A flavonoid chrysin suppresses hypoxic survival and metastatic growth of mouse breast cancer cells

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Abstract. Tumor hypoxia commonly occurs in solid tumors, and correlates with metastasis. Current cancer therapies are inefficient in curing metastatic disease. Herein, we examined the effect of Thai propolis extract and its major constituent, chrysin, on hypoxic survival of 4T1 mouse breast cancer cells in vitro, and investigated its underlying mechanism. In vivo effect of chrysin on metastatic progression of cancer cells was studied, both as a single agent and in combination with another antimetastatic agent, agonistic monoclonal antibody targeting the DR5 TRAIL receptor (DR5 mAb). Thai propolis extract and chrysin decreased survival of 4T1 cells after exposure to hypoxia (1% O\textsubscript{2}), for 2 days. Immunoblot analysis revealed that chrysin inhibited hypoxia-induced STAT3 phosphorylation without affecting HIF-1\textbeta protein level. Chrysin also abrogated hypoxia-induced VEGF gene expression as determined by qRT-PCR. The in vivo effect of chrysin was determined in a spontaneous metastasis mouse model of breast cancer, either alone or in combination with DR5 mAb. Daily oral administration of chrysin in Balb/c mice implanted with 4T1 cells significantly suppressed growth of lung metastatic colonies. Moreover, antimetastatic activity of DR5 mAb was enhanced when given in combination with chrysin. We demonstrate that chrysin has potential in controlling metastatic progression.

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Introduction

Metastasis is the spread of cancer in the body, by which cancer cells in the primary tumor travel to distant sites, and form metastatic colonies. Most cancer deaths are caused by the growth of metastases to form clinically harmful tumors, rather than primary tumor growth. Since current cancer therapies are inefficient in eradicating metastases, any strategy that can prevent metastatic progression or inhibits the growth of metastases in distant sites benefit patients (1). An agonistic monoclonal antibody (DR5 mAb) targeting a receptor of endogenous death ligand (DR5 TRAIL receptor) can reduce the number of metastatic colonies in mouse models (2). However, the effect of combining DR5 mAb with other anti-metastatic natural products has not been investigated.

A unique feature of solid tumors is the occurrence of low oxygen levels (hypoxia) in some regions within the tumor as it grows. This has received much attention as an attractive target for anticancer drug development (3). Clinical studies indicate correlation between tumor hypoxia, tumor progression and development of distant metastases (4,5). The presence of hypoxia is not restricted to the primary tumor, but also appears in metastatic sites. Increasing evidence obtained from various animal models indicates avascular micrometastases (<1 mm in diameter) are hypoxic, while macrometastases (1-4 mm in diameter) are vascularized and not hypoxic (6-8). Tumor-induced angiogenesis is known to contribute to metastasis promotion and growth of micrometastases (9). Vascular endothelial growth factor (VEGF) is one of the most potent angiogenic factors, and its production is upregulated in hypoxic cells, further inducing angiogenesis in hypoxic tissues (10). Hypoxic adaptation is mediated by hypoxia-inducible factor (HIF)-1 which is a heterodimeric transcription factor composed of HIF-1\textalpha and HIF-1\textbeta subunits. Although HIF-1 is the master regulator of hypoxic response, other signaling pathways including STAT3, Akt, ERK, and NF-\textkappaB, are also activated...
by hypoxia and participate in hypoxia-induced VEGF gene expression (10,11). VEGF also acts as an autocrine survival factor for breast cancer cells under hypoxia (12). Moreover, inhibition of VEGF gene expression in breast cancer cells can suppress metastasis in a mouse model (13).

Propolis (bee glue) is a resinous material that honeybees collect from various plants, and is a rich source of bioactive compounds. Several HIF-1 modulating compounds have been isolated from Brazilian green propolis, indicating the potential of propolis as a source of hypoxic modulating compounds (3). We have isolated a flavonoid chrysin (5,7-dihydroxyflavone) from Thai propolis extract as a major constituent. Chrysin has also been shown to possess multiple bioactivities. Inhibition of subcutaneous tumor growth by chrysin has recently been demonstrated in mouse models of breast cancer and hepatoma (14,15). Chrysin has also been shown to suppress hypoxia-induced VEGF expression in human prostate cancer cells in vitro (16), and abrogates tumor-induced angiogenesis in vivo (15,16). However, until now, there has been no report on the effect of chrysin on hypoxic survival and metastatic progression of cancer cells.

