Parthenolide, a Natural Inhibitor of Nuclear Factor-κB, Inhibits Lung Colonization of Murine Osteosarcoma Cells

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Abstract Purpose: The transcription factor nuclear factor-κB (NF-κB) regulates the expression of several genes important for tumor metastasis and is constitutively active in the highly metastatic murine osteosarcoma cell line LM8. Parthenolide, a sesquiterpene lactone, was reported to inhibit the DNA binding of NF-κB. The purpose of this study is to investigate the usefulness of parthenolide as a target for antimetastatic therapies.

Experimental Design: We examined the effect of parthenolide on metastasis-associated phenotypes in vitro and in murine experimental lung metastasis models by s.c. and i.v. inoculation of LM8 cells.

Results: We found that parthenolide strongly induced apoptosis and inhibited cell proliferation and the expression of vascular endothelial growth factor in vitro. In the in vivo metastasis models, parthenolide treatment suppressed lung metastasis when treatment was initiated concurrently with s.c. or i.v. inoculation of tumor cells, whereas lung metastasis was not reduced when parthenolide was given after the homing of tumor cells. The growth of s.c. tumors that developed at the inoculation site was not suppressed by parthenolide. We also found that the genetic inhibition of NF-κB activity by expressing mutant IκBα suppressed lung metastasis in vivo but not s.c. tumor growth. This supports our notion that the metastasis-preventing effect of parthenolide is mediated at least in part by inhibition of NF-κB activity.

Conclusions: These findings suggested that NF-κB is a potential molecular target for designing specific prophylactic interventions against distant metastasis and that parthenolide is a hopeful candidate for an antimetastatic drug.

Osteosarcoma is the most common primary bone malignancy affecting children and young adults. The biggest difficulty with osteosarcoma is its high propensity for pulmonary metastasis. Patients with extremity lesions in whom lung metastases are detected at the time of diagnosis have a very poor prognosis. Modern multidisciplinary therapy has improved their outlook; however, up to half of the patients with osteosarcoma are still not cured. Thus, clarification of the molecular mechanism of pulmonary metastasis of osteosarcoma and introduction of a new intervention method are still needed (1).

Previously, we showed that LM8, a highly metastatic subclone of the Dunn murine osteosarcoma cell line, overexpressed valosin-containing protein (also known as p97) and exhibited increased nuclear factor-κB (NF-κB) activity (2). The transfection of valosin-containing protein in Dunn osteosarcoma cells could activate NF-κB and its metastatic potential. We also reported that the expression of vascular endothelial growth factor (VEGF) and matrix metalloproteinase, which were regulated by NF-κB, was up-regulated in LM8 and in Dunn cells transfected with valosin-containing protein compared with original Dunn cells (3). These findings suggested that therapy against NF-κB activity is a prime target and has the potential to improve the prognosis of osteosarcoma.

NF-κB is an inducible dimeric transcription factor that belongs to the Rel/NF-κB family of transcription factors. NF-κB consists of two major polypeptides, p50 and p65 (4). In resting cells, NF-κB is sequestered in the cytoplasm by IκB proteins. Stimulus-mediated phosphorylation and subsequent proteolytic degradation of IκB allows the release and nuclear translocation of NF-κB, where it transactivates several target genes (5).

NF-κB plays a key role in the malignant behavior of tumors. It has been shown to regulate a whole cadre of genes important for angiogenesis, invasion, and metastasis (6–8). Studies of cell lines in vitro have shown that NF-κB is constitutively activated in many malignancies (9, 10). In addition, some investigators have shown that inhibition of NF-κB by insertion of mutated IκB into cancer cell lines causes decreased tumor growth and metastasis in vivo (11–14).
Parthenolide is one of the main sesquiterpene lactones responsible for the bioactivities of feverfew, a traditional folk remedy that has been used for various inflammatory conditions, such as rheumatoid arthritis, fever, and migraine (15, 16). Several studies have proposed that the effect of parthenolide is due to its ability to inhibit NF-κB activation. In addition, parthenolide was also reported to have microtubule-interfering properties (17) that induce apoptotic cell death by multiple pathways, including oxidative stress, endoplasmic reticulum stress, intracellular thiol depletion, caspase activation, and mitochondrial dysfunction (18, 19); inhibit 5-lipoxygenase and cyclooxygenase (20); and sensitize cancer cells to chemotherapeutic drugs, such as paclitaxel and CPT11 (21). Despite its potential as an anticancer drug, few studies have clarified the effect of parthenolide on metastasis (22) or the role of antitumor activities in the course of metastasis in vivo. We showed that parthenolide suppressed lung metastasis in LM8, a highly metastatic murine osteosarcoma cell line, by interference with tumor cell integration in lung tissue.

