Age-related accumulation of Maillard reaction products in human articular cartilage collagen

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Non-enzymic modification of tissue proteins by reducing sugars, the so-called Maillard reaction, is a prominent feature of aging. In articular cartilage, relatively high levels of the advanced glycation end product (AGE) pentosidine accumulate with age. Higher pentosidine levels have been associated with a stiffer collagen network in cartilage. However, even in cartilage, pentosidine levels themselves represent < 1 cross-link per 20 collagen molecules, and as such cannot be expected to contribute substantially to the increase in collagen network stiffness. In the present study, we investigated a broad range of Maillard reaction products in cartilage collagen in order to determine whether pentosidine serves as an adequate marker for AGE levels. Not only did the well-characterized AGEs pentosidine, N^{ϵ}-(carboxymethyl)lysine, and N^{ϵ}-(carboxyethyl)lysine increase with age in cartilage collagen (all $P < 0.0001$), but also general measures of AGE cross-linking, such as browning and fluorescence (both $P < 0.0001$), increased. The levels of these AGEs are all higher in cartilage collagen than in skin collagen. As a functional measure of glycation the digestibility of articular collagen by bacterial collagenase was investigated; digestibility decreased linearly with age, proportional to the extent of glycation. Furthermore, the arginine content and the sum of the hydroxylysine and lysine content of cartilage collagen decrease significantly with age ($P < 0.0001$ and $P < 0.01$ respectively), possibly due to modification by the Maillard reaction. The observed relationship between glycation and amino acid modification has not been reported previously *in vivo*. Our present results indicate that extensive accumulation of a variety of Maillard reaction products occurs in cartilage collagen with age. Altogether our results support the hypothesis that glycation contributes to stiffer and more brittle cartilage with advancing age.

Key words: advanced glycation end product, aging, cross-linking, N^{ϵ}-(carboxymethyl)lysine, pentosidine.

INTRODUCTION

During aging, long-lived proteins such as collagen and eye lens proteins are non-enzymically modified by reducing sugars. The major initial product is fructose–lysine (FL) [1,2], which results from glycation of ϵ-amino groups on lysine residues. In subsequent Maillard or browning reactions, products known as advanced glycation end products (AGEs) are formed from FL [3,4], and accumulate with age in long-lived proteins [1,2,5–8]. These AGEs include structurally characterized adducts, such as N^{ϵ}-(carboxymethyl)lysine (CML) [1–3] and N^{ϵ}-(carboxyethyl)-lysine (CEL) [9], fluorescent cross-links, such as pentosidine formed between lysine and arginine residues [5,10], as well as chemically unidentified compounds which result in protein-bound browning or fluorescence, and cross-linking [11–14].

In comparison with other collagen-rich tissues such as skin, cartilage contains relatively large amounts of pentosidine [5,15]. Pentosidine levels in articular cartilage increase linearly with age [6–8], as was previously described for skin collagen [16] and lens proteins [10]. Pentosidine is also present in the proteoglycans in articular cartilage [17], but appears to be localized predominantly in the collagen component of the tissue [7]. Age-related accumulation of AGE cross-links in articular cartilage collagen may result in increased stiffening of the collagen network, as was shown after *in vitro* ribosylation of cartilage [7]. Thus AGE cross-linking *in vivo* may contribute to the age-related impairment of the ability of articular collagen to resist mechanically induced damage and eventually cartilage degeneration [18–20].

Even though pentosidine levels in cartilage collagen are high compared with the levels found in other long-lived proteins [5,15], the absolute number of pentosidine cross-links is less than 1 cross-link per 20 collagen molecules [7,8], and as such cannot be expected to substantially increase collagen network stiffness. In the present study, we investigated a range of Maillard reaction products in human articular cartilage collagen to determine whether the extensive accumulation of pentosidine in articular cartilage can be confirmed by other AGEs.

