Effects of 2-deoxy-D-glucose and quercetin on cytokine secretion and gene expression of type I collagen during osteoblastic differentiation in irradiated MC3T3-E1 cells

Haeng-Un Song, Hyoun-Suk Ahn, Sang-Rae Lee*, Kwang-Joon Koh

Department of Oral and Maxillofacial Radiology, School of Dentistry, and Institute of Oral Bio Science, Chonbuk National University
**Department of Oral and Maxillofacial Radiology, College of Dentistry, Kyung Hee University*

ABSTRACT

Purpose : To characterize the effects of 2-deoxy-D-glucose (2DG) and quercetin (QCT) on cytokine secretion of IL-6, TGF-β and gene expression of Col I in irradiated MC3T3-E1 cells

Materials and Methods : The MC3T3-E1 cells were cultured in an α-MEM supplemented with 5 mM 2DG or 10 mM QCT and then the cells were incubated 12h before irradiation with 2, 4, 6, and 8 Gy X-ray using a linear accelerator delivered at a dose rate of 1.5 Gy/min. Level of IL-6 and TGF-β was determined by ELISA. Also expression of Col I was examined by RT-PCR.

Results : In accordance with the radiation dose, the amount of TGF-β was not different in RA+QCT, but it showed a peak value in control and RA+2DG at 4 Gy on the 3rd day. However, all groups showed a decreasing tendency dose-dependently in RA+QCT on the 7th day ($p < 0.01$). In accordance with the radiation dose, the amount of IL-6 increased dose-dependently in all groups on the 3rd day. On the 7th and 21st day, all groups showed peak values at 4 Gy. RA+QCT showed a slightly increased amount of IL-6 at 2 Gy, but it showed a slightly decreased amount at 4, 6, and 8 Gy. In accordance with the period of culture after irradiation, the expression of Col I increased dose-dependently in RA+QCT.

Conclusion : The result showed that QCT acted as radiosensitizer in the secretion of TGF-β and gene expression of Col I during differentiation in irradiated MC3T3-E1 cells at the cellular level. (***Korean J Oral Maxillofac Radiol 2005; 35 : 191-8***)

KEY WORDS : 2-deoxy-D-glucose; Quercetin; Radiation; MC3T3-E1 Cells

Introduction

Ionizing radiation is commonly used to treat carcinomas of the head and neck. In spite of its therapeutic value, there are drawbacks to its use, especially in terms of maintenance of normal bone function such as delayed fracture healing and osteonecrosis.[1-3] These adverse side effects are consistent with the decreased osteocyte number, suppressed osteoblastic activity, and diminished vascularity observed histologically.[4,5]

Up to now, many related studies showing how bone cells respond to high or low doses of radiation have been reported. A review of pertinent literatures showed that ionizing radiation suppressed normal osteoblast proliferation.[1-3,6] It was noted that irradiated cells exhibited elevated alkaline phosphatase activity, decreased transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF) protein levels.[7] In another study, it was observed that there was an elevation in TGF-β receptor expression in irradiated cells.[2] Interleukin-6 (IL-6) has multifunctional cytokine involved in osteoclast recruitment and differentiation into mature osteoclasts, but also osteoblast-derived IL-6 is crucial to bone remodeling.[8] Szymczyk et al.[1] confirmed the observation that ionizing radiation inhibited proliferation of osteoblast-like cells. Robak and Gryglewski[9] reported that the expression of type I collagen (Col I) increased significantly 1 day after irradiation when compared with the control group.

2-deoxy-D-glucose (2DG), a non-metabolizable glucose analogue, is known as an inhibitor of glucose transport and glycolysis.[10] It has been shown to enhance the radiomodifying effects. Quercetin (QCT), one of the most abundant flavonoids, has comes into the spotlight as a cancer-preventive substance.[9,11] In recent studies,[12,13] it was reported as being an inhibitor of radiation effects.

