Soluble Elastin Decreases in the Progress of Atheroma Formation in Human Aorta

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Background: The serum levels of soluble elastin increase in patients with aortic dissection, but its distribution and characteristics are unclear.

Methods and Results: The 173 aortic specimens were categorized into 4 groups under microscopy (non-atherosclerotic aorta, n=13; fiber-rich plaque, n=77; lipid-rich plaque, n=66; ruptured plaque, n=17). Soluble elastin was abundant within the intima of both the non-atherosclerotic aorta and fiber-rich plaque, rather than in the media, and was decreased within the intima of lipid-rich and ruptured plaques. Soluble elastin levels decreased with progress of atherosclerosis (6.0±1.3μg/mg protein in non-atherosclerotic aorta; 5.8±0.2μg/mg protein in fiber-rich plaque; 4.9±0.2μg/mg protein in lipid-rich plaque; 2.8±0.4μg/mg protein in ruptured plaque, P<0.05). As well, both matrix metalloprotease-9 activity and elastin mRNA expression showed inverse distribution against soluble elastin (r=−0.437, P<0.0001; r=−0.186, P<0.05, respectively). Multivariable analysis revealed a decrease in the level of soluble elastin in ruptured plaque (2.8±0.4μg/mg protein in ruptured plaque, n=18; 5.5±0.2μg/mg protein in non-ruptured plaque, n=155, P<0.01). Furthermore, western blot showed soluble elastin consists of heterogeneous molecular pattern proteins.

Conclusions: Both the synthesis and degradation of elastin may be enhanced in active atherosclerotic lesions.

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atherosclerosis. To this end, we examined (1) the morphological characteristics of soluble elastin, (2) the relationship of the distribution patterns of soluble elastin and elastin mRNA expression, (3) the relationship between the level of soluble elastin and the histological features of atherosclerotic plaque, and (4) the relationship between the soluble elastin level and MMP-9 activity in atherosclerotic plaque.

Methods

Tissue Specimens

We obtained 173 fresh specimens of human aortic tissue from 9 autopsies (8 men, 1 woman; 64.0±3.4 years, range 53–84) within 12 h post mortem. The subjects consisted of 4 cancer cases and 5 non-cancer cases. The aorta was opened longitudinally, and each specimen was cut into 3 sections: the central section was reserved for histology, and the 2 adjacent sections were frozen in liquid nitrogen and stored at −80°C until use in protein assay and reverse transcriptase polymerase chain reaction (RT-PCR) study. The institutional review board approved the research protocol (approval no. 263).

Classification of Plaques

The specimens were diagnosed into 8 groups (types I, II, III, IV, Va, Vb, Vc, and VI) according to the classification issued by the Committee on Vascular Lesions set up by the Council on Atherosclerosis of the American Heart Association (AHA classification). Briefly, type I has an adaptive intimal thickening without accumulated foamy macrophages; type II has accumulated foamy macrophages within the intima without extracellular lipid deposition; type III has accumulated foamy macrophages with small extracellular lipid deposits and necrotic tissue within the intima (lipid constituents); type IV has large amounts of lipid constituents within the intima, and has a superficial, thin fibrous cap; type Va has large amounts of lipid constituents within the intima, but has a superficial, thick fibrous cap; type Vb has large amounts of lipid constituents, but also has mineral deposition within the intima; type Vc has large amounts of vascular smooth muscle cells, collagen, and elastin fibers (fibrocellular constituents) instead of lipid constituents in the intima; type VI exhibits intraplaque hemorrhage, fibrin deposition, fissure of the fibrous cap or thrombus formation, in addition to the accumulation of lipid constituents in the intima.

After diagnosis based on the AHA classification, we further categorized each specimen into 4 groups:19,20 ‘non-atherosclerotic aorta’ (type I), ‘fibris-rich plaque’ (types II, III, and Vc), ‘lipid-rich plaque’ (types IV, Va, and Vb), and ‘ruptured plaque’ (type VI). The intima of the fiber-rich plaque is basically composed of fibrocellular constituents, whereas that of the lipid-rich plaque is composed of lipid constituents.

In addition, each specimen was evaluated for the presence of 7 histological features, previously shown to be associated with plaque instability: plaque necrosis, macrophage infiltration into the fibrous cap, thin fibrous cap (<65 μm in thickness), intraplaque fibrin, intraplaque hemorrhage, plaque rupture, and mineral deposition.1,3,4,20,21 Fibrin deposition or hemorrhage was defined as a red-colored non-structural substance or aggregations of red blood cells outside the intimal microvessels using the Masson trichrome procedure. All evaluations were performed more than 3 times independently by 2 observers who were blinded to the results of the biochemical analyses.

