X-ray radiation at low doses stimulates differentiation and mineralization of mouse calvarial osteoblasts

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Radiotherapy is considered to cause detrimental effects on bone tissue eventually increasing bone loss and fracture risk. However, there is a great controversy on the real effects of irradiation itself on osteoblasts, and the mechanisms by which irradiation affects osteoblast differentiation and mineralization are not completely understood. We explored how X-ray radiation influences differentiation and bone-specific gene expression in mouse calvarial osteoblasts. Irradiation at 2 Gy not only increased differentiation and mineralization of the cells, but also upregulated the expression of alkaline phosphatase, type I collagen, osteopontin, and osteocalcin at early stages of differentiation. However, irradiation at higher doses (>2 Gy) did not stimulate osteoblast differentiation, rather it suppressed DNA synthesis by the cells without a toxic effect. Additional experiments suggested that transforming growth factor-beta 1 and runt-transcription factor 2 play important roles in irradiation- stimulated bone differentiation by acting as upstream regulators of bone-specific markers. [BMB Reports 2012; 45(10): 571-576]

INTRODUCTION

Radiotherapy is a useful treatment for oral cancer in combination with surgery. However, irrecoverable damage occurs to intact bone tissue after therapeutic irradiation depending on the quantity of ionizing radiation used. Persistent irradiation damage causes osteoradionecrosis followed by a loss of bone mass and an increase in bone fracture risk (1, 2).

Numerous studies have attempted to determine the mechanisms by which irradiation affects bone differentiation and mineralization. Considerable findings have shown that irradiation leads to detrimental effects on cells, as evidenced by decreased viability and/or DNA synthesis in bone-like (3-5), leukemia (6), and mesen-

chymal stromal cells (7). Irradiation also results in single or double-stranded DNA breaks and eventually blocks cell cycle progression (6). Therefore, it is believed that irradiation predominantly induces DNA damage with attendant growth inhibition and that this is responsible for suppressing osteoblast differentiation and mineralization. However, there is a great controversy on the actual effects of irradiation on bone, in which irradiation stimulates osteoblast differentiation (5, 8). This stimulation appears to be associated with increasing the levels of bone-specific regulatory markers such as alkaline phosphatase (ALP), transforming growth factor-beta 1 (TGF-β1), and runt-related transcription factor 2 (Runx2) (3, 8, 9). mRNA expression of bone differentiation-related proteins, such as type I collagen (COL I), osteocalcin (OCN), and osteopontin (OPN), is dynamically affected by irradiation, and the resulting effects differ according to the quantity of radiation employed and the times of testing after irradiation (5, 10, 11). In addition, the cellular mechanisms by which X-ray radiation affects differentiation and mineralization of osteoblasts are still unclear. Furthermore, almost all previous experiments used bone-like cell lines instead of primary osteoblast cultures.

In this study, we investigated how X-ray radiation influences differentiation and mineralization of mouse calvarial osteoblasts. We determined mRNA and/or protein expression patterns of ALP, COL I, OCN, OPN, TGF-β1, and Runx2 to explore the possible mechanisms involved in irradiation-stimulated osteoblastic differentiation.

RESULTS

Ionizing irradiation increases ALP activity and stimulates mineralization in calvarial osteoblasts based on quantity

