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Ginsenoside Rh2 Induces Bcl-2 Family Proteins-Mediated Apoptosis *In Vitro* and in Xenografts *In Vivo* Models

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ABSTRACT

The cancer chemoprevention effects of ginseng saponins have been demonstrated against a variety of experimental tumors; however, their molecular mechanisms *in vitro* and in *in vivo* models are not well studied. This study was undertaken to gain insights into the molecular mechanisms of ginsenoside Rh2 (Rh2)-induced cell death in human breast cancer cell lines as well as in *in vivo* xenografts. Rh2 treatment significantly inhibited viability of both MCF-7 and MDA-MB-231 human breast cells in a concentration-dependent manner, which correlated with mitochondria-mediated apoptosis. Rh2-induced apoptosis was accompanied by the down-regulation of antiapoptotic proteins Bcl-2, Bcl-xL, and Mcl-1. It also caused induction of the proapoptotic members Bak, Bax, and Bim leading to mitochondrial translocation of Bax and activation of caspases. Moreover, Rh2-induced apoptosis was partially, yet significantly protected by transient transfection of MCF-7 cells with Bax- and Bak-targeted siRNAs. Oral gavage of 5 mg Rh2/kg of mouse (three times a week) significantly caused apoptosis of MDA-MB-231 xenografts. An increase in Bax and Bak and a decrease in Bcl-2 and Bcl-xL transcript levels, in accordance with their protein expression, were observed in tumor tissue. Tumors from Rh2-treated mice exhibited a markedly higher count of apoptotic bodies and reduced proliferation index compared with control tumors. Our data suggest that Rh2 used in traditional oriental medicine for the treatment of various ailments, may be an attractive agent for the treatment and/or prevention of human breast cancers. J. Cell. Biochem. 112: 330–340, 2011.

KEY WORDS: GINSENOSIDE RH2; BREAST CANCER; Bcl-2 FAMILY PROTEINS; APOPTOSIS; XENOGRAFT

Epidemiologic studies have continuously demonstrated that dietary intake of natural products may be protective against the risk of various types of malignancies [Verhoeven et al., 1996; Cohen et al., 2000]. The root of *Panax ginseng* has been used worldwide for thousands of years as a traditional medicine for treatment of diverse ailments, including liver dysfunction, hypertension, atherosclerosis, and postmenopausal symptoms [Yun and Meyer, 2001]. More recent studies have indicated that purified ginsenoside saponins isolated from the root of *P. ginseng* C.A. Meyer can inhibit growth of cancer cells in culture and *in vivo* [Abe et al., 1979; Odashima et al., 1985; Lee et al., 1996; Oh et al., 1999; Kim and Jin, 2004; Kim et al., 2004; Cheng et al., 2005; Kitts et al., 2007; Huang et al., 2008]. Ginsenoside Rh2 (Rh2) belongs to the protopanaxadiol family and has drawn attention to chemopreventive research. For example, crude ginsenosides caused phenotypic reverse transformation in Morris hepatoma cells, and purified Rh2 inhibited growth of B16 melanoma cells increasing melanogenesis [Abe et al., 1979; Odashima et al., 1985]. Treatment with Rh2 caused repression of matrix metalloproteinase genes in human astroglioma cells [Kim et al., 2007]. The combination of Rh2 and paclitaxel synergistically inhibited growth of LNCaP human prostate cancer cells [Xie et al., 2006]. Furthermore, Rh2 enhanced *in vivo* antitumor activity of cyclophosphamide against B16 melanoma cells [Wang et al., 2006].

Recent studies, including those from our laboratory, have revealed that Rh2-mediated suppression of cancer cell proliferation correlates with G_1 phase cell cycle arrest and apoptosis induction. Elucidation of the mechanism responsible for Rh2-mediated apoptosis and cell cycle arrest has been the topic of intense research in the past few years [Lee et al., 1996; Oh et al., 1999; Kim and Jin, 2004; Kim et al., 2004; Cheng et al., 2005; Kitts et al., 2007; Choi et al., 2009]. Rh2-induced apoptotic cell death in neuroblastoma cells was caused by activation of caspase-1 and caspase-3 and up-regulation of Bax [Kim and Jin, 2004]. Apoptosis induction resulting from Rh2 exposure in PC-3 and LNCaP human prostate cells correlated with modulation of mitogen-activated protein

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kinases [Cheng et al., 2005]. Rh2 treatment blocked cell cycle progression of SK-HEP-1 cells at the G_1-S boundary by selectively inducing expression of $p27^{kip1}$ but without affecting the levels of cyclin E, cyclin-dependent kinase 2 (Cdk2), and $p21^{WAF1}$ [Lee et al., 1996]. The G_1-phase arrest caused by Rh2 treatment in MCF-7 human breast cancer cells was accompanied by induction of $p21^{WAF1}$ [Oh et al., 1999]. Our previous research also showed that Rh2-mediated G_0–G_1 phase cell cycle arrest in MCF-7 and MDA-MB-231 human breast cancer cells was caused by $p15^{Ink4B}$- and $p27^{Kip1}$-dependent inhibition of kinase activities of G_1-S-specific Cdk/cyclin complexes [Choi et al., 2009].

Breast cancer is a major cause of cancer deaths in women worldwide. Although significant advances have been made in screening techniques and treatment modalities, various strategies for reduction of breast cancer risk must be considered [Jemal et al., 2006]. The known risk factors for breast cancer include family history, Li-Fraumeni syndrome, typical hyperplasia of the breast, late-age at first full-term pregnancy, early menarche, and late menopause. Some of these risk factors are not easily modifiable (e.g., genetic predisposition) [Kelsey et al., 1993; Hulka and Stark, 1995; Kelsey and Bernstein, 1996]. In addition, selective estrogen receptor (ER) modulators (e.g., tamoxifen) appear promising for the prevention of breast cancer; this strategy is largely ineffective against ER-negative breast cancers [Fisher et al., 1998; Cuzick et al., 2002]. Moreover, selective ER modulators have other side effects, including increased risk of uterine cancer, thromboembolism, cataracts, and perimenopausal symptoms [Fisher et al., 1998; Cuzick et al., 2002]. Therefore, identification and clinical development of agents that are relatively safe but can suppress growth of both ER-positive and ER-negative human breast cancers is highly desirable. This study was undertaken to determine the efficacy of Rh2 against human breast cancer cells. We show that Rh2 exhibits significant growth-inhibitory effects against MCF-7 (estrogen responsive) and MDA-MB-231 (estrogen-independent) human breast cancer cells irrespective of their estrogen responsiveness or p53 status. Rh2-induced apoptosis was associated with induction of proapoptotic Bcl-2 family members Bax, Bak and Bim, and down-regulation of antiapoptotic proteins Bcl-2, Bcl-xL, and Mcl-1, leading to activation of caspases. Knockdown of Bak and Bax using siRNA confers partial yet significant protection against cell death caused by Rh2. Moreover, Rh2 administration significantly caused apoptotic cell death of MDA-MB-231 cells *in vivo*, which is accompanied with reduced cell proliferation. The results of this study merit clinical investigation to determine the efficacy of Rh2 against human breast cancers.

