Induction of Apoptosis by *Radix Paeoniae Alba* Extract through Cytochrome c Release and the Activations of Caspase-9 and Caspase-3 in HL-60 Cells

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*Radix Paeoniae Alba* has been used as a constituent of herbal medicine prescriptions for the treatment of inflammation, cancer, and other diseases. The aim of this study was to investigate the mechanism of *Radix Paeoniae Alba* extract (RPAE)-induced apoptosis in HL-60 leukemic cells. We observed that RPAE induced apoptotic changes in a dose-dependent manner, which was confirmed by DNA fragmentation and poly-(ADP-ribose) polymerase (PARP) cleavage. We also found release of cytochrome c from mitochondria to the cytosol in RPAE-treated HL-60 cells. The caspases, caspase-9 and -3, but not caspase-8, were found to be activated in response to RPAE treatment, and the caspase-3 inhibitor, Ac-DEVD-CHO, and the caspase-9 inhibitor, z-LEHD-FMK, but not the caspase-8 inhibitor, z-IETD-FMK, attenuated RPAE-induced DNA fragmentation and PARP cleavage. These results suggest that RPAE-induced apoptosis is stimulated by the release of cytochrome c to the cytosol, by procaspase-9 processing, and via a caspase-3 dependent mechanism.

Key words *Radix Paeoniae Alba*; apoptosis; HL-60 leukemic cell

**Radix Paeoniae Alba** (RPA) is the root of *Paeonia lactiflora* Pallas, which belongs to the Ranunculaceae family. RPA has been commonly used in traditional medicine to tonify the blood, to alleviate pain, and to treat inflammation and cancer. A recent study reported that RPA inhibits hepatocellular carcinoma cell growth and induces their apoptosis.1) Several constituents isolated from RPA have also been found to have immunologically active.2,3) However, details of the mechanism underlying the activity of RPA are not understood.

Apoptosis is a highly regulated process that involves the activation of a series of molecular events that lead to cell death, and is characterized by morphologic changes, chromatin condensation, and apoptotic bodies, which are associated with DNA cleavage and ladder formation.4,5) There are two major apoptotic pathways, namely, intrinsic and extrinsic apoptosis signaling, in cells responsive to apoptotic stimuli.6–9) The intrinsic apoptosis pathway involves mitochondrial signaling, and caspase-9 has been suggested to be the predominant initiator caspase in this pathway.7,9) In contrast, the extrinsic apoptosis pathway is mediated by death receptors, such as, Fas receptors and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), and the extrinsic pathway is initiated by caspase-8.5,8) The activated initiator caspases-8 and -9 starts a proteolytic cascade by cleaving downstream effector caspases, such as, caspase-3, -6, and -7.7) Of these, caspase-3 is believed to be the main executioner caspase and its activation has been shown to be essential for both intrinsic and extrinsic apoptotic cell death.10,11)

To study the mechanism underlying the anticancer activities of RPA, we examined the antiproliferative effect of RPA extracts (RPAE) in HL-60, a human promyelocytic leukemia cell line. Our results demonstrated that RPAE induces apoptosis in a dose-dependent manner in HL-60 cells. We further demonstrate that the apoptotic effects induced by RPAE are accompanied by intrinsic pathway features, i.e., the release of cytochrome c into cytosol and the activation of caspase-9, but not by extrinsic pathway features, and eventual caspase-3 activation.

**MATERIALS AND METHODS**

**Culture Conditions** Human leukemia HL-60 cells were purchased from the American Type Culture Collection. Cells were placed in 75 cm² tissue culture flasks and grown at 37 °C under a humidified, 5% CO₂ atmosphere in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum and 2 mM glutamine, 10,000 units/ml of penicillin, 10 mg/ml of streptomycin, and 2.5 µg/ml of amphotericin B.

**Preparation of Herbal Extract** *Radix Paeoniae Alba* was identified by Min-Kyo Shin, keeper of the herbarium at the Department of Physiology, School of Oriental Medicine, Wonkwang University (Voucher samples were preserved for reference; Ref. No. Omcpby 2001-80). To produce the extract, 200 g of RPA was added to 1800 ml of water, boiled for 2 h, filtered, and concentrated to 200 ml. The sterile extract (41.97 g) was stored at 70 °C.