Materials and Methods

Cell culture. The cloned murine osteosarcoma cell line LM8, which shows a high metastatic incidence to the lung after s.c. inoculation into the back of mice, was cultured in DMEM containing 10% fetal bovine serum (Life Technologies, Gaithersburg, MD) in an air incubator with 5% CO₂ at 37°C. In some cultures, parthenolide (Sigma Chemical, St. Louis, MO) was added at final concentrations of 0.2 to 200 μg/mL.

Animals. C3H male mice, ages 5 weeks, were purchased from Japan Oriental Yeast Co., Ltd. (Tokyo, Japan). All mice were housed under specific pathogen-free conditions with a 12-h light and dark cycle. The housing care rules and experimental protocol were approved by the Animal Care and Use Committee of Osaka University.

Electrophoretic mobility shift assay. Nuclear extracts of cells were prepared with the NE-PER nuclear extraction reagent (Pierce Biotechnology, Rockford, IL). Biotin end-labeled double-stranded oligonucleotides 5’-AGTTAGGGGACCTTCCCACGGC-biotin-3’ and 5’-CTACTCCCTGAAAGGTCCG-biotin-3’ were purchased from Invitrogen (Carlsbad, CA). The binding reactions contained 7.5 μg nuclear extract protein, buffer [10 mmol/L Tris (pH 7.5), 50 mmol/L KCl, 5 mmol/L MgCl₂, 1 mmol/L DTT, 0.05% NP40, 2.5% glycerol], 1 μg poly(dexonosinic-deoxycytidylid acid), and 2 nmol/L biotin-labeled DNA. The reactions were incubated at room temperature for 20 min. The reactions were electrophoresed on a 10% precast Tris-borate-EDTA gel at 100 V for 1 h in 100 mmol/L Tris-borate-EDTA buffer and transferred to a nylon membrane. The biotin-labeled DNA was detected with a LightShift chemiluminescent electrophoretic mobility shift assay (EMSA) kit (Pierce Biotechnology). We repeated the assay for four times.

Luciferase assays. LM8 cells seeded in 24-well plates were transfected with pNFκB-Luc (firefly luciferase driven by a TATA box with five NFκB sites in the enhancer element; Stratagene, La Jolla, CA) by jetPEI (Polyplus Transfection, Biopark, France). Normalization of transfection efficiency was done by cotransfection with pRL-TK (Promega, Madison, WI) that contained cDNA encoding Renilla luciferase. Luciferase activities were quantified at 24 h using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer’s instructions. We did the assay for four times.

Western blotting. LM8 cells in a 10-cm dish were starved with serum-free DMEM and incubated with various concentrations (0, 0.2, 2, 20, and 200 μg/mL) of parthenolide for 48 h. Ten-fold concentrated conditioned media were subjected to electrophoresis on a 12% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose filters.
parthenolide on tumor cell homing in lung tissue. LM8 cells were additionally labeled by the membrane dye PKH using the fluorescence cell linker kit PKH26 (Sigma Chemical). Then, a total of $2 \times 10^5$ cells was suspended in $200 \mu$L PBS and injected into the tail vein of C3H mice ($n = 15$). Parthenolide treatment at a dosage of $1 \text{mg/kg}$ daily was initiated concomitantly with injection of LM8. At 4 ($n = 5$), 24 ($n = 5$), and 48 h ($n = 5$) after cell injection, the mice were killed and the lungs were removed. The lungs were then homogenized in PBS. The fluorescence of the released PKH26 was measured at 551 nm. Data were expressed as units of PKH26 released (1 unit = 1 unit of fluorescence at 551 nm) per milligram of protein in each culture. The assay was done in triplicate.

