Advanced Glycation Endproducts in the Development of Osteoarthritis: Cartilage Synthesis and Degradation

Jeroen de Groot

STELLINGEN

behorende bij het proefschrift

ADVANCED GLYCATION ENDPRODUCTS IN THE DEVELOPMENT OF OSTEOARTHRITIS: CARTILAGE SYNTHESIS AND DEGRADATION

1. De mate van kraakbeenglycering bepaalt mede de gevoeligheid voor artrose *in vivo*.
   [Dit proefschrift]
2. De observatie dat de leeftijdsgerelateerde daling in proteoglycaan synthese in kraakbeen beter wordt voorspeld door de mate van glycering dan door de biologische leeftijd van de kraakbeendonor, suggereert dat het niveau kraakbeen glycering fungeert als ouderdomsmeter.
   [Dit proefschrift]
3. Verminderde kraakbeen proteolyse als gevolg van toegenomen glycering is een ambivalent fenomeen: zowel de essentiële fysiologische als de ongewenste pathologische matrixafbraak worden geremd.
   [Dit proefschrift]
4. Een toename in niet-enzymatische glycering van eiwitten vermindert de proteolytische afbraak, terwijl een toename in glycosylering van aggrecan bijdraagt aan efficiënte proteolyse. Deze discrepantie illustreert eens te meer dat glycering geen glycosylering is.
   [Dit proefschrift, Pratta *et al*, 2000]
5. Verhoging van glyceringsniveaus *in vitro* in remt collageensynthese bij gelijkblijvende totale eiwitsynthese: het algemene proces van glycering kan selectief celfuncties beïnvloeden.
   [Dit proefschrift]
6. Resultaten uit studies die de bijdrage van proteinases in kraakbeendestructie bestuderen in jonge dieren met lage glyceringsniveaus kunnen niet klakkeloos worden geëxtrapoleerd naar de volwassen humane situatie met hoge glyceringsniveaus.
   [Dit proefschrift]
7. De verminderde proteolytische afbreekbaarheid van oude, geglyceerde weefsels verstoort assays die gebruik maken van proteinases en kan daardoor studies naar veranderingen tijdens veroudering danig verstoren.
8. Aangezien in kweken van kraakbeen explants effecten van *in vitro* geïntroduceerde matrix modificaties niet te onderscheiden zijn van directe effecten van de behandeling op de chondrocyten, zullen experimenten met geïsoleerde cellen altijd hun plaats houden in studies naar chondrocyt-matrix interacties.
9. De karakteristieke geelbruine verkleuring van weefsel als gevolg van een toename in glycering maakt dubbelblind onderzoek naar glyceringsniveaus in weefsels schier onmogelijk.
10. Abstracte *abstracts* nodigen in de regel niet uit tot lezen, concrete *abstracts* des te meer.
11. De belangstelling van de media voor het bericht dat teniet doen van glycering verjonging teweeg brengt doet vermoeden dat oud worden nog steeds een teer punt is.
    [Asif *et al*, 2000; NOVA, maart 2000]
12. De metafoor 'het rondje om de kerk', die in de discussies over de NS frequent wordt gebruikt, wekt ten onrechte de suggestie dat elk dorp per trein te bereiken is.
13. Kanoën in een wedstrijd-K1 is als lopen: het duurt even voor je het onder de knie hebt, maar in de regel verleer je het nooit.

Jeroen de Groot
19 juni 2001

Advanced Glycation Endproducts in the Development of Osteoarthritis: Cartilage Synthesis and Degradation

NIET-ENZYMATISCHE GLYCERING IN HET ONSTAAN VAN ARTROSE:
KRAAKBEEN SYNTHESE EN AFBRAAK
(met een samenvatting in het Nederlands)

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geboren op 24 december 1972, te Eindhoven

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CONTENTS

ABBREVIATIONS

ACLT	**A**nterior **C**ruciate **L**igament **T**ransection
AGE	**A**dvanced **G**lycation **E**ndproduct
ANOVA	**An**alysis **O**f **Va**riance
APMA	4-**A**mino**P**henyl**M**ercuric **A**cetate
CEL	N^{ε}-(**C**arboxy**E**thyl)**L**ysine
CML	N^{ε}-(**C**arboxy**M**ethyl)**L**ysine
COMP	**C**artilage **O**ligomeric **M**atrix **P**rotein
CRP	**C**-**R**eactive **P**rotein
CS	**C**hondroitin **S**ulfate
ECM	**E**xtra**C**ellular **M**atrix
EGF	**E**ndothelial **G**rowth **F**actor
ER	**E**ndoplasmatic **R**eticulum
FCD	**F**ixed **C**harge **D**ensity
FL	**F**ructose-**L**ysine
GAG	**G**lycos**A**mino**G**lycan
G1	first (N-terminal) **G**lobular domain of aggrecan
G2	second **G**lobular domain of aggrecan
G3	third (C-terminal) **G**lobular domain of aggrecan
GOLD	**G**ly**o**xal-**L**ysine **D**imer
HP	**H**ydroxylysyl**P**yridinoline
(RP-) HPLC	(**R**eversed **P**hase-) **H**igh **P**erformance **L**iquid **C**hromatography
IGD	**I**nter **G**lobular **D**omain
IL-1ß	**I**nter**L**eukin-1ß
KS	**K**eratan **S**ulfate
LDH	**L**actate **D**e**H**ydrogenase
LP	**L**ysyl**P**yridinoline
MOLD	**M**ethylgly**o**xal-**L**ysine **D**imer
MMP	**M**atrix **M**etallo**P**roteinase
NEG	**N**on**E**nzymatic **G**lycation
NFC-1	**N**on-**F**luorescent **C**rosslink 1
OA	**O**steo**A**rthritis
PBS	**P**hosphate **B**uffered **S**aline
RA	**R**heumatoid **A**rthritis
RAGE	**R**eceptor for **AGE**s
RFU	**R**elative **F**luorescence **U**nits
SEM	**S**tandard **E**rror of the **M**ean
SF	**S**ynovial **F**luid
TIMP	**T**issue **I**nhibitor of **M**atrix metallo**P**roteinase

GENERAL INTRODUCTION

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1. ARTICULAR CARTILAGE

Articular cartilage is a highly specialized connective tissue that covers the ends of bones within synovial joints. By means of its unique composition and structure, articular cartilage meets its functional requirements of withstanding compressive and shear forces and distributing these forces onto the subchondral bone.[214] In addition, cartilage provides a lubricated surface that facilitates smooth joint movement, even under load. At first sight, cartilage seems a simple inert tissue, but upon closer inspection, it contains an elaborate, highly structured extracellular matrix that is deposited and maintained by a relatively small number of highly specialized cells: the chondrocytes (Figure 1).

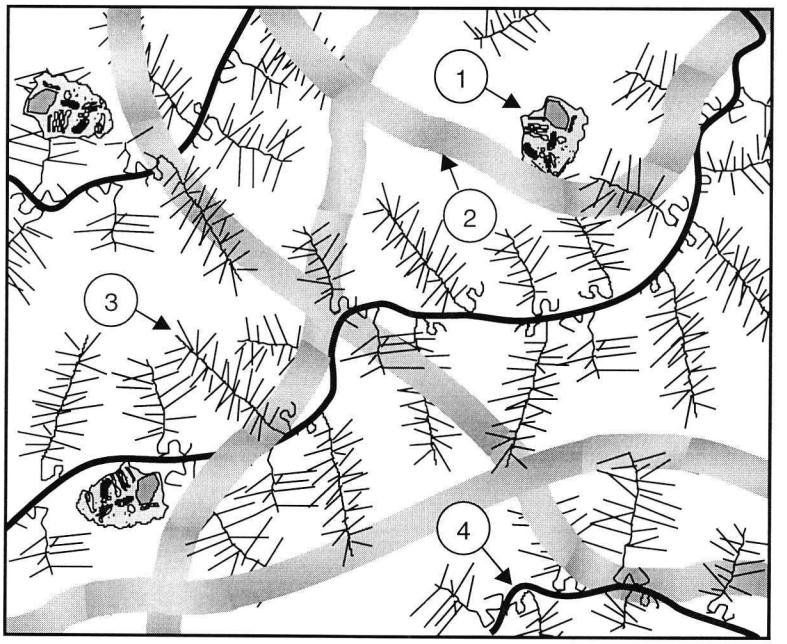

Figure 1. Articular cartilage. The main constituents of articular cartilage are depicted. Sparsely present chondrocytes (1) are embedded in a highly hydrated gel of proteoglycans and collagens. The collagen fibrils (2) form a network that gives cartilage its tensile strength. Within the collagen network proteoglycans (3) form large aggregates by binding to hyaluronan chains (4).

Surprisingly, the major component of the extracellular matrix of articular cartilage is water (70-75% of the weight in adult tissue), while collagen and proteoglycans account for the major portion of the dry weight (17-19% and 5-10% of the wet weight, respectively).[55,192] The collagen network defines the form and tensile strength of articular cartilage, while the highly hydrophilic proteoglycans are responsible for the resilience of cartilage. These proteoglycans are immobilized in the collagen network,

which results in fixation of a large negative charge within the cartilage matrix. To balance this negative charge, cations are drawn into the tissue thus creating a large osmotic potential. Consequently, water is imbibed into the tissue thereby generating a large swelling force. In the unloaded condition, swelling of the tissue is constrained by the tensile stiffness of the collagen network.[213] On compressive loading of the joint, water is squeezed out of the cartilage. During unloading, because of the osmotic pressure of the proteoglycans, this water is imbibed again, thus providing the unique resilience of articular cartilage (the ability to resume its original shape and texture after a deformation).[55,214] Thus, the mechanical properties of articular cartilage are highly dependent on the integrity of the collagen network, the retention within the network of a high concentration of proteoglycans, and the capacity of chondrocytes to maintain this extracellular matrix integrity.

1.1. CHONDROCYTES

Chondrocytes are mesenchymal cells that proliferate and differentiate during development. After growth has ceased (in adult cartilage) there is no detectable cell proliferation. In adult human cartilage, the chondrocytes occupy 1-5% of the tissue volume, the remainder being the extracellular matrix.[159,214] Since articular cartilage is avascular, nutrition of chondrocytes depends on diffusion from the synovial fluid, which is facilitated by intermittent loading of the cartilage.[189,214] Consequently, chondrocytes are adapted to exist under oxygen tensions as low as 1% and preferentially use anaerobic metabolism.[214,281] Chondrocytes are the sole cells responsible for the production and assembly of the constituents of the extracellular matrix of cartilage and are thus essential for maintaining the integrity of the tissue. To this purpose chondrocytes adjust their behavior in response to external signals. These signals include cytokines,[39,82,166] growth factors,[177] nitric oxide,[284] vitamins,[210,276] and expectedly matrix degradation products.[132,234] In addition, changes in mechanical and hydrostatic loading patterns influence the function of the chondrocytes.[213,279]

1.2. EXTRACELLULAR MATRIX

1.2.1. COLLAGENS

Collagens consist of three polypeptide chains (α-chains), each possessing the characteristic tripeptide sequence glycine-X-Y in which proline most often occupies the X position and hydroxyproline the Y position.[195] Three α-chains tightly entwine into a triple helix for which the presence of glycine on every third position is a prerequisite (only glycine is small enough to fit inside the helix).[195] In articular cartilage, approximately 95% of the collagen is type II collagen. Its polymers are the fibrils that form the basic cohesive framework of the tissue, which physically entraps aggregating proteoglycans.[159]

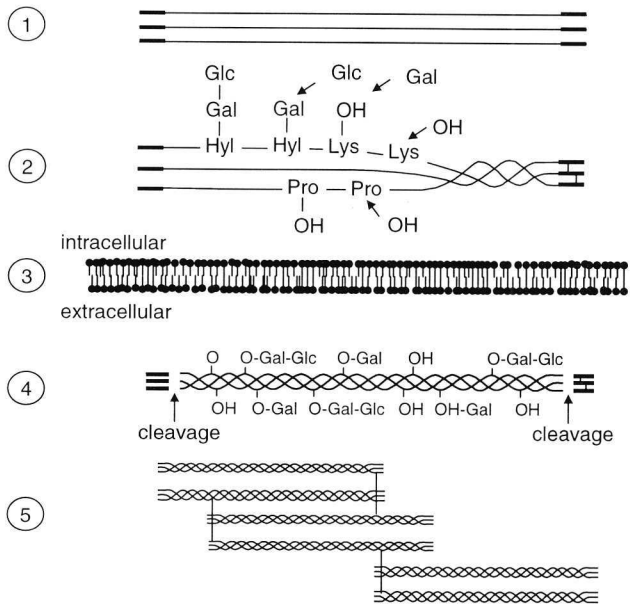

Figure 2. Collagen synthesis. Collagen is synthesized as propeptide-containing α-chains (1) that are posttranslationally modified by hydroxylation of lysyl and prolyl residues and by glycosylation of hydroxylysyl residues (2). These modifications cease when three α-chains entwine to form a collagen triple helix (2). Triple helical collagen molecules are secreted from the cell (3) and the propeptides are cleaved off extracellularly (4). Subsequently, collagen molecules spontaneously assemble into fibrils with quarter-staggered overlap of the individual triple helices. Finally, the fibrils are stabilized by formation of intermolecular pyridinoline crosslinks (5)

Collagen type II belongs to the fibril forming collagens (class I or interstitial collagens) and is a homotrimer of α1(II) chains, the product of the COL2A1 gene.[316] The collagen biosynthesis involves several unique posttranslational modifications (Figure 2). After translation of the procollagen α-chain, specific proline and lysine residues are hydroxylated by prolyl and lysyl hydroxylase, respectively.[153] Hydroxyproline is needed for the formation and stabilization of the triple helix by the formation of hydrogen bonds with proline residues. Hydroxylysine residues serve as sites for enzymatically regulated O-linked glycosylation and crosslinking of collagen.[152] Glycosylation of hydroxylysine residues is catalyzed by two specific glycosylation enzymes, hydroxylysyl galactosyltransferase and galactosylhydroxylysyl glucosyltransferase.[152,239] The function of collagen glycosylation is not yet clear but seems to play a role in decreasing the susceptibility of collagen to proteolytic degradation and thus contributes to collagen stability.[329] A function for collagen glycosylation in determining fibril diameter has also been postulated.[49,329] Glycosylation levels in type II collagen are the highest found in interstitial collagens, reaching levels of ~20 residues per collagen molecule in human articular cartilage (i.e. ~45 percent of the hydroxylysine residues is modified).[23] Hydroxylation and glycosylation cease when the α-chains entwine to form the procollagen triple helix which is subsequently secreted and processed for incorporation into the extracellular network.

In the extracellular space, the terminal propeptide regions are released by procollagen peptidases.[73,173,240] This results in a decrease in solubility, forcing the triple helical collagen molecules to self-assemble into fibrils. In the fibrils, the individual collagen molecules are orientated in parallel with quarter-staggered overlap. This highly

organized collagen arrangement results in the characteristic banded pattern seen by electron microscopy.[89] After fibril formation, intermolecular pyridinoline crosslinks are formed. This enzymatic crosslinking process is initiated by oxidation of hydroxylysyl residues in the telopeptide to form aldehyde precursors that spontaneously condense into difunctional crosslinks.[274] Subsequently, two difunctional crosslinks react to form a trifunctional crosslink that is responsible for the ultimate structural integrity of the collagen network. In cartilage, hydroxylysylpyridinoline (HP; derived from three hydroxylysine residues) is found at 30-50 fold higher levels than lysylpyridinoline (LP; derived from two hydroxylysine residues and a lysine residue at the triple-helical crosslinking site).[22,91]

The minor collagens present in cartilage are mainly type IX and XI collagen.[90] Type IX collagen is present on the surface of the type II collagen fibrils and may function as an interfibrillar connector facilitating the formation and organization of the three-dimensional network.[118,138] Type XI collagen is present within the interior of the fibrils and is suggested to contribute to fibril formation and to determine the final diameter of the fibril.[159,200] In addition, type VI collagen (<2% of the total collagen) forms a separate microfibrillar network in the pericellular matrix that acts as a bridge between the chondrocyte and the interterritorial matrix.[237,243]

1.2.2. PROTEOGLYCANS

The predominant proteoglycan in articular cartilage is aggrecan, comprising ± 90% of the cartilage proteoglycans. Aggrecan is a large, highly polyanionic macromolecule that forms large aggregates of ± 100 MDa. Aggrecan consists of a central core protein with a multi-domain structure, each domain providing the molecule with specific characteristics (Figure 3).[125]

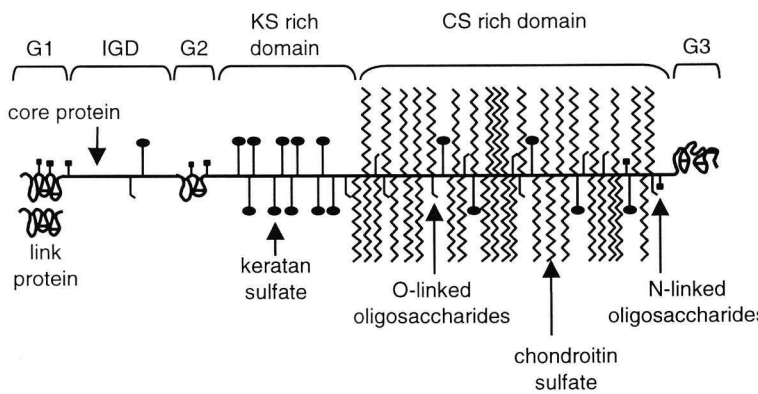

Figure 3. Schematic representation of the structure of an aggrecan monomer and link protein. Two of the globular domains (G1 and G2) are separated by the interglobular domain (IGD). Between G2 and the third globular domain (G3) a keratan sulfate (KS) rich domain and a chondroitin sulfate (CS) rich domain can be identified in the core protein. Furthermore, the core protein contains O- and N-linked oligosaccharides. The link protein stabilizes the interaction of the G1 domain with hyaluronan.

The N-terminal region contains two globular domains (G1 and G2) separated by an interglobular domain (IGD) that includes several proteinase cleavage sites. The G1 domain contains an immunoglobulin fold and two proteoglycan tandem repeat structures by which it interacts with hyaluronan (a long chain glycosaminoglycan).[100] One hyaluronan chain can bind up to 200 aggrecan monomers thus forming large proteoglycan aggregates. The aggrecan-hyaluronan interaction is stabilized by link protein, a 40 kDa globular protein with high homology to the G1 domain of aggrecan, in a 1:1 molar ratio (aggrecan : link protein).[123,159] At the C-terminal end of the aggrecan core protein a third globular domain is present (G3) that is highly conserved between different species and mediates interactions with other matrix constituents.[222] In between the G2 and G3 domains, the highly negatively charged chondroitin sulfate (CS) and keratan sulfate (KS) glycosaminoglycans are attached to the core protein. This results in the characteristic brush-like structure. Each core protein contains ± 50 KS chains, attached to the KS-rich region and ± 100 CS chains, attached to the CS-rich region. In addition, keratan sulfate chains are also sparsely present in the IGD domain and the CS-rich region.[125] The presence of keratan sulfate chains in the IGD is required for aggrecanase cleavage of the core protein and thus involved in the stability of the aggrecan molecule.[238]

In addition to aggrecan, small leucine-rich proteoglycans such as decorin, fibromodulin and biglycan are present in cartilage. They represent only a small fraction of the total mass of proteoglycans within the cartilage extracellular matrix, but being small sized they represent molar contents similar to or exceeding that of aggrecan.[236,277] Decorin and fibromodulin interact with collagen type II and are suggested to play a role in the formation and maintenance of the collagen fibrils.[118,169] Biglycan is found primarily in the pericellular matrix and may interact with type VI collagen.[286] For many other small proteoglycans, the function is not yet known.

1.2.3. NONCOLLAGENOUS, NONPROTEOGLYCAN MATRIX CONSTITUENTS

In addition to the collagens and proteoglycans, several minor constituents that are neither collagens nor proteoglycans are present in articular cartilage. The putative functions of these proteins range from involvement in matrix assembly and matrix-cell interactions to modulation of the chondrocyte phenotype. For some of these proteins no functional role has yet been identified.[180,216] As an example, a prominent noncollagenous, nonproteoglycan matrix molecule is COMP (cartilage oligomeric matrix protein) which associates with collagen and is believed to play a role in collagen fibrillogenesis in the pericellular matrix.[252]

1.3. EXTRACELLULAR MATRIX TURNOVER

Cartilage is generally viewed as an inert tissue that merely deteriorates after skeletal maturity has been reached ("wear-and-tear" theory). Indeed, the half-life of type II

collagen in healthy human cartilage is estimated to be more than 100 years,[193] implying that collagen turnover is virtually absent in normal adult cartilage. However, since the turnover of all matrix constituents is determined by the balance between synthesis and degradation, the presence of type II collagen propeptides in adult articular cartilage suggests that remodeling, though slowly, does take place.[218] In addition to the synthesis of collagen, its proteolytic degradation determines the rate of its turnover. Matrix metalloproteinase (MMP)-mediated collagen degradation is observed in adult human cartilage.[42] The MMPs form a family of Zn^{2+}- and Ca^{2+}-dependent enzymes of which the expression is transcriptionally regulated by growth factors, hormones and cytokines. MMPs are synthesized as inactive precursors, the proMMPs. The proteolytic activities of the MMPs are regulated by the tightly controlled activation of proMMPs, and by the efficient inhibition of active MMPs by endogenous inhibitors such as tissue inhibitors of matrix metalloproteinases (TIMPs) and α2-macroglobulin.[215]

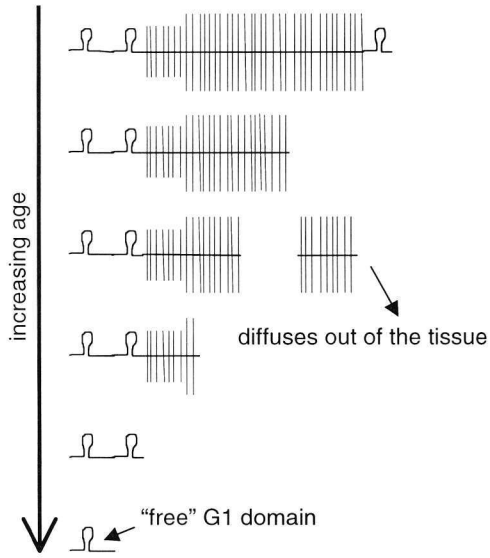

Figure 4. Schematic representation of the turnover of the aggrecan monomer. Degradation of the aggrecan monomer with age results in loss of C-terminal fragments from the tissue and accumulation of the smaller fragments that remain bound to hyaluronan through their G1 domain.

The turnover of the aggrecan fractions in healthy adult human cartilage is much faster than the collagen turnover: its half-life has been estimated to range from 3 to 24 years.[191] Degradation of aggrecan monomers is mediated by MMPs (e.g. MMP-1, -2, -3, -8 and -13[63,101,103]) and by aggrecanases such as aggrecanase-1 and –2.[1,63,293] Degradation of aggrecan occurs at multiple sites in the core protein thus resulting in the formation of aggrecan molecules of different length.[294] C-terminal fragments diffuse out of the tissue whereas the N-terminal fragments, independent of their length, remain bound to the hyaluronan via their G1 domain.[123,191] With increasing age, this

results in heterogeneous collection of aggrecan monomers, with the smallest N-terminal fragments having the highest residence time in the matrix (Figure 4).[191]
Synthesis of proteoglycans in cartilage is seen throughout the adult tissue[164] and must be coordinated with proteoglycan degradation to ensure that the extracellular matrix content of proteoglycans remains constant.[123] Feedback mechanisms that enable the chondrocyte to detect the proteoglycan content of its surrounding matrix are responsible for maintaining the proteoglycan homeostasis.[182]
The turnover of the hyaluronan chains in articular cartilage is less well studied. Coordinated synthesis of hyaluronan and aggrecan has been shown for normal bovine cartilage suggesting similar turnover rates for these molecules.[211] Degradation of hyaluronan is accomplished via receptor-mediated endocytosis and subsequent intracellular degradation.[134]

1.4. CELL - MATRIX INTERACTIONS

Cartilage homeostasis is regulated by soluble factors (e.g. cytokines, growth factors), and by the interactions of the chondrocytes with the extracellular matrix. When taken out of the extracellular matrix, chondrocytes rapidly dedifferentiate and start producing inappropriate, non-cartilage-specific proteins.[40,41,314] The interactions between the extracellular matrix and the chondrocytes are mediated by several classes of highly specific transmembrane receptors. The type II collagen is connected to the chondrocytes via integrin receptors and anchorin CII (=annexin V). In addition, the integrins provide chondrocytes with binding sites for other matrix constituents such as fibronectin.[176] Integrin-mediated chondrocyte-matrix interactions are essential in chondrocyte differentiation, survival, and response to mechanical stimuli.[128,130]
Hyaluronan-binding by chondrocytes is mediated by the CD44 receptor, thereby providing a link between the proteoglycans and the cells.[14,124] The binding of hyaluronan to CD44 is essential in the formation and retention of the pericellular matrix.[140,154] Loss of this interaction results in depletion of aggrecan from the matrix.[65]
In addition, chondrocytes respond to specific degradation products of matrix constituents. Fragments of collagen,[156] fibronectin,[132] and link protein[196] have been shown to influence the biosynthetic activity of chondrocytes. Since these products are derived from the extracellular matrix, they can be considered markers that communicate the health status of the surrounding extracellular matrix to the chondrocyte.[131,196]

2. CARTILAGE AGING

During aging of articular cartilage, both the extracellular matrix and the chondrocytes are susceptible to change. Many age-related changes in the structure and composition of human articular cartilage aggrecan have been described. With increasing age, the aggrecan KS content steadily increases while the CS content remains constant, which

results in an increase in the proportion of KS to CS.[192] Furthermore, an increase in CS 6-sulfation relative to 4-sulfation and a relative increase in the protein content of aggrecan have been demonstrated.[192,232] Some of these changes may be explained by the age-related decrease in the size of the aggrecan monomers, which is the result of C-terminal degradation fragments diffusing out of the tissue, while the remaining hyaluronan-bound N-terminal fragments accumulate with age (Figure 4).[30,123,191]

Despite an increase in hyaluronan concentration in articular cartilage with age,[129] the rate of incorporation of aggrecan monomers into aggregates is much slower in mature cartilage than in tissue from young individuals.[31] This may contribute to the decreased capacity to assemble large aggregates with increasing age.[305] In addition, the ratio of newly synthesized link protein to aggrecan decreases with age, which may result in a higher proportion of link protein-deficient, less stable aggregates with advancing age.[44]

The net effect of all combined age-related changes in aggrecan composition is an increase in the fixed charge density (FCD) of human articular cartilage with age, which results in an increase in osmotic swelling pressure.[115] Based on this age-related increase in swelling pressure, the water content of cartilage is expected to increase with age. However, the measured water content of human articular cartilage decreases somewhat with age.[249,304] The water content of unloaded cartilage is the result of the balance between the tendency of the highly hydrophilic proteoglycans to imbibe water and the tensile stresses in the collagen network that entraps the proteoglycans. Thus, the observed age-related decrease in water content of human articular cartilage, in combination with an increase in the osmotic swelling pressure of the proteoglycans, indicates that the stiffness of the collagen network must increase with age.[115]

Indeed, Basser et al. showed an age-related increase in the tensile stiffness of the articular cartilage collagen network.[29] This increased stiffness cannot be explained by a change in enzymatic collagen crosslinking, (both HP and LP concentrations in cartilage collagen are constant with age[22,91]) nor by the slight age-related decrease in collagen content.[22,304] As far as the relative contribution of different collagen types is concerned, a decrease in the content of collagen types IX and XI is observed with advancing age, which may modify the properties of cartilage but is not expected to increase the stiffness of the collagen network.[90] Yet, crosslinks derived from the process of nonenzymatic glycation increase with age in cartilage and could explain the increased tissue stiffness (see paragraph 4).[295,303]

Age-related changes have also been observed in the structure or content of some of the minor cartilage matrix constituents. For example, the contents of lumican and some of the noncollagenous, nonproteoglycan matrix molecules in human articular cartilage increase with age.[114,180,198] Reports on the effect of aging on the amounts of decorin, biglycan, and fibromodulin are less consistent: both increased and decreased levels of biglycan and decorin have been reported.[253,311] Furthermore, the structure of the KS chains that are attached to fibromodulin change with age.[169] The possible effects of these age-related changes in the amount or composition of the minor

cartilage molecules on tissue function are not yet known.
In addition to changes in the extracellular matrix with increasing age, the number and behavior of the chondrocytes are subject to age-related changes. The chondrocyte number is reported to decline with age for some joints,[310] whereas others have demonstrated stable cell numbers with increasing age.[197,280] Cell functions are generally believed to slightly decline with advancing age but unambiguous evidence to support this is lacking.[56] Some age-related changes in chondrocyte function have been described, such as a decrease in link protein mRNA expression[43] and a change in sulfation of the nonreducing terminal mono- and disaccharides on aggrecan CS.[232]

3. OSTEOARTHRITIS

Osteoarthritis (OA), or cartilage degeneration, is a widely prevalent chronic disabling condition. Clinically, OA is characterized by joint pain, tenderness, limitations of movement, crepitus, occasional effusion, and local inflammation secondary to the disease process.[230] The main pathological characteristic of OA is the centripetal progressive destruction of the articular cartilage. In many cases, subchondral cysts and sclerosis, osteophyte formation and mild synovial inflammation are observed.[135] Ample evidence suggests a multifactorial etiology of OA by combinations of biomechanical, biochemical and genetic factors.[94,96] The initial event that triggers the pathological process is unclear and it is still being debated whether the initial changes occur in the cartilage, the subchondral bone or even in the synovium.[81] Risk factors that are identified for OA are diverse and can be separated in systemic factors that increase the susceptibility to OA (e.g. gender, race, genetical predisposition) and in factors that determine the site and severity of OA such as joint trauma, obesity, congenital or acquired anatomical deformities, chondrocalcinosis and occupation.[74,93,96,97,167] By far the single greatest systemic risk factor for the development of OA is age.[76,97,299] Although age is recognized as the main risk factor for OA, the mechanism by which aging is involved in the etiology of OA remains largely unknown. In general, the disease can be initiated by age-related changes in the shape or stability of the joint, in the structure of the supporting tissues (e.g. bone), and/or in the integrity of the extracellular matrices of cartilage, bone or ligaments.[57] In our view, age-related changes in articular cartilage are likely to be causally involved in the pathogenesis of OA. These age-related changes in articular cartilage predispose to the development of OA but are fundamentally different from the changes that occur in cartilage during the disease process.[115]
One of the earliest features of cartilage degeneration is the increase in swelling of the cartilage.[190] Since the water content of articular cartilage depends on the balance between the swelling pressure of the proteoglycans and the restraining force of the collagen network, swelling of the tissue indicates loss of integrity of this network.[192] The increase in cartilage swelling is corroborated by a decrease in collagen network stiffness and both are highly correlated with the amount of degraded collagen.[28]

Collagen damage early in the process of cartilage degeneration could result from fatigue failure of the collagen network,[106,107] from mechanical trauma,[110] or from proteolytic degradation of collagen by MMPs.[34,42,269] Presumably in an attempt to repair the damage, type II collagen synthesis is increased in OA cartilage.[218] In addition, synthesis is observed of collagen types that are normally not present in articular cartilage or only at very low levels, such as type III, VI, and X collagen. Due to the different biomechanical properties of these aberrant collagen types, this results in an extracellular matrix that does not meet its functional requirements and repair is therefore ineffective.[3,243,315]

Similar to what is found for collagen, the synthesis and degradation of proteoglycans are increased in OA cartilage. The release of proteoglycans is higher from OA cartilage than from normal cartilage,[162] which is consistent with increased MMP and aggrecanase activity in OA cartilage and synovial fluid.[11,34,42,175] Furthermore, proteoglycan synthesis, measured as the rate of sulfate incorporation, is increased two- to four-fold both in the early, preclinical stage and in the late stage of OA.[162,254,288,301] In addition, slightly different proteoglycans are synthesized in OA cartilage: the sulfation pattern of CS chains in OA cartilage proteoglycans is different from normal adult cartilage and resembles that in cartilage at adolescence.[231] The enhanced proteoglycan synthesis does not compensate for the increased release: a net loss of proteoglycans is observed in OA cartilage. This is likely due to the decreased retention of newly synthesized proteoglycans,[162] possibly caused by the damaged collagen network in OA cartilage.[160]

These osteoarthritic changes in articular cartilage are completely different from those observed during aging, but may very well be initiated by age-related changes in the cartilage matrix that increase the susceptibility to damage, hence OA. One of the most important age-related changes in articular cartilage is the accumulation of products from the Maillard reaction.

