In Vivo Complex Formation of Oxidized \(\alpha_1\)-Antitrypsin and LDL

Shinichi Mashiba, Youichiro Wada, Motohiro Takeya, Akira Sugiyama, Takao Hamakubo, Akio Nakamura, Noriko Noguchi, Etsuo Niki, Akashi Izumi, Mika Kobayashi, Kazuo Uchida, Tatsuhiko Kodama

Abstract—An inactivated form of \(\alpha_1\)-antitrypsin (AT) and LDL coelutes in gel permeation chromatography. To characterize and to quantify the amount of this fraction of AT, a monoclonal antibody was established against chloramine T–oxidized AT and named OxAT-4. OxAT-4 recognized the oxidatively modified AT, including hexylaldehyde- or 4-hydroxy-2-nonenal–modified AT, but neither normal active AT nor trypsin/AT complex. Comigration of apoB and oxidized AT was demonstrated by Western blotting analysis of AT-LDL by means of anti-apoB monoclonal antibody and OxAT-4. A complex of oxidized AT and LDL (AT-LDL) was isolated from human plasma LDL by affinity column with an OxAT-4 antibody–coated carrier. AT-LDL was degraded 4 times more effectively by mouse peritoneal macrophages, but this was not mediated by scavenger receptor class A type I. Localization of AT-LDL was detected in human atherosclerotic lesions of the coronary artery, but distribution of it was not completely identical to that of macrophages. In situ hybridization revealed AT expression by macrophages, which were present in intimal layers of the coronary artery. From these findings, we concluded that AT is produced and oxidized by macrophages, then attached to LDL in the intimal layer of the arterial wall. Although AT-LDL that escapes into the blood stream can be cleared by hepatocytes, the remaining AT-LDL may be taken up by macrophages and contribute to the lipid accumulation in arterial wall cells as the early stage of atherogenesis. (Arterioscler Thromb Vasc Biol. 2001;21:1801-1808.)

Key Words: \(\alpha_1\)-antitrypsin ■ LDL ■ complex ■ monoclonal antibody ■ macrophage

Serine proteinase activity is regulated by a group of inhibitors of the serine proteinases (also called serpin). \(\alpha_1\)-Antitrypsin (AT) is one of the serpin family proteins. It is produced mainly in the liver, and partly by macrophages stimulated by interleukin-6 or lipopolysaccharide.1–5 At inflammatory sites, AT protects the tissue against damage caused by proteinases derived from white blood cells; in the lung, AT is produced by monocytes, which are stimulated by endotoxin, interleukin-1, and tumor necrosis factor-\(\alpha\) and protect the alveolar microstructure against excess activity of proteinases.6,7

After formation of complexes of AT with serine proteinase, AT is cleaved at the site of the serpin reactive loop and changed into the inactive form.8,9 The resulting 4-kDa carboxy-terminal fragment of 36 residues (C-36) still binds the complex noncovalently, but under denaturing conditions in vitro, the C-36 fragment dissociates from the complex.10,11 Recent studies have elucidated the biological activities of the C-36 fragment: upregulation of expression of the LDL receptor in HepG2 cells12–14 and induction of cytokine production and CD36 expression in monocytes.15 Furthermore, it is involved in neutrophil recruitment.16,17 Thus, not only AT itself but also the byproducts of its enzymatic inhibition possess biological activities at the locus of inflammation.

Oxidized AT is another modified form of \(\alpha_1\)-AT, which is found in inflammatory exudates at levels of \(~5\%\) to 10\% of total AT18,19 and inflammatory synovial fluids.20–21 AT contains methionine at the “P1 position,” which is located in the reactive site, and determines the specificity of inhibition. Various oxidant radicals such as peroxide, the hydroxyl radical, hypochloride, chloramine, and peroxynitrite22,23 change the methionine into methionine sulfoxide, and such modified forms of AT lose the inhibitory activity against proteinases. Furthermore, such an oxidized form of AT has the function of monocyte activation.24 Thus, oxidized AT promotes tissue destruction not only by the loss of proteinase inhibitory activity but also by recruiting and activating monocytes in the lesion. This suggests that the amount of oxidized AT reflects the degree of tissue destruction in inflammatory diseases.
For the purpose of developing an ELISA system to assay the amount of oxidized AT, we have established a monoclonal antibody (OxAT-4) that cross-reacts with chloramine T–oxidized AT, but not with native AT. The OxAT-4 ligand colocalized with apolipoprotein B (apoB) in vivo, and a complex of oxidized AT and LDL was isolated from human plasma by OxAT-4 affinity column. The complex was degraded by macrophages. In the present study, we evaluated the pathophysiological relevance of this complex to atherogenesis in the arterial wall.

