Oxidized low-density lipoprotein impairs the anti-coagulant function of tissue-factor-pathway inhibitor through oxidative modification by its high association and accelerated degradation in cultured human endothelial cells

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We have examined whether oxidized low-density lipoprotein (ox-LDL) affects the function of tissue-factor-pathway inhibitor (TFPI), an anti-coagulant regulator in the extrinsic pathway of coagulation, in cultured human umbilical vein endothelial cells (HUVEC). Treatment of culture medium of HUVEC with ox-LDL, but not with native or acetylated LDLs, drastically decreased the reactivity of TFPI to its antibody specific for Kunitz domain 1 or one specific for the conformation between Kunitz 1 and 2 of TFPI, and caused a rapid, concentration-dependent decrease in the functional activity of TFPI to inhibit Factor X activation. When 5 ng of recombinant TFPI (rTFPI) was mixed with 10 μg of ox-LDL for 30 min, almost all of the rTFPI was detected in the ox-LDL fraction and no free rTFPI was observed on non-denaturing PAGE, in contrast with the virtual absence of rTFPI in the native LDL fraction. Ox-LDL decreased the antigen level of TFPI in the lysate of HUVEC in a time-dependent manner. It did not affect the mRNA level, but ox-LDL-dependent reduction of the TFPI antigen level in HUVEC was reversed by the simultaneous treatment of ox-LDL with bafilomycin A1, an inhibitor of the lysosomal proton pump. These results indicate that ox-LDL lessens the anti-coagulant function of TFPI through both oxidative modification and accelerated degradation of the molecule outside and inside HUVEC respectively.

Key words: atherosclerosis, Factor X, lactacystin, lysophosphatidic acid.

INTRODUCTION

Vascular endothelial cells have a crucial function in the regulation of thrombosis and haemostasis. Recent studies have shown that the extrinsic pathway of blood coagulation has a role in the progression and clinical expression of atherosclerosis. Tissue-factor-pathway inhibitor (TFPI), a Kunitz-type protease inhibitor, is the major physiological inhibitor of the extrinsic coagulation system through the direct inhibition of Factor X (FX) activation and the subsequent formation of an activated FX (FXa)-dependent quaternary FXa/TFPI/FVIIa/tissue factor (TF) complex that inactivates FVIIa/TF [1–4]. In 1987, Bajaj et al. [5] reported that TFPI is synthesized mainly in endothelial cells and that the antigen is present in the culture medium of human umbilical-vein endothelial cells (HUVEC). They also found a high expression of TFPI mRNA in endothelial cells and suggested that plasma TFPI was derived from endothelial cells [6]. Most circulating TFPI has been found to be associated with plasma lipoproteins, especially low-density lipoprotein (LDL) [7,8].

Oxidized LDL (ox-LDL) is thought to be important in atherogenesis [9–11] and it exhibits a wide variety of biological properties such as alterations of coagulation pathways, the formation of foam cells and fatty streaks, the induction of gene expression, and arterial vasomotor properties [12]. Ox-LDL has been found in atherosclerotic plaques and the expression of TF has also been observed in ruptured atherosclerotic plaques, where it might trigger fibrin formation and thrombosis [13]. It has been reported that ox-LDL induces the expression of TF [14] and we have shown that ox-LDL decreases the expression of thrombomodulin (TM) [15], an anti-coagulant protein C activator localized on the surface of endothelial cells. Crawley et al. [16] reported that particularly high TFPI expression was detected in macrophages located on the rim of the lipid-rich core of atherosclerotic plaques, where it was co-localized with TF; they suggested a role of TFPI in the local down-regulation of TF-mediated procoagulant activity. Therefore the activity of TFPI at the site of local endothelial cells seems to be important in controlling the level of endothelial thrombin potential via the extrinsic pathway, especially in a procoagulant environment.

The present study was designed to determine whether or not the increase in thrombosis of atherosclerotic lesions is associated in part with a decrease in TFPI activity. We investigated the effect of ox-LDL on the interactions of TFPI and its synthesis and degradation in HUVEC. We found that TFPI associated quite rapidly with ox-LDL but not with native LDL, and that the ox-LDL-associated form of TFPI lost the ability to interact with some monoclonal antibodies specific for the Kunitz 1 domain of TFPI, the conformation between the Kunitz 1 and 2 domains, and the Kunitz 3 domain. It is supposed that ox-LDL modulates the structure of TFPI through an oxidative process and simultaneously decreases the catalytic function to inhibit FX

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; FX, Factor X; FXa, activated Factor X; HUVEC, human umbilical-vein endothelial cells; TFPI, tissue-factor-pathway inhibitor; h-rTFPI, human recombinant TFPI; LDL, low-density lipoprotein; lyso-PA, lysophosphatidic acid; lyso-PtdCho, lysophosphatidylcholine; ox-LDL, oxidized low-density lipoprotein; ox-PtdCho, oxidized phosphatidylcholine; TBARS, thiobarbituric-acid-reactive substances; TF, tissue factor; TM, thrombomodulin.

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activation on the outside of HUVEC. Within the cells, in contrast, ox-LDL accelerates TFPI degradation through the lysosomal pathway in HUVEC.

