Short Communication

Marked Increase in Hyaluronan and Dermatan Sulfate in Cancellous Bone Accompanying Progress of Osteoporosis

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Abstract: Glycosaminoglycans are extracellular matrix constituents suggested to function in a variety of cellular and tissue regulations including bone mineralization and demineralization. Since osteoporosis results from a deviation of those remodeling balance, glycosaminoglycans in osteoporotic bones were analyzed. Both cancellous and compact bones contained chondroitin 4-sulfate as a predominant glycosaminoglycan. Hyaluronan and dermatan sulfate were also detected. The content of hyaluronan and dermatan sulfate in cancellous bone was significantly higher in samples of advanced osteoporosis than in less osteoporotic specimens. The results may indicate that the progress of osteoporosis involves a compositional change in cancellous bone-associated glycosaminoglycans.

Keywords: osteoporosis, bone matrix, glycosaminoglycan, hyaluronan, dermatan sulfate

INTRODUCTION

Bone is calcified tissue, comprising mineral and non-mineral (or matrix) materials. The matrix, which is of critical importance in the dynamic remodeling of bone, is composed mainly of collagen and also contains substantial amounts of glycosaminoglycans (GAGs). Hyaluronan (HA) is present in the form of a free sugar chain and the other GAG subspecies are integrated into proteoglycans, sugar chain-bearing proteins. Those macromolecules have been suggested to play roles in a variety of cell and tissue phenomena, including those related to bone regulation: Parathyroid hormone, which regulates bone mineral absorption, exerts an increase in HA synthesis by bone organ culture and osteosarcoma cell culture. Vitamin D deficiency leading to rachitis results in a several-fold increase in bone-associated HA. HA aberrantly increases in the bone and the culture of fibroblasts from patients of osteogenesis imperfecta (OI). In this connection, HA is a strong inhibitor of in vitro hydroxyapatite growth, while an increase in chondroitin 4-sulfate (C4S) coincides development of mineralization in cartilage. It has been reported that C4S enhances mineralization of bone-derived cell culture. Suggestively, a decrease in production of chondroitin sulfate-bearing proteoglycans has been observed with cultured osteoblasts from OI patients. Taken together, it is conceivable that the GAGs in the bone may play a role in the regulation of osteogenesis and bone remodeling.

Little, however, has been addressed to the relationship of bone GAGs with osteoporosis, a disease resulting from an imbalance of the bone remodeling processes. Here we report a pilot analysis on the bone-associated GAGs with subjects from aged and osteoporotic patients. The results clearly show that HA and dermatan sulfate (DS) in cancellous bone increase with progress of osteoporosis. An implication thereof is a pathogenic significance of the alteration of bone-associated GAGs in osteoporosis.

MATERIALS AND METHODS

Clinical materials. Patients (4 men and 8 women; the age ranging from 75 to 92 years) with femoral neck fractures were subjected to the internal fixation using compression hip screw and plate (the surgery excising pieces of the lateral cortex and cancellous bone underneath from the proximal femur). Resulted bone specimens were used in this study. The patients were
examined by roentgenography to determine the grading of osteoporosis according to Singh (categorizing into 6 grades, with grade 6 indicating no mineral loss and grade 1 most advanced osteoporosis). It was found that they all had osteoporosis (2 cases at grade 1; 5 cases at grade 2; 4 cases at grade 3; and 1 case at grade 4). Samples from a 32-year-old normal male (a bone specimen from the lateral femoral condyle excised at open reduction and internal fixation) and from a 12-year-old male with OI (a bone specimen excised at correction osteotomy of the femur) were also analyzed for reference. Three samples from patients of subtrochanter fracture and one from an intertrochanter fracture patient were examined as well (1 case at grade 1 and 2 cases at grade 2). Informed consent was obtained from all the patients.

**Preparation and analysis of GAGs.** Bone specimens were mechanically separated into cancellous bone and compact bone. After being smashed into small pieces samples were washed well in a saline to remove any attached bone marrow constituents. Then they were dried and weighed. To ensure efficient degradation of the tissue, samples were pretreated as follows: incubation in 0.5 N NaOH at 4°C overnight, neutralization, addition of HCl to a final concentration of 0.5 N, and incubation at a room temperature for 3 hours. Thereafter GAGs were purified and analyzed as previously specified in detail. Briefly, the samples were digested with a