We initially explored the effects of X-ray radiation on mineralization of osteoblasts. Exposure of the cells to low-dose radiation increased the formation of bone-like nodules at 21 days after radiation (Fig. 1A). The number of bone-like nodules was augmented up to 131% and 134% in the 1 and 2 Gy-irradiated groups, respectively, compared to that in the non-irradiated controls (Fig. 1B). However, irradiation at > 2 Gy did not stimulate cells mineralization. In parallel, the deposition of calcium ion was significantly higher in the groups exposed to 1 or 2 Gy radiation than those exposed to 4 or 8 Gy radiation as compared to that in the contrzol groups (Fig. 1C). The groups irradiated with 2 Gy, but not 4 Gy, revealed a significant in-
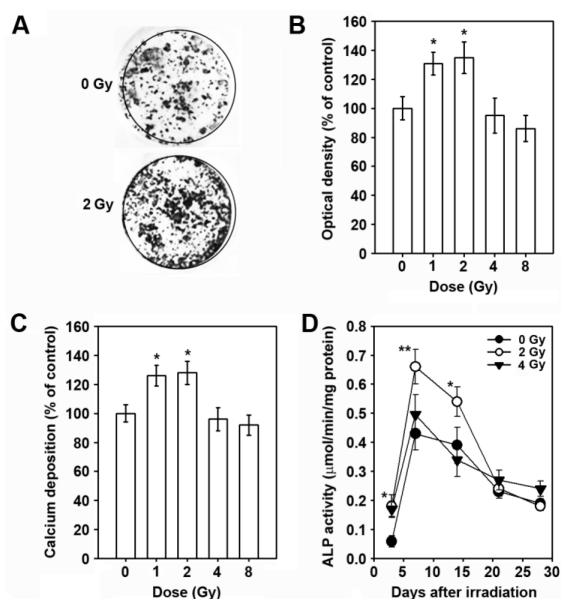
Fig. 1. Effects of ionizing irradiation on bone-like nodule formation and alkaline phosphatase (ALP) activity in calvarial osteoblasts. (A) Cells were exposed to X-ray radiation and processed for Alizarin Red staining at 21 days post-irradiation. A representative result from triplicate experiments is shown. (B) Optical density of the cultures stained with Alizarin Red was measured, and the results are expressed as a percentage of non-irradiated control levels. (C) Deposition of calcium ions in irradiated cells was determined using a Calcium C kit on post-irradiation day 21. (D) ALP activity in cell layers was determined at various times (3, 7, 14, 21, and 28 days) post-irradiation. *P < 0.05 and **P < 0.01 vs. the non-irradiated control values.

crease in ALP activity at 3, 7, and 14 days post-irradiation (Fig. 1D).

Ionizing irradiation stimulates mRNA expression of bone-specific markers in primary osteoblasts

The results from real time reverse transcription polymerase chain reaction (RT-PCR) analyses revealed that mRNA expression of osteoblast differentiation-related genes, such as ALP, COL I, and OPN, was stimulated 7 days post-irradiation, and that 2 Gy radiation led to the most prominent increase in ALP, COL I, and OPN mRNA levels (Fig. 2). The irradiation-mediated increase in ALP and COL I mRNA levels also occurred at 14 days after 2 Gy irradiation. However, exposure of the cells to 4 Gy irradiation caused a significant decrease in mRNA expression specific to ALP and OCN at 21 days after radiation.

Ionizing irradiation increases TGF-β1 and Runx2 mRNA expression at the early stages of osteoblastic differentiation

Results from the RT-PCR analysis showed that irradiation increased TGF-β1 and Runx2 mRNA expression levels at 3 days post-irradiation (Fig. 3A). These findings were supported by the results of real time-PCR in which X-ray radiation dramatically facilitated mRNA expression of both TGF-β1 and Runx2 at 3 days post-irradiation (Fig. 3B, C). When mRNA expression levels were determined at 7 days post-irradiation, TGF-β1 level had increased only in 4 Gy-irradiated cells, whereas the Runx2 mRNA level was augmented in cells irradiated with 1 or 2 Gy. Runx2 and TGF-β1 mRNA expression was not affected by X-ray radiation at 14 days post-irradiation, whereas the levels were significantly suppressed in 4 Gy-irradiated cells 21 days after radiation.

Ionizing irradiation at low doses promotes secretion of cytokines from osteoblasts without cytotoxicity

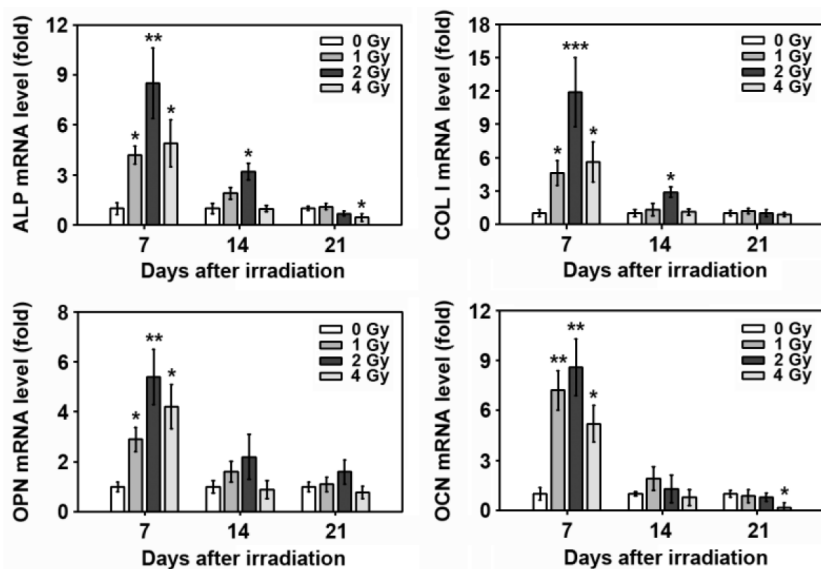TGF-β1 levels were determined in conditioned media at various

