Glycoconjugate Journal (1997) **14:** 917–923

Nonenzymatic glycation of cartilage proteoglycans: an *in vivo* and *in vitro* study

Hemlata K. Pokharna* and Lawrence A. Pottenger

Section of Orthopedic Surgery, University of Chicago Medical Center, MC 6032, 5841 S. Maryland Ave, Chicago, IL 60637, USA

In this study we have investigated whether proteoglycans (aggrecan) are modified by nonenzymatic glycation as in collagen. Purified human aggrecan from osteoarthritic and normal human knee articular cartilage was assayed for pentosidine, a cross-link formed by nonenzymatic glycation, using reverse-phase HPLC. In addition, an *in vitro* study was done by incubation of purified bovine nasal cartilage aggrecan with ribose. Pentosidine was found in all the purified human aggrecan samples. 2–3% of the total articular cartilage pentosidine was found in aggrecan. Purified link protein also contained penosidine. The *in vitro* study led to pentosidine formation, but did not appear to increase the molecular size of the aggrecan suggesting that pentosidine was creating intramolecular cross-links. Similar amounts of glycation were found in osteoarthritic and normal cartilage. Like collagen, aggrecan and link proteins are crosslinked by nonenzymatic glycation in normal and osteoarthritic cartilage. Crosslinking could be reproduced, *in vitro,* by incubating aggrecan with ribose.

***Keywords*: aggrecan, link protein, pentosidine, cross-links, nonenzymatic glycation**

1. Introduction

Age related changes in the extracellular matrix have been attributed to changes in the intra- or intermolecular collagen cross-links [1–3]. Stable covalent cross-links are known to be formed in collagen by both enzymatic and nonenzymatic processes. One such enzymatic process is initiated by lysyl oxidase and results in the formation of pyridinoline crosslinks.

In addition, spontaneous chemical reactions between proteins and sugars lead to nonenzymatically formed cross-links that appear to increase with age [4]. The process of nonenzymatic glycation of proteins is a very complicated and poorly understood. Lack of guidance of glycation by enzymes results is the creation of multiple advanced glycosylated endproducts (AGE) of which pyrraline and pentosidine have been identified in extracellular matrix proteins [5, 6]. Pentosidine is a pentose mediated cross-link between lysine and arginine. It has been shown to be present in increasing amounts with age and diabetes, in plasma proteins [7], lens crystallins [8] and collagen-rich tissues [9–11]. The age related changes caused by AGE cross-linking in collagen-rich tissues, result in loss of elasticity, thickening and scleroses [12]. At a physiochemical level, collagen becomes less soluble and less digestible by collagenase [13], increasingly thermostable [1], and acquires covalently bound fluorophores [14].

Large cartilage proteoglycans (aggrecan) are long lived components of cartilage [15]. Aggrecan consists of a protein backbone to which multiple chains of chondroitin sulfate and keratan sulfate are attached. At the N-terminal end of the protein there is a region that is capable of simultaneously binding hyaluronic acid and link proteins. Link proteins are also capable of binding hyaluronic acid [16]. In articular cartilage, aggrecan aggregates form when as many as 100 individual aggrecan bind to one long hyaluronic acid chain with link proteins assisting in binding of individual aggrecan.

Although aggrecan are long lived proteins and therefore potentially susceptible to postsynthetic nonenzymatic modifications, there is no information in the literature about AGE in proteoglycan macromolecules. In this study, we analyzed purified human aggrecan for the presence of pentosidine. Since *in vitro* incubation of proteins with either hexose sugars, pentose sugars, or ascorbic acid under oxidative conditions has been shown to produce pentosidine cross-linking [17], we attempted to produce pentosidine crosslinking *in vitro* by incubating young bovine aggrecan with glucose, ribose and ascorbic acid.

Materials and methods

Tissue preparation

Cartilage samples were obtained from patients undergoing surgery for knee replacement due to osteoarthritis, and from

*To whom correspondence should be addressed. Tel: (773) 702-6308; Fax: (773) 702-4378; E-mail: hpokharn@surgery.bsd.uchicago.edu

0282–0080

individuals at autopsy. The mean age of patients from whom articular OA cartilage was obtained was 69.4 years (range = 50–92 years, $n = 9$). Control articular cartilage obtained from knee at autopsy was from subjects with mean age of 52.5 years (range 28–80 years, $n = 8$). Only macroscopically normal appearing cartilage from OA and control cartilage was used in the study. Cartilage for *in vitro* incubation studies was collected from bovine calf nasal septum obtained from a slaughterhouse and kept at $-20\,^{\circ}C$ until extraction.

