BONE

Radiocarbon dating reveals minimal collagen turnover in both healthy and osteoarthritic human cartilage

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The poor regenerative capacity of articular cartilage presents a major clinical challenge and may relate to a limited turnover of the cartilage collagen matrix. However, the collagen turnover rate during life is not clear, and it is debated whether osteoarthritis (OA) can influence it. Using the carbon-14 (^{14}C) bomb-pulse method, life-long replacement rates of collagen were measured in tibial plateau cartilage from 23 persons born between 1935 and1997 (15 and 8 persons with OA and healthy cartilage, respectively). The ^{14}C levels observed in cartilage collagen showed that, virtually, no replacement of the collagen matrix happened after skeletal maturity and that neither OA nor tissue damage, per se, influenced collagen turnover. Regional differences in ^{14}C content across the joint surface showed that cartilage collagen located centrally on the joint surface is formed several years earlier than collagen located peripherally. The collagen matrix of human articular cartilage is an essentially permanent structure that has no significant turnover in adults, even with the occurrence of disease.

INTRODUCTION

Articular cartilage comprises a collagen matrix consisting mainly of type II collagen, and this matrix provides tensile strength to cartilage and restrains the swelling pressure created by the hydrophilic proteoglycans that fill the spaces of the matrix (*1*). Cartilage represents a major clinical challenge owing to its poor regenerative capacity after injury and the high prevalence of osteoarthritis (OA) (*2*). The understanding of the pathogenesis is limited, and even today, it is unclear to what extent cartilage tissue is replaced during life in human joints, due to lack of direct measurements. Indirect attempts to estimate tissue turnover of cartilage have suggested that turnover of collagen is low, whereas that of proteoglycans (mainly aggrecan) is proposed to be somewhat higher (*3*–*8*). These turnover estimates are largely based on measurements of the time-related accumulation of D-aspartate (D-Asp) or advanced glycation end products (AGEs) (*4*–*6*). However, the accuracy of these approaches is limited because time is not the only factor influencing the rate of D-Asp and AGE accumulation (*4*, *9*–*11*). The effect of OA on cartilage tissue turnover has mainly been studied by measuring the incorporation of radioactive tracers in cartilage in vivo in animals and ex vivo in humans. With this approach, it has been observed that OA leads to a general increase in cell activity and protein synthesis (including collagen) (*12*–*16*). Thus, the current indirect knowledge suggests that collagen turnover is slow but that OA will increase collagen synthesis because of disease-initiated activation of chondrocytes. It is questionable whether this increase in protein synthesis leads to any actual renewal of the load-bearing collagen matrix.

Here, our aims were to accurately determine the life-long cartilage collagen turnover in healthy and OA tissue and, secondarily, to estimate the turnover rate of glycosaminoglycans (GAGs; main component of aggrecan) by the carbon-14 (^{14}C) bomb pulse method. This method has been applied to several tissues (*17*–*22*) and has been used to demonstrate an almost negligible life-long tissue renewal in, for instance, the eye lens and tooth enamel (*19*, *21*) and a high renewal rate in tissues like fat and skeletal muscle (*17*, *18*). The method takes advantage of the marked increase in atmospheric ^{14}C levels peaking in 1963 due to the testing of nuclear bombs in the 1950s and 1960s, and the subsequent exponential decline after the testing of nuclear bombs was banned (*23*). This marked change of the atmospheric ^{14}C is called the "^{14}C bomb pulse." Because all living organisms incorporate ^{14}C from the atmospheric CO_2 (via plants and animals in the diet), equilibrium exists between levels of ^{14}C in the atmosphere and in the organism. Tissues that are constantly renewed (high turnover) will contain amounts of ^{14}C close to the current atmospheric level of ^{14}C, whereas tissues with very slow turnover will retain ^{14}C from the years close to the formation of the tissue (*17*, *21*, *24*). In a very early study right at the time of the ^{14}C peak in the atmosphere, Libby *et al.* published data on two individuals, indicating very slow tissue turnover in cartilage in old age (70+ years) (*3*).

Here, we found that the human cartilage collagen matrix is essentially permanent in adulthood and that this is not influenced by the presence of cartilage disease or by differences in mechanical loading of the cartilage. Thus, major tissue damage or osteoarthritic disease is unlikely to result in any repair of major collagen matrix structures and regeneration of the cartilage tissue. Furthermore, it implies that OA research should focus on mechanisms behind the mechanical disruption of the cartilage, rather than on factors adjusting collagen turnover.

RESULTS

Tissue damage and composition in healthy and osteoarthritic human cartilage

Human healthy and OA joint cartilages were sampled from tibial plateaus obtained during knee joint replacement surgeries performed because of either OA or malignant bone tumors (healthy cartilage and no involvement of the tibia in the disease) and, in one case, from a university body donation program. To assess the effect of OA, as well