FL, CML, CEL and pentosidine were measured in cartilage collagen, and their levels were compared with those previously reported for skin collagen and lens protein [1,2,21]. In addition, general measures of glycation-derived cross-links, i.e. browning and Maillard-type fluorescence [12], were determined. Modification of arginine, lysine and hydroxylysine residues in cartilage collagen was also determined as a possible measure of overall glycation and AGE formation. The digestibility of articular collagen by bacterial collagenase was explored as a functional measure of cross-linking.

EXPERIMENTAL

Cartilage samples

Macroscopically normal human articular cartilage was obtained *post mortem* from femoral condyles within 18 h of death. Patients had no clinical history of joint disorders. Cartilage from a total

Abbreviations used: AGE, advanced glycation end product; CEL, N^{ϵ}-(carboxyethyl)lysine; CML, N^{ϵ}-(carboxymethyl)lysine; FL, fructose–lysine.

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of 60 donors was used; ages ranged from 2.5 to 103 years. Different subsets of samples were used for the various assays. In a few cases ($n = 6$), paired cartilage and skin samples were collected. All tissue samples were stored at −20 °C until analysis.

Purification of collagen

Articular cartilage collagen was purified by depleting the tissue of all proteoglycans and other non-collagenous proteins by sequential enzymic treatment with chondroitinase ABC (Sigma), trypsin (Boehringer Mannheim), and *Streptomyces* hyaluronidase (Sigma) at 37 °C, as described by Schmidt et al. [22]. This procedure resulted in the removal of more than 97 % of the total glycosaminoglycans with a minimal loss of collagen (< 1 %, measured as hydroxyproline; results not shown). Skin collagen was isolated by sequential extraction for 24 h at 4 °C with 1 M NaCl, chloroform/methanol (2:1, v/v), and 0.5 M acetic acid as described previously [23].

Analytical procedures

For analysis of FL, samples of cartilage collagen (1 mg in 1 ml of 6 M HCl) were hydrolysed for 24 h at 110 °C under nitrogen. CML and CEL were measured separately in 2 mg samples that were first reduced overnight at 4 °C in 500 μl of 0.1 M $NaBH_4$ (Sigma) in 0.1 M sodium borate buffer (pH 9.0). To remove excess $NaBH_4$, samples were washed three times with 5 ml of deionized water and then hydrolysed in 1 ml of 6 M HCl, as described above. An aliquot (30 %) of the reduced hydrolysates was removed for pentosidine and amino acid analysis, described below. The FL, CML, CEL and lysine contents of the collagen hydrolysates were measured as their *N*-trifluoroacetyl methyl esters by isotope-dilution selected ion monitoring GC–MS [2,9], using deuterated internal standards. The FL, CML and CEL content of the collagen samples is expressed as mmol per mol of lysine residues. Historical data for lens protein and skin collagen have been determined using the same methodology as described above [1,2,9,21].

Pentosidine content and amino acid composition were determined by HPLC as described previously [24,25]. In short, dried hydrolysates were dissolved in internal standard solution containing 10 μM pyridoxine (Sigma) and 2.4 mM homoarginine (Sigma). For pentosidine analysis, samples were diluted fivefold with 0.5 % (v/v) heptafluorobutyric acid (Fluka) in 10 % (v/v) acetonitrile (Rathburn, Walkerburn, Scotland, U.K.) and analysed by HPLC [25]. Pentosidine was generously given by Professor V. M. Monnier (Case Western Reserve University, Cleveland, OH, U.S.A.), and calibrated against our pentosidine standard [10]. For amino acid analysis, an aliquot of the pentosidine samples was diluted 50-fold with 0.1 M borate buffer (pH 11.4), derivatized with 9-fluorenylmethylchloroformate (Fluka) and analysed by HPLC [24]. The pentosidine content of the collagen samples is expressed as mmol per mol of lysine residues. The quantities of arginine and the sum of the primary amines hydroxylysine and lysine, i.e. (hydroxy-)lysine, are expressed as mol per mol of collagen, assuming 300 hydroxyproline residues per triple-helical collagen molecule [25].

