Ganoderma lucidum suppresses motility of highly invasive breast and prostate cancer cells

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Abstract

A dried powder from basidiomycetous fungi, Ganoderma lucidum, has been used in East Asia in therapies for several different diseases, including cancer. However, the molecular mechanisms involved in the biological actions of Ganoderma are not well understood. We have recently demonstrated that phosphatidylinositol 3-kinase (PI 3-kinase) and nuclear factor-κ B (NF-κB) regulate motility of highly invasive human breast cancer cells by the secretion of urokinase-type plasminogen activator (uPA). In this study, we investigated the effect of G. lucidum on highly invasive breast and prostate cancer cells. Here we show that spores or dried fruiting body of G. lucidum inhibit constitutively active transcription factors AP-1 and NF-κB in breast MDA-MB-231 and prostate PC-3 cancer cells. Furthermore, Ganoderma inhibition of expression of uPA and uPA receptor (uPAR), as well secretion of uPA, resulted in the suppression of the migration of MDA-MB-231 and PC-3 cells. Our data suggest that spores and unpurified fruiting body of G. lucidum inhibit invasion of breast and prostate cancer cells by a common mechanism and could have potential therapeutic use for cancer treatment.

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The fungi Ganoderma lucidum (Reishi, Mannentake, or Lingzhi) has been used for centuries in East Asia to cure various human diseases such as hepatitis, hepatopathy, hypertension, nephritis, bronchitis, and cancers [1–3]. Its dried powder was especially popular as a cancer chemotherapy agent in the Imperial Court of ancient China [4]. Some of the triterpenes, such as ganoderic and lucidic acids, recently isolated from Ganoderma demonstrated cytotoxicity against mouse sarcoma and mouse lung carcinoma cells in vitro [5]. Intraperitoneal administration of water-soluble polysaccharides isolated from Ganoderma inhibited growth of sarcoma-180 solid tumors in mice [6,7]. In addition, polysaccharides from Ganoderma also potentiated production of cytokines, which subsequently suppressed proliferation of HL-60 and U937 leukemic cell lines [8].

Tumor invasion and metastasis are multifaceted processes involving cell adhesion, proteolytic degradation of tissue barriers, and cell migration [9]. The urokinase-type plasminogen activator uPA and the uPA receptor (uPAR) play a crucial role in cancer metastasis (for review, see [10]). uPA is a serine protease that cleaves the extracellular matrix and is also involved in cell adhesion and migration [11]. Therefore, uPA can stimulate cell migration directly through its proteolytic activity by activating transforming growth factor-β (TGF-β) and fibroblast growth factor (FGF) [12,13], or alternatively, uPA lacking proteolytic activity stimulated cell migration directly through interaction with uPAR [14,15]. In addition, antibodies preventing binding of uPA to uPAR inhibited uPA-induced cell
migration [16,17]. Both uPA and uPAR are overexpressed in different tumors, and the correlation between uPA and uPAR concentration and poor prognosis is consistent with the idea that uPAR-bound uPA at the surface of cancer cells is responsible for the invasiveness of cancer cells and metastasis [10]. Therefore, inhibition of uPA activity or expression of uPA and uPAR drastically reduced tumor invasiveness [18–20].

Breast cancer and prostate cancer are the most common malignancy in women and men and in the United States account for about 30% of all cancers diagnosed in females and males, respectively [21]. Breast cancer often progresses from the estrogen-dependent, nonmetastatic, antiestrogen-sensitive phenotype to the estrogen-independent, antiestrogen- and chemotherapy-resistant phenotype with highly invasive and metastatic growth properties [22]. Prostate cancers also progress from androgen-dependent to androgen-independent, highly metastatic carcinomas, which are responsible for the relapse of prostatic cancers to androgen ablation therapies [23]. As mentioned above, uPA and uPAR are overexpressed in cancers and their levels correspond to poor prognosis [10]. Furthermore, the expression of uPA and uPAR is controlled by transcription factors AP-1 and NF-κB [24–26], and constitutive activation of NF-κB and AP-1 has been detected in some highly invasive breast and prostate cancer cell lines [22,27–29]. In addition, NF-κB can further induce transcription of AP-1-regulated genes through the interaction of NF-κB with AP-1 [30]. Finally, we have recently demonstrated that constitutively active AP-1 and NF-κB controlled secretion of uPA from highly invasive breast cancer cells [31,32].

