The mechanical properties of human adipose tissues and their relationships to the structure and composition of the extracellular matrix

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Alkhouli N, Mansfield J, Green E, Bell J, Knight B, Liversedge N, Tham JC, Welbourn R, Shore AC, Kos K, Winlove CP. The mechanical properties of human adipose tissues and their relationships to the structure and composition of the extracellular matrix. Am J Physiol Endocrinol Metab 305: E1427–E1435, 2013. First published October 8, 2013; doi:10.1152/ajpendo.00111.2013.—Adipose tissue (AT) expansion in obesity is characterized by cellular growth and continuous extracellular matrix (ECM) remodeling with increased fibrillar collagen deposition. It is hypothesized that the matrix can inhibit cellular expansion and lipid storage. Therefore, it is important to fully characterize the ECM’s biomechanical properties and its interactions with cells. In this study, we characterize and compare the mechanical properties of human subcutaneous and omental tissues, which have different physiological functions. AT was obtained from 44 subjects undergoing surgery. Force/extension and stress/relaxation data were obtained. The effects of osmotic challenge were measured to investigate the cellular contribution to tissue mechanics. Tissue structure and its response to tensile strain were determined using nonlinear microscopy. AT showed nonlinear stress/strain characteristics of up to a 30% strain. Comparing paired subcutaneous and omental samples (n = 19), the moduli were lower in subcutaneous: initial 1.6 ± 0.8 (means ± SD) and 2.9 ± 1.5 kPa (P = 0.001), final 11.7 ± 6.4 and 32 ± 15.6 kPa (P < 0.001), respectively. The energy dissipation density was lower in subcutaneous AT (n = 13): 0.1 ± 0.1 and 0.3 ± 0.2 kPa, respectively (P = 0.006). Stress/relaxation followed a two-exponential time course. When the incubation medium was exchanged for deionized water in specimens held at 30% strain, force decreased by 31%, and the final modulus increased significantly. Nonlinear microscopy revealed collagen and elastin networks in close proximity to adipocytes and a larger-scale network of larger fiber bundles. There was considerable microscale heterogeneity in the response to strain in both cells and matrix fibers. These results suggest that subcutaneous AT has greater capacity for expansion and recovery from mechanical deformation than omental AT.

adipose; biomechanics; extracellular matrix

* Both of these authors have contributed equally to this article.

http://www.ajpendo.org
weight (38). Each adipocyte is closely encapsulated by an extracellular matrix that has been described as consisting of a basement membrane rich in type IV collagen surrounded by a matrix rich in fibrous collagens (4). There is also a second, larger-scale matrix component in the form of large assemblies of collagen fibers forming interlobular septa associated with vasculature and nerves (4, 7). There have been relatively few studies on the mechanical properties of AT. Most of these have focused on its behavior in compression (11, 21), which is relevant to its function as a shock absorber but not to its function in fat storage. We are aware of only a single group of studies relating the mechanical properties of AT to its structure (6–8). This work, based on uniaxial compression and tear testing of porcine subcutaneous AT, concluded that the interlobular septa make little contribution to the mechanical properties and that the tissue can be modeled as a closed cell foam filled with an incompressible inviscid fluid.

Extension of these studies to human tissue is urgently required to underpin clinical research on obesity and its management. The aims of the present work were to 1) determine the viscoelastic properties of human AT, 2) compare the mechanical properties of subcutaneous and omental ATs [these deposits of fat tissues have different physiological functions, and it is important to understand how these are related to mechanical properties; subcutaneous AT, a triglyceride storage organ, is considered the normal physiological depot for fat in the form of triglycerides (16), whereas omental AT, as part of visceral tissue, has a greater role as a shock absorber surrounding the abdominal viscera, protecting them from physical injury (40)], and 3) relate the mechanical properties to the composition and organization of the extracellular matrix of human AT taking advantage of nonlinear microscopy to visualize structure and dynamic responses to mechanical loading in fresh, unstained tissues (23).

