RESEARCH ARTICLE

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

Kazem Nejati-Koshki1,2*, Abolfazl Akbarzadeh2*, Mohammad Pourhasan-Moghaddam1, Alireza Abhari3, Hassan Dariushnejad1

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 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

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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 Diagnostik ar 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% CO₂.

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 IC₅₀ of drugs on T47D was determined on graph.

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⁻ΔΔCT 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 controls and treated cells were calculated by the Student t-test method.

Table 1. Primers used for Real-time PCR Amplifications

<table>
<thead>
<tr>
<th>Primer</th>
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<td>Leptin</td>
<td>22</td>
<td>CACCCAAACCCCTCATCAAGACA</td>
<td>80</td>
</tr>
<tr>
<td>OB-Ra</td>
<td>21</td>
<td>CAAGATTTGTCTGGGCACA</td>
<td>110</td>
</tr>
<tr>
<td>OB-Rb</td>
<td>21</td>
<td>ACTGTTGGGAAATGTGGCACA</td>
<td>114</td>
</tr>
<tr>
<td>B-actin</td>
<td>20</td>
<td>TGGACCTCGAGCAAGAGAT</td>
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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
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control group containing 0.1% DMSO as vehicle control.
the control and treated cells were analyzed by one-way ANOVA, followed by Dunnett’s multiple comparison test. A p value <0.05 was considered as significant.

**Results**

**MTT assay**

Data analysis of cytotoxicity assay showed that IC\textsubscript{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 (Garcia-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.
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.

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References


