Basic Science Report

Neutrophil Elastase in Human Atherosclerotic Plaques
Production by Macrophages

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Background—Catabolism of the extracellular matrix (ECM) contributes to vascular remodeling in health and disease. Although metalloenzymes and cysteinylin proteases have garnered much attention in this regard, the role of serine-dependent proteases in vascular ECM degradation during atherogenesis remains unknown. We recently discovered the presence of the metalloproteinase MMP-8, traditionally associated only with neutrophils, in atheroma-related cells. Human neutrophil elastase (NE) plays a critical role in lung disease, but the paucity of neutrophils in the atheromatous plaque has led to neglect of its potential role in vascular biology. NE can digest elastin, fibrillar and nonfibrillar collagens, and other ECM components in addition to its ability to modify lipoproteins and modulate cytokine and MMP activity.

Methods and Results—Fibrous and atheromatous plaques but not normal arteries contained NE. In particular, NE abounded in the macrophage-rich shoulders of atheromatous plaques with histological features of vulnerability. Neutrophil elastase and macrophages colocalized in such vulnerable plaques (n=7). In situ hybridization revealed NE mRNA in macrophage-rich areas, indicating local production of this enzyme. Freshly isolated blood monocytes, monocyte-derived macrophages, and vascular endothelial cells in culture produced active NE and contained NE mRNA. Monocytes produced NE constitutively, with little regulation by cytokines IL-1β, TNF-α, or IFN-γ but released it when stimulated by CD40 ligand, a cytokine found in atheroma.

Conclusions—These findings point to a novel role for the serine protease, neutrophil elastase, in matrix breakdown by macrophages, a critical process in adaptive remodeling of vessels and in the pathogenesis of arterial diseases.

Key Words: atherosclerosis ■ remodeling ■ arteries ■ vessels ■ inflammation

Proteolysis and elastolysis contribute to the development of both occlusive and aneurysmal arterial diseases.1 A broad spectrum of elastolytic enzymes exists, including metalloelastases and cysteine proteases such as the cathepsins.2–4 We recently demonstrated that nonmyeloid cells found in atheroma express MMP-8, a collagenase previously associated only with neutrophils, and their inhibitors.5 This finding suggested to us that neutrophil elastase (NE) might also play a broader role than its name implies.

Myeloid precursor cells produce and activate NE in the bone marrow, and neutrophils store this 218-amino acid glycoprotein in lysosomal granules.6 It is predominantly present in neutrophils and has an important role in defense against infection.7 NE degrades not only elastin but also fibronectin, laminin, collagen III, IV, and VI, and proteoglycans.8 NE swings the proteolytic balance in favor of matrix breakdown by activating MMP-2, MMP-3, and MMP-9 and by inactivating their inhibitor, TIMP-1.8–10 In addition to matrix breakdown, NE also regulates the activity of cytokines such as IL-β, TNF-α, and IL-811 and cleaves the TNF-α 75-kDa receptor and T-cell surface proteins CD2, CD4, and CD8.6 NE has other actions of potential importance in the vasculature. It modulates the activation of nonmyeloid cells such as platelets, promoting aggregation, and augments both thrombosis and fibrinolysis by cleavage of clotting factors and their inhibitors.12 NE also has a proatherogenic role in lipid metabolism by virtue of its ability to modify apolipoproteins resulting in HDL destruction, enhanced LDL uptake, and foam cell formation.13,14 NE catastrophically promotes lung diseases such as emphysema. Cigarette smoking, which augments NE release in the lung, also contributes to atherothrombosis.15,16 Inherited deficiency of α1-proteinase inhibitor (α1-PI), the principle extracellular inhibitor of NE, increases risk of severe early-onset emphysema. Although less well recognized, humans deficient in α1-PI can also have fibromuscular dysplasia in...
the carotid and intracranial arteries, indicating an additional role for this enzyme in arterial biology.17

Subpopulations of mononuclear phagocytes, “proinflammatory” (P) monocytes, express enzymes such as myeloperoxidase, traditionally associated with granulocytes. This peroxidase-positive subpopulation of monocytes also contains NE and cathepsin G in the subcellular peroxidase granules. Surprisingly, however, some lesional macrophages can express myeloperoxidase, traditionally regarded as a marker for neutrophils.19

Thus, a subpopulation of plaque macrophages might express NE and function not only in proteolysis but also in cytokine activation and migration of macrophages, thus influencing plaque stability. We tested the hypothesis that human atheroma contain NE despite the paucity of neutrophils in these lesions. We examined the cellular localization of NE at various stages of atherosclerosis and investigated the ability of atheroma-associated cells to produce this enzyme, a property previously considered restricted to myeloid precursors in the bone marrow.20 In addition, we tested whether CD40 ligand (CD40L), a cytokine found in atheroma, could regulate the release of NE from mononuclear phagocytes.

