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Induction of G_1/S Phase Arrest and Apoptosis by Quercetin in Human Osteosarcoma Cells

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Quercetin (3,3',4',5,7-pentahydroxyflavone) is a polyphenolic flavonoid compound and is found in a variety of plants. Potential biological activities including antioxidant and anticarcinogenesis have been reported. The antiproliferative effect and apoptosis inducing effect of quercetin in human osteosarcoma cells was evaluated in this study. The IC_{50} values were 290 μM and 160 μM at 24 h and 48 h incubation, respectively. Antiproliferative action of quercetin appeared to be linked to apoptotic cell death based on increase in the sub-G_1 apoptotic cell population analyzed by flow cytometric analysis. Prior to apoptosis induction, quercetin caused cell cycle arrest at G_1/S phase. The G_1/S phase arrest was accompanied by down regulation of cyclin D1, one of the cyclins required for advance from G_1 to S. Subsequent apoptosis was induced by the gradual activation of caspase-3 and the cleavage of PARP.

Key words: Quercetin, G_1/S arrest, Apoptosis, Cyclin D1, Caspase-3, Human osteosarcoma cells

INTRODUCTION

Many plant-derived compounds and their derivatives have been identified useful in the treatment of cancer including paclitaxel (from *Taxus brevifolia* L.), vincristine (*Catharanthus roseus* G. Don), podophyllotoxin (*Podophyllum peltatum* L.), and camptothecin (*Camptotheca acuminata*) (Lilenbaum and Green, 1993; Pezzuto, 1997; Bertrand and Sané, 1999). Naturally occurring cytotoxic compounds have been added to the list, such as neolignans from *Saururus chinensis*, squamocin from the *Annona reticlata* seed, and momordin I from *Amelopsis japonica* – all of which effective against bladder and other types of cancer (Kim et al., 2002; Hahm et al., 2005; Yuan et al., 2006). Recently, it was reported that whole apple extract supressed DMBA-induced mammary tumor in rats (Liu et al., 2009). And the phenolic fraction of Duchesnea induced apoptosis and cell-cycle arrest in cervical cnacer *in vitro* and *in vivo* (Peng et al., 2009). A compound, 4-nerolidylcatechol purified from *Pothomorphe umbellate* induced apoptosis in melanoma cell lines through G1 cell cycle arrest and inhibition of MMP-2 activity (Brohem et al., 2009).

Quercetin (3,3',4',5,7-pentahydroxyflavone), a polyphenolic flavonoid compound, is a common plant polyphenol found in a variety of plants including caper (*Capparis spinosa* L.), lovage (*Levisticum officinale*), and apple (*Malus domestica*) (Yang et al., 2001). There are many natural flavonoids distributed among the plants and they have been consumed as human diet and medicinal herbs for centuries. Flavonoids including quercetin are attracted great interests for their potential biological activities such as antioxidant, antiproliferative, anti-inflammatory or anticarcinogenesis activities (Lamson and Brignall, 2000; Ramos, 2007). However, the effect of quercetin in human osteosarcoma cells and the mechanism of action are not well-studied. Therefore, antiproliferative effect of quercetin and the apoptosis induction mechanism were evaluated and reported in this study.

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MATERIALS AND METHODS

Cell culture sample treatment

Human osteosarcoma cells (HOS, ATCC 1543) were maintained in the logarithmic phase of growth in Dulbecco's modified Eagle's media (Gibco) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics in a humidified incubator with 5% CO_2/95% air at 37°C. Logarithmically growing HOS cells were used for all experiments.

Antiproliferation analysis

Exponentially growing HOS cells were seeded at 5×10^4 cells/well in a 96-well plate and treated with quercetin (Sigma) or vehicle, as indicated. Cytotoxicity of quercetin was estimated by the MTT assay (Hansen et al., 1989). All experiments were performed in triplicate.

Flow cytometry analysis

The effects of quercetin on cell proliferation were evaluated by measuring the distribution of the cells in the different phases of the cell cycle by flow cytometry. Cells were treated with quercetin for 48 h at the indicated concnetrations and harvested by centrifugation at $750\times g$ for 5 min. Cells were prepared as described previously (Piao et al., 2001) and analyzed on a fluorescence-activated cell sorter flow cytometer (FACScaliber, Becton Dickinson). Results shown are an example of 3 different experiments.

