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Xanthorrhizol, a natural sesquiterpenoid from *Curcuma xanthorrhiza*, has an anti-metastatic potential in experimental mouse lung metastasis model

Min-Ah Choi[a,b], Seong Hwan Kim[a,1], Won-Yoon Chung[b], Jae-Kwan Hwang[d], Kwang-Kyun Park[a,b,c,*]

[a] *Brain Korea 21 Project for Medical Science, Yonsei University, Seoul 120-752, Republic of Korea*
[b] *Department of Oral Biology, Yonsei University, Seoul 120-752, Republic of Korea*
[c] *Oral Science Research Center, College of Dentistry, Yonsei University, Seoul 120-752, Republic of Korea*
[d] *Department of Biotechnology, Yonsei University, Seoul 120-752, Republic of Korea*

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Abstract

Xanthorrhizol is a sesquiterpenoid compound isolated from the rhizome of *Curcuma xanthorrhiza*. In this study, the anti-metastatic activity of xanthorrhizol was evaluated by using an in vivo mouse lung metastasis model and a tumor mass formation assay. Interestingly, xanthorrhizol dramatically inhibited the formation of tumor nodules in the lung tissue and the intra-abdominal tumor mass formation. Next, to examine the mechanism of the anti-metastatic action of xanthorrhizol in the mouse lung metastasis, expression patterns of the several intracellular signaling molecules were evaluated using the lung tissues with tumor nodules. Higher expression levels of cyclooxygenase-2 (COX-2), matrix metalloproteinase-9 (MMP-9), and phosphorylated extracellular signal-regulated kinase (ERK) were observed in the metastatic group compared with control, but these were attenuated by the treatment of xanthorrhizol. In conclusion, xanthorrhizol exerts anti-metastatic activity in vivo and this effect could be highly linked to the metastasis-related multiplex signal pathway including ERK, COX-2, and MMP-9.

Keywords: Xanthorrhizol; In vivo mouse lung metastasis; COX-2; MMP-9; ERK

One of the major causes of death in cancer patients is due to the ability of tumor cells to metastasize. Metastasis is a complex process that requires malignant cells to leave the primary tumor and proliferate at a distant site. This process includes separation from their primary site by penetrating the stromal tissue, circulation through the blood vessels or the lymph nodes, adhesion to the basement membrane, and invasion at the target organ of distinct metastasis [1–4]. Any drug that can inhibit one of the steps in the cascade will be useful in the inhibition of cancer metastasis [5].

Many studies have been trying to develop a new anticancer drug from natural products, which can prevent the metabolic activation of procarcinogens and inhibit or delay the processes of tumor initiation, promotion, and progression in vitro and/or in vivo [6]. *Curcuma* species, a medicinal plant in Indonesia, has been shown to exert diverse physiological functions [7–13]. Curcumin (diferuloylmethane), a major yellow pigment isolated from the rhizome of *Curcuma* species, has been reported to have the cancer chemopreventive activity by attenuating the over-expression of cyclooxygenase-2 (COX-2)

* Corresponding author. Fax: +82 2 364 7113.
E-mail address: biochelab@yumc.yonsei.ac.kr (K.-K. Park).
[1] Present address: Bio-Organic Science Division, Korea Research Institute of Chemical Technology, P.O. Box 107, Yuseong-gu, Daejeon 305-600, Republic of Korea.

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and the induction of inducible nitric oxide synthase (iNOS) activity in cancer cells [7]. Curcumin has inhibitory function towards a broad range of tumors such as mammary adenocarcinoma, forestomach, duodenal and colon cancer as well as 12-*O*-tetradecanoyl-13-phorbol ester (TPA)-induced skin tumors in mice [8–10]. In addition, curcumin prevented mouse pulmonary lung metastasis of B16F10 melanoma cells [11]. Pharmacologically, curcumin has been found to be safe and human clinical trials indicated no dose-limiting toxicity when administered at doses up to 10 g/day. All of these studies suggest that curcumin has enormous potential in the prevention and therapy of cancer [12,13].

