Plasminogen Activator Inhibitor-1 in Patients with Kawasaki Disease: Diagnostic Value for the Prediction of Coronary Artery Lesion and Implication for a New Mode of Therapy

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ABSTRACT

Kawasaki disease (KD) in children takes the form of acute systemic vasculitis, which causes coronary artery dilation and aneurysm formation in 10% to 15% of the patients. We have recently shown that matrix metalloproteinases (MMPs) are intimately involved in coronary arterial wall destruction and the resultant formation of coronary artery lesions (CALs) in this disease. Plasminogen activators (PAs) are known to be a major pathway of MMP activation, and this suggests that their inhibitor, plasminogen activator inhibitor-1 (PAI-1), also plays important roles in the development of CALs in KD. The present study was conducted to test the hypothesis that circulating levels of PAI-1 are related to CAL formation in KD. Plasma levels of PAI-1 were measured by enzyme-linked immunoassay in 37 KD patients without CALs (group 1) and 7 KD patients with CALs (group 2). Blood samples were obtained before and after i.v. gammaglobulin therapy (IVGG), and in the convalescent stage. Levels of PAI-1 were significantly higher in KD patients before IVGG than in 18 age-matched healthy control subjects ($p < 0.01$). More importantly, both pre-IVGG and post-IVGG levels of PAI-1 were significantly higher in group 2 than in group 1 ($p < 0.01$). Furthermore, PAI-1 levels of 9 patients from group 1

who showed pre-IVGG PAI-1 levels higher than the minimum PAI-1 level in group 2 significantly decreased after IVGG, whereas PAI-1 levels of group 2 patients remained persistently elevated, further suggesting a close association between PAI-1 and CAL development in KD. Thus, PAI-1 may be useful as a predictive marker for CAL development in KD. Studies of the effects of PA inhibition on coronary outcome may provide evidence that PA is a viable therapeutic target for the prevention of KD-related CALs. (*Pediatr Res* 53: 983–988, 2003)

Abbreviations

MMP, matrix metalloproteinase
PA, plasminogen activator
PAI-1, plasminogen activator inhibitor-1
KD, Kawasaki disease
CAL, coronary artery lesion
IVGG, i.v. administration of gammaglobulin
TIMP, tissue inhibitor of matrix metalloproteinase
TNF, tumor necrosis factor
IL, interleukin

The extracellular matrix of the arterial wall provides a strong structural safety net that resists arterial expansion in a healthy individual. Arterial aneurysm development is thought to be the consequence of degradation of these structural proteins by proteolytic enzymes derived mainly from inflammatory cells infiltrating into media and adventitia (1–3). Matrix metalloproteinases (MMPs) have been implicated in this degradation

process, and are thought to play dominant roles in the formation of abdominal aortic aneurysms (3–6).

Plasminogen activators (PAs) and plasmin (a serine protease) are known to be activated in response to several inflammatory conditions and to act as triggers activating the MMP pathway (7, 8). In addition, recent study of aortic aneurysm in a rat model has demonstrated that blocking PA activity *via* local overexpression of plasminogen activator inhibitor-1 (PAI-1) prevents formation of arterial aneurysms by inhibiting MMP activation, suggesting a critical role for PA-mediated MMP activation in arterial wall destruction and resultant aneurysm formation (6, 9).

Kawasaki disease (KD) in children takes the form of acute systemic vasculitis, which causes coronary artery dilation and aneurysm formation in 10% to 15% of patients during its acute

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stage (10, 11). We have recently shown that, in KD, MMPs may also be intimately involved in coronary arterial wall destruction and the resultant formation of coronary aneurysms (12). Given the inhibitory effects of PAI-1 on MMP activation and our previous data indicating important roles for MMP in the formation of coronary artery lesions (CALs) in KD, it is possible that PAI-1 is also involved in the development of CALs in KD. The present study was conducted to test the hypothesis that circulating levels of PAI-1 are related to CAL formation in KD.