MATERIALS AND METHODS

REAGENTS

Ginsenoside Rh2 (purity ~98.7%) was purchased from LKT Laboratories (St. Paul, MN). Stock solution of Rh2 (final concentration 0.1 M) was prepared in DMSO, stored at −20°C, and diluted with fresh complete medium immediately before use. An equal volume of DMSO (final concentration <0.1%) was added to the controls. Tissue culture media, fetal bovine serum (FBS), trypsin–EDTA solution, antibiotic mixture, sodium pyruvate, and non-essential amino acids were obtained from GIBCO (Grand Island, NY). Cell Death Detection ELISA PLUS kit and mitochondrial isolation kit was from Roche (Mannheim, Germany) and Pierce (Rockford, IL), respectively. Protease inhibitor cocktail was from BD Biosciences PharMingen (San Diego, CA). The phosphatase inhibitor cocktail and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) reagent were from Sigma (St. Louis, MO). Antibodies specific for detection of Bak, Bax, and Bcl-xL were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Bim, caspase-3, caspase-9, and poly(ADP-ribose) polymerase (PARP) were obtained from Cell Signaling Technology (Beverly, MA). Bax protein undergoes conformational changes after translocation. Anti-6A7 Bax antibody that recognizes an epitope of the activated Bax protein was purchased from BD PharMingen. Antibodies against Bcl-2 and proliferating cell nuclear antigen (PCNA) were from DakoCytomation (Glostrup, Denmark). Bak- and Bax-targeted siRNAs were from Santa Cruz Biotechnology. Control nonspecific siRNA (UUCUCCGAACGUGUCACGUdTdT) and HiPerFect transfection reagent were from Qiagen (Germantown, MD). H&E stain was from Anatech Ltd (Battele Creek, MI). ApopTag Plus Peroxidase In situ Apoptosis kit was from Chemicon (Temecula, CA).

CELL CULTURE AND VIABILITY ASSAY

Monolayer cultures of MCF-7 cells were maintained in MEM supplemented with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1.5 g/L sodium bicarbonate, 10% (v/v) FBS, and antibiotics. MDA-MB-231 cultures were maintained in RPMI 1640 medium supplemented with 10% (v/v) non-heat-inactivated FBS and antibiotics. Each cell line was maintained in an atmosphere of 95% air and 5% CO_2 at 37°C. The effect of Rh2 on cell viability was determined by the MTT colorimetric method. Briefly, desired cell lines were plated in 96-well culture plates and allowed to adhere by overnight incubation. The cells were then treated with DMSO (control) or desired concentration of Rh2 for 24 h. At the end of the incubation, 20 μl MTT (5 mg/ml) was added for 4 h at 37°C. After incubation, the supernatant were removed carefully and 150 μl DMSO was added to each well. After 10 min incubation and vibration, the absorbance was read at 490 nm wavelength. Results are expressed as the ratio of the number of viable cells with Rh2 treatment relative to that of DMSO-treated control.

APOPTOTIC DNA FRAGMENTATION ASSAY

Rh2-mediated apoptotic cell death, characterized by cytoplasmic histone-associated DNA fragmentation, in MCF-7 and MDA-MB-231 cells was determined with a Cell Death Detection ELISA PLUS kit according to the manufacturer's instructions. The plated and attached cells in 96-well plates were treated with DMSO (control) or desired concentrations of Rh2 for 24 h. Both floating and attached cells were collected and processed for analysis of cytoplasmic histone-associated DNA fragmentation as described by the manufacturer.

IMMUNOBLOTTING

Cells were treated with desired concentrations of Rh2 for specified time intervals as indicated in figures and lysed as we previously described [Choi et al., 2009]. The cell lysate was cleared by

centrifugation at 12,000 rpm for 20 min, and the supernatant fraction was used for immunoblotting. Proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membrane. After blocking with 5% nonfat dry milk in tris-buffered saline containing 0.05% Tween 20, the membrane was incubated with the desired primary antibody for 1 h at room temperature or overnight at 4°C. The primary antibodies such as mouse anti-β-actin, anti-Bcl-2 anti-Bcl-xL, anti-Bax, anti-Bim, anti-caspase 3, anti-caspase-9, and anti-PARP were used at 1 μg/ml concentration. Rabbit anti-Bak and anti-Mcl-1 antibodies were used at 0.5 μg/ml concentration in TBST containing 5% BSA. The membrane was then washed and treated with horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (diluted 1:3,000) for 2 h at 22°C. The immunoreactive bands were visualized by enhanced chemiluminescence method. Each membrane was stripped and re-probed with anti-actin antibody to ensure equal protein loading. Mitochondrial and cytosolic fractions from control (DMSO-treated) and Rh2 treated MCF-7 cells were prepared using a mitochondria isolation kit according to the manufacturer's instructions. Each fraction was collected and used for immunoblotting of Bax and/or cytochrome *c* (BD PharMingen).

IMMUNOCYTOCHEMISTRY OF BCL-2 AND BAX

MCF-7 cells were cultured on coverslips and treated with DMSO (control) or 20 or 40 μM Rh2 for 6 and 12 h. After washing with PBS, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. The cells were incubated with normal goat serum (1:20 dilution) in PBS for 45 min. Subsequently, the cells were treated with anti-Bcl-2 and/or Bax antibody (1:500 dilution) for 2 h. The cells were washed with PBS and incubated with Alexa Fluor 488 or Alexa Fluor 594-conjugated secondary antibody (1:1,000 dilution, Molecular Probes) for 1 h. After washing, cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 10 ng/ml) for 10 min. The cells were visualized under a fluorescence microscope.

RNA INTERFERENCE

The MCF-7 cells were seeded in 60-mm plates and when reached 50% confluency the cells were transfected with a control siRNA and specific siRNA duplex targeting Bak and/or Bax (5 nM) using HiperFect transfection reagent according to the manufacturer's recommendations. After 24 h of transfection, the cells were treated with DMSO or 40 μM Rh2 for 24 h. The cells were then collected and processed for immunoblotting and analysis of apoptotic DNA fragmentation. Results are expressed as enrichment of cytoplasmic histone-associated DNA fragments relative to DMSO-treated control.

SEMI-QUANTITATIVE REVERSE TRANSCRIPTION-PCR FOR BAK, BAX, BCL-2, AND BCL-XL

Tumors were obtained from animals treated with PBS (control) or Rh2 (2 or 5 mg) and RNA was extracted using BCP phase Separation Reagent (MRC, Inc., Cincinnati, OH) following the manufacturer's instruction. cDNA was synthesized by reverse transcription using Maxime RT premix kit containing oligo (dT) primer from Intron Biotech. (Seoul, Korea). The PCR exponential phase was determined between 22 and 30 cycles to allow semi-quantitative comparisons of target. cDNAs was prepared with one master mix (Intron Biotech) containing 2 μl RT transcript, reaction buffer, 30 pM of primer, 2.5 mM each dNTP and Taq polymerase (2.5 U). The primers and PCR conditions are shown in Table I. PCR products were electrophoresed on 1% agarose gel and visualized under ultraviolet light after ethidium bromide staining of the gel.