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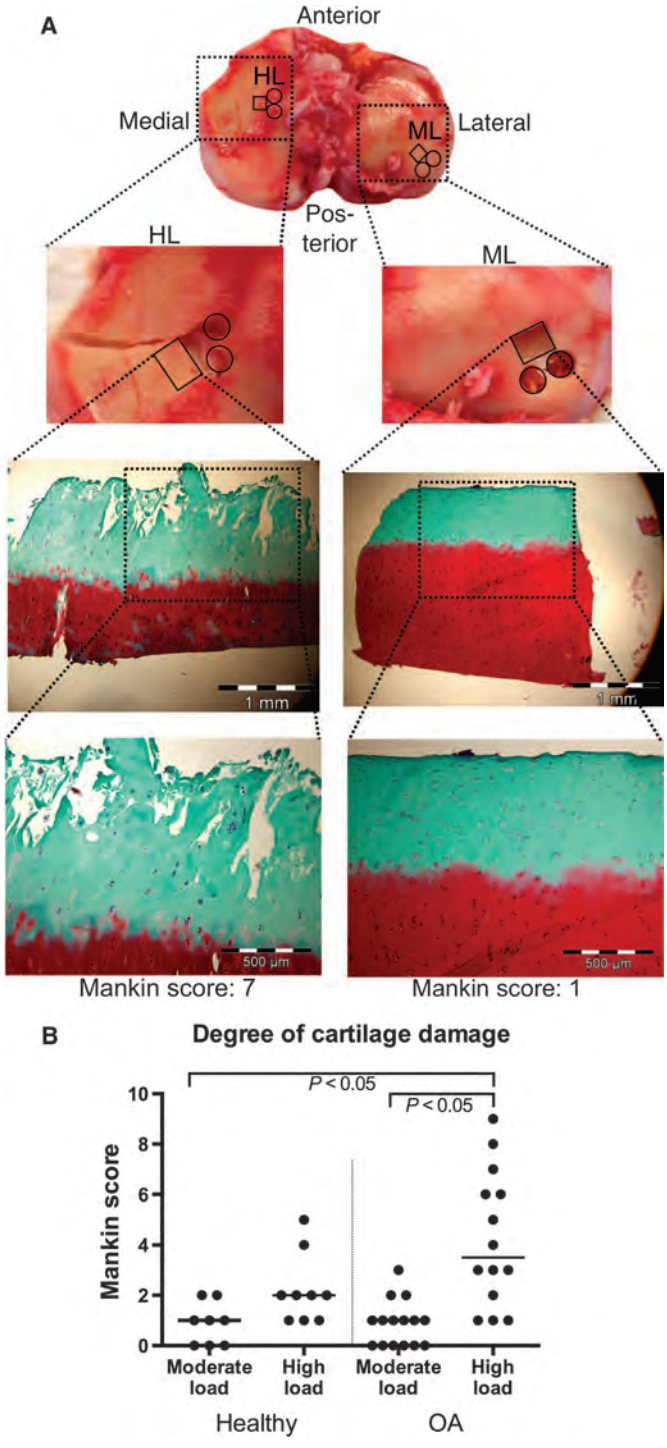

Fig. 1. Sampling and assessment of damage in differentially loaded regions of healthy and OA cartilage. (**A**) Example of sampling locations on the tibial plateau of subject 12. Cartilage was sampled from a moderately loaded (ML) area (peripherally on the lateral condyle) and from a highly loaded (HL) area (centrally on the medial condyle). Two punch biopsies (circles) were obtained from each area, and a square sample for histology (black squares) was cut out adjacent to the biopsies. Cryosections, stained with Safranin-O and Fast Green, were used for assessment of tissue damage using a modified version of the Mankin score (ranging from a minimum of 0 to a maximum of 11). Scores of 7 and 1 were given for HL and ML, respectively, for this subject. Cartilage from punch biopsies was used for measurements of tissue composition, ^{14}C levels, and stable isotope levels. Values for ^{14}C, stable isotopes, and Mankin score for these exact samples can be found in table S1 (subject 12). (**B**) Cartilage damage scores shown as single observations for all subjects within the groups of healthy and OA cartilage from moderately and highly loaded areas (n = 8 to 15). Horizontal lines indicate median values. P values were determined by Kruskal-Wallis one-way analyses of variance (ANOVAs) on ranks.

as the effect of mechanical loading, we analyzed the cartilage sampled from highly and moderately loaded areas of both healthy and OA cartilage (Fig. 1A). Tissues were stained with Safranin-O and Fast Green to assess tissue damage, based on structure, cellularity, and loss of GAGs (Fig. 1A). The level of tissue damage according to the Mankin score (*25*) was significantly greater in the highly loaded OA tissue compared to both moderately loaded healthy and moderately loaded OA tissues (Fig. 1B and table S1). A relatively large variation in the degree of tissue damage was observed in highly loaded OA cartilage, with several samples showing low damage scores (<4). This variation is presumably related to the fact that areas of very severe damage with great loss of cartilage could not be chosen for sampling, because this would make it difficult to obtain sufficient amounts of tissue for the planned analyses.

The cartilage samples contained concentrations of collagen, GAGs, and water content within the expected ranges (Fig. 2) (*26*). Cartilage from highly loaded areas (middle of condyle) contained higher GAG and lower collagen levels compared to tissue taken from the moderately loaded areas (closer to the edge of the plateau) (Fig. 2, A and B). Consequently, the GAG/collagen ratio was significantly greater in tissue from highly loaded areas compared to those from moderately loaded areas (Fig. 2C). No significant differences in tissue composition (Fig. 2, A and B), DNA content (Fig. 2D), or water content (Fig. 2E) were found between healthy and OA cartilage.

The main goal of the investigation was to identify the turnover rate of the collagen matrix of cartilage. Therefore, a collagen purification procedure was performed to remove GAGs and other noncollageneous substances from the cartilage. The collagen concentration was increased by the purification procedure (Fig. 2A), and the GAG content was reduced by about 90% (Fig. 2B). Furthermore, the atomic carbon/nitrogen (C/N) ratio decreased from 3.78 ± 0.06 in raw cartilage to 3.49 ± 0.03 (means ± SEM; n = 12) after purification ($P < 0.01$, t test for paired samples). This drop in C/N ratio serves as further confirmation of GAG removal because GAGs have a high C/N ratio owing to their high carbohydrate and low protein content (table S1).

A small difference in the collagen content between highly and moderately loaded tissues remained after purification (Fig. 2A), and the concentration of remaining GAGs was slightly but significantly higher in the OA compared to healthy cartilage (Fig. 2B), which was also reflected in the GAG/collagen ratio (Fig. 2C).