MATERIALS AND METHODS

The use of human tissue conformed to the principles of the Declaration of Helsinki. AT collection was approved by the Exeter Tissue Bank Steering Committee in accordance with their ethical approval requirements (ref. no. 11/SW/0018). Subjects who underwent gynecological, abdominal, and subcutaneous biopsy procedures were recruited by the nurses of the National Institute for Health Research (NIHR) Exeter Clinical Research Facility, and written informed consent was obtained. A medical history was obtained and height and weight measured. Patients with the following conditions were excluded from the study: cancer or suspected cancer, acute or chronic inflammatory disease, steroid treatment in the last 3 mo, and altered mechanical properties. In most cases the AT sample was subdivided for use in all experiments, but this was not always practicable.

Mechanical Testing

Mechanical testing was performed using a custom-built one-dimensional tensile-testing apparatus (shown in Fig. 1, A and B). The hydrated dimensions of the specimen were measured using a micrometer prior to testing, and the specimen, ~8–17 mm in length, 3–6.5 mm in width, and 1.5–3.5 mm in thickness, was attached using a small quantity of high-viscosity superglue gel (Everbuild, Stick 2 Superglue Gel) that was applied to two metal paddles. Approximately 3 mm of the sample at both ends was glued to the paddles. In preliminary experiments, this procedure resulted in less slippage or tissue tearing than clamping. One paddle was attached to a micrometer driven by a pulse generator-controlled stepper motor (Radio Spares, 1.8 Deg/pulse), whereas the other was attached to a rigidly mounted force transducer (WPI, FORT100). Readings from the transducers were logged to a computer using a PicoLog unit (Pico ADC-200). The sample lay horizontally, completely immersed in phosphate-buffered saline (PBS), pH 7.4, at room temperature.

Prior to each measurement, the initial length of the sample between the ends of the paddles was measured at the point where the sample was just straight, and the recorded force first rose above zero. Strain was calculated with reference to the sample length at zero force, and stress was related to geometric area of the sample calculated from the sample’s width and depth. Stress-strain curves were obtained from most specimens; these samples were then subdivided into two groups, one for quantifying the energy dissipation density and the other for osmotic challenge. Energy dissipation density is defined as the irrecoverable energy loss during tensile testing of the AT samples, and is

Table 1. Patient details

<table>
<thead>
<tr>
<th>Bariatric Surgery (Subcutaneous and Omental; n = 5)</th>
<th>Elective Surgery</th>
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<tbody>
<tr>
<td>BMI (range)</td>
<td>Subcutaneous (n = 28)</td>
</tr>
<tr>
<td>44.2 ± 9.5 (36.3–57.7)</td>
<td>30.2 ± 6.4 (21–43.6)</td>
</tr>
<tr>
<td>Sex</td>
<td>25 females</td>
</tr>
<tr>
<td>5 females</td>
<td></td>
</tr>
<tr>
<td>Age, yr (range)</td>
<td>48.3 ± 12.2 (32–82)</td>
</tr>
<tr>
<td>49.4 ± 6.7 (38–55)</td>
<td>8 current smokers</td>
</tr>
<tr>
<td>Smoking status</td>
<td>1 current smoker</td>
</tr>
<tr>
<td>Medical history</td>
<td>4 type 2 diabetes</td>
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</tbody>
</table>

Whole study. Values are means ± SD. BMI, body mass index in kg/m². All patients had no known cardiovascular disease.
the area enclosed within the hysteresis loop of a stress-strain curve, which is calculated from the difference between the area under the sample’s extension curve and its subsequent relaxation curve. The samples for stress relaxation formed a third separate group, as the protocols for osmotic challenge, energy dissipation density, and stress relaxation were not compatible. In a small number of individuals, the tissue samples were sufficiently large to be divided into up to four samples. Measurements on these samples were compared with assessed intrasubject variability, and the mean values for the patient were used in subsequent analysis.

Two groups of experiments were performed.

