Expression of Tissue Inhibitor of Metalloproteinases-3 in Human Atheroma and Regulation in Lesion-Associated Cells

A Potential Protective Mechanism in Plaque Stability

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Abstract—Atherosclerotic plaque stability depends on the structural integrity of its extracellular matrix skeleton. The balance between degradation by matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) may regulate plaque stability. Although MMP expression in atheroma is well documented, localization and control of expression of TIMPs in these lesions is incomplete. Extracts of atheroma (n=14) had 5-fold higher levels of TIMP-3 than nonatherosclerotic tissue (n=10). Plaques (n=24) contained abundant TIMP-1, -2, and -3 in macrophages in plaque shoulders, intimal-medial borders, and areas overlying the lipid core, as well as in medial smooth muscle cells, albeit in lesser amounts. These observations suggested that macrophages, a cell type not heretofore known to express TIMP-3, did so in atheroma in vivo. Further studies in vitro established the human macrophage as a novel source of TIMP-3 mRNA and protein. Human smooth muscle cells constitutively expressed TIMP-1, -2 and -3 proteins; platelet-derived growth factor and transforming growth factor-β augmented levels of TIMP-1 and TIMP-3 but not TIMP-2. These findings suggest that regulated expression of TIMP-3, in addition to the presence of TIMP-1 and TIMP-2, counteracts MMP activity in atheroma and hence influences plaque stability. (Circ Res. 1998;83:270-278.)

Key Words: tissue inhibitor of metalloproteinases ■ atherosclerosis ■ macrophage ■ smooth muscle cell

Rupture of atherosclerotic plaques frequently causes myocardial infarction. The stability of an atherosclerotic plaque largely depends on the structural integrity of its fibrous cap, which is composed mainly of ECM components rich in collagen. The deposition of ECM proteins by intimal SMCs contributes to the tensile strength of the atherosclerotic plaque, rendering it resistant to rupture. Thus, dissolution of the ECM by MMPs provides a likely contributing mechanism of plaque rupture. In support of this hypothesis, we and others have demonstrated increased expression of MMPs such as collagenases, stromelysins, and gelatinases in atherosclerotic tissue versus normal tissue. Together, these MMPs can degrade all the ECM components in the plaque.

Endogenous inhibitors known as TIMPs hold MMP activities in check under usual circumstances. The TIMP family consists of TIMP-1, TIMP-2, TIMP-3, and a more recently described TIMP-4. TIMP-1 and TIMP-2 are the better-characterized members of the group and are secreted in a soluble form by cultured cells. TIMP-3, however, once secreted, binds to and forms an insoluble complex with constituents of the ECM. TIMP-1, -2, and -3 all have similar inhibitory activities against MMPs. TIMPs exhibit sequence homology and share domains of identical protein structures consisting of a highly conserved N-terminal region, considered critical for inhibition of the enzymes, and a more diverse C-terminal domain of unknown function. In addition to binding active MMPs, TIMP-1 and TIMP-2 also bind the zymogen forms of MMP-9 and MMP-2, respectively, a process that may contribute to maintaining stability of the enzymes. The balance between MMPs and TIMPs critically regulates ECM homeostasis. Excessive MMP activity likely contributes to a number of disease processes, including cancer invasion and metastasis, rheumatoid arthritis, and atherosclerosis.

TIMP-1, -2, and -3 can be secreted by SMCs from various species in vitro. Although a number of studies have demonstrated increased expression of MMPs in atherosclerotic tissue, only a few of these studies examined the expression of TIMPs. We have reported the constitutive expression of TIMP-1 and TIMP-2 in both normal and diseased arteries. Additionally, Nikkari and coworkers demonstrated TIMP-1 expression in diseased arteries, although they were only able to detect weak staining in normal arteries. The presence of TIMP-3 in atherosclerotic arteries in vivo, however, remains undetermined. The present study investigated whether human atheroma contain this ECM-associated form of TIMP, in addition to TIMP-1 and TIMP-2. Moreover, we examined whether growth factors and cytokines that are present in atherosclerotic tissue modulate expression of TIMPs in vitro.
Selected Abbreviations and Acronyms

