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Enhancement of parthenolide-induced apoptosis by a PKC-alpha inhibition through heme oxygenase-1 blockage in cholangiocarcinoma cells

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Abbreviations: CC, cholangiocarcinomas; FCM, flow cytometry; HO-1, heme oxygenase-1; PTL, parthenolide; Ro, Ro317549; TUNEL, (TdT)-mediated dUTP nick end-labeling; ZnPP, zinc protophorphyrin IX

Abstract

Cholangiocarcinoma (CC) is a chemoresistant intrahepatic bile duct carcinoma with a poor prognosis. The aims of this study were to identify molecular pathways that enhance sesquiterpene lactone parthenolide (PTL)-induced anticancer effects on CC cells. The effects of PTL on apoptosis and hemoxygenase-1 (HO-1) induction were examined in CC cell lines. The enhancement of PTL-mediated apoptosis by modulation of HO-1 expression and the mechanisms involved were also examined in an *in vitro* cell system. Low PTL concentrations (5 to 10 μM) led to Nrf2-dependent HO-1 induction, which attenuated the apoptogenic effect of PTL in Choi-CK and SCK cells. PTL-mediated apoptosis was enhanced by the protein kinase C-alpha inhibitor Ro317549 (Ro) through inhibition of expression and nuclear translocation of Nrf2, resulting in blockage of HO-1 expression. Finally, HO-1 silencing resulted in enhancement of apoptotic cell death in CC cells. The combination of PTL and Ro efficiently improved tumor growth inhibition compared to treatment with either agent alone in an *in vivo* subcutaneous tumor model. In conclusion, the modulation of HO-1 expression substantially improved the anticancer effect of PTL. The combination of PTL and Ro could prove to be a valuable chemotherapeutic strategy for CC.

Keywords: cholangiocarcinoma; drug therapy; heme oxygenase-1; parthenolide; protein kinase C-α

Introduction

Cholangiocarcinoma (CC), a malignant tumor derived from the bile duct epithelium, currently accounts for approximately 15% of all cases of liver cancer worldwide, and its incidence is rising (Blendis and Halpern, 2004; Shaib *et al*., 2004). The prognosis for CC is quite poor, with an average five-year survival rate of 5-10% due to the lack of early diagnosis (de Groen *et al*., 1999) and relative resistance of the tumor to chemotherapy (Mittal *et al*., 1985; Pitt *et al*., 1995). Surgery is potentially curative, but only 25% of patients have resectable tumors at diagnosis, and a majority of these patients relapse within two years (Vauthey and Blumgart, 1994). Generally, chemotherapeutic drugs exert their antitumor effects by inducing apoptosis in cancer cells. Parthenolide (PTL), the major sesquiterpene lactone found in medicinal plants such as feverfew (*Tanacetum parthenium*) is known to inhibit IL-1- and TNFα-mediated NF-κB activation; these are responsible for PTL's anti-inflammatory activity (Hwang *et al*., 1996; Bork *et al*., 1997). PTL also exhibits effective anticancer effects, including the induction of apoptosis and growth arrest in sarcomatous hepatocellular carcinoma cells. Oxidative stress has been shown to contribute to PTL-induced apoptosis in a glutathione-sensitive manner (Wen *et al*., 2002). Subsequent investigation of PTL activity has confirmed that it can induce apoptosis, indicating potential as an anticancer agent (Guzman *et al*., 2005; Sweeney *et al*., 2005; Oka *et al*., 2007). Previously, we found that the sesquiterpene lactone, PTL, effectively induced apoptosis in CC cells through oxidative stress and that susceptibility of CC cells to PTL is modulated by the Bcl-2-related family of proteins (Kim *et al*., 2005), however, the molecular mechanism behind PTL-induced apoptosis remains unclear. Recently, we observed that heme oxygenase-1 (HO-1) was highly expressed during PTL-induced apoptosis in CC cells, and we propose that this induction may contribute to cellular resistance against chemo-oxidative stress. A high dose of PTL alone or a low dose of PTL in

combination with an effective inhibitor of HO-1 induction efficiently induces apoptosis by inhibition of HO-1 expression.

HO-1 catalyzes the conversion of heme to carbon monoxide, iron, and biliverdin. It represents a prime cellular defense mechanism against oxidative stress *via* the antioxidant function of its catalytic products, such as bilirubin and carbon monoxide, and concomitant induction of iron-sequestering ferritin (Ryter and Choi, 2002). HO-1 overexpression in human cancers may offer cancer cells a growth advantage and provide cellular resistance against chemotherapy and photodynamic therapy (Tanaka *et al*., 2003; Fang *et al*., 2004a). HO-1 induction by stress-related agents has been reported to play a role in resistance to apoptosis in several types of human cancer cells (Liu *et al*., 2004; Sasaki *et al*., 2005). Similarly, inhibition of HO-1 has been shown to reduce tumor growth and increased sensitivity to chemotherapy (Fang *et al*., 2003, 2004b).

In the present study, we examined the molecular mechanisms by which PTL induces apoptosis in CC cells through the modulation of HO-1 expression and explored which molecular pathways could be targeted to enhance this susceptibility.

