Ageing and zonal variation in post-translational modification of collagen in normal human articular cartilage

The age-related increase in non-enzymatic glycation affects biomechanical properties of cartilage

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A biomechanical failure of the collagen network is postulated in many hypotheses of the development of osteoarthritis with advancing age. Here we investigate the accumulation of non-enzymatic glycation (NEG) products in healthy human articular cartilage, its relation to tissue remodelling and its role in tissue stiffening. Pentosidine levels were low up to age 20 years, and increased linearly after this age. This indicates extensive tissue remodelling at young age, and slow turnover of collagen after maturity has been reached. The slow remodelling is supported by the finding that enzymatic modifications of collagen (hydroxylysine, hydroxylysylpyridinoline, and lysylpyridinoline) were not related to age. The high remodelling is supported by levels of the crosslink lysylpyridinoline (LP) as a function of distance from the articular surface. LP was highest at the surface in mature

cartilage (> 20 years), whereas in young cartilage (< 10 years) the opposite was seen; highest levels were close to the bone. LP levels in cartilage sections at age 14 years are high at the surface and close to the bone, but they are low in the middle region. This indicates that maturation of cartilage in the second decade of life starts in the upper half of the tissue, and occurs last in the tissue close to the bone. The effect of NEG products on instantaneous deformation of cartilage was investigated as a functional of topographical variations in pentosidine levels *in vivo* and in relation to *in vitro* induced NEG. Consistently, higher pentosidine levels were associated with a stiffer collagen network. A stiffer and more crosslinked collagen network may become more brittle and more prone to fatigue.

INTRODUCTION

The principal components of articular cartilage are collagen type II and proteoglycan (aggrecan). Aggrecan has a high affinity for water by virtue of its high negative fixed-charge density and is trapped in a three-dimensional network of type II collagen fibrils. The combination of the properties of the aggrecan and the collagen network endows cartilage with the capacity to take up and distribute loads and to present a low-friction surface [1–3]. The high tensile stiffness of the collagen network is responsible for the resistance of cartilage to compression and fatigue [4–8]. A deterioration in the mechanical properties of the collagen network is thought to play an important role in the aetiology of osteoarthritis (OA) [4–10]. OA occurs in a considerable proportion of the elderly population, indicating that age is a major ‘risk factor’. However, the mechanism by which ageing is involved is largely unknown. Studies regarding age-related changes of the structure of the collagen network and its mechanical properties should therefore provide insight into the processes leading to the development of OA. In the present work we have focussed on the fibrillar component of the collagen network.

Biosynthesis of collagen is a multistep process characterized by extensive modifications of the protein. Many of these modifications are unique to collagen. During synthesis, hydroxylation of proline and lysine residues results in the formation of hydroxyproline (Hyp) and hydroxylysine (Hyl), respectively.

After secretion into the extracellular space, the C- and N-terminal propeptides are removed by proteinases, enabling the molecules to aggregate into fibrils. Subsequently, collagen molecules become covalently crosslinked. All these modifications are mediated by enzymes [11,12]. In most mature tissues, collagen molecules have, once they are incorporated into the extracellular matrix, an exceptionally long life-time (> 200 years) [13,14], making them susceptible to non-enzymatic glycation (NEG) via the so-called Maillard reaction [15,16]. The accumulation of NEG products is supported by the well-known colour change of articular cartilage from bluish in young age to yellow/brownish in the elderly. NEG results in increased crosslinking (such as pentosidine formation), which is of a different nature from the enzymatic crosslinks formed during the assembly of collagen fibrils [17,18]. Crosslinks play an important role in the tensile stiffness and strength of the collagen fibrils [11,12,18–22]. Thus, biochemical changes in the collagen network must have a considerable effect on the biomechanical properties of cartilage. Yet, to date only limited information is available on this topic. Hydroxylysylpyridinoline (HP) crosslink levels were found not to change with age [23,24]; in addition, HP levels were found to be lowest near the surface of cartilage, gradually increasing with increasing depth towards bone ($n = 1$) [25]. A previous investigation suggested that there was an increase of the pentosidine level in cartilage with advancing age [24], but data of the first two decades of life were not provided. Clearly, the biochemistry of the collagen network of cartilage has not been

Abbreviations used: HFBA, heptafluorobutyric acid; Hyl, hydroxylysine; Hyp, hydroxyproline; HP, hydroxylysylpyridinoline; ID, instantaneous deformation; LP, lysylpyridinoline; NEG, non-enzymatic glycation; OA, osteoarthritis; RP-HPLC, reversed-phase HPLC.