Methods

Materials
Human recombinant IL-1β, IFN-γ, and TNF-α were from Endogen, neutrophil elastase was from CalBiochem, and recombinant CD40L was from Leinco Technologies. BD Pharmingen and Athens Research and Technology supplied mouse monoclonal (immunohistochemistry) and rabbit polyclonal antibodies (Western) to NE, respectively. DAKO supplied monoclonal mouse anti-human CD31, CD4, and CD68. Mouse IgG1 was from Sigma, and rabbit IgG1 was from Santa Cruz Biotechnology Inc.

Cell Culture
We isolated human vascular endothelial cells (ECs) and smooth muscle cells (SMCs) from saphenous veins, as described previously.21 Cells were stimulated for 24 hours by cytokines with TNF-α and IL-1β both at 10 ng/mL and IFN-γ (500U/mL). Mononuclear phagocytes were isolated from fresh human peripheral blood monocytes by density gradient centrifugation with Cappell LSM Lymphocyte Separation Media (ICN Biomedical) and adherence on plastic dishes for 2 hours in RPMI (BioWhittaker). This technique yields >92% monocytes.3 Experiments used fresh monocytes or macrophages differentiated in culture in RPMI with 2% human serum (Sigma) for 3 to 10 days; 24 hours before and during stimulation, cells were incubated in serum-free media as previously described.3,21

Venous blood was diluted 9:1 with 4% sodium citrate (Sigma) for neutrophil isolation. Cells were separated on neutrophil isolation medium gradients (Cardinal Associates Inc) and red cells were lysed with 155 mmol/L NH₄Cl, 20 mmol/L NaHCO₃, and 166 μmol/L EDTA. The preparation contained >95% neutrophils as assessed by staining of cytospin preparations.

HL-60 cells (ATCC) were maintained in RPMI with 10% FCS and RPMI with 20% FCS and 1.25% DMSO, respectively. Human foreskin fibroblasts (ATCC) were maintained in DMEM with 5% FCS.

Preparation of Human Arterial Extracts and Cellular Extracts
We harvested surgical specimens of human carotid atheroma as well as nonatherosclerotic arteries (aortic fragments from cardiac transplantation donors) according to protocols approved by the Human Investigation Review Committee at Brigham and Women’s Hospital (n=7). Atherosclerotic plaques were dichotomized into fibrous and atheromatous subsets by morphological criteria, as described previously.21 Specimens, homogenized under liquid nitrogen, underwent protein assay (Pierce) and storage at −70°C.22

Western Blot Analysis
Protein was separated by SDS-PAGE and applied to Western blot analysis, as previously described, and probed with anti-NE polyclonal Ab (1 in 6000), and goat anti-rabbit horseradish peroxidase conjugated monoclonal antibodies diluted 1 in 3000 (Bio-Rad). No cross-reaction with 1 μg cathepsin G, Proteinase 3, a-1-PI, MMP-8, or MMP-9 occurred (data not shown). Immunoreactive proteins were visualized with the use of enhanced chemiluminescence (PerkinElmer Life Sciences Boston MA).3,21 Bands were analyzed by densitometry using Gel Pro Analyzer Software (Media Cybernetics).

Immunohistochemistry
Immunohistochemistry was performed on cryostat sections (6 μm) cut, air-dried, and fixed in ice-cold acetone (5 minutes). After blocking with 0.3% hydrogen peroxide and PBS with 4% species-appropriate normal serum, sections were processed according to the manufacturer’s recommendations (Universal Dako LSAB kit, DAKO). Species-appropriate biotinylated secondary antibodies (Vector Laboratories) were applied and sections were incubated with avidin-biotin-peroxidase complex (Vectorstain ABC kit, Vector Laboratories). Colocalization was proved by fluorescent double immunostaining. The NE antibody was applied for 90 minutes, followed by biotinylated goat-anti-rabbit secondary antibody for 45 minutes and Texas-red conjugated streptavidin (Amersham). After application of the avidin-biotin blocking kit (Vector), sections were incubated overnight at 4°C with anti-CD31, anti-CD68, or anti-HHF35, as appropriate. Biotinylated horse anti-mouse antibody was applied for 45 minutes, followed by streptavidin-FITC (Amersham).