METHODS

Forty-four consecutive KD patients were prospectively recruited. These subjects were subdivided into 2 groups: 37 patients without CALs (group 1), and 7 patients with CALs (group 2). The blood samples were obtained between 9 and 10 am by nontraumatic needle aspiration from the antecubital vein. Immediately after samples were centrifuged, plasma was cooled to -80°C and stored at that temperature until assays were performed. Plasma levels of PAI-1 were measured by enzyme-linked immunoassay (ELISA) using MAb (13). Because endogenous PAs (mostly tissue PA) are saturated by PAI-1 and circulate predominantly in complex with PAI-1 (14), the PA/PAI-1 complex level reflects the level of PA (14). Therefore, we estimated the level of PA from PA/PAI-1 complex levels measured by ELISA.

All KD patients fulfilled the revised 1984 KD criteria of the Kawasaki Disease Research Committee of Japan (15). Coronary arteries with diameters of 4 mm or greater were considered to exhibit CALs (15). All KD patients were treated with i.v. administration of gammaglobulin (IVGG, 400 mg/kg/d for 5 consecutive days) combined with oral aspirin (50 mg/kg/d). Data were obtained before and after gammaglobulin treatment, and near the time of hospital discharge (convalescent stage). These data were compared with those from 18 age-matched afebrile control subjects (group 3). Group 3 included patients who had undergone reparative surgery for total anomalous pulmonary venous connection, small subpulmonary ventricular septal defect, or atrial septal defect more than 1 y before this study. None of the afebrile controls were receiving medication, and the results of their echocardiogram, ECG, laboratory tests and physical examination were all normal. Written informed consent was obtained from the parents of all patients, and the procedures were approved by The Saitama Medical School Committee on Clinical Investigation.

Statistical analysis

Data are presented as mean $\pm$ SEM. Comparisons among groups were made by ANOVA followed by Bonferroni correction. Differences in PAI-1 and PA/PAI-1 complex levels between time points were tested by repeated measures of ANOVA. $p < 0.05$ was considered to indicate statistical significance.

RESULTS

Table 1 summarizes patient characteristics of each group. The term "days of illness" indicates the points during the time course of illness at which blood samples were obtained, and there was no significant difference in these time points between groups 1 and 2 ($p = \text{NS}$). We defined onset of illness as the day on which the patient developed fever. The only significant difference in data between groups 1 and 2 was that group 2 had a higher C-reactive protein level than group 1 after IVGG therapy. None of the group 2 patients had CAL either before IVGG or before the second samples were taken. The average time at which CALs developed was 13.9 ± 1.7 d of illness.

Figs. 1A and B demonstrate circulating levels of PAI-1 and PA/PAI-1 complex in the afebrile control group and in KD patients before IVGG therapy. The values obtained for the afebrile control group (PAI-1, 19.3 ± 2.5 ng/mL; PA/PAI-1 complex, 5.1 ± 0.1) were quite similar to those previously reported for healthy adults (16). Therefore, although group 3 subjects were not truly healthy subjects, they can be considered comparable to healthy children for the purposes of the present study. The levels of PAI-1 and PA/PAI-1 complex in KD patients were significantly elevated compared with those of group 3 ($p < 0.01$). More importantly, the PAI-1 level of group 2 patients was significantly higher than that of group 1 (150 ± 21 versus 77 ± 10 ng/mL, $p < 0.01$), whereas groups 1 and 2 had similar PA/PAI-1 complex levels, suggesting that KD patients with CALs have an imbalance between plasminogen activation and inhibition at the very early stage of illness. To further elucidate the balance of this fibrinolytic system, we compared the ratio of PA/PAI-1 complex to PAI-1 among the 3 groups (Fig. 1C). This ratio was significantly lower in group 2 (0.11 ± 0.02) than in groups 1 (0.24 ± 0.03) and 3 (0.30 ± 0.04). The ratio was lower in group 1 than in the controls, but the difference was not statistically significant.