Interestingly, xanthorrhizol, one of sesquiterpenoid compounds isolated from rhizome of *Curcuma* species, has been used as a folk medicine for treatment of various ailments, including rheumatic and stomachic remedy, and reported to have anti-microbial activity [14–16]. In addition, xanthorrhizol suppressed the activities of COX-2 and iNOS in mouse macrophage cells [17], and attenuated the induction of both COX-2 and iNOS genes in cisplatin-induced hepatotoxicity [18]. Although xanthorrhizol is isolated from the same species and has shown similar cellular action to attenuate the levels of COX-2 and iNOS as curcumin, the physiological function of xanthorrhizol on the cancer metastasis has not been studied yet.

Therefore, here we evaluated the effect of xanthorrhizol on cancer metastasis using an in vivo experimental mouse lung metastasis model. In addition, to investigate whether its effect is associated with the signaling cascade such as mitogen-activated protein (MAP) kinases including extracellular signal-regulated kinase (ERK), the c-Jun N-terinal kinases/stress-activated protein kinases (JNK/SAPK), and the p38 MAP kinase, and the activities of matrix metalloproteinases (MMPs), the Western blot analysis, and gelatin zymography were performed.

Materials and methods

Experimental animals. Six-week-old male Balb/c mice and C57BL6 mice (Korea Laboratory Animals, Taejon, South Korea) were used for the in vivo studies. They were housed in specific pathogen-free conditions and fed standard chow pellets and water ad libitum.

Cell culture. The murine colon cancer CT26 cells, murine melanoma B16BL6 and human colon cancer HT29 cells (Korean Cell Line Bank, Seoul, Korea) were maintained in Dulbecco's modified Eagle's media (DMEM) containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 (KOMA, Japan). For the in vivo experiments, the cells were detached using trypsin–EDTA for 1 min and washed twice with phosphate-buffered saline (PBS). The cells were then resuspended with Hanks' balanced salt solution (HBSS) and counted using a hematocytometer. All materials for cell culture were purchased from Life Technologies (NY, USA).

In vivo tumor growth assay. Twelve C57BL6 male mice were divided into four groups; control group ($n = 3$), cell alone-treated group ($n = 3$), and two groups of xanthorrhizol and cell-treated groups ($n = 3$ each). The control mice were treated with the same quantity of PBS without xanthorrhizol. The viable B16BL6 cells (2×10^5) were suspended in 0.2 ml HBSS and injected onto the peritoneal region of C57BL6 mice. The mice were treated daily for 2 weeks with an intraperitoneal (i.p.) administration of 0.2 and 0.5 mg/kg, BW of xanthorrhizol that was diluted with PBS (Fig. 2). Each mouse was sacrificed at the end of the treatment period and the weight of the melanoma mass on the intra-peritoneal region was measured.

In vivo experimental metastasis assay. To determine the inhibitory effects of xanthorrhizol (Fig. 1) a pulmonary colonization assay was carried out as described by Fidler [19]. Briefly, the CT26 cells were trypsinized, washed, and resuspended in HBSS at a concentration of 5×10^5 cells/ml. Aliquots of 200 μl were injected into the tail vein of 6-week-old male Balb/c mice using a 27-gauge needle. The Balb/c mice were divided into six groups; a control group ($n = 10$), a cell alone-treated group ($n = 10$), and four groups of xanthorrhizol (0.1, 0.2, 0.5, and 1.0 mg/kg, BW), and cell-treated groups ($n = 10$ each). Thirty minutes before the intravenous injection of the CT26 cells, the mice were pre-treated with PBS as a control and xanthorrhizol diluted with PBS, and then injected in the intra-peritoneal region with a daily dose (Fig. 2). Two weeks after tumor inoculation, the mice were sacrificed and the lobes of the lungs were separated and fixed overnight in a Bouin's solution. In addition, the total numbers of superficially visible colonies per lung were counted using an ocular micrometer. The lung tissues with tumor nodules were homogenized by grinding in liquid nitrogen and stored at −70 °C until used.

