Radiation-induced Reduction of Osteoblast Differentiation in C2C12 cells

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Bone morphogenic protein 2 (BMP-2)/Heparin/Alkali phosphatase activity/Real-time PCR.

Therapeutic radiation causes bone damage and may increase fracture risks in treatment for head-and-neck cancer and in pelvic irradiation. These properties can also be used for prevention of heterotopic ossification in hip arthroplasty. To evaluate the effects of ionizing radiation on osteoblast differentiation, C2C12 cells were directed into an osteogenic lineage by treatment with a combination of bone morphogenic protein 2 (BMP-2) (100 ng/ml) and heparin (30 μg/ml) 6 h after irradiation (2 and 4 Gy). Osteoblast differentiation was evaluated based on alkal phosphatase (ALP) activity and expression of mRNA encoding ALP and collagen type I. Ionizing radiation suppressed the growth of C2C12 cells and decreased expression of ALP and collagen type I mRNAs with concomitant reduction of the ALP activity. Although further studies are needed to elucidate the molecular mechanism, our findings suggest that ionizing radiation at therapeutic doses interferes with bone formation by reducing ALP activity and expression of mRNA encoding ALP and collagen type I.

INTRODUCTION

Therapeutic radiation causes bone damage and may increase the risk of fracture. In treatment for cancer of the oral cavity, osteoradionecrosis of the mandible is reported as a common late effect, though combined external beam radiotherapy is considered to be one of standard treatment approaches.1,2) Pelvic irradiation has also been reported to increase the risk of pelvic fracture in older women.3) At the same time, irradiation has been used for preventing heterotopic ossification, which is a common postoperative complication of total hip arthroplasty.4) However, the exact mechanism of radiation effect on bone formation has not been elucidated and clarification of the radiation effects on osteoblast differentiation may improve the efficacy of therapeutic radiation.

The mouse myoblast cell line, C2C12, is a well-defined model of osteoblast differentiation upon exposure to bone morphogenic protein 2 (BMP-2).5–7) BMPs exert their activity by facilitating assembly of members of two subfamilies of transmembrane serine/threonine kinases, referred to as the type I and type II receptors. In the resulting complex, the type II receptors phosphorylate the type I receptors, causing propagation of the differentiation signal.8) Runx2, a downstream effector of BMPs, is an important transcription factor that regulates osteoblast and chondrocyte differentiation. Runx2 can be viewed as a marker gene for the BMP signaling pathway, and the coordinated action of Runx2 and Smads, which play central roles in intracellular signaling by BMPs, leads to the induction of osteoblast-specific gene expression.9)

Many extracellular molecules regulate the biological activities of BMPs in vivo and in vitro, including sulfated polysaccharides such as heparin and heparan sulfate, which are macromolecules associated with the cell surface and the extracellular matrix.10) These highly negatively charged polysaccharides interact directly with a number of growth factors, including BMPs. Sulfated polysaccharides including heparin, heparan sulfate and dextran sulfate, but not desulfated heparin, enhance osteoblast differentiation. A similar level of the alkal phosphatase (ALP) activity, a typical marker of osteoblast differentiation, is attained by treatment of cells with 300 ng/ml BMP-2 alone and with after treatment with 100 ng/ml BMP-2 and 30 μg/ml heparin. These molecules may protect BMP-2 from degradation and from inhibition by BMP-2 antagonists, thereby increasing its biological activity.11,12) In fact, BMP-2 activity is not inhibited by noggin, a BMP-2 antagonist, when heparin is present, and increased expression of noggin...
mRNA continues for at least 72 h after induction of osteoblast differentiation by BMP-2.\(^{11,12}\)

Ionizing radiation has no effect on osteoblast differentiation when C2C12 cells are induced into an osteogenic lineage through BMP-2 treatment just after irradiation,\(^{13}\) but radiation-induced reduction of alkali phosphatase (ALP) activity does occur when cells are treated with BMP-2 6 h before and after irradiation.\(^{14}\) As noted above, the effect of BMP-2 has to be evaluated together with the extracellular matrix such as heparin when examining the effect of radiation on the osteoblast differentiation. Therefore, we evaluated in irradiated C2C12 cells in the presence of heparin, which enhances BMP-2-induced osteoblast differentiation.\(^{11,12}\)

