

# Inhibition of gamma ray-induced apoptosis by stimulatory heterotrimeric GTP binding protein involves Bcl-xL down-regulation in SH-SY5Y human neuroblastoma cells

So-Young Kim<sup>1</sup>, Miran Seo<sup>1</sup>, Jung-Min Oh<sup>1</sup>, Eun-Ah Cho<sup>1</sup> and Yong-Sung Juhn<sup>1,2</sup>

<sup>1</sup>Department of Biochemistry and Molecular Biology  
Cancer Research Institute, Seoul National University  
College of Medicine, Seoul 110-799, Korea

<sup>2</sup>Corresponding author: Tel, 82-2-740-8247;  
Fax, 82-2-744-4534; E-mail, juhnn@snu.ac.kr

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Abbreviations: G $\alpha$ s, alpha subunit of stimulatory G proteins; GPCR, G protein-coupled receptor

## Abstract

Heterotrimeric GTP-binding proteins (G proteins) transduce extracellular signals into intracellular signals by activating effector molecules including adenylate cyclases that catalyze cAMP formation, and thus regulate various cellular responses such as metabolism, proliferation, and apoptosis. cAMP signaling pathways have been reported to protect cells from ionizing radiation-induced apoptosis, but however, the protective mechanism is not clear. Therefore, this study aimed to investigate the signaling molecules and the mechanism mediating the anti-apoptotic action of cAMP signaling system in radiation-induced apoptosis. Stable expression of a constitutively active mutant of G $\alpha$ s (G $\alpha$ sQL) protected gamma ray-induced apoptosis which was assessed by analysis of the cleavages of PARP, caspase-9, and caspase-3 and cytochrome C release in SH-SY5Y human neuroblastoma cells. G $\alpha$ sQL repressed the gamma ray-induced down-regulation of Bcl-xL protein, but transfection of Bcl-xL siRNA increased the gamma ray-induced apoptosis and abolished the anti-apoptotic effect of G $\alpha$ sQL. G $\alpha$ sQL decreased the degradation rate of Bcl-xL protein, and it also restrained the decrease in Bcl-xL mRNA by increasing the stability following ionizing irradiation. Furthermore, prostaglandin E2 that activates G $\alpha$ s was found to protect gamma ray-induced apoptosis, and the protective effect was abolished by treatment with prostanoid receptor antagonist specific to EP2/4R subtype. Moreover, specific agonists for adenosine A1

receptor that inhibits cAMP signaling pathway augmented gamma ray-induced apoptosis. From this study, it is concluded that G $\alpha$ s-cAMP signaling system can protect SH-SY5Y cells from gamma ray-induced apoptosis partly by restraining down-regulation of Bcl-xL expression, suggesting that radiation-induced apoptosis can be modulated by GPCR ligands to improve the efficiency of radiation therapy.

**Keywords:** apoptosis; bcl-x protein; cyclic AMP; gamma rays; heterotrimeric GTP-binding proteins; GTP-binding protein  $\alpha$  subunits, Gs; receptors, G-protein-coupled

## Introduction

The heterotrimeric GTP-binding proteins (G proteins) are composed of  $\alpha$ ,  $\beta$  and  $\gamma$  subunits, and are classified, based on the primary sequences of  $\alpha$  subunit of G protein (G $\alpha$ ) subunits, into four main families: G $\alpha$ s, G $\alpha$ i, G $\alpha$ q, and G $\alpha$ 12. A wide range of external stimuli, including neurotransmitters, growth factors, hormones, light, odorants, and certain taste ligands, can activate specific members of G proteins-coupled receptor (GPCR) family, which induce a conformational change of the coupled G protein to cause exchange of GDP with GTP in the  $\alpha$  subunit, and thus dissociation of  $\alpha$  and  $\beta\gamma$  heterodimer (G $\beta\gamma$ ) (Neer, 1995). The GTP-bound G $\alpha$  and G $\beta\gamma$  regulate a wide variety of intracellular signaling pathways including adenylate cyclases, phospholipases, phosphodiesterases, and ion channels to transduce extracellular signals into intracellular signals (Gilman, 1987). The G $\alpha$ -bound GTP is hydrolyzed to GDP by intrinsic GTPase, a process that is regulated by RGS (regulator of G-protein signaling), which leads to reassociation of the G $\alpha\beta\gamma$  heterotrimer and termination of the activation cycle (McCudden *et al.*, 2005).

Radiation therapy, together with surgery and chemotherapy, forms the major treatment modality for various cancers, and it can be used alone or together with chemotherapy to produce cure of localized tumors and control of the primary site of tumors that have disseminated. Ionizing radiation induces cancer cell death via various mechanisms

including apoptosis. However, development of radioresistance of tumor cells results in unsuccessful treatment, and thus understanding the mechanisms for cell death and development of radioresistance is essential for improving the efficiency of radiotherapy for cancer. Ionizing radiation has been shown to regulate expression and activity of various molecules involved in cell death including Bcl-2 family proteins in a variety of cancer cell lines (Findley *et al.*, 1997). Bcl-2 family proteins are classified according to their effects on cellular apoptosis, as anti-apoptotic proteins and pro-apoptotic proteins. The anti-apoptotic members include Bcl-2, Bcl-xL, Bfl-1, Bcl-W, and Mcl-1, and the pro-apoptotic members include Bax, Bak, and Bik. Ionizing radiation induces an increase in Bax expression, and the activated Bax/Bak induces apoptosis by allowing outer mitochondrial membrane permeabilization and release of cytochrome C which leads to activation of caspase-9 and caspase-3 (Morrison *et al.*, 2005). Ionizing radiation also induces a decrease in Bcl-2 and Bcl-xL, which can inhibit the apoptosis induced by various cytotoxic drugs (Huang *et al.*, 1997).

Apoptosis is regulated by various cellular signaling systems including cAMP signaling pathway. Production of cAMP from ATP is catalyzed by adenylate cyclases, which are activated by stimulatory G proteins (Gs) but inhibited by inhibitory G proteins (Gi). cAMP binds and activates cAMP-dependent protein kinase (PKA), which phosphorylates various target proteins that contribute to regulation of a variety of cellular responses including apoptosis (Koyama *et al.*, 2001). Increased cAMP levels were reported to exert protective effects against radiation-induced apoptosis (Lehnert, 1975), and the G $\alpha$ s-mediated signaling also protected reactive oxygen species induced apoptosis (Lewerenz *et al.*, 2003). Inhibition of PKA enhanced the cytotoxic effects of ionizing radiation in radioresistant cells by inhibiting prosurvival signaling molecules such as Bcl-2 (Tortora and Ciardiello, 2002; Chin *et al.*, 2005).