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investigated in detail (e.g. zonal and topographical differences in relation to age) and has never been related to its biomechanical properties.

The present study was designed to characterize the biochemical changes of the articular collagen network with age (extensive age range 2.5–92 years) and to relate them to the stiffness of cartilage. Therefore, we investigated, as a function of depth, topography, and age, the extent of enzymatic modifications of collagen in healthy human articular cartilage specimens [levels of lysyl hydroxylation, Hyl, and the amount of the crosslinks HP and lysylpyridinoline (LP)]. Pentosidine crosslinks, resulting from the NEG reaction of reducing sugars with collagen [16], were also included. Although pentosidine accounts for a small proportion of the total Maillard crosslinks, it serves as a sensitive marker for the accumulation of NEG products. In addition, we studied the effect of enhanced NEG formation *in vitro* on the stiffness of articular cartilage. Using a ribosylation procedure the NEG level of young tissue was increased, and changes in the collagen network stiffness were assessed by measuring the ‘instantaneous deformation’ (ID) of cartilage specimens in unconfined compression [26,27].

METHODS

Cartilage

Macroscopically normal human articular cartilage was obtained from intact knee joints at surgery for bone tumours not involving the joint space, or *post mortem* within 18 h after death from patients without predisposing conditions for joint disorders. The 64 donors ranged in age from 2.5 to 92 years. Representative samples of full-thickness cartilage excluding the underlying bone were taken from the femoral condylar surface. Samples were derived from the lateral and medial parts of the posterior and anterior regions of the femoral condyle, and from the patello-femoral groove. Our sampling thus covered the complete joint, including load- and non-load-bearing areas. Zonal variations were assessed in successive 200 μm sections from 4 mm diameter full-depth plugs. Donors for the assessment of zonal variations were 9, 14, 30 and 43 years of age. All tissue collection was approved by the Local Ethical Committee; all material was stored lyophilized until analysis.

Crosslink and amino acid analysis

Cartilage samples (routinely 1–10 mg dry weight) were hydrolysed in 5 ml Teflon sealed glass tubes, containing 800 μl 6 M HCl, in an oven at 110 °C for 20–24 h. After drying (Speed Vac, Savant), the hydrolysates were dissolved in water containing the internal standards pyridoxine (10 μM ; Sigma, St. Louis, MO, U.S.A.) and homoarginine (2.4 mM; Sigma); for every mg tissue (dry weight) 95 μl solution was used. Samples used for zonal variation studies were dissolved in 200 μl solution regardless of their dry weight (usually < 500 μg).

For crosslink analysis, treated samples were diluted 5-fold with 0.5% (v/v) heptafluorobutyric acid (HFBA) (Fluka, Buchs, Switzerland); 100 μl of the diluted sample was injected into the HPLC system. In this way, material derived from 210 μg cartilage (dry weight) is applied onto the column, together with 200 pmol pyridoxine (internal standard). The crosslinks HP, LP, and pentosidine were quantified with a standard containing pyridoxine, pentosidine (a kind gift from Professor V. M. Monnier, Cape Western Reserve University, Cleveland, OH, U.S.A.) and HP and LP purified by CF-1 cellulose from demineralized bovine bone [28].

For amino acid analysis the same hydrolysates were used: aliquots of the 5-fold diluted sample were diluted 50-fold with 0.1 M sodium borate buffer, pH 8.0; 200 μl was derivatized at room temperature for 10 min with 200 μl acetone containing 1.5 mg 9-fluorenylmethyl chloroformate/ml (Fluka, Buchs, Switzerland). Termination of the reaction and removal of excess reagent and acetone was performed by extraction with 600 μl pentane. After two additional extractions, 400 μl 25% (v/v) acetonitrile in 0.1 M sodium borate buffer, pH 8.0, was added. A 50 μl aliquot of the derivatization mixture was injected into the HPLC system. In this way material derived from 0.70 μg cartilage (dry weight) is applied onto the column, together with 160 pmol homoarginine (internal standard). Calibration was performed with an amino acid standard for collagen hydrolysates (Sigma).

The quantities of crosslinks as well as Hyl are expressed as mol per mol collagen, assuming 300 HP residues per triple-helical collagen molecule [29].