**MTT Cell Viability Assays** Cultured cell viabilities were determined by assaying the reduction of MTT to formazan.12) In brief, after incubation with RPAE, cells (10⁴/well) in 96-well plates were washed twice with PBS and MTT (100 µg/0.1 ml of PBS) was added to each well. The cells were then incubated at 37 °C for 1 h, and DMSO (100 µl) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm with a spectrophotometer (E-MAX, Molecular Devices, U.S.A.).

**Detection of Cytosolic Cytochrome c** The release of...
mitochondrial cytochrome c was determined by Western blotting, as described previously. Briefly, after the various treatments, cells (1.5×10^7 cells) were washed with PBS, and resuspended in ice-cold homogenization buffer (250 mM sucrose, 20 mM Hepes-KOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mg/ml aprotinin, 1 µg/ml leupeptin). After 30 min incubation on ice, cells were homogenized with a glass Dounce homogenizer (30 strokes). The homogenates obtained were then subjected to a series of centrifugations at 10000×g for 60 min and cytosolic fractions were collected. Thirty micrograms protein aliquots were loaded on 15% SDS gel, and after electrophoretic separation, proteins were transferred to nitrocellulose membranes (Millipore, Bedford, MA, U.S.A.) using a semi-dry blotting apparatus (Bio-Rad, Munich, Germany). The blots obtained were incubated with mouse anti-cytochrome c antibody (Pharmingen, San Diego, CA, U.S.A.). Prior to incubation with antibodies against Fas, a horse radish peroxidase-coupled secondary antibody was added. The protein bands were visualized using an enhanced chemiluminescence (ECL) system (Bio-Rad, Munich, Germany).

**Caspase Activity Assay** After designated treatments, cells were washed with ice-cold PBS and lysed in Triton X-100 buffer (0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris–HCl, pH 7.5) for 30 min on ice. Cell lysates were then mixed with caspase assay buffer (10% glycerol, 2 mM DTT, and 20 mM HEPES, pH 7.5) containing 20 µM Ac-DEVD-AFC (substrate specific for caspase-3), 50 µM Ac-IETD-AFC (specific for caspase-8), or 50 µM Ac-LEHD-AFC (specific for caspase-9), and incubated for 1 h at 37 °C. The enzyme catalyzed release of AFC was monitored using a spectrophotometer (F-2500, Hitachi, Japan) at an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

**Western Blot Analysis of Caspases and PARP Cleavage** Cell extract proteins were separated by SDS-PAGE, and subsequently transferred onto nitrocellulose membranes (Millipore) using a semi-dry blotting apparatus (Pharmacia Biotech). Prior to incubation with antibodies against Fas, FasL, caspase-3, -8, -9 or PARP (Pharmingen, San Diego, CA, U.S.A.), membranes were blocked with 2% BSA for 60 min and cytosolic fractions were collected. Thirty micrograms protein aliquots were loaded on 15% SDS gel, and after electrophoretic separation, proteins were transferred to nitrocellulose membranes (Millipore, Bedford, MA, U.S.A.) using a semi-dry blotting apparatus (Bio-Rad, Munich, Germany). The blots obtained were incubated with mouse anti-cytochrome c antibody (Pharmingen, San Diego, CA, U.S.A.). Prior to incubation with antibodies against Fas, a horse radish peroxidase-coupled secondary antibody was added. The protein bands were visualized using an enhanced chemiluminescence (ECL) system (Bio-Rad, Munich, Germany).

**Detection of DNA Fragmentation by Gel Electrophoresis** Cell pellets (3×10⁶) were resuspended in 500 µl of lysis buffer (0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris–HCl, pH 8.0) at room temperature for 15 min and centrifuged at 16000×g for 10 min. DNA was then extracted twice using phenol/chloroform (1 : 1), precipitated with ethanol, and resuspended in Tris/EDTA buffer (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA). DNA was analyzed after separation by gel electrophoresis (2% agarose).

**Protein Determinations** Protein concentrations were determined using the Bradford method with bovine serum albumin as standard. All samples were assayed in triplicate.

**Statistical Analysis** Statistical analysis was performed using the Student’s t-test and ANOVA. Differences with p values of <0.05 were considered statistically significant.

