Induction of Plasminogen Activator Inhibitor Type 1 Synthesis by Cadmium in Human Vascular Endothelial Cells in Culture

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Cadmium is a unique heavy metal that stimulates the secretion of plasminogen activator inhibitor type 1 (PAI-1) from vascular endothelial cells. However, it has been incompletely understood whether cadmium stimulation of PAI-1 secretion actually results in a reduction of endothelial fibrinolytic activity and whether the stimulation results from an induction of endothelial PAI-1 synthesis. To address these questions, human umbilical vein endothelial cells were cultured with cadmium chloride in the presence or absence of actinomycin D, H-7 or HA1004. The activity of tissue type and urokinase type plasminogen activators (t-PA and u-PA, respectively) in the conditioned medium was analyzed by fibrin zymography and mRNAs coding t-PA, u-PA and PAI-1 were determined by quantitative reverse transcription-polymerase chain reaction. Results of the experiments indicate that cadmium reduces the activity of both t-PA and u-PA in vascular endothelial cells through induction of PAI-1 synthesis which is mediated by protein kinase C activation. Since the PAI-1 induction by cadmium was observed in neither human vascular smooth muscle cells nor human fibroblasts, it was suggested that vascular endothelial cells are a particular cell type of which PAI-1 synthesis is stimulated by cadmium.

Key words —— cadmium, endothelial cell, fibrinolysis, plasminogen activator inhibitor, endothelial cell, vascular

INTRODUCTION

Vascular endothelial cells overspread the inner surface of blood vessels in the monolayer and play an important role in the regulation of the blood coagulation-fibrinolytic system. Fibrinolysis is a phenomenon where plasmin degrades fibrin. Vascular endothelial cells synthesize and secrete tissue type and urokinase type plasminogen activators (t-PA and u-PA, respectively) that convert plasminogen to plasmin.[1,2] The cells also express plasminogen activator inhibitor type 1 (PAI-1),[3] so that fibrinolytic activity in blood depends on the balance between plasminogen activators and PAI-1. Although u-PA lacks fibrin-binding capacity whereas t-PA activity is markedly enhanced upon binding to fibrin,[4,5] both activators contribute to the fibrinolytic activity in the liquid phase.

Cadmium is a toxic heavy metal that has been experimentally and epidemiologically implicated in vascular disorders such as atherosclerosis.[6–9] Since functional damage of vascular endothelial cells and intimal hyperplasia of vascular smooth muscle cells are crucial events of atherosclerosis, we studied the effects of cadmium on vascular endothelial and smooth muscle cell functions using a cell culture system. It was found that the two cell types are highly sensitive to the cytotoxicity of cadmium;[10,11] as the result, cadmium destroys the monolayer of vascular endothelial cells.[12] At noncytotoxic levels, cadmium stimulates the proliferation of vascular smooth muscle cells.[13] These results support the hypothesis that cadmium can directly promote the progression of atherosclerosis.

There is a strong interrelationship between atherosclerosis and fibrinolysis. For example, expression of PAI-1 is increased in atherosclerotic human arteries,[14–17] and it is suggested that the excess PAI-1 is involved in the progression of atherosclerosis.[18,19] In our previous study, it was shown that cadmium does not influence the secretion of t-PA but promotes that of PAI-1 in cultured human vascular endothelial cells.[20] However, it has been unclear whether or

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not the activity of either t-PA or u-PA is actually reduced by the excess accumulation of PAI-1 in the conditioned medium. In addition, it is also incompletely understood whether or not stimulation of the PAI-1 secretion by cadmium is a reflection of induction of the synthesis.

In the present study, we first determined the activity of t-PA and u-PA in the conditioned medium of vascular endothelial cells after exposure to cadmium and confirmed that the activity of both plasminogen activators were reduced by the metal. We next investigated the synthesis of t-PA, u-PA and PAI-1 in the cells. It was suggested that cadmium induces the synthesis of PAI-1 through activation of protein kinase C in vascular endothelial cells.

