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European Journal of Pharmacology 553 (2006) 46–53
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Anthraquinone derivative emodin inhibits tumor-associated angiogenesis through inhibition of extracellular signal-regulated kinase 1/2 phosphorylation

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Received 5 January 2006; received in revised form 12 September 2006; accepted 12 September 2006
Available online 23 September 2006

Abstract

An anthraquinone derivative, emodin, suppresses tumor development both in vitro and in vivo. In this study, we examined the anti-angiogenic activity of emodin and its modifying effect on the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2. In cell cultures, emodin inhibited endothelial cell proliferation, migration, and tube formation in a dose-dependent manner. In addition, the mouse dorsal air sac assay revealed the vivo anti-angiogenic potential of emodin. Matrix metalloproteinase-9 (MMP-9) expression, which is critical for the angiogenic process, including migration and tube formation, decreased after exposure to emodin, as determined by polymerase chain reaction with reverse transcription (RT-PCR) and gelatin zymography. Moreover, the phosphorylation of ERK 1/2 decreased after exposure to emodin in a dose-dependent manner. These observations suggest that emodin has the potential to inhibit several angiogenic processes and that these effects may be related to suppression of the phosphorylation of ERK 1/2.

Keywords: Emodin; Angiogenesis; Human umbilical cord vein endothelial cells; ERK 1/2

1. Introduction

Tumor cell proliferation and tumor progression are considered to largely depend on angiogenesis (Folkman et al., 1971). In angiogenesis, endothelial cells proliferate, lyse the basement membrane and the extracellular matrix, migrate into the surrounding stroma, and finally mature as new tubular vessels (Folkman and Shing, 1992; Carmeliet, 2000). In a healthy adult, new vessel formation is strictly restricted to the local the environment, including wound healing, acute inflammation, and menstruation. In contrast, angiogenesis in tumors is not controlled. Tumor cells continuously form abnormal vessels that are irregular, extensive, and circuitous. Expansion of a microtumor beyond 1–2 mm in size requires a continuous blood supply (Seno et al., 2002). Once tumor cells acquire the ability to induce angiogenesis, the tumor grows aggressively, which promotes invasion and metastasis. Angiogenesis involves many angiogenic molecules, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and interleukin-8 (IL-8), and anti-angiogenic molecules, including angiostatin, thrombospondin-1, and endostatin.

Because the angiogenic response is restricted in adults, although with a few exceptions, and normal endothelial cells are quiescent, resistance to anti-angiogenic therapy rarely develops, in contrast to tumor-targeted therapy. Therefore, inhibition of angiogenesis is considered a promising approach for cancer therapy or prevention.

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is derived from the rhizome of *Rheum palmatum L.* (Tsai and Chen, 1992; Liang et al., 1995). *R. palmatum L.* has been used since ancient times, to treat inflammatory diseases such as peptic ulcers and as a strong laxative. It has been documented that emodin, which suppresses bacterial and tumor growth, has a vasorelaxant effect (Koyama et al., 1988; Huang et al., 1991a,b). Although, inhibitory effects of emodin on NF-κB, protein tyrosine kinase, protein

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doi:10.1016/j.ejphar.2006.09.026

kinase C, and activator protein-1 (AP-1) have been reported (Zhang and Hung, 1996; Huang et al., 2004b; Yang et al., 2004), its anti-angiogenic effect has not been well studied. Recently, it was reported that emodin suppressed angiogenesis induced by VEGF-A by blocking VEGFR2 phosphorylation (Kwak et al., 2006). However, whether emodin inhibits tumor-associated angiogenesis remains unclear. Therefore, the focus of this article was on the effect of emodin on angiogenesis. When searching for an inhibitor of tumor-associated angiogenesis, we found that emodin had strong anti-angiogenic activity. In the present study, we examined the in vitro effect of emodin on four important angiogenic processes: endothelial cell proliferation, migration, differentiation, and matrix degradation. Furthermore, we demonstrated the anti-angiogenic effect of emodin in vivo.

2. Materials and methods

2.1. Cell culture and reagents

Human umbilical cord vein endothelial cells (HUVECs) were cultured in HuMedia EB2 (Kurabo, Industries Ltd., Osaka, Japan) with endothelial cell growth supplement (containing 2% fetal bovine serum (FBS), 10 ng/ml human epidermal growth factor (hEGF), 1 μg/ml hydrocortisone, 50 μg/ml gentamicin, 50 ng/ml amphotericin B, 5 ng/ml bFGF, and 10 μg/ml heparin). Human colon carcinoma cell line HT29 was cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% heat inactivated FBS (BioWest, Nuaille, France). Both cell lines were maintained in humidified 5% CO_2 at 37 °C. Hypoxic conditions were generated in an Astec ACM-165 multigas incubator (ASTEC Co., Ltd., Fukuoka, Japan) with 1% O_2, 5% CO_2, and 94% N_2. Emodin (Sigma) and mitogen-activated protein kinase (MAPK) kinase (MEK) inhibitor PD098059 (Calbiochem, San Diego, CA, USA) were dissolved in dimethylsulfoxide (DMSO; Sigma).

2.2. Proliferation assay

Proliferation was measured using a 3-(4,5-Dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay kit (Roche Diagnostic Corp., Indianapolis, IN, USA). HUVECs were seeded onto a 96-well plate (2.0×10^3 per well) and incubated in HuMedia-EB2 with endothelial cell growth supplement overnight for cell attachment. Then the medium was replaced by fresh medium containing various concentrations of test compounds. After a 48-h incubation, the cells were treated with 10 μl of MTT reagent for 4 h at 37 °C and then incubated with solubilization solution for several hours. Cell proliferation was measured using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The formazan products were quantified. The results are expressed as a ratio compared to control.