Western blot analysis. The total protein contents of the lung tissue with tumor nodules were determined using the BCA method. The collected tissues were lysed in 120 μl of an ice-chilled 1× RIPA buffer for 40 min. The lysates were centrifuged at 12,000*g* for 30 min, and aliquots of the supernatant containing 30 μg of the protein were boiled in a SDS sample-loading buffer for 5 min prior to electrophoresis on a 12% SDS–polyacrylamide gel. The blots were transferred from the SDS–polyacrylamide gel to a PVDF membrane (Amersham, IL, USA), blocked with a 5% fat-free dried milk-PBST buffer. The membranes were incubated with a 1:1000 dilution of murine COX-2 polyclonal and COX-1 monoclonal antibodies (Cayman Chemical, MI, USA), ERK/phosphorylated (p)-ERK, p38/p-p38, and JNK/p-JNK antibodies (Santa Cruz Biotechnology, CA, USA), and anti-β-actin and tubulin antibodies (Sigma, MO, USA) for 1 h at a room temperature. The blots were washed with PBST three times for 5 min and incubated with a 1:5000 dilution of anti-mouse horseradish peroxidase-conjugated secondary antibodies. After 1 h hybridization, the blots were washed three times with PBST and then developed with an ECL detection kit (Amersham, IL, USA), according to the manufacturer's

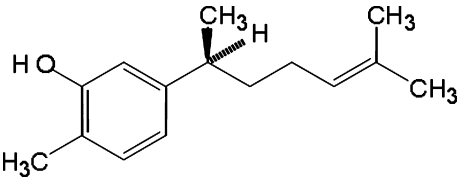

Fig. 1. Chemical structure of xanthorrhizol.

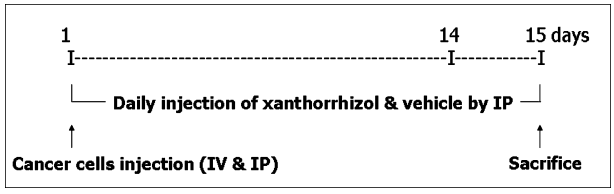

Fig. 2. Experimental design on the mouse lung metastasis model.

protocol. The densities of bands were measured by RFLP scan version 2.1 software program (Scanalytics, USA).

Gelatin zymography. The proteins (10 μg) of tumor invaded lung homogenates were mixed with a non-reducing sample buffer containing 0.5 M Tris (pH 6.8), 5% SDS, 20% glycerol, and 1% bromophenol blue in a 1:1 ratio. The samples were loaded onto the 10% SDS–polyacrylamide gels (SDS–PAGE) co-polymerized with 0.2% gelatin (Sigma, MO) and electrophoresed under non-reducing conditions. After electrophoresis, the gels were washed twice for 30 min at room temperature in a 2.5% (v/v) Triton X-100 solution, and incubated for 18 h in a zymogram incubation buffer (50 mM Tris–HCl (pH 7.5), 10 mM $CaCl_2$, and 0.15 M NaCl) at 37 °C. The gels were stained for 30 min with 0.25% (w/v) Coomassie Brilliant Blue R250 (Bio-Rad, CA, USA) in 10% (v/v) glacial acetic acid and destained with 10% (v/v) glacial acetic acid/40% (v/v) isopropyl alcohol. The proteolytic activity of MMP was detected as a clear band against a blue background. The serum-starved medium of the HT1080 cells, which is known to release MMP-2 and MMP-9 into the culture medium, was used as the standard control.

Statistical analysis. Statistical analysis of the data was performed using a Student's *t* test. The *p*-value $\leqslant 0.05$ was considered significant.

Results

First, the effect of xanthorrhizol on metastasis was investigated using tumor mass formation model. The C57BL6 mice were inoculated with 2×10^5 B16BL6 melanoma cells and then treated with xanthorrhizol (0.2 and 0.5 mg/kg, BW/day) for 2 weeks by i.p. administration. In the mice injected with cells alone, the melanoma mass was produced in the whole peritoneal region, but the treatment of xanthorrhizol suppressed the formation of intra-abdominal tumor mass in a dose-dependent manner (71% and 97% reduction; 0.2 and 0.5 mg/kg, BW/day of xanthorrhizol, respectively) (Fig. 3).