Methods

Reagents

The 7D4 antibody was purchased from American Tissue Culture Collection. Proteins were determined by the method of Lowry et al. For protein detection, a Silver Stain II Kit was purchased from Wako Pure Chemical Industries, Ltd.

Gel Filtration on Sephacryl S-200 Column

Human plasma (1 mL) was applied to a Sephacryl S-200 column (1×100 cm) equilibrated with phosphate buffer, pH 7.4, containing 0.15 mol/L NaCl. It was eluted at a speed of 10 mL/h, and 2-mL fractions were collected. Each fraction was used to assay the protein level of AT-LDL complex, and 100-times-diluted solutions of each fraction were used to determine the amount of AT and apoB. ApoB was detected by ELISA using an anti–human apoB-100 monoclonal antibody (Cappel) as the first antibody and human apoB-100 polyclonal antibody (Biostripe) as the second antibody.

Preparation of Complex of Oxidized LDL and AT

Oxidized LDL and AT complex was prepared by coincubating 500 g/mL oxidized LDL and 50 μg/mL purified AT for 16 hours at 37°C.

Establishment of Monoclonal Antibody OxAT-4

AT was treated with chloramine T to oxidize the methionine in the functional domain of proteinase inhibitors. Oxidized, inactivated AT was used as an antigen, and antibodies were established by use of B-cell hybridoma as previously described. From several antibodies obtained, an antibody that did not react with native AT, trypsin-AT complex, or elastase-AT complex but did react with chloramine T–treated AT and oxidized LDL-AT complex was selected and named OxAT-4.

Characterization of the Monoclonal Antibody OxAT-4

Specificity was derived from the inhibition of the binding of this antibody to immobilized artificial AT-LDL by means of different competing soluble ligands, including normal active AT, trypsin/AT complex, neutrophil elastase/AT complex, glycolaldehyde (C2)–modified AT, malondialdehyde (C3)–modified AT, hexylaldehyde (C6)–modified AT, and 4-hydroxy-2-nonenal (C9)–modified AT. The association constant for immobilized modified AT and maximal number of binding sites per AT-LDL molecule were calculated from the amount of bound fluorescence-labeled antibodies to immobilized oxidized AT.

Preparation of OxAT-4–Conjugated Affinity Carrier

CNBr-activated Sepharose 4B (Pharmacia) was prepared according to the manufacturer’s protocol, and 3 mg of OxAT-4 or 3 mg of 7D4 monoclonal antibody was coupled with the Sepharose 4B carrier. Briefly, 0.15 g of CNBr-activated Sepharose 4B (Pharmacia) was treated with 2 mL of 1 mmol/L HCl at 4°C, transferred to a new column, and washed with 40 mL of 1 mmol/L HCl. All subsequent operations were carried out at 4°C unless otherwise stated. The coupling solution containing the OxAT-4 antibody with the gel was mixed in a stoppered vessel and rotated end-over-end overnight.

Excess antibody was washed away by 2 mL of coupling solution (0.1 mol/L NaHCO3 [pH 8.3], 0.5 mol/L NaCl). By transfer of the gel to 0.2 mol/L Tris-glycine buffer (pH 8.0) and mixing at room temperature for 2 hours, the remaining active groups were blocked. The gel was washed with 20 mL of 0.1 mol/L acetate buffer (0.5 mol/L NaCl, pH 4.0), 0.1 mol/L NaHCO3 buffer (0.5 mol/L NaCl, pH 8.3), and PBS (3 mol/L potassium thiocyanate). Finally, the gel was suspended in PBS (0.01% EDTA-2K, pH 7.4).

Immunoprecipitation

Immunoprecipitation was performed as described previously. Briefly, 0.3 mL of OxAT-4–conjugated affinity carrier (6 mg protein/mL gel) was applied to 4 mg protein LDL, dissolved at a concentration of 2 mg protein/mL in PBS with 0.01% EDTA-2K, and incubated for 30 minutes at 4°C. As a control, mouse monoclonal antibody 7D4–conjugated affinity carrier was used and applied to LDL solution in the same manner. All subsequent operations were carried out at 4°C unless otherwise stated. The OxAT-4 carrier was collected as a pellet after centrifugation at 29g for 5 minutes. The pelleted carrier containing the immunoprecipitate was washed on a rotator with 10 mL PBS (1 mol/L NaCl, 0.01% EDTA-2K), followed by 3 additional washes. Then 1 mL PBS containing 0.01% EDTA-2K and 3 mol/L potassium thiocyanate was added to the carrier and mixed gently for 15 minutes. By centrifugation at 89g for 5 minutes, supernatant was collected separately from the carrier, and the buffer was changed to LDL buffer A (150 mmol/L NaCl, 0.24 mmol/L EDTA-2Na [pH 7.4]) on a PD-10 (Pharmacia) column. Finally, the AT-LDL–containing solution was concentrated from 1.5 mL to 0.1 mL with a Centriprep spin column (Millipore).