Total pyridinoline and pentosidine was determined in intact cartilage samples and in residual cartilage after extraction with 4 M GdnHCl, as well as, purified proteoglycan aggregates and monomers.

Isolation and purification of proteoglycans

Proteoglycans were extracted from 1 mm pieces of cartilage following procedures described by Kahn *et al.* [18]. The cartilage pieces were associatively extracted for 24 h at 4 °C with 10 volumes of buffer containing protease inhibitors. Extracted proteoglycans were allowed to aggregate by dialysis against nine volumes of buffer without GdnHCl, and then purified by CsCl density gradient centrifugation under associative conditions. The bottom fractions (A1) containing proteoglycan aggregates were collected. Purified aggrecan monomer (A1D1D1) were obtained from A1 fractions by two CsCl density gradient centrifugations under dissociative conditions [19]. Aggrecan fractions were lyophilized after dialysis against water. Aggrecan concentrations were estimated by uronic acid analysis [20].

Isolation of link proteins

Link protein was isolated separately from cartilage obtained from four patients undergoing knee replacement. Link protein was isolated from fractions A1D5 and further purified by two gel permeation chromatographies on a Sephacryl S-200 column. The column effluent was monitored for protein by reading $\mathbf{A}_{280}$. Midportion of the peak, which was found to be pure by SDS gel electrophoresis, was analyzed for pentosidine. The extinction coefficient $E_{1\,cm}^{0.1\%}$ at A^{280} of purified human link protein was found to be 1.14 following the procedure described by Tang *et al.* [21].

In vitro crosslinking of proteoglycans

Bovine calf proteoglycan A1A1 fraction was incubated in triplicates in the presence of oxygen at 37 °C with either 500 mM ribose, or 25 mM ascorbic acid, or 100 mM glucose. Since zinc is known to associate link proteins [22], 100 μM $ZnCl_2$ was added to another 500 mM ribose incubation. Incubation proceeded over 4 weeks with weekly sampling. In another experiment aggregates were incubated with various concentrations of ribose for 4 weeks. Some aggregates from each group were incubated under antioxidative conditions in the presence of 1 mM diethylenetriaminepentaacetic acid (DTPA) and 1 mM phytic acid and nitrogen [17]. Control groups contained only aggregates in buffer. All incubations were done in 0.1 M phosphate buffer at pH 7.4 with a drop of toluene to prevent bacterial growth. After incubation, fractions were subjected to dissociative centrifugation (D1) and analyzed for the presence of pentosidine.

Column chromatography

Aggrecan (A1D1D1) from the *in vitro* incubation with ribose were placed on a Sepharose CL-2B column (120×0.8 cm), and eluted with buffer (pH 7.0) at a rate of 1.2 ml h^{-1} [18]. The column effluent was monitored for uronic acid. Fractions from the aggrecan peak were collected as five pools for pentosidine analysis.

Pyridinoline and pentosidine determination

Samples in distilled water were lyophilized and then hydrolyzed in 6 N HCl at 110 °C for 24 h. Hydroxyproline was assayed by the modified method of Stegemann and Stadler [23]. Pyridinoline and pentosidine were quantified by reverse phase high performance liquid chromatography (HPLC) as described by Eyre *et al.* [24].

The presence of pentosidine in the HPLC run was confirmed in representative samples using dual chromatographic system by collecting fractions eluting from HPLC using a Waters automated column switching valve set to collect fractions eluting from 2 min prior to and 2 min post elution of standard pentosidine [7]. The 5 ml fractions were concentrated under reduced pressure with speedvac system and then rehydrated with loading buffer for HPLC, using cation exchange column (protein pak SP-5PW, Waters Chromatography) with a gradient of NaCl from 0–40 min in 0.02 M sodium acetate buffer at pH 4.7. Pentosidine eluted at 25.3 min. Pyridinoline and pentosidine standards were kindly provided by Dr Vincent Monnier (Case Western Reserve University, Cleveland, OH).

