Age-related changes of scleral hydration and sulfated glycosaminoglycans

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Abstract

Human scleras were analyzed to determine if tissue hydration changed in an age-related fashion, and whether this change could be related to decreased levels of glycosaminoglycans. The anterior half of normal human scleras were examined with donor ages ranging from newborn to 99 years ($n = 40$). Tissue hydration was found to decrease by 1.06% per decade ($P = 0.0012$). Glycosaminoglycans were extracted from scleras with guanidine hydrochloride, purified by anion exchange chromatography and quantitated using dimethylene blue and selective enzymatic digestion. The glycosaminoglycans of the sclera were primarily dermatan sulfate and chondroitin sulfate with small quantities of hyaluronic acid. While no loss of chondroitin sulfate with age was detected, a significant age-related loss of dermatan sulfate was found ($P = 0.0006$, $n = 14$). An examination of the relationship between glycosaminoglycan concentration and scleral hydration showed that increased levels of sulfated glycosaminoglycans were associated with increased levels of scleral hydration ($P < 0.02$, $n = 14$). No improvement in the correlation could be achieved by considering either dermatan sulfate or chondroitin sulfate individually. Our results suggest that, as in other connective tissues, aging is associated with a loss of glycosaminoglycans, and their loss is coincident with decreased tissue hydration.

1. Introduction

The age-related changes that occur in the composition and character of soft connective tissues are well-documented in the literature. While many of these changes

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are tissue-specific, others occur in a wide variety of tissues. Elastin fibers undergo morphologic and compositional changes with age [1,2]. There is a general increase in collagen content and the extent to which it is cross-linked [3,4]. In contrast, the glycosaminoglycan (GAG) content decreases in many tissues with age [5]. Little work has been done to relate these age-related changes to the properties of the connective tissues. The observed loss of GAGs is likely to influence tissue hydration as GAGs are highly hydrated [6]. While some evidence suggests a marked loss in extracellular and extravascular water with age [3,7], other studies [8] suggest only a minor decrease. In cartilage, however, hydration appears to increase with age [9].

To date, only limited analyses of biochemical changes in the human sclera with age have been conducted. Hydration of the sclera, defined as weight percent water, has been measured and determined to be 68% [10]. Previous analyses of scleral GAGs have been limited to quantifying hexosamines or uronic acid [11]. In this paper, we demonstrate that scleral hydration decreases with age. The decrease in hydration is accompanied by a decrease in total sulfated glycosaminoglycans, specifically dermatan sulfate.

2. Materials and methods

2.1. Materials

Whole human eyes were obtained from the New England Eye Bank or the National Disease Research Interchange (Philadelphia, PA). Guanidine HCl was obtained from Gibco BRL (Gaithersburg, MD). Dimethylmethylene blue and benzamidine hydrochloride hydrate was purchased from Aldrich Chemical Company (Milwaukee, WI). Q-Sepharose (fast flow), sodium acetate, heparinase III, chondroitinases AC Flavo and ABC, keratan sulfate and heparan sulfate were obtained from Sigma Chemical Company (St. Louis, MO). Chondroitin sulfates A, B and C (super special grade standards), and keratanase II were purchased from Seikagaku America (MD). Endo-beta-galactosidase was purchased from Boehringer Mannheim (Indianapolis, IN). AG 501-XB mixed ion exchange resin was purchased from Bio-Rad (Richmond, CA). The hyaluronic acid kit was purchased from Pharmacia Diagnostics (Uppsala, Sweden). Metaphore agarose was purchased from FMC (Vallensbaek Strand, Denmark)

2.2. Tissue preparation

Analyses were carried out on the anterior portion of the scleras. Fat and muscle tissue were trimmed from the outside of the eyeball. Samples were dissected in a ring around the cornea from the limbus to the four rectus muscle insertions. After blotting, the wet weight of the tissue was recorded. Samples were lyophilized and the dry weight measured. Tissue hydration was defined as the weight percent of water in the sample.

2.3. Dimethylmethylene blue colorimetric assay

Total sulfated GAGs were quantitated colorimetrically using the dimethylmethylene blue (DMB) assay [12]. Briefly, samples were mixed with the DMB reagent

(1:25) and absorbance (525 nm) was immediately read. Concentrations were determined from standard curves of highly purified specific GAGs.