**MATERIALS AND METHODS**

**X-ray Irradiation**

X-ray irradiation was performed using an X-ray generator (MBR-1520R, Hitachi Medical Corporation, Tokyo, Japan) operating at 150 kVp and 20 mA with 0.5-mm aluminum and 0.1-mm copper filters.\(^{15}\) The upper surface of the culture medium was placed 55 cm from the X-ray source, and the dose rate was 85–90 cGy/min.

**Cell Cycle Analysis**

C2C12 cells were cultured in DMEM supplemented with 10% FBS and a high concentration of glucose (4.5 g/l). The cells were seeded at a density of \(2 \times 10^4\) cell/cm\(^2\) on a 100-mm dish and cultured for 24 h. After irradiation at 2 or 4 Gy, the cells were incubated for 6 h, washed with PBS(−), and then collected using trypsinization and centrifugation. The cells were then washed with PBS(−) and fixed in 70% ethanol/ PBS(−) at −20°C. On the day of flow cytometric analysis, the fixed cells were washed with PBS(−), treated with 250 μg/ml of RNase A (Roche Diagnostics, Basel, Switzerland) at 37°C for 15 min, and stained with 10 μg/ml of propidium iodide (Sigma, St. Louis, MO) for 15 min on ice. DNA content was analyzed with a flow cytometer (EPICS XL; Beckmann Coulter Inc., Fullerton, CA). More than 10,000 cells were analyzed per sample, and three independent experiments were conducted. Fluorescence data were displayed as dot plots to estimate the percentage of cells in the G0/G1, S, and G2/M phases, using ModFit LT software version 2.0 (Verity Software House, Topsham, ME).

**Cell Number**

The cells were seeded at a density of \(2 \times 10^4\) cell/cm\(^2\) on a 60-mm dish in triplicate and cultured for 24 h. After irradiation at 2 or 4 Gy, cell numbers were estimated at 6, 24 and 48 h after irradiation using a cell and particle counter (Coulter Z1; Beckmann Coulter Inc., Fullerton, CA).

**Osteoblast Differentiation**

For the osteoblast differentiation study, cells were seeded at a density of \(2 \times 10^4\) cell/cm\(^2\) on 12- or 24-well culture plates and cultured for 24 h. They were then irradiated with X-rays at 2 or 4 Gy and subsequently cultured for 6 h. To induce osteoblast differentiation, the culture medium was changed to DMEM supplemented with 2% FBS, 100 ng/ml recombinant human BMP-2 (R&D Systems, Minneapolis, MN), and/or 30 μg/ml heparin (Sigma). The culture for induction of differentiation was continued for an additional 3 days.

**ALP Activity**

After differentiation into osteoblasts, the cells were collected using a cell scraper and extracted with CellLytic M (Sigma) according to the manufacturer’s instructions. Cell extracts were frozen and thawed, and ALP activity was measured using an alkaline-phospha-B-test Wako kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan), which was used according to the manufacturer’s instructions with some modifications. The optical density was recorded using a microplate reader (Sunrise™ Basic; Tecan, Zurich, Switzerland) at a wavelength of 405 nm (620 nm reference). ALP activity was standardized using the total protein content, which was measured with a BCA™ Protein Assay Kit (Pierce, Rockford, IL).

**RNA Extraction and DNase I Treatment**

After differentiation into osteoblasts, the cells were scraped and total RNA was prepared using an RNaseasy® Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions. The amount of RNA was measured using an ultraviolet-visible spectrometer at 260 nm, and the purity of the RNA was determined from the absorbance ratio at 260 nm/280 nm. A ratio of >1.8 was used following DNase I treatment, which was performed using RQ1 RNase-free DNase (Promega, Madison, WI) according to the manufacturer’s instructions.