In this study, we demonstrate the inhibitory effect of chrysin on hypoxic survival of mouse breast cancer cells in vitro, and also investigate its mechanism of action. We also studied the in vivo effect of chrysin in a spontaneous metastasis model of breast cancer, both as a single agent and in combination with DR5 mAb.

Materials and methods

Chemicals and antibodies. Protease inhibitor cocktail for use with mammalian cells and tissue extracts was obtained from Sigma-Aldrich (St. Louis, MO, USA). Chrysin was purchased from Alexis Biochemicals (San Diego, CA, USA). Specific inhibitors of STAT3 (Cucurbitacin-I), PI3K (LY294002), ERK (PD98059), and NF-κB (IKK-2 inhibitor IV) were obtained from Calbiochem (San Diego, CA, USA). For in vitro experiments, test compounds were dissolved in dimethyl sulfoxide (DMSO) and kept as a stock solution at -20˚C. The final concentration of DMSO was kept below 0.2% throughout the study. Primary antibodies specific to Tubulin, STAT3, Akt, ERK1/2, and phosphorylated forms of STAT3, ERK (Thr202/Tyr204) were purchased from Cell Signaling Technology (Beverly, MA, USA). Primary antibody specific to HIF-1α (H1a67) was obtained from Novus Biologicals (Littleton, CO, USA). Agonistic monoclonal antibody targeting mouse DR5 TRAIL receptor (DR5 mAb or MD5-1) was obtained from the Department of Immunology, Juntendo University School of Medicine, Japan.

Propolis extraction and isolation of compounds. Thai propolis was harvested from a hive of Apis mellifera collected in Chiangmai province, Thailand, in August 2006. A voucher specimen (SWU 0212) was deposited at the Faculty of Pharmacy, Srinakharinwirot University, Nakhon Nayok province, Thailand. Two major compounds, chrysin and tectochrysin, were isolated. Propolis (1 kg) was extracted with methanol (MeOH) under sonication yielding 517 g extract. Part of the extract (140 g) was fractionated by silica gel column chromatography using dichloromethane (CH₂Cl₂), with increasing concentration of MeOH, to obtain 16 fractions. Fraction 5 (3.0 g, 1-1.5% MeOH-CH₂Cl₂ eluate) and fraction 8 (9.5 g, 2% MeOH-CH₂Cl₂ eluate) was left overnight to obtain the crystals of tectochrysin (314 mg) and chrysin (732 mg), respectively. Their structures were identified by comparison of physical and spectroscopic data with authentic standard and the published data (17,18).

Cell culture. 4T1 murine mammary carcinoma cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in DMEM supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS) and antibiotics, and maintained at 37˚C in a humidified atmosphere of 5% CO₂; these conditions were used as normoxic condition (21% O₂). For hypoxic condition (1% O₂), the cells were incubated at 37˚C, in a humidified atmosphere of 1% O₂ and 5% CO₂ maintained by a regulated supply of N₂ and CO₂ gas inside a hypoxic chamber (C-Chamber with ProOX and ProCO₂ controllers, Biospherix, NY, USA).

Hypoxic cell survival assay. Cells were seeded into 96-well plates (5x10³ cells/well) and left overnight in a CO₂ incubator. The cells were pretreated by adding medium containing test compound to the wells, and incubated for 30 min, then the plates were further incubated under hypoxia (1% O₂) for 48 h. Number of surviving cells after treatment was determined by crystal violet staining method as previously described (19). Briefly, the cells were washed three times with phosphate buffer saline (PBS), fixed with 95% ethanol, and stained with crystal violet solution (0.5% w/v in 25% MeOH). After extensive washing by tap water, the stained cells were lysed with 0.1 N HCl in MeOH, after which absorbance was determined at 550 nm using a microplate reader. Assay was performed in triplicate wells, and results were expressed as percent survival compared with control (DMSO).