ELISA for Soluble Elastin

The levels of soluble elastin in the protein extracts were measured by sandwich ELISA utilizing the 2 mAbs (HASG-61-1 and HASG-30) as previously described.17 The standard curve was obtained using 8 points for concentrations ranging from 0.69 to 500 ng/ml of soluble human elastin fragments (Elastin Products Co). The minimum detection limit for soluble elastin concentration was 0.5 ng/ml.

MMP-9 Activity

The level of MMP-9 activity in the protein extracts was measured using an MMP-9 activity assay system kit (RPN2634; Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer’s instructions. Briefly, 100 μl of protein extract was embedded in an immobilized 96-well microplate and incubated overnight at 4°C. After washing, 50 μl of either p-aminophenylmercuric acetate solution or assay buffer was added to wells containing the standard or the protein extract, respectively. After incubation for 1.5 h at 37°C, 50 μl of the detection reagent was embedded in all wells and the reaction was measured at 405 nm after 1 h of incubation.

Histological Staining

After fixation in 10% phosphate-buffered formalin (pH 7.3) for 24 h, histological specimens were paraffin-embedded, sectioned, and stained using hematoxylin–eosin, elastica van Gieson (EVG) or Masson trichrome.

Immunohistochemistry

Deparaffinized formalin-fixed sections were stained using the polymer–peroxidase method (EnVision+HRP; DAKO Cytomation, Glostrup, Denmark) with mouse mAbs (CD68 for macrophages [Dako Cytomation; diluted 1:200], α-smooth muscle actin for vascular smooth muscle cells [Dako Cytomation; diluted 1:200], HASG-61-1 or HASG-30 for soluble elastin, each at a final concentration of 0.4 μg/ml). In the absorption experiment, tissue sections were treated with each primary antibody in the presence of antigen
peptide (200μg/ml). For the negative control, the incubation step with the primary antibody was omitted.

Immunoelectron Microscopy
Immunohistochemical electromicroscopic analysis was performed as previously described. The sections were incubated with HASG-61-1 and with HRP-Linked F(ab')2 antibody (1:30; Amersham Bioscience) for 48 h at 4°C.

Total RNA Extraction and Semi-Quantitative RT-PCR for Elastin mRNA
RT-PCR was performed as previously described, using an amplification reagent kit (TagMan EZRT-PCR kit; Applied Biosystems, Alameda, CA, USA) with human elastin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers. For elastin, the primers and TagMan probe used were: 5’-GCG AAT TCC TGG AAT TGG A-3’ (sense), 5’-TGC TTC TGG TGA CAC AAC CC-3’ (antisense), and 5’-CAT CGC AGG CGT TGG GAC TCC-3’ (TagMan probe). The cDNA amplification product was predicted to be a 256-bp fragment from position 861–1116 in the cDNA of human elastin (GenBank M36860). The reaction master mix contained 900 nmol/L elastin primers and 200 nmol/L TaqMan probe.

In Situ Hybridization (ISH) for Elastin mRNA
ISH for elastin mRNA using deparaffinized formalin-fixed sections was performed as previously described. The human elastin cDNA probe used was a 430-bp fragment (obtained from positions 298–727 in the cDNA of human elastin) subcloned into the EcoR I site of a pGEM-T Easy Vector (Promega, Madison, WI, USA). Antisense probes and corresponding sense probes were labeled with biotin using SP6 and T7 polymerases, respectively (RNA labeling kit; Boehringer Mannheim, Mannheim, Germany). Hybridization was performed overnight at 42°C in 50% (vol/vol) deionized formamide, 5X Denhardt’s solution, 5% (W/vol) dextran sulfate, 2X SSC, 0.3 mg/ml human placenta DNA (D3287; Sigma-Aldrich), 20 mmol/L EDTA, and 2 μg/ml biotin-labeled probes.

Western Blot Analysis
Equal amounts (50 μg) of protein extracts were separated by 7.5% SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Hybond-N+: Amersham Biosciences, Piscataway, NJ, USA) as described previously. Each mAb (clone no. HASG-61-1 or HASG-30) was used at a final concentration of 0.4 μg/ml. In the absorption experiment, the transferred membrane was treated with each primary antibody in the presence of antigen peptide (200 μg/ml).

Statistical Analysis
Data are presented as mean±SEM. Comparisons between 2 groups were performed using the Mann-Whitney U-test. Comparisons among 4 groups were performed using ANOVA with a post-hoc Games-Howell test. Multivariable analyses were performed using multiple regression analysis. Differences were considered significant at P<0.05.