EXPERIMENTAL

Materials

TFPI was purified from culture medium of Hep G_2 cells by precipitation with $CdCl_2$ followed by chromatography on DEAE-Sephacel, heparin-agarose and anti-($TFPI_{239–265}$-IgG-Sepharose) in the presence of protease inhibitors (1 mM benzamidine hydrochloride, 0.1 mM PMSF and 20 units/ml aprotinin). Human recombinant TFPI (h-rTFPI) was donated by the Chemo-Sero-Therapeutic Research Institute (CSTRI), Kumamoto, Japan. Polyclonal antibody against full-length TFPI and a monoclonal antibody specific for Kunitz domain 1 of TFPI (Imubind total TFPI ELISA kit) were obtained from American Diagnostica (Greenwich, CT, U.S.A.). Another total TFPI ELISA kit, from CSTRI (Kaketsuken kit), containing polyclonal antibody against full-length TFPI coated on a plastic plate and horseradish-peroxidase-labelled monoclonal antibody specific for the conformation between the Kunitz domains 1 and 2 of TFPI were obtained from CSTRI. Solutions of these antibodies were gifts from the same institute. An ELISA kit for measuring free TFPI, consisting of anti-(human TFPI) polyclonal antibody and monoclonal antibody specific for Kunitz domain 3, was also obtained from CSTRI. Polyclonal antibody against a C-terminal fragment (peptide 239–265, provided by Daiichi Pure Chem. Co., Tokai Research Laboratory) of TFPI was prepared by immunization of a rabbit. Oxidized phosphatidylcholine (ox-PtdCho) [17] and its aldehydic products were donated by Dr Itabe (Faculty of Pharmaceutical Sciences, Teikyo University, Kanagawa, Japan); other phospholipids were obtained from Avanti Polar Lipids.

Cell cultures

Endothelial cells were isolated from human umbilical-cord veins and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 % (v/v) heat-inactivated fetal calf serum (FCS) containing antibiotics as described previously [15]. For measuring TFPI activity, the medium was changed to HuMedia-EG2 (Kurabo Biomedical) containing 2 % (v/v) FCS in the absence of heparin before the addition of lipoprotein samples. Although a cytotoxic effect of ox-LDL on the proliferation of endothelial cells has been reported [18], cell viability was more than 95 % after treatment with ox-LDL (200 μg/ml protein) for 24 h in the present study, as confirmed by means of the Trypan Blue dye-exclusion test.

Modification of LDL

LDL was prepared by the method of Redgrave et al. [19]. Ox-LDL was prepared by the incubation of LDL (200 μg/ml protein) with 10 μM $CuSO_4$ for 12 h at 37 °C. At the end of the incubation, 200 μM EDTA was added and the ox-LDL sample was obtained after dialysis against PBS. Thiobarbituric-acid-reactive substances (TBARS) were measured by a reported method [20]. The average TBARS value of ox-LDL samples prepared was 13.4 ± 2.8 nmol malonaldehyde/mg of protein (mean $\pm$ S.D.). Immunoblot analysis of apoB-100 in the native LDL and ox-LDL samples was performed with anti-(apoB-100) (ICN Pharmaceuticals) after SDS/PAGE. Acetylated LDL was prepared by the method of Basu et al. [21]. The extent of acetylation was confirmed by agarose-gel electrophoresis followed by Oil Red O staining, and more than 50 % of the acetylation in acetylated LDL was at lysine residues, as measured by the method of Steinbrecher [22]. Through all the present experiments, the amount of LDL in preparations was consistent in terms of protein content measured by Lowry's method [23], because the ratio of protein to cholesterol was unchanged, irrespective of its oxidation.

Measurement of TFPI antigen level

A secondary culture of HUVEC was maintained until confluence in DMEM containing 20 % (v/v) FCS; the medium was then replaced with fresh medium. The culture medium of HUVEC containing TFPI secreted for 24 h was recovered into polystyrene tubes containing 0.2 % BSA and protease inhibitors; the medium was then incubated with various concentrations of native LDL, ox-LDL or acetylated LDL. HUVEC cultured in the second fresh medium were treated with these lipoproteins for 24 h. After treatment, the cells were washed twice with PBS containing protease inhibitors and solubilized in the same solution containing 0.2 % BSA and 0.5 % Triton X-100 for 30 min at 4 °C. The TFPI antigen level was measured with two kinds of sandwich ELISA kit: Imubind total TFPI ELISA kit and Kaketsuken kit. Both ELISA systems can measure all forms of TFPI. To measure free TFPI content, the free TFPI ELISA kit from CSTRI was used. In addition we used a pair of antibodies, such as the polyclonal antibody against a C-terminal fragment (peptide 239–265) of TFPI and monoclonal antibody specific for Kunitz domain 1. In this combination, 0.6 M NaCl was added to the assay system to avoid the interaction of negatively charged lipoproteins with the C-terminus of TFPI. This ELISA system can be used to detect full-length TFPI (intact TFPI), because TFPI is trapped by the two antibodies. Our results suggested that TFPI secreted from HUVEC was the intact molecule containing the C-terminus and was a free form, not associated with lipoproteins, under the experimental conditions used.

Measurement of inhibitory activity of TFPI

Inhibitory activity on FX activation by the quaternary complex of FVIIa/TF/FXa/TFPI was measured with a two-stage assay with a chromogenic substrate by the method of Sandset et al. [24], with a slight modification. In the first stage, 25 μl of sample was incubated for 10 min at 37 °C with 100 μl of a mixture containing thromboplastin (at a final concentration of 2 %, v/v), FVIIa (0.2 nM), FXa (0.8 nM) and $CaCl_2$ (15 mM) in 25 mM Tris/HCl, pH 7.5, containing 100 mM NaCl, 10 mM trisodium citrate, 0.02 % NaN_3 and 0.2 % BSA. In the second stage, 25 μl of bovine FX (85 nM) was added and FXa generated in the reaction mixture was measured with S-2222 as a substrate. The TFPI activity in the culture medium of HUVEC at 24 h incubation was defined as 100 % activity. The activity of FXa direct inhibition by TFPI was measured by the method of Wesselschmidt et al. [25].

