N-Acetyl-Cysteine Decreases the Matrix-Degrading Capacity of Macrophage-Derived Foam Cells
New Target for Antioxidant Therapy?

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Background—Atherosclerotic plaque destabilization triggers clinical cardiovascular disease and thus represents an attractive therapeutic target. Weakening of tissue through the action of matrix-degrading enzymes, called matrix metalloproteinases (MMPs), released by resident macrophages was previously implicated in unstable vascular syndromes.

Methods and Results—We used a hypercholesterolemic rabbit model of atherosclerosis to investigate the gelatinolytic activity associated with macrophage-derived foam cells (FCs). Gelatinolytic activity and expression of MMP-9 but not of MMP-2 cosegregated with macrophage FCs in aortic lesions. Macrophage-derived gelatinases were further investigated in vitro. MMP-9 was identified as the main macrophage-derived gelatinase in cells isolated from aortic lesions and from granuloma induced in the same rabbits to increase cell yield. Importantly, detection of activated MMP-9 in the FC culture medium supports the notion that these cells can independently initiate processing of secreted MMP zymogens to active enzymes. We further examined whether FC gelatinolytic activity is dependent on the presence of reactive oxygen species (ROS). We found that treatment (1 to 5 days) with 1 to 10 mmol/L N-acetyl-L-cysteine (NAC), an ROS scavenger, decreased not only gelatinolytic activity but also gelatinase expression by FCs. Similarly, NAC treatment of explanted lesions abolished in situ gelatinolytic activity and MMP-9 expression.

Conclusions—Macrophage FCs are an abundant source of gelatinolytic activity that can be inhibited in vitro and in situ by NAC. This newly described action of antioxidant therapy might prove useful to inhibit matrix degradation and to improve vascular stability. (Circulation. 1998;97:2445-2453.)

Key Words: atherosclerosis ■ metalloproteinases ■ free radicals ■ antioxidants

Macrophage-derived FCs, which figure prominently in areas of atherosclerotic plaques prone to rupture, are a likely source of plaque instability.1-3 Many recent studies focusing on various aspects of the active macrophage FC involvement in the weakening of the vascular matrix scaffold suggest that it is a major factor determining plaque vulnerability. Activated macrophages release cytokines, which increase the repertoire of matrix-degrading enzymes, called MMPs, secreted in vitro by vascular cells4-6 and produce their own MMPs.7 Furthermore, we and others have shown that macrophage-derived FCs resident in human and experimental atherosclerotic lesions are also associated with matrix-degrading activity due to active MMPs.8-11 To digest matrix components, however, latent cell-secreted MMPs require posttranslational processing to active forms, a step acting as a key regulatory mechanism of matrix degradation by MMPs.12 In our search for MMP activation mechanisms relevant to atherosclerosis, we recently found that ROSs can trigger activation of MMP precursors released by vascular SMCs.13 Similar ROSs are produced by macrophage-derived FCs; thus, in addition to modulating vascular MMP gene transcription via release of stimulatory cytokines, these cells may regulate MMP enzymatic activation via release of ROSs. In addition, macrophage-derived FCs most likely contribute with their own MMPs to matrix degradation. Certain conditions enhance production of MMPs by monocytic cells and cell lines,7 and the intracellular lipid accumulation characteristic of monocyte-derived macrophages residing in atheroma could be one of them.14 We found previously that the macrophage-derived FCs isolated from aortic lesions of hypercholesterolemic rabbits secrete in vitro precursors of the inducible MMPs interstitial collagenase and stromelysin.14 However, we did not detect generation of their active forms, the only ones capable of matrix degradation. We also did not investigate the expression of the macrophage-derived gelatinases MMP-9 and MMP-2. These MMPs, specialized in digestion of basement membrane collagens and elastin,15 have since been implicated in weakening of vascular tissue in unstable coronary syndromes10 and in aortic aneurysms.16 Thus, in the present study, we sought to characterize gelati-
nase production by in vivo differentiated macrophage FCs, which to the best of our knowledge has not yet been investigated. We also explored the hypothesis that gelatinase activity in atheroma areas rich in macrophage-derived FCs is ROS-dependent and thus inhibitable by ROS scavengers. For this purpose, we used an experimental hypercholesterolemic rabbit model that develops macrophage FC–rich aortic lesions and allows isolation of in vivo differentiated macrophage FCs for in vitro studies.