4. THE MAILLARD REACTION

4.1. CHEMISTRY

Nonenzymatic glycation (NEG) is a common modification of proteins, and of DNA and lipids.[53,54,247] In the classical view (Figure 5), protein glycation is initiated by the nonenzymatic condensation of a reducing sugar with the ε-amino group of a lysine residue or the ε-guanidino group of an arginine residue to form a reversible Schiff base (e.g. fructose-lysine), that is subsequently stabilized by Amadori rearrangement. This Amadori product undergoes Maillard or browning reactions, that result in the formation of advanced glycation endproducts (AGEs).[247]

More recently, alternative pathways for formation of the Amadori product and AGEs have been proposed, e.g. through metal-catalyzed glucose auto-oxidation[326] or as the result of lipid peroxidation reactions.[223] This has resulted in the appreciation that there are multiple sources and mechanisms of AGE formation *in vivo*. Due to the highly

diverse reaction pathways leading to AGE formation, AGEs with a variety of chemical structures have been identified. Some AGEs are protein-adducts, whereas others present protein-protein crosslinks.[33,51,247] All proteins are prone to AGE formation, and since AGEs cannot be removed from proteins, accumulation of AGEs occurs in long-lived proteins such as lens crystallins and tissue collagens.[22,85,86,266] A summary of AGEs that have been identified in tissue proteins *in vivo* is provided in Table 1.

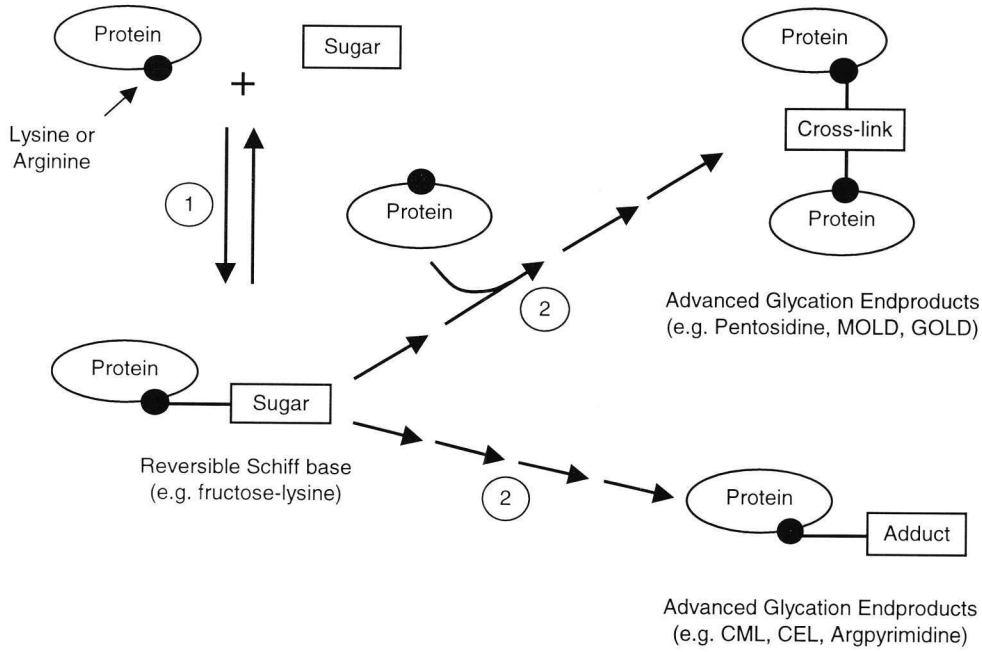

Figure 5. Classical view of AGE formation. Reducing sugars such as glucose or fructose react spontaneously with lysine or arginine residues in proteins. Initially a reversible Schiff base (e.g. fructose-lysine) is formed (1). Subsequently, Amadori rearrangement and Maillard browning reactions (2) result in the formation of stable AGEs: some form protein-protein crosslinks, whereas others present protein adducts.

The range of methods employed to analyze AGEs reflects the diversity of AGEs that are identified *in vivo*. These methods vary from high-performance liquid chromatography (e.g. for pentosidine[24]) and gas chromatography - mass spectrometry (e.g. for CML and CEL[2,86]) to the use of specific antibodies (e.g. for pyrraline[202]). In contrast, general measures of AGE crosslinking, that do not require sophisticated equipment or antibodies, have successfully been used. These measures include the

analysis of protein-bound browning (absorbance at 340 nm) or fluorescence at 370/440 nm,[207,265] and the analysis of the susceptibility of AGE-modified protein to enzymatic digestion,[262] the latter providing to a certain extent a functional measure of crosslinking.

Table 1. AGEs that have been identified in human tissue proteins

AGE	Crosslink/adduct	Present in:
Pentosidine	crosslink	many tissues such as: dura mater,[266] skin,[88] articular cartilage,[22] brain,[150] Bruch's membrane[122]
CML (N^{ε}-(carboxymethyl)lysine)	adduct	many tissues such as: lens,[86] skin,[85] brain,[150]
CEL (N^{ε}-(carboxyethyl)lysine)	adduct	lens[2]
Pyrraline	adduct	kidney[202]
Imidazolium salts: MOLD (methylglyoxal-lysine dimer) GOLD (glyoxal-lysine dimer)	crosslink	lens,[50] skin[50]
Vesperlysine A (LM-1)	crosslink	lens[289]
Argpyrimidine	adduct	cornea,[268] diabetic kidney[225]
Imidazolones	adduct	diabetic aorta,[221] diabetic kidney,[221] diabetic retina[121]
NFC-1 (non-fluorescent crosslink 1)	crosslink	diabetic skin,[19] aorta[273]

4.2. EFFECTS OF AGES ON TISSUE, CELL AND PROTEIN FUNCTION

Connective tissue strength is highly dependent upon the amount of crosslinks present in collagen. Controlled formation of enzymatic crosslinks (e.g. pyridinolines), balances tissue stiffness and strength with physiological functions such as load bearing. This is vividly illustrated by pathologies in which crosslinking is disturbed: defective crosslinking in bone of Bruck syndrome patients or in fibrotic skin severely affects tissue function.[27] In this view, it is clear that the mechanical properties of tissues are

adversely affected by the accumulation of spontaneously formed AGE crosslinks. Accumulation of AGEs is correlated with increased tissue stiffness in arteries,[273] lens,[20] skin,[246] tendon,[157] and articular cartilage.[22] Moreover, an increase in AGE levels makes tissues increasingly brittle, and thus more prone to mechanical damage. This effect has been shown for human lens capsules[20] and cortical bone.[61,292] For articular cartilage, a decrease in strength is observed with increasing age,[145,319] coinciding with an increase in AGEs in the tissue.[22] Although no direct correlation between AGE levels and brittleness has been shown, these data suggest that also for articular cartilage the level of AGEs determines its resistance to mechanical trauma or fatigue failure.[139]

In addition to affecting the mechanical properties of tissues, AGEs also interfere with cellular processes. It has been suggested that many of the effects of matrix glycation are mediated by specific AGE receptors (for review see 290). AGE-binding receptors include: scavenger receptors types I and II,[255] the receptor for AGE (RAGE),[151,217,258] oligosaccharyl transferase-48 (OST-48, AGE-R1),[174] 80K-H phosphoprotein (AGE-R2),[330] and galectin-3 (AGE-R3).[312] AGE receptors are found on a variety of cell types, including macrophages, endothelial cells and neural cells.[290] Cell activation in response to binding of AGE-modified proteins results in activation of key cell signaling molecules, such as NF-κB, and changes in gene expression.[151,259] Depending on the cell type, this can be associated with cell proliferation, oxidative stress, or apoptosis.[142,201,258] In addition, AGE-modified proteins affect the synthesis of matrix components: AGE-modified albumin has been reported to stimulate collagen type IV synthesis in glomerular mesangial cells.[9,72] In contrast, mesangial cells grown in the presence of glycating sugars or on glycated matrix show decreased synthesis of collagen types I and IV.[272] Type I collagen synthesis is also inhibited in endothelial cells and in fibroblast cell lines upon exposure to glycated albumin.[70,224] Furthermore, AGE-modified albumin has been shown to inhibit matrix metalloproteinase-2 mRNA production by mesangial cells while stimulating TIMP-1 expression, which likely results in decreased extracellular matrix proteolysis.[9] In addition to effects mediated by specific AGE receptors, accumulation of AGEs in extracellular matrix proteins also directly interferes with cell-matrix interactions. A decreased adhesion of cells to the extracellular matrix, possibly mediated by glycation of the arginine residue in the Arg-Gly-Asp motif that provides the binding site for integrins, has been demonstrated for osteosarcoma and fibrosarcoma cells.[119,226]

Besides cell-mediated effects of AGEs on matrix turnover, accumulation of AGEs also directly affects matrix turnover by changing the physical and chemical properties of proteins. AGE-modification of proteins changes their structure such that it may interfere with highly specific enzyme-substrate interactions[5] and thus prevent e.g. proteolysis. In addition, increased inter- and intramolecular crosslinks may diminish the release of degraded cartilage constituents,[18] which may result in chondrolysis due to the stimulation of chondrocytes by unremoved matrix degradation products.[131] Alternatively, modification of lysine and arginine residues changes the charge

distribution of the protein,[116] thereby influencing its tertiary structure as well as its interactions with other proteins. Because of these changes in protein conformation and interactions, the accessibility of the extracellular matrix for proteinases may be altered, thus affecting proteolysis. Additionally, the susceptibility of matrix proteins for proteolytic degradation may be changed by conformational changes in the matrix proteins. Indeed, a decrease in the susceptibility of extracellular matrix proteins to proteolytic degradation has been reported at elevated AGE levels. Degradation of AGE-modified collagen by matrix metalloproteinases is impaired compared to unmodified collagen.[212] Furthermore, the proportion of pepsin-released skin collagen decreases with age,[262] which coincides with the age-related increase in AGE levels in skin.[88]

5. HYPOTHESIS

The most important risk factor for the development of OA is age,[76,97,299] but the mechanism by which aging is involved in the etiology of OA remains thus far largely unknown. The spontaneous age-related accumulation of AGEs in articular cartilage could provide such a mechanism. In comparison to other collagen-rich tissues such as skin, cartilage contains relatively large amounts of AGEs such as pentosidine.[208,266] Pentosidine levels in articular cartilage increase linearly with age.[22,295] Analogous to the effects observed in other tissues, the age-related accumulation of AGEs is likely to affect the biomechanical, biochemical and cellular properties of cartilage. Increased cartilage stiffness due to AGE accumulation is expected to result in increased brittleness and therefore increased susceptibility of the tissue to mechanical damage. Indeed, a preliminary study suggests that an increase in AGEs results in increased cartilage stiffness.[22] In addition, similar to effects on mesangial cells, fibroblasts and endothelial cells, AGEs are expected to decrease extracellular matrix synthesis by chondrocytes and impair proteolytic tissue degradation.[70,212,224,262,272] This would result in a decreased capacity of chondrocytes to remodel and/or repair their extracellular matrix which expectedly leads to cartilage that does not possess the optimal structure and integrity to meet its functional demands of load bearing and distribution. The cartilage is therefore more prone to development and progression of damage.

Thus, the age-related accumulation of AGEs in articular cartilage is expected to increase cartilage brittleness and decrease the chondrocytes' capacity to remodel their extracellular matrix. In combination, these mechanisms are hypothesized to render the cartilage more prone to damage and thus to the development and progression of osteoarthritis.

6. OUTLINE OF THIS THESIS

The hypothesis that the age-related accumulation of AGEs in articular cartilage is causally involved in the etiology of OA was investigated using a variety of experimental approaches. The results from these studies are described in two theses. In *Advanced*

Glycation Endproducts in the Development of Osteoarthritis: Cartilage Biochemistry and Biomechanics, Nicole Verzijl describes in detail the variety of AGEs present in articular cartilage, the effects of protein turnover on the accumulation of these AGEs, the effects of AGEs on cartilage biomechanics, and a cross-sectional study on the relation between AGE levels and the occurrence of cartilage degeneration.[307] The present thesis focuses on the question:

WHAT ARE THE EFFECTS OF AGE ACCUMULATION ON THE SYNTHESIS AND DEGRADATION OF THE EXTRACELLULAR MATRIX OF ARTICULAR CARTILAGE AND HOW DO THESE EFFECTS INFLUENCE THE SUSCEPTIBILITY FOR OA *IN VIVO*?

The individual chapters each address different aspects of this question. The capacity of chondrocytes to maintain extracellular matrix integrity and composition is essential for articular cartilage to remain fully adapted to the functional demands. Generally, chondrocyte function is believed to decline with increasing age, but strong evidence to support this is lacking.[56] In **chapter 2** age-related changes in the synthesis of proteoglycans were studied and related to the level of extracellular matrix glycation, both in an extensive age range of human articular cartilage and after *in vitro* enhancement of glycation levels.

In addition to proteoglycan synthesis, the degradation of matrix constituents is an important part of the turnover of the tissue. **Chapter 3** focuses on the effects of AGE accumulation on both the synthesis and degradation of proteoglycans in human articular cartilage. To this purpose, cartilage AGE levels were enhanced *in vitro* using a variety of reducing sugars to mimic the diversity of AGEs found *in vivo*. Possible interfering effects of age-related changes besides AGE accumulation were eliminated using this approach of *in vitro* glycation.

The integrity of the collagen network presents an important aspect of the functioning of articular cartilage. The collagen network provides shape and tensile strength to the tissue. Damage to the collagen network is one of the first characteristics of the development of OA. Some studies suggest that collagen damage is irreversible and presents a point of no return in the development of the disease. Therefore, the regulated synthesis and degradation of the collagen network by chondrocytes is an important factor in the maintenance of the cartilage integrity. In **chapter 4**, the alginate culture system was employed to study the effect of AGE accumulation on the turnover (synthesis and MMP-mediated degradation) of collagen by bovine articular chondrocytes.

The progressive proteolytic destruction of cartilage is a hallmark of OA. Elevated levels of MMPs have been detected in the synovial fluid of OA patients. These MMPs can potentially degrade all the main cartilage components (i.e. collagen, aggrecan). Since the structure of the matrix influences its degradation, age-related AGE modification of extracellular matrix proteins likely affect the susceptibility to proteolytic damage. Therefore, the role of AGE accumulation on the synovial fluid-mediated degradation of

cartilage was studied in **chapter 5**.

In vitro studies into effects of AGEs on extracellular matrix metabolism can provide detailed information on specific, isolated processes that are influenced by matrix glycation. In addition, predictions can be made on how these processes may affect the development of OA *in vivo*. However, more often than not, *in vitro* data cannot directly be translated to the *in vivo* situation, in which the combination of many (unknown) processes determines the final effects of AGE accumulation. Therefore, in **chapter 6**, a study is described that was designed to obtain insight in the role of AGE accumulation on the development of OA in an *in vivo* model. To this purpose, AGE levels were enhanced in articular cartilage of young Beagle dogs by intra-articular injections with ribose. Subsequently, OA was induced by anterior cruciate ligament transection and the severity of OA was compared between normal animals and those with enhanced AGE levels. Using this approach, only effects of differences in AGE levels were studied, excluding possible interference of other age-related changes in the articular cartilage.

In the final chapter of this thesis (**chapter 7, Summary and Discussion**), the results from the individual studies are integrated into a general concept that describes the role of AGE accumulation in the development of osteoarthritis.

AGE-RELATED DECREASE IN PROTEOGLYCAN SYNTHESIS OF HUMAN ARTICULAR CHONDROCYTES: THE ROLE OF NONENZYMATIC GLYCATION

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ABSTRACT

Objective. *To examine the effect of nonenzymatic glycation of cartilage extracellular matrix on the synthetic activity of chondrocytes.*

Methods. *Proteoglycan-synthesis rate ($^{35}SO_4^{2-}$ incorporation) and levels of advanced nonenzymatic glycation (determined by high-performance liquid chromatography measurement of pentosidine) were evaluated in human articular cartilage of 129 donors, varying in age from 25 to 88 years, and in cartilage with enhanced levels of advanced glycation endproducts (AGEs) resulting from incubation with ribose.*

Results. *Cartilage showed a strong age-related increase in pentosidine levels ($r = 0.97$, $p < 0.0005$) and, concomitantly, a decrease in proteoglycan synthesis ($r = -0.98$, $p < 0.0002$). This decrease in proteoglycan synthesis correlated with the increase in pentosidine ($r = -0.95$, $p < 0.02$). Moreover, the elevation of pentosidine levels in the in vitro ribosylated cartilage was proportional with the decrease in proteoglycan synthesis ($r = -0.95$, $p < 0.005$).*

Conclusion. *In both aged and in vitro AGE-enriched cartilage, the rate of proteoglycan synthesis was negatively correlated with the degree of glycation. This suggests that the age-related increase in cartilage AGE levels may be responsible, at least in part, for the age-related decline in the synthetic capacity of cartilage.*

INTRODUCTION

Age is a major risk factor for the occurrence of osteoarthritis (OA), but the mechanism by which age is involved in the etiology of OA is largely unknown.[56,76,120] Age-related changes in the cartilage extracellular matrix (ECM) may increase its susceptibility to damage, but evidence to support this is lacking.[56,76] The progression of cartilage degeneration after severe damage indicates that from the point of damage onward, chondrocytes fail to uphold the integrity of the matrix.[47,76] They start producing inappropriate, non-cartilage-specific matrix constituents.[3,220] Inasmuch as loss of proteoglycans from articular cartilage is an early event in OA,[83] chondrocytes should be able to replenish lost proteoglycans to ensure effective maintenance of the matrix. Since cell functions are generally believed to decline with advancing age,[56] it is important to know whether chondrocytes retain their proteoglycan synthetic capacity with advancing age. If not, maintenance of cartilage ECM integrity may be impaired which could ultimately lead to the development of OA.

Cell function is highly dependent on interactions with the ECM.[14,137,257] When chondrocytes are taken out of the tissue, they change their synthesis of matrix components and easily dedifferentiate.[40,41,314] Effects of ECM composition on chondrocyte function are, for instance, mediated by specific plasma membrane proteins such as CD44,[154,155] integrins,[37,67] and anchorin.[120,159] Considering that articular cartilage ECM undergoes extensive changes during aging, it is likely that

chondrocytes experience different regulatory signals from their ECM in aged cartilage than in young tissue. Age-related changes in articular cartilage include an increase in noncollagenous proteins such as decorin and biglycan,[159,253] changes in glycosaminoglycan (GAG) composition,[30] and an increase in hyaluronic acid concentration.[129]

Another prominent feature of aging is nonenzymatic glycation (NEG): reducing sugars, such as glucose or fructose, spontaneously react with free amino groups of proteins to form early glycation products that are eventually converted to advanced glycation endproducts (AGEs).[206,295] All proteins are sensitive to NEG, but AGEs accumulate in matrix constituents with slow turnover rates, such as collagens.[205,207,256] Pentosidine, a crosslink resulting from lysine, a sugar, and arginine, is one of the best-characterized AGEs and is considered an adequate marker for the process of NEG.[266] In human cartilage, levels of pentosidine increase 50-fold from age 20 to age 80.[22] The present study was designed to investigate the hypothesis that this extensive age-related accumulation of AGE products in articular cartilage influences the chondrocytes' capacity to maintain the matrix through proteoglycan synthesis.

MATERIALS AND METHODS

Donors. Macroscopically normal human articular cartilage was obtained *post-mortem* at autopsy within 24 hours after death of the donor (ages 25-88 years). Cartilage was obtained from femoral knee condyles (n = 77) or from humeral heads (n = 52). Previous studies have shown that this cartilage is biochemically normal, even if focal OA lesions are present elsewhere in the joint.[163,165]

Cartilage culture. Full-thickness slices of cartilage were cut aseptically, kept in phosphate buffered saline (pH 7.4), and processed within 1 hour after dissection. Cartilage slices were cut into square pieces (10-15 mg), which were randomly divided over experimental conditions (10-12 explants per condition), and individually cultured and handled. Cartilage explants were cultured for 4 days in round-bottom, 96-well microtiter plates (200 µl/well, 37°C in 5% CO_2 in air) containing Dulbecco's Modified Eagle's Medium (DMEM, GibcoBRL, Breda, The Netherlands) supplemented with ascorbic acid (0.85 mM, Sigma, Zwijndrecht, The Netherlands), glutamine (2 mM, GibcoBRL), sodium benzylpenicillin (100 IU/ml), streptomycin sulfate (100 IU/ml), and 10% (volume/volume) heat-inactivated pooled human adult male AB-positive serum.[165]

***In vitro* enhancement of NEG.** In cartilage, AGE levels were enhanced *in vitro*. For this purpose, D(-)-ribose (0-50 mM, Sigma) was added to the culture medium of femur condyle cartilage tissues for 4 or 12 days. Culture medium was refreshed every 4 days. In some experiments, after 4 days of exposure to ribose, cartilage was washed 3 times for 1 hour in 200 µl culture medium with 1% (v/v) human AB-positive serum without ribose, and subsequently cultured for another 4 days without ribose, as described above.

Proteoglycan synthesis rate. As measure of proteoglycan synthetis activity, the rate of sulfate incorporation was determined (8-10 explants per condition) using carrier-free $^{35}SO_4^{2-}$ (NEN Dupont, Hoofddorp, The Netherlands) during the last 4 hours of culture. After labeling, tissue digestion with papain (Sigma), and precipitation of GAGs with cetylpyridiniumchloride (Sigma), the incorporation of

$^{35}SO_4^{2-}$ was analyzed by liquid scintillation counting. Sulfate incorporation was calculated from the $^{35}SO_4^{2-}$ incorporation and the specific activity of the culture medium, and was expressed as nmoles of sulfate incorporated per hour per gram wet weight of tissue.[298]

DNA content. In a separate set of donors (n = 26, ages 46-103 years), cartilage DNA content was determined after tissue digestion with papain (Sigma) and DNA staining (Hoechst 33258, Sigma) by measuring fluorescence (DNA fluorometer TKO100; Hoefer Scientific Instruments, San Fransisco, CA), using calf-thymus DNA (Sigma) as a reference.[165]

Pentosidine levels. Levels of pentosidine in the tissue (1-3 explants per condition) were determined by reverse-phase high-performance liquid chromatography (RP-HPLC) after acid hydrolysis as described previously.[26] In short, hydrolyzed cartilage (6 N HCl for 18 hours at 110°C) was dried (Speed Vac; Savant, Holbrook, NY) and dissolved in internal standard solution. For pentosidine analysis, samples were diluted 5-fold and analyzed by RP-HPLC. For hydroxyproline levels, aliquots of the 5-fold-diluted samples were diluted 50-fold, derivatized with 9-fluorenylmethyl chloroformate (Fluka, Zwijndrecht, The Netherlands), and analyzed by RP-HPLC.[25] Pentosidine levels were calculated as mmoles/moles triple-helical collagen, assuming 300 residues of hydroxyproline per triple helix.

Cell death. As measure of chondrocyte death, release of lactate dehydrogenase (LDH) into culture medium was assessed with a cytotoxicity detection kit (Boehringer Mannheim, Almere, The Netherlands). Maximum release was induced by overnight addition of 1% (v/v) Triton X-100 (Sigma).

Statistical analysis. Statistical evaluation was performed using SPSS software version 6.1.4. (SPSS, Chicago, IL). Data are presented as the mean ± SEM. Correlations between parameters were determined by multiple linear regression analysis. *P* values less than 0.05 were considered statistically significant.

RESULTS

Ribosylated cartilage. The accumulation of AGEs *in vivo* is the result of the reaction of a reducing sugar with amino groups of proteins (i.e., lysine residues). *In vitro* acceleration of glycation can be accomplished by incubation with reducing sugars.[87,113] Of the sugars studied, ribose rapidly reacts with lysine and arginine to form the AGE pentosidine.[21,45,58] Therefore, cartilage was incubated with ribose (0-50 mM for 12 days) to study the effects of enhanced NEG of the ECM on proteoglycan-synthesis rate of the chondrocytes. This 'artificial aging' resulted in a 9-fold increase in pentosidine levels, which occurred in a dose-dependent manner (Figure 1).
A concomitant dose-dependent decrease in the cartilage proteoglycan-synthesis rate was observed (Figure 2). The increase in pentosidine levels was strongly correlated with the decrease in proteoglycan-synthesis rate (Figure 3) ($r = -0.95$, $p < 0.005$). A similar trend was observed after 4 days of culture with ribose ($r = -0.99$, $p < 0.01$; proteoglycan synthesis in the presence of 50 mM ribose declined to 26.7% of control levels). These *in vitro* data suggest that the level of ECM glycation affects chondrocyte function.