Chromatography

Reversed-phase high performance liquid chromatography (RP-HPLC) of crosslinks were performed on a Micropak ODS-80TM column (150 mm $\times$ 4.6 mm; Varian, Sunnyvale, U.S.A.). The elution conditions were: time 0–17 min 0.15% (v/v) HFBA in 24% (v/v) methanol (elution of pyridoxine, HP and LP); time 17–30 min 0.05% (v/v) HFBA in 40% (v/v) methanol (elution of pentosidine); time 30–40 min 0.1% (v/v) HFBA in 75% (v/v) acetonitrile (washing); time 40–50 min 0.15% HFBA in 24% methanol (equilibration). Chromatography was performed at room temperature at a flow rate of 1 ml/min. Fluorescence was monitored with a programmable fluorimeter (model 821-FP, Jasco): 0–22 min 295/400 nm (for pyridoxine, HP and LP); 22–45 min 328/378 nm (for pentosidine).

RP-HPLC of amino acids were performed as described elsewhere [30]. Fluorescence was monitored at 254/630 nm.

Ribosylation procedure

Healthy full-depth cartilage plugs (6.5 mm diameter) were cored from a 25-year-old human femoral head and immersed in 0.15 M NaCl for 24 h at 4 °C. NEG was carried out in 0.05 M potassium hydrogen phosphate buffer, pH 7.2, containing 0.15 M NaCl, 0.6 M ribose (Sigma), and proteinase inhibitors di-sodium-EDTA, (25 mM), ϵ -amino-n-hexanoic acid (25 mM), benzamidine (5 mM) and *N*-ethylmaleimide (10 mM) for 86 h at 37 °C. As a control, adjacent plugs were incubated in the same solution, but without ribose. After ribosylation, samples were washed three times for 24 h in 0.15 M NaCl at 4 °C. Samples were subjected to unconfined compressive loading before and after ribosylation.

ID

The surface of the ribosylated and control plugs (age 25 years) under consideration, as well as plugs obtained from different locations from the femoral head of a 53-year-old subject, was pricked with a circular pin and marked with Indian ink to show the split line pattern (related to the orientation of the collagen fibrils). The specimens were subjected to unconfined compressive step-loading with a purpose-built apparatus [26,27]. During the experiment, the specimen was immersed in normal saline at 4 °C in a transparent glass cell. The top surface of the specimen was compressed against a transparent rigid plunger and viewed with a microscope to which a photo camera is attached. Prior to loading, a preload corresponding to approx. 0.09 atm (9119 Pa)

was applied to ensure contact between the surface of the specimen and the plunger. The specimen was then loaded so that the initial pressure on its top surface was 7.56 atm (766×10^3 Pa). The ID, expressed as percentage change in diameter of the surface perpendicular to the prick line, was measured within 1 min after loading.

Data analysis

The paired Student's *t* test (for ID data) and linear regression analyses (for age-related changes of biochemical parameters) were performed with StatWorks version 2.1 for Macintosh; $P < 0.05$ was considered to represent statistically significant differences.

RESULTS

Chromatography of amino acids and crosslinks

Figure 1(A) shows a typical chromatographic analysis of pre-column derivatized amino acids present in cartilage hydrolysates. With the exception of Ile and Leu, all amino acids are adequately separated. The amino acids quantified, Hyp, Pro and Hyl, are all baseline separated from the other amino acids, thus allowing accurate determination of their absolute amounts. Homo-arginine, used as internal standard, eluted at the beginning of the chromatogram just ahead of Hyp.

Ion-pair HPLC for crosslink analysis of cartilage hydrolysates showed no interfering peaks in the region where HP, LP, pentosidine, and the internal standard pyridoxine elute, thus enabling easy quantification (Figure 1B). As cartilage matures (Figure 1C), an increase is seen in pentosidine. In contrast to most other findings reported, LP was detectable in all samples analysed. Clearly, LP levels were much lower than HP levels (ratio 1:30). To confirm its identity, the peak eluting at the LP position was collected and its excitation and emission spectra were determined at 400 and 290 nm, respectively. No differences were found between these spectra and the spectra obtained with purified LP from bone hydrolysates (data not shown).