Coefficient of variation

A piece of cartilage for one subject was cut into four pieces, these were hydrolyzed and analyzed separately for hydroxyproline, pyridinoline and pentosidine. The coefficient of variation for each was calculated, using $CV = (\sigma/X)100$ where CV is the coefficient of variation, σ is the standard deviation, and X is the observed mean [25]. CV for hydroxyproline was 5.5%, for pyridinoline 5% and for pentosidine 3.4%.

Statisical analysis

For the differences among the groups nonparametric analysis was used because of nonparametric distribution of data. The Mann-Whitney rank sum test was used for comparison between two groups using Sigma plot software. Linear

equations, and regression coefficients were generated using Microsoft Exel software. *p*-values less than 0.05 were considered significant.

Results

Analysis of cartilage specimens

Five samples of control and four samples of OA cartilage were analyzed in terms of original wet weight. The average amount of pentosidine in the control and OA samples was 11.8 pmol mg^{-1} (range 2.4–33.0) and 9.4 pmol mg^{-1}(range 6.7–15.1), respectively. In the same tissues, the concentrations of pentosidine in purified aggrecan were determined. Extrapolating the concentrations of pentosidine in aggrecan to the total amount of aggrecan as determined by uronic acid gave an estimate of the total pentosidine in aggrecan to be 0.13 pmol mg^{-1} (range 0.02–2.30) in control cartilage and 0.21 pmol mg^{-1} (range 0.03–0.46) in OA cartilage. The amounts of pentosidine calculated as percentage of total cartilage pentosidine in aggrecan was 2.77% (range 0.57–11.0) for control tissue and 2.83% (range 0.32–6.89) in OA cartilage.

In a larger group of specimens the residual cartilage after extraction with 4M GdnHCl was studied to estimate the amount of pentosidine (Table 1). The mean concentrations of pyridinoline and pentosidine did not differ significantly in normal and OA cartilage. Studies of OA human articular cartilage by Takahashi *et al.* [10] using similar methods found average pyridinoline concentrations of 3.6 mmol mol^{-1} hydroxyproline and average pentosidine levels of 0.107 mmol mol^{-1} hydroxyproline. These findings are in agreement with our findings. In our study, pentosidine was approximately 3–5% of pyridinoline present in the residual cartilage.

Age dependence of the amount of cross-linkage

Figure 1 shows the correlation of age of the subjects from whom the cartilage was obtained with molar concentrations of pyridinoline and pentosidine per mole hydroxyproline in the hydrolysates of residual cartilage after 4 M GdnHCl extraction. Pyridinoline from OA and normal cartilage showed no correlation with age ($y = 0.012x + 3.16$, $R^2 = 0.004$ and $y = -0.019x + 5.32$, $R^2 = 0.063$, respectively), whereas pentosidine increased with age ($y = 0.008x -$

