Stimulatory heterotrimeric G protein augments gamma ray-induced apoptosis by up-regulation of Bak expression via CREB and AP-1 in H1299 human lung cancer cells

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Abstract

**Stimulatory heterotrimeric GTP-binding proteins (Gs protein) stimulate cAMP generation in response to various signals, and modulate various cellular phenomena such as proliferation and apoptosis. This study aimed to investigate the effect of Gs proteins on gamma ray-induced apoptosis of lung cancer cells and its molecular mechanism, as an attempt to develop a new strategy to improve the therapeutic efficacy of gamma radiation. Expression of constitutively active mutant of the α subunit of Gs (GαsQL) augmented gamma ray-induced apoptosis via mitochondrial dependent pathway when assessed by clonogenic assay, FACS analysis of PI stained cells, and western blot analysis of the cytoplasmic translocation of cytochrome C and the cleavage of caspase-3 and ploy(ADP-ribose) polymerase (PARP) in H1299 human lung cancer cells. GαsQL up-regulated the Bak expression at the levels of protein and mRNA. Treatment with inhibitors of PKA (H89), SP600125 (JNK inhibitor), and a CRE-decoy blocked GαsQL-stimulated Bak reporter luciferase activity. Expression of GαsQL increased basal and gamma ray-induced luciferase activity of cAMP response element binding protein (CREB) and AP-1, and the binding of CREB and AP-1 to Bak promoter. Furthermore, prostaglandin E2, a Gαs activating signal, was found to aug-

ment gamma ray-induced apoptosis, which was abolished by treatment with a prostanoid receptor antagonist. These results indicate that Gαs augments gamma ray-induced apoptosis by up-regulation of Bak expression via CREB and AP-1 in H1299 lung cancer cells, suggesting that the efficacy of radiotherapy of lung cancer may be improved by modulating Gs signaling pathway.**

Keywords: apoptosis; bcl-2 homologous antagonist-killer protein; cyclic AMP; gamma rays; heterotrimeric GTP-binding protein; receptors, G-protein-coupled

Introduction

Lung cancer is the leading cause of cancer deaths in many countries including Korea (Greenlee *et al.*, 2000). Non-small cell lung cancer (NSCLC) represents approximately 65-70% of primary lung cancers, and most NSCLC is treated with combined-modality treatment with surgery, radiotherapy, and chemotherapy (Potti and Ganti, 2006). However, development of resistance to chemotherapy and radiotherapy is the major limitation of efficient therapy of human lung cancer (Decker and Wilson, 2008). Radiotherapy is one of the major treatment modality for various solid tumors including lung cancer, and induces DNA damages and various types of cell death including apoptosis. Thus, the enhancement of apoptosis-inducing efficiency of radiation might contribute to improvement of radiotherapy of lung cancer.

Apoptosis is a type of programmed cell death that is used by multicellular organisms to dispose of unwanted cells, and plays an important role in normal development and maintenance of tissue homeostasis (Taylor *et al.*, 2008). Abnormal regulation of apoptosis is implicated in neurodegenerative disorders, autoimmune diseases and cancer. Two apoptotic pathways are widely studied: extrinsic pathway and intrinsic pathway. The extrinsic pathway is triggered by activation of tumor necrosis factor receptor family, and the intrinsic pathway is triggered by various forms of stress such as radiation and cytotoxic drugs. The intrinsic pathway

involves mitochondrial outer membrane permeation (MOMP), which results in releases of apoptosis-inducing molecules including cytochrome c from intermembrane space of mitochondria. The released cytochrome c induces sequential activation of caspase-9 and caspase-3, which in turn hydrolyzes cellular components to induce apoptosis.

Heterotrimeric GTP-binding proteins (G proteins) are activated by G protein-coupled cell surface receptors, and activate various effectors including second messenger-forming enzymes and ion channels (Brown *et al.*, 1991). G proteins are composed of α, β and γ subunits, and the α subunits are classified into 4 families: Gαs, Gαi, Gαq, Gα12 (Clapham and Neer, 1993; Conklin and Bourne, 1993; Daaka *et al.*, 1997). The α subunit of stimulatory G protein (Gαs) stimulates adenylate cyclases to increase cAMP level, which activates cAMP-dependent protein kinase (PKA). PKA phosphorylates various target molecules to regulate metabolism and gene expression. The cAMP response element binding protein (CREB) is phosphorylated by PKA, and then binds to the cAMP response element (CRE) to induce transcription of approximately 4000 target genes, including genes regulating apoptosis (Zhang *et al.*, 2005; Siu and Jin, 2007). Recently, stimulatory G proteins were reported to modulate apoptosis induced by hydrogen peroxide and gamma rays by regulating Bcl-2 family expression in human neuroblastoma cells. Thus, in an attempt to develop a novel strategy to improve the therapeutic efficacy of radiation by modulating the cAMP signaling system, this study was aimed to investigate the effect of the Gαs signaling system on gamma ray-induced apoptosis of lung cancer cells and its underlying mechanism. From this study, Gαs-cAMP signaling pathway was found to augment gamma ray-induced apoptosis by up-regulation of Bak expression through CREB and AP-1 in H1299 lung cancer cells.

