RESEARCH ARTICLE

Inhibition of Leptin and Leptin Receptor Gene Expression by Silibinin-Curcumin Combination

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Abstract

Leptin and its receptor are involved in breast carcinogenesis as mitogenic factors. Therefore, they could be considered as targets for breast cancer therapy. Expression of the leptin receptor gene could be modulated by leptin secretion. Silibinin and curcumin are herbal compounds with anti-cancer activity against breast cancer. The aim of this study was to assess their potential to inhibit of expression of the leptin gene and its receptor and leptin secretion. Cytotoxic effects of the two agents on combination on T47D breast cancer cells was investigated by MTT assay test after 24h treatment. With different concentrations the levels of leptin, leptin receptor genes expression were measured by reverse-transcription real-time PCR. Amount of secreted leptin in the culture medium was determined by ELISA. Data were statistically analyzed by one-way ANOVA test. The silibinin and curcumin combination inhibited growth of T47D cells in a dose dependent manner. There were also significant difference between control and treated cells in leptin expression and the quantity of secreted leptin with a relative decrease in leptin receptor expression. In conclusion, these herbal compounds inhibit the expression and secretion of leptin and it could probably be used as drug candidates for breast cancer therapy through leptin targeting in the future.

Keywords: Leptin - silibinin - curcumin - breast cancer - T47D cell line

Introduction

Breast cancer is the most common cancer in women worldwide with the high mortality rate (Siegel et al., 2012). Many factors contribute to breast cancer development that one documented risk factor is leptin (García-Robles et al., 2013). Leptin, a 167 protein with a molecular mass of 16 kDa, expressed mainly by adipose tissues (Go et al., 2013). It has central roles in the energy expenditure, food intake, many reproductive processes, regulation of energy homeostasis, neuroendocrine function, and metabolism (Kelesidis et al., 2010). Beside the synthesis by adipose tissue as the main source (Go et al., 2013), there have been indentified other sources of leptin in the body including testicles (Soyupek et al., 2005), ovaries (Löffler et al., 2001), placenta (Maymó et al., 2011), cartilage and bone cells (Morroni et al., 2004), skeletal muscle (Solberg et al., 2005) and stomach (Mix et al., 2004). Furthermore, the mitogenic, transforming or migration-induced properties of leptin have been revealed in many different cell types such as smooth muscle cells (Oda et al., 2001), normal and neoplastic colon cells (Hardwick et al., 2001; Liu et al., 2001); and also normal and malignant mammary epithelial cells (Dieudonne et al., 2002; Laud et al., 2002). It has been shown that leptin induces growth and transformation in T47D breast cancer cells unlike normal breast epithelial cells (Hu et al., 2002). Leptin acts through binding to its receptor known leptin receptor (ObR), a type I cytokine receptor, located in the target cell membrane. Two main leptin receptor isoforms dominate: the short leptin receptor isoform (OB-Ra) and the long leptin receptor isoform (OB-Rb). OB-Rb contains the full-length intracellular domain and is believed to be the main leptin signaling receptor. Ob-Ra contains a truncated intracellular domain and has been shown to participate in signaling through JAK-dependent activation of MAPK but cannot activate STAT (Cottrell and Mercer, 2012).

Significantly higher levels of both leptin and ObR expression have been found in cancer tissue relative to non-cancer epithelium (Ishikawa et al., 2004). Also, numerous breast cancer cell lines such as MCF-7 and T47D could express leptin and ObR (Yom et al., 2013). All these observations confirm that leptin can act not only by endocrine and (or) paracrine action on mammary tumor cells, but also via an autocrine pathway. Additionally, a significant positive correlation has been obtained between leptin and ObR expressions with breast cancer tissue (Koda et al., 2007). Therefore, this paracrine-autocrine

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leptin axis could become a target for leptin-inhibiting drugs in cancer treatment and prevention. Silibinin, a flavonolignan prepared from milk thistle, has cancer chemopreventive activity in preclinical models of prostate and colorectal cancer. Curcumin (diferuloylmethane) is the chief component of the spice turmeric and is derived from the rhizome of the East Indian plant Curcuma longa. Curcumin has several of biological activities that finally make this molecule a possible anti-cancer drug, as both chemopreventive and chemotherapeutic. Considering important roles of leptin and leptin receptor in the breast cancer biology, in this study we investigated the possible variations in the leptin secretion and expression as well as expression of leptin receptor in the T47D breast cancer cell line after its treatment with silibinin, curcumin and their combination.

