RESEARCH ARTICLE

Glaucocalyxin A Activates FasL and Induces Apoptosis Through Activation of the JNK Pathway in Human Breast Cancer Cells

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Abstract

This study was conducted to analyze the molecular mechanisms responsible for anti-proliferation effects of glaucocalyxin A in cultured MCF-7 and Hs578T breast cancer cells. The concentration that reduced cell viability to 50% (IC50) after 72 h treatment was derived and potential molecular mechanisms of anti-proliferation using the IC50 were investigated as changes in cell cycle arrest and apoptosis. Gene and protein expression changes related to apoptosis were investigated by semi-quantitative RT-PCR and western blotting, respectively. Involvement of phosphorylated mitogen-activated protein kinases and JNK signaling in regulation of these molecules was characterized by western blotted. Cell viability decreased in a concentration-dependent manner and the IC50 was determined as 1 μM in MCF-7 and 4 μM in Hs578T cell. Subsequently, we demonstrated that the GLA-induced MCF-7 and Hs578T cell death was due to cell cycle arrest at the G2/M transition and was associated with activation of the c-jun N-terminal kinase (JNK) pathway. We conclude that GLA has the potential to inhibit the proliferation of human breast cancer cells through the JNK pathway and suggest its application for the effective therapy for patients with breast cancer.

Keywords: Glaucocalyxin A - cell arrest - cell death - human breast cancer - FasL - JNK pathway

Introduction

Breast cancer is the most common malignant tumor in women in recent years. It is estimated that more than one million new cases of breast cancer are diagnosed every year in the world, and that about half of these patients would die of this disease. Estrogen therapy is frequently used in the early-stage of breast cancer (Jemal et al., 2011). However, because of the drug resistance, breast cancer is highly resistant to estrogen as the pathology progresses (Miyoshi et al., 2010). Radiotherapy is also used after breast-conserving surgery to decrease the chance of recurrence (Veronesi et al., 2010). Nevertheless, novel therapies and chemo-therapeutic drugs are urgently needed to be developed for the treatment of breast cancer.

Apoptosis, which is characterized by cytoplasmic shrinkage, chromatin condensation and DNA fragmentation, is an active form of cell death that occurs in response to several agents, including anticancer chemotherapeutic drugs (Lawen et al., 2003). Many biomarkers and events, such as the caspase family proteins and Bcl-2 family members, could be considered as the determinants of apoptosis (Antonsson et al., 2000). The abnormal production of the molecule may trigger redox signaling pathways, such as oxidative stress, cell cycle arrest and apoptosis (Zhang et al., 2013).

Natural products have been considered as major sources of chemotherapeutic drugs in the treatment of breast cancer (Bishayee et al., 2011). Rabdosia japonica (R. japonica) is a perennial herb that is distributed widely in East Asia, and the entire R. japonica extract has been used traditionally as a folk medicine for treating gastrointestinal disorders, tumors, and inflammatory diseases (Sun et al., 2006; Hong et al., 2008; Kim et al., 2013). In this study, the anti-cancer effect of Glaucocalyxin A (GLA) and the underlying mechanisms involved were investigated in breast cancer cells.

The c-Jun N-terminal kinases (JNKs) are members of the mitogen activated protein kinase (MAPK) family that regulate multiple cellular processes, including development, differentiation, proliferation and apoptosis, in response to extracellular signals, metabolic status and environmental cues (Weston et al., 2002). The kinases are encoded by three distinct genes; JNK1 and JNK2 are ubiquitously expressed, while JNK3 is present primarily in the brain (Nakano et al., 2006). In general, the JNK and p38 cascades are activated by chemicals and environmental stress and are usually involved in cell growth and apoptosis.

JNKs have been proposed to function both as tumor suppressors and mediators of many malignant cell proliferation, survival and resistance to chemotherapy,
such as glioma (Wagner et al., 2009), breast cancer (Lee et al., 2013). However, the functional role of JNK in breast cancer after treated with GLA has not been investigated. In this study, we used pharmacologic and molecular approaches to investigate the role of JNK in the GLA-induced death of breast cancer cell.

Materials and Methods

Chemicals, reagents and instruments

A 1 mM stock solution of GLA was obtained by dissolving GLA in DMSO. RPMI-1640 medium (Gibco, CA, USA), TRIZol (Gibco, CA, USA), MTT (Sigma, St. Louis, USA), Annexin V FITC Kit (Byotime, Nantong, Jiangsu, China). The following primary antibodies were used: p-JNK, Bcl-2, Bax, p-c-jun (Cell Signaling Technology, Beverly, MA, USA), p53, p-Erk and p-c-jun (Santa Cruz Biotechnology, Inc. Dallas, Texas, USA), and β-actin (Sigma, St. Louis, USA).