The purpose of this study was to characterize the effects of

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Correspondence to : Prof. Kwang-Joon Koh
Department of Oral and Maxillofacial Radiology, School of Dentistry, 634-18, Keum-Am Dong, Duk-Jin Gu, Jeon-Ju, Chonbuk 561-712, Korea
Tel) 82-63-250-2023, Fax) 82-63-250-2081, E-mail) radkoh@chonbuk.ac.kr

2DG and QCT on the cytokine secretion of interleukin-6 (IL-6) and TGF-β; both which are produced during cell proliferation and gene expression of Col I in irradiated MC3T3-E1 cells.

Materials and Methods

1. Chemicals and laboratory wares

Unless otherwise specified, chemicals and laboratory wares 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 a 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and antibiotics. The cultures were maintained at 37°C with a gas/air mixture of 5% CO_2/ 95% O_2. Subcultures were performed with 0.05% trypsin-0.02% EDTA in Ca^{2+}, Mg^{2+}-free phosphate buffered saline (DPBS; Gibco BRL Co., USA). 1×10^6 cells/ml were resuspended in either a 2 ml or 100 ml 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 differentiation induction

MC3T3-E1 cells were replated in 6- or 96-well flat-bottomed plates at a dose of 5×10^5 cells/ml and the final contents of media in each plate were to be recommended as follows: 6-well plate, 2 ml, 24-well plate, 500 ml and 96-well plate, 200 ml. After the cell numbers reached a level of 70-80% confluence, culture media was changed with α-MEM and supplemented with 5 mM 2-DG or 10 mM QCT. The cells were then incubated 12h before irradiation with 2, 4, 6, and 8 Gy using X-ray delivered at a dose rate of 1.5 Gy/min using a linear accelerator (Mevaprimus, Germany). After that, cells were incubated in α-MEM and supplemented with 10% FBS, 5 mM β-glycerol phosphate, and 50 mg/ml ascorbic acid. Fresh medium was supplied to cells at 3 day intervals depending on experimental purpose. At various times after irradiation, the cells were processed for the analyses of cytokine production and gene expression of bone-related protein, Col I.

4. Measurement of cytokine levels

The amount of cytokines produced by the control or irradiated MC3T3-E1 cells was determined by ELISA which was provided by the Bank for Cytokine Research (Chonbuk National University, Jeonju, Korea) as described previously. MC3T3-E1 cells cultured in 24-well tissue culture plates were exposed to various doses (2, 4, 6, and 8 Gy) of X-ray, and at various times the culture supernatants were collected. Levels of cytokines such as IL-6 and TGF-β were determined by ELISA. The amount of cytokines produced was calculated from standard curves generated using known concentrations of recombinant cytokine proteins.

5. RNA preparation and RT-PCR

Total RNA was isolated from control and irradiated MC3T3-E1 cells according to the manufacturer's instructions. After treating with RNase-free deoxyribonuclease I, 1 μg of total RNA was reverse-transcribed using an RNA PCR kit according to the instruction manual. The reaction time was 30 min at 42°C. Aliquots from 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, USA) under the following conditions : initial denaturation step at 94°C for 5 min and 30 cycles of denaturation at 94°C for 30-60s, annealing at 52-60°C for 30-60s, and extension at 72°C for 60-120s. 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. Amplification reaction specific for Col I 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 followed.

-GAPDH (450 bps)
Forward primer (5′-3′) CAC CAC CAT GGA GAA GGC CG
Reverse primer (5′-3′) GAA CAC GGA AGG CCA TGC CA

-Type I collagen (950 bps)
Forward primer (5′-3′) ACA CTT TCA CTC CAATCG TCC
Reverse primer (5′-3′) TGC CCT TTC CGT TGT TGT CC