However, the underlying molecular mechanism of the protective effect of G $\alpha$ s-cAMP signaling pathways against ionizing radiation-induced apoptosis is not clearly known. Thus, we aimed to investigate the molecular mechanism for G $\alpha$ s-cAMP signaling pathways to inhibit radiation-induced apoptosis in this study, and found that G $\alpha$ s inhibits gamma ray-induced apoptosis by restraining Bcl-xL down-regulation in SH-SY5Y human neuroblastoma cells.

## Materials and Methods

### Cell culture and reagents

SH-SY5Y human neuroblastoma cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and maintained in DMEM containing 10% FBS (JBI, Korea) and 100 U/ml penicillin/streptomycin, in a 5% CO<sub>2</sub> incubator at 37°C. SH-SY5Y cells stably expressing constitutively active mutant G $\alpha$ s (G $\alpha$ sQ227L) were established in previous study (Jang and Juhnn, 2001). N-acetyl-L-cysteine (NAC), actinomycin D, AH-6809, AH23848, 2-chloro-N(6)-cyclopentyladenosine (CCPA), cycloheximide, DMSO, prostaglandin E2 (PGE2), R-phenylisopropyl-adenosine (R-PIA) and SC19220 were purchased from Sigma (St. Louise, MO).

### Measurement of cAMP accumulation

cAMP levels were determined by competitive binding with [3H]cAMP to a cAMP binding protein, the regulatory subunit R1 $\alpha$  of cAMP dependent protein kinase which was expressed in *E. coli*. In brief, the culture medium was removed from a 12-well plate containing the SH-SY5Y neuroblastoma cells, and cAMP was extracted by immediate addition of 2.5 M perchloric acid. After the acid extract was neutralized with 4.2 M KOH, cAMP levels were determined by competitive binding with [3H]cAMP to the cAMP binding protein, and cAMP levels were normalized to the amount of acid-insoluble protein.

### Irradiation with gamma ray

Cells were plated in 10-cm dishes and incubated until 80% confluent. Cells were then exposed to gamma-rays from a <sup>137</sup>Cs source at a delivering dose rate of 246.5 cGy/min.

### Cell viability assay

Cells (5,000 cells/well in 96-well plates, 100  $\mu$ l) were exposed to 0-30 Gy of gamma ray. After 24 h, cells were incubated with 0.5 mg/ml MTT for 2 h. Absorption at 595 nm was measured after solubilization of the formazan crystals with DMSO. Assays were performed in triplicate, and mean cell viability was compared to DMSO-treated controls.

### Flow cytometric analysis of Annexin V stained cells

Apoptosis was quantified using Annexin V-FITC apoptosis kits (BD Biosciences, San Diego, CA) according to the manufacturer's instructions.

Stained cells were analyzed using a FACSCalibur flow cytometer and the CellQuest analysis program (BD Biosciences, NorthRyde, Australia).

### Construction and transfection of Bcl-xL small interference RNA (siRNA)

Double strand DNA coding Bcl-xL siRNA was prepared by annealing the chemically synthesized oligonucleotides having complementary sequences; a forward primer 5'-CACCGCAGGGACAGCA-TATCAGAGCGAACTCTGATATGCTGTCCCTG-3', a reverse primer 5'-AAAACAGGGACAGCATAT-CAGAGTTCGCTCTGATATGCTGTCCCTGC-3'. The double strand DNA was inserted into pENTR/H1/TO vector to generate the pENTR/H1/TO-Bcl-xL siRNA construct using BLOCK-iTTM Inducible H1 RNAi Entry Vector Kit (Invitrogen, Carlsbad, CA). Transfection of pENTR/H1/TO-Bcl-xL siRNA was performed by electroporation using a Gene Pulser II (Bio Rad, Hercules, CA) at 250 V/950  $\mu$ F, and the transfected cells were allowed to grow at 37°C for 48 h before gamma ray irradiation.

### Immunoblot analysis

Total cell lysates (50  $\mu$ g of protein) was separated by 10 or 15% SDS-PAGE, transferred to nitrocellulose paper, and analyzed with specific antibodies. Antibodies against Bcl-2, caspase-9 and  $\beta$ -actin were from Santa Cruz Biotechnology (Santa Cruz, CA), antibodies against Bcl-xL, Bad, Bax, Bak, cleaved caspase-3 (Asp175) and poly-(ADP-ribose) polymerase (PARP) from Cell Signaling Technology (Beverly, MA). cytochrome C release into cytoplasm was analyzed by subcellular fractionation (Ahn *et al.*, 2000) followed by immunoblotting using antibody from BD Biosciences (San Diego, CA). The proteins were visualized by incubation with an enhanced chemiluminescence substrate mixture (Pierce) and then exposed to X-ray film (AGFA Curix RPI). The densities of visualized bands were quantified using a NIH Image J software and relative band densities were expressed as percentages of corresponding control densities.

### Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was performed as described previously (Cho *et al.*, 2007). Total RNA was isolated from the harvested cells using acid guanidinium thiocyanate-phenol chloroform extraction method. First-strand cDNA was synthesized with oligo-dT as primers, using SuperScript First-Strand Synthesis System according to manu-

facturer's protocol (Invitrogen, Carlsbad, CA). PCR was performed with specific primers: Bcl-xL; a forward primer 5'-TTGGACAATGGACTGGTTGA-3', a reverse primer 5'-GTAGAGTGGATGGTCAG-TG-3', GAPDH; a forward primer 5'-ACCACAGTC-CATGCCATCAC-3', a reverse primer 5'-TCCACC-ACCCTGTTGCTGTA-3'. Real-time RT-PCR was performed in a 25  $\mu$ l mixture composed of 200 nM each of forward and reverse primers, and iQTM SYBR Green Supermix (Roche, Basel, Switzerland) using iCycler and iQ software (Bio Rad, Hercules, CA). After 40 cycles of PCR, average threshold cycle (Ct) values for Bcl-xL from the triplicate PCR reactions were normalized against the average Ct values for GAPDH from the same cDNA sample.

