Activation of Primary Human Monocytes by the Oxidized Form of α1-Antitrypsin*

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The oxidation of methionine residues in many proteins, including the serine proteinase inhibitor α1-antitrypsin (AAT), can result in functional inactivation. In this study we investigated the pro-inflammatory properties of oxidized AAT (oxAAT), specifically its ability to activate human monocytes in culture. Monocytes stimulated with oxAAT at concentrations up to 0.2 mg/ml for 24 h showed significant elevation in monocyte chemoattractant protein-1, cytokine interleukin-6, and tumor necrosis factor-α expression and increased NADPH oxidase activity. Monocytes activated with oxAAT showed surprising effects on lipid metabolism. Expression of low density lipoprotein (LDL) receptors increased by up to 76% compared with controls but was not accompanied by any changes in 125I-labeled LDL binding and, paradoxically, decreased LDL uptake, degradation, and intracellular cholesterol synthesis. oxAAT also down-regulated the scavenger receptor CD36, which takes up and is up-regulated by oxidized LDL and is down-regulated by cholesterol efflux. As a by-product of oxidative events accompanying inflammation, oxAAT has multiple effects on cytokine expression, generation of reactive oxygen species, and on intracellular lipid metabolism. The up-regulation of monocyte-derived reactive oxygen by oxAAT could potentially result in self-amplification of AAT oxidation and, thereby, the other effects deriving from it. This implies that there are as yet unidentified regulatory processes that control this cycle.

Proteinases are normally tightly regulated by their naturally occurring inhibitors, but in some pathological conditions, proteinase activity may overwhelm inhibitory capacity as a result of proteolytic or oxidative inactivation of the inhibitor (1–3). α1-Antitrypsin (AAT) is an acute phase protein and one of the major serine proteinase inhibitors in human plasma. It is synthesized primarily in the liver but also in extra-hepatic tissues and cells including neutrophils, monocytes, and macrophages, alveolar macrophages, intestinal epithelium cells, breast carcinoma cells, and the cornea (4–8). Local regulation of AAT may be important in maintaining the protease-antiprotease balance and preventing tissue damage induced by proteases in the microenvironment of injury or inflammation. For instance, several studies have demonstrated that local release of bacterial endotoxin and/or early production of inflammatory mediators such as interleukin-1 (IL-1) and tumor necrosis factor α (TNFα) in lung tissue may up-regulate AAT expression in monocytes and, thereby, serve an important regulatory role in preventing protease destruction in the lung microenvironment (9, 10). Enhanced plasma levels of α1-AAT are also known to be correlated with severity of inflammatory processes associated with coronary atherosclerosis and have been suggested to play an important part in protecting endothelial cells against the degradative effects of proteases released from activated phagocytes (11, 12).

It has previously been shown that inflammatory exudates contain AAT in diverse molecular forms including native inhibitory form and several inactive, noninhibitory forms such as complexed with protease, cleaved, polymerized, and oxidized (13–15). AAT is known to be inactivated by cleavage and complex formation with target protease, such as leukocyte elastase, by cleavage by certain nontarget matrix metalloproteases, by oxidation of the reactive site methionine, and by polymerization induced by various factors such as oxidation, low pH, and interactions with other molecules (1, 16–18). Our understanding of AAT function has been derived primarily from studies of native, functionally active AAT, whereas possible biological roles of oxidized, polymerized, and post-cleavage, noninhibitory forms of AAT have not been thoroughly investigated. Inactivation of AAT with subsequent enhanced proteolysis, particularly by neutrophil elastase, has been invoked in the pathogenesis of lung disease, such as emphysema and lung matrix degradation in adult respiratory distress syndrome as well as in rheumatoid arthritis (19, 20). It has also been proposed that fragmented and complexed AAT promotes an increase in synthesis of AAT in human monocytes and mediates neutrophil chemotaxis (21, 22), which suggests that proteolytically inactivated AAT may play multiple roles at sites of inflammation. In our previous work, we examined the effects of the proteolytically modified, cleaved form of AAT on Hep2 cells and also the effects of the amyloidogenic C-terminal fragment (C-36, corresponding to amino acid sequence 358–396) of AAT on human monocyte culture. We showed that these forms of AAT induce significant changes in lipid catabolism in both cell types and a remarkable stimulation in pro-inflammatory cytokine and free radical production and also up-regulate scavenger receptor CD36 in primary human monocyte cultures (23–26). This led us to propose that under inflammatory conditions, AAT might play not only a role as an inhibitor of proteases but also as a protease substrate and a reservoir of physiologically active degradation products.