**Viscoelasticity Measurements**

Force-extension curves were acquired by applying strain at a rate of 5 μm/s up to a 30% strain. The sample was then immediately relaxed at the same rate, allowing the energy dissipation density from straining the tissue to 30% strain to be quantified from the area between the loading and relaxation curves. Preliminary experiments showed that there was a higher risk of samples tearing, which is easily recognized as a decrease in the gradient of the curve, above a 30% strain; therefore, a 30% strain was chosen as the maximum applied strain.

To determine the time course of stress relaxation, samples were strained at a rate of 26 μm/s up to a 30% strain and held at this strain until stress relaxation was complete. Using KaleidaGraph (Synergy Software, Reading, PA), the time course of stress relaxation was fitted by a double exponential function of the form $F(t) = A_1e^{-k_1t} + A_2 + A_3e^{-k_2t}$, where $k_1$ and $k_2$ are the rate constants, $A_1$ and $A_3$ are the amplitudes of the two exponential components, and $A_2$ is the asymptote.

**Osmotic Challenge**

To investigate the contribution of the cells to the tissue mechanics, cell volume was modified osmotically. After an initial stress-strain measurement, the tissue was equilibrated at 30% strain and the PBS in
the bath replaced by deionized water. After the stress had reequilibrated, stress-strain and stress relaxation measurements were performed as described above.

**Multiphoton Microscopy**

Multiphoton microscopy allows confocal imaging of collagens, elastin, and lipids in fresh tissues without sectioning, staining, or other preparation (23). A combination of three imaging modalities was used: two-photon fluorescence (TPF) to visualize elastin fibers using their intrinsic auto fluorescence, second harmonic generation (SHG) for collagen fibers, and coherent anti-Stokes Raman scattering (CARS) for lipids by exciting the Raman modes of the C-H bonds in the lipid hydrocarbon chains.

The microscopy system has been described in detail elsewhere (23). In brief, multiphoton autofluorescence and SHG were excited using 800 nm of light from a mode-locked femtosecond Ti:sapphire oscillator (Mira 900 D Coherent). Picosecond excitation required for using the 816-nm output picosecond oscillator (High-Q Laser Production). For CARS imaging, a combination of three imaging modalities was formed as described above.

**Visualization of Structural Responses to Strain and Osmotic Challenge**

Mechanical testing was combined with multiphoton microscopy to visualize the structural changes that occur during tensile loading and osmotic challenge of AT. The samples were mounted on a custom-built tensile loading rig that was designed to fit onto the microscope stage. Samples were mounted on paddles as described above and laid horizontally, completely immersed in PBS, pH 7.4, at room temperature. Strain was applied using micrometers (Thorlabs MT1/M) with an accuracy of 10 μm, and the force was measured using a 5N force transducer (model 31, RDP). A long-working distance objective (UMPlanFLN 20 × 0.5 NA Olympus) was used to enable the samples to be imaged while immersed in medium.

Image J software (rsweb.nih.gov; National Institutes of Health) was used to analyze the image stacks acquired under different conditions of strain or osmotic pressure.

**Analysis of AT Composition**

Following mechanical testing, the lipid content, content of fibrous proteins, and relative amounts of collagen and elastin were estimated for patients in whom samples of both omental and subcutaneous tissue had been obtained. The specimens were blotted dry and their weight recorded. Lipids were extracted by overnight incubation in excess chloroform-methanol (2:1 vol/vol), followed by washing in three changes of deionized water. The dry weight of the residue was measured, and then it was placed under the multiphoton microscope. Four areas of each sample were imaged with a ×60 objective in TPF and SHG to image the elastin and collagen fibers. The ratio of TPF to SHG was calculated per field of view, and an average value was taken for each sample to provide a relative measurement of the ratio of elastin to collagen.

**Data Analyses and Statistics**

Data are expressed as means ± SD. P < 0.05 was considered significant. Nonparametric tests were used for data analysis. The Wilcoxon matched-pair signed-rank test was used to compare paired samples, and the Mann-Whitney U-test was used to compare unpaired samples. Pearson correlation coefficients were used to examine relationships between variables.