Age-related variations of post-translational modifications

The extent of enzymatic modification of collagen (Hyl, HP and LP) remains constant throughout the entire life-span and is not correlated with age (Figures 2A–2C). The mean values ($\pm$ S.D.) as calculated for ages > 25 years are 43.3 ± 3.9 (Hyl), 1.52 ± 0.20 (HP) and 0.051 ± 0.018 (LP) (residues per triple helix); the respective ranges are 36.3–54.5, 1.1–2.2 and 0.018–0.112 ($n = 34$). During the first two decades of life, similar levels of enzymatic modification are present, albeit with a larger inter-individual variation at ages over 25 years (Figures 2A–2C). In immature cartilage (< 18 years) pentosidine levels are as low as 0.001 crosslinks per triple helix (Figure 2D), but after maturity (18–20 years), pentosidine levels start to increase linearly with age ($r = 0.934$, $P < 0.001$; Figure 2D). Cartilage from individuals aged 80–95 years contains 50 times more pentosidine than 20-year-old cartilage.

Zonal variations of post-translational modifications

Typical graphs of the concentration of Hyl, HP, LP and pentosidine versus distance from the articular surface are presented in Figures 3A–3D. At all ages investigated, lysyl hydroxylation and HP-crosslinking were lowest at the surface, and increased gradually with increasing depth (Figures 3A, 3B). In the lower half of the cartilage, the amount of HP was relatively constant and

Figure 1 Amino acid and crosslink profile in hydrolysates of native cartilage

(A) RP-HPLC of pre-column derivatized amino acids from hydrolysed cartilage (material derived from 0.70 μ g dry weight injected). The peaks are labelled by one-letter codes for amino acids; IS = internal standard (homoarginine), OK = Hyl, OP = HP. Fluorescence was monitored at 254/630 nm. (B, C) RP-HPLC of the crosslinking amino acids HP, LP and pentosidine from hydrolysed cartilage (material derived from 210 μ g dry weight injected) aged 20 and 42 years, respectively. Fluorescence was monitored at 295/400 nm (0–22 min) and 328/378 nm (remaining time). Pyridoxine serves as internal standard. Note the increase of pentosidine levels with age.

decreased again in the deepest regions close to the subchondral bone. In mature cartilage (> 20 years), LP-crosslinking showed a pattern opposite to that of lysyl hydroxylation: it was highest at the surface and gradually declined with depth (Figure 3C, closed symbols). In contrast, the zonal profile of LP in young cartilage (9 years) resembled that of Hyl: it increased steadily throughout the depth of cartilage (Figure 3C, open circles). Interestingly, an intermediate situation was observed for the subject of intermediate age (14 years): in the region 1 mm from the surface LP levels tend to decrease, thereafter LP remains constant up to a depth of approximately 2.5 mm; this is followed

Figure 2 Amounts of Hyl and mature crosslinking residues in articular cartilage collagen as a function of age

(A–C) The relation between enzyme-mediated post-translational modifications of collagen with age (Hyl, HP and LP); (D) relationship between NEG (pentosidine levels) and age. Full depth femoral condylar cartilage of 62 subjects in the age range 2.5–92 years was analysed; data are expressed as mol of residues/mol of collagen assuming 300 Hyp residues per triple helix. A significant correlation ($r = 0.934$; $P < 0.001$) between age and pentosidine content was observed; no other correlations were significant ($P > 0.05$). Closed circles: subjects > 25 years for Hyl, HP and LP and > 18 years for pentosidine. Open circles: subjects < 25 years for Hyl, HP and LP and < 18 years for pentosidine. Linear regression analysis was performed on closed circles only.

by an increase in the deepest part of the cartilage (Figure 3C, open square). Pentosidine levels remained the same throughout the tissue depth except for the oldest age investigated: at age 43

Figure 3 Zonal variations in articular cartilage collagen

(A–C) Changes in the level of enzymatically mediated post-translational modifications of collagen with depth; (D) extent of NEG (pentosidine) versus depth. Representative profiles of full-depth plugs are shown for different ages (9, 14, 30 and 43 years). Zonal variation was assessed by slicing full-depth femoral condylar cartilage into 200 μm slices. Open symbols: zonal variations at young age (9 and 14 years; circles and squares, respectively); closed symbols: zonal variations at older ages (30 and 43 years, circles and squares, respectively).

the level was on average slightly higher in the lower than in the upper half of cartilage (note the logarithmic scale of Figure 3D ordinate).