**Reverse Transcription (RT) and Real-Time Polymerase Chain Reaction (PCR)**

cDNA synthesis was performed using random 6mers as primers and ExScript™ RTase (TaKaRa Bio, Shiga, Japan) according to the manufacturer’s instructions. Semi-quantitative PCR was performed using a SYBER® Premix Ex Taq™ kit (TaKaRa Bio) and a Smart Cycler® II System (Cepheid, Sunnyvale, CA) according to the manufacturers’ instructions. In this system, PCR products were quantified using SYBR Green I as an intercalating dye. Cycle conditions were as follows: after an initial incubation at 95°C for 10 s, 40 cycles of denaturation at 95°C for 5 s and primer annealing at 60°C for 20 s were performed. Fluorescence was measured at 60°C. The primers used in the study are summarized in Table 1. Collagen type I and ALP were used as markers of osteoblast differentiation, Runx2 was used to detect early osteoblast differentiation, and noggin was used...
as a signal to evaluate negative feedback of osteoblast differentiation. The expression level of β-actin was used to normalize the amount of template cDNA in the semi-quantitative PCR reaction.

Statistical analysis
Statistical comparisons were performed using a Bonferroni correction for multiple comparisons. Each experiment was repeated independently at least three times.

RESULTS

Cell cycle distribution and cell number after treatment of C2C12 cells with ionizing radiation
Few studies have evaluated the effects of ionizing radiation on C2C12 cells, and therefore we first analyzed the cell cycle distribution and cell number following treatment with ionizing radiation (Fig. 1). Representative flow cytometry histograms are shown in Fig. 1A. A decrease in the G0/G1 peak and an increase in the G2/M peak were observed at 2 Gy, and these changes were even more enhanced at 4 Gy. Quantitation of cell phases by ModFit LT software (Fig. 1B) showed a significant and dose-dependent increase in the population of cells in G2/M phase and a significant decrease in the S phase population in irradiated cells, as compared with non-irradiated cells. A significant decrease in the G0/G1 phase population was observed 6 h after irradiation at 4 Gy. The growth of cells was suppressed as shown in Fig. 1C. The subsequent osteoblast differentiation studies were performed using radiation doses of 2 and 4 Gy.

Effects of ionizing radiation on osteoblast differentiation
The effects of ionizing radiation on osteoblast differentiation were evaluated by measuring the ALP activity (Fig. 2A) and alterations in the levels of mRNA encoding ALP and collagen type I (Figs. 2B and C). ALP activity in non-irradiated C2C12 cells was slightly, but not significantly, increased by treatment with 100 ng/mL BMP-2 alone (Fig. 2A, BMP-2 (+) and heparin (−)). This result is consistent with previous work demonstrating the requirement of more than 300 ng/mL BMP-2 for effective osteoblast differentiation.16 In contrast, the same 100 ng/mL BMP-2 induced a significant increase in the ALP activity in the presence of 30 μg/mL heparin (Fig. 2A, BMP-2 (+) and heparin (+)), and this was also consistent with previous reports.11,12 In addition, the ALP activity was not detected when C2C12 cells were treated with heparin alone or with 4 mM HCl containing 0.1% BSA (the reconstitution solution for BMP-2).

A significant and dose-dependent reduction of ALP activity was observed in C2C12 cells exposed to ionizing radiation and then directed to osteoblast differentiation by BMP-2 and heparin (Fig. 2A, BMP-2 (+) and heparin (+)). The ALP activity was slightly suppressed in osteoblast differentiation induced by BMP-2 alone following irradiation, but the level of reduction was not significant (Fig. 2A, BMP-2 (+) and heparin (−)).