**RESULTS**

**Mechanical Testing**

*Viscoelasticity.* At strains of ≤30%, AT produced nonlinear stress-strain curves, as shown in Fig. 1, C–E. Beyond this, plastic deformation or damage occurred. The value of strain at which this damage occurred varied widely between specimens. In consequence, no quantitative analysis was undertaken beyond a strain of 30%. The tissues were characterized, as indicated in Fig. 1E, by an initial elastic modulus taken as the tangent to the curve at zero strain, by a final elastic modulus taken as the tangent modulus at 30% strain, and by the transition strain (the intercept of the final modulus with the strain axis). In preliminary experiments, the reproducibility of the stress-strain curves over repeated cycles was investigated. The initial and final moduli calculated from the first and fifth cycles differed by <1.2% (n = 4). The energy dissipation density decreased slightly over five cycles, as found in many soft tissues (10). All reported data are for the first cycle.

Measurements were made on 23 omental samples (BMI = 31.1 ± 8.2 kg/m²) and 26 subcutaneous samples (BMI = 32.5 ± 8.8 kg/m²). In 20 patients, paired samples were obtained (BMI = 31.9 ± 8.5 kg/m²).

In a small number of subjects, up to four samples per patient could be tested. The intrasubject average standard deviation for the initial modulus was 1 kPa [mean initial moduli 2.2 kPa, coefficient of variation (CV) = 43%], and for the final modulus it was 12.1 kPa (mean final moduli 23.1 kPa, CV = 48%).

Subcutaneous and omental tissues were compared in individuals where both tissues were obtained as paired samples. As shown in Table 2, both the initial and final moduli were significantly lower in subcutaneous than omental AT samples (P ≤ 0.001, n = 19; BMI 31.9 ± 8.7 kg/m²). The area of the energy dissipation density loop, a measurement of energy dissipation over the stretch-relaxation cycle, was also significantly lower in subcutaneous than omental AT paired samples (P = 0.006, n = 13; BMI 30.9 ± 6.3 kg/m²).

As illustrated in Fig. 1G, the stress was relaxed over ~50 min and followed a two-exponential course. Measurements made in 12 unpaired specimens (Table 3) showed that for omental tissue the two time constants differed by almost one

<table>
<thead>
<tr>
<th>Value</th>
<th>Subcutaneous</th>
<th>Omental</th>
<th>P Value</th>
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<tbody>
<tr>
<td>Initial modulus, kPa</td>
<td>1.6 ± 0.8</td>
<td>2.9 ± 1.5</td>
<td>0.001</td>
</tr>
<tr>
<td>Final modulus, kPa</td>
<td>11.7 ± 6.4</td>
<td>32 ± 15.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Energy dissipation density, kPa</td>
<td>0.1 ± 0.1</td>
<td>0.3 ± 0.2</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 13. AT, adipose tissue. For the the participants from whom the initial and final moduli were measured, BMI = 31.9 ± 8.7 kg/m² (n = 19). For the participants from whom the energy dissipation density was measured, BMI = 30.9 ± 6.3 kg/m².
order of magnitude but were similar in amplitude. In subcutaneous tissue the two time constants also differed by almost one order of magnitude, but the dominant component had a time constant intermediate between the two omental time constants, and the second component in subcutaneous was extremely variable and small in amplitude. There were no significant differences between the time constants of omental and subcutaneous AT samples.

**Osmotic challenge.** When PBS was exchanged for deionized water while the tissue was held at 30% strain, there was a decrease in the force of 31 ± 9% ($P = 0.005$, $n = 10$) in omental and 31 ± 10% ($P = 0.002$, $n = 12$) in unpaired samples of subcutaneous AT. There were also changes in the stress-strain characteristics, as illustrated in Fig. 1F and summarized in Table 4. There was an increase in the initial modulus by 66 ± 88% in omental and 120 ± 235% in subcutaneous and a significant increase in the final modulus by 182 ± 10% in omental and 259 ± 236% in subcutaneous, with a decrease in the transition strain of ~10% in omental and an increase of 10% in subcutaneous AT samples.

**Multiphoton Microscopy**

Preliminary experiments compared the structure of fresh tissue with specimens that had been either frozen and thawed or formalin fixed. Both procedures caused cell shrinkage, which served to reveal the pericellular matrix, which contained both collagen and elastin but presumably distorted its normal architecture (Fig. 2A).

