Mechanism of Hypochlorite-Mediated Inactivation of Proteinase Inhibition by α_2-Macroglobulin†

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ABSTRACT: The proteinase−proteinase inhibitor balance plays an important role in mediating inflammation-associated tissue destruction. α_2-Macroglobulin (α_2M) is a high-affinity, broad-spectrum proteinase inhibitor found abundantly in plasma and interstitial fluids. Increased levels of α_2M and proteinase−α_2M complexes can be demonstrated in patients with sepsis, emphysema, peridontitis, rheumatoid arthritis, and other inflammatory diseases. Despite these increased levels, proteolysis remains a significant problem. We hypothesized that a mechanism for inactivating α_2M-mediated proteinase inhibition must exist and recently demonstrated that α_2M isolated from human rheumatoid arthritis synovial fluid is oxidized and has decreased functional activity. The oxidant responsible for α_2M inactivation and the mechanism of such destruction were not studied. We now report that while hypochlorite and hydroxyl radical both modify amino acid residues on α_2M, only hypochlorite can abolish the ability of α_2M to inhibit proteinases. Hydrogen peroxide, on the other hand, has no effect on α_2M structure or function. Protein unfolding with increased susceptibility to proteolytic cleavage appears to be involved in α_2M inactivation by oxidation. The in vivo relevance of this mechanism is supported by the presence of multiple cleavage fragments of α_2M in synovial fluid from patients with rheumatoid arthritis, where significant tissue destruction occurs, but not in patients with osteoarthritis. These results provide strong evidence that hypochlorite oxidation contributes to enhanced tissue destruction during inflammation by inactivating α_2M.

α_2-Macroglobulin (α_2M)[1] is a 720 kDa glycoprotein found in plasma at high concentrations (2−4 mg/mL) (reviewed in ref *1*). It is composed of four identical 180 kDa subunits, two of which are covalently linked by disulfide bonds. It was first described as a proteinase inhibitor with the unique ability to inhibit proteinases from all mechanistic classes (that is, serine, metallo, cysteine, and aspartate) by a distinct trapping mechanism. Proteinases entrapped within the α_2M cage are shielded from high-molecular mass substrates but are still able to cleave small proteins and peptides that can diffuse into the cage. Inhibition of proteinases by α_2M involves first the entry of a proteinase into the internal cavity of α_2M. Subsequent cleavage of a specific amino acid sequence in α_2M called the "bait region" results in rupturing of an internal β-cysteinyl−γ-glutamyl thioester bond and the formation of a covalent linkage between α_2M and the attacking proteinase (*2*). Concomitantly, α_2M undergoes an extensive compaction resulting in exposure of its receptor recognition site which binds to the low-density lipoprotein receptor-related protein (LRP), for proteinase clearance (*3*, *4*), and a newly described signaling receptor for cell regulation (*5*, *6*).

A number of studies have demonstrated the importance of α_2M in maintaining the proteinase−proteinase inhibitor balance in inflammation. α_2M has been found at increased levels in plasma and/or extracellular fluids in patients with emphysema (*7*), periodontitis (*8*), rheumatoid arthritis (*9*), and pneumonia (*10*). Complexes of α_2M and elastase, collagenase, plasmin, thrombin, kallikrein, and others have all been found in disease fluids (*2*). In earlier studies of disseminated intravascular coagulation, the onset of shock correlated precisely with the depletion of plasma α_2M (*11*). More recent studies employing genetically engineered mice that are α_2M deficient suggest that α_2M is involved in tissue remodeling such as during pregnancy and wound repair (*12*). The fact that no complete human deficiency has been found to date highlights the biological importance of this molecule.

Despite the elevated levels of α_2M in extracellular fluid in disease, proteolytic tissue destruction occurs readily during inflammation. A large body of literature has demonstrated the role of oxidation in the inflammatory response to bacterial invasion and autoantigens (*13*, *14*). Estimated concentrations of neutrophil-derived oxidants released during an oxidative burst can be as high as the millimolar level (*15*). It has been speculated that oxidation of proteinase inhibitors such as α_1-protein inhibitor (also known as α_1-antitrypsin), secretory leukoprotease inhibitor, and α_2M may contribute to an increased level of proteolysis during inflammation (*15*). Consistent with this hypothesis, we found recently that oxidized and functionally defective α_2M is present in the

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[1] Abbreviations: α_2M, α_2-macroglobulin; α_2M*, α_2-macroglobulin−methylamine or α_2-macroglobulin−proteinase; DNPH, dinitrophenylhydrazine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); LRP, low-density lipoprotein receptor-related protein; OA, osteoarthritis; PVDF, polyvinylidene fluoride; RA, rheumatoid arthritis; TNBS, trinitrobenzenesulfonate.

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knee joint synovial fluid of patients with rheumatoid arthritis (*16*). We found, in addition, that oxidized α_2M is more able to bind to proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), and interleukin-6 (IL-6), and less able to bind to tissue-repair growth factors such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and nerve growth factor (NGF) (*16*). The ability of α_2M to regulate the activities of these growth factors may play a significant role in inflammation. Despite these interesting and potentially important findings, the pathophysiologically relevant oxidant(s) capable of mediating α_2M oxidation and the molecular mechanism(s) involved in its inactivation are, however, unknown.

