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Ganodermanontriol (GDNT) exerts its effect on growth and invasiveness of breast cancer cells through the down-regulation of CDC20 and uPA

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ABSTRACT

Ganoderma lucidum is a medicinal mushroom that has been recognized by Traditional Chinese Medicine (TCM). Although some of the direct anticancer activities are attributed to the presence of triterpenes—ganoderic and lucidenic acids—the activity of other compounds remains elusive. Here we show that ganodermanontriol (GDNT), a *Ganoderma* alcohol, specifically suppressed proliferation (anchorage-dependent growth) and colony formation (anchorage-independent growth) of highly invasive human breast cancer cells MDA-MB-231. GDNT suppressed expression of the cell cycle regulatory protein CDC20, which is over-expressed in precancerous and breast cancer cells compared to normal mammary epithelial cells. Moreover, we found that CDC20 is over-expressed in tumors when compared to the tissue surrounding the tumor in specimens from breast cancer patients. GDNT also inhibited invasive behavior (cell adhesion, cell migration, and cell invasion) through the suppression of secretion of urokinase-plasminogen activator (uPA) and inhibited expression of uPA receptor. In conclusion, mushroom GDNT is a natural agent that has potential as a therapy for invasive breast cancers.

1. Introduction

Due to the invasive behavior of breast cancer cells, breast cancer is the second leading cause of cancer death in the United States [1]. Cancer metastasis consists of several interdependent processes including uncontrolled growth of cancer cells, their invasion through surrounding tissues, migration to the distant sites of the human body, and adhesion, invasion and colonization of other organs and tissues [2]. Therefore, prevention of growth and invasiveness of cancer cells could lead to the inhibition of cancer metastasis that would eventually increase survival of breast cancer patients. Recent epidemiological studies suggested that mushrooms could protect against gastric, gastrointestinal, and breast cancer, respectively [3–6]. *Ganoderma lucidum* is a mushroom that has been recognized by Traditional Chinese Medicine (TCM) and is commonly used in the forms of tea, powder, and dietary supplements [7]. The botanical characterization, description and therapeutic effects of *G. lucidum* are summarized in the American Herbal Pharmacopoeia [8]. While the anticancer activity of *Ganoderma* polysaccharides is associated with the stimulation of an immune response [9], triterpene extracts or isolated *Ganoderma* triterpenes have a direct inhibitory effect on cancer cells through a variety of mechanisms including the inhibition of proliferation, cell cycle arrest, suppression of invasive behavior, induction of apoptosis, and autophagy [10–17]. Moreover, some of these triterpenes demonstrated anti-metastatic activities *in vivo* [18–20]. However, these anticancer activities have usually been attributed to the presence of different ganoderic or lucidenic acids.

In the present study, we evaluated the effect of ganodermanontriol (GDNT), a *Ganoderma* alcohol, on growth (proliferation and colony formation) and invasive behavior (cell adhesion, migration, and invasion) on highly invasive, metastastic, and therapy-resistant human breast cancer cells. We found that GDNT inhibits expression of CDC20, a protein that controls mitotic chromosome segregation during cell proliferation and, as recently suggested, a potential therapeutic target [21,22]. In addition, we found that CDC20 is over-expressed in tumors when compared to the tissue surrounding the tumor in specimens from breast cancer patients. Finally, we found that GDNT inhibited invasiveness of highly metastatic breast cancer cells through the suppression of uPA secretion and the inhibition of uPAR expression.

2. Materials and methods

2.1. Reagents

GDNT was purchased from ChromaDex (Santa Ana, CA, USA) and Planta Analytica (Danbury, CT, USA). DMSO was purchased

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from Sigma (St. Louis, MO). GDNT was dissolved in DMSO at a concentration of 50 mM and stored at −20 °C. Anti-CDC20 (p55 CDC), uPAR, and anti-β–actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). uPA antibodies were from Calbiochem (San Diego, CA, USA).

2.2. Cell culture

Primary human mammary epithelial cells (HMEC) were obtained from Lonza (Walkersville, MD) and grown in Mammary Epithelial Cell Growth Medium (MEGM, Lonza) with supplements according to manufacturer's protocol. Non-tumorigenic MCF-10A human mammary epithelial cells and human breast cancer cells MCF-7 and MDA-MB-231 were obtained from ATCC (Manassas, VA). MCF-10A cells were maintained in DMEM/F12 medium containing 5% horse serum (HS), insulin (10 μg/ml), epidermal growth factor (EGF, 20 ng/ml), cholera toxin (100 μg/ml), hydrocortisone (0.5 μg/ml), penicillin (50 U/ml), and streptomycin (50 U/ml). MCF-7 and MDA-MB-231 cells were maintained in DMEM 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). HS and FBS were obtained from Hyclone (Logan, UT).