**Stable transfection of LM8 cells with IκBαM and control vector.** LM8 cells were transfected with pCMV-IκBαM expression vector or control pCMV-Iκ vector (Clontech, Mountain View, CA) using jetPEI. The IκBαM gene differed from wild-type IκBα by serine-to-alanine mutations at residues 32 and 36. These mutations protected IκBα from ubiquitination and proteasome-mediated proteolysis. Thus, the product of pCMV-IκBαM blocked NF-κB activation in a dominant-negative fashion. Cells were selected with standard medium containing G418 (Life Technologies). Two clones (M7 and M9) that overexpress IκBαM and a control clone (CTL), which was transfected with control pCMV-Iκ vector, were selected.

**Statistical analysis.** Data are presented as mean ± SD. Groups were compared by one-way ANOVA and individual groups were compared using the Mann-Whitney U test for unpaired analysis. Differences between treatment groups were considered significant at $P < 0.05$.

**Results**

**NF-κB DNA binding and transcription activity of NF-κB was inhibited by parthenolide.** To assess the influence of parthenolide on NF-κB signaling in LM8 cells, EMSAs for NF-κB were done. We added parthenolide or vehicle to subconfluent LM8 cell cultures for 1 h and nuclear protein was extracted. As shown in Fig. 1A, NF-κB DNA binding was inhibited by parthenolide dose dependently. Additionally, we determined the effect of parthenolide on the transactivational activity of NF-κB in LM8 cells by promoter reporter assay. As shown in Fig. 1B, NF-κB transcriptional activity was inhibited by parthenolide in a dose-dependent manner. The NF-κB activity in the cells incubated with high-dose parthenolide (200 μg/mL) was 10-fold lower than the control.

**VEGF expression was inhibited by parthenolide.** When cultured in serum-free medium, LM8 cells released VEGF in culture medium as determined by Western blotting. Parthenolide

![Fig. 1.](image-url)

**Fig. 1.** In vitro effect of parthenolide on murine osteosarcoma cell line LM8. **A,** EMSA for NF-κB. LM8 cells were pretreated with parthenolide for 1 h. Nuclear extracts were subjected to EMSA. Parthenolide inhibits the DNA binding of NF-κB. **B,** transcriptional activity of NF-κB. A Luciferase reporter assay for NF-κB transcriptional activity was done as described in Materials and Methods. NF-κB transcriptional activity was inhibited by parthenolide dose dependently. Representative data from four different experiments. C, VEGF secretion was decreased by parthenolide. Conditioned medium of LM8 cell cultures treated by parthenolide was analyzed for VEGF expression by Western blotting. **D,** parthenolide reduced the invasiveness of LM8. Invasiveness was determined using a Matrigel-precoated cell culture insert ($n = 4$). Representative data from three different experiments. Columns, mean; bars, SD. ***,** $P < 0.05$ to controls without parthenolide (ANOVA).
treatment inhibited the secretion of VEGF in a dose-dependent manner (Fig. 1C).

**Invasive potential was inhibited by parthenolide.** Invasive potential of LM8 cells after incubating with parthenolide was determined using the Matrigel invasion assay. Figure 1D shows that tumor cell invasion was decreased by parthenolide in a dose-dependent manner.

**Parthenolide inhibited cell growth and induced apoptosis in LM8 cell culture.** We determined the effect of parthenolide on the cell cycle of LM8 cells using FACS analysis. Parthenolide treatment reduced the growth fraction (S + G2-M) and increased the apoptosis fraction (sub-G1) at 4, 12, and 24 h. At 48 h, parthenolide treatment abolished the G1 peak and no cells seemed to be viable under the microscope (Fig. 2).