Figure 2 shows the serial changes in PAI-1, PA/PAI-1 complex and the ratio of PA/PAI-1 to PAI-1 in KD patients. After IVGG, levels of PAI-1 remained elevated in both KD

Table 1. Patient characteristics

	Baseline (before IVGG)			After IVGG		Convalescent stage	
	Group 1	Group 2	Group 3	Group 1	Group 2	Group 1	Group 2
Age (y)	2.5 ± 0.3	2.4 ± 0.4	2.6 ± 0.5				
Days of illness (d)	5.0 ± 0.2	4.8 ± 0.8	—	11.4 ± 0.5	12.1 ± 2.0	19.5 ± 2.5	22.4 ± 4.1
WBC ($10^3/\mu\text{L}$)	$16.7 \pm 2.0^*$	$13.6 \pm 1.8^*$	5.9 ± 0.4	$9.8 \pm 0.8^*$	8.3 ± 1.2	6.6 ± 0.4	6.8 ± 1.1
CRP (mg/dL)	$12.5 \pm 1.4^*$	$8.6 \pm 1.9^*$	0.1 ± 0.0	$2.1 \pm 0.4^*$	$4.0 \pm 0.8^{* \#}$	0.1 ± 0.1	0.3 ± 0.1

IVGG, intravenous gamma globulin; WBC, white blood cell count; CRP, C-reactive protein. * $p < 0.05$ vs. afebrile controls (group 3), # $p < 0.05$ vs. group 1 by unpaired *t*-test.

Figure 1. Circulating levels of PAI-1 (A), PA/PAI-1 complex (B), and the ratio of PA/PAI-1 complex to PAI-1 (C) for each group before i.v. gammaglobulin therapy. * $p < 0.05$ vs. group 3, # $p < 0.05$ vs. group 1.

Figure 2. Serial changes in PAI-1 (A), PA/PAI-1 complex (B), and the ratio of PA/PAI-1 complex to PAI-1 (C) in Kawasaki disease patients. ○, group 1; ●, group 2; 1, before IVGG; 2, after IVGG; 3, convalescent stage; * $p < 0.05$ vs. group 1; # $p < 0.05$ vs. before IVGG.

groups, and PAI-1 was significantly higher in group 2 than in group 1 (168 ± 16 versus 65 ± 9 ng/mL, $p < 0.01$). In the convalescent stage, PAI-1 levels of the 2 KD groups fell to similar levels ($p = \text{NS}$), but those levels were still significantly higher than those of group 3. In both KD groups, changes in the levels of PA/PAI-1 complex showed patterns similar to those of PAI-1 levels. There was no difference in PA/PAI-1 complex levels between the 2 KD groups at any time during the disease course. Thus, in group 2, the ratio of PA/PAI-1 complex to PAI-1 remained low and was significantly lower than that of group 1 after IVGG, suggesting persistent fibrinolytic imbalance after IVGG in KD patients with CALs.

To further elucidate the pathologic significance of the PA/PAI-1 system in CAL development, we compared individual changes in PAI-1 and the ratio of PA/PAI-1 complex to PAI-1 between group 2 patients and group 1 patients whose PAI-1 levels before IVGG were higher than the lowest value of group

2 (80 ng/mL; 9 patients) or whose ratio of PA/PAI-1 complex to PAI-1 before IVGG was lower than the highest value of group 2 (0.21; 15 patients). Mean values of PAI-1 and ratio of PA/PAI-1 complex to PAI-1 for these group 1 patients were similar to those of group 2 patients (PAI-1, 138 ± 14 ng/mL; ratio of PA/PAI-1 complex to PAI-1, 0.11 ± 0.02 ; $p = \text{NS}$ versus group 2). As shown in Fig. 3, PAI-1 levels of these group 1 patients significantly decreased after IVGG (85 ± 18 ng/mL, $p < 0.01$ versus before IVGG), whereas those of group 2 patients remained unchanged. Thus, PAI-1 after IVGG was significantly higher for group 2 patients than for this subset of group 1 patients ($p < 0.01$). A PAI-1 level of 80 ng/mL or greater, both before and after IVGG, had a sensitivity of 100% and specificity of 70% for prediction of development of CALs. Similarly, the ratio of PA/PAI-1 complex to PAI-1 markedly increased after IVGG in these group 1 patients (0.23 ± 0.02 , $p < 0.01$ versus before IVGG), but not in group 2, indicating a

Figure 3. Individual changes in PAI-1 and the ratio of PA/PAI-1 complex to PAI-1 in selected group 1 patients (open circles) and group 2 patients (closed circles), before and after i.v. gammaglobulin therapy. Mean changes are indicated by triangles. ○, group 1; ●, group 2; △, group 1 mean; ▲, group 2 mean; * $p < 0.05$ vs. group 1; # $p < 0.05$ vs. before IVGG.

significantly higher ratio of PA/PAI-1 complex to PAI-1 in these group 1 patients than in group 2 ($p < 0.01$). Thus, persistent fibrinolytic imbalance that favors inhibition of the PA/plasmin pathway appears to be closely related to CAL development in KD.