TUMOR XENOGRAFTS MODEL

Female nude mice (6–8 weeks old) were purchased from Central Lab. Animal, Inc. (Seoul, Korea) and housed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC, Hallym 2008-18) of Hallym University. The use of mice for studies described herein was approved by the IACUC. The mice were randomly divided into three groups of five mice per group. The mice were gavaged orally with either 0.1 ml vehicle or vehicle containing 2, or 5 mg Rh2/kg body weight thrice per week for 2 weeks before tumor cell implantation. After 2 weeks of pretreatment, exponentially growing MDA-MB-231 cells (2×10^6, suspended in 0.1-ml PBS) were injected subcutaneously onto the right back of each mouse. Tumors were let to grow for 90 days. Tumor size was measured thrice a week using a caliper and tumor volume was estimated as $V = (a \times b^2)/2$, where a is the large dimension and b is the perpendicular diameter. Mice of each group were also monitored for weight loss and other symptoms of side effects, including food and water withdrawal and impaired posture or movement. At the

TABLE I. PCR Primers

Target genes		Primer sequences	Size (bp)	Annealing temperature (°C)	Cycle
Bax	Sense	5′-GGGTTTCATCCAAGGATCGAGCAGG-3′	476	50	25
	Antisense	5′-ACAAAGATGGTCACGGTCTGCC-3′			
Bak	Sense	5′-TGAAAAATGGCTTCGGGGCAAGGC-3′	642	54	24
	Antisense	5′-TCATGATTTGAAGAATCTTCGTACC-3′			
Bcl-2	Sense	5′-GACTTCGCCGAGATGTCCAGC-3′	440	60	30
	Antisense	5′-CCTCACTTGTGGCCCAGATAGG-3′			
Bcl-xL	Sense	5′-AGGATACAGCTGGAGTCAG-3′	416	54	26
	Antisense	5′-TCTCCTTGTCTACGCTTTCC-3′			
GAPDH	Sense	5′-CCTCCAAAATCAAGTGGGGCGATG-3′	525	56	[a]
	Antisense	5′-CATATTTGGCAGGTTTTTCTAGAC-3′			

[a]The cycle of GAPDH as an internal control was adjusted to that of each target gene.

termination of the experiment, the tumor tissues were harvested and used for immunohistochemistry or RT-PCR.

HISTOLOGIC ANALYSIS OF APOPTOTIC BODIES

Tumor tissues from control and Rh2-treated mice were fixed in 10% neutral-buffered formalin, dehydrated, embedded in paraffin, and sectioned at 4- to 5-μm thickness. Apoptosis in tumor sections of control and Rh2-treated mice was visualized by terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay using ApopTag Plus Peroxidase In situ Apoptosis kit according to the manufacturer's recommendations. Apoptotic bodies in the tumor sections were quantitatively observed by counting the number of TUNEL-positive cells in randomly selected, nonoverlapping fields (magnification, 200×).

H&E STAINING AND IMMUNOHISTOCHEMICAL ANALYSIS FOR BCL-2, BAX, AND PCNA

Representative tumor sections from control and Rh2 groups were fixed in paraformaldehyde for 10 min at 4°C. After endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 15 min, the representative sections were incubated with the primary anti-Bcl-2 (1:200 dilution), anti-Bax (1:200 dilution), or anti-PCNA antibody (1:500 dilution) for 1 h at room temperature and washed with TBS. Polymer-horseradish peroxidase conjugated anti-mouse IgG (1:3,000) was applied for 30 min at room temperature. Color was developed by 10-min incubation with 3,3′-diaminobenzidine (DakoCytomation). The sections were counterstained with H&E. The slides were examined under a Zeiss Axiovert microscope (Zeiss, Hamburg, Germany). At least three nonoverlapping representative images of each tissue were captured from each section using the camera attached to the microscope.

STATISTICAL ANALYSIS

Statistically significant difference in measured variables between control and Rh2-treated groups was determined by one-way ANOVA followed by Dunnett's or Bonferroni's test for multiple comparisons. Difference was considered significant at a P-value of <0.05.

RESULTS

GINSENOSIDE Rh2 TREATMENT CAUSED APOPTOTIC DNA FRAGMENTATION IN BREAST CANCER CELLS

We have previously shown that Rh2 (see Fig. 1A for the chemical structure of Rh2) treatment decreases viability of MCF-7 (estrogen-responsive and wild-type p53) and MDA-MB-231 (estrogen-independent and p53 mutant) human breast cancer cells at concentrations of 20–80 μM, as judged by the trypan blue dye exclusion assay [Choi et al., 2009]. We confirmed the effect of Rh2 treatment on cell viability by the MTT assay. Survival of MCF-7 and MDA-MB-231 cells was significantly decreased after a 24-h exposure to Rh2 in a concentration-dependent manner, with an IC_{50} of 50 μM. These results indicate that Rh2 treatment decreases the viability of breast cancer cells irrespective of their estrogen responsiveness. Next, we addressed the question of whether Rh2-mediated suppression of MCF-7 and MDA-MB-231 cell viability was due to apoptosis induction by determining the effect of Rh2 treatment on cytoplasmic histone-associated DNA fragmentation.

