Human Neutrophils Express the α1-Antitrypsin Gene and Produce α1-Antitrypsin

By Roland M. du Bois, Jean-Francois Bernaudin, Paavo Paakko, Richard Hubbard, Hideki Takahashi, Victor Ferrans, and Ronald G. Crystal

The potent serine protease, neutrophil elastase (NE), is stored in neutrophil azurophilic granules, where it is available to degrade phagocytosed material and can be released by the cell to assist in tissue migration and help clear tissue debris. While neutrophils carry NE, they cannot produce it; the NE gene is expressed only in bone marrow granulocyte precursor cells. Protection of normal tissues from the destructive capacity of NE is provided by α1-antitrypsin (α1AT), a 52-Kd serine antiprotease produced by hepatocytes and mononuclear phagocytes. In the context of the broad destructive capacity of NE, we evaluated the concept that human neutrophils may be able to modulate the extracellular activity of NE by synthesizing and secreting α1AT. Immunocytochemical analysis demonstrated that the neutrophil contains α1AT. Northern analysis and in situ hybridization with α1AT-specific probes demonstrated the presence of α1AT messenger RNA transcripts within neutrophils. 

α1-antitrypsin (α1AT), a 52-Kd glycoprotein, present in plasma of normal individuals at levels of 20 to 48 μmol/L. The α1AT gene is known to be expressed in liver hepatocytes and mononuclear phagocytes. The liver is the major source of plasma α1AT. α1AT is capable of diffusing from the circulation through tissues, although its molecular mass of 52 Kd retards its movement such that tissue levels are lower than those in plasma. The critical importance of α1AT in suppressing NE activity is highlighted by the hereditary disorder α1AT deficiency, in which mutations of the α1AT gene result in low serum levels of α1AT and progressive destruction of the lung parenchyma by NE.

In the context of the danger posed by uninhibited NE in the extracellular milieu, the aim of the present study was to evaluate the hypothesis that the neutrophil may be capable of modulating at least some of its own extracellular NE activity by synthesizing and secreting α1AT. This hypothesis was based on the knowledge that: (1) despite the fact that neutrophils are regarded as terminally differentiated cells and cannot synthesize NE, they can synthesize a variety of proteins and (2) immunohistochemical studies have demonstrated α1AT in the neutrophil. The data demonstrate that neutrophils are clearly capable of expressing the α1AT gene and secreting newly synthesized α1AT, at least some of which is functional and capable of complexing with NE.

MATERIALS AND METHODS

Cell preparations. Neutrophils were separated from mononuclear cells in heparinized blood of normal individuals using lymphocyte separation medium (Organon Teknika Corp, Durham, NC) and neutrophils isolated from the resulting cell pellet using plasmagel (Cellular Products, Buffalo, NY). The resulting populations of neutrophils and mononuclear cells were resuspended in Dulbecco’s modified Eagle’s medium (DMEM). Viability, assessed by Trypan blue exclusion, was always greater than 95% for the neutrophils and greater than 90% for the mononuclear cells. Differential counts of 500 cells performed after Diff-Quik
(American Scientific Products, McGaw Park, IL) staining of the neutrophil preparation typically showed 97% neutrophils, 2% to 3% eosinophils, and 0% to 1% lymphocytes. To further differentiate neutrophils from monocytes by cytochemistry, a naphthyl acetate esterase staining was used to identify monocytes and naphthol AS-D chloroacetate esterase was used to identify neutrophils (both reagents from Sigma, St Louis, MO).26 Neutrophil preparations never contained more than 0.2% monocytes.

Detection of α1AT in neutrophils by immunohistochemistry. The cells were evaluated for the presence of intracellular α1AT using cytospin preparations of purified neutrophil suspensions. After fixation for 1 minute in buffered formalin acetone, they were incubated with either a goat (Sigma) or a rabbit (Boehringer Mannheim, Indianapolis, IN) polyclonal IgG antiantithrombin α1AT antibody. These primary antibodies were shown by either rabbit antigoat or goat antirabbit phosphatase-labeled IgG antibody (Kirkegaard), visualized with fast red violet alkaline-naphthol AS-BI alkaline solution (Sigma). Incubations with a nonimmune normal rabbit or goat serum, a rabbit polyclonal IgG antiantihuman α2-macroglobulin (Dako, Carpinteria, CA), or the secondary antibody alone were used as controls.