Collagen-linked fluorescence and browning were measured in cartilage and skin collagen (1–5 mg) digested for 2 h at 65 °C with 2.5 units/ml of papain (from Papaya latex, Sigma) in 300 μl of papain buffer [50 mM phosphate buffer (pH 6.5), 2 mM L-cysteine and 2 mM EDTA] [26]. Fluorescence of the papain digests was measured at $\lambda_{ex} = 360$ nm and $\lambda_{em} = 460$ nm in a Cytofluor II Multi-Well Plate Reader (PerSeptive Biosystems), because those filters were available for the plate reader. A high correlation was found between these fluorescence measurements and the fluorescence measured at 370 nm/440 nm (the excitation–emission maxima for AGE fluorescence [12]; $r = 0.99$, $P < 0.0001$; results not shown) in an SFM-25 fluorometer (Kontron, Milan, Italy). Browning was measured as absorption at 340 nm in a Titertek Multiskan MCC/340 plate reader (Labsystems, Helsinki, Finland). The measurements were made against papain buffer. An aliquot of the papain digests was hydrolysed in 1 ml of 6 M HCl at 110 °C for 20–24 h for hydroxyproline analysis [27,28]. Both fluorescence and browning were normalized to the hydroxyproline content of the digest (papain contributed < 1 % of the hydroxyproline in the digests).

Collagen digestibility was measured following the digestion of cartilage collagen for 6 h at 37 °C with *Clostridium histolyticum* collagenase (CLS 2; Worthington Biochemical Corp., Freehold, NJ, U.S.A.) at a final concentration of 5 μg of collagenase per mg of collagen in a 50 mM Tris buffer containing 5 mM $CaCl_2$, 0.15 M NaCl, 1 μM $ZnCl_2$, 0.02 % (w/v) NaN_3, and 0.01 % (v/v) Brij 35. After incubation, the supernatant and remaining tissue were separated and hydrolysed in 1 ml of 6 M HCl at 110 °C for 20–24 h. The relative amount of collagen in the supernatant was estimated by measuring the amount of hydroxyproline in the hydrolysates of both the supernatant and the remaining tissue [27,28]. The mean collagenase digestibility was calculated from duplicate analyses. In a subset of the donors ($n = 12$), an additional cartilage collagen sample was digested with papain for measurement of browning and AGE fluorescence and pentosidine content (after hydrolysis of the papain digest) as described above, in order to correlate collagenase digestibility of articular collagen with levels of AGE cross-linking.

Statistical analysis

Linear regression analyses and paired Student's *t* tests were performed with SPSS version 8.0 for Windows (SPSS, Chicago, IL, U.S.A.); $P < 0.05$ was considered to represent statistically significant differences.

RESULTS

Age-related variation in levels of Maillard reaction products in articular cartilage collagen

In articular cartilage from healthy individuals, a slight, but statistically significant, age-related increase in collagen glycation (measured as FL) was found ($r = 0.37$, $P < 0.05$; Figure 1A). The glycation of lysine residues in cartilage collagen was about 5 mmol of FL/mol of lysine in a sample from a 2.5-year-old and increased only by an additional 23 % up to the age of 80 years. Thus the extent of modification of lysine residues in cartilage collagen by FL ranged from 0.5 to 0.7 % over the entire human life span.

In contrast with the marginal age-related increase in the concentration of FL, AGE concentrations per amount of lysine residues increased considerably with age in cartilage collagen. In immature cartilage (< 20 years), AGE levels are very low, but after maturity (> 20 years) levels of CML ($r = 0.87$, $P < 0.0001$; Figure 1B), CEL ($r = 0.76$, $P < 0.0001$; Figure 1C), and pentosidine ($r = 0.88$ and $P < 0.0001$; Figure 1D) in cartilage collagen increased 27-fold, 6-fold and 33-fold with age respectively. When expressed per collagen triple helix, similar correlations and increases of CML, CEL and pentosidine levels with age were found (results not shown). Summed levels of CML, CEL and pentosidine reached 5.6 mmol/mol of lysine in 80-year-