No turnover of collagen after maturity in healthy and OA cartilage

The ^{14}C data give an immediate indication of very slow collagen turnover because a clear imprint of the bomb pulse was retained in the samples of purified cartilage collagen (Fig. 3A). The measured levels of ^{14}C were comparable to the elevated atmospheric levels occurring up to at least 40 years before sampling (Fig. 3A and table S1). In cartilages

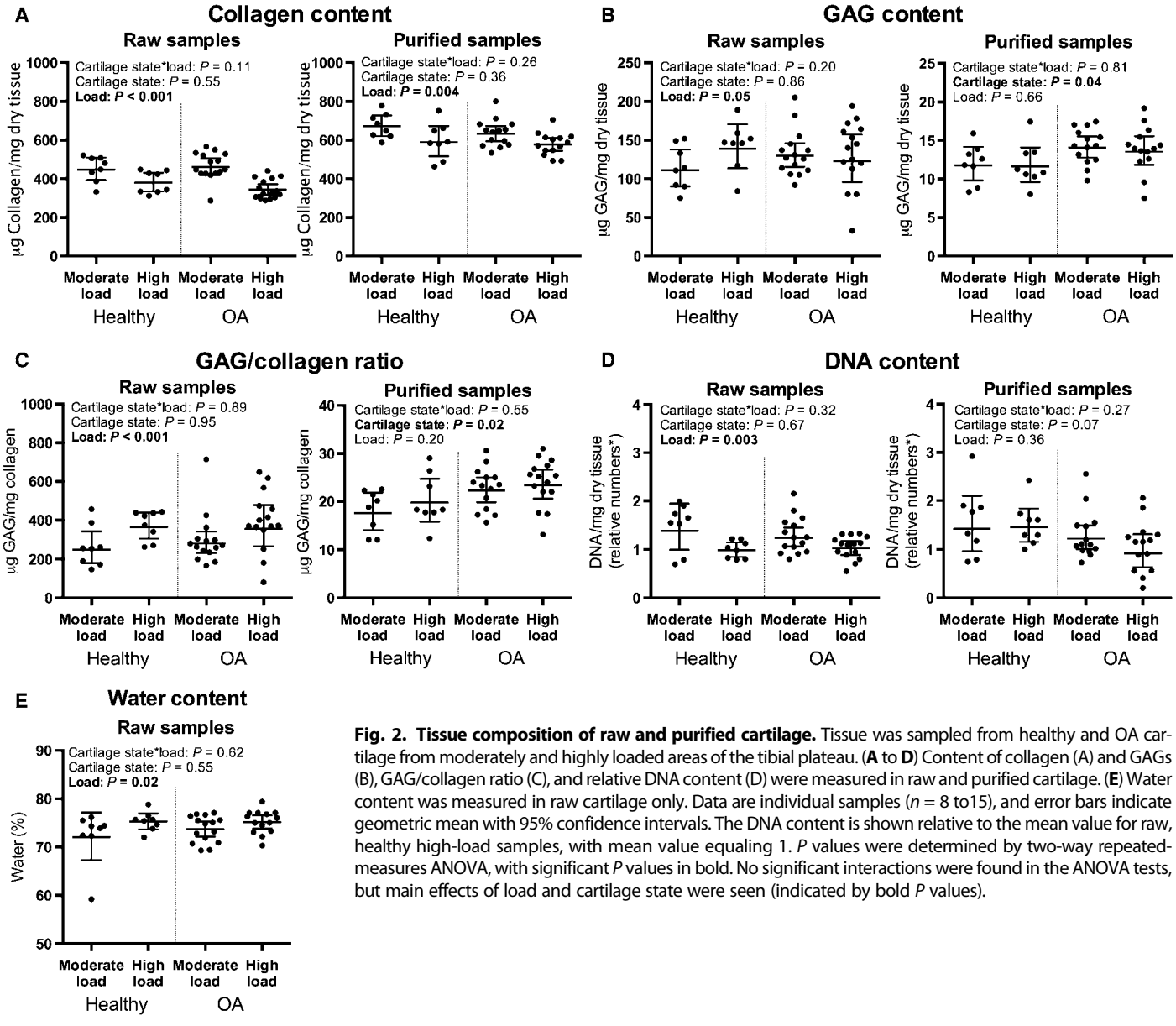

Fig. 2. Tissue composition of raw and purified cartilage. Tissue was sampled from healthy and OA cartilage from moderately and highly loaded areas of the tibial plateau. (**A** to **D**) Content of collagen (A) and GAGs (B), GAG/collagen ratio (C), and relative DNA content (D) were measured in raw and purified cartilage. (**E**) Water content was measured in raw cartilage only. Data are individual samples (n = 8 to15), and error bars indicate geometric mean with 95% confidence intervals. The DNA content is shown relative to the mean value for raw, healthy high-load samples, with mean value equaling 1. P values were determined by two-way repeated-measures ANOVA, with significant P values in bold. No significant interactions were found in the ANOVA tests, but main effects of load and cartilage state were seen (indicated by bold P values).

from persons born after the bomb pulse peak in 1964 (where a good time resolution can be obtained because of the high ^{14}C starting point and subsequent gradual decrease with time), we found levels of ^{14}C corresponding to atmospheric levels present at 11 years (median) after birth in highly loaded (centrally located) samples and 13 years (median) after birth in moderately loaded (peripherally located) samples (Fig. 3A and table S1). Similarly, samples from donors born close to the beginning of the ^{14}C rise (births from 1948 to 1955) had ^{14}C levels corresponding to atmospheric levels present at 8 and 11 years (median) after birth in highly loaded/centrally located and moderately loaded/peripherally located samples, respectively (Fig. 3A and table S1). These data indicate that a major part of the collagen matrix formation takes place before maturity.

Cartilage collagen from the oldest donor, born in 1935, had maintained low, prebomb levels of ^{14}C, indicating that very little of the bomb pulse ^{14}C rise was integrated into the collagen and, therefore, that no significant renewal of the collagen matrix had taken place after the age of 20 years in this person. Similarly, cartilage collagen from the donor born in 1944 had limited incorporation of bomb pulse ^{14}C (Fig. 3A). It should be mentioned that the levels of ^{14}C found in the two oldest donors could, in principle, also occur if the entire collagen matrix of their cartilage was newly synthesized, because atmospheric ^{14}C levels at the time of sampling (2012–2014) were close to prebomb pulse levels (present until 1955). However, considering the high ^{14}C levels preserved in cartilage from the donors born close to the bomb pulse peak, this is an unlikely explanation. In combination, the observations of ^{14}C levels in cartilage from donors within a large range of birth years give clear evidence that collagen formation/renewal is very limited in adults.