Evaluation for α1AT messenger RNA (mRNA) transcripts. Neutrophils were evaluated for the presence of α1AT mRNA transcripts using Northern analysis of total RNA extracted from purified neutrophils and a 32P-labeled full-length human α1AT cDNA probe as previously described.20-21 RNA extracted from blood monocytes purified by adherence to plastic and the Jurkat T-cell line, a cell that does not express the α1AT gene, were also evaluated. As a further control, γ-actin mRNA was evaluated using a 32P-labeled human fibroblast γ-actin cDNA.21 Confirmation of the presence of α1AT mRNA transcripts in neutrophils was provided by in situ hybridization using a 35S-labeled α1AT cRNA antisense and sense probes. To accomplish this, cytocentrifuge preparations of purified neutrophils (2 x 106 cells/slide) were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), for 1 minute and kept at 4°C under 70% ethanol until used. Hybridization was performed as described by Harper et al22 with minor modifications as previously described.23 High specificity 35S-labeled single-stranded antisense and sense α1AT-specific cRNA probes were transcribed from a full-length α1AT cDNA subcloned into the transcription vector pGEM-3Z (Promega, Madison, WI).20 To generate small probe fragments (mean size 150 bp) permitting maximal access to the in situ mRNA, the transcription products were hydrolyzed using the method of Cox et al.27 Each hybridization reaction used 2 x 106 dpm of probe. Autoradiography and cell staining were performed as previously described.28

α1AT synthesis and secretion. To evaluate de novo synthesis and secretion of α1AT by neutrophils, different concentrations of neutrophils were placed in polypropylene tubes in “culture medium” (DMEM containing 10% heat-inactivated fetal calf serum [FCS], 2 mmol/L glutamine, 100 U/mL penicillin G, and 100 μg/mL streptomycin) at 20 x 106 cells/mL. After 30 minutes at 37°C, the cells were washed twice (4°C) in “pulse medium” (DMEM without methionine, 10% heat-inactivated FCS that had been dialysed [48 hours] against PBS, pH 7.4, 2 mmol/L glutamine, 100 U/mL penicillin G, and 100 μg/mL streptomycin), resuspended in pulse medium containing [35S]methionine (500 μCi/mL; >1,000 Ci/ mmol; Amersham, Arlington Heights, IL) and incubated for 3 to 6 hours as indicated. Following incubation, the supernatants were collected (200g; 5 minutes, 4°C). The supernatants were evaluated for the presence of α1AT mRNA transcripts by immunoprecipitation with anti-α1AT antibody bound to sepharose 4B beads followed by sodium dodecyl sulfate (SDS)-acylamide gel analysis and fluorography, as previously described.29 To confirm that the newly synthesized α1AT detected in the supernatants was released from neutrophils and not the very few monocytes present in some preparations, various concentrations of blood mononuclear cells were evaluated to compare relative neutrophil and monocyte synthesis of α1AT using the methods described above. The blood mononuclear cells were composed of 28% ± 3% monocytes and 72% ± 3% lymphocytes.

Function of α1AT synthesized and secreted by neutrophils. To determine whether the α1AT synthesized and secreted by neutrophils was capable of complexing with NE produced by the same neutrophils, neutrophils were evaluated under three conditions: (1) at rest; (2) following cytochalasin B priming and stimulation with N-formyl-methionyl-leucyl-phenylalanine (FMLP), conditions under which stored NE is released from the cell by degranulation;21; and (3) following stimulation with lipopolysaccharide (LPS), a stimulus that does not cause significant NE release.10

To evaluate neutrophils under resting conditions, neutrophils were incubated in pulse medium for 6 hours, 37°C and the supernatants evaluated for the presence of [35S]methionine-labeled α1AT as described above. To assess neutrophils under conditions in which there was maximal NE release, the neutrophils were incubated with [35S]methionine for 6 hours, 37°C as described above, but were then primed with cytochalasin B (5 μg/mL, 10 minutes, 37°C), followed by FMLP (10–7 mol/L, 1 minute, 37°C). The supernatants were collected and evaluated as described above. The autoradiograms were deliberately overexposed to determine if the major 52-Kd α1AT band was accompanied by 80-Kd NE-α1AT complexes. The relative positions of native α1AT and NE-α1AT complexes were identified by parallel experiments using standards of α1AT and α1AT that had been allowed to react with NE. To confirm that FMLP stimulation was capable of releasing NE in excess of the amount of α1AT produced, supernatants of parallel neutrophil cultures not labeled with [35S]methionine were evaluated for the presence of active NE using the NE-specific substrate methoxy succinyl-alanyl-alanyl-prolyl-valyl-nitroanilide.30

To evaluate neutrophils under a mild inflammatory stimulus where only low levels of NE are released, the neutrophils were incubated with [35S]methionine for 16 hours, 37°C in the presence of 1 μg/mL LPS (Escherichia coli serotype 0127:B8; Difco). The supernatants were then collected and evaluated as above. To determine if there was any “reserve” α1AT present that could combine with an additional low level burden of NE, the parallel supernatants were incubated with NE (0.5 μg/mL; 5 minutes, 23°C) and then evaluated as above.

