Cytochalasin D promotes pulmonary metastasis of B16 melanoma through expression of tissue factor

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Abstract. Cytochalasin D (CytD) targets actin, a ubiquitous protein in eukaryotic cells. Previous studies have focused mainly on the antitumor effects of CytD. We previously found CytD to promote lung metastasis in B16 melanoma cells, which we had not anticipated, and, therefore, in the present study we investigated the possible underlying mechanisms. B16 melanoma cells were co-cultured with CytD and other agents and used to establish a lung metastatic model. In this B16 melanoma metastatic model, significantly increased lung metastasis and lung weight were found in CytD-treated mice, which was almost completely suppressed by tissue factor (TF) RNA interference expressed via lentivirus. The results of northern and western blot, and real-time RT-PCR analysis showed that the expression of TF was significantly upregulated in B16 cells treated with CytD but was significantly inhibited by TF RNA interference. In addition, upregulation and phosphorylation of mitogen-activated protein kinase p38 were also found in the metastatic lung tissues treated with CytD and in the B16 cells co-cultured with CytD and factor VIIa (FVIIa), but not in cells cultured with CytD, dimethyl sulfoxide or FVIIa alone. These results indicate that CytD stimulates the expression of TF in B16 melanoma cells, activating both coagulation-dependent and -independent pathways via binding to FVIIa, eventually promoting lung metastasis. TF interference is a potential approach to the prevention of B16 melanoma metastasis.

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Introduction

Malignant tumors develop initially as localized clones of tumor stem cells. These subsequently become invasive and migrate through the blood and lymphatic vessels to generate metastatic tumors in other organs (1). Since mortality due to malignant tumor is most often induced by metastatic rather than primary tumors, studies that are concerned with elucidating the metastatic process are critical to cancer research. The results of previous studies have demonstrated that various molecular mechanisms are involved in the progression to the metastatic state, which results in tumor cells that differ from their progenitors in several ways (2-4). At present, tissue factor (TF) has been implicated in the metastatic process. TF is a transmembrane protein that can function as a receptor for clotting factor VII (FVII) and form a complex with FVII/FVIIa (zymogen and protease forms of clotting FVII) to initiate blood coagulation (5). Blood coagulation occurs at the end of a cascade of serial zymogen activations, which lead to the formation of a fibrin network (5). Various early clinical observations have shown a close connection between cancer and blood coagulation (6,7). In addition, some experimental studies have found high-level expression of TF in metastatic human melanoma cells relative to their nonmetastatic counterparts (8,9). Blocking coagulation activity by monoclonal anti-TF antibodies, TF pathway inhibitor, or *in vivo* delivery of anti-TF short interfering RNA (siRNA) has been reported to inhibit experimental lung metastasis in some preclinical studies (10-12). However, the exact mechanism underlying the relationship between TF and tumor metastasis is not solely dependent on the blood coagulation cascade; other intracellular signal pathways are also involved in the progression of tumor metastasis (13).

Cytochalasins are major metabolites that are extracted from various fungi abundant in tropical regions. Cytochalasins can permeate cell membranes, bind to actin and alter its polymerization, thereby altering cellular morphology, gene expression, and even cellular functions such as cell division and apoptosis. Of the various types of cytochalasins, cytochalasins B and E have been shown to possess the most evident anticancer effects (14,15). However, cytochalasin D (CytD) is considered the most specific to the actin cytoskeleton (16). Functionally, CytD binds to the barbed end of growing actin microfilaments

