Abstract. Esculetin, a phenolic compound, has been shown to inhibit the growth of colon tumors in animal studies. However, the roles of signaling pathways and cell cycle regulation in the esculetin-induced inhibition of cancer cell growth, remain to be elucidated. The present study suggests a novel mechanism for the Ras/ERK1/2 pathway in esculetin-treated human colon cancer HCT116 cells. The treatment of cells with esculetin resulted in significant growth inhibition and G1 phase cell cycle arrest, which led to the down-regulation of cyclin and cyclin-dependent kinase (CDK) expressions. This G1 phase cell cycle arrest was associated with the up-regulation of p27KIP expression. In addition, ERK1/2 was activated by esculetin. The pre-treatment of cells with the MEK1/2-specific inhibitor, PD98059, blocked the p27KIP expression induced by esculetin. Blockage of the ERK1/2 function consistently prevented the inhibition of cell proliferation and decreased G1 phase cell cycle protein levels. Furthermore, Ras activation was increased by the esculetin treatment. Transient transfection of the dominant negative Ras (RasN17) mutant gene abolished both the ERK1/2 activity and p27KIP expression induced by esculetin. Finally, the overexpression of RasN17 suppressed the esculetin-induced reduction in cell proliferation and cell cycle proteins. In conclusion, these results indicate that the molecular action of esculetin has therapeutic potential for the treatment of colon malignancies.

Introduction
Colon cancer is a major cause of cancer incidence and mortality worldwide, so its pathogenesis and chemoprevention are areas of intense investigation (1,2). Environmental factors, such as dietary habits and lifestyle, are associated with the risk of colon cancer (3). Epidemiological and experimental studies have suggested that genetic defects in adenomatous polyposis coli and p53 (tumor suppressor gene), play a role in the process of colon cancer progression (4,5). Possible mechanisms of the anti-proliferative properties of current chemotherapeutic colon cancer agents include inhibitory effects on DNA synthesis, the induction of apoptosis, modulation of cell cycle control, and the regulation of signal transduction pathways (6-9). However, a number of studies have demonstrated that widely used, naturally-occurring compounds are more effective and safer chemopreventive agents, in comparison to the severe toxicity and side-effects associated with current chemotherapeutic agents for colon cancer (10).

Eukaryotic cell cycle progression involves the regulation of the sequential activation and inactivation of cyclin-dependent kinases (CDKs), which is associated with their cyclin-regulatory subunits (cyclins) at different phases (11). The cyclins positively regulate the activities of CDKs, and CDK inhibitors (CDKIs) negatively control the cyclins and CDKs (12,13). The impairment of cell growth has been shown to induce the down-regulation of cyclin/CDK complexes, such as cyclin D1/CDK4 and cyclin E/CDK2 (13). CDKIs, such as p27KIP, obstruct the kinase activities of the cyclin/CDK complexes, which subsequently block the G1 cell cycle progression (12).

Mitogen-activated protein kinases (MAPKs) have been involved in the regulation of several cellular responses, including cell proliferation and cell growth inhibition (14-16). In mammalian cells, many studies have identified three distinct MAPKs: Extracellular signal-regulated kinase 1/2 (ERK1/2),
c-Jun N-terminal kinase (JNK) and p38 MAPK (14). In general, the ERK1/2 signaling pathway has been implicated in the proliferation of cells by a variety of proliferative factors (17-19). However, other studies have demonstrated that the inhibition of cell growth induced by cell stressors (pro-inflammatory cytokines, UV radiation, heat and osmotic shock) is associated with the up-regulation of ERK1/2 signaling (20-22). In addition, it has been found that the Ras expression regulates the activation of the ERK1/2 pathway (23). The Ras/ERK1/2 signal transduction pathway reportedly acts as a main factor in both cell growth (23,24) and cell death (25,26) in many cell lines.

Esculetin (6,7-dihydroxycoumarin), a plant phenol exhibiting many pharmacological effects, is known to be an inhibitor of lipoxigenase (27). Previous studies have reported the suppression of xanthine oxidase activity, antioxidant activity, platelet aggregation, the inhibition of human leukemia cell growth and anti-atherogenic activity (28-33). Although the inhibition of cell growth by esculetin has been observed in cancer cells (34), the mechanism by which esculetin is involved in the cross-link between the Ras/ERK1/2 signaling pathway and cell cycle regulation is not well understood.