RESULTS

α1AT in neutrophils. Immunocytochemical evaluation of neutrophils with either the goat or rabbit antiantihuman α1AT antibody demonstrated the presence of α1AT in the cytoplasm of the majority of neutrophils with a granular pattern (Fig 1). By contrast, lymphocytes present in the preparation were negative, as were neutrophils when using the different control antibodies (not shown). Importantly, no staining was present in neutrophils after incubation with an antibody against α2-macroglobulin, a protein known not to be present in neutrophils.26

α1AT mRNA in neutrophils. Extraction of total RNA from neutrophils yielded less RNA than from monocytes and Jurkat T cells (neutrophils, 1 μg/106 cells; monocytes, 10 μg/106 cells; Jurkat T cells, 7 μg/106 cells). However, using an α1AT CDNA probe, Northern analysis of RNA isolated from neutrophils showed easily detectable 1.8-kb mRNA α1AT transcripts (Fig 2, lane 1). In separate experiments, Northern analysis of RNA isolated from
monocytes also showed the presence of 1.8-kb mRNA α1AT transcripts; however, the signal was strikingly lower than that observed when testing neutrophil RNA (Fig 2, lane 2). Therefore, when testing the total RNA, the signal, i.e., the number of α1AT transcripts, is higher when analyzing neutrophils compared with monocytes. However, because of the much lower amount of total RNA for neutrophils when compared with monocytes, this result suggests that the ratio of specific α1AT mRNA transcripts is higher in neutrophils but with a lower number of copies per cell. In contrast, parallel evaluation of equivalent amounts of RNA isolated from the T-cell line Jurkat demonstrated no α1AT transcripts (Fig 2, lane 3). However, the detection of γ-actin mRNA transcripts gave a similar result when analyzing the RNA from neutrophils, monocytes, or Jurkat cells (Fig 3).

The presence of α1AT mRNA transcripts in neutrophils was confirmed by in situ hybridization analysis using °S-labeled antisense and sense α1AT cRNA probes. As a control, monocytes evaluated using these probes demonstrated the presence of silver grains with the antisense α1AT cRNA but not with the sense cRNA. Neutrophils hybridized with the °S-labeled antisense α1AT cRNA probe exhibited autoradiographic grains above the cells (Fig 4A). Use of the °S-labeled sense α1AT cRNA probe under the same conditions showed only very few grains (Fig 4B). As controls, lymphocytes observed on the same slide (approximately 1% of the cells) showed an average of 0.4 grains/cell with both the antisense and sense probes.
Neutrophils were considered as having a positive signal attributable to α1AT mRNA when the cells, after hybridization with the antisense probe, exhibited more grains (range 1 to 9) than when hybridized with the sense probe. α1AT mRNA was detected in 22% of neutrophils based on the presence of silver grains.

*De novo* synthesis and secretion of α1AT by neutrophils. [35S]methionine labeling and anti-α1AT immunoprecipitation and analysis clearly demonstrated the capacity of the neutrophil to synthesize α1AT de novo and secrete the newly synthesized α1AT molecules. The secreted [35S]methionine-labeled α1AT had the expected molecular mass of 53 Kd (Fig 5, lane 1). The identity of the labeled protein as α1AT was confirmed by blocking its immunoprecipitation with an excess of unlabeled α1AT (Fig 5, lane 2) and by parallel migration with an α1AT standard.

To interpret these results in the context of blood cells known to secrete α1AT, variable numbers of monocytes, incubated in parallel under identical conditions, synthesized α1AT molecules of identical size to the α1AT found in neutrophil supernatants. On the average, 20 × 10⁶ neutrophils were required to synthesize the equivalent amounts of α1AT as 2.5 to 5 × 10⁶ monocytes in monocyte-lymphocyte mixtures, i.e., whereas neutrophils synthesize and secrete α1AT, they do so to a lesser extent (40- to 80-fold) than do monocytes. However, because monocytes always represented no more than 0.2% of the neutrophil preparations, the α1AT synthesized by the neutrophil preparations was clearly greater than that which could be accounted for by the few monocytes present.

