Age-related changes in the physical properties, cross-linking, and glycation of collagen from mouse tail tendon

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Running title: *Changes in collagen crosslinks and glycation with age*

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Abstract

Collagen is a structural protein whose internal cross-linking critically determines the properties and functions of connective tissue. Knowing how the cross-linking of collagen changes with age is key to understanding why the mechanical properties of tissues change over a lifetime. The current scientific consensus is that collagen cross-linking increases with age and that this increase leads to tendon stiffening. Here, we show that this view should be reconsidered. Using MS-based analyses, we demonstrate that during aging of healthy C57BL/6 mice, the overall levels of collagen cross-linking in tail tendon decrease with age. However, the levels of lysine glycation in collagen, which is not considered a cross-link, increased dramatically with age. We found that in 16-week-old diabetic db/db mice, glycation reaches levels similar to those observed in 98-week-old C57BL/6 mice, while the other cross-links typical of tendon collagen either decreased or remained the same as those observed in 20 week old WT mice. These results, combined with findings from mechanical testing of tendons from these mice, indicate that overall collagen cross-linking in mouse tendon decreases with age. Our findings also reveal that lysine glycation appears to be an important factor that contributes to tendon stiffening with age and in diabetes.

Main

The literature surrounding the mechanical and chemical properties of collagen and changes which occur with age is extensive. The general consensus is that as collagen ages there is an increas

disagreement[5,6] with respect to the exact chemical structure the analysed compound represents within collagen, for the purposes of this publication it can be considered an indicator of aldol crosslinks present in collagen before analysis.

These crosslinks are early structures in the enzymatic crosslinking process and are often described as immature crosslinks. The immature crosslinks can go on to form irreversible crosslinks[1] (pyridinolines) often described in the literature as "mature" crosslinks through further reactions. It has been noted in numerous papers that the number of immature crosslinks per mole of collagen decreases with age and that the increase in mature pyridinoline crosslinks does not seem to match this decrease[7,8,9], implying that the loss of immature crosslinks cannot be entirely explained by their conversion into mature crosslinks. In papers describing mature crosslink formation the emphasis is usually on the increase i

crease

be between 0.04% and 0.06% collagen lysine content per day in tail tendon showing little change with age. (Figure S1c).

Profiling changes in the physical properties of tail tendon with age. The stress-strain profiles for multiple tail tendons from 5 mice from a range of ages were measured using a tensile stress stage. There are 4 tendons for each vertebral bone in the tail which run from the base of the tail to each bone insertion point in four bundles of tendons. The individual tendons were carefully dissected out from the bundles for use. Individual tendons were stretched to breaking point at a rate of 1mm/min and the stress measured every 0.5s. The stress-strain profiles (Figure 2) for the fibres from younger animals were more homogeneous across the population than those from older animals. Tendon from 10 week old mice showed an initial stretching phase followed by a long plastic extension phase before breaking. The stress-strain profile of tendons from older animals had a much greater spread, some fibres still showing a plastic extension phase, with others breaking earlier at a lower strain with only a modest plastic extension phase. With increasing age a greater proportion of the tendons showed an increase in the gradient of the initial stretching phase, that is the stiffness (Youngôs Modulus) of the tendons increases. The initial stretching phase in animals of all ages extended to about 5% strain before transitioning into the plastic phase. Young adult tendons in the 20 week group frequently reached a greater strain before breaking than that seen in either younger tendons or tendons greater than 1 year. The picture of changes seen here is more subtle than the impression usually presented in the

that seen in the 20 week WT mice. The level of irreversible crosslinks in the db/db mice, both the mature and AGE classes, were lower than those seen for 20 week WT mice.

Discussion

Within the literature of ageing collagen the focus is usually on the increase of mature crosslinks formed through mature covalent bonding as the reason for the changing properties observed. What we have seen here shows that the situation is more complex than this and that both the loss of reversible crosslinks and increase in lysine glycation are important changes to consider in the overall picture of how tendon collagen functions.

The often stated view that tendon stiffens with age due to an increase in crosslinking does not fit the entire data set shown here, and in the light of the db/db data it would appear that the mechanical changes do not have to be linked to an increase in AGEs such as glucosepane or pentosidine. With the marked decrease in crosslinking in db/db mice it might be expected that the tendons would be less stiff than older WT mice, however, the stiffness of the db/db tendons is similar to that of older WT mice. The one clear factor that could give rise to the observed stiffness of db/db tendon is the increased glycation of lysine residues which is similar to that found in tendon from 98 week mice. It is apparent from the kinks and wobbles in the db/db stress-strain profiles that there are faults in the tendon structure. It is likely that these faults are due to the lower levels of crosslinking explaining why the diabetic tendon is often unable to maintain structure under stress.