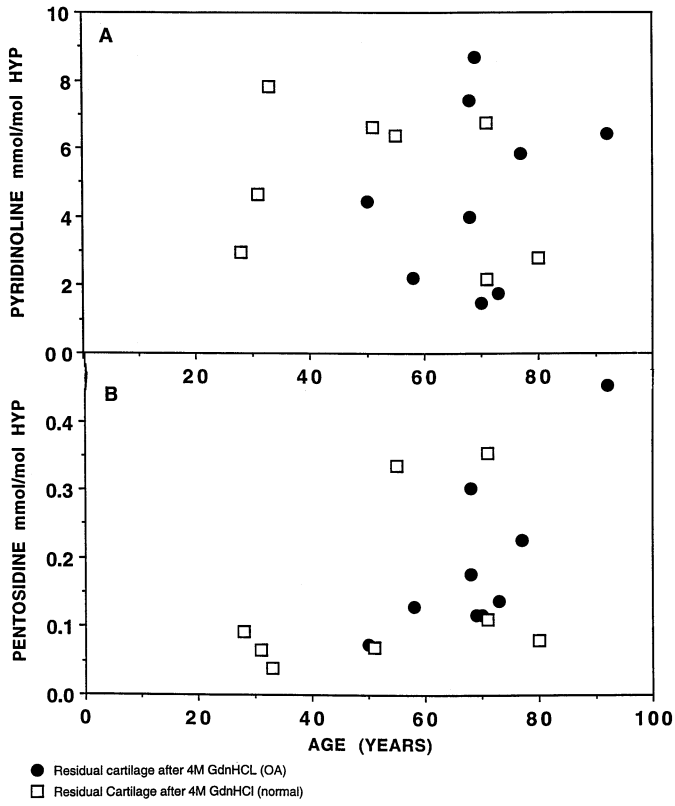

Figure 1. Age-related changes in the content of pyridinoline (A) and pentosidine (B) in residual cartilage obtained after extraction with 4 M GdnHCl extraction. Cartilage was obtained from knees of patients with OA during surgery and normal cartilage at autopsy. Cartilage samples were hydrolyzed in 6 N hydrochloric acid and crosslinks were estimated by reverse phase HPLC.

Table 1. Analysis of *in vivo* crosslinks in human cartilage fractions

	Pyridinoline		*Pentosidine*	
	Normal (n = 8)	*OA (n = 9)*	*Normal (n = 8)*	*OA (n = 9)*
	(mmol mol^{-1} hydroxyproline)		*(mmol mol^{-1} hydroxyproline)*	
Residual cartilage after 4 M GndHCl extraction	4.31 ± 0.55	3.58 ± 0.74	0.14 ± 0.04	0.19 ± 0.04
			(mmol mol^{-1} uronic acid)	
Aggrecan aggregates			0.011 ± 0.002	0.005 ± 0.001
Aggrecan monomers			0.006 ± 0.002	0.005 ± 0.002

Data are given as mean ± SEM.
There is no significant difference in pentosidine between normal and OA samples as *p* was >0.1 in all comparisons.

0.365, $R^2 = 0.621$ for OA and $y = 0.0024x + 0.016$, $R^2 = 0.155$ for normal) consistent with continued modification of the collagen after maturity.

Detection of pentosidine in proteoglycans

Both purified aggrecan aggregates and monomers from human specimens contain measurable amounts of pentosidine (Table 1). There is no difference in pentosidine between normal and OA samples ($p > 0.1$). No pyridinoline or hydroxyproline was detected in the same samples, which indicates that the pentosidine found in the purified aggrecan aggregates was not due to contamination by collagen. As shown in Figure 2, there is a large variation in pentosidine levels which is largely due to different amounts of pentosidine present in the tissue as the coefficient of variation of the assay itself is very small. The concentration of pentosidine in purified monomers from OA and normal cartilage showed no significant correlation with age ($y = 0.0001x + 0.0006$, $R^2 = 0.021$ and $y = -0.0001x + 0.009$, $R^2 = 0.096$ respectively).

Pentosidine concentration in the electrophoretically pure link protein pooled from 4 patients was 34.7 mmol mol^{-1} protein, assuming a molecular weight of 50kD.

In vitro glycation of proteoglycans

Figure 3 shows the effect of incubating aggrecan aggregates from bovine calf nasal cartilage with different glycating agents. After incubation, aggrecan monomers were obtained by dissociative CsCl density gradient centrifugation. Incubation of aggrecan with either glucose or ascorbic acid, formed no detectable pentosidine, while ribose incubation led to the appearence of significant amounts of pentosidine within the first week, which did not change significantly over the next three weeks. The addition of zinc to the ribose incubation, reduced pentosidine synthesis to levels significantly less than ribose alone ($p < 0.001$) in 4 weeks. This inhibition of pentosidine formation was unexpected since zinc is known to cause link proteins to associate [22].

Figure 4 shows the effect of incubating bovine nasal aggregates with various concentrations of ribose. Pentosidine increased significantly at 100 mM ribose concentration ($p < 0.002$) after 4 weeks of incubation compared to control. There was an unexpected decrease ($p < 0.005$) in pentosidine at 500 mM ribose concentration as compared to pentosidine at 100 mM ribose.

Incubation of aggrecan with ribose under antioxidative conditions led to the formation of only 3.5% of the pentosidine formed in presence of oxygen.