Immunoblot analysis. After treatment, cells were rinsed with cold PBS, scraped and lysed in RIPA lysis buffer containing phosphatase inhibitors (20 mM β-glycerophosphate, 1 mM Na₃VO₄, 10 mM NaF) and protease inhibitor cocktail. Cell lysates were fractionated by electrophoresis in 7.5% or 10% SDS-PAGE, and electrophoretically transferred to PVDF membrane (Immobilon-P nylon, Millipore, Bedford, MA, USA). After blocking with BlockAce (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan), the membranes were probed with the indicated primary antibodies overnight, followed by horseradish peroxidase-conjugated secondary antibodies. Bands were visualized using WesternBright ECL reagent (Advansta, Menlo Park, CA, USA).

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). RNA extraction and first-strand cDNA synthesis were performed as previously described (20). Real-time PCR was run on a LightCycler® 2.0 Instrument (Roche), using QuantiTect™ SYBR Green PCR Master mix (Qiagen, Valencia, CA, USA). Sequences of PCR primers were obtained from following references; mouse VEGF (21); mouse β-actin (22). VEGF primer (fwd) 5'-GAGATGAGCTTCCTAAGATGAC-3'; and VEGF primer (rev) 5'-TCACCGCCTTGGAGAGAC-3'.
groups was analyzed by analysis of variance (ANOVA), and the in vivo determine the significance of differences between groups. For as mean ± SD, and analyzed by Student’s two-tailed t-test to Statistical analysis.

mean ± SD of 10 mice per group. total volume of lung metastatic colonies. Data are expressed as and fixed in 10% formalin. After sacrificing the mice on day 32, tumors and metastatic colonies was measured using a caliper inoculation. Primary tumors were excised on day 18, weighed inoculation. Primary tumors were excised on day 18, weighed and fixed in 10% formalin. After sacrificing the mice on day 32, lungs were collected and fixed in Bouin's solution (0.9% picric acid, 9% formaldehyde, 5% acetic acid). Number of metastatic colonies on surface of the lungs was counted. Size of primary tumors and metastatic colonies was measured using a caliper. Daily oral administration (p.o.) of chrysin suspended in 0.5% carboxymethyl cellulose began on day 1 after tumor inoculation. DR5 mAb (10 µg/mouse) was injected intraperitoneally (i.p.) on day 0. Tumors were measured every other day (length and width) using a caliper. Pretreatment with inhibitors for 30 min before exposure to hypoxia (1% O \(_2\)) for 2 days. Number of surviving cells after treatment was determined by crystal violet staining method. Data are expressed as mean ± SD from three independent experiments, and significant differences from control are shown by ***p<0.001. (B) Chemical structure of chrysin and tectochrysin.

Spontaneous metastasis mouse model. Balb/c mice (6-week-old female) were purchased from Sankyo Lab Service Co. (Hamamatsu, Japan). Experiments were conducted in accordance with institutional guidelines and approval of University of Toyama Animal Experimental Ethics Committee. Cell suspensions (5x10\(^5\) cells) were injected into mammary fat pads on day 0. Tumors were measured every other day (length and width) using a caliper. Daily oral administration (p.o.) of chrysin suspended in 0.5% carboxymethyl cellulose began on day 1 after tumor inoculation. DR5 mAb (10 µg/mouse) was intraperitoneally (i.p.) injected on day 10 and day 14 after tumor inoculation. Primary tumors were excised on day 18, weighed and fixed in 10% formalin. After sacrificing the mice on day 32, lungs were collected and fixed in Bouin's solution (0.9% picric acid, 9% formaldehyde, 5% acetic acid). Number of metastatic colonies on surface of the lungs was counted. Size of primary tumors and metastatic colonies was measured using a caliper square along the longer axes (a) and shorter axes (b), and tumor volume was calculated by the following formula; tumor volume (mm\(^3\)) = ab\(^2\)/2. The volume of metastatic colonies was categorized into 3 sizes; small (<0.5 mm\(^3\)), medium (0.5-1.5 mm\(^3\)), and large (>1.5 mm\(^3\)). Metastatic burden was determined from total volume of lung metastatic colonies. Data are expressed as mean ± SD of 10 mice per group.