Materials and Methods

Chemicals and reagents

Silibinin (Sigma, Germany), Curcumin (Sigma, Germany), MTT (Sigma, Germany), Leptin ELISA kit (Labor Diagnostika nord gmbh & co. kg, Germany), Fetal bovine serum (Gibco, USA), Phenol-red free RPMI 1640 with L-glutamine (Gibco, USA), T47D cells (Pasteur Institute of Iran), Sodium bicarbonate (Merck, Germany), Penicillin (SERVA, Germany), Streptomycin (Merck, Germany), Amphotericin B(Merck, Germany), TRIZOL Reagent (Invitrogen, USA), First-Strand Synthesis kit (Fermentas, USA), Syber Green-I reagent (Fermentas, USA).

Cell culture

T47D cells were cultured in RPMI1640 (with glutamine) supplemented with 10% FBS, penicillin, streptomycin and amphotericin B and incubated at 37°C in a humidified atmosphere containing 5% CO_2.

MTT assay and cell treatment

The cytotoxic effect of silibinin, Curcumin and silibinin curcumin combination on T47D cells was studied by 24h MTT assays. Briefly, 2500 cell/well were cultivated in a 96 well culture plate. After 24h incubation in 37°C cells were treated with different concentrations of drugs (0-120 μM) for 24h in the quadruplicate manner. Then, medium of all wells were removed carefully and 50μl 2mg/ml MTT was added to each well and incubated in dark for 4.5h, followed by addition of 200μl DMSO. Thereafter, Sorensen's' glycine buffer was added and absorbance of each well was read at 570 nm during 15-30 minutes. For data analysis, mean OD of each well was calculated. Then, percent of cells viability was calculated according to this formula: percent of cells viability=mean OD of test wells/mean OD of control wells×100. Finally, a graph was plotted using SPSS 16.0 and IC_{50} of drugs on T47D was determined on graph.

For studying the inhibitory effect of silibinin and curcumin mixture on leptin and leptin receptor expression as well as leptin secretion, $1×10^5$ cells/wells were treated in a 6-well plate with different sub-toxic concentrations of these compounds (0, 10, 20, 40, 60 and 80μM) for 24h. A control group containing 0.1% DMSO as vehicle control.

Isolation of total RNA and cDNA synthesis

Total RNA was extracted directly from attached cells using TRIZOL Reagent according to the manufacturer's instructions. The concentration of prepared RNA was measured using a NanoDrop spectrophotometer (Termoscientific, USA) and its integrity was confirmed by electrophoresis on 1.2% agarose gel containing 1% formaldehyde.

After RNA preparation, cDNA was synthesized using the First-Strand Synthesis kit according to the manufacturer's instructions. The synthesized cDNA was immediately used in a real-time PCR or stored at -70°C for later use.

Real-time PCR

The real-time PCR was used for measurement of leptin and leptin receptor expression levels in the control and treated cells. β-actin gene expression was used as the internal control.

The real-time PCR reaction was done using the Syber Green-I reagent in the Rotor Gene TM 6000 system (Corbett research, Australia) according to the manufacturer's instructions in a triplicate manner. The amplification conditions were as follows: leptin (2 min at 95°C and a two-step cycle of 95°C for 15 s and 60°C for 40s for 40 cycles), OB-Ra (5 min at 95°C and a two-step cycle of 95°C for 30s and 58°C for 40s for 40 cycles), and OB-Rb (5 min at 95°C and a two-step cycle of 95°C for 15s and 59°C for 40s for 40 cycles). Sequences of used primers were shown in Table 1.

Changes in leptin, OB-Ra and OB-Rb expression levels between the control and treated T47D cells were calculated by the $2^{-\Delta\Delta CT}$ method.

Measurement of the secreted leptin

For analysis of possible effect of silibinin Curcumin compound on amount of secreted leptin in the treated cells compared with the control cells, leptin concentration was measured in the supernatant media of cells using a human leptin ELISA kit according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed with SPSS 18.0 software. Data are expressed as mean±standard deviation. All experiments were performed in triplicate. The differences in expression levels of leptin, OB-Ra and OB-Rb as well as quantity of secreted leptin between

Table 1. Primers used for Real-time PCR Amplifications

Primer	Primer length	Sequence (5´ to 3´)	Product size (bp)
Leptin Forward	22	CACCAAAACCCTCATCAAGACA	80
Leptin Reverse	24	CTTTCTGTTTGGAGGAGACTGACT	
OB-Ra Forward	21	CAAGAATTGTTCCTGGGCACA	110
OB-Ra Reverse	20	ACTGTTGGGAAGTTGGCACA	
OB-Rb Forward	21	CAAGAATTGTTCCTGGGCACA	114
OB-Rb Reverse	21	TCAGGCTCCAAAAGAAGAGGA	
B-actin Forward	20	TGGACTTCGAGCAAGAGATG	137
B-actin Reverse	20	GAAGGAAGGCTGGAAGAGTG	

the control and treated cells were analyzed by one-way ANOVA, followed by Dunett's multiple comparison test. A p value <0.05 was considered as significant.

