulation of PAI-1 Protein

IL-1 β increased PAI-1 protein accumulation in a concentration-dependent fashion at 24 h (Fig. 1). Peak effects were seen at an IL-1 β concentration of 1 ng/ml (2.1 $\pm$ 0.2 fold over the control). Increased accumulation secondary to IL-1 β was

Fig. 4. Effects of mevalonic acid lactone on the mevastatin (Mev)-mediated reduction of PAI-1 accumulation in the conditioned medium of HepG2 cells. Upon reaching 80–90% confluence, cells serum starved for 24 h and then incubated with fresh serum-free media containing IL-1 β (1 ng/ml), IL-6 (1 ng/ml), Mev (10 μ mol/l), and/or mevalonic acid lactone (1 mmol/l) 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 the means $\pm$ SD of the fold increase over the controls without agents (n = 4). * $p < 0.05$ between the two groups indicated. A representative blot from four separate experiments is shown in the upper panel.

evident at 0.01 ng/ml, and induction was somewhat diminished at 3 ng/ml. In contrast to IL-1 β , IL-6 increased PAI-1 protein accumulation only modestly (Fig. 2). 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-1 β (1 ng/ml) and IL-6 (1 ng/ml) increased PAI-1 protein accumulation by 2.4 ± 0.2 fold over the control (Fig. 3). Total protein content in the conditioned media was not altered by IL-1 β or IL-6 (results not shown).

Effects of Mevastatin and Mevalonic Acid Lactone on Accumulation of PAI-1 Protein

Mevastatin (10 μ mol/l) significantly decreased the IL-1 β -, IL-6-, and IL-1 β + IL-6-induced accumulation of PAI-1 protein into the conditioned media at 24 h ($p < 0.05$) (Fig. 3). The degree of decrease was $23 \pm 3\%$ for the IL-1 β -induced PAI-1 accumulation and $30 \pm 3\%$ for the IL-6-induced PAI-1

Fig. 5. Effects of IL-1 β , IL-6, and mevastatin (Mev) on the expression of PAI-1 mRNA in 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-1 β (1 ng/ml), IL-6 (1 ng/ml), or mevastatin (10 μ mol/l) for 3 h. Cells were harvested and expressions of PAI-1 mRNA and β -actin were assayed by Northern blotting as described in the Methods section. The amount of PAI-1 mRNA was normalized to the amount of β -actin mRNA. Values are the means $\pm$ SD of the fold increase over the controls without agents (n = 3). * $p < 0.05$ between the two groups indicated; # $p < 0.01$ compared with the values in untreated control cells. A representative blot from three separate experiments is shown in the upper panel.

accumulation. Mevastatin decreased the PAI-1 accumulation induced by the combination of IL-1 β and IL-6 by $47 \pm 4\%$. Mevastatin alone did not affect PAI-1 protein accumulation. Addition of mevalonic acid lactone (1 mmol/l) was able to reverse the mevastatin-mediated reduction of PAI-1 accumulation (Fig. 4). Mevalonic acid lactone alone did not noticeably modify PAI-1 accumulation (results not shown).

Effect on Expression of PAI-1 mRNA

An increase in PAI-1 mRNA expression was seen in HepG2 cells treated with IL-1 β (1 ng/ml) and IL-6 (1 ng/ml) as assessed by Northern blotting (Fig. 5). Both the 3.2 kb and 2.2 kb products were increased. The combination of IL-1 β (1 ng/ml) and IL-6 (1 ng/ml) also increased PAI-1 mRNA. Mevastatin (10 μ mol/l) decreased the PAI-1 mRNA expression induced by IL-1 β , IL-6, and IL-1 β + IL-6 significantly

(Fig. 5). Both the 3.2 kb and 2.2 kb products were decreased.

Markers of Fibrinolysis and Inflammation in Hypertensives

Systolic blood pressure levels of hypertensives and normotensives were 139 ± 20 mmHg and 118 ± 13 mmHg, respectively ($p < 0.01$), and diastolic 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 AU/ml, $p < 0.05$). The plasma PAI-1 antigen level was higher in hypertensives than in normotensives (30.9 ± 22.4 ng/ml vs. 24.4 ± 13.3 ng/ml, $p < 0.05$). 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 ng/ml, $p < 0.05$), 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 pg/ml). The plasma insulin level was higher in hypertensives than in normotensives (6.1 ± 2.9 μ U/ml vs. 4.6 ± 1.7 μ U/ml, $p < 0.05$).

Discussion

In this study we evaluated the effects of two representative proinflammatory cytokines, IL-1 β and IL-6, and the HMG-CoA reductase inhibitor, mevastatin, on PAI-1 mRNA expression and accumulation of PAI-1 protein into conditioned media. The results showed that both IL-1 β 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-1 β it caused a significant induction of PAI-1 protein production and mRNA expression. On the other hand, mevastatin significantly diminished the effects of IL-1 β 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.

In this study 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 (14), and the liver is a major source of acute-phase protein production (15). 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 (16), but other cytokines such as IL-1 also contribute to the regulation. Our results demonstrated that PAI-1 production was increased by the proinflammatory cytokines IL-1 β and IL-6 in HepG2 cells; this finding was consistent with those of previous studies (17).

HMG-CoA reductase inhibitors (statins) exert various beneficial clinical effects on coronary diseases beyond their effects on serum cholesterol levels (18). Recent studies have focused on the pleiotropic effects of statins, and especially on inflammation and the fibrinolytic system (19, 20). Although the effects of statins on PAI-1 in vascular cells (21) and hematocytes (9, 22) have been studied previously, it remains unclear whether statins affect the hepatic PAI-1 expression under stimulation by proinflammatory cytokines. Our study showed that mevastatin decreased the PAI-1 production induced by IL-1 β 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 (23). Our results suggest that PAI-1 may become a major target of statin therapy.

IL-1 β is a prototypic multifunctional cytokine (24), but its effect on PAI-1 is not well understood. Although, in a previous study, 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 (25). Unlike lymphocyte and colony-stimulating growth factors, IL-1 β 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 (26). We have already reported that PAI-1 levels are increased in an animal model of atherogenic metabolic derangement (3). 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 (13, 27), was increased 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. Plasma PAI-1 levels may be regulated by a diverse range of mechanisms. High prevalence and incidence of diabetes is readily recognized in Japan (28), and hyperinsulinemia and diabetes are predictors of the development of hypertension (29, 30). Because insulin can stimulate PAI-1 synthesis in the liver (5), the increased insulin levels observed in hypertensives in the present study may have contributed to the increase in plasma PAI-1. Conversely, because the angiotensin converting enzyme inhibitor can attenuate plasma PAI-1 levels (31), 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 (32, 33). This may have been the reason for the only modest increase of plasma IL-6 levels in hyper-

tensives receiving the angiotensin converting enzyme inhibitor and statin in our study.

It has been suggested that there is a close link between diminished fibrinolysis and atherogenic metabolic derangement (34). Inadequate activation of endogenous fibrinolysis may contribute to microcirculatory dysfunction by inducing fibrin deposition and formation of microthrombi in coronary circulation (10, 35). Thus, the poor cardiovascular prognosis associated with hypertension may be a reflection, in part, of microvascular damage. Our findings suggest that 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.

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