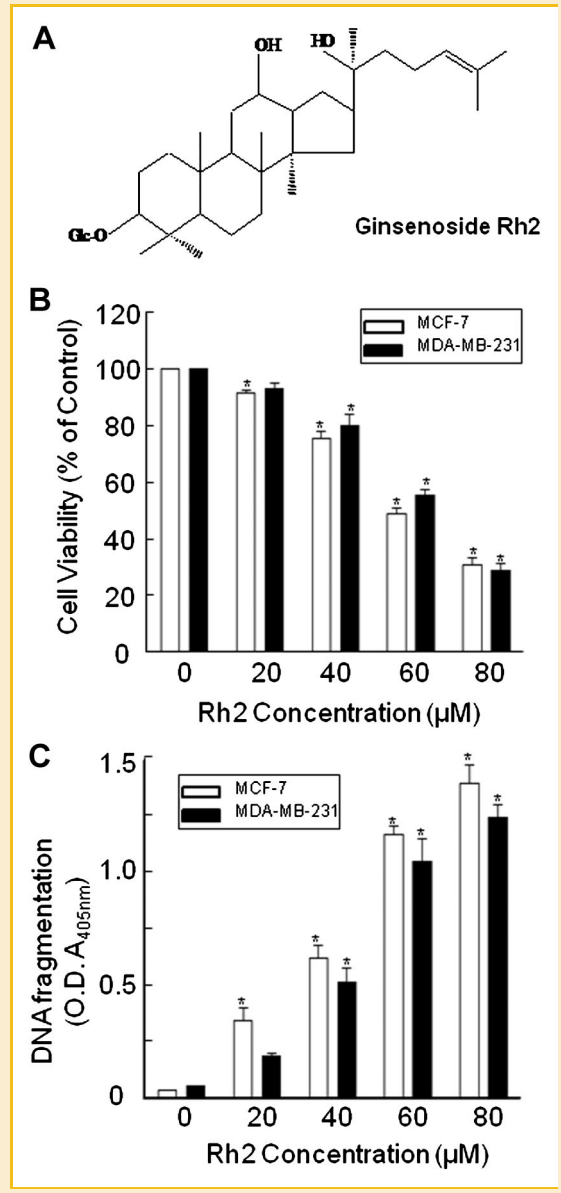

Fig. 1. Ginsenoside Rh2 treatment caused apoptotic DNA fragmentation in human breast cancer cells irrespective of their estrogen responsiveness or p53 status. A: Chemical structure of Rh2. B: Effect of Rh2 treatment on viability of MCF-7 (estrogen responsive and wild-type p53) and MDA-MB-231 (estrogen-independent, mutated p53) cells as determined by the MTT assay. C: Effect of Rh2 treatment on apoptosis induction in both MCF-7 and MDA-MB-231 cells, assessed by quantitation of cytoplasmic histone-associated DNA fragmentation. Human breast cancer cell lines were exposed to DMSO (control) or desired concentration of Rh2 for 24 h. Both floating and adherent cells were collected and processed for apoptotic DNA fragmentation assay. Data are presented as mean ± SEM ($n = 3$). $^*P < 0.05$, significantly different compared with control by one-way ANOVA followed by Dunnett's test. Similar results were observed in replicate experiments.

Figure 1C shows that Rh2 treatment increased cytoplasmic histone-associated DNA fragmentation over DMSO-treated control in both cell lines in a concentration-dependent manner. For example, the cytoplasmic histone-associated DNA fragmentation was increased by ~8.9- to 35.7-fold upon a 24-h treatment of MCF-7 cells with Rh2, compared with DMSO-treated control (Fig. 1C). Consistent with these results, Rh2 treatment also caused apoptotic cell death in MDA-MB-231 (Fig. 1C). These results indicate that Rh2 causes apoptotic DNA fragmentation in breast cancer cells irrespective of their estrogen responsiveness or p53 status.

Rh2 TREATMENT ALTERED LEVELS OF BCL-2 FAMILY PROTEINS IN BREAST CANCER CELLS

The Bcl-2 family proteins play critical roles in regulation of apoptosis by functioning as either promoters (e.g., Bax and Bak) or inhibitors (e.g., Bcl-2 and Bcl-xL) of cell death [Hockenbery et al., 1990; Adams and Cory, 1998; Chao and Korsmeyer, 1998]. We proceeded to determine the effect of Rh2 treatment on levels of Bcl-2 family proteins to gain insights into the mechanism of apoptosis in our model. Figure 2A (MCF-7) and 2B (MDA-MB-231) shows that Rh2 treatment (50 μM) resulted to a modest increase (1.1- to 2.2-fold increase over control) in protein levels of multidomain proapoptotic Bcl-2 family members Bax and Bak especially at the 16- to 24-h time points. Rh2-treated MCF-7 cells exhibited a 50–80% decrease in levels of antiapoptotic proteins Bcl-2 and Bcl-xL relative to control (Fig. 2A). Bcl-2 and Bcl-xL levels in MDA-MB-231 were modestly decreased on treatment with Rh2 (Fig. 2B). Exposure of MCF-7 and MDA-MB-231 cells to Rh2 also resulted in alteration of BH3 only protein such as Bim and Mcl-1 (Fig. 2A,B). The most conspicuous effect of Rh2 treatment was the repression of Mcl-1 in both cells. In MCF-7 cells, the Rh2 treatment caused a time-dependent and significant increase in the protein levels of Bim-s and Bim-L (Fig. 2A). The Rh2-treated MDA-MD-231 cells exhibited increased level of Bim-s as well as Bim-EL (Fig. 2B). Together, these results indicate that Rh2 treatment cause an increase in the levels of proapoptotic (Bak, Bax, and Bim) and decrease of antiapoptotic (Bcl-2, Bcl-xL, and Mcl-1) Bcl-2 family proteins in human breast cancer cells.

The effect of Rh2 treatment on MCF-7 cells was further observed by immunocytochemistry (Fig. 3). In control (DMSO-treated) cells,

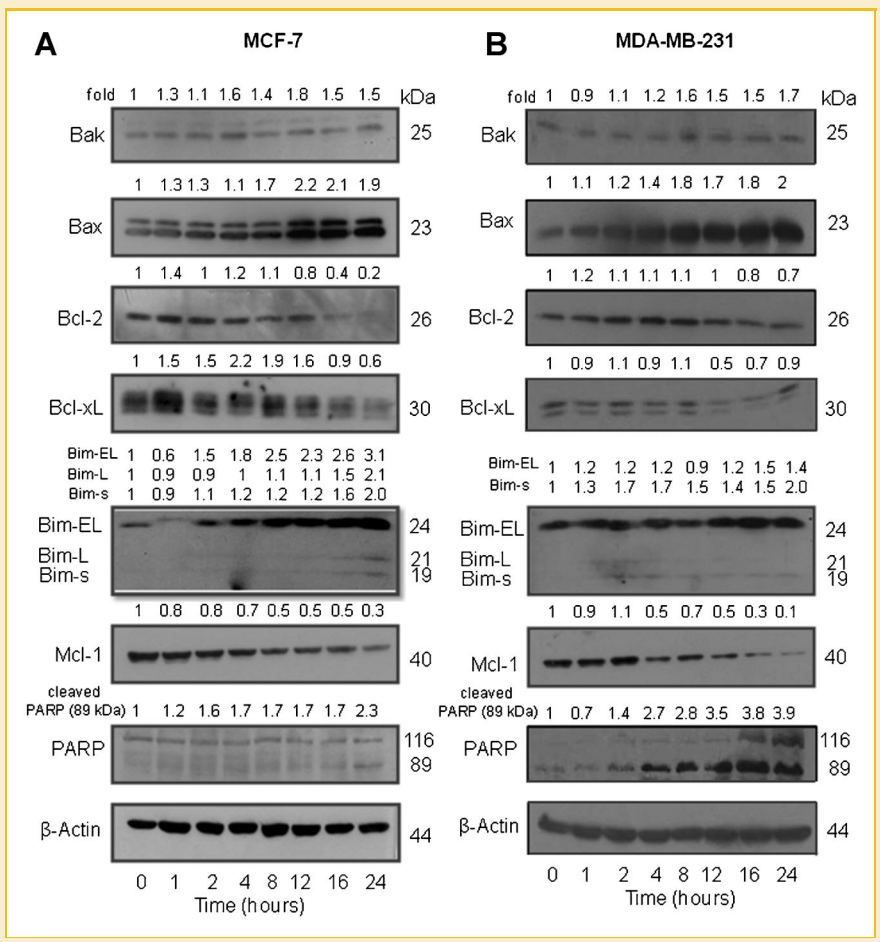

Fig. 2. Rh2 treatment alters levels of Bcl-2 family proteins in MCF-7 and MDA-MB-231 cells. Immunoblotting for Bcl-2 family proteins using lysates from MCF-7 cells (A) and MDA-MB-231 cells (B) treated with DMSO (control) or 50 μM Rh2 for the indicated time period. The blots were stripped and reprobed with anti-actin antibody to correct for differences in protein loading. Densitometric scanning data are shown on top of each band. Immunoblotting for each protein was done at least twice using independently prepared lysates, and the results were consistent.