The findings of the present study demonstrate that esculetin suppresses the growth of colon cancer HCT116 cells, and this is mediated via p27KIP-mediated G1 cell cycle arrest, by the activation of the Ras/ERK1/2 signaling pathway.

Materials and methods

Materials. Esculetin was purchased from Sigma (St. Louis, MO). Polyclonal antibodies to cyclin E, CDK2 and 4 were obtained from Santa Cruz (Santa Cruz, CA). Polyclonal antibodies to cyclin D1, p21WAfi1, p53, p27KIP, ERK1/2, phospho-ERK, p38 MAPK, phospho-p38 MAPK, JNK and phospho-JNK were obtained from New England Biolabs. The anti-Ras antibody was obtained from Transduction Laboratories. PD98059 was obtained from Calbiochem (San Diego, CA). The pCMV vector encoding dominant-negative Ras (RasN17) was from Clontech.

Cell cultures. Human colon cancer HCT116 cells were obtained from the American Type Culture Collection. These explants were grown in DMEM containing 10% FBS, 2 mM glutamine, 50 μg/ml gentamicin, and 50 μg/ml amphotericin-B at 37°C in a humidified 5% CO2 atmosphere.

Cell viability assay. Subconfluent, exponentially growing cells were incubated with esculetin in 24-well plates for various lengths of time. Cell viability was determined using a modification of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously (35).

[3H]Thymidine incorporation. Cells were grown to near confluence in 24-well tissue culture plates, treated with esculetin, and then used in the [3H]thymidine-incorporation experiments, as described previously (36).

Cell cycle analysis (FACS). Cells were harvested, fixed in 70% ethanol, and stored at -20°C. Cells were thawed and then washed twice with ice-cold PBS, followed by incubation with RNase and propidium iodide, a DNA-intercalating dye. Cell cycle phase analysis was performed using a Becton-Dickinson FACStar flow cytometer equipped with Becton-Dickinson CellFit software.

Immunoprecipitation, immunoblotting and immune complex kinase assays. Cells were treated with esculetin for various time periods at 37°C in the presence of 10% FBS. Cell lysates were prepared, and immunoprecipitation, immunoblotting and immune complex kinase assays were performed as described previously (36).

Transient transfection. Cells were plated at 3x10^5 cells ml^-1 (2 ml per well) in 6-well culture plates for 24 h, at which time they had reached ~80% confluence. Cells were washed once with PBS and once with serum-free DMEM, and then 0.8 ml of serum-free OPTI-MEM I medium was added to each well. The DNA PLUS-Lipofectamine reagent complex was prepared according to the instructions of the manufacturer (Gibco-BRL). The amount of transfected plasmid added to each well was 2 μg. The DNA PLUS-Lipofectamine reagent complex (0.2 ml per well) was added to each well, followed by incubation at 37°C for 5 h. After transfection, the cells were washed twice with PBS and were then maintained in DMEM containing 10% FBS until they were used in the experiments in which cells were incubated with esculetin for various times.

Affinity precipitation of Ras active form (Ras-GTP). Cells seeded on 100-mm dishes were serum-starved to sub-confluence (5x10^6 cells/cm^2) and treated with 100 μg/ml of esculetin for the indicated times. After washing once with ice-cold PBS, the cells were lysed by adding 500 μl of lysis buffer [25 mM HEPES, 10 mM EDTA, 1% Igepal CA-630, complete protease inhibitor cocktail (from Roche Diagnostics), 1 mM sodium orthovanadate and 10% glycerol]. The lysate was clarified by centrifugation for 15 min at 14,000 g and the protein concentration of the lysate was determined by a BCA assay (Pierce, Rockford, IL). Equal amounts of cell lysates (500 μg) were subjected to affinity precipitation for Ras-GTP with 10 μl of an agarose suspension conjugated with GST fusion protein, which corresponds to the human Ras-binding domain of e-Raf (GST-RBD) (Upstate Biotechnology, Lake Placid, NY). After 1-h incubation at 4°C, the agarose was washed three times with lysis buffer and boiled with 30 μl SDS sample buffer. The product was resolved by 15% SDS-PAGE, followed by immunoblotting with the anti-Ras antibody.