Non-denaturing PAGE

TFPI purified from Hep G_2 culture medium or h-rTFPI (5 ng) was incubated with 10 μg of either ox-LDL or native LDL at 37 °C in the presence of protease inhibitors; the mixture was subjected to non-denaturing PAGE with a non-linear gradient (2.4–15.2 %, w/v) polyacrylamide gel (Multi gel lipo; Daiichi Pure Chemical Co.). Proteins were transferred to a nitrocellulose filter and bands were detected by applying anti-TFPI polyclonal IgG (prepared by CSTRI) followed by staining with a Konica Immunostain kit.

Northern blotting

Northern blot hybridization was performed as described previously [26]. Human TFPI cDNA probe was prepared by PCR with HUVEC cDNA as a template. The PCR product (935 bp) was subcloned into pBluescript II SK and the sequence was confirmed with the use of a 373S DNA sequencer (Applied Biosystems). The radioactivity in the hybridized band was measured with a bioimaging analyser (BAS 1500; Fuji Film).

Bafilomycin A1 and lactacystin treatments

HUVEC were maintained until confluence in HuMedia-EG2 containing 20% (v/v) FCS in the absence of heparin; the medium was then replaced with fresh medium. The cells were treated with 50 nM bafilomycin A1 or 10 μg/ml lactacystin for 12 h in the presence or absence of 200 μg/ml ox-LDL.

Separation of ox-LDL components and treatments of lyso-PtdCho, lysophosphatidic acid (lyso-PA) and ox-PtdCho

Native LDL and ox-LDL were fractionated into aqueous and organic phases by the method described previously [15]. Organic-solvent-soluble components of ox-LDL were further fractionated into representative lipid fractions, such as triacylglycerol, cholesterol, fatty acid and phospholipid, by TLC on a silica-gel plate with a mobile phase of light petroleum (boiling range 30–60 °C)/diethyl ether (1:1, v/v). Each fraction obtained by extraction of the spot on the silica-gel plate with chloroform/methanol (19:1, v/v) was evaporated under nitrogen gas and used after dispersal in PBS by sonication. Lyso-PtdCho (1-oleoyl), lyso-PtdCho (crude extract) and lyso-PA (1-oleoyl) were dispersed in PBS and an amount equivalent to the total phospholipid content in ox-LDL was added to the culture medium of HUVEC. The concentration of phospholipid in ox-LDL was determined by measuring P_i content after dialysis against 50 mM Tris/HCl, pH 7.4, containing 0.1 M NaCl.

RESULTS

Effect of ox-LDL on antigenicity of TFPI in culture medium of HUVEC

The culture medium of HUVEC (approx. 10 ng of TFPI per 10^5 cells at 24 h incubation) was collected into a polystyrene tube and directly incubated with various concentrations of ox-LDL; the TFPI antigen level in the culture medium was then measured with two kinds of ELISA kit (Figures 1A and 1B). Both ELISA systems are known to detect the LDL-associated form of TFPI in addition to the free form; the TFPI antigen level was measured in the presence of native LDL in the present study. On the addition of native LDL, a small increase in the TFPI level compared with that in PBS was observed; this increase might have been derived from the LDL fraction. Ox-LDL, but not native or acetylated LDLs, drastically decreased the TFPI antigenicity when the levels were measured with the American Diagnostica kit (Figure 1A) or the Kaketsuken kit (Figure 1B). These decreases in TFPI antigenicity occurred in a concentration-dependent manner up to 10 μg/ml ox-LDL. In the time-course study with the same sample, it was found that the antigenicity of TFPI was decreased within 1 h of treatment with 100 μg/ml ox-LDL, whereas a slower decrease in activity was observed when a lower concentration of ox-LDL (5 μg/ml) was used (results not shown). From these results it is apparent that the remarkable decrease in TFPI level in the culture medium of HUVEC exposed to ox-LDL (results not shown) was due mainly to decreased reactivity rather than a decrease in TFPI secretion from HUVEC. The results suggest further that care is required in the measurement of TFPI antigen level in plasma with the monoclonal antibody, because LDL might be oxidized during storage.

Dose-dependent decrease in the activity of TFPI exposed to ox-LDL

Various concentrations of ox-LDL, acetylated LDL and native LDL were added to culture media derived from HUVEC (Figure 2A) or TFPI purified from cultures of Hep G_2 cells (Figure 2B), and the dose-dependent changes in TFPI activities were measured by a two-stage assay after incubation for 3 h at 37 °C. With increasing concentration of ox-LDL, a marked decrease in the Xa inhibitory activity of TFPI was recognized in both TFPI sources, even though essentially no changes in the activities were observed in the presence of native and acetylated LDLs (Figure 2). The lack of a further decrease in TFPI activity at a higher concentration of ox-LDL in both sources might be ascribed to the limited linear range of the assay system rather than to the presence of a different TFPI form that could be resistant to ox-LDL, because TFPI purified from Hep G_2 cells was homogeneous