Methods

Animal Model of Atherosclerosis

Experimental atherosclerotic lesions were induced in the double-injury rabbit model, in which aortic intimal lesions are rich in lipid-laden macrophages. We induced aortic lesions in New Zealand White rabbits (n = 8), as previously described in detail, by balloon angioplasty 1 week after initiating a hypercholesterolemic diet (0.5% cholesterol and 4.5% coconut oil added to Purina chow). The diet was maintained for the following 8 weeks. Three weeks before the animals were killed, subcutaneous granulomas were induced in the same rabbits by implantation of 2 or 3 sterile polyurethane sponges (Baxter Scientific) per animal under the dorsal skin. A similar procedure was used simultaneously to induce subcutaneous granuloma formation in normocholesterolemic New Zealand White rabbits on regular Purina chow. All animals were euthanized with 100 mg/mL pentobarbital. Aortas were harvested for morphological processing, biochemical analysis, and isolation of FCs. Sponges were removed under sterile conditions and used for isolation of macrophages. The protocol for animal use was approved by the Emory University Committee on Institutional Animal Care and Use.

Isolation of Macrophages

Aortic lipid-laden macrophages were isolated from rabbit atheromas as described previously. The aortica intima was separated and minced in ice-cold sterile HBSS. Tissue was incubated with agitation at 37°C in sterile HBSS containing collagenase (type I, Worthington), elastase, and soybean trypsin inhibitor (Sigma Chemical Co), then filtered through sterile nylon mesh. Granuloma macrophages were collected by gentle squeezing of sponges. Macrophages were further isolated by metrizamide-density centrifugation as previously described and plated in Opti-MEM (Gibco-BRL). Macrophage purity was assessed by immunocytochemical staining as described below.

Cell Culture Experiments

Macrophages and macrophage-derived FCs were maintained in culture in serum-free Opti-MEM for up to 5 days. In some experiments, NAC (100 μmol/L to 10 mmol/L) was added to the culture medium of granulomatus macrophages. We harvested cell-conditioned culture media and cell lysates obtained by use of ice-cold 10 mmol/L phosphate buffer/150 mmol/L sodium chloride containing 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, and 0.2% sodium azide. Cell viability was assessed at the end of various treatments by the “Live-dead” fluorescent kit (Molecular Probes). Computer-assisted image analysis was performed with ImagePro Plus 2.0 software (Media Cybernetics).

In Situ Treatment With ROS Scavengers

Paired aortic rings (of abdominal, thoracic, or aortic arch) were incubated with or without 10 mmol/L NAC for up to 4 days. Tissues were then processed for immunocytochemistry or extracted with the lysis buffer and analyzed for gelatinolytic activity by SDS-PAGE as previously described. Conditioned culture media were harvested and compared for expression and activity of secreted gelatinases.

SDS-PAGE Zymography

In this method, a gelatin substrate was included in the composition of the polyacrylamide/SDS gels, and samples were separated according to their apparent molecular weight by electrophoresis. Areas of lysis appeared as white after renaturing and staining of gels with colloidal Brilliant Blue G-250 (Fisher Scientific). MMPs with gelatinolytic activity were identified and compared in samples of culture media harvested from explanted lesions or cultured macrophages. An increase in the intensity of gelatinolytic bands with lower apparent molecular weight and/or generation of new gelatinolytic bands relative to bands representing thezymogens is interpreted as gelatinase activation. Gelatinolytic bands were quantified after scanning densitometry with NIH Image 1.57 software. Statistical significance was investigated by Student’s t test with one-tailed distribution by use of Microsoft Excel 5.0 software.