In total, 14 omental and 14 subcutaneous unpaired fresh samples were imaged (BMI 35.6 ± 10.4 kg/m²).

All tissue samples displayed considerable heterogeneity on a microscopic scale, but two types of structure were generally observed in the same specimen. Throughout the bulk of the tissue (Fig. 2C), the cells were almost completely space-filling with smooth outlines, which obscured the very fine pericellular matrix shown in Fig. 2A, but a network of fine collagen fibers spanned the region with sparse elastic fibers. However, there were also regions containing a much higher proportion of matrix, with coarser collagen fibers and some elastin fibers (Fig. 2E). The diameter of the elastin fibers varied, with some regions showing fibers ≤6 μm and others showing fibers <1 μm in diameter. These features were seen in both the intact tissues and digested samples.

**Visualization of Responses to Strain and Osmotic Challenge**

Mechanical testing of five subcutaneous and five omental samples (BMI 26 ± 4 kg/m²) was performed on the stage of the multiphoton microscope. Changes in cell and matrix organization were observed as incremental strains were applied to the tissue up to a 30% strain.

These changes differed between regions, with some regions showing deformations in the cells and pericellular collagen, whereas others showed larger-scale reorientations, with the interfibrillar collagen taking most of the tension. Both modes of deformation were observed in both omental and subcutaneous AT. Comparing Fig. 2, D and C, shows that both the cells and their pericellular matrices have deformed when the sample is strained by 30%. Even within this microscopic field there is substantial heterogeneity in the response. Some cells retain their circular outline, whereas others elongate by ~45.5% in the direction of applied strain, with an average deformation of 21%. We also see the cells translate and reorientate with respect to one another within the field of view. We presume that the changes in cellular boundaries correspond to changes in pericellular matrix, as close inspection of the images reveals collagen fibers following the cell boundaries. In contrast, two blood vessels running through this region of tissue do not appear to be under tension.

Comparison of Fig. 2, E and F, shows that in this region the strain is taken mostly by the interfibrillar collagen fibers and the cells remain circular at 30% applied strain. Instead, the tissue deforms on a larger scale as a gap opens up between the two groups of cells within the image. At 30% strain, interfibrillar collagen fibers connecting the two areas of cells are seen to be under tension. In addition, some fibers straighten out and change angle, revealing their branched structure, and are seen to be connecting to the interfibrillar collagen network. It was noteworthy in all of the images examined that tension in the collagen fiber network was not distributed evenly. Whereas many of the fibers in the strained tissues were straight, indicating that they were under tension, others remained convoluted.

| Table 3. Parameters for the time course stress relaxation equation |
|-------------------|-------------------|-------------------|-------------------|
|                   | Omental            | Subcutaneous       |
| $A_1$ (mN)        | $6.3 \pm 2.6       | $1.7 \pm 0.5^*    |
| $k_1$, s⁻¹        | $0.008 \pm 0.005   | $0.004 \pm 0.003  |
| $t_1$, min        | $2.1 \pm 3.3       | $4.2 \pm 5.6      |
| $A_2$, mN         | $12.1 \pm 5.4      | $4.2 \pm 2.8^*    |
| $A_3$, mN         | $6.6 \pm 3.5       | $-0.4 \pm 3.2^*   |
| $k_2$, s⁻¹        | $0.001 \pm 0.0003  | $0.0004 \pm 0.001 |
| $t_2$, min        | $16.7 \pm 55.6     | $41.7 \pm 16.7    |
| $C_{ch}$          | $2.785 \pm 3393    | $247 \pm 158      |
| $r$               | $0.99 \pm 0.01     | $0.98 \pm 0.01    |

Values are means ± SD. Chisq, chi-squared distribution. For omental samples, BMI = 26.1 ± 2.5 kg/m², ($n = 6$) and for subcutaneous samples BMI = 30.9 ± 6.9 kg/m², ($n = 6$); $k_1$ and $k_2$ are the rate constants, and $t_1$ and $t_2$ are the time constants (min). $A_1$ and $A_2$ are the amplitudes of the 2 exponential components, $A_3$ is the asymptote and $r$ is the goodness-of-fit coefficient. *$P < 0.05$.