In the study presented here, we investigated the effects of three oxidants that are classic mediators of inflammation—hypochlorite (HOCl), hydroxyl radical (•OH), and hydrogen peroxide (H_2O_2)—on α_2M structure and function. We found that while hypochlorite and hydroxyl radical oxidation in vitro can both increase the protein carbonyl content in α_2M, only hypochlorite can abrogate the ability of α_2M to inhibit proteinases at biologically relevant concentrations. Hydrogen peroxide, on the other hand, has no effect on α_2M structure or function. Further investigation into the mechanism of such inactivation showed that structural fragmentation and intersubunit cross-linking characterize hypochlorite oxidation but are not essential for its decreased functional activity. Instead, the generation of additional proteolytic cleavage sites leading to enhanced degradation by proteinases appears to be critical for its inactivation. Additional studies using synovial fluid from patients with rheumatoid arthritis (RA), but not osteoarthritis (OA), confirmed that α_2M fragments are generated during inflammatory disease processes. These results provide strong evidence that hypochlorite is a specific oxidant involved in α_2M inactivation and may contribute to enhanced tissue destruction during inflammation.

EXPERIMENTAL PROCEDURES

Reagents. Bovine serum albumin (BSA), sodium hypochlorite, L-methionine, 2,4-dinitrophenylhydrazine (DNPH), and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma (St. Louis, MO). Hydrogen peroxide (30% active) was purchased from EM Science (Gibbstown, NJ). BS^3 [bis(sulfosuccinimidyl)suberate] cross-linker and the QuantiCleave Protease Assay kit were purchased from Pierce (Rockford, IL). Tris base was purchased from Boehringer Mannheim (Indianapolis, IN). The PD-10 column was purchased from Pharmacia (Uppsala, Sweden). ^{125}I-labeled Bolton Hunter reagent for protein iodination was obtained from New England Nuclear (Boston, MA). All other reagents were of the highest quality commercially available.

Preparation of α_2M. Human α_2M was purified according to a previously published protocol (*17*). α_2M was at least 95% active against proteinases as determined by thioester titration using the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) assay (*18*). The concentration of α_2M was determined using an $A_{280}^{(1\%/1cm)}$ of 8.93 and a molecular mass of 719 kDa (*19*). α_2M was iodinated using ^{125}I-labeled Bolton Hunter reagent according to the manufacturer's specified protocol. The specific activity of ^{125}I-labeled proteins varied from 500 to 700 cpm/ng.

Oxidation of α_2M. α_2M was oxidized by sodium hypochlorite, hydrogen peroxide, or hydroxyl radical according to previously published protocols (*16*, *20*). In brief, α_2M at a concentration of 0.25 mg/mL was incubated at 37 °C with various concentrations of oxidants (from 1 μM to 10 mM) for 15 min in phosphate-buffered saline (PBS) at pH 7.4. At the end of the incubation, L-methionine was added at concentrations corresponding to oxidant concentrations to quench residual oxidants. Oxidant concentrations were determined by iodometric analysis (*21*), or by measuring the spectrophotometric absorption of the hydrogen peroxide solution at 240 nm ($\epsilon = 43.6\ M^{-1}\ cm^{-1}$) and the hypochlorite solution at a wavelength of 292 nm ($\epsilon = 206\ M^{-1}\ cm^{-1}$) at pH 7.4 (*22*).

Protein Carbonyl Content Determination. Measurement of the extent of oxidation in α_2M was performed using 2,4-dinitrophenylhydrazine (DNPH) derivatization of protein carbonyls as previously described (*23*) with modifications. One hundred micrograms of α_2M in 800 μL of PBS was added to 200 μL of 10 mM DNPH in 2.0 M HCl and incubated at 25 °C for 1 h. Following incubation, proteins were precipitated with 150 μL of 70% trichloroacetic acid (TCA) and placed on ice for 10 min. Proteins were then centrifuged at 16000*g* for 5 min. Protein pellets were washed with ethyl acetate/ethanol (1:1, v:v), and the centrifugation/washing process was repeated two more times before final solubilization in 6 M guanidine hydrochloride (pH 7.4). The absorbances of each protein sample at wavelengths of 276 and 370 nm were measured using a Beckman DU-640 spectrophotometer (Arlington Heights, IL). Nonspecific absorption was determined by parallel experiments performed in the absence of DNPH and was subtracted from the total.

Polyacrylamide Gel Electrophoresis (PAGE). Nondenaturing, nonreducing gradient (5 to 15%) PAGE and reducing or nonreducing SDS−PAGE (7.5 or 5 to 15% gradient) were performed to determine the effects of oxidation on α_2M structure. Following electrophoresis, gels were fixed in acetic acid with Coomassie brilliant blue. Oxidant concentrations of up to 250 μM had no effect on the ability of α_2M bands to be stained. Oxidation at concentrations of >250 μM results in a decreased level of α_2M staining (*24*, *25*).

Determination of the Proteinase Inhibitory Activity of α_2M. The ability of oxidized α_2M to inhibit proteinases was determined using QuantiCleave Protease Assay II from Pierce according to the manufacturer's suggested protocol. In brief, oxidized α_2M at a concentration of 0.25 mg/mL was added to 0.02 mg/mL thermolysin (in a 1:1 molar ratio) and the mixture incubated for 30 min at 37 °C. Following incubation, 25 μL of the reaction mixture was added in duplicate to 96-well plates containing 100 μL of buffer [10 mM Tris and 2 mM $CaCl_2$ (pH 8.0)] either alone or with 2 mg/mL succinylated casein and the mixture incubated for an additional 1 h at 37 °C. At the end of the incubation, 50 μL of trinitrobenzenesulfonate (TNBS) solution was added to each well for 10 min and the plates were read on a microplate reader at a wavelength of 450 nm. The total α_2M proteinase inhibitory activity was determined by measuring the absorbance difference between samples containing thermolysin alone or with nonoxidized α_2M. Residual inhibitory activity is measured as a percentage of the total activity.