Results

HO-1 induction is associated with resistance of CC cells to PTL-induced apoptosis

We previously found that 10 μM PTL effectively induced apoptotic cell death in a time- and dose-dependent manner in CC cells in which oxidative stress plays a pivotal role in PTL-induced apoptosis (Kim *et al*., 2005). We examined whether HO-1 expression is correlated with susceptibility of CC cells to PTL. To do this, we selected two CC cell lines: Choi-CK cells with low HO-1 expression and SCK cells with high HO-1 expression. PTL effectively triggered apoptotic cell death in a dose-dependent manner in both cell lines (Figure 1A); 72 h treatment with 10 mM PTL induced cell death in 19.2% ± 0.2% of the Choi-CK cells and in 22.7% ± 0.7% of the SCK cells. Unexpectedly, apoptotic cell death of SCK cells, which constitutively express HO-1, was significantly more pronounced than that of Choi-CK cells, suggesting that other molecular mechanism(s) may be involved in PTL-mediated apoptosis. At a PTL concentration of 40 μM, the fraction of apoptotic cells abruptly increased to 55.7% in Choi-CK cells and 79.8% in SCK cells. During apoptosis, PTL induced Nrf2-mediated HO-1 expression in a dose-dependent manner, except for in the case of treatment with high concentrations of PTL (Figure 1B). HO-1 induction was abruptly inhibited to basal levels or below in CC cells treated with 40 μM PTL. Because this abrupt decrease may have resulted from the inhibition of Nrf2 expression or from its nuclear translocation, we examined whether PTL treatment is associated with the nuclear translocation of Nrf2, an upstream transcriptional factor, in cells. The nuclear accumulation of Nrf2 peaked in cells treated with 5 to 10 μM PTL and decreased with higher concentrations. Cytoplasmic accumulation of Nrf2 was greater at lower concentrations, however, and less attenuated at the higher concentrations in both cell lines (Figure 1C). These results suggest that PTL modulates nuclear translocation of Nrf2 at high concentrations of PTL and the expression of Nrf2 at low concentrations of PTL. To determine whether ectopic expression of HO-1 modulated PTL-mediated apoptosis in CC cells, Choi-CK cells that stably expressed HO-1 were established and treated with the indicated concentrations of PTL (Figure 1D). At 40 μM PTL, the fraction of apoptotic cells increased in the vector control cells but not in transfectants that stably express HO-1 (55.8% ± 3.6% *versus* 34.0% ± 4.0%). Ectopic overexpression of HO-1 appears to contribute to the resistance of CC cells to high PTL concentrations.

PKCα inhibitor Ro317549 induced apoptosis and inhibited Nrf2-dependent HO-1 expression

To identify an agent that could simultaneously induce apoptosis and down-regulate HO-1, we examined various protein kinase inhibitors including PI3K inhibitor LY294002, MEK1 inhibitor U0126, ERK inhibitor PD98059, and PKCα inhibitor Ro317549 (Ro), because several upstream signaling kinases, including PKC, PI3K, and MAPKs are known to be involved in Nrf2 regulation (Martin *et al*., 2004; Kobayashi and Yamamoto, 2005). Choi-CK cells were treated with each of these inhibitors for 72 h. The PKCα inhibitor Ro induced apoptosis in a dose-dependent manner much more effectively than did other inhibitors (Figure 2A) and decreased Nrf2 expression in a dose-dependent manner (Figure 2B). Ro did not enhance HO-1 expression in Choi-CK cells, although other inhibitors did (Figure 2C). PI3K and MAPK inhibitors induced HO-1 expression, which seems to have resulted from the stress of signal inhibition. Furthermore, Ro decreased Nrf2/HO-1 expression in a dose-dependent manner in SCK cells (Figure 2D). These results point to Ro as an ideal candidate for enhancement of PTL-mediated apoptosis in CC cells.