Results
Characteristics of Soluble Elastin in the Non-Atherosclerotic Aorta
The EVG staining and immunohistochemistry for soluble elastin using serial cross-sections and immunoelectron-microscopic analyses of non-atherosclerotic aortic specimens are shown in Figures 1a–h. Soluble elastin was present both within the intima (Figures 1b, d) and around mature elastin within the media (Figures 1a, g), showing that soluble elastin is distributed both within the extracellular matrix of the intima (Figures 1a–e) and around mature elastin within the media (Figures 1a, b, f–h). Soluble elastin appeared to be more abundant in the intima than in the media, and also appeared to be different from that of mature elastin itself (Figures 1b, d, e, g, h).

Distribution Patterns of Soluble Elastin in Plaques
Each specimen was diagnosed as 1 of 8 groups: type I, n=13; type II, n=34; type III, n=29; type IV, n=21; type Va, n=23; type Vb, n=22; type Vc, n=14; type VI, n=17. The specimens were then categorized as non-atherosclerotic.
aorta (n=13), fiber-rich plaque (n=77), lipid-rich plaque (n=66) or ruptured plaque (n=17).

**Figure 2** is representative images of advanced atherosclerotic plaques (fiber-rich plaque in a,d,g,j,m; lipid-rich plaque in b,e,h,k,n; ruptured plaque in c,f,i,l,o). In the advanced atherosclerotic aorta, soluble elastin was also dense in the intima rather than in the media, as in non-atherosclerotic aorta, and was noted to be dense within the extracellular matrix of the intima in the fiber-rich plaque (**Figure 2g**) and in the fibrous cap of lipid-rich plaques and ruptured plaques (**Figures 2h,i**). As well, the immunoreactivity for soluble elastin within the intima was weaker in the core of both lipid-rich plaques (**Figure 2h**) and ruptured plaques (**Figure 2i**) compared with the fiber-rich plaque (**Figure 2g**). A focal area of strong staining was noted in the intima of the fiber-rich plaque (**Figure 2g**, inset high-power view: **Figure 2m**), and in the rim of the intima adjacent to the atheroma formation in lipid-rich plaque (**Figure 2h**, inset; high-power view: **Figure 2n**). The areas densely positive for soluble elastin mostly coincided with those stained with resorcin fuchsin with EVG (**Figures 2d,e,j,k**). In addition, there was less stained with resorcin fuchsin and with HASG-61-1 in the ulceration in ruptured plaque (**Figures 2f,i,l,o**).
Level of Soluble Elastin in Protein Extracts
In the ELISA examination of 173 specimens, soluble elastin levels showed a stepwise decrease during the progress of atherosclerosis. The soluble elastin levels in ruptured plaques (2.8±0.4 μg/mg protein, n=17) were significantly lower than those in the non-atherosclerotic aorta (6.0±0.3 μg/mg protein, n=13), fiber-rich plaque (5.8±0.2 μg/mg protein, n=77) or lipid-rich plaque (4.9±0.2 μg/mg protein, n=66; P<0.05 in each case; Figure 3a).

Level of MMP-9 Activity in Protein Extracts
The level of MMP-9 activity was significantly higher in ruptured plaque (3.0±0.6 ng/mg protein, n=17) than in the non-atherosclerotic aorta (0.0±0.0 ng/mg protein, n=13), fiber-rich plaque (0.3±0.1 ng/mg protein, n=76) or lipid-rich plaque (0.8±0.2 ng/mg protein, n=67; P<0.05 in each case; Figure 3b). Thus, a stepwise increase in the MMP-9 activity was seen during the progress of atherosclerosis. Indeed, the plot of MMP-9 activity against soluble elastin level showed an inverse distribution (Figure 3c).

Figure 3. Ruptured plaque has lowest level of soluble elastin (a), but highest MMP-9 activity (b); that is, soluble elastin and MMP-9 activity shows an inverse distribution pattern in human aortic tissue (c). As with MMP-9 activity, elastin mRNA expression is highest in ruptured plaque (d). *P<0.05; data are mean±SEM. MMP, matrix metalloprotease.