2.3. Chemotactic migration assay

The migration of HUVECs was determined with the modified Boyden chamber method described previously (Basaki et al., 2001; Tan et al., 2003). The chemotactic response of the cells was assayed using 24-well chemotaxis chambers with upper and lower wells with 8 μm-pore size filters placed in between (Becton Dickinson, Franklin Lakes, NJ, USA). HUVECs were seeded into the upper well of each chamber (5×10^4 cells) and 900 μl of FBS-free conditioned medium from HT29 cells, which were treated under 1% O_2, was added to the lower wells. The indicated concentrations of emodin, DMSO, and 30 μM PD098059 were added to the upper chamber. Cell migration was assessed by counting the number of cells that had migrated from the upper side to the lower side of the filter membrane after an 8-h incubation. Migrated cells were fixed with methanol and stained with Giemsa (Merck KGaA, Darmstadt, Germany) solution. The number of migrated cells was counted in 15 fields. Cells on both the upper and lower sides of the filter membrane were also counted in another 15 fields to determine whether the effect of emodin on cell migration was due to apoptosis.

2.4. Tube formation assay

In vitro angiogenesis of HUVECs was assessed using a tube-like network formation assay. A Matrigel-coated 48-well plate (Becton Dickinson) was incubated at 37 °C for 30 min to solidify the gel. HUVECs (5×10^4 cells) were then plated on the Matrigel and cultured in HuMedia EB-2 medium containing different concentrations of emodin, 30 μM PD098059, and DMSO for 24 h. The enclosed networks of complete tubes from five different fields were counted under a microscope.

2.5. Dorsal air sac assay

Four-week-old male ICR mice were obtained from SLC (Sizuoka, Japan). All mice were maintained under standard conditions in accordance with the guidelines for animal experiments provided by the University of the Ryukyus.

The mouse dorsal air sac assay was as described previously (Oikawa et al., 1997; Aonuma et al., 1998). In brief, a Millipore chamber (pore size: 0.45 μm, Millipore Corporation, Billerica, MA, USA) containing 0.2 ml of HT29 cell suspension (5×10^7 cells/ml) was implanted into a pre-formed air sac on the back of male ICR mice. The mice were divided into three groups (each group included five mice) and drugs were administered from day 1 to day 5. On day 6, the implanted chambers were removed and new blood vessels were recorded and measured with an Image-Pro Plus version 4.0 (Media Cybernetics, Inc., Silver Spring, Maryland, USA). The data are expressed as the area of new vessels.

2.6. RT-PCR analysis

HUVECs, at about 80%–90% confluence, were treated with the indicated concentrations of emodin for 12 h. Total RNA was isolated from cultured cells by a standard acid guanidinium thiocyanate-phenol-chloroform method.

cDNA was synthesized using reverse transcriptase (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA) and 0.5 μg of total RNA was primed with random hexamers. cDNA amplification was performed as follows: initial denaturation at 94 °C for 4 min; followed by denaturation at 94 °C for 1 min, annealing at 55 °C

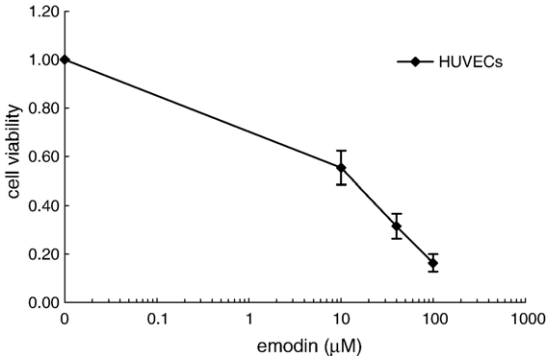

Fig. 1. Effects of emodin on proliferation of HUVECs. HUVECs (2000 cells/well) were exposed to the indicated concentrations of emodin (0–100 μM) for 48 h and cell viability was determined using MTT assay. Values represent the means±S.D. obtained from three independent experiments.

for 90 s, extension at 72 °C for 90 s for 35 cycles; and a final elongation step at 72 °C for 7 min for matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) amplification. For GAPDH amplification, there were a total of 20 PCR cycles. The PCR products were electrophoresed on a 2% agarose gel and visualized by staining with ethidium bromide.

The primers used were MMP-2, sense: 5′-GGGGCCTC-TCCTGACATT-3′, antisense: 5′-TCACAGTCCGCCAAATG-AA-3′; MMP-9, sense: 5′-GAGACCGGTGAGCTGGATAG-3′, antisense: 5′-GTACACGCGAGTGAAGGTGA-3′; GAPDH, sense: 5′-ATCATCTGCCCCCTCTGCT -3′, antisense: 5′-GCC-TGCTTCACCACCTTCTTG-3′.

2.7. Gelatin zymography

MMP-2 and MMP-9 gelatinase activities were determined by gelatin zymography as described elsewhere (Liu et al., 2005). Briefly, the supernatants from HUVECs treated with various concentrations of emodin for 12 h were collected and concentrated. They were then mixed with Tris-glycine SDS sample buffer and loaded onto a 10% SDS-polyacrylamide gel containing 1 mg/ml gelatin (Sigma). Then the gels were washed in 2.5% Triton X-100 for 30 min at room temperature and incubated in developing buffer (50 mM Tris-HCl (pH 7.5), 0.2 M NaCl, 5 mM $CaCl_2$, 0.02% Brij 35, and 0.02% NaN_3) overnight at 37 °C. Gels were subsequently stained with 0.5% Coomassie brilliant blue R-250, 5% methanol, and 7.5% acetic acid, and then destained with 25% methanol and 7.5% acetic acid. The zone of gelatinase activity appeared as clear bands against a dark blue background where the gelatinase had digested the substrate.

2.8. Western blot analysis

HUVECs were cultured with the indicated concentrations of emodin for 12 h. Whole protein was extracted using cell lysis solution containing 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholic acid sodium salt, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, and 1 μg/ml aprotinin. Protein concentration was determined using the Bradford assay (Bio-Rad). Equal amounts of protein (50 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to the polyvinylidene difluoride (PVDF) membrane. Blots were incubated with anti-phosphorylated or anti-total p44/42 ERK Antibody (1:1000 dilution; Cell Signaling Laboratories, Bervely, MA), anti-phosphorylated or anti-total Jun-*N*-terminal kinase/stress-activated protein kinase (JNK/SPAK) Antibody and p38 mitogen-activated protein (MAP) kinase Antibody (1:500 dilution; Cell Signaling Laboratories). Blots were incubated with horseradish-peroxidase-conjugated secondary antibodies (Amersham Biosciences Corp., Piscataway, NJ, USA). Protein expression was detected using an ECL Plus™ Western Blotting Detection system (Amersham Biosciences Corp.).