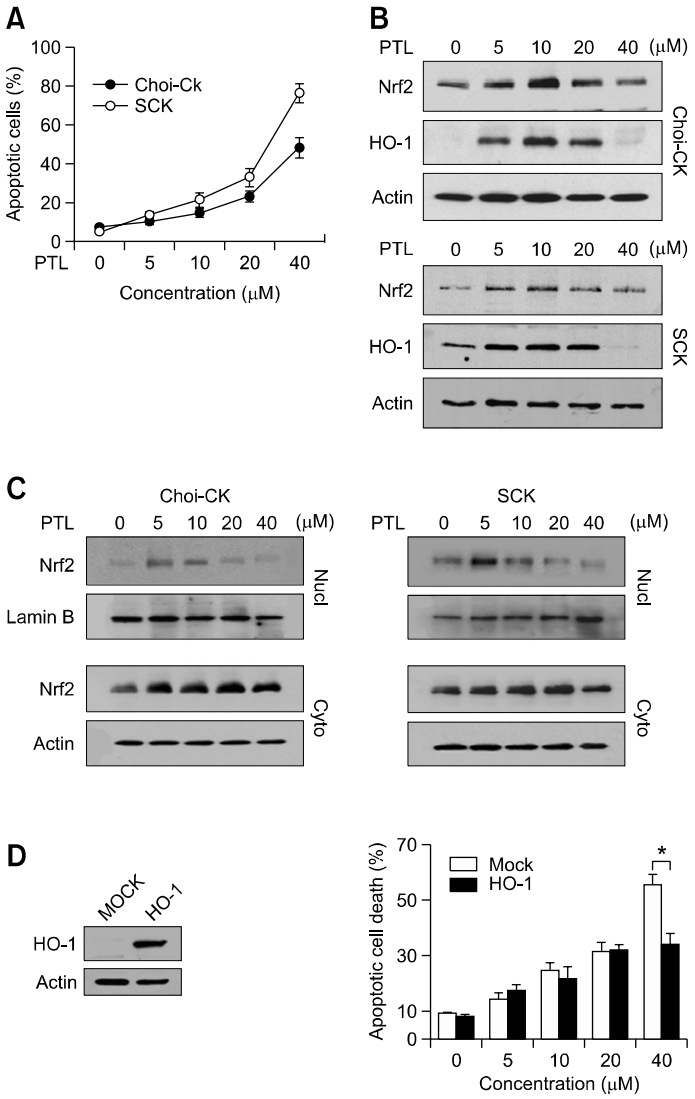

Figure 1. HO-1 expression is involved in apoptotic cell death of CC cells. (A) Apoptotic cell death in Choi-CK and SCK cells treated with the indicated concentrations of PTL for 72 h. Cells were stained with Hoechst 33258, and the fraction of apoptotic cells was determined using fluorescence microscopy. Each data point represents the mean ± SE of three independent experiments performed in duplicate. (B) Nrf2 and HO-1 expression during apoptotic cell death of Choi-CK and SCK cells treated for 72 h with the indicated concentration of PTL (C) Nuclear and cytoplasmic Nrf2 expression in Choi-CK and SCK cells treated with various concentrations of PTL for 48 h. Cellular lysates were subjected to immunoblot analysis. Nucl, nuclear fraction; Cyto, cyctoplasmic fraction. (D) Ectopic overexpression of HO-1 (*left*) and apoptotic cell death (*right*) in stably transfected Choi-CK cells expressing HO-1. Cells were treated with the indicated doses of PTL for 72 h. Each data point represents the mean ± SE of three independent experiments performed in duplicate. *, $P < 0.01$.

PKCα inhibitor Ro enhanced PTL-mediated apoptosis in CC cells

Next, we treated CC cells with PTL alone or in combination with 5 μM Ro. Treatment with 2.5 μM Ro did not significantly increase the apoptotic cell fraction in CC cells, as compared with other inhibitors. Ro significantly enhanced PTL-mediated apoptogenic effects in both Choi-CK (64.5% ± 3.2% *versus* 47.4% ± 0.8%) and SCK cells (88.5% ± 9.5% *versus* 78.5% ± 5.2%) (Figure 3A). We also used FACScan analysis to determine the apoptotic cell fraction in Choi-CK cells treated with

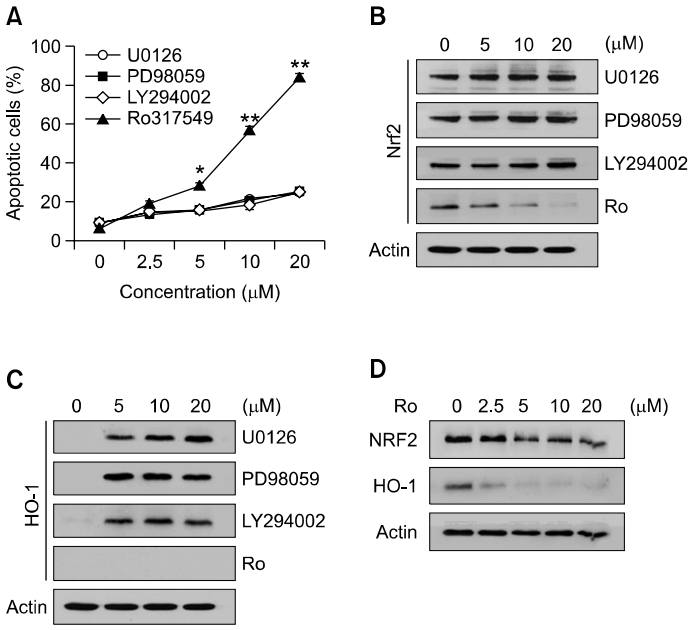

Figure 2. Apoptotic cell death and Nrf2 and HO-1 expression in CC cells treated with various inhibitors. (A) Apoptotic cell death in Choi-CK and SCK cells treated with the indicated inhibitors for 72 h and then stained with Hoechst 33258. The fraction of apoptotic cells was determined using fluorescence microscopy. Each data point represents the mean ± SE of three independent experiments performed in duplicate. *, $P < 0.05$; **, $P < 0.01$. (B) Nrf2 expression in Choi-CK cells treated for 72 h with various inhibitors at the indicated concentration. (C) HO-1 expression in Choi-CK cells treated with various inhibitors at the indicated concentrations for 72 h. (D) Expression of Nrf2 and HO-1 in SCK cells treated with Ro at the indicated concentrations for 72 h. For each blot, initial immunodetection was performed, and then the blots were stripped and reprobed with a monoclonal antibody to actin as a loading control. All immunoblot experiments were performed at least three times, and the result of one representative experiment is shown.

