Effects of 2-deoxy-D-glucose and quercetin on the expression of osteonectin and osteopontin during the differentiation of irradiated MC3T3-E1 osteoblastic cells

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ABSTRACT

Purpose : To characterize the effects of 2-deoxy-D-glucose (2-DG) and quercetin (QCT) on gene expression of osteonectin (ON) and osteopontin (OP) in irradiated MC3T3-E1 cells.

Materials and Methods : When MC3T3-E1 osteoblastic cells had reached 70-80% confluence, cultures were transferred to a differentiating medium supplemented with 5 mM 2-DG or 10 µM QCT and then irradiated with 2, 4, 6, and 8 Gy. At various times after irradiation, the cells were analyzed for the expression of bone mineralization genes such as ON and OP.

Results : The mRNA expression of both ON and OP was increased according to the culture time in the differentiation medium, and the increase of the genes peaked at 14 days after the differentiation induction. In the case of OP, the increase of mRNA expression was maintained to 28 days after the differentiation, while the mRNA level of ON was reduced to the basal level at the same time. Irradiation adding 2-DG showed a significant peak value in the expression pattern of ON at 4 Gy 7 days after irradiation. Irradiation adding QCT increased the mRNA expression of ON and OP in a dose-dependant manner, but irradiation adding 2-DG did not show any differences between the control and experiments 14 days after irradiation. Irradiation adding QCT increased significantly the expression patterns of ON 21 days after irradiation.

Conclusion : The results showed that QCT acted as a radiosensitizer in the gene expression of ON and OP during differentiation of the late stage of irradiated MC3T3-E1 osteoblastic cells in vitro. (*Korean J Oral Maxillofac Radiol 2008; 38 : 195-202*)

KEY WORDS : Osteonectin; Osteopontin; 2-deoxy-D-glucose; Quercetin; Irradiation

Introduction

Several radiomodifiers have been investigated to achieve a control over radiation damage in tumor versus normal cells. 2-DG has been suggested as a suitable candidate to achieve this objective.[1] 2-DG has been shown to enhance radiation-induced chromosome aberrations, micronucleus formation and lethality in tumor cells and to reduce these in normal tissue.[2] Latz et al.[3] showed that 2-DG could indeed increase the radiation-induced tumor cell loss, tumor regression and survival in mice with tumors. QCT, one of the most abundant of flavonoids, possesses many biological effects including cardiovascular protection,[4] anti-cancer activity,[5] and anti-inflammation.[6] QCT and other flavonoids have been shown to scavenge free radicals.[7] Kahraman and Inal[8] has found that the antioxidant QCT

protects liver cells in rats from the harmful effects of ultraviolet light or, at least, it reduces the damage. QCT may be useful for reducing or preventing photobiologic damage. Its ultraviolet protective and antioxidant effects could be associated with an inhibitory action on irradiation damage.

The biochemical and histochemical observations have indicated the expression patterns of bone-related proteins, which are regulated in a temporal manner during successive developmental stages including proliferation, bone matrix formation, maturation, and mineralization.[9] The expression of cell-cycle or growth-related genes and extracellular matrix genes increased and were followed by bone mineralization genes such as osteocalcin, ON, OP, and bone sialoprotein during bone formation.

ON and OP are the most well-known bone phosphoproteins. ON, a secreted protein acidic and rich in cysteine, is a secreted Ca^{2+}-binding glycoprotein.[10] It shows high affinity binding to type I collagen, and is considered to be associated with tissue remodeling rather than calcification.[11] Kato et al.[12] showed that high ON production in the acidic medium is thought to be an

important potential for tumor invasive behavior. OP is associated with the transformation process, and shows high affinity for binding to hydroxyapatite. It has been identified as a gene inducible by tumor promoters and growth factors in a variety of cultured mouse cell lines.[13] Kitahara et al.[14] showed that a deficiency of OP induces the activation of parathyroid hormones.

Ionizing irradiation has an influence on the production of cytokines during the differentiation of MC3T3-E1 cells and changes the proliferation of the osteoblasts and the calcification of the extracellular matrix.[15] However the effects of 2-DG or QCT on gene expression of bone formation-related factors in irradiated osteoblastic cells are poorly understood. The present work was undertaken to characterize the effects of 2-DG or QCT on the gene expression of ON and OP.

Materials and Methods

1. Chemicals and laboratory materials

Unless otherwise specified, chemicals and laboratory materials were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA), respectively.

