Regional Atherosclerotic Plaque Properties in ApoE^{−/−} Mice Quantified by Atomic Force, Immunofluorescence, and Light Microscopy

H.N. Hayenga^a A. Trache^{a,b} J. Trzeciakowski^b J.D. Humphrey^c

^aDepartment of Biomedical Engineering, Texas A&M University, and ^bDepartment of Systems Biology and Translational Medicine, Texas A&M Health Science Center, College Station, Tex., and ^cDepartment of Biomedical Engineering, Yale University, New Haven, Conn., USA

Key Words

Apolipoprotein E • Material stiffness • Lipid • Atherosclerosis

Abstract

Elucidating regional material properties of arterial tissue is fundamental to predicting transmural stresses and understanding how tissue stiffness influences cellular responses and vice versa. Atomic force microscopy (AFM) was used to measure point-wise the axial compressive stiffness of healthy aortas and atherosclerotic plaques at micron level separation distances. Cross sections of plaques were obtained from a widely used animal model of atherosclerosis (ApoE^{−/−} mice). Median point-wise values of material stiffness were 18.7 and 1.5 kPa for the unloaded healthy wall (n = 25 specimens) and plaque (n = 18), respectively. When the healthy wall was distended uniformly during AFM testing, two mechanically distinct populations emerged from comparisons of normal cumulative distributions, with median values of 9.8 and 76.7 kPa (n = 16). The higher values of stiffness may have been due to extended elastin, which was not present in the plaques. Rather, most plaques were identified via standard and immunofluorescent histology to be largely lipid laden, and they exhibited a nearly homogeneous linear elas-

tic behavior over the small AFM indentations. Understanding the mechanics and mechanobiological factors involved in lesion development and remodeling could lead to better treatments for those lesions that are vulnerable to rupture.

Copyright © 2011 S. Karger AG, Basel

Introduction

Atherosclerotic plaques are complex materials consisting of aberrant accumulations of smooth muscle cells, foam cells, collagen, calcium, and necrotic debris [1]. The distribution and material properties of these different constituents can be initiators or indicators of plaque vulnerability. For example, it has been suggested that plaques rupture more frequently within regions of mechanical stress concentrations, often located along the border of soft lipid pools [2, 3] or near deposits of calcium [4]. Many such predictions stem, however, from continuum models that account for differences in mechanical properties (material stiffness) based on lumped plaque types: primarily fibrous, lipid laden, or calcified [5, 6]. There is, therefore, a pressing need for additional measurements of the stiffness of individual constituents within a plaque both to improve mathematical modeling and to exploit

advances in medical imaging. In particular, advances in imaging promise to better identify plaque composition *in vivo*, i.e. to distinguish regions containing calcium, dense fibrous tissue, lipids, smooth muscle cells, and even increased metabolic activity [7, 8]. Moreover, techniques such as elastography are emerging as potentially useful tools for estimating mechanical strain and stiffness within plaques *in vivo*, though there remains a need for a better correlation between plaque composition and stiffness to enable such methods to predict possible vulnerability [9, 10]. Indeed, composition must be correlated with stiffness before we can reliably predict the structural integrity of a plaque based on any method, which would represent a significant clinical advance.

In addition to data collected on human lesions, obtained primarily at autopsy, ApoE^{-/-} mice provide a unique opportunity to study the natural history of atherosclerotic disease progression and to identify underlying molecular mechanisms. ApoE^{-/-} mice do not express apolipoprotein E, an important ligand for receptor-mediated removal of lipoproteins from the plasma; lack of ApoE causes low-density lipoproteins (or LDLs) to accumulate within the arterial wall, which leads to atherosclerosis. Atherosclerotic plaques in ApoE^{-/-} mice exhibit many characteristics found in humans [5, 11], such as, for example, compensatory enlargement, medial thinning, and elastolysis [12–14]. ApoE^{-/-} mice have also been used for studying balloon angioplasty and intravascular stenting because the overall morphology and procedural success rates are similar to those for humans postintervention [15]. Nevertheless, there has been little attention directed towards quantifying the mechanical properties of plaques in the ApoE^{-/-} mouse, even though computational models can now predict stresses in these lesions [16]. Because the area of a typical ApoE^{-/-} aortic plaque is only ~100 mm² by 56 weeks [16], one of the challenges in quantifying potential regional differences in mechanical properties is the smallness of the plaques. We address this need in this paper by combining atomic force microscopy (AFM) with standard and immunofluorescent histology to quantify potential regional differences in the axial compressive stiffness of both healthy aorta and atherosclerotic aorta in ApoE^{-/-} mice.

Methods

Animal Model

All animal protocols were approved by the Institutional Animal Care and Use Committee at Texas A&M University. ApoE^{-/-} breeder mice were purchased from Jackson Laboratories and

maintained by the Comparative Medicine Program at Texas A&M University for up to 56 weeks. The colony was switched from a 9% fat chow diet to a western-type diet consisting of 21.2% fat and 0.2% cholesterol (Harlan Tekland, Madison, Wisc., USA) after an initial period of 16 weeks. Heterozygous and wild-type mice from a fibrillin-1-deficient colony, with a C57/s129 background, were also used to study mechanical properties of the non-atherosclerotic aortic wall.