![Fig. 1](image)  
**Fig. 1** Bone glycosaminoglycans separated by electrophoresis. **A**: Cu²⁺, electrophoresis with 0.1 M cupric acetate; Ca²⁺, electrophoresis with 0.3 M calcium acetate; H⁺, electrophoresis with 0.1 M sulfuric acid; Ba²⁺, electrophoresis with 0.1 M barium acetate. **B**: electrophoresis with 0.1 M pyridine/0.47 M formic acid. The number for each sample denotes the grading of osteoporosis (1, 2, advanced; 3, 4, intermediate). I canc, grade 1 cancellous bone; I comp, grade 1 compact bone; O, osteogenesis imperfecta specimen; Y, young control specimen. Vertically aligned samples in this figure were from the same patients. S, a mixture of standard GAGs; Ch, chondroitin; C4S, chondroitin 4-sulfate; C6S, chondroitin 6-sulfate; DS, dermatan sulfate; HA, hyaluronan; HP, heparin; HS, heparan sulfate; KS, keratan sulfate; LCS, low sulfated chondroitin sulfates; ori, electrophoretic starting position.
protease, GAG moieties were precipitated with cetylpyridinium chloride, and aliquots of GAG preparations were subjected to 1) carbazole reaction to determine the total hexuronate content and to 2) electrophoresis to determine the relative proportion of each GAG species. After staining of electrophoretic membranes with Alcian blue the amount of GAGs in each spot was quantitated by densitometry. To assess the presence or absence of individual GAG sub-species this study employed electrophoretic analyses with a variety of electrolyte systems which include 0.1 M sulfuric acid, 0.3 M calcium acetate, 0.1 M cupric acetate, 0.1 M barium acetate, and 0.1 M pyridine/0.47 M formic acid. Quantitative data were evaluated by utilizing analysis of variance and nonparametric tests.

RESULTS
The methods in this study achieved apparently complete degradation of bone tissues, since little solid materials remained after protease digestion. Pretreatment with an acid appears effective but might be caustic in particular for N-sulfate-bearing GAG species. Authentic GAG standards identically processed in a control experiment exhibited no change in electrophoretic mobilities suggesting little vulnerability of the GAGs to the treatment.

Purified GAG samples were then analyzed by electrophoresis with a variety of electrolyte systems (Fig. 1A). A cupric acetate system produced electrophoretic patterns suggesting the presence of C4S, DS and HA in the bone GAG preparations whereas little keratan sulfate or chondroitin was observed therein. A calcium acetate system showed chondroitin 6-sulfate lacking and a barium acetate system showed HS and heparin lacking in the preparations. The latter system also suggested the absence of low sulfated chondroitin. Taken together C4S, DS, and HA were found to be main GAGs but the other species accounted for little, if anything, of Table 1. Composition of glycosaminoglycans prepared from osteoporotic bones

<table>
<thead>
<tr>
<th>Sample</th>
<th>GAG species</th>
<th>[1&amp;2]</th>
<th>[3&amp;4]</th>
<th>Ratio</th>
<th>Significance</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancellous bone</td>
<td>Total GAGs</td>
<td>920±358</td>
<td>792±255</td>
<td>1.16</td>
<td>p=0.54 ns</td>
<td>540 520</td>
</tr>
<tr>
<td></td>
<td>C4S</td>
<td>590±160</td>
<td>684±262</td>
<td>0.86</td>
<td>p=0.49 ns</td>
<td>310 460</td>
</tr>
<tr>
<td></td>
<td>DS</td>
<td>153±87</td>
<td>62±19</td>
<td>2.47</td>
<td>p=0.043*(#)</td>
<td>90 40</td>
</tr>
<tr>
<td></td>
<td>HA</td>
<td>156±113</td>
<td>44±22</td>
<td>3.55</td>
<td>p=0.029*(#)</td>
<td>140 20</td>
</tr>
<tr>
<td>Percentage of the total GAGs</td>
<td>C4S</td>
<td>66.4±7.4</td>
<td>85.4±7.3</td>
<td>0.78</td>
<td>p=0.002**</td>
<td>58 88</td>
</tr>
<tr>
<td></td>
<td>DS</td>
<td>15.9±4.7</td>
<td>8.0±2.3</td>
<td>1.99</td>
<td>p=0.010*</td>
<td>16 8</td>
</tr>
<tr>
<td></td>
<td>HA</td>
<td>17.7±5.2</td>
<td>6.8±5.0</td>
<td>2.60</td>
<td>p=0.006**</td>
<td>25 4</td>
</tr>
<tr>
<td>Compact bone</td>
<td>Total GAGs</td>
<td>626±177</td>
<td>694±220</td>
<td>0.90</td>
<td>p=0.57 ns</td>
<td>680 560</td>
</tr>
<tr>
<td></td>
<td>C4S</td>
<td>534±158</td>
<td>646±222</td>
<td>0.83</td>
<td>p=0.34 ns</td>
<td>570 520</td>
</tr>
<tr>
<td></td>
<td>DS</td>
<td>61±66</td>
<td>24±9</td>
<td>2.54</td>
<td>p=0.18 ns(#)</td>
<td>60 30</td>
</tr>
<tr>
<td></td>
<td>HA</td>
<td>30±32</td>
<td>26±15</td>
<td>1.15</td>
<td>p=0.81 ns</td>
<td>50 10</td>
</tr>
<tr>
<td>Percentage of the total GAGs</td>
<td>C4S</td>
<td>85.6±10.4</td>
<td>92.4±5.4</td>
<td>0.93</td>
<td>p=0.24 ns</td>
<td>84 93</td>
</tr>
<tr>
<td></td>
<td>DS</td>
<td>10.1±11.0</td>
<td>3.4±1.1</td>
<td>2.97</td>
<td>p=0.15 ns(#)</td>
<td>9 5</td>
</tr>
<tr>
<td></td>
<td>HA</td>
<td>4.3±2.7</td>
<td>4.2±4.4</td>
<td>1.02</td>
<td>p=0.96 ns</td>
<td>8 3</td>
</tr>
</tbody>
</table>

