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Inhibitory Effects of Cultivated Wild Ginseng on the Differentiation of 3T3-L1 Pre-adipocytes

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Wild ginseng has been used as a traditional medicine for thousands of years and for increase physical strength in Korea, China and Japan. This study reports that cultivated wild ginseng (CWG) inhibits adipocyte differentiation of 3T3-L1 pre-adipocytes in a concentration-dependent manner. Inhibition of adipocyte differentiation is one possible anti-obesity strategy. CWG inhibits the expression of the adipocyte differentiation regulator peroxisome proliferators-activated receptor (PPAR)γ and CCAAT/enhancer-binding protein α mRNA. It also inhibited the expression of PPARγ and adiponectin at the protein level during the differentiation of pre-adipocytes into adipocytes. Additionally, CWG blocked the cell cycle at the sub-G_1 phase transition, causing cells to remain in the pre-adipocyte state. These results indicate that CWG inhibits adipocyte differentiation and adipogenesis through pre-adipocyte cell cycle arrest in cultured 3T3-L1 cells.

Keywords: *Panax ginseng*, Cultivated wild ginseng, 3T3-L1 cells, Adipocyte differentiation, Adipogenesis, Cell cycle arrest

INTRODUCTION

Adipocytes play an important role in the regulation of energy balance and an array of endocrine functions. Their major role is the storage of lipids during periods of energy excess [1]. Adipocytes are also known as endocrine cells that secrete several biological active molecules including a variety of growth factors, cytokines, and hormones [2-3]. Disorders of lipid metabolism are associated with various diseases such as obesity, type 2 diabetes mellitus, hypertension, cardiovascular disease, and cancer [4]. Obesity, a major risk factor for metabolic diseases, is a complex multifactorial disorder characterized by increased fat cell size and number [5]. Adipocyte differentiation has often been a target of anti-obesity strategies because obesity is caused not only by hypertrophy of adipocytes but also by adipocyte hyperplasia [1]. The 3T3-L1 cell line is a well-established and widely-used *in vitro* model of obesity for studying adipocyte differentiation. Pre-adipocytes differentiate in monolayer cultures, changing their morphological and biochemical characteristics during adipocytes maturation [6]. Peroxisome proliferator-activated receptor (PPAR)γ and CCAAT/enhancer-binding protein (C/

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EBP)α play essential roles in adipogenic differentiation by promoting the transcription of various genes responsible for fat transport and accumulation [4-5]. Additionally, two major proteins regulate adipocyte differentiation such as adiponectin and PPARγ [6-7], and are involved in obesity and diabetes. PPARγ is activated during adipocyte differentiation [8]. The inhibition of PPARγ expression with specific ligands can successfully induce anti-obesity effects. Therefore, treating obesity by supplementation with an active compound is important in the prevention of various obesity-related diseases.

Ginseng has been used in traditional herbal medicine for over 2,000 years in Asian countries including Korea, China, and Japan, and is believed to exert beneficial effects. Wild ginseng is relatively rare and increasingly endangered due in large part to high demand in recent years which has led to the wild plants being sought and harvested in an unsustainable way. Cultivated wild ginseng (CWG) is dilatory in growth and more sensitive to environmental changes. CWG grows naturally; however, it is not cultivated. It displays a preference for areas with fluctuating daily temperatures and minor exposure to direct sunlight. These differences may result in a variation of the bioactive compounds present in CWG. Currently, various ginseng extracts have been studied for a wide range of biological and biochemical actions [9]. Ginseng saponins and *Panax ginseng* berry extract are candidates for reducing obesity and hyperlipidemia [10-11]. However, the effect of CWG on the cellular and molecular mechanisms responsible for the differentiation and regulation of adipocytes has not been reported.

The focus of this study was to investigate decreasing fat accumulation of CWG following adipocyte differentiation. The mechanism of CWG action was evaluated with respect to alterations in PPARγ and C/EBPα mRNA expression. PPARγ and adiponectin protein levels which might potentially lead to inhibiting the progression of obesity were also examined.