The present study was undertaken to elucidate the effect of Ganoderma on motility and constitutively active signaling pathways in highly invasive human breast and prostate cancer cells. In this report we show that Ganoderma can suppress constitutive migration of both breast MDA-MB-231 and prostate PC-3 cells. Furthermore, Ganoderma decreased constitutive activation of AP-1 and NF-κB in the reporter gene assay. Finally, secretion of uPA as well as expression of uPA and uPAR in MDA-MB-231 and PC-3 cells were suppressed by Ganoderma. Taken together, our data suggest that the antitumor activity of Ganoderma can be caused by the inhibition of transcription factors AP-1 and NF-κB, resulting in the suppression of expression of uPA and uPAR and the secretion of uPA, followed by the inhibition of cell motility of highly invasive breast and prostate cancer cells.

Materials and methods

Ganoderma lucidum. The spores and fruiting body powder from G. lucidum (Ganoderma) were purchased as a medical supplement called ZhongKE from Nanjing Zhongke Biochemical Technology, China. The authentication and morphological characterization (with Nikon 200 TE inverted microscope) of spores and fruiting body were provided by Dr. Nancy W.Y. Ho, Purdue University. The spores (GS) and fruiting body (GFB) of Ganoderma were dissolved in boiled water at a concentration of 50 mg/ml. The extracts were stored at 4°C and reheated to 70°C for 10 min before every experiment.

Cell culture. The human breast cancer cell line MDA-MB-231 and human prostate cancer cell line PC-3 were obtained from ATCC (Manassas, VA). MDA-MB-231 was maintained in Dulbecco’s modified Eagle’s medium (DMEM) and PC-3 cells were maintained in F-12 medium containing penicillin (50 U/ml), streptomycin (50 U/ml), and 10% fetal bovine serum (FBS). Media and supplements came from Gibco BRL (Grand Island, NY). FBS was obtained from Hyclone (Logan, UT).

Cell migration assay. MDA-MB-231 and PC-3 cells were harvested and incubated for 4 and 24 h, respectively, with either GS or GFB from Ganoderma, as indicated in the text. Chemokinesis was assessed in Transwell chambers in DMEM for MDA-MB-231 cells or in the F-12 medium containing 10% FBS for PC-3 cells, as previously described [33]. After fixing and staining, we counted the number of migrating cells from at least four random fields using a microscope at 20× magnifications [33]. Data points represent the average ± SD of individual filters within one representative experiment repeated at least twice.

Cell viability. MDA-MB-231 and PC-3 cells were harvested and incubated with Ganoderma for 4 and 24 h, respectively, as described for the cell migration assay. Cell viability was determined with trypan blue stain according to the standard protocol [34]. Data points represent the average ± SD in one representative experiment repeated at least twice.

DNA transfection and chloramphenicol acetyltransferase (CAT) assay. Both MDA-MB-231 and PC-3 cells were transfected with either NF-κB-CAT or AP-1-CAT reporter constructs and β-galactosidase expression vector pCH110, as previously described [31]. Twenty-four hours after transfection, cells were treated with either GS or GFB for an additional 24 h at 37°C, as indicated in the text. CAT assays were performed as described [31]. Data points represent the average ± SD of 3–6 independent transfection experiments.

Gel electrophoretic mobility shift assay (GEMSA). MDA-MB-231 and PC-3 cells were treated for 24 h with GS and GFB, and nuclear extracts were prepared as described previously [35]. A GEMSA was performed with 32P-labeled AP-1 and NF-κB according to the standard protocol [31]. Oligonucleotide probes containing consensus sequences for AP-1 and NF-κB were purchased from Promega (Madison, WI). Supershift analyses were performed with anti-NF-κB p50 antibodies (Upstate Biotechnology, Lake Placid, NY).

Expression of IkBα and uPA secretion. MDA-MB-231 and PC-3 cells were treated for 24 h with GS and GFB, and whole cell extracts were prepared [36]. IkBα (inhibitor κBα) and actin expression were determined in the extracts (25 μg) by Western blot analysis [31]. The secretion of uPA was determined after treatment with GS and GFB for 24 h in concentrated DMEM from MDA-MB-231 cells or concentrated F-12 medium from PC-3 cells as described [31].

