HIROSAKI UNIVERSITY

Radiation Emergency Medicine 2013 Vol. 2, No. 1 43-49

REM
Radiation Emergency Medicine

Effect of X-ray Irradiation on Extracellular Matrix Components of Rat Osteoblasts and Femurs

Komei Tanaka[1], Shinichiro Suto[2], Yoshiyuki Kuroda[1,2], Takashi Ishikawa[1], Kyoko Nakano[1], Mitsuru Chiba[1,3], Naoki Nanashima[1,3], Manabu Nakano[1,3], Koichi Ito[1,3] and Toshiya Nakamura[1,2,3*]

[1]*Department of Biomedical Sciences, Division of Medical Life Sciences, Hirosaki University Graduate School of Health Sciences 66-1 Hon-cho, Hirosaki, Aomori 036-8564, Japan*
[2]*Department of Glycomedicine, Hirosaki University Graduate School of Medicine 5 Zaifu-cho, Hirosaki, Aomori 036-8562, Japan*
[3]*Research Center for Biomedical Sciences, Hirosaki University Graduate School of Health Sciences 66-1 Hon-cho, Hirosaki, Aomori 036-8564, Japan*

Received 6 September 2012; revised 28 October 2012; accepted 15 November 2012

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 5Gy. After 1-3 months, the rats were euthanized 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 ΔDi-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

*Toshiya Nakamura: Department of Biomedical Sciences, Division of Medical Life Sciences, Hirosaki University Graduate School of Health Sciences, 66-1 Hon-cho, Hirosaki, Aomori 036-8564, Japan.
E-mail: toshiyan@cc.hirosaki-u.ac.jp

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[1]. The main components of bone extracellular matrix are type-I collagen and proteoglycans[2-4]. 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[5]. Glycosaminoglycan chains essentially consist of repeating disaccharide units of uronic acid and hexosamine[6]. Hyaluronan also appears to regulate bone remodeling by controlling osteoclast, osteoblast, and osteocyte behavior[7]. 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[3, 8-10]. 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 matrix metalloproteinase (MMP)-13 and MMP-2, which are associated with increases in bone matrix degradation[11].

It is known that cancer patients who receive radiotherapy tend to suffer from osteoporosis as a side effect several months later[12, 13]. 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[14, 15]. During exploratory missions, astronauts face not only microgravity but also radiation from cosmic and solar sources[16, 17]. Therefore, elucidation of the mechanism of radiation-induced osteoporosis is an important issue.

Bone metabolism is regulated by the balance of osteoblasts and osteoclasts[18]. Mainly osteoblasts are involved in bone formation by producing type-I collagen and small proteoglycans such as decorin, which are the main collagen and proteoglycan in the extracellular matrix of bone, respectively. On the other hand, osteoclasts are involved in bone resorption and bone degradation. If the balance of activity of osteoblasts and osteoclasts is perturbed, bone structure is altered.

Osteoporosis is a common age-related disorder manifested clinically by skeletal fractures, especially fractures of the vertebrae, hip, and distal forearm. The major cause of these fractures is low bone mass, although an increase in trauma due to falls in the elderly also contributes. There are multiple causes for the low bone mass which, in any given individual, may contribute differently to the development of osteopenia. In normal young adults, the processes of bone resorption and bone formation are tightly coupled so that bone balance is maintained. However, during age-related bone loss, there is a remodeling imbalance with a relative or absolute increase in resorption over formation[19]. However, the relationship between radiation and any decrease in bone strength is unknown and poorly studied.

In this study, the effects of X-irradiation on extracellular matrix components and their related molecules were investigated using cultured rat fetal osteoblasts and rat femurs.