**Parthenolide had a distinct effect on lung metastasis but no effect on tumorigenesis in the prophylactic treatment model.** We evaluated the effect of parthenolide on tumors in vivo using a murine LM8 model of metastasis. After s.c. inoculation of tumor cells, all mice developed large primary tumors at the injection site. In the prophylactic treatment model, the primary tumors treated with parthenolide of >1 μg/kg/d tended to be smaller than the vehicle-treated control tumors but the differences were not statistically significant (Fig. 3A). However, we observed that pulmonary metastatic nodules were dramatically suppressed by parthenolide. There were significant differences in the proportional area of lung metastasis between vehicle-treated and parthenolide-treated mice (Fig. 3B-F). Especially, treatment with parthenolide of >1 μg/kg/d effectively suppressed lung metastasis. Immunohistochecmical analysis showed strong p65 expression in the lung metastatic nodules of the untreated mice. However, metastatic tumors treated with parthenolide of 1 mg/kg/d were negative for p65 immunolocalization. Immunohistochemistry also showed that VEGF expression of metastatic lung tumors and surrounding lung tissue was clearly suppressed by parthenolide treatment compared with untreated metastatic tumors of similar size (Fig. 3G-N).

**Parthenolide had no effect on lung metastasis or tumorigenesis in the therapeutic treatment model.** Next, we did the experiment to determine whether parthenolide can decrease pulmonary
Fig. 3. The effect of parthenolide on tumor metastasis in the prophylactic model. LM8 cells (1 × 10^5) were injected s.c. into the back of mice. The mice were treated with i.p. injection of parthenolide or vehicle (PBS) starting from the day of tumor cell inoculation. A, the primary tumors treated by parthenolide tended to be smaller than the controls but no statistically significant difference was found. B, pulmonary metastatic nodules were dramatically suppressed by parthenolide. n = 4–14 from three different experiments. Columns, mean; bars, SD. *, P < 0.05 from PBS control. C to F, microphotographs of the lungs treated by PBS (C) and by parthenolide at a dosage of 1 μg/kg/d (D), 100 μg/kg/d (E), and 1 mg/kg/d (F). Bar, 1 mm. Magnification, ×40. Immunohistochemical analyses of the tumor nodules in lung tissue treated with PBS (G–J) and with parthenolide at a dosage of 1 mg/kg/d (K–N). The expression of p65 was suppressed in parthenolide-treated mice (L) compared with control mice (H). The expression of VEGF was also suppressed in the parthenolide-treated mice (M) compared with control mice (I). An intrinsic positive control of VEGF was vascular endothelial wall. G and K, H&E staining. Arrowheads, blood vessels; arrows, tumor nodules. J and N, negative controls without primary antibodies. Bar, 100 μm. Magnification, ×100.
lung nodules after the development of lung metastasis. The primary tumor volumes and the proportional area of lung metastasis showed no significant difference between vehicle-treated and parthenolide-treated mice (Fig. 4).

**Parthenolide interferes with early events of tumor cell colonization in lung.** To clarify where parthenolide works during the multistep metastatic process, we tested its effect in a lung metastasis model by tail vein injection. The lung metastasis of mice that received parthenolide concomitantly with tumor cell inoculation was significantly reduced compared with other groups (Fig. 5A-D). In the mice treated with parthenolide starting from 48 h after the injection of tumor cells, the lung metastasis was not suppressed (Fig. 5B and D), suggesting that parthenolide inhibited metastasis not by working on the steps before extravasation but by working on early events of lung colonization that occur within 48 h after cell intravasation. Then, we examined the effect of parthenolide on the homing of tumor cells to lung tissue. After 4 or 24 h, there was no significant difference in the fluorescence of labeled LM8 cells in the lung between the control group and the parthenolide-treated group. However, the fluorescence in the mice treated with parthenolide gradually decreased with time and, at 48 h, it was significantly lower than that in mice that were treated with PBS of 48 h (Fig. 5E).

