Effect of Lead on the Synthesis of Tissue Plasminogen Activator by Vascular Endothelial Cells in Culture

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To investigate the effect of lead on the synthesis of fibrinolytic proteins by vascular endothelial cells, cells obtained from human umbilical vein were exposed to the metal in a culture system. It was found that the secretion of tissue plasminogen activator (t-PA) is inhibited by lead but that of plasminogen activator inhibitor type 1 (PAI-1) was unaffected by the metal. However, gene expression of t-PA as well as PAI-1 was unaffected by lead when evaluated by quantitative reverse transcription-polymerase chain reaction. The inhibition of t-PA secretion by lead was observed even in the presence of actinomycin D, an inhibitor of mRNA synthesis. The inhibition of t-PA secretion by lead was also observed in the presence of the adenylate cyclase activator forskolin but was suppressed and disappeared in the presence of either 8-bromo adenosine 3′, 5′-cyclic monophosphate that is a congener of adenosine 3′, 5′-cyclic monophosphate which is resistant to degradation by phosphodiesterase or the phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine. On the other hand, both the incorporation of [^{14}C]leucine into the acid-insoluble fraction of endothelial cells and the leakage of lactate dehydrogenase from the cells were unchanged by lead, indicating that the metal did not cause inhibition of general protein synthesis and nonspecific cell damage. The present data suggest that lead selectively inhibits t-PA synthesis by vascular endothelial cells at the post-transcriptional level and the inhibition is mediated by activation of the cyclic AMP-dependent pathway as a result of a lower activity of phosphodiesterase.

Key words —— endothelial cell, lead, tissue plasminogen activator, plasminogen activator inhibitor type 1, fibrinolysis

INTRODUCTION

Vascular endothelial cells overspread the inner surface of blood vessels in a monolayer and play an important role in the regulation of the blood coagulation-fibrinolytic system. The cells in normal blood vessels exhibit antithrombogenic properties by synthesizing and secreting anticoagulant and fibrinolytic substances such as prostacyclin[1] which potently inhibits platelet aggregation, anticoagulant heparan sulfate,[2] thrombomodulin[3] and plasminogen activators.[4] Fibrinolysis in normally circulating blood depends on the balance between tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1) secreted from vascular endothelial cells[5]; t-PA is bound to fibrin[6] and converts plasminogen to plasmin which degrades fibrin.

Lead is a heavy metal which has been experimentally and epidemiologically shown to induce vascular lesions including atherosclerosis and hypertension.[7-9] Since atherosclerosis, hypertension and thrombosis have a strong interrelationship and an involvement of endothelial injury in the pathogenesis of these vascular lesions is postulated, we have investigated the functional damage of vascular endothelial cells caused by lead using a cell culture system. It was shown that lead inhibits the recovery of the wounded monolayer of the cells[10] through inhibition of their proliferation.[11] In addition, lead was shown to decrease the synthesis of heparan sulfate chains bound to a large core protein, probably

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perlecan core.[12,13])

We have also found that lead decreases the secretion of t-PA by vascular endothelial cells.[14]) In our recent study, it was shown that lead perturbs the secretion of not only t-PA but also PAI-1 in vascular smooth muscle cells and fibroblasts,[15]) suggesting that the secretion of PAI-1 by vascular endothelial cells may be also affected by the metal. Since only a negligible amount of t-PA and PAI-1 is bound to the cell layer, secretion of the fibrinolytic proteins is postulated to reflect their synthesis. It appears to be possible that the inhibition of endothelial t-PA synthesis by lead occurs at the transcriptional and/or the post-transcriptional level. In addition, it is also possible that the adenosine 3′, 5′-cyclic monophosphate (cyclic AMP) pathway is involved in the inhibition of endothelial t-PA synthesis since the second messenger mediates the suppressive regulation of t-PA synthesis.[16])

The present study was undertaken to address these questions from the viewpoint of the toxic effect of lead on the regulation of vascular endothelial cell functions.