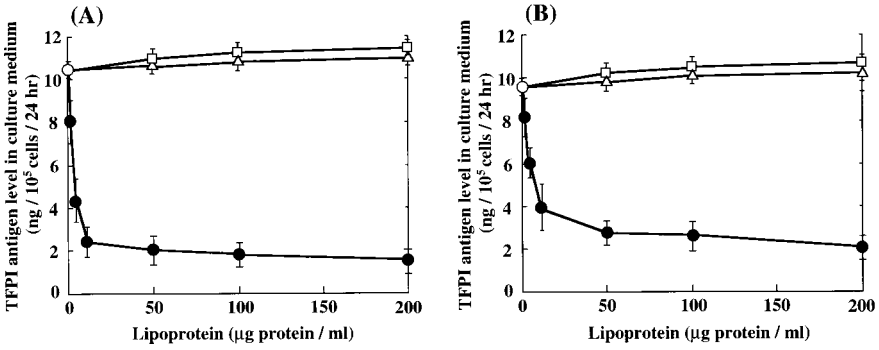

Figure 1 Ox-LDL decreases the reactivity of TFPI in ELISA systems

The culture medium of HUVEC containing TFPI secreted for 24 h was recovered in a polystyrene cup and the medium was incubated with PBS alone (○) or with various concentrations of native LDL (□), ox-LDL (●) or acetylated LDL (△) for 3 h and the TFPI antigen levels were measured by two kinds of sandwich ELISA. Anti-human TFPI polyclonal antibody and monoclonal antibody specific for Kunitz domain 1 of TFPI were used as first and second antibodies respectively in the American Diagnostica ELISA kit (**A**), or anti-human TFPI polyclonal antibody and monoclonal antibody specific for the conformation between the Kunitz 1 and 2 domains of TFPI respectively were used in the Kaketsuken ELISA kit (**B**). The average TBARS value corresponded to 2.7 nmol of malonaldehyde/ml of culture medium when 200 μg/ml ox-LDL was added. Results are means ± S.D. from four experiments.

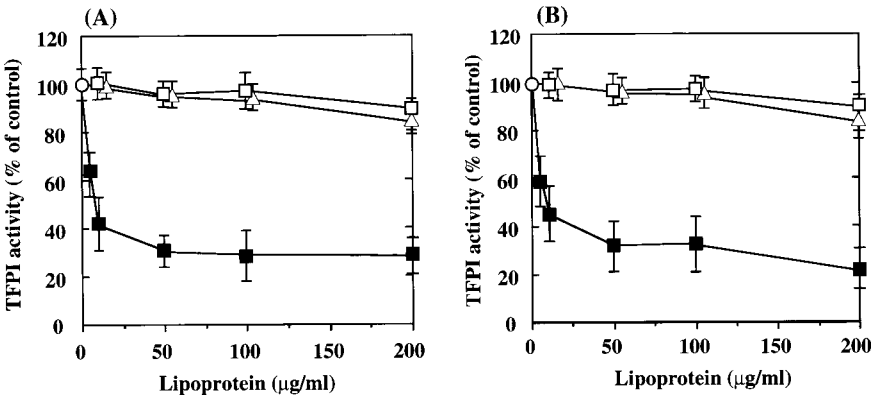

Figure 2 Effect of ox-LDL on TFPI-dependent inhibitory activity of FX activation

TFPI obtained from culture medium of HUVEC (**A**) or purified from Hep G_2 cells (**B**) was incubated with various concentrations of native LDL (□), ox-LDL (■) or acetylated LDL (△) for 3 h. The FXa inhibitory activities were measured by a two-stage assay based on the ability of TFPI samples to inhibit TF/FVIIa catalytic activity in the presence of FXa. The activity of TFPI in a sample incubated for 3 h without LDLs (○) was defined as 100% activity in each case. Results are expressed as means ± S.D. from three independent experiments. Similar results were observed when TFPI activity was measured in terms of the direct Xa inhibitory activity.

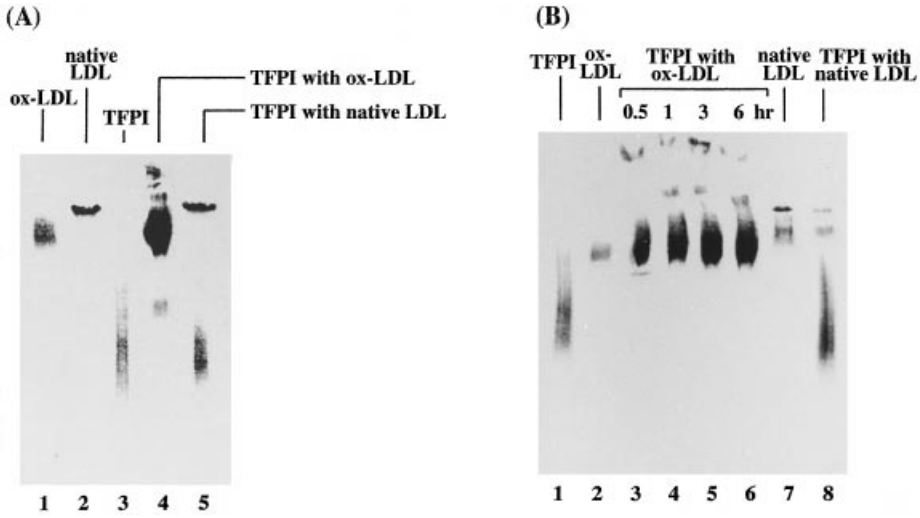

Figure 3 TFPI co-migrates with ox-LDL on non-denaturing PAGE after incubation with ox-LDL

(**A**) h-rTFPI was incubated with or without ox-LDL or native LDL for 3 h and subjected to non-denaturing PAGE. Bands were transferred to a nitrocellulose filter and TFPI was detected with anti-human TFPI polyclonal antibody. The ratio of LDL to TFPI on a molar basis was approx. 300:1, which is one-fifth of that in normal human plasma (in which the ratio is approx. 1000:1 to 2000:1). Lane 1, 10 μg of ox-LDL alone; lane 2, 10 μg of native LDL alone; lane 3, 5 ng of rTFPI; lane 4, 10 μg of ox-LDL plus 5 ng of rTFPI; lane 5, 10 μg of native LDL plus 5 ng of rTFPI. (**B**) h-rTFPI was incubated with ox-LDL for 0.5, 1, 3 and 6 h (lanes 3–6) or with native LDL for 6 h (lane 8) at 37 °C. Each sample containing rTFPI alone (lane 1), ox-LDL alone (lane 2) or native LDL alone (lane 7) was also incubated for 6 h. After the incubation, samples were subjected to non-denaturing PAGE and detected as described above. Lane 1, 5 ng of rTFPI; lane 2, 10 μg of ox-LDL alone; lanes 3–6, 10 μg of ox-LDL plus 5 ng of rTFPI; lane 7, 10 μg of native LDL alone; lane 8, 10 μg of native LDL plus 5 ng of rTFPI.

in amino acids, as confirmed by immunoblotting with polyclonal antibodies against TFPI. However, it is possible that a small amount of ox-LDL-resistant TFPI, such as the C-terminal truncated form, was formed in both sources during the incubation period.