## RESULTS

**Growth Inhibition and Apoptosis Induction by RPAE** Initially, the effect of RPAE on cell growth was examined using MTT assays. As shown in Figs. 1A and B, RPAE inhibited HL-60 cell growth in a dose- and time-dependent manner. The IC₅₀ of RPAE with respect to cell growth was less than 1.0 mg/ml. However, normal peripheral blood mononuclear cells were little affected cell viability by 2.5 mg/ml RPAE treatment (data not shown). To characterize the cell death induced by RPAE, DNA fragmentation was observed by agarose gel electrophoresis, as a marker of apoptotic cell death. Genomic DNA extracted from HL-60 cells after treatment with various concentrations of RPAE for 24 h displayed a dose-dependent characteristic ladder pattern of discontinuous DNA fragments (Fig. 2A). We also evaluated the cleavage of poly (ADP-ribose) polymerase (PARP), another hallmark of apoptosis, and again treatment of HL-60 cells with various concentrations of RPAE for 24 h caused a dose-dependent disappearance of the full size 116 kDa PARP, and an accumulation of its cleavage product (85 kDa) (Fig. 2B).

**Caspase-3 Activation by RPAE** Based on the observation of increased apoptosis in RPAE-treated cells, we examined the involvement of caspase-3, which plays a major role in the execution of apoptotic events. Caspases are activated in a sequential cascade of cleavages from their inactive forms. To monitor the enzymatic activity of caspase-3, we used the specific caspase-3 fluorogenic substrate, Ac-DEVD-AFC. As illustrated in Figs. 3A and B, RPAE induced a sig-
significant increase in caspase-3 enzyme activity in HL-60 cells in a time and dose dependent manner. This result was confirmed by Western blot analysis of caspase-3 protein (Fig. 3C), thus demonstrating that the RPae-induced proteolytic cleavage of procaspase-3 occurs in a time-dependent manner.

**Examination of the Contributions of the Extrinsic and Intrinsic Apoptotic Pathways**  The activation of caspase-9 was examined using a specific fluorogenic substrate for caspase-9, Ac-LEHD-AFC, and RPae were found to induce caspase-9 enzyme activity in a dose- and time-dependent fashion (Figs. 4A, B). The cleavage of procaspase-9 in cells treated with RPae was observed after 12 h of treatment (Fig. 4C, upper panel). These observations corresponded to caspase-3 activation (Fig. 3). We then investigated cytochrome c release in the cytosolic fraction following RPae treatment. Cytochrome c is released from the mitochondrial intermembranous space, and is a key event in the activations of caspase-9 and subsequently of caspase-3.13) Immunoblot analysis of the cytosolic fraction of RPae (1.2 mg/ml) treated HL-60 cells showed an increase in the level of cytochrome c after 6—24 h of treatment versus untreated control cells (Fig. 4C, lower panel). Subsequently, the components of extrinsic pathways, i.e., caspase-8, Fas, and FasL expression were examined in RPae-treated cells. As shown in Fig. 5, RPae was found to have no effect on caspase-8 activation or FasL and Fas expressional variations.

**Effects of Caspase Inhibitors on RPae-Induced Apoptosis**  To determine whether the activations of caspase-9 and caspase-3 are required for the induction of apoptosis by RPae, we pretreated cells with caspase-3-, -8, or -9 inhibitors. Ac-DEVD-CHO (a caspase-3 inhibitor) and z-LEHD-FMK (a caspase-9 inhibitor) abolished both the PARP cleavage (Fig. 6A, lane 3, lane 5) and DNA fragmentation (Fig. 6B, lane 3, lane 5) induced by RPae, but the caspase-8 inhibitor, z-IETD-FMK, did not block apoptotic events in RPae-treated HL-60 cells (Figs. 6A, B, lane 4). When cells were pretreated with the general caspase inhibitor, z-VAD-FMK, apoptotic patterns were completely abolished (Figs. 6A, B, lane 6).