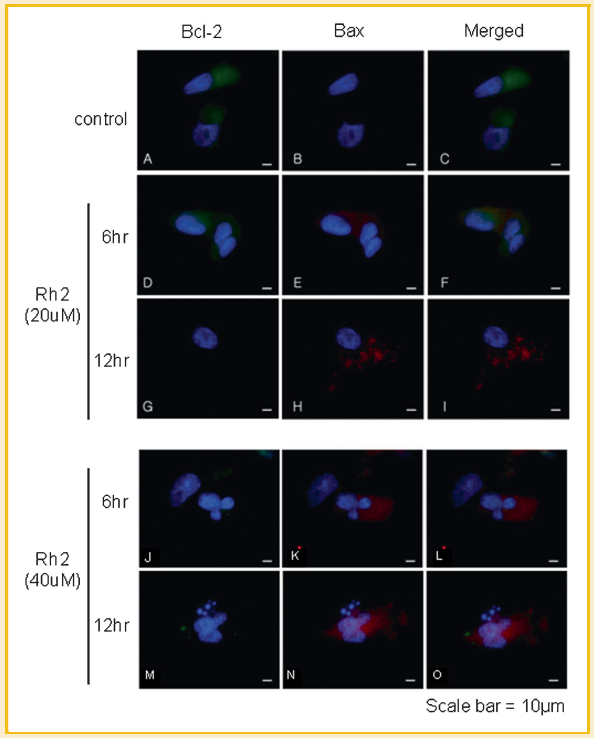

Fig. 3. Rh2 treatment caused alteration of expression level of Bcl-2 and Bax proteins in MCF-7 cells. Immunocytochemical analysis of Bcl-2 and Bax proteins in MCF-7 cells following 6- or 12-h exposure to DMSO, 20 or 40 μM Rh2. Green, red, and blue fluorescence represent staining for Bcl-2, Bax, and nucleus, respectively. Images were merged to detect distribution of Bcl-2 and Bax proteins dependent on Rh2 treatment.

Bcl-2 protein was predominantly stained as evidenced by a green fluorescence (specific Bcl-2 green staining); however, Bax protein was not marked. Cells were additionally reacted with DAPI (blue staining), which clearly shows DNA fragmentation and is also used to detect apoptosis. DAPI staining revealed abundance of cells with condensed and fragmented DNA in Rh2-treated MCF-7 cells, which were rare in DMSO treated controls. Consistent with the results of altered Bcl-2 and Bax protein immunoblotting, Rh2 treatment increased the level of Bax red staining over DMSO-treated control in a concentration- and time-dependent manner. Representative images for Bcl-2, Bax, and DAPI staining in DMSO-treated MCF-7 cells are shown in Figure 3 (top). These results clearly indicate that Rh2-induced apoptosis in MCF-7 cells is associated with alteration in expression level of Bcl-2 family proteins, and that both Bax and Bcl-2 were required for this effect.

Rh2 TREATMENT CAUSED MITOCHONDRIAL TRANSLOCATION OF BAX, LEADING TO ACTIVATION OF CASPASES

In normal cells, Bax protein, which exists in the cytosol, can be translocated to the mitochondria in response to a variety of apoptotic stimuli [Wolter et al., 1997; Adams and Cory, 1998; Yamaguchi and Wang, 2001]. The activated Bax protein oligomerizes on the outer mitochondrial membrane and induces release of the apoptogenic molecule, cytochrome *c* to the cytoplasm [Liu et al., 1996; Wolter et al., 1997; Adams and Cory, 1998; Green and Reed, 1998; Susin et al., 1999; Du et al., 2000; Hengartner, 2000; Yamaguchi and Wang, 2001]. Once in the cytosol, cytochrome *c* binds to apoptosis protease activation factor-1 (Apaf-1) and recruits and activates procaspase-9 in the apoptosome [Green and Reed, 1998; Thornberry and Lazebnick, 1998; Wolf and Green, 1999; Hengartner, 2000]. Active caspase-9 cleaves and activates executioner caspases, including caspase-3 [Thornberry and Lazebnick, 1998; Wolf and Green, 1999]. Next, we determined the effect of Rh2 treatment on cytosolic release of cytochrome *c* by immunoblotting to determine if apoptosis induction in MCF-7 cells was associated with translocation of Bax into the mitochondria. Figure 4A,B shows that Rh2 treatment resulted in release of cytochrome *c* from mitochondria to the cytosol, which was evident as early as 1 h after treatment, corresponding with the decrease of cytochrome *c* in the mitochondrial fraction. We also observed that the proapoptotic Bax was increased in the mitochondrial fraction with a corresponding decrease of cytochrome *c*. The time-dependent induction of Apaf-1 protein expression was shown in Figure 4C. The Rh2-mediated increase in Apaf-1 protein level was evident as early as 4 h after treatment. Because cytochrome *c* release was observed in Rh2-treated MCF-7 cells, we determined the effect of Rh2 treatment on proteolytic cleavage of caspase-9 and caspase-3 by immunoblotting. Figure 4C shows that Rh2 treatment caused proteolytic cleavage of both caspase-9 and caspase-3. In time course experiments using 50 μM Rh2, proteolytic cleavage of caspase-9 was evident as early as 1 h after treatment, whereas caspase-3 cleavage was not observed until 12 h (Fig. 4C).

Activation of caspase-3 leads to cleavage and inactivation of key cellular proteins, including the DNA repair enzyme poly(ADP-ribose) polymerase (PARP). We therefore determined the effect of Rh2 treatment on cleavage of PARP to confirm caspase-3 activation. Figure 2A shows that PARP cleavage was observed in Rh2-treated MCF-7 and MDA-MB-231 cells.