In Situ Hybridization
In situ hybridization with streptavidin peroxidase amplification was performed on 4% paraformaldehyde-fixed cells and sections, using an Iso-IHC detection kit (Innogenex) according to the manufacturer’s instructions (hybridization time of 2 hours at 37°C). A 39-mer oligonucleotide probe was commercially synthesized and 5’-labeled with fluorescein (IDT). Sense probe for NE was GTA AAC TTG CTT AAC GAC ATC ATC GTC CTC CAC AAT (positions 372 to 410, GenBank M34379). The antisense probe was GTT GAG CTT AAT CAC CAG CTC GTC CTT GAT CAA GGT TTC in situ in all negative tissues, the preservation of RNA was confirmed by use of a poly-A-positive control probe (Innogenex).

Reverse Transcriptase–Polymerase Chain Reaction
Total cellular RNA was prepared from cells by using Qiagen RNeasy extraction columns according to the manufacturer’s instructions. RNA (100ng) was reverse-transcribed by random hexamers and superscript II reverse transcriptase (Gibco-BRL). Samples were amplified for 2 minutes at 95°C, then for 38 cycles at 95°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute. Primers for NE were CCC TCA CGA GAG TCG AGA CGT T and CGT AAA CTT CTT GCT CAA CGA CAT C. PCR products were run on 2% agarose gels, stained with ethidium bromide, and analyzed by densitometry using Gel Pro Analyzer Software. NE results were normalized to the GAPDH result from the same reaction.23 RNA from HL60 cells and fibroblasts served as a positive and negative control, respectively.

Elastase Activity Assay
Cells were harvested in HBSS containing 1 mol/L NaCl and 0.04% Triton. Extracts or NE standards (Calbiochem) were incubated for 3 hours at 37°C with 200 μmol/L MeO-Succ-Ala-Pro-Val-ACF (Enzyme System Products) in 0.2 mol/L Tris HCl (pH 8.5) containing 0.15 mol/L NaCl and 0.02 mol/L CaCl₂, Liberated 7-aminofluorescin methyl coumarin was quantified (excitation wavelength, 400
nm; emission wavelength, 505 nm) by fluorometry. Results are expressed as nanograms of active NE per microgram of total protein. A specific inhibitor of NE; 400 μmol/L N-(methoxysuccinyl)-Ala-Ala-Pro-Val-chloromethyl ketone (Enzyme System Products); a general synthetic serine protease inhibitor, 5 mmol/L phenylmethylsulfonyl fluoride, (Sigma); and a general synthetic inhibitor of metalloenzymes, 1 mmol/L 1,10 phenanthroline (Sigma) explored the nature of the activity.

Statistics
Data are presented as mean±SEM. Differences between two experimental groups were examined by use of the Student’s t test. ANOVA with post hoc Dunnett’s test for comparison of groups with control condition was used for multiple comparisons between groups of parametric data and Dunnett’s C for nonparametric data.

Results
Expression of NE in Cells Found in Human Atheroma In Situ
Previous studies have localized NE in abdominal aortic aneurysms but not in atheroma. Atheromatous plaques (n=7) showed robust staining for NE with localization to the lesion’s shoulder (Figure 1A), a region prone to rupture and provoke acute coronary syndromes. Further observations determined the cell types that contained NE in plaques. As expected, atheromatous plaques showed vivid staining for macrophages (CD68) in the shoulder regions (Figure 1B). Atheromatous plaques contained NE within the macrophage-rich areas, whereas normal arteries had only low levels of this enzyme (Data Supplement Figure 1). Some but not all of the CD68-positive macrophages in atheroma expressed NE, consistent with its detection in subsets of macrophages and monocytes.