### Data analysis

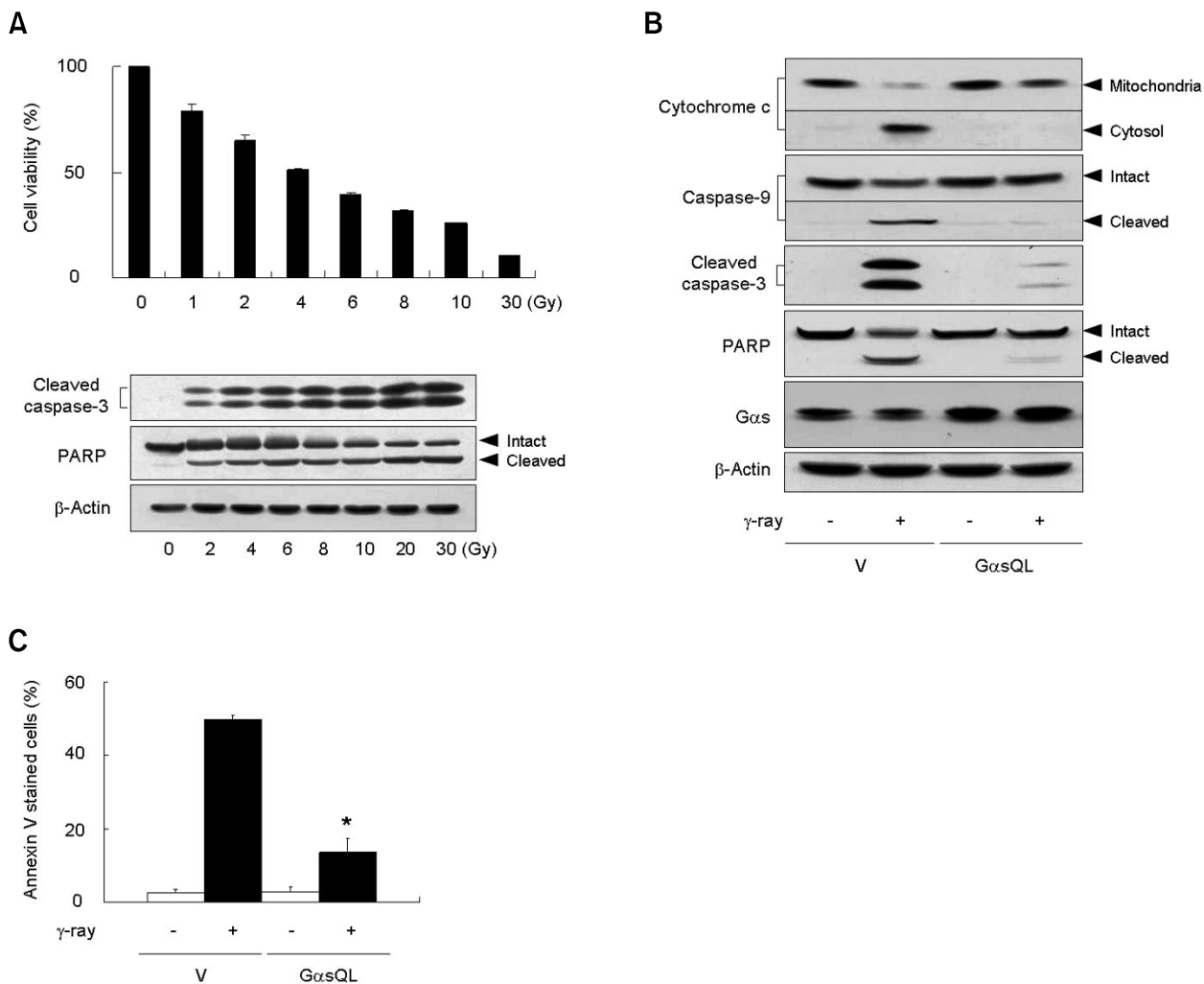
At least three or more independent experiments were conducted for all the analysis. The non-parametric Mann-Whitney U test was used to analyze mean values, and a *P* value of < 0.05 was considered statistically significant.

## Results

### G $\alpha$ s protected SH-SY5Y neuroblastoma cells from gamma ray-induced apoptosis

To investigate the role of G $\alpha$ s in gamma ray-induced apoptosis, we used a SH-SY5Y human neuroblastoma cell line, which stably expresses a mutant form of G $\alpha$ s (G $\alpha$ sQL) that constitutively activates adenylate cyclases (Jang and Juhn, 2001). The expression of mutant G $\alpha$ s was confirmed again in this study by Western blot analysis of G $\alpha$ sQL expression using antibodies specific for G $\alpha$ s, HA-tag, or phosphorylated CREB, and by measuring the basal cAMP levels; in the G $\alpha$ sQL expressing cells cAMP level was increased from  $3.4 \pm 0.6$  pmol/mg protein to  $28.5 \pm 0.5$  pmol/mg protein (*P* < 0.05) in vector-transfected cells (data not shown).

When SH-SY5Y cells were irradiated with varying dose of gamma ray from 1 to 30 Gy, the cell viability decreased and cleavages of caspase-3 and PARP increased in a dose-dependent manner (Figure 1A). Gamma ray-irradiation (4 Gy) induced the cleavages of caspase-9, caspase-3, and PARP in SH-SY5Y cells, and the expression of G $\alpha$ sQL decreased the cleavages of caspase-9 ( $18.5 \pm 1.4\%$ , *P* < 0.05), caspase-3 ( $23.6 \pm 4.4\%$ , *P* < 0.05) and PARP ( $28.3 \pm 1.8\%$ , *P* < 0.05), and cytochrome C release ( $17.3 \pm 2.9\%$ , *P* < 0.05) into cytosol versus vector-transfected cells