2.9. Statistical analysis

ANOVA followed by the Tukey test or Dunnett's test, or the Kruskal–Wallis test followed by a Tukey-type test were used to analyze the significance of any difference between different groups. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Effect of emodin on proliferation of HUVECs

The proliferation of HUVECs was determined with the MTT assay. The growth of HUVECs was inhibited by emodin in a

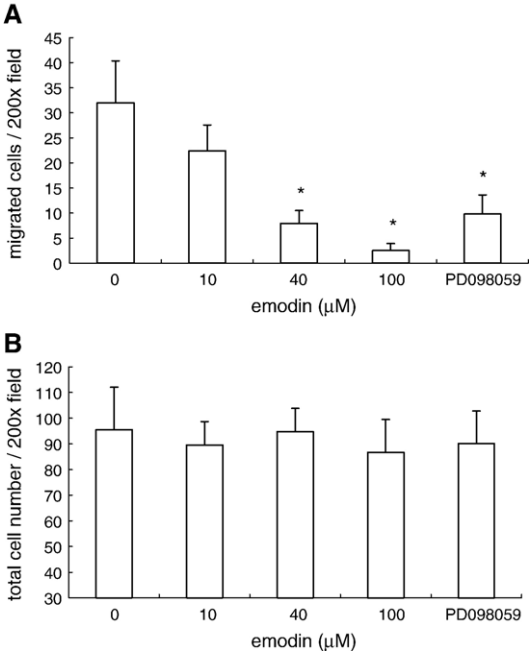

Fig. 2. Effects of emodin on chemotactic migration of HUVECs. Vehicle, emodin or PD098059 was added to the upper well of modified Boyden chambers containing HUVECs, while conditioned medium from HT29 cells that were grown under hypoxic conditions was added to the lower well. (A) Migratory activity of HUVECs was evaluated by counting the number of cells that had migrated to the lower side of 8 μM-pore size polyethyleneterephthalate filters. (B) To evaluate whether emodin inhibits endothelial cell migration by inducing apoptosis, both migrated and nonmigrated cells were counted. Values expressed the means±S.D., * $P<0.001$ vs. control by Kruskal–Wallis test and Tukey-type test.

dose-dependent manner (Fig. 1). The 50% growth inhibitory concentration (IC_{50} value) of emodin on HUVECs was about 20 μM. To determine the time-dependent nature of the inhibitory effect of emodin on cell growth, we performed a time-course study. HUVECs growth was suppressed by emodin in a dose- and time-dependent manner. These suppressive effects lasted for 72 h (data not shown).

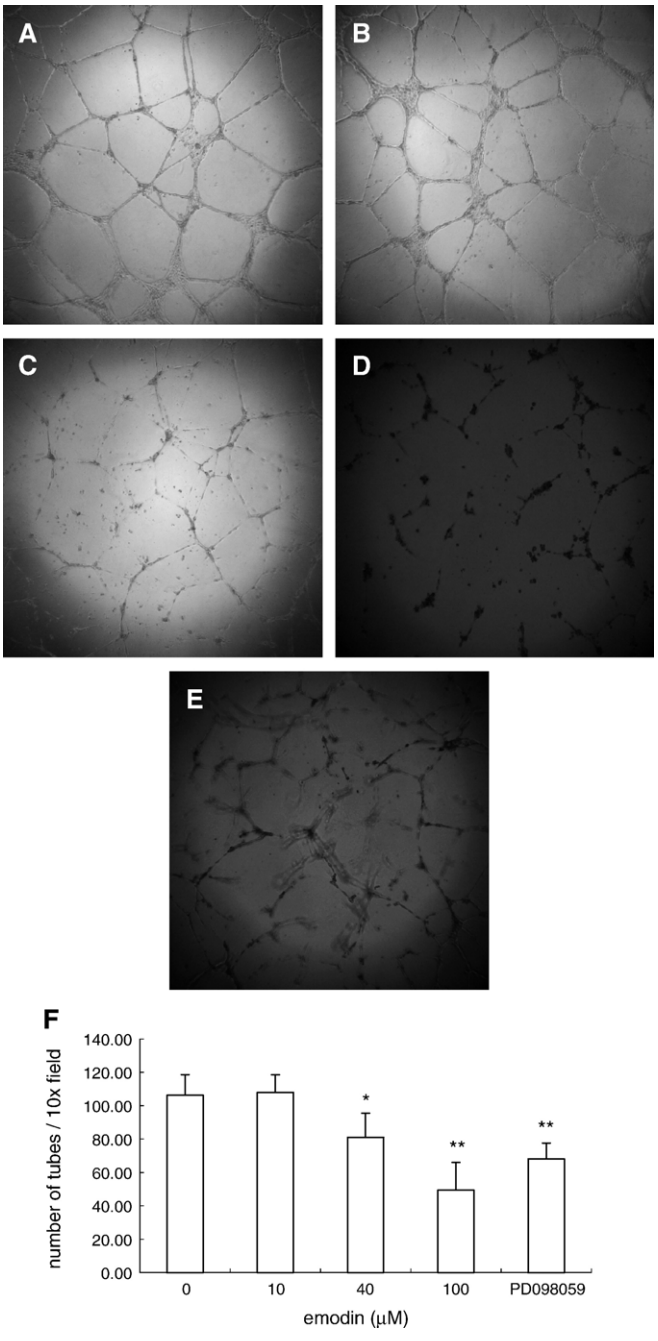

Fig. 3. Effects of emodin on in vitro angiogenesis. The angiogenic response of HUVECs was evaluated in the presence or absence of emodin or PD098059 by examining tube formation on Matrigel. Panel A: Vehicle control; Panel B: treatment with 10 μM emodin; Panel C: treatment with 40 μM emodin; Panel D: treatment with 100 μM emodin; Panel E: treatment with PD098059. (F) Quantitative analysis of in vitro tube formation and the effect of emodin. The number of capillary-like tubes was counted under light microscopy (×10 field). Results represent the means ± SD from three independent experiments. *$P<0.05$ vs. control, **$P<0.001$ vs. control by ANOVA and Tukey test.