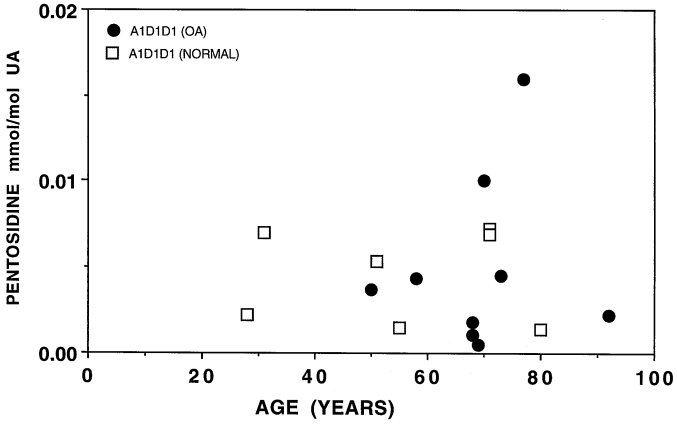

Figure 2. Changes in the content of pentosidine in purified aggrecan (A1D1D1) extracted from OA and normal human cartilage of different ages. Aggrecan was purified by 4 M GdnHCl extraction followed by centrifugation under associative and dissociative conditions and analyzed for pentosidine estimation.

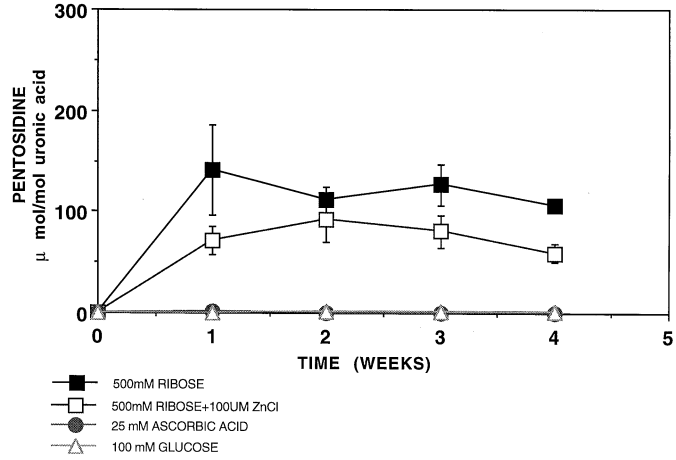

Figure 3. *In vitro* glycation of proteoglycans: Effect of incubating bovine proteoglycan aggregates with different glycating agents including ribose, ascorbic acid, glucose and ribose with zinc chloride, over a period of 4 weeks. Proteoglycans extracted from bovine nasal cartilage were incubated in presence and absence of different glycating agents. After incubation, fractions were subjected to dissociative centrifugation and analyzed for the presence of pentosidine.

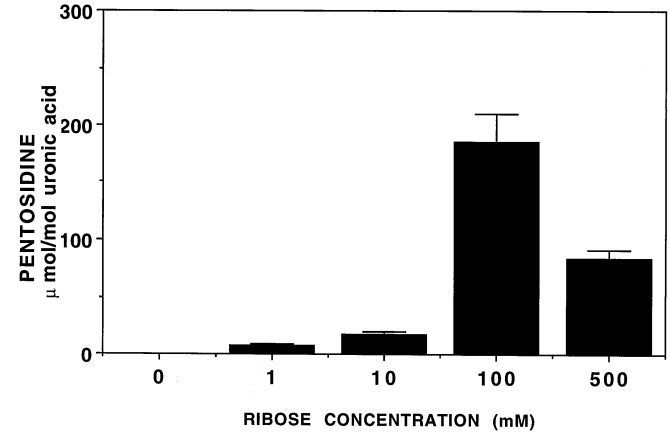

Figure 4. Effect of ribose concentration on pentosidine formation in aggrecan over a period of 4 weeks.