Sample Preparation

AFM force-indentation testing was performed on cross sections of nondiseased aortic wall and atherosclerotic plaques (thickness 0.5 mm, outer diameter 0.5–1.5 mm). The suprarenal aorta (i.e. between the diaphragm and renal arteries) was harvested by blunt dissection from ApoE^{-/-} mice at ages ranging from 42 to 56 weeks ($n = 13$ animals, $n = 18$ plaques), with at least 1 plaque detected visually in each animal (fig. 1a, b). Next, the aorta was embedded in a 2% agarose gel, just prior to solidification, and sectioned into 0.5-mm-long segments using a vibratome (Vibratome Series 1000; The Vibratome Company, St. Louis, Mo., USA). Each tubular segment was then affixed to a 60-mm diameter petri dish with RP30 instant adhesive (Adhesive Systems, Inc., Frankfort, Ill., USA); 1.7 ml of phosphate-buffered saline (PBS) was then added to the petri dish to submerge the sample prior to AFM testing (fig. 1d).

Aortic samples were prepared from nondiseased mice in a similar fashion, with a few modifications. Following blunt dissection, the infrarenal aorta was either embedded in 2% agarose gel while pressurized at approximately 100 mm Hg ($n = 15$ animals, $n = 20$ aortic walls; in 5 cases 2 aortic wall samples were obtained from 1 animal) or while unpressurized ($n = 30$ animals, $n = 30$ aortic walls). Pressurization was maintained by cannulating the proximal aorta (near the renals), injecting dyed agar antegrade through the aorta, ligating the distal aorta (near the iliacs), and bringing the pressure to 100 mm Hg as determined by a pressure transducer (Living Systems Instrumentation, Burlington, Vt., USA). After solidification of the agar by cooling to room temperature, the aortic segment was sectioned, affixed to the petri dish, and immersed using PBS as described above.

AFM Testing

Force-indentation data (fig. 1c) were acquired using a Bioscope System AFM (Veeco Instruments, Santa Barbara, Calif., USA) equipped with a Nanoscope IIIa controller and mounted on a Zeiss Axiovert 100 TV inverted optical microscope (Carl Zeiss, Thornwood, N.Y., USA). A 5- μ m diameter spherical tip (borosilicate glass bead) attached to a silicon-nitride cantilever having a manufacturer-specified spring constant of either 60 or 120 pN/nm (Novascan, Ames, Iowa, USA) was used to indent the samples. A spring constant ~60 pN/nm allows increased sensitivity in the approach curve for softer samples; in contrast, a spring constant of ~120 pN/nm can be useful for stiffer samples. We purchased the latter in anticipation of stiff plaques, but pilot tests revealed that the 60 pN/nm cantilevers were sufficient for all specimens and thus were used in most experiments, including the data reported herein. The calibrated spring constant for these cantilevers was measured to be 67.5 ± 8.3 pN/nm (mean $\pm$ SD) using the reference cantilever method and FCL calibration probes (Applied NanoStructures, Inc., Santa Clara, Calif., USA). Deflection sensitivity was also determined for each tip. Afterward, the sample was

Fig. 1. **a, b** Plaque was detected by gross examination of the suprarenal aorta from ApoE^{-/-} mice aged 42–56 weeks. **d** The excised aortic segment was embedded in 2% agarose gel, sectioned with a vibratome at a thickness of 0.5 mm, mounted onto a 60-mm diameter petri dish, and mechanically tested via nanoindentations in the axial direction. **c** Representative mean force versus mean ($\pm$ SD error bars) indentation curve during AFM testing.

located within the petri dish and visualized in real time using a Zeiss 32X objective and video camera (TM 34KC; Pulnix, Yokohama, Japan). The AFM cantilever tip approached the sample at a frequency of 0.5 Hz with a z-scan size of 800 nm (speed 0.8 μ m/s). After recording typically 30 consecutive indentation force-depth curves at each location on the artery/plaque, the tip was retracted to its original position. Additional curves were recorded similarly over regions of $80 \times 80 \mu\text{m}^2$ spaced about 20 μ m apart achieved using the x and y offset controller. To move to a different macroscopic region, the sample and petri dish were moved together via the x-y stage controller. The AFM tip was then brought into contact with the surface of the sample and force curves were acquired over another $80 \times 80 \mu\text{m}^2$ region. This process was repeated until the entire region of interest was mapped.

