Myricetin induces G2/M phase arrest in HepG2 cells by inhibiting the activity of the cyclin B/Cdc2 complex

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Abstract. Myricetin, a naturally occurring flavonol, has been shown to inhibit the proliferation of human hepatoma HepG2 cells and to induce G2/M phase arrest. However, the underlying mechanisms of Myricetin activity have yet to be revealed. The aim of the present study was to clarify the molecular mechanisms of cell cycle arrest induced by myricetin in HepG2 cells. The MTT assay confirmed that exposure of HepG2 cells to myricetin triggered G2/M phase arrest. Western blot analysis showed that myricetin increased the protein levels of the p53/p21 cascade, and markedly decreased Cdc2 and cyclin B1 protein levels in HepG2 cells. Additionally, myricetin treatment resulted in the up-regulation of Thr14/Tyr15 phosphorylated (inactive) Cdc2 and p27, and the down-regulation of CDK7 kinase protein, as well as CDK7-mediated Thr161 phosphorylated (active) Cdc2. These data indicate that a decrease in cyclin B/Cdc2 complex activity mediated G2/M phase arrest induced by myricetin in HepG2 cells. This novel finding provides insight into the potential applications of myricetin in the treatment of hepatocellular carcinoma.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignancy and the third most common cause of cancer-related death worldwide (1,2). Surgical resection is a generally accepted therapeutic modality for the treatment of HCC (3). However, most patients with HCC are diagnosed at a late stage, by which time surgical resection is not a viable option. Chemotherapy is therefore the sole available treatment for these patients. However, the development of drug resistance following treatment is a major obstacle to the successful management of HCC. Therefore, the development of alternative therapeutic agents is required.

Natural products derived from plants are a rich source of novel anti-cancer drugs (4,5). Myricetin is a naturally occurring flavonol that is widely presents in plants, including fruits, vegetables, tea, berries and red wine (6). It has been reported that myricetin has anti-carcinogenic properties (7-9), including the inhibition of cell proliferation and the induction of cell cycle arrest in human heptoma HepG2 cells (10). However, the underlying mechanisms of myricetin activity have yet to be defined. The aim of the present study was to elucidate these underlying mechanisms. To this end, we detected the expression of cell cycle-related proteins. Our data demonstrated that the cell cycle arrest of HepG2 cells induced by myricetin was mediated by the down-regulation of cyclin B/Cdc2 complex activity.

Materials and methods

Cell culture. HepG2, a human HCC cell line, was obtained from the Cell Bank of Shanghai, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Cells were cultured in RPMI-1640 medium (Gibco, Invitrogen Corp., NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sijiqing, Zhejiang, China), 100 U/ml benzyl penicillin and 100 U/ml streptomycin at 37˚C in a humidified 95% air/5% CO_2 incubator. Cells from exponentially growing cultures were used in the experiments. Myricetin was dissolved in DMSO to make a 200-mM stock solution and stored at -20˚C. The working solution was freshly prepared in the cell culture medium with a final DMSO concentration of <0.1%. Control cultures contained the same concentration of DMSO as that used in the experiment cultures.

Colorimetric MTT assay. The MTT assay assesses cell viability by measuring the cellular redox environment. MTT, a yellow tetrazolium salt that is reduced to a blue formazan, was obtained from Sigma, USA. Three thousand cells were seeded onto flat-bottom 96-well plates. After a 24-h incubation, the growth medium was replaced by fresh medium containing 10% fetal calf serum. Following exposure to various concentrations of myricein for 24, 48 and 72 h, MTT (20 μg per well) was added and the mixture was incubated at 37˚C for another 4 h. The culture medium was then discarded and 0.1 ml

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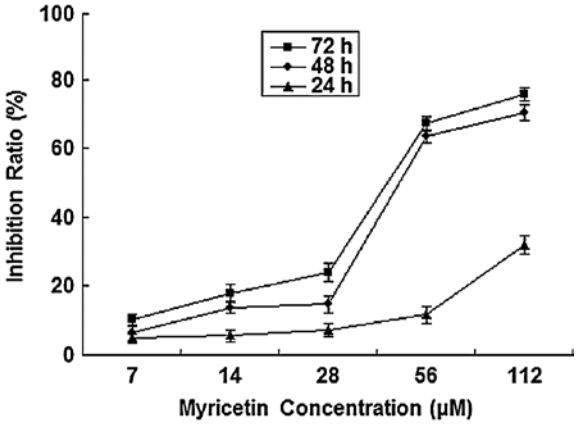

Figure 1. Inhibition of HepG2 cell proliferation by myricetin. HepG2 cells were treated with the indicated concentrations of myricetin for 24 h (▲), 48 h (●) and 72 h (■), and the inhibitory effect was determined by the MTT assay. Data represent the means ± SD of three independent experiments performed five times.

