# Ginsenosides as Apoptosis Inducers

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*Panax ginseng* is a medicinal plant that is cultivated in Korea, Japan, China, and Russia (Yun, 1996; Nah et al., 1995). In Asian countries, it is used as a treatment for various illnesses and also as a daily supplement. Although these putative therapeutic effects have prompted tremendous efforts to reveal the cellular mechanism of its action (Yokazawa et al., 1993; Yoo et al., 1990), this remains poorly understood. A number of components of *Panax ginseng* have been isolated and characterized (Yoo et al., 1990); Over 30 different ginseng saponins (gensenosides) that have been isolated from white ginseng and red ginseng were structurally identified.
Several ginsenosides (Rh2, Rg3, Rg5, Rs3, Rs4 and several other ginsenoside metabolites) have been shown to have cell-growth suppressive effect on various cancer cell. Thus our study was aimed to examine the possible molecular action mechanisms by which these ginsenosides can suppress and induce apoptosis in animal cells.

Ginsenoside-Rg5 suppresses cyclin E-dependent protein kinase activity via up-regulating $p21^{Cip/wafl}$ and down-regulating cyclin E in SK HEP-1 cells: Anticancer Research 17,1067 (1997)-- In the present study, we report that ginsenoside-Rg5 (G-Rg5), a newly discovered diol-containing ginsenoside, blocks the cell cycle of human hepatoma SK-HEP-1 cells via the down-regulation of cyclin E-dependent kinase activity. The results from flow cytometric analysis show that G-Rg5 arrests the cell cycle of SK-HEP-1 cells at the G1/S transition phase via the down-regulation of cyclin E-dependent kinase. The cyclin E-dependent kinase activity that has been immuno precipitated with cyclin E-specific antibody is down-regulated in response to G-Rg5. The results from immunoblottings show that the down-regulation of cyclin E-dependent kinase activity is related to incresed protein levels of $p21^{Cip1/WAF1}$ and to decreased protein levels of cyclin E, cdk2, and CDC25A in the cells. However, the down-regulation of the kinase activity appears to be caused mainly by highly induced $p21^{Cip1/WAF1}$ and decreased cyclin E, since the altered protein levels of $p21^{Cip1/WAF1}$ and cyclin E were much higher than those of cdk2 and CDC 25A. Collec-

tively, these data suggest that G-Rg5 blocks cell cycle of SK-HEP-1 cells at the G1/S transition phase by down-regulating cyclin E-dependent kinase activity and that the down-regulation of cyclin E-dependent kinase activity is caused mainly by induced CDK2 inhibitor, $p21^{Cip1/WAF1}$ and decreased levels of the CDK2 regulatory subunit, cyclin E.

Ginsenoside-Rs3, a new diol-type ginseng saponin, selectively elevates protein levels of p53 and $p21^{Cip/waf1}$ leading to induction of apoptosis in SK HEP-1 cells: Anticancer Research 19, 487 (1999)-- In this paper, we present evidence that Ginsenoside-$Rs_3$ (G-Rs3), a new diol-type of ginseng saponin isolated from the roots of *Panax ginseng*, efficiently arrests the cell cycle at the G1/S boundary at lower doses, 0.1-5 μM, but induces apoptosis at higher doses, 10-25 μM, the effects of which were associated with selectively elevating protein levels of p53 and $p21^{Cip1/WAF1}$ in SK-HEP-1 cells. The cell growth suppressive and apoptosis inducing effects were confirmed by MTT assays together with analyses of flow cytometry, morphological changes and DNA fragmentation. Immunoblottings showed that G-Rs3 significantly elevated protein levels of p53 and $p21^{Cip1/WAF1}$ prior to inducing apoptosis, while it did not elevate those of cyclin E, cyclin A, $p27^{Kip1}$, and PCNA. Immune complex kinase assays showed that G-Rs3 downregulated the activities of both cyclins E- and A-associated kinases. Collectively, we suggest that G-Rs3 selectively elevates protein levels of p53 and $p21^{Cip1/WAF1}$ and hence downregulates the activities of the cyclin-dependent kinases, resulting in cell cycle arrest at the G1/S boundary. We also propose that apoptosis induced by G-Rs3 is related to the elevations of p53 and $p21^{WAF1/Cip1}$ in the cells.

Ginsenoside-Rs4, a new type of ginseng saponin concurrently induces apoptosis and selectively elevates protein levels of p53 and $p21^{Cip/waf1}$ in human hepatoma cell SK HEP-1 cells: Eur. J. cancer vol. 35, No. 3, 507 (1999)-- In this paper, we present evidence that Ginsenoside-$Rs_4$ (G-Rs4; an acetylated analog of Ginsenoside-$Rg_5$), a new ginseng saponin isolated from *Panax ginseng*, elevates protein levels of p53 and $p21^{WAF1}$, which are associated with induction of apoptosis in SK-HEP-1 cells. Flow cytometric analyses showed that G-Rs4 initially arrested the cell cycle at the G1/S boundary, but consequently induced apoptosis as evidence by generating apoptotic peak. The inductive effect on apoptosis was confirmed by results of DNA fragmentation assays and alterations in cell morphology after treatment of the cells with G-Rs4. Immunoblot assays showed that G-Rs4 significantly elevated protein levels of p53 and $p21^{WAF1}$, which were temporally well related to the down-regulation of both cyclins E- and A-dependent kinase activities and induction of apoptosis. Collectively, we suggest that G-Rs4 induces apoptosis, the effect of

which is closely related to the down-regulation of both cyclins E- and A-dependent kinase activity as a consequence of selectively elevating protein levels of p53 and $p21^{WAF1}$ in SK-HEP-1 cells.