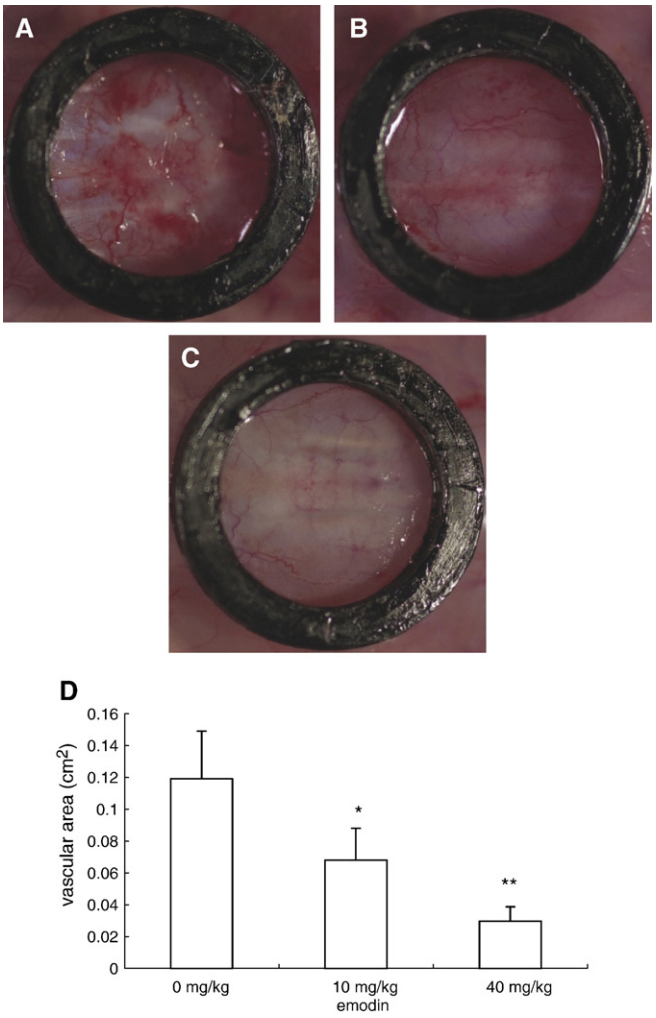

Fig. 4. Effect of emodin on HT29 cell-induced angiogenesis. Panel A, mice were implanted with a chamber containing HT29 cells and treated with vehicle. Panel B and C, mice were implanted with a chamber containing HT29 cells and then injected i.p. with 10 mg/kg or 40 mg/kg emodin for 5 consecutive days from implantation, respectively. The number of newly formed blood vessels with a characteristic zigzag shape was reduced by administration of emodin. (D) represents the area of newly formed vessels. The experiments were conducted in duplicate and gave similar results. *$P<0.05$ vs. control, **$P<0.001$ vs. control by Kruskal–Wallis test and Tukey-type test.

3.2. Emodin inhibits migration of HUVECs

Treatment with 10, 40, and 100 μM emodin reduced the migration of HUVECs by 30%, 75.2%, and 92%, respectively, compared to control (Fig. 2A). In particular 40 μM and 100 μM emodin significantly inhibited endothelial cell migration ($P<0.001$). We also examined the effect of PD098059 on cell migration. Like emodin, PD098059 significantly reduced endothelial cell migration ($P<0.001$). Furthermore, to explore whether the inhibition of endothelial cell migration by emodin was due to apoptosis, we counted the number of migrated and nonmigrated cells in each group (Fig. 2B). There was no

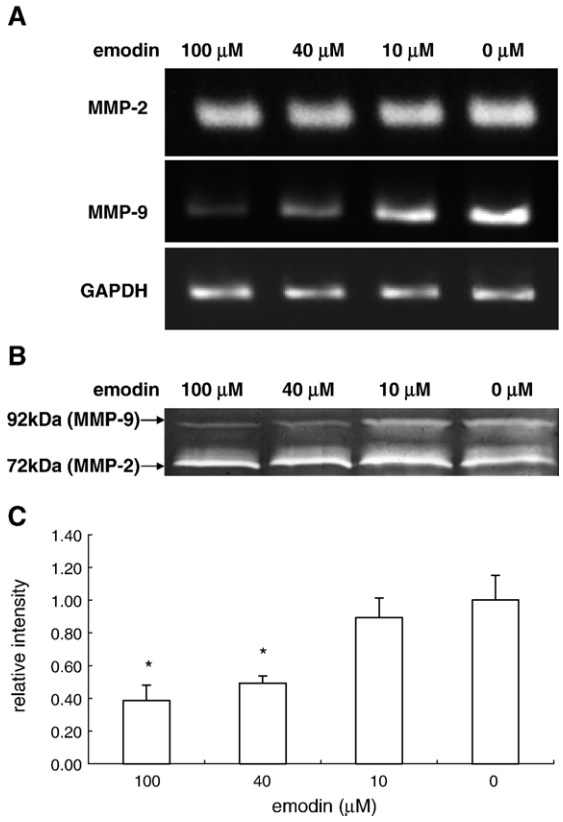

Fig. 5. Inhibition of matrix metalloproteinase-9 expression and gelatinase activity in endothelial cells. HUVECs were treated with the indicated concentrations (0–100 μM) of emodin for 12 h. (A) The mRNA expression of MMP-2 and MMP-9 was determined by RT-PCR. (B) Gelatinase activity of MMP-2 and MMP-9 was detected by gelatin zymography. (C) Quantification of matrix metalloproteinase-9 gelatinolytic activity. The data are expressed as means ± S.D., *$P<0.001$ vs. control by ANOVA and Tukey test.

significant difference observed between the control and treated groups.