2. Materials and methods

2.1. X-irradiation of osteoblasts and rats

Osteoblasts from fetal rat calvarias were purchased from Takara Bio, Ohtsu. 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% CO_2-95% 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_

Table 1. Substrate specificities of mucopolysaccaridases

Mucopolysaccharidases	substrates	products
Str. hyaluronidase[20]	Hyaluronan	Unsaturated tetrasaccharide Unsaturated hexasaccharide
Chondroitinase AC-I[21]	Hyaluronan	Unsaturated disaccharide
	Chondroitin	Unsaturated disaccharide
	Chondroitin sulfate	Unsaturated disaccharide
Chondroitinase ABC[21]	Hyaluronan	Unsaturated disaccharide
	Chondroitin	Unsaturated disaccharide
	Chondroitin sulfate	Unsaturated disaccharide
	Dermatan sulfate	Unsaturated disaccharide
Heparitinase[22]	Heparan sulfate	Unsaturated disaccharide

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 $CaCl_2$ 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 $MgCl_2$. 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 mucopolysaccharidases[20-22] 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 al[23]. 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 (8.0 mm × 300 mm; Shoko, Tokyo) with 0.2 M NaCl at a flow rate of 1.0 ml/min at 30°C. For detection, the eluates were monitored at excitation and emission wavelengths of 320 and 400 nm, as described previously[24].

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 CH_3COONa) 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

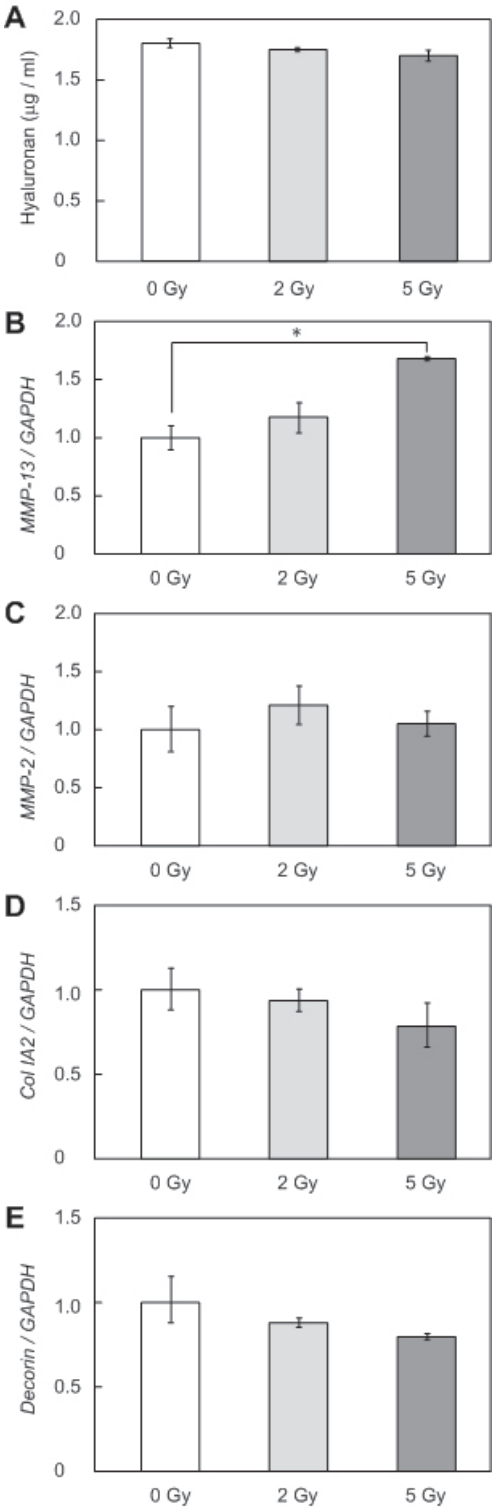

Fig. 1. Effect of X-irradiation on hyaluronan synthesis and the expression of the genes related to extracellular matrix components in cultured rat osteoblasts.
Cultured rat osteoblasts were X-irradiated at 2 or 5 Gy. After 48 h of culture, hyaluronan secreted into the medium was determined (A). Total RNA was extracted at 24 h after exposure, and real-time RT-PCR was performed to examine gene expression of MMP-13 (B), MMP-2 (C), collagen α-2(I) chain (*Col1A2*) of type-I collagen (D) and decorin (E). Values were expressed as mean of two separate experiments each assayed in duplicate (Student's *t* test, $^{*}P<0.05$).