and at last causes inhibition or disruption of actin microfilaments by altering actin polymerization (17). Several previous studies have been carried out to determine the effects of CytD on cell and tissue morphology and its functions *in vitro* and *in vivo*. These include comparisons of normal and cancer cells (18-20). The effects of CytD on cellular functions, such as adherence, motility, secretion, and drug efflux, indicate that CytD may induce important responses in experimental cancer chemotherapy model systems, either as an individual drug or, more likely, as an amplifier of known antitumor agents. In addition, a study by Milsom and Rak (21) showed that CytD treatment can alter cellular architecture and increase expression of TF and multiple angiogenic effectors, such as VEGF, TSP-1, TSP-2 and Ang-1. Recently, we isolated abundant CytD from a strain of endophytic fungus in an endangered plant (*Cephalotaxus hainanensis*). We found it to have antitumor effects in some tumor cell lines (22). However, we also found something that we had not anticipated; in a B16 melanoma model CytD did not induce anti-metastatic effects but rather promoted tumor metastasis. We further investigated the possible mechanisms by which this may occur and found that CytD can stimulate TF expression in melanoma cells. This promotes melanoma metastasis in combination with FVIIa by activation of the mitogen-activated protein kinase (MAPK) p38 signal pathway.

Materials and methods

Cell culture and preparation for tail vein injections. Murine melanoma cell line B16 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The B16 cells were cultured in DMEM medium (Gibco) and supplemented with 10% fetal bovine serum (FBS), 2 mmol/l glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere with 5% CO_2. The logarithmic phase cells were washed twice and detached with trypsin. Following serum inactivation of the trypsin, cells were washed twice in PBS and finally resuspended in PBS at a concentration of $2x10^6$/ml. Viability of the injected cells was assessed by trypan blue staining and was typically >95% for subsequent experiments.

Establishment of lung metastatic model and CytD treatment in vivo. A lung metastatic model was established as in our previous study (23). Female C57BL/6N mice 4 weeks old were obtained from Hainan Provincial Animal Center (Hainan, China) and housed (5 mice/cage) in macrolon cages in a laminar flow cabinet. They were provided with food and water *ad libitum* prior to and during the experiments. To establish the lung metastatic model, 6-week-old mice were injected with 200 μl $2x10^6$/ml B16 cells into the tail vein. On the second day after injection, the mice were randomly divided into two groups (n=5-10 in each group), a CytD treatment group and a dimethyl sulfoxide (DMSO) control group. CytD group mice were i.v. injected with CytD 50 mg/kg dissolved in 100 μl DMSO every other day, and DMSO group mice were i.v. injected with DMSO 100 μl only. Eighteen days after injection with B16 cells, all mice were euthanized by cervical dislocation to measure the weight of the lungs and to count the number of metastatic nodules on the lung surfaces. The animal protocols used in this study were approved by the College's Animal Care and Use Committee (approval ID: HNMCE10012-4).

Cell treatment with CytD in vitro. Stock concentration (1 mg/ml) of CytD (Sigma-Aldrich, USA) was prepared in DMSO. B16 cells cultured in logarithmic growth were treated with CytD (5 μg/ml) for 24 h. This was established through extensive dose-response and time course testing (data not shown). For control, B16 cells were also treated with DMSO only for 24 h. Thereafter, cells were detached with trypsin and washed twice in PBS following serum inactivation of the trypsin. These cells were used to isolate the total RNA for subsequent experiments.

Western blot analysis. Western blot analysis was performed as previously described (23,24). In brief, lysates of cells treated with chemical agents (CytD and DMSO) or tumor-tissue homogenate proteins were separated by 12% SDS-PAGE. Gels were further transported onto a polyvinylidene difluoride membrane (Bio-Rad, USA) by a Mini Trans-Blot system (Bio-Rad). The membrane blots were blocked at 4˚C in 5% nonfat dry milk, washed, and probed with antibodies against corresponding target molecules [TF, MAPK p38 and phosphorylated-p38 (P-p38) MAPK, all purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA] at 1:500. They were then detected using the enhanced chemiluminescence system (Amersham) as previously reported (25). Digital images were acquired and analyzed with a gel imaging system (Bio-Rad Gel Doc 1000; Bio-Rad). The resultant protein levels of age-comparable normal mice were set as 1 and those of mice in other groups were compared against this standard. Data are expressed as fold values.

RNA isolation and northern blot analysis. Total RNA was isolated directly from cultured cells and lung tumor masses using TRIzol reagent (Gibco-BRL/Invitrogen, Gaithersburg, MD, USA) as recommended by the manufacturer. For northern blot analysis, RNA was transferred to Hybond-N^+ membranes and then hybridized with full-length cDNA probes for murine TF (mTF) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in PerfectHyb™ Plus hybridization buffer (Sigma-Aldrich) according to the manufacturer's instructions.