ECM = extracellular matrix  
IL-1α = interleukin 1-α  
MMP = matrix metalloproteinase  
PDGF = platelet-derived growth factor  
PMA = phorbol myristate acetate  
RT-PCR = reverse transcription–polymerase chain reaction  
SMC = smooth muscle cell  
TBS-T = Tris-buffered saline/0.1% Tween  
TGF-β = transforming growth factor-β  
TIMP = tissue inhibitor of metalloproteinases

We present evidence herein of increased TIMP-3 expression in atherosclerotic tissue versus normal arteries. In atheroma, TIMP-3 localized mainly with intimal macrophages in areas previously reported to be rich in MMP activities. The present study also demonstrates that fibrogenic mediators such as PDGF and TGF-β, which are present in atherosclerotic tissue, can increase expression of TIMP-1 and TIMP-3 but not TIMP-2 in human SMCs in vitro.

Materials and Methods

Materials

Monoclonal anti-human macrophage CD68 antibody was obtained from Dako Corp, polyclonal rabbit anti-human TIMP-3 antibody was purchased from Chemicon International Inc, and polyclonal rabbit anti-human TIMP-1 and TIMP-2 antibodies were obtained from Triple Point Biologics. Human recombinant PDGF-BB and TGF-β were purchased from Genzyme. Human recombinant IL-1α was a generous gift from Hoffmann La Roche (Nutley, NJ). PMA was obtained from Sigma Chemical Co.

Cell Culture

SMCs were prepared from medial explants of human saphenous vein. Cultures were maintained in Dulbecco’s Modified Eagles Medium (DMEM, Gibco) supplemented with 10% FCS in a humidified chamber of 5% CO2/95% air. Human aortic SMCs were isolated enzymatically and cultured as described previously. For stimulation experiments, cells were switched to serum-free medium consisting of DMEM/F12 (1:1) supplemented with 1 mmol/L insulin and 5 mg/mL transferrin serum-free medium.22 After 72 hours, SMCs were treated with the agent under investigation. Monocytes were isolated from freshly prepared human peripheral blood mononuclear cells obtained from plateleterapheresis residues of healthy volunteers (kindly provided by Steve K. Clinton, Dana-Faber Cancer Institute and obtained from plateletpheresis residues of healthy volunteers (kindly provided by Steve K. Clinton, Dana-Faber Cancer Institute) by Ficoll density gradient centrifugation as previously described. After adherence on the monolayer, nonadherent cells were removed. The remaining matrix was rinsed twice in PBS, followed by several washes in distilled water, air dried, then solubilized in 1% nonreducing Laemmli sample buffer and stored at −20°C before further analysis.

Preparation of ECMs and Human Arterial Extracts

Cells were washed twice in PBS and then lysed in 0.5% Triton X-100 in phosphate buffer (pH 7.4) at room temperature for 10 minutes, followed by a 10-minute incubation in 100 mmol/L NH4OH to remove the nuclei and cytoskeleton. The remaining matrix was rinsed twice in PBS, followed by several washes in distilled water, air dried, then solubilized in 1X nonreducing Laemmli sample buffer and stored at −20°C before further analysis.

Specimens of human carotid atheroma and aorta were obtained by protocols approved by the Human Investigation Review Committee at Brigham and Women’s Hospital. The source of nonatherosclerotic tissue was either the thoracic aorta of transplant donors or carotid arteries of healthy individuals at autopsy. Diseased tissue was from carotid endarterectomy specimens obtained at surgery. All tissues were stored at −70°C. Specimens were ground under liquid nitrogen with a mortar and pestle, and the resulting fine tissue powder was solubilized in buffer containing 20 mmol/L NaCl, 100 mmol/L Tris-HCl, pH 7.6, and 10% SDS. Insoluble material was precipitated by centrifugation, and soluble extracts were collected and stored at −20°C before further analysis.

