Mechanical Properties of the Extracellular Matrix of the Aorta Studied by Enzymatic Treatments

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ABSTRACT  The microarchitecture of different components of the extracellular matrix (ECM) is crucial to our understanding of the properties of a tissue. In the study presented here, we used a top-down approach to understand how the interplay among different fibers determines the mechanical properties of real tissues. By selectively removing different elements of the arterial wall, we were able to measure the contribution of the different constituents of the ECM to the mechanical properties of the whole tissue. Changes in the network structure were imaged with the use of two-photon microscopy. We used an atomic force microscope to measure changes in the mechanical properties by performing nanoindentation experiments. We show that although the removal of a key element of the ECM reduced the local stiffness by up to 50 times, the remaining tissue still formed a coherent network. We also show how this method can be extended to study the effects of cells on real tissues. This new (to our knowledge) way of studying the ECM will not only help physicists gain a better understanding of biopolymers, it will be a valuable tool for biomedical researchers studying processes such as wound healing and cervix ripening.

INTRODUCTION

In addition to the different fibers and other components that make up a tissue, the microarchitecture of the different components is crucial to the mechanical properties of a tissue. In recent years, various in vitro studies on reconstituted components of the extracellular matrix (ECM) have shed light on the physical principles that determine the mechanical properties of tissues. In studies using a bottom-up approach, one or two different ECM components were combined to make a gel, and the results revealed a rich interplay among fibers, linkers, and cells (1−4).

In the study presented here, we used a top-down approach to understand how the interplay among different components determines the mechanical properties of real tissues. By selectively removing different elements of the arterial wall (e.g., removing the collagen by collagenase), we were able to measure the contribution of the different constituents of the ECM to the mechanical properties of the tissue as a whole. Similar proteolytic treatments of the arterial wall were previously used to study the spatial organization of the fibers within the tissue via immunolabeling (5) and scanning electron microscopy (SEM) (6). Despite the difficulties posed by the complexity of real tissues, this method allows the microarchitecture present in real tissues to be studied. Ultimately, one could extend this method to study the effects of cells on the ECM, e.g., by studying the effects of the contents of neutrophils on the ECM. We imaged changes in the network structure using two-photon microscopy. Previous studies used enzymatic digestions to differentiate between the contributions of the different constituents of the ECM and the mechanical response of the whole tissue (7−11). In this study, we used an atomic force microscope used to measure changes in mechanical properties on the submicrometer scale by performing nanoindentation experiments.

MATERIALS AND METHODS

All experiments were performed on porcine aorta to minimize biological variation among the samples. Whole porcine aortas were collected within 18 h after slaughter, sliced in 15-mm pieces, and snap-frozen in liquid pentane. The tissue was cryosliced to 50 μm and stored at −80°C until it was used.

The aortic wall is comprised of multiple layers consisting of different components, as is shown in Fig. 1. By studying the tunica adventitia, the collagen-rich outer layer of the aorta, and the neighboring tunica media, which is rich in elastin and also contains some collagen fibers, we were able to examine the proteolytic effects on two different networks within one sample.

After thawing, the samples were placed in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4; all from Sigma-Aldrich, Zwijndrecht, The Netherlands) for 5 min to reconstitute. After the PBS was removed, the samples were placed in PBS with penicillin-streptomycin (50 U/ml) and the enzymes in an oven overnight at 37°C, except for the elastin digestion, which was performed overnight at 25°C. The concentrations of the enzymes (listed in Table 1) were chosen such that the specific components were removed but the rest of the tissue remained intact (8). The contents of the neutrophils, activated by 100 nM formyl-Met-Leu-Phe (Sigma), were prepared as described previously (12). After the proteolytic treatment, the buffer with enzymes was changed to PBS and the sample was used for either two-photon imaging or atomic force microscopy (AFM) nanoindentation.

Second-harmonic generation was used to image the organization of the collagen and the elastin fibers (13). To detect collagen, we used filter
Biological tissues tend to stiffen when they are deformed (3,4), and the Young’s modulus that is independent of the applied force is not met. The assumption of the Hertz model of an isotropic, smooth substrate with settings to be sensitive for frequency doubling, whereas to detect elastin we relied on its autofluorescence. The two-photon microscopy was performed on a Zeiss 710 NLO (Jena, Germany) upright confocal microscope equipped with a Spectra-Physics Deep See MP laser (Spectra-Physics, Mountain View, CA). The images were obtained with an excitation wavelength of 800 nm, and emitted light with wavelengths of 371–425 nm and 471–532 nm was detected for the collagen and elastin signals, respectively. Confocal stacks were processed for maximum-intensity projections with ImageJ (NIH, Bethesda, MD).