Delipidation and Immunoblot

Before SDS-PAGE, AT-LDL was delipidated as described elsewhere, and the protein fraction was dissolved with 60 μL SDS-PAGE sample buffer (0.0625 mol/L Tris-HCl buffer [pH 6.8], containing 5% [vol/vol] 2-mercaptoethanol, 4% SDS, 10% [vol/vol] glycerol, 0.001% bromphenol blue). It was then boiled for 5 minutes and subjected to 2% to 15% polyacrylamide gel (Daiichi Pure Chemical Industries Co Ltd) and electrophoresed at 40 mA for 1 hour. The proteins were transferred in blotting buffer (0.025 mol/L Tris-HCl buffer, 0.192 mol/L glycine, 20% [vol/vol] methanol) at 50 V over a period of just 2 hours by means of a blotting apparatus (Nihon-eido) from the gel to the polyvinylidine difluoride (PVDF) membranes, which had earlier been dipped into Tris-buffered saline (0.01 mol/L Tris-HCl, 0.15 mol/L NaCl [pH 7.5]) for 5 minutes with shaking and treated with 30 mL of 1× Block Ace (Dainippon Pharmaceutical Co Ltd) at 4°C overnight. PVDF membranes were incubated with anti–human AT mouse monoclonal antibody (Calbiochem) or anti–human apoB-100 monoclonal antibody (ICN) at room temperature for 1 hour on the rotator. Bound antibodies were visualized with peroxidase-conjugated goat F(ab)2, anti-mouse immunoglobulins (Biosource) using the Supersignal CL-HRP Substrate System (Pierce) according to the manufacturer’s instructions. Gels were calibrated with prestained molecular weight markers (Bio-Rad). Filters were exposed to Fuji medical x-ray film (Fuji Photo Film) at room temperature for the indicated periods.
Preparation of AT-LDL Complex From LDL

OxAT-4—conjugated carrier (2.5 mL) was applied to a new column body and equilibrated with PBS (0.01 mol/L phosphate buffer, pH 7.4) containing 0.15 mol/L NaCl, 0.01% (wt/vol) EDTA-2K, and treated with 1% (wt/vol) analytical grade Chelex 100 resin (No. 142-2832, Bio Rad Co Ltd). Then 0.001% (wt/vol) N,N-bis(2-hydroxybenzyl) ethylenediamine-N,N-diacetic acid (Dojindo Laboratories) was added after preparation. The following procedures were performed at 4°C unless otherwise stated. Two milligrams protein of LDL were dissolved with 4 mL of PBS and applied to an affinity column, followed by 3 more applications. After addition of 2.5 mL PBS, the elution fraction was collected and designated as the flow-through fraction. After treatment with 50 mL of washing solution (the same PBS as described previously, containing 1 mol/L NaCl), 20 mL of eluting solution (PBS containing 3 mol/L potassium thiocyanate) was applied to the column. The elution fraction was collected in a new tube and designated the bound fraction. Both the flow-through and bound fractions were concentrated to 1 mL with a Centriprep-10 (Amicon Inc) at 2000g, and the buffer was changed to 1 mL of LDL buffer (0.15 mol/L NaCl containing 0.01% [wt/vol] EDTA-2K [pH 7.4]). The bound fraction was concentrated to 50 μL by a Micronex at 8944g and designated the AT-LDL fraction. After measurement of protein content, these 2 fractions were subjected to the following experiments.

Western Blotting

Five microliters of both the flow-through and the AT-LDL fraction solutions were diluted by an equal volume of SDS-PAGE sample buffer. Then they were developed in 2% to 15% polyacrylamide gel and transferred to a PVDF membrane in the manner described previously. The protein band was visualized by silver staining according to the manufacturer’s instructions. Then the PVDF membrane was dipped in Tris buffer for 5 minutes and a blocking buffer solution (2% to 15% polyacrylamide gel) was applied to the PVDF membrane and stained with brilliant blue for 5 minutes, at room temperature for 2 hours. After 3 washes with washing solution (0.1 mol/L Tris-HCl buffer [pH 8.0], 0.15 mol/L NaCl, 0.05% [wt/vol] Tween 20) and 1 with PBS, each for 5 minutes, the PVDF membrane was treated with the second antibody solution, diluted 500 times, at room temperature for 2 hours. As the second antibody, peroxidase-conjugated swine anti-rabbit immunoglobulins (No. 0217, Dako) were used as anti-apoB polyclonal antibody, and peroxidase-conjugated mouse anti-rabbit immunoglobulins (No. 0260, Dako) to OxAT-4. After 3 washes with washing solution (the same contents as used for the first antibody) and 1 with distilled water, the membrane was treated with substrate solution (4CN PLUS [No. NEL300], NEN Life Science Products, Inc) at room temperature for 30 minutes in a dark room, and the reaction was stopped by a washing with distilled water.