Western Blotting

Culture media were separated on 10% SDS-PAGE minigels and transferred onto nitrocellulose (Bio-Rad Laboratories). Incubation with anti–MMP-2 or anti–MMP-9 monoclonal antibodies (Oncogene Science), recognizing both the latent and active forms, was followed by incubation with secondary antibodies coupled to horseradish peroxidase and development of a chemiluminescent reaction (ECL kit from Amersham International). Signals (positive bands) were quantified and analyzed as described above.

Histological Characterization of Aortic Tissue and Isolated Cells

Immunostaining was performed on frozen tissue specimens embedded in O.C.T. compound (Miles) to identify macrophages (anti–RAM-11, Dako Corp) and to detect MMP-9 and MMP-2 (Oncogene). Staining was developed with the LSAB staining kit (Dako), and sections were counterstained with Gill’s hematoxylin (Sigma). Isolated cells were stained by single or double immunofluorescence using species-specific secondary antibodies coupled to fluorochromes (FITC or Texas Red, Jackson ImmunoResearch). Nuclei were counterstained for 2 minutes with 0.5 μg/mL bisbenzamide (Calbiochem). Intracellular lipid accumulation was revealed by staining of cells with 5 μg/mL Nile red (Molecular Probes).

In Situ Zymography

In situ gelatinolytic activity was detected in frozen aortic tissue specimens as previously described, with gelatin coupled to a green fluorochrome. Briefly, tissues were processed for obtaining unfixed frozen sections, which were placed on microscope slides previously coated with fluorescent gelatin. The specimens were then incubated at 37°C for 2 days and examined with a Zeiss fluorescence microscope to reveal areas of active lysis of the gelatin substrate.

Results

Gelatinolytic Activity Is Associated With Increased MMP-9 Expression and Macrophage-Derived FCs in Experimental Atheroma

Neointimal lesions developed in the double-injury model of atherosclerosis were composed predominantly of macrophage-derived FCs (Figure 1) identified by immunohisto-
chemistry with a macrophage-specific antibody. The gelatinolytic activity, examined by in situ zymography, was restricted to the macrophage-rich intimal area. Immunostaining for the two possible sources of gelatinolytic activity, MMP-2 (gelatinase A) and MMP-9 (gelatinase B), showed that expression of MMP-9 was specifically associated with the neointimal lesion, whereas MMP-2 staining was diffuse. This observation suggested that the macrophage-derived gelatinolytic activity was due mainly to the presence of macrophage-derived MMP-9.

Characterization of Lipid-Laden Macrophages

Further experiments were performed to confirm that the lipid-laden macrophage was the source of MMP-9 and gelatinolytic activity. However, to further investigate expression and activation of gelatinases in culture, we had to find an alternative source of macrophage-derived FCs, the yield of aortic FCs being very limited (3×10^5 to 10^6 cells per aorta). We therefore produced and isolated in vivo differentiated macrophage-derived FCs from subcutaneous granulomas of hypercholesterolemic rabbits. To be able to assess possible differences associated with the FC phenotype, we also produced and studied, in parallel, non–lipid-laden macrophages by implanting subcutaneous sponges in normocholesterolemic rabbits. Isolated cells were positively identified as being of macrophage origin by immunofluorescence with the RAM-11 antibody (Figure 2) and were used for the in vitro experiments. Macrophage purity and viability were >98%.

Figure 1. Experimental atheroma in aorta of hypercholesterolemic rabbits. Detection of macrophages, gelatinolytic activity, and expression of gelatinases MMP-2 and MMP-9. Top four panels illustrate consecutive sections of lesions in abdominal aorta; bottom four panels, in thoracic aorta. Top, Staining with cell-specific antibody (RAM-11) identifies numerous macrophages in intimal region. Same region contains gelatinolytic activity, as shown by processing of a consecutive section by in situ zymography (gelatinolytic activity is indicated by disappearance of green fluorescent gelatin substrate used to coat microscope slide). Immunostaining for MMP-2 and MMP-9 gelatinases shows colocalization of macrophage accumulations with MMP-9 expression but not with MMP-2. Bottom, Macrophages, MMP-9 expression, and gelatinolytic activity colocalize in a thoracic aorta lesion. Control specimens were processed without primary antibody (Ab). Bars=100 μm.
Nile red staining confirmed intracellular lipid accumulation in macrophages from hypercholesterolemic rabbits. Granuloma FC yield was an order of magnitude higher than aorta (2\times10^7 to 3\times10^7 per rabbit). These FCs produced MMP-3 (Figure 2), similar to macrophage-derived FCs isolated from aortic lesions.14