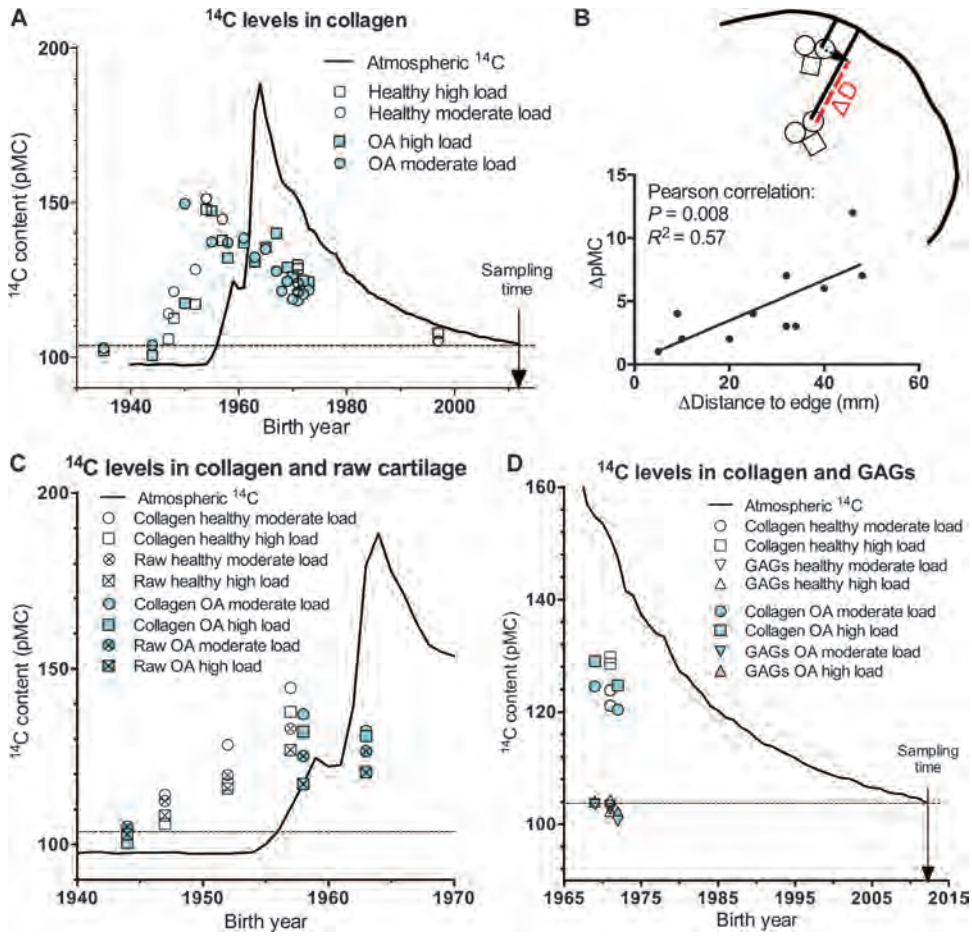

Fig. 3. ^{14}C content in healthy and OA cartilage. (**A** to **D**) The ^{14}C bomb pulse curve shows the chronological atmospheric concentration of ^{14}C (black line) shown as percent modern carbon (pMC) based on data up to 2001 in (*33*) and from 2002 in (*34*) (A, C, and D). In (C) and (D), shorter time spans of the bomb pulse curve are shown. The horizontal dashed line indicates the approximate atmospheric ^{14}C level at the period of tissue sampling (November 2012 to January 2014). (A) ^{14}C concentrations in purified collagen from paired samples of moderately and highly loaded cartilage sampled from both healthy and OA tibial plateaus (n = 23). The trend of the data points mirrors the bomb pulse ~10 years after birth. Vertically aligned symbols represent data from the same individual (except for the data points in 1971 that represent two healthy and one OA donors). (B) Schematic illustration of the measurement of Δdistance to the edge of the joint surface (curved black line) between central and peripheral samples. The shortest distance between sample site and joint edge was always measured. (See also Fig. 1 for explanation of sample dissection.) The graph shows the difference in ^{14}C content (ΔpMC) between the centrally and the peripherally located samples plotted versus their difference in distance to the edge of the joint surface (Δdistance) (n = 11). Only data for individuals born after the peak (1964) are shown. P value was determined by Pearson linear regression. (C) ^{14}C concentrations in intact cartilage ("raw") compared to that in purified cartilage collagen ("collagen") from which GAGs had been removed. Data are shown for paired samples of moderately and highly loaded cartilage, sampled from both healthy and OA tibial plateaus (n = 6). Vertically aligned symbols represent data from the same individual. (D) ^{14}C concentrations in GAG fractions isolated during the collagen purification procedure. Data are shown for paired samples of moderately and highly loaded cartilage from two healthy and two OA donors. The ^{14}C content in purified collagen from the same persons are shown for comparison (n = 4). Vertically aligned symbols in years 1969 and 1972 represent data from one individual in each year, whereas the 1971 data represent two donors.

When comparing ^{14}C levels in collagen from OA and healthy cartilage, we observed no distinct difference in the pattern of ^{14}C in relation to birth year (Fig. 3A and table S1). If OA had led to a substantial renewal of the collagen matrix, the OA samples would appear "younger"—in other words, have ^{14}C levels lower than healthy samples owing to incorporation of the modern, low levels of ^{14}C. Furthermore, there was no indication that the highly loaded (more damaged) tissue from healthy or OA cartilage had experienced higher levels of collagen turnover. For all donors born after 1964 (living during a continuous decline in atmospheric ^{14}C), the highly loaded samples had higher ^{14}C levels than the moderately loaded ones, indicating either slower turnover of highly loaded regions or an earlier developmental formation of the collagen in the damaged/highly loaded cartilage compared to the moderately loaded cartilage (discussed below). In further support of unchanged collagen turnover in OA cartilage, the OA samples from the two oldest donors born in 1935 and 1944 (Mankin scores of 5 and 6, respectively) maintained low levels of ^{14}C, indicating that no new collagen has been added to the collagen matrix for decades despite OA. In table S1, corresponding values of cartilage damage (Mankin score), donor age, and ^{14}C levels are shown for each sample.