Gelatin Zymography. Gelatin zymograms were prepared by adding 0.1% gelatin to either a native Tris-borate or SDS Tris-glycine-containing acrylamide gel matrix. Protein samples were electrophoresed at 110 V for 1.5 h followed by

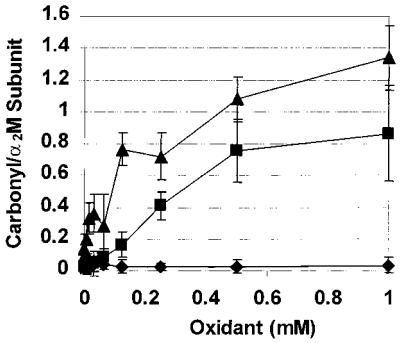

FIGURE 1: Oxidant-mediated generation of protein carbonyl in α_2M. α_2M was treated with the indicated concentrations of HOCl (■), •OH (▲), and H_2O_2 (◆) according to Experimental Procedures and subsequently incubated with DNPH for 1 h at 25 °C. Following incubation, trichloroacetic acid was added and unreacted DNPH was removed by washing. The protein was then resuspended in guanidine hydrochloride (pH 7.4), and the absorbances at 276 and 450 nm (λ) were read on the spectrophotometer. The data represent the mean ± the standard error of the mean of three independent experiments performed in duplicate.

incubation in a 2.5% Triton X-100 solution for 30 min at 25 °C (for SDS gels only). The gel was then rinsed with dH_2O to remove excess Triton X-100 and then transferred into a digestion buffer containing 20 mM glycine, 10 mM $CaCl_2$, and 1 μM $ZnCl_2$ (pH 8.3) for 16 h at 25 °C (for both native and SDS gels). Following incubation, the gel was stained with Coomassie brilliant blue for 30 min and destained to optimize the appearance of zones of lysis.

Assessment of Oxidation-Induced Proteolytic Cleavage Sites in α_2M. α_2M (0.25 mg/mL) oxidized with various concentrations of oxidant was incubated in the absence or presence of a 5-fold molar excess of trypsin for 15 min at 37 °C. Following incubation, a 5% solution of TNBS was added to each tube and the mixture incubated for an additional 20 min at 25 °C. The absorbance of each sample at a wavelength of 450 nm was subsequently read. The extent of specific trypsin-mediated cleavage was determined by measuring the difference in absorbance between samples without trypsin and samples with trypsin. The absorbance of trypsin alone was less than 10% of the total absorbance in any sample ($n = 3$).

Collection of Synovial Fluids from Patients with Rheumatoid Arthritis. Synovial fluids from four patients fulfilling the American College of Rheumatology's revised criteria for the classification of RA (*26*), and from four patients fulfilling the criteria for OA, were obtained from the Rheumatology Clinic of Duke University Medical Center. Synovial fluids were aspirated and anticoagulated with 5 mM EDTA or 10 units/mL heparin, and cell debris was removed by centrifugation. The samples were collected as discarded human fluid and were frozen immediately at −70 °C for storage. Prior to analysis, synovial fluids were thawed and treated with a mixture of proteinase inhibitors to give final concentrations of 2 mM phenylmethanesulfonyl fluoride (PMSF), 2 mM 3,4-dichloroisocoumarin (DCI), 5 mM 1,10-phenanthroline, and 2 μM E-64.

Western Blotting of Rheumatoid Synovial Fluid and Purified α_2M. RA or OA synovial fluids were diluted 3-fold with PBS and loaded onto a native gel along with plasma-derived α_2M and α_2M−methylamine as controls and electrophoresed for 1 h at 150 V. Following electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore Corp., Bedford, MA) using a Bio-Rad (Hercules, CA) Trans-Blot semi-dry transfer cell and blocked in 0.2% BSA for 2 h at 25 °C. Rabbit anti-human α_2M polyclonal antiserum purchased from Accurate Chemicals (Westbury, NY) was added at a dilution of 1:1000 and allowed to incubate overnight at 25 °C. Following incubation and washing, goat anti-rabbit IgG polyclonal antibody (2 mg/mL) conjugated to horseradish peroxidase was added at a dilution of 1:1000 for 2 h. The membrane was then washed and developed using the 4-chloronaphthal insoluble substrate. In Western blotting experiments involving purified RA and OA synovial fluid α_2M, the protein samples were purified using polyclonal anti-α_2M antibody according to a previously published protocol (*16*).

Data Analysis. The carbonyl content of oxidized α_2M per subunit of α_2M was calculated according to a previously published formula:

$$\text{carbonyls}/\alpha_2\text{M subunit} = (ab)/22000 \times (c - 0.43b)$$

where a is the molecular weight of the α_2M subunit, b is the absorbance value of the DNPH-modified α_2M at 370 nm (λ), and c is the absorbance value at 276 nm (λ) (*25*). The extinction coefficient of hydrazone is 22 000 M^{-1} cm^{-1} (*23*), and 0.43 is the correction factor for the contribution of hydrazone absorbance to protein absorbance.