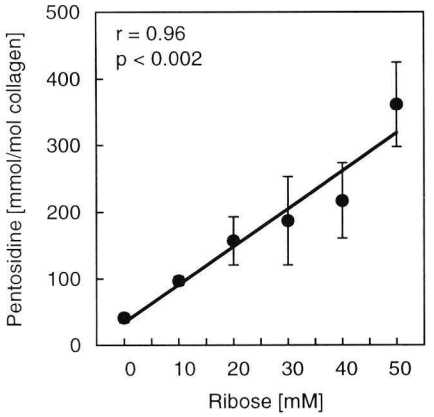

Figure 1. Levels of the glycation crosslink pentosidine in human articular cartilage after incubation with ribose (for 12 days at 37°C). Bars show the mean ± SEM of 3 independent experiments, each performed with at least 2 cartilage explants (mean age 71.7 ± 3.4 years). Incubation with ribose resulted in a concentration-dependent increase in pentosidine levels.

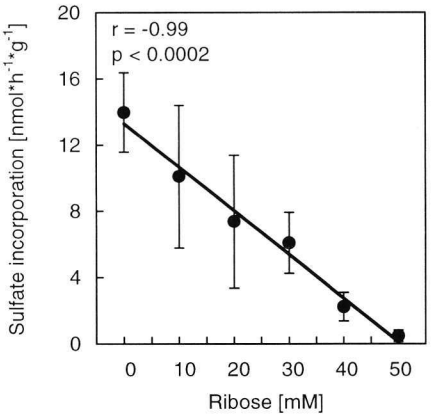

Figure 2. Proteoglycan synthesis (rate of sulfate incorporation) in human articular cartilage as a result of incubation with ribose. Bars show the mean ± SEM of 3 independent experiments, each performed with at least 8 cartilage samples cultured individually (mean age 71.7 ± 3.4 years). Incubation with ribose resulted in a concentration-dependent decrease in sulfate incorporation.

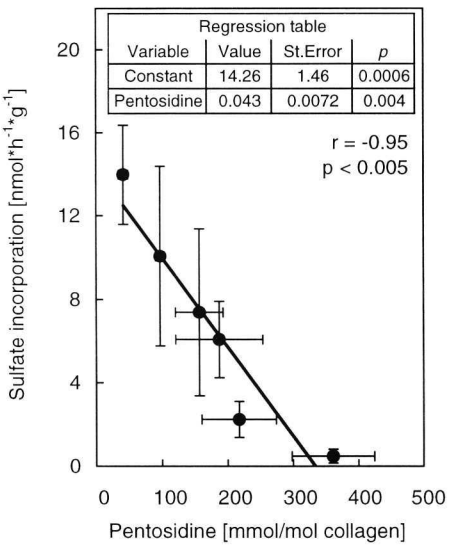

Figure 3. Influence of glycation of human articular cartilage (pentosidine) on the rate of proteoglycan synthesis (sulfate incorporation). Cartilage was cultured in the presence of increasing concentrations of ribose (see Figures 1 and 2). Bars show the mean ± SEM of 3 independent experiments (mean age 71.7 ± 3.4 years). Cartilage proteoglycan synthesis showed a high correlation with the levels of cartilage pentosidine. Linear regression analysis parameters are specified in the inset.

To ensure that ribose-derived AGEs, and not ribose itself, were responsible for this decline in the rate of proteoglycan synthesis, ribose cytotoxicity was assessed by measurement of the plasma-membrane integrity (release of LDH into the culture medium). LDH release in cartilage incubated with ribose (50 mM for 4 days) was not elevated compared to control cultures (16.7% of maximal release versus 10.4%; $p > 0.05$), suggesting that ribose is not cytotoxic under the present conditions.
In addition, the contribution of direct (NEG independent) effects of ribose on the observed inhibition of proteoglycan synthesis was studied. Cartilage proteoglycan synthesis following a 4-day incubation with ribose (50 mM) was suppressed to 20% of control levels ($p < 0.001$). At day 4, cartilage was washed to remove ribose, but not irreversibly bound AGEs. After the ribose washout, proteoglycan synthesis increased to steady levels (reached at 4 days after washout), but remained significantly inhibited compared with control cultures (45% of control, $p < 0.001$). Moreover, in these washout experiments (4-day incubation with ribose, then washout for 4 or 20 days) the rate of proteoglycan synthesis was negatively correlated with the level of glycation (data not shown). Altogether this suggests a lasting inhibitory effect of the NEG-modified ECM on chondrocyte metabolism following short-term exposure to ribose.

***Ex vivo* effect.** To further substantiate the finding that the level of NEG of the ECM influenced chondrocyte activity, the relationship between matrix glycation (pentosidine levels) and proteoglycan synthesis was studied with *ex vivo*-cultured cartilage specimens. Pentosidine levels in articular cartilage increased linearly with age (Figure 4) ($n = 36$, $r = 0.97$, $p < 0.0005$), consistent with previous findings.[22] The proteoglycan-synthesis rate showed a significant decrease with increasing age (Figure 5) ($n = 129$, $r = -0.98$, $p < 0.0002$). This was not due to a decrease in cell number with age (ages 46-103 years), since the DNA content (mean ± SD 0.17 ± 0.007 µg DNA/mg cartilage, $n = 26$) was constant with age ($r = 0.22$, $p = 0.27$). A strong relationship was found between the amount of cartilage pentosidine and the proteoglycan-synthesis rate (Figure 6) ($n = 36$, $r = -0.95$, $p < 0.02$). The higher the levels of pentosidine in cartilage, the lower the rate of proteoglycan synthesis.
Outcome of these experiments was not influenced by the sex of the donor nor by the joint origin of the cartilage (data not shown). Using multiple linear regression analysis, pentosidine levels fully explained the observed age-related decrease in proteoglycan synthesis shown in Figure 5 ($p < 0.02$), thus indicating no significant contribution of age, *per se*, in this age-related decline (Figure 6, inset)($p > 0.05$).
In both *in vitro* and *ex vivo* experiments, a strong relationship was found between the level of pentosidine and the rate of proteoglycan synthesis. Regression models for both relationships were not statistically significantly different ($p = 0.08$) (Figure 3 and Figure 6).

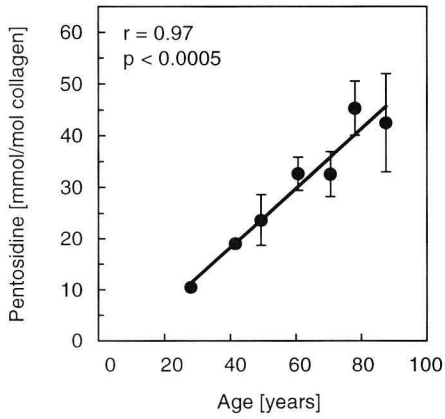

Figure 4. Pentosidine levels in human articular cartilage versus the age of the cartilage donor. Bars show the mean ± SEM level in 36 donors, clustered in 10-year age intervals (ages 25-34 [n = 2], 35-44 [n = 2], 45-54 [n = 3], 55-64 [n = 10], 65-74 [n = 11], 75-84 [n = 6] and 85-88 [n = 2]). With increasing age, articular cartilage shows a significant increase in pentosidine levels.

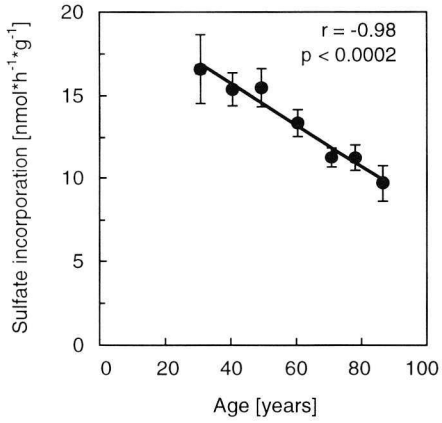

Figure 5. Proteoglycan synthesis in human articular cartilage as a function of the age of the cartilage donor. Bars show the mean ± SEM sulfate incorporation rates in donors (ages 25-34 [n=7], 35-44 [n = 11], 45-54 [n = 9], 55-64 [n = 30], 65-74 [n = 44], 75-84 [n = 21], and 85-88 [n = 7]). With increasing age, articular cartilage shows a significant decrease in proteoglycan synthesis.

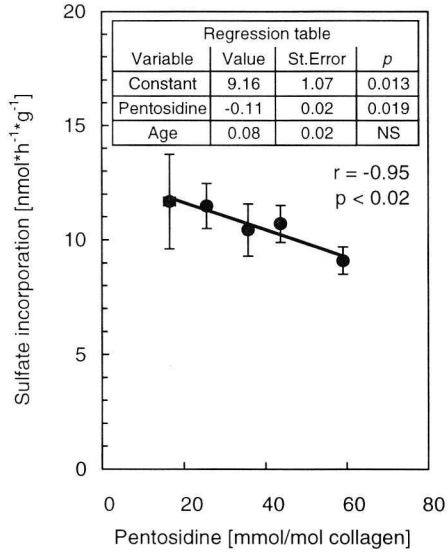

Figure 6. Pentosidine levels and proteoglycan synthesis in human articular cartilage. Bars show the mean ± SEM values for 36 donors, on which both proteoglycan synthesis (see Figure 5) and (see Figure 4) pentosidine data were available. Donors were homogeneously clustered by pentosidine levels (10-20 [n = 10], 21-30 [n = 6], 31-40 [n = 10], 41-50 [n = 6], and 51-65 [n = 5] mmoles/moles collagen). With increasing levels of pentosidine, articular cartilage shows a significant decrease in the sulfate-incorporation rate. Multiple linear regression analysis (inset) shows no significant correlation between age and proteoglycan synthesis. NS = not significant.

DISCUSSION

Many factors (biochemical, mechanical, and physical) have been shown to affect chondrocyte proteoglycan synthesis.[187] Reports on the effects of age on proteoglycan synthesis of human cartilage are rare.[194,306] Isolated chondrocytes in agarose culture show diminished aggrecan synthesis with age.[306] Femoral head cartilage shows, depending on localization within the joint, either a slight increase or a decrease in sulfate incorporation with increasing age.[194] In the present study, we show a surprisingly strong age-related decrease in articular cartilage proteoglycan synthesis (Figure 5). The mechanism by which age influences proteoglycan synthesis is unknown. No change in cartilage DNA content was observed in our samples. Moreover, chondrocyte number is reported to remain constant with aging in full thickness humeral head and femoral condyle cartilage.[197,280] Therefore, factors other than decreased cellularity must have played a role in the decreased proteoglycan synthesis rate observed. A possible explanation for this age-related reduction in synthetic activity is the level of matrix AGEs.

The age-related increase in NEG occurs in virtually all tissues, including cartilage.[22,207,256] Our data demonstrate a strong correlation between increased cartilage pentosidine levels at advanced age and the synthetic capacity of chondrocytes. In both the *in vitro* experiments with cartilage from a single age group and the *ex vivo* experiments with cartilage from different age groups, this correlation was shown to exist. Although in the *in vitro* study, ribose may have had a direct effect on cell function, the LDH data indicated that cell-death was not involved. Short-term incubation with ribose and subsequent washout demonstrated that ribose exerted a lasting inhibition of proteoglycan-synthesis rate, independent of a direct effect of ribose on proteoglycan synthesis. Together the *ex vivo* and *in vitro* data strongly suggest an influence of AGE levels on chondrocyte metabolism.

A plausible candidate mechanism for the observed effects is that increased NEG crosslinking of ECM components leads to altered signaling to the chondrocyte. In the kidney, matrix glycation has been shown to interfere with integrin-mediated cell-matrix interactions.[158] Upon binding to specific AGE receptors, glycated albumin stimulates fibronectin, laminin and collagen type IV synthesis.[71,241] Human fibroblasts exhibit a decrease in type I procollagen messenger RNA and type I collagen synthesis after exposure to glycated β_2-microglobulin.[224] In addition, the degradation of glycated collagen was shown to be dependent on interaction of AGEs with their receptor.[285] Recently, galectin-3[332] and RAGE (Grande DA, unpublished data; Clancy RM, Rediske J, TeKoppele JM, unpublished data), both receptors for AGEs, have been identified on chondrocytes. The combination of the high pentosidine levels in cartilage, which were 6-fold higher than in most other tissues,[266] and the presence of AGE receptors suggest that effects of NEG on chondrocyte activity can be mediated by these receptors. The mechanism by which such effects are exerted merits further investigation.

In conclusion, NEG may contribute to the process of OA. Elevated NEG levels result in a stiffer matrix[22] and in a decreased synthetic capacity of the chondrocytes, thus diminishing the capacity of chondrocytes to uphold the matrix integrity after injury. This may contribute to the progression of cartilage defects. These findings suggest that the increased sensitivity for damage and the impaired capacity of the chondrocytes to maintain the matrix integrity by NEG may contribute to the high incidence of OA in the elderly.

ACKNOWLEDGEMENTS

This study was supported by grants from the Dutch Arthritis Association and the Netherlands Organization for Scientific Research.

ACCUMULATION OF ADVANCED GLYCATION ENDPRODUCTS REDUCES CHONDROCYTE-MEDIATED EXTRACELLULAR MATRIX TURNOVER IN HUMAN ARTICULAR CARTILAGE

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ABSTRACT

Objective. *The prevalence of osteoarthritis increases with age and coincides with the accumulation of advanced glycation endproducts (AGEs) in articular cartilage, suggesting a causal role for these glycation products in the development of OA. This study was designed to examine the effects of accumulation of AGEs on the turnover of the extracellular matrix of human articular cartilage.*

Design. *Chondrocyte mediated cartilage degradation (GAG release, colorimetric) was measured ex vivo in human articular cartilage of donors aged 19-82 yr. (n=30, 4-day culture). In addition, to mimic the age-related increase in AGE levels in vitro, cartilage was cultured in the absence or presence of glucose (3, 10, 30 mM), ribose (3, 10, 30 mM) or threose (3, 10 mM). Cartilage degradation and proteoglycan synthesis ($^{35}SO_4^{2-}$ incorporation) were measured and related to the degree of cartilage AGE levels (fluorescence at 360/460 nm).*

Results. *Chondrocyte-mediated degradation of articular cartilage (i.e. ex vivo GAG release) decreased with increasing age of the cartilage donor ($r = -0.43$, $p < 0.02$). In vitro incubation of cartilage with glucose, ribose or threose resulted in a range of AGE levels that was highly correlated to the chondrocyte-mediated cartilage degradation ($r = -0.77$, $p < 0.001$, $n = 26$). In addition, in these in vitro glycated cartilage samples, a decrease in proteoglycan synthesis was observed at increasing AGE levels ($r = -0.54$, $p < 0.005$, $n = 25$).*

Conclusions. *This study shows that an increase in AGE levels negatively affects the proteoglycan synthesis and degradation of articular cartilage. In combination, these two effects reduce the turnover of the cartilage and thereby the maintenance and repair capacity of the tissue. By this mechanism, the age-related increase in cartilage AGE levels may contribute to the development of OA.*

INTRODUCTION

Osteoarthritis (OA) is one of the most common diseases of the elderly: at age >60 years the majority of the population has radiological osteoarthritis in one or more joints.[76,97] Although its role in the pathophysiology is largely unknown, age has been recognized as one of the main risk factors for the development of OA. Age-related changes in articular cartilage are therefore likely to play a role in the etiology of OA.

One of the major age-related changes in articular cartilage is the accumulation of advanced glycation endproducts (AGEs), resulting from the spontaneous reaction of reducing sugars with proteins.[247] The initial step in this reaction is the formation of a Schiff base through the condensation of a sugar aldehyde with an ε-amino group of (hydroxy)lysine or arginine residues in proteins. After a cascade of largely unknown reactions, the Schiff bases are ultimately converted into a heterogeneous collection of fluorophores and chromophores, collectively known as advanced glycation

endproducts (AGEs).[18,247] Once formed, AGEs can only be removed from the body when the protein involved is removed. Consequently, though all proteins are prone to AGE formation, AGEs especially accumulate in those with slow turnover.[309] From age 20 onward (skeletal maturity), levels of AGEs increase linearly with age in articular cartilage.[22,80,308,309] This increase in AGE levels coincides with the age-related increase in the incidence of OA, suggesting a causal role for AGEs in the disease process.

Accumulation of AGEs is correlated with impaired biomechanical properties in many tissues including arteries, lens, tendon, skin and articular cartilage.[10,20,22,209,245,327] In addition, AGE affect the biochemical properties of proteins: extracellular matrix susceptibility to degradation by purified proteinases is decreased at elevated AGE levels in lens capsule, basal membrane and skin.[212] On cellular level,[181,199,262] AGE-modified proteins affect processes such as the synthesis of matrix components. AGE-modified albumin has been reported to stimulate collagen type IV synthesis in glomerular mesangial cells.[9,72] In contrast, mesangial cells grown in the presence of glycating sugars or on glycated matrix show decreased synthesis of collagen types I and IV.[272] Type I collagen synthesis is also inhibited in endothelial cells and in fibroblast cell lines upon exposure to glycated albumin.[70,224]

The combined effects of AGEs on synthesis and degradation of matrix constituents indicate that extracellular matrix turnover (i.e. matrix synthesis and degradation) is affected by AGEs. Since articular cartilage is one of the tissues containing the highest amounts of AGEs in the body, AGE-related effects on extracellular matrix turnover are likely to be of major importance in this tissue.[22,266,308,309] Almost a decade ago, Kuettner already suggested that an age-dependent increase in AGE levels could result in enhanced resistance of cartilage collagen fibrils to enzymatic breakdown: evidence for this hypothesis is lacking thus far.[160] Therefore, the present study was designed to investigate the relation between AGE levels and chondrocyte-mediated turnover of human articular cartilage.

MATERIALS AND METHODS

Cartilage samples. Macroscopically normal cartilage was obtained from femoral condyles (n = 30; 19-82 yr.) and humeral heads (n = 7; 46-64 yr.) *post-mortem* at autopsy within 24 hours after death of the donors. Previous studies have shown that this cartilage is biochemically normal, even if focal OA lesions are present elsewhere in the joint.[164] Full thickness cartilage was cut into square pieces (5-15 mg), which were randomly divided over experimental conditions and that were all individually handled. The cartilage explants were cultured in Dulbecco's Modified Eagle's Medium (GibcoBRL) supplemented with 0.85 mM ascorbic acid (Sigma), 2 mM glutamin (GibcoBRL), 100 IU/ml sodium benzylpenicillin, 100 IU/ml streptomycin sulfate and 10% (v/v) heat inactivated adult pooled human male AB^+ serum (200 µl/well, 37°C, 5% CO_2 in air).[80] To circumvent disturbing effects of the initial tissue processing, cartilage was always precultured for 16-24 hours in culture medium. After preculture, medium was replaced by fresh culture medium and cartilage samples were cultured for 4 days.

Cartilage degradation. To confirm that in our system GAG release is largely chondrocyte-dependent, the chondrocyte-mediated release of glycosaminoglycans (GAGs) into the culture medium (humeral head cartilage of 4 donors; 55 ± 4 yr.) was compared to the loss of GAGs from non-viable cartilage (n = 3; 56 ± 4 yr.). Cartilage was rendered non-viable by freezing at -20°C followed by thawing at room temperature, and this cycle was repeated three times.[238] Subsequently, cartilage was washed and incubated according to standard culture conditions.

***In vitro* enhancement of AGE levels.** Humeral head cartilage (n = 3 donors; 46, 52, 58 yr., n = 12 samples per condition per donor) was incubated with glucose (3, 10 and 30 mM), D(-)-ribose (3, 10 and 30 mM) or threose (3 and 10 mM) to enhance AGE levels. Each of these sugars has a different potency of glycation and therefore this approach results in a quantitative and qualitative range of AGE levels, mimicking the diversity of AGE found *in vivo*.[87,170]

AGE measurements. Cartilage AGE levels were determined as described previously.[308] Cartilage samples (3 samples per condition per donor) were reduced overnight, sequentially treated with chondroitinase ABC (Sigma), trypsin (Boehringer Mannheim) and *Streptomyces* hyaluronidase (Sigma), and solubilized by digestion with papain. In papain digests, AGE specific absorption at 340 and 414 nm (Titertek Multiskan MCC/340) and fluorescence (ex: 360 nm, em: 460 nm; CytofluorII, PerSeptive Biosystems) were determined.[308] Modification of the amino acids arginine and (hydroxy)lysine was determined in acid hydrolysates (6 N HCl, 18 hrs, 110°C) of the papain digested cartilage samples by RP-HPLC of 9-fluorenylmethyl chloroformate derivatized amino acids, as described previously.[24] NEG parameters were expressed per amount of hydroxyproline (absorption and fluorescence) or per collagen triple helix (amino acid modification; assuming 300 hydroxyproline residues per triple helix).[27]

Proteoglycan release. Cartilage degradation (n = 9 samples per donor per condition) was assessed by colorimetric assessment of glycosaminoglycan (GAG) release into the culture medium.[250] GAGs were precipitated and stained with Alcian Blue dye solution (Sigma). Staining was quantified photometrically by the change in absorbance at 620 nm. Shark cartilage chondroitin sulfate (Sigma) served as a standard. GAG release was normalized to the wet weight of the cartilage samples.[250]

Proteoglycan synthesis. As measure of proteoglycan synthetic activity the rate of sulfate incorporation was determined (9 explants per donor per condition) using carrier free $^{35}SO_4^{2-}$ (NEN Dupont) during the last 4 hours of the 4-day culture period. After labeling, tissue digestion (papain) and precipitation of glycosaminoglycans (cetylpyridiniumchloride, Sigma), $^{35}SO_4^{2-}$ incorporation was analyzed by liquid scintillation counting. Sulfate incorporation was calculated from the $^{35}SO_4^{2-}$ incorporation rate and the specific activity of the culture medium and was expressed as nmol of sulfate incorporated per hour per gram wet weight of tissue.[80,250]

Cytotoxicity. Release of LDH into the culture medium was determined as a measure of possible sugar-induced cell death (Cytotoxicity kit, Boehringer). LDH activity measurements were corrected for inhibition of LDH activity as a result of glycation of LDH, using purified LDH (Boehringer) incubated for 4 days in the presence or absence of glucose (3, 10 and 30 mM), D(-)-ribose (3, 10 and 30 mM) or threose (3 and 10 mM) in culture medium.

Statistical analysis. Statistical evaluation was performed using SPSS software version 10.0 (SPSS, Chicago, IL.). Data are presented as mean ± SEM. Correlations between AGE parameters were determined by bivariate correlation analysis (Pearson). Relations between cartilage AGE levels and proteoglycan synthesis or degradation were tested by linear regression analysis. Differences between groups were analyzed by Wilcoxon tests. P values < 0.05 were considered to represent statistically significant differences.

RESULTS

Effect of aging. The capacity of chondrocytes to remodel their extracellular matrix is an important feature in the maintenance of cartilage integrity. The age-related increase in OA incidence suggests that with increasing age, the capacity to maintain this integrity decreases. Since degradation of extracellular matrix components is an essential part of matrix remodeling, age-related changes in cartilage degradation may be of importance in the etiology of OA. Therefore, cartilage degradation was studied in human knee cartilage of donors of various ages. With increasing donor age, a significant decrease in chondrocyte-mediated GAG release was observed in human articular cartilage ($r = -0.43$, $p < 0.02$, $n = 30$, 19-82 years, Figure 1).

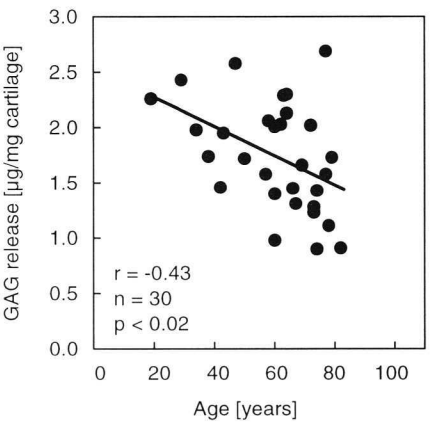

Figure 1. GAG release decreases with donor age. Chondrocyte-mediated release of GAGs from human femoral condyle cartilage decreases with increasing donor age (n = 30, 19-82 yr.). Each symbol represents the average of at least 8 individually handled cartilage samples.

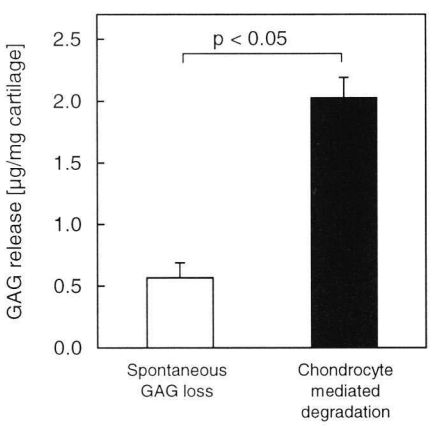

Figure 2. Spontaneous loss of GAGs *vs.* chondrocyte-mediated cartilage degradation. GAG release into culture medium was determined as a marker for matrix degradation in cartilage of which cellular activity was abolished by freeze-thawing (spontaneous GAG loss: n = 3 donors, 56 ± 4 yr.; 9 samples per donor) and in normally cultured explants (chondrocyte-mediated degradation: n = 4 donors, 55 ± 4 yr.; 9 samples per donor). Bars represent mean values ± SEM. The age did not differ between both groups ($p > 0.8$). Chondrocyte-mediated release of GAGs is 4-fold increased compared to the spontaneous GAG loss ($p < 0.05$).

To ensure that mainly chondrocyte-mediated cartilage degradation rather than spontaneous loss of GAGs was measured, cartilage degradation was compared between normally cultured explants *vs.* age-matched explants, which were rendered

non-viable by freeze-thawing. Chondrocyte-mediated GAG release was 4-fold higher than spontaneous GAG release ($p < 0.05$, Figure 2), emphasizing that in the human cartilage explant culture system GAG release is mainly (>75%) chondrocyte-mediated. The age-related decrease in GAG release (Figure 1) coincides with the frequently observed age-related increase in cartilage AGE levels; a 50-fold increase is observed between age 20 and 80 years.[22,80,308] To investigate whether this age-related increase in cartilage AGE levels is causally involved in the observed decrease in matrix loss, *in vitro* enhancement of cartilage AGE levels was employed to exclude possible interference from other age-related changes.

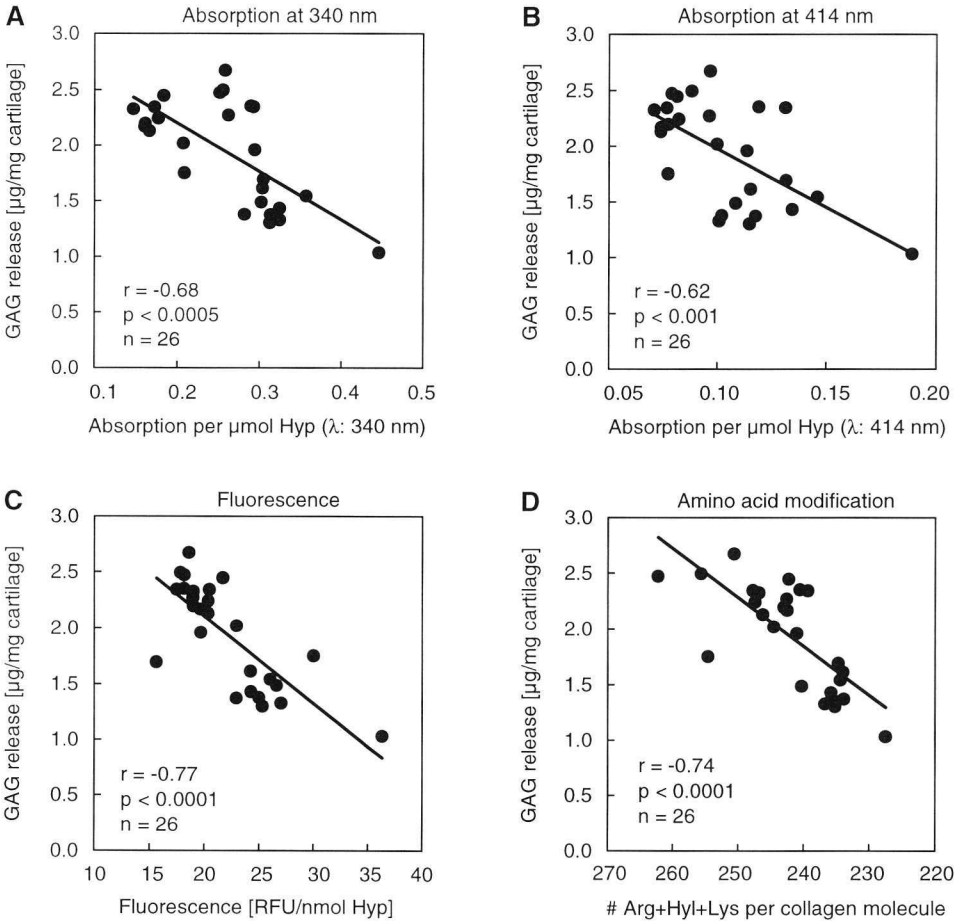

Figure 3. Chondrocyte-mediated GAG release decreases with cartilage AGE levels. Each data point represents the mean of n = 9 samples for humeral head cartilage degradation (GAG release) and n = 3 samples for AGE levels. GAG release decreases linearly with increasing AGE levels, either determined as AGE related absorption at 340 nm (A), at 414 nm (B), fluorescence at 360/460 nm (C), or amino acid modification (D). The correlation between matrix degradation and AGE levels is highly significant for all these AGE measures (A-D; $p < 0.001$).