**DISCUSSION**

Herbal medicines and dietary agents are being increasingly utilized as effective means of managing the treatments of various cancers. RPA has been commonly used in Asia as a traditional medicine to treat inflammation and cancer. Lee et al. recently reported that RPae inhibits cell growth and induces apoptosis in HepG2 human hepatocellular carcinoma cells.1) Other studies have also revealed that RPA, in herbal prescriptions, is effective at inhibiting malignancy recurrence and cancer progression.19,20) However, the molecular mechanism underlying the anti-cancer effects of RPae is not understood. The present study shows, that RPae induces the apoptosis of human promyelocytic leukemia cells via the activations of caspase-9 and caspase-3 and that this process involves cytochrome c release by mitochondria into the cytoplasm.
It is known that caspase-3, the main executioner caspase, can be activated by caspase-8 and/or caspase-9. In the two major apoptotic pathways, the extrinsic pathway involves the activation of initiator caspase-8 by death receptor signaling, whereas the intrinsic pathway involves the activation of initiator caspase-9 by cytochrome c release from mitochondria. In the present study, RP AE was found to induce the cleavage and activation of initiator caspase-9 (not caspase-8) and executor caspase-3 in a dose- and time-dependent manner, indicating the involvement of an intrinsic pathway. Many anti-cancer drugs trigger the extrinsic pathway in which Fas receptor-mediated signals induce caspase-8 activation. The involvement of the Fas/FasL pathway in apoptosis remains controversial. Even though some drugs induce apoptosis by up-regulating the expression of Fas/FasL, Fas/FasL expression change does not appear to be required to mediate drug-induced apoptosis. As shown in Fig. 5C, no change in Fas/FasL expression was observed in RP AE-treated cells, thus suggesting that RP AE-induced apoptosis is not mediated.

Fig. 4. Effects of RP AE on the Intrinsic Caspase Pathway
HL-60 cells (5×10^6) were treated with either a range of concentrations (0—2.5 mg/ml) of RP AE for 24 h (A), or 1.2 mg/ml RP AE for 3, 6, 12, 18 or 24 h (B, C). Cytosolic extracts were prepared and assayed for caspase-9 activity (A, B), cleavage and cytochrome c release (C). Values are means±S.E.M. of four independent experiments. *p<0.05, **p<0.01 vs. control.

Fig. 5. Effects of RP AE on the Extrinsic Caspase Pathway
HL-60 cells (5×10^6) were treated with either a range of concentrations (0—2.5 mg/ml) of RP AE for 24 h (A), or 1.2 mg/ml RP AE for 3, 6, 12, 18 or 24 h (B, C). Cytosolic extracts were prepared and assayed for caspase-8 activity (A, B), cleavage, and Fas/FasL expression (C). Values are the means±S.E.M. of four independent experiments.
by the Fas/FasL pathway. This result was confirmed by pretreating with the specific caspase-8 inhibitor, z-LEHD-FMK (Fig. 6), which did not affect the RPAE-induced cleavage of PARP or DNA fragmentation.

Mitochondria have been shown to be involved in the integration of different pro-apoptotic pathways by releasing cytochrome c into the cytosol.24 This released cytochrome c complexes with Apaf-1 and procaspase-9 and further initiates the activation of the caspase cascade leading to apoptosis.25 Consistent with these reports, Western blot analysis of cytosolic fractions, in the present study, showed that RPAE increased cytochrome c release from mitochondria into the cytosol. RPAE also increased the cleavage of procaspase-9, which was accompanied by an increase in caspase-9 activity. The involvement of caspase-9 activation by cytochrome c was further demonstrated by the specific caspase-9 inhibitor, z-LEHD-FMK, as pretreatment with this inhibitor completely abolished RPAE-induced apoptotic signs, such as, PARP cleavage and DNA fragmentation in HL-60 cells.

The activation of apoptosis by RPAE is independent of the p53 pathway, because RPAE can induce apoptosis in p53-deficient HL-60 cells.26 Another group concluded that RPAE-induced apoptosis is p53-independent based on results obtained in non-p53-expressing Hep3B cells.27 We also observed that RPAE induced caspase-9 activity and cytochrome c release into cytosol in HepG2 cells, which possesses wild-type p53 (data not shown).

In conclusion, the present study describes the RPAE-induced apoptotic signal transduction pathway in HL-60 cells. Specifically, the pathway involves DNA fragmentation by PARP cleavage through the activations of caspase-9 and caspase-3 and the mitochondrial release of cytochrome c. Moreover, the study demonstrates that these mechanisms are independent of the Fas/FasL/caspase-8 pathway and p53.

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