Table. Relationship Between Level of Soluble Elastin and Histological Features

<table>
<thead>
<tr>
<th>Histological feature</th>
<th>Soluble elastin level, μg/mg protein*</th>
<th>Univariate† P value</th>
<th>Multivariable†† P value</th>
</tr>
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<tr>
<td>Present n</td>
<td>Absent n</td>
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<td>Plaque necrosis</td>
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<td>&lt;0.01</td>
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<tr>
<td>Intra-plaque hemorrhage‡‡</td>
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<td>&lt;0.01</td>
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<tr>
<td>Mineral deposition</td>
<td>4.8±0.4</td>
<td>5.4±0.2</td>
<td>0.13</td>
</tr>
</tbody>
</table>

*Data are mean±SEM.
†Univariate, Mann-Whitney U-test; ††multivariable, multivariable regression analysis; ‡fibrous cap was considered to be thin when thickness <65 μm; ‡‡fibrin deposition or hemorrhages were recognized as red-colored non-structural substances or aggregations of red blood cells outside the intimal microvessels (Masson trichrome procedure).
Univariate analysis showed that soluble elastin levels were significantly lower in plaque with histological features associated with instability (viz. macrophage infiltration into the fibrous cap, thin fibrous cap, intraplaque fibrin, intraplaque hemorrhage, and plaque rupture) than in plaques without these features (Table). There was no significant difference in soluble elastin levels between plaques with and without plaque necrosis or between those with and without mineral deposition. Multivariable analysis between soluble elastin level and histological features showed that the soluble elastin level was significantly lower in plaques showing evidence of rupture than in those without this feature (Table, P=0.01).

**ISH and Semi-Quantitative RT-PCR for Elastin mRNA**

Representative high-power images of serial sections of fiber-rich plaque, lipid-rich plaque and ruptured plaque are shown in Figure 4 (Masson trichrome procedure, a–c; immunohistochemistry for vascular smooth muscle cells, d–f; immunohistochemistry for macrophages, g–i; immu-
nohistochemistry for soluble elastin, j-l; ISH for elastin mRNA, m-o. In the ISH, grains of elastin mRNA were observed within the cytoplasm of vascular smooth muscle cells in fiber-rich plaque (Figure 4m, white arrows) and macrophages in fiber-rich plaques (Figure 4m), in the rim of the intima adjacent to the atheroma formation in lipid-rich and ruptured plaque (Figures 4n, o).

Semi-quantitative RT-PCR for elastin mRNA was performed on 147 of the 173 samples. PCR products were detected at 256 bp (data not shown). Elastin mRNA expression showed evidence of a stepwise increase (as with MMP-9 activity) during the progress of atherosclerosis. The ratio of elastin mRNA over GAPDH mRNA was significantly higher for ruptured plaque (4.7±1.0, n=16) than for the non-atherosclerotic aorta (1.6±0.2, n=13), fiber-rich plaque (2.7±0.3, n=62), or lipid-rich plaque (3.1±0.5, n=56; P<0.05, in each case, Figure 3d).

Western Blot Analysis
Figure 5 shows the results of western blot analysis for soluble elastin. At least 5 bands ranging from 20 to 65 kDa, including tropoelastin, were noted in the 4 types of plaque (Figure 5a). Thus, soluble elastin consists of tropoelastin, a pre-cursor of elastin (65 kDa), and several heterogeneous low-molecular-mass proteins (35–45 kDa). It seemed that low-molecular-mass soluble elastins decreased in the progress of atherosclerosis. Figure 5b shows the ratio of low-molecular-mass soluble elastin over tropoelastin in the 4 types of plaque. The ratio of tropoelastin/low-molecular-mass soluble elastin was significantly higher in ruptured plaque (1.0±0.1) than in the non-atherosclerotic aorta (0.5±0.0), fiber-rich plaque (0.4±0.1) and lipid-rich plaque (0.5±0.0) (Figure 5b, P<0.05, all).

Discussion
Although elastin degeneration and synthesis are involved in atherosclerosis, there have been few reports investigating the relationship between elastin and atherosclerosis. Recently, Krettek et al reported that macrophages, as well as vascular smooth muscle cells, express elastin mRNA. Concerning elastolysis, MMP-9 is reportedly involved in elastin degeneration and Goncalves et al reported that intermediate fractions of elastin increase as atherosclerosis progresses.

Figure 5. Western blot analysis shows soluble elastin has at least 5 bands of proteins with heterogeneous molecular weights (a), including tropoelastin (65 kDa), and (b) low-molecular-mass soluble elastins ranging from 35 to 45 kDa. Data are mean±SEM. *P<0.05. Band at extreme left in (a) shows that detection of soluble elastin were almost negligible in the absorption experiment with non-atherosclerotic aorta on the same membrane (arrowheads).
progresses. Those reports indicated that elastin degradation and/or degradation products increase during the progression of atherosclerosis, according to the presence of various kinds of proteases including MMP-9. However, the soluble elastin that we investigated in the present study of aortic specimens showed a decreasing pattern, which is different from the other elastin-associated proteins previously described.

