Elastogenesis in Human Arterial Disease
A Role for Macrophages in Disordered Elastin Synthesis

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Objective—Elastin, an extracellular matrix protein, constitutes about 30% of the dry weight of the arteries. Elastolysis induced by inflammatory processes is active in chronic arterial diseases. However, elastogenesis in arterial diseases has received little attention. In this work we hypothesized that disordered elastogenesis is active in matrix remodeling in atheroma and abdominal aortic aneurysm (AAA).

Methods and Results—Human AAA and atheroma have 4- to 6-fold more tropoelastin protein than nondiseased arteries. The smooth muscle cell–containing media and fibrous cap of atherosclerotic arteries contain ordered mature elastin, whereas macrophage (MΦ)-rich regions often have disorganized elastic fibers. Surprisingly, in addition to smooth muscle cells, MΦs in diseased arteries also produce the elastin precursor tropoelastin, as shown by double immunostaining, in situ hybridization, and reverse transcription–polymerase chain reaction for tropoelastin mRNA. Cultured monocyte-derived MΦs can express the elastin gene. AAA have 9-fold but atheroma only 1.6-fold lower levels of desmosine, a marker for mature cross-linked elastin, than normal arteries.

Conclusions—This study demonstrates ongoing but often ineffective elastogenesis in arterial disease and establishes human macrophages as a novel source for this important matrix protein. These results have considerable import for understanding mechanisms of extracellular matrix remodeling in arterial diseases. (Arterioscler Thromb Vasc Biol. 2003;23:582-587.)

Key Words: atherosclerosis ■ AAA ■ elastin ■ macrophages

The extracellular matrix (ECM) protein elastin constitutes about 30% of the dry weight of the arteries. Elastolysis induced by inflammatory processes is active in chronic arterial diseases, notably atherosclerosis1-2 and abdominal aortic aneurysm (AAA).3-5 However, elastogenesis (ie, the de novo synthesis of elastin) in arterial diseases has received much less attention.6

Elastin production begins with the synthesis and secretion of the soluble precursor tropoelastin. Different isoforms (67.5 kDa, 65 kDa, and 62 kDa) arise from translation of alternatively spliced mRNA transcripts.7,8 Microfibrillar components create a “scaffold” onto which the tropoelastin is attached. Subsequent cross-linking by lysyl oxidase yields insoluble amorphous elastin–laminin receptor on monocytes in atherosclerotic plaques17 may mediate cell death by apoptosis or by oncosis.18

Impaired elastogenesis, or disruption of the elastin gene, contributes to human disorders, such as supravalvular aortic stenosis,19 cutis laxa,20 Hurler disease,21 and Costello syndrome.22 Targeted inactivation of the elastin gene has established an essential role for elastin in arterial morphogenesis.23,24 Homozygous elastin-deficient mice die of obstructive arterial disease after birth,23 whereas the arteries of mice with one allele for elastin show increased elastic lamellae.23

During arterial development, the number of elastic lamellae increases.24 Alterations in elastin content also accompany atherosclerotic plaque development,25 flow-induced dilatation,26 and nonatherosclerotic intimal thickening.27 Several pathological stimuli may evoke elastogenesis,28-30 for example, reduplication of elastic lamellae in healed arteritis and some atheroma.

We therefore hypothesized that dysregulated synthesis of elastin accompanies atherogenesis and aneurysm formation, processes that were heretofore associated with elastin breakdown. This study investigated the expression of elastin in...
arterial diseases and of tropoelastin in human macrophages (MΦ) in vitro. Our results showed augmented elastogenesis during both atheroma and AAA formation but favoring accumulation of immature rather than fully processed and cross-linked elastin. AAA particularly showed this defect in elastin maturation. We also identified macrophages as a novel source for elastin. These results have considerable import for understanding mechanisms of ECM remodeling in arterial diseases.