Oxidized AAT is a modified form of AAT found in inflammatory exudates at levels of about 5–10% that of total AAT (27, 28). The amino acid at position P1 in the reactive site of each
inhibitory serpin primarily determines the specificity of inhibition and, thereby, its biological activity. P1 in AAT is methionine, the most readily oxidized amino acid of proteins, which is converted by oxidation to methionine sulfoxide. Met can be attacked by various oxidants produced in biological systems, such as peroxide, hydroxyl radicals, hypochloride, chloramines, and peroxynitrite (29, 30). Evidence that this occurs in vivo comes from the observation that inactive AAT purified from inflammatory synovial fluid contains methionine sulfoxide residues (31, 32). Also, oxidative inactivation of the AAT can be induced in vitro by incubating AAT with purified myeloperoxidase or stimulated phagocytes (33). This oxidation results in a change in the functional activity of AAT and probably promotes local inflammatory processes, including uncontrolled degradation of connective tissues. Oxidative inactivation of AAT with subsequent enhanced proteolysis, particularly by neutrophil elastase, has been invoked in the pathogenesis of pulmonary emphysema (34) and rheumatoid arthritis (15). That AAT oxidation and proteolysis occur is supported by findings that, on average, 41% of total AAT in rheumatoid arthritis synovial fluid is inactive (31). Recently Scott et al. demonstrate that oxidation of AAT promotes AAT-immunoglobulin A complex formation in vitro. IgA-oxidized AAT complexes isolated from synovial fluid of rheumatoid disease patients were suggested to protect the oxidized AAT molecule from proteolytic cleavage by free elastase (35).

Leukocytes, neutrophils, and macrophages, which secrete large quantities of oxidants at sites of inflammation, were shown to induce oxidative inactivation of AAT in vivo and to result in perturbed protease-antiprotease balance. Although oxidized AAT plays a pro-inflammatory role at sites of inflammation because of its loss of inhibitor activity toward proteases, it cannot be excluded that oxidized AAT may also have other biological activities related to inflammation. In this study, we have examined whether oxidized AAT can stimulate monocyte activation. We show that oxidized AAT induces monocyte chemoattractant protein-1 (MCP-1) and pro-inflammatory cytokine expression, activates NADPH oxidase, decreases LDL uptake and degradation and intracellular cholesterol synthesis, increases LDL receptor number, and decreases scavenger receptor CD36 expression.

MATERIALS AND METHODS

Preparation and Characterization of Oxidized AAT—Native, purified AAT was a gift from Prof. C.-B. Laurell, Department of Clinical Chemistry, MAS, Malmo, Sweden. Native AAT was oxidized with N-chlorosuccinimide (Sigma) as described (32). Briefly, a reaction between AAT and N-chlorosuccinimide in a molar ratio 1.25 in a 0.1 M Tris-HCl buffer, pH 8.0, was allowed to proceed at room temperature for 30 min, and oxidized AAT was recovered after passing the reaction mixture through a Sephadex G-25 column (2 cm x 15 cm) that had been equilibrated in 50 mM NH4HCO3 or by using a centrifugal microconcentrator. The oxidized AAT was also tested for capacity to form covalent complex with pancreatic elastase (EC 3.4.21.36) (Sigma). Samples of oxidized AAT or native AAT were digested with pancreatic elastase at a 1:2.1 molar ratio for 15 min at room temperature. The reaction was stopped by adding SDS sample buffer, and mixtures were analyzed by 7.5% SDS-PAGE without reducing agent and stained with Coomassie Blue.

The endotoxin content in the oxAAT preparations used was tested by limulus amebocyte lysate (LAL), Comatrace® Chromo-LAL assay (Chromogenix, AB, Sweden) according to the manufacturer’s instructions. Endotoxin standard solutions in intravenous doses, 0.05 enzyme units/ml and tested samples were placed into a microplate (preincubated at 37 °C), mixed with substrate, and incubated in a reader (ThermoMax, Molecular Devices, Inc) at 37 °C for 1 h. Negative controls (endotoxin-free water) were included in every set of assays. Absorbance measurements at 405 nm were collected with time after the addition of chromo-LAL and analyzed by the software program. Assay sensitivity was 0.005 enzyme units/ml. According to this assay, the endotoxin levels ranged between 0.006 and 0.079 enzyme units/ml in all oxAAT preparations used in our experiments.