Histology and Immunofluorescence

A 0.5- to 1.0-mm-long aortic section adjacent to that tested with the AFM was fixed with 4% paraformaldehyde for 1 h at room temperature and then placed in 30% sucrose overnight at 4°C for cryoprotection. The sample was then embedded in ‘optimum cutting temperature’ compound, flash-frozen with liquid nitrogen, and cut into 5- μ m-thick cross sections for standard and immunofluorescent histology. Serial sections were stained with hematoxylin and eosin (H&E) for general morphology, Verhoeff-van Gieson (VVG) for elastin (Verhoeff) and collagen (van Gieson), Masson’s trichrome (TRI) for collagen, oil red O (ORO) for lipids, von Kossa for calcium, alpha smooth muscle actin (α SMA) for the contractile apparatus in cells, and von Willebrand factor (vWF) for endothelial cells. Optimal conditions for the α SMA stain were 1:100 dilution of primary antibody (ab5694; Abcam) for 1 h at room temperature in PBS followed by heat-induced (pressure cooker) sodium citrate antigen retrieval and then expo-

sure to a 1:500 dilution of Alexa Fluor[®] 594 secondary antibody (A11012; Invitrogen) for 30 min. Optimal conditions for the vWF stain were 1:100 dilution of the primary antibody (ab6994; Abcam) for 1 h at room temperature in PBS, with heat-induced (pressure cooker) sodium citrate antigen retrieval and then exposure to a 1:500 dilution of Alexa Fluor[®] 594 secondary antibody (A11012; Invitrogen) for 30 min.

Point-Wise Elasticity Measurement

AFM force curves were analyzed as described by Costa et al. [17]. The applied force, f^{sphere} (pN), was derived from the measured cantilever deflection, d (V), via

$$f^{sphere} = kd_s d$$

where k is the spring constant (pN/nm) for the cantilever and d_s is the deflection sensitivity (nm/V); the cantilever deflection was determined in volts from the laser position on the photodetector. Cantilever deflection resulting from the approach/retraction cycle was monitored as a function of piezo movement, namely

$$\delta = z - d_s d$$

where δ is the indentation depth (nm), or deformation of the aortic wall or plaque, and z is the piezo displacement (nm) during testing. One can calculate an effective Young’s modulus (E), a measure of material stiffness in response to a small strain, using the Hertzian relationship for the indentation of a flat, homogeneous, semi-infinite elastic material by a rigid spherical probe [18]. In this case,

$$f^{sphere} = \frac{4}{3} \left(\frac{E}{1 - \nu^2} \right) \sqrt{R \delta^3}$$

where ν is Poisson's ratio, often assumed to be 0.5, and R the radius of the sphere. The simplifying assumptions associated with the Hertzian equation (including infinitesimal strains and rotations) generally do not apply for cells or biological soft tissues due to the finite strains and rotations nearest the probe. Therefore, to accommodate possible nonlinearities we calculated the point-wise apparent modulus ($\tilde{E}_i$), or material stiffness, at each point (i) along the approach curve (i.e. for all 512 points along the 800-nm indentation path), namely [17]

$$\tilde{E}_i = \frac{3}{8} \left(\frac{f_i^{\text{sphere}}}{\sqrt{R\delta_i^3}} \right)$$

where f_i^{sphere} is the force (pN) at each data point, and $\sqrt{\delta_i^3}$ is computed from the indentation depth (nm) at each data point.

The initial point of contact was first determined by fitting a bidomain polynomial algorithm to the AFM force curve [see 17 for details]. Using a linear least squares regression, error was minimized between a line fitted through the precontact region and a cubic polynomial fitted to the postcontact region of the indentation, $\sqrt{\delta^3}$, versus approach force, $kd_s d$, curve. If the goodness of fit (i.e. R^2 value) fell below 0.9, it was typically due to premature convergence on a local contact point caused by a sharp change in deflection during contact. To accommodate these cases, and to ensure we found the global contact point, a computational loop within the algorithm changed the initial contact point incrementally, within 30 points out of the 512 points constituting the indentation curve, until the R^2 value leveled out, typically at around 0.99. The local apparent point-wise elastic modulus was then calculated from each curve. Mean values of both this modulus and the force at each point, for all curves acquired at each location, were then plotted versus the mean indentation. All point-wise values of stiffness converged to an asymptotic value after the probe attained a certain indentation depth, typically less than 30 nm.

Statistical Analysis

To minimize noise, we removed point-wise values corresponding to indentations ≤ 10 nm of the approach curve. Approximately 750 curves for the wall and 1,650 curves for a plaque were analyzed per sample (i.e. ~ 30 curves per location, with ~ 25 locations per aortic wall sample and ~ 55 locations per plaque sample). All asymptotic values of point-wise stiffness for each condition (unloaded aortic wall, pressurized aortic wall, or plaque) were examined graphically using histograms. Doane's formula (proposed in 1976 as a variation of Sturges' formula to account for skewness) was used to calculate the bin size for each histogram. The data were transformed from a right-skewed distribution to one more Gaussian in appearance using the natural logarithm. The median, mean, and standard deviation were calculated for original data and data transformed using the natural logarithm. Differences in the transformed populations were evaluated using an unpaired 2-sample *t* test.