3.3. Effect of emodin on tube formation by HUVECs

HUVECs that were seeded onto a Matrigel-coated 48-well plate formed capillary-like tube networks after a 24-h incubation at 37 °C (Fig. 3). As shown in Fig. 3A, the control group formed an enclosed network of tubes. In the emodin- and PD098059-treated groups, the length of the tubes was decreased compared to that of the control (Fig. 3B, C, D and E). In this study, we counted several capillary-like structures per field to evaluate angiogenesis. Emodin at 40 μM and 100 μM and 30 μM of PD098059 inhibited tube formation by HUVECs by 23.7%, 53.5%, and 35.9%, respectively, compared to control (Fig. 3F). Emodin at 40 μM and 100 μM reduced tube formation significantly ($P<0.05$ and $P<0.001$, respectively) compared to control, as did PD098059 ($P<0.001$).

3.4. In vivo anti-angiogenic activity of emodin

The in vivo angiogenic response of tumor cells was examined in the mouse dorsal air sac assay. Using this assay, we evaluated the ability of emodin to suppress tumor-induced angiogenesis. The control group showed newly formed, well-developed microvessels characterized by a zigzag shape. As shown in Fig. 4, emodin inhibited the angiogenic response in a dose-dependent manner. Treatment with 10 mg/kg emodin and 40 mg/kg emodin significantly inhibited the formation of new microvessels, compared with control ($P<0.05$ and $P<0.001$, respectively). The inhibition rate for 10 mg/kg and 40 mg/kg emodin was 42.9% and 75.0%, respectively.

3.5. Decrease in MMP-9 mRNA expression and enzyme activity

The effect of emodin on MMP-9 expression in HUVECs was examined by RT-PCR. HUVECs that were treated with emodin for 12 h had a reduced expression of MMP-9 at the mRNA level (Fig. 5A). In addition, MMP-9 enzyme activity was reduced significantly when HUVECs were treated with 100 μM and 40 μM emodin (Fig. 5B, C) ($P<0.001$). However, emodin had almost no effect on MMP-2 mRNA expression and gelatinase activity (Fig. 5A,B).

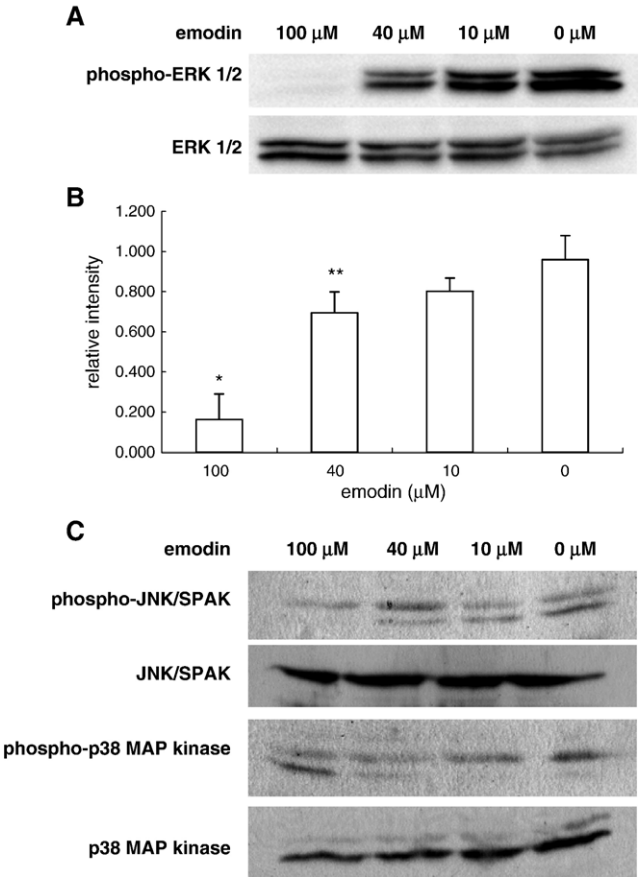

Fig. 6. Emodin blocks ERK 1/2 phosphorylation in endothelial cells. HUVECs were treated with the indicated concentrations (0–100 μM) of emodin for 12 h (A and C). The phosphorylation and expression of ERK 1/2, JNK/SPAK, and p38 MAP kinase were analyzed by Western blotting. (B) The histograms represent the relative intensity of ERK 1/2 phosphorylation, as determined by densitometric analysis. The data represent the means ± S.D. from three independent experiments. *$P<0.01$ vs. control, **$P<0.05$ vs. control by ANOVA and Dunnett's test.

3.6. Emodin reduces the phosphorylation of ERK 1/2

To evaluate the effect of emodin on intracellular signal transduction, we examined the phosphorylation level of ERK 1/2, JNK/SPAK, and p38 MAP kinase in HUVECs, using Western blotting after 12 h of treatment with emodin. As shown in Fig. 6A, 100 μM emodin virtually inhibited the phosphorylation of ERK 1/2. Emodin, at 40 μM or 10 μM, also decreased the phosphorylation of ERK 1/2 in a dose-dependent manner. Treatment with 100 μM, 40 μM, and 10 μM emodin reduced the phosphorylation of ERK 1/2 by about 80%, 25%, and 20%, respectively, compared to control (Fig. 6B). Emodin at 100 μM and 40 μM significantly reduced the phosphorylation of ERK 1/2 ($P<0.01$ and $P<0.05$, respectively). However, virtually no effect of emodin on the phosphorylation of JNK/SPAK and p38 MAP kinase was observed (Fig. 6C).