Results

Gαs augments gamma ray-induced apoptosis of H1299 human lung cancer cells

To study the effect of Gαs on gamma ray-induced apoptosis of lung cancer cells, we screened the effect of transient expression of constitutively active GαsQL in H1299 human lung cancer cells, which showed that expression of GαsQL augmented gamma ray-induced apoptosis of H1299 cells (data not shown). Thus, GαsQL was expressed stably in H1299 cells to investigate in detail the mechanism by which Gαs augments the apoptosis. The stable expression of GαsQL was confirmed by western blot analysis using an antibody specific to Gαs, and resulted in a large increase in CREB phosphorylation, indicating that the cAMP signaling pathway was activated by GαsQL expression (Figure 1).

The effect of Gαs on gamma ray-induced apoptosis was examined using H1299 cells that stably express GαsQL, which showed that GαsQL augmented apoptosis of the H1299 cells when assessed by clonogenic assay, by FACS analysis of propidium iodide stained cells, and by western blot analysis of cytoplasmic translocation of cytochrome C and the cleavage of caspase-3 and poly (ADP-ribose) polymerase (PARP). Expression of GαsQL decreased survival fraction of H1299 cells treated with various dose of gamma ray (Figure 2A), increased the fraction of cells that contained sub G1 DNA content (Figure 2B). GαsQL expression also increased the cleavage of caspase-3 and PARP (Figure 2C), and the translocation of mitochondrial cytochrome c to cytosol (Figure 2D).

Gαs augments gamma ray-induced apoptosis by up-regulation of Bak expression in H1299 cells

To elucidate the underlying mechanism by which Gαs augmented gamma ray-induced apoptosis in H1299 cells, the effect of Gαs on the expression of Bcl-2 family proteins that mediate both the pro-apoptotic and the anti-apoptotic responses was examined. Expression of GαsQL increased the gamma ray-induced expression of pro-apoptotic molecule, Bak (Figure 3A). The expression level of Bak protein increased to 2.1-fold in gamma ray exposed GαsQL-expressing cells in comparison to vector-transfected control cells ($P < 0.05$, Figure 3B). The expression of Bax, another pro-apoptotic
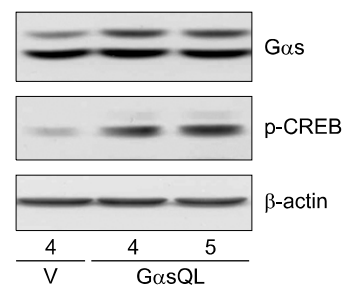

