Deguelin inhibits human hepatocellular carcinoma by antiangiogenesis and apoptosis

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Received February 14, 2008; Accepted March 26, 2008

Abstract. Deguelin is a rotenoid isolated from several plant species, which has been reported to have chemopreventive effects in skin, mammary, colon and lung cancers. The effects of deguelin on the proliferation and apoptosis of hepatic cancer cells were assessed by MTT assay and flow cytometric analysis. The growth of hepatic cancer cells (HepG2, Huh7 and SK-Hep1) was inhibited by deguelin in a dose-dependent manner. HepG2 cells of all the cell lines were the most sensitive to deguelin (IC_{50} = 0.62 μM). The proportion of sub-G1 apoptotic cells increased from 5.19 to 41.27% by deguelin (0.01-10 μM) treatment for 3 days in the HepG2 cells. The effects of deguelin on anti-angiogenesis of the HepG2 cells were assessed by using Western blot and RT-PCR analysis. Treatment of HepG2 cells with deguelin for 16 h under hypoxia conditions reduced the expression of the hypoxia-inducible factor 1α protein and vascular endothelial growth factor mRNA in a dose-dependent manner. In order to investigate whether deguelin shows antiangiogenic activities, we performed *in vitro* and *in vivo* angiogenesis assays. In a tube formation assay, deguelin remarkably reduced the capillary network formation of human umbilical vein endothelial cells (HUVECs) on Matrigel beds. Furthermore, deguelin markedly decreased the migration of HUVECs compared to the control and reduced angiogenesis on the CAM of chick embryos. These results suggest that deguelin is potentially useful as a chemotherapeutic agent in hepatocellular carcinoma.

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Key words: deguelin, hepatocellular carcinoma, HepG2, apoptosis, angiogenesis

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related death worldwide (1). HCC may be one of the most common fatal cancers, especially in east Asian countries (2,3). HCC has characteristics of rapid growth rate, strong malignancy, easy invasion, metastasis and poor prognosis and the occurrence of hepatoma presented in an ascending trend. However, the curative effect of current therapies in liver cancer is not perfect (4). Therefore, the development of chemotherapeutic or chemopreventive agents for HCC is very important in reducing the mortality caused by this disease.

Deguelin is a rotenoid of the flavonoid family isolated from several plant species including *Mundulea sericea* (*Leguminosae*) (5) and a naturally occurring insecticide. It has previously been reported as an inhibitor of activated Akt with chemopreventive properties (5,6). Deguelin has been shown to be a potential chemopreventive agent against breast, skin and colon cancers (6,7). Deguelin has also been reported to effectively prevent tobacco carcinogen-induced lung carcinogenesis by blocking Akt activation (5,6) and has shown antiproliferative and apoptotic activities in certain non-small cell lung cancer (NSCLC) cell lines *in vitro* (8,9). Furthermore, deguelin has recently shown potential to be an angioprevention and antiangiogenic therapeutic agent in human umbilical vein endothelial cells (HUVECs) and cancer targeting hypoxia-inducible factor 1α (HIF-1α) (6,10,11).

Although, deguelin has shown potential chemopreventive activities against several types of cancers, the effects of deguelin in hepatic cancer cells have not been defined. In the present study, we examined the effects of deguelin on the proliferation in HCC cells and then investigated the apoptosis and angiogenesis for the mechanism. As a result, we found that deguelin inhibits the progression of HCC by the anti-angiogenesis and apoptosis pathway.

Materials and methods

Cells and materials. The human HCC cell lines HepG2, Huh7 and SK-Hep1 were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). These cell lines were cultured in

Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. FBS, cell culture media, antibiotics and all other reagents used in cell culture studies were purchased from Seoulin Science Co. (Seoul, Korea). Cultures were maintained at 37˚C in a CO_2 incubator with a controlled humidified atmosphere composed of 95% air and 5% CO_2.

For hypoxic stimulation, dishes were placed in an incubator with 1% O_2, 5% CO_2 and 94% N_2 in a humidified atmosphere for the times indicated. HUVECs were grown in a gelatin-coated 75-cm^2 flask in an M199 medium containing 3 ng/ml basic fibroblast growth factor (bFGF), 5 U/ml heparin and 20% FBS at 37˚C in a humidified atmosphere of 5% CO_2/95% air. The cells used in this study were from passages 5 to 6.