RESULTS

Determination of the Protein Carbonyl Content in Oxidized α_2M. Oxidative modification of proteins results in the alteration of a number of solvent-exposed amino acid residues such as cysteine, methionine, tyrosine, tryptophan, and lysine (*13*). A general marker of protein oxidation is the appearance of carbonyl groups by deamination of the amino terminus and lysine side chains (*20*). To determine which oxidant is likely to be responsible for α_2M inactivation in vivo, we first compared the ability of hypochlorite, hydroxyl radical, and hydrogen peroxide to modify α_2M by measuring the number of protein carbonyl groups generated following oxidation. As shown in Figure 1, both hypochlorite and hydroxyl radical are capable of increasing the carbonyl content in α_2M at concentrations of <0.1 mM, with hydroxyl radical exhibiting slightly larger effects than hypochlorite. Hydrogen peroxide, on the other hand, has no ability to generate protein carbonyl on α_2M. Hydroxyl radical induced this modification with an EC_{50} of 0.2 mM, while the EC_{50} for hypochlorite was 0.3 mM, demonstrating that both of these oxidants modify α_2M with high potency. These findings are relevant particularly in inflammation since the concentrations of oxidants in an inflammatory milieu can be as high as the millimolar level (*15*).

Structural Disruption of Oxidized α_2M. Given the fact that hydroxyl radical and hypochlorite are both effective in generating protein carbonyls in α_2M, we compared the ability of these oxidants to alter α_2M structure. Hypochlorite oxidation can fracture the α_2M tetramer along its dimeric axis (*25*). As shown in Figure 2 (top panel), α_2M oxidation by hypochlorite at ≥0.1 mM resulted in a protein that migrates significantly faster electrophoretically compared with nonoxidized protein. This position corresponds to the position of α_2M dimers generated by SDS treatment (data

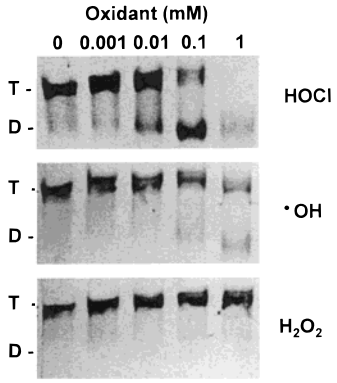

FIGURE 2: Structural disruption of oxidized α_2M. α_2M was oxidized with the indicated concentrations of HOCl (top panel), •OH (middle panel), and H_2O_2 (bottom panel) according to Experimental Procedures and subsequently electrophoresed via 5 to 15% nondenaturing nonreducing PAGE. T represents tetrameric α_2M, and D represents dimeric α_2M. The decreased level of protein staining in 1 mM HOCl-oxidized α_2M has been reported previously (*25*). The gel strips show the representative data of three independent experiments.

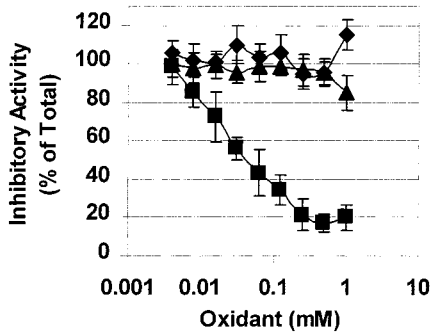

FIGURE 3: Determination of the ability of oxidized α_2M to inhibit proteinases. α_2M oxidized with H_2O_2 (◆), •OH (▲), or HOCl (■) at the indicated concentrations was incubated with a 2:1 molar ratio of thermolysin to α_2M at 37 °C for 30 min. The residual proteolytic activity was measured using the QuantiCleave protease assay according to the manufacturer's suggested protocol. The data represent the mean ± the standard error of the mean of four independent experiments performed in duplicate.

not shown) and not the migration of conformationally transformed α_2M which migrates only slightly faster (by 2 mm) than tetrameric α_2M in the same gel system (*18*). Hydroxyl radical-mediated oxidation also generated α_2M dimers (middle panel); however, a significantly higher concentration was necessary to achieve this effect. Hydrogen peroxide (bottom panel) has no ability to generate α_2M dimers, consistent with the results depicted in Figure 1. The decreased level of staining by Coomassie brilliant blue is evident in 1 mM hypochlorite-oxidized α_2M. Additional verifications using Western blotting analysis and autoradiography of radiolabeled proteins ($n = 3$ for each assay) show equivalent protein quantities in all lanes.