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 American Type Culture Collection (Rockville, MD, U.S.A.); HuMedia-EG2, a growth medium for the 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 (Osaka, Japan); tissue culture dishes and plates were from Iwaki (Chiba, Japan); commercially available enzyme immunoassay kit for t-PA and PAI-1 and the standards of t-PA and 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, CA, U.S.A.); Transfer membranes (Hybond N^+) were from Amersham (Little Chalfont, U.K.); L-[^{14}C(U)]leucine (10.8 GBq/mmol) was from New England Nuclear (Boston, MA, U.S.A.); 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrasolium bromide (MTT) was from Chemicon International (Temecula, CA, U.S.A.); thrombin, actinomycin D, 8-bromo cyclic AMP, forskolin and 1-methyl-3-isobutylxanthine (IBMX) were from Sigma (St. Louis, MO, U.S.A.); the lactate dehydrogenase kit and other reagents were from Wako Pure Chemical Industries (Osaka, Japan).

Determination of t-PA and PAI-1 —— Vascular endothelial cells were cultured in HuMedia-EG2 in 24-well culture plates at 37℃ 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 incubated at 37℃ for 24 h in fresh serum-free ASF 301 medium in the presence of lead chloride (2, 5 or 10 μM) combined with or without actinomycin D (2 μM), 8-bromo cyclic AMP (10 mM), forskolin (100 μM) or IBMX (100 μM). After incubation, the conditioned medium was harvested and used for the determination of t-PA and PAI-1 by enzyme immunoassay. The cell layer was used for the MTT assay to determine the cell number. The secretion of t-PA and PAI-1 was expressed as ng/10^6 cells.

Fibrin Zymography —— To examine the fibrinolytic activity of the liquid phase of vascular endothelial cells after exposure to lead, electrophoretic fibrin zymography was performed according to the method of Matsuo *et al.*[17]) Briefly, confluent cultures of endothelial cells were exposed to lead chloride (1, 2 or 5 μM) in the serum-free ASF 301 medium. The conditioned medium was harvested and incubated at 37℃ for 1 h 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 h and incubated at 37℃ for 48 h 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[18]) from confluent cultures of vascular endothelial cells after exposure to lead chloride (1, 2 or 5 μM) for 24 h and analyzed by quantitative RT-PCR.[19]) PCR was conducted under quantitative conditions, which were determined by plotting signal intensities as functions of template amounts and of cycle num-

bers. Oligonucleotide primers were designed against human t-PA and PAI-1 mRNAs as follows: Sequences of the upstream and downstream primers and of the internal probe were 5′-CGAAGGATT-TGCTGGGAAGT-3′, 5′-TGCGGTTCTTCAGCACG-TGG-3′ and 5′-TACGAGGACCAGGGCTACAG-3′, respectively, for detecting t-PA mRNA[20] and 5′-ATGGGATCAAGATTGATGA-3′, 5′-TCAGTATA-GTTGAACTTGTT-3′ and 5′-AGAGAGCCAGATT-CATCAAT-3′, respectively for detecting PAI-1 mRNA.[21] After quantitative RT-PCR, an aliquot of the reaction mixture was electrophoresed on a 2% agarose gel containing 0.1 μg/ml ethidium bromide. Size of the PCR products for t-PA and PAI-1 was 521 and 452 base pairs, respectively. Sequences of primers and a probe for detecting β-actin mRNA were according to Ninomiya *et al.*[22]

