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Effect of age on pyridinoline and pentosidine matrix cross-links in the desert sand rat intervertebral disc

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Spondylosis in the desert sand rat (*Psammomys obesus*) has been studied as a model for intervertebral disc degeneration. Reducing sugars, which react with protein amino groups to form a diverse group of moieties with fluorescence and cross-linking properties, have been implicated in the structural and functional alterations of proteins that occur during aging and long-term diabetes. This study was undertaken to determine the changes in two matrix cross-links of the intervertebral disc and to study their association with aging. Two types of cross-links were studied: the physiological cross-link, pyridinoline, which is initiated by lysyl oxidase; and the non-enzymatically initiated cross-link, pentosidine. A significant increase in pentosidine, but not pyridinoline, was observed in the intervertebral disc with aging. Radiological, histological and biochemical findings support a hypothesis that subchondral bone responses, marked by increased bone density, contribute to alterations in the intervertebral disc. Cross-link changes in the structural proteins of the disc may contribute to the progressive fibrocartilage degradation typical of intervertebral disc disease as an effect of age.

Keywords: collagen, intervertebral disc degeneration, pentosidine, pyridinoline

Introduction

Intervertebral disc degeneration is a leading cause of musculoskeletal disability in humans [1]. Changes associated with disc aging and degeneration are characterized by intervertebral cartilage thinning, fissuring, decreased water content, alterations in collagen morphology and marked pigmentary changes. Collagen cross-links are crucial for the maintenance of collagen integrity. Several known cross-links are formed through non-enzymatic glycosylation, a series of covalent reactions that result in addition of sugar residues to the lysine side-chains of long-lived extracellular proteins. These Maillard or browning reactions result in brown fluorescent products and are proposed to cross-link proteins, and thus have been implicated in changes in collagen occurring with age [2]. Indeed, age-related changes in disc coloration are observed, from natural white color during early age, to yellow by middle age, and to a definite brown by old age [3, 4]. It seems likely that much of this coloration is due to the ensuing products of non-enzymatic glycosylation in collagen [3, 5].

This present investigation was initiated to characterize the association of morphologic and biochemical changes related to intervertebral disc disease in the desert sand rat (*Psammomys obesus*). This animal, indigenous to the eastern Mediterranean region, develops degenerative changes of the intervertebral disc [6]. In this study, changes in two types of cross-links in the inter-

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vertebral disc matrix were correlated with radiologic and histologic findings, as functions of aging.

Materials and methods

Desert sand rats (*P. obesus*) were obtained from the breeding colony raised at Case Western Reserve University. Animals were housed under controlled conditions, including an ambient temperature of 26°C and a 12 h light and 12 h dark photoperiod. Six groups of animals were studied at time periods 3, 6, 12, 18 and 24 months, six animals per group.

Roentgenographic analysis
Roentgenographic studies were performed as previously described [6] on six animals from each age group, sedated with ketamine (1.2 mg/100 g) and xylosine (2.16 mg/100 g) administered intraperitoneally. Radiographs were assessed separately by three observers in a blinded fashion using the following grading criteria. Since a majority of the degenerative changes occur in the lumbar interspace areas, grading was based on this area of the spine. Disc space narrowing was graded as: grade 0 = normal; grade 1 = possibly abnormal (changes are minimal but differ clearly from normal); grade 2 = definite disc space narrowing, 25–75% narrowing observed; grade 3 = disc space narrowing as in grade 2, with additional osteophyte lipping, ligamentous calcification and/or subchondral sclerosis. Agreement among observers within 1 unit of the scale occurred in 95% of specimen readings. The average of three observer readings was used as the grading severity score for each animal.

Blood was collected through the orbital sinus before sacrifice, and plasma concentrations of glucose were measured in duplicate by the enzymatic hexokinase method (Sigma, St Louis, MO, USA) on specimens of each age group; the same animals were used for study of the intervertebral discs.

Histopathologic analyses. Animals were sacrificed by asphyxiation in carbon dioxide. Spinal columns were removed and then further dissected free of paraspinal soft tissues. For histopathologic studies, entire lumbar vertebral columns were collected, decalcified and stained with hematoxylin and eosin, and Safranin-O with fast green as a counter stain.