2. Cell culture

The murine osteoblastic MC3T3-E1 cells (ATCC, CRL-2593) were cultured in an alpha-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and antibiotics. The cultures were maintained at 37°C with a gas mixture of 5% CO_2/95% air and subcultures were performed with 0.05% trypsin-0.02% EDTA in Ca^{2+}, Mg^{2+}-free phosphate buffered saline (DPBS; Gibco BRL Co., USA). One million cells per milliliter were resuspended in either 2 mL or 100 μL media for spreading onto either 6-well or 96-well flat-bottomed plates (Falcon, Becton Dickinson, NJ, USA), respectively. Cultures were switched to a fresh batch of the same medium two times per week.

3. Cellular irradiation and treatment

MC3T3-E1 cells were divided into 6- or 96- well flat-bottomed plates at a dose of 5×10^5 cells/mL and the final contents of the media in each plate are recommended as follows: 6 well plate, 2 mL; 24 well plate, 500 μL; and 96 well plate, 200 μL. After the cell numbers reached a level of 70-80% confluence, the culture media were exchanged for α-MEM supplemented with 10% FBS, 5 mM β-glycerol phosphate,

and 50 μL/mL ascorbic acid (differentiating medium) to induce cellular differentiation. Then, the cells were treated with 2, 4, 6, and 8 Gy using X-ray irradiation delivered at a rate of 1.5 Gy/min in the presence and absence of 5 mM 2-DG or 10 μM QCT. Fresh medium was supplied to the cells at 3-day intervals depending on the experimental purpose. At various times after the irradiation, the cells were analyzed for the expression of bone mineralization genes such as ON and OP.

4. RNA preparation and RT-PCR

Total RNA was prepared from cells cultured for 3, 7, 14, 21 and 28 days after irradiation according to the manufacturer's instructions (SV Total RNA Isolation System, Promega, Madison, WI, USA). After treatment with RNase-free deoxyribonuclease I, 1 μg of total RNA was reverse-transcribed using an RNA PCR kit according to the instruction manual (the Access RT-PCR System, Promega). The reaction time was 30 min at 42°C. Aliquots of the obtained complementary DNA (cDNA) pool were subjected to PCR and amplified in a 20-μL reaction mixture using Taq polymerase. The amplifications were performed with a DNA thermal cycler (PTC-100, MJ Research, Watertown, MA) under the following conditions: an initial denaturation step at 94°C for 30-60 s, annealing at 52-62°C for 30-60 s, and extension at 72°C for 60-120 s. Positive standards and reaction mixtures lacking reverse transcriptase were used routinely as controls for each of the RNA samples. No PCR product was detected in the absence of reverse transcriptase during the RT step, indicating that the RNA preparations were free from intact genomic DNA. Specific amplificatory reaction for ON and OP cDNAs was performed. PCR products were electrophoresed in 1-2% agarose gels, and the amplified DNA fragments were visualized by ethidium bromide staining under UV light. The PCR primer sequences were used as follows.

- Osteonectin (~ 480 bps)
Forward primer 5'-TGGTCACCTTGTACGAGAGA-3'
Reverse primer 5'-GGTGTTAGCAGCTTATCCAC-3'
- Osteopontin (~ 450 bps)
Forward primer 5'-TGGTCACCTTGTACGAGAGA-3'
Reverse primer 5'-GGTGTTAGCAGCTTATCCAC-3'
- GAPDH (~ 450 bps)
Forward primer 5'-CACCACCATGGAGAAGGCCG-3'
Reverse primer 5'-GAACACGGAAGGCCATGCCA-3'

5. Statistical analysis

Data were expressed as the mean ± the standard deviation.

One-way analysis of variance (ANOVA) was used for multiple comparisons. A value of P<0.05 was considered significant.

Results

1. Expression patterns of osteonectin and osteopontin during differentiation of MC3T3-E1 cells

Fig. 1 and Table 1 show the expression patterns of ON and OP during the differentiation of MC3T3-E1 osteoblastic cells. The mRNA expression of both ON and OP was increased according to the culture time in the differentiation medium, and increased of the genes peaked at 14 days after the differentiation induction. In the case of OP, the increase of mRNA expression was maintained to 28 days after the differentiation, while the m RNA level of ON was reduced to the basal level at the same time.