While tendon does

to the vertebral bone. Tendon was isolated sequentially one vertebral bone at a time, working up the tail from the tip to the base. Tendon fibres were detached from the tail vertebrae with a scalpel. Cellular and soluble extracellular components were removed from tail tendon by washing in 3% triton in a continuous flow system (~200ml / 24 hours) for 3 days, followed by washing in water for 2-3 days (~200ml / 24 hours).

Of note here is that that the chemistry of the immature crosslinks is potentially reversible, any processing that changes chemical environment before reduction could perturb the equilibrium and impact the results[16,17]. For example, when we incubated skin with a 50% glycerol solution in PBS pH 7.4 for 2 hours we found after reduction there was a 37% drop in the level of HLNL and a 27% drop in Lys glycation. Figure S3 shows the impact on tendon collagen of using TES/Tris buffer as commonly used[14] when compared to PBS that we use in this work. It can be seen that in TES/Tris buffer, HLNL is 13tiused

$^{13}C_6$ Hx-Lysine, $^{12}C_6$ Hx-Lysine and $^{12}C_6$ cHx-lysine were made in house as standards to enable calibration curves to be constructed. The response of the $^{13}C_6$ Hx-Lysine ISD was found to be the same as that for $^{12}C_6$ Hx-Lysine. The response of $^{12}C_6$ cHx-lysine was found to be a

mixture of *N*-glycated lysine (by NMR) which looked the same as endogenous glycated lysine by mass spectroscopy. It is likely that some racemisation occurred at chiral centres in this synthesis and this accounts for some or all of the additional complexity seen in the NMR spectra. This synthesis enabled a $^{13}C_6$ internal standard to be synthesised which behaved well in the mass spectroscopy assay.

N -benzyloxycarbonyl-L-lysine (200mg, 1eq) was added to glucose (1.55g, 12eq) in methanol (30ml). The reaction was refluxed for 4 hours and then allowed to cool. Sodium borohydride (324mg, 12eq) was then added and the reaction left to stir overnight. 1M Hydrochloric acid (3ml) was then added and the solution concentrated to an oil. Water (30ml) was added and the pH adjusted to 4. The solution was loaded onto a 5g C18 SPE cartridge (BondElute, Varian), washed with 5mM HCl (20ml) and the glycated materials eluted with 10% acetonitrile. The solution was then freeze dried. HPLC showed a number of peaks which were analysed by mass spectroscopy. The largest peak had the correct mass of 445.5 (MH+). Starting with the crude glycated material (80 mg), pure mono glycated Z-Lys (14mg) was isolated after 3 rounds of purification on a C18 Luna 10x250mm column with a 5% to 50% B gradient (A:9819 (A)15.00001 (:9819 (5 Td[r)-272.019 (i)-4.00363 10182 (M HC)5i2_612v/TT1 11.04 TT1 11.04 Tf73 (

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Author contributions

JC, M.Stammers, II, IN designed and performed experiments and analysed data. AS-P carried out statistical analysis. JC wrote the paper and directed the research. M.Streeter and DAS synthesised and provided the ^{12}C and ^{13}C labelled glucosepane standards.

Competing interests

The authors declare no competing financial interests.

Data availability

All data are available from the corresponding author upon reasonable request.

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Glycated (linear) hydroxylysine (Hly-Hx)	327.18	82.08	30	0.2
pentosidine	379.21	187.1	40	0.2
Deoxypyridinoline (DPD)	413.2	84.08	40	0.3
Pyridinoline (PYD)	429.2	82.08	40	0.3

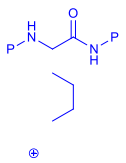

Figure 1 The structures of LNL, HLNL, and DHLNL analysed by mass spectroscopy after treatment with sodium borohydride. The relationship to lysine and hydroxylysine, and the intermediate imine crosslink structures found in collagen are also shown above.

Figure 2 Plots showing representative stress-strain profiles from multiple tendon fibres at four ages. Each age group was sampled from five mice.