Lipoprotein Isolation and Labeling—LDL was isolated by sequential preparative Ultracentrifugation using an Optima® XL-100 Ultracentrifuge (Beckman) according to the procedure described previously (25). Native LDL density range (1.034–1.054 kg/liter) was used to prepare LDL for the experiments. LDL was labeled with 125I by the iodine monochloride method (36). Unbound 125I was removed by chromatography on Sephadex G-25 PD-10 columns (Amersham Pharmacia Biotech) followed by extensive dialysis against 0.15 M NaCl, 1 mM EDTA, and 0.03 M KI and further dialysis against 0.15 M NaCl, containing 1 mM EDTA. The specific activity of LDL ranged between 229 and 433 cpm/ng of LDL protein. The endotoxin content in the lipoprotein preparations used was tested by LAL assay (E-TOXATE, Sigma). LDL samples were diluted 1:10 in endotoxin-free water, heated at 65 °C for 5 min to inactivate the LDL inhibitor found in plasma, and incubated at 37 °C for 1 h. The positive test performed using endotoxin standard dilutions (0.25, 0.125, and 0.06 enzyme units/ml) was formation of a hard gel, which permits complete inversion of the tube. Absence of hard gel formation was found in all our tested LDL samples, which are endotoxin-free, as assessed by this assay.

Isolation and Culture of Monocytes—Human monocytes were isolated from buffy coats obtained from pooled plasma of different donors by the standard Ficoll-Hypaque procedure as described previously (26). A monocyte isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) was used to obtain a highly pure monocyte population. Cell purity was >90%, as determined on an AC900® AutoCounter (Swellab Instruments, AB); cell viability was analyzed by 0.4% trypan blue staining. Monocytes were plated at a density of 2 × 10^5 cells/ml into plastic plates or dishes. After removal of nonadhering cells, the remaining adherent monocytes were cultured in RPMI 1640 (Life Technologies, Inc.) supplemented with 2 mM N-acetyl-L-alanyl-L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 1% (by volume) nonessential amino acid, 2% (by volume) sodium pyruvate, and 20 µg Hepes (Fluka, Chemie AG) without serum at 37 °C in a 5% CO2. Experiments were performed within 24 h after plating of monocytes. For experiments, monocytes were incubated alone or with the addition of native or oxAAT (0.05, 0.1, and 0.2 mg/ml) or native LDL (100 µg/ml) separately or together.

LDL Uptake and Degradation Assays—Monocytes seeded into 12-well plates (Nunc), 2 × 10^5 cells/well were incubated in 1 ml of RPMI 1640 medium without fetal calf serum (see above) without or with various concentrations of native or oxAAT and with 125I-LDL (3.4 µg of LDL protein/mg of cell protein) for 24 h at 37 °C in 5% CO2. The medium was aspirated for subsequent determinations of LDL degradation, measured as the trichloroacetic acid-soluble noniodine 125I radioactivity in the medium (37). Cells were washed 3 times with PBS and scraped into 1 ml of 0.5% NaOH for 125I-LDL uptake measurement (the sum of bound and internalized 125I-LDL) and for cell protein determination. The radioactivity was determined in an LKB 1271 automatic gamma counter (Wallac, Turku, Finland). The results are expressed as ng of LDL protein taken up or degraded per mg of cell protein.

LDL Binding Assay—The binding of LDL to monocytes was performed at 4 °C. Cells in serum-free medium were incubated with several concentrations of oxAAT (up to 0.2 mg/ml) alone or in the presence of LDL (100 µg/ml). Binding studies at 4 °C were performed by washing the cells three times with 1 ml of ice-cold PBS followed by precooled for 20 min at 4 °C in 1 ml of ice-cold RPMI medium containing 0.5% human serum albumin. After the addition of 125I-LDL (3.5 mg/liter), the cells were incubated for 2 h at 4 °C, then washed 3 times with 1 ml of ice-cold PBS. New medium containing 10 g/liter dextran sulfate (Mw ~ 500 000, Amersham Pharmacia Biotech) was added (polymer was added for osmotic balance), and the cells were subjected to a second incubation for 1 h at 4 °C in a rotary shaker at 60 rpm. Dextran sulfate is known to release receptor-bound LDL from the surface of the cell (38). Medium containing dextran sulfate-released 125I-LDL was then aspirated, and the radioactivity was measured in a gamma counter.