Real-time quantitative PCR. The total RNA extracted from B16 cells and lung tumor masses was stored in ethanol at -80˚C and cDNA synthesis was performed with First Strand cDNA Synthesis Kit (Pharmacia Biotech, Inc.). Real-time quantitative PCR was performed in a Multi-Color Real-Time PCR Detection System (Bio-Rad) to detect of mTF and GAPDH levels using TaqMan Gene Expression Assay reagents and Universal PCR Master Mix (Takara). The total reaction volume in each well of the 96-well MicroAmp Optical reaction plates was 10 μl, including 1 μl of cDNA. The reaction was carried out using a standard two-step protocol (step 1, 10 min at 95˚C; step 2, 40 cycles of 15 sec at 95˚C plus 1 min at 60˚C). The Ct value of each amplification reaction was determined using a threshold value 0.03. For data analysis, mTF-specific Ct values were normalized against the house-keeping gene GAPDH, whose expression was determined in a similar manner. The experiments were performed in triplicate and the results were

averaged. The resultant mRNA levels of age-comparable normal mice were set as 1 and those of other experimental mice were compared against this standard. Data are expressed as fold values.

Preparation of lentiviruses expressing siRNA against TF and tumor cell infection. A siRNA known as mTF223i (5'-GCAUUCCAGAGAAAGCGUUUA-3') has been shown to have a potent interfering effect (10). We chose mTF223i to construct the plasmid that we would use to produce lentiviral vectors expressing siRNA against mTF (termed iTF in this study). We prepared the lentivirus as in a previous study (26). Briefly, the sequences of mTF223i and control negative siRNA (5'-GUCAGAGUGUGCCUUGACUTG-3') were cloned into pTY-linker plasmids for the lentiviral delivering system. These pTY-linker vectors and three other packaging plasmids (transfer vector, packaging vector, and envelope vector) were mixed at ratios of 3, 3, 2 and 1 and co-transfected into 293T cells (Invitrogen). Forty-eight hours after transfection, lentiviral particles in the supernatant were concentrated with Microcon YM-100 Centrifugal Filter Unit (Millipore, USA) and stored at -80˚C for subsequent experiments.

For infection of tumor cells, B16 cells at the logarithmic phase were cultured in DMEM supplemented with 10% FBS. Fresh medium was replaced after 2 h and the lentiviral particles were added at a multiplicity of infection (MOI) of 50 overnight before being injected into the recipient mice to establish tumor models.

Cell treatment by FVIIa in vitro. CytD (5 μg/ml) was preincubated with the resuspended B16 cells for 60 min at 37˚C and then stimulated with recombinant FVIIa (Novo Nordisk). Subsequently, cells were washed and extracted in cold lysis buffer, normalized for protein content, and subjected to western blot analysis to detect total p38 MAPK and P-p38 MAPK.

Data and statistical analysis. All experiments were conducted in triplicate (at least) with similar results. Representative images are included. Student's t-test for independent samples was performed for pairwise comparisons of mean values of variables. In calculating two-tailed significance levels for equality of means, equal variances were assumed for the two populations. P-values <0.05 were considered to indicate statistically significant differences.

Results

Effects of CytD treatment on lung metastasis in vivo. C57BL/6N mice were injected with B16 cells into the tail vein to establish a lung metastatic model. These mice were randomly divided into two groups (n=5-10 in each group) and i.v. injected with CytD (50 mg/kg dissolved in 100 μl DMSO) or DMSO (100 μl) every other day. Eighteen days after injection with B16 cells, all mice were sacrificed by cervical dislocation. Relative to the DMSO-treated control group, significant lung metastatic colonies were observed on the lung surfaces of the mice treated with CytD (Fig. 1A). The average lung weight of the CytD-treated mice was significantly greater than that of the DMSO-treated mice, 1.46±0.15 vs. 0.82±0.18 g (Fig. 1B)