Determination of the Ability of Oxidized α_2M To Inhibit Proteinases. Given the fact that hypochlorite and hydroxyl radical are both capable of inducing amino acid modifications and structural fragmentation in α_2M subunits, we compared the ability of these oxidants to inactivate α_2M proteinase inhibition. As shown in Figure 3, hypochlorite was the only oxidant capable of destroying the proteinase inhibitory activity of α_2M. This is quite surprising since there was no significant difference in the modification of α_2M between

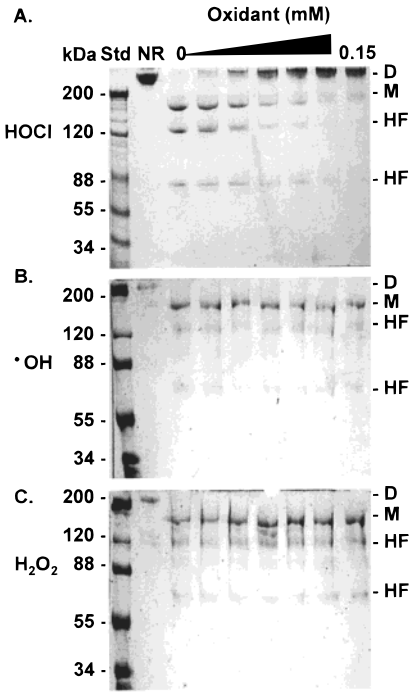

FIGURE 4: Structural cross-linking of oxidized α_2M. α_2M (5 μg per lane) was oxidized with the indicated concentrations of HOCl (A), •OH (B), and H_2O_2 (C) and electrophoresed via denaturing, reducing PAGE for 1 h at 110 V prior to staining. The lane that is labeled with NR contained nonreduced α_2M. The lane that is labeled with Std contained broad-range molecular weight standards. D represents dimeric α_2M; M represents monomeric α_2M, and HF represents autolytic heat fragments generated when α_2M is boiled in a reducing agent. The gels show the representative data of three independent experiments.

hypochlorite and hydroxyl radical as evidenced by protein carbonyl generation (Figure 1). The EC_{50} for α_2M inactivation by hypochlorite was approximately 0.03 mM, a concentration that is much lower than that needed to generate protein carbonyl in α_2M.

Intersubunit Cross-Linking of α_2M by Oxidation. Since hypochlorite but not hydroxyl radical or hydrogen peroxide can abolish proteinase inhibition by α_2M, we investigated the mechanism of this process. Inactivation of α_2M typically involves modification of the α_2M bait region leading to decreased reactivity between proteinase and α_2M, or cleavage of the internal thioester bond resulting in premature trap closure (*1*, *2*). Earlier studies by our colleagues have shown no effect of hypochlorite oxidation on α_2M bait region or thioester bond integrity (*25*), suggesting that another mechanism(s) must be involved in α_2M inactivation. Our results in Figure 2 showed that structural fragmentation from tetramer to dimer may lead to ineffective proteinase entrapment which may explain the inability of α_2M to inhibit proteinase. On the other hand, hypochlorite is also uniquely able to generate intersubunit cross-linking in α_2M (Figure 4). This modification may result in α_2M inactivation by preventing the closure of the α_2M "trap".

Chemical Cross-Linking of α_2M with BS[3]. To study the role of intersubunit cross-linking in mediating α_2M inactivation, we generated a chemically cross-linked α_2M using BS^3, a nonoxidative cross-linker, to mimic oxidation-induced protein cross-linking. We found, interestingly, that this cross-linked α_2M also undergoes tetramer to dimer fragmentation (Figure 5A, top panel) which is similar to oxidation with

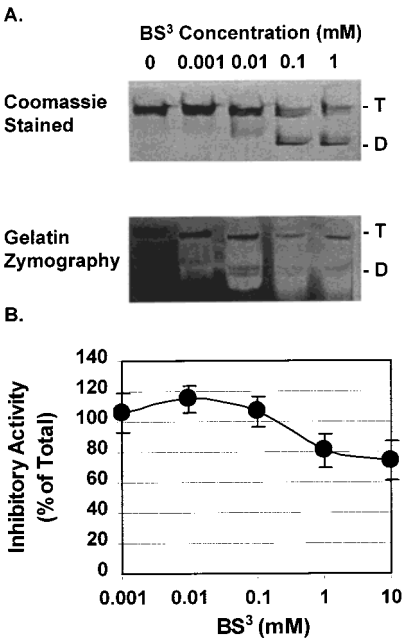

FIGURE 5: Determination of the effects of BS^3 cross-linking on α_2M structure and function. (A) α_2M was incubated with the indicated concentrations of BS^3 and electrophoresed via 5 to 15% nondenaturing, nonreducing PAGE for 3 h at 150 V followed by Coomassie brillant blue staining (top panel). Some of the BS^3-treated α_2M was incubated with a 2:1 molar ratio of trypsin and then electrophoresed in a nondenaturing, nonreducing gelatin zymogram to determine its ability to bind to trypsin (bottom panel). T represents the α_2M tetramer, and D represents the α_2M dimer. (B) α_2M treated with the indicated concentrations of BS^3 cross-linker was incubated with trypsin at 37 °C for 30 min. The residual proteolytic activity was measured using the QuantiCleave protease assay as described in Experimental Procedures. The data represent the mean ± the standard error of the mean of three independent experiments performed in triplicate.

