The Thermal Stability of Collagen in Diabetic Rats: Correlation with Severity of Diabetes and Non-Enzymatic Glycosylation

D. K. Yue¹, S. McLennan³, L. Delbridge², D. J. Handelsman¹, T. Reeve² and J. R. Turtle¹

Departments of ¹Medicine and ²Surgery, University of Sydney and ³Department of Endocrinology, Royal Prince Alfred Hospital, Sydney, New South Wales, Australia

Summary. The thermal stability of collagen fibres from rat tail tendon was studied in both normal and diabetic rats. Results were correlated with the age of the animals, glycosylated haemoglobin level and the degree of non-enzymatic glycosylation of the collagen. Age was found to be the most important single determinant of the thermal rupture time of collagen fibres ($r=0.87$, $p<0.005$). Thermal rupture time was also increased in diabetes and showed good correlation with glycosylated haemoglobin ($r=0.69$, $p<0.005$) and non-enzymatic glycosylation of collagen ($r=0.51$, $p<0.005$). The correlation of glycosylated haemoglobin level and thermal rupture time

was significant, independent of age and non-enzymatic glycosylation of collagen. Experiments in vitro showed that incubation of collagen fibres with glucose increased the thermal stability of collagen. These results demonstrated an independent effect of severity of diabetes on the physical properties of collagen. The role of non-enzymatic glycosylation in causing the changes in collagen properties in diabetes remains to be fully elucidated.

Key words: Diabetes, rat collagen, thermal stability, glycosylated haemoglobin, ageing, non-enzymatic glycosylation.

Collagen is the major macromolecule of most connective tissues and has a wide variety of important functions [1–4]. It undergoes characteristic changes during the process of ageing and this has been the subject of many previous investigations [3, 4]. In both man and animals, diabetes leads to changes in collagen fibres resembling those that occur with increasing age [5–7]. It has been suggested that these effects of diabetes represent an acceleration of the normal ageing process [5]. However, the relationship between these two conditions is far from certain. In both diabetes and ageing an increased amount of hexosyllysine, formed by the process of non-enzymatic glycosylation, can be recovered from the collagen [8–10]. This post-translational modification of proteins has also been demonstrated in other tissue and is probably a general phenomenon [11–13]. Its possible role in the alteration of collagen properties remains to be fully evaluated. Further understanding of this area is important as disturbance of collagen metabolism could have serious effects on its many physiological functions, and may thus be of fundamental importance in the development of diabetic complications.

In this study the thermal stability of collagen fibres from the tail tendon of streptozotocin-induced diabetic rats was investigated. Results were correlated with the age of the animals, glycosylated haemoglobin level and

the extent of non-enzymatic glycosylation. The possible significance of direct effects of glucose on collagen was further explored by in vitro incubation experiments.

Materials and Methods

Animals

Inbred female Wistar rats (weight: 205 ± 8 g) were obtained from the Atomic Energy Commission of Australia, Lucas Heights. Diabetes was induced by IV injection of streptozotocin (65 mg/kg) and confirmed by blood glucose measurement (Dextrostix) of > 22 mmol/l 3 days later. In all 39 animals (28 diabetic and 11 littermate non-diabetic control rats) were studied in two groups.

Group 1: 12 diabetic animals in which SC insulin treatment (4 U/day) was commenced 2 weeks after induction of diabetes and continued for 2–6 weeks duration before sacrifice. Age-matched untreated diabetic rats ($n=6$) and normal littermates ($n=6$) were killed at the same time as controls. The duration of diabetes in this group was 4–8 weeks.

Group 2: Six diabetic rats were treated with insulin (4 U/day) for 2 to 6 weeks after diabetes had been present for 22 weeks. Again four age-matched untreated diabetic and five normal littermates were used as controls and killed at the same time as the insulin-treated diabetic rats. The duration of diabetes in this group was 24–28 weeks.