2.3. Cell proliferation and colony formation

Proliferation of MDA-MB-231 cells treated with GDNT (0–50 μM) for 24–72 h was determined as we previously described [23]. Colony formation of MDA-MB-231 cells incubated in the presence of GDNT (0–50 μM) was evaluated as described [24]. Data points represent mean ± SD in one experiment repeated at least twice.

2.4. Human specimen

Samples of human breast tumors and the tissue surrounding the tumors were collected from breast cancer patients by the Methodist Research Institute Biorepository according to HIPAA and approved IRB protocol # 1011004282 (04-093).

2.5. Western blot analysis

Whole cell extracts, from HMEC, MCF-10A, MCF-7, and MDA-MB-231 cells treated with GDNT (0–50 μM) for 24 h, and extracts from human specimens were prepared, and Western blot analysis with CDC20 antibodies was performed as previously described [23]. Western blots were quantified with HP-Scanjet 550c and analyzed by UN-SCAN-IT software (Silk Scientific, Orem, UT).

2.6. Cell invasiveness

Cell adhesion of MDA-MB-231 cells treated with GDNT (0–50 μM) for 24 h was performed as we described [25]. Cell migration of MDA-MB-231 cells treated with GDNT (0–50 μM) for 5 h was evaluated as previously described [25,26]. Cell invasion of MDA-MB-231 cells treated with GDNT (0–50 μM) for 24 h was performed as we described [25]. Data points represent the mean ± SD of three individual filters within one representative experiment repeated at least twice.

2.7. uPA secretion and uPAR expression

uPA secretion from MDA-MB-231 cells treated with GDNT (0–50 μM) for 24 h was evaluated as we previously described [25]. uPAR expression in MDA-MB-231 cells treated with GDNT (0–50 μM) was evaluated by immunocytochemistry as described [27].

2.8. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Statistical comparison between groups of data was carried out using ANOVA. $P < 0.05$ was considered to be significant.

3. Results

3.1. GDNT inhibits proliferation and colony formation of highly invasive breast cancer cells MDA-MB-231

We have recently found that ganodermanontriol (GDNT, Fig. 1A), a biologically active triterpene alcohol isolated from the medicinal mushroom *G. lucidum*, inhibits growth of human colon cancer cells [28]. To evaluate whether GDNT affects growth of highly invasive human breast cancer cells, MDA-MB-231 cells were treated with GDNT (0–50 μM) for 24–72 h, and the proliferation was determined as described in Section 2. As seen in Fig. 1B, GDNT significantly inhibited cell proliferation of MDA-MB-231 cells with IC_{50} 42.0, 15.7, and 11.6 μM at 24, 48 and 72 h, respectively. In addition, chemically synthesized GDNT also suppressed proliferation of poorly invasive breast cancer MCF-7 cells[1] but only slightly affected proliferation of MCF-10A human mammary epithelial cells.[2]

Cancer cells can grow under the nonadhesive condition and this anchorage-independent growth (colony formation) is correlated with *in vivo* oncogenic potential of cancer cells. Because colony formation is a key parameter for cells to acquire a metastatic potential [29], we evaluated effects of GDNT on colony formation of highly invasive MDA-MB-231 cells. In agreement with its effect on cell proliferation, GDNT decreased the number of colonies of MDA-MB-231 cells (Fig. 1C). In summary, GDNT suppresses the anchorage-dependent (cell proliferation) as well as anchorage-independent (colony formation) growth of invasive breast cancer cells.

3.2. CDC20 is over-expressed in human breast tumors and GDNT down-regulates CDC20 expression in MDA-MB-231 cells

To identify molecular targets of GDNT in human breast cancer cells, we performed DNA-microarray analysis of MDA-MB-231 cells treated with GDNT (data not shown). One of the genes in which expression was significantly down-regulated by the GDNT treatment was CDC20, which controls the mitotic chromosome segregation during cell proliferation [21,22]. To confirm that CDC20 is over-expressed in human breast tumors, we analyzed expression of CDC20 in tumor specimens from breast cancer patients. As seen in Fig. 2A, CDC20 was more expressed in tumors when compared to the tissue surrounding the tumor in specimens from breast cancer patients. In addition, we found increased expression of CDC20 in precancerous and breast cancer cells compared to normal mammary epithelial cells (Fig. 2B). To determine whether GDNT inhibits expression of CDC20 in MDA-MB-231 cells, these cells were treated with GDNT and Western blot analysis was performed. As seen in Fig. 2C, GDNT treatment markedly suppressed expression of GDNT, suggesting that CDC20 is a suitable target for GDNT.

3.3. GDNT inhibits invasive behavior of breast cancer cells

To evaluate whether GDNT suppresses invasive behavior, MDA-MB-231 cells were treated with GDNT (0–50 μM) and cell adhesion, cell migration, and cell invasion was determined as described in Section 2. As seen in Fig. 3A, GDNT markedly suppressed adhesion of MDA-MB-231 cells to the extracellular

[1] Kennedy et al. (submitted for publication).
[2] Thyagarajan-Sahu (unpublished).