***In vitro* formation of AGEs.** Non-enzymatic glycation is initiated by many different carbohydrates *in vivo* and results in a heterogeneous collection of AGEs, few of which have been characterized.[247] To mimic this *in vivo* situation, different reducing sugars can be used to initiate the AGE formation *in vitro*. Consequently, general measures for quantification of AGE levels (rather than a specific AGE) must be employed to examine the effects of AGEs.[171] In the present study, humeral head cartilage was cultured with three different reducing sugars (glucose, ribose and threose) to initiate the AGE formation. This approach resulted in a range of extracellular matrix glycation levels in the different samples. AGE specific yellowing ranged from 0.15 to 0.45 (absorbance at 340 nm per µmol hydroxyproline), a 3-fold increase between the lowest and highest glycated samples. A comparable increase in AGE levels was observed for the absorbance at 414 nm (0.07 to 0.19; 2.7-fold increase) and the AGE specific fluorescence (15.6 to 36.3 RFU/nmol hydroxyproline; ~2.5-fold increase). The AGE modification resulted in a 15% loss of arginine and (hydroxy)lysine residues (unmodified: 262 residues per collagen molecule; maximally modified: 228 residues).

AGE effects on matrix degradation. Culture of human humeral head cartilage for 4 days resulted in the release of ~2.5 µg GAG per mg cartilage into the culture medium, in control conditions. This release was decreased at increasing AGE levels, down to ~1.0 µg GAG per mg cartilage at the highest AGE levels (Figure 3A-D).
All the AGE measures, absorption at 340 and 414 nm (Figure 3A and 3B), fluorescence (Figure 3C) and amino acid modification (Figure 3D) showed a highly significant, negative linear correlation with GAG release ($p < 0.005$ for all parameters). All AGE measures showed highly significant correlation to one another, indicating that in this study with *in vitro* enhancement of AGE levels, these parameters are interchangeable (bivariate Pearson correlation coefficients; Table 1).

Table 1. Pearson correlation coefficients of *in vitro* enhanced AGE levels in articular cartilage (by exposure to reducing sugars). All AGE measures (absorption, fluorescence and amino acid modification) show strong and highly significant correlations.

	Amino Acid Modification	Absorption at 340 nm	Absorption at 414 nm
Absorption at 340 nm [per µmol hydroxyproline]	$r = 0.56$ $p < 0.005$		
Absorption at 414 nm [per µmol hydroxyproline]	$r = 0.66$ $p < 0.0005$	$r = 0.92$ $p < 0.0001$	
Fluorescence at 360/460 nm [RFU per nmol hydroxyproline]	$r = 0.46$ $p < 0.02$	$r = 0.52$ $p < 0.01$	$r = 0.48$ $p < 0.02$

AGE effect on proteoglycan synthesis. In addition to matrix degradation, synthesis of cartilage constituents is an important factor in the turnover of the tissue. The effect of the heterogeneous collection of cartilage AGEs on the capacity of chondrocytes to synthesize proteoglycans was therefore determined by measuring the incorporation of the radioactive tracer $^{35}SO_4^{2-}$. Concomitant with the sugar-induced increase in AGE levels, a decrease in proteoglycan synthesis was observed (Figure 4; $r = -0.54$, $n = 25$, $p < 0.005$, AGE-related fluorescence). These data are consistent with previous observations using both a different experimental approach (using only a concentration range of ribose) and a different AGE marker (pentosidine).[80]

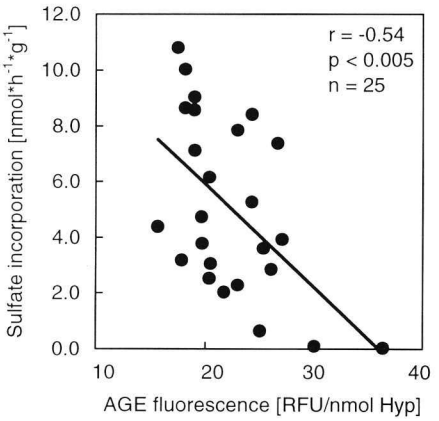

Figure 4. Proteoglycan synthesis in human humeral head cartilage after incubation with different sugars to enhance extracellular matrix AGE levels. Each data point represents the mean of n = 9 samples for proteoglycan synthesis (rate of $^{35}SO_4^{2-}$ incorporation) and n = 3 samples for AGE levels (AGE fluorescence). The decrease in proteoglycan synthesis is significantly correlated to the increase in AGE-fluorescence ($p < 0.005$).

Chondrocyte viability. Previous studies showed that sugars are not cytotoxic in the concentrations used.[72,80,111] In the present study, to ensure that sugar-derived AGEs (and not the sugars themselves) were responsible for the decline in chondrocyte-mediated cartilage turnover, cell death was assessed by measurement of the plasma membrane integrity. At the sugar concentrations used, no cytotoxicity was observed (nor by comparison of different treatment groups by ANOVA $p > 0.05$; nor by correlating the LDH release to the AGE parameters: $p > 0.05$ for all parameters).

DISCUSSION

The maintenance of extracellular matrix integrity in articular cartilage depends on the balance between synthesis and degradation of matrix components. The chondrocytes residing within the cartilage matrix are responsible for maintaining this tight equilibrium. Due to the avascular nature of cartilage, no other cell types contribute to the internally regulated maintenance of the matrix.[214] Changes in the capacity of chondrocytes to preserve their extracellular matrix will therefore directly affect the quality of the tissue.

With increasing age, the capacity of articular cartilage to synthesize proteoglycans

decreases.[32,80,305] Concomitantly, the AGE levels of the tissue increase linearly with age from 20 years onward.[22,80,308] Previously, we showed that the level of a specific AGE (i.e. pentosidine) is highly correlated with the capacity of cartilage to synthesize proteoglycans.[80] Employing a different approach by using different AGE precursors (glucose, ribose and threose that generate a wide variety of AGEs that are quantified with general AGE measures), the present study shows that *in vitro* enhancement of AGE levels results in decreased proteoglycan synthesis.

In addition to the effects of AGEs on proteoglycan synthesis, the present data show that chondrocyte-mediated degradation of the cartilage matrix is severely impaired at increased AGE levels. As yet, the exact mechanism by which AGE accumulation results in diminished GAG release remains unclear. Accumulation of AGEs may render proteins less susceptible to degradation by interfering with proteinase-substrate interactions: AGEs change protein charge and conformation and/or may cause sterical hindrance and thereby interfere with proteinase-substrate interactions. In addition, increased inter- and intramolecular AGE crosslinks may diminish the release of degraded cartilage constituents.[18]

Both synthesis, degradation, and removal of matrix constituents such as collagens and proteoglycans are important features of physiological tissue turnover. In response to external stimuli, chondrocytes adjust the surrounding matrix to new requirements (e.g. response to differences in loading regime).[46,213,279] This adaptation of the articular cartilage structure is essential for the maintenance of a healthy tissue capable to withstand the repetitive loading forces intrinsic to the normal use of a joint. Decreased matrix turnover due to the increased AGE levels may lead to a less adequate response to stimuli, thus inferior cartilage quality and consequently to enhanced susceptibility to damage.

In addition to being indispensable for maintaining the cartilage integrity under physiological conditions, synthesis and degradation of matrix components also play a role in the capacity of the tissue to repair damage during pathophysiology. Degradation and removal of matrix proteins is essential in repair processes after tissue injury. Damaged tissue constituents are proteolytically cleaved, removed and replaced by new, intact molecules. If removal and replacement are hampered by the presence of AGEs, repair will be slowed, and tissue damage is more likely to progress. Moreover, AGEs can even be hypothesized to amplify cartilage degeneration: due to AGE accumulation, fragments of matrix constituents are less readily removed and since cartilage degradation products have been shown to stimulate chondrolysis, this may enhance cartilage degradation.[132,234]

The increase in AGE levels has been shown to affect the mechanical properties of articular cartilage: increased tissue stiffness has been observed at increased AGE levels, possibly leading to increased susceptibility towards mechanically induced damage.[22] The increased susceptibility towards mechanical damage, in combination with an AGE-dependent decrease in matrix turnover as demonstrated in the present study, may predispose to the development of OA with increasing age.

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ACCUMULATION OF ADVANCED GLYCATION ENDPRODUCTS DECREASES COLLAGEN TURNOVER BY BOVINE CHONDROCYTES

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Experimental Cell Research, in press

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ABSTRACT

The integrity of the collagen network is essential for articular cartilage to fulfil its function in load support and distribution. Damage to the collagen network is one of the first characteristics of osteoarthritis. Since extensive collagen damage is considered irreversible, it is crucial that chondrocytes maintain a functional collagen network. We investigated the effects of advanced glycation endproducts (AGEs) on the turnover of collagen by articular cartilage chondrocytes. Increased AGE levels (by culturing in the presence of ribose) resulted in decreased collagen synthesis ($p < 0.05$) and decreased MMP-mediated collagen degradation ($p < 0.02$). The latter could be attributed to increased resistance of the collagen network to MMPs ($p < 0.05$) as well as the decreased production of MMPs by chondrocytes ($p < 0.02$). Turnover of a protein is determined by its synthesis and degradation rates and therefore these data indicate that collagen turnover is decreased at enhanced AGE levels. Since AGE levels in human cartilage increase ~50 fold between age 20 and 80, cartilage collagen turnover likely decreases with increasing age. Impaired collagen turnover adversely affects the capacity of chondrocytes to remodel and/or repair its extracellular matrix. Consequently, age-related accumulation of AGE (via decreased collagen turnover) may contribute to the development of cartilage damage in osteoarthritis.

INTRODUCTION

In articular cartilage, chondrocytes are surrounded by an extracellular matrix that accounts for >95% of the total volume of the tissue.[214] This matrix is composed of a three-dimensional network of type II collagen fibrils, which determines the shape of the tissue and endows the cartilage with resistance towards tensile forces.[214] Within this collagen network highly negatively charged proteoglycans are embedded that generate a large osmotic pressure and thereby maintain tissue hydration.[125] The capacity to withstand compressive loading is determined by the balance between the swelling capacities of the proteoglycans and the tensile stiffness of the collagen network. The integrity and organisation of the collagen network is therefore essential in the functioning of the tissue.[28]. Failure of the collagen network is one of the first characteristics of the degenerative joint disease osteoarthritis.[28,190] The cartilage extracellular matrix cannot be regenerated when the collagen network is extensively damaged, whereas proteoglycan depletion can be restored.[92,141] In this respect, collagen damage is a critical event in the development of OA and expectedly indicates a point-of-no-return in the development of the disease. To maintain a healthy collagen network chondrocytes continuously remodel the extracellular matrix, albeit slowly.[42,214,218] Changes in the capacity of the chondrocytes to maintain the collagen network are likely to affect the integrity of the cartilage matrix and thereby influence the resistance to cartilage degeneration.

Osteoarthritis (OA) is one of the most common diseases of the elderly, with a large impact on the patient's quality of life.[97,127] The most prominent feature of OA is the progressive destruction of the articular cartilage in the joint resulting in impaired joint motion, severe pain and ultimately disability.[76] The incidence of OA increases with increasing age and although the aetiology of OA remains largely unknown, age is recognised as the main risk factor.[97,127] One of the major age-related changes in articular cartilage that is suggested to play a role in the development of OA, is the accumulation of advanced glycation endproducts (AGEs). AGE formation is initiated by the spontaneous condensation of a reducing sugar with an amino group of (hydroxy)lysine or arginine residues in proteins.[247] Subsequent Amadori rearrangement and Maillard browning reactions ultimately result in the generation of a range of fluorophores and chromophores, collectively known as AGEs.[18,247] All proteins are prone to AGE formation and, once formed, AGEs cannot be removed from proteins unless the protein is removed. Consequently, AGEs accumulate in all tissues with age, but this accumulation is most pronounced in long-lived proteins such as human articular cartilage collagens ($t_{1/2}$ ~117 yr.).[309] Levels of pentosidine - a well-characterised AGE that is considered an adequate marker for glycation - increase >50-fold in human cartilage collagen between age 20 and 80 years.[308]

In tissues other than cartilage, accumulation of AGEs has been shown to affect collagen synthesis. AGE-modified albumin has been reported to stimulate collagen type IV synthesis by glomerular mesangial cells.[9,72] In contrast, others demonstrate decreased synthesis of collagen types I and IV by mesangial cells grown in the presence of glycating sugars or on glycated matrix.[272] Also fibroblasts and endothelial cells show decreased collagen synthesis in the presence of glycated albumin.[70,224] In addition, the susceptibility of collagen to degradation by purified proteinases is decreased at elevated AGE levels in basal membrane and skin.[212,262] Since the turnover of a protein is determined by its synthesis as well as its degradation, these data indicate that accumulation of AGEs lowers collagen turnover by a variety of cell types. In articular cartilage, collagen turnover by the chondrocytes is essential to maintain a functional collagen network. With increasing age the incidence of OA increases, which coincides with an increase in AGEs in the cartilage. We hypothesise that the age-related increase in cartilage AGE levels, via reduction of collagen turnover, contributes to the development of OA. In the present study, the effect of AGEs on collagen turnover by bovine chondrocytes was investigated. Insight in such effects may help elucidating the aetiology of OA.

MATERIALS AND METHODS

Cell isolation and culture. Chondrocytes from the metacarpophalangeal joint of calves (6-9 months old, local slaughterhouse) were isolated by collagenase digestion as described previously.[37-39] Cells were entrapped in 1.2% alginate beads (Keltone® LVRC, Monsanto Pharmaceutical Ingredients,

Chicago, USA) at a concentration of 2.5 x 10^6 cell/ml and cultured in Dulbecco's Modified Eagle's Medium with Glutamax (GibcoBRL) supplemented with ascorbic acid (0.28 mM, Sigma), penicillin (100 IU/ml; Bio Whitaker), streptomycin (100 µg/ml Bio Whitaker) and 10% FCS (GibcoBRL). Cells were cultured at 10 alginate beads per well in 24-well culture plates (Costar) in 0.5 ml medium at 37°C in humid atmosphere (5% CO_2 in air). The medium was refreshed twice weekly. Culture medium was supplemented with 20 mM D(-)-ribose (Sigma) to enhance cartilage AGE levels.

Amino acid analysis. Amino acid content was determined after acid hydrolysis (6 N HCl, 18 hrs, 110°C) of papain digested alginate beads (2 hours at 65°C in 250 µl 50 mM phosphate buffer (pH 6.5) containing 2 mM L-cysteine (Sigma), 2 mM EDTA and 2% papain (Sigma)) by reversed-phase HPLC of 9-fluorenylmethyl chloroformate (Fluka) derivatised amino acids, as described previously.[24] Total protein content was calculated from the amino acid content (assuming an average molecular weight of 115 g/mol for each amino acid) and normalised to the cellularity of the bead. DNA levels were determined in papain digested alginate beads using the fluorescent dye Hoechst 33258 (Sigma) and calf thymus DNA (Sigma) as a reference. Collagen content was calculated assuming 300 residues hydroxyproline per triple helix and a molecular weight of 300000 g/mol. Collagen content was normalised to the DNA content.[27]

Glycation levels. Pentosidine levels were determined as a marker for AGE levels by reversed-phase HPLC after acid hydrolysis as described previously.[24,80] Pentosidine levels were expressed per µg protein.

Collagen degradation. MMP-mediated collagen degradation was induced as described previously.[39] In short, alginate beads (cultured for three weeks) were transferred to 48 well plate (4 beads/well), washed 3 times for 1 hour with serum-free culture medium containing 1% human serum albumin, and next stimulated with 20 ng/ml IL-1ß (PeproTech EC Ltd. London, UK) for 48 hrs at 37°C. Subsequently, proMMPs were activated with 1 mM *p*-aminophenyl mercuric acetate (APMA, Sigma, St Louis) in Tris buffer pH7.5 (50 mM Tris, 5 mM $CaCl_2$, 150 mM NaCl, 1 µM $ZnCl_2$, 0.01% Brij-35, 0.02% NaN_3) for 4 hrs. Release of hydroxyproline from the bead (as a percentage of the total hydroxyproline content of the bead) was determined as a measure for collagen degradation. Hydroxyproline levels were determined using the colorimetric assay described by Creemers *et al.*[78,278]

MMP activity. Gelatinase activity was assessed using fluorogenic substrate TNO211-F as described previously.[35] MMP-1 activity was determined with the selective fluorogenic substrate TNO113-F (2.5 µM, Dabcyl-Gaba-Pro-Cha-Abu-Smc-His-Ala-Cys(Fluorescein)-Gly-Lys-NH_2; Cha, cyclohexylalanine; Abu, aminobutyric acid; Smc, S-methyl-cysteine), in the presence of EDTA-free general proteinase inhibitor cocktail Complete™ (Roche) to prevent conversion of TNO113-F by proteinases other than MMPs. Further improvement of the assay specificity for MMP-1 was achieved by addition of the MMP inhibitor RS102,223 (final concentration 1 µM; a generous gift of Dr. R Martin, F Hoffmann-La Roche Ltd.). MMP-1 is not inhibited by RS102,223 (IC_{50} > 700 nM), while other MMPs are strongly inhibited at 1 µM RS102,223 (IC_{50} values for MMP-3, -8, -9, -12, -13, and -14 are below 1 nM).[35,36] This assay is considered to represent MMP-1 activity.

Collagenase-3 incubations. Human recombinant proMMP-13 (a generous gift of Dr. P Mitchell, Pfizer Central Research, Groton, CT, USA) was activated for 2 hours at 37°C with 2 mM APMA. The amount of active enzyme was calibrated by active-site titration with TIMP-1 (generous gift from Dr. G. Murphy, University of East Anglia, Norwich, UK). Activated MMP-13 was diluted to a concentration of 20 nM in Tris buffer, (see above). Alginate beads (control and glycated, n = 5 independent incubations) were incubated for 2 days at 37°C with 100 µl 20 nM MMP13. Subsequently collagen degradation was measured as hydroxyproline release into the buffer as described above.

Statistical analysis. Statistical evaluation was performed using SPSS software version 10.0 (SPSS, Chicago, IL.). Data are presented as means ± SEM. Differences in bead composition during culture were analysed using trend analysis (paired T-tests). Differences between control and AGE groups were evaluated using Student T-tests. Multiple group comparisons were tested by ANOVA, followed by Tukey *post hoc* tests. *P* values less than 0.05 were considered to indicate statistically significant differences.

RESULTS AND DISCUSSION.

Effect of AGEs on collagen synthesis. Previously, we have shown that the alginate culture system is an adequate model to study synthesis and degradation of extracellular matrix by articular cartilage chondrocytes.[37,38] To study the effects of accumulation of AGEs on collagen turnover by chondrocytes, alginate beads were cultured in the presence or absence of 20 mM ribose over a culture period of 17 days (Figure 1A). The amount of pentosidine increased 50-fold in alginate beads cultured with ribose. As expected, beads cultured in the absence of ribose showed no pentosidine accumulation ($p < 0.02$ for control *vs.* ribose cultures).

The synthesis of collagen was clearly affected by the increased glycation levels. On average, incubation with ribose caused ~20% reduction of collagen content (Figure 1B, $p < 0.05$). This decease in collagen synthesis was not due to a general decrease in protein synthesis rate: total protein content was similar in the presence or absence of ribose, indicating the absence of generalised cytotoxic effects of ribose on cell function (Figure 1C, $p > 0.1$). These data are in agreement with a similar decrease in collagen synthesis by fibroblasts, endothelial cells and mesangial cells at increased AGE levels,[70,224,272] while total protein synthesis and cell proliferation were unaffected by glycating sugars.[272]

The mechanisms by which AGEs reduce collagen synthesis are unknown as yet. AGEs haven been shown to induce IL-1 production,[203,323] a pro-inflammatory cytokine that reduces extracellular matrix synthesis in cartilage.[324] AGE receptors, such as RAGE and galectin-3 on chondrocytes[80] may be responsible for this effect. Alternatively, AGE modification of the extracellular matrix has been shown to decrease integrin-mediated cell attachment to the matrix,[226] indicating disturbed cell-matrix interactions that may also affect collagen metabolism. In addition, we have previously shown that the level of crosslinking of the extracellular matrix affects collagen synthesis: decreased enzymatic crosslinking (pyridinolines) resulted in increased collagen synthesis.[37] A shift in the distribution of collagen over the pericellular and interterritorial matrix was observed: less collagen was deposited in the pericellular matrix at decreased crosslink levels.[37] These data suggested that chondrocytes continue to produce collagen until the collagen network surrounding the cell is dense enough to cause feedback to the chondrocytes and thereby inhibits collagen synthesis. Following this reasoning, increased crosslinking will retain the collagen in the pericellular matrix, which causes decreased collagen synthesis by cell-matrix

interaction. Indeed, the present data demonstrate that collagen synthesis is inhibited upon enhanced matrix crosslinking through the formation of AGEs.

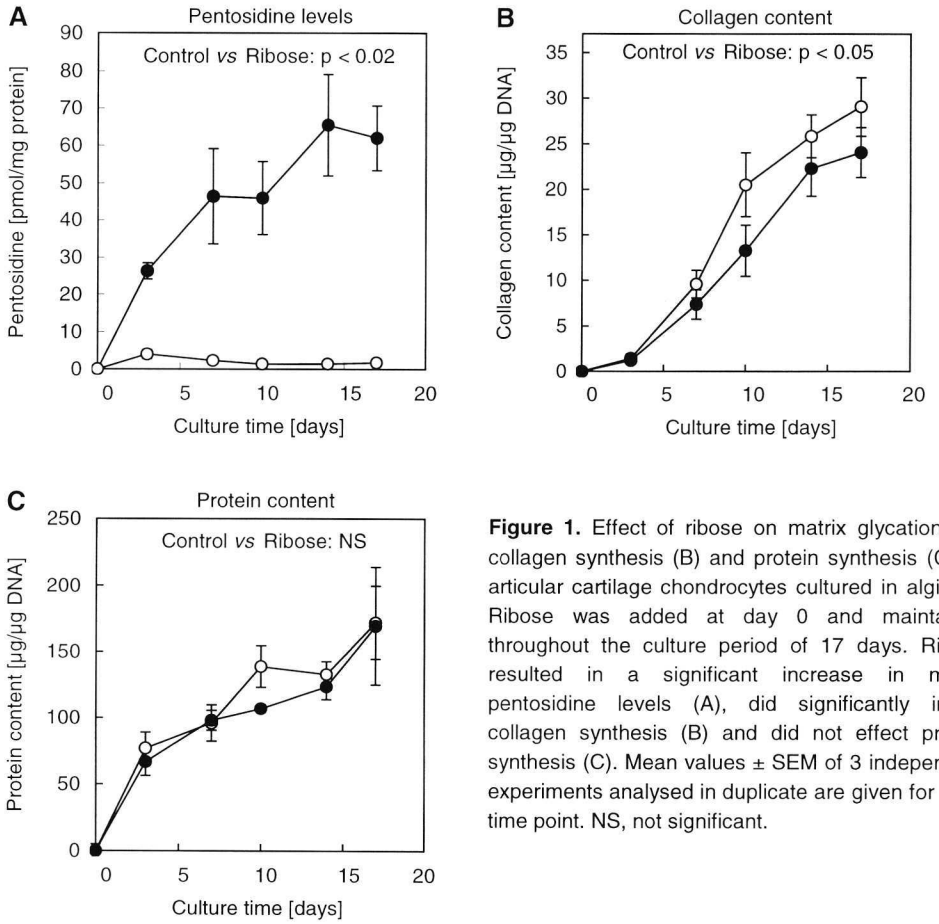

Figure 1. Effect of ribose on matrix glycation (A), collagen synthesis (B) and protein synthesis (C) by articular cartilage chondrocytes cultured in alginate. Ribose was added at day 0 and maintained throughout the culture period of 17 days. Ribose resulted in a significant increase in matrix pentosidine levels (A), did significantly inhibit collagen synthesis (B) and did not effect protein synthesis (C). Mean values ± SEM of 3 independent experiments analysed in duplicate are given for each time point. NS, not significant.

Collagen degradation model. To study the effect of glycation on MMP-mediated degradation of collagen, alginate beads were stimulated with IL-1ß to induce proMMP production and subsequently incubated with APMA to convert proMMPs into active enzymes.[39] Collagen degradation was monitored as the release of hydroxyproline. In control cultures (without ribose), treatment with IL-1ß and APMA increased collagen release 3-fold (Figure 2A; $p<0.001$ *vs.* control beads treated with IL-1ß only). This effect was completely inhibited by the addition of the MMP inhibitor BB94 ($p<0.005$ *vs.* IL-1ß/APMA and $p>0.9$ *vs.* control beads treated with IL-1ß only). These data indicate that collagen release after IL-1ß/APMA incubation indeed represents MMP-mediated collagen degradation. This was further supported by MMP activity measurements. IL-1ß induced proMMP production and consequently, collagenase (MMP-1) and

gelatinase (MMP-2 and -9) activity were undetectable (Figure 2B and 2C). Addition of APMA converted proMMPs into active proteinases, which was confirmed by the significant increase in detected MMP activity (Figure 2B and 2C). This elevation was completely inhibited by addition of the MMP inhibitor BB94 (Figure 2B and 2C).

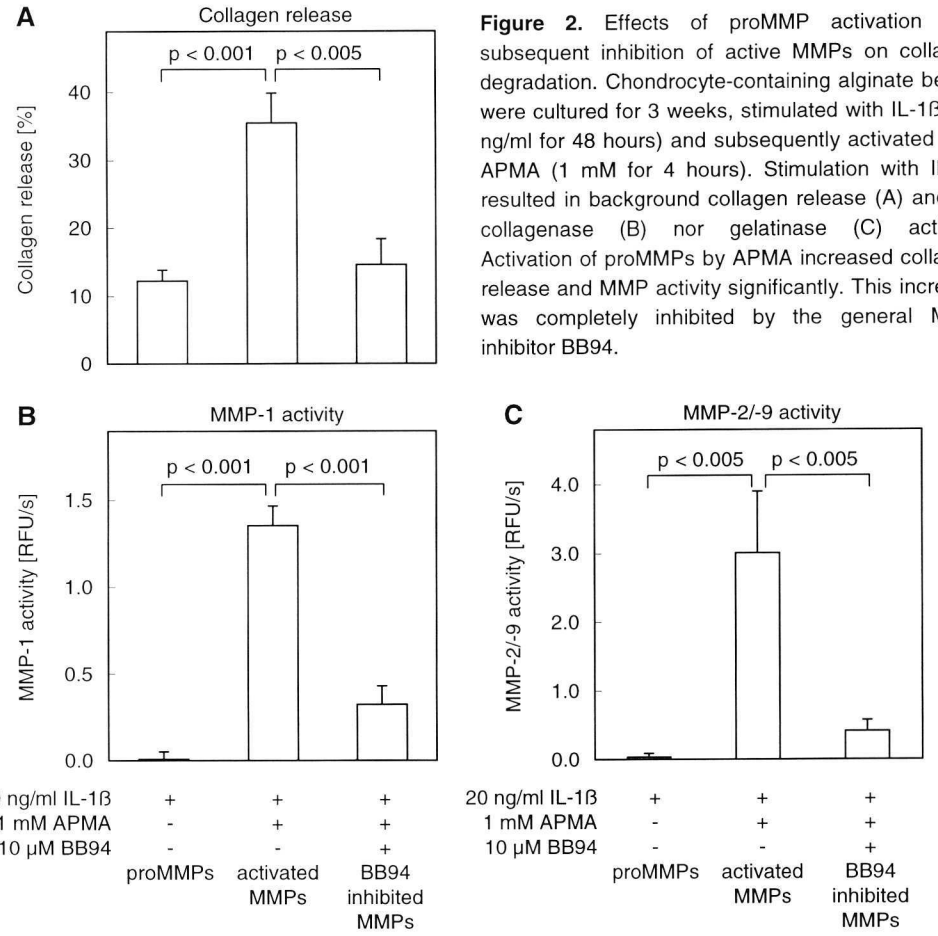

Figure 2. Effects of proMMP activation and subsequent inhibition of active MMPs on collagen degradation. Chondrocyte-containing alginate beads were cultured for 3 weeks, stimulated with IL-1ß (20 ng/ml for 48 hours) and subsequently activated with APMA (1 mM for 4 hours). Stimulation with IL-1ß resulted in background collagen release (A) and no collagenase (B) nor gelatinase (C) activity. Activation of proMMPs by APMA increased collagen release and MMP activity significantly. This increase was completely inhibited by the general MMP inhibitor BB94.