Western blot analysis

After quercetin treatment, cells were prepared as as described previously (Piao et al., 2001). Blots were probed with mouse monoclonal antihuman cyclin D1 (Santa Cruz Biotechnology), mouse monoclonal antihuman caspase-3 (Transduction Laboratory), rabbit monoclonal antihuman PARP (Santa Cruz Biotechnology) and mouse monoclonal anti-β-actin (Santa Cruz Biotechnology) antibodies. Immunoreactivity was detected using either an antimouse (Santa Cruz Biotechnology) or antirabbit (Amersham Biosciences) peroxidase-conjugated secondary IgG antibody and an enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences). Experiments are repeated at least three times.

RESULTS

Antiproliferative effect of quercetin on HOS cells

The chemical structure of quercetin (3,3',4',5,7-pentahydroxyflavone) is shown in Fig. 1. The antiproliferative effect of quercetin on a human osteosarcoma cell line, HOS was evaluated with the MTT assay. When cells were treated with 0, 10. 100, 200, 500 and 1000 μM of quercetin for 24 h or 48 h, the relative cell proliferation progressively decreased in a dose-dependent manner, as shown in Fig. 2. When the incubation time was increased to 48 h, the relative cell survival rate was further decreased. The IC_{50} value for quercetin on HOS cells was approximately 290 μM at 24 h incubation and 160 μM at 48 h incubation.

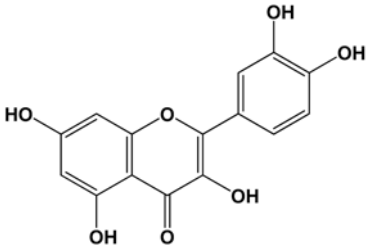

Fig. 1. Structure of quercetin (3,3',4',5,7-pentahydroxyflavone)

Flow-cytometric analysis

We further investigated the effects of quercetin on the progression through the cell cycle and on the induction of apoptotic bodies. HOS cells were cultured for 48 h in the presence or absence of quercetin and analyzed by a flow cytometry. As shown in Fig. 3, quercetin induced a dose-dependent accumulation of HOS cells in the G_1/S phase of the cell cycle. Relative population of cells in G_1/S phase for untreated control was 65.5%. Treatment of quercetin increased the relative population of cells in G_1/S to 66.6%, 71.0%, 71.4%, 70.1% and 73.7% at 10, 100, 200, 500 and 1000 μM of concentration (Fig. 3B). Progression of the cell

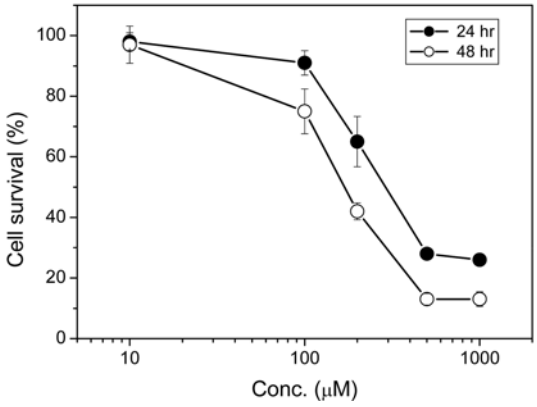

Fig. 2. Antiproliferative activity of quercetin in HOS cells. HOS cells were incubated with 0, 10, 100, 200, 500 and 1000 μM of quercetin for 24 h (●) and 48 h (○). Cell survival rate was measured by the MTT assay. Data are presented as means ± S.D. The percentage of cell growth in the control group was designated as 100%.

cycle was unchanged in cells treated with medium containing DMSO alone (0 μM in Fig. 3). After cell cycle arrest at the G_1/S phase, quercetin-treated cells underwent apoptosis.

Cells with sub-G_1 levels of DNA were scored as apoptotic. At 0 μM treatment about 2.4% of cells were localized in apoptotic/hypodiploid peak (Fig. 3A and C). Of the total number of cells, 9.0% and 24% were contained in the apoptotic region at 10 and 100 μM of quercetin treatment, respectively. At 200, 500 and 1000 μM of quercetin, 31.3%, 41.2% and 51.3% of the cells underwent apoptosis. In addition, the relative ratio of cells in the apoptosis/hypodiploid peak corresponded proportionately to the dose of quercetin used.

Effect of quercetin on cyclin D1

Since G_1/S arrest was observed in quercetin treated HOS cells by flow cytometry analysis, it was of interest to test the effect of quercetin on cyclin D1 which is one of the cyclins required for advance from G_1 to S.