| Table 4. Effects of osmotic challenge on mechanical parameters |
|-------------------|-------------------|-------------------|
|                   | Omental            | Subcutaneous       |
|                   | PBS                | Deionized water    | $P$ value |
|                   | PBS                | Deionized water    | $P$ value |
| Initial modulus   | $2.7 \pm 1.3       | $3.6 \pm 1.2       | 0.074     |
| Final modulus     | $24.1 \pm 15.5     | $61.2 \pm 36.6     | 0.005     |
|                   | $1.2 \pm 0.6       | $1.7 \pm 0.9       | 0.028     |
|                   | $8 \pm 6.5         | $20.9 \pm 12.9     | 0.002     |

Values are shown as percentage changes from the values in PBS. For omental tissue BMI = 29.8 ± 10.8 kg/m² ($n = 10$), and for subcutaneous BMI = 30.6 ± 11.52 kg/m² ($n = 12$).
In tissue exposed to osmotic challenge with deionized water while under strain on the stage of the microscope (n = 3), CARS imaging did not show any change in the appearance of cells, presumably because contrast was derived largely from the lipid droplets rather than the plasma membrane. Careful examination of matrix fibers also failed to reveal any reorientation under osmotic stress (data not shown).

**AT Analysis**

The content of fibrous proteins was analyzed in paired samples from 11 subjects (BMI = 28.3 ± 6.5 kg/m²). In six of these subjects (BMI = 28.2 ± 7.7 kg/m²) the ratio of elastin to collagen was also determined.

Omental AT samples had a higher content of fibrillar proteins than subcutaneous AT samples (8 ± 4.1% in omental, 4.5 ± 1.9% in subcutaneous, P = 0.026). Multiphoton microscopy showed that the majority of the fibers were collagen. However, there was no difference in the elastin/collagen ratio between omental and subcutaneous AT (TPF/SHG ratio 0.073 ± 0.043 in omental and 0.072 ± 0.04 in subcutaneous).

The correlations between the initial and final moduli and the compositional data on individual specimens were investigated. A significant relationship was found between the ratio of initial to final modulus and the elastin/collagen ratio, which reached statistical significance in subcutaneous AT (r = 0.87, P = 0.025) but not in omental tissue, where the individual data points were more widely scattered.

**DISCUSSION**

The nonlinear stress-strain behavior we observed in tensile testing of human adipose tissue was qualitatively similar to those reported in animal tissues under a variety of testing regimes, including trouser tear tests and confined and uniaxial compression tests (6–8, 11). To investigate the viscoelastic properties of AT, strains of up to 30% were applied based on preliminary experiments, which showed that there was a higher risk of samples tearing at strains greater than 30%. However, there are also indications that AT is exposed to such high strains in humans in vivo (21).

We compared the mechanical properties of subcutaneous and omental fat. These tissues have different physiological roles. In addition to fat storage, subcutaneous AT provides thermal insulation and acts as a shock absorber, providing padding at various anatomic sites (2, 20), whereas omental AT is one of the visceral AT depots, surrounding the intestines superficially (41) and among other things cushioning and protecting inner organs from physical injury (40). As a fat storage organ, subcutaneous AT should have greater capacity for expansion with lipid storage than omental AT. The greater low-strain compliance of subcutaneous AT and its greater distensibility may be appropriate in its role as the normal physiological depot for fat in the form of triglycerides (16). Adipocytes need to be able to expand to store excess triglycerides during periods of positive energy balance or overeating and also to decrease in size when the triglycerides are released during negative energy balance or decreased dietary intake (41). It is increasingly accepted that the capacity for expansion of AT with its storage capacity may not only modify the obesity phenotype but determine the amount of storage of surplus triglycerides as ectopic fat deposition and with it determine the risk of obesity-associated complications such as diabetes (18, 24, 30, 34).