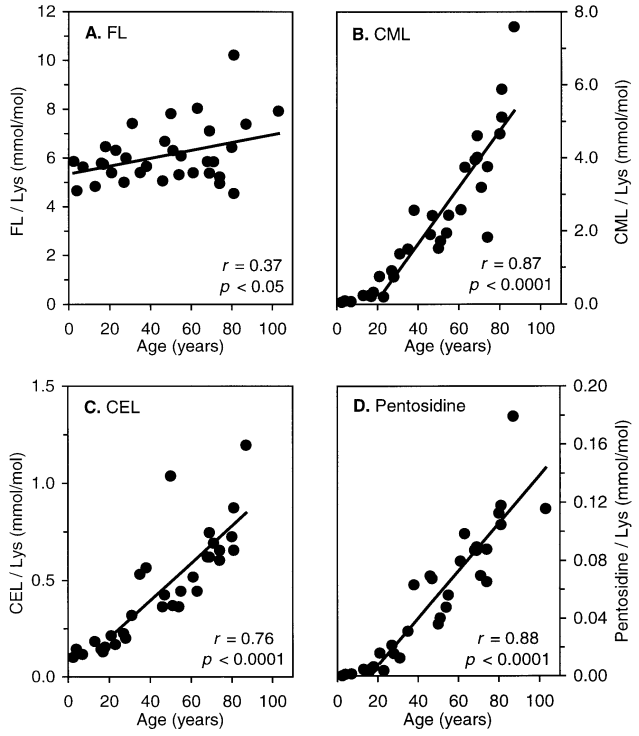

Figure 1 AGEs in articular cartilage collagen increase markedly with age

(**A**) Levels of the initial glycation product FL in cartilage collagen; (**B**–**D**) levels of the AGEs CML, CEL and pentosidine in cartilage collagen as a function of age. Normal full-depth femoral condylar cartilage of 33 subjects in the age range 2.5–103 years was analysed; data are expressed as mmol of residues/mol of lysine. Linear regression analysis was performed on all data for FL and on data > 20 years for CML, CEL and pentosidine.

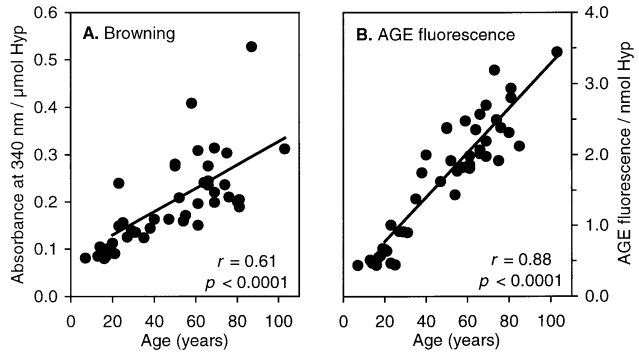

Figure 2 General measures of Maillard-type cross-linking of articular cartilage collagen increase linearly with age

(**A**) Browning (absorbance at 340 nm) and AGE fluorescence (**B**) in cartilage collagen versus age. Normal full-depth femoral condylar cartilage of 45 subjects in the age range 7–103 years was analysed; data are expressed as relative units per hydroxyproline. Linear regression analysis was performed on data > 20 years.

old cartilage collagen (approx. 0.6% of the total lysine residues in cartilage collagen).

Besides the concentrations of the chemically characterized AGEs, the levels of general measures of Maillard-type cross-linking were also determined. Both browning (absorbance at 340 nm) and AGE fluorescence increased significantly with age in cartilage collagen ($r = 0.61$, $P < 0.0001$; Figure 2A; and $r = 0.88$, $P < 0.0001$; Figure 2B respectively). The threefold increase in browning and the fivefold increase in fluorescence from age 20 to 80 were less pronounced than the age-related increases in CML, CEL, and pentosidine (Figures 1B–1D).