Tests were also performed to determine the presence or lack of multiple stiffness populations within each transformed population (i.e. the unpressurized normal wall stiffness, pressurized normal wall stiffness, or unpressurized plaque stiffness population). A cumulative distribution of stiffness showed deviations from normality. Likewise, computing the probability density estimate based on a normal kernel function with a bandwidth of 0.25 further illustrated the presence or lack of multiple stiffness populations.

Histomorphometry

To spatially register real-time AFM images, obtained during experiments that assessed regional values in material stiffness, with standard and immunofluorescent histological images of the adjacent sample, we scaled the histological images using GIMP-shop 2.2.8. That is, due to the extra handling and occasional loss of information from the histological sample (additional processing often resulted in a loss of ~ 100 μm of material), there were slight geometrical differences between the otherwise adjacent samples. After resizing to obtain the best match, however, the histological sample generally aligned well with the AFM image, thus permitting AFM measured values of stiffness to be overlaid on histological images.

Results

Plaques were observed in the suprarenal aorta in all ApoE $^{-/-}$ animals ($n = 13$, 42–56 weeks) but were rare in the descending thoracic aorta and infrarenal aorta. Recalling figure 1, a ventral view of the aorta from a 45.6-week-old male ApoE $^{-/-}$ mouse, gross examination revealed that flat plaques tended to form eccentrically on the ventral side, whereas smaller plaques taking on the shape of blisters protruding into the lumen formed on the dorsal side. Figure 2 shows 4 representative stiffness curves (from softest to stiffest) collected at 4 of the 40+ locations on a representative plaque plus the associated histology. As noted earlier, most of the curves representing point-wise moduli only depended on depth during the first 10 nm of the indentation. The associated early decrease in stiffness may have been due to noise as the probe transitioned from fluid to a sample surface with slight irregularities [19]. Nevertheless, the generally asymptotic values of stiffness revealed that this plaque behaved almost linearly elastically over the small indentations (< 800 nm), that it was very soft, and that it exhibited only slight regional differences (fig. 2b). By overlaying the stiffness values on the gross image of the plaque (fig. 2b), it was seen that the few localized regions of increased stiffness occurred near the shoulders of lipid regions (revealed by ORO). In particular, values of stiffness near 9 kPa corresponded approximately with contractile cells (revealed by αSMA). Values of stiffness were mostly less than 5 kPa, however; these values corresponded well with the overall histology that revealed that the plaque consisted primarily of lipid and some dispersed collagen, with few smooth muscle and possibly endothelial cells but no elastin or calcium (fig. 2c). Finally, it is important to note that positive vWF was found mostly in the endothelial layer that lined the entire lumen, with some positive intraplaque staining for vWF in otherwise

Fig. 2. **a** Four representative measured regional axial compressive stiffness versus indentation depth results (symbols and SD error bars) and calculated asymptotic values (solid lines) of 0.9, 3.9, 6.0, and 9.2 kPa are shown on the same scale (0–15 kPa for modulus and 0–200 nm for depth). **b** Overlaid data, acquired via an inverted microscope and CCD camera plus the AFM, outline the opaque ApoE^{−/−} plaque, arterial wall, and mechanically tested locations as well as asymptotic point-wise stiffness at these locations. The location of the stiffness curves in **a** is shown by the markers A, B, C, and D to the right of their location in **b**. **c** Histological examination of the face adjacent to that tested via AFM reveals a lipid-laden (ORO), fibrocellular plaque without calcifications (von Kossa stain). Collagen was revealed throughout (TRI) with localized fragmentation and straightening of the elastic lamina in the aortic wall (VVG). Cells in the plaque and adjacent wall exhibited a less contractile phenotype (α SMA). Endothelial cells lined the luminal surface (vWF) but also appeared regionally within some plaques.

acellular areas (as indicated by DAPI counterstaining, not shown).

Figure 3 shows results from another representative plaque, with stiffness values overlaid on the AFM microscopic image and each of the histological images. All asymptotic values of stiffness were below 10 kPa, except at 2 locations near the plaque border on the right where the values were 15 and 27 kPa. It is not clear what yielded these higher values of stiffness, but it is interesting that the histological samples had artifact-related tears near both sites, suggesting that a material discontinuity (i.e. compliant-stiff border) may have contributed both to the tearing during sectioning and to the larger values of AFM-measured stiffness.

Values of stiffness were also found for the aortic wall from both wild-type and heterozygous mice from a fibrillin-1 colony, which share a common background with the ApoE^{−/−} mice. Figure 4 shows both actual and natural log-transformed distributions for all asymptotic values of

stiffness acquired over a nondiseased aortic wall in both unloaded (fig. 4a, b) and loaded (fig. 4c, d) configurations. The actual values displayed a right-skewed distribution (fig. 4a, c): the unpressurized walls were predominately right-skewed with a long tail (median 18.7 kPa) and the pressurized walls were more bimodal (median 9.8 and 76.7 kPa; mean $\pm$ SEM 12.3 ± 0.59 and 76.4 ± 3.22 kPa). Median values of stiffness for actual data correspond to the back-transformed value of the natural log mean. Comparing all values of (axial compressive material) stiffness from unloaded and loaded walls showed, however, that the unloaded group was significantly stiffer overall in axial compression ($p = 0.0015$; 18.7 vs. 11.4 kPa).