Effect of AGEs on collagen degradation. The effect of enhanced AGE levels on the degradation of the collagen network by MMPs was studied in alginate beads that were cultured for three weeks in the presence or absence of 20 mM ribose. As expected this resulted in a ~50-fold increase in pentosidine levels in beads cultured in the presence of ribose compared to control cultures (Figure 3A, $p < 0.001$; cf. Figure 1A). The increase in pentosidine levels was accompanied by a 50% decrease in MMP-mediated collagen degradation upon stimulation with IL-1ß and APMA (Figure 3B, $p<0.02$), suggesting that increased AGE levels affect collagen degradation. This observation is supported by data from Mott *et al*, who demonstrated decreased proteolytic degradation of collagen type IV at elevated AGE levels.[212]

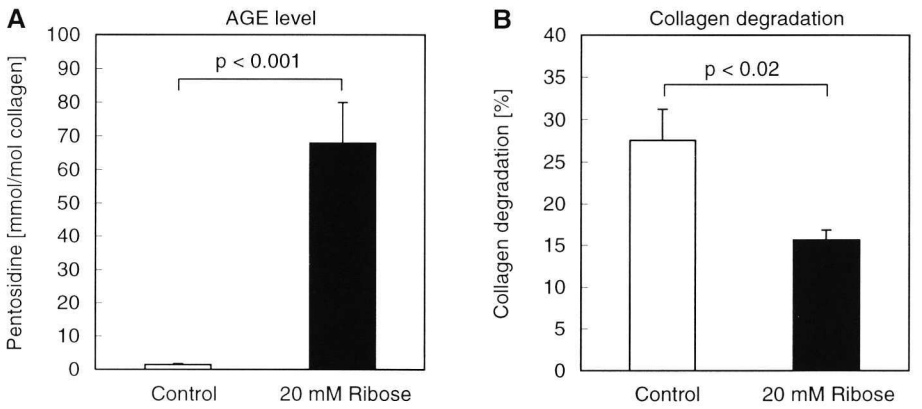

Figure 3. Effect of matrix glycation on MMP-mediated collagen degradation. Alginate beads cultured for 3 weeks in the presence of 20 mM ribose contain ~50-fold increased pentosidine levels (A). MMP-mediated collagen degradation after stimulation with 20 ng/ml IL-1ß (48 hours) and subsequent activation with 1 mM APMA (4 hours) was significantly decreased in alginate beads with enhanced glycation (20 mM ribose) compared to control beads (B).

The mechanisms by which accumulation of AGEs can interfere with collagen degradation are divers. AGE-modification of proteins changes their structure such that it may interfere with highly specific enzyme-substrate interactions.[5] In addition, increased inter- and intramolecular crosslinks may diminish the release of degraded collagen fragments from the alginate bead or the crosslinks may interfere with the accessibility of the matrix for proteinases. Alternatively, AGE modification of lysine and arginine residues changes the charge distribution of proteins,[116] thereby influencing their tertiary structure as well as the interactions with other proteins. To test whether AGE modification of collagen increases the resistance of the collagen towards MMP-mediated degradation, purified collagenase (MMP-13; collagenase-3) was added to alginate beads with low (control) and high (20 mM ribose) AGE levels. The degradation of the collagen matrix was ~20% decreased in beads with high AGE levels compared to control beads (Figure 4, $p < 0.05$), indicating that glycation of the collagen network *per se* interferes with its proteolytic degradation.

In addition to changing the properties of collagen, AGEs may reduce collagen degradation by decreasing the production of MMPs by the chondrocytes. Therefore MMP activity was compared between IL-1ß/APMA stimulated control alginate beads and beads with enhanced AGE levels. Both the collagenase (MMP-1) and the gelatinase (MMP-2 and MMP-9) activity were ~50% decreased at high AGE levels (Figure 5A and 5B, $p < 0.02$ and $p < 0.002$ respectively).

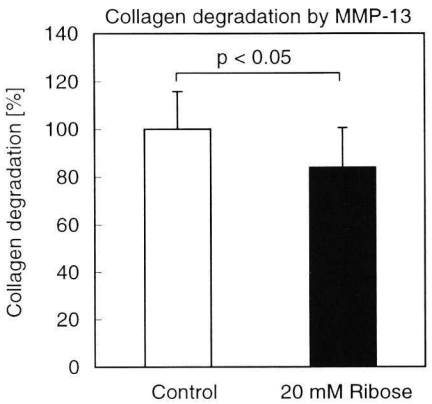

Figure 4. Effect of AGE levels on collagen degradation by purified MMP-13. Alginate beads cultured in the presence of 20 mM ribose (containing ~50 fold increased pentosidine levels, see Figure 3A) are significantly more resistant to degradation by purified MMP13 (20 nM, 48 hours at 37°C) than control beads. Bars represent mean values ± SEM of 5 independent incubations.

Thus, the observed decrease in collagen degradation after ribose incubation is partly caused by the decreased synthesis of MMPs by the chondrocytes that are surrounded by a glycated matrix. These data are in concordance with observations by others that exposure of fibroblasts to glycated proteins results in decreased MMP production and activation.[204,248] In addition, glycation of active MMPs may occur *in vivo* (and not in our in vitro system where the ribose was washed away before IL-1-mediated proMMP production) and this could lead to decreased proteolytic activity. Indeed, incubation of purified MMP-13 with ribose (to enhance AGE levels) dose-dependently decreased its proteolytic activity ($r = -0.571$, $p < 0.0005$; Figure 6). Altogether, decreased collagen degradation at increased AGE levels is caused by a combination of decreased proMMP production, inactivation of MMPs and increased matrix resistance to proteolysis.

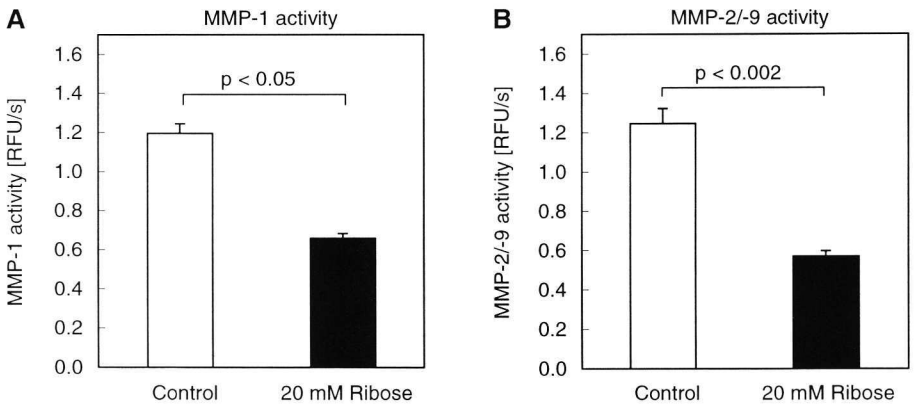

Figure 5. MMP-1 (A) and MMP-2/-9 (B) activity in beads cultured in the presence of 20 mM ribose and subsequently stimulated with IL-1ß/APMA, was significantly decreased compared to control beads. MMP activities were determined using fluorogenic peptide substrates as described in the materials and methods section. Bars represent mean values ± SEM of 3 independent experiments, each with 3 wells per condition.

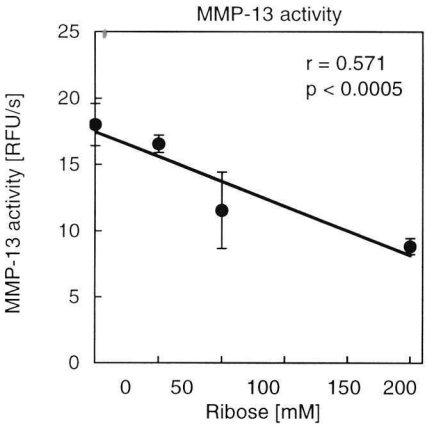

Figure 6. MMP-13 activity is decreased following a 2-day incubation with ribose at 37°C. Purified MMP-13 (10 nM) was incubated with increasing concentrations of ribose (48 hours, 37°C). Subsequently, MMP activity was measured using fluorogenic substrate TNO211-F. For each ribose concentration, mean values ± SEM of 3 incubations each analysed in triplicate are shown.

Implications for the *in vivo* situation. The present data clearly show that culturing in the presence of ribose increases extracellular matrix AGE levels. Concommitantly, a reduction in both synthesis and degradation of collagen is observed, which strongly suggests that collagen turnover by articular cartilage chondrocytes is adversely affected by an increase in matrix AGE levels. Since AGE-levels increase strongly with age in articular cartilage, the present data suggest that cartilage collagen turnover decreases with increasing age. The decreased collagen turnover impairs the capacity of the chondrocytes to remodel their extracellular matrix. Consequently, the glycated cartilage may not be fully adapted to meet its functional demands (load bearing and distribution) which may eventually result in an increased risk to develop damage.

Age-related accumulation of AGEs has previously been shown to increase the stiffness and brittleness of articular cartilage and thereby render the tissue more prone to damage, hence OA.[22] The present data suggest an additional role for the age-related accumulation of AGEs in the development of OA: accumulation of AGEs results in impaired cartilage collagen turnover. Decreased collagen turnover reduces the capacity of chondrocytes to maintain the integrity of the collagen network and to repair this damage. By this mechanism, the age related increase in cartilage AGE levels may contribute to the age-related increase in the incidence of osteoarthritis.

ACKNOWLEDGEMENTS

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AGE-RELATED DECREASE IN SUSCEPTIBILITY OF HUMAN ARTICULAR CARTILAGE TO MATRIX METALLOPROTEINASE-MEDIATED DEGRADATION: THE ROLE OF ADVANCED GLYCATION ENDPRODUCTS

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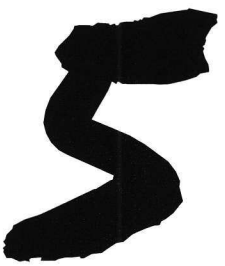
Submitted for publication



ABSTRACT

Objective. *Progressive destruction of articular cartilage is a hallmark of osteoarthritis (OA) and rheumatoid arthritis (RA). Age-related changes in cartilage may influence tissue destruction and thus progression of the disease. Therefore, the effect of age-related accumulation of advanced glycation endproducts (AGEs) on cartilage susceptibility to proteolytic degradation by matrix metalloproteinases (MMPs) present in synovial fluid (SF) of OA and RA patients was studied.*

Methods. *Cartilage was incubated with APMA-activated SF obtained from OA or RA patients and tissue degradation was assessed by colorimetric measurement of glycosaminoglycan (GAG) release. Cartilage degradation was related to the level of AGEs in cartilage from donors of different ages (33-83 yr.) and in cartilage with in vitro enhanced AGE levels (by incubation with ribose). MMP activity in SF was measured using a fluorogenic substrate. AGE levels were assessed by HPLC measurement of the glycation product pentosidine.*

Results. *In cartilage from donors aged 33 to 83 years a strong correlation was found between the age-related increase in pentosidine and the decrease in MMP-mediated tissue degradation ($r=-0.74$, $p<0.002$). Multiple regression analysis showed pentosidine to be the strongest predictor of the decreased GAG release ($p<0.0005$); age did not contribute ($p>0.8$). In addition, decreased MMP-mediated GAG release was proportional to increased pentosidine levels after in vitro enhancement of glycation ($r = -0.27$, $p < 0.01$). This was demonstrated for both OA and RA SF (control vs. glycated: $p < 0.002$ for all SF samples tested).*

Conclusion. *Increased cartilage AGEs resulted in decreased cartilage degradation by MMPs from SF, indicating that aged cartilage is less sensitive than young cartilage to MMP-mediated cartilage degradation, such as occurs in OA and RA. Therefore, the level of cartilage glycation may influence the progression of these diseases.*

INTRODUCTION

Osteoarthritis (OA) and rheumatoid arthritis (RA) are joint diseases of different etiology, but with a similar feature: the progressive degradation of articular cartilage that leads to joint dysfunction, disability and pain.[76,126] In both pathologies, erosion of the cartilage matrix is thought to be due primarily to the increased synthesis and activation of proteinases.[275] Although all classes of proteinases may be involved, the matrix metalloproteinases (MMPs) are considered to play a pivotal role in the cartilage destruction.[68,215] Both the chondrocytes and the inflamed synovium contribute to the production of MMPs in OA and RA.[168,183,229] MMP activity is increased in synovial fluid (SF)[34,36,69,318] and cartilage[79] of OA and RA patients compared to that of healthy controls.

Overall, the MMPs are capable of degrading the major cartilage matrix components such as collagen,[328] aggrecan,[102,168] link protein[219] and cartilage oligomeric protein.[282] Neoepitopes that result from the MMP-mediated cleavage of collagen and aggrecan have been detected in OA and RA cartilage.[42,79,168] Levels of degradation products of these cartilage constituents are increased in SF of OA and RA patients[102,178,179] and inhibition of MMPs reduces cartilage degradation *in vitro*[59] and *in vivo.*[172]

Proteolytic degradation of tissues is generally influenced by the structure of the extracellular matrix and its individual components. One of the major changes in the extracellular matrix of cartilage is the age-related accumulation of advanced glycation endproducts (AGEs). AGEs result from nonenzymatic glycation: the spontaneous reaction of a reducing sugar with proteins. After the initial addition of a sugar to the amino group of a (hydroxy)lysyl or argininyl residue, the resulting Schiff base undergoes Amadori rearrangement and Maillard browning reactions to form a variety of stable endproducts.[247] Some of these AGEs form protein-protein crosslinks whereas others are protein adducts.[247] All proteins are prone to AGE formation and, once formed, AGEs cannot be removed from proteins unless the protein is removed.[247] Consequently, AGEs accumulate in all tissues with age, but this accumulation is most pronounced in tissues with long-lived proteins.[309] Articular cartilage is a tissue with relatively low turnover[193,309] and therefore contains high AGE levels compared to other tissues.[266,308] Pentosidine, an AGE crosslink, is one of the few characterized AGEs and is considered an adequate marker for the many AGEs that are formed *in vivo.*[266] Pentosidine levels in articular cartilage increase as much as 50-fold from age 20 to 80 years.[22,80]

Accumulation of AGEs is known to affect physical and chemical properties of proteins.[247] AGEs change the structure of proteins and thereby affect highly specific enzyme-substrate interactions.[5] Modifications of (hydroxy)lysine and arginine residues change the charge distribution of a protein, thereby influencing its tertiary structure as well as its interactions with other proteins.[116] Moreover, these changes in cartilage matrix structure may influence the accessibility of the matrix and thereby interfere with the enzymatic degradation of matrix constituents. In skin and renal basal membrane, susceptibility to degradation by pepsin[181,262] or MMPs[212] is indeed decreased at elevated AGE. Decreased susceptibility to degradation by bacterial collagenase was observed with increased AGE levels in articular cartilage.[308]

Thus far, most studies on the effects of AGE accumulation focus on mechanical properties of tissues or on cellular functions.[15,224,246,247,267,272] Almost no data are available on the effect of AGEs on the proteolysis of the extracellular matrix. Cartilage contains very high AGE levels and MMP-mediated cartilage degradation is an important feature of disabling diseases such as RA and OA. Therefore in the present study we addressed the effects of age-related AGE accumulation in articular cartilage on its susceptibility to MMP-mediated degradation.

MATERIALS AND METHODS

Cartilage. Macroscopically normal human articular cartilage was obtained *post mortem* at autopsy within 24 hours of the death of the donor. Cartilage was obtained from femoral knee condyles (n=18; 33-83 yr., age-range study) or from humeral heads (n=7; 50-76 yr., *in vitro* glycation experiments). Previous studies have shown that this cartilage is biochemically normal, even when focal OA lesions are present elsewhere in the joint.[161] Full thickness slices of cartilage were cut aseptically, kept in phosphate-buffered saline (pH 7.4), and processed within 1 hour of dissection. Cartilage slices were cut into square pieces (5-30 mg), randomly divided over experimental conditions, and handled individually. For some *in vitro* glycation experiments (when indicated), bovine nasal cartilage (local slaughterhouse) was used. In all experiments, chondrocytes were killed by three consecutive freeze–thaw cycles (1 hour at -20°C alternated by 1 hour at room temperature) to exclude cellular effects.[238]

***In vitro* enhancement of pentosidine levels.** To increase AGE levels, cartilage (n= 7 donors, mean age 67.1 ± 4.8 yr., 8 explants per condition per donor, handled individually) was incubated with D(-)-ribose (0-250 mM; Sigma, St. Louis, MO, USA) in phosphate buffered saline (PBS, 4 days, 37°C).[87] Free ribose was washed out with PBS (3 times for 1 hr at 37°C). Subsequently, cartilage was incubated in PBS (8 days, 37°C) to allow early glycation products to mature into AGEs. When indicated, a mixture of D-(-)-ribose (1 M) and L-(+)-threose (50 mM, Sigma) was used instead of ribose alone. AGE-enriched cartilage was stored at -20°C until use.

AGE levels. Tissue pentosidine levels (3-4 explants per condition) were determined by reversed-phase high-performance liquid chromatography (RP-HPLC) after acid hydrolysis as described previously.[80] In short, hydrolyzed (6 N HCl, 18 hrs, 110°C) cartilage was dried (Speed Vac, Savant, Holbrook, NY, USA) and dissolved in internal standard solution (2.4 mM homo-arginine, 10 µM pyridoxine (Fluka, Buchs, Switzerland) in water). For pentosidine analysis, samples were diluted 5-fold in 0.5% (v/v) heptafluorobutyric acid (Fluka) in 10% (v/v) acetonitrile (Rathburn, UK) and analyzed by RP-HPLC. To determine hydroxyproline levels, aliquots of the 5-fold diluted samples were diluted 50-fold in 0.1 M borate buffer pH 11.4, derivatized with 9-fluorenylmethyl chloroformate (Fluka) and analyzed by RP-HPLC.[25] Pentosidine levels were expressed as mmol/mol triple helical collagen, assuming 300 residues of hydroxyproline per triple helix.[309]

Synovial fluid incubations. Knee synovial fluid (SF) was obtained from 21 RA patients fulfilling the 1987 ACR criteria[13] and from 2 OA patients. SF samples were centrifuged to remove cells (10 min. 1000 g) and stored at -20°C until use. SF samples from 1 RA patient and both OA patients were used separately. When indicated, pooled RA SF of the other 20 RA patients was used. Prior to use, SF was thawed and proMMPs were activated with 0.5 mM aminophenyl mercurial acetate (APMA, at 37°C for 2 hours, Sigma). Subsequently, SF was diluted with PBS (final concentration 10% or 20% v/v) and added to cartilage explants for 4 days at 37°C. Incubations with PBS, or with 20% APMA-activated pooled RA SF supplemented with 25 mM EDTA (MMP inhibitor) served as controls. In one set of experiments, *Clostridium histolyticum* collagenase (CLS 2, Worthington Biochemical Corporation, Lakewood, NJ; up to 0.2 mg/ml in 50 mM Tris, 5 mM $CaCl_2$, 150 mM NaCl, 1 µM $ZnCl_2$, 0.01% Brij-35, 0.02% NaN_3) was used instead of SF.

Proteoglycan release. Cartilage degradation was assessed by colorimetric assessment of glycosaminoglycan (GAG) release into the incubation medium: in a papain (Sigma) digest of the incubation medium, GAGs were precipitated and stained with Alcian Blue dye solution (Sigma)[251] or with 1,9-dimethyl-methylene blue (Blyscan kit, Biocolor Ltd., Belfast, N.Ireland). Staining was quantified photometrically by measuring the change in absorbance at 620 nm. Shark cartilage chondroitin sulfate served as a standard. GAG release was corrected for GAGs present in the SF samples prior to the incubation, and normalized to the wet weight of the cartilage samples.[298]

MMP activity. MMP activity was assessed using a fluorogenic substrate as described previously.[35] In short, conversion of the fluorogenic substrate TNO211-F (2.5 µM, Dabcyl-Gaba-Pro-Gln-Gly-Leu-Cys(Fluorescein)-Ala-Lys-NH_2) was measured in the presence of EDTA-free general proteinase inhibitor cocktail Complete™ (Roche) to prevent conversion of substrate TNO211-F by proteinases other than MMPs. Further improvement of the assay specificity for MMPs was achieved by determining the difference in substrate conversion in the presence or absence of MMP inhibitor BB94 (10 µM). Since TNO211-F is not cleaved by aggrecanases, this approach detects only MMP-mediated substrate conversion and reflects the overall MMP activity in the SF samples.

Statistical analysis. Statistical evaluation was performed using SPSS software version 8.0. (SPSS, Chicago, IL, USA). Data are presented as means ± SEM. Correlation between parameters was determined by multiple linear regression analysis on individual data. Differences between groups were analyzed by 1-way analysis of variance (ANOVA) followed by Tukey *post hoc* tests. Glycated *vs.* control comparisons for individual OA and RA SF samples were tested by unpaired Student's t-test. *P* values less than 0.05 were considered to indicate statistically significant differences.

RESULTS

MMP-mediated cartilage degradation. Synovial fluid from OA patients contains elevated MMP levels compared to healthy controls. MMP levels in RA SF are even further increased.[34,36,69,318] Cartilage degradation by such synovial fluid MMPs was determined by incubation of cartilage with APMA-activated SF from OA (n=2) and RA (n=1) patients. Cartilage destruction was monitored as the release of GAGs. As expected, incubation with SF increased GAG release compared to control incubations with PBS (Figure 1A). The release of GAGs induced by the OA SF was lower in than by RA SF. This is corroborated by the MMP activity levels in the SF samples that reflect the GAG release data (Figure 1B). Incubation with APMA has been shown to eliminate aggrecanase activity,[12,136] and therefore - in our experimental setup - GAG release is unlikely to be caused by aggrecanases. Moreover, MMP activity in the SF samples was highly correlated with the release of GAGs from the cartilage (Figure 1C, $r = 0.97$, $p < 0.05$). Since the fluorogenic substrate used for the MMP activity measurements is not cleaved by aggrecanases, these data support that MMPs from the SF are responsible for the observed cartilage degradation. MMPs can cleave the aggrecan core protein and thereby release GAGs.[102,168] Alternatively, proteinase-mediated damage to the collagen network can result in release of GAGs. This was supported by incubation of cartilage with bacterial collagenase; in this setup only triple helical collagen is cleaved, which resulted in a dose-dependent increase in GAG release (Figure 1D, $r = 0.98$ $p < 0.05$).

To further characterize the SF-mediated cartilage degradation, human articular cartilage was incubated in PBS, pooled RA SF (10 and 20%) or 20% pooled RA SF supplemented with 25 mM EDTA. Incubation of cartilage in PBS resulted in a background release of 0.68 ± 0.09 µg (mean ± SEM) GAG per mg cartilage over a

4-day period (Figure 2A). Incubation with RA SF increased GAG release in a dose-dependent manner (82% increase at 10% SF and 130% increase at 20% SF; $p < 0.001$ compared to PBS control, Figure 2A). Addition of EDTA completely inhibited the SF-induced GAG release (0.89 ± 0.14 µg/mg; $p > 0.65$ and $p < 0.001$ compared to PBS and 20% SF, respectively; Figure 2A), supporting MMP involvement in the SF-induced cartilage degradation. Again, cartilage degradation data were reflected by MMP levels: no MMP activity was detected in control conditions (PBS and 20% RA SF + 25 mM EDTA), whereas 10% and 20% RA SF showed a dose dependent increase in MMP activity (Figure 2B, $p < 0.0001$).

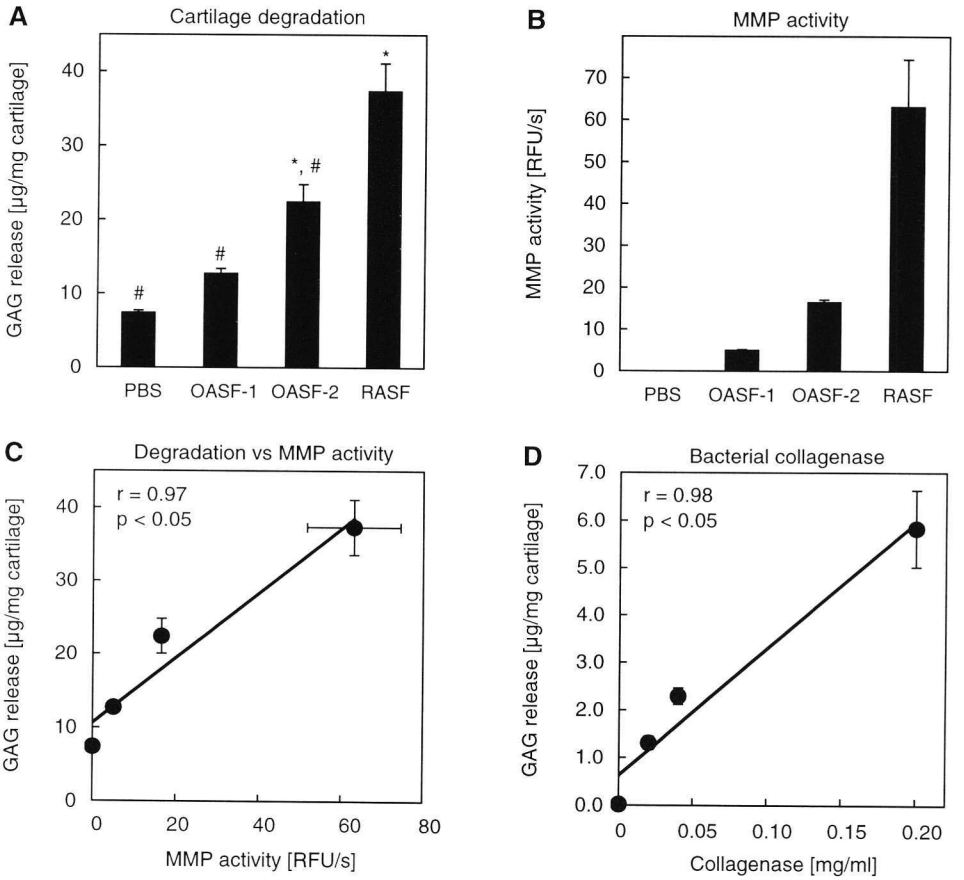


Figure 1. MMP mediated cartilage degradation. Bovine nasal cartilage was incubated for 4 days at 37°C with PBS or 20% APMA-activated SF from OA (n = 2) or RA (n = 1) patients. Bars show mean ± SEM of 6 samples per condition. Incubation with SF resulted in an increase in GAG release compared to PBS control for all three SF samples tested (A). MMP activity in the SF samples (mean ± SEM of duplicate analyses) (B). Moreover, MMP activity showed significant correlation with GAG release (C). Incubation of human articular cartilage with bacterial collagenase resulted in a collagenase-dose-dependent increase in GAG release, illustrating that proteolytic damage to the collagen network results in release of GAGs (D). * and # indicates $p < 0.001$ compared to PBS and RA SF, respectively.

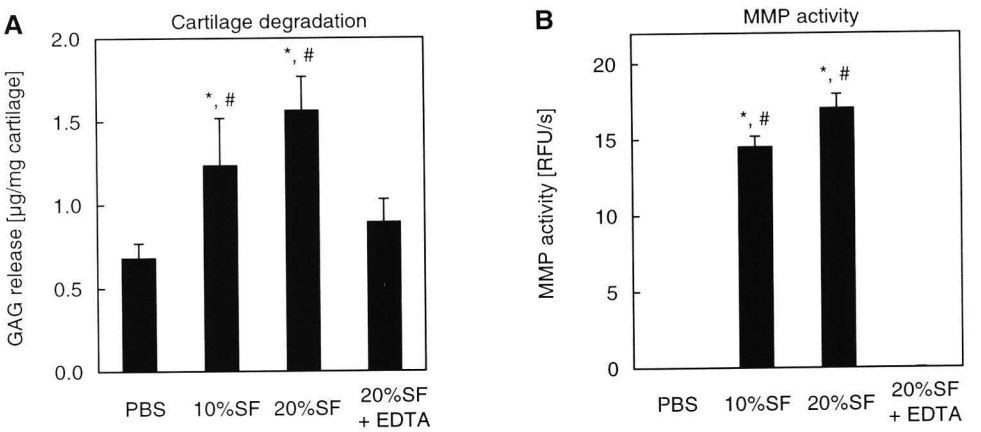


Figure 2. (A) GAG release of human articular cartilage incubated with PBS, APMA-activated RA SF (10% and 20% v/v), or APMA-activated RA SF in the presence of 25 mM EDTA for 4 days at 37°C. Bars show the mean (± SEM) of 3-7 independent experiments, each performed with 5-9 cartilage samples incubated individually (mean age 67 ± 5 years). APMA-activated pooled RA SF dose-dependently increased GAG release more than 2-fold at 20% SF. Addition of EDTA completely inhibited the GAG release induced by 20% SF. MMP activity in the incubation medium is shown in panel B. * and # indicate $p < 0.0001$ compared to PBS and EDTA controls, respectively.