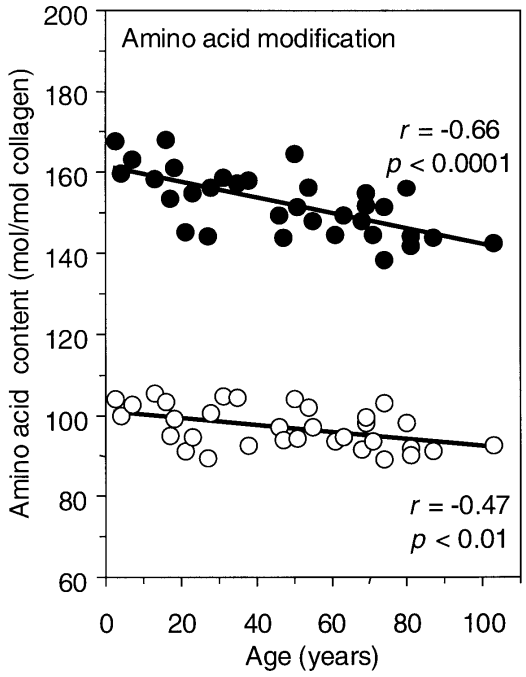

Figure 3 Arginine and (hydroxy-)lysine content of articular cartilage collagen decrease with age

Arginine (●) and (hydroxy-)lysine (○; sum of hydroxylysine and lysine) content of cartilage collagen as a function of age. Normal full-depth femoral condylar cartilage of 33 subjects in the age range 2.5–103 years was analysed; data are expressed as mol of residues/mol of collagen, assuming 300 hydroxyproline residues per triple helix. Linear regression analysis was performed on all data.

With increasing age, a decrease in the arginine, hydroxylysine and lysine content of cartilage collagen was observed. While the decrease in lysine content was not significant, the arginine content and the sum of the primary amines decreased significantly with age ($r = -0.66$, $P < 0.0001$ and $r = -0.47$, $P < 0.01$, respectively, Figure 3). Over the entire human life span (0–80 years), 15 arginine residues (9.5%) and seven (hydroxy-)lysine residues (6.9%) are lost per cartilage collagen molecule, suggesting that they have been subjected to modification during aging. Both the sum of three well-characterized AGEs (CML, CEL and pentosidine) and the AGE fluorescence correlated significantly with the mean degree of modification of arginine, hydroxylysine and lysine residues in collagen ($r = 0.58$, $P < 0.001$ and $r = 0.52$, $P < 0.05$ respectively).

Besides the age-related increase in Maillard reaction products, we also explored functional properties of articular collagen (i.e. enzymic digestibility) as a measure of glycation cross-links. An age-related decline in digestibility of cartilage collagen by bacterial collagenase of 0.3% per year was observed ($r = -0.75$, $P < 0.0001$; Figure 4A), which may well be indicative of increased AGE cross-linking with advancing age. Correspondingly, the collagenase digestibility showed a strong negative correlation with the pentosidine content ($r = -0.87$, $P < 0.0005$), browning ($r = -0.82$, $P < 0.005$), and AGE ($r = -0.87$, $P < 0.0005$; Figure 4B) fluorescence of the collagen.

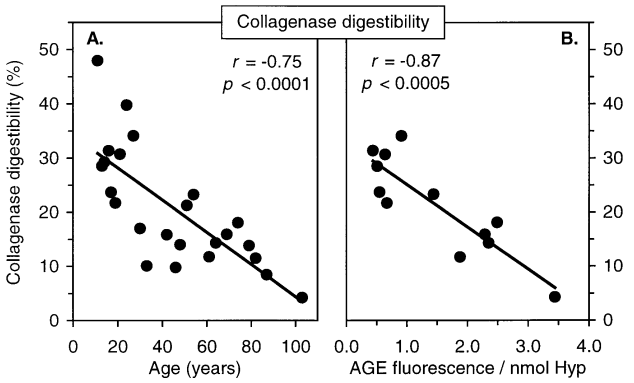

Figure 4 Digestibility of articular cartilage collagen by bacterial collagenase decreases with age and with AGE cross-linking

The proportion of cartilage collagen digested by *C. histolyticum* collagenase (**A**) as a function of age ($n = 24$, age range 11–103 years) and (**B**) as a function of AGE cross-linking determined as Maillard-type fluorescence (see the Experimental section; $n = 12$, age range 13–103 years). Linear regression analysis was performed on all data.