**Blocking NF-κB activation inhibited VEGF expression and lung metastasis in vivo.** We established LM8 cell clones that were stably transfected with an IκBα mutant expression vector pCMV-IκBαM. Two clones (M7 and M9) were selected. Nuclear proteins from transfectants and wild-type cells were analyzed by EMSA for NF-κB binding activity. DNA binding of NF-κB was inhibited in both mutant clones (Fig. 6A). Consistent with these results, the NF-κB-luciferase reporter assay (n = 4) showed that the transcription activity of NF-κB was significantly reduced in those clones compared with control clone, CTL (Fig. 6B).

Next, we tested the metastatic potential of those clones in vivo using a spontaneous metastasis model to determine whether selective inhibition of NF-κB suppresses lung metastasis. As shown in Fig. 6C, the primary tumor volumes were not statistically different between the mice implanted with IκBαM clones and the mice implanted with CTL (n = 6). However, we found that pulmonary metastatic nodules in the mice implanted with the clones overexpressing IκBα mutant were dramatically suppressed, especially in the M7 clone with the lowest NF-κB activity. There were significant differences in the proportional area of metastasis between the mice with CTL and the mice with M7 or M9 (Fig. 6D-G).

**Discussion**

Suppression of various genes that are involved in tumor cell invasion and angiogenesis on blockade of NF-κB activity has been reported in cancer cells (12, 23–27). Although previous studies claimed the involvement of several cytokines under the regulation of NF-κB, such as interleukin-8, tumor necrosis factor-α, and VEGF, in the regulatory mechanisms (12, 22–25, 28, 29), little has been known when and how NF-κB is implicated during the multistep cascade of events in the development of metastasis. In addition, although parthenolide, an inhibitor of NF-κB, has also shown antimetastatic activity in vitro (21, 30), there have been few reports about its in vivo antimetastatic activity or its mode of action (22). Our in vivo experiments showed the following: (a) lung metastasis was suppressed when parthenolide was given...
Parthenolide Decreases Lung Metastasis

Fig. 5. The effect of parthenolide on lung metastasis in a tail vein injection model. The H&E staining of lung tissue of the mice that received PBS (A), parthenolide starting from 48 h after cell injection (B), and parthenolide concomitantly with the injection of LM8 (C). The lung metastasis of mice that received parthenolide concomitantly with the injection of tumor cells was significantly reduced compared with other groups (n = 7, D). LM8 cells were labeled by the membrane dye PKH and injected into the tail vein. Parthenolide treatment at a dosage of 1 mg/kg daily was initiated concomitantly injection of cells. At 4, 24, and 48 h after cell injection, the fluorescence of the released PKH in lung tissue was measured. At 4 and 24 h, there was no significant difference in the fluorescence of labeled cells in the lung between the control group and the parthenolide-treated group. However, the fluorescence treated with parthenolide gradually decreased with time and was significantly lower than that treated with PBS at 48 h (n = 5; E). Points, mean; bars, SD. * P < 0.05 to PBS control.

Parthenolide blocks the development of lung metastasis.

Parthenolide decreases the area of metastatic tumor in lung (%). In the i.v. tumor cell inoculation model, parthenolide treatment starting from 48 h after cell inoculation, when solitary tumor cells are beginning to extravasate and proliferate (39). These observations were consistent with our results in the i.v. tumor cell inoculation model, which revealed failure in the blockade of metastasis with parthenolide treatment starting from 48 h after cell inoculation. This supports our notion that the metastasis-preventing effect of parthenolide is mediated at least in part by inhibition of VEGF secretion.

Although the inhibition of NF-κB activity by expressing Lck-BeM has been shown to suppress metastasis in a variety of tumors, the reports differ about the effect on the growth of primary tumors (11, 12, 14, 25). Our study showed that parthenolide effectively blocked the development of lung metastasis derived from tumor cells in lung tissue gradually decreased over time with parthenolide treatment with a significant difference at 48 h, suggesting that parthenolide, at least in part, induced tumor cell death before the cells can form multicellular micrometastatic foci.