Considering data from all plaques, 85.6% of the asymptotic values of stiffness were below 5 kPa, which was significantly less than for the normal aortic wall (fig. 5) and consistent with the predominantly lipid-laden composition of the plaques (fig. 2, 3). However, the maximum value of stiffness found in 1 plaque was 37.2 kPa. A small

Fig. 3. Representative overlay showing mechanically tested locations and associated values of stiffness mapped over each histological image as well as the original AFM image (upper left corner). The histological stains are the same as in figure 2 (i.e. ORO, α SMA, von Kossa, H&E, vWF, VVG, and TRI).

cluster of values near this site, with a median stiffness of 24.3 kPa, was found to correspond to a slightly calcified region (not shown). This calcification was found in a plaque from a 56-week-old female ApoE $^{-/-}$ mouse and was the only one found in the 18 plaques examined. Albeit not shown, plaques from females and males were not statistically different overall; both had fairly large lipid-laden plaques with a median stiffness of around 1.5 kPa. Both genders ranged from 42 to 56 weeks of age, with slightly more plaques tested from females than males ($n = 11$ and $n = 7$, respectively). Although collagen was present in most plaques (fig. 2, 3), it did not appear to have a significant effect on the (axial compressive) stiffness. The lack of increased stiffness may have been due to a diminished organization, cross-linking, or density of collagen fibrils in the plaques or simply due to the axial compressive nature of the testing (collagens I and III exhibit high stiffness primarily in tension).

Discussion

Using a 5- μ m diameter spherical AFM tip, we were able to quantify regional material properties within cross sections of atherosclerotic plaques from the suprarenal aorta of ApoE $^{-/-}$ mice. The size of tip was chosen to provide an averaged mechanical property, consistent with underlying continuum assumptions, without compromising the specificity needed to characterize individual structural constituents within the plaques. Previous techniques that have been used to characterize plaque components include uniaxial tensile testing of larger (31.7 $\times$ 1.2 mm) human aortic intimal plaques lumped into 3 groups (i.e. cellular, hypocellular, and calcified [20]), compressive cyclic loading and stress-relaxation testing on 5 $\times$ 5 mm² square plaque samples [21], and torsional rheometer testing of synthesized lipid pools 0.3–1 mm thick [20]. Each of these techniques required either re-

Fig. 4. Histograms of asymptotic axial compressive stiffness over the wall of unloaded ($n = 25$ animals) (**a**, **b**) and pressurized to ~ 100 mm Hg ($n = 16$ animals) (**c**, **d**) nondiseased arteries. **a**, **c** The distributions of stiffness were originally right skewed (bin size 15, skewness 8.6 and 3.2 for unloaded and loaded, respectively). **b**, **d** After a natural log transformation, the distributions were nearly Gaussian (bin size 14 and 13, skewness -0.3 and 0.3 for unload-

ed and loaded, respectively). **c** Normal distributions constructed from the mean and standard deviations are outlined by solid curves for the softer and stiffer populations of the pressurized aortas. The compressive axial stiffness of the unloaded wall was statistically different from that of the pressurized wall (unequal 2-sample t test, $p = 0.00042$).

moving the plaque from its natural environment and treating it as a homogenized (lumped) material or synthesizing a single constituent, yet the complexity in geometry and composition (possible calcium deposits, fibrous tissue, fatty substances, smooth muscle cells, elastin fragments, and so forth) demands methods that can better discriminate individual contributions by the different constituents. Indeed, stress analyses reveal that the most important aspect of plaque properties may be material discontinuities – the close proximity of stiff and compliant materials can lead to stress concentrations and surfaces where rupture may occur [4].

The plaques from ApoE $^{-/-}$ mice studied here consisted primarily of lipid-laden pools plus dispersed collagen and noncontractile cells; the associated median axial compres-

sive stiffness was 1.5 kPa. None of the plaques ruptured prior to or during testing. Rupture is a complex mechanical process dictated by plaque composition and morphology as well as the applied loads. Using computational models, Finet et al. [22] showed that a modest increase in the stiffness of a lipid core (from 1 to 2–30 kPa) results in mechanically stable plaques (where peak cap stress is brought below a 300 kPa threshold assumed for the rupture of collagen). Using computational stress analyses, Vengrenyuk et al. [16] calculated peak circumferential stress in nonruptured ApoE $^{-/-}$ plaques to be higher than the estimated rupture threshold of humans (minimum 300 kPa, average 545 kPa). Thus, despite having a thin cap and large lipid pool, the lack of rigid inclusions (calcification) was suggested to render these plaques relatively stable.

Fig. 5. Histograms of asymptotic values of the axial stiffness of plaques ($n = 18$ plaques, $n = 12$ animals). Originally, the distributions were right skewed (bin size 19, skewness 5.36) (a). After a natural log transformation, the distributions were nearly Gaussian (bin size 15, skewness 0.58) (b). The axial stiffness of plaques was statistically different from that of the normal wall (unloaded, 2-sample t test, $p < 0.0001$; pressurized, 2-sample t test, $p < 0.0001$).