Protein Synthesis and Nonspecific Cell Damage——Confluent cultures of vascular endothelial cells in 6-well plates were incubated at 37℃ for 24 h in serum-free ASF 301 medium in the presence of lead chloride (1, 2 or 5 μM) and labeled with [^{14}C]leucine (15 kBq/ml) during the last 3 h of the incubation. The medium was then harvested and analyzed for the activity of lactate dehydrogenase leaked from the cells. The cell layer was washed twice with Ca, Mg-free phosphate-buffered saline (CMF-PBS) and was scraped off with a rubber policeman in the presence of CMF-PBS. After collecting the cell suspension, the well was washed with CMF-PBS and the wash was combined with the cell suspension. The cell homogenate was prepared by sonication and the incorporation of the radioactivity into the 5% trichloroacetic acid-insoluble fraction of the cell homogenate was determined by liquid scintillation counting. A portion of the cell homogenate was analyzed for DNA content by the fluorometric assay.[23]

RESULTS

Fig. 1 shows the secretion of t-PA and PAI-1 by vascular endothelial cells after exposure to lead. The secretion of t-PA was significantly decreased but that of PAI-1 was unaffected by lead, indicating that the metal selectively inhibits the t-PA synthesis in this cell type.

Fig. 2 shows the fibrin zymography of the conditioned medium of endothelial cells after exposure to lead. The activity of t-PA was reduced by lead at 5 μM, however, that of urokinase was unaffected by the metal.

Fig. 3 shows the steady-state levels of t-PA and PAI-1 mRNAs of vascular endothelial cells after exposure to lead. Lead failed to change the

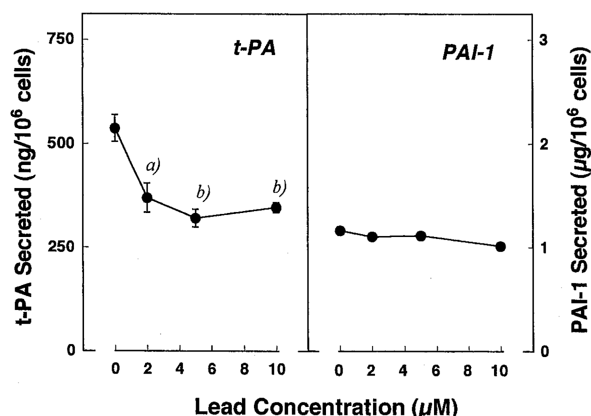

Fig. 1. Secretion of t-PA and PAI-1 from Vascular Endothelial Cells after Exposure to Lead

Confluent cultures of human umbilical vein endothelial cells were incubated at 37℃ for 24 h in the presence of lead chloride (1, 2 or 5 μM). Values are means ± S.E. of six samples. Significantly different from the corresponding control, *a*) $p<0.01$; *b*) $p<0.001$.

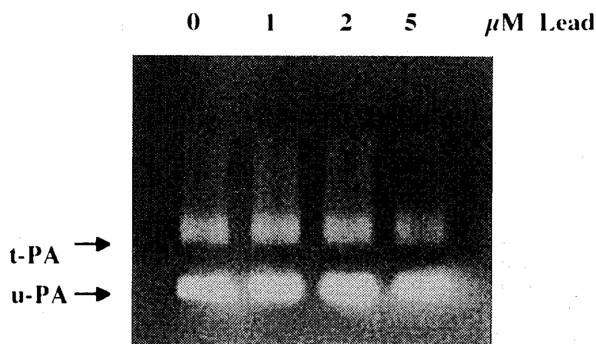

Fig. 2. Electrophoretic Enzymography of the Conditioned Medium of Cultured Vascular Endothelial Cells after Exposure to Lead

Confluent cultures of human umbilical vein endothelial cells were incubated at 37℃ for 24 h in the presence of lead chloride (1, 2 or 5 μM).

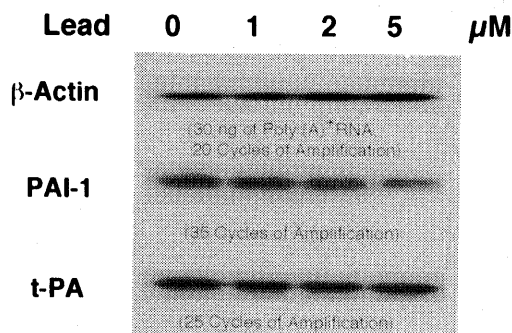

Fig. 3. Quantitative RT-PCR Analysis of t-PA and PAI-1 mRNAs in Vascular Endothelial Cells after Exposure to Lead

Confluent cultures of human umbilical vein endothelial cells were incubated at 37℃ for 24 h in the presence of lead chloride (1, 2 or 5 μM).