Radial cartilage formation during growth

The reason for sampling at the central and peripheral parts of the joint surface was originally to obtain, respectively, damaged (highly loaded) and nondamaged (moderately loaded) tissues (Fig. 1) to investigate whether load/damage directly affects tissue turnover. This sampling strategy has, by chance, added an extra dimension to the findings of this study, because the ^{14}C data reveal that the collagen from the central part of the tibial condyle is formed at an earlier time point compared to the collagen located at the periphery (Fig. 3A), indicating radial cartilage growth during skeletal maturation. This is shown by the observation that in persons born before 1964, all but one sample taken from the central part of the condyle (squares in Fig. 3A) had a lower content of ^{14}C compared to the samples taken from the peripheral part of the condyle (circles in Fig. 3A). In persons born after 1964, this pattern is reversed, and samples from the central part (high load) had the highest ^{14}C content (Fig. 3A). This indicates that the cartilage of the central part was formed first, as less of the ^{14}C rise was added to this tissue in the persons born before 1964, whereas more of the ^{14}C peak remained in this tissue in persons born after 1964 (tissue formed early while ^{14}C was high). The difference in the timing of collagen formation in the central and peripheral cartilage becomes clear when looking only at individuals born after 1964. In these persons, a large distance between the centrally and peripherally located samples (Δdistance) coincided with a large difference in ^{14}C (ΔpMC) level, and without exception, the higher ^{14}C level was in the central rather than peripheral sample, indicating earlier formation (Fig. 3B).

GAGs have markedly faster turnover than collagen

We measured the ^{14}C content in paired samples of raw cartilage and purified collagen from both highly and moderately loaded areas in six persons. The raw samples generally contained lower levels of ^{14}C compared to the corresponding samples of purified collagen, suggesting that the GAGs (present in the raw samples only) contributed with a low ^{14}C level (Fig. 3C). This effect was not discernible in the samples with early birth years (1944 and 1947), where the collagen fraction in itself had a very low ^{14}C level (in other words, close to prebomb levels). A generally low ^{14}C concentration in the GAG fraction would support the idea of constant and relatively fast turnover of these molecules, with constant incorporation of the present-day low atmospheric ^{14}C levels.

To further test this hypothesis, we measured the ^{14}C level in a purified fraction of GAGs extracted from cartilage from four persons born between 1969 and 1972. A high GAG content in these extracts was confirmed by the very high C/N ratio found in the material [7.32 ± 0.62 (mean ± SEM); $n = 8$] (table S1). The ^{14}C levels in the GAG fractions were all close to the atmospheric level at the time of sampling (2012–2014), whereas the ^{14}C levels in the matching collagen fractions had retained ^{14}C levels corresponding to atmospheric levels present during the 1980s (Fig. 3D). These differences in ^{14}C levels confirm a continuous, fast turnover of the GAGs combined with a negligible collagen turnover.

Loss of tissue mass during extraction

The purification procedure led to a loss of mass from the cartilage samples, and the percentage loss was significantly higher for the samples taken from highly loaded areas (Fig. 4A). The loss of mass correlated with the amount of collagen found in the supernatants from the purification procedure (Fig. 4B), but only 20% of the lost mass can be explained by collagen loss (Fig. 4C). Despite the difference in loss of mass from the moderately and highly loaded samples, the C/N ratios of the extracted collagen samples were similar [3.53 ± 0.04 and 3.57 ± 0.06 (mean ± SEM); $n = 21$] for moderate and high loads, respectively ($P = 0.6$, paired t test), indicating that the composition of the extracted tissue from moderately and highly loaded areas was similar (table S1). Thus, there is no indication of a systematically greater loss of certain cartilage molecules from the highly loaded samples. The greater loss may be explained by the fact that the highly loaded cartilage was generally more damaged and fragile, and therefore, small tissue fragments were more easily lost in the extraction procedure.

DISCUSSION

Our study reveals that the collagen matrix of human cartilage is an essentially permanent structure with no significant replacement in adult life and that the occurrence of disease, such as OA, does not increase collagen turnover. Conversely, GAGs, which intersperse the permanent collagen matrix, have a fast rate of renewal. Additionally, our data suggest that collagen located in the central part of the cartilage tibial plateau is formed at an earlier time in life compared to the cartilage located peripherally, giving evidence for radial growth of cartilage on the joint surface during skeletal growth. These observations solve an important question in cartilage research, because previous studies, based on relatively indirect measurements of acute collagen synthesis, suggested an increased collagen turnover in OA cartilage (*12*–*14*, *16*). The ^{14}C bomb pulse method gave us a unique opportunity to answer these questions with good precision, without such limitations. The method was introduced in 1964, where two samples of cartilage collagen showed no reflection of the atmospheric ^{14}C rise, suggesting that no addition of new collagen had occurred (*3*). Here, in a more comprehensive study that included both moderately loaded (nondamaged) and highly loaded (damaged) articular cartilage from both healthy and OA knee joints of persons ranging in age from 16 to 78 years, we demonstrate that integration of new collagen stops around the age of skeletal maturity and that ^{14}C levels in cartilage collagen corresponded to the atmospheric level present at 8 to 13 years of age, reflecting a mass-weighted average of the ^{14}C levels accumulated during the growth of the original collagen structure.