*, significant; ***, highly significant; ns, not significant; (#), unequal variances.
GAG contents are shown as Mean ± SD for the groups [1&2] (Grade 1 and 2 specimens pooled) and [3&4] (Grade 3 and 4 specimens pooled). The donor ages are 83.4±3.7 years for [1&2] and 79.6±7.1 years for [3&4]. Data for the specimens from an osteogenesis imperfecta patient (OI; 12-year-old male) and a young control donor (YC; 32-year-old male) are shown for reference.
bone GAGs. We therefore performed quantitative analyses by using a pyridine/formic acid system, which is most capable of separating those 3 species.

C4S was the predominant GAG in all the samples but the amounts of HA and DS varied greatly among the specimens (Fig. 1B). This variation is more prominent in cancellous bone. In compact bone the relative proportion of HA or DS was as constantly low as 1-5% in most samples (Table 1). We then analyzed as to what is the factor relating to that change in the bone GAGs. When GAG composition was compared between the groups of intermediate osteoporosis (grade 3 and 4) and advanced osteoporosis (grade 1 and 2), progressing osteoporosis was found to significantly relate to an increase in the amounts of HA and DS (as well as their percentage in the total GAGs) in cancellous bone (Table 1). In addition, a highly significant correlation ($r=0.941; p<0.0001$) was detected between the amounts of HA and DS in cancellous bone (not shown), suggesting a physiological link of their regulations. However, no statistical significance was found with the osteoporosis-related differences in compact bone. As for the other factors, an analysis of correlation was performed for each GAG composition versus the age of the donors, but no combination was found to be significant (estimated probabilities were all 0.35 or greater), although it should be evaluated with subjects from a wider range of age to draw a conclusion. Neither sex-related difference was observed.

The results of the reference samples were suggestive (Table 1): The OI patient-derived bone exhibited a GAG composition similar to that of severely osteoporotic bones, while young patient-derived bone was similar to intermediately osteoporotic bones in their GAG composition. In addition, 3 specimens from the patients of subtrochanter and intertrochanter fractures had a GAG pattern concordant with those of the femoral neck fracture samples (not shown).

**DISCUSSION**

This is the first study having addressed the relationship of bone GAGs with osteoporosis. The results clearly show that HA and DS increase (and the relative proportion of C4S decreases accordingly) with progress of osteoporosis in cancellous bone.

Since the analysis used the whole material remaining after washing and therefore included some gelatinous material associated with the surface of mineralized tissue. The material might be a complex mixture of substances produced by bone-related cells attaching to the bone surface or those absorbed from the surrounding microenvironment. Being at the site of active bone remodeling, however, the material would be adequate to be integrated with the bone matrix in the present research concerning the bone regulatory system, although analyzing GAGs in the attached soft material and the mineralized tissue separately is certainly important.

In relation to the tissue mineralization, an increase in the amount or synthesis of HA has been observed under the experimental or physiological conditions where matrix mineralization is impaired (such as exposure to parathyroid hormone, vitamin D deficiency, and OI). Although we do not know the reason thus far, HA and DS fluctuate coincidentally in some cases, instancing a decrease during aging in skin and an increase at aneurysm-affected areas of aorta. The change in bone GAGs observed herein may be another example. HA and DS have been described to affect mineralization: Exogenously added HA and DS cause a significant increase in calcium release from bone organ cultures, and HA inhibits in vitro crystallization. On the contrary, the mineralization process might involve a function of chondroitin 4-sulfate.

Given the situation, one may be tempted to speculate as follows: The overwhelming C4S ensures efficient mineralization in normal bone. Upon development of osteoporosis, not yet identified mechanisms start up to induce an increase in HA and DS. Since these GAGs favor demineralization rather than mineralization, a further loss of the bone mineral would proceed. It is noteworthy that such a change in the GAG composition takes place mainly in cancellous bone, where mineral loss accompanying osteoporosis is marked.

This study is a small pilot examination. The prompting results therefrom, however, urge us to organize more large-scaled and detailed surveys on the bone matrix GAGs, which will certainly
contribute to elucidate the pathogenic process of osteoporosis.

REFERENCES


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