RNA Isolation—Total RNA from monocytes was isolated as outlined by Davis et al. (39). Cold GT buffer (4 mM guanidine thiocyanate, 3 mM sodium acetate, pH 6, and 7% β-mercaptoethanol) was added directly to monocytes in culture dishes. After the addition was added, the cells were layered onto a 4-ml 5.7 M CsCl cushion and centrifuged at 100,000 × g for 16 h. The pellet was washed with ethanol (95%), suspended in diethylpyrocarbonate-treated distilled water (dH2O), precipitated in 95% ethanol at pH 5.0, and stored at −20 °C.

Reverse Transcription Polymerase Chain Reaction—LDL receptor mRNA was quantified by reverse transcription polymerase chain reac-
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Monocytes were cultured for various time points alone or with the addition of oxAAT and/or LDL as described above. Culture medium was collected, and MCP-1 expression was assayed by a quantitative sandwich immunoassay technique according to manufacturer’s instructions (R&D Systems Europe Ltd, Abingdon, UK). The optical density was determined using a microplate reader at 450 nm. The readings at 570 nm were subtracted from the readings at 450 nm for wavelength correction. The duplicate readings for each standard, control, and samples were averaged, and the average zero standard optical density was subtracted.

Assay for Detection of NADPH Oxidase-generated Superoxide by Cyclchrome c Reduction—Superoxide produced from the NADPH oxidase was monitored by the superoxide dismutase-inhibitable rate of cytochrome c reduction (40). Monocytes (2 × 10^6/ml) were incubated for various time points up to 3 h at 37 °C, with 1.5 mg/ml cytochrome c and oxAAT in 2 ml of air-saturated PBS containing 0.5 mM MgCl₂, 0.7 mM CaCl₂, and 0.1% glucose in the presence or absence of 300 units/ml superoxide dismutase. After incubation, the cells were removed by centrifugation at 400 × g for 5 min, and the reduced cytochrome c in the supernatant was measured at 550 nm. The rate of superoxide production is given by the difference in the rate of cytochrome c reduction in the absence and presence of superoxide dismutase.

[^HT]Thymidine Incorporation Assay—Cells were incubated with and without added oxidized or native AAT for 20 h. [^HT]Thymidine (Amerham Pharmacia Biotech) was then added to the cells (0.2 μCi/ml) for a further 4-h incubation at 37 °C. After the medium was aspirated, the cells were washed twice with 0.5 mM NaCl and incubated for 5 min with 5% trichloroacetic acid. Cells were then washed with water, dissolved in 1 ml 0.5 M NaOH, and neutralized with 200 μl of HCl, and radioactivity was determined in a β-counter (Packard 300CD liquid scintillation spectrometer; Packard Instrument Co.).

Statistical Analysis—The differences in the means in experimental results were analyzed for their statistical significance with independent sample two-sided t test and/or one-way analysis of variance combined with a multiple comparisons procedure (Scheffé multiple range test) with the overall significance level of α = .05. Statistical Package for Social Sciences (SPSS for Windows, Version 6.0) was used for the calculations (41).

RESULTS

Oxidative Inactivation of AAT—AAT can be rendered inactive toward elastase by at least two known mechanisms: either by oxidation of the reactive center Met-358 or by protease cleavage of peptide bonds close to the reactive center (1, 31). In our experimental model, purified native AAT was oxidized with N-chlorosuccinimide and characterized for its ability to interact with pancreatic elastase. Samples of native and oxAAT alone or incubated with pancreatic elastase were subjected to 7.5% SDS-PAGE. In Fig. 1 a single band with similar molecular mass is seen for both native and oxAAT. Interaction between...
native AAT and elastase results in the generation of cleaved AAT and formation of the higher molecular weight, SDS stable AAT-elastase complex (83 kDa). The oxidation of AAT drastically diminishes its ability to interact with elastase. No complex formation between oxAAT and elastase is observed under the same conditions, and only low molecular weight bands are visible on the gels. This is consistent with observations of other investigators showing that oxidation of AAT has a profound effect on its ability to interact with most serine proteinases, including pancreatic elastase (42).

**Activation of Production of NADPH Oxidase-derived Superoxide by Oxidized AAT-stimulated Monocytes**—Phagocytic cells, including monocytes, carry a plasma membrane-bound NADPH oxidase that catalyzes production of superoxides (43). Superoxide produced from NADPH oxidase can be monitored by the superoxide dismutase-inhibitable rate of cytochrome c reduction. To test whether stimulation of monocytes with oxAAT results in the activation of plasma membrane NADPH oxidase, we treated monocytes with various concentrations of oxAAT (up to 0.2 mg/ml) and monitored superoxide generation by ferricytochrome c reduction at 30 min. As shown in Table I, the presence of oxAAT resulted in increases of from 2- to 4.2-fold (p < 0.01) in superoxide production after incubation for 30 min. In contrast, native AAT showed no significant effects on oxidase activity (data not shown). The generation of reactive oxygen species due to stimulation of the NADPH oxidase activity causes direct oxidative damage to biomolecules, including AAT. In this way, oxAAT, which activates NADPH oxidase, promotes the oxidative inactivation of AAT.