MATERIALS AND METHODS

Reagents and cells

3T3-L1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). Dexamethasone (DEX), 1-methyl-3-isobutyl xanthine (IBMX), insulin, Oil Red O and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO, USA). Antibodies against PPARγ and adiponectin were purchased from Assay Designs (Ann Arbor, MI, USA).

Preparation of cultivated wild ginseng

CWG (50 g, *Panax ginseng* C. A. Meyer) was purchased from a market in Daegu Yagryong and originated from the Andong province (Korea). The CWG was estimated to be 9 to 11 years old and identified by the Korean Ginseng Center for Most Valuable Products and Ginseng Genetic Resource Bank (Yongin, Korea). The CWG was washed, segmented, lyophilized, and powdered in the Oriental Medicinal Material and Resource Laboratory of Kyung Hee University (Yongin, Korea). The voucher specimen has been deposited in same laboratory. The powdered CWG was stored -20°C until to use.

Cell culture and differentiation of adipocytes

3T3-L1 cells were maintained in DMEM containing 10% fetal bovine serum and 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 37°C in 5% CO_2. Two days after the cells reached confluency (day 0), the cells were stimulated with differentiation medium (DM) containing 0.5 mM IBMX, 0.25 μM DEX, and 10 μg/mL insulin added to DMEM containing 10% FBS for two day (day 2). Cells were then maintained in 10% FBS/DMEM medium with 10 μg/mL insulin for two day (day 4) which was refreshed every two day. Approximately 8 d after the induction of differentiation, >90% of cells displayed the characteristic lipid-filled adipocyte phenotype under an inverted microscope.

Oil Red O staining

To determine the state of adipose differentiation by visual inspection, the cells were washed with phosphate-buffered saline twice, fixed with 10% formalin at room temperature for 10 min, and stained with 0.5% Oil Red O for 1 h. After staining, the cultures were rinsed several times with 70% ethanol. Pictures were taken using an inverted microscope (Olympus, Tokyo, Japan).

Cytotoxicity assay

Cytotoxicity of CWG was determined by MTT assay. 3T3-L1 cells were seeded in 96-well plates at a density of 1×10^4 cells/well. At 24 h after plating, all media were removed from the wells and the cells were then treated with different concentration of CWG (0, 50, 100, 200, 250, 500, and 1,000 μg/mL) dissolved in DMEM. After incubation for 24 h, 100 μL of yellow MTT solution (5 mg/mL) was added followed by an additional 2 h of

incubation to stain the living cells. Afterwards, the supernatant was removed and 100 μL of dimethylsulfoxide was used to dissolve the formazan crystal. The cell viability was calculated by reading the absorbance of each well at 570 nm (Bio-Tek, Winooski, VT, USA).

RT-PCR analysis of C/EBPα and PPARγ mRNA

The cells were treated with CWG (250, 500 and 1,000 μg/mL), and then the total RNA was extracted with easy-BLUE (iNtRON Biotechnology, Seoul, Korea). An aliquot of 2 μg of total RNA was used to produce cDNA using a reverse transcription polymerase chain reaction (RT-PCR) system (Bioneer, Daejeon, Korea). The RT was performed at 42°C for 1 h and heated to 95°C for 5 min. The following primers were used: glyceraldehyde-3-phosphate dehydrogenase, forward 5'-AGCCATGTACGTAGCCATCC-3' and reverse 5'-CTCTCAGCTGTGGTGGTGAA-3'; PPARγ, forward 5'-GGTGAAACTCTGGGAGATTC-3' and reverse 5'-CAACCATTGGGTCAGCTCTT-3'; C/EBPα, forward 5'-AGGTGCTGGAGTTGACCAGT-3' and reverse 5'-CAGCCTAGAGATCCAGCGAC-3'. The standard amplification program included 30 to 40 cycles which involved heating the product to 94°C with a 30s hold, annealing at 56°C to 60°C with a 30 s hold, and extending at 72°C for 45-60 s, and final elongation step at 72°C for 10 to 15 min. The PCR products were separated by electrophoresis on a 1% agarose gel, visualized by ethidium bromide staining under ultraviolet light using Eagle Eyes image analysis software (Stratagene Co., La Jolla, CA, USA).

