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Sensitization of the Apoptotic Effect of γ-Irradiation in Genistein-pretreated CaSki Cervical Cancer Cells

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Radiotherapy is currently applied in the treatment of human cancers. We studied whether genistein would enhance the radiosensitivity and explored its precise molecular mechanism in cervical cancer cells. After co-treatment with genistein and irradiation, the viability, cell cycle analysis, and apoptosis signaling cascades were elucidated in CaSki cells. The viability was decreased by co-treatment with genistein and irradiation compared with irradiation treatment alone. Treatment with only γ-irradiation led to cell cycle arrest at the G_1 phase. On the other hand, co-treatment with genistein and γ-irradiation caused a decrease in the G_1 phase and a concomitant increase up to 56% in the number of G_2 phase. In addition, co-treatment increased the expression of p53 and p21, and Cdc2-tyr-15-p, supporting the occurrence of G_2/M arrest. In general, apoptosis signaling cascades were activated by the following events: release of cytochrome *c*, upregulation of Bax, downregulation of Bcl-2, and activation of caspase-3 and -8 in the treatment of genistein and irradiation. Apparently, co-treatment downregulated the transcripts of E6*I, E6*II, and E7. Genistein also stimulated irradiation-induced intracellular reactive oxygene, species (ROS) production, and co-treatment-induced apoptosis was inhibited by the antioxidant *N*-acetylcysteine, suggesting that apoptosis has occurred through the increase in ROS by genistein and γ-irradiation in cervical cancer cells. Gamma-irradiation increased cyclooxygenase-1 (COX-2) expression, whereas the combination with genistein and γ-irradiation almost completely prevented irradiation-induced COX-2 expression and PGE_2 production. Co-treatment with genistein and γ-irradiation inhibited proliferation through G_2/M arrest and induced apoptosis *via* ROS modulation in the CaSki cancer cells.

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Cervical cancer is a major cause of cancer-related death in women. Human papillomaviruses (HPVs) can infect basal epithelial cells of the skin or inner lining of tissues and are categorized as cutaneous or mucosal types [2, 5]. During malignant progression, the HPV genome frequently integrates into the host DNA, resulting in the expression of two viral oncoproteins, E6 and E7, which bind and induce the degradation of the p53 and pRb tumor suppressor proteins, respectively [22]. A large number of cancer patients received radiotherapy as part of their treatment [26] and it is currently applied in the treatment of a wide array of human cancers including cervical cancer. Radiation kills cells by inducing apoptosis, generating free oxygen species, and causing DNA strand breaks [15]. Several agents and drug combinations including misonidazole, hydroxyurea, cisplatin, carboplatin, fluorouracil, mitomycin C, and, more recently, taxanes have been used as radiation sensitizers in cervical cancer [29].

Various dietary agents have been classified as chemopreventive agents because of their ability to delay the onset of the carcinogenic process, and they have been extensively studied. Genistein (4',5,7-trihydroxyisoflavone), a major phytoestrogen in soybeans and other kinds of legumes, has a structural similarity to estrogens [24]. The initial observation that genistein is a specific inhibitor of tyrosine protein kinase, an enzyme frequently overexpressed in cancer cells, sparked numerous investigations to unravel its chemopreventive mechanisms [1]. *In vitro* and *in vivo* experimental studies have shown that genistein can inhibit the growth of various cancer cell lines through the modulation of genes that are related to the homeostatic control of cell cycle and apoptosis [25]. Genistein inhibits the activities of

NF-κB and Akt, resulting in the inhibition of cancer cell growth and the induction of apoptosis [25]. Genistein enhances radiosensitivity by suppressing the radiation-induced activation of survival AKT signals, upregulated expressions of Bax and $p21^{WAF1}$, and also downregulated Bcl-2 levels in the TE-2 (p53, wild) cell line, a human esophageal squamous cancer cell line [1]. Previous studies have shown that genistein inhibits the invasion of tumor cell lines *in vitro* [21] and reduces metastasis of tumors in animal models *in vivo*. Cancer cells treated with various agents exhibited the features of apoptosis, as demonstrated by DNA fragmentation, release of cytochrome *c* from mitochondria, and activation of caspase-3-like enzyme [14, 18, 19].

The ultimate target of the G_2 checkpoint signaling pathway is the cyclin-dependent kinase (Cdk) complex, Cdk1-cyclin B1. Cdc2, also known as Cdk1, forms a heterodimeric complex with cyclin B1, which is maintained in an inactive form by phosphorylation of residues Thr-14 and Tyr-15 in the ATP-binding domain of Cdc2 by Wee1 kinase, and is converted to an active form by dephosphorylation of these residues by the dual specificity phosphatase, Cdc25C. This dephosphorylation/activation is an absolute requirement for the onset of mitosis [7]. In fact, it was already reported that genistein treatment resulted in an increased ROS level [12]. Recent studies provide evidence that the increase in ROS stress can induce various biological responses such as increase in cellular proliferation, growth arrest, apoptosis, and necrosis [27]. Under physiological conditions, the maintenance of an appropriate level of intracellular ROS is important in keeping redox balance and signaling cellular proliferation. However, an overproduction of ROS or a suppression of cellular ability to eliminate ROS from the cells would result in a significant increase of intracellular ROS, leading to cellular damage, including lipid peroxidation, oxidative DNA modifications, protein oxidation, and enzyme inactivation. These damages can ultimately cause cell death if the ROS stress is severe and persistent [27, 28]. Although phenolic phytochemicals unequivocally have antioxidant activity, they can paradoxically have pro-oxidant activity under certain experimental conditions. Phenolic phytochemicals are able to generate ROS [20]. Recent reports suggest that radiation results in upregulation of cyclooxygenase (COX) and a concomitant increase in prostaglandin E_2 (PGE_2) synthesis, which is related to radioresistance [32]. Moreover, there has been accumulating evidence for the association between inflammatory tissue damage and the process of cancer development [10]. Numerous dietary phytochemicals are shown to downregulate COX-2 expression, whereby they exert chemopreventive activity. In addition, genistein was shown to be a potent inhibitor of the transcriptional activation of COX-2 [16]. Most recently, attention has focused on the use of chemical modifiers as radiosensitizers in combination with low-dose irradiation and it may increase the therapeutic efficacy by overcoming a high apoptotic threshold. Interaction of genistein with anticancer agents or radiation has been examined, and an enhancing effect of genistein on radiation-induced cell killing has already been observed [1]. However, the precise mechanism of genistein on radiation-induced apoptosis in cervical cancer cells has not been well elucidated. Therefore, the purpose of the present study was to evaluate the potential effects of genistein on enhancement of radiosensitivity as a therapeutic agent and to explore its molecular mechanism in cervical cancer cells.

