The effect of X-irradiation on extracellular matrix components was examined in cultured rat fetal osteoblasts and rat femurs. The osteoblasts were X-irradiated at 2 Gy or 5 Gy, and gene expression of matrix metalloproteinase (MMP)-2, MMP-13, type-I collagen and decorin were analyzed by real-time polymerase chain reaction. Hyaluronan in the medium was also determined after 48 h of culture. Expression of the MMP-13 gene was found to be increased, whereas those of the type-I collagen and decorin genes tended to be decreased. Synthesis of hyaluronan and expression of the MMP-2 gene were not significantly affected. We then subjected Sprague-Dawley rats to X-irradiation at 2 Gy or 5 Gy. After 1-3 months, the rats were euthanatized and the glycosaminoglycan fractions were purified from their femurs. These fractions were analyzed for their uronic acid content, sensitivity to mucopolysaccharidases, glycosaminoglycan chain length, and unsaturated disaccharide units. It was found that the uronic acid content was decreased by X-irradiation. The main glycosaminoglycan in rat femur was identified as dermatan sulfate, and its chain length was not affected by X-irradiation. The unsaturated disaccharide unit of the glycosaminoglycan was found to be ADi-4S, and no apparent structural alteration of the glycosaminoglycan was observed after X-irradiation. The core protein and dermatan sulfate chain of decorin have been reported to bind with type-I collagen and to type-IX collagen, which binds to type-I collagen, respectively. The present results thus suggest that the decrease in both decorin and type-I collagen levels in rat bone after X-irradiation may contribute to a loss of mechanical strength.

Key words: bone, X-irradiation, extracellular matrix, glycosaminoglycan, decorin, osteoporosis
1. Introduction

Bone is composed of large amounts of extracellular matrix, the major role of which is considered to be its contribution to biomechanical strength. The main components of bone extracellular matrix are type-I collagen and proteoglycans. Decorin, a proteoglycan comprising a single glycosaminoglycan chain bound to a core protein, is known to be one of the main proteoglycans in bone tissue. Glycosaminoglycan chains essentially consist of repeating disaccharide units of uronic acid and hexosamine. Hyaluronan also appears to regulate bone remodeling by controlling osteoclast, osteoblast, and osteocyte behavior. It has been reported that the core protein and dermatan sulfate chain of decorin binds with type-I collagen and also to type-IX collagen, which binds to type-I collagen, respectively. Therefore, interaction between the sugar chain of decorin and type-IX collagen seems to play a very important role in determining the strength of bone tissue. In addition, osteoblasts are known to produce metalloproteinase (MMP)-13 and MMP-2, which are associated with increases in bone matrix degradation.

It is known that cancer patients who receive radiotherapy tend to suffer from osteoporosis as a side effect several months later. The improved survivorship of cancer patients who receive radiotherapy increases the importance of understanding the causal mechanisms and long-term effects of radiation-induced bone loss. The incidence of hip fractures is significantly increased following targeted radiotherapy for cancer. This decline in bone health can have a severe impact on the patient’s functional capabilities. On the other hand, astronauts on the International Space Station have reported 0.8 - 1.5% bone loss per month in the vertebrae and femora. During exploratory missions, astronauts face not only microgravity as above, and after 1, 2, and 3 months postexposure were euthanatized. Their femurs were extracted and stripped of any surrounding connective tissues. The cartilages at both ends of the femur were removed and the bone marrow was washed out with phosphate-buffered saline. These femurs were then used for glycosaminoglycan extraction.

2. Materials and methods

2.1. X-irradiation of osteoblasts and rats

Osteoblasts from fetal rat calvarias were purchased from Takara Bio, Otsu. The cells were cultured in RPMI1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Biofluid, Rockville, MD) and antibiotics (100 units/ml penicillin G + 100 μg streptomycin sulfate, Gibco) in a humidified atmosphere of 5% CO₂-95% air at 37°C. Cells at confluency in 60-mm dishes were washed with calcium- and magnesium-free phosphate-buffered saline, and serum-free media were added. The cells were then X-irradiated at 2 or 5 Gy (150 kVp, 20 mA; 0.5-mm Al and 0.1-mm Cu filters at a dose rate of approximately 1.0 Gy/min) using an X-ray generator (MBR-1520R-3, Hitachi, Tokyo), and culture was continued. After 24 h or 48 h, the conditioned medium was recovered and the cells were collected by trypsin treatment.