2.4. Hexosamine analysis

Equivalent amounts (3 mg) of dry scleral tissue were hydrolyzed in 6 N HCl for 6 h in vacuo. Glucosamine and galactosamine determination was carried out with a Beckman 6300 amino acid analyzer. The dried hydrolyzate was resuspended in a sodium citrate buffer (pH 4.5) and eluted through a cation exchange column. Standards were used to establish retention times and to quantify the hexosamines.

2.5. GAG extraction

Tissue samples were homogenized using a Polytron (Brinkman Inst., Inc., Westbury, NY) in de-ionized water, lyophilized and stored at −70°C until extraction. The dried samples were extracted overnight at 4°C (in an excess of 100 v/w) in a solution of 4 M guanidine HCl containing 0.05 M acetate pH 6.0 and the following protease inhibitors: 0.1 M ε-aminocaproic acid, 1 mM benzamidine HCl, 1 mM phenylmethylsulfonylfluoride, 10 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM N-ethylmaleimide [13]. The extracts were centrifuged for 30 min at $4 \times 10^4 \times g$ and the pellet was reextracted until GAG was no longer detected in the supernatant by DMB analysis. The extracts were pooled and dialyzed exhaustively against de-ionized water, lyophilized and resuspended in de-ionized 7 M urea [14].

2.6. Purification

Extracts were loaded on a 2-ml Q-sepharose Fast Flow anion exchange column equilibrated with the urea buffer. The conductivity and absorbance at 280 nm of the column eluent were constantly monitored. The non-bound fractions were collected and analyzed for hyaluronic acid using a [125I]protein radiometric assay (Pharmacia, Sweden). The proteoglycans were eluted using a linear 0–1.5 M NaCl gradient and 1.3-ml fractions were collected. The GAGs eluted as a single peak between 0.50 M and 0.70 M NaCl. The elution profile of the proteoglycans was assessed using DMB. Fractions containing GAG were pooled, concentrated and desalted by dialysis followed by lyophylization.

2.7. Specific GAG determination

Purified GAGs were digested with selective polysaccharide lyases. Digestion conditions were optimized according to time, temperature, and concentration. Specificity studies were performed on each enzyme and highly purified GAG standards. Aliquots of each sample were subjected to digestion for 3 h at 37°C in 70 mM Tris−HCl. The concentration of GAG in the digestion mixture was adjusted to 80 μg/ml (monitored with DMB analysis) and the pH was adjusted to the particular enzyme: chondroitinase ABC (1 U/ml) pH 8.0; chondroitinase AC (0.5 U/ml) pH 7.3; keratanase II; endo-beta-galactosidase (0.1 U/ml) pH 5.9 and heparinase III [10 U/ml] pH 7.0. A nitrous acid degradation procedure was also conducted to measure heparan sulfate content on some samples [15]. The specific GAG content was deter-

mined (using DMB analysis) as the concentration difference between each digest and a control lacking enzyme.

2.8. Agarose gel electrophoresis

Purified proteoglycans were digested for 18 h with papain 10:1 in 0.5 M sodium acetate, 0.1 M potassium phosphate, 0.01 M EDTA and 0.01 M cysteine, pH 6.7 at 64°C for 18 h to release the GAG chains [16]. Electrophoresis of both digested and undigested proteoglycans was conducted using 4% low viscosity agarose (Metaphore agarose, FMC). The gel was fixed for 1 h in 1:9 acetic acid/95% ethanol. Gels were stained with DMB solution for 1 h [12] (DMB concentration was increased 10-fold) and destained with de-ionized H_2O.

2.9. Statistics

Correlations between measured variables (hydration, chondroitin sulfate concentration, dermatan sulfate concentration) and age were performed using standard linear regression and a two-sided t-test. Correlations between two measured variables (each assumed to have a comparable relative error of measurement) were analyzed as a problem in 'errors and variables', and thus a linear regression was performed using a loss function that minimized the sum of the area of the right triangle formed by each data point and the regression line [17]. The P-value was found using the standard error of the line slope, determining a t-value, and performing a two-sided t-test.