4. Discussion

It has been reported that emodin inhibits (+/-)-(*E*)-4-methyl-2-[(*E*)-hydroxyimino]-5-nitro-6-methoxy-3-hexenamide (NOR-1) +12-*O*-tetradecanoyl phorbol-13-acetate (TPA)-induced mouse skin carcinogenesis and induces apoptosis and G1 cell cycle arrest in human hepatoma cell line (Koyama et al., 2002; Kuo et al., 2002; Shieh et al., 2004). Therefore, emodin seems to possess anti-tumor activity. In addition, emodin sensitizes chemoresistant breast cancer or lung cancer to chemotherapy by inhibiting $p185^{neu}$ tyrosine kinase activity in a synergistic manner (Zhang and Hung, 1996; Zhang et al., 1999). However, knowledge of the inhibitory effects of emodin on tumor-associated angiogenesis is limited.

In this study, the potential of emodin to suppress angiogenesis was shown in in vitro and in vivo experiments. In vitro studies helped to determine the efficacy of emodin against angiogenesis, but results needed to be confirmed in in vivo experiments. Thus, we examined the effect of emodin on the proliferation, migration, and differentiation of endothelial cells in independent experiments. In addition, we investigated the anti-angiogenic activity of emodin in vivo, using the mouse dorsal air sac assay. We found that emodin inhibited the main angiogenic stages, including proliferation, migration, and tube formation of endothelial cells in vitro, and suppressed angiogenic activity in vivo. Most of these effects were dose dependent.

Endothelial cell migration was assessed by using a modified Boyden chamber method, which has been reported to be useful for testing the activity of angiogenic substances including stimulators and inhibitors (Auerbach et al., 2003). In this study, emodin inhibited the chemotactic response of endothelial cells in a dose-dependent manner. To clarify the nature of these effects, we compared the total number of cells in both the control and treated groups in the migration assay. No apparent decrease in total cell number was observed in either group. This result suggests that emodin suppresses cell migration by a mechanism other than apoptosis.

The inhibition of the formation of capillary-like tube structures by HUVECs on Matrigel-coated culture plates suggested that emodin might suppress other functions of endothelial cells during angiogenesis beside suppression of cell proliferation. This method, by which endothelial cells form three-dimensional capillary-like structures, is one of the most reliable in vitro models for evaluating angiogenesis (Auerbach et al., 2000). In our experiments, both emodin and PD098059 significantly inhibited tube formation by HUVECs grown on Matrigel. In addition, tumor-associated angiogenesis in vivo, as determined in the dorsal air sac assay, which is the best method for monitoring progressive changes occurring in the microenvironment (Auerbach et al., 2000), was dramatically reduced by emodin treatment. This was a very strong and dose-dependent response.

Because emodin significantly inhibited the angiogenic processes of tube formation and migration, we tried to investigate the inhibitory mechanism of angiogenesis using HUVECs. We tested the effect of emodin on the expression of matrix metalloproteinase. MMP-9 is a type IV collagenase that is implicated in angiogenesis and tumor development (Handsley and Edwards, 2005). It plays a major role in angiogenesis because the MMP-9 inhibitor, GM6001, significantly decreases human cerebral endothelial cell proliferation and migration (Yao et al., 2006). Further, a positive correlation between MMP-9 expression and microvessel density has been reported in human hepatocellular carcinoma (Sun et al., 2005), and antisense MMP-9 inhibits tumor-cell-induced angiogenesis (Rao et al., 2005). This study verified that treatment with emodin for 12 h decreased the expression and gelatinase activity of MMP-9. Inhibition of MMP-9 expression and endothelial cell migration may cooperate to decrease the formation of tube-like structures by endothelial cells on the Matrigel matrix.

Furthermore, we assessed the effects of emodin on the growth factor production of human colon carcinoma cell line HT29. It has been reported that HT29 cells have the potential to express VEGF after hypoxic stress (Welsh et al., 2002; Cianchi et al., 2005). However, the expression of mRNA for VEGF, bFGF, IL-8, and Hypoxia Inducible Factor-1α (HIF-1α) in HT29 cells was not affected by emodin (data not shown). These data suggest that the anti-angiogenic activities of emodin are mediated by a VEGF-independent mechanism.

Since there are many unanswered questions regarding the mechanism of the inhibition produced by emodin, we focused on the modifying effects of emodin on tyrosine phosphorylation (Chan et al., 1993; Zhang et al., 1995; Zhang and Hung, 1996). The inhibitory effect of emodin on ERK phosphorylation is well documented (Huang et al., 2004a; Zhou et al., 2006). Emodin suppresses ERK phosphorylation either by inhibiting the kinase activity (Jayasuriya et al., 1992; Kim et al., 2005) or by preventing the binding of ATP to PTK (Jayasuriya et al., 1992). MAPK signal transduction regulates angiogenic processes including endothelial cell migration, tube formation, and expression of MMP-9 (Gum et al., 1996; Klemke et al., 1997; Maru et al., 1998). Emodin inhibits these endothelial cell functions through suppression of MAPK signaling by inhibiting ERK phosphorylation. Although emodin was able to block the chemotactic migration and tube formation of HUVECs, these effects were, to some extent, associated with growth inhibitory effects, as determined with the MTT assay. Furthermore, it has been documented that MAP kinase signaling events are critical for the induction of cell migration (Klemke et al., 1997). Klemke at al. demonstrated that cell migration was associated with increased MAP kinase activity followed by myosin light chain kinase and myosin light chain activation, and that cell

migration was blocked by the MEK inhibitor, PD098059. Matrigel induces an integrin-mediated Ras signal that results in MAPK activation. MAPK signal inhibition by PD098059, but not by a PI3 kinase inhibitor, wortmannin, or a protein kinase C inhibitor, GF 109203X, results in a strong inhibition of tubulogenesis (Maru et al., 1998). In this study, we demonstrated that PD098059 affected both endothelial cell migration and tube formation, and that these effects were similar to those of emodin, which suppressed phosphorylation of ERK. Although we tested MMP-2 expression as well as MMP-9 expression, MMP-2 mRNA expression and enzyme activity were not affected by emodin treatment. It has been reported that MMP-2 (gelatinase A) activity is not diminished by PD098059, whereas MMP-9 gene expression is regulated by c-Jun-*N*-terminal kinase and extracellular signal-regulated kinase (Gum et al., 1996). In addition, MMP-2 mRNA expression is not affected by emodin or PD098059 (Huang et al., 2004b). Our findings are consistent with these reports and show that emodin inhibits ERK phosphorylation and suppresses endothelial cell proliferation, migration, tube formation, and MMP-9 expression. In the present study, MMP-9 mRNA expression and gelatinase activity, and the phosphorylation of ERK 1/2 were suppressed by 40–100 μM of emodin. This suggests that suppression of ERK phosphorylation affects MMP-9 expression and its activity.