Statistical analysis. For in vitro experiments, data are expressed as mean ± SD, and analyzed by Student's two-tailed t-test to determine the significance of differences between groups. For in vivo experiments, the significance of differences between groups was analyzed by analysis of variance (ANOVA), and the means were compared by using Tukey, with a p-value <0.05 being considered significant.

Results

Inhibitory effect of Thai propolis extract and chrysin on hypoxic survival of breast cancer cells. We first examined the effect of Thai propolis extract against hypoxic survival of 4T1 cells. Pretreatment with propolis extract (10-100 µg/ml) for 30 min before exposure to hypoxia (1% O \(_2\)) for 2 days decreased hypoxic cell survival in a concentration-dependent manner (Fig. 1A). Next, the two major constituents present in Thai propolis extract, chrysin and tectochrysin (Fig. 1B) were examined. Chrysin treatment showed a concentration-dependent decrease in hypoxic cell survival, with 28-74% inhibition observed at 40-100 µM chrysin, while tectochrysin did not show inhibition at the highest soluble concentration (60 µM) (Fig. 1C). These results indicate that chrysin contributes to the inhibitory effect of Thai propolis extract on hypoxic cell survival.

Effect of chrysin on hypoxia-induced activation of multiple cell signaling pathways and VEGF expression. We further explored effect of chrysin on hypoxia-induced activation of multiple cell signaling pathways that contribute to cell survival, including HIF-1, STAT3, Akt, ERK, and NF-κB. 4T1 cells were pretreated with 60-100 µM chrysin for 30 min before exposure to hypoxia for 6-24 h. HIF-1α protein stabilization and hypoxia-induced phosphorylation of ERK and p65 NF-κB were not inhibited by chrysin treatment (Fig. 2A). In contrast, hypoxia-induced STAT3 tyrosine phosphorylation was markedly inhibited by chrysin, in a dose- and time-dependent manner, while marginal decrease in Akt phosphorylation was observed only at the highest concentration test (100 µM) (Fig. 2A), suggesting that STAT3 was primary target of chrysin rather than Akt.

Specific inhibitors of STAT3 (cucurbitacin-I, 2 µM), PI3K (LY294002, 25 µM), ERK (PD98059, 25 µM), and NF-κB (IκK-2 inhibitor IV, 10 µM), were used to evaluate the contribution of the signaling pathways on hypoxic survival of 4T1 cells. Pretreatment with inhibitors for 30 min before hypoxic exposure for 2 days revealed that hypoxic cell survival was
inhibited by inhibitors of STAT3, Akt, and NF-κB pathways, while ERK inhibitor showed marginal effect (Fig. 2B). These results indicate the important role of STAT3, Akt, and NF-κB pathways in hypoxic survival of 4T1 cells. Moreover, STAT3 inhibitor treatment mimics the effect of chrysin, suggesting that STAT3 inhibition is the mechanism underlying inhibitory effect of chrysin on hypoxic survival of 4T1 cells.

VEGF has been shown to be an autocrine survival factor for breast cancer cells under hypoxia (12). We examined the effect of chrysin on hypoxia-induced VEGF gene expression in 4T1 cells, in comparison to a STAT3 inhibitor (cucurbitacin-I). The cells were pretreated with chrysin (60 µM) or STAT3 inhibitor (2 µM) for 30 min before exposure to hypoxia for 24 h. qRT-PCR analysis revealed that hypoxia increased VEGF mRNA levels in 4T1 cells by 16-fold higher than normoxic cells (Fig. 2C). Chrysin decreased VEGF mRNA levels in hypoxic 4T1 cells, similar to that observed with STAT3 inhibitor treatment, by 71 and 81% inhibition, respectively, compared with control hypoxic cells (Fig. 2C). The reduction of VEGF expression by chrysin and STAT3 inhibitor was correlated with decrease in STAT3 tyrosine phosphorylation levels under hypoxia (Fig. 2D). Taken together, the results indicate that STAT3 inhibition is likely to be a mechanism of action of chrysin for decreasing hypoxia-induced VEGF expression in 4T1 cells, and this might contribute to the effect of chrysin on hypoxic survival.