Table 1 Comparison of cartilage collagen levels of Maillard reaction products in elderly subjects with previously reported data for skin and lens

Levels of Maillard reaction products are expressed as mmol/mol of lysine (means ± S.D., if available). Cartilage collagen data are from the present study, and represent an age range of 61–87 years ($n = 12$). FL data for skin collagen and lens protein (mean for ages 60–85 years) are taken from [1,2]. The levels of AGEs in skin collagen (85-year-old donor pool) and lens protein (ages 60–85 years) are reproduced from [21].

	Content (mol/mol)		
	Cartilage collagen	Skin collagen	Lens protein
FL	6.36 ± 1.60	5.12	1.57
Pentosidine	0.098 ± 0.030	0.025 ± 0.004	0.004 ± 0.002
CML	4.24 ± 1.51	1.70 ± 0.31	4.95 ± 0.50
CEL	0.70 ± 0.19	0.50 ± 0.09	3.78 ± 0.28
CML/CEL	6.06	3.40	1.31

Comparison of Maillard reaction products in cartilage collagen with those in skin collagen and lens protein

Levels of the chemically well-characterized AGEs in cartilage collagen from elderly donors were compared with previously reported data on human skin collagen and lens protein [1,2,9,21]. The pentosidine level in aged cartilage collagen was fourfold higher than in skin collagen and 25-fold higher than in lens protein from donors of a comparable age (Table 1). This comparison confirms previously published data that pentosidine levels in cartilage are extremely high [5,15]. Furthermore, CML and CEL concentrations in cartilage collagen are higher than in skin collagen (2.5-fold and 1.4-fold respectively, Table 1). As a general measure of AGE cross-linking, Maillard-type fluorescence was measured in paired samples of cartilage and skin collagen of aged donors ($n = 6$, age 76.4 ± 4.4 years; means ± S.D.). The fluorescence in cartilage collagen (36.6 ± 3.7 relative fluorescence units/nmol of hydroxyproline) was about 2.5-fold higher than in skin collagen (14.4 ± 6.3 relative fluorescence units/nmol of hydroxyproline, $P < 0.001$, Figure 5). Thus AGE levels in general are severalfold higher in cartilage collagen than in skin collagen. Although pentosidine levels in cartilage collagen are as much as 25-fold higher than in the lens, CML and CEL levels in aged cartilage collagen are comparable with (CML) or even lower (CEL) than in lens protein from elderly donors.

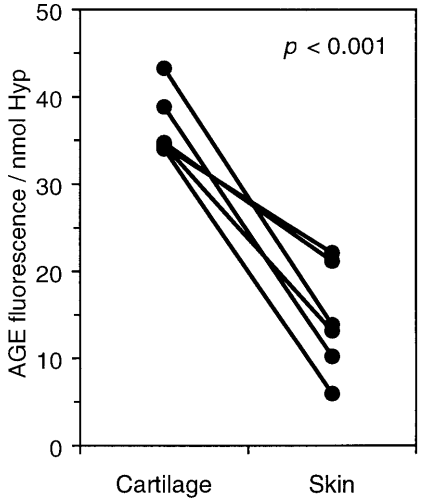

Figure 5 Maillard-type fluorescence is higher in cartilage compared with skin collagen

AGE fluorescence was measured in paired samples of cartilage and skin collagen of aged donors ($n = 6$, age 76.4 ± 4.4 years; mean ± S.D.). Data are expressed as relative units per hydroxyproline. The significance of the difference between cartilage and skin collagen was tested using a paired Student's t test.

In addition to their absolute levels in tissues from elderly donors, the mean CML/CEL ratio was compared between cartilage collagen, skin collagen and lens protein, because this ratio may yield insight into the chemical origin of these lysine modifications in proteins. The highest CML/CEL ratio (6.06) was measured in cartilage collagen, being 1.8-fold higher than in skin collagen (3.40) and 4.6-fold higher than in lens protein (1.31; Table 1).