Non-denaturing PAGE of TFPI treated with ox-LDL

To understand how ox-LDL affects the reactivity of TFPI, the binding between ox-LDL and TFPI was studied in comparison with native LDL (Figure 3). rTFPI migrated faster than the lipoproteins and a single but relatively broad band of rTFPI was observed after non-denaturing PAGE followed by immunoblotting with a polyclonal antibody against full-length TFPI (from CSTRI). It is plausible that heterogeneous bands of rTFPI on non-denaturing PAGE might have been due to molecules with different glycosylation levels [27]. Ox-LDL migrated slightly faster than native LDL, as confirmed by lipid staining of the gel with Sudan dye (results not shown) and the lipoprotein bands coincided completely in position on the filter (detected by the antibody against TFPI), as shown in Figure 3(A), lanes 1 and 2. Both native LDL and ox-LDL contained small but significant amounts of TFPI. On incubation of rTFPI with ox-LDL for 3 h, substantially all the rTFPI migrated with slow mobility, which corresponded to that of ox-LDL alone, on non-denaturing PAGE, although no essential change in the

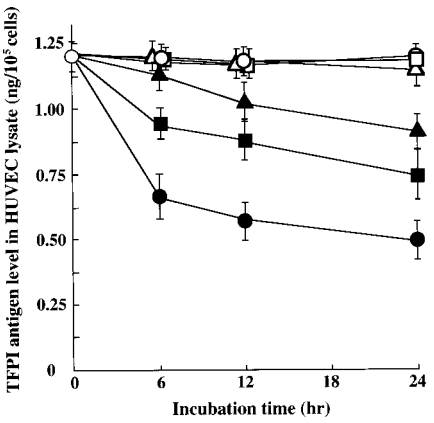

Figure 4 Time-dependent decreases in antigen levels of TFPI in the lysates of HUVEC treated with native and modified LDLs

A secondary culture of HUVEC was treated with PBS alone (○), native LDL (200 μg/ml) (□), ox-LDL (▲, 50 μg/ml; ■, 100 μg/ml; ●, 200 μg/ml) or acetylated LDL (200 μg/ml) (△) for 24 h and TFPI antigen levels in the cell lysates were measured with an American Diagnostica ELISA kit consisting of a human TFPI polyclonal antibody and a monoclonal antibody specific for Kunitz domain 1 of TFPI as the first and second antibodies respectively. Results are means ± S.D. from four experiments.

migration of rTFPI was observed after incubation of rTFPI with native LDL for 3 h (Figure 3A). The results suggest a high association of TFPI with ox-LDL. Essentially the same result was obtained when TFPI purified from Hep G_2 culture medium was used instead of rTFPI (results not shown).

The time course of the association of rTFPI with ox-LDL was studied (Figure 3B). A maximum amount of rTFPI was found in the ox-LDL fraction as early as 30 min after mixing rTFPI with ox-LDL. Therefore it is considered that TFPI associates with ox-LDL very quickly, in accordance with its oxidation process. It is supposed that the ox-LDL-dependent decrease in the reactivity between TFPI and its monoclonal antibodies, as shown in Figure 1, is attributable to a change in the conformation of the TFPI molecule as a result of its association with ox-LDL.

Effect of ox-LDL on TFPI antigen levels in HUVEC

To investigate whether or not ox-LDL results in a decrease in the antigen level of TFPI in HUVEC, we determined the changes in the content in lysate of HUVEC, that had been washed after treatment with ox-LDL, with the American Diagnostica kit (Figure 4). With ox-LDL, but not native LDL or acetylated LDL, a decrease in TFPI antigen level in the lysates of HUVEC was observed. TFPI antigen of HUVEC exposed to ox-LDL decreased slowly in a time-dependent and concentration-dependent manner, in contrast with the decrease in antigenicity of TFPI in the culture medium, which occurred rapidly owing to the association with ox-LDL as described in the previous section. Therefore the decrease in the antigen could not account for the decrease in reactivity towards anti-TFPI antibody. A similar result was obtained when the Kaketsuken kit was used instead of the American Diagnostica kit (results not shown).