Immunohistochemistry. MDA-MB-231 and PC-3 cells were seeded and grown in a Chamber Slide System (Lab-Tek II, Nalgene Nunc). After 24 h of treatment with GS and GFB, the cells were washed twice with phosphate-buffered saline (PBS) and air-dried without chemical fixation at room temperature. Samples were washed for 30 min in PBS and incubated for 15 min in a moisture box with 25 μl of uPA (Cedarlane, Ontario, Canada) and uPAR (American Diagnostica, Greenwich, CT) antibody, respectively. After washing with PBS (3 × 10 min), the samples were incubated for 15 min with 25 μl anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) (Protos ImmunoResearch, San Francisco, CA). The samples were subsequently rinsed three times with PBS. After the last wash, slides were reacted with propidium iodide (50 μg/ml) for 15 min. Finally, the slides were washed three times (10 min) and coverslipped. The expression of uPA and uPAR and the nuclei staining were detected by epi-illumination in a Leitz DMRB microscope. The expressions of uPA and uPAR were semiquantitatively evaluated for the intensity of immunoreactivity
Ganoderma inhibits migration of highly invasive human breast and prostate cancer cells

We have recently demonstrated that cell migration of highly invasive and chemotherapy-resistant human breast cancer cells MDA-MB-231 is dependent on constitutively active AP-1 and NF-κB and secretion of uPA [31,32]. Because extracts from Ganoderma have been used for cancer treatment in Asian countries for centuries [4], we wanted to elucidate the mechanisms involved in its antitumor activities. The morphology of Ganoderma samples was confirmed by microscopy (Fig. 1A). To assess the role of Ganoderma on the metastatic motility of human breast cancer cells, we preincubated MDA-MB-231 cells for 1 h with spores (GS) and fruiting body (GFB) and determined cell motility after an additional 3 h under standard cell migration assay conditions [33]. As seen in Fig. 1B, both spores and fruiting body (0.5–2.5 mg/ml) significantly inhibited cell motility of breast cancer cells in a dose-dependent manner.

To determine whether Ganoderma also inhibits motility of highly invasive prostate cancer cells, we preincubated PC-3 cells with GS and GFB (0.5–2.5 mg/ml) for 1 h and determined cell migration after an additional 23 h of incubation (although prostate cancer cells PC-3 are highly invasive they need longer incubation time in migration assays than the highly invasive breast cancer cells MDA-MB-231). The cell motility of Fig. 1C shows that both spores and fruiting body significantly inhibited constitutive migration of PC-3 cells. Therefore, Ganoderma was able to suppress constitutive migration of the highly invasive breast cancer cells MDA-MB-231 as well as the highly invasive prostate cancer cells PC-3.

To determine whether the inhibition of cell motility is caused by the cytotoxic effect of Ganoderma, we incubated both MDA-MB-231 and PC-3 cells with GS and GFB under the same conditions as the cell migration assay, and cell viability was determined with trypan blue stain. Spores or fruiting body did not demonstrate any cytotoxicity for either MDA-MB-231 or PC-3 cells at the highest used concentration, 2.5 mg/ml (data not shown).

Ganoderma suppresses constitutively active AP-1 and NF-κB in breast and prostate cancer cells

Constitutive DNA-binding activity of NF-κB has been reported previously in primary breast cancers [22], and we have recently demonstrated that inhibition of PI 3-kinase and protein kinase C (PKC) suppresses constitutive transactivation of NF-κB and AP-1 in the highly invasive breast cancer cells MDA-MB-231...
[31,32]. To examine whether Ganoderma can inhibit transactivation of NF-κB and AP-1, we transiently transfected MDA-MB-231 cells with either NF-κB-CAT or AP-1-CAT reporter gene plasmids and treated the cells with spores and fruiting body for 24h. As seen in Fig. 2A, treatment with both spores and fruiting body inhibited constitutive activation of NF-κB in a dose-dependent manner (0.5–2.5 mg/ml). We also observed the same effect on the constitutive activation of AP-1 where increased concentration of spores and fruiting body inhibited AP-1 in MDA-MB-231 cells (Fig. 2B). Because we have recently shown that the activity of NF-κB and AP-1 was suppressed only at the transactivation level without the changes in DNA-binding activity in MDA-MB-231 cells [31,32], we were interested to see whether Ganoderma inhibits constitutive NF-κB and AP-1 activity by the same mechanism. Nuclear extracts from MDA-MB-231 cells treated with spores or fruiting body for 24h were subjected to gel-shift analysis with either NF-κB and AP-1-labeled probes; the specificity of NF-κB and AP-1 binding in MDA-MB-231 cells was confirmed previously by competitive and supershift gel analysis [31,32]. As seen in Fig. 2C, pretreatment of spores and fruiting body at a concentration of 2.5 mg/ml markedly reduced the constitutive DNA-binding activity of NF-κB. However, the constitutive DNA-binding activity of AP-1 was not affected by Ganoderma treatment (Fig. 2D). Therefore, the decrease in the constitutive NF-κB activity in a reporter gene assay by Ganoderma reflects the suppression of the NF-κB DNA binding, whereas constitutive AP-1 activity is suppressed at the transactivation level without changing AP-1 DNA-binding.