Sprague-Dawley rats (SD rats; six weeks, male; CLEA Japan Inc, Tokyo) were acclimatized for 1 week before irradiation, and provided food and water ad libitum. Before irradiation, the rats were placed individually in rectangular polystyrene boxes with air holes. Three rats were X-irradiated simultaneously under the same conditions as above, and after 1, 2, and 3 months postexposure were euthanatized. Their femurs were extracted and stripped of any surrounding connective tissues. The cartilages at both ends of the femur were removed and the bone marrow was washed out with phosphate-buffered saline. These femurs were then used for glycosaminoglycan extraction.

2.2. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was purified from osteoblasts using an RNEasy Mini Kit (QIAGEN K.K., Tokyo), and cDNA was prepared from the total RNA by reverse transcription reaction using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies Japan, Tokyo). Gene expression of matrix metalloproteinase (MMP)-13, MMP-2, type-I collagen, and decorin was measured by real-time PCR using TaqMan assay (Applied Biosystems; Rn01448194_m1 for MMP-13, Rn01538170_m1 for MMP-2, Rn01526721_
m1 for type-I collagen (collagen α-2(I) chain; Col1A2), Rn01503161_m1 for decorin). PCRs were performed in a StepOnePlus Real-Time PCR system (Applied Biosystems) using 40 cycles of 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min.

2.3. Preparation of the glycosaminoglycan fraction from rat femurs

Rat femurs were cut into small pieces, and crushed using a Micro Smash MS-100R (Tomy Seiko, Tokyo) at 3,000 rpm for 5 min at 4°C in 0.5 M disodium ethylenediaminetetraacetate (EDTA-Na). The samples were decalcified in the same solution for a week, replacing the solution with fresh 0.5 M EDTA-Na on the 3rd day. The decalcified samples were collected by centrifugation at 3,000 rpm for 10 min, and then delipidated in chloroform-methanol (2:1) solution for 24 h with shaking. The samples were collected on filter paper and delipidated again under the same conditions. Decalcified and delipidated samples were dried in a desiccator. Actinase digestion was then performed in 0.1 M Tris-HCl buffer (pH 8.0, 10 mM CaCl₂ at 50°C for 48 h with addition of actinase at 24 h incubation. Actinase digestion was stopped by boiling the reaction solution at 100°C for 10 min, followed by centrifugation at 3,000 rpm for 10 min. Four volumes of ethanol saturated with NaCl were added to the supernatant and the mixture was then left to stand at -30°C for 1 h. After centrifugation at 5,000 rpm for 10 min, the supernatant was discarded and the pellet recovered. The pellet was dissolved in 0.04 M NaCl and treated with 5% cetylpyridinium chloride. The solution was centrifuged after 30 min, and the resulting pellet was dissolved in 2 M MgCl₂. Four volumes of ethanol saturated with NaCl were added to the supernatant and the mixture was then left to stand at -30°C for 1 h. After centrifugation at 5,000 rpm for 10 min, the supernatant was discarded and the pellet recovered. The pellet was dried with absolute ethanol and ether, and used as the glycosaminoglycan fraction.

### 2.4. Cellulose acetate membrane electrophoresis

Glycosaminoglycan fractions were incubated with chondroitinase ABC (Proteus vulgaris), chondroitinase AC-I (Arthrobacter aurescens), hyaluronidase (Streptomyces hyalurolyticus), and heparitinase (Flavobacterium heparinum), respectively, according to the procedures described in the manufacturer’s protocol (Seikagaku Biobusiness, Tokyo). Substrate specificities of these mucopolysaccharidases²⁰-²² are shown in Table 1. The reaction products were electrophoresed on cellulose acetate membrane (Separax, Jokoh, Tokyo) in 0.1 M formic acid-pyridine buffer (pH 3.0) at 1 mA/cm for 30 min. The glycosaminoglycans on the membrane were then visualized by alcian blue staining.

### 2.5. High-performance liquid chromatography (HPLC)

Reducing terminals of sugar chains in the glycosaminoglycan fractions were labeled with 2-aminopyridine, followed by incubation with cellulose (Aspergillus niger; Sigma, St. Louis, MO, USA) by the method of Takagaki et al²³. HPLC (Hitachi L-7100) connected to a fluorescence detector (Hitachi L-7480) was used. The fluorescence-labeled glycosaminoglycans were analyzed on a Shodex OHpak SB-804 HQ gel-permeation column (4.6 mm × 250 mm; YMC Co., Kyoto). For detection, the eluates were monitored at excitation and emission wavelengths of 320 and 400 nm, as described previously²⁴.