Our results also reveal that subcutaneous AT has lower energy dissipation density than omental AT and slower stress relaxation. This is compatible with omental AT’s function as a good shock absorber to protect the viscera, but in subcutaneous AT there is the additional requirement that it retains its shape to maintain the outer contour of the body. The energy dissipation density depends both on the rate of strain and the amount of strain. Therefore, our numerical values depend on the conditions under which the experiments were performed, and until we know how to replicate the conditions to which the tissue is exposed in vivo, their main point is to draw attention to the fact that AT dampens mechanical responses.

The time constants demonstrate that stress relaxation occurs over minutes to tens of minutes, with no significant differences between omental and subcutaneous depots. Therefore, the stress-relaxation properties are likely to be important only in situations where AT is subject to sustained compressive loading. Although the structural bases of stress relaxation are not presently clear, these are likely factors determining, for example, the risk in the generation of bed sores.

As we discuss later, the structure of the tissue samples and their mechanical properties were heterogeneous on a microscopic scale. To account for potential artifacts relating to shape variation and deformation during the mechanical studies, we performed preliminary work in developing and validating our methodology. First, we compared various methods of mounting the specimens and found that gluing was most effective in not introducing local strains into the tissue. Second, we placed fiducial markers on the tissue surface and showed that the longitudinal strain distribution was acceptably uniform. Third, using equine adipose tissue where we could obtain large quantities of relatively uniform samples, we investigated the relationship between sample dimensions and calculated modulus and found no significant correlation. Therefore, we believe that although the changes in sample dimensions, etc., may contribute to the scatter in our measurements and are reflected in the quoted standard deviations, they do not amount to order of magnitude uncertainties.

There is another more fundamental concern in our relating mechanical properties to geometrical sample size, because as we show from the microscopy, stresses and strains are very heterogeneous distributed between components and between

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**Fig. 2.** Multiphoton images of human AT using coherent anti-Stokes Raman scattering (CARS), 2-photon fluorescence (TPF), and second harmonic generation (SHG), with lipids shown in red (CARS), collagen in blue (SHG), and elastin in green (TPF). A: section of previously frozen AT showing pericellular matrix (containing both collagen and elastin) around the cells. B: subcutaneous AT from an area rich in fibrous proteins showing dense elastin and collagen fibers alongside adipocytes. C and D: a subcutaneous AT sample studied at 0 and 30% strain, respectively. In this region, many cells are deformed and displaced under strain, and the matrix fibers reorientate. Two blood vessels (V) are seen running through the field of view. E and F: an omental AT sample imaged at 0 and 30% strain, respectively. In this sample the adipocytes retain their circular cell shape, and the interlobular collagen fibers are seen to experience the most strain, as a gap opens up between 2 groups of adipocytes in the top and bottom left of the image.
regions of tissue. Making the appropriate adjustments is a task for the future, but we feel that the indices we use allow us to compare the bulk mechanical properties of the different tissues.

Whole sample analysis demonstrated that omental tissue had a higher proportion of fibrous proteins than subcutaneous, and in both, the proportion of collagen greatly exceeded that of elastin. However, in paired samples from the same individual, there was no consistent difference in the proportion of collagen to elastin between the two depots. In individual tissue specimens a greater proportion of elastin corresponded to higher distensibility and a greater difference between the initial and final moduli, although overall, the relationship was statistically significant only in subcutaneous tissue. In many soft tissues it has been found that the initial modulus reflects the application of loads to the elastin network, and the final modulus represents the transfer of loads to the collagen network (14, 31). The correlation we observe between the initial and final moduli and the microscopically derived elastin/collagen ratio suggests that the same is true of adipose tissue. Despite the suggestion that subcutaneous and visceral adipose tissue depots from obese subjects have a higher degree of fibrosis than tissues from lean subjects when assessed by picrosirius red staining of immunohistological preparations (35), no correlation could be established between mechanical properties and the BMI of individual patients (data not shown), although a larger study covering a wider range of BMIs may be able to do so. At a microscopic level there was considerable heterogeneity in both the structure and strain response of the tissue. This heterogeneity may be responsible for the large standard deviations found in most of the macroscopic mechanical parameters. Multiphoton imaging was consistent with previous light and electron microscopic studies that have drawn attention to the rich networks of collagen fibers in both the pericellular and interlobular matrices in AT (4, 5, 7, 17, 26, 29). However, it is clear that elastin also constitutes a small but potentially important component of these networks. The two proteins have very different mechanical properties; elastin fibers are extremely flexible and give connective tissue resilience and flexibility (19), complimenting the function of collagen fibers, which are very stiff and provide the tissue with tensile strength (19), generally forming the main load-bearing component in the tissue (25). The elastin fibers provide long-range elasticity (28) and appear to contribute both to the initial elastic modulus and the more rapid phase of stress relaxation. At a cellular level, elastin may be important in allowing the pericellular matrix to accommodate changes in cell volume and thereby may play an important role in fat storage. In this connection it is important to note that, in our unfixed tissue, the pericellular fibers are in much closer apposition to the cell than it appears in most fixed preparations.