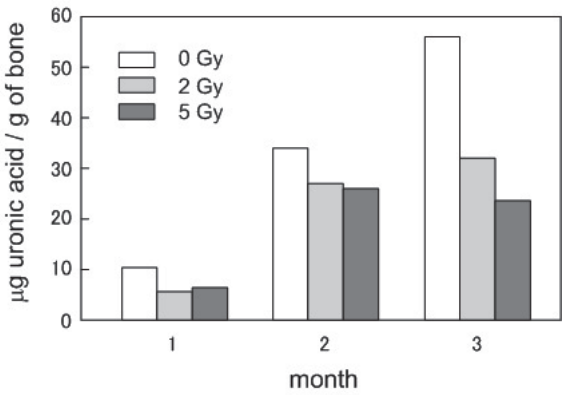

Fig. 2. Effect of X-irradiation on uronic acid content of rat femurs.
SD rats were X-irradiated at 2 or 5 Gy. After 1-3 months, glycosaminoglycan fractions were purified from their femurs and the uronic acid content was determined. A total of six pooled femurs were used in each determination, and values were expressed as means of two separate experiments.

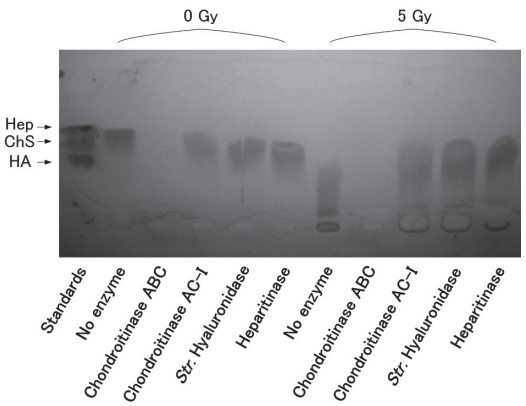

Fig. 3. Identification of main glycosaminoglycan in rat femurs on cellulose acetate membrane electrophoresis.
SD rats were X-irradiated at 5 Gy, and glycosaminoglycan fractions were purified from their femurs at 3 months after exposure. The fractions were electrophoresed on cellulose acetate membranes before and after incubation with mucopolysaccharidases. Lane 1, standard heparin, chondroitin sulfate and hyaluronan; lanes 2 & 7, glycosaminoglycan fractions without enzyme treatment; lanes 3 & 8, incubation with chondroitinase ABC; lanes 4 & 9, incubation with chondroitinase AC-I; lanes 5 & 10, incubation with *Streptomyces* hyaluronidase; lanes 6 & 11, incubation with heparitinase.

solution A (16 mM NaH_2PO_4) and solution B (1 M NaH_2PO_4) 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[25]. Hyaluronan was determined by an ELISA-like assay using a Hyaluronan Assay Kit (Seikagaku Biobusiness).

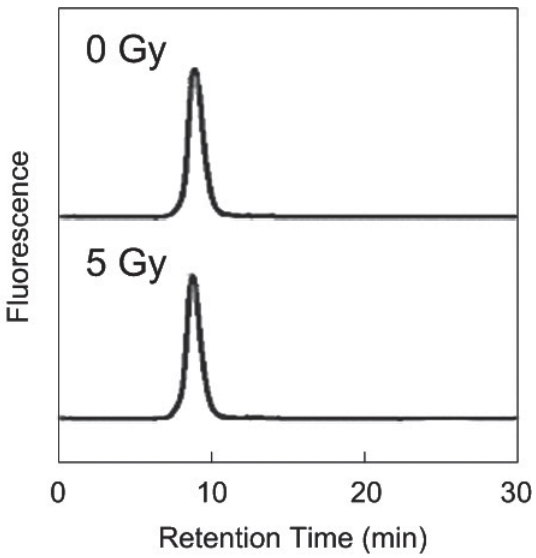

Fig. 4. Gel permeation chromatography of glycosaminoglycan in rat femurs.
SD rats were X-irradiated at 5 Gy, and glycosaminoglycan fractions were purified from their femurs at 3 months after exposure. The glycosaminoglycan fraction was pyridylaminated and subjected to HPLC on a Shodex OHpak SB-804 HQ column.