Animals were anaesthetized with halothane and exsanguinated by cardiac puncture. The degree of hyperglycaemia was assessed by

Table 1. Thermal rupture time in normal and diabetic animals

Thermal rupture time (min)			
	Normal rats	Diabetic rats	Insulin-treated diabetic rats
Group 1	7.5 ± 1.4 (n=6)	13.6 ± 2.2 ^a (n=6)	11.9 ± 2.2 ^a (n=12)
Group 2	29.2 ± 2.1 (n=5)	58.0 ± 14.3 ^b (n=4)	52.9 ± 11.0 ^b (n=6)

Results expressed as mean ± SD. The number of animals in each group is indicated in parentheses. The duration of diabetes was 4–8 weeks in Group 1 and 24–28 weeks in Group 2. Significance of differences from normal rats: ^a $p < 0.0002$ ^b $p < 0.004$

measurement of glycosylated haemoglobin. Tail tendons were removed as described below for measurement of thermal stability and non-enzymatic glycosylation.

Assessment of Thermal Stability of Collagen

Thermal stability was assessed by measuring the thermal rupture time of collagen fibres in urea (7 mol/l) at 40 °C using a technique modified from Boros-Farkas and Everitt [14]. After sacrifice, intact tendons were serially removed from the tail with bone crushers, dried in air on filter paper, and subsequently stored at –4 °C until required. Individual tendons were cut to a standard length of 10 cm and intact collagen fibres, in the weight range of 3.5–4.5 mg, obtained by gentle teasing of the tendon. Each fibre, with a 3 g weight attached to the bottom, was suspended by alligator clip and immersed in a glass tube containing urea solution (7 mol/l) kept in a water bath maintained at 40 °C. Breaking times were measured by an electronic timing apparatus. Four strips of collagen fibres from each tail tendon were studied and the mean breaking time taken as the thermal rupture time. The mean coefficient of variation of measurement in quadruplicate was 13.7%.

Measurement of Glycosylated Haemoglobin

Glycosylated haemoglobin in the normal, diabetic and insulin-treated diabetic rats was measured by a method of m-aminophenylboronic acid chromatography described previously [15]. In this method glycosylated haemoglobin is retained by m-aminophenylboronic acid immobilized on solid phase and subsequently eluted by excess sorbitol. The normal range of this method was 5%–10% and the coefficients of variation of the high, middle and low control samples over 30 consecutive measurements were 10.7, 8.4 and 9.2%, respectively. This technique is not affected significantly by the reversible aldime phase of the glycosylation reaction.

Measurement of Non-enzymatic Glycosylation of Collagen

Non-enzymatic glycosylation of collagen was measured by m-aminophenylboronic acid chromatography using an adaptation of a method described by Vlassara et al. for the measurement of non-enzymatic glycosylation of peripheral nerve protein [16]. In this method collagen fibres (approximately 40 mg weight) obtained from rat tail tendon were minced finely with scissors and suspended in phosphate buffer A (50 mmol/l, pH 7.0) to a concentration of 20 mg/ml (dry weight). Tritiated sodium borohydride (NaB^3H_4 , 200 μl , specific activity 228–484 mCi/mmol) was added to 1 ml of the suspension and allowed to react for 40 min at room temperature. The reduction was stopped by placing the incubation mixture on ice. Afterwards the reaction product was dialyzed against 5 litres of phosphate buffer A with four changes over 24 h; a procedure shown to remove NaB^3H_4