KNOCKDOWN OF BAK OR BAX CONFERRED PARTIAL PROTECTION AGAINST Rh2-INDUCED DNA FRAGMENTATION

Because Rh2 treatment resulted in a marked increase in the Bax protein expression, despite modest induction of Bak protein (Fig. 2), we designed experiments using Bax- and Bak-targeted siRNAs to test the role of these proteins in regulation of Rh2-induced apoptosis. Transient transfection of MCF-7 cells with Bax- or Bak-targeted siRNAs resulted in >80% knockdown of each protein (Fig. 5A,B) compared with cells transiently transfected with a control nonspecific siRNA. Consistent with the results in untransfected MCF-7 cells (Fig. 1C), a 24-h exposure of nonspecific siRNA-transfected MCF-7 cells to 50 μM Rh2 resulted in statistically significant increase in cytoplasmic histone-associated DNA fragmentation compared with DMSO-treated control (Fig. 5C). On the other hand, the Bax or Bak protein depletion conferred partial yet significant protection against Rh2-mediated cytoplasmic histone-associated DNA fragmentation when compared with MCF-7 cells transfected with the control nonspecific siRNA. These results indicate that Bax and Bak are targets of Rh2-induced apoptosis, and

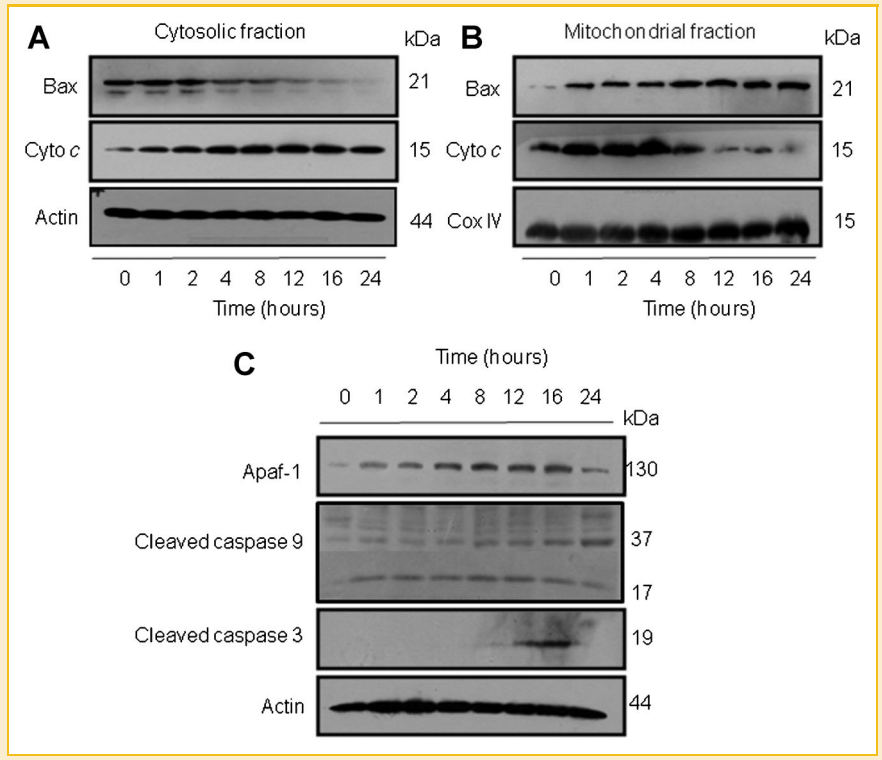

Fig. 4. Rh2 treatment caused an intrinsic apoptotic pathway mediated by mitochondrial Bax translocation, release of cytochrome *c*, and induction of Apaf-1, leading to activation of caspase-9 and caspase-3 in Rh2-treated MCF-7 cells. A: Immunoblotting for Bax and cytochrome *c* using cytosolic fractions. B: Immunoblotting for Bax and cytochrome *c* in mitochondrial fractions. Each fraction was prepared from MCF-7 cells following treatment of DMSO (control) or 50 μM Rh2 for the indicated time. C: Immunoblotting for cleavage of caspase-9, caspase-3, and Apaf-1 using lysates from MCF-7 cells treated with 50 μM Rh2 for the specified time intervals. Blots were stripped and reprobed with anti-actin or CoxIV antibody to normalize for protein loading. Immunoblotting for each protein was done at least twice, and results were comparable.

induction of proapoptotic proteins is a critical event in Rh2-mediated apoptosis in human breast cancer cells.

MODULATION OF APOPTOSIS-ASSOCIATED GENE EXPRESSION IN MDA-MB-231 XENOGRAFTS TREATED WITH Rh2

To gain insights into the mechanism of Rh2-mediated apoptosis in MDA-MB-231 xenograft *in vivo*, we compared the levels of *bcl-2*, *bak*, *bax*, and *bcl-xL* mRNA in tumors from control and Rh2-treated mice. Representative RT-PCRs for *bcl-2*, *bak*, *bax*, and *bcl-xL* mRNA using tumor tissue from control and Rh2-treated mice are shown in Figure 6A. Change in mRNA level was quantified by densitometric scanning of the cDNA bands and normalized with GAPDH.

As can be seen in Figure 6B, the level of each mRNA examined, including Bcl-2 and Bcl-xL, is relatively lower in tumors from Rh2-treated mice compared with control tumors (0.3- to 0.5-fold decrease over control); however, Bax and Bak expression appeared to be 2.2- and 1.7-fold up-regulated, respectively, following Rh2 treatment (5 mg/kg concentrations). These RT-PCR results were well correlated with immunoblotting results.

Rh2-mediated apoptosis *in vivo* through the regulation of Bcl-2 family protein expression was confirmed by using tumor tissues of MDA-MB-231 xenografts from control and Rh2-treated mice, which were processed for immunohistochemical analysis of Bcl-2 and Bax expression.

Although the expression levels of Bcl-2 in Rh2-treated mice did not show much difference between samples treated with different amount of Rh2 (2 or 5 mg), Rh2 treatment certainly caused decrease in Bcl-2. Rh2 treatment also resulted in significant increase in Bax protein levels in a concentration-dependent manner compared with control tumor tissue (Fig. 6C).

Rh2 ADMINISTRATION SUPPRESSED CELL PROLIFERATION AND CAUSED APOPTOSIS IN TUMORS

To test the significance of *in vitro* observations *in vivo*, we determined the effect of Rh2 administration by oral gavage (thrice a week) on MDA-MB-231 xenograft growth. The dose and route of Rh2 administration was selected based on previous studies documenting *in vivo* efficacy against various cancer cells [Jia et al., 2004; Wang et al., 2006; Xie et al., 2006; Musende et al., 2009]. The average body weights of the control and Rh2-treated mice did not differ significantly throughout the study (data not shown).

The Rh2-treated mice did not exhibit signs of distress such as impaired movement or posture, indigestion, and areas of redness or swelling. Tumor tissues of MDA-MB-231 xenografts from control and Rh2-treated mice were processed for H&E staining, immunohistochemical analysis of PCNA expression, and TUNEL assay. Data from a representative mouse of each group are shown in Figure 7.