Isolation of the 52-kDa Protein From AT-LDL Complex and Amino Acid Sequencing

The bound fraction from the OxAT-4 affinity column was treated with 2% SDS and 5% 2-mercaptoethanol at 100°C for 5 minutes and fractionated with a Sephacryl S-200 gel filtration column (Pharmacia Biotech). The AT-containing fraction was concentrated with a Centriprep (Amicon, Inc) and purified with an affinity column with an anti-human AT antibody. Fifteen nanograms of the purified fraction was separated in 10% SDS-PAGE, and a protein of 52 kDa was visualized by silver staining. The purified fraction was transferred to the PVDF membrane and stained with brilliant blue for 5 minutes. The visible 52-kDa band was extracted and applied to an air-phase amino acid sequencer (PQSP-23, Shimazu). The amino acid sequence obtained was checked for homology by use of a FASTA sequence similarity search (http://www fasta. genome.ad.jp).

Cellular Uptake of Modified LDL: Degradation Assay of AT-LDL by the Macrophage

The proteolytic degradation of 125I-labeled flow-through and LDL by mouse resident peritoneal macrophages was measured by assay of the amount of 125I-labeled trichloroacetic acid–soluble (noniodine) material formed by the cells and excreted into the culture medium. Briefly, both transgenic and wild-type murine resident peritoneal macrophages were seeded in a 24-well plate at a cell density of 5×10^5 cells/well and grown for 72 hours in 400 mL Ham F12 containing 10% FCS at 37°C in a 5% CO_2 atmosphere. After iodide labeling, high-performance liquid chromatography and protein assay were performed to measure the protein and cholesterol contents of each fraction, and the comparative radioactivity of the labeled ligands was calculated. Just before the addition of 125I-labeled AT-LDL and competitors, plates were washed gently with pre-warmed PBS 3 times, and adheres cells were cultivated in EGM-2 medium with 2% rabbit serum added. Protein and cholesterol measurement of each fraction showed that flow-through LDL contains 0.693 mg protein/mg cholesterol and AT-LDL and 0.94 mg protein/mg cholesterol. Because the attachment of oxidized AT-LDL increases the molecular protein weight of AT-LDL, molecular cholesterol weight was used as a standard. With these values taken into account, final concentrations of 4 μg protein/mL (5.77 μg cholesterol/mL) of the flow-through fraction and 5.44 μg protein/mL (5.77 μg cholesterol/mL) of the AT-LDL fraction were applied to the macrophages. With regard to the competitors, 100, 200, and 400 times the density of the nonlabeled ligands were used. After 5 hours of cultivation, trichloroacetic acid–soluble (noniodine) radioactivity was counted. The data obtained from the cultivation with competitor at 200 times greater density were used as the baseline.

Immunohistochemistry

Human coronary artery specimens were obtained from autopsy cases with the informed consent of the families. The specimens were fixed in a 2% periodate-lysine-paraformaldehyde fixative for 4 hours, washed with PBS, and frozen in liquid nitrogen. Frozen sections were immunostained by the indirect immunoperoxidase method. After endogenous peroxidase activity had been blocked, the sections were incubated with OxAT-4 for 2 hours. An anti-macrophage antibody, KP-1 (CD68), was also used to detect macrophage distribution in serial sections. The sections were then reacted with anti-mouse IgG (Fab')_2 conjugated with peroxidase (Amersham) for 1 hour. To visualize peroxidase activity, 3,3'-diaminobenzidine was used as the substrate. Counterstaining was performed with hematoxylin.

In Situ Hybridization

Fresh-frozen sections were mounted on silane-coated glass slides. In situ hybridization was performed with an anti-sense probe of human α1-AT. The sections were fixed in 4% paraformaldehyde for 20 minutes, treated with proteinase K, and acetylated with 0.25% acetic anhydride in 0.1 mol/L triethanolamine-HCl buffer (pH 8.0) at room temperature for 10 minutes. After the sections had been dehydrated and dried in air, they were treated with hybridization solution containing the probe and incubated at 50°C for 16 hours in a humidified chamber. The sections were then washed briefly in 2× concentrated standard saline citrate (SSC) (1× SSC contains 0.15 mol/L NaCl and 0.015 mol/L sodium citrate) and in 50% formamide with 2× SSC at 4°C for 30 minutes. After reaction with a digoxigenin-labeled blocking reagent for 1 hour, the sections were exposed to anti-digoxigenin antibody overnight at 4°C. Hybridized digoxigenin-labeled probes were detected by use of the nucleic acid detection kit (Boehringer-Mannheim) containing nitro blue tetrazolium chloride, 5-bromo-4-chloro-3-indolyl phosphate, and toluidine salt, and the sections were kept in a dark place for 24 hours. After the development of color, the sections were rinsed in 10 mmol/L Tris-HCl (pH 8.0) and 1 mmol/L EDTA and were stained with methyl green for nuclear visualization. The specificity of the oligonucleotide probe used for this procedure was verified by comparison with slides treated with a sense probe.

