The Control of Human Immunosystem by Using Paony Root Drug

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Abstract

Paoniflorin (PF), isolated from paony root, has been used as a herbal medicine for more than 1200 years in China, Korea and Japan for its anti-allergic, anti-inflammatory and immunoregulatory effects. In this study, we found that PF induces apoptosis in both murine T-lineage cells and human T-cell leukemia Jurkat cells. This apoptosis was mediated through the reduction of mitochondrial membrane potential, activation of caspase and fragmentation of DNA. Interestingly, PF induced generation of reactive oxygen species (ROS) and a reducing agent, dithiothreitol (DTT), and a ROS scavenger, N-acetyl cysteine (NAC), successfully attenuated the PF-induced apoptosis. Additionally, PF induced the phosphorylation of three mitogen-activated protein (MAP) family kinases, extracellular signal-regulated kinase, c-Jun amino-terminal kinase (JNK) and p38 MAP kinase. Curcumin, an anti-oxidant and JNK inhibitor, inhibited PF-induced apoptosis, suggesting the possible involvement of curcumin-sensitive JNK or other redox-sensitive elements in PF-induced apoptosis. These results partially explain the action mechanism of PF-containing paony root as a herbal medicine.

Keywords: paoniflorin; paony root; apoptosis; reactive oxygen species (ROS); redox; CD8+ CD122+ regulatory T cells

Introduction

Paony root (Paeoniae radix; Shakuyaku in Japanese) is one of the most well-known herbs in China, Korea and Japan and has been used as a medicine for more than 1200 years. Several ingredients have been extracted from the paony root, including oxypaeoniflorin, albiflorin, benzoylpaeoniflorin and paoniflorin [12, 22, 29]. Among them, paoniflorin (PF), a glucoside, is known to be one of the principle bioactive components of paony root [31]. PF has been reported to have immunoregulatory [16], anti-allergic [34], anti-inflammatory [34], cognition-enhancing [23], neuromuscular-blocking [13, 14], anti-convulsant [2], anti-hyperglycemic [10], anticoagulant [11], and sedative [30] effects. Preparations of more than 40 kampo (Chinese and Japanese traditional) medicines contain paony root. Although paony root or PF as its principle component is one of the main constituents of many kampo medicines, the mechanism by which it elicits immunoregulatory action has not been elucidated. In this study, we found that PF induces apoptosis in both murine T-lymphocytes and human T-cell leukemia Jurkat cells through a redox-linked mechanism. PF-induced apoptosis is mediated by the activation of caspase followed by the fragmentation of DNA. We also found that PF induces reduction of mitochondrial membrane potential and production of reactive oxygen species (ROS), which may act as a second messenger for transducing the PF-mediated pro-apoptotic signals.

Materials and Methods

Cells, Reagents and Antibodies

PF [C29H36O11, MW: 480.47, purity: 93.0% (HPLC), LD50: 9530mg/kg] was purchased from Wako Biochemicals (Osaka, Japan). Single cell suspensions of murine thymocytes and splenocytes were prepared from 6-8-wk-old C57BL/6 strain mice. Splenic T cells (a T-cell-enriched fraction) were obtained by passing spleen cells through a nylon wool column.

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according to the technique described previously [19]. A Jurkat cell line (human T-cell leukemia cells), a NIH 3T3 cell line (murine fibroblasts), a SK Hep1 cell line (human hepatoma cells) and a COS-7 cell line (monkey kidney fibroblasts) were also used. The cells were cultured in RPMI-1640 for thymocytes, splenocytes and Jurkat cells or in DMEM for other cell lines (Nissui Pharmaceutical Co., Tokyo, Japan), each supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 µg/ml penicillin and 100 µg/ml streptomycin (complete RPMI-1640 or DMEM) at 37 °C in a humidified atmosphere of 5 % CO₂ and 95% air. Thymocytes or splenocytes (5.0 × 10⁶/well) and cell lines (1.0 × 10⁶/well) were placed in each well of a 6- or 12-well plate containing 1 ml complete RPMI-1640 or DMEM in the presence or absence of PF dissolved in PBS. PBS was added to control cultures. Curcumin, dithiothreitol (DTT), N-acetyl cysteine (NAC), hydroethidine (HE) and carbamoyl cyanide m-chlorophenyl hydrazone (mCCCP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and 3,3′-dihexyloxacarbocyanine iodide (DiOC₆ (3)) was obtained from Molecular Probes (Eugene, OR). Polyclonal anti-phospho-p42/44MAPK (ERK), anti-phospho-JNK, anti-phospho-p38 and anti-cleaved caspase 3 antibodies were purchased from New England Biolabs (Beverly, MA), and the monoclonal anti-pan ERK antibody was purchased from Transduction Laboratories (Lexington, KY). Monoclonal anti-p38 and polyclonal anti-JNK antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and polyclonal anti-poly (ADP-ribose) polymerase (PARP) antibody was obtained from Upstate Biotechnology (Lake Placid, NY).