Expression levels of mRNA encoding ALP and collagen type I were slightly, but not significantly, increased by 100 ng/mL BMP-2 alone in non-irradiated cells (Figs. 2B and C, BMP-2 (+) vs. BMP-2 (−) and heparin (−)). These results are consistent with the measured ALP activity and with previous reports of only slight induction of osteoblast differentiation at this concentration of BMP-2, as mentioned above.16 In parallel with the ALP activity, 100 ng/mL BMP-2 and 30 μg/mL heparin increased the expression levels of mRNA encoding ALP and collagen type I in non-irradiated cells. Treatment with heparin alone caused a slight, but non-significant decrease in expression of collagen type I mRNA, compared to treatment with 4 mM HCl containing 0.1% BSA.

Following exposure of C2C12 cells to ionizing radiation and subsequent induction of osteoblast differentiation with

Table 1. Sequence of primers for PCR.

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<th>Gene</th>
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<td>5’-TCACCCGAGTGGTAGTCACAAATG-3’</td>
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<td>collagen type I</td>
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<td>5’-GGAGGCGCTGCGTGAGCAATTA-3’</td>
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<td></td>
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Fig. 1. Effects of ionizing radiation on cell cycle distribution and cell number. Cell cycle distributions were analyzed 6 h after treatment with ionizing radiation at 2 and 4 Gy. A representative flow cytometry histogram (A) and cell cycle analysis by ModFit software (B) are shown. (C) The number of cells was analyzed 6, 24 and 48 h after treatment with ionizing radiation at 2 and 4 Gy. Data are shown as means ± SD from three independent experiments. ** P < 0.01, * P < 0.05.

Fig. 2. Effects of ionizing radiation on osteoblast differentiation. Osteoblast differentiation was induced over 3 days by 100 ng/ml BMP-2 and/or 30 μg/ml heparin, starting 6 h after treatment with ionizing radiation. ALP activity (A) and the expression levels of mRNA encoding ALP (B) and collagen type I (C) are shown. Data are shown as means ± SD from three independent experiments. ** P < 0.01.
Reduction of Osteoblast Differentiation


When a combination of BMP-2 and heparin, the levels of mRNA for ALP and collagen type I were significantly reduced. However, induction of osteoblast differentiation by BMP-2 alone following irradiation caused only a slight, and not significant, decrease in mRNA for ALP and collagen type I.

Effects of ionizing radiation on expression of Runx2 and Noggin mRNAs

To examine the possible mechanisms underlying the effects of ionizing radiation on the expression of genes related to osteoblast differentiation, we measured the expression of mRNA encoding Runx2, the earliest marker for osteoblast differentiation, and noggin, a BMP antagonist that blocks BMP signaling in the extracellular space (Fig. 3). There was no difference between the expression of Runx2 mRNA in irradiated and non-irradiated cells following osteoblast differentiation induced by a combination of BMP-2 and heparin. In contrast, an effect of ionizing radiation was observed when cells were induced to osteoblasts by BMP-2 alone both at 100 and 300 ng/ml (Fig. 3A). The expression of noggin in non-irradiated C2C12 cells differentiated by the combined treatment with BMP-2 and heparin was more pronounced than that in cells induced by BMP-2 alone (Fig. 3B, BMP-2 (+) and heparin (+) vs. BMP-2 (+) and heparin (−)). Ionizing radiation had no effect on the expression of noggin mRNA in C2C12 cells treated with BMP-2 alone or with a combination of BMP-2 and heparin (Fig. 3B).

DISCUSSION

In this study, we first analyzed the cell cycle distribution and cell number following treatment of C2C12 cells with ionizing radiation. These results were used to determine the radiation doses for a subsequent analysis of osteoblast differentiation. We observed a dose-dependent increase in the G2/M phase population and a decrease in the G0/G1 phase population at the higher dose of irradiation (Figs. 1A and 1B), consistent with previous work.17 We also found a decrease in the S phase population (Figs. 1A and 1B), and noted that a similar ionizing radiation-induced decrease in this population has been reported in other cell lines.18–21