Results

MTT assay

Data analysis of cytotoxicity assay showed that IC$_{50}$ of silibinin, curcumin and their combination on T47D breast cancer cell line was 110, 30 and 20μM for 24h MTT assays, respectively (Figure 1) .

Quantitative real-time PCR

Real-time PCR results showed a significant decrease in leptin expression in the treated cells with silibinin, curcumin and also, much more with their combination compared to the control cells (p value <0.05). We, also, measured the expression levels of OB-Ra and OB-Rb in the treated and control cells. Although, OB-Ra and OB-Rb expression levels between the treated and the control cells was relatively decreased But this decrease was not significant (Figure 2). Data analysis revealed a positive correlation between leptin gene expression and OB-Ra and OB-Rb gene expression level.

Measurement of secreted leptin

Amounts of secreted leptin were evaluated using ELISA. A significant difference was found between the control and treated cells in term of secreted leptin (Figure 4). This finding was in accordance with inhibition of leptin gene expression by silibinin Curcumin compound.

Discussion

This study demonstrates that silibinin and curcumin can inhibit leptin gene expression and secretion in T47D breast cancer cells and this decrease in leptin gene expression and secretion has link with OB-Ra and OB-Rb gene expression. These results indicate that silibinin and curcumin have strong potential to interact with the expression of leptin gene, which has significant roles in carcinogenesis and proliferation of breast cancer cells (García-Robles et al., 2013). Regarding to the critical role of leptin in breast carcinogenesis, there are many attempts to inhibit leptin function and secretion. Gonzalez et al. (2009) inhibited growth of murine mammary cancer cell and xenograft tumor model of human breast cancer cell lines by leptin peptide antagonist (Gonzalez et al., 2009). In addition, leptin analog mimicking its action (Peters et al., 2007) and anti-leptin receptor monoclonal antibody (Fazeli et al., 2006) are also other approaches for interfering with the leptin function. In the other studies, administrations of some compounds including b3-adrenoreceptor agonist, conjugated linoleic Acid, isoflavone, resveratrol and bitter melon lead to decreased secretion and lower levels of serum leptin (Ray and Cleary, 2010). The current work, however, aimed to direct inhibition of leptin expression and secretion in human T47D breast cancer cell line using herbal compounds. Due to the significant anti-cancer effects of silibinin and curcumin on various types of cancers such as prostate, skin, colon, bladder and breast (Kaur and Agarwal, 2007; Sareen et al., 2013), they can be used as chemotherapeutic agents for breast cancer therapy.

Lin et al. (2009) found that silibinin blocks mammalian target of rapamycin signaling with a concomitant reduction in translation initiation, thus inhibit growth of transformed cells. Rana et al. (2009) reported Silibinin/silymarin also inhibits the secretion of proangiogenic factors from tumor cells, and causes growth inhibition of endothelial cells. Furthermore, Chiu and Su, (2009) have shown that curcumin inhibits the migratory activity of breast cancer
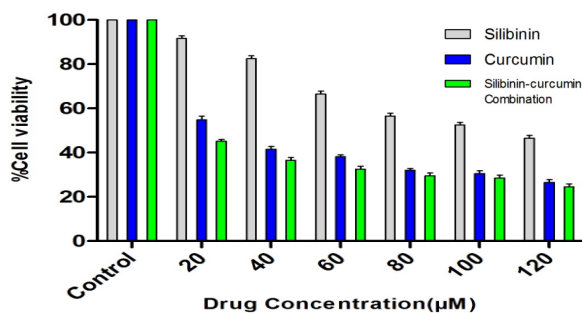

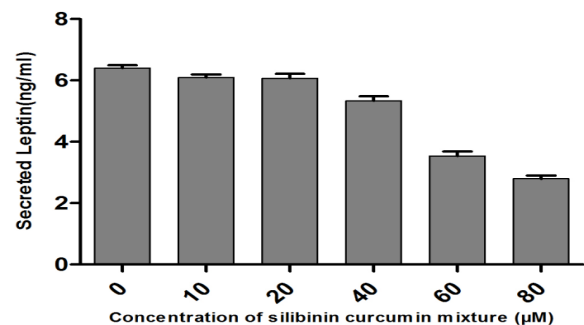**Figure 1. The Cytotoxic Effect of Silibinin, Curcumin and Their Combination on T47D Cells during 24h MTT Assays**