Materials and Methods

Materials

Human keratinocyte cell line HaCaT and cervical cancer cell line CaSki were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, South Logan, UT, U.S.A.) as previously described [4]. DMEM was purchased from GIBCO/BRL (Rockville, MD, U.S.A.). DMEM complete medium was supplemented with 10 mM L-glutamine, 24 mM $NaHCO_3$, 10 mM Hepes, 100 units/ml penicillin, and 100 μg/ml streptomycin (GIBCO/BRL). All of the antibodies used were as follows: anti-catalase (Calbiochem, San Diego, CA, U.S.A.), anti-α-tubulin (Sigma, St. Louis, MO, U.S.A.), anti-p21 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-p53 (Oncogene Science, Cambridge, MA, U.S.A.), anti-cyclin B1 (Santa Cruz Biotechnology), anti-phospho-Cdc2 (Cell Signaling Technology, Beverly, MA, U.S.A.), anti-bax (Santa Cruz Biotechnology), anti-bcl-2 (Santa Cruz Biotechnology), anti-cytochrome *c* (BD Biosciences, Oxford, UK, U.S.A.), anti-caspase-3 and -8 (Calbiochem), anti-COX-2 (Santa Cruz Biotechnology), horseradish-peroxidase-conjugated anti-rat IgG, horseradish-peroxidase-conjugated anti-goat IgG (Santa Cruz Biotechnology), and horseradish-peroxidase-conjugated anti-mouse IgG (Sigma). Hoechst 33342, 2',7'-dichlorofluorescein diacetate (DCFH-DA), NAC (*N*-acetyl cysteine), 12-*O*-tetra decanoyl phorbol-13-acetate (TPA), and genistein were purchased from Sigma. Stock solutions of genistein were prepared in dimethylsulfoxide (DMSO) and stored in the dark at −20°C. The final concentration of DMSO containing tested genistein was 0.05%. The PGE_2 assay kit was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.).

Cell Culture

An HPV 16-positive cervical cancer cell line, CaSki containing 60–600 copies of the HPV 16 genome [17], and human normal keratinocyte HaCaT cells were maintained in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 25 ng/ml amphotericin B, and 10% FBS at 37°C in a humidified incubator with 5% CO_2.

Gamma Irradiation

CaSki cells were seeded at an initial density of 3×10^5 cells in 60-mm culture dishes, allowed to adhere overnight, and exposed to γ-irradiation at room temperature with a γ-irradiator (dose rate, 435 rad/min; J.L. Shepherd & Associates, San Fernando, CA, U.S.A.). After γ-irradiation, cells were cultured in a 37°C incubator for further experiments.

Viability Assay

Cellular viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. In brief, cells were seeded at 3×10^5 cells in a 96-well culture plate containing the test compounds for the indicated time period, and then incubated with 10 μl of MTT solution (5 mg/ml in PBS) for 1 h at 37°C. The optical densities in the 96-well plates were determined with an ELISA reader (Molecular Devices, Sunnyvale, CA, U.S.A.) at 595 nm.

Apoptosis Detection Using Hoechst 33342 DNA Staining

Apoptosis was observed by chromatin staining with Hoechst 33342. Cells were seeded on cover glasses in a 60-mm culture dish. After treatment for the indicated time period, the supernatant was discarded and cells were fixed with 4% paraformaldehyde in PBS for 1 h at room temperature, and then washed three times in PBS and stained with Hoechst 33342 (Sigma) at 10 μM for 1 h at room temperature. Fluo-stained nuclei were observed using a Zeiss confocal microscope (Zeiss, Oberkochen, Germany) with the FITC filter set to detect the condensed nuclei, and apoptotic cells were observed.

Cell Cycle Distribution Analysis

Cells were incubated for 48 h with increasing concentrations of genistein alone or a combination of genistein and γ-irradiation, fixed with 70% ice-cold ethanol for overnight at −20°C, and treated with 150 mg/ml RNase A, and the DNA was labeled with 20 mg/ml propidium iodide. The DNA content of the cells was analyzed with a flow cytometer (FACS Calibur; Becton Dickinson, Rutherford, NJ, U.S.A.) as previously described [13]. The cell cycle distribution was calculated by the CELLQUEST software and the MODFIT program (Becton Dickinson).