The ^{14}C data here show that neither disease (OA) nor load/damage affects the inertia of the collagen matrix in any detectable way. The observation that OA does not initiate renewal of the collagen matrix is in apparent conflict with several previous studies that indicate an increase in collagen synthesis in OA compared to healthy cartilage (*12*–*14*, *16*). Most of these studies are based on the assessment of short-term collagen synthesis ex vivo (human tissue) or in vivo (animals), by incorporation of radioactive tracers (*13*, *14*, *16*) and/or formation of collagen II propeptide (*12*, *16*). However, our ^{14}C data may be compatible with ex vivo collagen synthesis data, assuming that OA leads to an activation of chondrocytes to produce new collagen molecules that are never incorporated into the collagen matrix. This would result in a measurable increase in the collagen synthesis but not in exchange with collagen molecules in the more permanent collagen matrix (that is, the fraction of insoluble collagen that was analyzed for ^{14}C concentration in this study). However, in one study (*14*), an increase in cartilage collagen synthesis, in response to experimental OA in dogs, was detected by in vivo incorporation of [^{3}H]proline into a cartilage collagen fraction that is presumably similar to the fraction analyzed for ^{14}C in the present study. If species difference does not explain this discrepancy, these data can only be reconciled with the present ^{14}C data if a small fraction

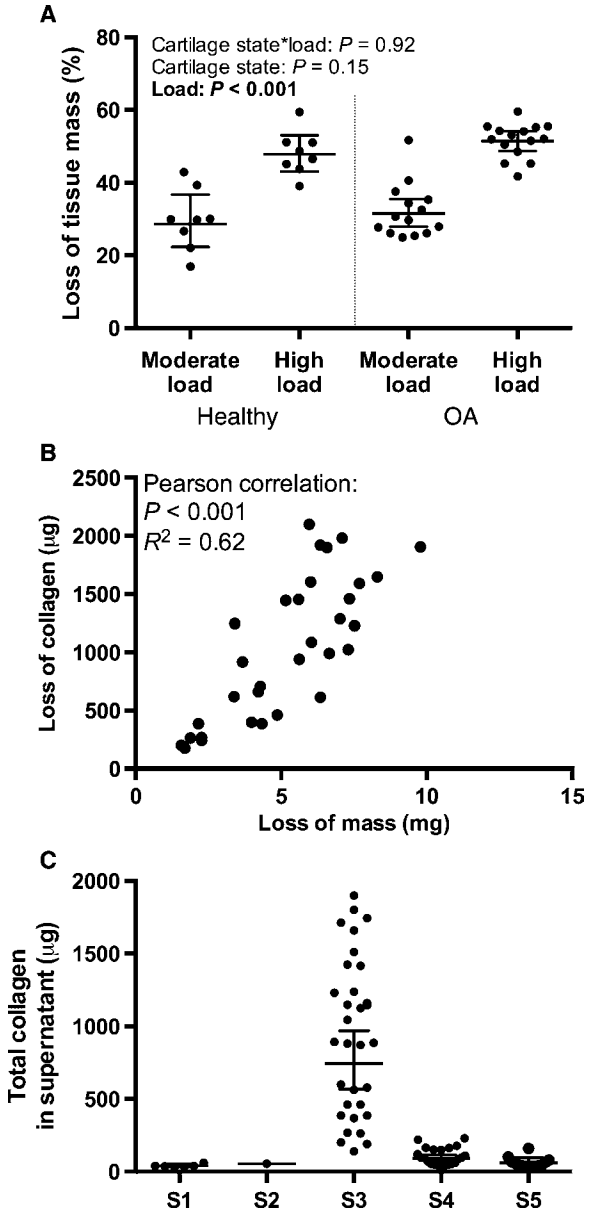

Fig. 4. Loss of tissue mass during collagen purification. (**A**) Percent loss of tissue dry mass during the collagen purification procedure in moderately and highly loaded cartilage sampled from healthy and OA tibial plateaus. Data are individual samples (n = 8 to 15), and error bars indicate geometric mean with 95% confidence intervals. P values were determined by two-way repeated-measures ANOVA, with the significant P value in bold text. (**B**) Correlation between loss of tissue dry mass and loss of collagen to the supernatants of the purification procedure. Data are individual samples (n = 32). P value was determined by Pearson linear regression. (**C**) Loss of collagen during extraction procedures to supernatants 1 to 5 (S1 to S5). S1, supernatant after hyaluronidase digestion; S2, supernatant after saltwater wash; S3, supernatant after trypsin digestion; S4, supernatant after phosphate-buffered saline (PBS) wash; S5, supernatant after KCl wash. Data are individual samples (n = 32), and error bars indicate geometric mean with 95% confidence intervals.

(5 to 10%) of the insoluble collagen matrix has a more dynamic nature. This is possible because a small fraction with higher turnover would not dilute the ^{14}C levels enough to abolish the remnants of the bomb pulse ^{14}C that are present in the cartilage samples analyzed in the present study [a similar theory was previously discussed for human tendon collagen (*17*)].

Our data revealed that damage does not affect cartilage turnover rates to any detectable degree. Instead, the location on the joint surface correlated with the age of the cartilage collagen, with collagen from the central areas being older than collagen from peripheral parts. This corresponds well with studies on bone, which show that lateral growth of the tibial plateau happens by addition of new bone to the periphery of the plateau (*27*, *28*). The relatively large differences in ^{14}C levels seen between some pairs of central and peripheral samples indicate that several years had passed between formation of the central cartilage and the peripheral cartilage, suggesting that the cartilage collagen in one location is formed over a relatively short period of time and that relatively little happens to the collagen matrix after it has been laid down. This phenomenon can also explain the large spread in ^{14}C values in samples from donors with a birth age compatible with skeletal growth during the rapid atmospheric ^{14}C rise that happened from 1955 to 1964. The ^{14}C levels in the cartilage from these donors would be expected to vary markedly depending on the exact location on the joint surface, owing to the extensive change in atmospheric ^{14}C during this period. Two important conclusions can be made from the observations in systematic regional differences in cartilage ^{14}C content. First, the age of the collagen is more dependent on the position on the joint surface than on the degree of damage, which provides good evidence that cartilage damage has no major effect on collagen turnover. Second, the fact that the large regional difference in collagen ^{14}C content across the joint surface can be maintained for decades serves as evidence that, at least, a large part of the collagen matrix has negligible turnover after its initial formation.