*Function of α1AT secreted by neutrophils.* Consistent with the knowledge that resting neutrophils do not release NE, no [35S]methionine-labeled α1AT-NE complexes were observed in the supernatants of resting neutrophils labeled with [35S]methionine, even when the autoradiograms were deliberately overexposed (Fig 6, lane 1). In contrast, after stimulation with fMLP, in addition to the major 52-Kd band

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**Fig 4.** In situ hybridization with an α1AT probe demonstrating α1AT mRNA transcripts in blood neutrophils. Shown are autoradiographs of cytocentrifuge preparations of blood neutrophils hybridized with [35S]-labeled antisense or sense α1AT cRNA probes. (A) [35S]-labeled antisense α1AT cRNA probe. Of the neutrophils shown in this field, most demonstrate two or more grains. (B) [35S]-labeled sense α1AT cRNA probe, as a negative control.

**Fig 5.** Synthesis and secretion of α1AT by neutrophils. Shown are fluorograms of SDS-acrylamide gel analysis of [35S]-labeled proteins in supernatants of neutrophils incubated for 3 hours with [35S]methionine and immunoprecipitated with an anti-α1AT antibody. Lane 1, neutrophil supernatant. Lane 2, similar to lane 1, but the immunoprecipitation was performed in the presence of excess unlabeled α1AT. The position of normal 52-Kd α1AT is shown.
Neutrophils Neutrophils Neutrophils

Resting + + +

LPS

neutrophils fMLP LPS +NE

kDa

1 2 3 4

Fig 6. Capacity of newly synthesized α1AT secreted by neutrophils to inhibit neutrophil elastase. Shown are fluorograms of SDS-acrylamide gel analyses of [35S]labeled proteins isolated from supernatants of neutrophils incubated under various conditions with [35S]methionine and immunoprecipitated with an anti-α1AT antibody. Lane 1, supernatant of resting neutrophils cultured for 6 hours with [35S]methionine. A 52-kDa α1AT protein is observed, but no α1AT-NE complexes. Lane 2, neutrophils incubated for 6 hours with [35S]methionine and then stimulated for 1 minute with fMLP. Note the presence of an additional 80-kDa band immunoprecipitated by the α1AT antibody. The 80-kDa band comigrates with α1AT-NE complexes. Lane 3, neutrophils cultured for 16 hours with [35S]methionine in presence of LPS. Note the small amount of 80-kDa α1AT-NE complexes. Lane 4, identical to lane 3 except that after collection of the supernatant, NE was added and the mixture incubated before immunoprecipitation. The 80-kDa band is now more prominent, demonstrating that some of the α1AT was able to combine with the added NE.

there was also present a higher molecular weight 80-kDa band comigrating with standard α1AT-NE complexes, demonstrating that at least some of the newly synthesized [35S]methionine-labeled α1AT was capable of forming complexes with neutrophil elastase from the same cells (Fig 6, lane 2). To confirm that neutrophils release active NE after fMLP stimulation, parallel evaluations of neutrophil supernatants demonstrated ample amounts of active NE as assessed by the NE-specific substrate methoxysuccinylalanyl-alanyl-prolyl-valyl-nitroanilide (not shown), thus confirming the concept that the fMLP stimulus is sufficient to have the neutrophils discharge enough NE to overwhelm the amount of α1AT also being produced by these cells. However, when the neutrophils were stimulated with LPS, a stimulus that does not initiate major degranulation, not only were α1AT-NE complexes observed, but at least some of the newly synthesized and secreted α1AT was capable of interacting with an additional burden of NE added to the supernatant. In this regard, analysis of the supernatants of neutrophils stimulated for 16 hours with LPS showed an 80-kDa band, consistent with the presence of α1AT-NE complexes (Fig 6, lane 3). Furthermore, when purified NE was added to the neutrophil supernatants an increase in the amount of complexed α1AT was observed (Fig 6, lane 4), providing further evidence that the higher molecular weight band is α1AT-NE complexes and that neutrophil supernatants contain newly synthesized α1AT capable of complexing with NE. In contrast to neutrophils, evaluation of activated monocytes using similar methods demonstrated that all of the α1AT was secreted in a free, active 52-kDa form. With addition of neutrophil elastase to the monocyte α1AT, however, greater than 90% of the α1AT formed a complex with the neutrophil elastase.