Statistical Analysis

Results are given as the mean±SEM. Statistical comparisons were performed with the 2-tailed Student’s paired t test. Results were considered significant at a value of P<0.01.
Results

Colocalization of AT and LDL

Distribution of AT in human plasma was analyzed by gel permeation chromatography using polyclonal antibody against AT, and 2 peaks of different molecular weight were detected (Figure 1). The major peak appeared in a range from 30 to 67 kDa, with the maximal peak at 40 kDa, and most of the AT in plasma appeared in the major peak. Another peak at 600 kDa remained, however, and the distribution of apoB was closely related to the peak. Furthermore, in the same fractions, a positive signal was detected by the sandwich ELISA using polyclonal anti-apoB antibody and polyclonal antibody against AT. The amount of AT that coeluted with the LDL fraction was estimated from these data at 1% of the total plasma AT. Because the molecular weight of native AT is 52 kDa, the major peak is a nonmodified type of AT. the presence of another peak at 600 kDa, however, suggested that a fraction of AT coexisted with other, larger molecules, including apoB.

Establishment of Antibody Against OxAT-4

To investigate the presence of oxidized AT in an inflammatory locus, monoclonal antibodies were developed as described in the Methods. One IgG antibody, which can bind oxidized AT or the artificial AT-LDL complex but not the native or trypsin-AT complex or the elastase-AT complex, was established and named OxAT-4. This monoclonal antibody was characterized by the inhibition assay of its binding to immobilized artificial AT-LDL in the presence of increasing concentrations of different soluble ligands. Figure 2 indicates that C6-modified AT and C9-modified AT did inhibit the binding of OxAT-4. From the group of aldehyde-modified AT, neither C2-modified AT nor C3-modified AT inhibited the binding. Normal active AT, the trypsin/AT complex, and the neutrophil elastase/AT complex were not able to inhibit the binding of OxAT-4 even at a concentration of 100 nmol/L. Greater than half inhibition was obtained with 48.9 nmol/L C6-modified AT and 64.3 nmol/L C9-modified AT. The association constant for the binding of OxAT-4 to immobilized oxidized AT was 64.1 \times 10^9 (mol/L)^{-1}, whereas its association constant C9-modified AT was 33.1 \times 10^9 (mol/L)^{-1}. The maximal number of binding sites per apoB molecule was \approx 2.

Presence of Oxidized AT in LDL Fraction of Human Plasma

Because colocalization of a fraction of AT with apoB was suggested, immunoprecipitation using OxAT-4 was performed to detect OxAT-4 ligand in the LDL fraction of human plasma without modification of apoB and the nonspecific binding of AT. As a control, the monoclonal antibody 7D4, which recognizes sterol regulatory element binding protein-2, was used. Figure 3a and 3b shows the Western blot of monoclonal antibody against AT and anti-apoB monoclonal antibody of the immunoprecipitated fraction from 4 mg human plasma LDL, respectively. In the lane of OxAT-4, there were 2 major bands, the first of \approx 200 kDa and another of \approx 50 kDa. The 200-kDa band may be caused by the oxidized AT contained in LDL or nonspecific binding of apoB and the nonmodified type of AT. The presence of another peak at \approx 600 kDa, however, suggested that a fraction of AT coexisted with other, larger molecules, including apoB.

Figure 1. Gel filtration on Sephacryl S-200 column of a plasma sample. Human plasma (1 mL) was applied to column and eluted at 10 mL/h. Fractions (2 mL) were collected, and each fraction was used to assay protein level of AT-LDL complex and 100 times diluted solution of each fraction for AT and apoB. Difference of absorbance at 450 nm minus 620 nm, measured by ELISA as described in Methods, was plotted vs fraction number. Gel filtration column was calibrated with thyroglobulin (660 kDa, peak at fraction 17), aldolase (158 kDa, peak at fraction 22), albumin (67 kDa, peak at fraction 26), and ovalbumin (43 kDa, peak at fraction 29).