Notwithstanding a lack of information on exact mechanisms and signaling pathways for force transduction, it is evident that cells sense the stiffness and geometric features of their substrates and respond accordingly [23–27]. For example, cells respond to their environment through integrin clustering, strengthening of integrin-cytoskeleton linkages, and even remodeling of the extracellular matrix [23, 24, 27]. Moreover, cells can change their morphology (e.g. round for soft and spread for stiff substrates), cytoskeletal organization (fewer stress fibers for soft and more stress fibers at focal adhesions for stiff), and stiffness (within a range) based on that of the substrate [23, 24, 26, 28]. Matrix stiffness also guides the differentiation of mesenchymal stem cells [29]. Because cells control the properties of evolving atherosclerotic plaques while the plaque material may, in turn, control cell phenotype locally, it is important to know regional plaque mechanical properties.

The main structural components of the normal aortic wall, in order of decreasing tensile stiffness, are collagen, elastin, and smooth muscle cells. Using a scanning microindentation tester, Masumoto et al. [30] measured the radial stiffness of the smooth muscle cell layer and elastic laminae of an unloaded porcine aorta to be 50 and 180 kPa, respectively. Oie et al. [31] used a tactile mapping system and found the axial stiffness of the media (com-

prised of smooth muscle cells and collagen fibrils) to be 17.0 ± 9.0 kPa (mean $\pm$ SD) and that of elastin to be 69.0 ± 12.8 kPa. Hence, although collagen is well known to be orders of magnitude stiffer than elastin in tension [32], it need not be stiffer in compression or at low pressures (e.g. previous studies suggest that collagen fibers contribute minimally to residual stresses [33]). Oie et al. [31] thus suggest that the medial stiffness of 17.0 ± 9.0 kPa results from a low density of collagen.

The bimodal distribution of axial stiffness revealed by figure 4, with medians of 9.7 and 76.7 kPa, may also suggest 2 material populations within the modestly pressurized nondiseased aortic wall. Albeit not statistically significant, a plot of (axial compressive) stiffness versus location revealed that most stiff values were within the inner two thirds of the aortic wall. This observation suggests that the stiffer population may have been due to elastin, for collagen primarily resides in the adventitia and elastin resides in the media. Costa et al. [17] also reported a bimodal distribution of stiffness for individual cells. Whereas they used nanometer diameter tips, able to distinguish cytoskeletal filaments (5.6 ± 3.5 kPa) and cytoplasm (1.5 ± 0.76 kPa), we used micron diameter tips that were able to distinguish material within the smooth muscle layer (thickness 10.1 ± 4.7 μ m, mean $\pm$ SD) from the elastic laminae (thickness 6.2 ± 2.1 μ m) in an un-

loaded section of the wall (total thickness $136.6 \pm 22.9 \mu\text{m}$). Excluding the upper 5% due to a small cluster of stiffness values at $\sim 175 \text{ kPa}$, stiffness ranged from 0.5 to 90 kPa with a median of 18.7 kPa for the unloaded aorta. In agreement with previously reported material properties, the upper range may have been due to elastin, the middle range may have been due to a mixture of smooth muscle and unloaded collagen, and the lower range may reflect cytoplasmic material. In the samples with a fixed pressure of $\sim 100 \text{ mm Hg}$, analyses indicated 2 significantly different populations of material with moduli of 12.3 ± 0.59 and $76.4 \pm 3.22 \text{ kPa}$. Nevertheless, similar to the unloaded case, excluding the upper 5%, stiffness ranged from 0.8 to 94 kPa with a median of 11 kPa for the pressurized aorta. The difference in material populations that arose when the vessels were pressurized may have been due to an increase in lamina stiffening resulting from elastin straightening. Of course, circumferentially oriented fibers (e.g. collagen and elastin) may not be load bearing in the unpressurized samples.

Contractile cells appeared to be absent in soft regions of the plaques as indicated by the lack of αSMA staining. Rather, these plaques appeared to be dominated by lipids, with over 90% of the asymptotic values of stiffness less than 10 kPa (fig. 5). A correlation between the cell function (i.e. contractility) and local stiffness has been suggested previously. For example, minimal stress fibers form in cells grown on soft surfaces (Young's modulus of 10 kPa) whereas many stress fibers form when grown on stiff surfaces (Young's modulus of 100 kPa) [34, 35]. We found, at the tissue level, that normal mixtures of collagen, elastin, and smooth muscle cells in the wall of a non-diseased aorta had a compressive axial stiffness around 18.7 kPa, which was well above that of the lipid-laden

plaques. Histology confirmed a high lipid content in the plaques as well as a low collagen content and a faint layer of contractile cells in the plaque cap, with expression of vWF lining the lumen (fig. 2). Thus without cell contractility and much of the structural matrix proteins that reside in the wall of normal arteries, stiffness values appeared to be dominated by lipids abundant in the plaques.