Next, the effect of xanthorrhizol on lung metastasis was investigated. The CT26 cells were injected into the tail vein of the Balb/c mice, and the mice were treated with xanthorrhizol (0.1, 0.2, 0.5, and 1.0 mg/kg, BW/day) by daily i.p. administration for 2 weeks. In cells-injected mice, 500 tumor nodules were colonized at lung, but the formation of tumor nodules was significantly attenuated by the treatment of xanthorrhizol (0.1, 0.2, 0.5, and 1.0 mg/kg, BW/day xanthorrhizol-treated group; 36%, 63%, 61%, and 52% reduction, respectively) as shown in Fig. 4. There was neither change in body weight nor any other adverse effects. Thus, no lethality of mice was observed during the experimental period.

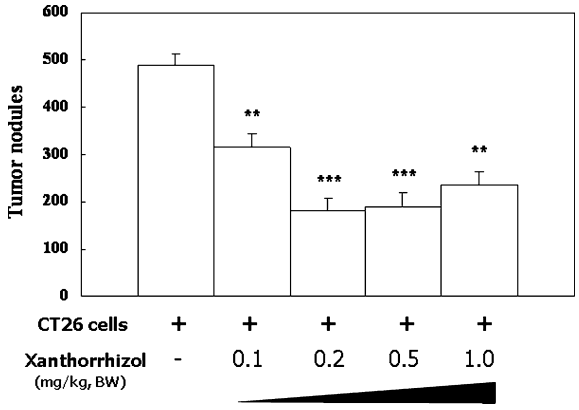

Fig. 4. The effect of xanthorrhizol on the formation of tumor nodules in the lung metastasis induced by CT26 cells. The mice were treated daily with xanthorrhizol for 2 weeks by an i.p. injection after the CT26 cell inoculation. The mice were sacrificed and the lung tissues with tumor nodules were removed. A number of pulmonary nodules were stained with Bouin's solution and counted using an anatomical microscope. The result was represented a number of tumor nodules in the individual mice. Statistical analysis was performed using a Student's *t* test and differences were considered significant at $p < 0.005$ (**) and $p < 0.0001$ (***), compared with the cells-alone injected group.