Figure 1. Stable expression of constitutively active GαsQL in H1299 human lung cancer cells. Expression of GαsQL and phosphorylation of CREB in GαsQL-transfected cells were confirmed by western blot analysis using a specific antibody against Gαs and phosphorylated CREB (Ser-133). Expression of β-actin was also analyzed as a loading control. V and GαsQL refer to vector-transfected cells and GαsQL-transfected cells, respectively, and the numbers refer the cell clones.
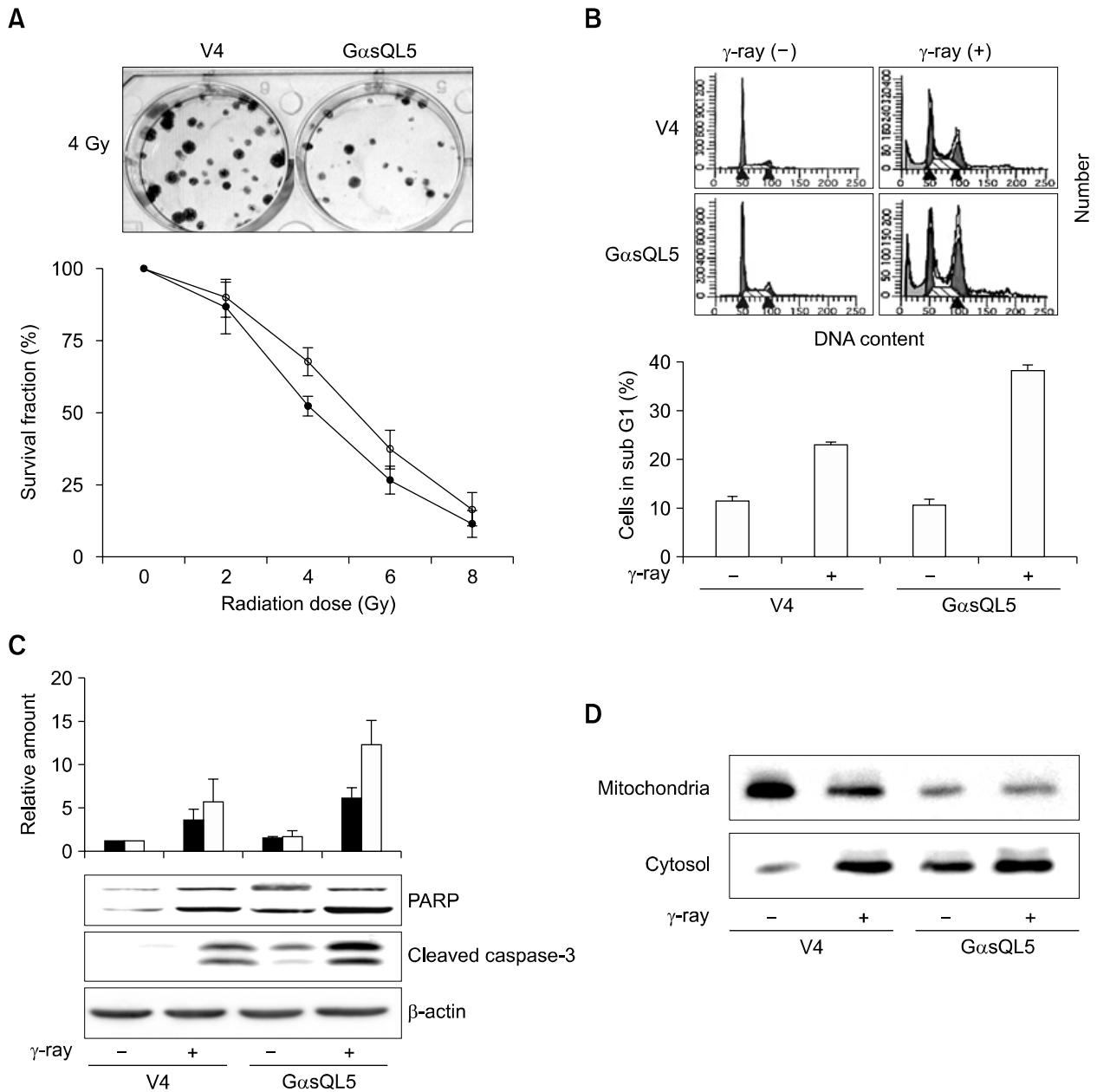
A

B

C

D

Figure 2. Gαs augmented gamma ray-induced apoptosis of H1299 human lung cancer cell. (A) Effects of GαsQL on survival of the gamma ray-irradiated cells. H1299 cells in 6-well plates (100 cells/well) were treated with gamma ray (0-8 Gy), and the cell viability was assessed after 14 days by colonogenic assay. Open circles represent vector transfected cell and closed circles represent GαsQL-transfected cell clones. (B) Effects of GαsQL on the DNA content of gamma ray-irradiated cells. The DNA content was assessed by FACS analysis of propidium iodide-stained H1299 cells 48 h after gamma ray-irradiation (10 Gy), and the sub G1 fraction (%) was presented as histograms. (C) Effects of GαsQL on the cleavage of caspase-3 and PARP in gamma ray-irradiated cells. Forty-eight hours after gamma ray irradiation (10 Gy), cleavages of capspase-3 (■) and PARP (□) were assessed by western blot analysis of vector- or GαsQL-expressing H1299 cells, and the densitometric results were presented as percentages of the vector-transfected control. (D) Effects of GαsQL on the cytosolic release of cytochrome c from mitochondria in gamma ray-irradiated cells. The cytochrome c release into cytosol was analyzed by western blotting after cytosolic and mitochondrial fractions were prepared by centrifugation. The blots shown are representative of at least three independent experiments. Histograms represent means ± SE, and the amounts are expressed as percentages of that in control cells.

Bcl-2 family protein was not changed, and the expression of anti-apoptotic Bcl-XL protein seemed to be slightly increased by expression of GαsQL.