Constitutive NF-κB activity was recently reported in androgen-independent prostate tumor cell lines [29]. In light of this finding, we wanted to examine the effect of Ganoderma on NF-κB and AP-1 activity in the highly invasive prostate cancer cells PC-3. Gel-shift analysis confirmed constitutive NF-κB DNA-binding activity in nuclear extracts from PC-3 cells, the specificity of which was determined by competitive and supershift assays with anti-NF-κB p50 antibodies (Fig. 3A). In addition, nuclear extracts from PC-3 cells demonstrated the high constitutive DNA-binding activity of AP-1 (Fig. 3B). To examine whether Ganoderma employ the same mechanism of inhibition of migration in both breast and prostate cancer cells, we transfected PC-3 cells with both NF-κB-CAT and AP-1-CAT reporter gene plasmids.

Fig. 2. Effects of *Ganoderma lucidum* on the constitutive transactivation and DNA-binding activity of NF-κB and AP-1 in breast cancer MDA-MB-231 cells. (A) NF-κB-CAT activity after treatment with Ganoderma. MDA-MB-231 cells were transfected with 1 μg NF-κB-CAT reporter construct and 3 μg β-galactosidase expression vector pCH110. Twenty-four hours after transfection, the cells were treated with spores (GS) or fruiting body (GFB) (0–2.5 mg/ml) for an additional 24h. CAT activity was measured as described under Materials and methods. Data are means ± SD of triplicate determinations. Similar results were obtained in at least two additional experiments. (B) AP-1-CAT activity after treatment with Ganoderma. MDA-MB-231 cells were transfected with 2.5 μg AP-1-CAT reporter construct and 3 μg β-galactosidase expression vector pCH110. Twenty-four hours after transfection, the cells were treated and CAT activity was determined as described in (A). Data are means ± SD of triplicate determinations. Similar results were obtained in at least two additional experiments. (C) NF-κB DNA-binding after treatment with Ganoderma. MDA-MB-231 cells were treated with spores (GS) or fruiting body (GFB) (2.5 mg/ml) for 24h and nuclear extracts were subjected to gel-shift analysis with specific NF-κB probe as described under Materials and methods. The results are representative of three separate experiments. (D) AP-1 DNA-binding after treatment with Ganoderma. MDA-MB-231 cells were treated as described for C and nuclear extracts were subjected to GEMSAs with specific AP-1 probe. The results are representative of three separate experiments.
and treated the cells with spores and fruiting body for 24 h as described above. As seen in Fig. 3C, both spores and fruiting body inhibited the constitutive activation of NF-κB in PC-3 cells in a dose-dependent manner (0.5–2.5 mg/ml). The constitutive activation of AP-1 was also inhibited by treatment with spores and fruiting body (Fig. 3D). Gel-shift analysis demonstrated that the DNA binding of NF-κB in PC-3 cells was also reduced by the treatment with spores and fruiting body of Ganoderma (Fig. 3E). Nevertheless, the DNA-binding activity of AP-1 was not affected by the same treatment with spores or fruiting body (Fig. 3F). Therefore, Ganoderma inhibited both DNA binding and transactivation of NF-κB, whereas the inhibition of AP-1 occurred only at the transactivation level. We can conclude from this that the same mechanism of inhibition is employed by both breast and prostate cancer cells.