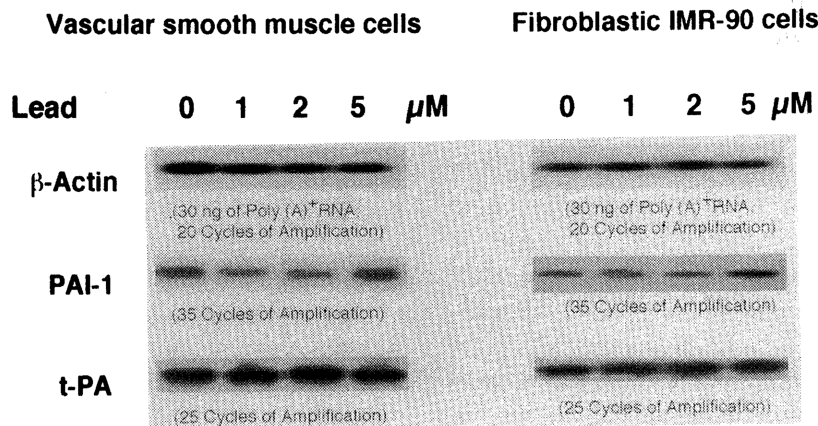

Fig. 4. Quantitative RT-PCR Analysis of t-PA and PAI-1 mRNAs in Vascular Smooth Muscle Cells (left) and Fibroblasts (right) after Exposure to Lead

Confluent cultures of human aortic smooth muscle cells and human fibroblastic IMR-90 cells were incubated at 37℃ for 24 h in the presence of lead chloride (1, 2 or 5 μM).

Table 1. Secretion of t-PA from Vascular Endothelial Cells after Exposure to Lead in the Presence or Absence of Actinomycin D

	t-PA secreted ($ng/10^6$ cells)
Absence of Actinomycin D	
Control	764 ± 30
Lead	641 ± 13[b]
Presence of Actinomycin D	
Control	797 ± 25
Lead	722 ± 16[a]

Confluent cultures of human umbilical vein endothelial cells were incubated at 37℃ for 24 h with lead chloride (5 μM) in the presence or absence of actinomycin D (2 μM). Values are means ± S.E. of six samples. Significantly different from the corresponding control, *a)* $p<0.05$; *b)* $p<0.01$.

level of t-PA mRNA as well as PAI-1 mRNA, suggesting that inhibition of t-PA synthesis by the metal is not due to a lower expression of t-PA gene but occurs at the post-transcriptional level.

It was shown that the secretion of t-PA by vascular smooth muscle cells and fibroblasts is inhibited by lead ; on the other hand, the secretion of PAI-1 is inhibited in vascular smooth muscle cells but promoted in fibroblasts after exposure to the metal.[15] Based on these previous results, the steady-state level of t-PA mRNA in these cell types after exposure to lead was also examined. However, as shown in Fig. 4, the level of t-PA and PAI-1 mRNAs was unaffected by lead in both vascular smooth muscle cells and fibroblasts.