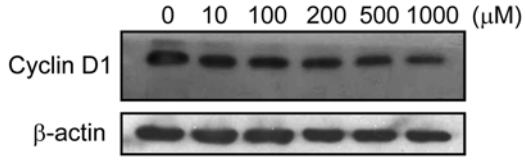

Fig. 4. Changes in the expression of cyclin D1 in quercetin-treated HOS cells. Cells were treated with quercetin for 48 h. Cell extracts were subjected to Western blotting to determine immunoreactivity levels of cyclin D1, as described in Materials and Methods. Representative Western blots are shown.

As shown in Fig. 4, the level of cyclin D1 was gradually decreased upon the treatment of quercetin in the range of the concentration tested in this study.

Effect of quercetin on caspase-3 activation and the cleavage of PARP

In order to investigate the mechanism by which quercetin causes apoptosis, we monitored the protein

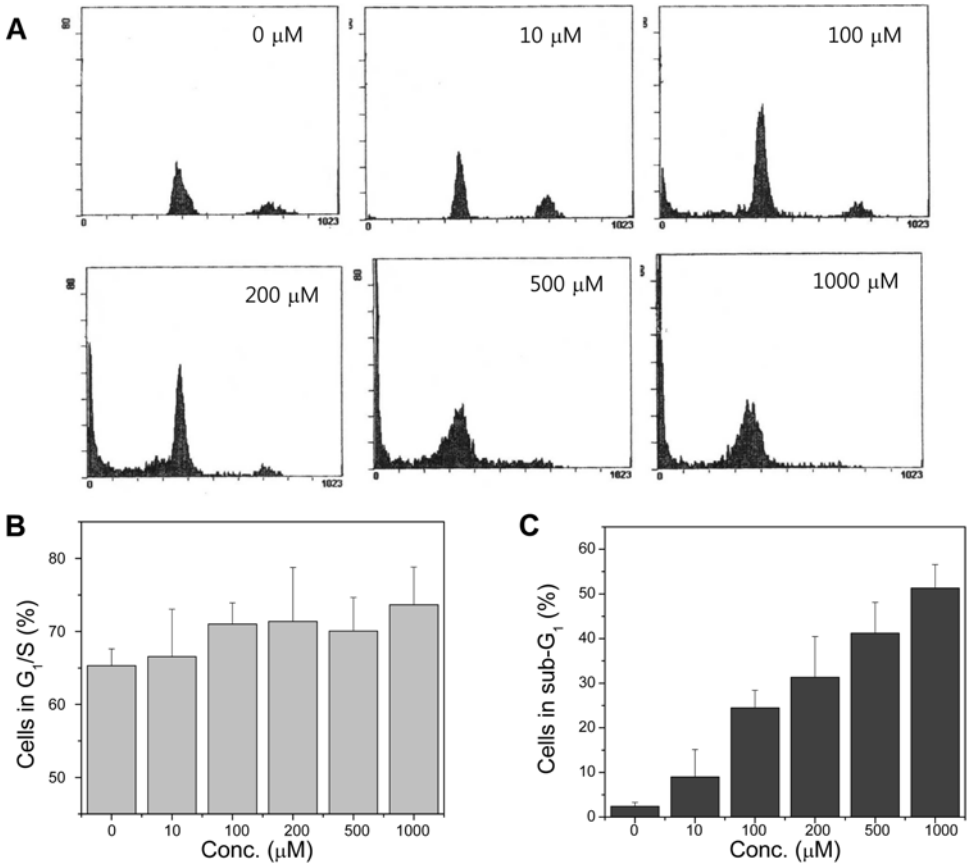

Fig. 3. Flow cytometric analysis of quercetin-treated HOS cells. HOS cells were treated with quercetin at concentrations of 0, 10, 100, 200, 500 and 1000 μM for 48 h. Propidiun Iodide stained cells were analyzed for DNA content using a flow cytometry (**A**). Representative histograms are shown. Relative percentage of cells in G_1 (**B**) and sub-G_1 (**C**) are shown. When cells were treated with quercetin at concentration of 0, 10, 100, 200, 500 and 1000 μM, cells in G_1/S phase were 65.5%, 66.6%, 71.0%, 71.4%, 70.1%, and 73.7%. At the same concentration of quercetin treatment, cells in sub-G_1 were 2.4%, 9.0%, 24%, 31.3%, 41.2%, and 51.3%.