SDS-PAGE and Immunoblotting

SDS-PAGE and immunoblotting were performed as described previously [1]. Briefly, cells were lysed by adding an equal volume of a two-fold concentrated sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 10% 2-ME, 20% glycerol), and proteins thus obtained were subjected to SDS-PAGE with 10% gel. The proteins were then transferred to a polyvinylidene difluoride membrane. The membrane was incubated with blocking buffer (5% non-fat dry milk or 3% bovine serum albumin) at 4 °C overnight and then incubated with a specific first antibody for 2-3 hours at room temperature. After washing, the membrane was again incubated with goat anti-rabbit or anti-mouse IgG conjugated to horse radish peroxidase (Tago, Burlingame, CA) for 1-2 hours at room temperature. The proteins in the membrane were visualized by Western Blot Chemiluminescence Reagent (DuPont-NEN, Boston, MA) according to the directions of the manufacturer. For reprobing, the membrane was stripped (2% SDS, 62.5 mM Tris, pH 6.8, 100 mM 2-ME, 50 °C, 30 min) and reprobed with the desired antibody. The molecular sizes of the developed proteins were estimated by comparison with prestained protein markers (New England Biolabs, Beverly, MA).

Analysis of DNA Fragmentation by Agarose Gel Electrophoresis

Cells were lysed in 100 µl of hypotonic lysing buffer (50 mM Tris-HCl, 0.5% SDS, 10 mM EDTA), followed by addition of 2 µl of proteinase K (20 mg/ml) and 6 µl of RNase (10 mg/ml). The resultant mixture was incubated at 55 °C for 1 h. A sample (10 µl) was mixed with 3 µl of 0.25% (w/v) bromophenol blue and 40% (w/v) sucrose and was then run on 1.5% agarose gel with 0.1 µg/ml ethidium bromide [1].

Flow Cytometric Analysis of Mitochondria Membrane Potential (ΔΨₘ) Disruption

Mitochondria membrane potential analysis was performed as described previously [17, 37, 38]. In short, cells were collected and washed twice with PBS. The cells were incubated in 500 µl of PBS containing 50 nM DiOC₆ for 30 min. This DiOC₆ cyanine dye accumulates in the mitochondrial matrix under the influence of the ΔΨₘ. DiOC₆ membrane potential-related fluorescence was analyzed using a FACScalibur flow cytometer.

Analysis of ROS Production by Flow Cytometry (FCM)

For measurement of the level of ROS production by PF, cells were incubated with or without 500 µg/ml PF for 1-4 h (thymocytes) or 2-6 h (Jurkat cells) at 37 °C. The cells were then stained with 2 µM hydroethidine (HE) for 15 min at 37 °C. Thereafter, cells were kept on ice until cytofluorometric analysis within 60 min. Analysis was performed by FCM (EPICS profile; Coulter, Hialeah, FL). Forward and side light scatters were gated on the major population of normal-sized lymphoid cells. Generation of superoxide anion was measured as described previously [26, 35].
Reproduction of the Experiments

At least three independent experiments were performed for each assay. Representative results of the experiments are shown in Figures 1-6.

Results

PF Induces Apoptosis with DNA Fragmentation in Murine T-Lineage Cells and Human T-cell Leukemia Jurkat Cells

We first investigated whether PF induces DNA fragmentation in murine T lymphocytes and human T-cell leukemia Jurkat cells. As shown in Figure 1A-1D, PF induced fragmentation of DNA in murine thymocytes, spleen cells, splenic T cells and Jurkat cells in a concentration-dependent manner. DNA fragmentation was also induced in some (Figure 1E) but not all non-T-cell lines (Figure 1F, 1G). These results suggest that sensitivity to PF depends on cell type and that T-lineage cells are included in the highly sensitive group.

PF Promotes Phosphorylation of MAPK Family Members

The members of the MAPK family that are activated by dual phosphorylation on both tyrosine and threonine residues have been implicated in the transduction of a wide variety of extracellular signals [8, 27, 28]. Therefore, we next tested whether PF promotes phosphorylation of three classes of MAPK family kinases (ERK, JNK, and p38). As shown in Figure 2, PF dose- and time-dependently increased the phosphorylation of ERK and JNK in murine thymocytes. We obtained the same results in Jurkat cells (data not shown). PF also promoted phosphorylation of p-38 MAPK in a manner similar to that for JNK (data not shown).