DMSO was used to dissolve the blue crystals. The absorbance was measured at 492 nm using a microplate reader, and absorbance values were expressed as a percentage of the untreated controls. Three independent assays were performed at least five times. The inhibition ratio (*I* %) was calculated using the equation: $I\ \% = (1 - A_{treated}/A_{control}) \times 100\%$. IC_{50} values were calculated according to the Logit method.

Cell cycle analysis by flow cytometry. Flow cytometric analysis was used to assess cell cycle phase distribution. Briefly, 2.5×10^5 HepG2 cells were seeded onto 6-well plates. After 24 h, the cells were treated with various doses of myricetin (0, 33, 66, 132 and 198 μM) for an additional 24 h. Cells were then trypsinized, washed in cold PBS and fixed in 70% cold ethanol overnight at -20˚C. Subsequently, cells were washed with cold PBS and stained with reagents for cell cycle analysis (Beckman Coulter) in the dark for 30 min. DNA content was determined using a FACScan laser flow cytometer (FACSCalibur, Becton Dickinson, USA). Data were analyzed using ModFit and CellQuest software.

Western blot analysis. HepG2 cells treated with the indicated concentrations of myricetin for 24 h were collected and lysed with ice-cold NP-40 lysis buffer (Beyotime Institute of Biotechnology, China) containing the protease inhibitor PMSF (1 nM) on ice. Lysates were then centrifuged at 10,600 x g for 5 min at 4˚C. The supernatant was collected and total protein concentrations were measured using the BCA protein assay (Beyotime Institute of Biotechnology). All lysates were boiled for 5 min before loading. Equal amounts of total protein were separated on 10% SDS-polyacrylamide gels and transferred to a PVDF membrane. After blocking with 5% non-fat dry milk for 1 h at room temperature, the membranes were incubated overnight at 4˚C with primary antibodies to β-actin, cyclin A1, cyclin B1, Cdc2, p-Cdc2 (Thr^{14}/Tyr^{15}), p-Cdc2 (Thr^{161}), CDK7 (Santa Cruz, CA, USA) and to p21, p27 and p53 (Cell Signaling Technology, USA). The following day, the membranes were incubated with the appropriate horseradish peridoxase-conjugated secondary antibodies (Boster Corporation, China). Signals were visualized using enhanced chemiluminescence reagent (Tiangen Biotech, China).

Statistical analysis. Data are expressed as the means ± SD of three independent experiments. Statistical analysis was performed with SPSS 11.0 software (Chicago, IL, USA). Cell cycle distribution was analyzed by the Student's t-test. A p-value of <0.05 was considered significant.

Results

Myricetin inhibits the viability of human HepG2 cells. To evaluate the effect of myricetin on HepG2 cell viability, exponentially growing cells were incubated in the absence or presence of various concentrations of myricetin for 24, 48 and 72 h. The MTT assay was used to assess cell growth at each time point. Myricetin significantly inhibited the viability of HepG2 cells. As shown in Fig. 1, the inhibitory effects of myricetin on HepG2 cells were both time- and concentration-dependent. The IC_{50} was estimated to be 95.80, 47.80 and 36.37 μM at 24, 48 and 72 h, respectively.