PTL alone or with 5 μM and confirmed that Ro significantly enhanced PTL-mediated apoptogenic effects in Choi-CK cells (64.8% ± 7.2% *versus* 49.3% ± 2.7%) (Figure 3B). Concomitant treatment with Ro and either 10 μM or 40 μM PTL attenuated Nrf2-dependent HO-1 expression in both Choi-CK and SCK cells (Figures 3C and D). Ro enhanced apoptosis in Choi-CK cells independently of HO-1 blockage at the highest concentration (40 μM) of PTL. In addition, Ro-mediated Nrf2 inhibition was less remarkable than HO-1 inhibition at the low concentration of PTL (10 μM) in SCK cells.

Nuclear PKCα regulate HO expression

We found that SCK cells showed higher levels of expression and activation of PKCα than other CC cell lines (Figure 4A). Moreover, a transcriptional inhibitor Bach1 expression was much lower in SCK cells than in other CC cells, suggesting that downregulation of Bach1 might be responsible for constitutive expression of HO-1. Accordingly, we examined the effect of Bach1 on the promoter activity of HO-1 in SCK cells, and found that Bach1 effectively downregulated promoter activity of HO-1 to 21.5% of that attained with the mock transfection. As a positive control, Nrf2 increased the promoter activity of HO-1 by 4.0-fold in Choi-CK cells (Supplemental Data Figure S1A). We also investigated whether ectopic expression of Bach1 altered the expression of other HO-1-modulating molecules. SCK cells stably expressing Bach1 showed suppressed expression of HO-1, but no change in the expression of PKCα or Nrf2 (Supplemental Data Figure S1B). The same results were obtained by immunofluoresence assay in SCK cells (Supplemental Data Figure S1C). The expression of PKCα was mainly localized in the cytoplasm of SCK cells (Figure 4B). PKCα and constitutively active PKCα (MyrPKCα) led to Nrf2 expression and its nuclear translocation and subsequent HO-1 expression (Figure 4C). These results indicate that nuclear PKCα translocation

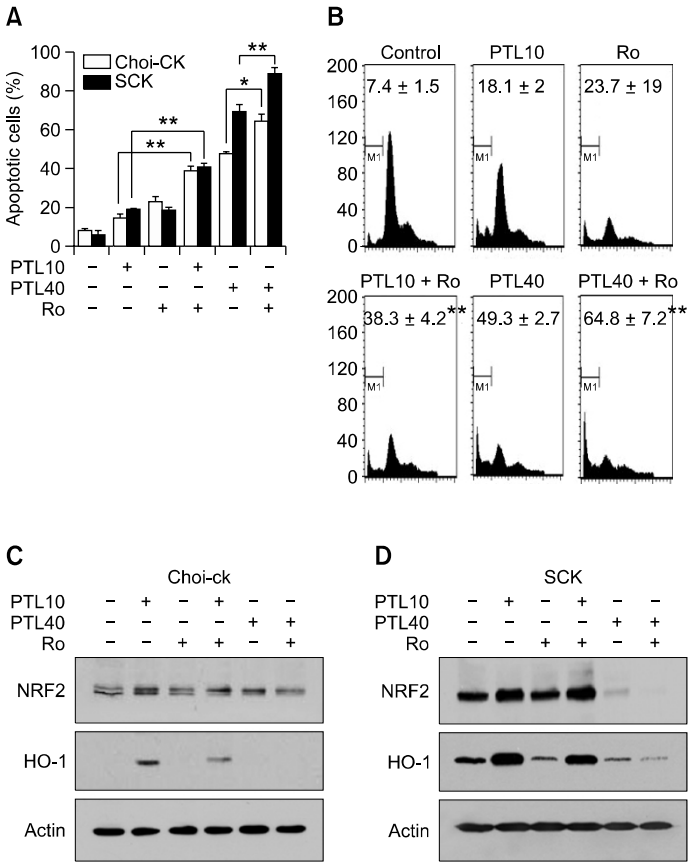

Figure 3. Apoptotic cell death and HO-1 expression in CC cells treated with PTL and/or Ro. (A) Apoptotic cell death in Choi-CK and SCK cells treated with PTL and/or 5 μM Ro for 72 h. The cells were stained with Hoechst 33258, and the fraction of apoptotic cells was determined using fluorescence microscopy. Each data point represents the mean ± SE of three independent experiments performed in duplicate. *, $P < 0.05$; **, $P < 0.01$. (B) Quantification of the apoptotic fraction by FCM analysis. Cells were treated with PTL and/or 5 μM Ro for 72 h. The sub G_1 fraction was estimated by gating hypodiploid cells in the histogram using the LYSIS II program. DNA content is plotted on the linear abscissa (M_1, apoptotic fraction). Each value represents the mean ± SE of three independent experiments performed in duplicate. (C) Expression of Nrf2 and HO-1 in Choi-CK cells treated with PTL and/or 5 μM Ro for 72 h. (D) Expression of Nrf2 and HO-1 in SCK cells treated with PTL and/or 5 μM Ro for 72 h. Immunoblot experiments were performed at least three times, and the result of one representative experiment is shown.

contributes to the constitutive expression of HO-1 in SCK cells.