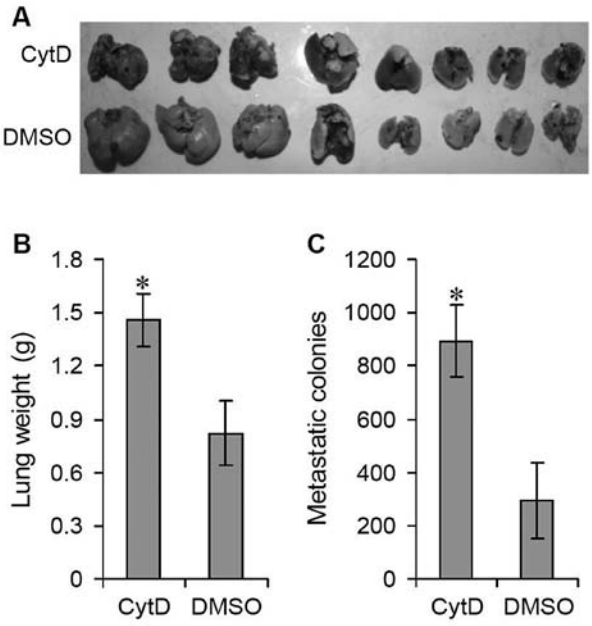

Figure 1. Increase in lung metastasis. B16 melanoma cells were injected into the mice via the tail vein. The mice were treated with CytD or DMSO. Lungs were removed 18 days after injection. (A) Direct observation of the lung organs. (B) Lung weight. (C) The number of the metastatic colonies on the lung surfaces. Results are presented as the means ± SEM. n=5, *P<0.001 vs. DMSO.

(P<0.001). In addition, the number of surface metastatic colonies was also significantly increased in CytD-treated mice relative to controls, 893.47±135.69 vs. 294.63±143.29 (Fig. 1C) (P<0.001).

Effects of CytD treatment on the expression of TF in vitro. CytD and DMSO were added to DMEM medium to culture the logarithmic-phase B16 cells for 24 h. Thereafter, the cells (including normal cultured cells not treated with CytD or DMSO) were collected to isolate total RNA for northern blot analysis and real-time quantitative PCR. B16 cells treated with CytD and B16 cells treated with DMSO were also subjected to western blot analysis to determine TF protein expression. Both the results of western blot analysis (Fig. 2A) and northern blot analysis (Fig. 2B) showed that TF protein and mRNA levels expressed in the CytD-treated cells were significantly higher than in the DMSO-treated cells. The quantitative value of the TF protein in the cells treated with CytD was 2.91±0.28 vs. 0.92±0.12 in the cells treated with DMSO (Fig. 2A) (P<0.001). The results of real-time quantitative PCR showed that the level of TF mRNA expression in the cells treated with DMSO (1.08±0.15-folds) was similar to that in the normal untreated cells (1-fold), whereas the level of TF mRNA in the cells treated with CytD was >3-fold (3.47±0.36) that of DMSO-treated and normal cells (Fig. 2B) (P<0.001).

Effects of CytD treatment on the expression of TF by tumor tissues in vivo. Total RNA was isolated from tumor tissues treated with CytD or DMSO and used to perform northern blot analysis and real-time quantitative PCR. Results similar to those observed in cultured cells were found. TF mRNA levels expressed in tumor tissues treated with CytD were signifi-