Gel Electrophoresis

Protein concentrations of arterial extracts were determined with the bicinchoninic acid kit. Equivalent amounts of protein (35 μg) were heat denatured in the presence of Laemmli sample buffer containing 5% to 10% 2-mercaptoethanol and electrophoresed on 12% to 15% SDS-PAGE gels. Proteins were then transferred onto polyvinylidene difluoride membranes (Millipore) in the presence of Towbin transfer buffer (25 mmol/L Tris, 192 mmol/L glycine, 20% methanol, pH 8.3) by use of a semi-dry electroblotting apparatus according to the manufacturer’s recommendations (Bio-Rad). Membranes were blocked in TBS-T containing 5% (wt/vol) nonfat dry milk for 1 hour, then incubated in fresh buffer containing primary antibody at the desired concentration. Membranes were washed in several changes of TBS-T followed by incubation with TBS-T/5% dry milk containing a 1:15 000 dilution of horseradish peroxidase–conjugated donkey anti-rabbit IgG as secondary antibody (Jackson Laboratories). Immunoreactive proteins were visualized with the enhanced chemiluminescence detection system (DuPont NEN) and exposure to x-ray film.

Reversed zymography was used to detect TIMP-3 activity in arterial extracts from atherosclerotic tissue as previously described.

N-Glycosidase F Treatment

Human macrophage–conditioned media and extracts were dialyzed overnight against buffer containing 20 mmol/L NaPO4, 0.1% SDS, and 10 mmol/L EDTA. Duplicate aliquots of sample were denatured in the presence of 1% 2-mercaptoethanol at 90°C for 5 minutes, followed by the addition of n-octylglucoside to a final concentration of 0.5%. Samples were subsequently incubated at 37°C overnight either in the presence or absence of N-glycosidase F (Boehringer Mannheim). The inhibitors were analyzed by PAGE and Western blotting with the specified antibody.

Immunohistochemistry

For immunostaining, cryostat sections (6 μm) were cut, air dried, and fixed in acetone at −20°C for 5 minutes. Sections were preincubated for 20 minutes with 0.3% hydrogen peroxide in Dulbecco’s PBS to reduce endogenous peroxidase activity and with 2.5% of normal serum in PBS for 20 minutes to avoid nonspecific binding of antibodies. Sections were incubated with primary antibodies, diluted in PBS with 2.5% appropriate normal serum at room temperature for 90 minutes. Species-appropriate biotinylated secondary antibodies (Vector Laboratories) were applied for 45 minutes at room temperature. After 30 minutes’ incubation with avidin-biotin peroxidase complex (Vectastain ABC kit, Vector Laboratories), staining was visualized with 3-amino-9-ethyl-carbazole (Sigma Chemical). Sections were counterstained with Gill’s hematoxylin (Sigma Diagnostics). Staining with type- and class-matched irrelevant immunoglobulin served as a negative control.

RT-PCR and Northern Blotting

Total cellular RNA was prepared from 100-mm dishes of human monocytes/macrophages and endarterectomy tissue specimens with Qiagen Rneasy RNA extraction columns and RNAzol B (Tel-test, Inc), respectively. RNA was quantified by absorbance at 260 nm, and 1 μg was reverse transcribed with random hexamers and Superscript II reverse transcriptase according to the manufacturer’s...
recommendations (Gibco BRL). Identical reactions were also set up in the absence of reverse transcriptase to eliminate the possibility of genomic contamination. Subsequent PCR reactions were performed with specific primers for TIMP-3 as previously described and β-actin primers as an internal control (Clontech). Aliquots of the PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining and UV transillumination.

For Northern analysis, 15 μg of total RNA was fractionated on 1% agarose/2.2 mol/L formaldehyde gels, then transferred onto nylon membranes (DuPont NEN) by capillary blotting with 10× SSC (1.5 mol/L sodium chloride and 300 mmol/L sodium citrate). The RNA was bound by cross-linking the membrane with UV irradiation. Prehybridization and subsequent overnight hybridization of the blots were performed at 42°C in buffer containing 40% formamide, 10% dextran sulfate, 4× SSC, 7 mmol/L Tris, pH 7.6, and 0.2 mg/mL salmon-sperm DNA. The probe used for hybridization was a 488-bp fragment of the TIMP-3 cDNA radiolabeled with [α-32P]dATP by random hexamer priming and Klenow enzyme (Boehringer Mannheim). Posthybridization of blots was performed in 2× SSC, 0.1% SDS at 50°C, followed by higher-stringency washes in 1× SSC, 0.1% SDS at 65°C. Autoradiography of membranes was performed with intensifying screens at −70°C for 24 to 72 hours.