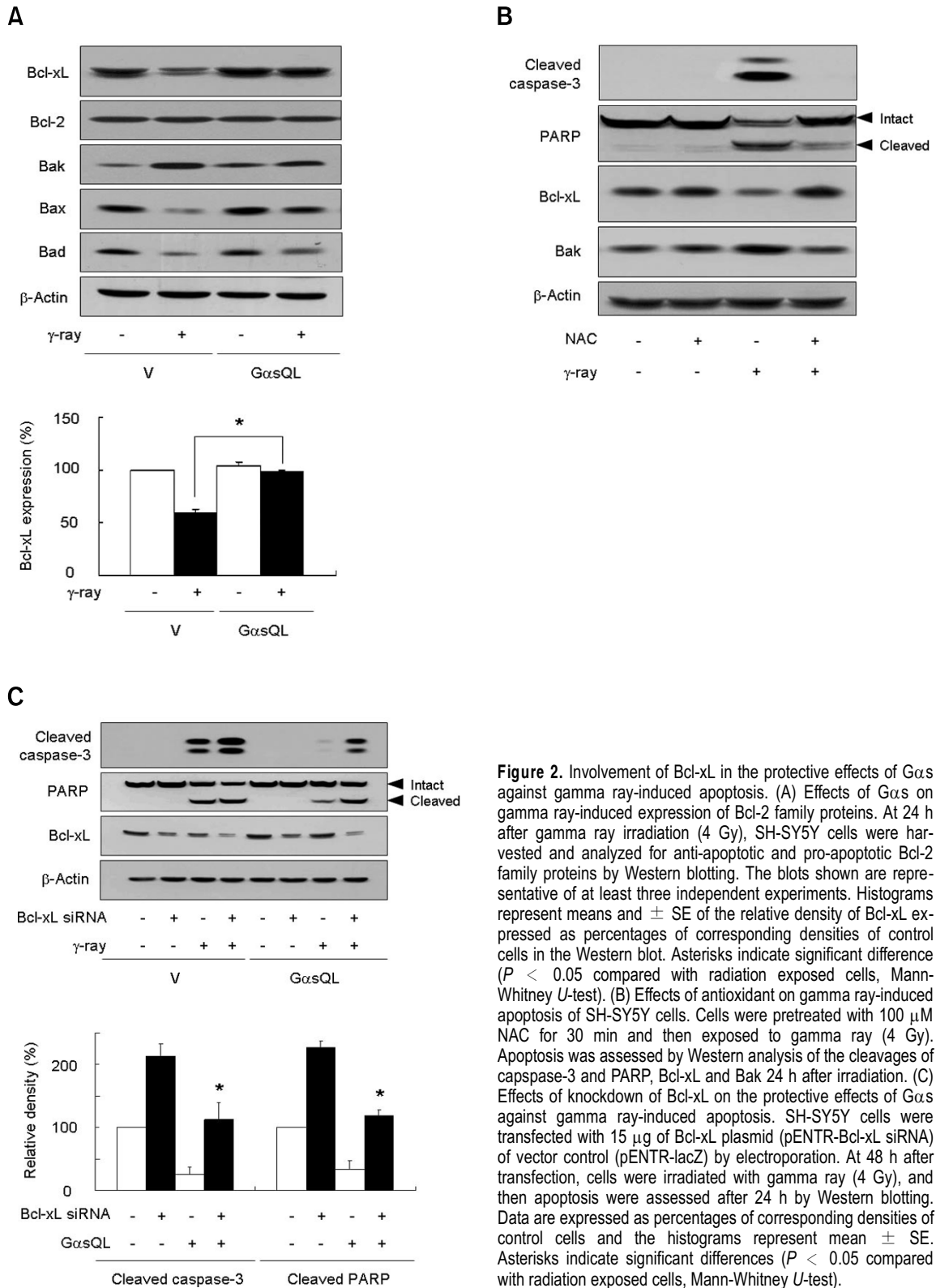


**Figure 1.** Effects of  $G\alpha s$  on gamma ray-induced apoptosis of SH-SY5Y human neuroblastoma cells. (A) Effects of gamma ray irradiation dose on apoptosis of SH-SY5Y cells. SH-SY5Y cells, plated in 96-well plates (5,000 cells/well), were exposed to varying dose of gamma ray (0-30 Gy). Then, the cell viability was assessed by MTT assay and apoptosis was assessed by Western analysis of the cleavages of caspase-3 and PARP 24 h after irradiation. (B, C) Effects of  $G\alpha sQL$  on gamma ray-induced apoptosis. At 24 h after gamma ray irradiation (4 Gy), apoptosis was assessed by Western analysis of the cleavages of caspase-9, caspase-3 and PARP (B) in vector (V)- or  $G\alpha sQL$ -expressing SH-SY5Y cells. The cytochrome C release into cytosol was analyzed by Western blotting after cytosolic and mitochondrial fractions were prepared by centrifugation. The histograms shows representative FACS analysis data of Annexin V stained cells (C). The blots shown are representative of at least three independent experiments. Histograms represent average and  $\pm$  SE. And the data are expressed as percentages of corresponding densities of control cells. Asterisks indicate significant difference ( $P < 0.05$  compared with radiation exposed cells, Mann-Whitney  $U$ -test).

24 h after irradiation (Figure 1B). Stable  $G\alpha sQL$  expression reduced Annexin V positive cells to  $13.6 \pm 3.7\%$  from  $49.9 \pm 1.0\%$  ( $P < 0.05$ ) in vector transfected control following gamma ray irradiation (Figure 1C). This result suggests that  $G\alpha s$  can protect SH-SY5Y neuroblastoma cells from gamma ray-induced apoptosis of SH-SY5Y cells.

**$G\alpha s$  inhibited gamma ray-induced apoptosis by restraining down-regulation of Bcl-xL protein in SH-SY5Y neuroblastoma cells**

To study the mechanism for  $G\alpha s$  to protect the neuroblastoma cells from gamma ray-induced apoptosis, the effects of  $G\alpha s$  on the expression of Bcl-2 family proteins following gamma ray irradiation was examined. Gamma ray irradiation decreased expression of anti-apoptotic Bcl-xL protein to  $59.4 \pm 1.8\%$  ( $P < 0.05$ ) without changing the expression of Bcl-2 protein. The radiation increased the expression of pro-apoptotic Bak



**Figure 2.** Involvement of Bcl-xL in the protective effects of Gαs against gamma ray-induced apoptosis. (A) Effects of Gαs on gamma ray-induced expression of Bcl-2 family proteins. At 24 h after gamma ray irradiation (4 Gy), SH-SY5Y cells were harvested and analyzed for anti-apoptotic and pro-apoptotic Bcl-2 family proteins by Western blotting. The blots shown are representative of at least three independent experiments. Histograms represent means and  $\pm$  SE of the relative density of Bcl-xL expressed as percentages of corresponding densities of control cells in the Western blot. Asterisks indicate significant difference ( $P < 0.05$  compared with radiation exposed cells, Mann-Whitney *U*-test). (B) Effects of antioxidant on gamma ray-induced apoptosis of SH-SY5Y cells. Cells were pretreated with 100  $\mu$ M NAC for 30 min and then exposed to gamma ray (4 Gy). Apoptosis was assessed by Western analysis of the cleavages of caspase-3 and PARP, Bcl-xL and Bak 24 h after irradiation. (C) Effects of knockdown of Bcl-xL on the protective effects of Gαs against gamma ray-induced apoptosis. SH-SY5Y cells were transfected with 15  $\mu$ g of Bcl-xL plasmid (pENTR-Bcl-xL siRNA) or vector control (pENTR-lacZ) by electroporation. At 48 h after transfection, cells were irradiated with gamma ray (4 Gy), and then apoptosis were assessed after 24 h by Western blotting. Data are expressed as percentages of corresponding densities of control cells and the histograms represent mean  $\pm$  SE. Asterisks indicate significant differences ( $P < 0.05$  compared with radiation exposed cells, Mann-Whitney *U*-test).

protein to  $3.1 \pm 0.3$ -fold ( $P < 0.05$ ), but decreased the expression of Bax ( $34.6 \pm 1.2\%$ ,  $P < 0.05$ ) and Bad proteins ( $44.1 \pm 1.4\%$ ,  $P < 0.05$ ). The stable expression of G $\alpha$ sQL restored the gamma ray-induced decrease in Bcl-xL expression to  $98.3 \pm 1.7\%$  of the un-irradiated control without affecting Bcl-2 expression. In addition, expression of G $\alpha$ sQL increased the basal level of Bak by  $1.5 \pm 0.2$ -fold ( $P < 0.05$ ), but reduced the gamma ray-induced expression of Bak to  $1.7 \pm 0.1$ -fold ( $P < 0.05$ ) of the untreated vector control level. The expression of G $\alpha$ sQL showed a tendency to increase the basal and gamma ray-induced expression of Bax and Bad proteins in comparison to vector-transfected control (Figure 2A). Because ionizing radiation induces production of ROS, the effect of ROS on the gamma ray induced apoptosis was analyzed. Pretreatment with an antioxidant N-acetyl-L-cysteine (NAC) effectively blocked the gamma ray-induced decrease in Bcl-xL expression and the increase in Bak expression (Figure 2B). NAC treatment also significantly reduced gamma ray-induced cleavages of caspase-3 and PARP, suggesting that ROS mediates the apoptotic cell death induced by the gamma ray irradiation.