MATERIALS AND METHODS

Materials —— Vascular endothelial cells obtained from human umbilical vein (HUV-CE-C) and fibroblastic IMR-90 cells obtained from human fetal lung were from the American Type Culture Collection (Rockville, MD, U.S.A.); HuMedia-EG2, a growth medium for endothelial cells, and ASF 301 medium were from Kurabo (Osaka, Japan) and Ajinomoto (Tokyo, Japan), respectively; vascular smooth muscle cells derived from human aorta were from Kurabo; tissue culture dishes and plates were from Iwaki (Chiba, Japan); a commercially available enzyme immunoassay kit for PAI-1 and the standards of PAI-1 were from Biopool (Umea, Sweden); plasminogen rich fibrinogen was from Daiichi Chemicals (Tokyo, Japan); reverse transcriptase was from Takara (Kyoto, Japan); AmpliTaq DNA polymerase was from Perkin Elmer (Foster City, CA, U.S.A.); transfer membranes (Hybond N^+) were from Amersham (Little Chalfont, U.K.); 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrasolium bromide (MTT) was from Chemicon International (Temecula, CA, U.S.A.); human thrombin and actinomycin D were from Sigma (St. Louis, MO, U.S.A.); the lactate dehydrogenase kit and other reagents were from Wako Pure Chemical Industries (Osaka, Japan).

Cell Culture —— Vascular endothelial cells were cultured in HuMedia-EG2 in 24-well culture plates at 37°C in 5% CO_2 in air until confluent. The medium was discarded and the cell layer was washed twice with serum-free ASF 301 medium. The cell layer was then incubated at 37°C for 24 hr in fresh serum-free ASF 301 medium with cadmium chloride (0.5, 1 or 2 μM) in the presence or absence of actinomycin D (2 μM), H-7 (50 μM) or HA1004 (50 μM). After incubation, the conditioned medium was harvested and used for either fibrin zymography or the determination of PAI-1 by enzyme immunoassay. The cell layer was used for the MTT assay to determine the cell number. The secretion of PAI-1 was expressed as ng/10^6 cells.

Fibrin Zymography —— To examine the fibrinolytic activity in the conditioned medium of vascular endothelial cells after exposure to lead, electrophoretic fibrin zymography was performed according to the method of Matsuo *et al.*[21] Briefly, confluent cultures of endothelial cells were exposed to cadmium chloride (0.5, 1 or 2 μM) in serum-free ASF 301 medium. The conditioned medium was harvested and incubated at 37°C for 1 hr with 0.125 M Tris–HCl buffer containing 4% sodium dodecyl sulfate, 20% glycerol and 0.002% bromophenol blue. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the medium was performed on a 7.5% polyacrylamide slab gel with fibrin matrix and plasminogen with a 4.5% stacking gel. The slab gel was washed with 2.5% Triton X-100 for 1 hr and incubated at 37°C for 48 hr in 0.1 M glycine-NaOH buffer (pH 8.3). After incubation, the gel was stained with 0.1% Coomassie brilliant blue and destained with 7.5% acetic acid containing 5% methanol until the lytic zones became clear. The plasminogen-dependent protease activities were identified as t-PA and urokinase activities on the basis of the molecular weight of standard t-PA and urokinase.

Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) —— Poly(A)$^+$ RNA was isolated from confluent vascular endothelial and smooth muscle cells and fibroblasts after exposure to cadmium chloride (0.5, 1 or 2 μM) for 24 hr and analyzed by quantitative RT-PCR. PCR was conducted under quantitative conditions, which were determined by plotting signal intensities as functions of template amounts and of cycle numbers. Oligonucleotide primers were designed against human PAI-1, t-PA and u-PA mRNAs as follows: Sequences of the upstream and downstream primers and of the internal probe were 5′-ATGGGATTCAAGATTGATGA-3′, 5′-TCAGTATAGTTGAACTTGTT-3′ and 5′-AGAGAGCCAGATTCATCATCAAT-3′ [nucleotides (nt) 379–398, 811–830 and 584–606, respectively] for detecting PAI-1 mRNA; 5′-CGAAGGATTTGCTGGGAAGT-3′, 5′-TGCGGTTCTTCAGCACGTGG-3′ and 5′-TACGAGGACCAGGGCATCAG-3′ (nt 245–264, 746–765 and 294–