**Induction of Chemokine MCP-1 and Pro-inflammatory Cytokines by Oxidized AAT**—It was previously demonstrated that proteolytically cleaved and native AAT have chemotactic activity (44, 26) and can induce expression of chemokine MCP-1 in monocytes. To determine the effects of oxAAT on MCP-1 protein expression, monocytes were incubated at 37°C for 24 h without and with the addition of a constant amount of oxAAT (0.10 mg/ml) or LDL (100 μg/ml) separately and together. As shown in Table II, both oxAAT and oxAAT + LDL significantly stimulated MCP-1 expression. oxAAT alone induced MCP-1 expression by about 106 times, whereas oxAAT + LDL induced MCP-1 expression by about 69 times (p < 0.001) compared with controls. We also examined the levels of pro-inflammatory cytokines in medium from monocytes cultured in the presence of oxAAT and LDL separately or simultaneously. Unstimulated or LDL-treated cells were negative controls. Cytokines tested (IL-6 and TNFα) showed a significant increase in response to oxAAT (Table II). In contrast, the addition of LDL and oxAAT simultaneously resulted in a significant decrease in MCP-1 levels (by about 1.3-fold, p < 0.01) and TNFα levels (by about 1.9-fold, p < 0.01) relative to those observed in cells stimulated only with oxAAT but had no effect on oxAAT-induced IL-6 levels. The origin of this large inhibitory effect of native LDL on oxAAT-induced MCP-1 and TNFα, but not on IL-6 levels, is not known, but it may be linked to the degree of activation of monocytes by oxAAT and to the fact that synthesis and release of these cytokines is known to be under independent control (46, 47). Another explanation might be also that LDL blocks the binding of oxAAT to a receptor and prevents it from acting on the sites specific to induction of MCP-1 and TNFα.

**Inhibition of LDL Uptake and Degradation in Monocytes by Oxidized AAT**—Previously it has been shown that human monocyte pro-inflammatory activation is associated with increased lipid uptake (48, 49). To evaluate the effects of oxAAT on LDL binding and internalization in human monocytes, cells were incubated with several different concentrations of oxAAT for 24 h. The oxAAT had no effect on LDL binding but significantly inhibited LDL uptake (33% ± 6.8, p < 0.05) and degradation (90.8 ± 19.4, p < 0.05) in a concentration-dependent manner (Fig. 1). Simultaneous treatment of monocytes with cold LDL (100 μg/ml) and oxAAT (0.1 mg/ml) reduced LDL uptake by 35.1% ± 2.5, p < 0.05, compared with LDL alone. In contrast to LDL uptake, the levels of LDL degradation products in the medium of the cells stimulated with oxAAT and LDL were found to be of the same magnitude as those of cells stimulated only with LDL (Fig. 2). The accumulation of lipids in monocytes treated with oxAAT or LDL alone or together was assessed qualitatively by Oil Red O staining. In support of the LDL uptake data, monocytes treated with LDL and oxAAT simultaneously showed much lower amounts of lipid droplets compared with monocytes treated with LDL alone, whereas control monocytes and those treated with oxAAT showed no lipid droplets at all (Fig. 3). These data together indicate that oxAAT inhibits lipid uptake and accumulation in human monocyte culture.

**Enhancement of LDL Receptor mRNA and Protein Levels by Oxidized AAT**—LDL receptor levels were determined to evaluate whether diminished LDL uptake and degradation in monocytes treated with oxAAT is related to a decrease in the number of receptors or in LDL receptor protein expression. Unexpectedly, treatment of monocytes with oxAAT (0.05 and 0.1 mg/ml) for 24 h caused significant and dose-dependent increases in expression of the LDL receptor mRNA (up to 177% ± 30.7, p < 0.05) compared with control (100% ± 0.6). Furthermore, to elucidate whether the stimulatory effect of oxAAT on LDL receptor synthesis could be inhibited by receptor-saturating concentrations of LDL, the cells were simultaneously treated with oxAAT and LDL (100 μg/ml). As shown in Fig. 4, the stimulatory effect of oxAAT on LDL receptor mRNA expression was reduced by about 69% when cells were treated with oxAAT and LDL together compared with oxAAT alone. This magnitude of suppression of mRNA expression corresponded to the reduction in the LDL receptor mRNA levels in cells treated only with LDL (Fig. 4). Consistent with this, LDL receptor protein levels in monocytes treated with oxAAT were increased by about 15% and decreased in monocytes treated with LDL (by about 15%) or both oxAAT and LDL (by 23%) (data not shown). Our data show that treatment of monocytes with oxAAT results in enhanced transcriptional expression and receptor numbers of LDL receptor but has no effect on 125I-LDL binding, and in contrast, reduces specific 125I-LDL uptake and degradation. The lack of correlation between LDL receptor mRNA levels and