Next, to confirm the role of Bcl-xL in the protective effect of G $\alpha$ s against gamma ray-induced apoptosis, the expression of Bcl-xL was knock-down by siRNA method. The transfection of Bcl-xL siRNA decreased the basal expression of Bcl-xL in both the G $\alpha$ sQL expressing cells ( $65.5 \pm 1.3\%$ ,  $P < 0.05$ ) and vector-transfected cells ( $58.5 \pm 1.4\%$ ,  $P < 0.05$ ), and it also augmented gamma ray-induced decrease in Bcl-xL expression in both the G $\alpha$ sQL expressing cells ( $36.4 \pm 3.2\%$ ,  $P < 0.05$ ) and vector-transfected cells ( $34.9 \pm 1.5\%$ ,  $P < 0.05$ ). Transfection of Bcl-xL siRNA increased gamma ray-induced caspase-3 cleavage from  $34.7 \pm 4.0\%$  to  $105.6 \pm 28.1\%$  ( $P < 0.05$ ) and PARP from  $23.6 \pm 3.4\%$  to  $117.7 \pm 11.7\%$  ( $P < 0.05$ ) in G $\alpha$ sQL expressing cells versus gamma ray irradiated vector-transfected control (Figure 2C). This result suggests that restraining down-regulation of Bcl-xL expression might be one mechanism for G $\alpha$ s to inhibit apoptosis induced by gamma ray irradiation in SH-SY5Y neuroblastoma cells.

#### **G $\alpha$ s restrained gamma ray-induced Bcl-xL down-regulation by inhibiting the degradation of both Bcl-xL protein and mRNA**

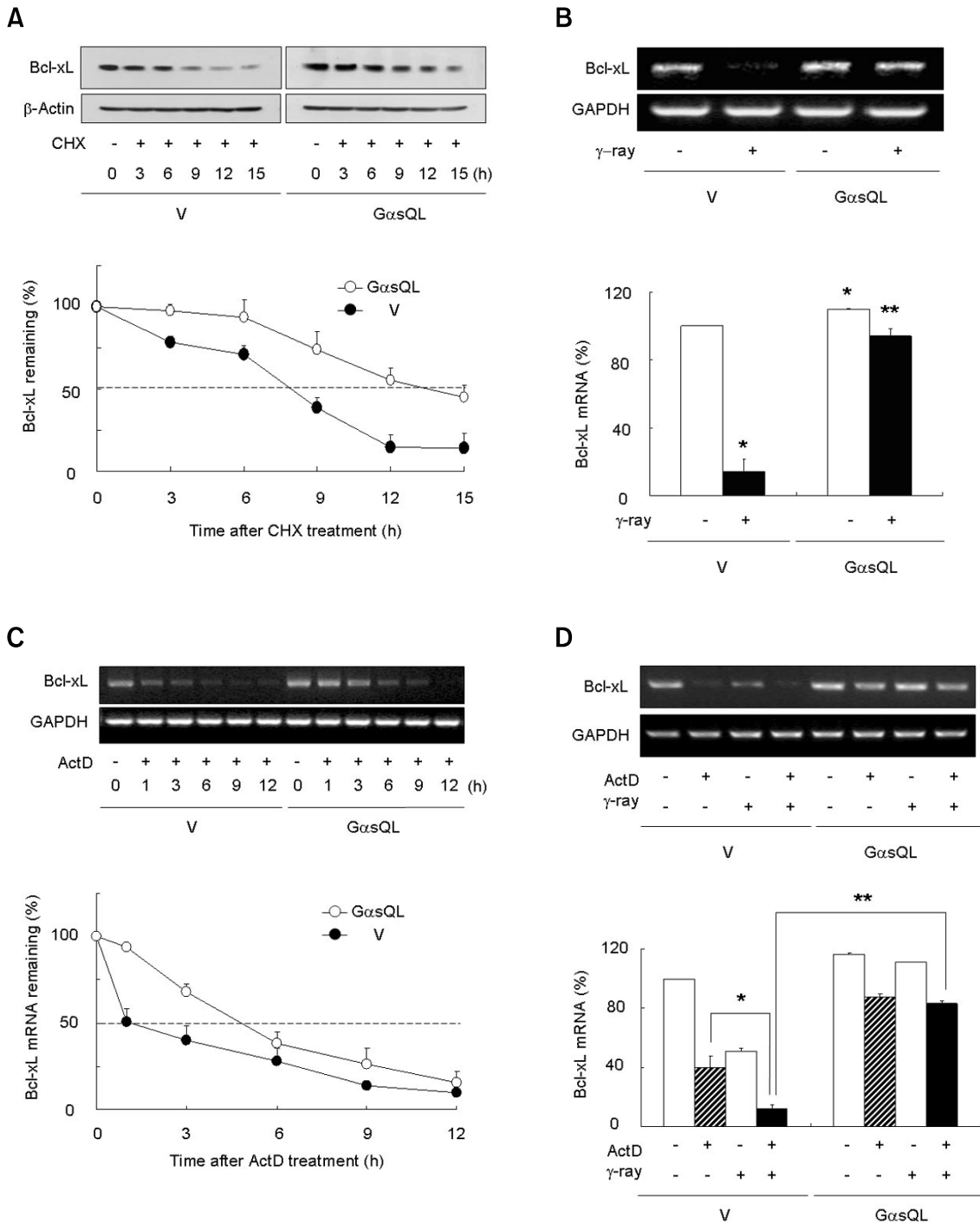
To investigate the mechanism whereby G $\alpha$ s restrained the gamma ray-induced Bcl-xL down-regulation, we first examined the degradation rate of Bcl-xL protein. The degradation rate of Bcl-xL protein in G $\alpha$ sQL expressing cells was significantly

slower than that in vector transfected cells, so the half-life of Bcl-xL protein after cycloheximide treatment increased to approximately  $14.4 \pm 0.2$  h ( $P < 0.05$ ) in G $\alpha$ s expressing cells from  $8.0 \pm 0.2$  h in vector transfected cells (Figure 3A). Next, the effect of G $\alpha$ s expression on the Bcl-xL mRNA level was examined. Gamma ray irradiation removed most of Bcl-xL mRNA, but the expression of G $\alpha$ sQL preserved most of Bcl-xL mRNA after 24 h following the irradiation. This result was confirmed by quantitative real time RT-PCR analysis, which showed that gamma ray irradiation decreased Bcl-xL mRNA level to  $14.4 \pm 6.8\%$  ( $P < 0.05$ ) of the untreated vector-transfected control, and that G $\alpha$ sQL expression increased the basal Bcl-xL mRNA level to  $116.6 \pm 0.6\%$  ( $P < 0.05$ ), and preserved almost intact Bcl-xL mRNA level ( $100.1 \pm 10.2\%$ ,  $P < 0.05$ ) following gamma ray irradiation (Figure 3B).