The proteoglycan fraction of articular cartilage is assumed to be more dynamic than the collagen matrix, based on Asp racemization and AGE accumulation studies (*6*, *8*). A dynamic GAG turnover is further supported by several studies in dogs, where moderate exercise and immobilization led to increased and decreased GAG content, respectively [reviewed in (*29*)]. Our ^{14}C data on both raw cartilage and purified GAGs confirm the dynamic nature of the GAG fraction and suggest that cartilage GAGs are essentially new, because their ^{14}C content was very close to the atmospheric levels at the time of tissue sampling. On the basis of AGE accumulation rates, it has been suggested that OA leads to an increased turnover rate of GAGs (*6*). However, with the ^{14}C bomb pulse method, it would not be feasible to detect differences in turnover rates in the range of a few years. Thus, we can only conclude that GAG turnover is very fast compared to collagen turnover, but we cannot detect potential differences in GAG turnover between healthy and OA with this method. Because the collagen matrix is inert and the GAGs are dynamic, the regulation of the mechanical properties of cartilage is most likely based on fluctuations in levels of GAGs. In addition, because the collagen matrix is finalized in youth, the conditions during this period of life, such as nutritional state and mechanical loading, will presumably determine the quality of the cartilage for the remaining lifetime. In relation to this, it should be mentioned that the present data do not rule out that damage occurring to the cartilage before maturity can be repaired.

We intended to provide a model for formation and turnover of cartilage collagen during human maturation and aging, as has been done for human Achilles tendon and eye lens crystallins (*17*, *21*). However, the

regional variation in collagen age across the joint surface adds an extra, unexpected dimension to this "model," and therefore, a more elaborate tissue sampling, with focus on the exact anatomical regions, is necessary in future studies to provide such a turnover model. Another limitation is that it was not possible to measure the age of the collagen that was lost during the purification procedure (because it was mixed with trypsin that contains modern carbon). This trypsin-digestible fraction may have contained newly synthesized collagen that is not incorporated/cross-linked into the stable collagen network, and although this collagen is unlikely to provide any significant mechanical strength to the tissue, it would have been interesting to know its age. Last, although we were successful in removing the GAGs from the cartilage (by purification), the collagen fraction was not pure collagen but likely contained other matrix proteins and cell debris. The ^{14}C data show that this composite material had extremely limited turnover, suggesting that other parts of the matrix, in addition to the collagen matrix, are also relatively inert.

The findings of this study support the view that a structurally permanent collagen matrix exists in human adult cartilage. Understanding tissue turnover is a first step in determining therapies for connective tissue disorders. The present findings do not demonstrate any direct pathogenesis of OA and indicate that OA is not associated with any increased turnover of collagen matrix. Instead, OA is most likely linked to mechanical or biochemical breakdown of major cartilage structures with no new collagen matrix formation. The findings are in line with the relatively poor clinical success in cartilage disease treatment attempting to transplant cartilage into diseased joints regions or to stimulate collagen matrix formation either pharmacologically or with stem cells [reviewed in (*30*)]. The findings do not exclude that treatments of OA, like physical training, can stimulate the production of proteoglycans (GAG's), and thus the water content of the cartilage or the release of potential chondroprotective substances, such as interleukins (*31*). The major focus in research on OA and cartilage repair should be on avoiding the development of cartilage disease and, once present, on counteracting further tissue damage by designing therapeutics that protect the remaining cartilage collagen structures and that target other molecules in the cartilage, such as GAGs, that have a high turnover and can beneficially modify the mechanical properties of the remaining cartilage.

MATERIALS AND METHODS

Study design

The primary aim of the study was to determine the turnover of the collagen matrix in cartilage with the use of the ^{14}C bomb pulse method. We hypothesized that the turnover of collagen would be very low in healthy adults, whereas OA would lead to increased turnover. On the basis of previous experience with the ^{14}C bomb pulse method (*17*) and pilot studies, we included a total of 15 OA patients with birth years distributed over the years before and after the ^{14}C bomb pulse peak. Tibial plateaus from these patients were obtained between November 2012 and January 2014 during knee arthroplasty operations due to primary OA at the Department of Orthopaedic Surgery, Bispebjerg-Frederiksberg Hospital, Copenhagen. In the same period, we included a total of eight persons (number was limited due to availability) without diagnosed OA as healthy controls. There were seven persons undergoing major bone resection [distal femur (n = 6) or total femur (n = 1)] and insertion of a mega-prosthesis due to primary malignant bone tumors (n = 5) or bone metastases (n = 2) (none had involvement of the tibia) at the Musculoskeletal Tumor Section, Department of Orthopaedic Surgery, Rigshospitalet, Copenhagen, Denmark, and one person through the body donation program of the Faculty of Health and Medical Sciences, University of Copenhagen. Ethical approval for obtaining waste tissue from knee arthroplasty and osteosarcoma surgery was obtained from the Ethical Committee of the Capital Region of Denmark (H-4-2012-131), and all persons gave informed written consent.

Cartilage samples

Samples were obtained in the form of whole tibial plateaus including the underlying bone. The samples were stored at −80°C until dissection. From the tibial plateaus, pairs of two full-depth punch biopsies (including underlying bone) (4- to 5-mm diameter) were taken both from a highly loaded part (located centrally on the medial or lateral condyle) and from a moderately loaded part (peripherally on the plateau) (Fig. 1A). From one tibial plateau, it was only possible to sample from the peripheral part of the plateau because insufficient amounts of cartilage remained in the central areas (because of OA) (subject 18; see table S1B). Cartilage was separated from the underlying bone by cutting as close as possible to the bone (<0.5 mm) with a scalpel. Cartilage biopsies intended for collagen purification (described below) were additionally cut in thin slices with a scalpel. All biopsy samples were then weighed, freeze-dried, weighed again, and kept at −80°C for later analyses.