Calcification in the intimal space is typical of advanced lesions in humans. In the ApoE $^{-/-}$ mice tested, only 1 of the 18 suprarenal aortic lesions exhibited even minimal calcification. Therefore, additional measures could be taken to better represent the heterogeneity of lesions seen in human plaques in suprarenal aortic lesions within ApoE $^{-/-}$ mice (fed a western diet). For example, dietary supplements of vitamin D, calcium, or warfarin can increase vascular calcification [36]. In addition, smooth muscle cell apoptosis [37] as well as matrix Gla protein- [38] and osteoprotegerin- [36] deficient models have also been shown to increase vascular calcification.

In summary, we presented a combined AFM-histological method that can be used to better correlate the relationships between (compressive material) stiffness and composition, even in small plaques such as those found in ApoE $^{-/-}$ mice. The overall lower stiffness of the plaques tested, relative to the normal arterial wall, was consistent with the predominant lipid-laden composition. Only in one case was calcification noted in a small region, which was detected by the AFM as well. There is a need, however, for modifications to produce more consistent calcifications as commonly found in human plaques. Correlations between regional variations and local composition promise to allow increased interpretation of medical images that focus on the latter.

References

- 1 Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM: Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* 2000; 20:1262–1275.
- 2 Richardson PD, Davies MJ, Born GV: Influence of plaque configuration and stress distribution on fissuring of coronary atherosclerotic plaques. *Lancet* 1989;2:941–944.
- 3 Huang H, Virmani R, Younis H, Burke AP, Kamm RD, Lee RT: The impact of calcification on the biomechanical stability of atherosclerotic plaques. *Circulation* 2001;103:1051–1056.
- 4 Vengrenyuk Y, Carlier S, Xanthos S, Cardoso L, Ganatos P, Virmani R, Einav S, Gilchrist L, Weinbaum S: A hypothesis for vulnerable plaque rupture due to stress-induced debonding around cellular microcalcifications in thin fibrous caps. *Proc Natl Acad Sci USA* 2006;103:14678–14683.
- 5 Lee RT, Grodzinsky AJ, Frank EH, Kamm RD, Schoen FJ: Structure-dependent dynamic mechanical behavior of fibrous caps from human atherosclerotic plaques. *Circulation* 1991;83:1764–1770.
- 6 Salunke NV, Topoleski LD: Biomechanics of atherosclerotic plaque. *Crit Rev Biomed Eng* 1997;25:243–285.
- 7 Groen HC, van Walsum T, Rozie S, Klein S, van Gaalen K, Gijzen FJ, Wielopolski PA, van Beusekom HM, de Crom R, Verhagen HJ, et al: Three-dimensional registration of histology of human atherosclerotic carotid plaques to in-vivo imaging. *J Biomech* 2010;43:2087–2092.
- 8 Riou LM, Broisat A, Dimastromatteo J, Pons G, Fagret D, Ghezzi C: Pre-clinical and clinical evaluation of nuclear tracers for the molecular imaging of vulnerable atherosclerosis: an overview. *Curr Med Chem* 2009;16:1499–1511.