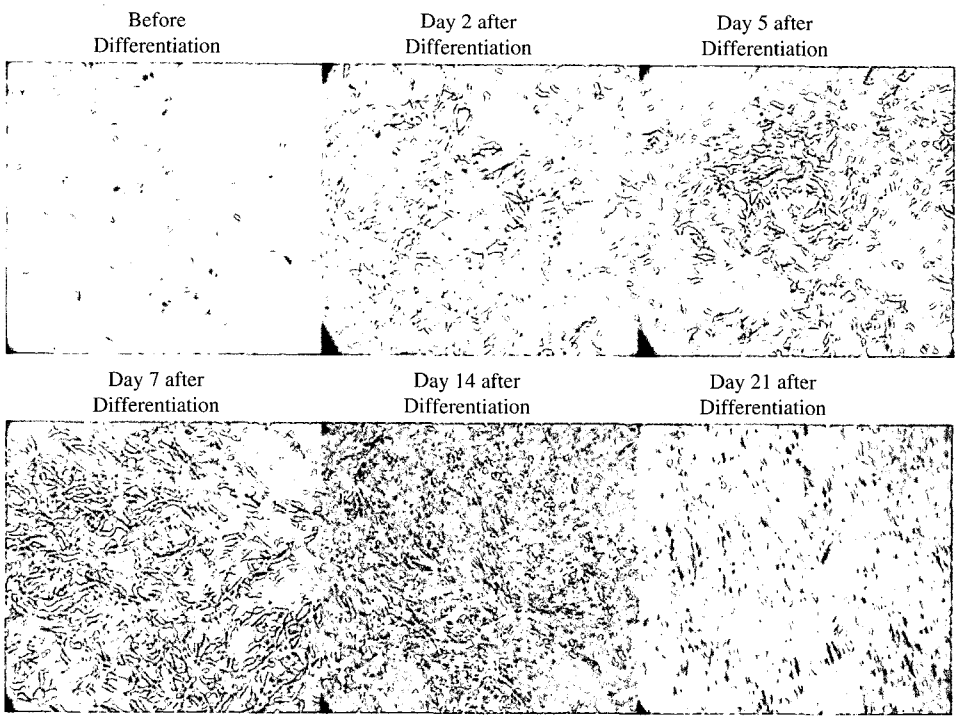

Fig. 1. Morphological changes according to differentiation.

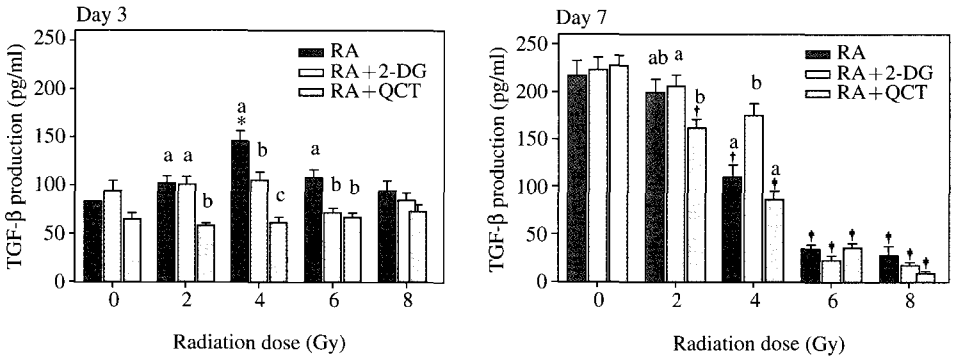

Fig. 2. Cytokine assay : TGF-β production (pg/ml) (RA : irradiation group, RA+2DG : irradiation group treated with 2DG, RA +QCT : irradiation group treated with quercetin).

6. Statistical analyses

The results were expressed as a mean±standard error (SE). One-way ANOVA using SPSS ver. 10.0 software was used for multiple comparisons. In addition, statistical analysis was performed using ANOVA, and followed by Duncan's test where a value of $p<0.05$ was considered significant.

Results

Fig. 1 showed morphological changes in accordance with the differentiation of murine osteoblastic MC3T3-E1 cells.

Fig. 2 showed the amount of TGF-β in accordance with the radiation dose. It showed a peak value on the 7th day after irradiation.