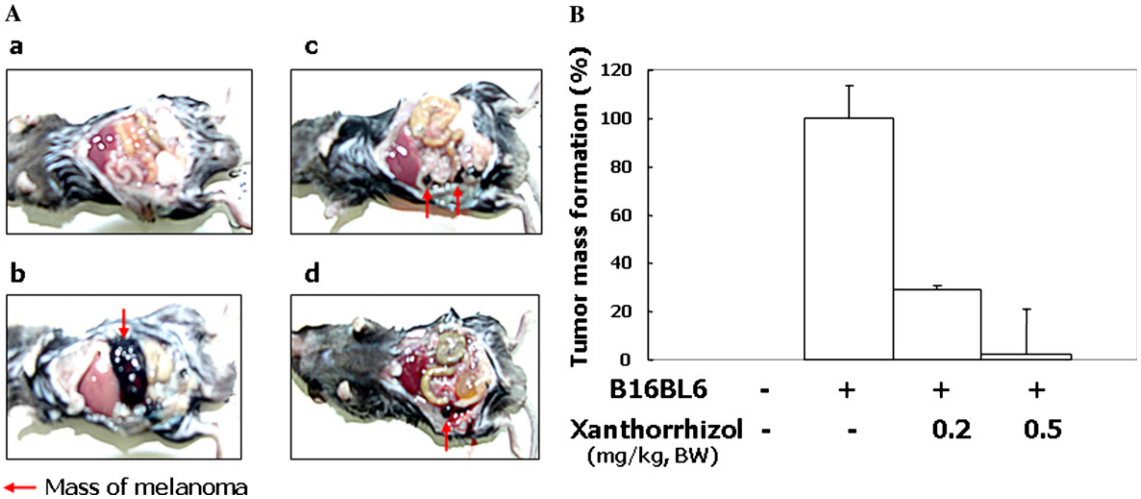

Fig. 3. The effect of xanthorrhizol on the tumor mass induced by an i.p. injection of B16BL6 melanoma cells in C57BL6 mice. The mice were treated daily with xanthorrhizol for 2 weeks by an i.p. injection after the inoculation of B16BL6 cells. The mice were sacrificed and the tumor was collected in the peritoneal region. (A) Arrows indicated the tumor mass in the peritoneal region: a, control group; b, cell alone-treated group; c, cell + 0.2 mg/kg, BW xanthorrhizol-treated group; d, cell + 0.5 mg/kg, BW xanthorrhizol-treated group. (B) The result was represented a percent of tumor mass weight to body weight of individual mice.

In addition, to investigate whether its effect is associated with the metastasis-related molecules such as COX-2 and MMPs, and signaling cascade such as MAP kinases, Western blot analysis and gelatin zymography were performed.

The expression of COX-2 protein in lung was highly induced in CT26 cells-injected mice, however, the xanthorrhizol treatment has markedly attenuated COX-2 expression as shown in Fig. 5A. This attenuation could be clarified by the ratio of COX-2/COX-1 (Fig. 5B). In cells-injected mice, COX-2/COX-1 ratio was increased over 1.5-fold compared to that observed in control mice, but it was attenuated at the basal level when treated with xanthorrhizol. The relative COX-2/COX-1 ratios in control mice, cells-injected mice, and 0.2, 0.5, and 1.0 mg/kg body weight xanthorrhizol-treated mice were 1, 1.65, 0.94, 0.84, and 0.93, respectively. The effect of xanthorrhizol on the expression of COX-2/COX-1 did not take place in a dose-dependent manner.

Densitometric analysis of the zymogram has shown that the degradation of gelatin substrate by MMP-9 had strongly induced MMP-9 activity in the lung metastasized group (Fig. 6). However, the increased level of MMP-9 activity was markedly attenuated by the treatment of xanthorrhizol. In this study, MMP-2 was not detected.

To further investigate the signaling mechanism for the functional action of xanthorrhizol in the lung metastasis model, the expression/activation of MAP kinases was evaluated using specific antibodies. As shown in Fig. 7, although the protein expression levels of ERKs were not changed compared with control, the phosphorylation of ERKs was strongly induced in cells-injected group. However, the ERK phosphorylation was attenuated by the treatment of xanthorrhizol in a dose-dependent manner. The expression levels of JNK and p38, the other subfamily members of MAP kinases, were induced in cells-injected group, and these proteins' expression was attenuated by the treatment of xanthorrhizol in a dose-dependent manner. The phosphorylation of JNK2 was induced in cells-injected group, but it was higher in 0.2 mg/kg, BW xanthorrhizol-treated group. Its induction showed to be slightly attenuated in 0.5