Gαs increased transcription of Bak gene through cAMP-PKA-CREB-dependent pathways

Next, the effect of Gαs on the level of Bak mRNA was examined, and the expression of Bak mRNA
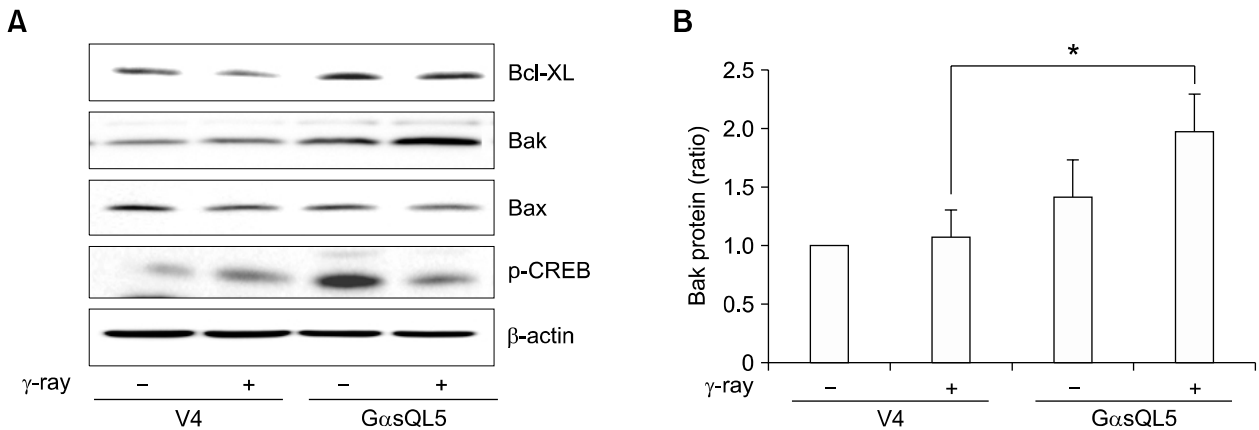
A

B

Figure 3. Gαs augments gamma ray-induced apoptosis by up-regulating the expression of Bak protein in H1299 cells. (A) Effects of GαsQL on the expression level of Bcl-2 family proteins in gamma ray-irradiated H1299 cells. (B) Effects of GαsQL on the expression level of Bak proteins in gamma ray-irradiated H1299 cells. Forty-eight hours after gamma ray irradiation (10 Gy), expression of Bcl-2 family proteins was analyzed by western blot analysis (A), and the densitometric results are presented as histograms (B). The blots shown are representative of at least three independent experiments, and histograms represent means ± SE. The expression was presented as a ratio to that of control cells, and asterisks indicate a significant difference from vector-transfected cells,) ($P < $ 0.05 Mann-Whitney *U*-test).

was also increased to 2.35-fold of the control by expression of GαsQL in H1299 cells when assessed by real time quantitative RT-PCR (Figure 4A). In a study to examine the effect of Gαs on the transcription of the Bak gene, expression of GαsQL increased Bak luciferase reporter activity to 3.1-fold from the control, and the luciferase activity was decreased by treatment with H89 (PKA inhibitor), SP600125 (JNK inhibitor), and a CRE-decoy (Figure 4B). This result suggests that Gαs increases Bak expression by up-regulating the transcription of the Bak gene, which is dependent on PKA, JNK, and CREB.

Gαs increased transcription of Bak gene by activations of CREB and AP-1 transcription factors

To investigate the mechanism how Gαs increased the transcription of Bak, the effect of GαsQL on the activity of transcription factors activated by gamma ray irradiation was analyzed. Irradiation with gamma ray increased the reporter luciferase activity under the control of AP-1 and CREB, but decreased luciferase activity under the control of NF-κB. The expression of GαsQL increased basal AP1-luciferase activity to 3.88 ± 0.08-fold ($P < $ 0.05) of the vector-transfected control, and gamma ray-induced AP-1 luciferase activity from 2.10 ± 0.08-fold to 8.59 ± 0.24-fold ($P < $ 0.02). GαsQL expression also increased basal CREB-luciferase activity to 3.69 ± 0.16-fold ($P < $ 0.02) of the vector-transfected control, and gamma ray-induced CREB-luciferase activity from 1.59 ± 0.07-fold to 6.10 ± 0.10-fold ($P < $ 0.05). The expression of GαsQL did

not cause significant change in basal and gamma ray-induced activities of NF-κB luciferase and NFAT luciferase (Figure 4C).

Next, to confirm that AP-1 and CREB mediate Gαs-induced increase in Bak transcription, the effects of Gαs on the binding of AP-1, CREB, and NF-κB to the Bak promoters were analyzed by EMSA in H1299 cells. The expression of GαsQL increased the basal and gamma ray-induced binding of CREB and AP-1 probes but inhibited NF-κB probe to the nuclear extract (Figure 4D).