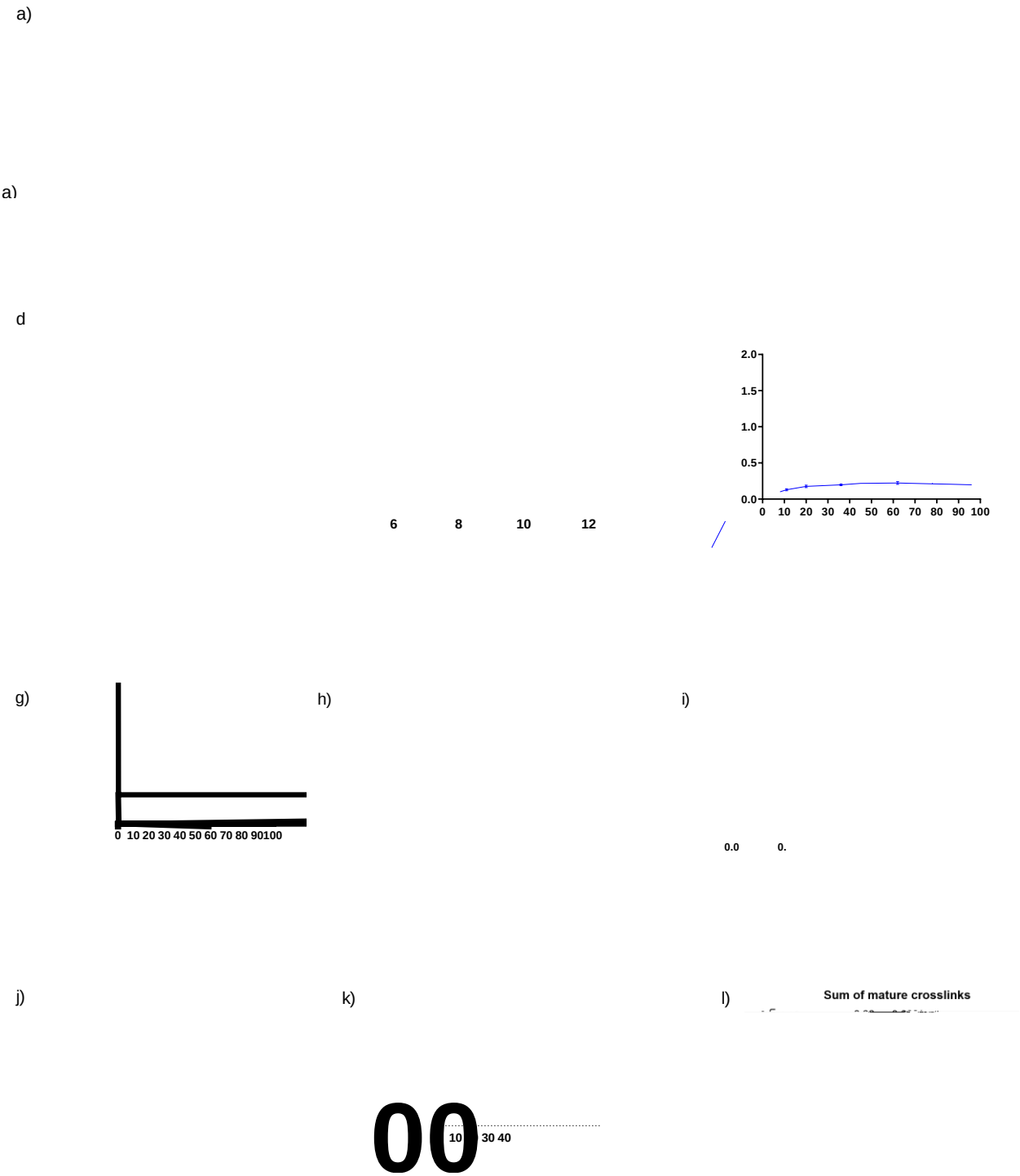

Figure 3a, b, c Graphs showing the levels of the immature crosslinks LNL, DHLNL and HLNL with age in the tendon fibres of C57BL/6 mice (n=5 per age group, in duplicate). Statistical analysis: Nonlinear regression fit shown in the HLNL plot (± SD). 3d Shows the change in the aldol product HHMD with age. Statistical analysis: ANOVA followed by Tukey's multiple comparisons tests (mean ± , n=5 to 10). 3e Shows correlations between HLNL and HHMD with age. Statistical analysis: linear regression. 3f Shows the levels of glycation products with age (mean ± , n=5, in duplicate). Lys glycation shown in green, Hly glycation shown in blue. 3g,h,j,k show the development of irreversible crosslinks with age in

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