Myricetin causes HepG2 cell arrest at the G2/M phase. Cell cycle arrest is commonly targeted as a cancer therapy, since cell cycle regulation is critical in the growth and development of tumors. Synchronized cells were treated with 0, 33, 66, 132 or 198 μM of myricetin in order to determine whether myricetin influences the cell cycle progression of HepG2 cells. Following 24 h of treatment with the various concentrations of myricetin, cell cycle phase arrest was determined by flow cytometry. As shown in Fig. 2A, myricetin induced an accumulation of cells in the G2/M phase of the cell cycle in a concentration-dependent manner, accompanied by a decrease in the G0/G1 phase fraction. As shown in further detail in Fig. 2B, the percentage of HepG2 cells in the G2/M phase after treatment with 66 μM myricetin underwent a statistically significant increase compared to the control ($p<0.05$, $n=3$). These results indicated that the growth inhibition of HepG2 cells by myricetin is associated with the induction of G2/M phase arrest.

Myricetin affects the protein expression of cyclins, Cdc2 and CDK7 in HepG2 cells. After exposure to the indicated concentrations of myricetin for 24 h, the levels of cyclin A1 and B1 were analyzed by immunoblotting. As shown in Fig. 3A, cyclin A1 and B1 proteins were down-regulated in a concentration-dependent manner. However, the changes in the levels of cyclins were not sufficient to explain the G2/M arrest induced by myricetin. Therefore, we subsequently investigated the effects of myricetin on Cdc2 protein expression, and found that Cdc2 was markedly reduced in myricetin-treated HepG2 cells (Fig. 3B). Furthermore, Cdc2 was not detectable in HepG2 cells exposed to a higher concentration (198 μM) of myricetin. Cell cycle progression is reported to be related to Cdc2 kinase activity, which is negatively regulated by the reverse phosphorylation of Thr^{14} and Tyr^{15}, and positively regulated by the CDK7-mediated phosphorylation of Thr^{161}. We next assessed the effect of myricetin on Cdc2 phosphorylation status and CDK7 protein levels. Immunoblot analysis