Statistical analysis. When appropriate, data were expressed as the means ± SE. Data were analyzed by factorial ANOVA and Fisher’s least significant difference test where appropriate. Statistical significance was set at P<0.05.

Results

Esculetin inhibits cell proliferation. We investigated the effect of esculetin on the proliferation of human colon cancer HCT116 cells using an MTT assay and [3H]thymidine incor-
poration. Esculetin inhibited the cell viability of HCT116 cells in a dose- (0-300 μg/ml) and time-dependent manner (0-24 h) (Fig. 1A and B). Moreover, as shown in Fig. 1C and D, DNA synthesis was also inhibited in a dose- and time-dependent manner, compared to the control cells. Neither the basal cell viability nor the [3H]thymidine incorporation were altered following the treatment of cells with the vehicle (DMSO) alone (data not shown).

**Esculetin-induced G1 phase cell cycle arrest.** In order to investigate the regulatory mechanism of HCT116 cells induced by esculetin, we examined the cell cycle distribution by flow cytometry. HCT116 cells were cultured with 100 μg/ml esculetin (the IC50 concentration as assessed by MTT assay and [3H]thymidine incorporation) for 24 h. After PI staining, the cell cycle distribution was determined (Fig. 2A-E). As shown in Fig. 2E, after esculetin treatment (100 μg/ml) for 24 h, DNA histogram analysis showed that esculetin induced a dose-dependent increase in the percentage of cells during the G1 phase, with a maximum of 70%. This increase was accompanied by a decrease in the percentage of proliferating cells in the S (18%) and G2/M phases (11%), respectively. This result suggests that esculetin inhibited HCT116 cell growth by inducing cell cycle arrest in the G1 phase.

Based on certain studies showing that cyclin/CDK complexes play important regulatory roles during G1 phase cell cycle progression, the next step was to examine the expression levels of cell cycle regulatory molecules after treatment with esculetin. The levels of cyclin D1 and E, and CDK2 and 4, were decreased 24 h after the treatment with 100 μg/ml esculetin (Fig. 3A). As the kinase activities associated with the CDKs are the essential controlling factors of the cyclin-CDK complexes (13), the CDK2- and 4-associated kinase activities were investigated in the esculetin-treated cells. The esculetin treatment significantly inhibited the activities of the CDK2 and CDK4-immunoprecipitates in the HCT116 cells (Fig. 3B). These results demonstrate that the esculetin-induced cell cycle arrest in the G1 phase is due to the suppression of cyclin D1/CDK4 and cyclin E/CDK2.

**Effect of p27KIP in G1 phase cell cycle arrest is induced by esculetin.** We then assessed the levels of p27KIP in the cells treated with esculetin, which is known to inhibit the entry of cells at the G1-S phase transition checkpoint (12). The results from the immunoblot analysis indicated that HCT116 cells treated with esculetin showed a marked increase in the expression of p27KIP (Fig. 3C). However, there were no changes in the expressions of either p21WAF1 or p53 tumor suppressor proteins under similar experimental conditions (Fig. 3C). In addition, immunoprecipitation analysis showed an increased association of CDK2 with p27KIP in the HCT116 cells treated with esculetin (Fig. 3D). The levels of the p27KIP/CDK4 complexes were also up-regulated in the esculetin-treated cells after 24 h (Fig. 3D). These results suggest that esculetin induces p27KIP induction by inhibiting CDK kinase activity, which leads to G1 phase cell cycle arrest in HCT116 cells.

**Esculetin induces ERK1/2 activation in HCT116 cells.** Stressful stimuli trigger cell growth inhibition via the activation of MAPKs. Therefore, we examined the possibility of ERK1/2, JNK and p38 MAPK activation in esculetin-treated cells. The results showed that esculetin activated ERK1/2 in the HCT116 cells (Fig. 4A). As shown in Fig. 4A, the treatment of cells with 100 μg/ml esculetin induced ERK1/2 activity to maximum effect after 12 h. However, JNK and p38 MAPK activation was not observed in the esculetin-treated cells (Fig. 4A). The increased ERK1/2
activation was suppressed by the treatment of cells with PD98059 (selective MEK inhibitor) in the esculetin-treated cells (Fig. 4B). These results suggest the possible role of ERK1/2 activation in the esculetin-induced inhibition of cell growth.