Chrysin suppresses growth of lung metastatic colonies in vivo. Since hypoxia and angiogenesis contribute to tumor progression and metastatic formation (7), chrysin was further studied in 4T1 spontaneous metastasis model. The experimental treatment protocol (Fig. 3A) did not affect body weight of the mice (data not shown). The effect of treatments was evaluated from both primary tumor growth and metastatic burden. Chrysin (100 and 250 mg/kg daily) had no significant effect on primary tumor growth (Fig. 3B and C). However, metastatic burden was largely decreased by chrysin, in a dose-dependent manner, and significant suppression was observed with the highest dose of
chrysin treatment (Fig. 3D). Reduction of metastatic burden might result from either decrease in metastatic rate (number of metastatic colonies) or metastatic growth (size of metastatic colonies), or both. Chrysin tended to decrease metastatic rate but without statistical significance (Fig. 3E). Suppressive effect of chrysin on metastatic growth was observed in a dose-dependent manner, and significant reduction was clearly observed with large size metastatic colonies (Fig. 3F). These results reveal the antimetastatic activity of chrysin in vivo by inhibiting the growth of metastatic colonies.

Chrysin potentiates antimetastatic activity of the DR5 agonistic mAb. In addition to therapeutic effect on primary tumor, DR5 mAb also expresses antimetastatic activity in mouse models (2). We evaluated the potential of chrysin as an adjuvant for antimetastatic activity of DR5 mAb. Since 4T1 cells are sensitive to apoptosis induced by DR5 mAb, low dose treatment of DR5 mAb (10 µg/mouse, x2) was used instead of therapeutic doses (200 µg/mouse, x3) used previously (2), in order to avoid metastasis reduction by massive elimination. DR5 mAb alone significantly suppressed primary tumor growth as shown by reduction in primary tumor size and weight, compared with control (Fig. 4B and C). Combination of DR5 mAb + chrysin (250 mg/kg/d) tended to be more suppressive, but no statistical difference between DR5 mAb alone and the combination treatment was observed (Fig. 4C). Antimetastatic activity of DR5 mAb was
revealed by significant decrease in metastatic burden and metastatic rate, and the combination treatment resulted in a significantly greater effect (Fig. 4D and E). DR5 mAb also expressed suppressive effect on metastatic growth, as shown by significant decrease in number of large size metastatic colonies, with greater effect being observed in combination treatment (Fig. 4F). Moreover, the effect of DR5 mAb on the number of small size metastatic colonies was significantly potentiated when DR5 mAb was combined with chrysin (Fig. 4F). Collectively, the results suggested that chrysin per se had a suppressive effect on metastatic growth, and could enhance antimetastatic activity of DR5 mAb in combination treatment.

Discussion

In the present study, we demonstrate the ability of chrysin to decrease hypoxic survival of cancer cells, by inhibiting hypoxia-induced STAT3 activation and VEGF expression. We also provide in vivo evidence of the suppressive effect of chrysin on metastatic growth, and a potentiating effect of chrysin when combined with another antimetastatic agent, DR5 mAb. To our knowledge, this is the first study on the antimetastatic effect of chrysin in a spontaneous metastasis mouse model.

Increased VEGF production has been shown to play a role in hypoxic survival of 4T1 cells, and treatment with VEGF neutralizing antibody can decrease hypoxic survival rate of cells (12).
Consistent with previous studies, our results showed that treatment with either chrysin or STAT3 inhibitor (cucurbitacin-I), which inhibited hypoxia-induced VEGF expression, could decrease hypoxic survival of 4T1 cells.