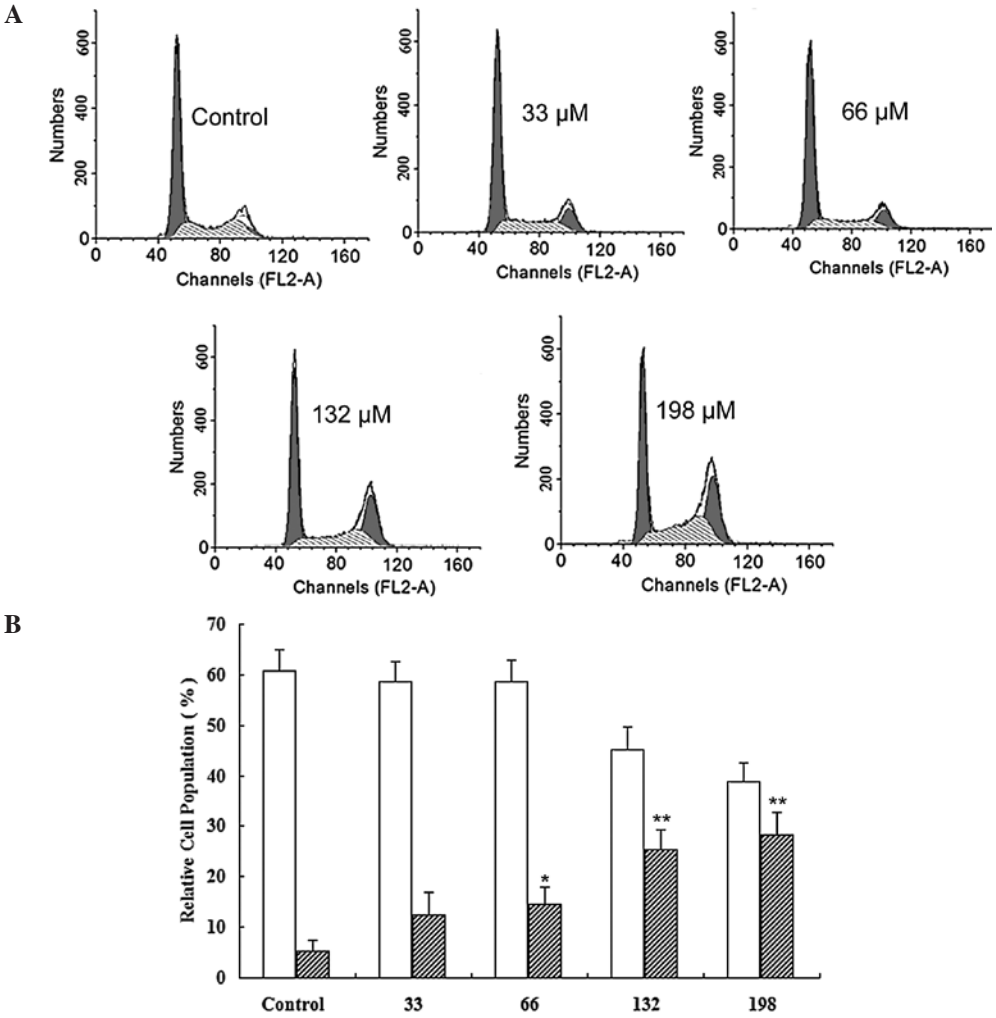

Figure 2. Myricetin induces G2/M arrest in HepG2 cells. Approximately 1x10^5 HepG2 cells were seeded in the wells of 6-well plates. After overnight incubation, the cells were treated with indicated concentration of myricetin for 24 h. (A) A marked concentration-dependent increase of the percentage of cells in the G2/M phase was observed in myricetin-treated cells. (B) Higher doses of myricetin (66-198 μM) caused a statistically significant increase in the percentage of cells in the G2/M phase (Student's t-test, *p<005; **p<001 compared to control). Data represent the means ± SD of three independent observations.

revealed that myricetin treatment resulted in a reduction in CDK7 protein and Thr161 phosphorylated Cdc2, and an increase in Thr^{14}/Tyr^{15} phosphorylated Cdc2 (Fig. 3B).

Myricetin affects the protein expression of CDK inhibitors p27, p21 and p53 in HepG2 cells. To further examine whether myricetin induces CDK inhibitor proteins, we investigated the effect of myricetin on p27 and p21 protein expression in HepG2 cells. The expression of p27 was significantly increased within 24 h of exposure to 33 μM myricetin (Fig. 3A). Myricetin treatment also induced a significant increase in p21 protein. Increases in p21 are reported to be regulated by either a p53-dependent or p53-independent mechanism. To determine whether the growth-inhibitory effect of myricetin was dependent on p53 status, p53 protein levels were detected. It was found that p53 was also up-regulated by myricetin in HepG2 cells.

Discussion

Traditional therapeutic methods for the treatment of cancer often result in serious side effects. Therefore, the use of dietary agents from natural resources is receiving increasing attention as a strategy to combat cancer (11,12). Myricetin, a naturally dietary agent, has been demonstrated to have strong anti-cancer activity (13,14). Recently, it was reported that the inhibition of HepG2 cell growth by myricetin is related to G2/M arrest (10), but the mechanisms of cell cycle arrest were not explored.

Cell cycle dysregulation is a hallmark of tumor cells, thus the regulation of proteins that mediate critical events of the cell cycle may be a useful anti-tumor therapy (15). Eukaryotic cell cycle progression is controlled by the action of cyclin-dependent kinases (CDKs) and their activating subunits, cyclins (16-18). Cyclins and CDK form heterodimeric complexes that phosphorylate downstream targets to drive the cell cycle (19). The protein kinase complex of CDK1, also known as Cdc2 (cell division cycle 2) and cyclin B are essential for the entry of cells into mitosis (20). Cdc2 is inactive as a monomer and must bind with cyclin B during the G2/M transition. Inhibition of Cdc2/p34 in mammalian cells has been shown to result in G2 phase arrest (21). In addition, cyclin A, known mainly for its role in G1/S transition, is also required for the entry of cells into mitosis (22). The present study demonstrated that