Figure 2. Characterization of OxAT-4. Interaction of OxAT-4 with artificial AT-LDL complex with various competing ligands. Artificial AT-LDL (10 ng/well) was plated antigen. Monoclonal antibody (OxAT-4) was added with and without competitor. Results are expressed as B/B0, where B is amount of OxAT-4 bound in presence of competitors and B0 amount bound in absence of competitor. Competitors were normal active AT, trypsin/AT complex, neutrophil elastase/AT complex, C2-modified AT, C3-modified AT, C6-modified AT, C9-modified AT, and chloramine T–modified AT.
Furthermore, the difference in the electrophoretic mobility suggested that the size of the 200-kDa band was greater than that found in lane 7D4. Because equal amounts of OxAT-4 and 7D4 antibody were applied for immunoprecipitation, we conclude that the difference in the intensity of the band at 200 kDa could be attributed to a binding effect of AT to apoB.

In Figure 3a, the OxAT-4 lane has a second band detected at 50 kDa. As shown in Figure 2, OxAT-4 did not recognize native AT, and the binding affinity of native AT and apoB is hardly recognized even in vitro. Furthermore, Figure 3b shows the carryover of apoB by detection of immunoprecipitate by OxAT-4. Taking these together, we conclude that the 50-kDa band is a modified form of AT that originally attached to apoB and was re-collected by OxAT-4, but later dissociated from apoB during procedures of immunoprecipitation or electrophoresis of SDS-PAGE.

Isolation and Identification of the 52-kDa Fragment
To identify the 50-kDa fragment, 15.0 mg of LDL was separated into the bound and the flow-through fractions by an affinity column filled with Sepharose 4B conjugated with OxAT-4. Figure 3c illustrates silver staining of SDS-PAGE in the reductive condition and Western blotting with anti-apoB polyclonal antibody and OxAT-4. Among the bands observable by silver staining, only 2 bands of ~50 kDa and 35 kDa were recognized by OxAT-4. Because they were detected only in the bound fraction of the affinity column, they were suggested to be fragments of oxidized AT. Of these 2 bands, the 52-kDa band was extracted and applied to an air-phase amino acid sequencer (PPSQ-23, Shimazu). The obtained amino acid sequence was searched for homology by use of a FASTA sequence similarity search (http://www.fasta.genome.ad.jp). The peptide sequence of the 52-kDa band was identical to that of the N-terminus of human $\alpha_1$-AT (Fig. I, please see http://atvb.ahajournals.org). Other bands >70 kDa were regarded as intact or fragmented apoB, because they were detectable in all the fractions.

Degradation of the AT-LDL Complex by Macrophages
The proteolytic degradation of the $^{125}$I-labeled AT-LDL and the flow-through fraction of affinity chromatography (flow-through LDL) by mouse resident peritoneal macrophages was measured by assaying the amount of $^{125}$I-labeled trichloroacetic acid–soluble (noniodide) material formed by the cells and excreted into the culture medium. The 50-kDa band was re-collected by OxAT-4, but later dissociated from apoB during procedures of immunoprecipitation or electrophoresis of SDS-PAGE.

Figure 3. a and b, Western blot of LDL fraction immunoprecipitated by OxAT-4 or 7D4. Monoclonal anti-human AT (a) and anti-human apoB-100 monoclonal antibody (b) were used as first antibody, respectively. c, Protein staining and Western blot analysis of total LDL fraction (lane 1), flow-through fraction of affinity column with OxAT-4 (lane 2), and AT-LDL (lane 3). Human LDL fraction was subjected to immunoaffinity chromatography with OxAT-4 and delipidated. Approximately 11% of LDL fraction was recovered. Lipoproteins were separated in SDS-PAGE (2% to 15%) in reductive environment and stained with silver. Western blots were performed with polyclonal antibody against apoB or OxAT-4. Molecular markers: myosin (blue), 218 kDa; $\beta$-galactosidase (magenta), 25 kDa; BSA (green), 78 kDa; carbonic anhydrase (violet), 44 kDa; and soybean trypsin inhibitor (orange), 32 kDa.

Figure 4. Degradation assay of AT-LDL and other LDL fractions was performed with mouse peritoneal resident macrophages. Radioactive iodide–labeled LDL was incubated with macrophages for 5 hours, monoiodide tyrosine was extracted, and radioactivity was assayed. a, Degradation of LDL adjusted to cholesterol weight. Wild indicates macrophages from wild-type mice; SRKO, macrophages from type A scavenger receptor–deficient mice. Flow-through indicates flow-through fraction of OxAT-4 affinity chromatography from LDL fraction. Note that AT-LDL was taken up by wild-type macrophages 4 times more effectively than that of flow-through LDL ($P<0.01$). Because the

Degradation of the AT-LDL Complex by Macrophages
The proteolytic degradation of the $^{125}$I-labeled AT-LDL and the flow-through fraction of affinity chromatography (flow-through LDL) by mouse resident peritoneal macrophages was measured by assaying the amount of $^{125}$I-labeled trichloroacetic acid–soluble (noniodide) material formed by the cells and excreted into the culture medium. The 50-kDa band was re-collected by OxAT-4, but later dissociated from apoB during procedures of immunoprecipitation or electrophoresis of SDS-PAGE.