313, respectively), for detecting t-PA mRNA; and 5'-ACCACCATCGAGAACCAGCC-3', 5'-AATCAGCTTCACAACAGTCA-3' and 5'-CTGGTTTGCGGCCATCTACA-3' (nt 2494–2513, 4174–4193 and 2514–2533, respectively) for detecting u-PA mRNA. Sequences of primers and a probe for detecting β-actin mRNA were according to Ninomiya *et al*.[22] After RT-PCR, 6 μl of the reaction mixture was electrophoresed on a 2% agarose gel containing 0.1 μg/ml ethidium bromide, transferred to a Hybond-N^+ nylon membrane and the membrane was hybridized with the respective ^{32}P-end labeled probes. The amounts of poly(A)$^+$ RNA templates (30 ng) and cycle numbers for amplification (35 cycles for PAI-1 mRNA, 25 cycles for t-PA and u-PA mRNAs and 20 cycles for β-actin mRNA) were chosen in quantitative ranges.

Determination of Cadmium and Metallothionein and Evaluation of Nonspecific Cell Damage —— Confluent cultures of vascular endothelial cells in 6-well plates were incubated at 37°C for 24 hr in serum-free ASF 301 medium in the presence of cadmium chloride (0.5, 1 or 2 μM). The medium was then harvested and analyzed for the activity of lactate dehydrogenase leaked from the cells as a marker of nonspecific cell damage. The cell layer was washed twice with Ca, Mg-free phosphate-buffered saline and was scraped off with a rubber policeman in the presence of 0.75 ml 0.25 M sucrose. After collecting the cell suspension, the well was washed with 0.75 ml 0.25 M sucrose and the wash was combined with the cell suspension. The cell homogenate was prepared by sonication and an aliquot was used for the direct determination of cadmium content by flameless atomic absorption spectrophotometry. A portion of the cell homogenate was analyzed for metallothionein content by cadmium-hemoglobin assay[23] with some modifications as follows: the cell homogenate (0.5 ml) was transferred into microtubes and mixed with 0.5 ml 0.1 M Tris–HCl buffer solution (pH 8.0). After addition of 50 μl of 10 μg/ml cadmium chloride and 0.1 ml of 2% bovine hemoglobin, the mixture was boiled for 2 min and centrifuged at $10000 \times g$ for 5 min. The addition of hemoglobin, the boiling and the centrifugation were repeated three times. The supernatant was analyzed for cadmium content by flameless atomic absorption spectrophotometry and the metallothionein content was calculated. Another portion of the cell homogenate was analyzed for DNA content by fluorometric assay.[24]

Statistical Analysis —— Data on the accumulation of PAI-1, cadmium and metallothionein, and the leakage of lactate dehydrogenase were analyzed for statistical significance by analysis of variance. $p < 0.05$ was considered a statistically significant difference.

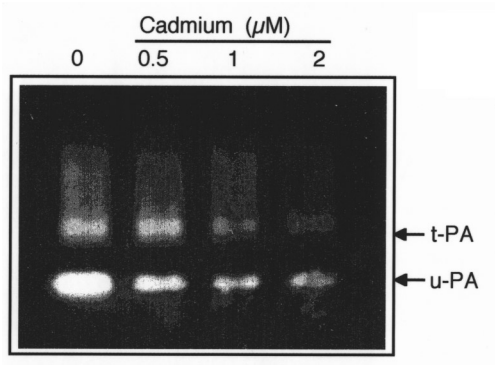

Fig. 1. Electrophoretic Enzymography (Fibrin Zymography) of the Conditioned Medium of Cultured Vascular Endothelial Cells after Exposure to Cadmium

Confluent cultures of human umbilical vein endothelial cells were incubated at 37°C for 24 hr in the presence of cadmium chloride (0.5, 1, or 2 μM).