2. Expression patterns of osteonectin and osteopontin during the differentiation of irradiated MC3T3-E1 osteoblastic cells

Fig. 2 and Table 2 show the expression patterns of ON and OP during the differentiation of irradiated MC3T3-E1 osteoblastic cells. The expression patterns of ON were not changed according to the indicated radiation doses and the incubation times. On the other hand, the expression patterns of OP showed significant differences at 2 and 4 Gy 7 days after irradiation and at 2, 4, and 8 Gy 14 days after irradiation, although there was no differences 21 days after irradiation.

3. Expression patterns of MC3T3-E1 cells treated with 2-DG or QCT

Fig. 3 and Table 3 show the effects of 2-DG or QCT on the mRNA expressions of ON and OP during the differentiation of MC3T3-E1 osteoblastic cells. According to the addition of 2-DG, the expression patterns of ON and OP were not changed during experimental periods. Otherwise, when the cells were treated with QCT, the mRNA level of ON was significantly increased, especially on day 14, whereas the mRNA level of OP was significantly lower than the basal level on day 21.

4. Expression patterns of ON and OP in MC3T3-E1 cells treated with 2-DG or QCT 7 days after irradiation with the indicated doses (Gy)

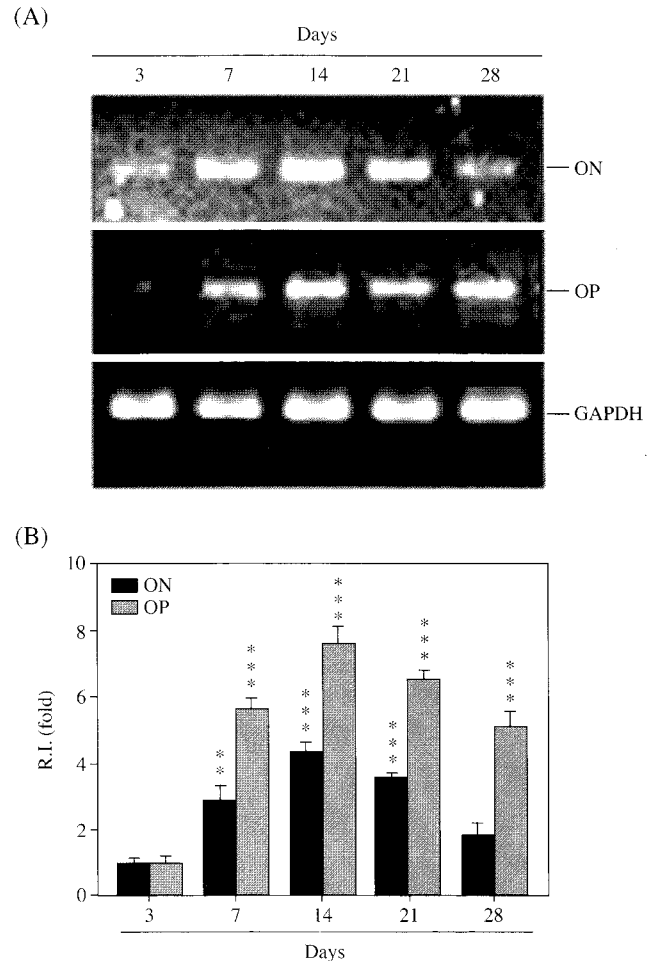Fig. 4 and Table 4 show expression patterns of ON and OP

(A)

(B)

Fig. 1. Expression patterns of ON and OP during the differentiation of MC3T3-E1 osteoblastic cells (ON, osteonectin; OP, osteopontin; R.I., relative intensity, $**P<0.01$, and $***P<0.001$ represent significant differences between the experimental and control values).

Table 1. Relative level of ON and OP mRNA expression during the differentiation of MC3T3-E1 osteoblastic cells

Days	ON	OP
3	1.00±0.16	1.00±0.20
7	2.90±0.45**	5.60±0.36***
14	4.30±0.35***	7.60±0.52***
21	3.50±0.24**	6.50±0.31***
28	1.80±0.43	5.10±0.51***

ON, osteonectin; OP, osteopontin; $**P<0.01$, and $***P<0.001$: represent significant differences between the experimental and control values.