DISCUSSION

The present results demonstrate that circulating levels of PAI-1 and PA/PAI-1 complex are markedly elevated in KD. More importantly, PAI-1 levels of KD patients with CALs were significantly higher than those of KD patients without CALs before IVGG and remained elevated after IVGG, with imbalance against PA/PAI-1 complex levels. These data suggest that circulating levels of PAI-1 and the ratio of PA/PAI-1 complex to PAI-1 are related to the severity of vascular damage during the acute phase of KD, and that KD patients with high PAI-1 levels (both absolute and relative to PA/PAI-1 complex) are susceptible to CALs.

The physiologic condition of extracellular matrix is maintained by a rigorously controlled balance between the activity of proteases of its component proteins and the activity of endogenous inhibitors of those proteases. MMPs and their inhibitors, known as tissue inhibitors of MMPs (TIMPs), play central roles in this process (3). Accelerated matrix breakdown caused by increased activity of MMPs and/or quantitative imbalance between MMPs and TIMPs is involved in several pathologic conditions, including rheumatoid arthritis (17), tumor metastasis (18), and heart failure (19, 20). There is also increasing evidence that MMP levels are increased in arterial walls of abdominal aortic aneurysms, and that this increase

contributes to wall degradation and aneurysm formation (4–6, 21, 22).

PAs convert plasminogen into plasmin. PAI-1 is the primary physiologic inhibitor of plasminogen activation (23). Because most MMPs are secreted as zymogens, MMP activity in the extracellular space is dependent on zymogen activation (3). *In vitro* studies suggest that the PA/plasmin system is a major pathway of MMP activation (7, 8). For example, plasmin can convert proMMP-3 into active MMP-3 (24, 25), which in turn activates MMP-1 and MMP-9 (25, 26). PA/plasmin also directly activates MMP-9, -12 and -13 (7, 8, 27). There have also been *in vivo* studies indicating that the PA/plasmin system plays a predominant role in regulating MMP activity and MMP-induced pathologic matrix degradation. In a mouse model of acute myocardial infarction, blocking plasminogen activation by temporal infusion of PAI-1 prevented rupture of ventricular aneurysm (28), in which ventricular wall destruction by increased MMP activity is thought to be a main mechanism (29). It has also been shown that adenoviral transfer of the PAI-1 gene reduces tumor metastasis (30); in metastasis, extracellular matrix lysis is necessary for movement of transformed cells in tissues. In addition, Allaire *et al.* have reported that, in a rat model of aortic aneurysm, blockade of PAs by local overexpression of PAI-1 prevents formation of aneurysms and arterial rupture by inhibiting MMP activation (9). The above findings indicate that the PA/Plasmin system can trigger extracellular matrix degradation by activating the MMP pathway of proteolysis, resulting in the pathologic conditions mentioned above.

Interestingly, PAI-1 levels have also been found to be elevated in patients with increased activity of PA/plasmin and MMPs (31, 32). Despite its presence in lesions where pathologic extracellular matrix lysis occurs, the concentration of PAI-1 may be inadequate for full blockade of the PA-MMP system and related lesion development.

A situation similar to that described above may be the increased PAI-1 levels in KD patients with CALs. In a previous study, we showed that MMPs may also be intimately involved in coronary arterial wall destruction and the resultant formation of coronary aneurysms in KD (12). Given the reported inhibitory role of PAI-1 in MMP activation, the present results, which indicate increased levels of PAI-1 in KD with CALs, suggest that PAI-1 levels increase in response to coronary arterial wall injury, and that PAI-1 helps to protect against arterial wall destruction and resultant CAL formation, although the protective effects of PAI-1 are insufficient in KD. This may be analogous to a situation observed in heart failure, where atrial and brain natriuretic peptides that are cardioprotective are elevated in proportion to the severity of heart failure but are not sufficient to prevent heart failure progression (33).

