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

Kazem Nejati-Koshki, Abolfazl Akbarzadeh, Mohammad Pourhasan-Moghaddam, Alireza Abhari, Hassan Dariushnejad

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

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 identified 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 (diferulolymethane) 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 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), Streptomyces (Merck, Germany), Amphotericerin B (Merck, Germany), TRIZOL Reagent (Invitrogen, USA), First-Strand Synthesis kit (Fermentas, USA), Syber Green-I reagent (Fermentas, USA), B-actin (Fermentas, USA), OB-Ra (Life Technologies, USA), OB-Rb (Fermentas, USA). All chemicals were purchased from Sigma-Aldrich.

Cell culture

T47D cells were cultured in RPMI1640 (with glutamine) supplemented with 10% FBS, penicillin, streptomyces 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 of 2mg/ml MTT was added to each well and incubated in 37°C for 30 minutes. For data analysis, mean OD of each well was calculated by the 2–ΔΔCT method.

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.

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 were calculated as a Student’s t-test.

Table 1. Primers used for Real-time PCR Amplifications

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer length</th>
<th>Sequence (5’ to 3’)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin Forward</td>
<td>22</td>
<td>CACCAAAAAACCTCATCAAGACA</td>
<td>80</td>
</tr>
<tr>
<td>Leptin Reverse</td>
<td>24</td>
<td>CTTCTGTTTGGAGGAGACTGACT</td>
<td>110</td>
</tr>
<tr>
<td>OB-Ra Forward</td>
<td>21</td>
<td>CAAGATGTGTTCCTGGCCACA</td>
<td>114</td>
</tr>
<tr>
<td>OB-Ra Reverse</td>
<td>20</td>
<td>ACGTTGGGAAATGGTGCCACA</td>
<td>120</td>
</tr>
<tr>
<td>OB-Rb Forward</td>
<td>21</td>
<td>CAAGATGTGTTCCTGGCCACA</td>
<td>114</td>
</tr>
<tr>
<td>OB-Rb Reverse</td>
<td>21</td>
<td>TCAGGCTCCAAAAGAAGAGGA</td>
<td>120</td>
</tr>
<tr>
<td>B-actin Forward</td>
<td>20</td>
<td>TGACCTCAGGAAGAGAGATG</td>
<td>137</td>
</tr>
<tr>
<td>B-actin Reverse</td>
<td>20</td>
<td>GAAGAAGGGCTGGAGAGATG</td>
<td>141</td>
</tr>
</tbody>
</table>
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_{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 cells.

![Figure 1. The Cytotoxic Effect of Silibinin, Curcumin and Their Combination on T47D Cells during 24h MTT Assays](image1)

![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](image2)

![Figure 3. Effect of Silibinin Curcumin Combination on Leptin Secretion in T47D Cells](image3)
The 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


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