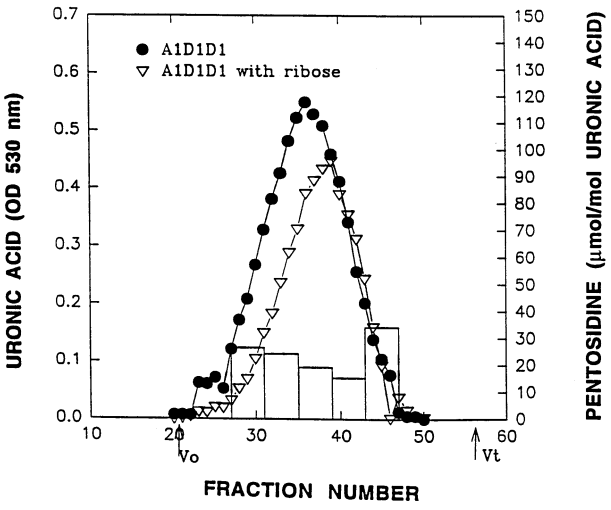

Figure 5. Sepharose CL-2B analysis of purified aggrecan monomers (A1D1D1) from bovine nasal cartilage incubated for 90 days in the absence and presence of 500 mM ribose at 37 °C. Elution profiles depict uronic acid content as measured by reaction with carbazole. Bar graph reflects concentration of pentosidine in pooled samples of ribose treated monomers.

Column chromatography

Aggrecan monomer fractions incubated with ribose were analyzed by Sepharose CL-2B chromatography to determine if *in vitro* glycation led to increase in the size of the monomers, which would suggest that the monomers were cross-linked together by pentosidine. As shown in Figure 5, monomers that were incubated with ribose did not appear to be larger than those incubated with saline alone, suggesting that the cross-linking occurred within individual monomers, rather than between monomers. There appeared to be a small shift in the ribose incubated profile to lower molecular weights, which may be due to sugar induced protein fragmentation reactions [17]. Pentosidine was present throughout the peak indicating the presence of pentosidine in large and small aggrecan molecules. This eliminates the possibility that pentosidine may have been in the purified aggrecan monomer as a contaminant of non-aggrecan proteins, which would have had lower molecular weight.

Discussion

In this study, we used pentosidine as a marker of the presence of AGE in human cartilage proteins. In general, pentosidine comprises only a small percentage of the total AGE produced [26] and the percentage of pentosidine compared to total AGE on specific proteins probably varies greatly. The relative positions of lysines and arginines on the protein may favor lysine-lysine cross-links or types of lysine-arginine cross-links other than pentosidine, and the array of AGE produced may be dependent upon the type of sugar participating in the reaction. In *in vitro* studies, the best estimate of total AGE may be found by measuring the amount of lysine and arginine residues in the molecules that have not been modified [27]. We used ribose in our *in vitro* glycation studies to accelerate the creation of pentosidine. Ribose has been found to be 130 times more reactive than glucose in producing browning reaction in proteins [28] and 50 times as reactive in producing pentosidine [29]. Pentoses are so much more reactive than hexoses that it has been hypothesized that the small amount of pentoses in the extracellular fluid may contribute significantly to AGE synthesis [14].

Since the prerequisite for a Maillard reaction is the presence of free amino groups, either on proteins or amino acids, and reducing sugar, all proteins containing arginine and lysine can undergo nonenzymatic glycation. This is the rationale used to propose the role of the Maillard reaction in the cellular and molecular damage that occurs with aging and diabetes [26]. The differences in the accumulation of pentosidine in different tissues as a function of age is thought to be due to different tissue protein turnover rates. Rapid turnover of most proteins may thus act as a natural mechanism to eliminate this sugar mediated damage, whereas the long lived proteins such as collagen succumb to AGE accumulation [27]. Studies by Takahashi *et al.* [10] quantifying amounts of pentosidine and pyridinoline in human articular cartilage from patients with bone and joint disorders have shown that pyridinoline remains constant after skeletal maturity, while pentosidine increases with age, indicative of on going chemical modification. Similar findings have been reported by Uchiyama *et al.* [11] in aging human articular cartilage obtained from patients, which was also the case in the present study. Studies of the Maillard reaction mediated cross-links in aging and osteoarthritic cartilage suggest pentosidine to be primarily associated with collagen [4, 6, 9–11, 30].

The present study shows the presence of pentosidine in aggrecan and link proteins. The accumulation of pentosidine in cartilage aggrecan can be attributed to the pool of proteoglycan which has a half life of many years as compared to the other aggrecan pool whose mean half life is about 1–2 years [15]. There was no significant difference in pentosidine levels in aggrecan derived from control and OA cartilage which appeared macroscopically normal. The fact that AGE appear to render collagen less digestible by proteases [13], suggests that AGE may be partially responsible for the increased longevity of proteoglycans if they block sites of proteolysis.