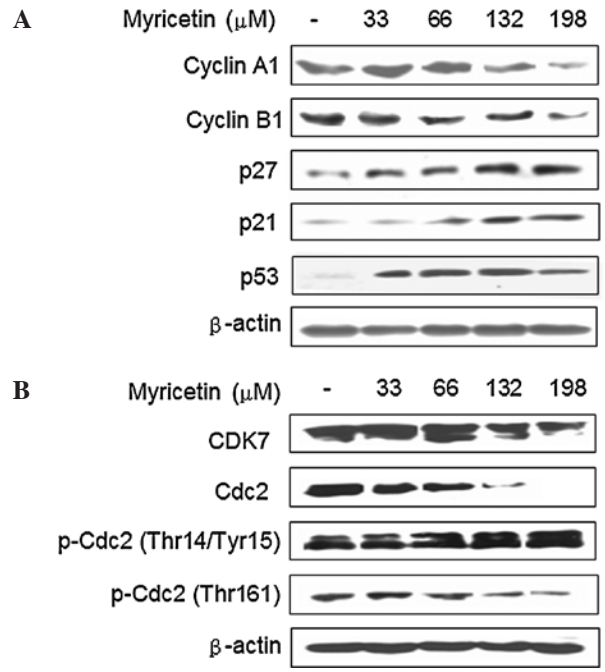

Figure 3. Effects of myricetin treatment for 24 h on CDK7, Cdc2 and phosphorylated Cdc2 proteins in HepG2 cells. Whole cell lysates were prepared and equal loading of protein was subjected to SDS-PAGE, followed by immunoblot analysis and chemiluminescence detection. (A) Expression of cell cycle regulatory proteins in HepG2 cells treated with the indicated concentrations of myricetin for 24 h. (B) Immunoblot analysis of cyclin A1, cyclin B1, p21, p27 and p53 proteins in HepG2 cells after a 24-h treatment with myricetin.

myricetin treatment led to the dose-dependent inhibition of cyclin A1 and B1 as well as of Cdc2 proteins in HepG2 cells (Fig. 3A and B). The down-regulation of these positive regulators of cell cycle progression impairs cyclin B/Cdc2 complex activity and hinders G2/M transition. However, the activity of the cyclin B/Cdc2 complex is also related to Cdc2 kinase phosphorylation status. It is known that the phosphorylation of either Thr^{14} or Tyr^{15} inhibits Cdc2/p34 kinase activity (23,24), while the phosphorylation of Cdc2-Thr^{161} enhances its activity. Thus, the hyperphosphorylation of Cdc2/p34 at Thr^{14}/Tyr^{15} and the dephosphorylation of Cdc2/p34 at Thr^{161} are responsible for G2 arrest (25). Inhibition of CDK7 may prevent mitotic entry, since phosphorylation of Thr^{161}Cdc2 was decreased in both a time- and dose-dependent manner (26). In the present study, Western blotting revealed that myricetin blocked the Tyr^{15} dephosphorylation of the Cdc2/p34 kinase, leading to inactive Cdc2/p34 kinase accumulation. We also found that myricetin resulted in the down-regulation of CDK7 and the dephosphorylation of Thr^{161}Cdc2 in HepG2 cells.

The function of the cyclin/CDK complex is negatively regulated by cell-cycle inhibitors, such as p21 and p27 proteins. It has been demonstrated that p21 contributes to the maintenance of the G2 phase by inhibiting Cdc2 activity. Tumor suppressor protein p53 is known to induce G2/M phase arrest in a p21-dependent manner. Moreover, it has been reported that the up-regulation of the p53/p21 cascade may contribute to the prolongation of G2/M phase arrest (27). In the present study, induction of p53 and p21 was observed in HepG2 cells exposed to myricetin. The CDK inhibitor p27 also has a critical role in the control of mammalian cell proliferation. It has been shown that p27 deficiency results in an increased mitotic index (28), and that p27 decreases Cdc2 protein levels (29). Moreover, p27 binds to the cyclin/CDK complex and inhibits its kinase activity (30). In this study, Western blot analysis showed that myricetin markedly enhanced p27 protein levels. The results indicate that the p53/p21 cascade and p27 proteins are involved in the G2/M phase arrest induced by myricetin.

In conclusion, myricetin-mediated G2/M phase arrest was linked to the inactivation of the cyclin B/Cdc2 complex through the phosphorylation of Thr^{14}/Tyr^{15} Cdc2 accumulation and dephosphorylation of Thr^{161} Cdc2 via the inhibition of CDK7. Moreover, the arrest of cell cycle progression at the G2/M phase by myricetin was related to the inhibition of the cyclin B/Cdc2 complex through the up-regulation of the p53/p21 cascade and of p27.

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