Inhibition of migration and NF-κB activation by Ganoderma is independent of IκBα

NF-κB can be inhibited by sequestering in the cytoplasm through assembly with the inhibitory protein IκBα [38] or by the alternative mechanism, independent

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Fig. 3. Effects of Ganoderma lucidum on the constitutive transactivation and DNA-binding activity of NF-κB and AP-1 in prostate cancer PC-3 cells. (A) DNA binding activity of NF-κB in PC-3 cells. Nuclear extracts were prepared from PC-3 cells and GEMSA was performed with 32P-labeled NF-κB probe in the presence or absence of cold NF-κB and AP-1 oligonucleotides as described under Materials and methods. Supershift experiments were performed with anti-NF-κB p50. The arrows indicate NF-κB, specific binding of NF-κB; SS, supershift. Results are representative of three separate experiments. (B) DNA binding activity of AP-1 in PC-3 cells. Nuclear extracts were prepared from PC-3 cells and GEMSA was performed with 32P-labeled AP-1 probe in the presence or absence of cold AP-1 and NF-κB oligonucleotides as described under Materials and methods. The arrow indicates AP-1-specific binding of AP-1. Results are representative of three separate experiments. (C) NF-κB-CAT activity after treatment with Ganoderma. PC-3 cells were transfected with 1 μg NF-κB-CAT reporter construct and 3 μg β-galactosidase expression vector pCH110. Twenty-four hours after transfection, the cells were treated with spores (GS) or fruiting body (GFB) (0–2.5 mg/ml) for an additional 24 h. CAT activity was measured as described under Materials and methods. Data are means ± SD of triplicate determinations. Similar results were obtained in at least two additional experiments. (D) AP-1-CAT activity after treatment with Ganoderma. PC-3 cells were transfected with 2.5 μg AP-1-CAT reporter construct and 3 μg β-galactosidase expression vector pCH110. Twenty-four hours after transfection, the cells were treated and CAT activity was determined as described in (C). Data are means ± SD of triplicate determinations. Similar results were obtained in at least two additional experiments. (E) NF-κB DNA binding after treatment with Ganoderma. PC-3 cells were treated with spores (GS) or fruiting body (GFB) (2.5 mg/ml) for 24 h and nuclear extracts were subjected to gel-shift analysis with specific NF-κB probe as described under Materials and methods. The results are representative of three separate experiments. (F) AP-1 DNA binding after treatment with Ganoderma. PC-3 cells were treated as described in (E) and nuclear extracts were subjected to GEMSA with specific AP-1 probe. The results are representative of three separate experiments.
Furthermore, we have recently demonstrated that although overexpression of IκBα suppressed migration of MDA-MB-231 cells, the inhibition of NF-κB transactivation by PI 3-kinase and PKC inhibitors was process independent of IκBα [31,32]. Therefore, we investigated whether Ganoderma would inhibit constitutive activation of NF-κB by increasing the levels of IκBα and by sequestering NF-κB in cytoplasm. MDA-MB-231 cells were treated with spores (0.5–2.5 mg/ml) and fruiting body (0.5–2.5 mg/ml) of Ganoderma for 24 h and whole cell extracts were subjected to SDS-PAGE. Western blot analysis with anti-IκBα antibody revealed that treatment with spores or fruiting body of Ganoderma did not increase the levels of IκBα (not shown). As expected, we observed the same behavior in highly invasive prostate cancer cells, where treatment with spores or fruiting body of Ganoderma also did not increase the levels of IκBα in PC-3 cells (not shown). Altogether, these data clearly demonstrated that Ganoderma inhibits migration and constitutive NF-κB activation in breast and prostate cancer cells by a distinct signaling mechanism independent of IκBα.

Ganoderma suppresses constitutive secretion of uPA from breast and prostate cancer cells

We have previously demonstrated that highly invasive breast cancer cells constitutively secrete uPA and that this secretion can be suppressed by the inhibition of PI 3-kinase and PKC [31,32]. To examine the effect of Ganoderma on uPA secretion from breast cancer cells, we treated MDA-MB-231 cells with spores (0.5–2.5 mg/ml) and fruiting body (0.5–2.5 mg/ml) of Ganoderma and concentrated media were subjected to Western blot analysis with anti-uPA antibody. As seen in Fig. 4A, both spores and fruiting body markedly inhibited secretion of uPA in a dose-dependent manner. Because uPA expression usually correlates with the aggressive phenotype of prostate cancer cells [40], we were interested in whether the highly invasive prostate cancer cells PC-3 also secrete uPA and whether Ganoderma inhibits this secretion. PC-3 cells were treated and analyzed as described above for MDA-MB-231 cells. Western blot analysis demonstrated that PC-3 cells secrete uPA and the treatment with spores and fruiting body clearly suppressed the uPA secretion from PC-3 cells in a dose-dependent manner (Fig. 4B). Therefore, Ganoderma inhibited secretion of uPA from both breast and prostate cancer cells.