Table 1 shows the secretion of t-PA by vascular endothelial cells after exposure to lead in the presence or absence of actinomycin D, an

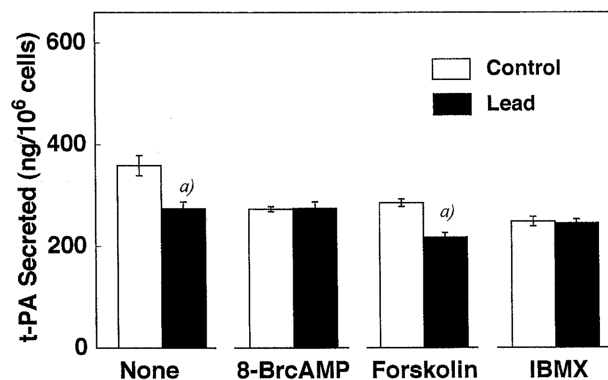

Fig. 5. Secretion of t-PA and PAI-1 from Vascular Endothelial Cells after Exposure to Lead in the Presence of Agents That Increase Intracellular Cyclic AMP

Confluent cultures of human umbilical vein endothelial cells were incubated at 37℃ for 24 h in the presence of lead chloride (5 μM) combined with or without 8-bromo cyclic AMP (10 mM), forskolin (100 μM) or IBMX (100 μM). Values are means ± S.E. of six samples. *a)* Significantly different from the corresponding control, $p<0.01$.

inhibitor of mRNA synthesis. Lead significantly decreased the t-PA secretion even in the presence of the inhibitor, supporting the hypothesis that inhibition of t-PA synthesis by the metal occurs at the post-transcriptional level.

Since the protein kinase C pathway mediates the promotion of endothelial synthesis of t-PA[24] and PAI-1[25] whereas the cyclic AMP pathway mediates the suppression of the synthesis,[16] the latter pathway is a candidate as the target for the inhibitory effect of lead on the synthesis of t-PA by the cells. Fig. 5 shows the secretion of t-PA by vascular endothelial cells after exposure to lead in the presence or absence of 8-bromo cyclic AMP, forskolin or IBMX. A lead-induced decrease in t-PA secretion was observed in the

Table 2. Incorporation of [^{14}C]Leucine into the Acid-insoluble Fraction and the Leakage of Lactate Dehydrogenase in Vascular Endothelial Cells after Exposure to Lead

	[^{14}C]Leucine incorporation (dpm/μg DNA)	Lactate dehydrogenase leakage (I.U./l)
Control	1886 ± 94	8.84 ± 0.17
Lead (1 μM)	1818 ± 155	6.48 ± 0.31
Lead (2 μM)	1975 ± 138	5.89 ± 0.73
Lead (5 μM)	1864 ± 107	9.38 ± 0.12

Confluent cultures of human umbilical vein endothelial cells were incubated at 37℃ for 24 h with lead chloride (1, 2 or 5 μM) and labeled with [^{14}C]leucine during the last 3 h of the incubation. Values are means ± S.E. of five samples.

presence of forskolin but disappeared in the presence of either 8-bromo cyclic AMP or IBMX.

The [^{14}C]leucine incorporation and the lactate dehydrogenase leakage after exposure to lead were determined as a marker of general protein synthesis and nonspecific cell damage, respectively. As shown in Table 2, both markers were unaffected by lead, suggesting that inhibition of t-PA synthesis by the metal is due to neither nonspecific inhibition of protein synthesis nor cytotoxicity of the metal.

DISCUSSION

Vascular endothelial cells synthesize and secrete two fibrinolytic proteins ; one is t-PA and the other is urokinase. Both t-PA and urokinase are capable of converting plasminogen to plasmin, however, urokinase has been implicated in processes involving tissue remodelling and tissue destruction, while t-PA appears to be active primarily in thrombolysis since t-PA has the capacity to bind fibrin and its activity is greatly potentiated in the presence of fibrin[26] whereas urokinase lacks fibrin binding capacity and circulates in blood.[27] On the other hand, PAI-1 associated with endothelium rapidly inhibits both t-PA and urokinase, while the protein released into the liquid phase has little anti-fibrinolytic activity.[28] Thus, fibrinolytic activity in the liquid phase mediated by vascular endothelial cells can be strongly dependent on the synthesis and secretion of t-PA by the cells. However, in our previous study, we showed that cadmium decreases the fibrinolytic activity in the conditioned medium of vascular endothelial cells through inhibition of PAI-1 secretion.[29] Although lead was shown to inhibit the secretion of t-PA by vascular endothelial cells,[14] it has been unclear whether the secretion of PAI-1 and urokinase is also affected by the metal or not. In the present study, it was indicated that t-PA is a particular fibrinolytic protein whose synthesis is inhibited by lead ; the activity of t-PA but not that of urokinase was reduced by the metal. It is thus postulated that lead diminishes the fibrinolytic activity in the liquid phase on vascular endothelium through inhibition of t-PA synthesis by endothelial cells.