Deguelin was manufactured from the natural product rotenone (Sigma-Aldrich, Milwaukee, WI, USA) via four steps and was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 20 mM and was stored at -20˚C.

Measurement of cell proliferation. To measure the effects of deguelin on cell proliferation, HepG2, Huh7 and SK-Hep1 cells were plated at a concentration of $1x10^3$ to $4x10^3$ cells/well in 96-well plates. After incubation for one day, cells were treated with either DMSO as a control or various concentrations of deguelin. The final concentration of DMSO in the medium was ≤0.5% (v/v); at this concentration, DMSO had no effect on cell growth. After the cells were incubated for 3 days, cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (12). Three replicate wells were used for each analysis. The drug concentration required to inhibit cell growth by 50% (IC_{50}) was determined by interpolation from dose-response curves.

Cell cycle analysis. HepG2 cells were plated in 100-mm culture dishes. The next day, cells were treated with various concentrations of deguelin or DMSO (0.5%) for 3 days. Floating and adherent cells were harvested and fixed with 70% ethanol overnight at 4˚C. After washing, the cells were subsequently stained with 50 μg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO, USA) and 100 μg/ml RNase A (Qiagen) for 1 h and subjected to flow cytometric analysis in order to determine the percentage of cells at specific phases of the cell cycle as described (13). Flow cytometric analysis was performed using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 488-nm argon laser. Events (~20,000) were evaluated for each sample and the cell cycle distribution was calculated by using CellQuest (Becton Dickinson) software. The results were presented as the number of cells versus the amount of DNA as indicated by the intensity of the fluorescence signal. Three independent experiments were performed.

RT-PCR analysis. To assess the effects of deguelin on mRNA expression by reverse-transcriptase polymerase chain reaction (RT-PCR) analyses, HepG2 cells were treated with various concentrations of deguelin in a complete medium for 16 h under hypoxic conditions.