7 days after irradiation in 2-DG- or QCT-treated MC3T3-E1 osteoblastic cells. When the cells were treated with 2-DG, the expression pattern of ON increased significantly at 4 Gy, whereas that of OP was not increased but markedly decreased at 8 Gy. In the case that QCT was added into the cells, the
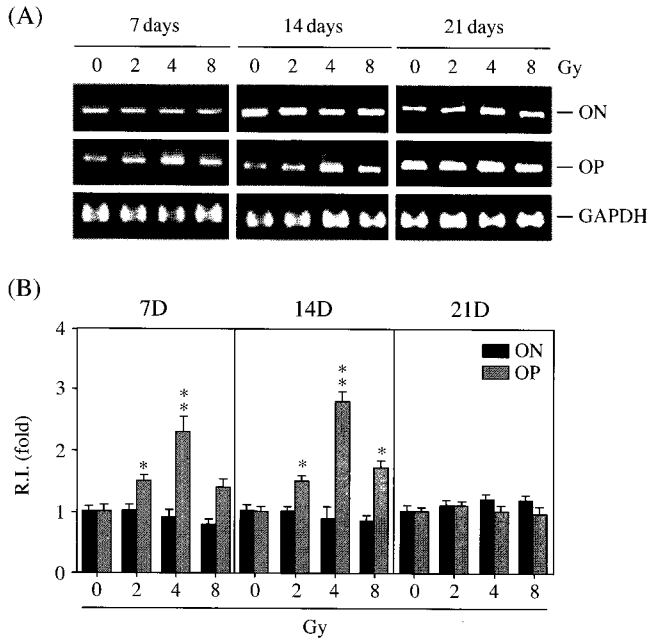
(A)

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Fig. 2. RT-PCR analysis of ON and OP mRNA during the differentiation of irradiated MC3T3-E1 osteoblastic cells (*$P < 0.05$ and **$P < 0.01$ represent significant differences between the experimental and control values).

Table 2. Relative intensity of gene expression of ON and OP during the differentiation of irradiated MC3T3-E1 osteoblastic cells

Days	Radiation dose (Gy)	ON	OP
7	0	1.00 ± 0.10	1.00 ± 0.12
	2	1.00 ± 0.12	$1.50 \pm 0.11*$
	4	0.90 ± 0.15	$2.30 \pm 0.26**$
	8	0.80 ± 0.07	1.40 ± 0.15
14	0	1.00 ± 0.12	1.00 ± 0.10
	2	1.00 ± 0.10	$1.50 \pm 0.12*$
	4	0.90 ± 0.19	$2.80 \pm 0.17**$
	8	0.85 ± 0.09	$1.70 \pm 0.15*$
21	0	1.00 ± 0.12	1.00 ± 0.08
	2	1.10 ± 0.12	1.10 ± 0.07
	4	1.20 ± 0.11	1.00 ± 0.12
	8	1.20 ± 0.08	0.95 ± 0.16

*$P < 0.05$ and **$P < 0.01$: represent significant differences between the experimental and control values.

expression pattern of OP showed a significant decrease at 8 Gy.

5. Expression patterns of MC3T3-E1 cells treated with 2-DG or QCT 14 days after irradiation

Fig. 5 and Table 5 show expression patterns of ON and OP 14 days after irradiation in 2-DG- or QCT-treated MC3T3-E1 osteoblastic cells. Irradiation adding QCT increased the mRNA expression of ON and OP in a dose-dependent manner. In
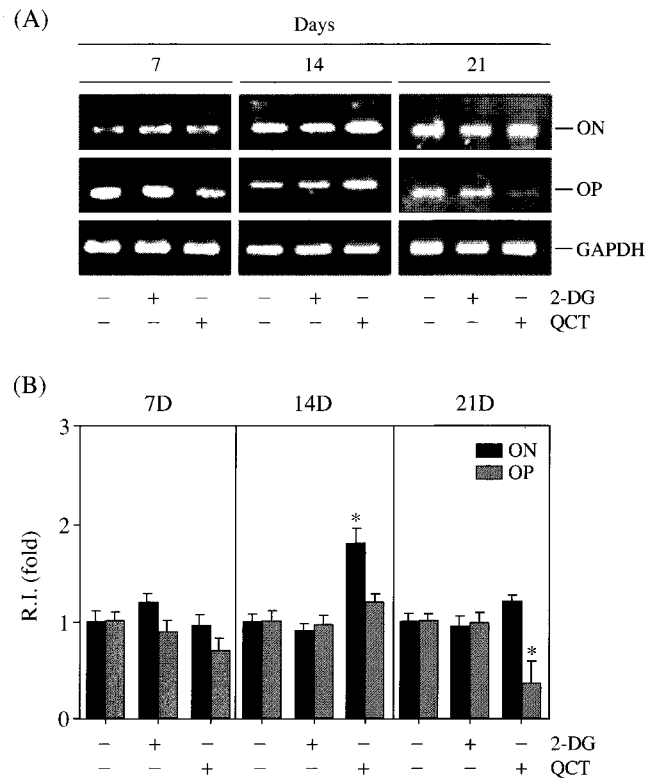
(A)