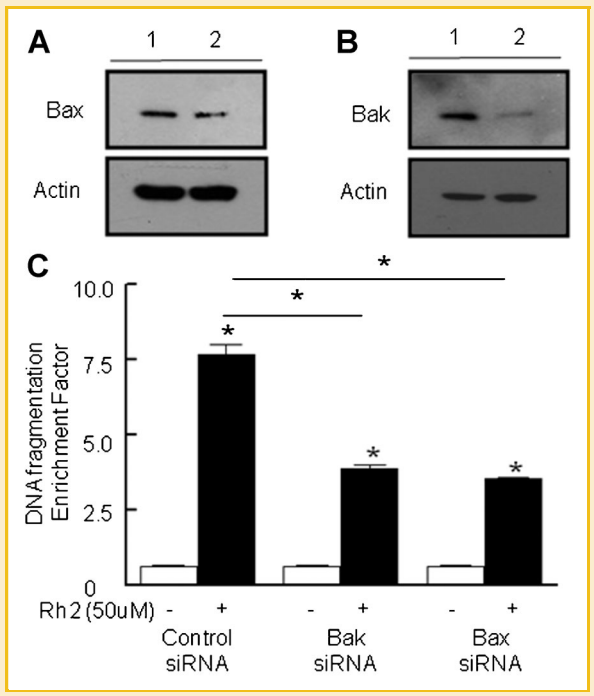

Fig. 5. Rh2-induced apoptotic DNA fragmentation was partially but significantly attenuated by Bax or Bak knockdown in MCF-7 cells. Immunoblotting for Bax (A) or Bak (B) using lysates from MCF-7 cells transiently transfected with a nonspecific control siRNA (lane 1) and Bax- or Bak-targeted siRNA (lane2). The blots were stripped and reprobed with anti-actin antibody to demonstrate equal protein loading. Transient transfection of MCF-7 cells with Bax and Bak siRNAs resulted in a marked knockdown of these proteins. C: Cytoplasmic histone-associated DNA fragmentation in MCF-7 cells transiently transfected with a control siRNA and Bax or Bak siRNA and treated for 24 h with either DMSO (control) or 50 μM Rh2. Data are presented as mean ± SEM ($n = 3$). $^*P < 0.05$, significantly different compared with corresponding control or between the indicated groups by one-way ANOVA followed by Bonferroni's multiple comparison test. Similar results were observed in two independent experiments.

H&E staining revealed a relatively higher nuclear to cytoplasmic ratio in the tumors from control mice compared with 2 or 5 mg/kg Rh2-treated mice. These results suggest a relatively higher proliferation index in control tumors than in tumors from Rh2-treated mice. We confirmed this speculation by immunohistochemical analysis for PCNA expression. PCNA is a marker for cellular proliferation and is expressed in >90% of in situ and invasive breast carcinomas [Terry et al., 2007]. The PCNA expression was markedly lower in tumors from Rh2-treated mice compared with tumors form control animal (Fig. 7, middle). Another supporting evidence is that the average tumor size in Rh2-treated mice was generally smaller compared with control mice on each day of tumor measurement, although the difference did not reach statistical significance at $P > 0.05$ (data not shown).

The apoptotic bodies in tumor sections of control and Rh2-treated mice were visualized by TUNEL staining, and representative micrographs are shown in Figure 7. The tumors from Rh2-treated mice exhibited a markedly higher count of apoptotic bodies compared with control tumors. Collectively, these results indicate that Rh2 administration causes suppression of cellular proliferation and increases apoptosis in tumors *in vivo*.

DISCUSSION

This study reveals that Rh2 causes apoptotic DNA fragmentation in human breast cancer cells irrespective of their estrogen responsiveness or p53 status. Proapoptotic response to Rh2 is possibly intensified by the presence of wild-type p53 because the MCF-7 cell line seems relatively more sensitive to Rh2-induced apoptosis compared with the MDA-MB-231 cell line, based on the quantitation of cytoplasmic histone-associated DNA fragmentation (Fig. 1C) although further studies are required to investigate this possibility.

In this study, we show that Rh2-mediated apoptosis in MCF-7 and MDA-MB-231 cells correlates with an increase in levels of proapoptotic proteins Bak, Bax, and Bim, and a decrease in levels of antiapoptotic proteins Bcl-2, Bcl-xL, and Mcl-1. In addition, Rh2-mediated suppression of MDA-MB-231 xenograft growth *in vivo* correlates with an increase in mRNA levels of Bax and Bak in the tumor specimen. The Bcl-2 family proteins have emerged as critical regulators of mitochondria-mediated apoptosis [Reed, 1997; Adams and Cory, 1998; Chao and Korsmeyer, 1998]. Rh2-mediated alteration in levels of Bcl-2 family proteins has been described in other cell types. For instance, apoptosis induction by Rh2 in HeLa human ovarian cancer cells correlated with up-regulation and translocation of Bax into the mitochondria which leads to mitochondria depolarization [Ham et al., 2006]. The present study revealed that Rh2 treatment causes mitochondrial translocation of Bax to trigger the release of cytochrome *c* from mitochondria to the cytosol, leading to activation of caspases. Rh2 treatment also causes cleavage of caspase-3 that coincides with cleavage of caspase-9 and subsequent inactivation of key cellular proteins such as PARP. These results indicate that Bcl-2 family proteins play an important role in the initiation of mitochondria-mediated apoptosis in our Rh2-treated breast cancer cells. However, several studies reported that Rh2 induces apoptosis independently of Bcl-2 family proteins in several cancer cell lines, such as human lung A549 [Cheng et al., 2005], hepatoma SK-HEP-1 cells [Park et al., 1997], and rat gliomal C6Bu-1 cells [Kim et al., 1999]. These results suggest that the molecular targets of Rh2-mediated apoptosis probably vary in different cancer cells. Nonetheless, this study points toward an important role of Bak, Bax, Bcl-2, and Bcl-xL in Rh2 induced apoptosis because the cytoplasmic histone-associated DNA fragmentation caused by this agent is partially yet significantly attenuated after knockdown of Bax or Bak protein.

Because preclinical *in vivo* efficacy testing of potential cancer therapeutic/preventive agents is a key step in their clinical development, we determined the effect of Rh2 administration on growth of MDA-MB-231 cells subcutaneously implanted in female nude mice. Growth inhibition of LLC, Lewis lung carcinoma, B16 melanoma, PC3 prostate, and HRA ovarian cancer cells in nude mice by Rh2 treatment had previously been reported [Nakata et al., 1998; Jia et al., 2004; Wang et al., 2006; Musende et al., 2009]. Consistent with the results of cellular studies, the growth of MDA-MB-231