All graphs in Figure 3 are based on samples derived from the medial anterior part of the femoral condyle. For all other locations studied (lateral and medial regions of the posterior part; lateral area of the anterior part) similar zonal variations and changes thereof with age were observed (data not shown). The same was the case for a sample derived from the patello-femoral groove, supporting the general applicability and consistent nature of the observed data for the entire knee condyles.

Biomechanical effects of NEG

To focus on the effects of accumulation of NEG products on the biomechanical properties of cartilage, eliminating all other complicated changes that take place during natural ageing, an *in vitro* ribosylation protocol was chosen. This enabled us to assess the changes in the stiffness of a cartilage sample by simply varying the NEG level, without introducing any other alterations. Cartilage treated with ribose acquired a yellowish colour (a characteristic feature of NEG), whereas control specimens treated identically in the absence of ribose remained bluish-white. Due to ribosylation, the mean pentosidine level rose from 0.001 to 0.055 ± 0.005 residues/triple helix, the latter value being characteristic of cartilage from 85-year-old subjects (Figure 2). Control samples showed no alternation in pentosidine levels.

Figure 4 Effect of ribosylation on the ID of articular cartilage

Full-depth femoral head cartilage plugs obtained from a 25-year-old donor were immersed in a phosphate buffer with 0.6 M ribose for 86 h at 37 °C. The instantaneous change in diameter of the surface specimen perpendicular to the orientation of the collagen was measured before and after incubation. The ID, expressed as percentage change in diameter, decreased in ribosylated specimens. The observed decrease in ID after NEG indicates that a stiffer collagen network is formed.

The incubation with ribose resulted in a decrease in ID of approximately 25% ($P < 0.001$) (Figure 4), whereas no significant changes in the ID were seen in control cartilage after treatment ($P > 0.6$). Thus NEG clearly leads to an increased cartilage stiffness as defined by ID. Since the latter has been shown in the past to be mainly dependent on the properties of the collagen network [26,27], the present results suggest that NEG formation leads to stiffening of the collagen network in cartilage.

We carried out further experiments in order to examine whether endogenous (*in vivo*) differences in pentosidine levels also correlate with the mechanical response of the tissue. Since we knew that large topographical differences exist in posttranslational modification of collagen (R. A. Bank and J. M. Tekoppele, unpublished work), we selected for testing 12 plugs from different locations on a 53-year-old femoral head. Pentosidine levels ranged from 0.020 to 0.035 residues per triple helix. As in the ribosylated specimens, an inverse relationship was found between the ID and the pentosidine content (Figure 5; linear regression: $r = 0.70$, $P < 0.05$), indicating that *in vivo* NEG appears to have the same biomechanical effect as NEG formed as a result of *in vitro* ribosylation.

The advantage of using samples from the same subject is that the genetic background is the same. A cautionary note should be added, however: in addition to variations in pentosidine levels, there are topographical differences in other tissue properties (e.g. hydration, proteoglycan content, proportion of total thickness occupied by the superficial zone) which may also influence the magnitude of the ID. This topic will be investigated in future studies.

Figure 5 Relationship of NEG on the ID of articular cartilage

Full-depth cartilage plugs were obtained from different locations on a 53-year-old femoral head. After equilibration of the plugs in 0.15 M NaCl, ID was measured perpendicular to the orientation of the collagen. After biomechanical testing pentosidine content was determined. An inverse relationship was found between ID and pentosidine levels, indicating a role for NEG in cartilage stiffening.

DISCUSSION

Interpretation of Hyl and crosslink data

Since Hyp is specific for collagen, this amino acid was used to calculate the amount of collagen present in the cartilage hydrolysates so that levels of Hyl and the crosslinks HP, LP and pentosidine could be expressed per mol of triple helical collagen. Measurements of Hyl, HP and LP are not influenced by proteoglycans in the cartilage, since the same results were obtained with cartilage from which proteoglycans were removed with guanidinium chloride or by enzymatic treatment (data not shown). Preliminary studies with guanidinium chloride-extracted cartilage indicated that less than 10% of the pentosidine present in the hydrolysates is derived from proteoglycans (not shown). Therefore, in the present study no attempt was made to remove proteoglycans before hydrolysis.