PGE2 augmented the gamma ray-induced apoptosis of H1299 lung cancer cells

Because Gαs was found to augments gamma ray-induced apoptosis of H1299 lung cancer cells, we examined whether PGE2, of which receptor activates Gαs to stimulate adenylate cyclases, can also stimulates gamma ray-induced apoptosis. Pretreatment with PGE2 increased gamma ray-induced cleavage of caspase-3 and PARP, and co-treatment of PGE2 together with AH6809, an EP1/EP2 prostanoid receptor antagonist, abolished the PGE2-induced increase in the cleavage of caspase-3 and PARP in gamma ray-irradiated H1299 cells (Figure 5). This result indicates that PGE2 that can activate Gαs may augment gamma ray-induced apoptosis via cAMP-dependent manners.

Discussion

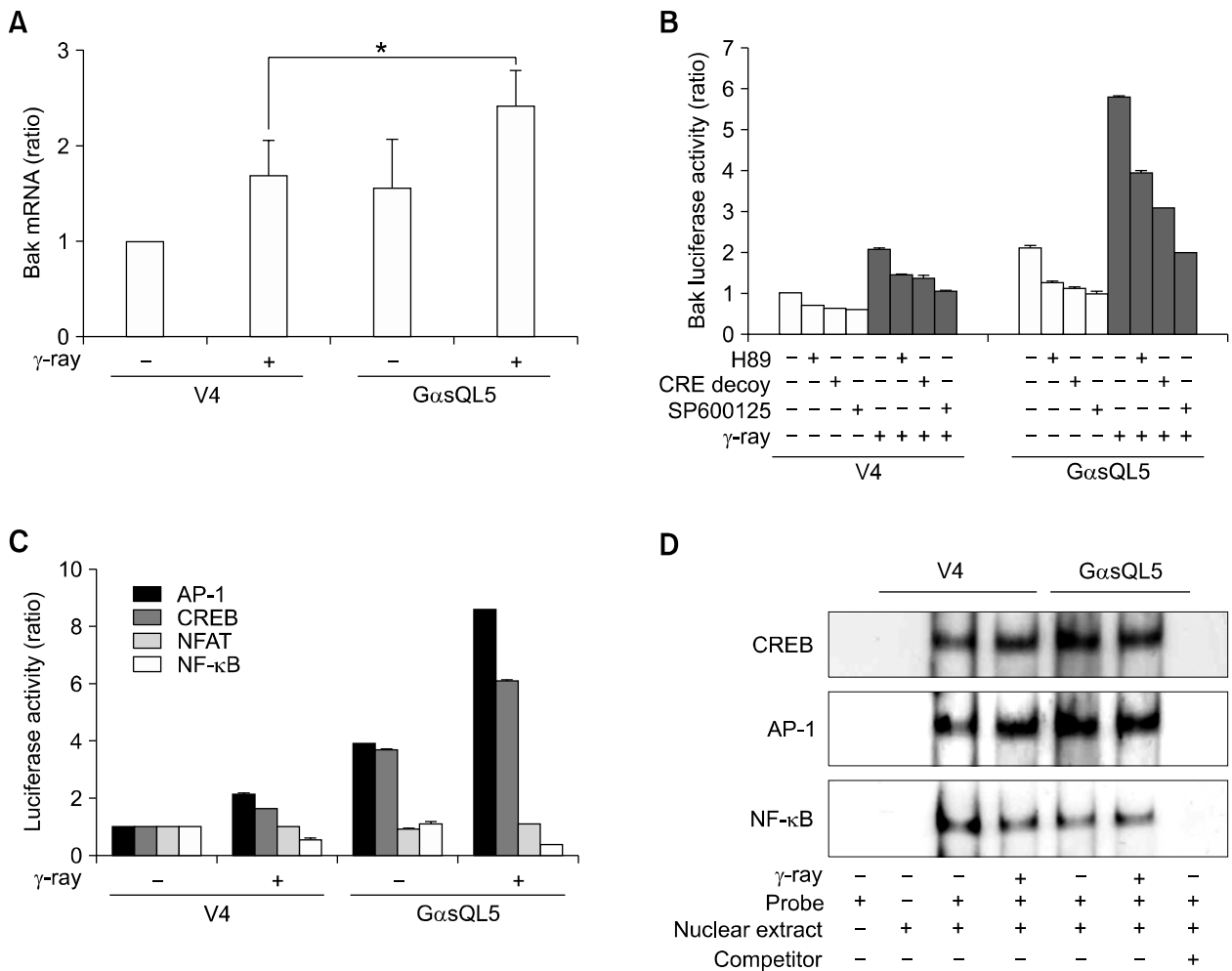This study was performed to examine the effect of