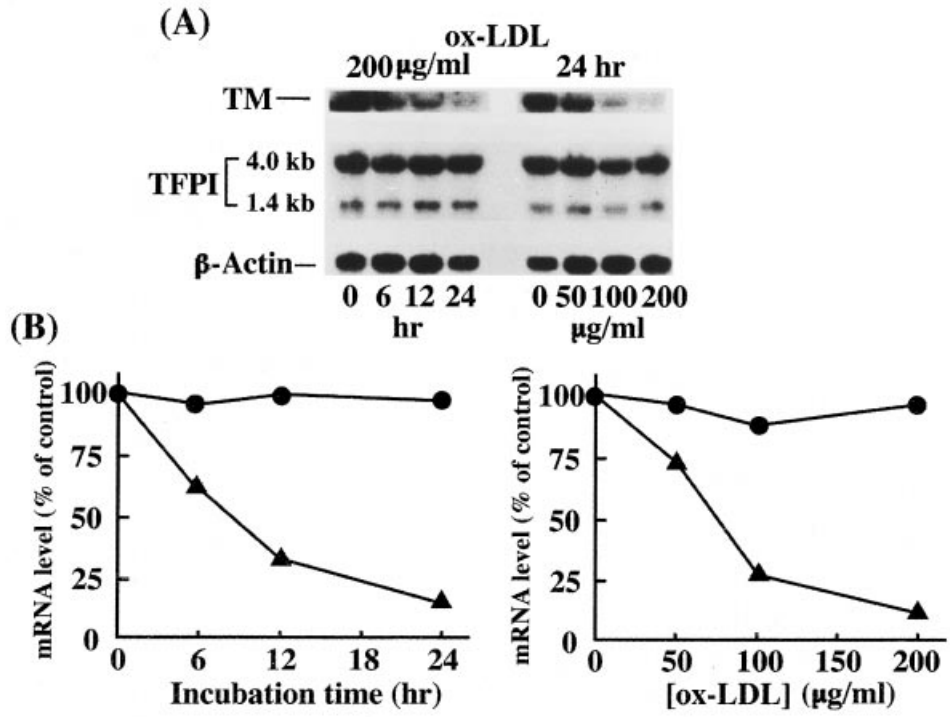

Figure 5 TFPI mRNA levels in HUVEC exposed to ox-LDL

HUVEC were treated with various concentrations of ox-LDL for 24 h and the mRNA levels of TFPI and TM were analysed by Northern blot as described in the Results section. Autoradiograms are shown in (**A**); the results in (**B**) are expressed as percentages of the control values after normalization to the actin mRNA signal. Measurements were made with a BAS-1500. Symbols: ●, TFPI, expressed as the sum of two signals (4.0 and 1.4 kb); ▲, TM.

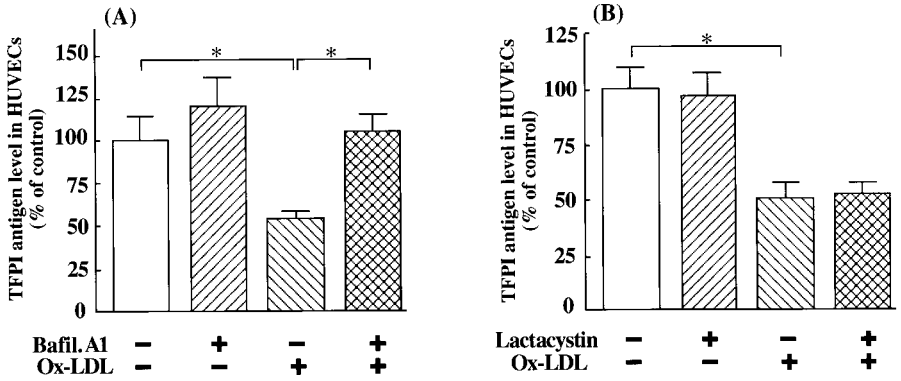

Figure 6 Effects of bafilomycin A1 and lactacystin on TFPI antigen levels in HUVEC exposed to ox-LDL

HUVEC cultured in HuMedia-EG2 containing 20% (v/v) FCS were treated with or without 200 μg/ml ox-LDL in the presence or absence of 50 nM bafilomycin A1 (**A**) or 10 μg/ml lactacystin (**B**) for 12 h. TFPI antigen level in the cell lysate was measured as described in the legend to Figure 4. Results are expressed as means $\pm$ S.D. from three independent experiments. The data were analysed by Student's *t* test; $^*P < 0.01$. The TFPI antigen level of HUVEC cells was 1.2 ng per 10^5 cells.

TFPI mRNA levels in HUVEC exposed to ox-LDL

To understand the mechanism by which ox-LDL decreases the TFPI level in HUVEC, total RNA was prepared from HUVEC treated with ox-LDL and the mRNA level was measured by Northern blotting (Figure 5A). Two bands corresponding to a major 4.0 kb and a minor 1.4 kb TFPI mRNA were detected in HUVEC, as reported previously [28]. No change in TFPI mRNA level was detected in studies of dependence on concentration and time, whereas an ox-LDL-dependent decrease in the mRNA level of TM was observed, as shown in our previous study [15]. These results indicate that although ox-LDL decreases the antigen levels of both TM and TFPI in HUVEC, the mechanisms are quite different from each other.

Degradation of TFPI via lysosomal and proteasomal pathways

The effect of bafilomycin A1 on the degradation of TFPI antigen level in HUVEC was investigated (Figure 6A). Bafilomycin A1 is an inhibitor of the lysosomal proton pump [29] but does not affect the internalization of ox-LDL [30]. HUVEC exposed to 200 μg/ml ox-LDL for 12 h showed a decrease in the TFPI antigen level to 55% of that in the untreated cells. Treatment with bafilomycin A1 (50 nM) alone tended to increase the TFPI antigen level compared with the control, so it was considered that TFPI was degraded via the lysosomal pathway in the cells. The decrease in the level in HUVEC exposed to the ox-LDL was abolished by co-incubation of the cells with bafilomycin A1; the recovery was significantly higher than that of the control treated with bafilomycin A1 alone. These results indicate that the ox-LDL-dependent decrease in the TFPI level in HUVEC is attributable to enhancement of the lysosomal degradation of TFPI.

The proteasome is known to decompose various types of proteins, such as regulatory proteins, in addition to abnormal proteins labelled with ubiquitin [31]. We studied the effect of lactacystin, a specific inhibitor of proteasome function [32], on the ox-LDL-dependent down-regulation of TFPI in HUVEC (Figure 6B). Treatment with lactacystin (1 μM) alone and with lactacystin plus 200 μg/ml of ox-LDL for 12 h did not change the TFPI antigen level compared with the respective control, suggesting that the cellular degradation of TFPI is independent of the proteasome pathway under normal and ox-LDL-exposed conditions.