For histology, square full-depth samples (not including the bone) were cut out with a scalpel right next to the two punch biopsies at both locations (Fig. 1A). These were embedded in Tissue-Tek (Sakura Finetek Europe), frozen by immersion in isopentane, precooled by liquid nitrogen, and stored at −80°C until sectioning. Methods for histology are described in the Supplementary Methods.

Collagen purification

The main goal of the investigation was to identify the turnover rate of the collagen matrix of cartilage by using the ^{14}C bomb pulse method. Therefore, we performed a collagen purification procedure to remove GAGs and other noncollagenous substances from tissue samples obtained from both highly and moderately highly loaded areas (Fig. 1). This was done with a modified version of the protocol used in (*32*) (modifications to the original protocol were suggested by D. R. Eyre). Samples (precut in thin slices during dissection) were subjected to overnight treatment with hyaluronidase (H3506, Sigma) [5 U/ml in 0.05 M sodium acetate and 1.15 M NaCl (pH 6)] at 37°C. After washing with isotonic salt water, trypsin (T8802, Sigma) (1 mg/ml in PBS) was added, and samples were incubated at 37°C overnight. To ensure complete removal of trypsin (to avoid contamination with modern carbon), samples were then washed for 1 hour with 0.7 M KCl and further washed three times with distilled water (complete removal of trypsin was confirmed by SDS–polyacrylamide gel electrophoresis in pilot studies). One-milliliter volumes were used in all steps of the extraction procedures, and supernatants from the extraction procedure were saved for later analyses. After the extraction procedure, the samples were again freeze-dried, weighed, and kept at −80°C for later analyses.

Isolation of GAGs for ^{14}C measurement

The secondary aim was to measure ^{14}C in purified GAGs in a subgroup of subjects. To isolate sufficient amounts of GAG, large samples of full-depth cartilage (165- to 255-mg wet weight) from two OA and two healthy tibial plateaus were cut out and subjected to the collagen

purification as described previously. The supernatant available after the hyaluronidase treatment, containing the major fraction of GAGs, was freeze-dried to isolate the GAG fraction. In addition to GAGs, this fraction contains 0.05 M sodium acetate and a very small amount of hyaluronidase (0.01 mg/ml), both containing "modern" carbon (carbon with a ^{14}C level corresponding to the currently low level in the atmosphere) and could potentially dilute the ^{14}C content of the GAG fraction. To make the acetate evaporate, 1 ml of 0.05 M HCl was added to the freeze-dried samples, and the samples were freeze-dried again. The small amount of hyaluronidase (0.01 mg) was considered insignificant. This freeze-dried material was used for isotope analyses (see the Supplementary Methods).

Hydroxyproline, GAG, and DNA measurement

Samples of both raw cartilage and purified collagen were digested with papain (P3125, Sigma) [papain (0.125 mg/ml) in 100 mM sodium phosphate buffer, 10 mM Na_2EDTA, and 10 mM L-cysteine (pH 6.5)] at 60°C overnight (*26*). This papain digest was used for analyses of hydroxyproline, GAG, and DNA content, as described in the Supplementary Methods.

Isotope analyses

For all subjects, samples of purified cartilage collagen from both highly and moderately loaded areas of the tibial plateau were analyzed for ^{14}C and stable isotopes (^{13}C and ^{15}N). Furthermore, from a subgroup of subjects, raw (nonpurified) cartilage samples (subjects 1, 3, 5, 10, 13, and 15), as well as isolated GAG fractions (subjects 6, 7, 19, and 22), were analyzed for ^{14}C and stable isotopes. These analyses are described in the Supplementary Methods.

Statistical analyses

A Kruskal-Wallis one-way ANOVA on ranks was used to test differences in Mankin scores. The data for Mankin scores are shown as individual data points with median values indicated. To approach normal distribution data, collagen, GAG, and DNA content as well as the GAG/collagen ratio were log-transformed before statistical analyses. A two-way repeated-measures ANOVA was performed on cartilage state (healthy versus OA) and load (moderate load versus high load) for collagen, GAG, and DNA content, as well as percent water content and percent loss of mass in extraction procedure. Only the main effects of cartilage state (healthy versus OA) and load (moderate load versus high load) are shown, because no significant interactions were found in the ANOVA tests. The relationship between loss of tissue mass and collagen content in supernatants from the collagen purification procedure was tested with Pearson linear regression. Pearson linear regression was also done to test the relationship between Δdistance (to edge of plateau) and ΔpMC and for highly loaded versus moderately loaded samples (within subjects). Differences were considered significant when $P < 0.05$. All statistical analyses were performed with SigmaPlot (version 12.5).

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/8/346/346ra90/DC1
Methods
Table S1A. ^{14}C concentration, stable isotope data, and Mankin scores for all healthy tissue samples.
Table S1B. ^{14}C concentration, stable isotope data, and Mankin scores for all OA tissue samples.
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Radiocarbon dating reveals minimal collagen turnover in both healthy and osteoarthritic human cartilage

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Editor's Summary

Cartilage claims a permament home

It has long been debated for many tissues in our bodies whether they are permanent or constantly refreshed as we go through life. Nuclear bomb testing in the 1950s and 1960s released a large amount of the carbon-14 isotope into the atmosphere, giving researchers the ability to determine the age and turnover of human tissues, ranging from the heart to the brain to, now, the cartilage. Heinemeier and colleagues used this so-called " ^{14}C bomb pulse" method to date cartilage from 23 individuals ranging from 18 to 76 years of age. They examined cartilage from knee joints, taking samples from both highly and moderately loaded areas, in both healthy individuals and those with osteoarthritis. The authors discovered that the collagen matrix of human cartilage is essentially permanent, with no major replacement even with disease. This finding has important implications for the tissue engineering and regenerative medicine fields, where the structural permanence of collagen will need to be contemplated when designing new cartilage repair strategies.