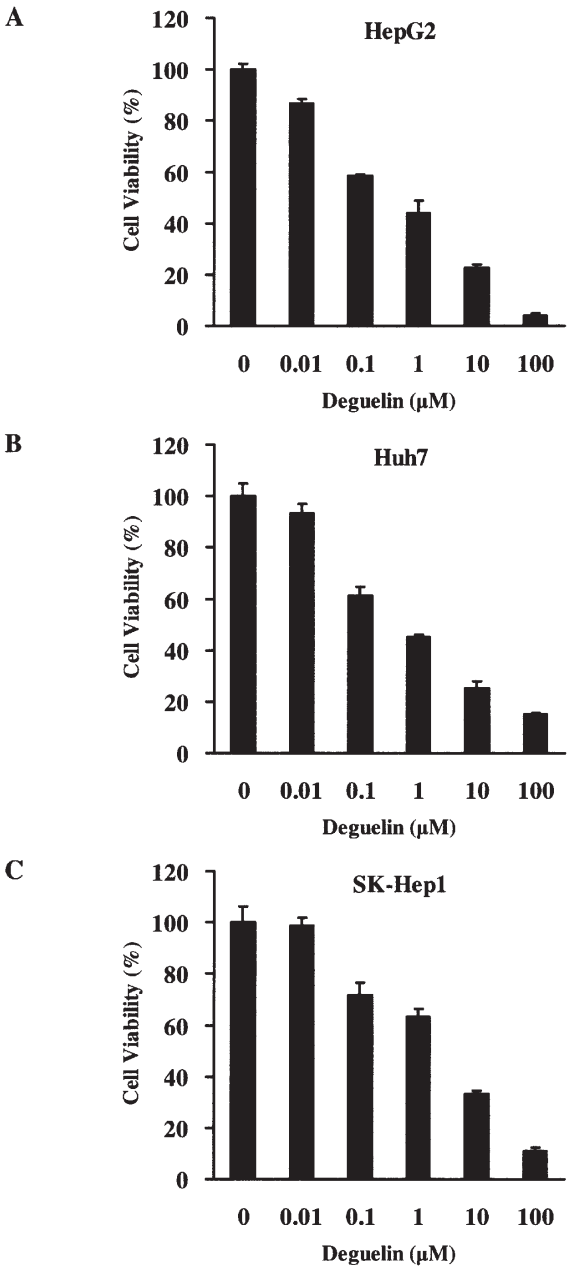
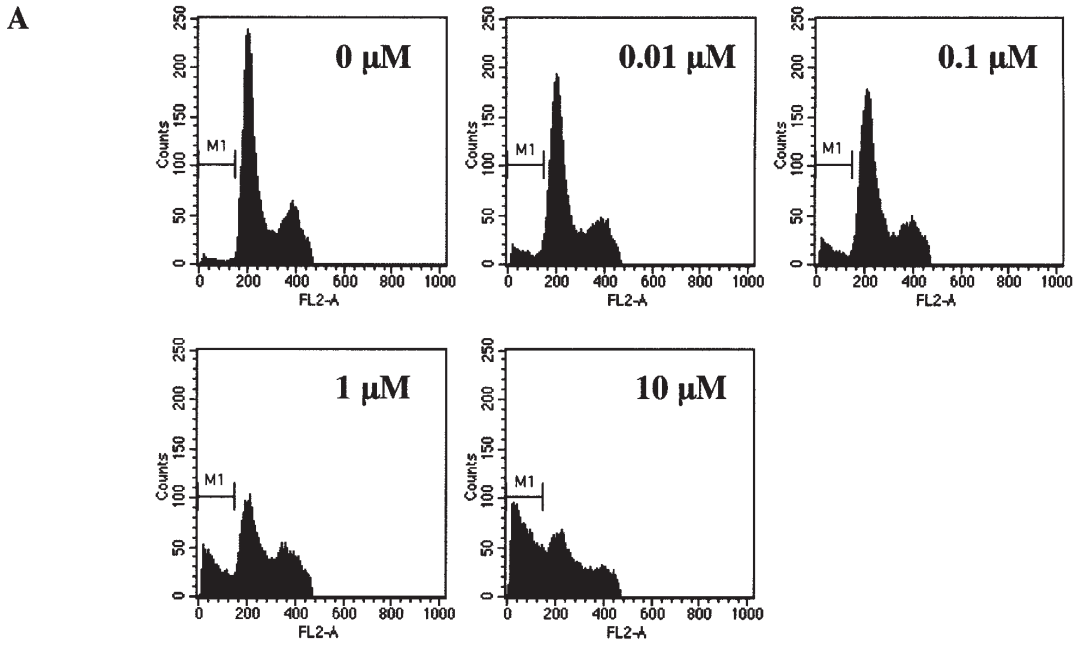


Figure 1. Effects of deguelin on the proliferation of human hepatocellular carcinoma (HCC) cells. HepG2 (A), Huh7 (B) and SK-Hep1 (C) cells were seeded in 96-well culture plates ($1x10^3$ to $4x10^3$ cells/well). After incubation for one day, the cells were treated with various concentrations of deguelin or with 0.5% dimethyl sulfoxide (DMSO) as a control. After incubation for 3 more days, they were subjected to MTT assay. Results are expressed as percent cell proliferation relative to the proliferation of DMSO-treated cells (control). Each value is the mean (± SD) from three identical wells.

Total RNA from the cells was isolated using Trizol reagent (Life Technologies) according to the manufacturer's instructions. First-stranded cDNA was synthesized by Moloney murine leukemia virus reverse transcriptase with 5 μg of each DNA-free total RNA sample and oligo(dT)[15] (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Equal amounts of cDNA were subsequently amplified by PCR in a 50 μl reaction volume containing 1X PCR buffer, 200 μM dNTPs, 10 μM specific primer for each gene and 1.25 units Taq DNA polymerase



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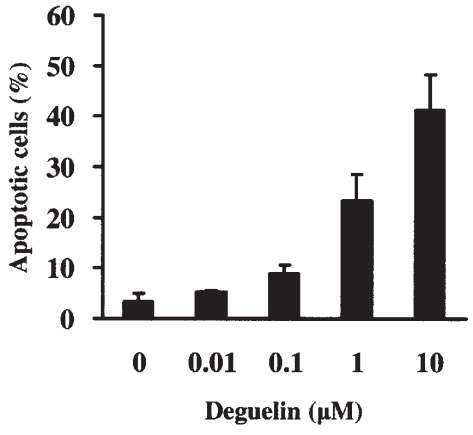