Fibrous plaques (n=7) had abundant smooth muscle cells (HHF35+) that did not contain immunoreactive NE (Figure 1H), although macrophages in this type of lesion colocalized with NE (Figure 1, E and F). Interestingly, luminal endothelial cells (CD31+) in these sections did not contain NE, whereas microvascular ECs in some areas did (data not shown). Double immunofluorescence study verified rigorously the cellular localization of NE within the plaque. NE (red fluorescence) localized to macrophages (CD68+, green

Figure 1. Macrophages of atheromatous and fibrous plaques contain neutrophil elastase. Frozen sections of atheromatous plaques (A, B, and C) and normal artery (D) were used for immunohistochemistry (NE Ab 1:200). Strongly positive NE staining (A) colocalizes with CD68 staining for macrophages (B). Isotype-matched control mouse IgG yields slight background staining (1:200) (C). Fibrous plaque (E through H) shows intense staining for NE (E, X-10; G, X40) colocalizing with CD-68 macrophage staining in an adjacent section (F). Areas of smooth muscle cells (HHF35) in close proximity to macrophages do not overlay regions of NE staining (H).
fluorescence) (Figure 2, A and B). Although absent from smooth muscle cells (Figure 2, C and D), NE colocalized with endothelial cells (Figure 2, E and F). ECs stained for NE only in the microvessels and not in the macrovascular ECs.

Biochemical studies corroborated the immunohistochemical findings of increased NE protein in atheroma and particularly in those with characteristics of vulnerability to rupture (Figure 3). Western blots showed abundant NE in extracts of atheromatous plaques (32.1±9.1) and an intermediate amount in fibrous plaques (11.7±3.2); normal arteries had low and somewhat variable levels (3.99±1.09). Thus, NE was 3-fold greater in fibrous plaques and 8-fold greater in atheromatous plaques than in normal vessels (P=0.03, 0.006 and 0.049 in normal versus fibrous, normal versus atheromatous and fibrous versus atheromatous respectively, n=9, Figure 3B).

In Situ Hybridization
Cells can take up NE from the extracellular milieu. Therefore, the presence of intracellular NE protein does not prove endogenous synthesis. Consequently, we sought the presence of mRNA encoding NE to affirm local synthesis in the plaque (Figure 4). Some but not all macrophage-rich areas of the plaque hybridized with the antisense probe, establishing the presence of NE mRNA (Panel A). This signal colocalized with CD68+ macrophages in the same plaque. Sense probes showed negligible signal (Panel D). Double immunohistochemical staining in the same section rigorously supported the cellular localization of NE to the macrophage (Data Supplement Figure II). Pretreatment of sections with RNase abolished all in situ hybridization signals (data not shown).

Expression of Active NE in Human Atheroma-Associated Cells
Monocyte extracts (day 0 and day 3 after isolation) contained NE (Figure 5). Exposure to CD40L but not IL1-β, TNF-α, or IFN-γ shifted recovery of NE from the cell lysate to the extracellular medium, suggesting that CD40 ligation stimulated secretion or release. A trend to increase in cellular NE after IFNγ stimulation did not achieve statistical significance. The levels of NE diminished as the cells differentiated into macrophages but remain robust at day 10. Fully differentiated monocyte-derived macrophages contained immunoreactive NE, although CD40L no longer provoked release of NE. Only a portion of the macrophage population stained positively for NE, consistent with previous descriptions. Smooth muscle cells did not express NE (data not shown). Endothelial cells constitutively contained NE protein, albeit at a lower level than monocyte/macrophages (data not shown).

Enzymatic activity of NE receded in lysates of macrophages as they differentiated. Macrophages at days 0, 3, and 10 had 0.29±0.02, 0.13±0.04, and 0.11±0.02 ng/μg total protein, whereas ECs had 0.2±0.05 ng/μg, n=4 (Data Supplement Figure III). Ultimately, levels declined to 100-fold less than in freshly isolated neutrophils. Macrophage (day 10) and endothelial cell lysates contained elastolytic activity.
activity. A chloromethyl ketone inhibitor specific for NE and PMSF, a general serine protease inhibitor, reduced the activity by 78.3 ± 0.9% and 87.2% (± 6.1), respectively, in day 10 macrophages (n=5). A broad-spectrum MMP inhibitor did not influence activity (Figure 6).