Activation of caspase-3 protease via a Bcl-2-insensitive pathway during the process of ginsenoside Rh2-induced apoptosis: Cancer Letters 121, 73 (1997)-- We have shown that ginsenoside Rh2 (G-Rh2), isolated from the root of *Panax ginseng* arrests the cell cycle at the G1/S transition phase by increasing protein levels of $p27^{Kip1}$ and thereby down-regulating cyclin E-dependent kinase activity in human hepatoma SK-HEP-1 cells. Here we demonstrate that G-Rh2 induced apoptosis of SK-HEP-1 cells, as evidenced by changes in cell morphology and DNA fragmentation. To gain insight into the molecular basis of the effect, we established bcl-2 transfectants, SK-Bcl-2/23 and SK-Bcl-2/126, which stably overexpress human Bcl-2 at different levels. We found that G-Rh2 equally induced DNA fragmentation of the bcl-2 transfectants. In contrast, the effect was completely blocked in SK-HEP-1 cells as well as in the transfectants by treatment with a protease inhibitor, such as iodoacetamide (IAA) or N-tosyl-L-phenylalanine chloromethyl ketone (TPCK). The results from immunoblottings revealed that G-Rh2 induced the proteolytic activation of CPP32/Yama/Apopain protease and subsequent cleavages of poly(ADP-ribose) polymerase (PARP) in wild-type cells as well as in bcl-2 transfectants. In summary, one of the biochemical mechanisms of G-Rh2-induced apoptosis in SK-HEP-1 cells may involve proteolytic activation of CPP32/Yama/Apopain protease and subsequent proteolytic cleavage of PARP, which are not prevented by the product of bcl-2.

Caspase-3 specifically cleaves $p21^{Cip/waf1}$ in the earlier stage of apoptosis in SK-HEP-1 human hepatoma cells: Eur. J. Biochem. 257, 242 (1998)-- We report here that $p21^{WAF1/CIP1}$, an inhibitor of cyclin-kinases, underwent proteolytic processing into a smaller fragment, p14, in the early stage of apoptosis in SK-HEP-1 cells. Apoptosis was induced by either staurosporine or ginsenoside Rh2 (G-Rh2), a ginseng saponin with a dammarane skeleton. Proteolytic processing was the result of caspase-3 activity, which accompanied the early changes in cell morphology and DNA fragmentation. $P21^{WAF1/CIP1}$ translated in vitro was cleaved into a p14 fragment when incubated with cell extracts obtained from either G-Rh2- or staurosporine-treated cells. Cleavage was equally inhibited in both cases by adding Ac-DEVD-cho, a specific caspase-3 inhibitor, but not by Ac-YVAD-cho, a specific caspase-1 inhibitor. Similarly, $p21^{WAF1/CIP1}$ was efficiently cleaved by recombinant caspase-3 overexpressed in E. coli. Moreover, the endogenous $p21^{WAF1/CIP1}$ of untreated-cell extracts was also cleaved by recombinant caspase-3 as measured by immunoblot-

ting. Mutation analysis allowed identification of two caspase-3 cleavage sites, DHVD$^{112}$/L and SMTD$^{149}$/F, which are located within, or near the interaction domains for cyclins, Cdks, and PCNA. Taken together, these results show that G-Rh2 as well as staurosporine increase caspase-3 activity, which in turn directly cleaves p21$^{WAF1/CIP1}$ during the early stages of apoptosis. We propose that proteolytic cleavage of p21$^{WAF1/CIP1}$ is a functionally relevant event that allows release of the cyclin/Cdk complex from the p21$^{WAF1/CIP1}$ inhibitor, resulting in the elevated levels of cyclin/Cdk kinase activity seen in the earlier stage of apoptosis.

Caspase 3-mediated cleavage of p21$^{Cip/waf1}$ associated with the cyclin A-dependent kinase 2 complex is a prerequisite for apoptosis in SH-HEP 1 cells: J. Biol. Chem. Vol. 275, no. 39, 30256 (2000)-- Apoptosis of human hepatoma cells SK-HEP-1 induced by treatment with ginsenoside-Rh2 (G-Rh2) is associated with rapid and selective activation of cyclin A-associated cyclin-dependent kinase 2 (Cdk2). Here we show that in apoptotic cells, the Cdk inhibitory protein p21$^{WAF1/CIP1}$, which is associated with the cyclin A/Cdk2 complex, undergoes selective proteolytic cleavage. In contrast, another Cdk inhibitory protein p27$^{KIP1}$, which is associated with cyclin A/Cdk2 and cyclin E/Cdk2 complexes, remained unaltered during apoptosis. Ectopic overexpression of p21$^{WAF1/CIP1}$ suppressed apoptosis as well as cyclin A/Cdk2 activity induced by treatment of SK-HEP-1cells with G-Rh2. The suppressive effects of p21$^{WAF1/CIP1}$ were much higher in the cells transfected with p21D112N, an expression vector that encodes a p21$^{WAF1/CIP1}$ mutant resistant to caspase 3 cleavage. Overexpression of cyclin A in SK-HEP-1 cells dramatically up-regulated cyclin A/Cdk2 activity and accordingly enhances apoptosis induced by treatment with G-Rh2. These up-regulating effects were blocked by coexpression of a dominant negative allele of cdk2. Furthermore, olomoucine, a specific inhibitor of Cdks, also blocked G-Rh2-induced apoptosis. These data suggest that the induction of apoptosis in human hepatoma cells treated with G-Rh2 occurs by a mechanism that involves the activation of cyclin A/Cdk2 by caspase 3-mediated cleavage of p21$^{WAF1/CIP1}$.