	SubG1	G0/G1	S	G2/M
0	3.40	66.26	11.27	19.51
0.01	5.19	65.01	11.82	18.44
0.1	9.07	65.07	8.90	17.34
1	23.40	42.76	15.62	18.91
10	41.27	34.13	13.90	11.06

Figure 2. Effect of deguelin on cell cycle distribution of HepG2 cells. (A) HepG2 cells were incubated with deguelin for 3 days, stained with propidium iodide (PI) and analyzed on a FACScalibur flow cytometer. M1, sub-G1. (B) The sub-G1 materials after 3 days of incubation were quantified and plotted against the concentrations of deguelin.

(Perkin-Elmer). GAPDH was used as an internal control cDNA amplification. Amplification products were separated on agarose gels and visualized by ethidium bromide staining under UV transillumination. The primer sequences were as follows: (sense) 5'-GGCCTCCGAAACCATGAACTTT CTG-3' and (antisense) 5'-CCTCCTGCCCGGCTCACCGC-3' for VEGF; (sense) 5'-ACCACAGTCCATGCCATCAC-3' and (antisense) 5'-TCCACCACCCTGTTGCTGTA-3' for GAPDH.

Western blot assay. To assess the effects of deguelin on protein expression by Western blot, HepG2 cells were treated with various concentrations of deguelin in a complete medium for 16 h under hypoxic conditions. Cells were lysed in a lysis buffer containing 40 mM Tris-HCl (pH 7.4), 10 mM EDTA, 0.1% NP-40 and 120 mM NaCl. Protein concentration was determined with the use of a BCA protein assay kit following the manufacturer's instructions (Sigma Chemical Co). Whole cell lysates were resolved in SDS-polyacrylamide gels followed by electrophoretically transferring them onto nitrocellulose membrane (Hybond™ ECL) and probed with anti-HIF-1α (BD Biosciences, San Diego) and anti-α-tubulin (InnoGenex) antibodies. The bands were visualized with the ECL plus system (ECL).

Tube formation assay. Matrigel (250 μl) (10 mg/ml) (BD Biosciences, New Jersey) was polymerized for 30 min at 37˚C. HUVECs ($5x10^4$ cells/48-well) were seeded on Matrigel and treated with deguelin (1 μM) for 8 h. The morphological changes of the cells and tubes formed were observed under a phase-contrast microscope and photographed at x200 magnification.

Wounding migration assay. HUVECs, plated on 60-mm culture dishes at 90% confluence, were wounded with a razor blade 2 mm in width and marked at the injury line. After wounding, the cultures were washed with a serum-free medium and further incubated in DMEM with 1% serum, 1 mM thymidine

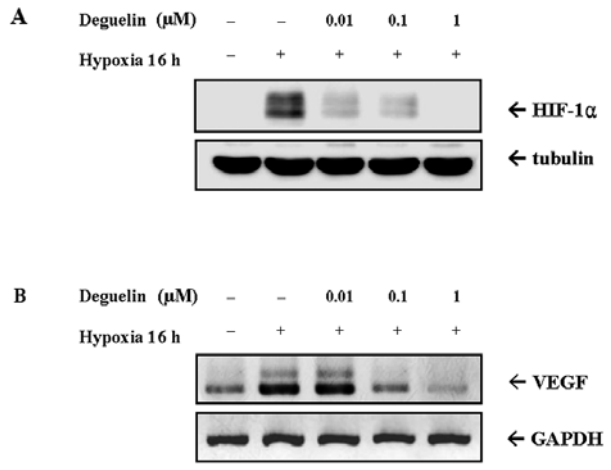

Figure 3. Deguelin inhibits HIF-1α and VEGF expression. (A) Western blot analysis on HIF-1α in HepG2 cells untreated (–) or treated (+) with deguelin. (B) RT-PCR analysis on VEGF mRNA expression in HepG2 cells untreated (–) or treated (+) with deguelin.

and/or deguelin (0.1-1 μM). HUVECs were allowed to migrate for 11 h and were rinsed with a serum-free medium, followed by fixing with absolute methanol and staining with Giemsa. Migration was quantitated with counting the number of cells that moved beyond the reference line.