RESULTS

We have reported that cadmium stimulates the secretion of PAI-1 but not that of t-PA from vascular endothelial cells.[20] Based on this result, we first confirmed whether or not the increased accumulation of PAI-1 actually results in a reduction of plasminogen activator activity in the conditioned medium using fibrin zymography. As shown in Fig. 1, the activity of either t-PA or u-PA was reduced by cadmium after a 24-hr incubation in a dose-dependent manner. This suggests that cadmium-induced PAI-1 secretion actually results in a reduction of plasminogen activator activity of vascular endothelial cells.

To address the question of whether cadmium induces the synthesis of PAI-1 in endothelial cells, steady state levels of PAI-1 mRNA were determined by quantitative RT-PCR (Fig. 2). It was shown that cadmium increased the PAI-1 mRNA level after a 24-hr incubation in a dose–dependent manner. However, the level of both t-PA mRNA and u-PA mRNA was unaffected by cadmium, suggesting that the metal selectively induces the synthesis of PAI-1 in vascular endothelial cells. On the other hand, cadmium failed to increase the PAI-1 mRNA level in either vascular smooth muscle cells or fibroblastic IMR-90 cells (Fig. 3). Thus, it is suggested that vascular endothelial cells are a unique cell type of which

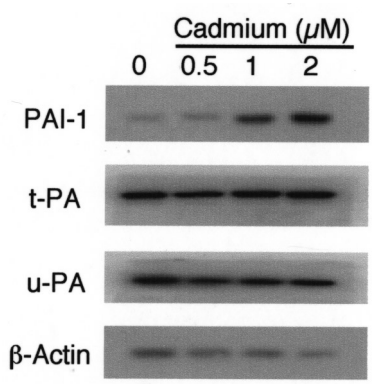

Fig. 2. Quantitative RT-PCR Analysis of PAI-1, t-PA and u-PA mRNAs in Vascular Endothelial Cells after Exposure to Cadmium

Confluent cultures of human umbilical vein endothelial cells were incubated at 37ºC for 24 hr in the presence of cadmium chloride (0.5, 1, or 2 μM).

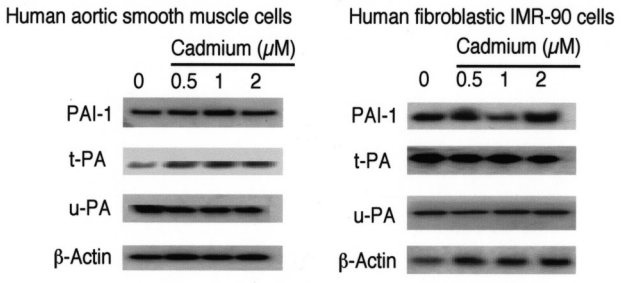

Fig. 3. Quantitative RT-PCR Analysis of PAI-1, t-PA and u-PA mRNAs in Vascular Smooth Muscle Cells and Fibroblasts after Exposure to Cadmium

Confluent cultures of human aortic smooth muscle cells and human fibroblastic IMR-90 cells were incubated at 37ºC for 24 hr in the presence of cadmium chloride (0.5, 1, or 2 μM).

PAI-1 synthesis is induced by cadmium.

Since it was suggested that cadmium induces the synthesis of PAI-1 at the transcriptional level in the cells, the PAI-1 secretion from the cells was investigated in the presence of actinomycin D that is an inhibitor of mRNA synthesis. It was shown that cadmium significantly stimulated the PAI-1 secretion but the stimulation disappeared in the presence of actinomycin D (Table 1), suggesting that the stimulatory effect of cadmium on the PAI-1 synthesis occurs at the transcriptional level.