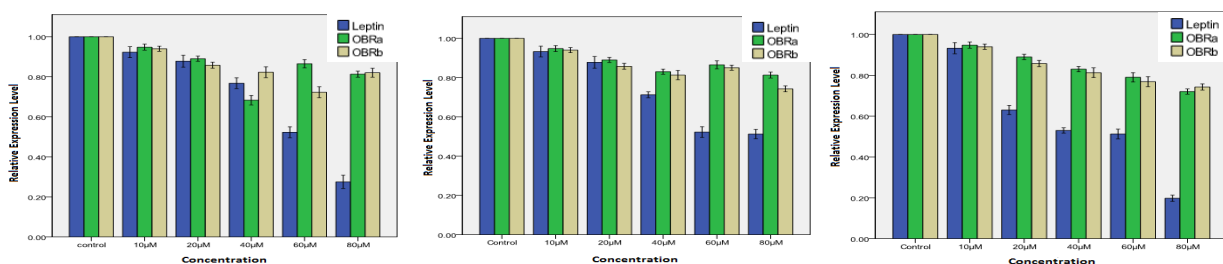**Figure 3. Effect of Silibinin Curcumin Combination on Leptin Secretion in T47D Cells**

Figure 2. Effect of A) Silibinin; B) Curcumin; and C) Silibinin Curcumin Combination, on Leptin, OB-Ra and OB-Rb Gene Expression in the T47D Cells. As the figure shows, there is a significant decrease in leptin gene expression levels with increasing in the Drug concentrations (p value<0.05), unlike leptin receptor genes expression levels

cells, proliferative rate, adhesion, and invasion through down-regulating the expression of NF-κBp65.

More recently, evidence is emerging that specific combinations of phytochemicals may be far more effective in protecting against cancer than isolated compounds (de Kok et al., 2008).

We found that curcumin and silibinin separately decrease the expression of both leptin and leptin receptor in T47D cells. Also, they showed more potent decreasing effect on the expression of leptin and its receptor when were used together. Thus, it seems that curcumin and silibinin intensify each other's inhibitory effects on leptin and its receptor genes expression and this intensifying effect may be through similar mechanisms. The mechanism for declining the expression of leptin was proposed by a recent study (Nejati-Koshki et al., 2012). This study showed that silibinin can decrease the expression of leptin in T47D cell line through increasing of ERβ/ERα (estrogen receptor β/ estrogen receptor α) gene expression ratio. Also, another study demonstrated the inhibitory effect of curcumin on expression of ERα, and thus, the increasing of ERβ/ERα gene expression ratio (Boominathan and Lakshmanane, 2009). Therefore, based on the results of these two studies (Boominathan and Lakshmanane, 2009; Nejati-Koshki et al., 2012), the decrease in the leptin gene expression by curcumin can be done through the decreasing in the ERα gene expression (increasing in the ERβ/ERα expression ratio).

Furthermore, with regarding to the fact that leptin and its receptors directly increase each other gene expression through autocrine cycle (Wazir et al., 2012), therefore, the decreasing in the leptin and or leptin receptors can inhibit this cycle, and down-regulate the expression of both leptin and its receptors. This can be considered as another mechanism for decreasing leptin expression. In our study, silibinin and curcumin as well as their combination inhibited the expressions of leptin and its receptors in T47D cell line. Therefore, it can be inferred that, beside down-regulation of leptin gene expression by decreasing of ERα gene expression, down-regulation of the mentioned genes by these compounds are done through interfering in the leptin-leptin receptors autocrine cycle. Briefly, these two mechanisms are possible mechanisms for inhibition of leptin gene expression through curcumin and silibinin in T47D cells.

Also, these mechanisms are linked to each other. A study showed that inhibition of leptin receptor by siRNA leads to down-regulation of ERα gene expression (Fusco et al., 2010). Thus, modulation of each involving gene (leptin, leptin receptors, ER) can cause interference in the entire pathway in ER+ cells and based on these mechanisms, the synergistic inhibitory effects of curcumin and silibinin on leptin expression are, therefore, due to their effects on the same targets.

Although we propose the possible mechanisms for inhibition of leptin gene expression, there are need to clarify the exact mechanism(s) for inhibition of leptin receptors genes expression by curcumin and silibinin in the future.

In conclusion, we demonstrated that silibinin-curcumin mixture could potently inhibit expression and secretion of leptin in T47D breast cancer cell line. Regarding to the significant roles of leptin and leptin receptor in breast carcinogenesis, its inhibition by curcumin and silibinin could be considered as a novel strategy for treatment of breast cancer in the future.

Acknowledgements

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