In cartilage, three collagen types are present in the network: collagen types II, IX and XI [31]. The main constituent is collagen type II, accounting for at least 95% of the total dry weight of collagen. Our data reflect predominantly posttranslational modifications of collagen type II, since type IX collagen (1–2% of articular collagen [31,32]) contains similar amounts of pyridinolines as type II collagen [33], and since pyridinolines are absent in type XI collagen [34].

Zonal variation of Hyl, HP, LP and pentosidine in mature cartilage

No variation was observed in the pentosidine levels with depth, except for the oldest age investigated. In contrast, a considerable

zonal variation was observed in Hyl, HP and LP (Figure 3). Both Hyl and HP increased with depth at all ages. HP is derived from three Hyl residues (two from the telopeptides and one from the α -helix). The simultaneous increase of both HP and Hyl implies that both the telopeptides and the triple helix are more heavily hydroxylated in the deeper zones of cartilage. Since hydroxylation of lysine is an entirely intracellular process [11,12], the observed zonal variation in lysyl hydroxylation patterns must be biosynthetic in origin, and must thus reflect phenotypic differences in chondrocytes located at different depths from the articular surface.

Turnover of the collagen network

The large linear increase in pentosidine levels from age 20 onwards suggests a low turnover rate of collagen in adult cartilage. This is consistent with the extremely long half-life reported for cartilage collagen (200–400 years, [14,35]). Furthermore, compared to other tissues, cartilage accumulates the highest pentosidine levels reported [36,37], another indication of the slow turnover of the articular collagen network. Taken together with the absence of age-related changes in HP and LP levels (age > 20 years; Figures 2A, 2B) it is tempting to speculate that the majority of the collagen deposited by the end of the second decade of life is not significantly renewed and is thus still present at older age, at the onset of OA.

The lack of accumulation of pentosidine during the first two decades of life suggests that the collagen matrix undergoes a relatively rapid turnover during this phase of life so that NEG products are efficiently removed and do not accumulate. This relatively high turnover is substantiated by the zonal variation in LP levels: the LP profile at age < 10 and age > 20 years are completely reversed (Figure 3C). LP-crosslinking in adult cartilage declines with depth, whereas in young cartilage a gradual increase with depth is seen. These opposite profiles indicate that the collagen synthesized in the first decade of life is completely re-modelled in the second decade. Interestingly, an intermediate situation in LP-crosslinking was observed at an intermediate age (14 years) (Figure 3C, open squares): LP levels slightly declined with depth (corresponding to the mature situation), and increased again towards the bone (as in the juvenile situation). This suggests that remodelling of the collagen network in the second decade of life is zonally regulated: the maturation process starts in the upper part and occurs last in the lower part of the tissue.

Effects of posttranslational modifications of collagen on biomechanical properties

Zonal variations

It is well known that the tensile stiffness and strength of cartilage varies considerably with distance from the articular surface, both being highest in the surface zone [4,38]. This variation has been explained in the past by the zonal variation in the orientation of the collagen fibrils, as well as by the differences in the concentration of collagen as a function of cartilage depth [4,38]. It is possible that differences in lysyl hydroxylation levels which we found with depth (low at the surface and high in the deep region) may also contribute to the zonal differences in the mechanical properties (high at surface, lower in the deep region). This is consistent with recent reports that bone containing collagen type I with a high level of lysyl hydroxylation had a low tensile stiffness and fracture threshold [39,40].

Ageing

In the present work we have tested the effect of *in vitro* and *in vivo* NEG formation on the stiffness of cartilage as measured by the instantaneous deformation in unconfined compression. The decrease in instantaneous deformation (Figures 4 and 5) indicates that NEG results in stiffer collagen fibrils. This is consistent with findings reported for other collagenous tissues, such as lens capsules [21] and vascular tissues [41]. In view of our present findings, it is likely that the age-related NEG accumulation is one of the factors contributing to the observed stiffening of the cartilage collagen network with advancing age [42].

A failure of the collagen network is the principal postulate in many mechanical hypotheses of OA (e.g. [10]). It is possible that, in addition to the stiffening, accumulation of NEG products makes cartilage collagen more brittle, as has been observed in the case of type IV collagen in lens capsules [21]. Such increased brittleness may contribute to the age-related decrease in the resistance of cartilage to fatigue [8,9] and thus be amongst the factors which predispose aged cartilage to damage and, finally, degeneration. If so, NEG could be one of the molecular events which cause age to be a major predisposing factor for the development of OA.

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