Inhibition of expression of uPA and uPAR by Ganoderma in breast and prostate cancer cells

As mentioned above, the expression of uPA and uPAR is controlled by transcription factors AP-1 and NF-κB [24–26], and we have demonstrated that Ganoderma inhibits both AP-1 and NF-κB in MDA-MB-231 and PC-3 cells. To examine the effect of Ganoderma on the expression of uPA and uPAR, we treated MDA-MB-231 cells with spores or fruiting body (2.5 mg/ml) for 24 h and immunostained the cells with anti-uPA antibodies. As seen in Fig. 5, both spores and fruiting body of Ganoderma significantly reduced expression of uPA and uPAR in MDA-MB-231 cells (Fig. 5A, a–c, Table 1). Immuno-staining with anti-uPAR antibodies demonstrated that uPAR expression was also decreased in MDA-MB-231 cells treated with both spores and fruiting body (Fig. 5A, d–f, Table 1). Similar to the results obtained with MDA-MB-231 cells, the expression of uPA and uPAR in PC-3 cells was also inhibited by spores and fruiting body (Fig. 5B, Table 2). Taken together, these data show that Ganoderma inhibited expression of uPA and uPAR in both highly invasive breast and prostate cancer cells.

Discussion

We have recently demonstrated that constitutively active AP-1 and NF-κB control secretion of uPA and that inhibition of AP-1 and NF-κB suppressed cell
motility and uPA secretion of highly invasive breast cancer cells [31,32]. In the present study, we examined the effect of the old Asian medicinal mushroom *G. lucidum* on highly invasive breast and prostate cancer cells. Here we show that both spores and fruiting body of Ganoderma inhibit constitutive activation of AP-1 and NF-κB. Furthermore, Ganoderma inhibits expression of uPA and uPAR as well as secretion of uPA and cell migration of MDA-MB-231 and PC-3 cells.
Although Ganoderma has been used in Asian medicine for centuries to treat and prevent various diseases and its purified spores or whole fruiting body are distributed as dietary supplements, the biological effects of Ganoderma remain elusive. The antitumor effects of Ganoderma were suggested to be caused by triterpenes [5], polysaccharides [6,7], and immunomodulatory proteins [41] and by the mechanisms involving inhibition of post-translational modification of the Ras oncoprotein [42], inhibition of DNA polymerase [4], or the stimulation of cytokine production [8]. Recently, the mitogen activated kinase (MAP) kinase signaling pathway, activated by Ganoderma extracts, was identified in the induction of neuronal differentiation and prevention of apoptosis [43]. In our study, we show that Ganoderma inhibited augmented cell motility of highly invasive breast and prostate cancer cells by inhibiting constitutively active AP-1 and NF-κB signaling. The inhibition of AP-1 and NF-κB by the Asian mushroom Ganoderma is of particular interest because recent studies suggest that AP-1 and NF-κB are potential targets for cancer treatment [44,45]. In addition, inhibition of NF-κB by proteasome inhibitor PS-341 is considered in clinical studies to be an adjuvant to systemic chemotherapy of cancer [46]. In our experiments, we used purified spores or whole fruiting body of Ganoderma, which are available as dietary supplements, and we found no significant difference in biological activity between the spores and the whole fruiting body of Ganoderma. The dose used, 0.5–2.5 mg/ml spores or fruiting body of Ganoderma per 1 million cancer cells, markedly inhibited migration as well as constitutive activation of AP-1 and NF-κB in breast and prostate cancer cells. The recommended dose, based on data from clinical studies with other fungi, should be 5–10 g fruiting body per day, with a linear enhancement in efficacy expected up to 30 g/day [47].