Then, to probe the mechanism of increased Bcl-xL mRNA levels, we examined the effects of G $\alpha$ s on the degradation of Bcl-xL mRNA after inhibition of transcription by treatment with actinomycin D. The degradation rate of Bcl-xL mRNA in G $\alpha$ sQL expressing cells was significantly slower than that in vector transfected cells, so the half-life of Bcl-xL mRNA increased to approximately  $5.5 \pm 0.1$  h ( $P < 0.05$ ) in G $\alpha$ s-expressing cells from  $1.1 \pm 0.3$  h in vector transfected cells (Figure 3C). Actinomycin D treatment caused a rapid decrease in Bcl-xL mRNA level to  $38.9 \pm 4.9\%$  ( $P < 0.05$ ) without gamma ray irradiation, and to  $12.0 \pm 2.4\%$  ( $P < 0.05$ ) of the vector-transfected control 2 h after irradiation. However, G $\alpha$ sQL expression maintained Bcl-xL mRNA level to  $87.6 \pm 1.9\%$  ( $P < 0.05$ ) of the untreated vector-transfected control after Actinomycin D treatment, and it also maintained Bcl-xL mRNA to  $83.1 \pm 1.9\%$  ( $P < 0.05$ ) following irradiation (Figure 3D). This result indicates that G $\alpha$ s restrains gamma ray-induced Bcl-xL down-regulation by slowing down degradation of both Bcl-xL mRNA and protein.

#### **GPCR ligands modulated the gamma ray-induced apoptosis of SH-SY5Y neuroblastoma cells**

G $\alpha$ s was found to protect gamma ray-induced apoptosis of SH-SY5Y neuroblastoma cells by restraining down-regulation of Bcl-xL, so, we examined whether PGE<sub>2</sub>, of which receptor activates G $\alpha$ s to stimulate adenylate cyclases, can also protect gamma ray-induced apoptosis. Pretreatment with PGE<sub>2</sub> reduced gamma ray-induced caspase-3 cleavage to  $28.3 \pm 9.4\%$  ( $P < 0.05$ ) and PARP cleavage to  $33.7 \pm 6.2\%$  ( $P < 0.05$ ) of untreated control (Figure 4A). Then, the subtype of

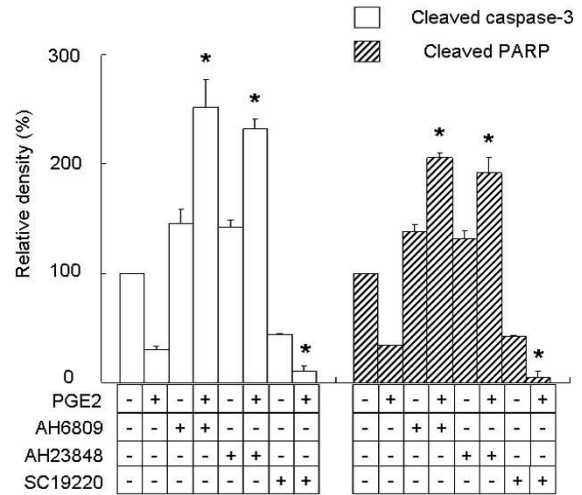
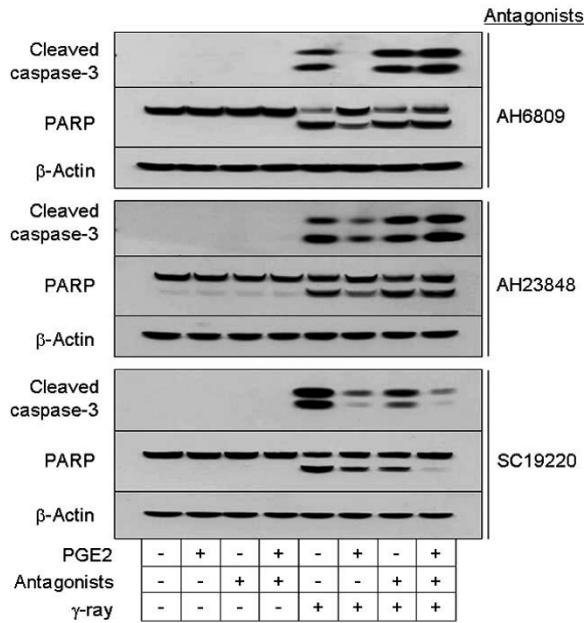


**Figure 3.** Effects of Gαs on the expression of Bcl-xL following gamma ray-irradiation in SH-SY5Y cells. (A) Effects of GαsQL on the degradation rate of Bcl-xL protein. SH-SY5Y cells were treated with 10 μg/ml cycloheximide (CHX), and Bcl-xL protein levels were quantified at various time intervals by Western blot analysis of total cell lysates. The graph shows the mean relative densities ± SE of Bcl-xL bands compared to the initial density. (B) Effects of GαsQL on the expression of Bcl-xL mRNA. The expression Bcl-xL mRNA was analyzed at 24 h after gamma ray irradiation by RT-PCR, and quantified by real time RT-PCR. The amount of Bcl-xL mRNA was normalized to that of GAPDH, and presented as ratio to that of vector transfected control. The values are mean ± SE of at least 4 independent experiments in triplicates. The asterisk (\*) indicates significant difference compared to that of un-irradiated vector-transfected cells, and double asterisks (\*\*) indicate significant difference compared to that of irradiated vector-transfected cells. (C) Effects of GαsQL on the degradation of Bcl-xL mRNA. The cells were treated with 1 μg/ml actinomycin D, and Bcl-xL mRNA levels quantified at various time intervals by RT-PCR analysis of total cell lysates. (D) Effects of GαsQL on the gamma ray-induced degradation of Bcl-xL mRNA. SH-SY5Y cells were irradiated with gamma ray (4 Gy), and then immediately treated with 1 μg/ml actinomycin D. Cells were harvested after 2 h for RT-PCR analysis. The graph shows the mean relative densities (± SE) of Bcl-xL bands compared to the initial density. The asterisk (\*) indicates significant difference compared to that of actinomycin D treated cells, and double asterisks (\*\*) indicate significant difference compared to that of actinomycin D-treated irradiated vector-transfected cells ( $P < 0.05$ , Mann-Whitney *U*-test).