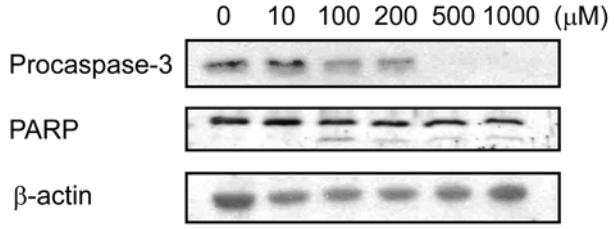

Fig. 5. Changes in the expression of apoptosis-related proteins in quercetin-treated HOS cells. HOS cells were treated with quercetin for 48 h. Cell extracts were subjected to Western blotting to determine immunoreactivity levels of procaspase-3, and PARP as described in Materials and Methods. Representative Western blots are shown.

level changes of apoptosis-related molecules, caspase-3 and PARP. First of all, we tested the effects of this compound on levels of caspase-3, an important execute molecule of apoptotic signaling pathways. We found that the level of procaspase-3 was decreased upon treatment of quercetin which implied that quercetin induced the proteolytic processing of caspase-3 as a dose-dependent manner (Fig. 5). Activation of caspase-3 leads to the cleavage a number of proteins, one of which is poly (ADP-ribose) polymerase (PARP). Although PARP is not essential for cell death, the cleavage of PARP is another hallmark of apoptosis. Quercetin treatment also induced a dose-dependent proteolytic cleavage of PARP, with concomitant appearance of the 85 kDa form and the disappearance of the full-size 116 kDa molecule (Fig. 5). Taken together, these findings suggest that quercetin induced G_1/S cell-cycle arrest and consecutive apoptosis through activation of caspase-3.

DISCUSSION

A significant number of naturally occurring substances have been shown to protect against experimental carcinogenesis. Thus, it is becoming increasingly evident that certain phytochemicals, particularly those included in the daily diet, have important cancer chemopreventive properties (Sanaha et al., 1997). It has been reported that quercetin was a potent inhibitor of proliferation and inducer of apoptosis in human colorectal cancer cells (Xavier et al., 2009), human prostate cancer cells (Aalinkeel et al., 2008; Lee et al., 2008), and human leukemia cells (Lee et al., 2006). Several mechanisms for apoptosis induction by quercetin were suggested. It was reported that quercetin decreased the Bcl:Bax ratio, and increased translocation and multimerization of Bax to the mitochondria membrane, thus caused the activation of caspase families (Lee et al., 2008). Quercetin also arrested leukemia U937 cells at G_2/M phase of the cell cycle and induced caspase-dependent apoptosis (Lee et al., 2006). In addition, it was observed that quercetin accelerated tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced and tumor necrosis factor alpha (TNF-α)-induced apoptosis (Kim et al., 2008; Son et al., 2008).

In this study, we observed that quercetin inhibited the proliferation of human osteosarcoma (HOS) cells and induced apoptosis through activation of caspase-3. Prior to apoptosis induction, treatment of quercetin caused cell cycle arrest; cells were arrested at G_1/S phase. Similar observation was made with human breast cancer cells; apoptosis induction after cell cycle arrest by quercetin (Choi et al., 2008). In other studies, quercetin-induced G_2/M phase block was observed in other human cancer cells including human leukemia cells, human oesophageal adenocarcinoma cells (Lee et al., 2006; Zhang et al., 2008). To further analyze the molecular mechanism by which quercetin cause cell cycle arrest, we evaluated the changes in cell cycle regulatory protein levels. Cyclin D1 is one of the cyclins required for advance from G_1 to S (Tashiro et al., 2007). It seemed that the reduction of cyclin D1 level is one of the main causes for the G_1/S block appeared in quercetin-treated HOS cells. These results demonstrated that quercetin induced transient cell cycle arrest at G_1/S phase in human osteosarcoma (HOS) cells through DNA damage led to decrease in the level of cyclin D1, a cell cycle regulatory molecule. Subsequent quercetin-induced apoptosis was observed through appearance of sub-G_1 population shown in flow cytometry analysis. Caspase-3 was activated by proteolytic cleavage as the decrease in the level of procaspase-3 in dose-dependent manner in quercetin-treated cells. The activity of casepase-3 was maintained at relatively higher doses of quercetin as evidenced by the cleavage of PARP were preserved. In conclusion, quercetin induced apoptotic cell death of human osteosarcoma (HOS) cells by blocking cells transiently at G_1/S phase and activiation the capspase-3pathway.

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