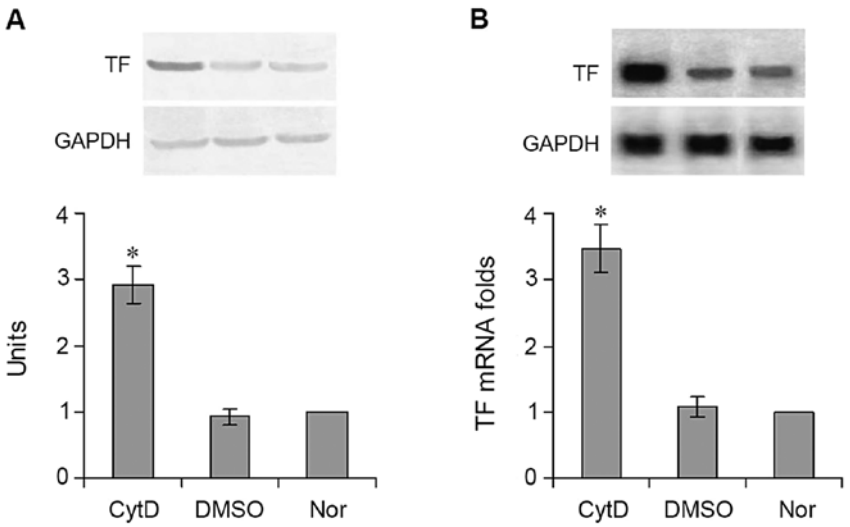

Figure 2. Increased expression of TF by CytD treatment *in vitro*. B16 melanoma cells were treated with CytD or DMSO. The cells (including normal cells) were separated by SDS-PAGE for western blot analysis, and total RNA was isolated for northern blot analysis and quantitative PCR. (A) Western blot analysis. (B) Northern blot analysis and real-time quantitative PCR. The results are presented as the means ± SEM. The value from the normal cultured cells was set as 1 and that of cells treated with CytD or DMSO was compared against this standard. *P<0.001 vs. DMSO.

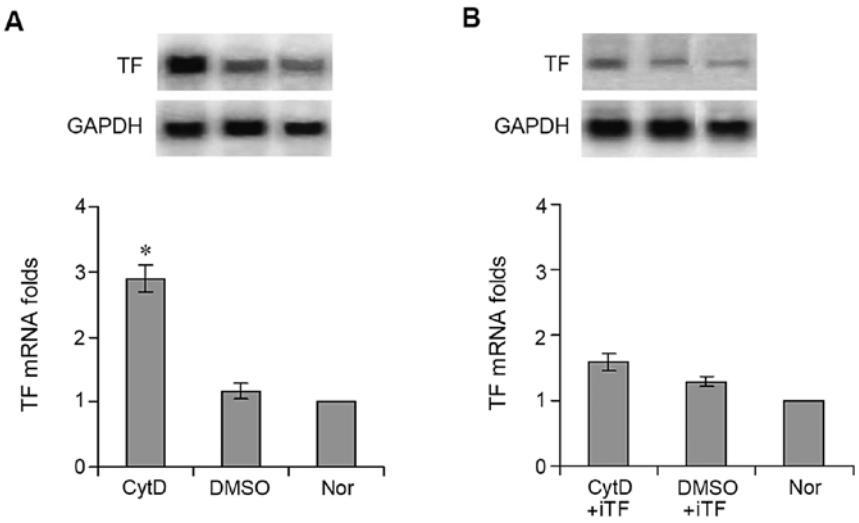

Figure 3. Increased TF expression by CytD treatment *in vivo* and decreased TF expression by TF interference *in vitro*. Total mRNA was extracted from metastatic lung tissues and from the cells treated with a combination of lentiviruses expressing siRNA against TF (iTF) and CytD (DMSO). (A) Lung metastatic tissues evaluated using northern blot analysis and real-time quantitative PCR. (B) Cells treated with iTF interference combined with CytD or DMSO. Results are presented as the means ± SEM. *P<0.001 vs. DMSO.

cantly higher than those expressed in tumor tissues treated with DMSO. TF mRNA expression in the tumor tissues treated with CytD was almost 3-fold (2.89±0.21) that (1.16±0.12) of tumor tissues treated with DMSO when mRNA expression in normal lung tissue was set as 1 (Fig. 3A) (P<0.001).

Effects of TF interference on TF expression and lung metastasis. In order to determine whether high levels of TF expression in the CytD-treated cells were involved in B16 tumor metastasis, we constructed a lentivirus expressing siRNA against TF (iTF-lentivirus) to interfere with TF expression both *in vitro* and *in vivo*. After B16 cells were transfected with the iTF-lentiviruses, CytD was added to the culture medium. As expected, results were opposite to those found with cultured cells not exposed to TF interference. TF mRNA expression in the cells treated with CytD was significantly decreased following interference, reaching a level almost identical to that of cells treated with DMSO, 1.58±0.13 vs. 1.29±0.07 (Fig. 3B) (P>0.05).