Fig. 2. Expression patterns of bone-specific markers in irradiated osteoblasts. mRNA expression of alkaline phosphatase (ALP), type I collagen (COL I), osteocalcin (OCN), and osteopontin (OPN) were analyzed by real-time RT-PCR at 7, 14, and 21 days after irradiation with the indicated quantities. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. non-irradiated control values.

times after radiation to further understand the effects of ionizing irradiation. As shown in Fig. 4A, radiating cells with 1 or 2 Gy increased TGF-β1 levels only at 3 days after radiation. A significant increase in TGF-β1 levels was observed in 4 Gy-irradiated cells 7 days after radiation, although its level was significantly reduced at

21 days after radiation. Moreover, a radiation-mediated stimulation of interleukin (IL)-6 secretion by osteoblasts was found in 4 Gy-irradiated cells at 3 and 7 days after radiation (Fig. 4B). Irradiation itself did not induce a significant decrease either in viability or cytotoxicity of the cells, as demonstrated by data from the MTT assay (Fig. 4C) and trypan blue staining (Fig. 4D). However, 8 Gy radiation significantly decreased TdR incorporation by the cells at 3 days after radiation (Fig. 4E). Irradiation augmented p21 protein levels in a dose-dependent manner, and its level was apparently higher in cells irradiated with 4 or 8 Gy than those irradiated with 2 Gy, compared to that in control cells (Fig. 4F).
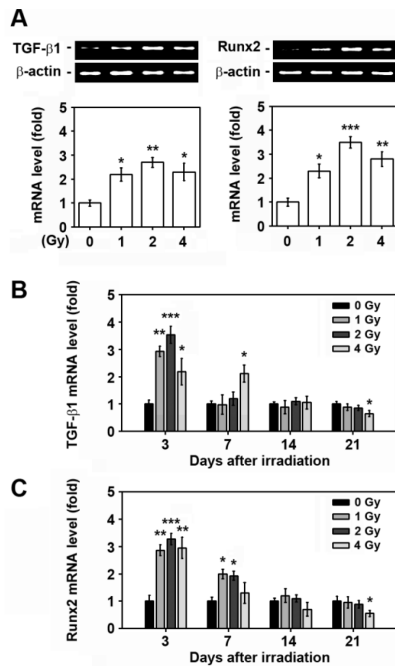
DISCUSSION

Although accumulated evidence emphasizes the detrimental effects of therapeutic irradiation on bone (1,12-15), there are reports showing positive effects on osteoblastic differentiation and bone-specific gene expression (5, 8, 16). In this study, we demonstrated that X-ray radiation at relatively low doses accelerated mineralization of calvarial primary osteoblasts with an attendant increase in ALP activity. ALP is one of the earliest markers expressed during the osteoblast differentiation process and its activity is often measured to determine osteoblast differentiation stage (8). Consistent with this observation, our present findings showed that ALP activity in calvarial primary osteoblasts peaked at 7 days after inducing differentiation and deceased gradually according to differentiation stage. As expected, irradiation also stimulated ALP activity in the cells, and such increase in the cells differed from previous reports showing that 4 Gy or more irradiation increases ALP activity in MC3T3-E1 cells (17) and ROS 17/2.8 osteoblasts (8). These findings support the notion that ionizing irradiation facilitates ALP activation, but that the resulting effects might differ according to the cell type examined.