On the 3rd day after irradiation, the irradiation group (RA) revealed a dose-dependent increasing amount at 4 Gy. But, there was no difference between the group of irradiation with 2DG (RA+2DG) and QCT (RA+QCT). RA and RA+2DG demonstrated higher levels of TGF-β than RA+QCT at 2 Gy ($p<0.05$). All groups showed peak values at 4 Gy ($p<0.05$).

RA at 6 Gy demonstrated an increasing amounts of TGF-β than other groups ($p<0.05$).

On the 7th day, RA showed different amount of TGF-β at 4, 6, and 8 Gy. Amounts decreased depending on the dose.

At 2 and 4 Gy, RA+2DG demonstrated a higher amount of TGF-β than RA. However it was decreased than in 6 and 8 Gy. RA and RA+QCT showed decreasing amounts of TGF-β at 4 Gy. But, there was no difference between each group at 6 or 8 Gy.

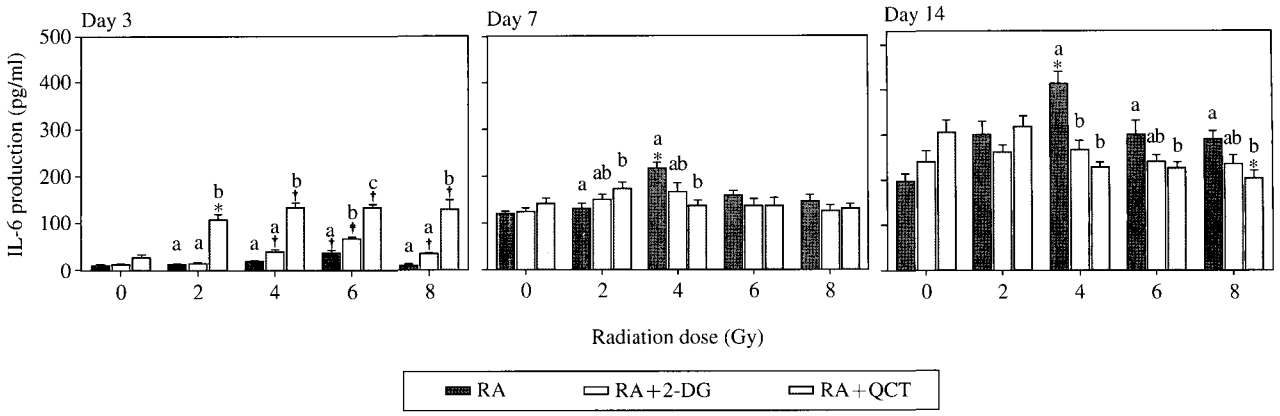

Fig. 3. Cytokine assay: IL-6 production (pg/ml).

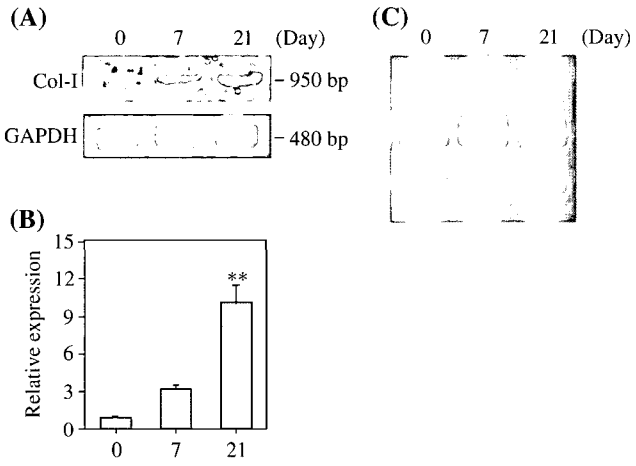

Fig. 4. RT-PCR of type I collagen (without irradiation).

On the 3rd day after irradiation, RA+QCT showed no specific variation in accordance with the radiation dose. However, on the 7th day after irradiation, the amount of TGF-β expression was less than other groups.