Figure 1. Western blot analysis of TIMP-3 expression in extracts of nonatherosclerotic arteries and advanced human atheroma. A, 35 μg of protein extracts from both nonatherosclerotic vessels and advanced human atheroma were subjected to Western blot analysis. B, Densitometric scanning of Western blots with NIH image software analysis. Data show 6 of 10 normal and 9 of 14 atherosclerotic arteries evaluated.

Results

Human Atheroma Contain Increased Amounts of TIMP-3

TIMP-3 expression was detected at various levels in extracts of all endarterectomy specimens examined, as demonstrated by Western blot analysis (Figure 1A). Although most samples (12 of 14) revealed moderate to strong expression, 2 samples (lanes 12 and 13) contained little or no TIMP-3 protein. By contrast, extracts of normal arteries exhibited a much weaker signal for TIMP-3 (Figure 1B). Densitometric scanning revealed an average 5-fold increase of TIMP-3 in endarterectomy specimens over nonatheromatous arteries (Figure 1B). In both normal and nonatherosclerotic tissue, 2 bands for TIMP-3 were observed on Western blots: a major band at 24 kDa and a minor band at ≈27 kDa, which is within the predicted molecular weight of the glycosylated species. Treatment with N-glycosidase-F demonstrated that neither the 24-kDa nor the 27-kDa band converted to a lower-molecular-weight species (Figure 2A). This observation sug-
gests that the 27-kDa band does not correspond to glycosylated TIMP-3 but indicates that the major band at 24 kDa is the unglycosylated form of TIMP-3. The minor 27-kDa band is also unlikely to represent unsecreted protein in tissue extracts, because the signal peptide for TIMP-3 has a predicted molecular weight of only 1 kDa. Human macrophage TIMP-1 positive control sample, which is a 28-kDa protein in its native form, yielded the expected 24-kDa deglycosylated product on treatment with N-glycosidase F (Figure 2B).

RT-PCR analysis was used to evaluate the presence of TIMP-3 mRNA in the extracted material (Figure 3A). An expected band at 488 bp was revealed in RNA from samples of advanced human atheroma. The β-actin internal control amplified the expected 800-bp product in all the samples tested. In addition, the control reactions (lacking reverse transcriptase) yielded the expected negative results, demonstrating no genomic contamination (not shown).

Reverse zymography of protein extracts from plaques in gelatin polyacrylamide gels revealed a 24-kDa metalloproteinase-inhibitory activity, demonstrating that TIMP-3 in atheroma is biologically active in blocking MMP (Figure 3B). A higher-molecular-weight band at 28 to 30 kDa and a weak band at 22 kDa were also observed, corresponding to TIMP-1 and TIMP-2, respectively (not shown).

Localization of TIMP-3 Within Nonatherosclerotic and Atherosclerotic Specimens

Immunohistochemistry was performed to investigate further the location of TIMP-3 expression in atherosclerotic specimens. All plaques studied were advanced fibrolipid plaques that contained a well-developed fibrous cap, clusters of macrophages in the shoulder regions, and a large lipid core. All atherosclerotic specimens contained TIMP-3. Strong staining was found in macrophages in plaque shoulders, intimal-medial borders at the base of the plaque, and areas overlying the necrotic core (Figure 4A, 4C, and 4E). Macrophages were identified by use of an antibody against the macrophage specific antigen CD68 (Figure 4B, 4D, and 4E). In addition, positive staining was observed in the necrotic core (Figure 4A). TIMP-3 was also found in SMCs within the media and the fibrous cap, albeit in lesser amounts than macrophages. Staining of nonatherosclerotic arteries (3 carotid arteries and 8 thoracic aortas) revealed little to moderate staining of TIMP-3 in smooth muscle cells in the media (Figure 4G and 4H). Nonimmune IgG negative controls yielded no immunoreactivity in either normal or atherosclerotic tissues (data not shown).