The osteoblast differentiation process is tightly controlled by bone-specific regulatory markers. In addition to ALP, COL I, the most abundant matrix protein, is an osteogenic marker expressed mostly at early stages of osteoblastic differentiation (18). Bone min-
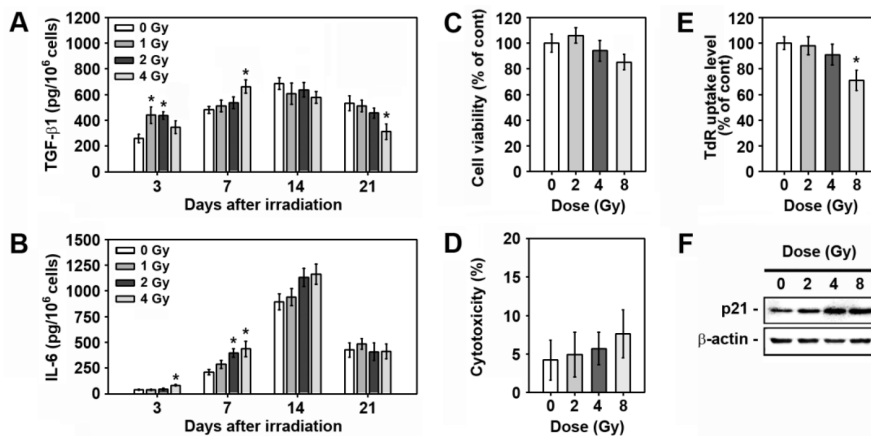

Fig. 3. Irradiation-mediated changes in mRNA expression patterns of transforming growth factor (TGF)-β1 and runt-related transcription factor 2 (Runx2) in mouse primary osteoblasts. (A) mRNA expression levels of TGF-β1 and Runx2 were determined by RT-PCR at 3 days after irradiation. Total RNA was extracted from the irradiated cells at the indicated times after irradiation and then subjected to real-time RT-PCR to analyze the expression patterns of (B) TGF-β1 and (C) Runx2. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the non-irradiated control values.

Fig. 4. Effects of irradiation on cytokine production, viability, cytotoxicity, DNA synthesis, and p21 protein levels in mouse primary osteoblasts. The levels of (A) transforming growth factor (TGF)-β1 and (B) interleukin (IL)-6 were analyzed using conditioned media collected at the indicated times after irradiation. The cells were also exposed to various doses (0-8 Gy) of X-ray radiation, and viability, cytotoxicity, DNA synthesis, and p21 protein induction were analyzed by (C) MTT assay, (D) trypan blue staining, (E) TdR uptake assay, and (F) Western blotting, respectively at 3 days after radiation. *P < 0.05 vs. the non-irradiated control values.

eralization proteins, such as OCN and OPN, also specifically increase during bone formation (10). OCN is secreted mostly in mature osteoblasts during the stage of matrix calcification (19). Consistent with previous reports (10, 20), dynamic and well-regulated mRNA expression patterns of these marker genes were observed depending on the differentiation stage of non-irradiated osteoblasts (data not shown). Our results also strongly support that the mRNA expression patterns of these bone-specific markers were affected differently according to the quantity of irradiation and the testing times examined (5). In particular, the current findings demonstrate that ionizing irradiation of <4 Gy increased bone differentiation and mineralization of calvarial osteoblasts by up-regulating bone-specific genes at the early stages of differentiation.

As TGF-β1 and Runx2 are key regulators of osteoblast differentiation and mineralization, we explored the effects of X-ray radiation on the expression of these regulators. Similar to the results from RT-PCR, the real-time PCR data revealed that irradiation strongly stimulated mRNA expression of both TGF-β1 and Runx2 at the initial stages of differentiation. However, the expression patterns of these genes were entirely different at 7 days post-irradiation depending on the doses used. TGF-β belongs to a family of multifunctional cytokines that regulate a variety of cellular events including proliferation, differentiation, mobility, and survival in many kinds of cells (21). There are few findings to show controversial effects of TGF-β1 on bone cells depending on the differentiation stage; it not only stimulates proliferation and differentiation of pre-osteoblasts (21, 22), but also inhibits the mineralization process in MC3T3-E1 cells (23). A temporal stimulation to proliferate is needed to enter the subsequent osteoblast differentiation process and this is accompanied by a coordinated expression of bone-specific marker proteins (24). These reports along with our present findings indicate that temporal stimulation in the expression of TGF-β1 is required for the early stages of osteoblast differentiation, but that its prolonged expression might disrupt the osteoblast mineralization step. It is important to note that irradiation at relatively high doses mostly induces DNA damage and eventually blocks cell cycle progression (6, 9). Our results show that exposure of calvarial osteoblasts to 4 Gy radiation significantly prevented DNA synthesis without cytotoxicity, and that the levels of cell cycle inhibitory protein p21 increased higher in cells irradiated at 4 or 8 Gy than those irradiated at 2 Gy during the early stages of differentiation. Although we cannot explain the mechanisms involved, it is postulated that irradiation at >2 Gy caused DNA damage and growth inhibition at early exposure times, which led to delayed recovery in cell cycle progression with the attendant stimulation of TGF-β1 expression even at 7 days post-irradiation in primary osteoblasts.