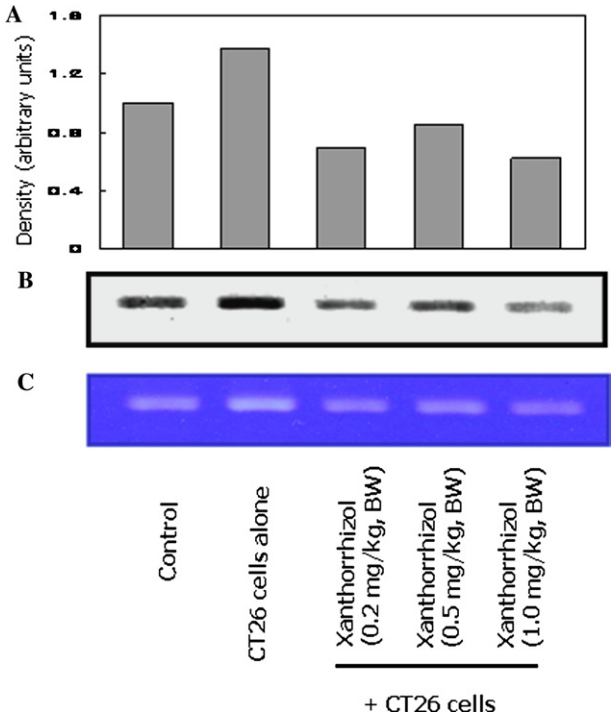

Fig. 6. The effect of xanthorrhizol on the MMP-9 expression in the lung metastasis induced by murine CT26 cells. The lung tissue homogenates were used to evaluate the expression levels of MMP-9 as described under Materials and methods. (A) The graph represents the relative density of the MMP-9/β-actin, as determined by densitometer. (B) Reverse image of gelatin zymogram of MMP-9. (C) Gelatin zymogram of MMP-9.

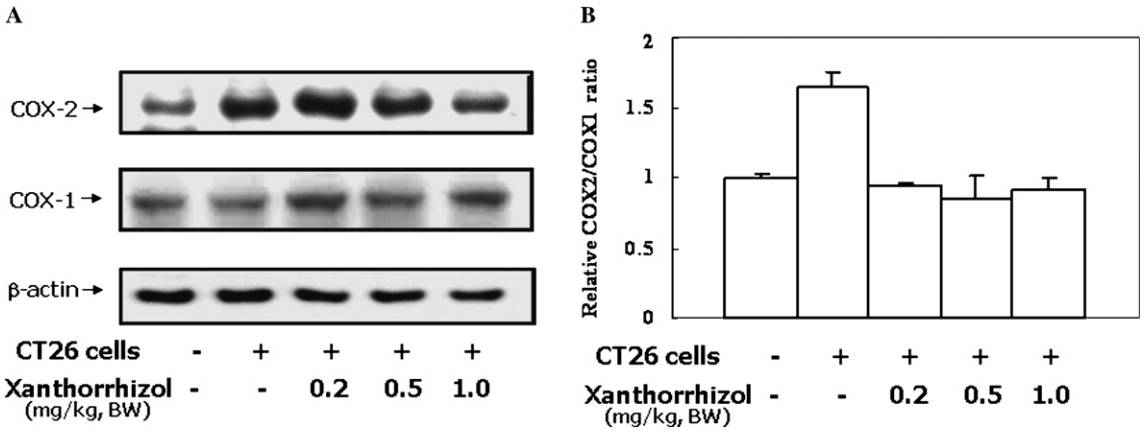

Fig. 5. The effect of xanthorrhizol on the expression of COX-2 and COX-1 in the lung metastasis induced by murine CT26 cells. The lung tissue homogenates were used to evaluate the expression levels of COXs as described under Materials and methods. (A) The Western blot images of COXs were represented. The β-actin was used as a control protein. (B) The relative COX-2/COX-1 ratio represented the level of COX-2 normalized to the relative COX-1/β-actin ratio.