Materials and Methods

Cell Culture
Monocytes isolated from leukopacks of healthy donors (n=4) by density gradient centrifugation (Cappel LSM Lymphocyte Separation Media, ICN Biomedical Inc., Aurora, Ohio) were cultured up to 14 days in RPMI-1640 (Gibco BRL, Gaithersburg, Md) containing 100 U/mL penicillin/streptomycin, 2 mmol/L L-glutamine, 1.26 mg/mL amphotericin B, 20 mmol/L sodium pyruvate, 0.1% NaHCO3, supplemented with 2% human serum and 10% fetal bovine serum. All media contained less than 40 pg endotoxin/mL as determined by the chromogenic Limulus Amebocyte Assay (Cambrex Biosciences, Walkersville, Md).

Tissue Samples
We obtained fresh surgical specimens of human carotid atheroma, nonatherosclerotic aorta (cardiac transplantation donors), as well as discarded aneurysmal tissue from AAA repair surgery according to protocols approved by the Human Investigation Review Committee at the Brigham and Women’s Hospital. Atherosclerotic plaques were dichotomized into fibrous (n=4) and atheromatous (n=5) subsets by morphological criteria as described previously.31 Specimens from nonatherosclerotic and AAA tissue contained the entire artery wall, whereas endarterectomy specimens (atherosclerotic plaques) only contained the intima and part of the media.

Immunohistochemistry
Serial cryostat sections (6 μm) of tissue from nonatherosclerotic arteries (n=4) fibrous (n=4), and atheromatous (n=5) plaques, as well as AAA (n=5), were fixed in acetone (−20°C for 5 minutes) and blocked with 0.3% hydrogen peroxide for 20 minutes at room temperature followed by 5% species-appropriate normal serum (Vector Laboratories, Burlingame, Calif). Subsequently, sections were incubated for 30 minutes with primary antibodies GA317 1:20 (Elastin Products, Owensville, Mo) and/or monoclonal mouse anti-elastin M5 1:10 (DAKO, Carpinteria, Calif). Competition experiments with or without insoluble bovine elastin and chicken tropoelastin verified the specificity of both antibodies (data not shown). Mouse IgG1 (M-9269; Sigma) and rabbit IgG1 (SC-2027; Santa Cruz Biotechnology Inc., Santa Cruz, Calif) at the same concentration as the primary antibodies served as negative controls. The reaction was visualized with 3-amin-9-ethyl carbazole. For double immunostaining, tissue sections were incubated with primary antihuman tropoelastin (GA)137 1:400 was applied for 90 minutes followed by biotin blocking kit (Vector Laboratories) was applied, followed by overnight incubation at +4°C with antihuman macrophage (CD68) 1:500. Finally, biotinylated horse anti-mouse secondary antibody was applied, followed by incubation with streptavidin-FITC (Amer sham Phamacia Biotech).

In Situ Hybridization for Tropoelastin mRNA
To colocalize tropoelastin mRNA with MΦ in tissues, we studied MΦ-rich shoulder regions of plaques (n=4). Sections were fixed with ice-cold acetone and analyzed with the Hyb-Probe Detection System according to the manufacturer’s instructions (Shandon/Lipshaw, Pittsburgh, Pa). Briefly, synthetic 40-mer 5′-fluorescein-labeled oligonucleotide probes were commercially synthesized (IDT, Coralville, Ind). The specificity was verified with the BLAST program (BLASTN. 2.2.2.).32

Antisense probes:

Elastin1481 CONTR (pos. 1481 to 1520) 5′-GGTTTAGTCTCTGGTGTTGCTGAT-3′;
Elastin1512 CONTR (pos. 1512 to 1551) 5′-GAGTTGGCCTGGCCCTCGTGAGTG-3′;
Elastin1584 CONTR (pos. 1584 to 1623) 5′-GAGTTGGTGGTGGCTCCTGCTGGAGT-3′;

Sense probes:

Elastin1481 5′-CGCAGAATCCAGGAGGAGGCGGAGGAGGACGAG-3′;
Elastin1512 5′-ACTCCAGGGACCCACACCAACCCAGGAGGACACGCCAATC-3′;
Elastin1584 5′-AGCGGCGGACCGACCAACCCGAGGCGCCGACGAG-3′.