Under tensile strain, significant loads were imposed on the coarser interlobular fibers, consistent with their presumed role in supporting the large-scale organization of the tissue. Lobules could be drawn apart, apparently reversibly, although whether this might occur under physiological loads is questionable. In other regions, strain was transmitted through the network of finer fibers that are presumably responsible for maintaining the coherence of the cellular clusters. The cells themselves showed a heterogeneous response to strain, with some remaining unaffected and others suffering both distortion and significant displacement. Whether this is associated with differences in the mechanics of individual cells or other aspects of their environment and is an important factor in the response of the tissue to mechanical loads is questions for further study. The physiological significance of the large strains applied is questionable, although they are seen in vivo during sitting (21); however, they are an invaluable guide in understanding the connectivity and mechanical interactions between the tissue components.

Cellular disruption by osmotic challenge altered the mechanical properties of the tissue. This observation conflicts with the only previous study of which we are aware, which attempts to relate the mechanical properties of AT to its structure and composition. On the basis of tear testing of porcine AT, Comley and Fleck (6–8) developed a mechanical model that discounted any contribution of cells to the observed mechanical properties. The nature of the osmotic disruption of the cells in our experiments was not clear. If the osmotic challenge is sufficient to rupture the cell membrane, as is the case with most mammalian cells, then an explanation for the observed decrease in force required to hold the samples at constant strain and the increase in the moduli may be that cell rupture might release tension in the pericellular matrix, resulting in a decrease in the stress at constant strain, and/or allow realignment in the direction of prevailing stress, giving a higher modulus when the sample is restretched. It is unfortunate that the pericellular matrix could be visualized only after cell shrinkage. This may in part be due to the relatively weak SHG signal and be accounted for by the presence of significant quantities of type IV collagen in the pericellular matrix, which does not generate a SHG signal (33). The CARS images did not reveal any changes in lipid distribution during osmotic challenge. However, these were dominated by the high concentration of lipid in the vacuole, so we cannot report on the integrity of the plasma membrane. Studies on the mechanics of isolated adipocytes are required further to clarify this issue and to obtain a platform for rigorous discussion of the hypothesis that the pericellular matrix limits adipocyte swelling in obesity. In this connection, it is noteworthy that multiphoton microscopy confirmed previous reports (36) that the adipocytes of subcutaneous AT are larger than omental. Therefore, a study of this sort may reveal additional biomechanical differences between the two tissues.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

N.A., J.M., J.B., B.K., N.L., J.C.T., R.W., and C.P.W. performed the experiments; N.A., J.M., and E.G. analyzed the data; N.A., J.M., E.G., A.C.S., K.K., and C.P.W. interpreted the results of the experiments; N.A. and J.M. prepared the figures; N.A. and C.P.W. drafted the manuscript; N.A., J.M., A.C.S., K.K., and C.P.W. edited and revised the manuscript; A.C.S., K.K., and C.P.W. approved the final version of the manuscript; K.K. and C.P.W. contributed to the conception and design of the research.
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