Figure 4. Gαs enhances cell gamma ray-induced transcription of Bak via CREB and AP-1 dependent pathways in H1299 lung cancer cells. (A) Effects of GαsQL on the expression level of Bak mRNA in gamma ray-irradiated H1299 cells. Expressions of Bak mRNA were measured by quantative real-time RT-PCR 24 h after gamma ray irradiation of H1299 cells. The amounts of Bak mRNA were normalized versus that of GAPDH, and presented as ratio to that of vector transfected controls. (B) Effect of inhibitors on GαsQL-induced expression of Bak-luciferase. H1299 cells were transfected with 2.5 μg of Bak-pLuc and 2.5 μg of a β-galactosidase construct, and after 24 h, the cells were irradiated gamma ray. Then, luciferase activities were measured versus β-galactosidase activity after 24 h, and were normalized and presented as ratios to vector-transfected controls. Asterisks indicate significant differences from that of vector-transfected cells ($P < 0.05$, Mann-Whitney U test). (C) Effects of GαsQL on the activation of transcription factors in gamma ray-irradiated H1299 cells. H1299 cells were transfected with CREB-, AP-1-, NF-AT-, or NF-κB-luciferase constructs (2.5 μg) and β-galactosidase plasmid. After 24 h, cells were irradiated with gamma ray, and then luciferase activities were analyzed after 48 h. (D) Effects of GαsQL on the binding of transcription factors to Bak promoter in gamma ray-irradiated H1299 cells. The bindings of transcription factors to Bak promoter were analyzed by EMSA using a chemiluminescent biotin 5' end-labeled DNA probe corresponding to the AP-1, CREB, NFAT, or NF-κB consensus sequences. For competition experiments, 100-fold excess of each unlabeled oligonucleotide was added.

Gαs on gamma ray-induced apoptosis of lung cancer cells and its underlying molecular mechanism, and we found that Gαs augments gamma ray-induced apoptosis by up-regulation of Bak expression through activation of CREB and AP-1 in H1299 human lung cancer cells. This conclusion is supported by the result that expression of constitutively active GαsQL mutant augmented radiation-induced apoptosis and up-regulated the expression of Bak protein through increasing transcription of Bak in a CREB- and AP-1-dependent pathway. Our finding agrees well with the report that overexpression of Gαs increased apoptosis of cardiac myocytes (Geng et al., 1999), and we also found Gαs protein to augment hydrogen peroxide-induced apoptosis of human fibroblast cells and endothelial cells (unpublished data). Furthermore, we showed that PGE2 augmented gamma ray-induced apoptosis of H1299 cells via Gαs-coupled receptor, as stimulation of beta-adrenergic
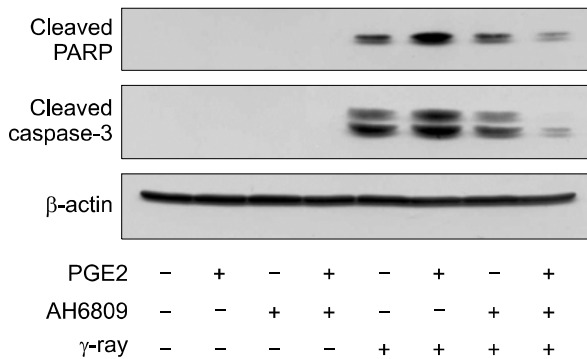
Figure 5. PGE2 augmented gamma ray-induced apoptosis of H1299 human lung cancer cells. H1299 cells were pretreated with 10 μM PGE2 in the presence or absence of AH6809 for 30 min before gamma ray irradiation (10 Gy), and then cells were harvested after 48 h. Apoptosis was assessed by western blot analysis for cleavages of caspase-3 and PARP, and the blots shown are representative of at least three independent experiments.

receptor induced cell death of thymocytes (Gu *et al.*, 2000). However, Gαs protein was reported to inhibit radiation- or hydrogen peroxide-induced apoptosis of SH-SY5Y human neuroblastoma cells (Kim *et al.*, 2007, 2008). Inhibitory G proteins were found to antagonize the anti-apoptotic effect of Gαs in hydrogen peroxide-treated SH-SY5Y human neuroblastoma cells (Kim *et al.*, 2008) and also to inhibit hydrogen peroxide-induced apoptosis by up-regulation of Bcl-2 via NF-κB in H1299 human lung cancer cells (Seo *et al.*, 2009). These reports indicate Gαs-cAMP signaling system exerts opposite effects depending on cell types, and Gαs-cAMP signaling system is also known to exert such opposite effects on cellular proliferation.