Since the effects of ionizing radiation on cell cycle distribution and cell number were observed at 2 and 4 Gy, these doses were selected for the next series of experiments. In addition, the time period between application of ionizing radiation and initiation of osteoblast differentiation was selected based on Pohl’s report, in which a radiation-induced reduction of ALP activity was found to take place when cells were treated with BMP-2 6 h after irradiation.14 We evaluated the effects of ionizing radiation on osteoblast differentiation in the presence of heparin, since heparin has been reported to enhance BMP-2-induced osteoblast differentiation in C2C12 cells.11,12 Our results indicate that ionizing radiation at 2 and 4 Gy reduces ALP activity when C2C12 cells are induced to osteoblast differentiation 6 h after irradiation using a combination of BMP-2 and heparin (Fig. 2A). The mRNA levels of osteoblast differentiation markers, ALP and collagen type I, were also reduced by ionizing radiation at 2 and 4 Gy (Figs. 2B and C). These results demonstrate that ionizing radiation reduces BMP-2-induced osteoblast differentiation, even in the presence of heparin.

The effects of ionizing radiation and heparin on BMP-2-induced osteoblast differentiation have been studied by other groups.11–14 Pohl et al. reported that ionizing radiation reduced ALP activity in C2C12 cells treated with BMP-2 alone when irradiation was performed within 6 h before or after BMP-2 treatment, and also found that the amount of BMP-2/receptor complex and activation (phosphorylation)
of Smad 1, not the level of Smad 1, were reduced by ionizing radiation. Based on these results, they concluded that radiation blocks cellular responsiveness to BMP-2-induced osteoblast differentiation, with the primary mechanism being down-regulation of the BMP-2/receptor complex.\textsuperscript{11} In contrast, heparin markedly enhanced BMP-2-induced osteoblast differentiation by prolongation of the half-life of BMP-2 in culture media, with increased BMP-2-induced phosphorylation of Smads 1, 5 and 8 after 24 and 48 h, and increased BMP-2-induced elevation of noggin mRNA after 72 h.\textsuperscript{11,12}

Based on these previous findings, we evaluated the effects of ionizing radiation on the expression levels of mRNAs encoding Runx2, which is a downstream effector of BMPs, and noggin, a BMP-2 antagonist, to assess the mechanisms underlying the reduced levels of osteoblastic markers observed in this study. Expression of Runx2 mRNA is upregulated during the first 6 h after BMP-2 treatment and then returns to the initial level after 24 h,\textsuperscript{22} whereas expression of noggin mRNA is prolonged for 3 days in osteoblast differentiation.\textsuperscript{12} Therefore, we evaluated expression of mRNAs for Runx2 and noggin after 6 h and 3 days, respectively, in the osteoblast differentiation culture.

Expression of Runx2 mRNA was unaffected by ionizing radiation when cells were induced by a combination of BMP-2 and heparin. In contrast, the Runx2 mRNA level decreased in irradiated cells induced by BMP-2 alone at both 100 and 300 ng/ml (Fig. 3A). The results for Runx2 mRNA in cells treated with BMP-2 alone are consistent with the prediction from previous work of Pohl et al., in which a decrease in the level of the BMP-2/BMP receptor complex and decreased activation of Smad 1 were observed under these conditions. This suggests that reduction of osteoblast differentiation induced by a combination of BMP-2 and heparin in irradiated cells may not be due to a decrease in the level of the BMP-2/BMP receptor complex. Ionizing radiation had no effect on the heparin-induced increase in noggin mRNA expression, suggesting that ionizing radiation does not affect the expression of BMP-2 antagonists or prolongation of BMP-2 activity by heparin.

In conclusion, our results demonstrate that treatment with ionizing radiation reduces ALP activity and expression of mRNA encoding ALP and collagen type I in cells treated with BMP-2 6 h after ionizing radiation. These findings suggest that ionizing radiations at therapeutic dose cause bone damage. These reductions may occur due to division delay, and/or suppression of osteoblast differentiation pathways. Further studies of the effects of ionizing radiation on BMP-2 signaling are in progress to delineate these mechanisms.

REFERENCES