PD98059, a specific inhibitor of MEK1/2, reverses the induction of p27KIP by esculetin. In order to investigate the physiological role of ERK1/2 in cell cycle regulation induced by esculetin, we examined the effect of a specific inhibitor of MEK1/2 (an upstream component of ERK1/2), PD98059. As
shown in Fig. 5A, the treatment of cells with PD98059 inhibited the esculetin-induced p27KIP expression. PD98059 alone did not alter the basal p27KIP expression (Fig. 5A). In addition, both CDK2 and CDK4 protein levels were reversed to basal levels after PD98059 treatment for 24 h (Fig. 5B). These results strongly suggest a relationship between the ERK1/2 signaling pathway and p27KIP expression, leading to G1 phase cell cycle arrest induced by esculetin.

Expression of dominant-negative Ras reverses the effects of esculetin on p27KIP expression, CDK levels and cell growth inhibition. The small GTP-binding protein has a pivotal role in the activation of ERK1/2 (23). We examined the activation of Ras, which is a small GTP-binding protein in esculetin-treated cells. As shown in Fig. 6A, the treatment with esculetin induced the activation of Ras in HCT116 cells, whereas the level of Ras protein did not affect the esculetin-treated cells. The involvement of Ras in the esculetin-induced ERK1/2 activation in HCT116 cells was investigated next, using a dominant-negative Ras plasmid (RasN17). HCT116 cells were transfected with a RasN17 or an empty vector (EV), followed by esculetin treatment. As shown in Fig. 6B, the treatment with esculetin induced ERK1/2 activation. The expression of RasN17 blocked the esculetin-induced ERK1/2 activation, whereas the EV transfectants did not inhibit the ERK1/2 activation induced by esculetin (Fig. 6B).

Finally, we investigated the role of Ras in the esculetin-induced p27KIP expression, CDK levels, and cell growth inhibition. As shown in Fig. 6C, D and E, the esculetin treatment resulted in increased p27KIP expression, reduced cell viability and [3H]thymidine incorporation, as well as a
diminished CDK2 and CDK4 expression. The cells transfected with RasN17 suppressed the esculetin-induced p27KIP expression (Fig. 6C). In addition, the RasN17 transfectants showed reverse effects on the esculetin-induced decrease in CDK expression, cell viability, and [3H]thymidine incorporation (Fig. 6C, D and E). There were no significant changes in p27KIP expression, cell viability, [3H]thymidine incorporation, CDK2 or CDK4 expression induced by esculetin when the cells were transfected with an EV (Fig. 6C, D and E). These results indicate that esculetin activates the Ras/ERK1/2 signaling pathway, thus producing p27KIP-mediated G1 phase cell cycle arrest, which leads to the inhibition of cell growth.

**Discussion**

Esculetin, a naturally-occurring coumarin compound, has been shown to have many pharmacological effects associated with cell proliferation (28-33). It is known to inhibit cancer cell proliferation in both in vitro and in vivo models (34,37). However, little is known about the activation and the possible role of the Ras/ERK1/2 signaling pathway that underlies cell cycle regulation in the esculetin-induced inhibition of cancer cell proliferation. The present study demonstrates that the Ras/ERK1/2 signaling pathway is involved in the esculetin-induced cell growth inhibition which is mediated by G1 phase cell cycle arrest via the regulation of p27KIP.

Previous studies have shown the inhibitory effect of esculetin on cell proliferation in many cell lines (31-34,37). The present study also shows that esculetin inhibits the growth of colon cancer HCT116 cells in a time- and dose-dependent manner, as is evident by cell viability and DNA synthesis. Moreover, esculetin induced cell cycle arrest in the G1 phase through the down-regulation of cyclin D1/CDK4 and cyclin E/CDK2, which are regulating factors governing the G1-S phase cell cycle progression. Furthermore, the esculetin treatment induced an increased expression of p27KIP, and thus suppressed CDK/cyclin complexes, which led to G1 phase cell cycle arrest. However, the expression of p21WAF1 and p53 did not affect the esculetin-treated cells, which suggests that G1 phase cell cycle arrest is dependent on the p27KIP pathway. The present study is the first report showing the role of the p27KIP and cyclin/CDK in the cell cycle arrest induced by esculetin in cancer cell lines, although a previous study has suggested that the p27KIP induction is associated with the inhibition of cell growth by esculetin in human leukemia HL-60 cells (31).