Chick chorioallantoic membrane (CAM) assay. Fertilized chick eggs were incubated under conditions of a constant humidified egg breeder at 37˚C. On the third day of incubation, ~3 ml of egg albumin were aspirated by an 18-gauge hypodermic needle in order to detach the developing CAM from the shell. At 4.5 days, sample-loaded thermanox coverslips (Nunc, Naperville, IL) were air-dried and applied to the CAM surface for the testing of angiogenesis inhibition by deguelin (4 μg/egg). Two days later, 1-2 ml of 10% fat emulsion (Intralipose) was injected into the chorioallantois and observed under a microscope.

Results

Inhibition of cell proliferation. To evaluate the inhibitory effect of deguelin on the growth of HepG2, Huh7 and SK-Hep1

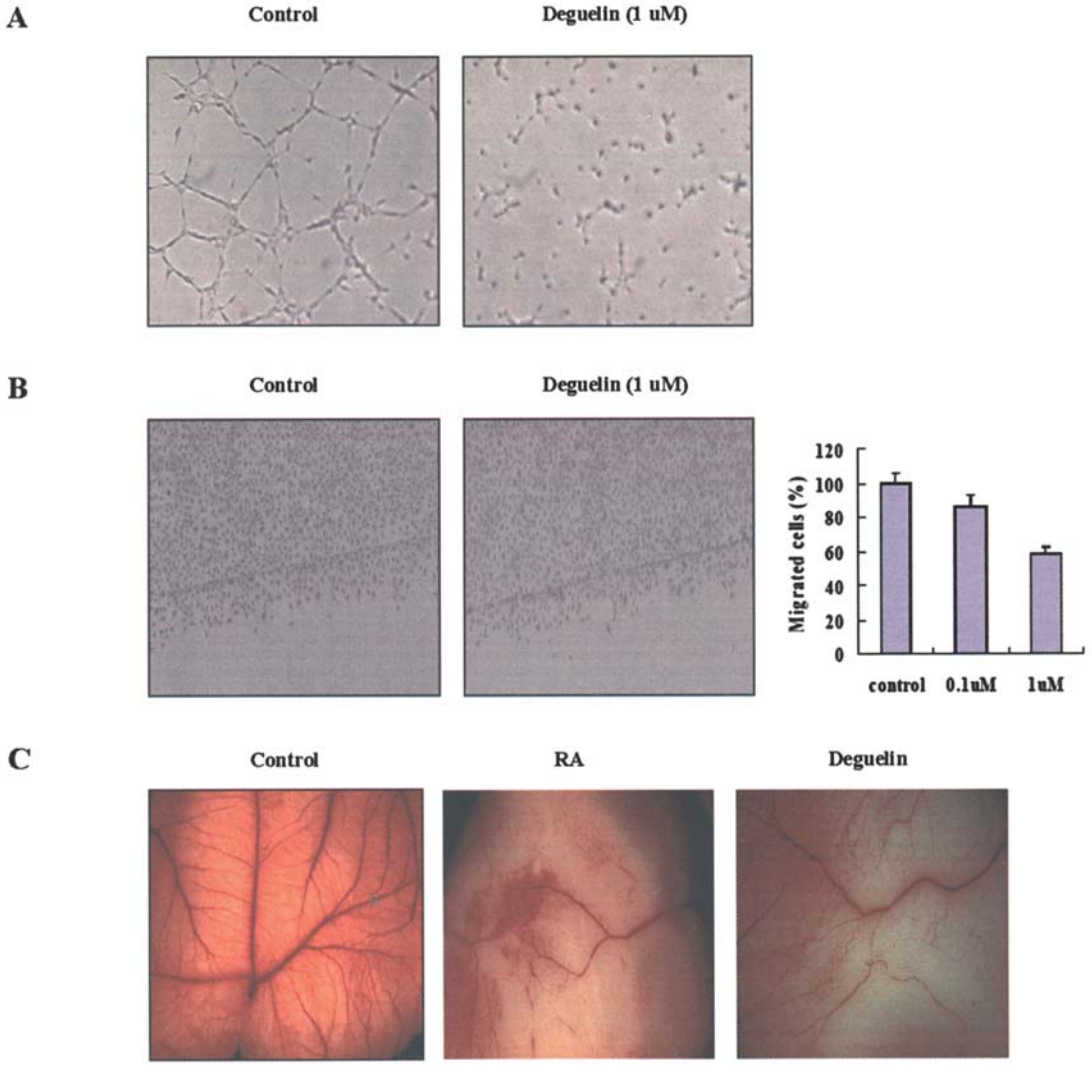

Figure 4. The effect of deguelin on the tube formation, migration *in vitro* and embryo vasculogenesis. (A) Representative images depicting the formation of capillary-like tube structures by HUVECs following 8 h of treatment with DMSO (control) or the indicated concentration of deguelin (1 μM). (B) Migration of deguelin-treated HUVECs compared with the control. (C) CAM surfaces were treated with vehicle (control), retinoic acid (RA) as a positive control, or deguelin (4 μg/egg), respectively.

cells, the culture was exposed to various concentrations of deguelin for up to 3 days. Deguelin was evaluated at five concentrations from 0.01 to 100 μM. It was found that cell growth was inhibited in a dose-dependent manner (Fig. 1). The IC_{50} of deguelin for 3 days of treatment were 0.62, 0.74 and 4.99 μM in HepG2, Huh7 and SK-Hep1, respectively. HepG2 cells of all the cell lines were the most sensitive to deguelin.

Deguelin induces apoptosis. Alterations of the cell cycle of HepG2 cells exposed to deguelin were investigated by flow cytometry. Cells incubated with different concentrations of deguelin for 3 days were analyzed for the distribution of sub-G1, G0/G1, S and G2/M phases of the cell cycle.