PF-Induced Apoptosis Accompanies the Reduction of Mitochondrial Membrane Potential ($\Delta \Psi_m$)

It has been reported that mitochondria play a key role in apoptosis induction, exhibiting major changes in their structure and function [6, 7, 21]. It is known that decrease in mitochondrial membrane potential ($\Delta \Psi_m$), which is caused by a local disruption of the outer mitochondrial membrane [17, 35, 37], is an early central event of apoptotic cell death [33]. We measured the level of in $\Delta \Psi_m$ cells treated with PF by the
uptake of a membrane potential-sensitive dye, DiOC₆. As shown in Figure 3, treatment of murine thymocytes or Jurkat cells with 500 µg/ml of PF for 4 hours induced reduction in mitochondrial membrane potential.

Figure 3. PF reduces mitochondrial transmembrane potential (ΔΨ₀). Thymocytes (A) and Jurkat cells (B) were treated with or without 500 µg/ml of PF for 4 hours at 37 °C, stained by incubation with DiOC₆(40 nM), and subjected to cytofluorometric analysis. To determine zero potential, cells were incubated with mCCCP, an uncoupler that almost completely abolished ΔΨ₀. The range shown by a horizontal line indicates “negative staining”.

PF Induces ROS Production

Apoptosis is frequently accompanied by the generation of reactive oxygen species (ROS), possibly in mitochondria of which membrane potential is reduced. ROS may act as a second messenger of the intracellular signal for the regulation of cell growth and death induced by a number of elements, including oxidative stresses, cytokines, and growth factors [3, 18, 24, 39]. Therefore, we performed an experiment to see whether PF induces ROS production in T-lineage cells. PF-treated and -untreated cells were labeled with HE, which is known to be oxidized by ROS to ethidium, for emitting red fluorescence. As shown in Figure 4, PF induced ROS production, which was sustained during an incubation period of 1~4 hours in murine thymocytes and for a period of 2~6 hours in Jurkat cells.

Figure 4. PF induces superoxide production. Thymocytes (A) or Jurkat cells (B) were incubated with or without 500 µg/ml PF for 1~4 hours (thymocytes) or 2~6 hours (Jurkat cells) at 37 °C. The cells were then labeled with HE (2 µM) for 15 min at 37 °C, and subjected to cytofluorometric analysis. The range shown by a horizontal line indicates “positive staining”.

PF Induces Caspase Activation

We next examined whether PF-induced apoptosis involves activation of caspase, a known key enzyme to mediate DNA fragmentation. As shown in Figure 5A, PF induced caspase-3 activation in murine thymocytes and splenocytes in a concentration-dependent manner, as demonstrated by degradation of PARP (poly(ADP-ribose) polymerase) (116 KDa), an intracellular substrate of caspase-3, to 85-KDa fragments. Correspondingly, PF time-dependently promoted cleavage of caspase-3 for activation in murine thymocytes (Figure 5B). PARP degradation was also induced in the T-lymphocytes-enriched fractions of spleen cells and Jurkat cells (Figure 5C and data not shown).

PF Induces Apoptosis through a Redox-Linked and Curcumin-Sensitive Pathway

The results we obtained suggested that production of ROS in linkage to mitochondrial dysfunction and activation of JNKs by ROS [32] are involved in signaling for PF-induced apoptotic cell death of T-lineage cells. To investigate the possible role of oxidative stress and JNK in PF-induced apoptosis, we treated
the Jurkat cells with a reducing agent, DTT, which clearly inhibited PF-induced ROS production (data not shown) and a JNK/oxidative stress-mediated signal inhibitor, curcumin [4, 5], before adding PF. As shown in Figure 5C, both DTT and curcumin clearly inhibited PF-induced PARP degradation.

Figure 5. PF induces PARP degradation and caspase 3 cleavage through a redox-linked mechanism. Thymocytes, splenocytes and Jurkat cells were incubated with or without the indicated concentration of PF at 37 °C for 10 hours. These cells were then lysed with sample buffer and subjected to immunoblot assay with anti PARP antibody (A and C) and anti-cleaved caspase 3 antibody (B). In some groups in (C), DTT (2 mM) or curcumin (20 µM) was added 1 h before the addition of PF. The positions of undegraded (116 KDa) and degraded (85 KDa) PARP molecules and cleaved caspase 3 molecules are shown on the right of the panel.

**Attenuation of ROS Production Inhibits PF-Induced Apoptosis**

To determine the role of ROS in PF-induced apoptosis, we pretreated the murine thymocytes or Jurkat cells with an anti-oxidant and ROS scavenger, NAC or DTT, before the addition of PF. As shown in Figure 6, pretreatment of cells with NAC or DTT, which inhibited PF-induced ROS production (data not shown), clearly attenuated PF-induced DNA fragmentation. These results suggested that PF induces ROS production as an early event for activating the apoptotic machinery of the cells.