Immunoblot analysis

The levels of two adipocyte specific proteins (PPARγ and adiponectin) were analyzed by immuno blot analysis. An aliquot of the protein sample (30 μg) was diluted in 2×sample buffer (50 mM Tris [pH 6.8], 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 5% β-mercaptoethanol) and heated for 5 min at 95°C before SDS-PAGE gel analysis (12%). Subsequently, the proteins were transferred to a PVDF membrane (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and incubated overnight with 5% skim milk at room temperature. The membrane was rinsed three to four times in Tris-buffered saline with Tween-20 (10 mM Tris–HCl, 150 mM NaCl, 0.1% Tween-20, pH 8.0) washing buffer. It was then incubated for 3 h with a blocking solution containing 1:200 dilution of primary antibody against PPARγ or adiponectin (Assay Designs). After being washed four times, the membrane was incubated again for 2 h in horseradish-peroxidase-conjugated anti-mouse IgG secondary antibody (1:1,000, Assay Designs) and developed using enhanced chemiluminescence (ECL Western blot analysis system kit; Amersham Biosciences, Piscataway, NJ, USA). The Western blot analysis was carried out by scanning with a UMAX PowerLook 1120 (Maxium Technologies Inc., Akron, OH, USA) and digitalized using image analysis software (KODAK 1D; Eastman Kodak, Rochester, NY, USA).

Flow cytometric analysis of cell cycle

3T3-L1 pre-adipocytes grown to post-confluency were treated with DM in the presence or in the absence of CWG for 48 h. The cells were then harvested and fixed with 70% ethanol at 4°C for 24 h. After removing of ethanol, cells were stained with a propidium iodide (Sigma Chemical) solution containing RNase (20 μg/mL, Sigma Chemical) for 30 min. Fluorescence activated cell sorting (FACS) analysis was performed with a Becton–Dickinson FACScantoII instrument and data analysis with FACSDiva software (Becton– Dickinson, San Jose, CA, USA).

Statistical analysis

All experiments were performed in triplicate and data are presented as mean±SD. Differences between groups were determined by ANOVA using the SPSS (SPSS Inc., Chicago, IL, USA). A *p*-value of 0.05 or less were considered significant.

RESULTS

Cell toxicity of cultivated wild ginseng on 3T3-L1 pre-adipocyte cells

The effects of CWG on cytotoxicity of 3T3-L1 pre-adipocyte cells are presented in Fig. 1. The various concentrations (0, 50, 100, 200, 250, 500, and 1,000 μg/mL) of CWG did not affect cell viability of the 3T3-L1 pre-adipocytes compared with the control (0 μg/mL concentration of CWG). Therefore, we concluded that CWG did not affect viability of 3T3-L1 pre-adipocyte as determined by the MTT assays.

Effect of cultivated wild ginseng on 3T3-L1 adipocyte differentiation

To test whether CWG inhibits adipocyte differentiation, DM containing insulin, dexamethasone, and IBMX was used to induce 3T3-L1 pre-adipocyte differentiation (Fig. 2) in the presence of CWG. During differentiation induction, CWG was added to the DM on day 0 to