HO-1 suppression increased susceptibility of SCK cells to PTL-mediated apoptosis

We assessed whether Bach1-mediated down-regulation of HO-1 or siRNA-mediated HO-1 silencing affected PTL-induced apoptosis in SCK cells. After treatment of SCK cells stably expressing Bach1 with 10 μM PTL, apoptotic cell death increased significantly ($P < 0.01$) to 34.3% or 31.3%, respectively, as compared to 13.8% or 16.2% in vector-treated control cells. Treatment with 20 μM PTL also significantly enhanced apoptotic cell death in SCK cells stably expressing Bach1 to 53.7% or 50.9%, respectively, as compared with 26.7% or 30.1% in vector control cells (Supplemental Data Figure S2). Furthermore, when the expression of HO-1 in SCK cells was inhibited to less than 20% of control levels using HO-1 siRNAs (Figure 5A), the level of apoptotic cell death following treatment with 10 μM PTL was significantly higher in HO-1-silenced cells than in cells treated with non-targeting siRNA (25.8% or 31.0% *versus* 16.6%; $P < 0.01$). Similar enhanced apoptotic cell death was observed following treatment with 20 μM PTL, as compared to cells trans-

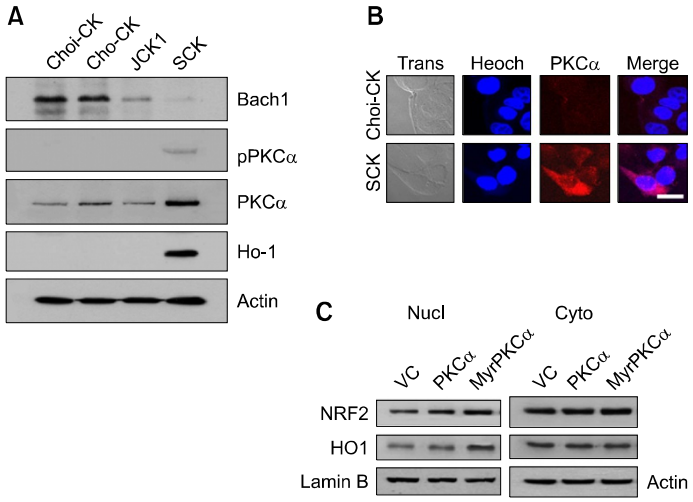

Figure 4. PKCα modulate HO-1 expression in CC cells. (A) Immunoblot analysis of Bach1, PKCα, phosphorylated PKCα, and HO-1 expression in the four CC cell lines. (B) Higher expression of PKCα (*red*) in SCK cells than in Choi-CK cells. Immunoreactivity was assayed by immunofluorescence. Trans, transmission. Bar, 20 μM. (*left panels*). (C) Nuclear Nrf2 and subsequent HO-1 expression was enhanced by PKCα in Choi-CK cells. The cells were transiently transfected with PKCα or constitutively active MyrPKCα, and their respective nuclear (*Nucl*) and cytoplasmic (*Cyto*) lysate fractions were subjected to immunoblot anlayses for Nrf2 and HO-1.

fected with non-targeting siRNA (50.5% or 53.1% *versus* 35.6%) (Figure 5B). These findings suggest that both Bach1-mediated downregulation of HO-1 and HO-1 silencing by siRNA enhance PTL-induced apoptosis in CC cells.

PKCα inhibitor Ro inhibited PTL-mediated Nrf2/HO-1 induction and enhanced apoptosis *in vivo*

To examine the effects of PTL and Ro on tumor growth *in vivo*, we used a xenograft nude mouse tumor model with subcutaneously implanted Choi-CK and SCK cells. The animals that were simultaneously treated with PTL and Ro displayed significantly less tumor growth than the animals that were treated with either PTL or Ro alone in the Choi-CK and SCK xenoplant models ($P < 0.01$ and $P < 0.05$, respectively, Figure 6A). We next investigated the effects of PTL and Ro on Nrf2 and HO-1 expression *in vivo*. PTL treatment induced Nrf2 and HO-1 expression, but concomitant treatment with Ro effectively inhibited PTL-mediated Nrf2 and HO-1 induction (Figure 6B). Because the downregulation of HO-1 seemed to contribute to

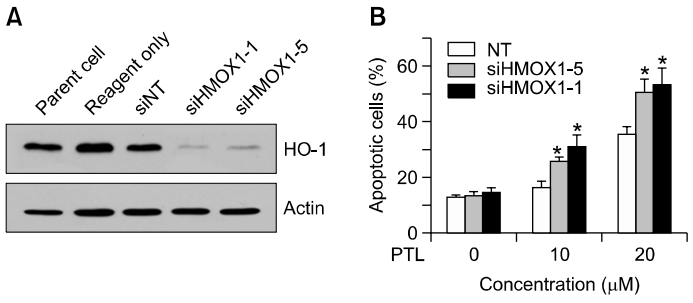

Figure 5. HO-1 suppression enhanced apoptotic cell death in SCK cells. (A) SCK cells were transfected with 50 nM siRNA targeting HO-1 (HMOX1-1 and HMOX1-5) or a non-targeting siRNA, and the efficiency of HO-1 silencing was determined by immunoblot analysis using actin as a loading control (*upper panels*). (B) Cells were transfected with 50 nM HO-1 siRNA for 24 h and subsequently treated with either 10 μM or 20 μM PTL for 72 h prior to staining with Hoechst 33258. The fraction of apoptotic cells was determined using fluorescence microscopy. Each value represents the mean ± SE of three independent experiments. *, $P < 0.01$.