**Table I**

| Stimulation of production of NADPH oxidase-derived superoxide by oxidized AAT in human monocytes |
|---|
| Oxidized AAT | Cytochrome c reduction | Mean S.E. | S.E. |
| mg/ml | nmol/30 min/10⁶ cells |
| 0 | 143 | 19.64 |
| 0.05 | 293 | 20.4 |
| 0.1 | 592 | 41.8 |
| 0.2 | 606 | 65.2 |

* Mean and S.E. of three experiments.

**Table II**

| Chemokine and cytokines produced by monocytes incubated with oxidized AAT together with LDL (100 μg/ml) for 24 h |
|---|
| MCP-1 (pg/ml) | IL-6 (pg/ml) |
| Mean S.E. | Mean S.E. |
| Control | 41.5 ± 0.93 | 19.23 ± 0.44 |
| oxAAT | 4550.8 ± 35.4 | 615.6 ± 1.99 |
| LDL | 50.9 ± 1.8 | 18.4 ± 0.86 |
| oxAAT + LDL | 3565.4 ± 106.3 | 631.8 ± 3.18 |

* Mean and S.E. of three experiments.
125I-LDL uptake and degradation rates in monocytes treated with oxAAT suggest that the turnover of LDL receptors might be suppressed in monocytes treated with oxAAT, or alternatively, LDL receptors might be blocked by oxAAT. Additional studies on these observations are therefore warranted.

Inhibition of Cholesterol Synthesis in Human Monocytes by Oxidized AAT—Although a significant decrease in LDL uptake and degradation rates in monocytes treated with oxAAT suggests that the turnover of LDL receptors might be suppressed in monocytes treated with oxAAT, or alternatively, LDL receptors might be blocked by oxAAT. Additional studies on these observations are therefore warranted.

Inhibition of Cholesterol Synthesis in Human Monocytes by Oxidized AAT—Although a significant decrease in LDL uptake and degradation in monocytes treated with oxAAT was expected to result in stimulation of intracellular cholesterol synthesis, our data surprisingly show that oxAAT added for 24 h to monocytes treated with oxAAT, or alternatively, LDL receptors might be blocked by oxAAT. Additional studies on these observations are therefore warranted.

Inhibition of Cholesterol Synthesis in Human Monocytes by Oxidized AAT—Although a significant decrease in LDL uptake and degradation in monocytes treated with oxAAT was expected to result in stimulation of intracellular cholesterol synthesis, our data surprisingly show that oxAAT added for 24 h to monocytes treated with oxAAT, or alternatively, LDL receptors might be blocked by oxAAT. Additional studies on these observations are therefore warranted.

Down-regulation of CD36 Scavenger Receptor Protein Expression by Oxidized AAT—Regulation of CD36 expression on monocytes involves cell surface adhesion molecules, soluble mediators, and cellular cholesterol levels (50, 51). Recently it has been demonstrated that CD36 expression is down-regulated by cholesterol efflux (52). Since in our experimental model we observed large alterations in intracellular cholesterol content in monocytes stimulated with oxAAT, we also sought to evaluate the effects of these changes on the expression of CD36 protein. Treatment of cells with various concentrations of ox-AAT (up to 0.1 mg/ml) for 24 h significantly reduces LDL uptake by monocytes and degradation.

FIG. 2. Effect of oxidized AAT on 125I-LDL uptake and degradation in monocytes. Each point represents the mean ± S.E. of three repeats from two independent experiments. oxAAT significantly, in a dose-dependent manner, decreases 125I-LDL uptake by monocytes and degradation.

FIG. 3. The accumulation of lipids in monocytes visualized by staining with Oil Red O. Monocytes were incubated with LDL (100 μg/ml) and oxAAT (0.1 mg/ml) separately or together for 24 h. The cells were fixed and stained with Oil Red (400×). The arrow indicate Oil Red O-stained vacuoles.