3. Results

Scleral hydration (% water in sclera) was found to decrease by 1.06% ($\pm$ 0.3% S.E.) per decade of life ($P = 0.0012$) (Fig. 1). No difference was found between scleral tissue obtained from males and females.

Limited hydrolysates conducted to examine the hexosamine content of the tissues did not reveal any age related change. The average hexosamine concentration was 6.2 $\pm$ 1.1 mg per g dry sclera. Selective enzymatic digestion demonstrated that the major sulfated GAG chains of the sclera were chondroitin sulfate and dermatan sulfate. All enzymes completely digested their respective purified substrate. Chondroitin sulfate was defined as GAGs susceptible to chondroitinase AC. Dermatan sulfate was defined as GAG susceptible to chondroitinase ABC but not chondroitinase AC. Heparan sulfate was determined to be negligible on the basis of two independent degradation procedures utilizing heparinase III and nitrous acid. Likewise, keratan sulfate was detected at negligible levels when samples were enzymatically digested with keratanase and endo-β-galactosidase. After chondroitinase ABC digestion, no sulfated GAGs were detected using the DMB assay.

After pooling, desalting and concentrating, HA was measured. Over 98% of the HA detected was present in the non-binding fractions, at an order of magnitude lower than the other GAGs (mean HA concentration of 41.3 μg/mg dry tissue). The detection limit of the radioimmunoassay was greater than 5 ng/ml.

Total sulfated GAGs (TSGAG) were found to decrease as a function of age:
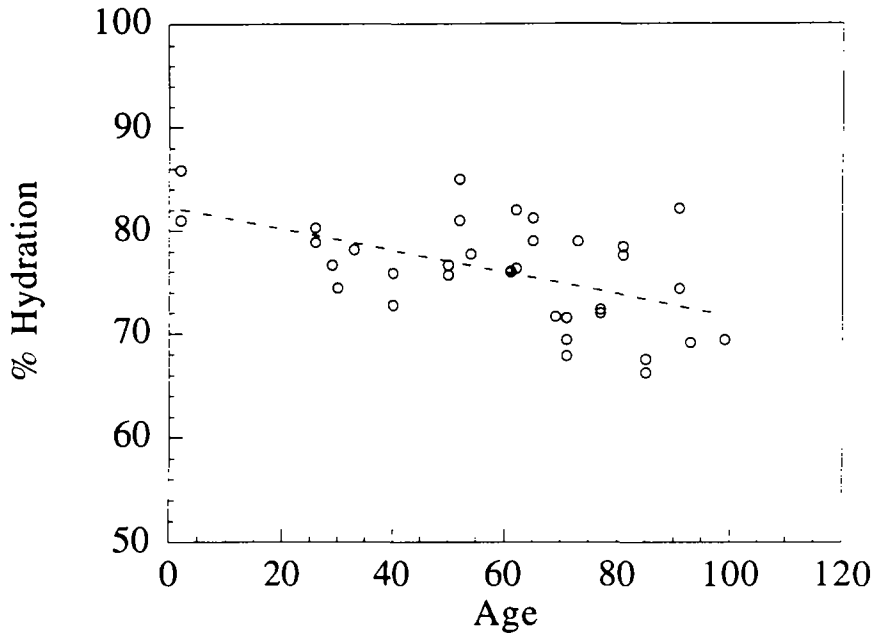
Fig. 1. Percent hydration of the human anterior sclera as a function of age. Line is best linear regression fit to the data: Hyd = 82.3 − 0.106 × age (P = 0.0012).
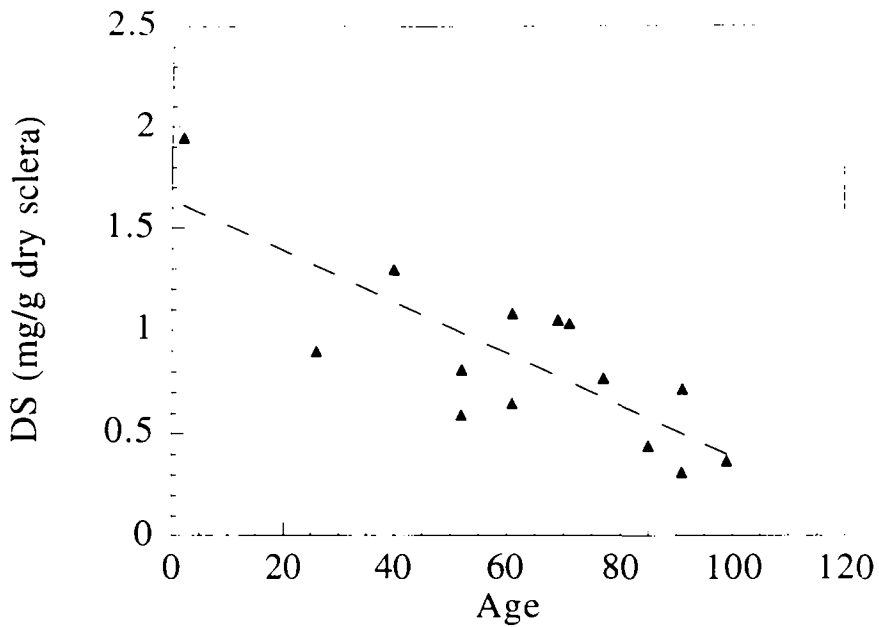