It is also important to note that, in addition to its inhibitory action against MMP activation, PAI-1 plays diverse pathophysiological roles in the process of tissue remodeling. Several previous studies using animal models of arterial injury have indicated that PAI-1 transiently impairs cell migration and thereby limits neointimal formation early after injury (34–36). However, PAI-1 subsequently promotes fibrin accumulation and cell proliferation, resulting in neointimal growth at later time points (34). Varying effects of PAI-1 during the time course of tissue remodeling have also been suggested by Heymans *et al.* who used a rat model of acute myocardial infarction (28). They reported that urokinase-type PA deficiency completely protected against cardiac rupture early after myocardial infarction, but those mice consequently died of cardiac failure due to impaired scar formation and infarct revascularization. Only temporary administration of PAI-1 or MMP inhibitor completely protected mice against rupture without aborting infarct healing (28). Therefore, although increased levels of PAI-1 in the early stage of KD may protect against vascular wall destruction, prolonged activation of PAI-1 at a later stage may produce undesirable effects by promoting cell proliferation and fibrosis. Furthermore, in plasma, PAI-1 is a critical determinant of endogenous thrombolytic activity (23). At later stages of KD, when platelet counts are markedly elevated, increased PAI-1 may also increase the risk of thrombotic events, particularly if CALs have already developed.

The various possible actions of PAI-1 at different stages of tissue remodeling may also have important implications in therapeutic strategy for preventing CALs in KD. Although our results suggest that blocking the PA/plasmin pathway is a viable therapeutic approach to preventing KD-related CALs, such therapeutic intervention may have to be limited to the very early stage of illness.

Because we do not have conclusive evidence that PAI-1 has inhibitory effects on MMP activities in KD, we must also consider the possibility that higher PAI-1 levels in patients

with CAL than in those without CAL are due to a causative role of PAI-1 in development of CAL. Therefore, further clinical studies of effects of PA inhibition on MMP activities and resultant coronary outcome are needed to define the roles of PAI-1 in CAL formation in KD. Data from such studies may support the use of PA/plasmin inhibitors for prevention of coronary artery complications in patients with KD.

The results of the present study indicate that KD patients with persistently elevated PAI-1 with imbalance against PA levels are highly susceptible to CAL development. The individual changes in PAI-1 shown in Fig. 3 strongly support this conclusion. Thus, circulating levels of PAI-1 and the ratio of PA/PAI-1 complex to PAI-1 may be useful as predictive markers for CAL development in KD. There is controversy over whether high-dose gammaglobulin therapy should be applied to all KD patients (37); consequently, the decision of whether to use gammaglobulin is often based on one of several risk stratification scores for development of CAL (38). PAI-1 level may provide additional information about the risk of CAL development, thus helping determine whether to use gammaglobulin. In addition, although high-dose IVGG therapy can reduce the risk of development of CAL, some patients who receive this treatment still develop CAL. Additional therapy, such as corticosteroids (39) or repeated administration of IVGG (40), may be required to further reduce the incidence of CAL development. The present results suggest that such additional therapy should be considered for KD patients with elevated PAI-1 that persists after IVGG therapy.

It is well known that levels of various cytokines, such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and IL-6, are elevated in KD patients (41, 42). Recent studies by Seki *et al.* indicate that IL-1 β plays a key role in inducing PAI-1 expression in response to local tissue injury (43, 44). Interestingly, Leung *et al.* reported that high levels of IL-1 were secreted from mononuclear cells in Kawasaki disease patients, and that IL-1 secretion remained high after gammaglobulin therapy in patients with CAL but fell to normal levels in patients without CAL (45). This is very similar to the pattern of PAI-1 expression observed in the present study; it suggests that there is a close link between IL-1 and PAI-1 in KD, and that both proteins have pathophysiological significance in KD. In addition, reports by Seki *et al.* suggest that TNF- α is important for the induction of PAI-1 (43). The above findings indicate the need for studies that directly examine relationships between these cytokines and PAI-1 in KD, and their contributions to development of CAL.

In summary, we found that circulating levels of PAI-1 are markedly elevated in KD patients and are closely related to CAL formation. The present data suggest that circulating levels of PAI-1 are useful as a predictive marker for CAL development, and that they can serve as a therapeutic target for prevention of CALs. Prospective studies could help to clarify the usefulness of these potential applications.

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