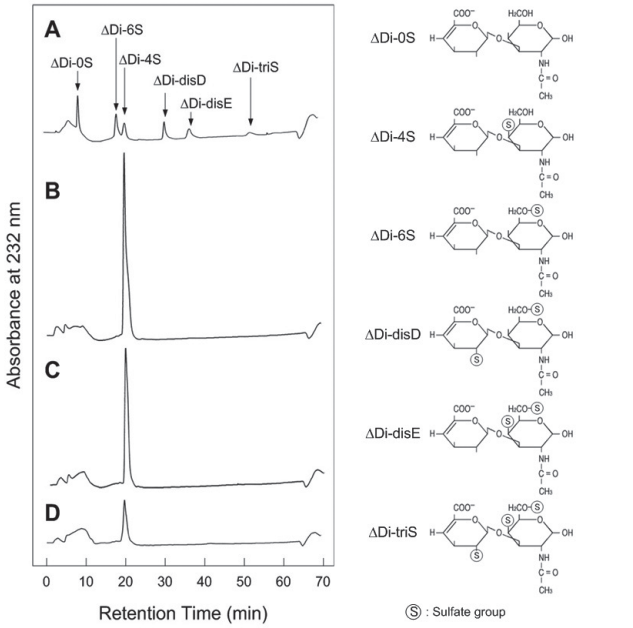

Fig. 5. Analysis of unsaturated disaccharide units in glycosaminoglycan fractions by HPLC.
Glycosaminoglycan fractions were purified from femurs of 5 Gy X-irradiated rats at 3 months after exposure. The fractions were digested with chondroitinase ABC and the resulting unsaturated disaccharides were separated by HPLC using a TSK gel Polyamine II column. A, Unsaturated disaccharide standards; B, 0 Gy; C, 2 Gy; D, 5Gy. Standard disaccharides were as follows: ΔDi-0S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔDi-disD, 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi-disE, 2-acetamido-2-deoxy-3-*O*-(2-O-sulfo-β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔDi-triS, 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-4,6-di-*O*-sulfo-D-galactose.

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, 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 showed bands corresponding to chondroitin sulfate, except for those that had been incubated with chondroitinase ABC (Fig. 3, lanes 3 & 8). This indicates that the main portion of the glycosaminoglycan was digested by chondroitinase ABC. According to the substrate specificities of mucopolysaccharidases shown in Table 1, the results indicate that the main glycosaminoglycan in the femur is dermatan sulfate, irrespective of X-irradiation.

The reducing terminals of glycosaminoglycans obtained 3 months after exposure to 5 Gy were labeled with the fluorescent reagent, 2-aminopyridine, and subjected to gel-permeation HPLC. Figure 4 shows that pyridylaminated glycosaminoglycans were eluted at the same retention time, indicating that the glycosaminoglycan chain size was not altered by X-irradiation. Next, in order to investigate the effect of X-irradiation on the sulfation content and sulfation position of glycosaminoglycans, glycosaminoglycan fractions obtained 3 months after exposure to 5 Gy were digested

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 ΔDi-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, 4]. 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[28]. 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*-acetyl-D-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

1. Scott JE (1992) Supramolecular organization of extracellular matrix glycosaminoglycans, in vitro and in the tissues. FASEB J 6: 2639-2645.
2. Toole BP and Linsenmayer TF (1977) Newer knowledge of skeletogenesis: macromolecular transitions in the extracellular matrix. Clin Orthop Relat Res 129: 258-278.
3. Scott JE (1988) Proteoglycan-fibrillar collagen interactions. Biochem J 252: 313-323.
4. Rodan GA and Noda M (1991) Gene expression in osteoblastic cells. Crit Rev Eukaryot Gene Expr 1: 85-98.
5. Waddington RJ, et al. (2003) Differential roles for small leucine-rich proteoglycans in bone formation. Eur Cell Mater 6: 12-21.
6. Rodén L (1980) Structure and metabolism of connective tissue proteoglycans. In: Lennarz WJ, ed. The Biochemistry of Glycoproteins and Proteoglycans. pp. 267-371, Plenum Press, New York.
7. Bastow ER, et al. (2008) Hyaluronan synthesis and degradation in cartilage and bone. Cell Mol Life Sci 65: 395-413.
8. Progany G and Vogel KG (1992) The interaction of decorin core protein fragments with type I collagen. Biochem Biophys Res Commun 189: 165-172.
9. Schonherr E, et al. (1995) Decotin-type I collagen interaction. Presence of separate core protein-binding domains. J Biol Chem 270: 8877-8883.