Runx2, also known as core binding factor alpha 1, plays an essential role in osteoblast differentiation through transcriptional regulation of bone-specific genes (25-28). A considerable number of findings suggest a relationship between TGF-β1 and Runx2 (29). TGF-β1 inhibits terminal osteoblast differentiation by repressing Runx2 transcriptional activity, which suppresses the formation of mineralized osteoblasts (30). Considering the functions of TGF-β1

on bone cells, its uncontrolled expression might affect Runx2 expression and eventually affect downstream bone-specific markers. Therefore, the irradiation-mediated prolonged stimulation of TGF-β1 might diminish the potential of osteoblasts to differentiate and mineralize by suppressing Runx2 and its downstream effectors. More detailed experiments using TGF-β1 over-expressing or knockout cells will be needed to clarify the relationship between TGF-β1 and Runx2 or other bone-specific genes in irradiated primary osteoblasts.

MATERIALS AND METHODS

Chemicals
Chemicals and laboratory ware were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA), respectively.

Cell culture
Primary osteoblasts were prepared from the mouse calvariae of 10 day-old BALB/c mice by repeated digestion with 0.05% trypsin and 0.1% collagenase. The use of mice in this experiment was approved by the Chonbuk National University Committee on Ethics and the Care and Use of Laboratory Animals. Primary osteoblasts were incubated in alpha-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and antibiotics and the cultures were switched to a fresh batch of the same medium every 3 days.

Osteoblastic differentiation and X-ray irradiation
Cells were added to various types of flat-bottomed culture plates at a concentration of 5×10^5 cells/ml, and the final contents of media in each plate were 2 ml, 500 μl, and 200 μl per 6-, 24-, and 96-well plates, respectively. After cells reached 70-80% confluence, culture media were changed with osteoblastic differentiating medium (α-MEM supplemented with 10% FBS, 5 mM β-glycerophosphate, and 50 μg/ml ascorbic acid) and the cells were exposed to 1, 2, 4, and 8 Gy at a rate of 1.5 Gy/min using a linear accelerator radiotherapeutic machine (Mevaprimus, Siemens, Munich, Germany). All irradiation was performed at room temperature in the Chonbuk National University Hospital, and the control samples were treated similarly except they were not irradiated. At various times (0-28 days) after irradiation, cells were processed for analyses of viability, cytotoxicity, mineralization, and induction of bone-formation related factors.

Alizarin Red staining
The degree of mineralization in calvarial primary osteoblasts was determined in 24-well plates by Alizarin Red staining. Irradiated osteoblasts were fixed with ice-cold 70% (v/v) ethanol 21 days after irradiation and were then stained with 0.2% Alizarin Red S in distilled water for 30 min. After destaining and air-drying, the cell culture plates were evaluated by light microscopy. The stain was solubilized with 10% acetylpyridinum chloride by shaking for 20 min and the absorbance was measured at 560 nm to quantify the red dye.

Determination of cellular calcium

The amount of calcium deposited in the cell layer was measured using a Calcium C kit (Wako Chemical Inc., Osaka, Japan), according to the manufacturer's instructions. Briefly, irradiated cells were washed with PBS at 21 days post-irradiation and incubated overnight with 1 ml of 2 N HCl with gentle shaking. The calcium ions in the samples were quantitated by the *o*-cresolphthalein complexone method using a Calcium C kit that was specific for calcium ions and had a limit of detection of 1 μg/ml.

Measurement of ALP activity

Cells were collected and resuspended in lysis buffer (50 mM Tris-HCl, pH 7.2, 0.1% Triton X-100, and 2 mM MgCl$_2$) at various times post-irradiation. ALP activity of each sample was determined by an established technique with *p*-nitrophenyl phosphate (*p*NPP) as the substrate after determining protein concentration. In brief, 200 μl of ALP yellow liquid substrate for the enzyme-linked immunosorbent assay (ELISA) was transferred to each well of 96-multiwell ELISA plates, and then each lysate (50 μl) was added to wells containing *p*NPP. The reaction was stopped by adding 50 μl of 3 N NaOH, and the optical density of the mixtures was measured at 405 nm using a SpectraCount ELISA reader. ALP activity was expressed as μmol/min/mg protein.