Here we show the inhibition of NF-κB in the DNA-binding activity and reporter gene assays in both breast and prostate cancer cells. In our previous studies [31,32] we demonstrate that inhibition of PI 3-kinase and PKC suppresses NF-κB in breast cancer cells only at the transactivation level. Therefore, Ganoderma probably employs a specific mechanism for NF-κB inhibition independent of the PI 3-kinase and PKC pathways. Although constitutive AP-1 transactivation was inhibited by Ganoderma in the reporter gene assay in both breast and prostate cancer cells, the DNA-binding activity of AP-1 was not changed. The inhibition of AP-1 at the transactivation level can be explained by the different mechanisms of NF-κB and AP-1 activation. Because NF-κB has been shown to induce transcription of AP-1 regulated genes through the interaction of NF-κB with AP-1 [30], it is possible that Ganoderma inhibits DNA binding and transactivation of NF-κB, which in turn inhibits AP-1 only at the transactivation level without changing the DNA-binding activity of AP-1.

The activity of NF-κB is controlled by NF-κB inhibitors, IkBs, a family of proteins that bind to NF-κB dimers, hiding their nuclear localization sequence and resulting in cytoplasmic retention of NF-κB [38,48]. In the present study, we show that although Ganoderma inhibits NF-κB, the levels of κBz are not changed, suggesting that NF-κB activity is regulated by a distinct pathway not involving IkB. Recently, the NF-κB activation pathway, separate from IkB degradation, was described in PI 3-kinase- and Akt-dependent signal transduction pathways [39,49,50]. In addition, our previous data demonstrate that inhibition of PI 3-kinase in MDA-MB-231 cells also suppresses cell migration through NF-κB pathway independent of IkB degradation [31].

A compelling body of evidence has accumulated showing that uPA and uPAR play an important role in cancer metastasis. The correlation between uPA and uPAR concentrations and poor prognosis is especially in agreement with the idea that uPAR-bound uPA at the surface of cancer cells is necessary for invasion by cancer cells and metastasis [10]. In addition, we have recently reported that uPA secretion is responsible for cell migration of highly invasive breast cancer cells and AP-1- and NF-κB-mediated secretion of uPA [31,32]. As shown above, Ganoderma inhibits AP-1 and NF-κB in both breast and prostate cancer cells. Therefore, we examined the effect of Ganoderma on uPA expression and secretion from MDA-231 and PC-3
cancer cells. Our data demonstrated that spores and fruiting body of Ganoderma markedly decreased expression and secretion of uPA from breast and prostate cancer cells. In addition, the expression of uPA receptor was also decreased after treatment with Ganoderma. As mentioned above, both uPA and uPAR contain AP-1 and NF-kB DNA binding sequences in their promoter regions. Therefore, Ganoderma is able to suppress expression of both uPA and uPAR in highly invasive breast and prostate cancer cells, suggesting that common mechanism can be employed to inhibit cancer metastasis.

In the last few years, the use of herbal therapies in alternative medicine has been increasing, and although the number of cancer patients using herbal dietary supplements is not exactly known, there is evidence of the increasing use of dietary supplements in cancer treatment [51]. *Ganoderma lucidum* is one of the herbs in the herbal mixture PC–SPES, which showed activity against hormone-refractory disease in two prostate cancer patients [52]. Extracts of PC–SPES demonstrated estrogenic effects [53] and decreased growth of hormone-sensitive as well hormone-insensitive prostate cancer cells [54]. Our data clearly demonstrate that Ganoderma inhibited transcription factors AP-1 and NF-kB, followed by the suppression of uPA and uPAR expression, and resulting in the inhibition of cell motility of highly invasive breast and prostate cancer cells. These data suggest Ganoderma’s anticancer biological activity in the herbal mixture PC–SPES. Although different biologically active compounds isolated from Ganoderma demonstrate anticancer activities [5–8], here we show that whole nonfractionated fruiting body or spores of *G. lucidum* can suppress cancer cell migration by inhibiting a specific signaling pathway. Further study will be required to isolate the most active components from Ganoderma and to identify their optimal ratio with the highest inhibitory activity against cancer cells.

In summary, our data demonstrate that *G. lucidum* inhibited the cell motility of highly invasive breast and prostate cancer cells by suppressing uPA and uPAR. Therefore, Ganoderma in the form of dietary supplement can be considered as an additional therapeutic intervention for the inhibition of metastasis of highly invasive breast and prostate cancers.

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