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Fig. 3. Effects of 2-DG or QCT on the mRNA expression of ON and OP during the differentiation of MC3T3-E1 osteoblastic cells (*$P < 0.05$ represents a significant difference between the experimental and control values).

Table 3. Relative intensity of ON and OP mRNA expression during the differentiation of MC3T3-E1 osteoblastic cells in the presence or absence of 2-DG and QCT

Days		ON	OP
7	No addition	1.00 ± 0.11	1.00 ± 0.10
	2-DG	1.20 ± 0.09	0.89 ± 0.12
	QCT	0.95 ± 0.12	0.68 ± 0.15
14	No addition	1.00 ± 0.08	1.00 ± 0.12
	2-DG	0.90 ± 0.07	0.96 ± 0.10
	QCT	$1.80 \pm 0.16*$	1.20 ± 0.09
21	No addition	1.00 ± 0.08	1.00 ± 0.08
	2-DG	0.95 ± 0.10	0.98 ± 0.12
	QCT	1.20 ± 0.07	$0.35 \pm 0.24*$

*$P < 0.05$: represents a significant difference between the experimental and control values.

contrast irradiation adding 2-DG did not show any differences between the control and experiments.

6. Expression patterns of MC3T3-E1 cells treated with 2-DG or QCT 21 days after irradiation

Fig. 6 and Table 6 show expression patterns of ON and OP 21 days after irradiation in 2-DG- or quercetin-treated MC3T3-
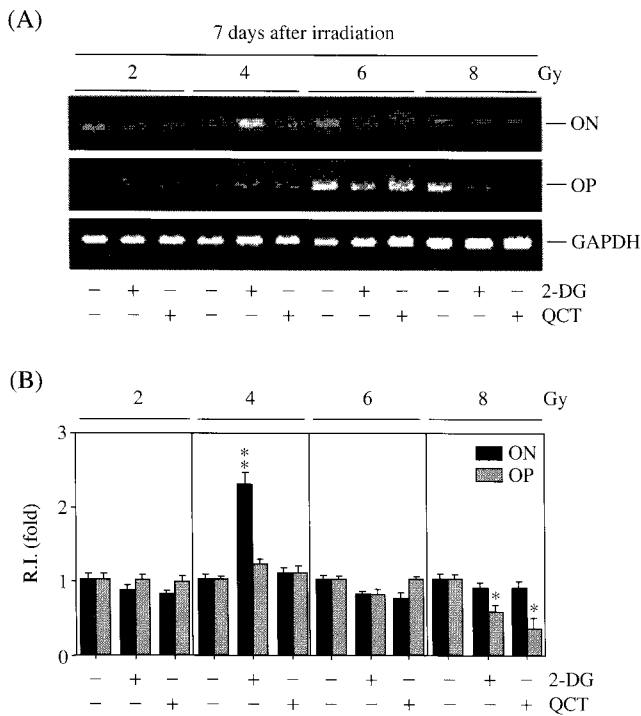
(A)

7 days after irradiation

(B)

Fig. 4. Expression patterns of ON and OP 7 days after irradiation in 2-DG or QCT-treated MC3T3-E1 osteoblastic cells (*$P < 0.05$ and **$P < 0.01$ represent significant differences between the experimental and control values).

(A)

14 days after irradiation

(B)

Fig. 5. Expression patterns of ON and OP 14 days after irradiation in 2-DG- or QCT-treated MC3T3-E1 osteoblastic cells (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ represent significant differences between the experimental and control values).