Cross-link estimation
For pyridinoline and pentosidine estimations, six lumbar discs were collected by carefully scraping each disc from its vertebrae. Skin was collected and scraped to remove outer hair coat and inner fat layer. Tendons from tails were obtained by detaching the skin and removing the distinctively shiny tendon fibrils. All three tissues (disc, skin and tail tendons) were processed for lipid extraction by treatment with a chloroform–methanol mixture (2:1). The tissues were then further processed for proteoglycan extraction with 4 M guanidine hydrochloride, followed by hydrolysis in 6 N HCl at 110°C for 24 h. The acid was evaporated using a Savant Speed Vac drier (Savant Instruments, Framingdale, NY, USA). All samples were reconstituted in 1.0 ml of water and filtered through 0.6 μm nylon filters (Ranin, Woburn, MA, USA).

Collagen estimation. Collagen was calculated by assaying for hydroxyproline by the method of Stegemann & Stadler [7]. The amount of collagen was determined assuming a content of 14% hydroxyproline by weight [8].

Pyridinoline estimation. Pyridinoline was quantified in acid-hydrolyzed disc cartilage samples by reverse phase high-performance liquid chromatography (HPLC) as described by Eyre *et al.* [9]. Acid-hydrolyzed samples were injected into a high-performance liquid chromatograph (Waters Chromatography Divisions, Millipore, Milford, MA, USA) equipped with a 0.46 × 25 cm Vydac C-18, 10 μm reverse phase column and an on-line fluorescence detector set at 297 nm excitation and 385 nm emission wavelengths. Pyridinoline eluted at 13.2 min (Figure 1B) and was based on a standard kindly provided by Dr D. Fujimoto (Tokyo University of Agriculture, Japan) by application of a linear gradient from 10 to 17% acetonitrile in 0.01 M *n*-heptafluorobutyric acid from 0 to 27 min. Molarity was calculated with same molar absorption coefficient at 297 nm. A standard was included with every run. Lumbar discs from each of six animals were used separately for cross-link estimation on the 3-, 6-, 12- and 18-month time points. Owing to degenerative loss of disc material on the 24-month time point, discs from six sand rats were pooled for the cross-link estimation.

Pentosidine estimation. Under similar HPLC conditions, pentosidine was eluted at 28.4 min with

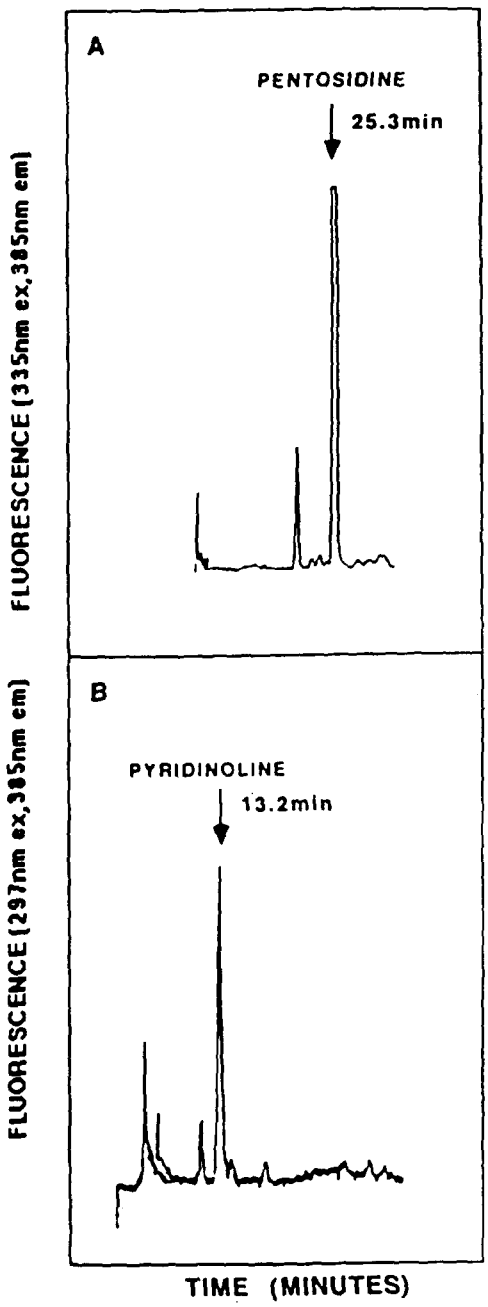

Figure 1. Reverse phase HPLC profile of fluorescence of 12-month-old sand rat intervertebral disc. (**A**) Pentosidine was eluted from a C-18 column and reinjected into a cation-exchange column, and fluoresence detected at 385 nm emission with excitation at 335 nm. (**B**) Pyridinoline was eluted from a C-18 column and detected at 297 nm excitation and 385 nm emission.

the fluorescence flow detector set at 335 nm excitation and 385 nm emission wavelengths. Pentosidine-containing fractions were collected using a Waters automated column-switching valve set to collect fractions eluting from 2 min prior to and 2 min post elution of standard pentosidine. The fractions (5 ml) were concentrated under reduced pressure with the Savant speedvac system, and then rehydrated with loading buffer for cation-exchange chromatography.