DISCUSSION

Maillard reaction products in articular cartilage collagen

There have been only limited studies of non-enzymic glycation and AGE accumulation in human articular cartilage [6–8,17,29]. Most of the relevant studies have focused on pentosidine, a well-known marker for advanced glycation reactions [5,10], which is present in cartilage collagen in relatively high amounts compared with other tissues [5,15]. Recently, the AGEs CML and CEL were shown to be present in human skin collagen and lens protein at concentrations considerably higher than that of pentosidine [1,2,9]. In the present study, we also demonstrate the presence and age-related accumulation of CML and CEL in cartilage collagen in a broader study of Maillard reaction products in articular cartilage collagen.

Glycation (measured as FL) of articular cartilage collagen is relatively constant throughout adult life. During the first two decades of life, low levels of the AGEs CML, CEL and pentosidine are measured. This suggests that cartilage collagen undergoes relatively rapid turnover during this phase of life, so that AGEs are efficiently removed and do not accumulate [7]. After maturity has been reached, the concentrations of the AGEs increase up to 30-fold with age, consistent with the slow turnover

reported for collagen in adult cartilage [7,30]. As expected, CML and CEL are present in cartilage collagen at considerably higher concentrations than pentosidine, as was previously described for skin collagen and lens protein [1,2,9]. Not only the well-characterized AGEs, but also general measures of AGE content, browning and Maillard-type fluorescence, increased with donor age.

Even though the levels of CML, CEL and pentosidine are relatively high in cartilage collagen compared with those in skin collagen [2,16], and the level of pentosidine is high compared with that in lens protein [1,9], it is not possible to draw conclusions on the extent of cartilage collagen modification solely based on the levels of these AGEs, because these analyses are limited to the few AGEs that are chemically characterized. Therefore we measured the cartilage collagen content of amino acids that become modified during glycation and AGE formation. Both the arginine and (hydroxy-)lysine content of cartilage collagen decrease significantly with age, implying an age-related increase in amino acid modification. The age-related modification of amino acids can be due to several processes, including glycation and lipid oxidation [31,32]. Furthermore, the modification of (hydroxy-)lysine residues can also be the result of enzymic collagen cross-linking. Mature pyridinoline cross-links comprise over 90 % of the presently characterized enzymic cross-links in type II collagen in articular cartilage [33]. Inasmuch as the extent of cross-linking of cartilage collagen by mature pyridinoline cross-links remains constant throughout the entire lifespan [7], it is expected that the age-related increase in modification of both arginine and (hydroxy-)lysine amino acids in cartilage collagen results from glycation or oxidation processes. Based on our present results, only 4.7 % of the age-related loss of (hydroxy-)lysine residues can be explained by the well-characterized AGEs CML, CEL and pentosidine. Still, this is consistent with data from *in vitro* incubations of cartilage collagen with ribose or threose in which respectively 3.9 % and 1.1 % of the modified (hydroxy-)lysine residues were identified as CML, CEL or pentosidine (N. Verzijl, J. DeGroot, R. A. Bank, A. Maroudas, S. R. Thorpe, J. W. Banes, J. W. J. Bijlsma, F. P. J. G. Lafeber and J. M. TeKoppele, unpublished work). In acid hydrolysates of skin collagen [34] and lens protein [1,35,36], no age-related changes in arginine and (hydroxy-)lysine content of the protein are detected, implying that, unlike cartilage collagen, the age-related accumulation of acid-stable modifications is not high in these proteins. Thus not only on the basis of the high AGE levels in cartilage collagen, but also on the basis of amino acid measurements, it is suggested that cartilage collagen is extensively modified by glycation.

The fact that the solubility of human skin collagen by enzymic digestion with pepsin decreases with age is attributed to the increased AGE cross-linking with advancing age [11,37]. Similarly, our data show that the digestibility of articular cartilage collagen by bacterial collagenase decreases substantially with age: from approx. 30 % in 20-year-old cartilage to 10 % in 80-year-old cartilage. Inasmuch as the collagenase digestibility of cartilage collagen is strongly correlated with its pentosidine content and AGE fluorescence (Figure 4B), the age-related decrease in digestibility is most likely due to the increase in AGE cross-linking with age.