In hypoxic cells, tyrosine phosphorylated STAT3 forms a complex with HIF-1α at the VEGF promoter, and this complex is important for upregulation of VEGF gene expression in hypoxic cells (11). Our results clearly show that chrysin, as well as a STAT3 inhibitor, could abrogate hypoxia-induced VEGF gene expression of 4T1 cells, through inhibition of STAT3 tyrosine phosphorylation, without decreasing HIF-1α protein levels. Chrysin has been shown to decrease HIF-1α protein levels in hypoxic DU145 human prostate cancer cells, thus abrogating insulin-induced HIF-1α protein stabilization in normoxic DU145 cells, via inhibition of Akt phosphorylation (16).

In a previous study, STAT3 signaling was not investigated, and the inhibitory effect of chrysin on Akt-modulated HIF-1α protein stability was shown only in the insulin-stimulated normoxic cells, but the mechanism was not tested in hypoxic cells (16). In 4T1 cells, the inhibition of hypoxia-induced VEGF expression and STAT3 activation by chrysin were observed at a concentration lower than that affecting Akt phosphorylation (Fig. 2A), suggesting that STAT3 was the primary target rather than Akt in our model. Additionally, a recent study shows that chrysin inhibits STAT3-mediated VEGF expression, which contributes to suppression of IL-6-induced angiogenesis by chrysin in human endothelial cells, as well as in ovo model (23). Collectively, evidence indicates that chrysin possesses ability to inhibit angiogenesis, at least partly, through inhibiting STAT3-mediated VEGF expression.

In our metastasis mouse model, daily oral administration of chrysin predominantly inhibited metastatic growth, rather than primary tumor growth. Chrysin has a low oral bioavailability in humans and rats, due to extensive metabolism to chrysin sulphate and chrysin glucuronide, and efflux into intestine (24). Thus, the concentration of chrysin within primary tumors might be low, especially during early treatment time, when the primary tumor is growing. Another low oral bioavailability flavonoid, genistein, also shows a significant suppressive effect on lung metastatic burden, while marginally decreasing primary tumor growth at mammary fat pads, in breast cancer-harboring mice receiving genistein-supplemented diet (25), similar to that observed in our chrysin treatment.

Our results revealed that chrysin significantly suppressed metastatic growth (size of metastatic colonies), while rate of metastasis (total number of metastatic colonies) was marginally decreased, suggesting that chrysin interfered with growth after the tumor cells reached their metastatic sites. Since progression of micrometastases to macrometastases requires angiogenesis (9), blocking VEGF-mediated angiogenesis may suppress metastatic growth. This is supported by results showing that treatment with a soluble VEGF decoy receptor (VEGF-Trap) leads to decrease in the size of established lung metastases of Wilms tumor cells, without changing the number of metastases (26). In another study, liver metastatic growth of colon adenocarcinoma was shown to be suppressed by rapamycin treatment, which affects both VEGF production of tumor cells and VEGF-response of endothelial cells (27). Taken together, we suggest that the suppression of metastatic growth by chrysin was, at least in part, due to interruption of angiogenesis in hypoxic micrometastases, by inhibiting hypoxia-induced STAT3 activation and VEGF expression.

Control of metastatic growth using hypoxia-selective chemotherapy as adjuvant appears to be an effective strategy, since a marked reduction of metastatic burden was found in lungs of KHT sarcoma-harboring mice receiving post-radiotherapy treatment with a hypoxia-activated cytotoxin NLCQ-1, when hypoxic micrometastases were evident in lungs (28). In our study, combination treatment of chrysin and DR5 mAb was more effective than each single agent in reducing both metastatic rate and metastatic growth, suggesting that chrysin might be useful as an adjuvant in controlling metastatic progression.

In conclusion, chrysin decreased hypoxic survival, inhibited STAT3 activation, and diminished VEGF expression in hypoxic cancer cells, leading to abrogation of hypoxia-induced angiogenesis and resulting in suppression of metastatic growth (Fig. 5). This study also suggests that chrysin is a potential chemopreventive as well as an adjuvant agent, that can be used in combination with other antimetastatic agents to control metastatic progression of tumors.

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