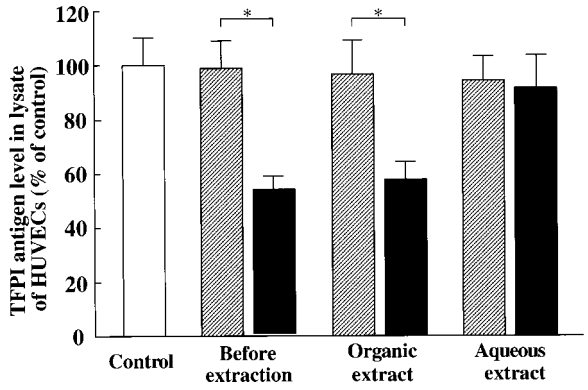

Figure 7 Effects of organic and aqueous extracts from ox-LDL on TFPI antigen levels in HUVEC

Aqueous and organic extracts were prepared from native LDL and ox-LDL as described in the Experimental section. After the culture medium of HUVEC had been changed, these extracts were added to cultures of HUVEC at a concentration equivalent to ox-LDL (200 μg/ml protein). After incubation for 6 h, TFPI antigen levels in the lysate of HUVEC were measured with an American Diagnostica ELISA kit. Results are expressed relative to the control treated with PBS only and are shown as means $\pm$ S.D. from three independent experiments. The data were analysed by Student's *t* test; $^*P < 0.01$. The TFPI antigen level of HUVEC was 1.2 ng per 10^5 cells. Open columns, PBS; hatched columns, native LDL; closed columns, ox-LDL.

Down-regulation of TFPI by ox-LDL components

To identify the active component(s) of ox-LDL that cause the down-regulation of TFPI, ox-LDL was fractionated into aqueous and organic phases and the effect of each extract on the TFPI antigen in HUVEC was investigated (Figure 7). An approx. 40% decrease in TFPI antigen, similar to that caused by ox-LDL, was observed on treatment with organic-solvent extracts for 6 h in the presence of an equivalent concentration of lipid to that in 200 μg/ml ox-LDL. In contrast, aqueous extracts of ox-LDL did not significantly decrease the TFPI content in HUVEC. Further study on the decrease in TFPI levels in HUVEC by lipid components obtained by TLC of the organic solvent extracts suggested that phospholipid might be a target substance. It is known that lyso-PtdCho is a major lipid component of ox-LDL [10,12] and that ox-PtdCho is the epitope for a monoclonal antibody that reacts to atherosclerotic lesions consisting of foam

cells derived from macrophages [17]. Therefore we investigated the effects of lyso-PtdCho, lyso-PA and ox-PtdCho, including its aldehydic products derived by the decomposition of lipid hydroperoxides [10,33], on the TFPI level in HUVEC by using concentrations of phospholipids comparable to that in ox-LDL. However, incubation of these phospholipids for 24 h resulted in no change in the TFPI content in HUVEC (results not shown).

DISCUSSION

TFPI inhibits the TF/FVIIa complex relatively slowly at the plasma concentration (2.4 nM) of TFPI [34]. However, in the present study TFPI secreted from HUVEC (2 ng/0.5 ml per 24 h per 2×10^4 cells) could inhibit the quaternary complex-dependent FX activation *in vitro*, even after the culture medium had been diluted 1:10. The final concentration of TFPI under the present assay conditions ranged from 0.01 to 0.1 nM. It has been widely accepted that the C-terminus of TFPI is a major site for interactions of heparin, lipoproteins and phospholipids; the significance of this region in anti-coagulant function has also been demonstrated [35,36]. Hansen et al. [37] have reported that the lipoprotein-associated form of TFPI, which was estimated to account for 70–85 % of the circulating TFPI in human plasma, does not possess anti-coagulant function when the activity is determined in terms of diluted prothrombin time. However, in the present study with antibodies recognizing the C-terminus of TFPI ($TFPI_{239–265}$) or specific for Kunitz domain 3, it was confirmed that TFPI secreted from HUVEC was the free form of the molecule having the C-terminal site. Further, only 5 % of the free form was converted into the LDL-associated form after mixing of TFPI secreted from HUVEC with native LDL for 24 h. It can therefore be considered that TFPI secreted from HUVEC serves as an essential regulator of anticoagulation in human plasma. It has been shown that ox-LDL and its lipid constituents impair endothelial cell functions [9–15]. Nevertheless, the ability of TFPI to inhibit FX activation after the exposure of HUVEC to ox-LDL is not well documented; in particular, it is not known whether or not ox-LDL causes qualitative and quantitative alterations of TFPI. In the present study we focused on (1) ox-LDL-dependent modification of TFPI outside HUVEC, including the binding between TFPI and ox-LDL, and (2) ox-LDL-dependent down-regulation of TFPI inside the HUVEC.