B16 cells were then further transfected with iTF-lentiviruses and injected by tail vein into C57BL/6N mice to establish a lung metastatic model. The mice were then treated with CytD or DMSO as above. Relative to the results of the *in vivo* experiments detailed above (Fig. 1), TF interference with iTF-lentiviruses significantly decreased the number of lung metastatic colonies on the surfaces of the lungs of CytD-treated mice (Fig. 4A) (P<0.0001). Following TF interference, treatment with CytD significantly suppressed

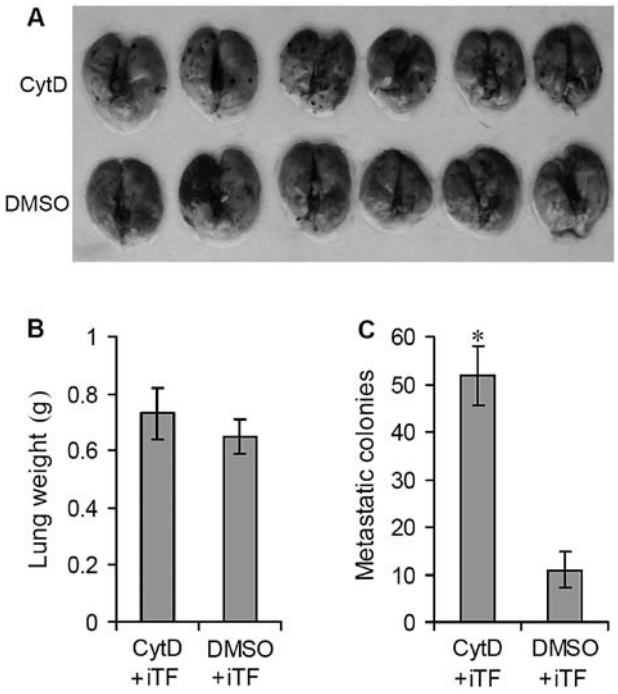

Figure 4. Inhibition of lung metastasis by interference against TF. B16 melanoma cells were transfected with lentivirus expressing siRNA against TF (iTF) injected into C57BL/6N mice and then with CytD or DMSO. (A) Direct observation of the lung organs. (B) Lung weight. (C) The number of the metastatic colonies on the lung surfaces. The metastatic colonies were significantly decreased relative to those not transfected with iTF (P<0.0001) (Fig. 1). The results are presented as the means ± SEM. n=6, *P<0.05 vs. DMSO.