Figure 2. Macrophage-derived FCs isolated from subcutaneous granuloma. Top, Cells isolated from subcutaneous granuloma of hypercholesterolemic rabbits are identified by immunofluorescence as macrophages (RAM-11 antibody). Inset, Negative control for immunostaining in which primary antibody (Ab) was eliminated. Middle, Intracellular lipid in macrophages from hypercholesterolemic rabbits is revealed by staining with Nile red. Inset, Nile red staining of macrophages (Mφ) from normocholesterolemic rabbits. Bottom, Macrophage-derived FCs of granuloma express stromelysin (MMP-3). Nuclei are counterstained with bisbenzamide. Magnification ×400.

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Macrophage-Derived FCs Release and Activate MMP-9
Analysis of gelatinolytic activity released in culture by different segments of atherosclerotic rabbit aorta by SDS-PAGE zymography showed that the aortic arch consistently produced the highest level of total gelatinolytic activity. The activity released by thoracic aorta was also higher than that of abdominal aorta (Figure 3). The gradual increase in gelatinolytic activity paralleled the extent of macrophage-rich lesions. Migration of higher-molecular-weight gelatinolytic activity was consistent with the presence of MMP-9, whereas gelatinolytic activity running at lower molecular weight was most likely due to MMP-2, the main MMP produced by vascular SMCs. Analysis of gelatinolytic activity released in culture by isolated aortic or granulomatous FCs showed that these cells produce copious amounts of the gelatinolytic activity associated with MMP-9. Because gelatinolytic profiles were identical for the FCs isolated from either source, we used granuloma macrophages for further in vitro experiments. Both lipid-laden and non–lipid-laden macrophages were maintained in culture up to 5 days.

Effect of NAC on Protein Expression and Activity of Macrophage-Derived MMP-9
Because our recent experiments showed that ROSs may function as activators of SMC-derived latent gelatinases,13 we hypothesized that activation of FC-derived pro–MMP-9 may be related to concomitant production of ROSs. To test ROS contribution, we treated macrophages with NAC, an ROS scavenger. Gelatinolytic activity in the culture media of untreated and NAC-treated cells was analyzed by SDS-PAGE zymography. We found that 24 hours of treatment with NAC reduced both the gelatinolytic activity consistent with migra-
tion of the MMP-9 zymogen and that of the faster band migrating at the expected position for active MMP-9 (Figure 4). To confirm the identity of MMP-9 and to further assess effects of treatment, we also analyzed the effect of NAC on MMP-9 protein level by immunoblotting (Figure 5). Comparing non–lipid-laden and lipid-laden macrophages, we identified the MMP-9 precursor in culture media conditioned by either macrophage population. Interestingly, only the culture media conditioned by lipid-laden macrophages contained the fully activated MMP-9, migrating around 66 kDa as previously reported.19,20 The same anti–MMP-9 antibodies did not recognize SMC-derived MMP-2, which has a similar apparent molecular weight (not shown), confirming that this faster band was indeed generated through processing of latent MMP-9. Culture media from macrophage FCs treated with 10 mmol/L NAC for 48 hours had significantly lower levels of the precursor and active forms of MMP-9. NAC treatment also inhibited expression of the MMP-9 precursor by non–lipid-laden macrophages. We also confirmed that the decreased gelatinase production was not due to a cytotoxic effect of NAC. Viability tests performed at the end of each experiment showed that after 5 days in culture, FC viability was 79.3±6.8% live cells in untreated versus 72.4±11.9% live cells treated with 10 mmol/L NAC (n=8, P=NS).