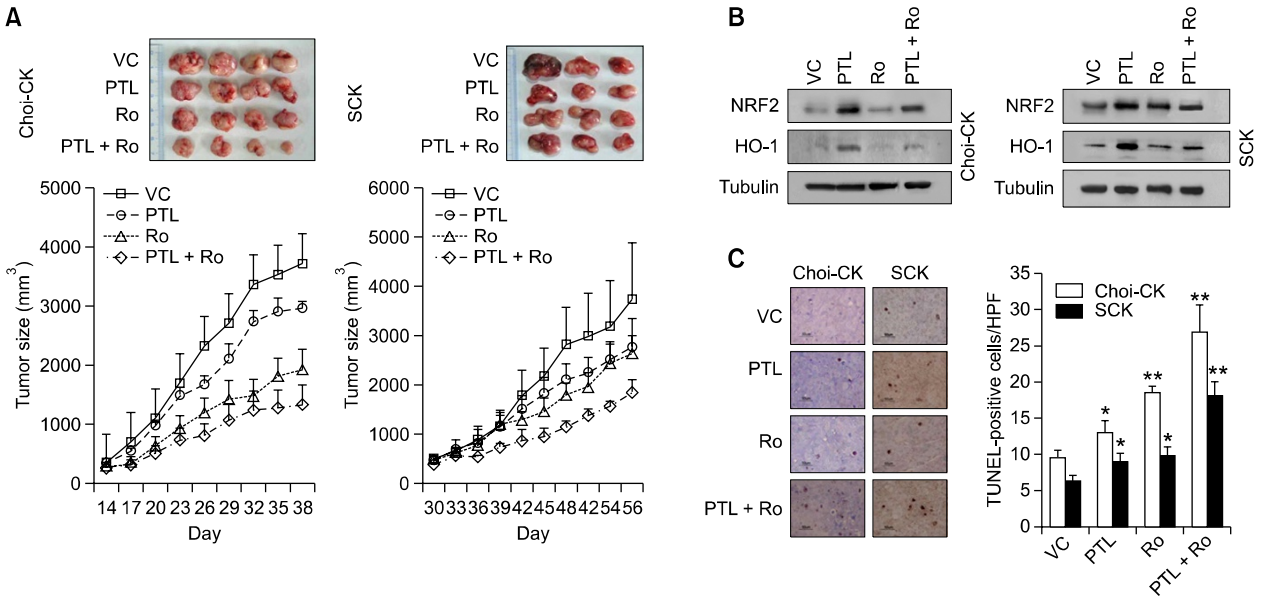

Figure 6. Impact of PTL and PKCα inhibitor combination on tumor growth in a subcutaneous xenograft model. (A) Choi-CK (*left*) and SCK cells (*right*) were subcutaneously implanted in nude mice. Mice bearing HCC xenografts were randomized into one of four treatment groups. Each treatment group consisted of 6-8 mice treated with vehicle (VC), 2.5 mg/kg PTL, 2.5 mg/kg Ro, or PTL plus Ro for 38-56 days. Tumor growth was measured and calculated. Each value represents the mean tumor volume ± SE. The differences seen were statistically significant ($P < 0.01$). (B) Nrf2 and HO-1 protein levels were determined by immunoblot analysis in tumor tissues treated with either PTL or Ro, or in combination. (C) Tumor sections from animals were analyzed by TUNEL assay for apoptotic cell death. The number of apoptotic (TUNEL-positive) cells in tumors was elevated by combination therapy (*, $P < 0.05$ *versus* control; **, $P < 0.01$ *versus* monotherapy).

apoptotic cell death by PTL-mediated oxidative injury *in vitro*, representative tumors were analyzed with the TUNEL assay for the measurement of apoptotic cells (Figure 6C). Tumor tissues from animals treated with PTL plus Ro displayed drastically more positively stained apoptotic cells, as compared to those from animals treated with either PTL or Ro alone. Thus, both *in vitro* and *in vivo*, Ro was able to suppress Nrf2 and HO-1 induction and subsequently induce apoptotic cell death in tumor tissues.

Discussion

CC cell sensitivity to PTL appears to be correlated with oxidative stress, which results mainly from GSH depletion and ROS generation (Kim *et al*., 2005). Overexpression or deletion of antioxidant enzymes can alter the sensitivity of CC cells to PTL, substantiating the notion that the anticancer effects of PTL can be enhanced by suppression of antioxidant enzyme expression. In the present study, we showed that typical CC cells express low levels of HO-1, whereas sarcomatous SCK cells constitutively express HO-1. Susceptibility to PTL was independent of HO-1 basal level expression, and instead appeared to be associated with HO-1 expression that was inducible by drugs. In A549 cells, induction of HO-1 by oxidant chemicals or transfection contributes to a cytoprotective effect (Lee *et al*., 1996; Speit and Bonzheim, 2003). In our study, a high dose of PTL caused inhibition of the expression and nuclear translocation of Nrf2 with subsequent blockage of HO-1 expression and enhanced apoptotic cell death in CC cells.