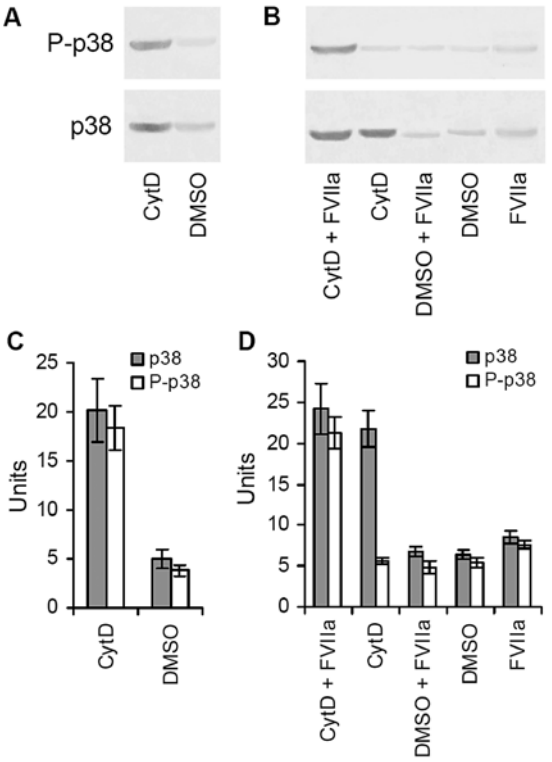

Figure 5. Activation of the MAPK p38 pathway. Metastatic lung tissues and cells cultured with separate or combined CytD, DMSO and FVIIa were separated by SDS-PAGE for western blot analysis to detect MAPK p38 (p38) and phosphorylated-p38 (P-p38) using corresponding antibodies. The results are presented as the means ± SEM. (A and C) Western blot analysis of tumor tissues treated with CytD or DMSO. (B and D) Western blot analysis of B16 melanoma cells treated with single or combination with CytD, DMSO and FVIIa. The results are presented as the means ± SEM.

the growth and formation of metastatic colonies on the lung surfaces. The average lung weight of the CytD-treated mice was similar to that of the DMSO-treated mice, 0.73±0.09 vs. 0.65 ± 0.06 g (Fig. 4B) (P>0.05). However, the number of surface metastatic colonies showed a significant decrease relative to the DMSO-treated controls, 51.73±6.32 vs. 10.86±3.74 (Fig. 4C) (P<0.05). These results indicate that TF interference can prevent B16 lung metastasis and that the promotion of TF expression by CytD may play an important role in B16 lung metastasis.

Effects of TF activation of the MAPK p38 pathway on lung metastasis. In order to determine the possible mechanisms by which upregulation of TF expression increases metastatic potential, we detected MAPK p38 (p38) and P-p38 in tumor tissues treated with CytD or DMSO by western blot analysis. Our results showed that both p38 and P-p38 were proportionally increased in the CytD-treated tumor tissues relative to those in the DMSO-treated tumor tissues (Fig. 5A). The quantitative units of both p38 and P-p38 in the CytD-treated tumor tissues were 20.17±3.27 and 18.39±2.19, whereas the corresponding quantitative units of both p38 and P-p38 in the DMSO-treated tumor tissues were 4.96±0.93 and 3.82±0.57 (Fig. 5C). This suggests that the p38 signaling pathway became activated in the CytD-treated tumor tissues.

We cultured B16 cells *in vitro* and added various doses of FVIIa accompanied by CytD or DMSO to the culture medium to detect p38 and P-p38. We found that B16 cells cultured with CytD or FVIIa alone did not induce P-p38, but B16 cells co-cultured with CytD and FVIIa not only significantly promoted TF expression but they also significantly induced P-p38 (Fig. 5B). The quantitative units of both p38 and P-p38 were 24.19±3.14 and 21.26±1.95 in the cells treated with both CytD and FVIIa, 21.73±2.19 and 5.62±0.42 in the cells treated with only CytD, 6.74±0.58 and 4.83±0.83 in the cells treated with both DMSO and FVIIa, 6.38±0.63 and 5.49±0.57 in the cells treated with only DMSO, and 8.52±0.69 and 7.64±0.53 in the cells treated with only FVIIa (Fig. 5D). These results indicate that TF promotes pulmonary metastasis of B16 melanoma cells through ligation with FVIIa (TF/FVIIa), which further activates the MAPK p38 signal pathway.

Discussion

CytD is a fungal metabolite that is capable of permeating cell membranes, binding to actin and altering its polymerization. At present, CytD is thought to be the cytochalasin most specific to the actin cytoskeleton (16). In addition, a number of studies have indicated that actin filaments transduce signals into cells by connection to focal adhesion molecules such as integrins, vinculin, and talin (27-30). Thus, disruption of actin filaments by CytD leads to severe impairment in cell function, even cell death (14,15,30,31). This also indicates that CytD may be able to induce significant responses in cancer chemotherapy model systems, either as an individual agent

or, more likely, as an amplifier of known antitumor drugs. We previously investigated the anti-metastatic effects induced by CytD or by its modified formula, with unexpected results. In a previous study, we found that CytD did not inhibit melanoma lung metastasis but rather promoted the process, as shown in our murine metastatic model induced by B16 melanoma cells. We designed the current study to investigate possible molecular mechanisms behind this process.