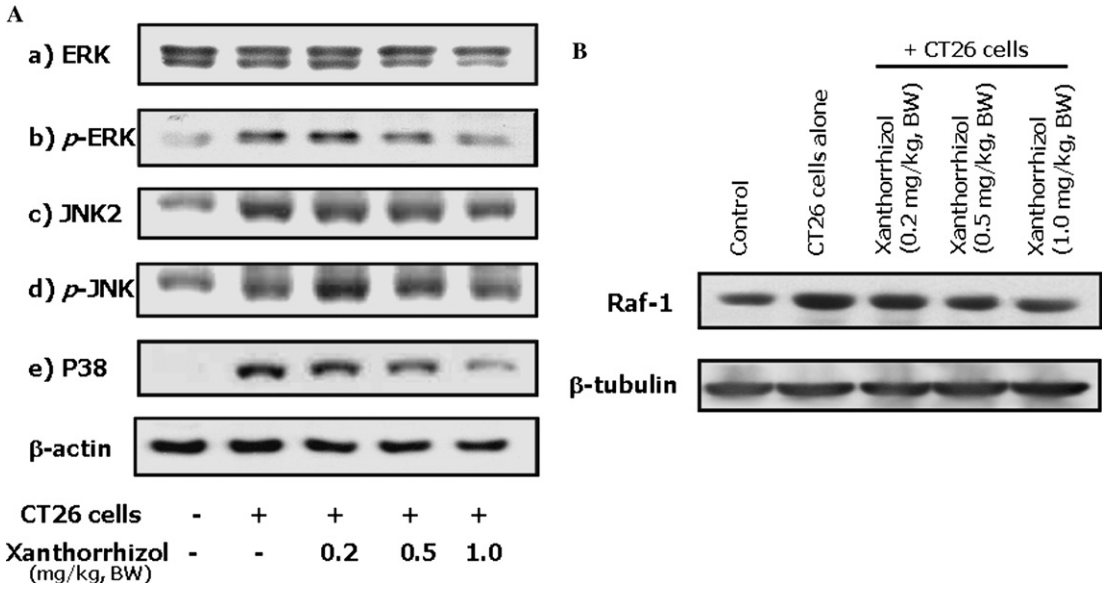

Fig. 7. The effect of xanthorrhizol on the expression of the MAP kinases in the lung metastasis induced by CT26 cells. The lung tissue homogenates were used to evaluate the expression levels of MAP kinases as described under Materials and methods. (A) The Western blot analysis for (a) the ERK, (b) the *p*-EKR, (c) the JNK, (d) the *p*-JNK), and (e) the p38. The β-actin was used as control protein. (B) The expression level of Raf-1. The tubulin was used as a control protein.

and 1.0 mg/kg, BW xanthorrhizol-treated groups, but its levels were higher than that in cells-injected group. No phosphorylated form of p38 was detected in any of the groups (data not shown). The protein expression of Raf-1, which can trigger the activation of ERK via the activation of MEK, was induced in cells-injected group, but it was also attenuated by the treatment of xanthorrhizol as similar with the expression pattern of the phosphorylated ERKs (Fig. 7C).

Discussion

Metastasis is the main factor causing the death of cancer patients. Recently, as part of a cancer chemoprevention study, the anti-metastatic effects of curcumin and cathechin in mice and the inhibitory effect of polyphenolic compounds on a pulmonary metastasis of B16F10 have been reported [11,20]. Although xanthorrhizol, one of sesquiterpenoid compounds isolated from rhizome of *Curcuma* species, has been reported to have a potential to regulate the expressions/activities of COX-2 and iNOS, which are highly linked to metastasis processes as well as inflammation [17,18], the effect of xanthorrhizol on metastasis has not been studied yet. Therefore, the effect of xanthorrhizol on metastasis processes was investigated in this study.

First of all, the effect of xanthorrhizol on the metastasis was evaluated using relatively short-term experimental animal model. The Balb/c mice were inoculated with colon cancer CT26 cells, which can induce a tumor in the lung, and then i.p. administrated with PBS or xanthorrhizol for 2 weeks. Interestingly, CT26 cells eventually induced the tumor nodules in lung, but the formation of tumor nodules was significantly attenuated by the treatment of xanthorrhizol in a dose-dependent manner. This demonstrates that xanthorrhizol has a potential to inhibit the metastatic activity of tumor cells. Moreover, the intra-abdominal melanoma mass formation has been abrogated by the treatment of xanthorrhizol, demonstrating that xanthorrhizol has an inhibitory activity on tumor growth as well as anti-metastatic activity in vivo. In the previous studies, the effects on the anti-tumor and anti-tumor promoter activity of sesquiterpenoids isolated from *Curcuma xanthorrhiza* have been reported [21,22]. In addition, xanthorrhizol exhibited the anti-proliferation of colon cancer cells, CT26 and HT29, in vitro and had a potential to increase the apoptosis-related protein, Caspase 9, Bid, and GSK3β, in our experimental mouse lung metastasis model (data not shown). These results suggest that xanthorrhizol may exert its anti-cancer effects on tumor cells directly or have a potential to induce apoptosis of cancer cells in part. Therefore, the anti-metastatic activity of xanthorrhizol shown in our experiments could be related to its pro-apoptotic potential for cancer cells. In addition, considering that curcumin isolated *Curcuma* species like as xanthorrhizol has been reported to have an anti-metastatic activity [7–11], this study presented strongly that *Curcuma* species have an opportunity to be developed as the new anti-cancer drug.