NF-κB is one of the most important upstream regulators of VEGF, a major angiogenic factor that induces endothelial cell proliferation. In our data, parthenolide clearly decreased VEGF secretion by LM8 cells in vitro. Moreover, in the spontaneous metastasis model, it suppressed the expression of immunoreactive VEGF at lung metastatic foci where intracellular accumulation of NF-κB was suppressed as well. These observations suggest that one of the mechanisms by which parthenolide inhibits lung metastasis is the suppression of VEGF expression via NF-κB inactivation. A recent study showed that disrupting VEGF signaling leading to vascular leakage by the pretreatment with pharmacologic inhibitors of VEGF could alleviate lung metastasis. It showed that the inhibitor could get the beneficial effect within the first days after tumor cell inoculation, when solitary tumor cells are beginning to extravasate and proliferate (39). These observations were consistent with our results in the i.v. tumor cell inoculation model, which revealed failure in the blockade of metastasis with parthenolide treatment starting from 48 h after cell inoculation. This supports our notion that the metastasis-preventing effect of parthenolide is mediated at least in part by inhibition of VEGF secretion.
metastasis of osteosarcoma but did not block the proliferation of the primary site tumor or established lung metastases. Despite its suppressive effect on the cell cycle in vitro, its poor effect on established tumors in vivo might spoil the importance of proliferation suppression among the putative mechanisms of metastasis inhibition by parthenolide. However, the pharmacodynamic properties of parthenolide are still under investigation (40) and there remains the possibility that parthenolide is not to be delivered in high enough concentrations to established tumors when given systemically.

Several antitumor studies on the inhibition of NF-κB activity in vivo used a gene therapy, including an IκB mutant. Parthenolide is currently used commonly as a food supplement for the treatment of migraines and reportedly has no severe side effects compared with the placebo group; therefore, it may be easier and more efficient than gene therapy in vivo (15). Mutation of p53 is a common genetic alteration in cancers that is closely associated with resistance to systemic therapy. A recent study revealed that one possible pathway through which mutants of p53 may induce loss of drug sensitivity is via the NF-κB pathway (41). Parthenolide directly blocks NF-κB activity regardless of p53 status and also induces apoptosis via the c-Jun NH2-terminal kinase pathway independent of NF-κB and p53. Thus, parthenolide could be expected to have an effect on tumors with the p53 mutation that poorly respond to anticancer drugs (30).

In conclusion, we showed that parthenolide, a sesquiterpene lactone derived from natural herbs, inhibited lung metastasis in a spontaneous metastasis model and an i.v. metastasis model using the highly metastatic murine osteosarcoma cell line LM8. Although extensive clinical studies are needed to clarify whether this is also the case in human patients with cancers, our results suggest that NF-κB is a potential molecular target for designing specific prophylactic interventions against distant metastasis.

Fig. 6. Suppression of NF-κB activity by the overexpressing mutant IκBα reduced VEGF secretion and blocked lung metastasis. An IκBαM construct was transfected to LM8 cells and two clones (M7 and M9) were selected according to the activity of NF-κB for in vitro and in vivo studies. EMSA of NF-κB DNA binding in M7 and M9 and in CTL, a clone that was transfected with control vector (A), and transcriptional activity of NF-κB by luciferase reporter assay (n = 4; B). Columns, mean; bars, SD. *, P < 0.05 to CTL without tumor necrosis factor-α (TNF-α). #, P < 0.01 to CTL with tumor necrosis factor-α. These clones were subjected to the spontaneous metastasis model for their metastatic potential. No statistically significant difference was observed in the volume of the primary tumors (C); however, the proportional area of lung metastasis in the mice inoculated with IκBαM clones was significantly smaller than that with the control clone (n = 6, D). Columns, mean; bars, SD. *, P < 0.05 to CTL. Representative microphotographs of the lungs of the mice injected with M7 (E), M9 (F), and CTL (G) cells. H&E staining. Magnification, ×40.
References


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