For unsaturated disaccharide analysis, the glycosaminoglycan fraction was incubated with chondroitinase ABC (0.1 unit) in 0.1 M Tris-HCl buffer (pH 8.0, 10 mM CH₃COONa) at 37°C for 24 h. The resulting unsaturated disaccharide units were analyzed by HPLC using a YMC-pack Polyamine II column (4.6 mm × 250 mm; YMC Co., Kyoto). The column was eluted with a combination of

### Table 1. Substrate specificities of mucopolysaccharidases

<table>
<thead>
<tr>
<th>Mucopolysaccharidases</th>
<th>substrates</th>
<th>products</th>
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<tbody>
<tr>
<td><em>Str.</em> hyaluronidase²⁰</td>
<td>Hyaluronan</td>
<td>Unsaturated tetrasaccharide</td>
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<tr>
<td></td>
<td></td>
<td>Unsaturated hexasaccharide</td>
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<tr>
<td>Chondroitinase AC-I²¹</td>
<td>Hyaluronan</td>
<td>Unsaturated disaccharide</td>
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<tr>
<td></td>
<td>Chondroitin</td>
<td>Unsaturated disaccharide</td>
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<tr>
<td></td>
<td>Chondroitin sulfate</td>
<td>Unsaturated disaccharide</td>
</tr>
<tr>
<td>Chondroitinase ABC²¹</td>
<td>Hyaluronan</td>
<td>Unsaturated disaccharide</td>
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<td></td>
<td>Chondroitin</td>
<td>Unsaturated disaccharide</td>
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<tr>
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<td>Chondroitin sulfate</td>
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<td></td>
<td>Dermatan sulfate</td>
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</tr>
<tr>
<td>Heparitinase²²</td>
<td>Heparan sulfate</td>
<td>Unsaturated disaccharide</td>
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solution A (16 mM NaH₂PO₄) and solution B (1 M NaH₂PO₄) delivered as a linear gradient (0-60 min, solution A 100-53% and solution B 0-47%) at a flow rate of 1.0 ml/min using a Hitachi L-7100. Unsaturated disaccharides were detected at 232 nm using a Hitachi L-7400.

2.6. Analytical methods

Uronic acid was determined by the method of Bitter and Muir. Hyaluronan was determined by an ELISA-like assay using a Hyaluronan Assay Kit (Seikagaku Biobusiness).
3. Results

3.1. Effect of X-irradiation on extracellular component of cultured rat osteoblasts

Rat osteoblasts were X-irradiated at 2 or 5 Gy. The cells were then cultured for 48 h and the amount of hyaluronan in the medium was determined. As shown in Fig. 1, A, the synthesis of hyaluronan in cultured osteoblasts was not affected by X-irradiation under the conditions employed (Fig. 1, A). The cells were then X-irradiated at 2 or 5 Gy, and cultured for 24 h. Total RNA were extracted from the cells and the expression of the MMP-13, MMP-2, type-I collagen (collagen α-2(I) chain) and decorin genes was analyzed by real-time RT-PCR. It was found that the expression of MMP-13 was dose-dependently increased by X-irradiation (Fig. 1, B), whereas the expression of type-I collagen and decorin was decreased (Fig. 1, C & D).

3.2. Effect of X-irradiation on glycosaminoglycan in rat femurs

Since expression of the decorin gene was shown to be decreased by X-irradiation in cultured osteoblasts, the effect of X-irradiation on glycosaminoglycans in rat femurs was examined. SD rats were X-irradiated at 2 or 5 Gy and then euthanatized at 1, 2 and 3 months after exposure. Glycosaminoglycan fractions were purified from the femurs, and the uronic acid content of each fraction was determined. As shown in Figure 2, the uronic acid content per unit bone weight was dose-dependently decreased by X-irradiation. In order to identify the main glycosaminoglycan in the femur, glycosaminoglycan fractions obtained 3 months after exposure to 5 Gy were electrophoresed on cellulose-acetate membranes before and after incubation with mucopolysaccharidases. The glycosaminoglycan fractions were digested with chondroitinase ABC and the resulting unsaturated disaccharides were separated by HPLC using a TSK gel Polyamine II column. A, Unsaturated disaccharide standards; B, 0 Gy; C, 2 Gy; D, 5 Gy. Standard disaccharides were as follows: ∆Di-0S, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; ∆Di-4S, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose; ∆Di-6S, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulfo-D-galactose; ΔDi-triS, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4,6-di-O-sulfo-D-galactose; ∆Di-5S, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-5-O-sulfo-D-galactose; ∆Di-5S, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-5-O-sulfo-D-galactose; ∆Di-triS, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4,6-di-O-sulfo-D-galactose.
with chondroitinase ABC, and unsaturated disaccharides in the reaction products were analyzed by HPLC. As shown in Figure 5, the main disaccharide unit of glycosaminoglycan in the femur was found to be ADi-4S, and this was not altered by X-irradiation (Fig. 5, B-D).