The mechanism of the anti-metastatic action of xanthorrhizol shown in mouse lung metastasis model could be highly linked to the molecules such as COX-2

and MMP-9, which were highly upregulated by the tail vein injection of tumor cells, however, their expression was attenuated by the treatment of xanthorrhizol.

Many studies on the close relationship between the expression of COX-2 and cancer have been reported [23–29]. COX-2 is over-expressed in malignant cancers of colon, breast, prostate, lung, and liver [30], and it also causes cancer when over-expressed in transgenic mice [31]. Additionally, COX-2 induced the resistance of cancer cells leading to resistance to apoptosis and increased the proliferation of cancer cells [24–26], and the over-expression of COX-2 protein induced invasion and metastasis of cancer cells [23,27–29]. However, the selective COX-2 inhibitor, celecoxib, inhibited the growth of ectopically implanted Lewis lung carcinoma cells and HT29 colon cancer cells and lung metastasis of HT29 cells [32].

MMPs produced by cancer cells play a pivotal role in the degradation of the basement membrane, which is essential for the invasiveness of cancer cells. It has been reported that cancers in lungs, ovary, prostate, breast, and pancreas with high activities of MMPs possess the highly invasive and metastatic potentials [33–36]. Among several MMPs, MMP-9 (gelatinase B, type IV collagenase) is one of important molecules in cancer metastasis since gelatinase-deficient mice have shown to inhibit metastasis [37,38].

The metastasis-related genes have been well-known to be regulated via MAP kinase signaling. MAP kinases are of the serine/threonine protein kinase family and generally divided into the ERK, JNK, and p38. These enzymes are regulated by a characteristic phosphorelay system in which a series of three protein kinases phosphorylate and activate one another [39]. Activated ERK pathway is functionally related with in vivo metastasis, in vitro invasion, and the production of MMP [40–42]. Likewise, the other studies have reported that ERK activity appears to be required for induction of COX-2 by Ras [43]. And it was clinically reported that there is a positive relation between ERK and lymph node metastasis in several cancers [44,45]. In this study, xanthorrhizol attenuated the cancer cell-induced expression of phosphorylated ERK and unphosphorylated p38 in a dose-dependent manner, but not JNK. These results suggested that ERK and p38 pathway could be responsible for the biological effect of xanthorrhizol on the suppression of CT26 cell metastasis. This involvement of ERK pathway could be supported by showing the suppression of the over-expressed Raf-1, which can trigger the activation of ERK via the activation of MEK, by the treatment of xanthorrhizol. However, contrary to our expectations, the expression of *p*-JNK was induced by low-dose xanthorrhizol. This could be observed by the effect of metastasized-tumor cells, not that of xanthorrhizol. In addition, this could be explained by the reports showing that the activation of JNK has a dual function for the inducing/inhibiting apoptosis of tumor cells [46–48], and recent study showing that the JNK pathway was activated in apoptotic cells and then apoptotic cells could induce compensatory cell proliferation through the JNK signaling pathway [48].

Taken together, xanthorrhizol possesses the anti-metastatic activity in vivo and its effect could be linked to the expression of COX-2 and the activity of MMP-9. In addition, the modulation in MAPK/ERK pathway may be, at least in part, an important signal cascade to inhibit the mouse lung metastasis by xanthorrhizol. Finally, because *Curcuma* species has been used traditionally and its safety as a food ingredient has been proven for a long time, xanthorrhizol might be developed as a chemopreventive agent or anti-cancer agent.

Acknowledgments

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