FIG. 4. The effect of oxAAT on the expression of LDL receptor mRNA in monocytes alone or in the presence of native LDL (100 μg/ml). Each bar represents the mean ± S.E. of three separate experiments. One-way analysis of variance and the Scheffe multiple-comparison test (α = 0.05) show that oxAAT, in a concentration-dependent manner, significantly induces the LDL receptor mRNA expression.

FIG. 5. The effect of oxAAT and LDL on cholesterol synthesis by monocytes. Each bar represents the mean ± S.E. of four independent experiments. One-way analysis of variance and the Scheffe multiple-comparison test (α = 0.05) show that oxAAT, in a concentration-dependent manner, significantly inhibits cholesterol synthesis from [2-14C]acetate. Cells incubated with LDL (0.1 mg/ml) or oxAAT (0.2 mg/ml) showed a decrease in cholesterol synthesis to a similar magnitude.
membranes were exposed to film for 20 s.

SDS-PAGE gel and analyzed by Western blotting with anti-CD36 antibody using the chemiluminescence Western blotting detection kit. The membranes were exposed to film for 20 s.

**FIG. 6.** Western blot analysis of CD36 scavenger receptor in cell lysates of monocytes. Cell lysates were separated on a 7.5% SDS-PAGE gel and analyzed by Western blotting with anti-CD36 antibody. The membranes were exposed to film for 20 s.

**FIG. 7.** 1% agarose electrophoresis, pH 8.6, of native LDL alone and incubated with oxidized or native AAT. LDL alone or together with various forms of AAT was incubated in monocyte culture medium without the cells for 24 h. The arrow shows the sample application slot. Lane 1, oxAAT; 2, native LDL; 3, native LDL + oxidized AAT; 4, native AAT; 5, native LDL; 6, LDL + native AAT.

Analysis revealed that the decrease in CD36 expression in monocytes paralleled the increased concentration of oxAAT, even in the presence of excess of LDL.

**DISCUSSION**

Much data support the widely accepted notion that a major function of AAT is the inhibition of overexpressed proteinases during inflammation. However, it is also known that the biological activity of AAT is affected by chemical modifications, including oxidation of the reactive-site methionine, intramolecular polymerization, and cleavage by unspecific proteases, all of which result in AAT inactivation and/or degradation. Previously, Matheson et al. (53) describe an important pathway whereby the levels of active circulating AAT could be reduced in normal, healthy individuals as a result of the enzymatic oxidation of the reactive-site methionine of AAT by neutrophil myeloperoxidase. In support, both oxidized and cleaved inactivated AAT have been detected (about 26–50% of total AAT) in the synovial fluid of patients with rheumatoid arthritis (31). Blood monocytes in inflammatory conditions are known to be activated to produce reactive molecular species such as oxygen-free radicals, nitric oxide, cytokines, proteinases, and proteinase inhibitors. Stimulated monocytes can abolish the inhibitory ability of AAT both through proteolysis by released proteinases and oxidatively by released reactive oxygen species. We hypothesize that generation of increased levels of inactivated forms of AAT, including oxidized AAT, during acute phase processes may be directly related to the development of oxidative stress and reflect the status of the inflammation.

In our previous work, we examined the effects of proteolytically cleaved AAT and the amyloidogenic C-terminal fragment (C-36, corresponding to amino acid sequence 358–396) of AAT on HepG2 cells and cultured monocytes and showed that these forms of AAT exert a significant change in intracellular lipid catabolism and mediate pro-inflammatory activation (23–26). Recent studies by Kataoka et al. (17) provide experimental evidence that the C-terminal fragment of AAT may also enhance tumor growth and invasiveness in vivo. Together these findings suggest that proteolytically inactivated AAT may play multiple roles at sites of inflammation.

The present study was designed to ascertain the biological relevance of the oxidized form of AAT using primary human monocyte cultures. We investigated the effects on cultured monocytes of oxAAT alone and in the presence of excess of LDL. We found that oxAAT in a concentration-dependent manner significantly activates NADPH oxidase, which is mainly responsible for the production of active oxygen species. Our findings indicate that oxAAT can potentiate the ability of mononuclear phagocytes to produce reactive oxygen species, which implies that oxAAT, through activation of the NADPH oxidase, promotes its own formation and thereby contributes to inflammation. Further studies are required to understand whether this effect of oxAAT can be attributed to the specific properties of oxAAT or to receptor-mediated interactions.