The AFM nanoindentation experiments were performed as described previously (14). In brief, we obtained measurements using a Molecular Imaging Picoscan atomic force microscope (Agilent Technologies, Palo Alto, CA) controlled with a custom scripting program written in Labview (National Instruments, Austin, TX) and Visual Basic 6 (Microsoft, Redmond, WA). The nanoindentation was performed with a 0.58 N/m cantilever (Veeco NP type; Veeco Metrology, Santa Barbara, CA) and recorded with a National Instruments card at 100 kS/s. We used MATLAB (The MathWorks, Natick, MA) to calculate the Young’s modulus for each indentation using the FIEL method of A-Hassan et al. (15). This method calculates the work needed for a certain deformation by integrating the force-distance curve. This measure for the stiffness is compared with the calculated values for the stiffness as a measure of the response of the tissue upon indentation. This effective Young’s modulus will reflect the local mechanical properties of the tissue under the set experimental conditions and can be used to compare indentations on different types of tissue.

By performing the indentations on a regular grid, we were able to obtain a stiffness map of the tissue with a corresponding stiffness distribution. All AFM measurements were performed in buffer at 37°C (control, collagenase, chondroitinase + hyaluronidase, and neutrophils) or 25°C (control and elastase). For every case, at least 2000 indentations at different locations were performed.

Samples for SEM imaging were prepared and incubated with enzymes according to the same method used for the AFM measurements. After overnight incubation with the enzymes, the samples were placed overnight in PBS with 2% glutaraldehyde (SIGMA) at 4°C. The samples were then critical-point dried and coated with a thin layer of gold-palladium (5 nm thick at maximum) and stored at room temperature until use. A field emission scanning electron microscope (JEOL JSM-6700F) was used at 5.0 kV.

**TABLE 1  Concentrations of enzymes used for the various proteolytic treatments**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control/PBS</td>
<td>–</td>
<td>25°C/37°C</td>
</tr>
<tr>
<td>Elastase (Worthington Biochemical, Lakewood, NJ)</td>
<td>5.5 µ/ml</td>
<td>25°C</td>
</tr>
<tr>
<td>Collagenase (type CLSPA; Worthington)</td>
<td>200 µ/ml</td>
<td>37°C</td>
</tr>
<tr>
<td>Chondroitinase ABC</td>
<td>1000 µ/ml</td>
<td>37°C</td>
</tr>
<tr>
<td>+ hyaluronidase (Sigma)</td>
<td>0.04 µ/ml</td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>10%</td>
<td>37°C</td>
</tr>
</tbody>
</table>

PBS was used as a buffer for all experiments except for that involving chondroitinase ABC + hyaluronidase, in which a 50 mM Tris, 10 mM sodium (both Sigma) acetate buffer was used for the incubation and all of the sample preparation steps. Incubation took place in the presence of penicillin (50 U/ml)-streptomycin (50 µg/ml; both Sigma) to prevent the growth of bacteria.
RESULTS AND DISCUSSION

Fig. 2 shows a stiffness map measured on the tunica media of a sample without any enzymes added. Each pixel of the stiffness map represents the stiffness calculated from a single indentation curve, with light colors indicating large stiffness. The stiffness map shows that stiffer pixels tend to be grouped in elongated patches, which have an orientation that coincides with the fibers that are visible in the optical microscope integrated with the AFM. We combined multiple stiffness maps for every sample treatment into a single histogram, which reflects the distribution of stiffnesses within a sample. These histograms of the media and the adventitia are plotted in Fig. 3 and show a clear change in the effective Young’s modulus for certain proteolytic treatments.

Fig. 4 shows the two-photon images of the adventitia and media for the different proteolytic treatments we performed. The two-photon images of the control show a difference in network structure between the media and adventitia. The adventitia consists of a densely woven network of collagen fibers, whereas the media consists mainly of parallel elastin fibers. The swelling stress of the proteoglycans present in both layers of the arterial wall puts the network, which is composed of collagen and elastin fibers, under tension (18,19). The small deformations made by the AFM tip mainly probe the tension on this combined network instead of the mechanical properties of the isolated fibers. The inhomogeneous distribution of proteoglycans across the vessel wall, showing a higher concentration in the media than in the adventitia (20,21), could give rise to the measured stiffer response in the media compared with the adventitia (22,23).

Digestion by elastase revealed the underlying collagen structure in the media and reduced the stiffness of the remaining tissue of the media, as expected given that elastin is one of its main components. However, the network structure and mechanics of the adventitia remained unaltered by the elastin removal. Because the removal of elastin required an incubation temperature of 25°C, a control with this temperature was also made. The small decrease in stiffness observed in the control sample left overnight at an incubation temperature of 37°C compared with 25°C is much smaller than the changes effected by the proteolytic treatments.

Collagenase treatment weakened both layers. The weakening of the adventitia (an ~45-fold decrease in stiffness) was stronger than in the media, where the stiffness decreased by a factor of ~3. Although two-photon imaging shows no collagen and elastin present in the digested adventitia, the AFM still reveals a coherent network. SEM imaging confirms the existence of a remaining coherent network. The remaining structures have a high similarity with the structure of proteoglycans, which have been reported to be present in both layers of the aortic wall (20,21).