Fig. 3 showed amounts of IL-6 in accordance with the radiation dose.

In comparison with the amount of IL-6 in accordance with the radiation dose, RA showed no variation at 2 or 4 Gy, but each group showed an increasing amount of IL-6 at 6 Gy on the 3rd day after irradiation. However, it decreased at 8 Gy. RA+2DG showed no changes at 2 Gy, but it increased at 4 and 6 Gy. RA+QCT demonstrated increasing amounts of IL-6 at 2, 4, 6, and 8 Gy.

On the 7th day after irradiation, each group showed increasing expressions of IL-6, especially at 2 and 4 Gy.

On the 14th day, it showed increasing expression of IL-6. In accordance with the radiation dose, RA demonstrated a peak value at 4 Gy. 2DG did not affect radiation effect, but QCT increased the expression of IL-6 at 2, 4, and 6 Gy. However it decreased at 8 Gy statistically.

In comparison, each group in accordance with the radiation dose, RA showed higher amounts of IL-6 than RA+2DG and RA+QCT at 4, 6, and 8 Gy.

Fig. 4 showed RT-PCR of Col I without irradiation.

On the 21st day, the expression of Col I increased remarkably.

Table 1. RT-PCR of relative expression level of type I collagen after irradiation

Day	Radiation dose (Gy)	RA	RA+2DG	RA+QCT
3	0	1.00±0.26	1.40±0.10	1.30±0.20
	2	1.00±0.26	1.53±0.23	1.97±0.31*
	4	1.00±0.20	1.67±0.25	5.07±1.36†
	6	1.00±0.30	1.10±0.26	1.50±0.26
	8	1.00±0.26	2.37±0.49*	2.63±0.31†
7	0	1.00±0.20	0.87±0.20	1.27±0.21
	2	1.00±0.20	1.17±0.32	1.87±0.21*
	4	1.00±0.34	1.47±0.31	3.03±0.47†
	6	1.00±0.26	0.83±0.23	1.33±0.23
	8	1.00±0.36	1.20±0.53	1.30±0.26
14	0	1.00±0.30	1.10±0.20	1.10±0.26
	2	1.00±0.30	1.30±0.44	1.40±0.20
	4	1.00±0.26	2.70±0.42	6.80±1.20†
	6	1.00±0.26	0.80±0.25	2.90±0.55†
	8	1.00±0.43	0.60±0.20	6.50±1.03†
21	0	1.00±0.35	0.90±0.31	1.10±0.26
	2	1.00±0.17	0.70±0.37	1.70±0.20*
	4	1.00±0.17	0.80±0.20	1.40±0.31
	6	1.00±0.26	0.80±0.20	2.40±0.36†
	8	1.00±0.17	0.60±0.20	1.23±0.15

RA : Irradiation group (control), RA+2DG : Irradiation with 2DG, RA+QCT : Irradiation with QCT, *,†,† : statistically significant ($p<0.05$, $p<0.01$, $p<0.001$).