**Discussion**

Our group and others have established the importance of metalloproteinases in the critical balance between extracellular matrix breakdown and synthesis that determines the plaque’s ultimate vulnerability to rupture and other aspects of vascular remodeling.21,27,28 We have also found other proteases such as Cathepsin S, a cysteine-dependent enzyme, in the plaque.2 In addition, we recently showed that a number of atheroma-associated cells can produce MMP-8, designated “neutrophil collagenase,” and localized MMP-8 to sites of collagen breakdown.5 The present work tested the conjecture that chronic inflammatory conditions in the atherosclerotic plaque might stimulate other cell types to produce proteases...
previously attributed to neutrophils and only considered synthesized in the bone marrow. 20

This study demonstrated the presence of NE in macrophages of the atherosclerotic plaque and localized NE in macrophages and ECs in the shoulders of the plaque and the microvessels, respectively. Atheromatous plaques with morphological features of vulnerability, such as a thin fibrous cap and macrophage-rich shoulder region, contained more NE than fibrous plaques or particularly normal arteries.

In vitro investigation of macrophages verified the ability of a subset of macrophages and ECs to produce NE mRNA and synthesize the protein. In light of previous dogma suggesting that NE synthesis does not occur outside the bone marrow, we used two methods (RT-PCR and in situ hybridization). In fact, earlier studies largely focused on macrophages from bronchial alveolar lavage samples that may have a different stage of activation and differentiation than those derived from circulating monocytes. 4 The monocyte-derived macrophages more closely parallel our results from human tissue.

In situ hybridization showed a subset of monocyte-macrophages (50% to 70%) positive for NE, likely to be the P monocytes previously described. The abundant P monocyte-macrophages in our cultures may result from the selection of monocytes by adherence, a procedure known to enrich this subset. 18 Biochemical studies established NE activity in monocyte-derived macrophages.

Figure 5. Western blot analysis of macrophage cell lysates and supernatants. Western blot analysis of NE protein in lysates and supernatants of monocytes—freshly isolated (day 0), partially differentiated (day 3), and fully differentiated into macrophages (day 10), showed little regulation of protein by IL-1β, TNF-α, or IFN-γ. Stimulation with CD40L caused release of NE from monocytes into supernatant. Graph shows mean integrated optical densitometry of Western blots and sample blots (50 μg protein/lane, n=5, *P<0.05 vs control).

Figure 6. NE activity in macrophages. Graph shows percentage activity of day 10 macrophages extracts with protease inhibitors compared with control, day 10 macrophages. Presence of a HLEC MK or PMSF suppressed activity by >78%, whereas 1,10 phenanthroline had no significant effect (n=5).
NE identified within the plaque may have important roles in positive arterial remodeling or in promotion of plaque rupture. In addition to its classic substrate, elastin, NE can also act on a number of other biologically important proteins, including collagens type I-IV, VI, and X.6 The MMPs probably predominate as collagenases in the plaque; however, to attain enzymatic activity, the zymogen precursors require activation. NE can activate MMPs -2, -3, and -9, enzymes found in the shoulders of vulnerable plaques. Thus, NE may contribute to collagenolysis indirectly through its ability to switch on these powerful proteases.8–10 Experiments in a model of bullous pemphigoid in NE and MMP-9 knockout mice have highlighted another complex interplay between the MMPs and NE. Lesion formation requires both NE and gelatinase B, but gelatinase B acts upstream to regulate NE activity by inactivating its inhibitor, α-1-PI.9 NE can also prevent inhibition of the MMPs by inactivating TIMP-1.9 This interaction between the MMPs and serine proteases suggests an important role for NE in remodeling processes within the atherosclerotic plaque.

NE may also contribute to a proinflammatory state in the plaque by cleaving pro-IL-1β to a truncated, more active form that could stimulate production of MMPs.6 NE can activate IL-8 through a similar proteolytic mechanism that may also contribute to inflammation in the arterial wall and promote macrophage accumulation.11 However, NE inactivates TNF-α and causes shedding of the 75-kDa TNF-α receptor that could counterbalance a proinflammatory state.

The unexpected finding reported in this study of the presence and production of NE in plaques provides a new perspective on the panel of ECM degrading enzymes in atheroma and the pathogenesis of vascular diseases. These results also extend the challenge to look beyond metalloproteinases in understanding the biology of and therapeutic strategies for arterial disease.
Acknowledgments
This work was supported grants from the British Cardiac Society to Dr Dollery and from the National Heart, Lung, and Blood Institute to Dr Libby (HL-56985). The authors acknowledge the skillful technical assistance of Eugenia Shvartz, Samantha LaClair, and Elissa Simon-Morrissey and Karen Williams’ editorial expertise.

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Circulation. 2003;107:2829-2836; originally published online May 27, 2003;
doi: 10.1161/01.CIR.0000072792.65250.4A
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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