Ion-exchange chromatography was done with the same HPLC apparatus as used for the reverse phase chromatography. A cation-exchange column (Protein Pak SP-5PW, Waters, Millipore) was used. The fractions collected from the C_{18} column were injected into the cation-exchange column by the application of a concave gradient of 0–0.06 M NaCl over a period of 40 min in 0.02 M sodium acetate buffer at pH 4.7. Under these conditions pentosidine eluted at 25.3 min (Figure 1A). Quantitation was done by comparison of peak area of samples to that of the standard.

Total pyridinoline and pentosidine were estimated without fractionating cartilage samples; pyridinoline and pentosidine results were expressed as nmol per mg of collagen and pmol per mg of collagen respectively.

Statistical analysis

Data were expressed as mean ± SD and statistical significance ($P < 0.05$) was calculated by Student's *t*-test using Stat View Software. Data were assessed by age (all lumber discs included per animal), and by upper and lower levels for separate assessment of combined upper (L1–2, L2–3, L3–4) and combined lower (L5–6, L6–7) lumbar levels.

Results

Roentgenographic findings

The extent of roentgenographically defined degeneration was different at various regions of the spine; for example, degeneration at level 6–7 was greater than that at level 1–2. Disc spaces were normal, or slightly narrowed at times, in 3-month-old animals. The frequency and severity of degenerative changes increased sequentially thereafter with aging (Table 1).

Gross and histologic findings

Gross pathological disc findings at 3–6 months were characterized by a light-yellow fibrocartilaginous appearance and the cartilages were easily removable on dissection. Discs of 12-, 18- and 24-month-old animals were darker and were more difficult to dissect cleanly.

At 3 months of age, all vertebral bodies were fully formed. The general histologic architecture at

Table 1. Changes in disc degeneration and glucose levels with age

	Age (months)				
	3	6	12	18	24
X-ray (grade of degeneration)	1 ± 0.5	1.7 ± 0.5	1.7 ± 0.4	1.5 ± 0.6	2.25 ± 0.3
Glucose (mg/dl)	169 ± 25	160 ± 61	105 ± 16.5	122 ± 47	124 ± 39

Number of animals = 6.

all ages showed a central nucleus pulposus composed of physaliform cells in a loose matrix. The nucleus pulposus was surrounded by variable amounts of hyaline cartilage with a linear arcade-like arrangement of chondrocytes. Fibrocartilaginous tissue constituting the annulus fibrosus surrounded the nucleus pulposus. In most of the 24-month-old animals, a tongue-like protrusion of the nucleus pulposus was seen posteriorly that indented slightly into the annulus.

Biochemical changes

In this study, as previously reported [6], blood glucose levels tended to be higher in younger animals (ages 3–6 months), although the differences did not reach statistical significance (Table 1). Levels of pentosidine in disc, tail tendons and skin were at their lowest (< 1 pmol/mg collagen) in 3-month-old animals; the levels in each tissue were not significantly different from each other. However, disc pentosidine levels increased markedly with age, from 0.68 to 4 pmol per mg of collagen, whereas pentosidine levels in skin and tail tendons remained unchanged (Figure 2A). Pyridinoline levels in 3-month-old animals, on the other hand, averaged 2.5 nmol per mg of collagen in discs, but were less than 0.5 nmol per mg of collagen in tail tendon (Figure 2B). No significant change in pyridinoline content in tail tendons and disc was observed with age. Pyridinoline was not detected in skin.

No significant differences in pentosidine or pyridinoline were noted when disc concentrations were compared by site of study in lower or upper lumbar regions.