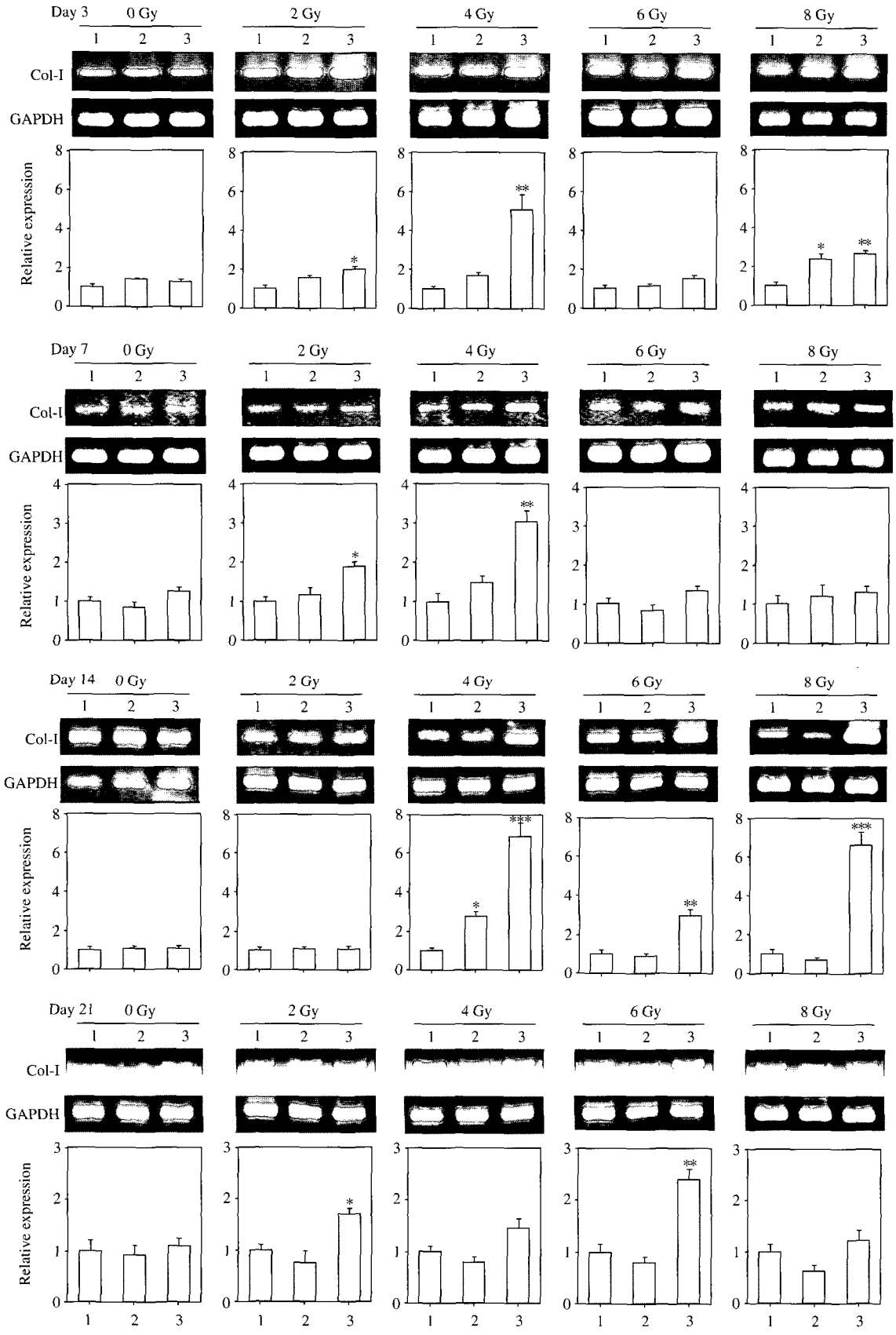

Fig. 5. RT-PCR of type I collagen after irradiation (1 : irradiation group, 2 : irradiation group treated with 2DG, 3 : irradiation group treated with quercetin).

Fig. 5 and Table 1 displayed RT-PCR of relative expression level of Col I after irradiation. On the 3rd day, each group showed similar expression levels than without irradiation group, but RT+QCT showed increasing expression of Col I at 2 and 4 Gy. This result was similar to that of the 7th day.

RA+2DG and RA+QCT showed statistical differences at 8 Gy.

On the 14th day, RA+QCT demonstrated increasing amounts of Col I with RT-PCR at 4, 6, and 8 Gy.

On the 21st day, RA+QCT showed an increasing expression of Col I at 2, 4, 6, and 8 Gy.