Isolation of Cytosolic Fraction

Cells were treated as indicated, harvested, and resuspended in a cytosolic extraction buffer (250 mM sucrose, 10 mM KCl, 1 mM EDTA, 20 mM Tris-HCl, 1 mM dithiothreitol, 1.5 mM $MgCl_2$, 100 μM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, and 2 μg/ml aprotinin, pH 7.2). The cells were incubated on ice for 5 min and microcentrifuged for 10 min at 4°C. The supernatant was saved as a cytosolic fraction.

Western Blot

Cells were rinsed twice with ice-cold PBS and scraped. After harvesting, the cells were lysed with ice-cold lysis buffer, including 50 mM Tris-HCl, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM NAF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin, pH 7.4. Solubilized proteins were centrifuged at 14,000 ×*g* for 30 min, and the supernatant was collected. The protein concentration was determined using the Bradford assay reagent (Bio-Rad, Hercules, CA, U.S.A.). Equal amounts of protein were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.). The membranes were blocked with 5% skimmed milk, and then incubated with antibodies against caspase-3, caspase-8, bcl-2, bax, cytochrome *c*, α-tubulin, p53, p21, cyclin B1, and phospho-Cdc2. Immunocomplexes were detected by subsequent incubation with appropriate horseradish-peroxidase-conjugated secondary IgG antibodies, and with enhanced chemiluminescence (ECL), according to the manufacturer's protocol (Amersham Pharmacia Biotechnology).

RNA Extraction and RT-PCR

RT-PCR was carried out to detect the expressions of the E6 and E7 genes from the total RNA of CaSki cells. Total RNAs were extracted from CaSki cells using the acid guanidium thiocyanate-phenol-chloroform method [9]. cDNA was synthesized from 5 μg of total RNA. The resulting cDNA was amplified using E6- and E7-specific primers as previously described and PCR was carried out through 30 cycles of the following three steps: 95°C for 1 min, 57°C for 1 min, and 72°C for 30 sec. The primer sequences used for E6 and E7 detection were 5'-ATG TTT CAG GAC CCA CAG GAG CGA-3' (forward) and 5'-TTA CAG CTG GGT TTC TCT ACG TGT TC-3' (reverse) and 5'-ATG CAT GGA GAT ACA CCT ACA TTG C-3' (forward) and 5'-TTA TGG TTT CTG AGA ACA GAT GGG GC-3' (reverse), respectively. The resulting cDNA was amplified using GAPDH- and β-actin-specific primers as described below. The GAPDH primer sequences were 5'-ACC ACA GTC CAT GCC ATC AC-3' (forward) and 5'-TCC ACC ACC CTG TTG CTG TA-3' (reverse), and the β-actin primer sequences were 5'-GCC ATG TAC GTT GCT ATC CAG GCT G-3' (forward) and 5'-AGC CGT GGC CAT CTC TTG CTC GAA G-3' (reverse). PCR products were analyzed on a 1.5% agarose gel.

Measurement of Intracellular ROS Production

ROS was measured using the oxidant-sensitive fluorescent probe DCFH-DA. DCFH-DA is cleaved intracellulary by nonspecific esterases, followed by further oxidation by ROS to form the fluorescent compound DCF (2',7'-dichlorofluorescein) [30]. CaSki cells (3×10^5 cells) were exposed to genistein alone or co-treated with γ-irradiation, and then pre-incubated with PBS in the presence of 20 μM DCFH-DA for 15 min at 37°C. The relative green DCF fluorescence within live cells was measured using a flow cytometer (Becton Dickinson).

PGE_2 Assay

CaSki cervical cancer cells were seeded at an initial density of 3×10^5 cells in 60-mm culture dishes, and allowed to adhear overnight. After 48 h of treatment with genistein alone or co-treatment with γ-irradiation, the supernatants of control and treated cell cultures were collected and the PGE_2 concentration was determined using a PGE_2 assay kit (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.).

Statistical Evaluation

The cellular viability data and ELISA data were analyzed by Student's *t*-test using Prism version 3.00 (GraphPad Software, San Diego, CA, U.S.A.).

RESULTS

Genistein Sensitized Apoptosis in γ-Irradiated CaSki Cells

CaSki cells were more resistant to γ-irradiation than the normal keratinocyte cell line HaCaT after exposure to 10 Gy of irradiation (data not shown). Therefore, CaSki cells were selected to evaluate the cancer therapeutic effect