10. Roughley PJ and Lee ER (1994) Cartilage proteoglycans: structure and potential functions. Microsc Res Tech 28: 385-397.

11. Kusano K, et al. (1998) Regulation of matrix metalloproteinases (MMP-2, -3, -9, and -13) by interleukin-1 and interleukin-6 in mouse calvaria: association of MMP induction with bone resorption. Endocrinology 139:1338-1345.

12. Rex C and Elsworth C (1998) Pelvic insufficiency fracture with diastasis of the pubic symphysis after irradiation: a case report. J Bone Joint Surg Br 80: 264-266.

13. Diwanji SR, et al. (2008) Septic arthritis of hip after radiotherapy for carcinoma of cervix. Singapore Med J 49: 142-144.

14. Lang T, et al. (2004) Cortical and trabecular bone mineral loss from the spine and hip in long-duration spaceflight. J Bone Miner Res 19: 1006-1012.

15. Tilton FE, Degioanni JJ and Schneider VS (1980) Long-term follow-up of Skylab bone demineralization. Aviat Space Environ Med 51: 1209-1213.

16. Cucionotta FA, et al.(2001) Space radiation and cataracts in astronauts. Radiation Res 156: 460-466.

17. Vernikos J and Nicogossian AE (1998) Strategic Program Plan for Space Radiation Health Research. Washington, DC: NASA Headquarters Space Radiation Health, p.1-71.

18. Riggs BL, Khosla S and Melton LJ 3rd (2002) Sex steroids and the construction and conservation of the adult skeleton. Endocr Rev 23: 279-302.

19. Riggs BL (1991) Overview of osteoporosis. West J Med 154: 63-77.

20. Ohya T and Kaneko Y (1970) Novel hyaluronidase from streptomyces. Biochim Biophys Acta 198: 607-609.

21. Yamagata T, et al. (1968) Purification and properties of bacterial chondroitinases andchondrosulfatases. J Biol Chem 243: 1523-1535.

22. Ototani N, et al. (1973) Unsaturated disaccharides in the enzymatic digests of heparan sulfates. J Biochem 94: 233-241.

23. Takagaki K, et al. (2001) Cleavage of the xylosyl serine linkage between a core peptide and a glycosaminoglycan chain by cellulases. J Biol Chem 277: 18397-18403.

24. Nakamura T, et al. (1990) Hyaluronidase assay using fluorogenic hyaluronate as a substrate. Anal Biochem 191: 21-24.

25. Bitter T and Muir HM (1962) A modified uronic acid carbazole reaction. Anal Biochem 4: 330-334.

26. Onodera S, et al. (2002) Macrophage migration inhibitory factor up-regulates matrix metalloproteinase-9 and -13 in rat osteoblasts. Relevance to intracellular signaling pathways. J Biol Chem 277: 7865-7874.

27. Quinn CO, Bizek GM and Agapova OA (2000) Induction of rat interstitial collagenase (MMP-13) mRNA in a development-dependent manner by parathyroid hormone in osteoblastic cells. Endocrine 12: 227-236.

28. Sawajiri M, et al. (2007) Expression of MMP-13 in osteoblast cells and rat tibia after exposure to Gamma rays or accelerated carbon ions. Phys Med 23: 73-79.

29. Lorenzo JA, et al. (1992) Production of both 92- and 72-kDa gelatinases by bone cells. Matrix 12: 282-90.

30. Hamilton SA, et al. (2006) A murine model for bone loss from therapeutic and space-relevant sources of radiation. J Appl Physiol 101: 789-793.