However, the steady-state level of t-PA mRNA was unchanged by lead in all cell types tested, suggesting that lead exhibits its toxicity on the synthesis of t-PA at the post-transcriptional level regardless of cell type-related differences. This hypothesis was based on the result that lead could inhibit the secretion of t-PA by vascular endothelial cells even in the presence of actinomycin D. In our recent study (submitted), it was confirmed that cadmium significantly increases the steady-state level of PAI-1 mRNA in vascular endothelial cells, suggesting that cadmium may induce PAI-1 synthesis at the transcriptional level and/or inhibit PAI-1 mRNA. Our study indicates that both cadmium and lead diminish the fibrinolytic activity in the liquid phase of vascular endothelial cells in different manners. In other words, cadmium and lead are toxic heavy metals which can cause vascular disorders such as atherosclerosis[7,30] but the mechanism of their toxic effect on endothelial regulation of fibrinolysis is different ; cadmium may act on the expression of PAI-1 gene and/or the degradation of PAI-1 mRNA whereas lead acts on the post-transcriptional process of t-PA synthesis.

Cyclic AMP is a second messenger which mediates the regulation of vascular endothelial

cell functions. The synthesis of t-PA is suppressed when the intracellular level of cyclic AMP rises.[16] Since lead failed to inhibit the secretion of t-PA by endothelial cells in the presence of 8-bromo cyclic AMP, the second messenger was suggested to mediate the inhibitory effect of the metal on the t-PA synthesis. Although lead inhibited t-PA secretion even in the presence of the adenylate cyclase activator forskolin but not in the presence of the phosphodiesterase inhibitor IBMX, suggesting that lead does not promotes the synthesis of the second messenger but inhibits the degradation of cyclic AMP. Although the possibility that protein kinase C was inhibited by lead and mediated the decrease in t-PA synthesis cannot be excluded, an assumption can be made that the increase in intracellular cyclic AMP by lead through inhibition of phosphodiesterase may be mediated by the suppression of t-PA synthesis by the metal. Both lead and cyclic AMP inhibit the proliferation of vascular endothelial cells[11,31] and the synthesis of glycosaminoglycans,[12,32] partly supporting the involvement of the cyclic AMP pathway in the inhibition of t-PA synthesis by lead.

The present data suggest the following effects of lead on the regulation of fibrinolytic protein synthesis by vascular endothelial cells: 1) Lead inhibits the synthesis of t-PA but not urokinase and PAI-1, resulting in a lower t-PA activity in the liquid phase. 2) Lead does not affect the steady-state level of t-PA mRNA and acts on the post-transcriptional level in the process of t-PA synthesis in vascular endothelial cells, vascular smooth muscle cells and fibroblast. 3) Lead inhibits the synthesis of t-PA *via* the intracellular cyclic AMP pathway as a result of lower degradation activity of the second messenger. On the other hand, lead not only induces functional damage of vascular endothelial cells but also enhances the cytotoxicity of cadmium.[33] In addition, lead promotes the proliferation of vascular smooth muscle cells,[34] suggesting that the metal can contribute to the intimal thickening of atherosclerosis. Taken together, lead is considered to contribute to the formation of vascular lesions such as atherosclerosis by the direct action on vascular cells and our present data will be useful for interpretation of the cellular mechanism.

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