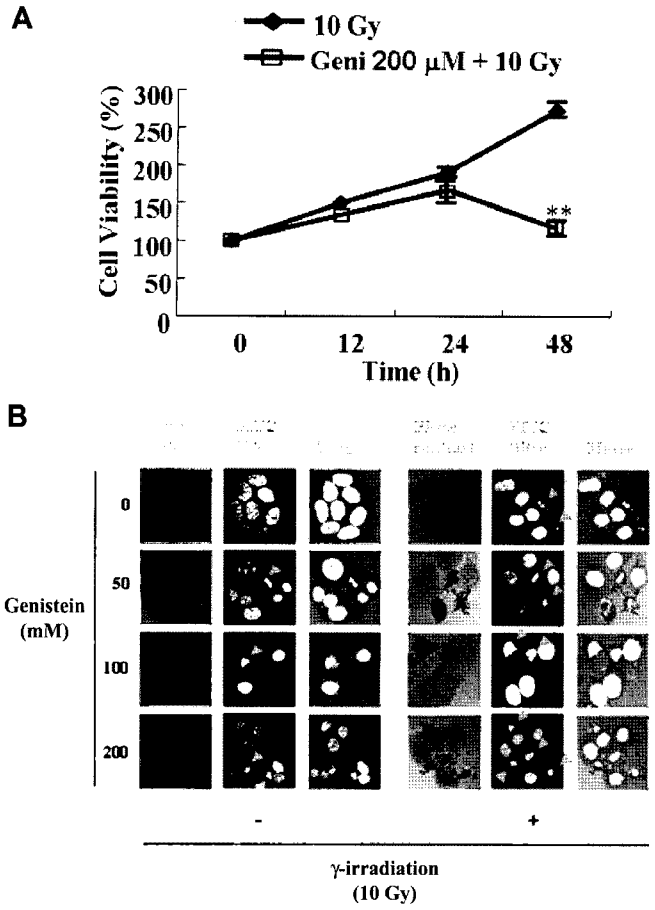

Fig. 1. The effects of genistein and γ-irradiation on the cell viability and apoptotic cell death in CaSki cells.
A. CaSki cells were pretreated (+ genistein) or non-treated (- genistein) for 1 h with 200 μM of genistein. Subsequently, the cells were irradiated with 10 Gy, and then the cell viability was measured by MTT assay as described in Materials and Methods. Measurements were done in triplicate, and the error bars represent SD. **, Significant difference from irradiation treatment ($p<0.01$). **B.** Cells were treated for 1 h with increasing concentrations of genistein prior to irradiation. The cells were fixed and stained with 10 μM of Hoechst 33342 as described in Materials and Methods.

of co-treatment of genistein and γ-irradiation. Pretreatment of CaSki cells with genistein rendered them hypersensitive to the death effect of γ-irradiation (Fig. 1A). Although MTT assay is a convenient method to measure cell death, it does not discriminate between apoptosis and necrosis. In order to investigate whether genistein and γ-irradiation would induce apoptotic cell death, CaSki cells were stained with Hoechst 33342. Hoechst staining clearly revealed the induction of apoptotic bodies by co-treatment of genistein and γ-irradiation (Fig. 1B).

Genistein Induced G_2/M Phase Arrest and Expression of Apoptotic Factors in CaSki Cells Treated with γ-Irradiation

The effects of genistein and irradiation on cell cycle distribution were investigated by FACS analyses (Fig. 2A). Genistein caused accumulation of cells in the G_2/M phase in a dose-response manner. However, co-treatment with 50 μM of genistein and γ-irradiation caused a decrease in the G_1 phase cells and a concomitant increase of up to 56% in the number of cells in the G_2 phase. In order to examine the expression pattern of intracellular proteins regulating cell cycle progression at the G_2/M boundary, the expressions of p53, p21, cyclin B1, and phospho-Cdc2 were analyzed (Fig. 2B). The cells were treated with genistein in a dose-response manner with or without irradiation, and the expression levels of cell cycle regulators were detected by Western blot analyses. The expressions of p53 and the cyclin kinase inhibitor p21 were upregulated in CaSki cells by a high concentration of genistein or the co-treatment, suggesting that p21 expression occurred in a p53-dependent manner in CaSki cells. There was no significant difference in cyclin B1 levels in CaSki cells treated with genistein, whereas there appeared to be a reduction in cyclin B1 in γ-irradiation treatment (Fig. 2B). Since the Cdc2-cyclin B1 complex is maintained in an inactive form by phosphorylation of residues Thr-14 and Tyr-15 on Cdc2, the phospho-Cdc2 level was analyzed by Western blotting and it was revealed that phosphorylation of residue Tyr-15 on Cdc2 was increased in CaSki cells when CaSki cells were treated with genistein or co-treated with γ-irradiation (Fig. 2B). In order to investigate the apoptotic signaling induced by treatment with genistein and γ-irradiation in CaSki cells, the expressions of several apoptosis-associated proteins were examined (Fig. 2C). Cytochrome *c* was released in a dose-response manner in the cytosol. Co-treatment with genistein and γ-irradiation decreased the expression level of anti-apoptotic bcl-2, whereas the pro-apoptotic bax protein level was increased. Western blot analyses were performed to confirm the participation of caspase-3 and -8. Their cleavages were induced by γ-irradiation and they were markedly enhanced after co-treatment with genistein in a dose-dependent manner (Fig. 2C), suggesting that the combination of genistein-pretreatment with or without γ-irradiation sensitized CaSki cells to apoptosis.

E6 and E7 Expressions were Downregulated in CaSki Cells Treated with Genistein and γ-Irradiation

In order to elucidate whether irradiation and genistein would modulate mRNA expression of E6/E7 viral oncogenes in the CaSki cell line, RT-PCR was performed and the expressions of E6, E6*I, E6*II, and E7 were detected in CaSki cells (Fig. 3). As previously reported [8], most of the E6 transcripts were in the spliced form E6*I, and full-length E6 and the other spliced form, E6*II, were detected in small amounts. Exposure to the high concentration of genistein resulted in downregulation of the expressions of E6*I, E6*II, and E7, whereas full-length E6 was unspliced. Gamma-irradiation treatment did not reduce these oncogenes,