It has been reported that protein kinase C activation mediates the up-regulation of PAI-1 synthesis in vascular endothelial cells.[25,26] Cadmium-induced increase in the PAI-1 secretion from the cells was diminished by H-7 which is an inhibitor of protein kinase C but not by HA1004 which is a structural analogue of H-7 with a low inhibitory activity against protein kinase C (Table 2); this suggests that the stimulatory effect of cadmium on endothelial PAI-1 synthesis is mediated by protein kinase C activation.

Table 3 shows the intracellular accumulation of cadmium and metallothionein and the leakage of lactate dehydrogenase in vascular endothelial cells. Cadmium and metallothionein accumulated within the cells in a dose–dependent manner, however, the

Table 1. Effect of Actinomycin D on the Accumulation of PAI-1 in the Conditioned Medium of Vascular Endothelial Cells after Exposure to Cadmium

	PAI-1 accumulation (μg/10^6 cells)	
	None	Actinomycin D
Control	0.746 ± 0.038	0.676 ± 0.036
Cadmium	0.905 ± 0.014	0.653 ± 0.012

Confluent cultures of human umbilical vein endothelial cells were incubated at 37°C for 24 hr with cadmium (1 μM) in the presence or absence of actinomycin D (2 μM). Values are means ± S.E. of six samples. The effect of cadmium on the PAI-1 accumulation in the absence of actinomycin D is significant ($p < 0.01$).

Table 2. Effect of H-7 and HA1004 on the Accumulation of PAI-1 in the Conditioned Medium of Vascular Endothelial Cells after Exposure to Cadmium

	PAI-1 accumulation (μg/10^6 cells)		
	None	H-7	HA1004
Control	0.604 ± 0.023	0.147 ± 0.007	0.704 ± 0.027
Cadmium	0.840 ± 0.023	0.153 ± 0.020	0.864 ± 0.031

Confluent cultures of human umbilical vein endothelial cells were incubated at 37°C for 24 hr with cadmium (1 μM) in the presence or absence of H-7 or HA1004 (50 μM). Values are means ± S.E. of six samples. The effect of cadmium on the PAI-1 accumulation in the absence of inhibitors is significant ($p < 0.01$), as its effect on the accumulation in the presence of HA1004 ($p < 0.05$). The effect of H-7 on cadmium-induced increase in the PAI-1 accumulation is also significant ($p < 0.05$).

Table 3. Intracellular Accumulation of Cadmium and Metallothionein in Vascular Endothelial Cells and Leakage of Lactate Dehydrogenase from the Cells after Exposure to Cadmium

	Cadmium (pmol/μg DNA)	Metallothionein (ng/μg DNA)	LDH (IU/L)
Control	N.D.	2.47 ± 0.25	2.55 ± 0.10
0.5 μM Cadmium	149 ± 5	4.45 ± 0.61	2.21 ± 0.14
1 μM Cadmium	167 ± 4	19.41 ± 1.34	2.06 ± 0.17
2 μM Cadmium	179 ± 8	33.39 ± 1.65	2.17 ± 0.12

Confluent cultures of human umbilical vein endothelial cells were incubated at 37°C for 24 hr with cadmium (0.5, 1 or 2 μM). Values are means ± S.E. of five samples. The effect of cadmium on the accumulation of intracellular cadmium and metallothionein is significant ($p < 0.01$).

lactate dehydrogenase leakage was unaffected by cadmium. This result suggests that intracellular cadmium influences PAI-1 synthesis but does not exhibit cytotoxicity by induction of metallothionein in the vascular endothelial cells used in the present study.