In the present study, we found that in the non-atherosclerotic aorta soluble elastin was (1) distributed both within the intima and around the mature elastin within the media, (2) different from mature elastic fiber (Figure 1), (3) had the lowest mRNA expression among the 4 histological groups (Figure 3d), (4) present in a low ratio to tropoelastin, which is conjectured to be a marker of elastin synthesis, compared with ruptured plaque (Figure 5), and (5) associated with the activity of MMP-9 among the 4 groups (Figure 3b). These results indicate that in the non-atherosclerotic aorta from subjects approximately 53–84 years of age, soluble elastin is densely distributed and neither endogenous elastogenesis or elastolysis is activated excessively, so soluble elastin may be deposited gradually and preserved within the intima and around the mature elastic fibers within the media over a number of years because the intima is not diseased. However, it needs to be clarified whether soluble elastin can be also detected in the non-atherosclerotic aorta of normal subjects younger than those entered in this study.

In advanced atherosclerotic lesions, soluble elastin was found to be present within the extracellular matrix of the intima in fiber-rich plaque (Figures 2g, 4j) and the fibrous caps of lipid-rich plaque (Figures 2h, 4k). In these plaques, moreover, focal areas of resorptive Fuschin staining with EVG (indicating the presence of mature elastin fibrils) mostly coincided with the areas that were strongly positive for soluble elastin (Figures 2d, g, j, m) fiber-rich plaque; Figures 2e, h, k, n: lipid-rich plaque). In those areas, elastin mRNA are also expressed in both vascular smooth muscle cells and macrophages in the fiber-rich plaque (Figures 4d, g, m), lipid-rich plaque (Figures 4e, h, n), and ruptured plaque (Figures 4f, l, o). In accordance with these histological observations, elastin mRNA expression showed a stepwise increase during the progress of atherosclerosis (Figure 3d). ELISA appeared to show less soluble elastin in fiber-rich and lipid-rich plaques than in the non-atherosclerotic aorta, although admittedly statistical significance was not reached (Figure 3a). MMP-9 activity, which indicates elastolytic activity in the arterial wall, displayed a pattern of change similar to that shown by elastin mRNA expression (Figures 3b, d) and an inverse relationship with soluble elastin (Figure 3c). Interestingly, western blot analysis showed the ratio of tropoelastin/low-molecular-mass soluble elastin was lowest in fibrous plaques and relatively higher in lipid-rich plaques, regardless there being no statistical significance (Figure 5). These results indicate that in both fiber-rich and lipid-rich plaques (1) the level of soluble elastin tended to decrease in response to increased activity of MMP-9, and (2) both elastogenesis and elastolysis may have been induced in activated vascular smooth muscle cells and macrophages.

In contrast, in the cores of both lipid-rich and ruptured plaques, soluble elastin (Figures 2h, i, 4k, l) and mature elastin fibrils (Figures 2e, f) were both scarcely detected. ISH showed that elastin mRNA was abundantly expressed within vascular smooth muscle cells and macrophages in both lipid-rich and ruptured plaques (Figures 4o, o), whereas MMP-9 activity and elastin mRNA expression were highest in ruptured plaques among the 4 tissue groups (Figures 3b, d). The ratio of tropoelastin/low-molecular-mass soluble elastin was highest in ruptured plaque. In addition, our ELISA data demonstrated that the level of soluble elastin was lowest in ruptured plaque among the 4 groups (Figure 3a). Furthermore, multivariable analysis showed that soluble elastin was significantly reduced in plaque that showed histological evidence of rupture (Table). These results indicate that (1) soluble elastin is significantly decreased in the core of both lipid-rich plaque and ruptured plaque, (2) elastogenesis and elastolysis may be excessively upregulated in the shoulder of both lipid-rich plaques and ruptured plaques, (3) soluble elastin may be transferred rapidly from the arterial wall to the systemic blood circulation by plaque rupture, and (4) based of these phenomena, the turnover of elastin may be enhanced in active atherosclerotic lesions (supporting the basic view of atherogenesis as a chronic inflammatory–repair process). We used postmortem aortic specimens, which is the limitation of the present study because we could not completely exclude the influence of postmortem elastolysis; however, used tissue samples obtained within 12h of postmortem. Further study needs to be undertaken to investigate the possible biological functions of soluble elastin.

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Disclosure/Conflict of Interest

None.

References