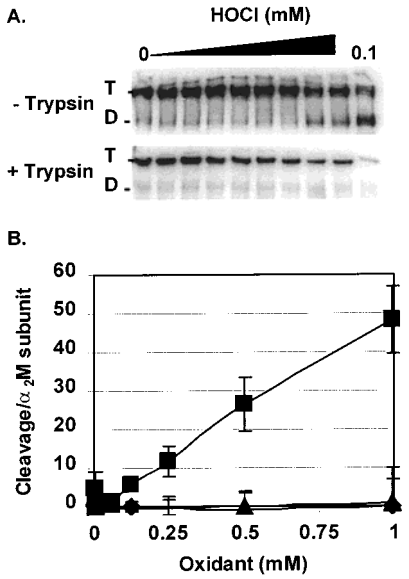

FIGURE 6: Determination of the susceptibility of hypochlorite-oxidized α_2M to proteolysis. (A) ^{125}I-labeled α_2M was oxidized with the indicated concentrations of HOCl and incubated in the absence (top panel) or presence (bottom panel) of a 5-fold molar excess of trypsin for 30 min at 37 °C. Following incubation, the mixture was electrophoresed via 5 to 15% nondenaturing, nonreducing PAGE and exposed to a Phosphorimager plate prior to development. T represents the α_2M tetramer, and D represents the α_2M dimer. (B) The presence of cleaved peptide bonds in trypsin-treated HOCl (■)-, •OH (▲)-, or H_2O_2-oxidized α_2M (◆) was measured by derivatization of the free amino terminus using TNBS. The number of bonds cleaved is expressed as a ratio to the number of α_2M subunits present. The data are the mean of three independent experiments performed in triplicate.

hypochlorite. To determine whether this cross-linked and structurally fragmented protein can react with proteinases, we incubated it with a 2-fold molar excess of trypsin and found that tetrameric, as well as dimeric cross-linked α_2M, is able to bind proteinases (Figure 5A, bottom panel). Additional studies (Figure 5B) show that the proteinase inhibitory activity of this cross-linked dimeric α_2M was essentially intact, in contrast to hypochlorite-oxidized α_2M.

Determination of the Susceptibility of Hypochlorite-Oxidized α_2M to Proteolysis. We investigated other mechanisms of α_2M inactivation given that neither protein cross-linking nor tetramer to dimer fragmentation was fully able to explain the decrease in the extent of α_2M proteinase inhibition with hypochlorite oxidation. Earlier studies demonstrated the ability of hypochlorite to generate partially unfold proteins, thereby increasing its susceptibility to proteolytic cleavage (*24*). We hypothesize that this increased sensitivity to proteolysis may, at least in part, explain the decreased ability of oxidized α_2M to inhibit proteinases. To test this possibility, we incubated hypochlorite-oxidized α_2M with a 5-fold molar excess of trypsin and measured the amount of protein degradation. The 5-fold excess of trypsin was used in these experiments to ensure that sufficient proteolytic activity is present to overcome proteinase inhibition by α_2M. We found that dimeric α_2M, which represents oxidized protein, is completely degraded in the presence of trypsin (compare the top and bottom panels of Figure 6A). This is in contrast to BS^3-cross-linked α_2M where dimeric α_2M retains the ability to bind trypsin without being degraded (Figure 5A, bottom panel). To quantify this increase in the susceptibility to proteolysis, we measured the number of peptide bonds cleaved following incubation of trypsin with oxidized α_2M. We found that the extent of this protein cleavage was linearly dependent on the concentration of hypochlorite used to oxidize α_2M and was specific only for hypochlorite and not hydroxyl radical or hydrogen peroxide (Figure 6B). As an additional confirmation to verify that chemical cross-linking alone does not generate such proteolytic cleavage sites, we also incubated BS^3-cross-linked α_2M with trypsin and measured the number of cleaved peptide bonds. We found no increase in the number of trypsin cleavages in the cross-linked α_2M compared with control, consistent with the results from Figure 5 (data not shown).

Gelatin Zymography of Trypsin-Treated Oxidized α_2M. These studies above demonstrate that α_2M inactivation by hypochlorite is most likely caused by increased sensitivity to proteolytic degradation. As additional support for this hypothesis, we asked whether this structural proteolysis can result in proteinase−α_2M complexes with different sizes and molecular weights. To answer this question, we performed gelatin zymography with trypsin-treated oxidized α_2M to detect the presence of degraded trypsin−α_2M complexes. As shown in Figure 7, incubation of trypsin with nonoxidized α_2M resulted in decreased free trypsin activity as well as some baseline degradation of trypsin−α_2M complexes. With an increasing level of α_2M oxidation, however, there was a significant increase in the number and intensity of bands

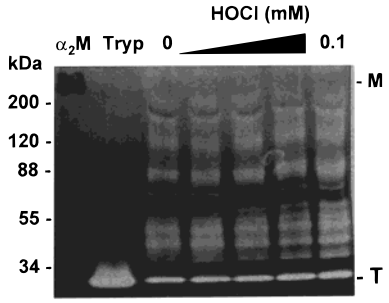

FIGURE 7: Gelatin zymography of HOCl-oxidized α_2M. α_2M oxidized with the indicated concentrations of HOCl was incubated with a 2-fold molar excess of trypsin for 20 min at 37 °C. At the end of the incubation, the mixture was electrophoresed in a denaturing gelatin zymogram (SDS−PAGE) and developed with Coomassie brilliant blue. α_2M represents nonoxidized α_2M added alone. Tryp represents trypsin added alone. The gel shows the representive data of three independent experiments performed in duplicate.

representing degraded trypsin−α_2M complexes. These data further support the fact that hypochlorite oxidation inactivates α_2M proteinase inhibition by increasing the susceptibility of α_2M to structural proteolysis.