Fig. 2. Dermatan sulfate content of the human anterior sclera as a function of age. Line is best linear regression fit to the data: DS = 1.64 − 0.0125 × age (P = 0.0006).

TSGAG (mg/g dry sclera) = 2.73 − 0.0186 × age (years) (P = 0.025). This decrease was found to be mostly due to a decrease in dermatan sulfate: DS (mg/g dry sclera) = 1.64 − 0.0125 × age (years) (P = 0.0006) (Fig. 2). No age-related decrease in chondroitin sulfate was detected (P > 0.1) (Fig. 3).

Fig. 4 shows scleral hydration as a function of total scleral sulfated GAGs. Hydration was found to vary with TSGAG as: HYD (%) = 66.0 + 6.43 × TSGAG (mg/g dry sclera) (P < 0.02). When comparing hydration against the concentration of either CS or DS, similar relationships were found, each with P < 0.02: HYD (%) = 68.6 + 15.9 × CS (mg/g dry sclera) and HYD (%) = 65.1 + 12.8 × DS (mg/g dry sclera). The relationship between the concentration of dermatan and chondroitin sulfate in each tissue sample was evaluated to determine whether there was a correlation (Spearman correlation test, rho = 0.58) (Fig. 5).

Extracts from samples representative of low and high hydrations were analyzed by gel electrophoresis to determine if the decrease in GAG quantity was accompanied by a decrease in the GAG chain size. Both intact proteoglycans and proteolytically released GAG chains were electrophoresed on 4% low viscosity agarose gels. All samples migrated as diffuse bands indicating heterogeneity of chain size. Little variation in electrophoretic mobility was detected between samples of low and high hydration (Fig. 6). Due to limited quantity, proteoglycans from the sclera with a hydration of 74% (lanes 4 and 10) were loaded at a concentration lower than could be detected with DMB.
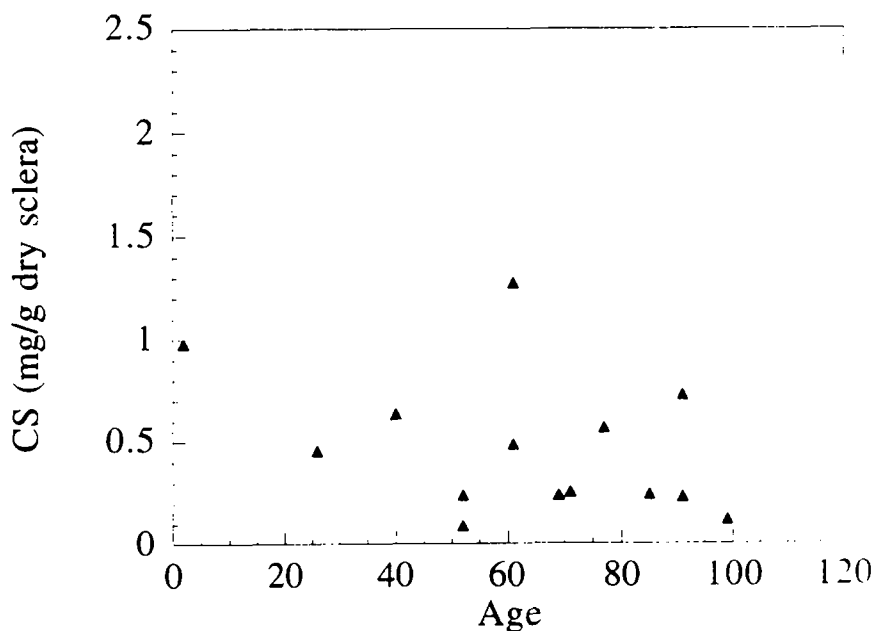