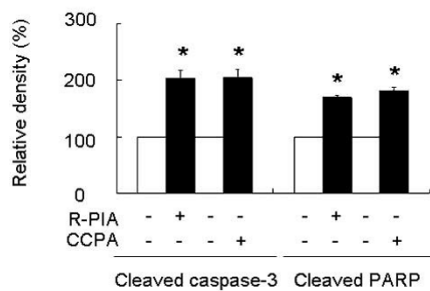
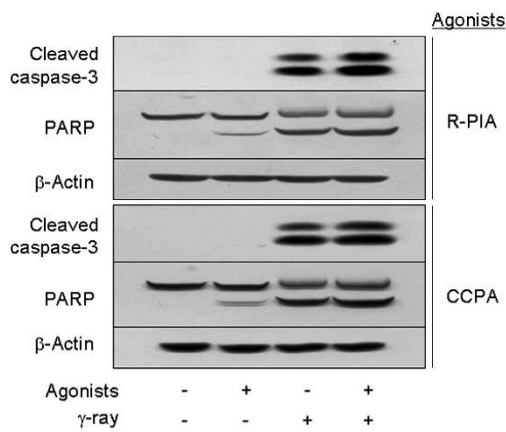
PGE2 receptors involved in anti-apoptotic effects of PGE2 was examined. Co-treatment of PGE2 together with AH6809, an EP1/EP2 mixed prostanoid receptor antagonist increased gamma ray-

induced caspase-3 cleavage to  $251.6 \pm 35.1\%$  ( $P < 0.05$ ), and PARP cleavage to  $205.6 \pm 5.3\%$  ( $P < 0.05$ ), respectively in SH-SY5Y cells. Similarly, co-treatment of PGE2 together with AH23848, an

**A**



**B**



**Figure 4.** GPCR ligands regulated the gamma ray-induced apoptosis of SH-SY5Y human neuroblastoma cells. (A) Effects of PGE2 on gamma ray-induced apoptosis. SH-SY5Y cells were pretreated with 10 μM PGE2, AH6809, AH23848 and SC19220 for 30 min before gamma ray exposure (4 Gy) and then cells were harvested after 24 h. (B) Effects of adenosine A1 agonists on gamma ray-induced apoptosis. SH-SY5Y cells were pretreated with 10 μM R-PIA or CCPA for 30 min before gamma ray irradiation (4 Gy) for 24 h. Apoptosis was assessed by Western analysis for cleavages of caspase-3 and PARP in SH-SY5Y cells, and the blots shown are representative of at least three independent experiments. Data are expressed as percentages of corresponding densities for gamma ray-irradiated cells and the histograms represent mean ± SE. Asterisks indicate significant differences ( $P < 0.05$  versus control cells, Mann-Whitney *U*-test).



EP4 prostanoid antagonist, increased gamma ray-induced caspase-3 cleavage to  $233.0 \pm 7.8\%$  ( $P < 0.05$ ), and PARP cleavage to  $192.1 \pm 13.9\%$  ( $P < 0.05$ ), respectively. On the other hand, co-treatment of PGE2 together with SC19220, an antagonist specific for EP1 prostanoid receptor that activates inhibitory G proteins (G $\alpha$ i2) to inhibit adenylate cyclase, decreased the gamma ray-induced caspase-3 cleavage to  $10.5 \pm 4.9\%$  ( $P < 0.05$ ) and PARP cleavage to  $4.6 \pm 6.0\%$  ( $P < 0.05$ ) in PGE2-treated cells (Figure 4A). Furthermore, treatment of SH-SY5Y cells with R-PIA and CCPA, agonists for adenosine A1 receptor that also activates G $\alpha$ i2 to inhibit adenylate cyclases, increased gamma ray-induced apoptosis. Pre-treatment with R-PIA augmented caspase-3 cleavage to  $203.9 \pm 13.0\%$  ( $P < 0.05$ ) and PARP cleavage to  $169.6 \pm 4.4\%$  ( $P < 0.05$ ), respectively, and pretreatment with CCPA also augmented caspase-3 cleavage to  $204.5 \pm 14.4\%$  ( $P < 0.05$ ), and PARP cleavage to  $181.6 \pm 6.7\%$  ( $P < 0.05$ ) (Figure 4B). This result indicates that GPCR agonists and antagonist coupled to G $\alpha$ s/G $\alpha$ i-cAMP signaling pathway can regulate gamma ray-induced apoptosis.

## Discussion

This study was performed to investigate the molecular mechanism for G $\alpha$ s-cAMP signaling pathways to inhibit radiation-induced apoptosis, and we found that G $\alpha$ s inhibits gamma ray-induced apoptosis by restraining down-regulation of Bcl-xL in SH-SY5Y human neuroblastoma cells. This finding is supported by the result that stable expression of constitutively active G $\alpha$ sQL inhibited gamma ray-induced apoptosis, which was assessed by cleavage of caspase-9, caspase-3 and PARP, cytochrome C release, and FACS analysis of Annexin V-stained cells. Second, expression of G $\alpha$ sQL preserved Bcl-xL level by reducing the degradation rate of both Bcl-xL protein and mRNA following gamma ray irradiation. Third, blocking of Bcl-xL expression by treatment with Bcl-xL siRNA abolished the inhibitory effects of G $\alpha$ s on gamma ray-induced apoptosis. Finally, pretreatment with PGE2 that activates G $\alpha$ s also inhibited gamma ray-induced apoptosis via G $\alpha$ s-coupled receptor subtype dependent pathways.

Ionizing radiation induces DNA damages to cause cell death by various mechanisms including apoptosis. During radiation-induced apoptosis, a variety of signaling pathways are activated to regulate the activity of apoptosis-related molecules. Ionizing radiation has been reported to

increase in the activity of pro-apoptotic Bax in contrast to decrease in that of anti-apoptotic Bcl-2 family including Bcl-2 and Bcl-xL (Findley *et al.*, 1997). cAMP signaling has been known to exert a protective effect against radiation-induced cell death for a long time (Lehnert, 1975), and has been reported to inhibit radiation-induced apoptosis by up-regulation of Bcl-2 expression which prevent activation of caspase-9 by inhibiting the release of cytochrome C from mitochondria into the cytosol (Srivastava *et al.*, 1998). The inhibition of PKA was reported to have a cooperative anti-tumor effect with radiotherapy and to cause inactivation of Bcl-2 which eventually leads to induction of apoptosis (Tortora and Ciardiello, 2002). The promoter of Bcl-2 gene contains cyclic AMP response element (CRE) that enables cAMP signaling pathway to up-regulate the Bcl-2 gene expression by CRE binding proteins (CREB) (Wilson *et al.*, 1996). However, the effects of cAMP signaling system on the expression of Bcl-xL, another anti-apoptotic Bcl-2 family protein, following exposure to ionizing radiation has not been reported yet, and in this study we shows that G $\alpha$ s-cAMP signaling system can protect radiation-induced apoptosis by up-regulating the expression of Bcl-xL, indicating another mechanism for cAMP signaling pathway to protect radiation-induced apoptosis. This finding agrees well with the report that treatment with a cAMP analogue, sp-5,6-DCI-BIMPS, was found to suppress apoptosis by restraining the Bcl-xL down-regulation in CD34(+) cells (Negrotto *et al.*, 2006). The alpha subunit of stimulatory G protein (G $\alpha$ s) activates adenylate cyclase to increase cellular cAMP, which in turn activates signaling pathway that has been reported to regulate apoptosis in a variety of cell types and neurons including SH-SY5Y human neuroblastoma cells. However, cAMP signaling system has been reported to stimulate apoptosis in some type of cells, suggesting that cAMP signaling system inhibits or stimulates apoptosis depending on the cell types and on the nature of apoptosis-triggering agents.