Identification α_2M Cleavage Products in RA and OA Synovial Fluids. Given the fact that α_2M in the synovial fluids of patients with RA is oxidized and has decreased functional activity (*16*) and that the mechanism of α_2M inactivation appears to be its increased susceptibility to structural proteolysis, we speculate that α_2M from RA synovial fluid may show evidence of proteolytic degradation by synovial fluid proteinases. To determine if this is indeed the case, we performed Western blotting analysis of synovial fluid α_2M purified from patients with RA and OA and compared these samples with α_2M isolated from control plasma. As shown in Figure 8A, two of the four α_2M samples from patients with RA have multiple lower-molecular weight bands representing degraded products. In samples from OA and control plasma patients, however, the intensities of these bands were decreased or the bands absent, consistent with our hypothesis that proteolysis may play an important role in α_2M inactivation in vivo. To determine whether the level of α_2M oxidation correlates with its susceptibility to proteolysis, we determed the extent of protein oxidation in these samples. As shown in Figure 8B, RA synovial fluid α_2M is significantly more oxidized (mean protein carbonyl ± the standard error of the mean) (7 ± 0.9 nmol/mg of protein) than OA (3 ± 0.4 nmol/mg of protein) or control plasma (2 ± 0.5 nmol/mg of protein).

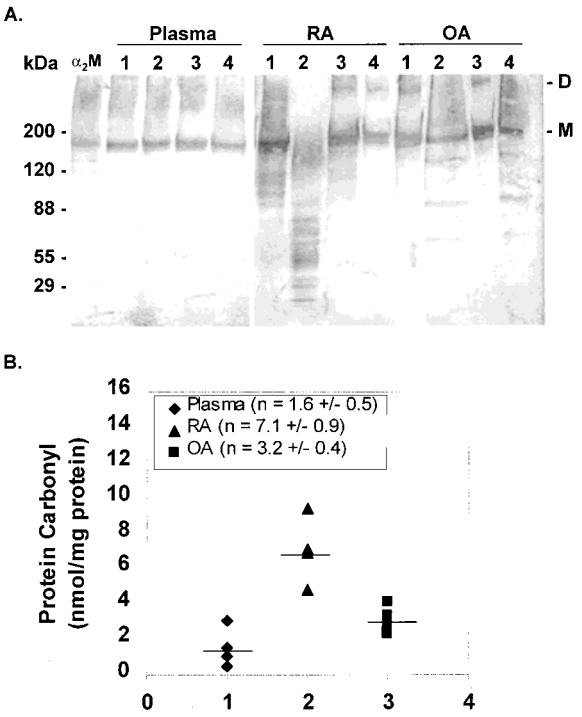

FIGURE 8: Identification of proteolytic cleavage products of synovial fluid and plasma α_2M by Western blotting. Purified synovial fluid α_2M from four patients with RA and four patients with OA, as well as plasma α_2M from four normal healthy volunteers, was isolated as described in Experimental Procedures. (A) Each sample was electrophoresed via 5 to 15% reducing SDS−PAGE. Plasma α_2M purified with a metal-chelating affinity column, labeled α_2M, was used as the α_2M standard. Following electrophoresis, proteins were transferred onto a PVDF membrane and blotted with rabbit polyclonal antisera against human α_2M. The Western blot shows the representative data of three independent experiments. (B) The protein carbonyl content of plasma (◆), RA (▲), and OA (■) synovial fluid α_2M was determined as described previously. Each data point represents the mean of three independent determinations per patient sample. The group mean ± the standard error of the mean is indicated in the inset legend.

DISCUSSION

In this study, we have found differential effects of hypochlorite, hydroxyl radical, and hydrogen peroxide oxidation on α_2M. Both hypochlorite and hydroxyl radical generate significant increases in protein carbonyl content; however, only hypochlorite is able to induce structural fragmentation and intersubunit cross-linking. Hydrogen peroxide, on the other hand, has no effect on α_2M. We also show that hypochlorite is the only oxidant of these three that can inactivate α_2M proteinase inhibition at physiologically relevant oxidant concentrations. We investigated further the mechanism of such inactivation and found that increased susceptibility to proteolysis is responsible for α_2M destruction, rather than intersubunit cross-linking or structural fragmentation. Finally, we provide evidence that α_2M oxidation may contribute to inflammatory tissue destruction by demonstrating the presence of oxidized and degraded fragments of α_2M in knee joint synovial fluid of patients with RA but not in patients with OA.

Inflammatory oxidation can modify protein structure and function with high potency (*14*, *20*, *27*). The physiologically relevant oxidant(s) capable of oxidizing α_2M has not been investigated in detail. A number of reactive oxygen species such as hydrogen peroxide, hydroxyl radical, superoxide, peroxy nitrite, singlet oxygen, alkyl oxides, and alkyl peroxides have all been shown to modify proteins and cells during inflammation (*13*, *28*). We chose three oxidants, hydrogen peroxide, hydroxyl radical, and hypochlorite, for these studies because these oxidants are extremely potent, are released in large quantities by either neutrophils or macrophages during oxidative burst, and have been shown to play important roles in a wide variety of processes of disease such as rheumatoid arthritis, bacterial infection, atherosclerosis, Alzheimer's disease, inflammatory bowel disease, and emphysema (*13*, *14*, *27*−*29*). It is possible that other oxidants such as superoxide anion, peroxy nitrite, singlet oxygen, alkyl oxides, alkyl peroxides, and reactive

nitrogen species may also be involved in α_2M oxidation in vivo. These oxidants, however, are extremely short-lived and are highly reactive with nearby plasma membrane lipids, limiting the likelihood of their interactions with α_2M. Further investigation into the ability of these oxidants to modify α_2M structure and function should provide a comprehensive understanding of the role of inflammation oxidation in α_2M inactivation (*13*).