DISCUSSION

The neutrophil is capable of causing massive tissue destruction through its powerful protease, neutrophil elastase.1-3,9,13-15 However, neutrophils cannot synthesize NE, and thus can vary the availability of this destructive enzyme only by modulating its secretion.4,5 Interestingly, the present study demonstrates that neutrophils contain α1AT, the natural inhibitor of NE, and are able to synthesize and secrete α1AT, some of which is capable of complexing with neutrophil elastase. Thus, neutrophils have the capacity to modulate some of the NE activity in its local milieu by producing a molecule capable of interacting with the released NE.

α1AT synthesis by neutrophils. Although the neutrophil has traditionally been considered a metabolically inactive,
differentiated cell incapable of significant de novo protein synthesis, the evidence that neutrophils can synthesize α1AT is consistent with recent reports demonstrating that neutrophils are capable of expressing a number of genes, including the heat shock proteins, the heavy chain of cytochrome b245, c-fos, the complement receptor CR1, the α-chain of the complement receptor CR3, the Fc receptor, major histocompatibility complex class I proteins, and actin.23-25,40,41

Although the neutrophil clearly synthesizes and secretes α1AT, comparison of the relative amounts of α1AT produced by neutrophils to those of other α1AT-synthesizing cells suggests that neutrophil α1AT is intended for local use, and not to provide α1AT for the entire body. Evidence from a variety of sources has demonstrated that the major site of α1AT gene expression is liver hepatocytes although mononuclear phagocytes, including blood monocytes and alveolar macrophages, also express this gene.17,18 The hepatocyte contains approximately 200 times more α1AT mRNA transcripts than the mononuclear phagocytes, and is thought to be the source of most of the α1AT in the circulation.49 Consistent with this concept, the α1AT phenotype in the serum following bone marrow transplantation is that of the recipient, ie, the recipient's liver rather than the donor's bone marrow (and thus donor mononuclear phagocytes and neutrophils).44 Putting the present study in the context of the other studies, it appears that the order of magnitude of α1AT synthesis is: hepatocytes > mononuclear phagocytes > neutrophils, ie, it is unlikely that either the neutrophil or the mononuclear phagocytes contribute significantly to "whole body" α1AT. Rather, the role of the α1AT-secretory products of both of these cells is likely a local modulation of the microenvironment of the cell producing the α1AT.

Neutrophil modulation of neutrophil elastase activity. Although circulating α1AT is clearly the body's major defense against NE, there is increasing evidence that α1AT diffusing from the circulation may not be able to inhibit NE released by neutrophils that have adhered to a surface.13-46 Consequently, the α1AT produced and secreted by neutrophils observed in the present study, and perhaps the nonglycosylated 42-Kd inhibitor of NE recently observed in the cytoplasm of neutrophils,47 may play a role in protecting tissues in the environs of neutrophils adhered to tissue, when neutrophils are not releasing major amounts of their stored NE. However, when the neutrophil is stimulated to release the majority of its stored NE, the neutrophil can easily overwhelm the α1AT it produces in vitro; not only is the amount of NE stored by neutrophils (1 to 2 pg/cell) likely to be far more than the α1AT the cell is capable of producing over the short time it takes the neutrophil to discharge its NE, but the stimulated neutrophil also releases large amounts of oxidants to the environs of the cell, agents that inactivate the α1AT secreted by the same cells.43 Thus, while active elastase is detected in the supernatant of fMLP-stimulated neutrophils, the supernatant contains significant amounts of newly synthesized α1AT, ie, α1AT that most likely has been rendered impotent when the neutrophil was activated. However, when the stimulus is mild, such as with LPS, the released NE is complexed and there is some additional newly synthesized α1AT that can combine with an additional burden of NE. Therefore, a likely role for neutrophil-produced α1AT is in modulating some of the NE released by the cell, such as during diapedesis through tissues, or leaked from the cell during phagocytosis, processes involving the discharge of NE into the local milieu.3,8-10,48-49 In this situation, when a neutrophil is firmly apposed to a substrate, plasma or mononuclear phagocyte-derived α1AT most likely cannot gain access to the microenvironment of the neutrophil, ie, only the neutrophil's own α1AT may limit any local tissue degradation by the NE it is secreting. However, this delicate balance will be rapidly changed in the presence of an acute inflammatory stimulus where the contribution of neutrophil α1AT to tissue defenses is negligible.

REFERENCES

Human neutrophils express the alpha 1-antitrypsin gene and produce alpha 1-antitrypsin

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