Therefore, growth inhibitory effects and disruption of MAPK signal transduction may contribute to the overall anti-angiogenic effect of emodin. However, further investigations are needed to find the precise mechanisms by which emodin inhibits endothelial cell function.

In this study, we demonstrated that emodin is a potent anti-angiogenic compound that inhibits endothelial cell proliferation, migration, and differentiation. Moreover, emodin inhibits the in vivo angiogenic response as well. Therefore, we presume that regular consumption of food rich in emodin will control or prevent angiogenic disease, including cancer.

Acknowledgments

We thank Kiyomi Kayo and Eriko Miyagi for their excellent technical assistance. This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare and a grant from the Takeda Science Foundation.

References

Aonuma, M., Iwahana, M., Nakayama, Y., Hirotani, K., Hattori, C., Murakami, K., Shibuya, M., Tanaka, N.G., 1998. Tumorigenicity depends on angiogenic potential of tumor cells: dominant role of vascular endothelial growth factor and/or fibroblast growth factors produced by tumor cells. Angiogenesis 2, 57–66.

Auerbach, R., Akhtar, N., Lewis, R.L., Shinners, B.L., 2000. Angiogenesis assays: problems and pitfalls. Cancer Metastasis Rev. 19, 167–172.

Auerbach, R., Lewis, R., Shinners, B., Kubai, L., Akhtar, N., 2003. Angiogenesis assays: a critical overview. Clin. Chem. 49, 32–40.

Basaki, Y., Chikahisa, L., Aoyagi, K., Miyadera, K., Yonekura, K., Hashimoto, A., Okabe, S., Wierzba, K., Yamada, Y., 2001. gamma-Hydroxybutyric acid and 5-fluorouracil, metabolites of UFT, inhibit the angiogenesis induced by vascular endothelial growth factor. Angiogenesis 4, 163–173.

Carmeliet, P., 2000. Mechanisms of angiogenesis and arteriogenesis. Nat. Med. 6, 389–395.

Chan, T.C., Chang, C.J., Koonchanok, N.M., Geahlen, R.L., 1993. Selective inhibition of the growth of ras-transformed human bronchial epithelial cells by emodin, a protein–tyrosine kinase inhibitor. Biochem. Biophys. Res. Commun. 193, 1152–1158.

Cianchi, F., Cortesini, C., Schiavone, N., Perna, F., Magnelli, L., Fanti, E., Bani, D., Messerini, L., Fabbroni, V., Perigli, G., Capaccioli, S., Masini, E., 2005. The role of cyclooxygenase-2 in mediating the effects of histamine on cell proliferation and vascular endothelial growth factor production in colorectal cancer. Clin. Cancer Res. 11, 6807–6815.

Folkman, J., Shing, Y., 1992. Angiogenesis. J. Biol. Chem. 267, 10931–10934.

Folkman, J., Merler, E., Abernathy, C., Williams, G., 1971. Isolation of a tumor factor responsible for angiogenesis. J. Exp. Med. 133, 275–288.

Gum, R., Lengyel, E., Juarez, J., Chen, J.H., Sato, H., Seiki, M., Boyd, D., 1996. Stimulation of 92-kDa gelatinase B promoter activity by ras is mitogen-activated protein kinase 1-independent and requires multiple transcription factor binding sites including closely spaced PEA3/ets and AP-1 sequences. J. Biol. Chem. 271, 10672–10680.

Handsley, M.M., Edwards, D.R., 2005. Metalloproteinases and their inhibitors in tumor angiogenesis. Int. J. Cancer 115, 849–860.

Huang, H.C., Chu, S.H., Chao, P.D., 1991a. Vasorelaxants from Chinese herbs, emodin and scoparone, possess immunosuppressive properties. Eur. J. Pharmacol. 198, 211–213.

Huang, H.C., Lee, C.R., Chao, P.D., Chen, C.C., Chu, S.H., 1991b. Vasorelaxant effect of emodin, an anthraquinone from a Chinese herb. Eur. J. Pharmacol. 205, 289–294.

Huang, Q., Shen, H.M., Ong, C.N., 2004a. Inhibitory effect of emodin on tumor invasion through suppression of activator protein-1 and nuclear factor-kappaB. Biochem. Pharmacol. 68, 361–371.

Huang, Q., Shen, H.M., Ong, C.N., 2004b. Inhibitory effect of emodin on tumor invasion through suppression of activator protein-1 and nuclear factor-kappaB. Biochem. Pharmacol. 68, 361–371.

Jayasuriya, H., Koonchanok, N.M., Geahlen, R.L., McLaughlin, J.L., Chang, C.J., 1992. Emodin, a protein tyrosine kinase inhibitor from *Polygonum cuspidatum*. J. Nat. Prod. 55, 696–698.

Kim, M.S., Park, M.J., Kim, S.J., Lee, C.H., Yoo, H., Shin, S.H., Song, E.S., Lee, S.H., 2005. Emodin suppresses hyaluronic acid-induced MMP-9 secretion and invasion of glioma cells. Int. J. Oncol. 27, 839–846.

Klemke, R.L., Cai, S., Giannini, A.L., Gallagher, P.J., de Lanerolle, P., Cheresh, D.A., 1997. Regulation of cell motility by mitogen-activated protein kinase. J. Cell Biol. 137, 481–492.

Koyama, M., Kelly, T.R., Watanabe, K.A., 1988. Novel type of potential anticancer agents derived from chrysophanol and emodin. Some structure-activity relationship studies. J. Med. Chem. 31, 283–284.