Effect of age on proteolysis. Since both OA and RA are affecting the elderly, the effect of age on the MMP-mediated cartilage degradation was studied. Cartilage from 18 donors (33-83 years) was incubated with pooled RA SF for 4 days. An increase in age of the donor was accompanied by a decrease in the proteolytic degradation of the cartilage (Figure 3A, $r = -0.701$, $p < 0.002$). All cartilage samples (freeze-killed to exclude cellular effects) were incubated with the same RA SF pool and the increased resistance towards proteolysis can therefore only be caused by age-related changes in the cartilage matrix. One of the most prominent age-related changes is the accumulation of AGEs. Therefore, levels of the AGE marker pentosidine were measured in the cartilage samples. Pentosidine levels increased with age in these cartilage samples (Figure 3B, $r = 0.928$, $p < 0.0001$), consistent with previous observations.[80,308] A strong correlation was observed between the increase in pentosidine levels and the decrease in cartilage susceptibility to proteolytic degradation (Figure 3C, $r = -0.741$, $p < 0.0005$). A 10-fold increase in cartilage AGE levels reduced the SF-induced GAG release by 60%. Multiple regression analysis showed that the pentosidine level was the strongest predictor for the observed decrease in degradation ($p < 0.0005$); the age of the cartilage donor did not contribute significantly ($p > 0.83$; Figure 3C, inset).

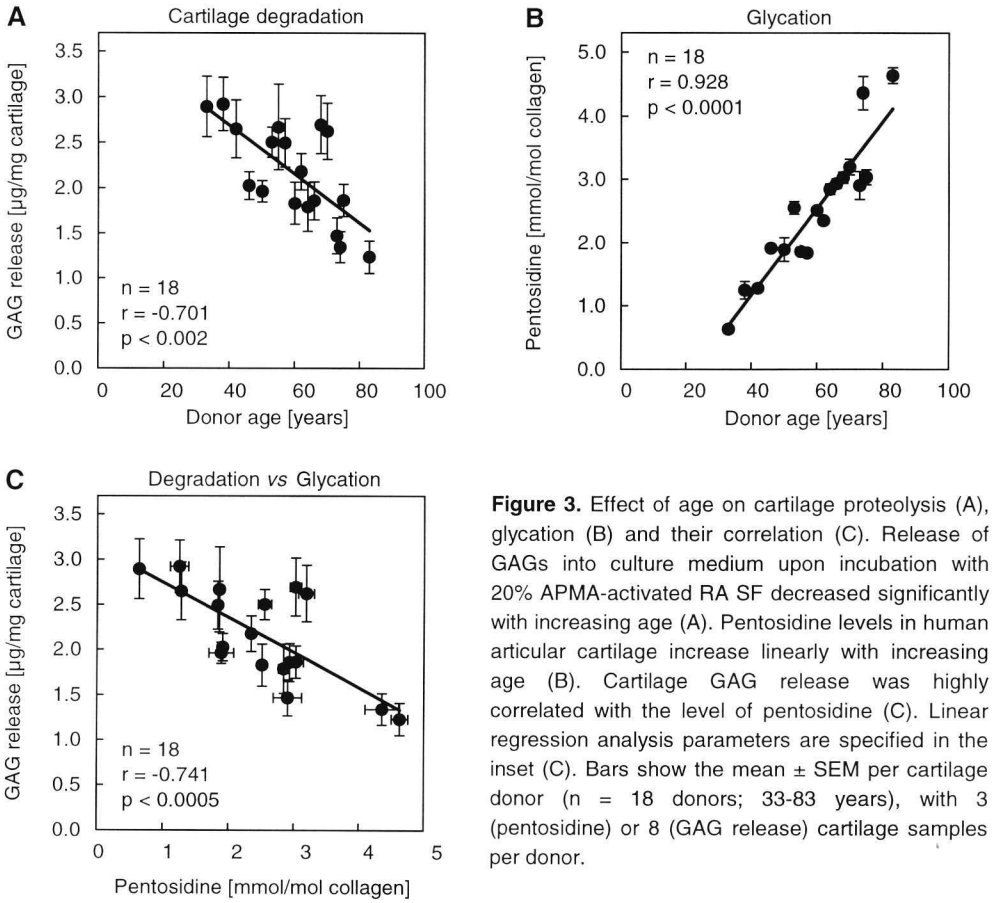


Figure 3. Effect of age on cartilage proteolysis (A), glycation (B) and their correlation (C). Release of GAGs into culture medium upon incubation with 20% APMA-activated RA SF decreased significantly with increasing age (A). Pentosidine levels in human articular cartilage increase linearly with increasing age (B). Cartilage GAG release was highly correlated with the level of pentosidine (C). Linear regression analysis parameters are specified in the inset (C). Bars show the mean ± SEM per cartilage donor (n = 18 donors; 33-83 years), with 3 (pentosidine) or 8 (GAG release) cartilage samples per donor.

Effect of *in vitro* increased AGEs on proteolysis. The role of AGEs in cartilage susceptibility to proteolysis was studied in cartilage with *in vitro* enhanced AGE levels (by incubation with ribose). Using this approach, only differences in AGE levels were induced, excluding other age-related changes that could affect GAG release. Ribose increased cartilage pentosidine levels proportional to the ribose concentration, and consistent with previous observations (Figure 4A, $r = 0.960$, $p < 0.0001$).[80] After exposure to 250 mM ribose, a 3- to 4-fold increase in pentosidine level was observed. The background release of GAGs (4 days at 37°C) was not influenced by the level of matrix glycation (Figure 4B, PBS, $r = -0.136$, $p > 0.05$). In contrast, incubation with 20% pooled RA SF showed a diminished GAG release (up to 30% decrease) with increasing glycation levels of cartilage (Figure 4B, RA SF, $r = -0.267$, $p < 0.01$). To test whether AGE levels affect cartilage degradation by MMPs from OA SF (containing lower levels of MMP than RA SF; cf. Figure 1B), bovine nasal cartilage was glycated with a mixture of ribose and threose and next, incubated with OA or RA SF. Incubation

with the reducing sugars resulted in a large increase in cartilage pentosidine levels (0.07 ± 0.04 mmol/mol in control *vs.* 28.7 ± 3.6 mmol/mol collagen in glycated samples, $p < 0.001$). As expected, all SF samples showed decreased cartilage degradation at enhanced AGE levels (Figure 5, control *vs.* glycated $p < 0.002$ for all SF samples). This confirms that AGE-modification of matrix proteins reduces their degradation by synovial fluid proteinases from OA and RA patients.

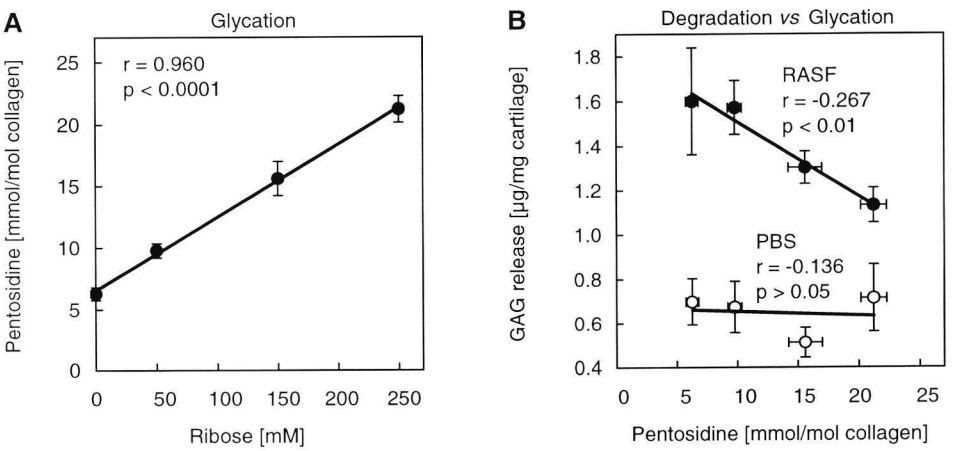


Figure 4. Levels of the NEG crosslink pentosidine in human cartilage incubated with ribose for 4 days at 37°C and subsequently for 8 days in PBS. Incubation with ribose resulted in a dose-dependent increase in pentosidine levels (A). GAG release from *in vitro* glycated cartilage upon incubation with PBS or APMA-activated RA SF (20% v/v) for 4 days at 37°C (B). Background GAG release (PBS) did not change with increased AGE levels whereas GAG release by APMA-activated MMPs (20% RA SF) decreased with increasing cartilage pentosidine levels. Mean values ± SEM of 6 independent experiments are shown, each performed with at least 3 (pentosidine) or 5 (GAG release) cartilage samples per condition (mean age 64 ± 4 years).

DISCUSSION

Remodeling of cartilage extracellular matrix is the net result of highly regulated matrix synthesis and degradation.[76] In pathological situations such as OA and RA, proteinase activity is elevated and the balance between matrix synthesis and degradation shifts towards matrix degradation, resulting in loss of articular cartilage.[325] The role of the different cell types (e.g. synoviocytes, inflammatory cells, chondrocytes) or proteinases (e.g. MMPs, aggrecanases, plasmin, cathepsins) in cartilage degradation has extensively been studied. To our knowledge, thus far no study has focussed on the extent in which age-related changes in the extracellular matrix of articular cartilage influence its degradation.

In skin and renal basal membrane, increased AGE levels have been shown to result in diminished protein solubility by pepsin.[181,262] Similar effects of AGE accumulation on extracellular matrix proteolysis by pepsin have been reported for diabetic or *in vitro* glycated rat tail tendon.[287,327] Only one study reports on the effects of matrix glycation on proteolytic degradation by (patho)physiologically relevant proteinases (MMPs) instead of pepsin: degradation of *in vitro* glycated collagen type IV in kidney glomerular membranes by MMP-3 and MMP-9 was impaired compared to that of non-glycated controls.[212] The present study is the first to show that AGE modification of the cartilage extracellular matrix proteins reduces their susceptibility to proteolytic degradation by MMPs. Increased AGE levels of cartilage were proportional to decreased release of GAGs by MMPs.

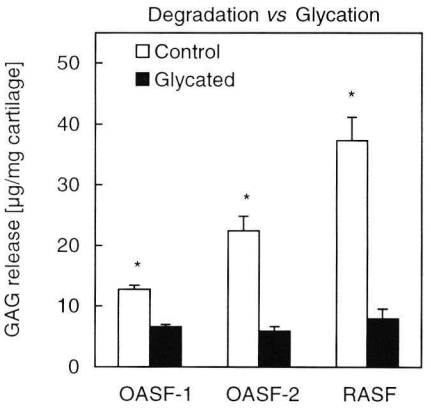


Figure 5. GAG release from control and glycated bovine nasal cartilage. Cartilage degradation by MMPs from OA and RA synovial fluid samples was decreased in cartilage with enhanced glycation levels. Bars shown mean values ± SEM of 6 independent samples. * indicates $p < 0.002$ for control *vs.* glycated comparison.

Although aggrecanases play an important role in aggrecan proteolysis, MMPs have been shown to contribute to GAG release as well. Direct cleavage of aggrecan core protein by MMPs has been demonstrated *in vitro* and *in vivo.*[101-103,168,219,297] MMP-mediated cleavage of aggrecan results in fragments that are aggrecanase-resistant, remain in the cartilage matrix and thereby possibly modulate cartilage homeostasis.[104] In addition, a damaged collagen network, due to the action of MMPs, results in loss of GAGs, since the depolymerized collagen network no longer retains the proteoglycans within the cartilage matrix.[28,160]

In our experimental set-up using freeze-killed cartilage, only the effect of extracellular matrix modifications on proteolysis by externally added MMPs was studied so that possible effects of age-related changes in chondrocyte activity were excluded. Other age-related changes in the extracellular matrix besides AGE accumulation may play a role in the decreased cartilage degradation with increasing age. For example, age-related changes in GAG content and/or composition may explain the observed age-related decrease in GAG release. However, GAG content does not decrease with age in adult articular cartilage,[44] and can therefore not cause the decreased GAG release.

Changes in the composition of the GAGs (e.g. chondroitin sulfate / keratan sulfate ratios) have been observed with increasing age.[192] These structural differences in GAG composition potentially contribute to the age-related decrease in degradation by MMPs. However, in our studies employing *in vitro* enhancement of glycation, incubation with ribose resulted in cartilage samples of a single age with different levels of AGE. Using this experimental approach no differences in GAG content, composition, or any other age-related change were present in the samples: only the glycation of the cartilage extracellular matrix differed. In these *in vitro* glycated cartilage samples, the increase in AGE levels was proportional with the decrease in degradation by MMPs. This indicates that AGEs in the extracellular matrix are indeed an important determinant for the matrix susceptibility to proteolytic degradation.

The exact mechanism by which AGEs impair cartilage degradation is as yet unclear. Formation of AGEs is a cascade, resulting in a variety of chemical structures, some of which are adducts (e.g. carboxymethyllysine), while others are crosslinks (e.g. pentosidine).[247] Adducts may interfere with the enzyme-substrate interaction and thus prevent proteolysis. In addition, increased inter- and intramolecular crosslinks may diminish the release of degraded cartilage constituents[18] or affect the accessibility of the matrix for proteinases. Alternatively, modification of lysine and arginine residues changes the charge distribution of the protein,[116] thereby influencing its tertiary structure as well as its interactions with other proteins. Which (combination) of these AGE-induced changes in the cartilage matrix is responsible for the observed effect has yet to be found out.

The present observation that the level of extracellular matrix glycation affects its proteolysis has an important implication for studies into the pathology of RA and OA. The contribution of proteinases in cartilage erosions is often studied using young animal cartilage (e.g. bovine, porcine) or rodent models (short life-span and therefore no extensive accumulation of AGEs[264]). Cartilage in these studies contains low glycation levels and the results can therefore not readily be extrapolated to the adult human situation in which cartilage contains high glycation levels.

In RA, these high AGE levels will possibly influence the progression of the cartilage erosion but are likely not causally involved in the etiology of the autoimmune disease. In OA however, accumulation of AGEs has previously been implicated in the etiology of the disease: accumulation of crosslinks is reported to increase cartilage stiffness and brittleness and renders the tissue more prone to mechanical damage.[22] In addition, AGEs reduce the capacity of chondrocytes to synthesize proteoglycans and thereby the capacity to remodel and/or repair the extracellular matrix.[80] The present data suggest an additional role for AGE accumulation: reduction of proteolytic degradation of articular cartilage proteins. Whether this decrease in proteolytic degradation is beneficial (cartilage destruction is diminished) or disadvantageous (because degradation products are no longer removed from the tissue and continue to stimulate chondrocyte proteinase production[131,234] merits further investigation.

ACKNOWLEDGEMENTS

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ACCUMULATION OF ADVANCED GLYCATION ENDPRODUCTS AS A MOLECULAR MECHANISM FOR AGE AS A RISK FACTOR IN OSTEOARTHRITIS

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ABSTRACT

Osteoarthritis, one of the most common diseases of the elderly, is characterized by destruction of articular cartilage that eventually leads to disability. The etiology of osteoarthritis is unknown, but age is recognized as the main risk factor. The age-related accumulation of advanced glycation endproducts (AGEs) adversely affects cartilage matrix turnover and impairs its mechanical properties. The hypothesis that accumulation of AGEs predisposes to the development of osteoarthritis in vivo was tested using a canine model for osteoarthritis.

Intra-articular injections with ribose enhanced cartilage AGE levels ~5 fold ($p<0.05$ vs. sham injected joints). Subsequent induction of osteoarthritis by anterior cruciate ligament transection resulted in significantly more pronounced osteoarthritis in dogs with enhanced AGE levels. This was observed as increased collagen damage and enhanced release of proteoglycans ($p<0.05$). The attempt to repair the matrix damage was impaired: proteoglycan synthesis ($p<0.02$) and retention ($p<0.05$) were decreased at enhanced AGE levels. Histological grading (Mankin score) also revealed more severe osteoarthritis in animals with enhanced AGE levels ($p<0.02$).

The present data demonstrate increased severity of experimental osteoarthritis at higher cartilage AGE levels and provide the first in vivo experimental evidence of AGE accumulation as a molecular mechanism by which aging may predispose to the development of osteoarthritis.

INTRODUCTION

Osteoarthritis (OA) is one of the most common diseases of the elderly, with a large impact on the patient's quality of life. The most prominent feature of OA is the progressive destruction of articular cartilage resulting in impaired joint motion, severe pain and ultimately disability.[76]

Articular cartilage derives its mechanical properties from its extracellular matrix. This matrix is composed of type II collagen, which forms a three-dimensional network providing the cartilage with resistance towards tensile forces.[214] Within this collagen network, highly negatively charged proteoglycans are embedded, which generate a large swelling force that facilitates load support (the resilience of cartilage).[123,296] One of the first characteristics of OA is the occurrence of damage to the collagen network, reflected in an increased swelling of the tissue and loss of proteoglycans.[28,108,190] Both the collagen damage and the loss of proteoglycans adversely affect the mechanical properties of the cartilage. The chondrocytes within the cartilage are essential in maintaining the integrity of the tissue. They respond to tissue damage by increasing proteoglycan and collagen synthesis in an attempt to repair.[162,218] If repair fails, progression of damage will eventually occur, leading to degeneration of the cartilage.

As yet, the etiology of OA remains largely unknown. The incidence of OA increases

strongly with age: >50% of the population over 60 years of age is affected.[97,127] Although age is identified as the main risk factor for the development of OA, the mechanism by which aging is involved remains unclear. Age-related changes in the articular cartilage are expected to play an important role in the susceptibility of cartilage to OA.

One of the major age-related changes in articular cartilage is the accumulation of advanced glycation endproducts (AGEs), resulting from the spontaneous reaction of reducing sugars with proteins: so-called nonenzymatic glycation.[247,261] The initial step in this reaction is the condensation of a sugar aldehyde with an ε-amino group of (hydroxy)lysine or arginine residues in proteins. Subsequently, the initially formed Schiff base is stabilized by Amadori rearrangement. The Amadori product is further stabilized by oxidation and molecular rearrangements, ultimately generating a range of fluorophores and chromophores, collectively known as advanced glycation endproducts (AGEs).[18,247] Most of these AGEs are not yet isolated nor characterized. Therefore, a few well-characterized AGEs are routinely used as marker for the process of nonenzymatic glycation. Pentosidine, a fluorescent AGE formed between lysine and arginine residues, is often used for this purpose.[266] AGEs are formed in all proteins, and since they can only be removed from the body when the protein is removed, AGEs accumulate in long-lived proteins such as collagens.[207,309] In human articular cartilage – a tissue with extremely slow turnover (half-life of type II collagen >100 yr.) – pentosidine levels increase 50-fold from age 20 to 80 years.[22,80,193,308,309]

AGEs are known to affect physical and chemical properties of proteins. In particular tissue strength is dependent upon the amount of crosslinks present.[18] Accumulation of AGEs is correlated with increased tissue stiffness in arteries, lens, skin, tendon and articular cartilage.[10,20,22,209,245,327] Moreover, an increase in AGE renders tissues increasingly brittle, and thus more prone to mechanical damage. This effect has been shown for human lens capsules and cortical bone.[20,292] For articular cartilage, a decrease in strength is observed with increasing age,[144] coinciding with an increase in AGEs in the tissue.[22,80,309] Although no direct correlation between AGE levels and brittleness has been shown, this suggests that also for articular cartilage, an increased level of AGEs reduces its resistance to mechanical trauma.

In addition to affecting the mechanical properties of tissues, AGEs also interfere with cellular processes such as adhesion of cells to the extracellular matrix, proliferation and gene expression.[119,142,259] Articular cartilage chondrocytes show decreased proteoglycan synthesis at increased AGE levels.[80] Degradation of AGE-modified collagen by matrix metalloproteinases is impaired compared to unmodified collagen.[212] Altogether, age-related accumulation of AGEs in articular cartilage increases tissue stiffness and decreases the capacity of the chondrocytes to remodel their extracellular matrix. In combination, these effects render the tissue more prone to damage that may eventually lead to the development of OA. In the present study, the hypothesis that accumulation of AGEs predisposes cartilage to the development of OA was tested in the established dog anterior cruciate ligament transection model of OA.[48,227,233]

ANIMALS, MATERIALS AND METHODS

Experimental design. Fifteen female Beagle dogs, obtained from the animal laboratory of the Utrecht University (~1 year old, 8-10 kg) were randomly divided into three treatment groups of 5 dogs each. After 1 week of acclimatization, dogs in the first group (= PBS/ACLT group) received intra-articular injections with PBS in the right knee joint (twice weekly for 7 weeks). Dogs in the other two groups (AGE/ACLT and AGE/Control) received injections with 350 mM D-(-)ribose (Sigma) in PBS to enhance cartilage AGE levels. All intra-articular injections were performed under Dormitor®/AntiSedan® (Pfizer Animal Health) sedation. After the last intra-articular injection, dogs were allowed to recover for 2 weeks, after which joint instability was induced by anterior cruciate ligament transection (ACLT) in the right knee joint of dogs in the PBS/ACLT and AGE/ACLT groups, as described below. The AGE/Control group served as a control with enhanced AGE levels but no surgery. Seven weeks after the ACLT surgery, dogs were terminated by intravenous injection of sodium pentobarbitone (Euthesate), both hind legs were amputated, and cartilage, synovium and synovial fluid were processed as described below. During the entire study, dogs were housed in groups of 5 dogs and were fed a standard commercial diet and water *ad libitum*. Weight of the animals was monitored during the study and did not change. The study was approved by the Ethical Committee on Animal Experiments of the Utrecht University.

Induction of joint instability; ACL transection. ACLT surgery was performed in the right knee joint as described previously; the other joint was left intact and served as a control.[300] The dogs were anesthetized with halothane in a mixture of oxygen and nitrous oxide, delivered endotracheally. A small anterolateral incision (< 2 cm) was made paralleling the *ligamentum patellae* to view the anterior cruciate ligament. The anterior cruciate ligament was lifted and transected with care not to damage other joint structures. Positive anterior drawer sign confirmed the completeness of the transection. The incision was sutured subcutaneously and cutaneously. Dogs received analgesics (Buprenorphine 0.01 mg/kg) and antibiotics (Amoxyllin 400 mg/kg) during the first 3 days after surgery. Exercise (twice daily for >30 minutes, five days/week) was started two days after surgery and continued until sacrifice.

Cartilage processing and AGE levels. Cartilage was obtained from the femoral condyles of control and experimental knee joints of all dogs according to standardized procedures.[164] Within 1 hour after dissection, full thickness cartilage slices (excluding the subchondral bone) were cut into square pieces (3-10 mg) and processed for histological and biochemical analysis.[300] Tissue pentosidine levels (2 cartilage samples per dog per joint) were determined by reversed-phase high-performance liquid chromatography after acid hydrolysis as described previously.[24,80] Pentosidine levels were expressed per collagen triple helix assuming 300 residues hydroxyproline per collagen molecule.[27]

Proteoglycan synthesis. *Ex vivo* proteoglycan synthesis was measured as the rate of sulfate incorporation (6 explants per joint per dog) using carrier free $^{35}SO_4^{2-}$ (NEN Dupont) during 4 hours of culture (after 1 hour preculture). Cultures were performed in 96-wells tissue culture plates in Dulbecco's Modified Eagle's Medium supplemented with 0.85 mM ascorbic acid, 2 mM glutamin, 100 IU/ml sodium benzylpenicillin, 100 IU/ml streptomycin sulfate and 10% (v/v) heat inactivated pooled Beagle serum (37°C, 5% CO_2 in air).[300] After labeling, tissue digestion (papain, Sigma) and precipitation of glycosaminoglycans (cetylpyridiniumchloride, Sigma), $^{35}SO_4^{2-}$ incorporation was analyzed by liquid scintillation counting. Proteoglycan synthesis was calculated from the $^{35}SO_4^{2-}$ incorporation and the specific activity of the culture medium, normalized to the initial wet weight of the explant and expressed as nmol $^{35}SO_4^{2-}$ incorporated per hour per gram wet weight of tissue.[80,300]

Proteoglycan retention. Cartilage samples (6 explants for each joint) that were labeled *ex vivo* for 4 hours using carrier free $^{35}SO_4^{2-}$ (NEN Dupont) were extensively washed to remove unincorporated $^{35}SO_4^{2-}$, and chased for three days to assess the retention of newly synthesized proteoglycans. Release

of radiolabeled proteoglycans into the culture medium was measured by Alcian Blue precipitation and liquid scintillation counting. Released proteoglycans were normalized to the remaining radiolabeled proteoglycans in the cartilage explant (measured by Alcian Blue precipitation after papain digestion).[300]

Proteoglycan release. Cartilage degradation was assessed by colorimetric assessment of glycosaminoglycan (GAG) release into the incubation medium over 3 days.[250] GAGs were precipitated and stained with Alcian Blue dye solution (Sigma). Staining was quantified photometrically by the change in absorbance at 620 nm (Vitalab10, Vital Science, Dieren, The Netherlands). Shark cartilage chondroitin sulfate (Sigma) served as a standard. GAG release was normalized to the wet weight of the cartilage samples.[298]

Collagen damage. Damage to the collagen network was assessed by selective proteolysis (by α-chymotrypsin) of denatured collagen as described previously.[26] Cartilage samples (2 samples per joint) were extracted twice with 4 M guanidinium chloride to remove proteoglycans. After washing, denatured collagen was digested overnight at 37°C with α-chymotrypsin. The supernatant was quantitatively separated from the insoluble collagen and hydroxyproline levels were determined colorimetrically in both fractions after acid hydrolysis.[78]

Cartilage histology. At sacrifice, cartilage (two samples per joint) were fixed in 4% formaldehyde (containing 2% sucrose), embedded in paraffin and sectioned at 3 μm. Histological grading of the extent of cartilage degeneration was carried out blinded in random order by light microscopy according to the slightly modified criteria of Mankin, on Safranin O stained sections.[300]

MMP activity. Synovial fluid (SF) was obtained from control and experimental knees by aspiration using 21 gauge needles. Typically, 25-50 μl SF was collected from control joints and > 150 μl from experimental knees. MMP activity in SF (final dilution 20-fold in 50 mM Tris (pH 7.5), 5 mM $CaCl_2$, 150 mM NaCl, 1 μM $ZnCl_2$, 0.01% Brij-35, 0.02% NaN_3) was determined with fluorogenic substrate TNO211-F (5 μM), in the presence of EDTA-free general proteinase inhibitor Complete™.[34-36] This assay is considered to represent overall MMP activity.

Synovium histology. At sacrifice, synovium samples (three infra-patellar samples per joint) were fixed in 4% formaldehyde, embedded in paraffin and sectioned at 3 μm. Synovial inflammation was quantified on hematoxilin/eosin stained tissue sections using the Goldenberg and Cohen score modified by Pelletier (synovial lining cell hyperplasia, villous hyperplasia, and cell influx).[112,228]

Statistical analysis. Statistical evaluation was performed using SPSS software version 10.0 (SPSS, Chicago, IL.). Data are presented as mean ± SEM. Differences between contralateral control knees of the 3 groups were analyzed by analysis of variance (ANOVA). Two-sided Student t-tests were performed for the comparison of control *versus* experimental knees (paired t-test) and PBS- *versus* ribose-injected experimental knees (unpaired t-test). *P* values < 0.05 were considered to indicate statistically significant differences.

RESULTS.

Enhancement of cartilage AGE levels. *In vivo*, AGE levels in articular cartilage increase with age after skeletal maturity has been reached. To study the effect of differences in cartilage AGE levels on the susceptibility to mechanically induced OA (without interference of other age-related changes besides AGE accumulation) five

dogs received 14 consecutive intra-articular injections with ribose in one knee joint (in 7 weeks) to enhance cartilage AGE levels. Five other dogs received control injections with PBS. The ribose injections induced a 5-fold increase in cartilage pentosidine levels compared to PBS injections (Figure 1). The increase in pentosidine was restricted to the injected joint: no changes in pentosidine levels were observed in the (untreated) contralateral control joint.