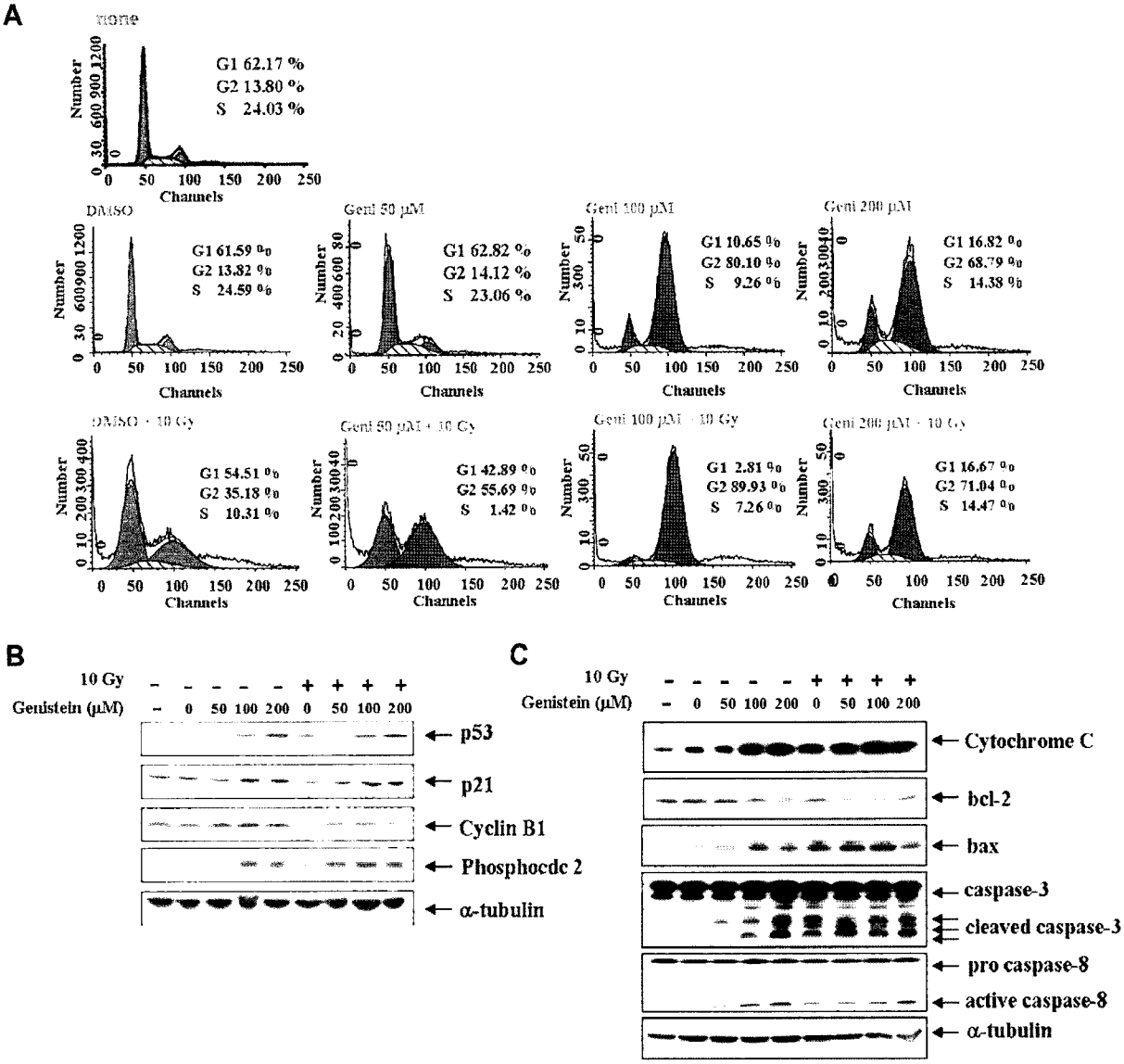

Fig. 2. The effect of genistein and γ-irradiation on the cell cycle and the expression of apoptotic factors in CaSki cells.
A. The effect of genistein and γ-irradiation on the cell cycle distribution in CaSki cells. Cells were treated for 1 h prior to irradiation with increasing concentrations of genistein. After 48 h, the DNA content was analyzed with a flow cytometer using PI staining, as described in Materials and Methods. The percentage of cells in each phase of the cell cycle was calculated using CellQuest Pro. **B.** The effect of genistein and γ-irradiation on the levels of G_2/M phase cell cycle regulatory proteins in CaSki cells. α-Tubulin was used as a loading control marker. **C.** The effect of genistein and γ-irradiation on the expression of apoptotic factors in CaSki cells. Cells were treated with increasing concentrations of genistein and/or 10 Gy of γ-irradiation. Equal amount of protein (100 μg) was subjected to SDS-PAGE and immunoblotted with antibodies against p53, p21, cyclin B1, and phospho-Cdc2 (Tyr-15). Cytosolic fractions were isolated as described in Materials and Methods. Apoptotic factors such as cytochrome c, cleaved forms of caspase-3 and caspase-8, bcl-2, and bax were analyzed by Western blot analyses. α-Tubulin was used as a loading control marker as in **B**.

whereas co-treatment with genistein and γ-irradiation downregulated the expressions of the E6*I, E6*II, and E7 genes.