- 9 Schaar JA, De Korte CL, Mastik F, Strijder C, Pasterkamp G, Boersma E, Serruys PW, Van Der Steen AF: Characterizing vulnerable plaque features with intravascular elastography. *Circulation* 2003;108:2636–2641.
- 10 Le Floch S, Ohayon J, Tracqui P, Finet G, Gharib AM, Maurice RL, Cloutier G, Pettigrew RI: Vulnerable atherosclerotic plaque elasticity reconstruction based on a segmentation-driven optimization procedure using strain measurements: theoretical framework. *IEEE Trans Med Imaging* 2009;28:1126–1137.
- 11 Glagov S, Weisenberg E, Zarins CK, Stankovic R, Koletis GJ: Compensatory enlargement of human atherosclerotic coronary arteries. *N Engl J Med* 1987;316:1371–1375.
- 12 Choudhury RP, Fayad ZA, Aguinaldo JG, Itskovich VV, Rong JX, Fallon JT, Fisher EA: Serial, noninvasive, in vivo magnetic resonance microscopy detects the development of atherosclerosis in apolipoprotein E-deficient mice and its progression by arterial wall remodeling. *J Magn Reson Imaging* 2003;17:184–189.
- 13 Lessner SM, Martinson DE, Galis ZS: Compensatory vascular remodeling during atherosclerotic lesion growth depends on matrix metalloproteinase-9 activity. *Arterioscler Thromb Vasc Biol* 2004;24:2123–2129.
- 14 Lutgens E, de Muinck ED, Heeneman S, Daemen MJ: Compensatory enlargement and stenosis develop in apoE(–/–) and apoE*3-Leiden transgenic mice. *Arterioscler Thromb Vasc Biol* 2001;21:1359–1365.
- 15 Ali ZA, Alp NJ, Lupton H, Arnold N, Bannister T, Hu Y, Mussa S, Wheatcroft M, Greaves DR, Gunn J, et al: Increased in-stent stenosis in ApoE knockout mice: insights from a novel mouse model of balloon angioplasty and stenting. *Arterioscler Thromb Vasc Biol* 2007;27:833–840.
- 16 Vengrenyuk Y, Kaplan TJ, Cardoso L, Randolph GJ, Weinbaum S: Computational stress analysis of atherosclerotic plaques in ApoE knockout mice. *Ann Biomed Eng* 2010;38:738–747.
- 17 Costa KD, Sim AJ, Yin FC: Non-Hertzian approach to analyzing mechanical properties of endothelial cells probed by atomic force microscopy. *J Biomech Eng* 2006;128:176.
- 18 Johnson K: *Contact Mechanics*. Cambridge, Cambridge University Press, 1985.
- 19 Elkin BS, Azeloglu EU, Costa KD, Morrison B 3rd: Mechanical heterogeneity of the rat hippocampus measured by atomic force microscope indentation. *J Neurotrauma* 2007;24:812–822.
- 20 Loree HM, Grodzinsky AJ, Park SY, Gibson LJ, Lee RT: Static circumferential tangential modulus of human atherosclerotic tissue. *J Biomech* 1994;27:195–204.
- 21 Topoleski LD, Salunke NV: Mechanical behavior of calcified plaques: a summary of compression and stress-relaxation experiments. *Z Kardiol* 2000;89(suppl 2):85–91.
- 22 Finet G, Ohayon J, Rioufol G, Lefloch S, Tracqui P, Dubreuil O, Tabib A: Morphological and biomechanical aspects of vulnerable coronary plaque. *Arch Mal Coeur Vaiss* 2007;100:547–553.
- 23 Discher DE, Janmey P, Wang YL: Tissue cells feel and respond to the stiffness of their substrate. *Science* 2005;310:1139–1143.
- 24 Janmey PA, McCulloch CA: Cell mechanics: integrating cell responses to mechanical stimuli. *Annu Rev Biomed Eng* 2007;9:1–34.
- 25 Nemir S, Hayenga HN, West JL: PEGDA hydrogels with patterned elasticity: Novel tools for the study of cell response to substrate rigidity. *Biotechnol Bioeng* 2010;105:636–644.
- 26 Solon J, Levental I, Sengupta K, Georges P, Janmey P: Fibroblast adaptation and stiffness matching to soft elastic substrates. *Biophys J* 2007;93:4453–4461.
- 27 Vogel V, Sheetz M: Local force and geometry sensing regulate cell functions. *Nat Rev Mol Cell Biol* 2006;7:265–275.
- 28 Engler AJ, Griffin MA, Sen S, Bonnemann CG, Sweeney HL, Discher DE: Myotubes differentiate optimally on substrates with tissue-like stiffness: pathological implications for soft or stiff microenvironments. *J Cell Biol* 2004;166:877–887.
- 29 Engler A, Sen S, Sweeney H, Discher D: Matrix elasticity directs stem cell lineage specification. *Cell* 2006;126:677–689.
- 30 Matsumoto T: Residual stress and strain in the lamellar unit of the porcine aorta: experiment and analysis. *J Biomech* 2004;37:807–815.
- 31 Oie T, Murayama Y, Fukuda T, Nagai C, Omata S, Kanda K, Yaku H, Nakayama Y: Local elasticity imaging of vascular tissues using a tactile mapping system. *J Artif Organs* 2009;12:40–46.
- 32 Gosline J, Lillie M, Carrington E, Guerette P, Ortlepp C, Savage K: Elastic proteins: biological roles and mechanical properties. *Philos Trans R Soc Lond B Biol Sci* 2002;357:121–132.
- 33 Greenwald SE, Moore JE Jr, Rachev A, Kane TP, Meister JJ: Experimental investigation of the distribution of residual strains in the artery wall. *J Biomech Eng* 1997;119:438–444.
- 34 Geiger B, Spatz JP, Bershadsky AD: Environmental sensing through focal adhesions. *Nat Rev Mol Cell Biol* 2009;10:21–33.
- 35 Walcott S, Sun SX: A mechanical model of actin stress fiber formation and substrate elasticity sensing in adherent cells. *Proc Natl Acad Sci USA* 2010;107:7757–7762.
- 36 Atkinson J: Age-related medial elastocalcification in arteries: mechanisms, animal models, and physiological consequences. *J Appl Phys* 2008;105:1643–1651.
- 37 Price PA, Faus SA, Williamson MK: Warfarin causes rapid calcification of the elastic lamellae in rat arteries and heart valves. *Arterioscler Thromb Vasc Biol* 1998;18:1400–1407.
- 38 Clarke MC, Littlewood TD, Figg N, Maguire JJ, Davenport AP, Goddard M, Bennett MR: Chronic apoptosis of vascular smooth muscle cells accelerates atherosclerosis and promotes calcification and medial degeneration. *Circ Res* 2008;102:1529–1538.