Uniform staining of TIMP-1 and TIMP-2 was found in the media and intima of normal carotid arteries, consistent with our previous findings (Figure 5A and 5B). The distribution pattern of TIMP-1 and TIMP-2 in atherosclerotic tissue resembled that of TIMP-3; staining was localized to plaque shoulders and macrophages overlying the necrotic core (Figure 5, C, D, and E). In addition, staining for TIMP-1 was also found in the necrotic core (Figure 5C), in agreement with the data of Nikkari and coworkers. Nonimmune IgG negative controls yielded no staining in either normal or atherosclerotic tissues (Figure 5F).
Figure 4. Immunolocalization of TIMP-3 in human atherosclerotic and nonatherosclerotic carotid arteries. All specimens examined contained features typical of advanced fibrolipid plaques, with a well-developed fibrous cap, clusters of macrophages in the shoulder regions, and a large lipid core. A, TIMP-3 immunostaining in the intima and media of 1 representative atherosclerotic plaque. B, Staining of serial section of A for macrophage surface marker CD68 showing prominent staining overlying the necrotic core in same areas as TIMP-3. C and D, Higher-power view of areas enclosed in boxes in A and B, demonstrating immunoreactive TIMP-3 and CD68, respectively. E, Shoulder of atheroma, demonstrating abundant staining of TIMP-3 in macrophage-rich areas and some medial staining of TIMP-3. F, TIMP-3 in macrophage-rich area of the lesion showing mainly intracellular staining. G, Immunolocalization of TIMP-3 in nonatherosclerotic human carotid arteries, revealing uniform staining in the media and intima. H, Higher-power view of G. Scale bar represents 500 μm in A, B, and E; 200 μm in C, D, and G; and 50 μm in F and H. Data are representative of 24 atherosclerotic arteries and 11 normal arteries (3 carotid and 8 thoracic aortas).
Human Macrophages but Not Monocytes Produce TIMP-3 In Vitro

TIMP-3 colocalized mainly with macrophages in the atherosclerotic plaque. Because no information exists on TIMP-3 expression by macrophages, we investigated whether isolated human monocyte-derived macrophages could produce this inhibitor. After 7 to 10 days in culture, Western blot analysis of macrophage ECM extracts revealed a major band at 24 kDa with TIMP-3 antibody (Figure 6A). RT-PCR analysis of macrophages showed TIMP-3 product that comigrated with that produced by PDGF-stimulated SMCs (Figure 6B). Northern analysis revealed that the TIMP-3 probe hybridized to the expected transcripts of \( \sim 5 \), 2.8, and 1.1 kb, further demonstrating the expression of TIMP-3 by macrophages (Figure 6C). Interestingly, freshly isolated monocytes did not express any TIMP-3 RNA (Figure 6B). As expected, macro-
phage-conditioned media contained no TIMP-3, and little TIMP-3 was found in cell lysates. The β-actin internal control amplified the expected 800-bp product in all samples tested. In addition, control reactions (minus reverse transcriptase) yielded the expected negative results, demonstrating no genomic contamination. We are unaware of any previous description of expression of TIMP-3 by human macrophages. Large amounts of TIMP-1 and TIMP-2 were found in media conditioned by macrophages, consistent with previous reports (data not shown).

Cytokines and Growth Factors Regulate the Synthesis of TIMP-1 and TIMP-3 but Not TIMP-2 in Human SMCs

Expression of TIMP-1 and TIMP-3 undergoes regulation in a number of cell types, whereas most cells express TIMP-2 constitutively with little variation. Growth factors such as PDGF and TGF-β can induce TIMP-3 in rabbit SMCs. To investigate further the in vitro regulation of TIMP-1, -2, and -3 in human SMCs, cells were exposed to various cytokines and growth factors, either alone or in combination. Western blot analysis revealed that human aortic SMCs express all 3 TIMP proteins constitutively (Figure 7). However, PDGF and the phorbol ester PMA increased expression of TIMP-1 (Figure 7A), whereas PDGF and TGF-β further increased TIMP-3 levels (Figure 7C). TIMP-2 levels were unaffected by the addition of exogenous stimuli (Figure 7B). An identical pattern of regulation was observed in human saphenous vein SMCs (data not shown).

To ascertain whether the increase in TIMP-1 and TIMP-3 by growth factors resulted from an increase in cell number, SMCs were dispersed by trypsin treatment from triplicate wells of a 6-well plate and counted with a hemocytometer (data not shown). The treatments did not change cell number. In addition, secretion of the constitutive protein TIMP-2 did not change, a further indication that a change in overall protein synthesis accounts for the effect of PDGF and TGF-β on TIMP-3 production (Figure 7B).