Fig. 3. Chondroitin sulfate content of the human anterior sclera as a function of age.
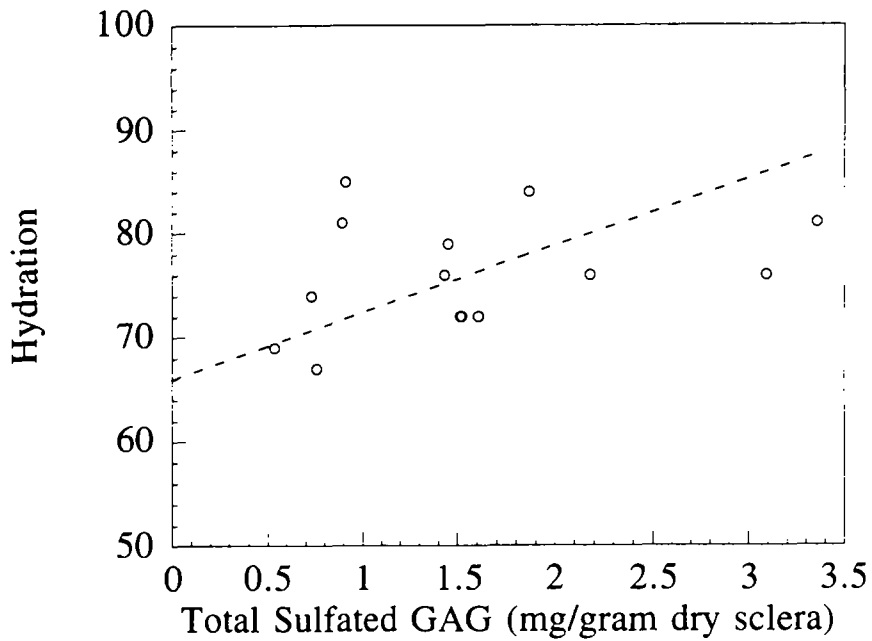
Fig. 4. Percent hydration of the human anterior sclera as a function of the total sulfate glycosaminoglycan content (TSGAG). Line is best linear regression fit to the data using a loss function that assumes the same relative error in both abscissa and ordinate: Hyd = 65.96 + 6.429 × TSG (P < 0.02).
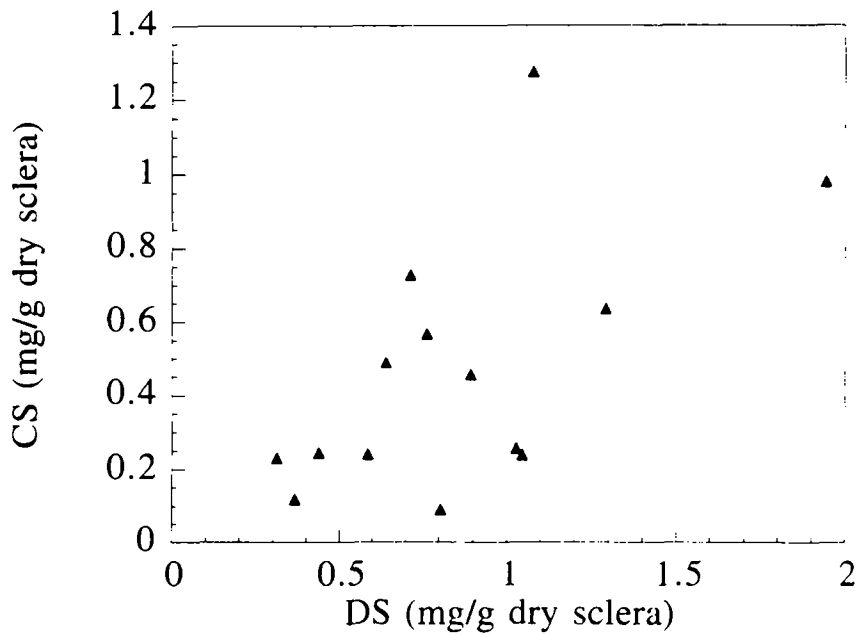