Figure 3. a and b, Western blot of LDL fraction immunoprecipitated by OxAT-4 or 7D4. Monoclonal anti-human AT (a) and anti-human apoB-100 monoclonal antibody (b) were used as first antibody, respectively. c, Protein staining and Western blot analysis of total LDL fraction (lane 1), flow-through fraction of affinity column with OxAT-4 (lane 2), and AT-LDL (lane 3). Human LDL fraction was subjected to immunoaffinity chromatography with OxAT-4 and delipidated. Approximately 11% of LDL fraction was recovered. Lipoproteins were separated in SDS-PAGE (2% to 15%) in reductive environment and stained with silver. Western blots were performed with polyclonal antibody against apoB or OxAT-4. Molecular markers: myosin (blue), 218 kDa; $\beta$-galactosidase (magenta), 25 kDa; BSA (green), 78 kDa; carbonic anhydrase (violet), 44 kDa; and soybean trypsin inhibitor (orange), 32 kDa.

Figure 4. Degradation assay of AT-LDL and other LDL fractions was performed with mouse peritoneal resident macrophages. Radioactive iodide–labeled LDL was incubated with macrophages for 5 hours, monoiodide tyrosine was extracted, and radioactivity was assayed. a, Degradation of LDL adjusted to cholesterol weight. Wild indicates macrophages from wild-type mice; SRKO, macrophages from type A scavenger receptor–deficient mice. Flow-through indicates flow-through fraction of OxAT-4 affinity chromatography from LDL fraction. Note that AT-LDL was taken up by wild-type macrophages 4 times more effectively than that of flow-through LDL ($P<0.01$). Because the

Figure 4. Degradation assay of AT-LDL and other LDL fractions was performed with mouse peritoneal resident macrophages. Radioactive iodide–labeled LDL was incubated with macrophages for 5 hours, monoiodide tyrosine was extracted, and radioactivity was assayed. a, Degradation of LDL adjusted to cholesterol weight. Wild indicates macrophages from wild-type mice; SRKO, macrophages from type A scavenger receptor–deficient mice. Flow-through indicates flow-through fraction of OxAT-4 affinity chromatography from LDL fraction. Note that AT-LDL was taken up by wild-type macrophages 4 times more effectively than that of flow-through LDL ($P<0.01$). Because the
latter fraction contains native LDL, these data suggested that AT-LDL may be taken up via a scavenger receptor pathway like acetylated LDL. Therefore, we used scavenger receptor type A–deficient mice to evaluate the contribution of this receptor in the uptake of AT-LDL by macrophages. As is shown in Figure 4a, no difference was observed between wild-type and scavenger receptor–knockout macrophages in term of uptake.

To assess the character of AT-LDL as a ligand for the macrophages, inhibition of degradation of AT-LDL by mouse peritoneal macrophages was assayed in the same manner with various concentrations of native, copper-oxidized, and acetylated LDL. Figure 4b shows that each competitor inhibited the uptake of AT-LDL in a concentration-dependent manner. Among them, native LDL was the most potent inhibitor; 100× LDL reduced the uptake of AT-LDL by 60%, and 200× LDL by 90%.

**AT Expression by Macrophages in the Arterial Wall**

Alveolar macrophages and peripheral monocytes are reported to produce AT abundantly, but it has not yet been demonstrated that macrophages in the arterial wall also produce AT. To elucidate the production pattern of AT in the arterial wall, we observed the gene expression by in situ hybridization. Figure 6 illustrates the immunostaining of KP-1 and in situ hybridization of AT in serial sections of human atherosclerotic lesions of the coronary artery. Significant positive staining of KP-1 was observed in the intimal layer (Figure 6a), and this suggests an accumulation of macrophages. In the same area of the intimal layer, positive staining of the antisense riboprobe of the AT gene was detectable (Figure 6b), but not of the sense riboprobe (Figure 6c). Furthermore, the AT signal was not detected in the medial layer but was faintly observed in the endothelial layer. Therefore, this finding suggests that AT was produced in the arterial wall, mainly by macrophages.

**Discussion**

**Presence of Oxidized AT-LDL Complex**

The present study revealed that by gel permeation chromatography, ~1% of AT coeluted with apoB (Figure 1). This fraction of AT was shown to be related to apoB by the sandwich ELISA with both polyclonal antibody against apoB and AT. Furthermore, this AT did not inhibit neutrophil elastase activity (data not shown); therefore, this AT was assumed to be an inactive form. Because the most frequent form of inactivated AT previously reported is cleaved, oxidized, or in a complex form with proteinase,12 this AT coeluted with the apoB is taken to be a modified form.