Table 4. Relative intensity of expression patterns of ON and OP 7 days after irradiation in 2-DG- or QCT-treated MC3T3-E1 osteoblastic cells

Radiation dose (Gy)		ON	OP
2	No addition	1.00±0.10	1.00±0.08
	2-DG	0.85±0.08	1.00±0.10
	QCT	0.81±0.06	0.98±0.08
4	No addition	1.00±0.10	1.00±0.06
	2-DG	2.30±0.15**	1.20±0.10
	QCT	1.10±0.09	1.10±0.10
6	No addition	1.00±0.08	1.00±0.07
	2-DG	0.80±0.07	0.80±0.09
	QCT	0.75±0.10	1.00±0.07
8	No addition	1.00±0.10	1.00±0.08
	2-DG	0.90±0.09	0.56±0.14*
	QCT	0.90±0.10	0.34±0.18*

*$P < 0.05$ and **$P < 0.01$: represent significant differences between the experimental and control values.

Table 5. Relative intensity of ON and OP mRNA expression 14 days after irradiation in 2-DG- or QCT-treated MC3T3-E1 osteoblastic cells

Radiation dose (Gy)		ON	OP
2	No addition	1.00±0.15	1.00±0.10
	2-DG	1.30±0.20	0.92±0.12
	QCT	1.50±0.23	1.60±0.13*
4	No addition	1.00±0.09	1.00±0.12
	2-DG	0.95±0.13	0.95±0.12
	QCT	1.10±0.14	1.50±0.11*
6	No addition	1.00±0.10	1.00±0.10
	2-DG	1.10±0.12	1.20±0.09
	QCT	3.10±0.30**	3.50±0.29**
8	No addition	1.00±0.13	1.00±0.12
	2-DG	0.90±0.11	0.95±0.11
	QCT	5.20±0.32***	5.80±0.26***

*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$: represent significant differences between the experimental and control values.

E1 osteoblastic cells. Irradiation adding QCT significantly increased the expression patterns of ON at 2, 4, 6, and 8 Gy, and those of OP at 6 Gy. Irradiation adding 2-DG increased the expression patterns of ON at 2 Gy and decreased those of OP at 8 Gy.
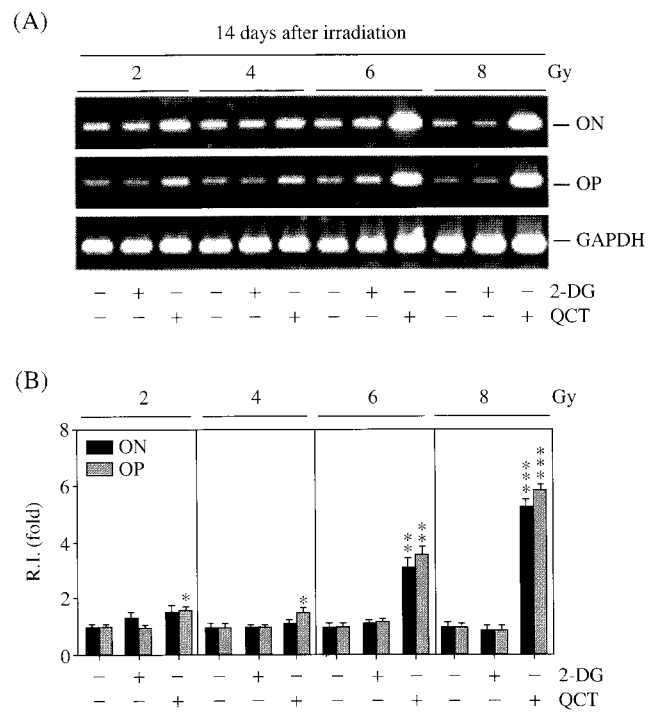
Discussion

Osteoblasts are bone-forming cells that are responsible for the production of the bone's extracellular matrix during intramembranous ossification, the remodeling or healing of bone.
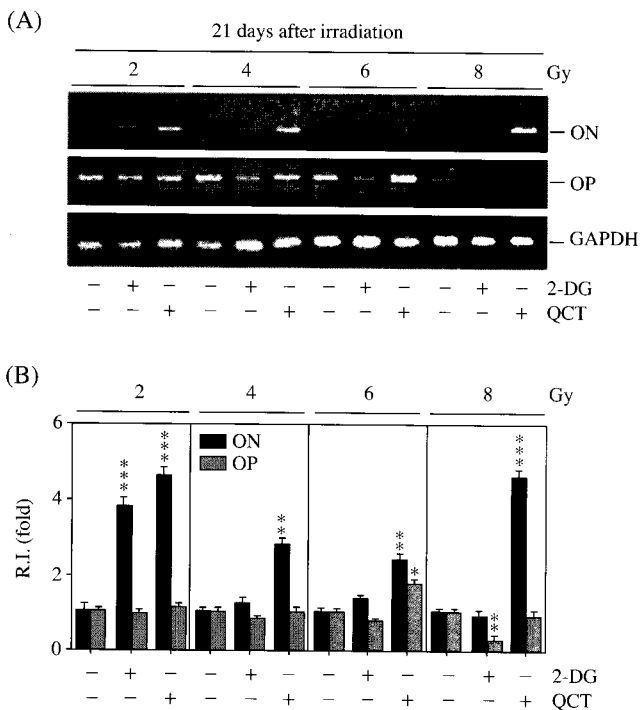
(A)