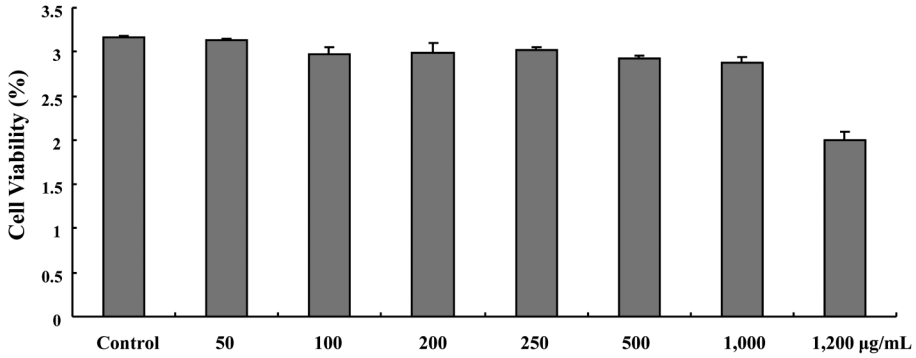

Fig. 1. Effects of cultivated wild ginseng (CWG) on the viability of 3T3-L1 pre-adipocyte cells treated with CWG for 24 h. Viability was determined with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. All values are means±SD of three experiments.

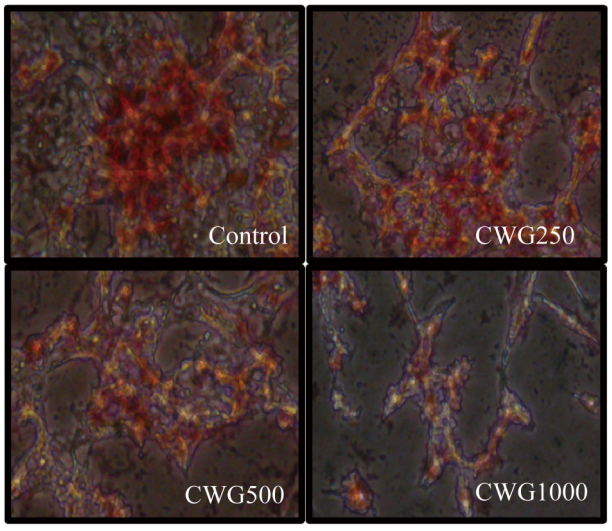

Fig. 2. Cultivated wild ginseng (CWG) inhibits adipocyte differentiation. 3T3-L1 pre-adipocytes were induced to differentiate in the presence of CWG (250, 500, and 1,000 μg/mL). On day 8, the morphological changes associated with adipogenesis were photographed after Oil Red O staining.

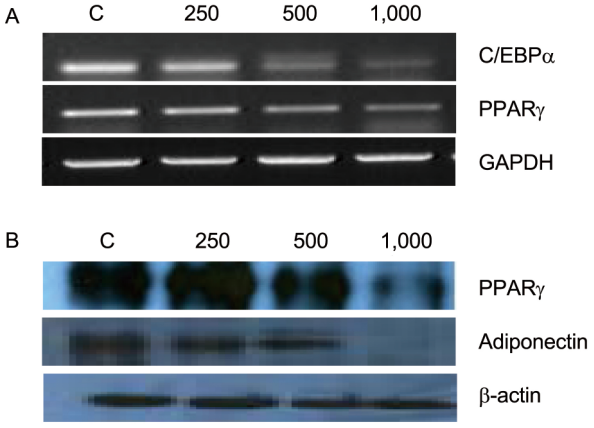

Fig. 3. The effect of cultivated wild ginseng (CWG) on mRNA expression of CCAAT/enhancer binding protein (C/EBP) α and peroxisome proliferator-activated receptor (PPAR)γ by reverse transcription polymerase chain reaction analysis (A) and protein expression of PPARγ and adiponectin by Western blot analysis in 3T3-L1 cells (B). Pre-adipocytes were cultured in growth medium until they reached confluency and then incubated in differentiation medium with or without 250, 500, and 1,000 μg/mL of CWG for 8 d. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

observe its effects on 3T3-L1 pre-adipocyte differentiation. Lipid accumulation and the development of the adipocyte phenotype were assessed by staining with Oil Red O on day 8. It was found that CWG treatment significantly reduced cell differentiation in a concentration-dependent manner compared to control cells. These results indicate that CWG is able to block adipocyte differentiation.