Discussion

Osteoblasts, bone-forming cells go through many phases in its life-span, from early commitment to organic-matrix production, mineralization and apoptosis or terminal differentiation into an osteocyte. And its gene-expression profile varies widely depending on the differentiation stage. In the process of bone formation, it is necessary for osteoblasts to proliferate, differentiate, and induce mineralization of the bone extracellular matrix.[14,15] This series of events is modulated by sequential phenotypic gene expression of the osteoblasts. For example, the expressions of cell cycle or growth-related genes such as histone, c-fos, c-myc, and extracellular matrix genes such as Col I, fibronectin, and TGF-β1 were increased and followed by the bone mineralization genes such as osteocalcin during the bone formation.[16,17]

Especially, TGF-β is a multifunctional cytokine and has been strongly implicated in the regulation of osteoblast proliferation, migration, and differentiation.[18] In addition, these growth factors have potent effects on extracellular matrix synthesis, deposition, and degradation. These effects are modulated through complex biologic interactions involving other growth factors and endogenous proteases. For example TGF-β1, the largest source of which is bone, is secreted by osteoblasts and stored in high concentrations in the bone extracellular matrix.[19] In vivo, higher levels of TGF-β1 secretion have been noted during the early stages of bone healing and during mandibular distraction osteogenesis.[18,20] Furthermore, the use of recombinant TGF-β1 protein augments osteogenesis by accelerating fracture healing and closing critically-sized bone defects.[21,22] Thus, the TGF-β family is an attractive target for investigation because alterations in TGF-β production may be, in part, responsible for or associated with the abnormal osteoblastic phenotype observed in irradiated tissues. Also Sporn et al.[23] have suggested that TGF-β works in conjunction with other cytokines to modulate their effects, be it stimulation or inhibition.

Duziak et al.[7] found that the TGF-β biologic activity of osteoblastic cells was dose-dependently decreased after high-dose irradiation.

In this study, the amount of TGF-β was not decreased in accordance with the radiation dose. On the 3rd day, it increased at 4 Gy, but it decreased on the 7th day.

RA+2DG showed a decreased amount of TGF-β at 2 and 4 Gy, and decreased significantly at 6 Gy. RA+QCT showed that it is dose dependent. This data supports that ionizing radiation has significant effects on the production of cytokines by osteoblasts and may provide a molecular mechanism by which altered bone-healing occurs in irradiated osseous tissues. It also showed the correspondence of this result with hypothesis that the TGF-β growth factors have been shown to act as potent modulators of osteoblastic differentiation by preventing terminal differentiation.

Interleukins were first identified as cytokines that regulate hemopoietic cell function and proliferation. In particular, IL-6 had multiple, partially overlapping functions, including effects on hemopoiesis and modulation of immune responses.[24] Osteoblast-derived IL-6 is crucial to bone remodeling-since excess IL-6 production predisposes to increased osteoclast number.[8] It is produced at high levels by cells of the stromal/osteoblastic lineage in response to stimulation by a variety of other cytokines and growth factors such as IL-1, tumor necrosis factor (TNF), TGF-β, platelet-derived growth factor (PDGF), and Insunlin-like Growth Factor-11 (IGF-11).[25,26]

Nakchbandi et al.[24] demonstrated that increased IL-6 production in response to PTH resulted in decreased circulating IL-11 levels due to down-regulation of IL-11 production. Kim et al.[27] studied that the expression levels of cytokines and growth factor genes from mesenchymal stem cells and their differentiated cell types. More recently, from a genetic approach, Sims et al.[28] demonstrated that IL-6 supports osteoblast generation through the gp-130-STAT 1/3 pathway. In addition, their study readdressed IL-6 function in bone formation in conditions of increased bone remodeling. Franchimont et al.[29] demonstrated that IL-6 was not essential for bone remodeling in physiological conditions, but this cytokine played a role in osteoblast generation in conditions of high bone turnover. Webb et al.[8] noted that p38 MAP kinase activity was increased in MG-63 cells in response to stimulation with TNF-α, and that the increased activity led to enhanced IL-6 secretion. These results may aid in the search for a better understanding of the cellular and molecular mech-

anism involved in IL-6 production in osteoblasts.