Genistein Sensitized CaSki Cells to Apoptosis Induced by γ-Irradiation *via* the Production of Reactive Oxygen Species

Several evidences suggested that cancer chemotherapeutic drugs induced apoptosis of tumor cells, in part, by inducing the formation of ROS [23]. To determine whether the ROS generation by genistein is involved in γ-irradiation-induced apoptosis, the intracellular ROS level was detected using the fluorescent probe DCFH-DA, which is readily oxidized to 2',7'-dichlorofluorescein (DCF) in the presence of ROS (Fig. 4). The intracellular ROS level was increased in a dose-dependent manner by genistein in γ-irradiated CaSki cells (Fig. 4A). In order to investigate whether or not co-treatment with genistein and γ-irradiation would induce apoptosis *via* production of ROS, cells were pretreated with antioxidant *N*-acetyl-L-cysteine (NAC), a broad ROS

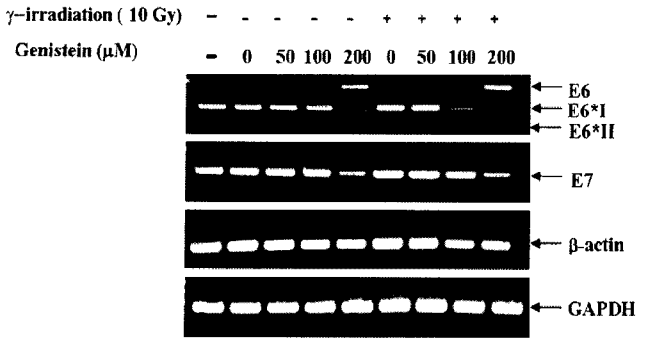

Fig. 3. Identification of E6 and E7 expressions in CaSki cells treated with genistein and γ-irradiation.
CaSki cells were pretreated with increasing concentrations of genistein for 1 h. After irradiation, E6 and E7 oncogenes were detected by RT-PCR using total RNA and combined primer sets, as described in Materials and Methods.

scavenger, before co-treatment, and then the p53, p21, and caspase-3 expressions were analyzed with Western blotting (Fig. 4B). Irradiation-induced p53 and p21 were inhibited and genistein-induced caspase-3 processing was slightly inhibited in NAC-treated cells. However, the failure of NAC to reduce the p53 and p21 induced by genistein might be due to an insufficient reduction of ROS by NAC at this concentration.

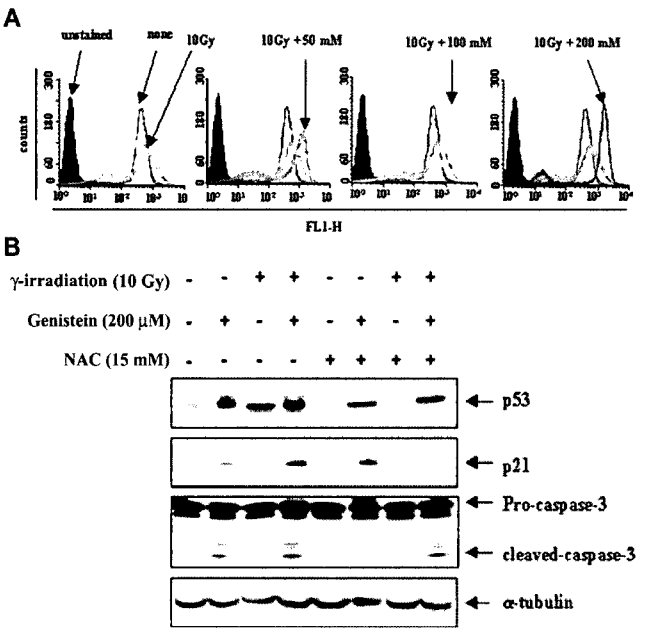

Fig. 4. The effect of genistein and γ-irradiation on the intracellular ROS level in CaSki cells.
A. CaSki cells were exposed to γ-irradiation alone or co-treated with γ-irradiation and increasing concentrations of genistein for 48 h. Intracellular ROS levels were detected using DCFH-DA, as described in Materials and Methods. **B.** The effect of NAC on p53/p21 expression and processing of caspase-3 in genistein-treated and/or γ-irradiated CaSki cells: CaSki cells were pretreated with 15 mM NAC for 1 h. These cells were exposed to genistein with or without γ-irradiation for 48 h, and then p53, p21, and caspase-3 levels were determined.

Co-Treatment of Genistein and γ-Irradiation Abrogated Irradiation-induced COX-2 Expression and PGE_2 Production

Gamma irradiation dose-dependently increases COX-2 protein expression accompanied by increased PGE_2 production, which may contribute to increase of radioresistance. This may explain why selective inhibition of COX-2 increases the radiosensitivity of COX-2-expressing cells. In this study, genistein inhibited COX-2 expression in a dose-dependent manner (Fig. 5A), whereas γ-irradiation treatment resulted in increased COX-2 expression and PGE_2 production. However, genistein treatment in γ-irradiated CaSki cells significantly inhibited COX-2 expression and PGE_2 production in a dose-response manner (Fig. 5B).

Genistein Inhibited TPA-induced COX-2 Expression and Enhanced the Expression of the Apoptotic Proteins in Irradiated CaSki Cells

Accordingly, the inhibitory effect of genistein was investigated on the TPA-induced COX-2 expression in irradiated CaSki cells. As a result, a high concentration (200 μM) of genistein inhibited TPA-induced COX-2 expression and PGE_2 production in irradiated CaSki cells (Fig. 6). In addition, the effect of TPA on apoptotic proteins such as p53 and