Bcl-2 family proteins are the master regulator of mitochondria-dependent apoptosis, and they are grouped into anti-apoptotic 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. The anti-apoptotic proteins and pro-apoptotic proteins form heterodimers and inhibit each others, and thus the balance between the anti-apoptotic and pro-apoptotic proteins determine the induction of MOMP, which releases cytochrome c to activate caspase-9 and caspase-3 to induce apoptosis (Adams and Cory, 2007; Youle and Strasser, 2008). The Gαs-cAMP signaling pathway has been reported to regulate apoptosis by modulating various Bcl-2 family proteins such as Bcl-2, Bcl-XL, Bax, Bak, and Bad (Lizcano *et al.*, 2000; Lewerenz and Methner, 2003; Yano *et al.*, 2003). Bak protein is a multidomain pro-apoptotic protein, and acts as a final gateway to intrinsic cell death pathways by

inducing MOMP (Chittenden *et al.*, 1995; Wei *et al.*, 2001), and ionizing radiation induces apoptosis by allowing MOMP through activation of Bax/Bak (Chen and Wang, 2002). In this study, we found that Gαs augment gamma ray-induced apoptosis of lung cancer cells by up-regulation of Bak expression. However, we have also reported previously that Gαs-cAMP signaling pathway inhibits apoptosis of SH-SY5Y neuroblastoma cells from hydrogen peroxide-induced apoptosis by repressing Bak induction (Kim *et al.*, 2008). Thus, Gαs seems to modulate the expression of Bak in opposite direction depending on the cell types: represses Bak induction in SH-SY5Y neuroblastoma cells, stimulates Bak induction in H1299 lung cancer cells. Gαs was found to up-regulate Bak expression by augmenting gamma ray-induced activation of CREB and AP-1 in H1299 cells, whereas Gαs inhibited hydrogen peroxide-induced activations of AP-1 in cAMP-PKA-dependent manner in SH-SY5Y neuroblastoma cells. Therefore, it is suggested that the different effect of Gαs on Bak expression might reflect the differential expression of molecules acting in Gαs-cAMP signaling pathway or the other interacting signaling pathways, which resulted from differentiation of specific tissues.

PGE2 is one type of prostaglandin, and it is involved in a variety of physiological responses and diseases including inflammation and cancer. PGE2 induces various cellular responses by binding and activating four G protein coupled receptor isotypes (EP1, EP2, EP3 and EP4), which differ in their PGE2 binding affinities and downstream signal transduction pathways. EP2 and EP4 are known to activate Gαs to stimulate adenylate cylclase, but EP3 activates Gαi to inhibit adenylate cyclase (Sugimoto and Narumiya, 2007). This study showed that PGE2 augmented radiation-induced apoptosis of H1299 lung cancer cells by activating EP2 that couples Gαs to stimulate adenylate cyclase. However, pre-treatment with PGE2 in the presence of AH6809, an EP2 antagonist, almost completely abolished radiation-induced apoptosis, which resulted in less apoptosis than the cell pre-treated AH6809 alone. Thus, it is speculated that the anti-apoptotic effect may result not only from the complete abolishment of the pro-apoptotic effect of PGE2 caused by blocking EP2 activation with the antagonist, but also from the stimulation of anti-apoptotic effect of exogenous PGE2 caused by activation of EP3 which couples Gαi and inhibits adenylate cyclase.

Radiotherapy of cancer induces DNA damage, which triggers intrinsic apoptosis by regulation of the activity of Bcl-2 family molecules (Baliga and Kumar, 2002). However, the development of resis-

tance to radiation-induced cell death is the major limitation for efficient radiotherapy, and thus development of strategies that prevent or avoid the radioresistance can improve the efficacy of radiotherapy of cancer. This study showed that stimulation of a Gαs-coupled receptor increased the efficiency of radiation-induced apoptosis of lung cancer cells, suggesting that modulation of Gαs-coupled receptor signaling pathway may be a potential strategy to kill the lung cancer cells efficiently and selectively.

From these results, it is concluded that Gαs signaling system augments gamma ray-induced apoptosis by up-regulation of Bak expression via CREB and AP-1 in H1299 human lung cancer cells. These results suggest that Gαs signaling pathway may be targeted to improve the efficiency of radiotherapy for lung cancer.