Cell Culture

Human breast cancer cell line MCF-7 and Hs578T purchased from ATCC (Manassas, VA, USA), were maintained in RPMI 1640 containing 10% fetal calf serum. All cell lines were cultured at 37°C, 5% CO₂.

Cell proliferation assays

As described previously (Chiang et al., 2007), the effects of glucocallyxin A on cell proliferation were examined by MTT method.

Briefly, 1x10⁴ cells were seeded into a 96 well plate, using 100 μL culture media per well. After cells have been incubated for 12 h, the medium was replaced by 100 μL fresh cell medium containing various concentrations of GLA (0, 1, 2, 4, 8, or 16 μM). For the measurement of cell viability after being incubated for 24 h, 48 h and 72 h, MTT assay was performed as described previously to examine the impact of drugs on cells. MTT solution (5 mg/mL in PBS) was added to the samples in the 96-well plates and the cells were allowed to incubate for 4 h at 37°C. And then, the cells and dye crystals (formazan) were dissolved by adding 100 μL DMSO and the absorbance was measured at 572 nm. The inhibition rate was calculated as follows: inhibition rate = 1 - (OD drug-treated/OD control) x 100%.

Table 1. The List of Primers Used in RT-PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sense primer</th>
<th>Antisense primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2</td>
<td>GGATTGTGGCCTTCTTGTGAG</td>
<td>CCAACTGAGCAGATTCCTTC</td>
</tr>
<tr>
<td>p53</td>
<td>GAAAGCCACAGTCGCAATGGA</td>
<td>GGTAGGTTTTCTGGGAGG</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>TGGAAATGTGGCTAATGTT</td>
<td>GGCAACCTGAAATATGCAA</td>
</tr>
<tr>
<td>c-fos</td>
<td>AAGGAAGATCCGAGGAAAAGGAAATAAGATGGCT</td>
<td>AGAGCAGGAAGGAAGGCGTGTAAGCAGTGAG</td>
</tr>
<tr>
<td>c-jun</td>
<td>GCTAGGAGGACCGCTACCGCTGGCAGCTTGCAAGTGAG</td>
<td>GGGCCACAGCTTCTCCACGTGACACACT</td>
</tr>
<tr>
<td>Fas</td>
<td>ATGCTGGGACATCGGACCT</td>
<td>TCTAGACAAAGGCTTGTGGT</td>
</tr>
<tr>
<td>FasL</td>
<td>CCTCAGAGCAGATGGTCC</td>
<td>ATCTGGGCTGTAGACTCTG</td>
</tr>
<tr>
<td>COX-2</td>
<td>TCTTAAATGAGATTGGGGAAT</td>
<td>AGATCATCTCCTGAGATATCTT</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>TCTCTGACTTCAAGCAGCACC</td>
<td>TCTACTCCCTTGGTGTCCTT</td>
</tr>
</tbody>
</table>

Reverse Transcription polymerase Chain Reaction (RT-PCR)

Total RNA was extracted using TRIZOL (Invitrogen, Carlsbad, CA, USA). RNA (2.5 μg) was reverse transcribed using a SuperscriptIII kit (Invitrogen), according to the manufacturer’s instruction. PCR amplification was carried out in a 50 μL PCR reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 20 pmol of each primer set, two units of Taq DNA polymerase (Transgene, Beijing, China), 0.2 mM dNTPs, and 2 μL of cDNA. The PCR conditions were described previously.
GLA suppressed the viability and growth of cells through G2/M phase arrest

Many studies have demonstrated that GLA exerted a potent cytotoxic activity and had a significantly inhibitory effect on several tumor cells, such as leukemia HL60 cell (Gao et al., 2011; Yang et al., 2013). Our results revealed that a marked anti-proliferative activity was observed in MCF-7 and Hs578T cells with IC50 value of 1 μM and 4 μM after treatment of GLA for 72 h, respectively (Figure 1).

To explore the mechanisms leading to the loss of MCF-7 and Hs578T cells proliferation by GLA, the effects of GLA treatment on cell cycle arrest were examined. MCF-7 and Hs578T cells were incubated with various concentrations of GLA for 72 h. A dose-dependent G2/M phase arrest was observed (Figure 2).