We have developed a monoclonal antibody (OxAT-4) that recognizes oxidized AT, but not native AT (Figure 2). In this report, 2 lines of findings were obtained with this antibody that oxidized AT associates with apoB in human plasma.
First, by immunoprecipitation, we could detect the complex of oxidized AT and apoB without a purification procedure by means of an affinity column with OxAT-4 (Figure 3a and 3b), and this result goes against the possibility of an artificial complex formation of oxidized AT and apoB in vitro. Second, using an affinity column with OxAT-4, we isolated the complex of oxidized AT and LDL from the LDL fraction of human plasma. A band of 52 kDa isolated from this complex under reduced conditions was identical to the N-terminal fragment of AT. Taking these findings together, we conclude that the presence of a complex of oxidized AT and LDL in human plasma became detectable by means of OxAT-4.

Uptake of AT-LDL by Macrophages
AT-LDL isolated from human plasma was taken up as ligands and degraded by the macrophages as in the case of modified LDL, including oxidized and acetylated LDL. Figure 4a indicates that wild-type macrophages took up AT-LDL 4 times more efficiently than the flow-through fraction of affinity column OxAT-4. Because the flow-through fraction is composed mainly of native LDL, this suggested that AT-LDL shares a characteristic with modified forms of LDL reported previously. As shown in Figure 4b, however, the class A scavenger receptor pathway makes no contribution to the uptake of AT-LDL. Furthermore, competition degradation assay (Figure 4b) showed that native LDL was the strongest competitor. The inconsistency may be explained by the idea that the uptake pathway of AT-LDL was mediated by some receptors that can associate with AT-LDL most effectively, and less with native LDL, but never with strongly modified LDL, including copper-oxidized or acetylated LDL. Although the expression of the LDL receptor is not significant in the macrophages, the uptake of complexes of AT and enzyme is reported to be mediated by LDL receptor–related proteins. Taken together, these may be candidate receptors of AT-LDL, but the involvement of such unidentified receptors remains to be elucidated.

Salvage Pathway of Oxidized AT and Involvement in Atherogenesis
By means of OxAT-4, AT-LDL was detected in human atherosclerotic lesions (Figure 5). As to the clearance of this complex, 2 pathways can be hypothesized. First, the AT-LDL complex can be taken up by hepatocytes in vitro, and escape the extracellular matrix of the arterial wall. But there are 2 difficulties with this idea. First, oxidized AT is salvaged rapidly by hepatocytes, and therefore, the incidence of complex formation with LDL may very low. Next, such modified forms of LDL may pass the endothelial layer only with great difficulty. Thus, it is simpler to think that the entire process, from AT production to AT-LDL formation, happens in the arterial wall. The supportive finding observed in this study is that AT was shown to be produced by macrophages in the intimal layer (Figure 6). Furthermore, previous studies have indicated that coinubation of native AT and activated macrophages caused oxidation of AT. These findings suggest that oxidized AT can be formed within the arterial wall in the presence of activated macrophages. In addition, LDL enters the extracellular matrix of the arterial wall freely and stays there for a certain amount of time. This situation may increase the likelihood of encounters of oxidized AT and LDL, and consequently, of formation of AT-LDL complex. By now, the precise process of AT-LDL formation is under active investigation. Furthermore, studies into the characterization of lipid profiles and the degree of modification of apoB of AT-LDL will show the pathological significance of AT-LDL.

Here, we report the establishment of an anti–oxidized α_1-AT monoclonal antibody (OxAT-4). A complex of oxidized AT and LDL formed in vivo could be detected and isolated by OxAT-4. Localization of AT-LDL in atherosclerotic lesions and enhanced degradation of AT-LDL by macrophages suggested the involvement of this complex in atherogenesis. The precise mechanism of complex formation in the arterial wall and the relationship between the plasma levels of AT-LDL and the incidence of ischemic disease remain to be elucidated.

Acknowledgments
This work was supported by The Japan Foundation for Aging and Health and the Research for the Future Program of the Japan Society for the Promotion of Science. The authors thank Dr Kevin Boru of Advanced Clinical Trials, Inc, for review of the manuscript.

References


In Vivo Complex Formation of Oxidized α₁-Antitrypsin and LDL
Shinichi Mashiba, Youichiro Wada, Motohiro Takeya, Akira Sugiyama, Takao Hamakubo, Akio Nakamura, Noriko Noguchi, Etsuo Niki, Akashi Izumi, Mika Kobayashi, Kazuo Uchida and Tatsuhiko Kodama

doi: 10.1161/hq1101.098232
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/21/11/1801

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2001/10/25/21.11.1801.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/