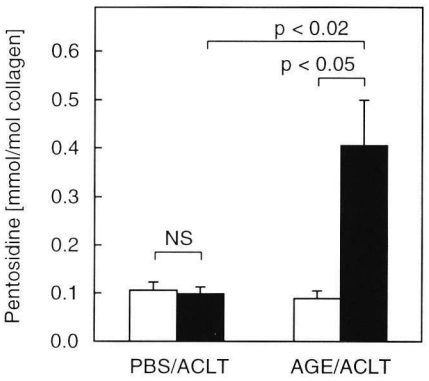

Figure 1. Cartilage AGE levels. Five beagle dogs received 14 injections with ribose (AGE/ACLT), whereas 5 control dogs received PBS injections (PBS/ACLT). Pentosidine levels were 5-fold increased upon ribose injections. Bars represent mean ± SEM of n = 5 animals per group, each analyzed in duplicate. White bars represent contralateral control knees, black bars represent experimental knees. NS, not significant.

Since synovial inflammation is regarded as a secondary process in OA, care was taken to minimize the synovial damage by the repeated injections. Histological assessment of joint inflammation indicated that such damage was successfully prevented: synovial tissue samples from five dogs that received 14 intra-articular ribose injections but no ACLT surgery showed no signs of inflammation. The modified Goldenberg/Cohen score for synovium inflammation was 0.6±0.4 in uninjected *versus* 1.7±0.5 in ribose-injected joints ($p > 0.1$; maximal score = 10).

Characterization of the test model: PBS/ACLT group. In our study design, the control group should show mild cartilage degeneration to allow a window for studying the aggravating effects of increased AGE levels. Induction of joint instability by transection of the anterior cruciate ligament induced indeed mild osteoarthritic features in the PBS injected animals 7 weeks post-surgery. Collagen damage, the first sign of OA, was increased by ~20% in the ACLT joint compared to the control joint (Figure 2).

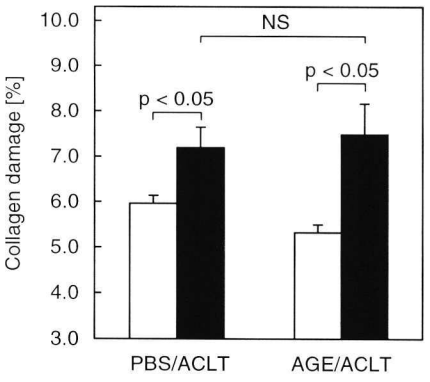

Figure 2. Effect of ACLT on cartilage collagen damage at low (PBS/ACLT) and high (AGE/ACLT) AGE levels. Collagen damage was increased in experimental knees 7 weeks after surgery (~20% and ~40% increase for PBS/ACLT and AGE/ACLT group, respectively). Bars represent mean ± SEM of n = 5 animals per group, each analyzed in duplicate. White bars represent contralateral control knees, black bars represent experimental knees. NS, not significant.

Consistent with the decreased matrix integrity, the release of glycosaminoglycans was ~30% increased in experimental knees (Figure 3A). In an attempt to restore tissue integrity, the chondrocyte repair mechanism was activated as is indicated by the observed increase in proteoglycan synthesis (~70 % increase; Figure 3B). This repair was clearly not effective, since the newly synthesized proteoglycans were poorly retained in the matrix: release of these newly produced molecules was increased by ~30% in the ACLT joint compared to the control joint (Figure 3C). Consequently, the level of GAGs in the matrix was ~14% reduced in the ACLT joint (36.5±1.1 µg/mg cartilage in control *vs.* 31.4±1.8 µg/mg experimental knees, not significant). In addition, minor synovial inflammation was observed (Goldenberg/Cohen score of 0.5±0.3 in control knees *versus* 2.5±0.4 in experimental knees; maximal score = 10; $p<0.02$).

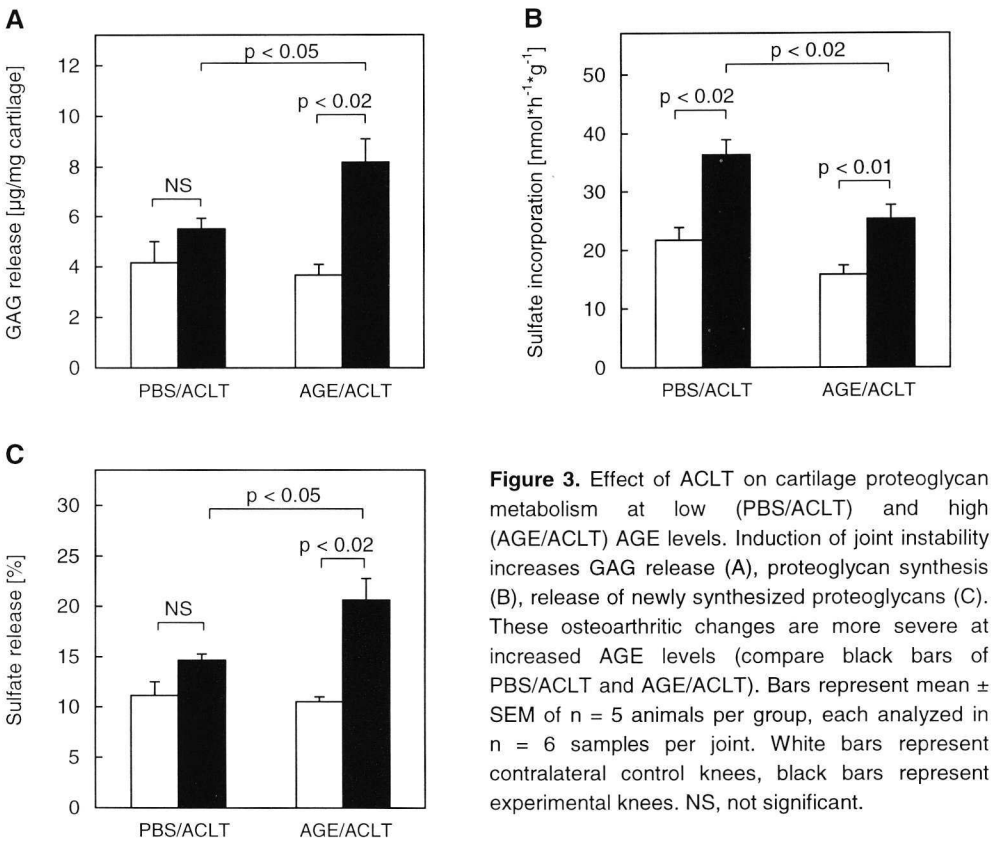

Figure 3. Effect of ACLT on cartilage proteoglycan metabolism at low (PBS/ACLT) and high (AGE/ACLT) AGE levels. Induction of joint instability increases GAG release (A), proteoglycan synthesis (B), release of newly synthesized proteoglycans (C). These osteoarthritic changes are more severe at increased AGE levels (compare black bars of PBS/ACLT and AGE/ACLT). Bars represent mean ± SEM of n = 5 animals per group, each analyzed in n = 6 samples per joint. White bars represent contralateral control knees, black bars represent experimental knees. NS, not significant.

The activity of MMPs (proteinases involved in the destruction of cartilage tissue in OA) in synovial fluid was increased in experimental knees (Figure 4). All these biochemical osteoarthritic changes were consistent with a ~50% increase in the histological Mankin score for OA (Figure 5). Altogether, these data indicate that a mild form of osteoarthritis has developed in PBS injected knees after ACLT surgery. Due to the

short follow-up (7 weeks), some of the above parameters did just not reach statistical significance. This mild degree of OA in the PBS/ACLT group is in good agreement with previous studies by others[6,186] and was essential for testing our hypothesis that an increased AGE level will increase the susceptibility for OA.

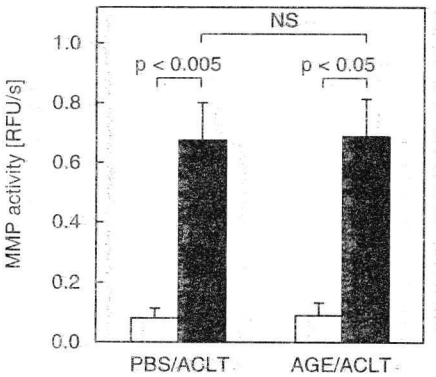

Figure 4. Effect of ACLT on synovial fluid matrix metalloproteinase activity at low (PBS/ACLT) and high (AGE/ACLT) AGE levels. Proteolytic activity in the synovial fluid was increased upon induction of joint instability but did not differ between experimental knees of PBS/ACLT *vs.* AGE/ACLT groups. Bars represent mean ± SEM of n = 5 animals per group, each analyzed in duplicate. White bars represent contralateral control knees, black bars represent experimental knees. NS, not significant.

Osteoarthritis at enhanced AGE levels: AGE/ACLT group. In animals with enhanced AGE levels (by ribose injections), all parameters of osteoarthritis were statistically significantly increased compared to the control knee (Figures 2 to 5). Collagen damage in AGE/ACLT animals was ~40% increased in the ACLT joint compared to the control joint (Figure 2). Release of glycosaminoglycans was more than doubled (2.2-fold higher) in the ACLT knee versus the contralateral control knee (Figure 3A). The chondrocyte repair mechanism was induced: proteoglycan synthesis increased by ~60% (Figure 3B). Matrix repair was not effective, since the release of the newly synthesized proteoglycans was almost doubled (190%) in experimental knees compared to contralateral control knees (Figure 3C). The combined matrix damage and ineffective repair response resulted in a ~13% decrease in the level of GAGs in the matrix (35.5±1.8 µg GAG/mg cartilage in control joints *vs.* 31.0±1.7 µg/mg in experimental knees; $p<0.05$). In addition, ACLT surgery resulted in a minor secondary synovial inflammation (Goldenberg and Cohen score of 0.3±0.2 in control knees *vs.* 3.5±0.4 in experimental knees; $p<0.01$), which was reflected by the concomitant increase in MMP activity in the experimental knee (Figure 4). The biochemical data were supported by a tripling of the histological (Mankin) score for cartilage damage (Figure 5).

Effect of AGE levels on OA: PBS/ACLT *vs* AGE/ACLT. The outcome parameters revealed that the grade of cartilage damage was more severe in the ribose injected group than in the PBS injected animals (compare black bars in Figures 2 to 4). A 20% increase in collagen damage due to the joint instability (i.e. control *vs.* experimental knee) was observed in the PBS/ACLT animal, whereas a 40% increase in damage

was found in the AGE/ACLT group (Figure 2). Similarly, the release of GAGs from the matrix was 4-fold more increased at high AGE levels (30% *vs.* 120% increase, $p<0.05$; Figure 3A). The repair response in the AGE/ACLT group was less effective than in the PBS/ACLT group: proteoglycan synthesis was significantly lower in the AGE/ACLT group ($p<0.02$; Figure 3B). Moreover, retention of these newly synthesized proteoglycans was impaired at high AGE levels compared to PBS injected animals ($p<0.05$; Figure 3C). Overall, this resulted in more severe OA in the AGE/ACLT group than in the PBS/ACLT group, as is underscored histologically by a significant increase in cartilage degeneration ($p<0.02$; Figure 5). These effects were not due to differences in synovial inflammation since both groups displayed only mild, not significantly different histological inflammation (2.5±0.4 in PBS/ACLT *vs.* 3.5±0.4 in AGE/ACLT; maximal score = 10; not significant). In addition, no differences were observed in the levels of synovial fluid MMP activity, which likely reflect the mild synovial inflammation (Figure 4).

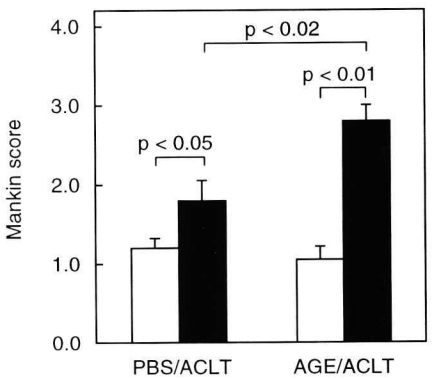

Figure 5. Effect of ACLT on cartilage degeneration at low (PBS/ACLT) and high (AGE/ACLT) AGE levels. Induction of joint instability induces osteoarthritic changes in the articular cartilage, quantified according to the modified Mankin criteria.[300] Cartilage degeneration was statistically significantly more severe at increased AGE levels. Bars represent mean ± SEM of n = 5 animals per group, each analyzed in n = 2 samples per joint. White bars represent contralateral control knees, black bars represent experimental knees.

Appropriate study controls. To ensure that the observed increase in OA severity was not caused by direct effects of the ribose injections, all the OA parameters were also determined in control animals, receiving only ribose injections but no ACLT surgery (AGE/Control group). In this group, AGE levels were 5-fold enhanced ($p<0.05$, control *vs.* experimental joint), similar to the AGE/ACLT group (see figure 1). In the AGE/Control group, none of the outcome parameters was significantly different from untreated contralateral control knees, indicating repeated injections with ribose do not induce cartilage degeneration in our model.

In addition, comparison of the severity of OA in two independent groups of animals (i.e. PBS/ACLT *vs.* AGE/ACLT) requires that the contralateral control joints of these two groups do not differ. Indeed, no changes were observed between the contralateral control knees of the PBS/ACLT *vs.* AGE/ACLT group nor between these two groups and the contralateral control knee of the AGE/Control group (not significant for all parameters tested).

DISCUSSION

Age is the most important risk factor for the development of OA. By which mechanism aging is involved in the development of this invalidating disease remains largely unknown. Fatigue failure of the cartilage collagen network due to repetitive loading has long been recognized as one of the mechanisms involved in the development of OA.[105,320] With increasing age, the strength of the collagen matrix to withstand loading diminishes. Therefore, age-related changes in articular cartilage that influence the composition and strength of the cartilage matrix are very likely involved in the development of OA.[144] One of such changes, the age-related accumulation of AGEs has previously been shown to increase tissue stiffness, to decrease extracellular matrix turnover (synthesis and degradation), and to affect many cellular processes.[22,80,247,261] In the present study, we demonstrate in an *in vivo* model that this process of non-enzymatic glycation is indeed causally involved in the age-related increase in susceptibility for OA.

Our study was designed such that by increasing the AGE levels in the cartilage matrix, the effect of nonenzymatic glycation on the susceptibility for OA could be studied. This approach clearly demonstrates that increased AGE levels predispose to the development of OA. As such, the spontaneous process of nonenzymatic glycation is the first molecular mechanism described today that is capable of, at least in part, explaining the strong age-dependency of OA incidence. Furthermore, the present data suggest that the rate of a generally occurring aging process (i.e. AGE formation occurs in all tissues but is especially important in tissues with slow turnover such as articular cartilage) may predispose to the development of an age-related pathology such as OA. These data are in line with observations from Sell *et al.,* who showed an inverse relation between the rate of glycation and the longevity of a species, which further supports the idea that AGE accumulation is an important process in aging and age-related diseases.[263,264]

The involvement of AGE accumulation in OA emphasizes the dual nature of sugars: on one hand they are essential for life as building blocks and cellular energy source, on the other hand they initiate the formation of potentially detrimental AGEs. The recognition of nonenzymatic glycation as a molecular mechanism that contributes to the development of OA provides new opportunities for therapies directed at prevention of OA by inhibiting or reversing AGE formation. Inhibition of AGE formation by prophylactic treatment with compounds such as aminoguanidine, pyridoxamine, tenilsetam, or simple amino acids (e.g. lysine or arginine) has been shown to prevent AGE-related pathologies such as vascular stiffening, heart collagen accumulation and protein crosslinking.[33,52,75,147,270] Alternatively, AGE-directed therapy can consist of so-called AGE-breakers.[84] Thiazolium compounds such as *N*-phenacylthiazolium bromide and phenyl-4,5-dimethylthiazolium chloride, reported to break dicarbonyl containing AGEs, showed efficacy in reversing AGE-related tendon crosslinking and cardiac stiffness.[15,302] Despite the fact that these therapies are relatively new, they provide

proof-of-principle that inhibition or reversal of AGE formation can have beneficial effects in AGE-mediated pathologies. The involvement of AGEs in the etiology of OA as is demonstrated by our data, in combination with the emerging possibilities for AGE-directed therapies provide a possible tool to prevent or postpone the development of OA. This is an important development since adequate therapy for OA is lacking while the number of people suffering from this invalidating disease will increase further because of the aging population.

Acknowledgements.

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Summary and Discussion

Osteoarthritis (OA) is one of the most prevalent diseases of the elderly, affecting >50% of the people over 60 years of age. Many factors are implicated in the development of OA but currently no mechanism has been described that provides an explanation for age as the major risk factor for OA. The studies presented in this thesis in combination with the studies described in the thesis by Nicole Verzijl in *Advanced Glycation Endproducts in the Development of Osteoarthritis: Cartilage Biochemistry and Biomechanics,*[307] demonstrate that the age-related accumulation of advanced glycation endproducts (AGEs) provides such a molecular mechanism, capable of (at least partly) explaining the age-related increase in the incidence of OA.

The presence of AGEs in cartilage was first demonstrated by Sell and Monnier following their discovery of pentosidine.[266] Subsequently, pentosidine levels were shown to increase in articular cartilage with advancing age.[22,295] Inasmuch as pentosidine represents only one specific AGE out of the variety of AGEs that are formed *in vivo*, pentosidine levels may function as a marker for the whole group of AGEs. Since many factors determine the formation of AGEs (e.g. pH, oxygen tension, type and amount of sugars), pentosidine is not *a priori* the single best marker for AGE accumulation in all tissues. Therefore, to gain insight in the diversity of AGEs present in articular cartilage, several AGE measures were determined in a wide age range of human articular cartilage samples. In addition to pentosidine, N^{ε}-(carboxymethyl)-lysine, N^{ε}-(carboxyethyl)lysine, absorption, fluorescence, amino acid modification and collagenase digestibility all demonstrated increased AGE levels with increasing age in articular cartilage (Figure 1A).[307] Moreover, the level of these AGEs is high in cartilage compared to other tissues such as skin, which is mainly caused by the very low turnover of the cartilage matrix proteins. The half-life of collagen in articular cartilage is ~117 years (compared to the half-life of skin collagen of ~15 years).[307]

Accumulation of AGEs in cartilage affects biomechanical, biochemical and cellular characteristics of the tissue. At the biomechanical level, increased AGE levels are accompanied by increased stiffness of the tissue (Figure 1B).[307] This was shown using the instantaneous deformation technique and by the osmotic stress technique. A recent study in collaboration with the group of Dr Sah (University of California, San Diego) showed that elevated AGE levels not only increase tissue stiffness but also increase cartilage brittleness,[64] indicating that AGE accumulation leads to enhanced susceptibility of articular cartilage to mechanical damage (Figure 1B).

On cellular level, accumulation of AGEs was shown to decrease proteoglycan[chapters 2 and 3] and collagen[chapter 4] synthesis by chondrocytes (Figure 1C). The mechanism that is responsible for this effect remains largely unknown. AGE accumulation in the extracellular matrix may affect extracellular matrix synthesis via AGE receptors (e.g. RAGE and galectin-3) present on chondrocytes. This is corroborated by the observation that RAGE-mediated signaling inhibits collagen synthesis in fibroblasts.[224] Alternatively, AGE modification of the extracellular matrix interferes with the normal cell-matrix signaling such as integrin-mediated signaling of collagens. Glycation of the arginine residue in the Arg-Gly-Asp (RGD) sequence of collagen that binds to integrin

receptors has been shown to decrease binding of osteo- and fibrosarcoma cells to the extracellular matrix[226] and may similarly influence chondrocyte attachment to the matrix and thereby cell function. The extracellular matrix acts as a mechanical signal transducer that receives input in the form of joint loading and generates a signal for the chondrocytes (e.g. cell deformation, osmotic pressure, fluid flow, nutrient gradient).[213]

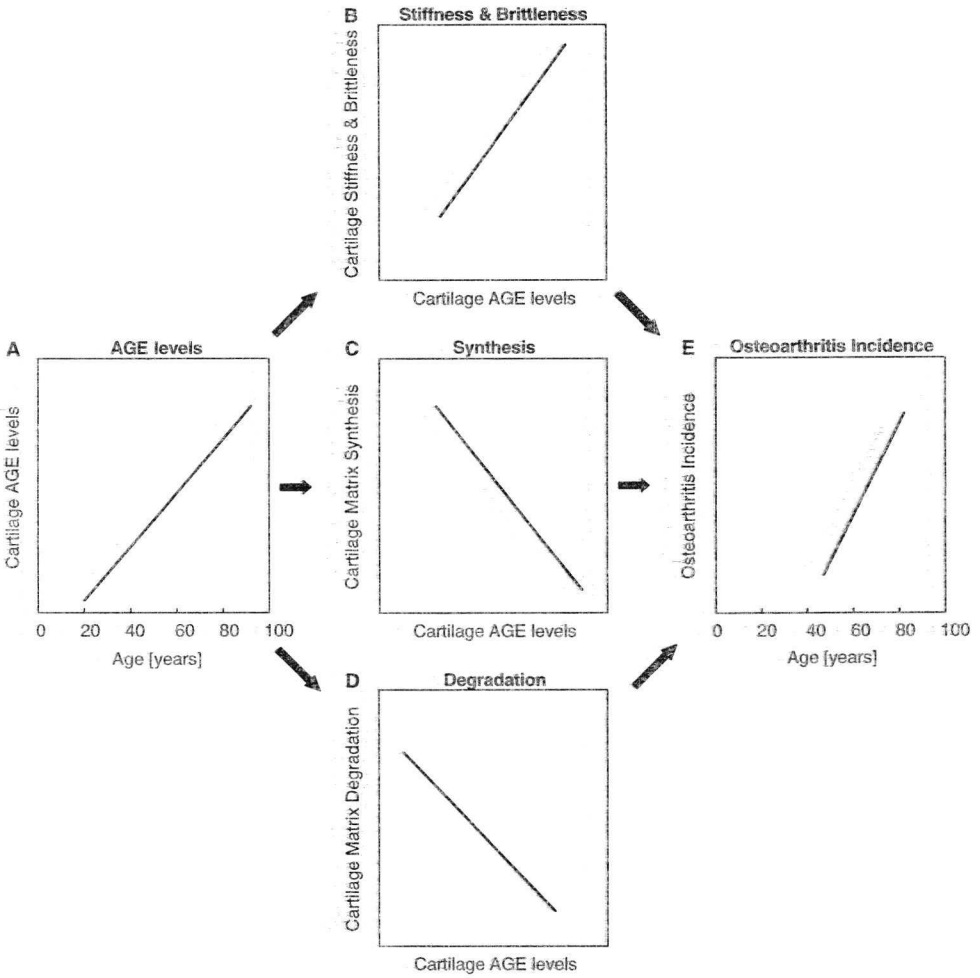

Figure 1. Model for the involvement of AGEs in etiology of OA. With increasing age, AGEs accumulate in articular cartilage (A). The accumulation of AGEs results in cartilage with increased stiffness and brittleness (B), decreased collagen and proteoglycan synthesis (C) and decreased collagen and proteoglycan proteolysis (D). In combination, these effects of AGE accumulation render the articular cartilage more prone to damage with increasing age, thus explaining the increased incidence of osteoarthritis with increasing age (E).

AGE crosslinking that results in a stiffer matrix interferes with this signal transduction and is therefore likely to affect chondrocyte function.[185] AGE accumulation can also occur intracellularly and thereby affect cell function.[143] Since chondrocytes are long living cells, such AGE-related modulation of cell function may be of particular importance for articular cartilage. Due to the close interaction between the extracellular matrix and the chondrocytes, effects of glycation on the extracellular matrix cannot readily be separated from effects of chondrocyte glycation. Experiments designed to separate these effects require isolation of chondrocytes and thereby destroy the structure of the extracellular matrix and the cell-matrix interactions that are both important in transmitting changes in the extracellular matrix to the chondrocytes and in maintaining chondrocyte differentiation.[40,41]

In addition to the decreased synthesis, accumulation of AGEs results in a decreased susceptibility towards proteinase-mediated cartilage degradation. Release of proteoglycan and collagen from the extracellular matrix by both chondrocyte-derived proteinases[chapters 3 and 4] and synovial fluid proteinases[chapter 5] was decreased at increased AGE levels (Figure 1D). Several mechanisms appear to contribute to this effect. Accumulation of AGE crosslinks in the extracellular matrix may decrease the release of degraded fragments from the tissue, thus explaining the decreased matrix degradation observed at increased AGE levels. Alternatively, many mechanisms interfere with the cleavage of the matrix constituents by the proteinases. Glycation of cartilage changes biophysical properties of the matrix proteins and thereby possibly affect the permeability of the matrix for proteinases.[8] In addition, AGE modification renders the matrix proteins increasingly resistant towards proteolytic degradation by changing proteinase cleavage and/or recognition sites. Also, production of MMPs by chondrocytes is decreased in a glycated environment (either as a consequence of different signals from the matrix or due to intracellular AGE accumulation). Activation of zymogens (such as proMMPs) into active enzyme may be decreased at increased AGE levels due to glycation of the proMMP itself or of components that are essential in the activation cascade. Finally, glycation of active proteinases decreases their proteolytic activity and thus impairs matrix degradation.

Decreased degradation of the extracellular matrix can be either an advantage or a disadvantage. In pathologies such as OA and rheumatoid arthritis proteolytic destruction of cartilage is reduced by the presence of AGEs and thereby progression of the disease is potentially reduced. On the other hand, impaired proteolysis will interfere with the clearance of matrix degradation products from the tissue. Degradation products derived from the extracellular matrix can function as signaling molecules that convey the integrity of the surrounding extracellular matrix to the chondrocyte.[131] Matrix degradation products stimulate chondrocytes[132,156,234] and several other cell types such as macrophages[149] and muscle cells[283] to produce proteinases. An impaired capacity to remove AGE-modified degradation products is likely to result in ongoing stimulation of chondrocytes to produce proteinases and consequently chondrolysis may occur. By this mechanism, that is thus far purely

hypothetical, accumulation of AGEs may even enhance matrix degradation and accelerate the progression of joint destruction.

Chondrocytes continuously remodel their extracellular matrix in response to external stimuli (loading, cytokines, growth factors).[213,214,242] In combination decreased extracellular matrix synthesis and degradation at increased AGE levels, implicates that chondrocytes in a glycated environment such as aged cartilage possess decreased capacity for this remodeling of their extracellular matrix. Consequently, in a glycated matrix the capacity of the chondrocytes to respond to external changes, such as a different loading pattern, is decreased. This may result in cartilage that does not possess the optimal composition and/or integrity to fulfil its function and therefore is more susceptible to damage.

In addition, decreased matrix turnover reduces the capacity of the chondrocytes to repair damage. Damaged matrix constituents are less well removed and new molecules are synthesized and incorporated in the matrix at a lower rate. Therefore, tissue damage in not readily repaired. During this period, stimuli that are normally not harmful can inflict more damage due to the decreased resistance of the (already damaged) tissue. Ultimately these processes could progress into degenerative changes and finally lead to development of clinical OA.

In combination, the increased tissue brittleness and decreased extracellular matrix turnover, due to the accumulation of AGEs, results in articular cartilage that is more prone to damage. By this mechanism, the age-related increase in cartilage AGEs (Figure 1A) can explain the age-related increase in the incidence of OA (Figure 1E). The concept, that AGE accumulation predisposes to the development of OA, was tested in the canine anterior cruciate ligament transection (ACLT) model for osteoarthritis.[233,300] By selectively enhancing AGE levels in articular cartilage of young animals, the role of AGE accumulation in the development of OA was studied in the absence of other age-related changes.[chapter 6] As expected, enhanced AGE levels resulted in more severe OA. Further support for the hypothesis that increased AGE levels predispose to the development of OA, was obtained in a cross-sectional study using human articular cartilage.[307] The rate of AGE formation is increased in healthy cartilage of donors that show degenerative (early osteoarthritic) changes elsewhere in the joint cartilage compared to the AGE formation in donors that do not show any signs of cartilage degeneration. In conclusion, both the canine ACLT study and the human cross-sectional study demonstrate that increased AGE levels predispose to the development of OA.