RNA preparation and real-time PCR

Total RNA was prepared from irradiated cells at various times (3-21 days) post-irradiation following the experimental procedures for the SV Total RNA Isolation System (Promega, Madison, WI, USA) and then was reverse-transcribed using an RNA PCR kit according to the instruction manual (the Access RT-PCR System, Promega). Real-time PCR amplification was performed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster, CA, USA) and the osteoblastic gene- specific primers; 5'-GCCCTCTCCAAGACATATA-3' and 5'-CCATGATC ACGTCGATATCC-3' for ALP, 5'-TCCTGCCGATGTCGCTATC-3' and 5'-CAAGTTCCGGTGTGACTCGTG-3' for COLI, 5'-T GGTCA CCTTGTACGAGAGA-3' and 5'-GGTGTTAGCAGCTTATCCAC-3' for OPN, 5'-TCTCTCTGCTCACTCTGCTG G-3' and 5'-ACCG TATAGGCGTTTGTAGGCG-3' for OCN, 5'-TCCCCGGGAACCA AGAAG-3' and 5'-GGTCAGAGAACAAACTAGGTTTAGA-3' for Runx2, 5'-ATGCCGCCCTCGGGGCTG-3' and 5'-CCA TTAGCA CGCGGGTGACT-3' for TGF-β1, and 5'-CCTGTGGC ATCCACG AAACT-3' and 5'-GAAGCATTTGGGGTGGACGA-3' for β-actin. The thermocycling conditions were as follows: predenaturation at 95°C for 10 min and 40 cycles of amplification using three-step cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30-60 seconds, and extension at 72°C for 30 seconds. Relative expression of the genes was obtained from relative standard curves run in triplicate after dividing each value by the β-actin value. In addition, aliquots of a cDNA pool were subjected to PCR and amplified in a 20-μl reaction mixture using Taq polymerase and the primers specific for TGF-β1 and Runx2. The amplifications were performed with a DNA thermal cycler (model PTC-100, Perkin Elmer, Waltham, MA, USA), and the resulting PCR products were electrophoresed on 1-2% agarose gels followed by ethidium bromide staining. Band intensity was calculated using a gel imaging system (model F1-F2 Fuses type T2A, BIO-RAD, Segrate, Italy).

Measurement of cytokines

Cytokine concentrations were determined by ELISA at the Bank for Cytokine Research (Chonbuk National University). Briefly, culture supernatants were collected from irradiated osteoblasts in 24-well culture plates at 3, 7, 14, and 21 days post-irradiation and then assessed by ELISA using TGF-β1-or IL-6-specific OptEIA kits. The cytokine levels produced were calculated from standard curves generated using known concentrations of recombinant cytokine proteins.

Measurement of DNA synthesis

The levels of DNA synthesis were measured by [methyl-^{3}H] thymidine deoxyribose (TdR; Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) incorporation assay. In brief, cells grown in 96-multiwell plates were treated with 1 μCi TdR/well at 3 days post-irradiation and then processed for an additional 12 h incubation. Cells were collected with a cell harvester (Inotech Biosystems International, Inc., Dietikon, Switzerland), and TdR contents were measured using a liquid scintillation counter (Packard Instrument Co., Downers Grove, IL, USA).

Western blot analysis

Cellular proteins were extracted using NP-40 lysis buffer (30 mM Tris-Cl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM PMSF, and protease inhibitor mixture containing 1 μg/ml aprotinin and leupeptin). Equal amounts of protein samples (30 μg/sample) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto PVDF membranes. The blots were probed with primary antibodies and incubated with a horse-radish peroxidase-conjugated anti-IgG in a blocking buffer for 1 h. Finally, the blots were developed by enhanced chemiluminescence (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and exposed to X-ray film (Eastman-Kodak, Rochester, NY, USA). The polyclonal antibody specific for p21 (sc-397) and the monoclonal antibody for β-actin (sc-47778) were obtained from Santa Cruz Biotechnology.

Statistical analyses

Data are expressed as mean ± standard deviation (SD) from five different samples. All experiments were performed in triplicate. A one-way analysis of variance (SPSS version 18.0 software, Chicago, IL, USA) followed by Scheffe's test was applied to determine significant differences between the groups. A P-value < 0.05 was considered significant.

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