INTRODUCTION

Type 2 Diabetes (non-insulin-dependent diabetes mellitus, T2D) has detrimental impacts on multiple organ systems including the renal, circulatory and nervous systems. Destructive effects are also apparent on the musculoskeletal system, although studies are limited in comparison. T2D likely affects bone and other collagen-based tissues through multiple mechanisms. Chronic hyperglycemia in T2D has been implicated in the formation of advanced glycation end products (AGEs) which form when reducing sugars react with free amino groups in proteins. The formation of AGEs in Type I collagen could lead to a stiffening of fibrils and, in the case of bone, this may lead to decreased toughness. However, few studies have directly investigated the collagen ultrastructure in diabetic tissues. The purpose of this work was to study the nanoscale morphology of Type I collagen in mineralized and non-mineralized tissue from normal and diabetic rats.

SIGNIFICANCE

Despite the importance of a healthy skeleton to overall health and well-being, it is still not clear how properties of the fundamental constituents of bone at the nanoscale ultimately translate into a stiff, strong and tough material that is capable of handling the daily demands that are placed on it. The contribution of this study will be to directly detect nanoscale changes in collagen in a model of T2D with known tissue and structural level mechanical defects.

METHODS

Zucker diabetic Sprague-Dawley (ZDSD) and control rats from the Charles River Laboratory (CD: Sprague-Dawley-derived) were used with prior approval (IAUC #3146). At 15 weeks of age, all animals were switched from regular chow (Purina 5008) to a high fat test diet (Test diet 5SCA) for 3 weeks to induce Type 2 diabetes in the ZDSD rats. The diet for all rats was switched back to regular chow at 18 weeks of age, and all rats remained alive until sacrifice by CO2 inhalation at 28 weeks of age. The left tibia and tail from 4 rats in each group were harvested, wrapped in saline-soaked gauze and stored at -20º until needed.

From the tibia of each rat, a 1.5 cm section from just distal to the tibia-fibula junction was sectioned, mounted to a steel disk (anterior side up), and a flat polished surface was created using a 3 μm diamond suspension. Each bone was treated for 20 minutes with 0.5M EDTA at a pH of 8.0 followed by sonication for 5 minutes in water. This process was repeated 4 times. From the tail of each rat, ~75 mm lengths of individual tendon fascicles were removed and placed in phosphate buffered saline (PBS). Each fascicle was placed on a glass slide and gently flattened with curved forceps. The fascicle was allowed to dry just long enough to adhere to the glass before imaging.

Samples were imaged using a Bruker Catalyst AFM in peak force tapping mode. Images were acquired from 4-5 locations in each bone using a silicon cantilever with a silicon probe (tip radius ~ 8 nm). At each location, 5-15 fibrils were analyzed on the nanoscale images (approximately 70 total fibrils in each of 4 samples per group). Images were acquired from 1-2 locations in at least 3 fascicles per tail using a silicon nitride cantilever with a silicon probe (tip radius ~ 2 nm). At each location, 5-15 fibrils were analyzed in 5 μm x 5 μm error images (approximately 90 total fibrils in each of 4 samples per group).

2D Fast Fourier Transform analysis was performed on individual fibrils and the first harmonic peak was analyzed to determine the value of the D-periodic spacing for that fibril. Values measured from individual samples were averaged, yielding a single value for that sample (n=4 for each tissue and treatment group). CD values were then compared with those from ZDSD in each tissue using a Student’s T-test. To investigate differences in fibril morphology distributions within each tissue, a Kolmogorov-Smirnov (KS) test was performed on the cumulative distribution function (CDF) in each group. For all tests, a value of p<0.05 was considered significant.

RESULTS

Measurements within each sample were averaged to yield the mean fibril spacing for that sample. In bone, the overall mean value was 66.1 ± 0.8 nm for CD and 66.5 ± 1.5 nm for ZDSD, respectively. A t-test indicated no statistical difference between groups (p=0.584). In tendon, the overall mean value was 68.4 ± 0.2 nm for CD and 68.6 ± 1.2 nm for ZDSD, respectively. A t-test similarly indicated no statistical difference between groups (p=0.699). A distribution of spacings existed in each tissue and group (Fig 1, 2). These distributions were statistically different between CD and ZDSD bone (Fig 3, n=274 in CD, n=294 in ZDSD, p=0.015) as well as between CD and ZDSD tendon (Fig 4, n=371 in CD, n=350 in ZDSD, p<0.001).

DISCUSSION

The ZDSD rat was chosen as a model of T2D in the current study. Based on the breeding method used to develop this strain, the ZDSD rat does not possess any leptin or leptin receptor defects. In response to dietary manipulation, ZDSD rats can gradually develop a T2D-like condition which simulates human adult-onset diabetes. Previous work has demonstrated marked decreases in mineral density and structural mechanical properties in the femur and L4 vertebral bodies of these rats, in addition to tissue-level changes in vertebral including decreased strength, modulus and toughness. Although the current results cannot be directly correlated with this previous work, alterations in collagen nanoscale structure observed here may be an important part of the mechanism for these changes. Changes in the D-period in collagen have been noted in bone with estrogen deficiency and with genetic changes in collagen leading to Osteogenesis Imperfecta. However, no differences have been noted between various normal tissues or between the bones of normal male and female mice. These findings support the conclusion that the structural changes in collagen in the bone and tendon of diabetic rats noted here are real and may be a manifestation of the presence of AGEs in the collagen structure. In this study, differences in the D-period in collagen were much more pronounced in tendon versus bone. In CD tendons, almost 74% of all fibrils fall within the mean ± 1 standard deviation (SD) versus about 55% for the ZDSD tendons over the same range. The ZDSD population is shifted to higher D-spacings, with 27% of fibrils above this range versus 12% in CD fibrils. Although the ZDSD fibril population in bone also is shifted higher, the change is more subtle. The presence of mineral in the bone samples likely makes the overall population spread wider and more variable, possibly masking the more dramatic change seen in the tendon samples.

In conclusion, although the average collagen D-periodic spacing in ZDSD bone and tendon did not differ from controls, both tissues in ZDSD rats had significant differences in spacing population distributions, with the difference in tendon being highly significant. Current studies are underway to investigate the nanomechanical impacts of these structural changes in air and in fluid, and to verify the presence of AGEs in these tissues.

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