TF is a transmembrane protease receptor for the zymogen FVII and the active enzyme form FVIIa. Binding of TF to FVIIa (TF/FVIIa) not only initiates the extrinsic coagulation cascade, which ultimately leads to thrombin generation, fibrin deposition, and platelet aggregation, but also mediates activation of various intrinsic cell signal pathways. These pathways influence various cell functions, including embryonic vessel development, tumor metastasis, and proinflammatory responses (11,13,21). Previous findings have indicated that TF is involved in tumor growth and metastasis in various types of cancer. Early clinical observations have shown that TF expression is strongly correlated with tumor progression (8,9,32,33). TF may promote tumor dissemination and metastasis through coagulation-dependent or -independent mechanisms (34). Activation of coagulation can capture tumor cells in fibrin-platelet clots and promote local tumor growth. This is the first important step in tumor dissemination and metastasis (35). Several products of the coagulation system, including thrombin and fibrin, can stimulate and promote tumor angiogenesis (35-37). This is also necessary for tumor growth and metastasis. Independent of the downstream coagulation factors, TF/FVIIa can also stimulate tumor-related angiogenesis by upregulating expression of certain angiogenic factors, such as vascular endothelial growth factor and IL-8 (38-40). Previous studies have indicated that the TF/FVIIa signal pathway resulting from the proteolytic activity of the complex is necessary for expression of such angiogenic factors and for TF-dependent metastasis (41-43). Scientists previously found evidence explaining why TF promotes melanoma metastasis by a pathway independent of blood coagulation, specifically the discovery of TF/FVIIa-induced calcium signal activation (44). Independent of the coagulation cascade, the binding of TF to FVIIa can lead to upregulation of growth factors, cytokines, and even induction of antiapoptosis, which may contribute to tumor growth and metastasis (45,46). Blocking TF function by administration of TF antibody, TF pathway inhibitor, inactivated FVIIa, or *in vivo* delivery of anti-TF siRNA has been found to decrease the rate of metastasis in murine metastasis models (10,11,47,48). The results of our present study show that B16 melanoma cells treated with CytD can increase expression of TF mRNA and protein both *in vitro* and *in vivo* (Figs. 2 and 4A) and significantly inhibit their expression by RNA interference against TF constructed in a recombinant lentivirus (Fig. 4B). In a murine lung metastatic model established by B16 melanoma cells, significantly increased lung metastasis was observed in mice treated with CytD (Fig. 1). This was almost completely suppressed by RNA interference against TF (Fig. 3). Western blot analyses demonstrated upregulation and phosphorylation of MAPK p38 in the lung metastatic tissues from the mice treated with CytD (Fig. 5A and C). In addition, upregulation and phosphorylation of MAPK p38 was also found in B16 melanoma cells co-cultured with CytD and recombinant FVIIa but not in B16 melanoma cells cultured with only CytD or other control agents (Fig. 5B and D).

Based on these findings, the binding of TF to FVIIa is the major step of the coagulation cascade. Since we found that the binding of TF to FVIIa could induce upregulation and phosphorylation of MAPK p38 in B16 melanoma cells cultured *ex vivo* and in metastatic tumor tissues *in vivo*, we conclude that both coagulation-dependent and -independent pathways may be involved in lung metastasis in B16 melanoma cells.

We also found that interference against TF can significantly inhibit lung metastasis (Fig. 4) when compared to the results shown in Fig. 1. These results are concordant with those of a previous report (10), and indicate that TF expression can play a critical role in the metastatic process in B16 melanoma cells. Collectively, these results suggest that interference against TF by lentiviruses expressing TF siRNA may become a clinical approach for the prevention of tumor metastasis.

In summary, our present study suggests that CytD can stimulate B16 melanoma cell expression of TF. The binding of TF to FVIIa may cause various signal activations via both coagulation-dependent and -independent pathways, which promote lung metastasis in B16 melanoma cells. Thus, it is necessary to reevaluate the value of CytD and its modified formula when considering it as an antitumor agent against melanoma.

Acknowledgements

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