Previous studies have shown that zinc protophorphyrin IX (ZnPP) inhibits HO-1 expression and reduces tumor growth in mice in a dose dependent manner, whereas treatment with cobalt protoporphyrin IX (CoPP) increases HO-1 expression and enhances tumorigenicity (Hirai *et al*., 2007). In the present study, we failed to enhance the PTL-mediated apoptogenic effect through the addition of ZnPP, which induced HO-1 expression in a dose-dependent manner (data not shown). Therefore, we tested several kinase inhibitors and found that the PKCα inhibitor effectively induced apoptosis and simultaneously inhibited Nrf2-dependent HO-1 expression, even at low concentrations of PTL. The PKC family of proteins comprises at least ten serine/threonine kinases (Rushworth *et al*., 2006). Among these, PKCα is known to phosphorylate Nrf2 at Ser^{40}, resulting in translocation of Nrf2 to the nucleus, where it forms a heterodimer and activates HO-1 gene expression (Bloom

and Jaiswal, 2003). In the present study, the PKCα inhibitor reduced both the expression and nuclear translocation of Nrf2, leading to HO-1 down-regulation. In contrast, neither PKCβ nor PKCδ inhibitor exerted effects on the inhibition of PTL-mediated HO-1 expression (data not shown). Other studies have shown that inhibition of PKCδ significantly decreases *HO-1* mRNA induction (Ogborne *et al*., 2008; Zhang and Forman, 2008), whereas PKCα- and β-specific inhibitors have no significant effect. The molecular mechanism of HO-1 modulation by PKC family members may be specific for each antioxidant. HO-1 induction also appears to be related to the downregulation of the transcriptional inhibitor Bach1, a basic leucine zipper mammalian transcriptional repressor that forms antagonizing heterodimers with members of the Maf-related oncogene family. These heterodimers bind to Maf recognition elements (MAREs) and suppress expression of various genes, including HO-1 and NQO1 (Sun *et al*., 2002, 2004). Suppression or silencing of HO-1 resulted in increased susceptibility of tumor cells to PTL. Similarly, specific inhibition of HO-1 expression increases the response of pancreatic cancer to anticancer treatment and enhances the cytotoxic effect of gemcitabine in urothelial cancer cells (Berberat *et al*., 2005; Miyake *et al*., 2010). Futhermore, PKC inhibition sensitizes TNFα- or TRAIL-induced apoptosis in cancer cells (Nishida *et al*. 2003; Shi *et al*., 2005). Therefore, specific inhibition of HO-1 expression may be a new option in cancer chemotherapy.

In summary, although CC cells are intrinsically sensitive to PTL, substantial cytoprotective molecular mechanisms attenuate this susceptibility. PKCα inhibitors effectively enhanced PTL-mediated apoptosis in CC cells by inhibition of the cytoprotective mechanisms both *in vitro* and *in vivo*. Combining a PKCα inhibitor and PTL may be an effective clinical chemotherapeutic application for CC.

Methods

Cell culture and transfection

Four distinct CC cell lines (Choi-CK, Cho-CK, JCK1, and SCK) were cultured as described previously (Kim *et al*., 2005; Yoo *et al*., 2009) and treated with 10 μM (or other concentrations as noted). PTL was dissolved in either dimethyl sulfoxide or absolute alcohol. Transfection of Choi-CK cells was performed using an expression plasmid vector encoding the human HO-1 gene or a control vector (Invitrogen, Carlsbad, CA). Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol, and transfectants were selected in the presence of 600 μg/ml G418 for 2-3 weeks.

Apoptosis assay by flow cytometric (FCM) analysis

Trypsinized monolayer CC cells and detached cells were collected at set intervals after treatment with PTL for analysis by FCM using a FACSCalibur (Becton Dickinson, San Jose, CA) with an argon laser at a wavelength of 488 nm. Propidium iodide and 40 μg/100 μl phosphate buffered saline (PBS) were added to 1×10^6 cells suspended in 800 μl PBS together with 100 μl RNase A (1 μg/ml), and the mixture was incubated at 37°C for 30 min prior to FCM analysis of 2×10^4 cells, as described previously (Sasaki *et al*., 2005). The cell cycle was analyzed using ModFit LT 3.0 software program (Verity Software House, Topsham, ME). The sub-G_1 fraction was estimated by gating the hypodiploid cells in the DNA histogram using the LYSIS II program.