(B)

Fig. 6. Expression patterns of ON and OP 21 days after irradiation in 2-DG or QCT-treated MC3T3-E1 osteoblastic cells (*$P<0.05$, **$P<0.01$, and ***$P<0.001$ represent significant differences between the experimental and control values).

Table 6. Relative intensity of expression patterns of ON and OP 21 days after irradiation in 2-DG or QCT-treated MC3T3-E1 osteoblastic cells

Radiation dose (Gy)		ON	OP
2	No addition	1.00±0.21	1.00±0.09
	2-DG	3.80±0.26***	0.96±0.08
	QCT	4.60±0.29***	1.11±0.11
4	No addition	1.00±0.12	1.00±0.10
	2-DG	1.20±0.12	0.81±0.10
	QCT	2.80±0.18**	1.00±0.14
6	No addition	1.00±0.13	1.00±0.13
	2-DG	1.30±0.12	0.75±0.09
	QCT	2.40±0.18**	1.80±0.12*
8	No addition	1.00±0.10	1.00±0.09
	2-DG	0.90±0.19	0.21±0.20**
	QCT	4.60±0.23***	0.86±0.20

*$P<0.05$, **$P<0.01$, and ***$P<0.001$: represent significant differences between the experimental and control values.

In the process of bone formation, it is necessary for osteoblasts to proliferate, differentiate and induce mineralization of the bone's extracellular matrix. This series of events is modulated by the sequential phenotypic gene expression of the osteoblasts, including those of type I collagen, alkaline phospha-

tase, OP, ON, and osteocalcin.[17-19] These sequential expressions are regulated by systemic and local factors such as parathyroid hormones, 1,25-dehydroxy vitamin D3, bone morphogenic proteins, transforming growth factor-β and fibroblast growth factor.[10,20-23] Owen et al.[10] found that the expression patterns of bone-related proteins were regulated in a temporal manner during the successive developmental stages, including proliferation (days 4-10), bone matrix formation/maturation (days 10-16), and mineralization stages (days 16-30). The alteration in this developmental progression was marked by distinct changes in the gene expression and biochemical parameters which are necessary for the cells to mature and organize into characteristic bone nodules.[21]

This study was carried out when cell proliferation was already 70-80% complete; therefore, the first 7 days were regarded as the maturation period and the period of day 14-21 was regarded as the calcification period.

Ionizing radiation may decrease successful osseous repair by altering cytokine expression profiles resulting from or leading to a change in the osteoblastic differentiation state.[24] These changes may, in turn, cause alterations in osteoblast proliferation and extracellular matrix formation.[25]

The exposure of MC3T3-E1 osteoblastic cells to ionizing radiation resulted in a dose-dependent decrease of cellular proliferation and a promoted cellular differentiation.[20,26] A review of pertinent literature showed that ionizing radiation suppressed normal osteoblast proliferation.[20] Choi et al.[9] showed that the expression pattern of ON was active around day 10 and maintained its level of expression throughout the culturing period (up to day 28 on MC3T3-E1 osteoblastic cells). The expression of these bone matrix proteins was very active in the intermediate period is between proliferation and mineralization.[9] In addition to the presence of the proliferation period, several reports indicate that the proper deposition of the extracellular matrix is required for bone cell differentiation. For example, if proliferation is inhibited by TGF-β_1, cells do not differentiate normally. If collagen is not produced properly, mineralization is not observed.[21]

OP is known to have an adhesive function. There is also the interesting possibility that OP may play a role in tumor cell invasion, metastasis, and processes in which adhesive interactions between tumor cells and the extracellular matrix are critical.[27] For example, there has been a report of increased plasma levels of OP in human breast cancer.[11] Yoshitake et al.[28] reported that OP knockout mice are resistant to ovariectomy-induced bone resorption compared with wild-type mice. The expression of OP reached 25% of its peak value on days 5

to 7, or the proliferation stage.[10] On the other hand, ON is a modular glycoprotein that is expressed in bone and other tissues undergoing active remodeling.[11,29] It can be regarded as a marker in the differentiation of bone-forming cells.[29] Although the precise role of ON in bone and other tissues has not been defined, in vitro studies suggest that ON has pleiotropic effects on gene expression.