mRNA expression of C/EBPα and PPARγ

To investigate the inhibitory mechanism of CWG during adipocyte differentiation, the expression levels of C/EBPα and PPARγ, key transcriptional factors for adipocyte differentiation, were examined. It is well-documented that during 3T3-L1 adipocyte differentiation, C/EBPα and PPARγ are activated by insulin, dexamethasone, and IBMX in DM. Total RNA was purified from 3T3-L1 cells at day 8 of differentiated and RT-PCR was carried out. It was found that the mRNA expression of both C/EBPα and PPARγ was strongly inhibited in a concentration-dependent manner by CWG, suggesting that CWG affects the signaling mechanism for adipocyte differentiation in 3T3-L1 (Fig. 3A).

Protein expression of peroxisome proliferators-activated receptor γ and adiponectin

PPARγ and adiponectin are known as key station proteins that are expressed early during adipocyte differentiation and adipogenesis. Western blot analysis confirmed that protein expression of PPARγ and adiponectin was significantly down-regulated in response to CWG treatment (Fig. 3B). These results suggest that CWG inhib-

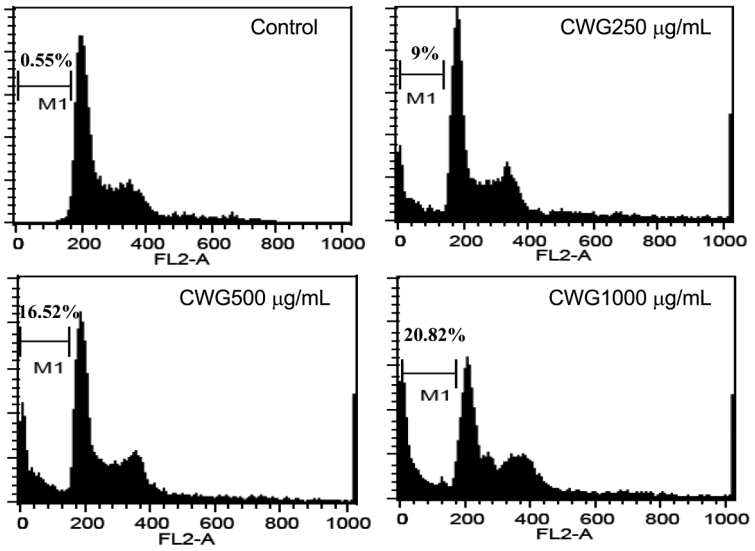

Fig. 4. Flow cytometric analysis of 3T3-L1 pre-adipocytes differentiation treated with cultivated wild ginseng (CWG; 250, 500, and 1,000 μg/mL). Cells stained with propidium iodide to analyze cell cycle distribution at 48 h after induction. M1, mitotic phase (sub-G_1 peak); FL2-A, fluorescein 2.

ited adipogenesis by the down-regulation of key adipogenic proteins.

CWG blocks cell cycle progression in 3T3-L1 cells

To prove the effect of CWG on cell mitosis after adipogenic induction, the 3T3-L1 cells were analyzed by FACS. Following CWG treatment, the cell cycle was blocked at the sub-G_1 phase in a concentration-dependent manner (Fig. 4). The percentages of cells in sub-G_1 phase were 9%, 16.52%, and 20.82% when treated with 250, 500, and 1,000 μg/mL of CWG for 48 h, respectively.