Removal of the proteoglycans by a mixture of chondroitinase ABC and hyaluronidase showed a reduced stiffness of
both the media and adventitia. This decrease is modest compared with that observed with collagenase treatment of the adventitia or elastase treatment of the media, but it is still quite significant, considering that the two-photon images do not reveal any significant change in the network structure.

Different studies have suggested that neutrophils are associated with degradation of the ECM. The various enzymes contained in neutrophils (24) have been shown to be capable of removing proteoglycans (25–27), collagen (28), and elastin (29) from the ECM. The visually unaltered collagen and elastin structure in the multiphoton images suggest that the concentration of collagen- and elastin-degrading enzymes is too low to alter these networks significantly. The AFM nanoindentation measurements, however, do show a weakening similar to the weakening of the tissue when the combination of chondroitinase ABC and hyaluronidase was applied. This could imply that there is a sufficient concentration of proteoglycan-degrading enzymes in the neutrophil extract to alter these components of the ECM. The larger spread in measured stiffnesses could be the result of ECM-degrading enzymes that locally damage the collagen and elastin network.

A close look at the individual nanoindentation curves, depicted in Fig. 5, reveals additional information about the mechanical interaction on the fiber level. Although untreated or unaltered tissue shows a steep increase in force upon indentation, we find that when a main component of the ECM is removed (e.g., collagen from the adventitia), we observe not only a decrease in the elasticity of the tissue but also many decreases in force, which we interpret as breaking events (denoted by asterisks in Fig. 5, b–d).

A large number of indentation curves of the neutrophil-treated adventitia show a piecewise linear increasing force upon indentation, indicating that the AFM tip pushes on an individual fiber instead of on a well-connected network. The piecewise linear curves of the neutrophil-treated adventitia show a peak in the stiffness distribution, at $10^4$ and at $10^5$ Pa, depending on the stiffness of the AFM cantilever. This shows that the Hertz model does not apply for these curves, because the slope of the force-distance curve is a measure for the ratio of the stiffness of the cantilever and the fiber on which it pushes, and not for the Young’s modulus of the sample.

A complicating factor of the neutrophil data arises from the fact that the action is consistently nonuniform. Only two out of five different $113 \, \mu m \times 113 \, \mu m$ grids (from two independent experiments) on which the nanoindentation measurements were performed showed a piecewise linear increasing force, revealing an inhomogeneity of the tissue on larger length scales. However, some of the indentations on the three other grids showed a piecewise linear decreasing force on the retracting part only of the indentation. The control measurements, taken at four different locations from two independent measurements, did not show any nonuniformity among the measurement locations.

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characterized by an inflammatory vessel wall containing a highly elevated number of neutrophils (30–32). The fact that an overnight incubation with the contents of activated neutrophils is enough to mimic the change in response from a healthy to an aneurysmatic tissue in terms of both the stiffness and shape of the indentation curve suggests that neutrophils may be responsible for the change in the mechanical properties of aneurysmatic aortic tissues in humans on the nanometer scale.

These measurements show how enzymes secreted by cells can alter the mechanical properties of their surroundings. Other studies have shown that mechanical strain affects the ECM and enzyme production of cells. For example, vascular smooth muscle cells produce more fibronectin, collagen (33), and proteoglycans (34) when strained, and the expression of the collagen-degrading matrix metalloproteinase 2 is also increased (33,35). These effects, which strongly depend on specific interactions between the cells and the ECM (36), have also been shown in skin tissue (37), synovial joints (38), and bone (39,40).

**CONCLUSION**

This study shows how the contribution of the individual components of the ECM to its mechanical properties is different for the adventitial and medial layers of the aorta. Although the removal of collagen, a key element of the ECM, is capable of reducing the local stiffness by up to 50 times, the remaining tissue still forms a coherent network. The revealed microarchitecture and stiffness of the individual network components can be used as a basis for further theoretical simulations to better understand the interplay between fiber mechanics and the network organization.

Furthermore, we believe the significance of this study is demonstrated by the effect of the contents of activated neutrophils on the aortic wall. An overnight incubation with the contents of activated neutrophils was able to reproduce the change in local mechanical properties observed in aneurysms. Although this does not explain the previously observed remodeling of the collagen fibers on a larger length scale (14), it is very possible that the local change in mechanical properties caused by the neutrophils triggers other cells to weave the new collagen fibers to repair the tissue in a different, less ordered way. Further studies are needed to elucidate which components of the ECM are altered by enzymes of the neutrophils, and how this gives rise to a distinct response upon indentation.

We believe that this new (to our knowledge) method for studying the mechanical properties of the ECM will not...
only help physicists gain a better understanding of the mechanical properties of biopolymers, it will also provide a valuable tool for biomedical researchers studying processes such as wound healing, atherosclerotic plaque development, and cervix ripening. These are all processes in which the interaction between cells and their environment results in a remodeling of the ECM.

**SUPPORTING MATERIAL**

A figure, additional methods, and three references are available at http://www.biophysj.org/biophys/supplemental/S0006-3495(12)00382-7.

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**REFERENCES**


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