The maximal levels of bone matrix protein mRNAs such as type I collagen, $TGF-\beta_1$, fibronectin, and ON are shown during the period of day 10-16.[9] It is likely that the expression of type I collagen and $TGF-\beta_1$ in bone formation may have an important role in the early and middle stages, whereas the expression of fibronectin and ON may have an important role in the late stage of osteoblast differentiation.[9] Choi et al.[9] reported that the expression of OP showed a peak value on day 21.

In this study, the expressions of OP and ON showed peak values on day 14. As irradiation was applied, the expression of OP showed significant differences, but the expression of ON showed no differences. Therefore 2, 4, and 8 Gy of irradiation affected the expression patterns of OP during the differentiation of MC3T3-E1 osteoblastic cells.

QCT belongs to a group of polyphenolic substances known as flavonoids.[30] Flavonoids, which are widely distributed in the plant kingdom, have recently received attention as cancer-preventive substances because of their strong antioxidative activity and free radical scavenging potency. In addition to their anticarcinogenic activity, flavonoids have demonstrated antimicrobial, anti-inflammatory-allergic, and antimutagenic properties in studies.[31] QCT, an inhibitor of heat-shock response, dose-dependently suppressed the p53 accumulation induced by X-rays.[32] Shimoi et al.[31] investigated the anticlastogenic effect of 12 flavonoids on micronucleus induction in γ-ray irradiated rats and showed that flavonoids have an inhibition mechanism on γ-ray irradiated rats.

2-DG is a glucose antimetabolite and an inhibitor of the glycolytic pathway. 2-DG has been shown to enhance radiation-induced chromosome aberrations, micronucleus formation and lethality in tumor cells and to reduce these in normal tissues.[33] Therefore, it has been suggested as a suitable candidate for radiotherapy. The exact mechanism underlying the differential modification of radiation damage by 2-DG is largely unknown.[34] Bidder et al.[35] reported that OP expression in cultured vascular smooth muscle cells is reciprocally regulated by glucose and 2-DG.

This study was undertaken to further investigate the effects of 2-DG or QCT on the expressions of ON and OP in irradi-ated in vitro MC3T3-E1 osteoblastic cells.

Irradiation adding QCT increased the expressions of ON and OP dose-dependently 14 days after irradiation, but irradiation adding 2-DG showed no difference. QCT also enhanced the expression of ON significantly 21 days after irradiation at 2, 4, and 8 Gy, but irradiation adding 2-DG showed no difference, except a significant increase in the expression pattern of ON at 2 Gy. Therefore, QCT may act as a radiosensitizer in the gene expression of ON and OP during the late stage of differentiation in irradiated MC3T3-E1 cells in vitro.

The expressions of ON and OP during the differentiation of irradiated MC3T3-E1 osteoblastic cells were modified by 2-DG and QCT. The expressions of ON or OP were higher in RA+QCT than in other groups. Thus, this suggests that QCT acted as a radiosensitizer in the expression of ON or OP in irradiated in vitro MC3T3-E1 osteoblastic cells.

Efficient and accurate rejoining of DNA breaks is crucial for the maintenance of genomic stability and for cell survival. The deleterious effects of ionizing radiation have been considered to result from the deposition of energy in the cell nucleus resulting in damage to the nuclear DNA with a major role in the induction of DNA double-strand breaks.[36]

In vitro studies cannot clarify whether the deleterious effects are the direct consequences of irradiation on osteoblasts or secondary effects from radiation damage to surrounding tissues.[36] In addition, osteoblasts undergo several stages of differentiation, which makes it difficult to investigate the direct effects of radiation on each stage.[9] Furthermore, when compared with in vitro studies, the in vivo data showed less damage per cell and fewer cells demonstrating chromosomal instability. This difference can be attributed largely to the cellular defence mechanisms that have evolved to recognize and remove aberrant cells.[37] Hereafter, in vivo study as well as in vitro study should be investigated considering variations such as radiation-induced genomic instability, radiation-induced bystander effects and cellular defence mechanisms.

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