Although aggrecans are long lived, the amount of pentosidine in aggrecan did not increase with age as in collagen, this could be attributed to the different pools of aggrecans which turnover at different rates. Also activity levels of joints are known to influence aggrecan metabolism. Temporary joint immobilization of the knee results in loss of aggrecan due to degradation and lowering of aggrecan synthesis [31]. Since the tissue samples in this study were

collected from arthritic knees at the time of surgery and from post mortem specimens, some of the joints from which the cartilage was obtained may have been relatively inactive for periods of time prior to the collection of the tissue. These factors may in turn contribute to the variation in amounts of aggrecan and its pentosidine content.

Incubation of bovine aggrecan with 500 mM ribose showed pentosidine synthesis within the first week which did not significantly increase over the next 3 weeks (Figure 3), whereas, glucose and ascorbic acid formed no detectable pentosidine after 4 weeks. This may be due to the lower reactivity of glucose and ascorbic acid compared to ribose [32]. Dyer *et al.* [33] also showed a similar low yield when incubating *N*-acetyl-arginine and *N*-acetyl-lysine with glucose or ascorbic acid. They suggest that the slower rate of synthesis of pentosidine by glucose as compared to ribose could possibly be due to the required autoxidation of hexoses into pentoses prior to formation of pentosidine.

An *in vitro* study of concentration dependence of ribose on pentosidine synthesis in aggrecan yielded the unanticipated result that pentosidine formation increased with increasing ribose concentration up to 100 mM, then decreased at 500 mM. The kinetic studies done by Khalifah *et al.* [34] also demonstrate inhibition of overall AGE formation by high concentrations of ribose. They explain this inhibition as possibly being caused by the interaction of ribose with a protein intermediate containing reactive Amadori products thus inhibiting the formation of the advanced glycosylated endproducts.

Another important area of future research is to determine if AGE are capable of cross-linking aggrecan to other proteins or to each other. The cross-linking reagent, dithiobis (succinimidyl propionate) (DTSP), which cross-links two lysine residues has been shown to cross-link link proteins to aggrecan in purified aggregates [35]. Ultrastructural studies of the extractable aggrecan showed that 45% of the aggrecan was cross-linked to form polymers. Even in the absence of DTSP 7–9% of monomers appeared to be in polymeric form. At the time of the study, there was no explanation for the existence of the polymers in non cross-linked cartilage. One possible explanation may be that the naturally occurring polymers are formed by pentosidine or other AGE.

When DTSP was added to purified aggrecan monomers (A1D1D1), cross-linking between monomers did not occur. Similarly in the present study Sepharose CL-2B chromatography showed no shift in the molecular weight of the monomers cross-linked after *in vitro* cross-linking with pentosidine. This suggests that pentosidine formed cross-links within monomeric aggrecan but not between aggrecan. It remains to be determined if AGE can form intermolecular cross-links of aggrecan in aggregates.

Cartilage aggrecan undergo several age related structural changes including the decrease in the length of chondroitin sulphate rich region of the molecules, increase in the variation of aggrecan length, and increase in the density of keratan sulphate chains. The number of aggrecan subunits per aggregate decrease, and percentage of aggrecan capable of aggregating also decrease [36]. What role AGE may play in altering the function and turnover of aggrecan and link proteins has yet to be determined. AGE could possibly block proteolytic enzymes or preserve the integrity of the proteins despite proteolytic cleavage. They might block new aggrecan monomers from aggregating, and they might further stabilize aggregates or connect aggregates by cross-linking aggrecan on different aggregates.

Acknowledgement

The authors thank Dr Nagraj Ramanakoppa for his assistance and advice. Ms Karen Craft provided excellent technical assistance.