DISCUSSION

Over the past few decades, obesity has become a global epidemic in both developed and developing countries. It is characterized by an increase in adipose tissue mass and is highly associated with various health risk factors [12]. Anti-obesity strategies are classified into four major categories: reducing food intake, blocking nutrient absorption, increasing thermo-genesis, and modulating fat or protein metabolism or storage [13]. The prevalence of obesity and obesity-related disorders has led to major research interests in the influence of adipose tissue mass [14]. Wang and Jones [15] reported that the decreased adipocytic lipogenesis is one of the proposed anti-obesity mechanisms. Utilization of anti-adipogenic compounds from natural sources could be helpful in the prevention of obesity, without incurring side effects.

Naturally-occurring wild ginseng has been found to have beneficial effects on health, and accumulated evidence has demonstrated its physiological properties along with anti-obesity and anti-diabetic effects in animals and humans [16]. Ginseng has been used traditionally in oriental countries to improve health. The whole extract of ginseng berries possesses both antidiabetic and anti-obesity activity in ob/ob mice [11]. Wild ginseng leaf extract supplementation to diabetic rats helps to control their blood glucose levels [17]. We have previously reported that the anti-obesity effects of wild ginseng mediated PPARγ, Glucose transporter type 4 (GLUT4) and Lipoprotein lipase (LPL) in ob/ob mice [18]. The present study showed that CWG suppresses adipocyte differentiation by inhibiting the expression of adipogenic genes. CWG treatment significantly reduced the levels of C/EBPα and PPARγ mRNA along with the protein expression of PPARγ and adiponectin.

At the molecular level, the adipocyte differentiation occurrence is regulated by transcriptional activators such as C/EBPα and PPARγ [4]. C/EBP (α, β, and δ) belongs to the basic leucine zipper family of transcription factors. PPARγ is a member of the nuclear receptor superfamily of transcription factors and both are predominantly expressed in adipose tissue. These transcription factors appear to function as dominant activators of adipocyte differentiation [4]. PPARγ is induced prior to transcriptional activation of most common adipocyte specific genes, and the expression of PPARγ is enough to induce growth arrest and initiate adipogenesis in exponentially-growing fibroblast cell lines [19]. Additionally, it was reported that C/EBPα is a likely candidate transcription factor for precise regulation

of adipocyte differentiation [20]. C/EBPα and PPARγ interchangeably bind to their genomic promoter regions to activate transcription and maintain their own activity [21]. In this study, we showed that CWG treatment significantly decreased C/EBPα and PPARγ mRNA expression in cultured 3T3-L1 cells. These observations suggest that CWG suppresses adipocyte differentiation through C/EBPα- and PPARγ-mediated mechanisms.

Several proteins that serve as markers for adipocyte differentiation have been well-documented. For example, PPARγ and adiponectin are also exclusively expressed in adipocytes [22,23]. PPARγ and adiponectin gene expression is turned on day 2 after the initiation of adipocyte differentiation and maintained at a relatively high level in mature adipocytes. Therefore, blockage of adipocyte differentiation is expected to reduce PPARγ and adiponectin expression as shown in this study.

This study examined the effect of CWG on cell cycle progression using flow cytometry. FACS data from cell cycle analysis demonstrated the effect of CWG on pre-adipocyte proliferation. Tang *et al*. [24] reported that DM caused the growth-arrested pre-adipocytes to initiate cell cycle progression; CWG blocked the cell cycle progression at the sub-G_1 phase. These results suggested that it was possible that CWG inhibited the differentiation of pre-adipocytes by preventing the cells from traversing the G_1 phase checkpoint of the cell cycle. This would cause the pre-adipocytes to remain at the sub-G_1 phase even when stimulated by adipogenic factors.

In conclusion, the results of this study demonstrated the inhibitory effects of CWG on the differentiation of 3T3-L1 pre-adipocytes. These inhibitory effects were mediated by decreased C/EBPα and PPARγ mRNA expression and also by decreased PPARγ and adiponectin protein levels. Thus, CWG extracts or the biologically active components of CWG may be new therapeutic candidates for the prevention and/or treatment of obesity and obesity-related diseases.

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