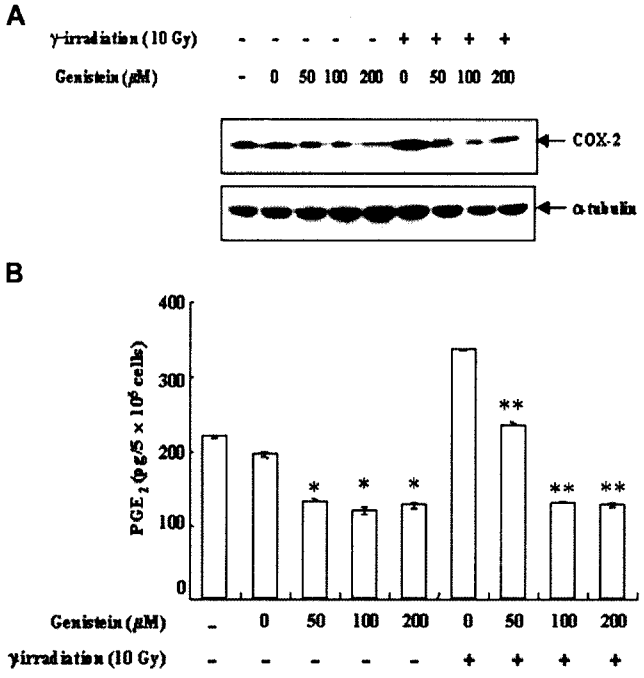

Fig. 5. The effect of genistein on COX-2 expression and PGE_2 production in γ-irradiated CaSki cells.
A. Cells were treated for 1 h prior to irradiation (10 Gy) with increasing concentrations of genistein. Equal amount of protein (100 μg) was analyzed by immunoblot analysis with anti-COX-2. α-Tubulin was used as a loading control. **B.** PGE_2 in the media was detected with an ELISA kit. Measurements were done in triplicate, and the error bars represent SD. *, Significant difference from non-treated control cells ($p<0.05$). **, Significant difference from irradiated cells ($p<0.01$).

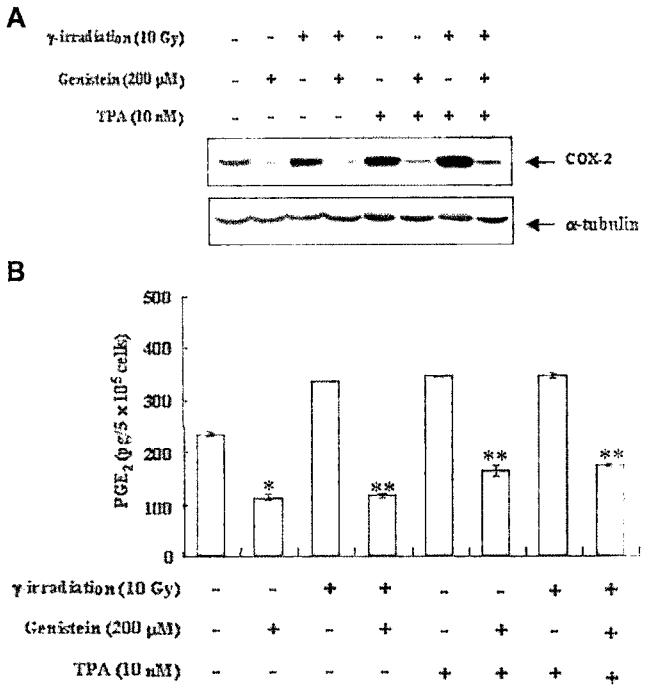

Fig. 6. The inhibitory effect of genistein on TPA-induced COX-2 expression and PGE_2 production in irradiated CaSki cells.
A. Cells were pretreated with 10 nM TPA for 1 h. These cells were then exposed to genistein with or without γ-irradiation for 48 h, and then COX-2 levels were detected. **B.** The amount of PGE_2 present in the supernatant of the culture medium was measured with an ELISA kit. Measurements were done in triplicate, and the error bars represent SD. *, Significant difference from non-treated control cells ($p<0.05$). **, Significant difference from TPA and/or γ-irradiation-treated cells ($p<0.01$).

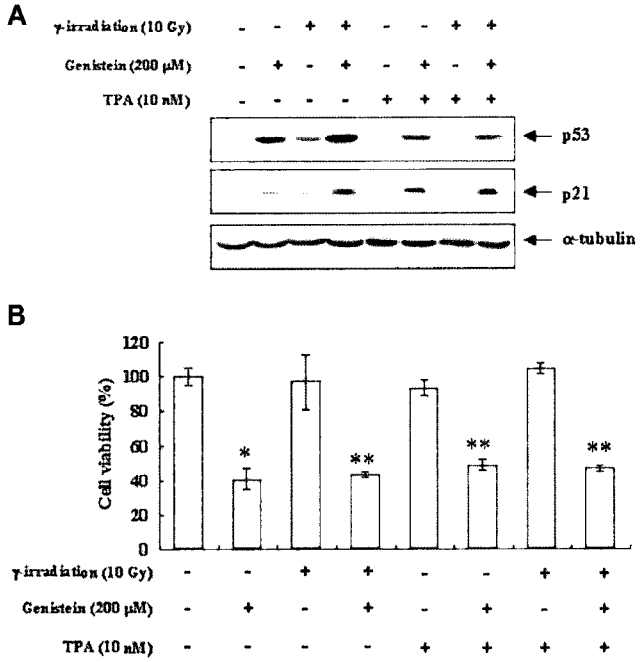

Fig. 7. The effect of genistein on the expressions of p53 and p21 in TPA-treated CaSki cells.
A. Cells were pretreated with 10 nM TPA for 1 h. These cells were exposed to genistein with or without γ-irradiation for 48 h, and then the levels of p53 and p21 were determined. **B.** The cell viability was measured by MTT assay. Data are expressed as percentage of viable cells relative to untreated control cultures. Experiments were performed in triplicate and repeated three times. Asterisks indicate significant level in the Student's *t*-test: * Significant difference from non-treated control cells ($p<0.05$). ** Significant difference from TPA and/or γ-irradiation-treated cells ($p<0.01$).

p21 was also investigated (Fig. 7A). TPA attenuated p53 expression induced by genistein with or without γ-irradiation treatment. However, genistein reversed the inhibitory effect of TPA, and decreased cell viability (Fig. 7B).