In humans, Bcl-x encodes two distinct species of mRNA designated Bcl-xL and Bcl-xS. The protein product of the longer transcript, Bcl-xL can inhibit apoptosis in tumor cell lines but, the smaller transcript, Bcl-xS can act as a dominant facilitator of cell death (Boise *et al.*, 1993). The anti-apoptotic Bcl-2 members including Bcl-xL prevent mitochondrial protein release by interacting with and inhibiting both Bak/Bax and BH3-only proteins. When Bad heterodimerizes with Bcl-xL in mammalian cells, it releases Bax from Bcl-xL complex, which results in the cytochrome C release to promote cell death (Oltvai *et al.*, 1993; Yang *et al.*, 1995, 2006). The

relative levels and competing dimerization between Bcl-2 family members can indirectly prevent the activation of executioner caspases by controlling cytochrome C efflux from mitochondria. In particular, Bax interacts with mitochondrial membrane allowing cytochrome c release, whereas Bcl-2 and Bcl-xL act in the opposite way, interfering with the activation of Bax (Yang *et al.*, 1995).

Interestingly, Bcl-xL but not Bcl-xS is highly expressed in neuroblastoma cell lines. Moreover, Bcl-xL contributes to pleiotropic drug resistance in neuroblastoma tumors by inhibiting chemotherapy-induced apoptosis, and Bcl-xL over-expression protects radiation-induced apoptosis in many cancer cell lines (Dole *et al.*, 1995; Taylor *et al.*, 1999). These reports indirectly support our finding that  $G\alpha_s$ -cAMP signaling system can exert a protective effect against ionizing radiation-induced apoptosis by inducing Bcl-xL expression.

In a study to probe the mechanism for  $G\alpha_s$  to restrain Bcl-xL down-regulation, we found that  $G\alpha_s$  slows down the degradation of both Bcl-xL mRNA and protein following gamma ray irradiation. Similar restraining of Bcl-xL down-regulation by cAMP signaling system was reported in serum deprivation-induced apoptosis in CD34+-derived megakaryocytes (Negrotto *et al.*, 2006).  $G\alpha_s$  was found to restrain the Bcl-xL mRNA down-regulation after blocking transcription by actinomycin treatment, and to slow down the degradation of Bcl-xL mRNA, suggesting that  $G\alpha_s$  increase stability of Bcl-xL mRNA to restrain Bcl-xL down-regulation. However, the mechanism for  $G\alpha_s$  to increase the stability of Bcl-xL mRNA needs to be elucidated. Furthermore, the effects of  $G\alpha_s$  on the transcription of Bcl-xL cannot be excluded because more mRNA was maintained in the cells that were not treated with actinomycin D, after gamma ray irradiation. In addition to stabilization of Bcl-xL mRNA,  $G\alpha_s$  signaling system was found to slow down the degradation of Bcl-xL protein, which also can contribute to maintain a higher protein level required for anti-apoptotic effect of  $G\alpha_s$ -cAMP system. Thus, this study shows that  $G\alpha_s$ -cAMP signaling system protects SH-SY5Y neuroblastoma cells by slowing down Bcl-xL mRNA and protein following gamma ray-irradiation. Moreover,  $G\alpha_s$  was found to repress ionizing radiation-induced up-regulation of Bak in SH-SY5Y human neuroblastoma cells, which also favors cell survival (Choi *et al.*, 2006), indicating  $G\alpha_s$ -cAMP signaling system protects cells against radiation-induced apoptosis by affecting multiple pro-apoptotic and anti-apoptotic Bcl-2 family proteins.

This study also shows that radiation-induced apoptosis can be modulated by the ligands for

GPCR that activates  $G\alpha_s$  or  $G\alpha_i$  to regulate adenylate cyclase activity and thus cAMP levels. This finding is supported by the result that treatment with PGE2 that binds to  $G\alpha_s$ -coupled receptor protected gamma ray-induced apoptosis in SH-SY5Y cells. PGE2 binds to four different receptor subtypes termed EP1, EP2, EP3 and EP4, among which EP2 and EP4 receptors couple to a  $G\alpha_s$  (Hata and Breyer, 2004). The protective effect of  $G\alpha_s$  against gamma ray-induced apoptosis was abolished by treatment with specific EP2/4R antagonist such as AH6809 and AH23848, but treatment with an antagonist of EP1 receptor that couples to  $G\alpha_i$  to inhibit adenylate cyclases decreased the apoptosis. In the same vein, treatment with R-PIA and CCPA, agonists for adenosine A1 receptor that also activate  $G\alpha_i$  to inhibit adenylate cyclases, augmented ionizing radiation-induced apoptosis of the SH-SY5Y neuroblastoma cells. Adenosine receptors are major targets of caffeine, and there is growing evidence that they could also be promising therapeutic targets in a wide range of conditions, including cancer (Jacobson and Gao, 2006). Our finding agrees well with the reports that PGE2 protects against apoptosis of human colon cancer cells (Sheng *et al.*, 1998), and thus EP receptors have been identified as potential targets for treatment and prevention of colorectal cancer. The anti-apoptotic effect of PGE2 and pro-apoptotic effect of adenosine A1 can result from the modulation of  $G\alpha_s$ / $G\alpha_i$ -cAMP signaling pathway that regulates expression of various apoptosis-related molecules including Bcl-xL. This finding suggests that the therapeutic efficiency of cancer radiotherapy can be improved by co-treatment with specific GPCR ligands that inhibits  $G\alpha_s$ -cAMP signaling system to reduce the resistance to radiation-induced apoptosis.

From this study, it is concluded that  $G\alpha_s$  can protect SH-SY5Y neuroblastoma cells from gamma ray-induced apoptosis partly by restraining Bcl-xL down-regulation, which can be modulated by the ligands of various receptors that couple to  $G\alpha_s$  or  $G\alpha_i$ . This finding provides a better understanding how  $G\alpha_s$  signaling pathways modulate gamma ray-induced apoptosis, and an important clinical implications that the efficiency of radiation therapy against cancer cells could be improved by modulation of  $G\alpha_s$ / $G\alpha_i$ -cAMP signaling system with agonists or antagonists for cancer-cell specifically expressed GPCR.

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