and free glucose completely. After NaB^3H_4 reduction and dialysis, 0.8 ml of treated protein was hydrolyzed in HCl (6 mol/l) for 16 h at 108 °C. The resultant mixture of amino acids was adjusted to pH 9.0 by the addition of NaOH (7 mol/l) and the amino acid concentration measured by Fluram (Roche, Switzerland), using L-leucine as standard. M-aminophenylboronic acid immobilized on Biogel P-6 (Affi-gel 601) was purchased from Bio-Rad Laboratory, Richmond, California, USA, and swollen in phosphate buffer B (50 mmol/l, pH 9.0). It was packed into a 2.0 × 1.0 cm column and equilibrated with 10 ml of phosphate buffer B. Tritiated amino-acids obtained by NaB^3H_4 reduction and acid hydrolysis of collagen (20 mg amino-acid) were applied to the column washed with 10 ml of phosphate buffer B and collected in one fraction. The eluting fluid was then changed to 10 ml of HCl (0.25 mol/l) and collected. Radioactivity in each fraction was monitored by counting 200 μl in 10 ml of Phase Combining System II liquid scintillation fluid. Radioactivity recovered in the acidic peak represents tritiated glycosylated amino acids retained by the column (by the reaction between m-aminophenylboronic acid and the 1,2 cis-diol group of the carbohydrate moiety) and is an estimate of non-enzymatic glycosylation. Reducible cross-links of collagen would not be retained due to the absence of carbohydrate groups. Results were expressed as radioactivity in the acidic peak (DPM/ μg amino-acid) loaded onto the column. Recoveries of radioactivity from the column were greater than 90%.

Glycosylation of Collagen Fibres In-vitro

Collagen fibres were obtained from a normal rat and divided into equal amounts (100 mg); each portion was incubated for 7 days at 37 °C in phosphate buffer A containing glucose up to a concentration of 50 mmol/l. Afterwards the fibres were dried on filter paper and thermal rupture time measured as described in previous section.

Data Analysis

All results are expressed as mean ± SD. Results were analysed by correlation coefficients after confirmation of a normal distribution. Because of the high degree of inter-correlation of thermal rupture time, age, glycosylated haemoglobin and non-enzymatic glycosylation, analysis of variance was not appropriate and the results were treated by an analysis of partial correlation coefficients [17]. Since the effect of age was dominant (79% of total variance) both diabetic and control rats were pooled to determine the regression parameters of thermal rupture time on age. Age adjustment of the thermal rupture time was carried out after confirmation of linearity in our own data. Analysis was performed by the BMDP6R programme implemented on a Digital 11/70 computer [18] (Digital Equipment Corporation, Maynard, Mass, USA).

Results

The thermal rupture times of collagen fibres from diabetic animals were greater than those of the controls in the two groups of rats with either short or long durations of diabetes ($p < 0.002$). In both groups the mean thermal rupture time was not significantly reduced by insulin treatment and remained elevated in comparison with controls ($p < 0.004$). Results are summarized in Table 1. When all animals were taken into consideration, age was the most important determinant of thermal rupture time ($r = 0.87$, $p < 0.005$). As expected glycosylated haemoglobin was not related to age ($r = 0.09$), but non-enzymatic glycosylation of collagen did correlate positively with increasing age ($r = 0.45$, $p < 0.002$) and with glycosylated haemoglobin ($r = 0.47$, $p < 0.002$).

Fig. 1. The relationship of glycosylated haemoglobin and non-enzymatic glycosylation of collagen with age-adjusted thermal stability of collagen ($r=0.69$, $r=0.51$ respectively, $p<0.005$ for each), ▲ = normal, ◆ = untreated diabetic, ■ = insulin-treated diabetic. DPM = Disintegration per min

Fig. 2. The dose-response relationship of glucose incubation on thermal stability of collagen. Incubation was at 37°C for 7 days

As age was such an important determinant of thermal rupture time ($r=0.87$) it was necessary to remove its influence before the independent effect of glycosylated haemoglobin or non-enzymatic glycosylation on the properties of collagen (i.e. above and beyond that due to age) of the 39 animals could be assessed as a group. The relationship between age-adjusted thermal rupture time with glycosylated haemoglobin and non-enzymatic glycosylation of collagen is shown in Figure 1. When

age was taken into consideration, thermal rupture time remained significantly correlated with glycosylated haemoglobin and non-enzymatic glycosylation of collagen ($r=0.69$ and 0.51 respectively, $p<0.005$).