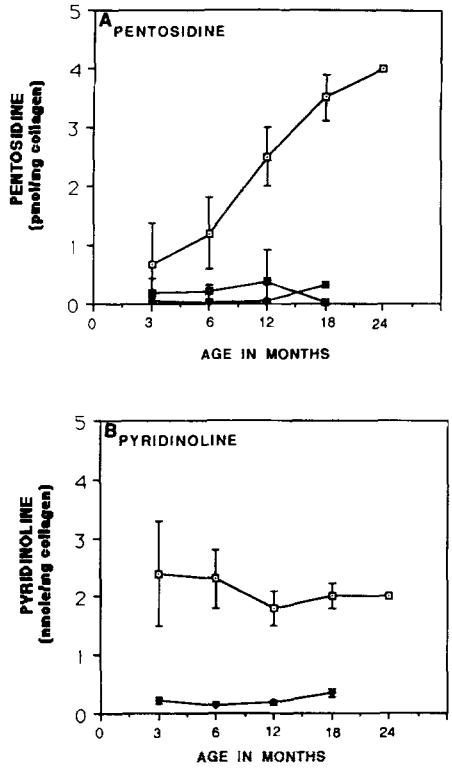

Figure 2. Effect of age on (**A**) pentosidine and (**B**) pyridinoline cross-links in the intervertebral discs, tail tendons and skin of sand rats. Pyridinoline was absent in skin. □, disc; ◆, tail tendon; ■, skin.

Discussion and conclusions

The purpose of our investigation was to study biochemical changes in matrix cross-links in the intervertebral disc of the desert sand rat as a function of aging, and to correlate these changes with radiologic and histologic findings. There was a significant progression of degenerative changes seen on radiographic study as the animals aged from 3 to 24 months; in contrast, minimal changes in disc histopathology were observed over the evaluation period. In a similar study by Silberberg *et al.* [10], distinctive degenerative changes were seen on histopathologic study in the desert sand rat only in animals aged 30 months or more, an age range greater than that studied in our series.

Of the biochemical changes in the discs studied, only levels of the non-enzymatically initiated cross-link, pentosidine, increased significantly with age.

Pyridinoline, the physiological collagen cross-link known to contribute collagen fiber stability, did not vary with age in disc tissue. Similarly, no changes in pyridinoline levels have been observed in rabbit articular hyaline cartilage with aging [11]. Pyridinoline content in tail tendons remained constant. Our pentosidine and pyridinoline results are consistent with those of Uchiyama *et al.* [4]. In our previous studies, the precise location of pentosidine, whether collagenous or non-collagenous, remained unclear [11]. Nevertheless, since pentosidine represents a marker for the overall Maillard reaction, the results of our studies support a role for Maillard reaction products in aging of extracellular matrix.

The increased levels of pentosidine in discs but not in tail tendon or skin was of interest; a similar increase in pentosidine was described in articular hyaline cartilage [11] and in human connective tissue [12] with age. The lack of sustained blood glucose elevation over the 24-month period in animals studied in the present investigation suggests that the progressive increase in pentosidine was due not to diabetes but solely to the consequences of aging.

The observation that the pentosidine concentration increased over time in the disc, but not in tendon or skin, supports a possible relationship between the advanced Maillard reaction, as represented by pentosidine, and the intervertebral disc degenerative process. However, a coincidental parallel increase in pentosidine and disc degeneration cannot be excluded. One possibility is that the lack of vascularity of the disc would contribute to a low turnover of disc proteins and accumulation of the precursors of the advanced glycosylated end products, resulting in increased pentosidine levels [13]. In contrast, higher turnover of tendon and skin collagen matrix would limit pentosidine accumulation.

No differences in pentosidine levels were observed among upper and lower lumbar levels, indicating similar responses to aging at all disc sites studied. The failure to demonstrate differences in pentosidine concentrations at different disc levels and lack of correlation with disc degeneration does not preclude a relationship between non-enzymatic cross-links and initiation of degeneration. In particular, the prominence of degenerative changes at the lower lumbar level may represent an interplay between mechanical stresses at that level and predisposing Maillard-related biochemical disc changes [14]. Sustained stresses interacting with proteins structurally modified by advanced Maillard products may eventually lead to fibrocartilage disc degeneration.

The parallel findings of intervertebral disc degeneration and an age-related increase in Maillard/advanced glycosylation end products (AGE) raise the question of whether the two are associated. Vlassara *et al.* [14] demonstrated that macrophage phagocytic uptake of proteins modified by the advanced Maillard reaction leads to release of the cytokines interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α). Activation of chondrocytes and osteoclasts by this mechanism has the potential to lead to age-related destruction of the intervertebral disc. In support of this concept, we have observed activation of rabbit articular chondrocytes to secrete IL-1β upon stimulation with bovine serum albumin-AGE (unpublished data). Further studies will be needed to define the proposed cause and effect relationship.

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