Discussion

Our studies demonstrate augmented TIMP-3 expression in atherosclerotic tissue compared with uninvolved arteries.
The present study localized TIMP-1, -2, and -3 in all these areas, particularly in macrophages in plaque shoulders and between the fibrous cap and necrotic core. The close proximity of enzyme and inhibitor in the plaque agrees with the hypothesis that TIMPs serve as an important counterregulatory mechanism limiting excessive MMP activity in vivo.

We also demonstrated that human monocyte-derived macrophages in vitro express TIMP-3 RNA and protein, establishing the macrophage as a novel source of TIMP-3. Moreover, the differentiation of monocytes into macrophages in culture involves induction of TIMP-3, as previously reported for MMPs. The present study further demonstrates that intimal and medial SMCs in normal and atherosclerotic tissue in vivo contain TIMP-3. Moreover, mediators such as PDGF and TGF-β present in atherosclerotic tissue increased TIMP-3 expression in both human saphenous vein and arterial SMCs. Taken together, our in vitro findings suggest possible mechanisms for induction of TIMP-3 in macrophages and SMCs in vivo.

Additionally, we found constitutive expression of TIMP-1 and TIMP-2 in SMCs, which was unaffected by cytokines such as IL-1 and tumor necrosis factor (data for tumor necrosis factor not shown), consistent with previous reports. However, the present study found that PDGF increased TIMP-1 expression in SMCs. Together with the findings for TIMP-3, this result demonstrates that a growth factor but not the inflammatory cytokine tested can modulate production of TIMPS in SMCs. Furthermore, we demonstrate that regulation of TIMP expression is identical in human saphenous vein SMCs and human arterial SMCs in culture.

Elevated levels of MMPs may regulate SMC migration after balloon injury of the rat and pig carotid artery. More recently, Forough and coworkers demonstrated a reduction in intimal thickening after local retrovirus-mediated gene transfer of rat SMCs overexpressing TIMP-1 into balloon-injured rat carotid arteries. In addition, arterial injury in rats increases expression of TIMP-2, as well as plasminogen activator inhibitor type 1. These various studies suggest an important counterregulatory role for TIMP-1 and TIMP-2 in the arterial response to injury.

As mentioned above, unlike TIMP-1 and TIMP-2, secreted TIMP-3 is unique in that it is bound to components of the ECM. The present study confirmed this finding in SMCs and macrophages in vitro. Although it is well known that SMCs synthesize abundant ECM proteins, little is known about the ability of macrophages to produce an ECM. A recent report demonstrated that macrophages can produce an ECM layer consisting mainly of chondroitin sulfate and also heparan sulfate and dermatan sulfate. These glycosaminoglycans were shown to be involved in the binding of oxidized LDL to the ECM. It is possible that these glycosaminoglycans can also account for the retention of TIMP-3 in macrophage ECM. Using high-power light microscopy to visualize immunoreactive areas of TIMP-3, we could not determine with certainty whether TIMP-3 was present extracellularly in vivo. However, it is very likely that TIMP-3, once secreted by plaque cells, is sequestered within the collagenous matrix of the fibrous cap, where it can serve as a long-term inhibitory pool, resisting local proteolysis. In this manner, the matrix can serve as an active protector in lesions of
fibrous character. It is unknown which of the ECM components bind TIMP-3. Advanced atherosclerotic lesions contain abundant collagen, proteoglycans, and elastin. Heparan sulfate proteoglycans can bind molecules such as fibroblast growth factor, lipoprotein lipase, and antithrombin III through specific interactions with its oligosaccharide side chains.31 Vimentin binds another proteinase inhibitor, type 1 plasminogen activator inhibitor.28 A similar mechanism may be used for TIMP-3 binding to the matrix.

The present results suggest that increased TIMP-3, in addition to the presence of TIMP-1 and TIMP-2 in rupture-prone sites of the atherosclerotic plaque, may serve as an important protective mechanism against plaque rupture. In addition, augmentation of TIMP-1 and TIMP-3 by PDGF and TGF-β present in atherosclerotic plaques is likely to favor decreased local proteolysis. Thus, the local balance between mediators that augment MMP expression (eg, proinflammatory cytokines) and those that also increase TIMP expression (eg, the fibrogenic mediators TGF-β and PDGF) may help determine the “vulnerability” of a given atheroma. This model aids the understanding of the considerable variability in the lesions of atherosclerosis, even within a single coronary arterial tree.

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