Cell lysis and immunoblotting

Cells were washed twice with cold PBS on ice and harvested by scraping with a rubber policeman. Cells were sedimented by centrifugation at 4°C and resuspended directly into Laemmli sample buffer containing 62.5 mM TrisHCl, pH 6.8, 2% SDS (w/v), 12% glycerol (w/v), and 5% mercaptoethanol (v/v). Extracted proteins were resolved by 12% SDS-PAGE and transferred to nylon membranes. Membranes were incubated at 4°C overnight with the primary antibody, washed in PBS/0.1% Tween and incubated for 45 min with the secondary antibody. Following incubation with the secondary antibody, the blots were washed three times with PBS/0.1% Tween and developed using a commercial chemiluminescence detection kit (Amersham ECL). Nrf2 (C-20) and Bach1 (c-20) polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HO-1 (SPA-896) polyclonal antibody was obtained from Stressgen (Ann Arbor, MI), and protein kinase C-α (PKCα) polyclonal antibodies (2056) were purchased from Cell Signaling Tech (Danvers, MA). Polyclonal antibody against phosphorylated PKCα (S657, 06-822) was obtained from Upstate (Temecula, CA). Monoclonal antibody to actin, anti-mouse IgG, anti-rabbit IgG, and anti-goat IgG were from Sigma (St. Louis, MO).

Immunofluorescence

Cells were grown on glass coverslips, fixed with 4% paraformaldehyde, permeabilized in PBS containing 0.2% Triton × 100, and blocked with 1% BSA. Transient transfection of a FLAG-PKCα fusion construct into Choi-CK cells was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were incubated with rabbit or mouse polyclonal antibodies against Nrf2 and HO-1, respectively, overnight at 4°C, washed, and incubated with tetramethylrhodamine isothiocyanate isomer R (TRITC)-conjugated anti-rabbit immunoglobulin and fluorescein isothiocyanate-conjugated (FITC)-conjugated anti-mouse immunoglobulin, respectively. After a final wash, the cells were stained for 15 min with 1 μg/ml Hoechst 33258 to visualize the nuclei and mounted in 50% glycerol

in PBS at 4°C. Cells were examined by laser scanning microscopy (LCM 510, Carl Zeiss, Jena, Germany).

Luciferase assay

The pGL3HO1/-4384-Luci construct, a human *HO-1* promoter-driven luciferase reporter construct spanning region -4384 to +24 of HO-1, was a gift from Prof. H.T. Chung (Wonkwang University School of Medicine, South Korea) (Lee *et al*., 2006). Cells were plated at 2×10^4 cells/well in 24-well plates and cultured for 18 h before a 16-h incubation at 37°C with 500 ng pGL3HO1/-4384-Luci plasmid, 50 ng pRL-TK plasmid (Promega, Madison, WI), and Lipofectamine 2000 (Invitrogen). Following transfection, cells were replenished with complete medium, lysed in 120 μl lysis buffer at the indicated time intervals, and stored at -20°C until assayed. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions and was normalized to Renilla luciferase activity.

RNA interference

Small interfering RNA (siRNA) against *HO-1* was procured from Qiagen (Hilden, Germany). Transfections with *HO-1* siRNAs HMOX1-1 (target sequence CACCAAGTTCAAGCAGCTCTA) and HMOX1-5 (target sequence CAGGCAATGGCCTAAACTTCA) were performed using HighPerFect Transfection Reagent (Qiagen) following the manufacturer′s protocol. Cells were also transfected with non-targeting, negative control siRNA (Qiagen, AllStar Negative Control siRNA) to assess target specificity and any non-specific gene silencing effects. Briefly, SCK cells were transfected with 50 nM siRNAs directed against *HO-1* for 72 h, and the cells were harvested and processed for cell lysate preparation.

Subcutaneous tumor models

Choi-CK (4.5×10^6) and SCK (6.5×10^6) cells were injected into nude mice (BALB/cByJ-*Hfh11*^{nu} KRIBB, Daejeon, South Korea). Mice were randomized and assigned to treatment groups and intraperitoneally injected every other day (200 μL) with diluent, PTL (2.5 mg/kg), Ro317549 (Ro, 2.5 mg/kg), or PTL + Ro, starting on days 14 and 28 day after tumor cell implantation (0.5 mm^3 tumor volume), respectively. Tumor diameters were measured at 3-day intervals, and tumor volumes were also calculated ($width^2 \times length \times 0.5$). The experiment was terminated on days 38 and 56, respectively, and the tumors harvested. All experiments were approved by the Chonbuk National University Animal Care and Use Committee. Apoptosis was measured quantitatively using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay (Li and Altieri, 1999). All components for this procedure were from the ApopTag *in situ* Apoptosis Detection kit (Chemicon, Temecula, CA), which was used according to the manufacturer's instructions. Four fields at 40 × magnification were selected at the proliferation front of each tumor, and TUNEL-positive cells were counted.

Quantification

Densitometric data were analyzed using a LAS3000 system (Fuji Photo Film, Tokyo, Japan). Expression of each gene was calculated by normalizing the expression level against the level of actin protein and then calculating the ratio of expression in treated cells as compared with that in control cells at the indicated time points.

Statistical analysis

Data are presented as mean ± SE of at least three independent experiments performed in duplicate. Representative blots are shown. All data were entered into Microsoft Excel 5.0, and GraphPad Software was used to perform two-tailed t tests or analysis of the variance, as appropriate. P values < 0.05 were considered significant.

Supplemental data

Supplemental Data include two figures and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-42-11-07.pdf.

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