In earlier studies, we and our colleagues determined the susceptibility of α_2M to hypochlorite oxidation and found that methionine, tryptophan, lysine, and histidine modifications occur readily (*24*, *25*). We show here that hydrogen peroxide and hydroxyl radical appear to be weaker oxidants than hypochlorite in modifying α_2M and that hypochlorite is the only oxidant capable of abolishing α_2M proteinase inhibition. Interestingly, the number of oxidative "hits" required to inactivate α_2M appears to be small since the protein becomes fully dysfunctional at a hypochlorite concentration that is barely able to generate any protein carbonyl. Oxidation with hypochlorite at a concentration as low as 200 μM can significantly diminish the ability of α_2M to inhibit proteinases. Given the fact that the in vivo concentration of hypochlorite generated by the neutrophil oxidative burst can be as high as the millimolar level, a large proportion of α_2M should be either partially or completely oxidized by hypochlorite. Indeed, using RA as an inflammatory disease model, we demonstrated previously that α_2M in the knee joint synovial fluid of patients with this disease is significantly oxidized and has decreased functional activity (*16*). This evidence strongly supports our hypothesis that oxidation is a potent modifier of α_2M functions in inflammation in vivo.

The current studies demonstrate further that hypochlorite is a specific oxidant for inactivating α_2M. It is unclear why hydroxyl radical, a highly reactive oxidant, is unable to abolish proteinase inhibition by α_2M. Differences in the reactivity of these oxidants toward proteins and the chemical diversity in the end products of these modifications (for example, generation of 3-chlorotyrosine and Schiff base by hypochlorite vs amino acid hydroperoxides and hydroxides by hydroxyl radical) are currently being investigated. These studies should reveal distinct patterns of protein modification that may help explain the difference in the susceptibility to oxidation between hypochlorite and hydroxyl radical.

Nevertheless, we have identified a unique mechanism of α_2M inactivation in this study that can explain the decrease in α_2M proteinase inhibitory activity in RA synovial fluid. Earlier studies of α_2M oxidation by us and others have hypothesized, without direct evidence, that intersubunit cross-linking and tetramer to dimer fragmentation may be responsible for α_2M inactivation because these modifications are expected to alter the ability of α_2M to entrap proteinases (*25*, *30*, *31*). We found, in this study, evidence that strongly opposes this hypothesis. Using BS^3, a nonoxidative cross-linker, we show that chemically cross-linked α_2M undergoes tetramer to dimer fragmentation but retains the ability to inhibit proteinases, indicating that neither structural cross-linking nor fragmentation is solely responsible for α_2M inactivation. Further analysis of the interaction between proteinase and hypochlorite-oxidized α_2M reveals that this protein is significantly more susceptible to proteolytic cleavage, a phenomenon that correlates directly with the decreased ability to inhibit proteinases. Additional experiments using BS^3-cross-linked α_2M confirm our hypothesis that resistance to proteolytic degradation is necessary for preserving proteinase inhibition by α_2M.

The specificity of hypochlorite oxidation in mediating α_2M degradation in RA raises interesting questions regarding the mechanism of tissue degradation during inflammation. Hypochlorite, a product of the hydrogen peroxide/myeloperoxidase/Cl^- system, is better known for its role in bacterial killing and protein degradation within the neutrophil phagolysosome (*29*) and is released in large quantities during inflammation along with neutrophil-derived proteinases (*15*, *29*). As an endogenous modulator of growth factor functions and a defense barrier against tissue proteolysis, α_2M can become oxidized and proteolyzed as a part of this inflammatory activity. This would lead not only to greater tissue damage but also to a disruption of the extracellular growth factor network that is tightly regulated in the presence of α_2M (e.g., clearance of α_2M−proteinase−PDGF complexes via LRP and protection of IL-6 against proteolytic degradation) (*32*, *33*). In RA, a similar mechanism may be at work, with the exception being that an autoantigen is believed to be responsible for eliciting this inflammatory cascade.

The contribution of α_2M inactivation to tissue destruction is not clearly defined, since recent studies have implicated extracellular matrix-bound metalloproteinases, which are not inhibited by α_2M, in a number of inflammatory diseases (*34*). Studies in which mice that were α_2M deficient were used showed increased protection against endotoxin challenge compared with that in wild-type mice, rather than an increased level of tissue destruction as our model would predict (*12*). This finding may be better explained by the ability of α_2M to regulate inflammatory cytokines and/or growth factors as we and our colleagues have demonstrated (*16*, *35*). Additional studies will be necessary to determine the significance of α_2M in preventing inflammatory proteolysis. The fact that collagenase−α_2M and plasmin−α_2M complexes are present in synovial fluid of patients with RA suggests that α_2M provides at least some protection against tissue degradation (*36*, *37*). The finding that hypochlorite potently inactivates α_2M toward proteinase inhibition suggests that selective scavengers of hypochlorite may confer some therapeutic benefits in inflammatory diseases. Indeed, a few of the existing antiinflammatory drugs used to treat RA (e.g., 5-aminosalicylic acid, D-penacillamine, and immunogold) all possess antioxidant activity. Further development of selective inhibitors against hypochlorite production or scavengers of hypochlorite activity may expand our current regimens against this and other inflammatory diseases.

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