4. Discussion

Although radiation exposure is known to induce osteoporosis, details of the mechanism involved have not been clarified. This study analyzed the weakening of bone resulting from exposure to X-irradiation, and its effects on extracellular matrix components. The main extracellular matrix component in bone is type-I collagen, which is known to be synthesized by osteoblasts\(^2\)\(^-\)\(^6\). Osteoblasts also produce MMP-13, the main collagenase in bone tissue\(^26\),\(^27\), which contributes to reconstruction of bone tissue.

It has been reported that expression of the MMP-13 gene in osteoblasts is increased by gamma-ray irradiation\(^29\). Our present study also demonstrated an increase in the expression of MMP-13 after X-irradiation (Fig. 1, B). The gene expression of MMP-2, the main gelatinase in bone tissue\(^29\), and the synthesis of hyaluronan, were not significantly affected by X-irradiation, while expression of the type-I collagen and decorin genes was reduced (Fig. 1). These results suggested that X-irradiation affected the metabolism of extracellular components of cultured osteoblasts, thus reducing the strength of bone tissue.

Next, we focused on the glycosaminoglycan of decorin. The glycosaminoglycan chain of decorin has been identified as dermatan sulfate, which has a fundamental repeating disaccharide unit consisting of L-iduronic acid and N-acetyld-galactosamine-4-sulfate. Therefore, we investigated the effect of X-irradiation on the structure of the decorin sugar chain. Hamilton et al.\(^30\) reported that significant loss of the trabecular bone volume fraction in C57BL/6 mice occurred at 110 days after irradiation with 2-Gy doses of gamma radiation, or accelerated proton, carbon, or iron ions. In our present study, SD rats were X-irradiated at 2 or 5 Gy. After 1-3 months, the rats were sacrificed and their femurs were extracted. Glycosaminoglycan fractions were purified from the femurs, and their uronic acid content was determined. The fraction was also subjected to analysis of glycosaminoglycan chain size and unsaturated disaccharide units. It was found that the uronic acid content was decreased, but the chain size was unaffected. The main glycosaminoglycan in rat femurs was totally digested with chondroitinase ABC, and analysis of the unsaturated disaccharide units revealed that its sulfate content was unaffected. These results indicate that the main glycosaminoglycan in the rat femur is dermatan sulfate, and that no structural alteration of the glycosaminoglycan occurs after X-irradiation.

In this investigation, it was found that X-irradiation induced alteration of the extracellular matrix component of rat bone. Gene expression of type-I collagen was decreased, while that of MMP-13 was increased in cultured rat osteoblasts. This alteration of gene expression may contribute to decreasing the amount of type-I collagen in bone. Furthermore, the gene expression of decorin core protein and the decrease of uronic acid content in the femur were found to be decreased by X-irradiation, which may weaken an affinity between the collagen fibers. X-irradiation affects stromal stem cells, which are osteoblast precursors, and may affect bone formation and growth. Therefore, the above-presented results suggest that the alteration of extracellular matrix components by X-irradiation may be caused mainly by the effect on osteoblasts. Although the structure of glycosaminoglycan chains in the femur appeared not to be significantly altered, the present results suggest that decreases in the levels of both decorin and type I-collagen in bone after X-irradiation may contribute to a loss of mechanical strength in bone tissue. The relationship between decorin and bone strength should be investigated in a future study.

Acknowledgement

This work was supported by a Grant for Co-medical Education Program in Radiation Emergency Medicine by the Ministry of Education, Culture, Sport, Science and Technology, and by a Grant for Hirosaki University Institutional Research.

References