DISCUSSION

Although both t-PA and u-PA have fibrinolytic activity to convert plasminogen to plasmin that degrade fibrin, it has been suggested that these two fibrinolytic proteins have different physiological functions. The activity of t-PA is markedly enhanced upon binding to fibrin[4,5] whereas u-PA lacks fibrin binding capacity, circulates in the blood and is found in large quantities in human urine.[27] This difference in fibrin binding capacity suggests that t-PA is mainly involved in fibrinolysis whereas the main role of u-PA is to regulate the metabolism of extracellular matrix. Nevertheless, it can safely be said that u-PA as well as t-PA can contribute to the fibrinolytic activity in the blood. In the present study, we determined the activity of both t-PA and u-PA by fibrin zymography. In the experiments, polyacrylamide slab gel contained fibrin and plasminogen, so that plasminogen activators converted plasminogen to plasmin and then degraded fibrin. It was shown that cadmium, in fact, reduced the activity of either t-PA or u-PA in the conditioned medium of vascular endothelial cells by induction of PAI-1 synthesis at the transcriptional level. The results support the hypothesis that pathogenesis of cadmium-induced vascular lesions such as atherosclerosis includes a reduction in fibrinolytic activity and an increase in PAI-1, which are involved in the progression of this condition,[14–19] derived from vascular endothelial cells after exposure to the metal.

It was also suggested that cadmium-induced PAI-1 synthesis is mediated by the protein kinase C pathway. It is unclear whether cadmium can activate protein kinase C in vascular endothelial cells or not. However, in other cell types including NRK-49F cells,[28] mouse 3T3/10T1/2 fibroblasts,[29] rat osteosarcoma (ROS 17/2.8) cells[30] and PC12 cells,[31] it has been suggested that cadmium can activate protein kinase C, although it is also possible that the metal may directly inhibit the kinase.[32] We postulate that endothelial protein kinase C can be activated by cadmium and then mediates the induction of PAI-1 synthesis in the cells. On the other hand, activation of protein kinase C in general results in an induction of both t-PA and PAI-1 synthesis. For example, phorbol ester,[25,26] diacylglycerol[26] and thrombin[26,33] activate protein kinase C and induce the synthesis of both t-PA and PAI-1. The mechanism by which cadmium induces the synthesis of PAI-1 alone remains to be elucidated. Since histamine stimulates the secretion of t-PA but not PAI-1,[34] it is suggested that there may be a downstream regulatory system of protein kinase C activation in the t-PA and PAI-1 synthesis; cadmium may act on the putative system.

Lead, as well as cadmium, is a unique heavy metal that inhibits not only the proliferation of vascular endothelial cells[35,36] but also the fibrinolysis derived from the cells. Cadmium stimulates the synthesis and secretion of PAI-1 as shown in the present study, while lead inhibits the synthesis and secretion of t-PA[37,38]; thus both heavy metals reduce the fibrinolytic activity of endothelial cells. It was suggested that stimulation of the PAI-1 synthesis by cadmium is mediated by the protein kinase C-dependent pathway whereas the inhibition of the t-PA synthesis by lead is mediated by the cyclic adenosine 5′-monophosphate (AMP)-dependent pathway.[37] On the other hand, cadmium and lead show different effects on the secretion of fibrinolytic proteins in vascular smooth muscle cells and fibroblasts.[39,40] Thus, it is suggested that the effects of heavy metals on the synthesis and secretion of fibrinolytic proteins depend on either the kind of metal or the cell type, although the mechanisms are unclear. The present data support the concept that cadmium is a particular heavy metal that induces PAI-1 synthesis in vascular endothelial cells, and that the cells are a particular type of which PAI-1 synthesis is induced by cadmium.

In conclusion, the present study confirmed that cadmium-induced PAI-1 synthesis results in a reduction of fibrinolytic activity derived from either t-PA or u-PA in vascular endothelial cells. In addition, the data suggest that induction of PAI-1 synthesis by cadmium is mediated by the protein kinase C pathway at the transcriptional level. Further studies should be performed to clarify (1) the mechanisms by which stimulation of protein kinase C selectively mediates endothelial PAI-1 synthesis but not the t-PA or u-PA synthesis in vascular endothelial cells, (2) the mechanisms governing the different effects of cadmium and lead on the synthesis of fibrinolytic proteins in vascular endothelial cells, and (3) the mechanisms by which different cell types

differently respond to either cadmium or lead in relation to the synthesis of fibrinolytic proteins.

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