Fig. 5. Relationship between dermatan sulfate and chondroitin sulfate measured in the human sclera.
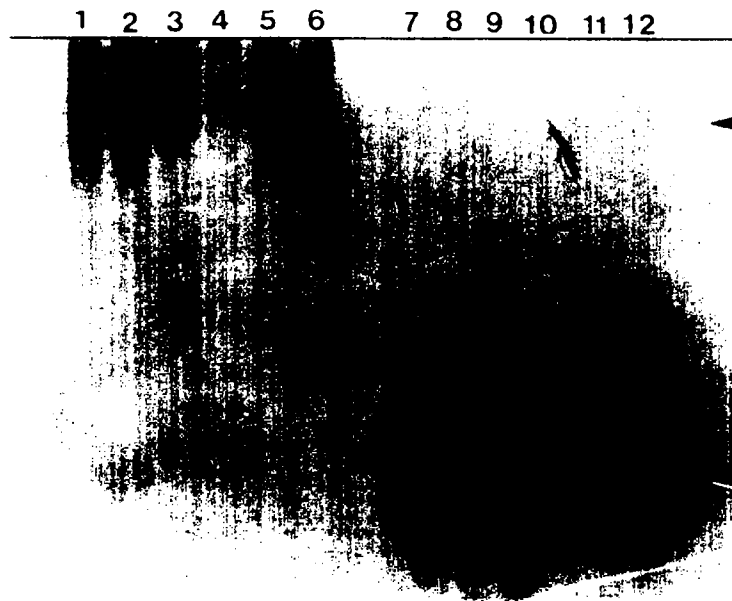
Fig. 6. Agarose gel electrophoresis of intact (lanes 1–6) and papain digested proteoglycans. Samples were from scleras of the following hydrations: 81% (lanes 1 and 6); 76% (lanes 2, 3, 8 and 9); 74% (lanes 4 and 10) and 72% (lanes 5, 6, 11 and 12).

4. Discussion

Substantial changes occur in the extracellular matrix with aging including a general increase in collagen content and the extent to which it is cross-linked [3,4]. The general level of glycosaminoglycans decreases with age in most connective tissues (skin [18], lung [19], intervertebral disk [20,21], aorta [22–24], myocardium [22,23], and trabecular meshwork in the eye [25]); the cornea appears to be unique in that no age-related loss of GAG occurs in this tissue [26]. Since glycosaminoglycans are thought to control tissue hydration, we reasoned that a similar loss of GAG in the sclera might translate into an age-related loss of hydration in the sclera. Our results are consistent with this hypothesis.

We found that human scleral glycosaminoglycans consisted primarily of chondroitin sulfate and dermatan sulfate with negligible amounts of hyaluronic acid and keratan sulfate. These values agree with earlier work in the bovine sclera [27] (Trinkaus-Randall, data not shown). While previous investigators [11] assumed that keratan sulfate was present in the sclera based on levels of glucosamine, we could only detect negligible levels of keratan sulfate with specific enzymatic digestion. Our extractions were from the anterior sclera and did not include muscle insertions or Tenon's capsule. The perilimbal scleral was omitted from the samples analyzed; however in other assays, we examined this region and did not detect keratan sulfate (unpublished data).