Methods

Cell culture and reagents

H1299 human non-small cell lung cancer cells were purchased from American Type Culture Collection (ATCC, Rockville, MD) and was maintained in DMEM medium containing 10% FBS (JBI, Korea) and 100 units/ml penicillin-streptomycin, in a CO_2 incubator at $37^\circ C$. A constitutively active α subunit of stimulatory G protein (GαsQ226L) in pcDNA3 vector (Invitrogen, Carlsbad, CA) was stably expressed in H1299 cells by transfection by electroporation using a Gene Pulser II (Bio-Rad). The H1299 cells expressing GαsQL were screened by growing in the medium containing G418 (500 μg/ml), and expression of GαsQL was confirmed by western blot analysis. Cycloheximide, PGE2, AH-6809, and DMSO were purchased from Sigma Chemicals (St. Louise, MO).

Treatment with gamma ray and clonogenic assay

H1299 cells of approximately 80% confluency in 10-cm dishes were irradiated with 10 Grey (Gy) gamma ray from a ^{137}Cs source at a rate of 246.5 cGy/min, which induced enough apoptosis for western blot analysis of the cleaved PARP and casapase-3. For clonogenic assay, H1299 cells (1×10^2/well) were seeded in 6 well plates and incubated for 24 h in DMEM medium. After exposure to gamma ray, the cells were incubated for 14 days before counting colonies. The colony forming efficiency was presented as the percentage of number of colonies formed after irradiation to that of untreated control group.

Flow cytometry of propidium iodide stained cells

Cellular DNA content was determined by flow cytometry of propidium iodide stained cells using a flow cytometer (Becton-Dickinson, San, Jose, CA) (Wang et al., 2007). This analysis was carried out using a double discriminator module, which distinguishes between signals coming from a single nucleus and those from two or more aggregated nuclei. Only signals from single nuclei were analyzed (104 nuclei/assay). The ploidy of nuclei was determined from the DNA content as described (Reid et al., 1987; Gong et al., 1993).

Real time quantitative RT-PCR

Total RNA for RT-PCR reactions was prepared with acid guanidinium thiocyanate phenol chloroform extraction method. First-strand cDNA was synthesized using oligo (dT) primers and the SuperScript First-Strand Synthesis System (Invitrogen). PCR was performed with specific primers: Bak, forward primer 5'-TGAAAAATGGCTTCGGG-GCAAGGC-3' and reverse primer 5'-TCATGATTTGAAGA-ATCTTCGTACC-3'; GAPDH, forward primer 5'-ACCACAG-TCCATGCCATCAC-3' and reverse primer 5'-TCCACCAC-CCTGTTGCTGTA-3'.

Immunoblot analysis

The expression of proteins was analyzed by western blotting using specific antibodies as described previously (Kim et al., 2007). Antibodies against β-actin and Bcl-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), antibodies against PARP, cleaved PARP, cleaved caspase-3, Bax, Bcl-XL, and Bak were obtained from Cell Signaling Technology (Beverly, MA). Cytochrome c released into the cytoplasm was analyzed by immunoblotting using an antibody purchased from Cell Signaling Technology (Beverly, MA) after subcellular fractionation. The blot image was developed by incubation with an enhanced chemiluminescence substrate mixture (Pierce), and captured by a luminescent image analysis system (LAS-3000, Fuji, Tokyo, Japan). Then, the density of protein bands was quantified using Multi Gauge v2.3 software, and the relative band density was expressed as a percentage or a multiple of corresponding densities of the control.

Luciferase activity assays and electrophoretic mobility shift assay (EMSA)

H1299 cells were transfected with plasmids containing a luciferase reporter gene under the control of Bak promoter by electroporation using a Gene Pulser II (Bio-Rad) at 200 V/950 microfarads. Luciferase activities were assayed using the Bioluminescent Reporter Gene Assay System (Tropix, Bedford, MA) according to the manufacturer's instructions. At least three independent experiments were performed in duplicate and promoter activities were normalized against β-galactosidase activity.

EMSA was performed using the LightShift Chemiluminescent EMSA Kit (Pierce) as described previously (Kim et al., 2008). Oligonucleotides containing AP-1, NFAT, NF-κB, or CREB binding site in the Bak promoter region were 5' end-labeled with biotin. Oligonucleotides sequences were as follows: AP-1 binding site, 5'-CTTTCCGTGA-CTAACGATGTGACC-3'; cAMP response element, 5'-GA-GTGCAGTGACGTCATCTCGGCT-3'; NF-κB binding site, 5'-GAAGTTGAGGGGGACTTTCCCAGG-3'.

Data analysis

All experiments were repeated independently at least three times, and data are presented as means $\pm$ standard error (SE). The nonparametric Mann-Whitney U test was used to analyze mean values, and P values < 0.05 were considered to be statistically significant.

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