In Situ Hybridization for Tropoelastin mRNA was applied to tissue sections. We studied MΦ, the tropoelastin mRNA signal was detected using alkaline phosphatase and NAMP/Fast Red substrate (Sigma) without counterstain. Negative controls were preincubated with 2 μg/μL RNase (Sigma) for 1 hour at 37°C before hybridization with antisense probes, or samples incubated either without probe or with sense probe.

Western Blot
Tissue extracts from nonatherosclerotic (n=8), fibrous (n=7), and atheromatous (n=9) plaques and from AAA (n=7) were prepared as described previously.33 Supernatants were removed from cultured MΦ and supplemented with the following proteinase inhibitors: 0.1 mmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride Hydrochloro-ride, 2 μg/mL aprotinin, 2 μg/mL leupeptin, and 1 μg/mL pepstatin A, Fifty micrograms of total protein per sample was analyzed under reducing conditions on 8% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes as described.34 The primary antibody was antihuman elastin (E4013) 1:500, and the secondary antibody was peroxidase-conjugated goat antimouse IgG (Jackson Immunoresearch Laboratories, West Grove, Pa) 1:10,000. Immuno-reactive bands were analyzed densitometrically using Gel Pro Analyzer Software (Media Cybernetics Inc., Des Moines, Ia). All results were normalized to total protein.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)
Total RNA was isolated from cultured MΦ with the Rneasy® Mini Kit (Qiagen, Valencia, Calif). Primers:
Elastin1a (positions 409 to 427) 5'-CTTACCGTGTCGTGGAT
Elastin1b (positions 704 to 727) 5'-CAGTGCCTGGGTGGTAGCG

Reverse transcriptase protocol: 10 minutes at 20°C, 15 minutes at 42°C, and 5 minutes at 99°C. Amplification protocol: 95°C at 2 minutes and then 36 cycles at 95°C for 1 minute, 64°C for 1 minute, and 72°C for 1 minute. PCR products (elastin 319 bp) were run on 1% agarose gels, stained with ethidium bromide, and analyzed as above.

Desmosine Analysis
MΦ from two donors was differentiated for 7 and 14 days and smooth muscle cells (SMCs; as positive control, n=2) were cultured for 7 days postconfluency (day 14 in culture). Cells were washed two times in PBS, and then cells and matrix were removed by scraping into 1 mL of distilled water. Samples were centrifuged for 5 minutes at 300g and pellets resuspended in 6 N HCl and hydrolyzed overnight at 110°C. Desmosine levels were determined by radioimmunoassay and normalized to total protein.

Statistics
Results were analyzed with the nonparametric Mann-Whitney test. Probability values of ≤0.05 (two-tailed tests) were regarded as statistically significant.

Results
Elevated Tropoelastin Levels in Human Atheroma and AAA
We found soluble tropoelastin protein in tissue extracts from normal artery, fibrous plaque, atheromatous plaque, and AAA by Western blotting using antihuman elastin antibody E4013. The predominant band migrated as the 67.5-kDa isoform (Figure 1A). Compared with normal arteries (n=3), fibrous (n=3) and atheromatous (n=3) plaques and from AAA (n=3) were dissolved in 6 N HCl and hydrolyzed overnight at 110°C. Desmosine levels were determined by radioimmunoassay and normalized to total protein.

Increased Tropoelastin Levels in Human Atheroma and AAA In Situ
Normal arteries had well-ordered elastin (Figure 2A, top). However, atheromatous tissue showed abundant but frequently disordered elastin (Figure 2A, middle left), but contained immunopositive tropoelastin (Figure 2A, middle right) both in SMC- (Figure 2A, bottom left) and MΦ-rich (Figure 2A, bottom right) areas. Verhoeff-van Gieson staining of AAA tissue showed occasional fragmented elastin fibers but no organized elastic laminae (see online Figure I, which can be accessed at http://atvb.ahajournals.org). These tissues contained scattered MΦ but almost no SMCs. Abundant tropoelastin, which was visualized with antitropoelastin GA317, localized mainly in MΦ-rich areas, whereas antielastin E4013 showed little staining. This finding suggests that tropoelastin and not mature elastin generates the signal observed in AAA tissue. Some acellular regions contained sparse elastin that may, given the paucity of cells, represent elastin formed earlier in the disease (data not shown). Lack of staining by mouse or rabbit IgG1 (data not shown) verified the specificity of the elastin/tropoelastin signal.