![Figure 6](image-url)  
**Figure 6.** Reducing agents inhibit PF-induced DNA fragmentation. Thymocytes (A) and Jurkat cells (B) were incubated with or without 500 µg/ml PF for 14 h (A) or 24 h (B) at 37 °C. In some groups, 2 mM DTT (A) or NAC (B) was added 1 h before the addition of PF. The cells were then lysed in hypotonic lysis buffer, and DNAs were then analyzed by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and photographed by UV illumination.

**Discussion**

In this study, we, for the first time, demonstrated that PF, the principal bioactive component of paony root, which is used in kampo medicine, induces apoptosis in both murine T-lineage cells and human T-cell leukemia Jurkat cells. This apoptosis occurred through activation of caspase (Figure 5) and fragmentation of DNA (Figure 1). The most intriguing finding of this study is that the pharmaceutical action of PF is linked to the action of oxidative stress. We found that just like arsenic [9] PF induced both reduction of mitochondrial membrane potential and ROS production as an early event for apoptosis induction. Inhibition of ROS production by anti-oxidants such as DTT (Figure 6A) and NAC (Figure 6B) almost completely attenuated PF-induced apoptosis, suggesting that ROS production is a prerequisite for PF-induced apoptosis. Moreover, we found that PF induces phosphorylation of MAPK family proteins, ERK, JNK, and p-38 MAPK, in a time- and concentration-dependent manner (Figure 2). It has been reported that phosphorylation (activation) of JNK is involved in oxidative stress-mediated induction of apoptosis of cells [25, 32] and that ASK1, an element upstream of JNK, is activated by oxidative stress [32]. Particularly because PF-mediated apoptosis was
prevented by curcumin as a JNK/oxidative stress-mediated signal inhibitor, the PF-triggered signal transduction cascade seems to include ROS production, ASK1 activation and JNK activation for subsequent mitochondrial membrane potential reduction, which leads to further ROS production, caspase activation, and DNA fragmentation. An earlier study showed that PF stimulates the adenosine A1 receptor, which in turn activates PI-3 kinase-dependent PKC [15]. Additionally, we have also observed that PF activates the PI-3 kinase-dependent Akt pathway. Therefore, PF seems to stimulate multiple signaling pathways for diverse cellular functions.

Is there any correlation between the demonstrated action of PF for inducing apoptosis in vitro and its pharmacological action in vivo? Our preliminary experiment in which C57BL/6 mice were given daily injections of PF (200 µg/mouse) for 7 days for measurement of cell population distribution in mice demonstrated that this amount of PF, which was not lethally toxic to mice, induced changes in the distributions of populations and subpopulations of lymphocytes and granulocytes in the spleen and bone marrow (Tsuboi et al., unpublished observation). These results suggest that PF affects the balance among the population and subpopulations of blood cells in mice, possibly through induction of apoptosis in different populations and subpopulations of blood cells to distinct extents. They may partially explain the mechanism of the anti-allergic or the anti-inflammatory or immunoregulatory effect of PF-containing paeony root. On top of these results, our recent study showed that PF also affects regulatory T cells of mice. In other study, we independently found and showed regulatory actions in murine CD8+CD122+ T cells [40]. With flow cytometry and cell sorting analysis, we recognized that PF promoted the regulating function of CD8+CD122+ T cells, which reduced IFN-γ production of CD8+CD122− (non-regulatory type) T cells, while activated IL-10 production in CD8+CD122+ T cells. And these regulatory actions of this CD8+CD122+ T cells were completely linked with the concentration of PF (Tsuboi et al., unpublished observation). These results suggest that there is high possibility of using PF as a new type medicine for treatment of some kinds of immuno diseases in future, by which control human immuno system. However, further study is needed to confirm the relationships between the demonstrated PF-induced signaling events and their ultimate pharmacological effects in vivo.

The concentrations of PF we used (100~500 µg/ml) in in vitro experiments do not seem to be attained in vivo when a herbal medicine is given to animals or humans. Paeony root is clinically used at 9~12 g/day for humans, and the content of PF in paeony root is 2~6%. Therefore, the lowest concentration that was effective in our in vitro experiments (100 µg/ml, which would correspond to 7g/70kg man) was 10-40-times higher than the clinical human dosage. It is, however, difficult to adequately compare the conditions for the chemicals to display their pharmacological actions in vivo and in vitro, particularly because herbal medicine is usually effective when it is administered for a long time as a mixture of various kinds of chemicals that may work synergistically even if concentrations of individual chemicals are low. It might thereby be that an unusually high concentration of PF is needed for displaying a pharmacological action in an in vitro system in which it affects cells for a short time in the absence of other potentially supporting chemicals. In fact, the effective concentration of PF for T-cell subpopulation change in the spleen in our preliminary in vitro experiments (200 µg/day/mouse) is within the calculated clinical human dosage of PF (80-300 mg/day/man).

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