The activation of ERK1/2 is known to have a central role in cell proliferation (17-19). Other studies have also demonstrated the involvement of ERK activation in the esculetin-induced inhibition of cell growth in human leukemia cells and VSMCs (32,38,39). Another study has indicated that esculetin induced apoptosis, through the activation of JNK in human leukemia cells (39). In this study, we show that esculetin activates ERK1/2 during the inhibition of cancer cell growth. However, this activation was not observed for p38 MAPK and JNK activation. Moreover, the esculetin-induced p27KIP was inhibited by the MEK inhibitor, PD98059. The present results suggest a link between ERK1/2 signaling and p27KIP expression in HCT116 cells induced by esculetin. In addition, the inhibition of ERK1/2 with PD98059 reversed the esculetin-induced decrease in cell growth.

**Figure 6.** Effect of a dominant-negative RasN17 (RasN17) mutant gene on esculetin-treated HCT116 cells. (A) Time course for esculetin-induced Ras activation. Cells were stimulated by esculetin for the indicated times, then cell lysates were extracted. The Ras active form (Ras-GTP) from the cellular content was analyzed by immunoblotting as described in Materials and methods. (B and C) Cells were transiently transfected with the RasN17 plasmid treated with esculetin for 12 h and then cell lysates were extracted. The expression of ERK1/2, p27KIP, CDK2 and CDK4 was determined by immunoblotting as described in Materials and methods. (D and E) The MTT assay and [3H]thymidine uptake experiments were assessed as described in Materials and methods. Results are the means ± SE of three experiments. **P<0.05 compared with esculetin treatment of HCT116 cells.
proliferation and the down-regulation of CDK2 and CDK4, which are essential factors in cell cycle regulatory proteins. These data provide evidence that esculetin induces the inhibition of cancer cell growth through the ERK1/2 signaling pathway.

It is well established that the ERK1/2 pathway requires the sequential activation of the small G protein, Ras, in growth factor-stimulated cells (23,24). The present study provides the first evidence of the esculetin-induced activation of Ras. Whether or not Ras, upstream from the ERK1/2 signaling pathway, is required for the esculetin-induced inhibition of cell growth, has not yet been elucidated. In order to determine whether the activation of Ras is required for the esculetin-induced inhibition of cell growth, HCT116 cells were transfected with a dominant-negative mutant of the RasN17 gene. The esculetin-induced ERK activation was suppressed by the transfection with the RasN17 gene in HCT116 cells, suggesting that Ras plays a key role in the esculetin-induced activation of the ERK signaling cascade in HCT116 cells. In addition, the esculetin-induced up-regulation of the p27KIP level was abolished in HCT116 cells transfected with RasN17. Furthermore, RasN17 transfectants reversed the inhibition of cell growth and the suppression of CDK2 and CDK4 expression which was induced by esculetin. These novel data suggest that the Ras/ERK1/2 signaling pathway could be required for the esculetin-induced inhibition of human colon cancer HCT116 cells.

The inhibition of cell proliferation is believed to be associated with the suppression of malignant cells. Many studies have shown that the Ras/ERK1/2 signaling cascade is required for the proliferative responses to diverse cellular stimulation in cancer cells (23,24). However, the present study suggests an essential role of the Ras/ERK1/2 signaling pathway in the inhibition of cell growth via cell cycle regulation in human colon cancer HCT116 cells. It is possible that the Ras/ERK1/2 signaling pathway, the best-known signaling cascade, is related to the esculetin-induced inhibition of cancer cell growth.

In conclusion, esculetin decreases cell proliferation by inducing G1 phase cell cycle arrest, which is associated with the down-regulation of cyclin D1/CDK4 and cyclin E/CDK2 complexes by the activation of p27KIP. Finally, the Ras/ERK1/2 signaling pathway is linked to the p27KIP induction in co-ordination with G1 phase cell cycle arrest, thus inhibiting cancer cell growth. These results suggest that esculetin could be a therapeutic molecule for the treatment of human colon cancer.

Acknowledgements

This study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (grant no. 2010-0001736).

References