References

1 Hamlin CR, Kohn RR (1971) *Biochim Biophys Acta* **236**: 458–67.
2 Eyre DR, Paz MA, Gallop PM (1984) [Review] *Annu Rev Biochem* **53**: 717–48.
3 Last JA, Armstrong LG, Reiser K (1990) *Int J Biochem* **22**: 559–64.
4 Reiser KM (1991) *Proc Soc Exp Biol Med* **196**: 17–29.
5 Miyata S, Monnier VM (1992) *J Clin Invest* **89**: 1102–12.
6 Sell DR, Monnier VM (1990) *J Clin Invest* **85**: 380–84.
7 Odetti P, Fogarty J, Sell DR, Monnier VM (1992) *Diabetes* **41**: 153–59.
8 Nagraj RH, Sell DR, Prabhakaran M, Ortwerth BJ, Monnier VM (1991) *Proc Natl Acad Sci* USA **88**: 10257–61.
9 Hormel SE, Eyre DR (1991) *Biochim Biophy Acta* **1078**: 243–50.
10 Takahashi M, Kushida K, Ohishi T, Kawana K, Hoshino H, Uchiyama A, Inoue T (1994) *Arth and Rheum* **37**: 724–28.
11 Uchiyama A, Ohishi T, Takahashi M, Kushida K, Inoue T, Fujie M, Horiuchi K (1991) *J Biochem* **110**: 714–18.
12 Kirk E, Kvorning SA (1949) *J Gerontol* **4**: 273–84.
13 Schnider SL, Kohn RR (1981) *J Clin Invest* **67**: 1630–35.
14 Sell DR, Monnier VM (1989) *J Biol Chem* **264**: 21597–602.
15 Maroudas A, Palla G, Gilav E (1992) *Connective Tissue Research* **28**: 161–69.
16 Neame PJ, Christner JE, Baker JR (1986) *J Biol Chem* **261**: 3519–35.
17 Fu MX, Wells-Knecht KJ, Blackledge, Lyons TJ, Thorpe SR, Baynes JW (1994) *Diabetes* **43**: 676–82.
18 Kahn A, Pottenger LA, Phillips FM (1991) *J Orthop Res* **9**: 777–86.
19 Hascall VC, Sajdera SW (1969) *J Biol Chem* **244**: 2384–96.
20 Bitter T, Muir HM (1962) *Anal Biochem* **4**: 330–34.
21 Tang LH, Rosenberg L, Reiner A, Poole AR (1979) *J Biol Chem* **254**: 10523–31.
22 Rosenberg L, Choi HU, Tang LH, Pal S, Johnson T, Lyons DA, Laue TM (1991) *J Bio Chem* **266**: 7016–24.
23 Stegeman, Stadler S (1967) *Clin Chim Acta* **18**: 267–73.
24 Eyre DR, Koob TJ, Ness KP (1984) *Anal Biochem* **137**: 380–88.

25 Stenesh J (1975) In *Dictionary of Biochemistry* pp 56. New York, Wiley Interscience Publication.
26 Monnier VM, Sell DR (1994) In *Maillard Reactions in Chemistry, Food, and Health*. (Labuza TP, Reineccius GA, Monnier VM, O'Brien J, Baynes J, eds) pp 235–243. Cambridge: The Royal Society of Chemistry.
27 Assoumani MB, Maxime D, Nguyen NP (1994) In *Maillard Reactions in Chemistry, Food, and Health*, Labuza TP, Reineccius GA, Monnier VM, O'Brien J, Baynes J, eds) pp 43–50. Cambridge: The Royal Society of Chemistry.
28 Monnier VM, Sell DR, Nagraj RH, Miyata S (1991) *Gerontology* **37**: 152–65.
29 Bailey AJ, Sims TJ, Avery NC, Halligan EP (1995) *Biochem J* **305**: 385–90.
30 Eyre D, Dickson I, Ness K (1988) *Biochem J* **252**: 495–500.
31 Behrens F, Kraft EL, Ogema TR (1989) *J Orthop Res* **7**: 335–43.
32 Kohn RR, Cerami A, Monnier VM (1984) *Diabetes* **33**: 57–59.
33 Dyer DG, Blackledge JA, Thorpe SR, Baynes JW (1991) *J Biol Chem* **266**: 11654–60.
34 Khalifah RG, Todd P, Booth AA, Yang SX, Mott JD, Hudson BG (1996) *Biochem* **35**: 4645–54.
35 Phillips FM, Pottenger LA, Hay RV (1990) *J Orthop Res* **8**: 189–98.
36 Buckwalter JA, Roughley PJ, Rosenberg LC (1994) *Microscopic Research and Technique* **28**: 398–408.

Received 1 November 1996, revised 6 December 1996, accepted 7 February 1997