Macrophages: A Novel Source of Tropoelastin
Tropoelastin protein colocalized with MΦ by single and double immunostaining of serial sections from human atheroma (Figure 2B). CD68 (blue color, Figure 2B, top right) and GA317 (red color, Figure 2B, bottom right) gave a mixed purple color when stained for both antigens (Figure 2B, left). Normal tissue had low levels of tropoelastin mRNA (Figure 3, top left) whereas the MΦ-rich shoulders of atheromatous plaques displayed abundant tropoelastin mRNA (Figure 3, right panels). Sense probes yielded no specific signal (Figure 3, bottom left). Colocalization of the MΦ marker CD68 (red-brown signal) with tropoelastin mRNA (purple signal) verified that MΦ can indeed transcribe the gene (see online Figure II, which can be accessed at http://atvb.ahajournals.org).

In vitro experiments extended these observations: primary human monocyte-derived MΦ, cultured for 10 days, expressed tropoelastin mRNA as determined by both in situ hybridization and RT-PCR (Figure 4A and 4B), whereas freshly isolated monocytes (1 day in culture) did not (data not shown). Negative controls using sense oligonucleotides (Figure 4A) and RNase-treated samples or no oligonucleotide (see online Figure III, which can be accessed at http://atvb.ahajournals.org) showed no signal.

Double immunofluorescent staining of MΦ cultured for 10 days verified that a subset of MΦ produce tropoelastin protein (Figure 4C). This observation agrees with localization of tropoelastin mRNA in situ (Figure 3). During differentiation in vitro, MΦ release soluble tropoelastin from days 3 through 12 (Figure 4D).
Biochemical Analysis of Desmosine Content Suggests the Presence of Cross-Linked Elastin in Diseased Arterial Tissue and Cell Cultures

When elastin assembles in vivo to form elastic fibers, extensive cross-linking yields a mature insoluble polymer that contains the rare amino acids desmosine and isodesmosine. Measurement of desmosine by radioimmunoassay determined whether MΦ could cross-link tropoelastin in vitro. Human monocyte-derived MΦ contained cross-linked elastin as determined by low but detectable levels of desmosine (6 pmol/mg protein), which were 6-fold less than desmosine levels in SMCs. Extracts from normal and diseased arterial tissue (Figure 5) showed higher desmosine levels in normal tissue (6529±707 pmol/mg protein) than in fibrous (3800±455 pmol/mg protein) or atheromatous plaques (3995±984 pmol/mg protein) or AAA (721±102 pmol/mg protein). Atheroma contained substantial desmosine although less than in normal tissue. Fibrous but not atheromatous plaques had a significant 1- to 7-fold reduction in desmosine (P=0.050). However, AAA tissue contained 9-fold less desmosine than unaffected arteries (P=0.050). This reduction in desmosine content indicates a paucity of mature, functional elastin in AAA, which may favor arterial ectasia.

Discussion

We provide here morphological, biochemical, and molecular evidence for ongoing but ineffective elastogenesis in human atheroma and AAA. Interestingly, these tissues expressed the precursor tropoelastin in MΦ-rich areas. These results may underestimate the actual tropoelastin expression because atherosclerotic plaques, harvested by endarterectomy, only contain part of the elastin-rich medial layer.

Previous morphological studies demonstrated duplicated elastic lamellae25,26 and signs of elastogenesis6,17 in atheroma. Early studies established a role for elastin in atherosclerosis,14,15 However, the cellular and molecular mechanisms of dysfunctional elastogenesis have received little attention.