Apoptosis can result in the progressive generation of particles corresponding to hypodiploid DNA content, which reflects DNA fragmentation (14-16). Therefore, the amount of apoptotic cells was calculated based on the appearance of cells in 'sub-G1' (17). Fig. 2A shows untreated HepG2 cells (control) at a normal cell cycle stage, consisting of a major diploid peak (G0/G1), a small hyperdiploid region (S) and a minor tetraploid peak (G2/M). However, deguelin pre-treatment (0.01-10 μM) led to the increase of the percentage of sub-G1, reflecting that cells had undergone apoptosis-associated DNA degradation, where sub-G1 apoptotic cells increased from 5.19 to 41.27% (Fig. 2B).

Effect of deguelin on the HIF-1α and VEGF expression in HCC. Hypoxia is the major stimulus for angiogenesis and HIF-1α is its key mediator (18). Since HIF-1 plays a crucial role in tumor progression by regulating the expression of key apoptotic and angiogenic factors, including VEGF (11), we examined whether deguelin influences the expression pattern of HIF-1α and VEGF. Treatment of HepG2 cells with 0.01-1 μM deguelin for 16 h under hypoxic conditions reduced the expression of HIF-1α protein (Fig. 3A) and VEGF mRNA (Fig. 3B) in a dose-dependent manner.

Deguelin inhibits angiogenesis in vitro and in vivo. To investigate whether deguelin shows antiangiogenic activities, we performed *in vitro* and *in vivo* angiogenesis assays. HUVECs were plated onto Matrigel where they normally aligned and formed a tube-like structure (19). However, in the tube formation assay, deguelin remarkably reduced the capillary network formation of HUVECs on Matrigel beds. The tube structures were not elongated and the cells were made a rump and did not develop into a tube network (Fig. 4A). Furthermore, deguelin markedly decreased the migration of HUVECs compared to the control (Fig. 4B). Moreover, we confirmed the antiangiogenic effect of deguelin *in vivo* with a chick embryo CAM assay. The chick embryos treated with retinoic acid (RA) as a positive control for the angiogenesis inhibitor showed a reduced vessel formation. Deguelin remarkably reduced angiogenesis on the CAM of chick embryos (Fig. 4C). These results strongly suggest that deguelin potently represses angiogenesis by inhibiting migration and tube formation of endothelial cells.