NAC Reduces the Gelatinolytic Activity and In Situ Expression of MMP-9 in Experimental Atherosclerotic Lesions

The results obtained with cultured macrophage-derived FCs and non–lipid-laden macrophages suggested the possibility of reducing production of active gelatinase by resident macrophages through NAC treatment. This effect was tested by incubating segments of atherosclerotic rabbit aorta with NAC in organ culture conditions. We found that the NAC treatment abolished the gelatinolytic activity released by aortic tissue and significantly decreased the level of MMP-9 protein detected by Western blotting (Figure 6). The fact that the effect of NAC was not restricted to inhibition of gelatinolytic activity but rather also affected FC MMP-9 expression was confirmed by the disappearance of MMP-9 immunopositive staining in the FC-rich lesions maintained in culture with NAC (Figure 7). This effect was specific to the signal associated with the presence of MMP-9, since it did not affect expression of macrophage markers also detected by immunostaining. In addition, detection of MMP-9 was not affected in paired untreated specimens that were maintained in culture and processed simultaneously (Figure 7). Thus, lack of MMP-9 detection in lesions after treatment with NAC suggests that the action of NAC is not restricted to inhibition of MMP-9 activity or cellular secretion but rather also involves suppression of macrophage MMP-9 synthesis.

Discussion

Recently, the action of MMPs has emerged as an important component of the natural history of atherosclerosis8,11,21 and of the vascular response to injury.22–24 Macrophage-derived FCs associate clinically with unstable human plaques1–3 and microscopically with vulnerable areas and active MMPs8 and thus may be responsible for compromising vascular tissue integrity through matrix degradation. Macrophages are also thought to be a major source of the redox stress that characterizes atherosclerotic vessels.25–27 In the present study, we investigated production and activation of gelatinases produced by macrophage FCs of hypercholesterolemic rabbits, a good model for study of macrophage-derived FCs resident in human atheroma. We also hypothesized that macrophage-derived FCs have a built-in redox-dependent mechanism leading to activation of their own MMPzymogens. We found that gelatinolytic activity in rabbit atherosclerotic lesions is restricted to the macrophage-rich areas and colocalizes with expression of MMP-9. Study of isolated cells
confirmed that macrophage-derived FCs were a major source of MMP-9 and detected the presence of active MMP-9. As mentioned, increases in MMP-9 activity were previously reported in vascular disease and a variety of other pathological situations with an inflammatory component, such as rheumatoid arthritis, as well as metastasis. To obtain sufficient material for meaningful in vitro studies with macrophage-derived FCs, we also induced formation of these cells in vivo in granulomas of hypercholesterolemic rabbits. Implantation of subcutaneous sponges triggers formation of granulomas whose macrophages accumulate lipid in hypercholesterolemic conditions. These macrophage-derived FCs from subcutaneous granulomas had the same MMP profile as the aortic FCs.

Observations made on isolated macrophages in culture showed the presence of active gelatinase, although all MMPs known so far are reportedly secreted by cells as zymogens only. This in vitro observation supports the notion that MMP-9 activation can occur independently of the classic plasmin-mediated pathway. The possibility that macrophage gelatinolytic activity is redox-dependent is suggested by our previous studies showing activation of gelatinase zymogens by ROSs known to be produced by macrophage FCs, as well as by our present detection of active MMP-9 in an isolated cell system along with inhibition of macrophage-derived gelatinolytic activity by treatment with NAC, an ROS scavenger. Activated macrophages, especially those of atherosclerotic lesions, are a major source of ROSs; thus, such an activation mechanism would result in activation of MMP zymogens secreted by the macrophages themselves as well as by the neighboring cells. However, our observations do not exclude the possibility that activation of MMP-9 might occur through other cell-dependent mechanisms, including the action of a group of cell membrane molecules called MT-MMPs. It was reported that these molecules activate the zymogens of MMP-2 and MMP-13, but it is not yet known whether monocytes/macrophages express MT-MMPs and whether pro–MMP-9, the gelatinase whose activity was investigated in this study, is a substrate for MT-MMPs.