AGE accumulation in non-cartilagenous tissues. Osteoarthritis is widely recognized as a disease of the joint, not merely of the articular cartilage.[235] Other structures in the joint include tendons and ligaments, synovial membrane, and (subchondral) bone. Age-related changes in these joint tissues may contribute to the development of osteoarthritis. Changes in subchondral bone have repeatedly been implicated in the etiology of OA.[188,244] However, it is still debated whether changes in

subchondral bone precede or follow those in articular cartilage.[4] As in articular cartilage, age-related accumulation of AGEs has been observed in human bone, and, similar to cartilage, bone becomes increasingly brittle with increasing AGE levels.[61] This age-related increase in brittleness of the subchondral bone can interfere with the distribution of forces from the articular cartilage into the bone. Consequently, these changes in bone may enhance the susceptibility for damage of the cartilage layer covering the bone. In addition to cartilage and bone, tendons and ligaments in joints become glycated with advancing age, which likely results in increased stiffness and therefore increased susceptibility to damage. Increased stiffness of the tendons and ligaments also affects the capacity of the joint to distribute forces over the cartilage surface and by that mechanism can contribute to the increased susceptibility for cartilage damage leading to OA.

Osteoarthritis in diabetes. The conclusion of this thesis that accumulation of AGEs predisposes to the development of OA implicates that (patho)physiological conditions such as diabetes, in which glycation is increased, are associated with an increase in the incidence of OA. Diabetic patients are exposed to high (fluctuating) blood glucose levels as a result of insufficient insulin production, peripheral resistance towards insulin[117] or both. Consequently, AGEs in many tissues are increased compared to non-diabetic, age-matched controls.[207,209] In pilot experiments we detected increased AGE levels in cartilage from both diabetic rats and diabetic patients compared to controls (unpublished data). These enhanced AGE levels are expected to result in stiff and brittle cartilage that is susceptible to damage. Indeed, one study showed increased cartilage hydration in diabetic rats compared to non-diabetic controls,[62] indicating increased damage to the cartilage collagen network.[28] Surprisingly, the proteoglycan levels that are responsible for maintaining cartilage hydration were decreased in these diabetic rats.[62] This indicates either loss of proteoglycans as a consequence of the OA process or reflects decreased proteoglycan synthesis caused by the insulin deficiency.[62,321] Insulin is repeatedly demonstrated to act as a growth factor for articular cartilage.[62,98,322]

Few biomechanical data on the stiffness of articular cartilage in relation to the diabetic status are available. One study, using a creep indentation technique that mainly assesses proteoglycan rather than collagen characteristics, demonstrated that diabetic cartilage is weaker and displays larger permeability than normal tissue.[16] This is consistent with the mentioned decreased proteoglycan content of diabetic cartilage.[62] No data are available on the effects of diabetes on the biomechanical characteristics of the collagen network that can explain the increased tissue hydration in diabetic rats.[62]

In epidemiological studies in the early 1960s, an increased incidence and severity of OA has been reported in patients with diabetes.[271,317] More joint space narrowing and subchondral cysts were observed in diabetics than in controls, but this effect did not reach statistical significance.[60] One study demonstrated increased plasma glucose

levels in patients with radiological OA compared to healthy controls,[66] whereas others did not find such effects.[109] Recent studies suggest a slight increase in risk for OA in diabetic patients, but fail to show a significant correlation.[7,17,93] The discrepancy between the different studies may be due to differences in experimental set-up, used definitions of OA and diabetes, or may reflect the increased glycemic control of the last decades. The absence of an obvious correlation between diabetes and OA can also be explained by diabetes-related changes that affect OA development and/or diagnosis. Bone differentiation is impaired in diabetic patients[99,322] and this is reflected by a reduced osteophyte formation in diabetic OA patients.[133] Since osteophytes are an important determinant of pain in OA patients[77] and also contribute to radiological OA grading,[95,146] a decreased osteophyte number decreases OA grades that include pain levels or are based on radiographs. Overall, the effects of diabetes on the development of osteoarthritis remain unclear.

Anti-AGE therapy as new avenue to prevent OA. The identification of AGE accumulation as a molecular mechanism contributing and/or predisposing to the development of OA opens new avenues for OA treatment and prevention. Therapies directed at prevention of AGE-related diabetic complications such as cataract, nephropathy, accelerated atherosclerosis and arterial stiffening are under development; OA patients may benefit from the development of these potential pharmacological treatments.

The recently developed soluble AGE receptor sRAGE, that binds to AGEs and thereby inhibits activation of cell surface AGE receptors, showed efficacy in treatment of vascular complications in animal models for diabetes.[260] Blocking AGE-chondrocyte interactions may have similar beneficial effects in prevention of OA. However, although the present thesis clearly demonstrates effects of AGE accumulation on chondrocyte behavior, it is still unclear whether these effects are mediated by specific AGE-receptors. Moreover, the relative contribution of chondrocyte-mediated (matrix turnover) *vs.* extracellular matrix-mediated (matrix stiffness/brittleness) effects of AGE accumulation in the development of OA remains unknown, which may largely determine the ultimate efficacy of sRAGE therapy in the prevention of OA.

An alternative approach towards anti-AGE therapy for OA is focussed at the prevention or reversal of AGE formation. Starting from the highly simplified Maillard reaction presented in the introduction of this thesis, intervention can be directed at different levels in the AGE formation (figure 2).

Strategies aimed at the inhibition of the first step in the glycation cascade, interfering with the formation of a Schiff base, could be directed at either the amino group of the protein or the reducing sugar involved. Modification of amino groups by non-sugars such as aspirin (acetylation) prevents amino groups from reacting with sugars.[247] Although beneficial effects of aspirin have been described in animal studies, its mode of action remains debated and inhibition of the later stages in the AGE formation due to its antioxidant properties appears to contribute largely to its efficacy. Moreover, the

approach of blocking a large number of amino groups in a tissue is not practical on therapeutical level[148] and may have adverse effects as well. Alternatively, inhibitors such as aminoguanidine, lysine, and arginine are directed at the reducing sugars and prevent AGE formation by reacting with the sugar. Similar to aspirin, aminoguanidine intervenes in AGE formation at different levels (it also reacts with dicarbonyl intermediates formed in the Wolff and Namiki pathways of AGE formation[148]).

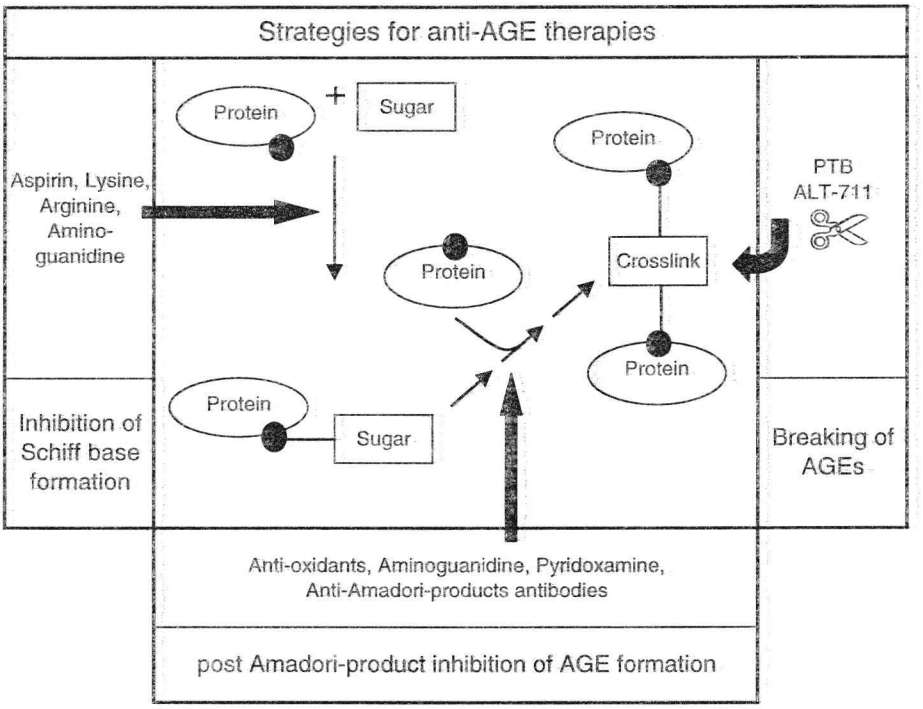

Figure 2. Inhibition of AGE formation based on the classical view of AGE formation. The initial formation of a Schiff base can be blocked by blocking the amino group (e.g. acetylation by aspirin) or by competitors for protein binding of the reducing sugar (e.g. aminoguanidine). Post Amadori-product inhibition can be accomplished by compounds such as pyridoxamine, aminoguanidine or anti-oxidants. Finally, recently discovered thiazolium bromide compounds are claimed to possess AGE cleavage capacity, and thereby break already formed AGEs.

A variety of compounds inhibit the post-Amadori formation of AGEs.[184,331] Some of these compounds are antioxidants (e.g. pyruvate, vitamin E, vitamin C); others present metal ion chelators (e.g. diethylenetriaminepenta-acetic acid, DTPA) or antibodies that bind Amadori products and thus prevent progression into AGEs.[184] Other compounds such as pyridoxamine inhibit post-Amadori progression into AGEs by trapping intermediates that are released from the protein or by reacting with Amadori products.[148]

Alternatively, anti-AGE therapy is directed at the final stage of the cascade. Mature dicarbonyl AGE-crosslinks are cleaved by thiazolium compounds such as PTB (*N*-phenacylthiazolium bromide) or ALT-711 (phenyl-4,5-dimethylthiazolium chloride) that thereby reverse a selection of the formed AGEs.[15,302,327] These compounds, although in a preliminary stage of development, suggest that reversal of AGEs can have beneficial effects on e.g. vascular compliance.[15] However, some studies debate the mode of action[148,291] and suggest that also thiazolium compounds interfere with AGE formation rather than breaking established AGEs. In addition, the crosslinking structures that are identified to date are thought to constitute only a small fraction of the AGE crosslinking occuring *in vivo*, with the major crosslinking structures still unidentified. Selective cleavage of these chemical structures may very well leave the majority of the AGEs unaffected and thus provides little therapeutic effect.

Although AGE formation is regarded as a side effect of the carbohydrate metabolism and mostly detrimental effects of AGE accumulation are studied, the possibility exists that AGEs play an essential role in life as a marker of the molecular age of a protein or cell[313] or as a modulator of the immune system (e.g. via the receptors of AGEs such RAGE[260]). These potentially beneficial effects of AGEs may eventually contribute to the final applicability and effectiveness of anti-AGE therapy.

The studies described in this thesis and the thesis by Nicole Verzijl[307] demonstrate that the spontaneous accumulation of AGEs in articular cartilage contributes to the development of OA. Considering this, prophylactic anti-AGE therapy for people who have increased risk to develop OA may be beneficial, but requires development of therapies that allow the removal of the variety of AGEs found *in vivo*.

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SAMENVATTING VOOR NIET-INGEWIJDEN

ARTROSE

Artrose of kraakbeenslijtage is een veelvoorkomende gewrichtsaandoening bij ouderen. Meer dan de helft van de 65-plussers heeft artrose in één of meer gewrichten. De belangrijkste klinische kenmerken van artrose zijn gewrichtspijn, bewegingsbeperkingen en soms zwelling van de gewrichten. Op weefselniveau wordt artrose gekenmerkt door de voortschrijdende afbraak van het gewrichtskraakbeen. Daarnaast treden botverdichting (subchondrale sclerose), abnormale vorming van nieuw bot (osteophyten) en milde gewrichtsontsteking op. Hoe de ziekte begint is tot dusver grotendeels onbekend. Wel is duidelijk dat artrose een multifactoriële aandoening is: zowel genetische als mechanische (bijvoorbeeld belasting) en ook biochemische factoren spelen een rol. Verreweg de belangrijkste risicofactor voor artrose is veroudering: het percentage mensen dat lijdt aan artrose neemt sterk toe met het toenemen van de leeftijd. Via welk mechanisme een toenemende leeftijd leidt tot artrose is echter grotendeels onbekend. Leeftijdsgerelateerde veranderingen in het gewrichtskraakbeen zijn mogelijk verantwoordelijk voor de leeftijdsgerelateerde toename in het aantal mensen met artrose.

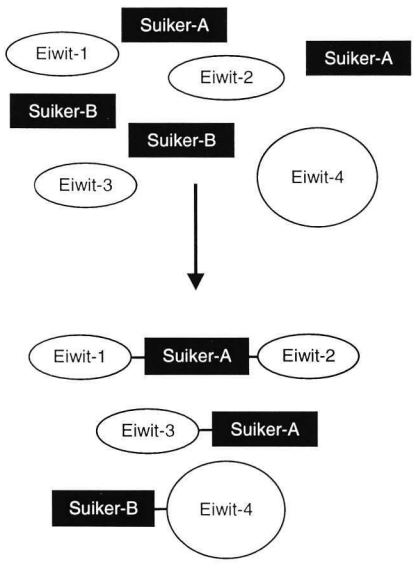
KRAAKBEEN

Kraakbeen is een gespecialiseerd bindweefsel dat als een dun laagje (enkele millimeters dik) het uiteinde van botten bekleedt binnenin de gewrichten. Het kraakbeen vervult een essentiële taak in het opvangen van belasting (schokdemping) en zorgt tevens voor een glad oppervlak, zodat de botuiteinden in de gewrichten bijna wrijvingsloos over elkaar kunnen glijden. Kraakbeen bestaat uit cellen (~1% van het weefsel), extracellulaire matrix (letterlijk: 'buiten de cellen gelegen matrix'; al wat tussen de cellen ligt; ~20%) en water (~80%; zie ook 'Figure 1' in de 'Introduction'). De kraakbeencellen zorgen voor de productie en het onderhoud van de extracellulaire matrix. Deze matrix is verantwoordelijk voor de unieke mechanische eigenschappen van het kraakbeen die ondermeer nodig zijn voor het opvangen van belasting. De verschillende componenten van de extracellulaire matrix hebben daarin elk hun eigen rol. De collageeneiwitten vormen een netwerk dat het kraakbeen vorm, samenhang en sterkte geeft. Binnen dit collageen netwerk liggen de zogenaamde proteoglycanen ingesloten. Deze proteoglycanen trekken heel sterk water aan en dat geeft het kraakbeen het vermogen om zware belastingen te weerstaan. Bij samendrukken van het kraakbeen tijdens normaal gebruik van het gewricht (bijvoorbeeld wanneer je een stap zet tijdens het lopen) wordt water uit het kraakbeen geperst (alsof een natte spons wordt ingedrukt en het water eruit komt). Op het moment dat de belasting wegvalt (het been wordt opgetild voor de volgende stap), wordt het water weer het kraakbeen ingezogen en neemt het kraakbeen de oorspronkelijke vorm aan (de spons

is weer "normaal"). Dit mechanisme van afwisselend water verliezen en weer opnemen geeft gezond kraakbeen zijn veerkracht en is van groot belang voor het goed functioneren van een gewricht. Deze functies kunnen niet of slechter uitgevoerd worden wanneer het kraakbeen beschadigd raakt of zelfs helemaal wegslijt. De botuiteinden in een gewricht rusten dan direct op elkaar met een ruw, niet-elastisch oppervlak: pijn en bewegingsbeperking zijn het gevolg, artrose.

NIET-ENZYMATISCHE GLYCERING

Het is goed voorstelbaar dat leeftijdsgerelateerde veranderingen in het gewrichtskraakbeen, die het weefsel gevoeliger maken voor schade, mogelijk één van de oorzaken zijn voor het ontstaan van artrose. Eén van de meest opvallende veranderingen in kraakbeen is de leeftijdsgerelateerde verandering in kleur: jong kraakbeen is wit terwijl oud kraakbeen een geel-bruine kleur heeft. Deze kleurverandering is toe te schrijven aan het accumuleren van niet-enzymatische glyceringsproducten in het kraakbeen. Niet-enzymatische glycering is de spontane reactie tussen eiwitten (de bouwstenen van de matrix zoals collageen en proteoglycanen) en suikers (de brandstof voor de cellen). Omdat er in kraakbeen veel verschillende eiwitten en suikers aanwezig zijn kunnen er heel veel verschillende glyceringsreacties optreden. Als gevolg van deze spontane reacties ontstaat dan ook een heel scala aan glyceringsproducten. Deze producten kunnen grofweg in twee groepen verdeeld worden: de dwarsverbindingen (crosslinks) en de aanhangsels (adducten) (zie figuur 1).



◀ Eiwitten (bouwstenen) en suikers (brandstof) zijn beide aanwezig in kraakbeen en kunnen spontaan met elkaar reageren: **niet-enzymatische glycering**

◀ Het resultaat van de glyceringsreacties is een scala aan producten: zowel dwarsverbindingen tussen eiwitten (crosslinks) als aanhangsels aan eiwitten (adducten) worden gevormd. Afhankelijk van welke suikers en eiwitten meedoen in de reactie kunnen heel verschillende glyceringsproducten gevormd worden

Figuur 1. Schematische weergave van de niet-enzymatische glycering

Beide typen glyceringsproducten zijn erg stabiel: ze kunnen niet uit het kraakbeen verwijderd worden tenzij het eiwit waaraan ze vastzitten wordt verwijderd. Aangezien de vernieuwingssnelheid van kraakbeeneiwitten erg laag is, worden er nauwelijks glyceringsproducten verwijderd. Daarentegen gaat de spontane vorming van de glyceringsproducten continu door, met als gevolg dat de glyceringsproducten met toenemende leeftijd ophopen in kraakbeen. Deze leeftijdsgerelateerde opstapeling van glyceringsproducten in kraakbeen gaat gelijk op met de leeftijdsgerelateerde toename in het aantal mensen dat leidt aan artrose. Dit suggereert dat het glyceringsproces mogelijk een rol speelt in het ontstaan van artrose.

DE VRAGEN

In het proefschrift van Nicole Verzijl, getiteld *'Advanced Glycation Endproducts in the Development of Osteoarthritis: Cartilage Biochemistry and Biomechanics'* wordt met name aandacht besteed aan de diversiteit aan glyceringsproducten in kraakbeen, de factoren die een rol spelen bij de vorming van deze producten, de effecten van de kraakbeen glycering op de mechanische eigenschappen van het kraakbeen en de relatie tussen glyceringsspiegels en het optreden van artrose in de mens. In mijn proefschrift staat de volgende vraag centraal: *Wat zijn de effecten van niet-enzymatische glycering op de aanmaak en afbraak van de extracellulaire matrix van gewrichtskraakbeen en hoe beïnvloeden deze effecten de gevoeligheid voor artrose?*

DE ANTWOORDEN

Beide proefschriften tonen aan dat het proces van niet-enzymatische glycering inderdaad een verklaring kan bieden voor de leeftijdsgerelateerde stijging in de incidentie van artrose. Een groot aantal bevindingen ondersteunt deze conclusie:
Om inzicht te krijgen in de diversiteit van glyceringsproducten in kraakbeen werd de aanwezigheid bepaald van een aantal glycerings-crosslinks en -adducten in kraakbeen varierend in leeftijd van 20 tot 90 jaar. Daarnaast werden algemenere glyceringsmaten gebruikt zoals bijvoorbeeld het meten van de gele verkleuring door de glyceringsreactie. Alle maten tonen een leeftijdsgerelateerde toename van glycering in gewrichtskraakbeen.[proefschrift N Verzijl, hoofdstuk 2] De glyceringsniveaus in kraakbeen blijken erg hoog te zijn ten opzichte van andere weefsels, zoals huid. Dit wordt voornamelijk veroorzaakt door de erg lage vernieuwingssnelheid van kraakbeen.[proefschrift N Verzijl, hoofdstuk 3 en 4]
De gevolgen van deze toename in kraakbeenglycering zijn divers. Op biomechanisch niveau gaat een toename in glycering vergezeld van een toename in de stijfheid: kraakbeen met een hoog glyceringsniveau vervormt minder onder belasting dan kraakbeen met een laag glyceringsniveau.[proefschrift N Verzijl, hoofdstuk 5] Tevens blijkt een

toename in kraakbeenglycering samen te gaan met een verhoogde broosheid van het weefsel: het kraakbeen kan minder ver uitrekken voordat het breekt. De toegenomen stijfheid en broosheid van het kraakbeen ten gevolge van de glycering maken het weefsel gevoeliger voor mechanische schade.
Naast de effecten op de mechanische eigenschappen van het kraakbeen blijken glyceringsproducten ook de activiteit van de kraakbeencellen te beïnvloeden. De aanmaak van proteoglycanen[hoofdstuk 2 en 3] en collagene eiwitten[hoofdstuk 4] door de kraakbeencellen vermindert als gevolg van toegenomen glyceringsniveaus. Het mechanisme dat hieraan ten grondslag ligt is vooralsnog onduidelijk. Glyceringsproducten kunnen direct aan cellen binden en beïnvloeden zo de activiteit van die cellen. Dit effect zou ook voor kraakbeencellen kunnen opgaan. Daarnaast staan kraakbeencellen continu in contact met de extracellulaire matrix om hen heen. Veranderingen in die matrix door de glycering kunnen de normale interacties tussen de kraakbeencellen en de matrix verstoren en daarmee de celfunctie beïnvloeden.
Naast effecten op de aanmaak van matrixeiwitten, blijkt een toename in glycering ook te leiden tot een verminderde afbraak van die matrix. Het vrijkomen van proteoglycanen[hoofdstuk 3 en 5] en collageen[hoofdstuk 4] uit de extracellulaire matrix als gevolg van de activiteit van proteinases ('matrix-afbrekende-enzymen') vermindert bij verhoogde glycering. De verminderde kraakbeenafbraak wordt waargenomen bij proteinases die door de kraakbeencellen zelf worden geproduceerd[hoofdstuk 3 en 4] en ook bij proteinases die gemaakt worden in ontstoken gewrichten en van buitenaf het kraakbeen afbreken.[hoofdstuk 5] Een aantal mechanismen is verantwoordelijk voor dit effect. Toegenomen glycerings-dwarsverbindingen maken dat afgebroken eiwitten via deze dwarsverbinding in de matrix vast blijven zitten. Een matrixeiwit dat wel geknipt is door een proteinase blijft via de dwarsverbinding toch vastzitten in de matrix (en dus komen er geen losse, detecteerbare afbraakfragmenten vrij). Daarnaast beïnvloeden glyceringsproducten ook direct de afbraak van de matrixeiwitten door proteinases. De glyceringsproducten veranderen de doordringbaarheid van de matrix en zorgen er ook voor dat een proteïnase de plek niet meer herkent waar geknipt moet worden (zoals een propje papier in een slot verhindert dat de sleutel er goed in past en omgedraaid kan worden). Daarnaast kunnen de proteïnases (dat zijn ook eiwitten) ook zelf geglyceerd raken en deze geglyceerde proteïnases zijn minder actief in het knippen van de matrix dan niet-geglyceerde proteïnases.
De verminderde afbraak van het geglyceerde kraakbeen heeft potentieel zowel goede als slechte kanten. In gezond kraakbeen is de gecontroleerde afbraak van kraakbeen noodzakelijk voor het correcte onderhoud en herstel van de kraakbeen matrix. Voor deze processen is het nodig dat bestaande eiwitten worden opgeruimd voordat er nieuwe eiwitten neergelegd kunnen worden (vergelijk met het verbouwen of renoveren van een huis: eerst de bestaande, soms vervallen, muren weghalen en dan pas nieuwe muren bouwen). Als glycering deze processen verstoort kan dit ertoe leiden dat het kraakbeen niet in de juiste conditie gehouden kan worden die nodig is voor het weerstaan van belasting. Dit negatieve effect van glycering maakt het kraakbeen

mogelijk gevoeliger voor schade, dus artrose. Daar tegenover staat een mogelijk positief effect van glycering. Tijdens het artroseproces slaan de kraakbeencellen op hol en worden grote hoeveelheden van allerlei proteinases gemaakt en treedt ongecontroleerde afbraak van het kraakbeen op. In een dergelijke situatie kan verminderde afbraak door toegenomen glyceringsniveaus het weefsel beschermen en het voortschrijden van de ziekte afremmen. Tot dusver is niet bekend welke van deze twee effecten (verminderde onderhouds- en herstelcapaciteit versus vertraging van overmatige afbraak) het belangrijkst is.
De combinatie van toenemende stijfheid en broosheid, en afnemende aanmaak en afbraak van de kraakbeenmatrix, als gevolg van toegenomen glycering in het kraakbeen, leidt tot een hogere gevoeligheid voor schade. Via dit mechanisme kan de leeftijdsgerelateerde toename in glycering de oorzaak zijn van de leeftijdsgerelateerde toename in artrose. Dit idee is getest in een hondenmodel voor artrose. In dit model wordt de voorste kruisband van de knie operatief doorgesneden, met als gevolg gewrichtsinstabiliteit die leidt tot artrose. Door kunstmatig de glyceringsniveaus in een aantal jonge honden te verhogen werd de invloed van glycering op het ontstaan van artrose bestudeerd in afwezigheid van andere leeftijsgerelateerde veranderingen.[hoofdstuk 6] Zoals verwacht ontwikkelen de honden met de kunstmatig verhoogde kraakbeenglycering ernstiger artrose dan honden met normale glyceringsniveaus. Ondersteuning van de hypothese dat glycering leidt tot artrose, werd ook verkregen uit een studie met van-de-mens-afkomstig kraakbeen.[proefschrift N Verzijl, hoofdstuk 6] Gezond kraakbeen van donoren die elders in het gewricht artrose hadden, bevat hogere glyceringsspiegels dan kraakbeen van donoren die geen enkel teken van artrose vertoonden.

De Conclusie

Samengevat laten zowel de hondenstudie als de studie met humaan kraakbeen zien dat een toename in kraakbeenglycering een factor is die bijdraagt aan het ontstaan van artrose. Deze observatie opent mogelijk nieuwe wegen voor therapiën voor de behandeling en preventie van artrose. Therapiën die worden ontwikkeld tegen complicaties bij diabetespatiënten (die vanwege hun verhoogde bloedsuikerspiegels ook verhoogde glyceringsniveaus hebben in veel weefsels), kunnen mogelijk ook tot nut zijn voor artrosepatienten. Echter, de grote variëteit in glyceringsproducten die gevormd wordt in het lichaam en de mogelijke bijeffecten van het remmen en/of verbreken van glycering maakt dat voor de ontwikkeling van anti-glycerings therapie als een nieuwe behandelmethode voor artrose nog veel onderzoek noodzakelijk is.

EPILOGUE

The 'dankwoord' or epilogue is certainly the part of a thesis that receives most attention, and therefore summarizing my research on this page would probably be the best way to share these exciting results with as many people as possible. However, I am afraid this would leave little room for the real purpose of this part of the thesis: the expression of my gratitude towards the many people that were essential in the production of this thesis. Therefore I would like to urge you all to read (after finishing this page) either the 'Summary and Discussion' or the 'Samenvatting voor niet-ingewijden'.

DANKJULLIEWEL

Aangezien het dankwoord of 'epilogue' het meest gelezen deel van een proefschrift is, zou het een slimme zet zijn om op deze pagina de belangrijkste resultaten en conclusies nog eens samen te vatten. Op die manier krijgen zoveel mogelijk mensen weet van de prachtige resultaten die beschreven zijn in dit proefschrift. Echter, zo'n aanpak laat weinig ruimte over om te zeggen waar het op deze bladzijde om gaat: het danken van alle mensen die hebben bijgedragen aan het tot stand komen van dit proefschrift. Ik wil jullie dan ook met klem verzoeken om na deze bladzijde de 'Summary and Discussion' of de 'Samenvatting voor niet-ingewijden' te lezen

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Jeroen, 19 juni 2001

CURRICULUM VITAE

Jeroen de Groot was born in Eindhoven on 24 December 1972. In 1991 he completed his secondary school education (Gymnasium ß) at the 'Jacob van Maerlantlyceum' in Eindhoven, the Netherlands. In September of the same year, he started his masters degree in Biology at the Leiden University, graduating *cum laude* in June 1996. During these studies, he obtained work experience at the Cell Biology section of the subfaculty of Biology, Leiden University (supervisors P.W. Schenk and Dr. B.E. Snaar-Jagalska), the department of Vascular and Connective Tissue Research, TNO Prevention and Health, Leiden (supervisors Dr. J.M. te Koppele, TNO and Dr. W. de Priester, Leiden University) and at the department of Genetics, Stockholm University, Sweden (supervisor Prof. Dr. E. Haggård-Ljungquist)
From July 1996 onward he was appointed as PhD student at the department of Rheumatology & Clinical Immunology of the University Medical Center Utrecht and at the Gaubius Laboratory, TNO Prevention and Health, Leiden. During this period, under the supervision of Dr. J.M. te Koppele, Dr. F.P.J.G. Lafeber and Prof. Dr. J.W.J. Bijlsma the work described in this thesis was performed. From July 1999 to June 2000, he worked at TNO on a research project titled 'MMP activity measurements using fluorogenic peptide substrates' sponsored by Pfizer Inc. After obtaining his PhD, he will continue to work at TNO as a scientist in the Matrix Biology group.

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