To assess the relative importance of glycosylated haemoglobin and non-enzymatic glycosylation of collagen acting alone as determinant of the tail collagen strength, the thermal rupture time (adjusted for both age and non-enzymatic glycosylation) was calculated. This remained highly correlated with glycosylated haemoglobin ($r=0.68$, $p<0.005$). By contrast, thermal rupture time, adjusted for age and glycosylated haemoglobin, was no longer significantly related to non-enzymatic glycosylation of collagen ($r=0.24$, $p=0.08$).

A clear dose-dependent effect on thermal rupture time was demonstrable when rat tail collagen was incubated with glucose. The thermal rupture time was found to lengthen progressively as glucose concentration was increased (Fig. 2).

Discussion

Collagen has been shown to undergo a number of biochemical and biophysical changes with increasing age [1–4]. The increased thermal stability of rat tail tendon collagen with ageing is one such parameter [14]. It is now apparent that diabetes also has profound effects on the properties of collagen in both man and experimental animal models of diabetes [5, 7]. In the present study the effects of ageing and diabetes on collagen were confirmed, with age being the single most important determinant of thermal stability. The changes induced by diabetes were rapid in onset, being clearly demonstrable after hyperglycaemia of only 4–8 weeks duration. In this study the collagen changes were not normalized by insulin treatment, raising the possibility that any established changes may not be easily reversible, due to the slow turnover of collagen. However the good correlation between thermal rupture time and glycosylated haemoglobin suggests that the changes in the properties of collagen are potentially preventable provided early and optimal metabolic control of diabetes can be achieved.

Precisely how ageing and diabetes affect the properties of collagen is not well established and it is uncertain if diabetes and ageing cause these alterations by the same mechanism. Age changes in collagen are related to the progressive formation of intermolecular cross-links between the terminal regions of neighbouring collagen molecules [1–4, 19]. Many factors have been postulated to be important in controlling this process: these include the many enzymes regulating the post-translational modification of collagen [4, 19]. Diabetes may modify collagen fibres by any of these mechanisms.

The relationship of non-enzymatic glycosylation to cross-linking in collagen is less clear. The increased

post-translational non-enzymatic glycosylation of protein in diabetes is not a phenomenon restricted to haemoglobin [9–13]: using a technique combining tritiated sodium-borohydride reduction with m-aminophenylboronic acid chromatography, we have confirmed an increase in non-enzymatic glycosylation of tail tendon collagen in diabetes and ageing. This correlates with glycosylated haemoglobin and increases progressively with age. It has been predicted on theoretical grounds that the hexosyl radicals which become attached to the ξ -NH₂ group of lysine cannot serve as a site of cross-linkage as its reactive group would no longer be available for binding [8]. Our finding that thermal rupture time, after adjustment for age and glycosylated haemoglobin, showed no significant correlation with non-enzymatic glycosylation of collagen could be construed as being consistent with this concept. However, we feel that a positive role of non-enzymatic glycosylation in this regard is not excluded. Non-enzymatic glycosylation of collagen and haemoglobin are inter-related and it is likely that adjustment of glycosylated haemoglobin would also remove any possible significance of non-enzymatic glycosylation of collagen. The *in vitro* incubation experiment in which thermal rupture time was increased by glucose supports the possibility that non-enzymatic glycosylation may indeed have effects on properties of collagen. Other workers have also reported the possible role of non-enzymatic glycosylation on the formation of cross-links [20] and further work is currently in progress to substantiate this point. It is noteworthy that glycosylated haemoglobin remains significantly correlated with thermal stability even after adjustment for age and non-enzymatic glycosylation of collagen. This would be consistent with the view that severity of diabetes has additional effects on thermal rupture time independent of non-enzymatic glycosylation. Whether this represents effects of enzymatic glycosylation, enzyme activity or other mechanisms remains to be explored.

Collagen is the major structural component of most tissues in the body. Alterations in its properties may be of fundamental importance in the aetiology of diabetic complications. The characteristics of collagen vary significantly in different organs and the effects of diabetes are likely to differ accordingly [7]. Further studies need to be performed before the significance of these changes in collagen in diabetes can be satisfactorily assessed.

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Dr. D. K. Yue
Department of Medicine
University of Sydney
Sydney, NSW, 2006
Australia