NAC is widely used as an antioxidant on the basis of its capacity to scavenge ROSs; however, its inhibitory effect on gelatinolytic activity might also involve a direct interaction with the enzyme. Although further biochemical studies will be necessary to differentiate between these two possible components of gelatinase inhibition, the capacity of NAC to inhibit gelatinolysis that we found is unquestionable. This is important because regulation of gelatinolytic activity ultimately determines the level of matrix degradation by vascular gelatinases. However, our results suggesting that NAC may also affect prior events in the pathway leading to secretion of...
MMP-9 are also interesting. For instance, we found that NAC treatment effectively inhibited the level of MMP-9 protein secreted in culture by macrophages. Findings from in situ observations of macrophage-derived FCs of NAC-treated experimental atheroma support NAC inhibition of MMP-9 expression. The hypothesis that redox stress modulates cellular MMP-9 expression is novel; thus, at present, the mechanism is still unknown. Possibly relevant information is provided by studies showing MMP-9 induction by cytokines and phorbol esters in a variety of cells, including vascular endothelial cells and SMCs. MMP-9 induction may require cooperation between AP-1–, NF-κB–, or SP-1–responsive elements. Conversely, monocyte/macrophages constitutively express pro–MMP-9, but its expression was shown to be enhanced by cellular differentiation, concanavalin A, or LPS stimulation. Interestingly, the effects of LPS, cytokines, and phorbol esters may occur via generation of ROS intermediates. In transformed cells, multiple pathways leading to activation of redox-sensitive transcription factors, such as NF-κB, SP-1, Ets, and AP-1, have been shown to increase expression of MMP-9. These additional considerations and the present results support the hypothesis that MMP-9 gene expression could be redox-regulated. As they become available, further experiments using species-specific molecular probes will be performed to confirm our observations at the messenger RNA level.

Beneficial cardiovascular effects of various antioxidant therapies were reported in human and experimental atherosclerosis or restenosis, and efforts to understand its mechanism of action are under way. Treatments with probucol and vitamins E and C have been shown to reduce intimal lesions after balloon injury in hypercholesterolemic animals. In addition to decreasing the lipid content and lesion area, antioxidants also appear to alter lesion cellularity, reducing primarily the monocyte-macrophage content. In addition to potentially reducing the extracellular effects of ROSs (eg, oxidative modification of lipoproteins and activation of MMPs), scavenging ROSs may prevent or diminish intracellular activation of redox-sensitive genes. Recently published results showed that after dietary supplementation with α-tocopherol, peripheral blood monocytes harvested from healthy volunteers produced fewer ROSs and interleukin-1 and displayed decreased adherence to endothelium when stimulated with LPS. Also, NAC was found to inhibit vascular cell adhesion molecule-1 expression in vitro and in vivo. Abatement of these effects of oxidative stress in incipient stages of atherosclerosis probably diminishes stimuli leading to monocyte recruitment and formation of macrophage-derived FCs. NAC was also found to inhibit the chemotactic and invasive activities of human tumor cells, most likely through inhibition of gelatinase activity, and it is currently considered to be a promising antioxidant and anticancer chemopreventive agent. Our study shows a new potential use for NAC as an inhibitor of MMP activity. Inhibition of MMP activity by antioxidants early in the course of atherosclerotic lesion development may limit inflammatory cell infiltration, cell movement, and proliferation, events that all require participation of active MMPs. Importantly, by showing the possibility of inhibiting the matrix-degrading capacity of macrophage FCs prevailing in advanced plaques, the present results suggest that treatment with ROS scavengers may be effective in late stages of atherosclerosis. This would contribute to restricting the weakening of vascular matrix, thought to be a major factor precipitating plaque destabilization. Although we extensively examined and report here the effects of NAC, we believe that the results are applicable to other antioxidants, such as vitamin E and probucol, as suggested by our preliminary experiments. Future in vivo experiments need to
be undertaken to confirm the possibility of using antioxidant therapy as a way to improve the stability of atherosclerotic plaques.

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