In this study, the amount of IL-6 increased during the culture period was similar to previous studies. In particular, on the 3rd day, RA+QCT showed a remarkable increased in the amount of IL-6. However on the 7th and 14th days, RA+QCT did not show differences. All groups demonstrated a peak value at 4 Gy. This result showed that QCT influenced production of IL-6 in the early stage of osteoblast differentiation.

In connective tissue, cell structure contributed to Col I expression,[30] especially the fully differentiated osteoblasts. They produced and secreted proteins that constituted the mineralized bone matrix under the control of the same cells. A major product of the bone-forming osteoblast is Col I. This polymeric protein was initially secreted in the form of a precursor which contained peptide extensions at both the amino-terminal and carboxyl ends of the molecule.[26] Choi et al.[9] showed that expression of Col I increased significantly 1 day after irradiation when compared with the control group. Quales et al.[31] examined the growth conditions necessary for osteoblast phenotype expression, and the time course of extracellular matrix accumulation. In their results, Col I was produced at the initial stage of development. This stage was characterized by cell proliferation and high level of Col I gene expression, biosynthesis, and secretion. In this study, the absolute value of expression of Col I could not measured. The authors examined an additive effect of 2DG and QCT to the irradiation effect.

2DG, a well-known glucose analogue, inhibits the glycolysis of glucose in a variety of normal and tumor tissue.[10] QCT, a well-known flavonoid, has recently been focused on as a cancer-preventive substance because of its strong antioxidative activity and free radical scavenging potency.[30,31] Also it plays a role radioprotective effect.[12,32,33]

Primarily, 2DG blocks glycolysis and inhibits protein glycosylation. It has been tested in multiple studies for possible application as an anticancer, or antiviral therapeutic. The inhibitory effect of 2DG on ATP generation made it a good candidate molecule as a calorie restriction mimetic as well.[33] Furthermore, 2DG selectively inhibits DNA repair in cells with high rates of glycolysis like cancer cells, thereby enhancing the radiation damage. The radiomodifying effects of 2DG could possibly be mediated by changes in the metabolic flux and reduction of energy flow due to the inhibition of glycolysis.[13]

However, in this study the irradiation effect was not influenced by 2 DG statistically. It is supposed that the cells used in this study were normal osteoblastic cells.

QCT belongs to a group of polyphenolic substances known as flavonoids. Krishnamutry and Simpson[34] have conducted studies with oxygen 18 on the action of dioxygenase on quercetin by Aspergillus flavus. The reports[35,36] demonstrated that QCT acted as a protective effect against ultraviolet. Bovy et al.[37] showed that ectopic expression of combinations of transcription factor genes was a powerful way to influence metabolic pathways to plants. Izzard et al.[38] proposed that given the involvement of DNA-PK, quercetin radiosensitize mammalian cells in culture. It has an attractive potential application for such inhibitory drugs was the enhancement of the efficacy of cancer chemotherapy and radiotherapy. Murata et al.[39] suggested that the antioxidative activities of the flavonoids were significantly higher that those of buthylated hydroxytoluene, α-tocopherol, ascorbic acid, and the antioxidative activity of quercetin to suppress the degradation of DHA-Et (ethyl 4, 7, 10, 13, 16, 19-docosahexaenoate) embedded in liposomes by irradiation with γ rays. Shimoi et al.[12] showed that the radioprotective effect of flavonoids in mice may be attributed to the hydroxyl radical scavenging potency in a direct or an endo-genous enzyme mediated manner.

Some reports, however showed that flavonoids caused an enhancement of X-ray induced cell damage; radiation toxicity in hepatoma cells.[40]

In this study, the expression of Col I was higher in RA+QCT than other groups. Thus, it is supposed that QCT acted as radiosensitizer in expression of Col I. It is uncertain whether it is related to the metabolism of QCT that work after digestion and absorption. Further investigation is required to clarify the mechanism and metabolic pathway of plant flavonoids in vivo. Also, it is required that a comparative study on the tumor cells as well as normal cells be made.

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