This study identified the human MΦ as an unexpected source of tropoelastin. Studies of tropoelastin protein and mRNA, both in vitro and in situ, verified this new function of mononuclear phagocytes. Immunohistochemical staining of elastin/tropoelastin protein in MΦ-rich areas could reflect signals from elastin fragments bound to the putative elastin–laminin receptor, but the detection of tropoelastin mRNA in MΦ using several indepen-
Figure 4. Human MΦs express tropoelastin in vitro. A, In situ hybridization: MΦs cultured for 10 days expressed tropoelastin mRNA (pink signal, upper left). Sense oligonucleotides showed specificity of the signal (Figure 4A and Figure III). B, RT-PCR: tropoelastin mRNA expression in MΦs that were cultured for 10 days. Negative control was RT-PCR run without RNA. C, Double immunofluorescent staining: MΦs cultured for 10 days that were stained for tropoelastin (red fluorescence, GA317, top) and MΦ (green fluorescence, CD68, bottom) showed that a subset of MΦs contain the precursor tropoelastin. D, Western blot: tropoelastin protein is secreted by MΦs into the supernatant from 3 to 12 days in culture.

The presence of LDL downregulates lysyl oxidase in vascular endothelial cells and the arterial wall of hypercholesterolemic pigs. 

The present study documents ongoing but often-ineffective elastogenesis in both human atherosclerotic disease and AAA formation. AAA particularly showed this defect in elastin maturation. We also identify MΦ as a novel source for this matrix protein. These results have considerable import for understanding mechanisms of ECM remodeling in arterial diseases.

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Figure 5. Diseased arteries contain desmosine, a marker for mature cross-linked elastin. Desmosine expression is reduced in atheroma and AAA. Normal (nonatherosclerotic), fibrous, and atheromatous plaques and AAA were analyzed for desmosine content. Diseased tissues showed a significant decrease in desmosine content, particularly in AAA. Results are shown as mean±SEM (n=3).

Addition, AAA have sparse cross-linked elastin (revealed by Verhoeff-van Gieson and antibody E4013), suggesting a so-called “maturation arrest” of elastogenesis. This maturation arrest could result from reduced expression or absence of the cross-linking enzyme lysyl oxidase or any of the components of the microfibrillar scaffold that are needed to create the elastic fiber. Recently, Huffman et al showed decreased lysyl oxidase expression in experimental abdominal aortic aneurysm. Inactivation of the lysyl oxidase gene in mice leads to aortic aneurysms and cardiovascular dysfunction. Thus, an increase in tropoelastin synthesis in AAA proves futile as elastin maturation fails and elastolysis predominates.

Surprisingly, both human fibrous and lipid-rich atheroma showed a 1.6-fold reduction in desmosine compared with normal arteries. Atheromatous plaques generally contain fewer matrix-producing cells and less ECM than fibrous plaques. Thus, mechanisms for preserving elastin operate to greater extent in atheroma than in AAA. Very recent observations show enhanced lysyl oxidase mRNA in transgenic atherosclerotic-hypertensive rats. Desmosine levels decline in atherosclerotic aortae of rats. However, the decreased desmosine level in atheroma compared with overall increased levels of tropoelastin suggest that the increased expression of tropoelastin does not correspond directly to the formation of cross-linked elastin. Much of the increased tropoelastin levels in these tissues may derive from MΦ, which appear to produce only partly cross-linked tropoelastin. Proteoglycan accumulation in atheroma may prevent tropoelastin integration into the functional elastic fiber. The presence of LDL downregulates lysyl oxidase in vascular endothelial cells and the arterial wall of hypercholesterolemic pigs.

The present study documents ongoing but often-ineffective elastogenesis in both human atherosclerotic disease and AAA formation. AAA particularly showed this defect in elastin maturation. We also identify MΦ as a novel source for this matrix protein. These results have considerable import for understanding mechanisms of ECM remodeling in arterial diseases.
References


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