Maillard reaction products in cartilage compared with skin and lens

It is assumed that the extent of glycation of long-lived tissue proteins is in equilibrium with the tissue glucose concentration [2]. This assumption is based on the 3–4-fold higher extent of glycation of skin collagen [2] compared with lens protein [1], which is consistent with the higher glucose concentration in plasma and skin extravascular fluid (approx. 5 mM) than in the lens (1–2 mM) [2,38–40]. In articular cartilage, the glucose concentration is equal to the plasma glucose concentration [41]. Therefore it was not surprising that the level of glycated lysine residues in cartilage collagen (5–7 mmol of FL/mol of lysine) is comparable with that in skin collagen [2] (Table 1).

Throughout life, skin collagen contains lower levels of CML and CEL compared with FL [2]. In lens, the CML and CEL content is considerably higher than its FL content [1,9]. As proposed by Dunn et al. [2], this indicates that skin collagen undergoes relatively more initial glycation than lens protein, but is exposed to less oxidative stress. The last finding is corroborated by the much lower levels in skin collagen compared with lens protein of *o*-tyrosine, a marker of oxidative damage to tissue proteins [42,43]. In cartilage collagen, as in skin collagen, lower levels of CML and CEL are present than of FL, suggesting that in cartilage oxidative processes may also play a less important role than in the lens. This is consistent with the low oxygen tension of articular cartilage, due to the avascular nature of the tissue [44,45]. In general, the results suggest that parameters determining concentrations of CML and CEL relative to the initial glycation product FL, such as tissue glucose concentration and oxidative stress, are relatively similar for cartilage and skin collagen, and different from lens protein. However, AGE levels are also dependent on protein turnover rates [5,15,46,47]. Lens protein [1,48] and cartilage collagen [30] essentially do not turn over, whereas skin collagen has a turnover faster than cartilage and lens [37]. While cartilage and skin collagen are relatively comparable in glucose concentrations and oxidative stress as determinants of AGE levels, the difference in protein turnover is clearly reflected in the higher CML, CEL and pentosidine levels in cartilage compared with skin collagen (Table 1).

Ahmed et al. [9] indicated that the CML/CEL ratio in different tissues provides insight into the chemical origin of these lysine modifications in proteins. In cartilage collagen a higher CML/CEL ratio (approx. 6.1) was observed compared with skin collagen (approx. 3.4) and lens protein (approx. 1.3). The approximate 1 : 1 ratio in lens proteins was close to the CML/CEL ratio obtained with 3-deoxyglucosone and ascorbate upon incubation of collagen with these carbohydrates *in vitro* [9]. In cartilage collagen the CML/CEL ratio is closer to the ratios found after *in vitro* incubation with ribose, glucosone and also glucose. A high CML/CEL ratio in cartilage and skin collagen compared with lens protein is consistent with the higher glucose concentration in plasma compared with the lens [2], and with the higher ascorbate than glucose concentration in the lens [38,39,49,50]. In addition, the ability of ascorbate to glycate and cross-link lens proteins is much greater than that of glucose [40]. These observations suggest that glycation and AGE formation in cartilage and skin collagen is more dependent on glucose, whereas ascorbate may be more important in these processes in the lens.

From our present results it is clear that, in human cartilage collagen, not only pentosidine levels, but also the levels of other well-characterized AGEs and general measures of AGE cross-linking, substantially increase with age. Predominantly on the basis of the 5–10 % decrease in arginine and (hydroxy-)lysine content of cartilage collagen during the human lifespan, we conclude that overall cartilage collagen modification by glycation is relatively high compared with other long-lived human proteins, and that pentosidine serves as an adequate marker for overall AGE levels. The age-related accumulation of high levels

of AGEs in articular cartilage collagen may contribute to the observed stiffening of the cartilage collagen network with advancing age [7,51]. The increased stiffness might make the cartilage collagen network more brittle, as described for vascular tissues [52], and as such impair the resistance of articular collagen to fatigue and predispose aged cartilage to damage and eventual degeneration. Thus the accumulation of AGEs represents a conceivable molecular mechanism whereby age is a predisposing factor for the development of osteoarthritis [7].

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