Expression of MCP-1, which is believed to be the major mediator of monocyte chemotactic migration, increases in response to several stimuli including cytokines, free radicals, and oxidized LDL, but it can also modulate other functions of monocytes (e.g., generation of reactive oxygen species) (54). We observed strong induction of MCP-1 protein release in cultured monocytes stimulated with oxAAT. Generation of oxidized AAT in inflammatory loci could thus be an indirect promoter of monocyte recruitment and activation. However, it remains to be elucidated whether oxAAT-induced MCP-1 levels in monocyte medium are dependent on de novo protein synthesis and also whether oxAAT induces MCP-1 expression directly or indirectly via stimulation of free radical and cytokine expression. It should also be noted that treatment of monocytes with oxAAT in the presence of native LDL resulted in significant reduction of the stimulatory effects of oxAAT on MCP-1 (by about 1.3-fold) compared with oxAAT alone. Native LDL-induced MCP-1 levels increased by only 22%. It is possible that binding of oxAAT to LDL receptors is essential for MCP-1 up-regulation.

Previously, Chidwick et al. (55) show that the secretion of TNFα from human peripheral blood mononuclear cells can be suppressed by native AAT in a dose-dependent manner, but on the other hand, a positive relationship between AAT inactivation and TNFα concentration was shown in the synovial fluids of patients with rheumatoid arthritis. We also found a significant increase in pro-inflammatory cytokine (IL-6 and TNFα) levels in medium from monocytes cultured with oxAAT. However, treatment of monocytes with oxAAT in the presence of LDL resulted in a large reduction in oxAAT-stimulated TNFα levels (by about 50%) but had no effect on the stimulated levels of IL-6. This may be related to the fact that TNFα and IL-6 expression are not always affected in parallel by the same stimulus, and specifically, that levels of TNFα, but not IL-6, are known to be directly co-ordinated with LDL receptor expression, which is generally regulated by uptake of LDL (48).

To test whether the oxidized form of AAT affects intracellu-
lar lipid catabolism and induces changes in human monocytes similar to those observed for the cleaved forms of AAT, we examined the effects of oxAAT on LDL binding, uptake, and degradation. We found that incubation of monocytes for 24 h without and with addition of oxAAT at various concentrations (up to 0.2 mg/ml) has no effect on LDL binding but significantly inhibits LDL uptake and degradation in a concentration-dependent manner. Since the uptake of most LDL cholesterol is mediated by the LDL receptor pathway, we also examined the effects of oxAAT on expression of LDL receptor mRNA and on protein levels. Interestingly, treatment of monocytes with oxAAT resulted in up-regulation of LDL receptor mRNA and protein expression. Although signaling pathways or effectors responsible for activation of LDL receptors are not completely understood, it is known that the principal pathway of LDL receptor regulation is controlled by cholesterol and its metabolites (57). Cellular uptake of cholesterol in the form of LDL is a receptor-mediated process, and additional factors, including oxidized LDL, but the cellular regulation of this multifunctional receptor has not been well studied (59, 60). Based on the results from our studies, we investigated its effects on the scavenger receptor CD36 protein expression in monocytes treated with oxAAT and LDL simultaneously showed no apparent differences in CD36 expression compared with monocytes cultured only with oxAAT. Consistent with this, we did not observe any increase of lipid accumulation in monocytes treated with oxAAT or oxAAT and LDL by staining for accumulated lipids with Oil red. These data suggest that alterations in cellular cholesterol induced by oxAAT may alter cellular events linked to CD36 expression and lipid accumulation.

Several recent studies have shown that decreased intracellular cholesterol levels are related to cell apoptosis and death (61). Based on the results from [3H]thymidine incorporation experiments performed on monocytes treated with oxAAT for 24 h, we conclude that oxAAT does not diminish DNA synthesis in monocytes and does not affect cell viability. It cannot be excluded that the observed pro-inflammatory activation and perturbed lipid homeostasis in monocytes treated with oxAAT are initial events leading to perturbed cellular functions. The extent of these effects of oxAAT on pro-inflammatory and defense mechanisms may determine whether cells survive or die.

The observed effects in this study of oxAAT on intracellular cholesterol homeostasis are totally different from that described for the cleaved forms of AAT. This suggests that the different molecular forms of AAT generated during inflammatory conditions have diverse functions, and which inflammation-mediated mechanisms are activated or suppressed may depend on which forms of AAT predominate in the inflammatory microenvironment. In summary, our data provide evidence that generation of oxAAT in inflammatory loci may play a role in modulating the inflammatory response. The mechanisms by which oxAAT induces monocyte activation and alterations in cholesterol homeostasis remain to be determined.

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