DISCUSSION

Phytoestrogen genistein, present in soybean, has a wide range of properties that may contribute to its cancer protective action, such as an inhibitory effect on tyrosine kinases, antiestrogenicity, antioxidant activity, antiangiogenesis activity, suppression of cell proliferation, induction of differentiation, and modulation of apoptosis [7]. Since the treatment of locally advanced disease by surgery combined with radiation therapy in various settings remains insufficient, genistein-modulated apoptosis may provide a novel basis for chemotherapy and benefit cervical cancer patients.

In this study, the possible roles of co-treatment with genistein and γ-irradiation on the human cervical cancer CaSki cells were elucidated. Our results revealed that the co-treatment with genistein and γ-irradiation inhibited cell proliferation, arrested the cell cycle at the G_2/M transition *via* p53, p21, cyclin B, and Cdc2-tyr-15-p checkpoints, and induced apoptosis *via* promoting cytochrome *c* release, processing of caspase-3 and -8, inhibition of Bcl-2 expression, enhancement of bax expression, downregulation of expressions of E6*I, E6*II, and E7, upregulation of intracellular ROS level, and downregulation of COX-2 expression and PGE_2 production. Members of the Bax/Bcl-2 family are important regulatory factors in the mitochondrial pathway that induce intrinsic activation of caspases. In particular, a decrease in the bcl-2 protein was related to genotoxic stress-induced apoptosis [11]. The transduction and execution of apoptotic signals require the proper signal transduction and the coordinated action of caspases. Caspase activity is responsible, either directly or indirectly, for cleavage of cellular proteins that are characteristically proteolyzed during apoptosis [25].

The HPV 16-type E6 gene encodes truncated E6 ORFs [E6*I (300 bp) and E6*II (150 bp)] that are generated as a result of differential splicing, in addition to the full-length E6 open reading frame (ORF: 450 bp). The E6*I-encoding mRNA is a major transcript in cervical carcinoma tissues, premalignant lesions, and cell lines derived from cervical cancer [8]. The commitment of HPV-positive CaSki cells to undergo apoptosis might be attributed to the downregulation

of E6 and E7, which cannot further promote p53 and pRb degradation, respectively. The p53 expression level in CaSki cell lines was increased under co-treatment with genistein and irradiation. These results might be indirectly implicated in the programmed cell death of CaSki.

It is likely that the decreased levels of E6 do not retain p53 in the cytoplasm anymore, as evidenced by an increase in nuclear p53 and an upregulation of $p21^{WAF1}$. It has been well established that cell cycle progression is triggered by cyclin-dependent kinases (cdks). The G_1/S phase progression is mainly regulated by cyclin E/CDK2 and cyclin D/CDK4, 6, whereas the G_2/M progression is controlled by cyclin B/Cdc2. The G_2/M phase checkpoint plays a key role in providing time for DNA repair, and it provides an alternative pathway to apoptosis to remove irreparably damaged cells [6]. Experimental studies have shown that E7 can bind to $p21^{WAF1}$ and neutralize its inhibitory effect on cyclin-CDK complexes [33]. In the present study, E6 and E7 downregulation likely allowed, at least partially, the restoration of p53 and $p21^{WAF1}$ functions. Additionally, the expressed $p21^{WAF1}$ binds to and inactivates most Cdk-cyclin complexes and contributes to their cycle arrest in G_2/M. The Cdc2/cyclin B complex has been well known as the regulators governing the G_2 to M progression or inducing apoptosis [6]. G_2 arrest is initiated *via* phosphorylation of Cdc25C on serine-216, and the maintenance of G_2 arrest is highly p53 dependent and involves its transcriptional targets p21 [31]. These results showed that genistein-induced G_2/M arrest in irradiated CaSki cells is closely related to the increased expression of p53 and p21, and Cdc2-tyr-15-p, indicating the occurrence of the G_2/M arrest.

In our present studies, we observed a significant decrease of the anti-apoptotic protein bcl-2, with an increase of the pro-apoptotic protein bax, a known p53-activated cell death inducer, which could be responsible for the enhanced release of cytochrome *c* in the cytosolic fraction with consequent activation of caspase-3 and apoptosis induction. Several anticancer agents for cancer treatment have been shown to cause increased cellular ROS generation, and one of the major effects is to generate increased intracellular ROS, causing loss of mitochondria membrane permeability [12]. Therefore, this study revealed that the co-treatment with genistein and irradiation significantly generated ROS, and induced apoptosis. The ROS scavenger NAC inhibited apoptosis in CaSki cells co-treated with genistein and irradiation. Recent reports have indicated that COX-2 inhibitors have cytotoxicity against cancer cells *via* p53 dependent or independent apoptotic pathways, and sensitize cancer cells to radiation therapy or drug-resistant chemotherapy [3]. However, the precise mechanism of blocking chemoresistance by the COX-2 inhibitors is not well understood. A relatively high level of COX-2 found in cervical adenocarcinoma was identified as a factor contributing to chemotherapy resistance [29]. TPA was shown to be a potent stimulator of COX-2 expression in various cell lines and in mouse skin [10]. In this study, we have found that co-treatment of genistein and irradiation abrogated irradiation-induced COX-2 expression.

Thus, these findings will help us to provide a molecular basis for developing the combination of genistein and irradiation as a potentially more effective chemotherapy regimen for treatment of patients with resistant cervical cancer, and lead to further investigation of *in vivo* studies in preclinical cervical cancer models with this combination therapy.

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