While many investigators have demonstrated that there is a tendency for the total concentration of GAG to decrease with age in different connective tissues, there are many tissue specific differences. For example, while the relative fraction of hyaluronic acid in many connective tissues decreases with age (coronary arteries [28], skin [18]), one notable exception is cartilage where the relative fraction of hyaluronic acid increases [29,30]. Along with the loss of hyaluronic acid, skin also shows a loss of dermatan sulfate with age [18]. Cartilage shows a loss of chondrotin sulfate relative to keratan sulfate, a decreased length of the chondroitin sulfate oligosaccharide chains, an increased ratio of chondroitin 6-sulfate (C-6-S) to chondroitin 4-sulfate (C-4-S), and an increased extent of sulfation [30–32]. In the coronary arteries, the relative GAG fraction of C-6-S, C-4-S and dermatan sulfate increased with age while those of hyaluronic acid and heparan sulfate decreased with age [28]. In the glomerular basement membranes, the concentration of total sulfated GAGs decreased with age [33]. We found that in the sclera, there is an age-related loss of dermatan sulfate with little or no loss of chondroitin sulfate (Figs. 2,3). This pattern appears to be distinct from other connective tissues.

A decrease in scleral hydration was associated with the loss of dermatan sulfate in the sclera (Fig. 1). Loss of tissue hydration is one of the hallmarks of aging [3,7]. The present work suggests that a decrease in glycosaminoglycans is at least in part responsible for this loss. To further explore this possibility, we examined the relationship between scleral hydration and the glycosaminoglycan content. While much literature exists on the relationship between tissue osmotic or swelling pressure and the glycosaminoglycan content of that tissue [6], we are not aware of any studies showing a direct relationship between tissue hydration and the tissue glycosaminoglycan content. We found a statistically significant correlation between the total sulfated glycosaminoglycan content of the sclera and its hydration (Fig. 4). We could not find an improved correlation between hydration and either the dermatan or chondroitin sulfate content of the sclera. However, since these two variables were highly correlated (Fig. 5), we cannot conclude that scleral hydration is determined by both GAGs.

Our results on age-related changes in sclera, linking loss of glycosaminoglycans to loss of tissue hydration, are consistent with the hypothesis that one of the hallmarks of aging is a progressive dehydration of the connective tissues. Dehydration likely decreases the permeability and hydraulic conductivity of the interstitium, possibly making transport of nutrients to cells more difficult with aging, and thus may be related to other deleterious effects of aging.

While the average hexosamine concentration was 6.2 ($\pm$ 1.1 S.E.) mg/g dry sclera, the concentration of sulfated GAGs following extraction, column chromatography and enzymatic digestion of DMB positive fractions ranged from 0.54 to 3.36 mg/g dry weight of sclera. Our hexosamine values are within the ranges given previously for adult bovine scleral tissue [34]. While our sulfated values are lower than the early studies in human and bovine sclera, respectively, their values ranged from 0.4–0.9% and were based on hexosamine and uronic acid content derived from limited hydrolysates [11,27]. We feel that the difference in the values depends on the inclusion of glycoproteins and low sulfated glycosaminoglycans in the samples.

The concentration of sulfated GAG in the cornea is approximately 16–19 mg/g

dry cornea [26]. Our finding of a sulfated GAG content in the sclera that is roughly an order-of-magnitude less than this is surprising since these two tissues have nearly the same interstitial hydraulic conductivity (cornea: $0.5-1.2 \times 10^{-12}$ cm^4/s/dyne; sclera: 1.4×10^{-12} cm^4/s/dyne; [35]). Glycosaminoglycans or their associated core proteins are currently hypothesized to be responsible for the bulk of the flow resistance that characterizes connective tissues [35,36]. This now appears an untenable hypothesis given the result of our study. The high concentration of hexosamine content of the sclera (non-GAG associated) suggests that glycoproteins or nonsulfated GAGs may be responsible for much of the scleral hydraulic flow resistance, a possibility first proposed for the cornea by Hedbys [37]. This conclusion should be examined in other non-cartilaginous connective tissues.

Age-related changes in the glycosaminoglycans appear to decrease tissue hydration. Further studies are needed to investigate how other properties of the sclera (transport and structural characteristics) are affected and thus shed more light on the nature of the aging process.

Acknowledgements

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