Koyama, J., Morita, I., Tagahara, K., Nobukuni, Y., Mukainaka, T., Kuchide, M., Tokuda, H., Nishino, H., 2002. Chemopreventive effects of emodin and cassiamin B in mouse skin carcinogenesis. Cancer Lett. 182, 135–139.

Kuo, P.L., Lin, T.C., Lin, C.C., 2002. The antiproliferative activity of aloe-emodin is through p53-dependent and p21-dependent apoptotic pathway in human hepatoma cell lines. Life Sci. 71, 1879–1892.

Kwak, H.J., Park, M.J., Park, C.M., Moon, S.I., Yoo, D.H., Lee, H.C., Lee, S.H., Kim, M.S., Lee, H.W., Shin, W.S., Park, I.C., Rhee, C.H., Hong, S.I., 2006. Emodin inhibits vascular endothelial growth factor-A-induced angiogenesis by blocking receptor-2 (KDR/Flk-1) phosphorylation. Int. J. Cancer 118, 2711–2720.

Liang, J.W., Hsiu, S.L., Wu, P.P., Chao, P.D., 1995. Emodin pharmacokinetics in rabbits. Planta Med. 61, 406–408.

Liu, S., Liang, Y., Huang, H., Wang, L., Li, Y., Li, J., Li, X., Wang, H., 2005. ERK-dependent signaling pathway and transcriptional factor Ets-1 regulate matrix metalloproteinase-9 production in transforming growth factor-beta1 stimulated glomerular podocytes. Cell. Physiol. Biochem. 16, 207–216.

Maru, Y., Yamaguchi, S., Takahashi, T., Ueno, H., Shibuya, M., 1998. Virally activated Ras cooperates with integrin to induce tubulogenesis in sinusoidal endothelial cell lines. J. Cell Physiol. 176, 223–234.

Oikawa, T., Sasaki, M., Inose, M., Shimamura, M., Kuboki, H., Hirano, S., Kumagai, H., Ishizuka, M., Takeuchi, T., 1997. Effects of cytogenin, a novel

microbial product, on embryonic and tumor cell-induced angiogenic responses in vivo. Anticancer Res. 17, 1881–1886.

Rao, J.S., Gondi, C., Chetty, C., Chittivelu, S., Joseph, P.A., Lakka, S.S., 2005. Inhibition of invasion, angiogenesis, tumor growth, and metastasis by adenovirus-mediated transfer of antisense uPAR and MMP-9 in non-small cell lung cancer cells. Mol. Cancer Ther. 4, 1399–1408.

Seno, H., Oshima, M., Ishikawa, T.O., Oshima, H., Takaku, K., Chiba, T., Narumiya, S., Taketo, M.M., 2002. Cyclooxygenase 2-and prostaglandin E(2) receptor EP(2)-dependent angiogenesis in Apc(Delta716) mouse intestinal polyps. Cancer Res. 62, 506–511.

Shieh, D.E., Chen, Y.Y., Yen, M.H., Chiang, L.C., Lin, C.C., 2004. Emodin-induced apoptosis through p53-dependent pathway in human hepatoma cells. Life Sci. 74, 2279–2290.

Sun, M.H., Han, X.C., Jia, M.K., Jiang, W.D., Wang, M., Zhang, H., Han, G., Jiang, Y., 2005. Expressions of inducible nitric oxide synthase and matrix metalloproteinase-9 and their effects on angiogenesis and progression of hepatocellular carcinoma. World J. Gastroenterol. 11, 5931–5937.

Tan, W.F., Lin, L.P., Li, M.H., Zhang, Y.X., Tong, Y.G., Xiao, D., Ding, J., 2003. Quercetin, a dietary-derived flavonoid, possesses antiangiogenic potential. Eur. J. Pharmacol. 459, 255–262.

Tsai, T., Chen, C., 1992. Ultraviolet spectrum identification of emodin in rabbit plasma by HPLC and its pharmacokinetics application. Asia Pac. J. Pharm. 7, 53–56.

Welsh, S.J., Bellamy, W.T., Briehl, M.M., Powis, G., 2002. The redox protein thioredoxin-1 (Trx-1) increases hypoxia-inducible factor 1alpha protein expression: Trx-1 overexpression results in increased vascular endothelial growth factor production and enhanced tumor angiogenesis. Cancer Res. 62, 5089–5095.

Yang, J., Li, H., Chen, Y.Y., Wang, X.J., Shi, G.Y., Hu, Q.S., Kang, X.L., Lu, Y., Tang, X.M., Guo, Q.S., Yi, J., 2004. Anthraquinones sensitize tumor cells to arsenic cytotoxicity in vitro and in vivo via reactive oxygen species-mediated dual regulation of apoptosis. Free Radic. Biol. Med. 37, 2027–2041.

Yao, J.S., Zhai, W., Young, W.L., Yang, G.Y., 2006. Interleukin-6 triggers human cerebral endothelial cells proliferation and migration: the role for KDR and MMP-9. Biochem. Biophys. Res. Commun. 342, 1396–1404.

Zhang, L., Hung, M.C., 1996. Sensitization of HER-2/neu-overexpressing non-small cell lung cancer cells to chemotherapeutic drugs by tyrosine kinase inhibitor emodin. Oncogene 12, 571–576.

Zhang, L., Chang, C.J., Bacus, S.S., Hung, M.C., 1995. Suppressed transformation and induced differentiation of HER-2/neu-overexpressing breast cancer cells by emodin. Cancer Res. 55, 3890–3896.

Zhang, L., Lau, Y.K., Xia, W., Hortobagyi, G.N., Hung, M.C., 1999. Tyrosine kinase inhibitor emodin suppresses growth of HER-2/neu-overexpressing breast cancer cells in athymic mice and sensitizes these cells to the inhibitory effect of paclitaxel. Clin. Cancer Res. 5, 343–353.

Zhou, X., Song, B., Jin, L., Hu, D., Diao, C., Xu, G., Zou, Z., Yang, S., 2006. Isolation and inhibitory activity against ERK phosphorylation of hydroxyanthraquinones from rhubarb. Bioorg. Med. Chem. Lett. 16, 563–568.