In immunoblotting, no proteolytic degradation product or truncated form of rTFPI was found when exogenously added rTFPI was incubated for 24 h with ox-LDL. However, the reactivity of TFPI towards the antibodies was decreased in the presence of ox-LDL. The ox-LDL-dependent decrease in the reactivity occurred in a dose-dependent manner up to 10 μg/ml ox-LDL; the decrease was correlated with the decrease in TFPI activity, which was measured in terms of inhibition of FX activation by the quaternary FXa/TFPI/FVIIa/TF complex. Because the direct FXa inhibitory activity of TFPI was also diminished in the presence of ox-LDL, the decrease in the reactivity of TFPI towards the antibodies might, at least in part, involve the Kunitz 2 domain of TFPI, which is known to bind FXa. Lesnik et al. [38] measured TFPI activities in the LDL fraction and in the fraction treated with copper or acetate, and demonstrated that the activity of TFPI in the LDL fraction of human plasma as an LDL-associated form was significantly decreased by both chemical oxidation and acetylation. In our experiment with TFPI secreted from HUVEC, the decrease in TFPI activity was ox-LDL-specific and was not observed with acetylated LDL. Although the experimental systems are quite different from each other, it seems clear that the expression of anti-coagulant activity of TFPI is decreased markedly as a consequence of the oxidative process. In this connection it has been widely accepted that the TFPI activity of the LDL-associated form is weak compared with that of the free form [34,37]. If this is so, the oxidative decrease in TFPI activity in the LDL fraction might not be important. We determined the TFPI activities of free and LDL-associated forms after incubation of partly purified TFPI from the culture medium of HUVEC in the presence of native LDL for 24 h, followed by separation on Sephacryl S-200. Though the ratio of bound to free forms was 1/20 at 24 h incubation of that in native LDL, TFPI in the LDL-associated form accounted for approximately one-third of the activity compared with free TFPI on an equimolar basis. These results suggest that anti-coagulant activity might be extensively lost in the oxidatively modified LDL fraction under physiological and pathological conditions.

We emphasize that TFPI associates quite rapidly with ox-LDL but not with LDL. This fact is consistent with the finding that only a small part of free TFPI was converted into an LDL-associated form and no significant decrease in TFPI activity was observed after 24 h exposure to native LDL. It has been shown that the C-terminus of TFPI is required for the interaction with apoB-100 [39]. ApoB-100 in the ox-LDL fraction was almost completely degraded in our experiment (results not shown), so the high association of TFPI with ox-LDL was not correlated with the presence of native apoB-100. It is known that the oxidation of lipoproteins leads to the formation of adducts between lipid peroxidation products and positively charged amino acids, particularly lysine residues of apoB-100, which might cause alterations in secondary and tertiary structure of lipoproteins [40]. Recently Ettelaie et al. [41] reported that a great suppression of the inhibitory potential of TFPI towards TF was observed when the positive charges of the lysine residues within TFPI were neutralized by acetylation of the molecules. As they suggested, it is conceivable that the oxidation of LDL might mask lysine and arginine residues within all Kunitz domains of TFPI. Further study is required to resolve the precise mechanism of the specific interaction between TFPI and ox-LDL by using various fragments of TFPI.

With regard to ox-LDL-dependent decreases in TFPI antigen inside HUVEC, we concluded that the down-regulation can be ascribed primarily to an enhancement of lysosomal degradation. Although the present paper has not clarified whether the incorporation of TFPI molecule is increased after association with ox-LDL, it is possible that the ox-LDL-associated form is recognized as a distinct molecule from the free or LDL-associated form in the cells. It is known that ox-LDL binds to the scavenger receptors on the surface of endothelial cells before its internalization [42]. To inhibit the binding of ox-LDL to one scavenger receptor, we examined the effect of antibody against CD36 [43] on the ox-LDL-dependent down-regulation of TFPI and found no effect (results not shown). It has been reported that endothelial cells express a new type of ox-LDL receptor in addition to the previously known receptors [44,45]. Further studies are required for an understanding of the kind of ox-LDL receptor that mediates the down-regulation of TFPI and for an explanation of how ox-LDL (or its metabolites) affects the degradation of TFPI. The oxidative process of LDL particles is associated with lipid peroxidation, the formation of conjugated dienes and the conversion of PtdCho into lyso-PtdCho by phospholipase A_2 in apoB-100, in addition to carbonyl modification of apoB-100 [10,12]. We found that organic extracts of ox-LDL decreased the TFPI antigen level of HUVEC to nearly the same extent as intact ox-LDL, but aqueous extracts did not. Further fractionation of the organic extracts of ox-LDL

suggested that the active component of ox-LDL was in the phospholipid fraction. Recently Sato et al. [46] reported that lyso-PtdCho decreased the synthesis of TFPI in HUVEC through a decrease in the TFPI mRNA levels. However, in our present study lyso-PtdCho and lyso-PA affected neither the TFPI antigen level nor the TFPI mRNA level of HUVEC, even if a concentration equivalent to that of total phospholipid was employed. Ox-PtdCho and its aldehydic compounds were also ruled out as the target phospholipids. The actual phospholipid constituent(s) of ox-LDL should be determined.

Oxidative modification of LDL is mediated *in vitro* by various types of cultured vascular cell and is thought to occur *in vivo* in the subendothelial area [10,12,47]. Ox-LDL might contribute to the atherogenic process in many ways; susceptibility to oxidation might, at least in part, determine the atherogenicity of LDL. Some clinical studies have revealed a positive correlation between plasma factors of LDL oxidation and atherosclerotic disease [48,49]. Because it has been reported that TFPI inhibits aortic smooth-muscle cell migration through its inhibitory action on the catalytic activity of TF/FVIIa complex [50], it could be suggested that ox-LDL-dependent decrease in TFPI activity causes the migration of smooth-muscle cells to atherosclerotic lesions. In HUVEC exposed to ox-LDL, a decrease in the cofactor activity of TM and an increase in the procoagulant activity of TF have been reported [16]. In addition, the present study suggests that ox-LDL promotes thrombosis in atherosclerotic lesions through a decrease in TFPI function in the bloodstream and on the vascular wall.

We can summarize the results of the present study as follows: (1) ox-LDL binds to TFPI rapidly and causes structural alteration and/or masking at various recognition sites of the TFPI molecule, (2) degradation of TFPI in the lysosomal pathway of endothelial cells in the presence of ox-LDL is faster than that in the absence of ox-LDL, and (3) the ox-LDL-dependent modification and accelerated degradation of TFPI outside and inside endothelial cells respectively decrease the total anti-coagulant ability of the vascular bed under atherosclerotic conditions.

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