Discussion

Many cancer chemopreventive agents, including naturally occurring and synthetic compounds, have been studied for their *in vivo* and *in vitro* antitumor efficacy (7). Most drugs currently available for the treatment of cancer are mechanistically based on the inhibition of cell proliferation and induction of apoptosis (20). Deguelin is a rotenoid of the flavonoid family isolated from several plant species and has shown potential as a chemopreventive agent against breast, skin, colon and lung cancers (6,7). In our studies, we examined effects of deguelin on the proliferation of various HCC cells. The growth of HepG2, Huh7 and SK-Hep1 cells were inhibited in a dose-dependent manner (Fig. 1). HepG2 cells of all the cell lines were the most sensitive to deguelin (IC_{50} = 0.62 μM). Considering that the IC_{50} value of deguelin for colon cancer was 0.43 μM (7), it seemed to have a strong efficacy in HCC.

We further investigated the mechanism by which deguelin inhibits cell proliferation. Chemotherapeutic agents causing apoptosis have been increasingly appreciated as ideal compounds for the management of cancer (21). Previous studies have reported that deguelin can trigger apoptosis in tumor cells *in vitro* (22). Deguelin promoted cell cycle arrest in the colon and lung cancer cells, which was followed by apoptosis induction (22). In colon cancer cells, deguelin arrested cells in the G0/G1 phase of the cell cycle at incubation for 24 h in the HT-29 cells and was associated with a sub-G1 peak at 72 h (7). Premalignant and malignant human bronchial epithelial (HBE) cells treated with deguelin accumulated in the G2/M phase of the cell cycle and underwent apoptosis in a dose- and time-dependent manner (6). Therefore, in order to investigate the possible pathway that was involved with the anticancer effect of deguelin, the apoptosis of HepG2 cells was analyzed *in vitro*. Although cell cycle arrest was not affected by deguelin treatment in our studies, we have demonstrated that the increase of a sub-G1 peak in a dose-dependent manner, which indicates that deguelin may be potentially mediating its actions by inducing apoptosis in these cells. These data support the potent apoptotic effects of deguelin on HCC.

Another possible pathway that was involved with the anticancer effect of deguelin *in vitro* and *in vivo* was examined in our study. Angiogenesis, the formation of new blood vessels from pre-existing endothelium, is integral to tumor growth and metastases (23). The angiogenic process is tightly controlled by a wide variety of positive or negative regulators, such as growth factors, cytokines, lipid metabolites and cryptic fragments of hemostatic proteins (19). In addition, hypoxia stimulates angiogenesis in order to support tumor growth by inducing the expression of angiogenic factors, such as insulin-like growth factor II and VEGF and HIF-1α is its key mediator (18,24). Recently, the drug development targeting hypoxia has evoked an extensive interest in cancer therapy (25). HIF-1α is a new target for the antiangiogenic therapy of HCC. Deguelin has been reported to be antiangiogenic targeting HIF-1α in lung cancer (11,26). Therefore, we investigated antiangiogenic activities of deguelin in HCC. As a result, deguelin inhibited hypoxia-induced angiogenesis via down-regulating the expression of the HIF-1α protein and VEGF mRNA in HepG2 cells. Furthermore, our study reveals that deguelin inhibits angiogenic features of HUVECs *in vitro* as revealed by a capillary-like tube formation and migration assays. In addition,

deguelin treatment significantly inhibited neovascularization *in vivo* in the CAM assay.

Most anticancer drugs such as tubulysin A (tub A), sulforaphane, phenethyl isothiocyanate (PEITC) and 6-(1-oxobutyl)-5,8-dimethoxy-1,4-naphthoquinone (OXO) induce apoptosis as well as inhibit angiogenesis to achieve therapeutic efficacy (20,27-29). Especially, curcumin, resveratrol, YC-1 and rapamycin have been reported to target HIF-1α in HCC (30-34). In our study, we demonstrated that deguelin induced apoptosis as well as inhibited angiogenesis in HCC and HUVECs. Therefore, we suggest that deguelin is potentially useful as a chemotherapeutic agent in HCC.

Taken together, our data indicated that deguelin had anticancer activity, which was correlated with the inhibition on angiogenesis and the induction of apoptosis in HCC. Deguelin may be a potential agent in inhibiting the progression of HCC by antiangiogenesis and the apoptosis pathway.

Acknowledgements

This study was supported by the Inha University Research Grant (INHA-33608-1).

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