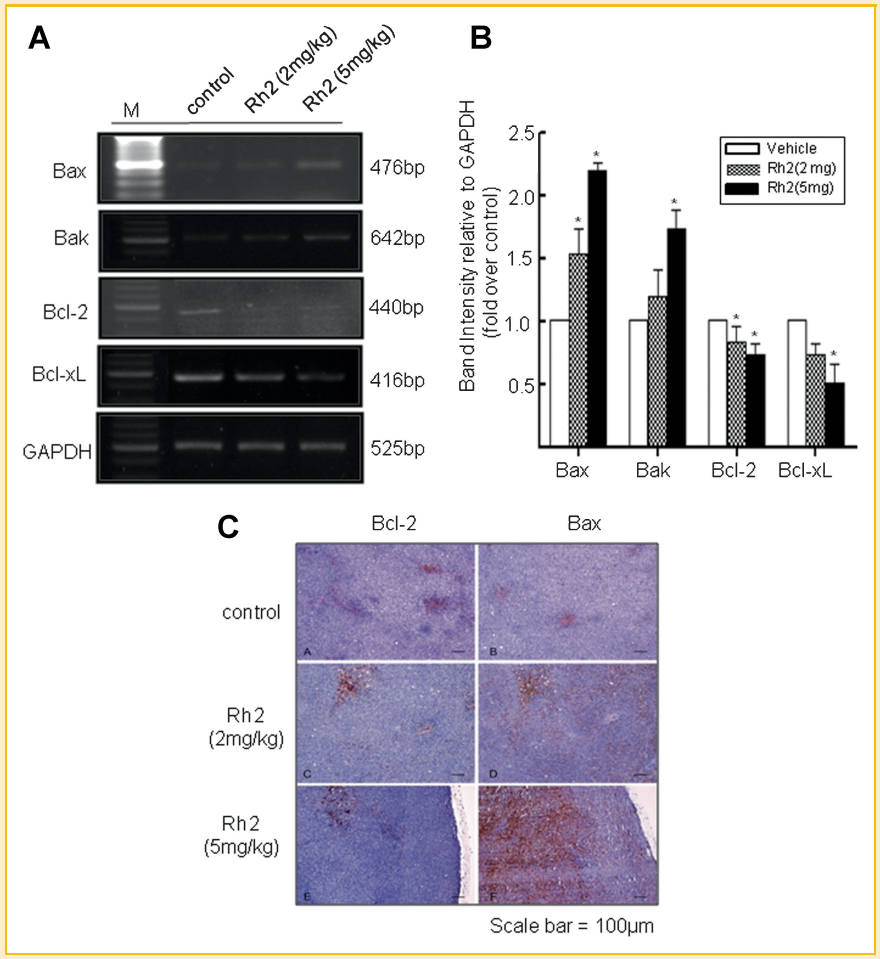

Fig. 6. Analysis of *Bax*, *Bak*, *Bcl-2*, and *Bcl-xl* mRNA levels in tumors harvested from control and Rh2-treated MDA-MB-231 xenograft mice. A: Effects of Rh2 on the Bcl-2 family protein transcript levels, as determined by semi-quantitative RT-PCR. Lane 1, 100 bp DNA marker; lane 2, control (DMSO); lanes 3 and 4, treatment group (2 or 5 mg Rh2/kg). RT-PCR for *GAPDH* was performed in parallel and showed an equal amount of total RNA in the sample. B: Densitometric scanning data for Bcl-2 family transcript levels in tumors from control and Rh2-treated mice. Tumor tissues from five mice of each group were used for RT-PCR. Data are presented as mean ± SEM ($n = 2–3$). $^{*}P < 0.05$, difference in gene expression of Bcl-2 family proteins between control and Rh2-treated group by one-way ANOVA followed by Dunnett's comparison test. C: Immunohistochemical examination was performed to measure the expression of Bcl-2 (A,C,E) and Bax (B,D,F) in tumor tissues derived from control and/or 2 or 5 mg Rh2/kg treated mice. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

xenograft tumor was suppressed by Rh2 administration at the 5 mg/kg dose, which correlated with decreased proliferation index (PCNA staining) and increased apoptosis (TUNEL-positive apoptotic bodies, Bax staining). These results indicate that apoptosis induction is a critical mechanism in Rh2-mediated suppression of MDA-MB-231 cell growth *in vivo*.

It is interesting to note that the Rh2-mediated changes in levels of Bcl-2 family proteins in cultured cells correlate with changes in mRNA level of Bcl-2 family *in vivo*. Especially, Rh2 treatment causes an increase in protein level of Bax in both cultured MDA-MB-231 cells and tumors from xenograft model. The level of Bcl-2 protein after Rh2 treatment was also decreased *in vitro* and *in vivo* xenograft model. However, the level of changes in xenograft model was subtle and not dose-dependent (Fig. 6C). Some possibilities exist to explain the inconsistencies of *in vivo* and *in vitro* results. One possibility may come from the difference in metabolism of Rh2 between cultured and *in vivo* cancer cells. Another possibility would be the variation in the optimal timing of tumor tissue collection for detecting Rh2-mediated *in vivo* changes as the level of Bcl-2 family proteins fluctuate. A time course analysis of the effect of Rh2 administration and the levels of apoptosis regulatory proteins including Bcl-2 family proteins *in vivo* may be needed to explore these possibilities.

In conclusion, our results show that Rh2 treatment suppresses survival of MCF-7 and MDA-MB-231 human breast cancer cells and induces apoptotic DNA fragmentation in both cells, irrespective of their estrogen responsiveness or p53 status. Rh2-mediated cell death in these cells is mediated by Bcl-2 family proteins through the mitochondrial apoptotic pathway. Furthermore, Rh2 also inhibits proliferation of MDA-MB-231 xenografts *in vivo*, in

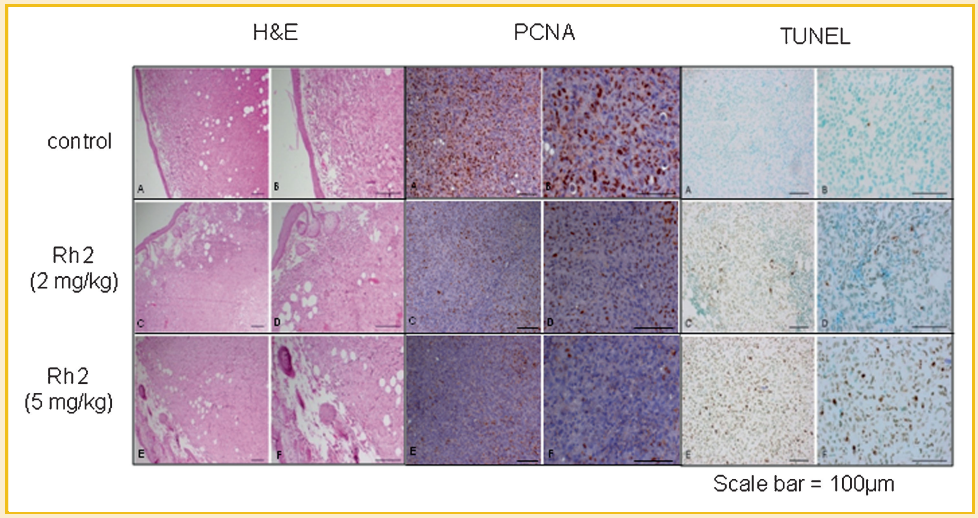

Fig. 7. Tumors harvested from Rh2-treated mice exhibited increased apoptosis and reduced proliferation compared with control tumors. Histologic analysis of MDA-MB-231 tumors from control and Rh2-treated mice (subcutaneous xenograft study) for H&E staining, PCNA expression, and TUNEL-positive apoptotic bodies. Representative staining patterns in tumor sections from a control mouse and 2 or 5 mg Rh2-treated mouse are shown. Magnification in each histologic section: A,C,E, 200×; B,D,F, 400×.

association with increased apoptosis as well as decreased proliferation index.

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