GLA induced apoptosis and regulated the expression of apoptosis related proteins

To further understand whether GLA-induced cell death is mediated by apoptosis or necrosis, we evaluated apoptotic cell death using annexin V/PI double staining, which specifically labels apoptotic cells. As shown in Figure 3a, treatment with GLA at concentrations of 8 μM for 24 h induced apoptosis in about 38% and 75% of the MCF-7 and Hs578T cells, respectively. Additionally, treatment with GLA activated caspase-3, which the significant proteolytic cleavage of caspase-3 was detected by RT-PCR and western blot.

Immunoblot analysis

Proteins were extracted by lysing cells in sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris–HCl [pH 6.8], 2% SDS) containing 1 mM phenylmethylsulfonyl fluoride, 10 mg/mL pepstatin, 12.5 mg/mL leupeptin, 2 mg/mL aprotinin, 1 mM sodium orthovanadate, and 1 mM sodium molybdate. Cell extracts were processed for western immunoblotting as described previously (Weston et al., 2002). The following antibodies used for immunoblotting were purchased from the indicated suppliers: p53 from Santa Cruz Biotechnology (Santa Cruz, CA, USA); phospho-JNK (Thr183/Tyr185), JNK1/2, and Bcl-2 from Cell Signaling Biotechnology (Beverly, MA, USA), β- actin from Sigma.

Statistical analysis

Experiments and in vitro assays were carried out at least in triplicate. Differences between mean values were assessed by Student’s t-test with two-sided P values, P < 0.05 was considered as statistically significant.
To confirm whether other apoptosis-related proteins, such as Bax, Bcl-2 and p53 protein was related to GLA-induced apoptosis, we treated MCF-7 cells with various concentrations of GLA (0, 2, 4, 8 μM) for 24 h and analyzed apoptosis-related proteins using Western blotting. As shown in Fig 3b, Bax, and p53 was significantly increased, however the expression of Bcl-2 was decreased.

GLA-induced apoptosis was attributed to the upregulation of Fas/FasL signaling pathway

To determine which signaling pathway was involved in cell apoptosis by GLA, expression of Fas and FasL in MCF-7 cells were examined by RT-PCR and Western blotting analysis.

In order to determine the effect of GLA on the Fas/FasL signaling pathway, we detected the gene expression of Fas and FasL at 24 h after treated with GLA, and the results showed that GLA increased the expression of FasL mRNA (Figure 3c and Figure 4c).

The roles of JNK signaling-related proteins in GLA-induced apoptosis of human breast cancer cell

Considerable evidence indicates that MAPK signaling cascades regulate not only cell growth, development and differentiation, but also apoptosis and cell growth arrest. To understand the mechanism by which GLA affects MAP kinase activation, the role of GLA in the activation of ERK, JNK and p38 MAP kinase was determined. Western blot showed that the phosphorylation of ERK and JNK was gradually, and significantly, increased after GLA treatment (Figure 4b and Figure 5). Interestingly, from Figure 4a, it can be seen that the JNK inhibitor SP600125 significantly restored cell apoptosis in response to GLA. At the protein level, the results were consistent with the results of flow cytometry and revealed that the SP600125 had a significantly opposite effect on the GLA-induced apoptosis-related proteins (Figure 5b to d). From the results obtained so far, it could be concluded that the cell apoptosis and G2 phase arrest of MCF-7 and Hs578T cells induced by GLA were mediated by activation of the JNK/MAPK signaling.

Discussion

In recent years, considerable emphasis has been given to identify new anti-cancer agents from natural sources, which could be useful for human beings. Glaucoalyxin A (GLA), a naturally occurring Rabdosia japonica (R. japonica) var. galucocalyx (Labiatae), has a wide range of pharmacologic effects, such as inhibition of cell proliferation, inhibition of cell cycle progression, and induction of apoptosis in various cancer cell lines. Metastatic spread of cancer is responsible for 90% of human cancer related deaths and thus remains one of the important impediments on cancer curing (Steeg et al., 2006). However, whether GLA exerts an inhibitory effect on tumor metastasis in breast cancer has not been elucidated previously.

In this report, we investigated the pro-apoptotic and cell arrest effect of GLA on MCF-7 and Hs578T cells, and found that the MCF-7 and Hs578T cells were highly sensitive to GLA. All the results showed that GLA efficiently inhibited the proliferation of MCF-7 and Hs578T cells. Treatment with GLA in MCF-7 and Hs578T...
cells resulted in G2 phase arrest in a dose-dependent manner. To the best of our knowledge, this study is the first to demonstrate the effect of GLA on MCF-7 and Hs578T cells.

Cell death signals from the extracellular environment or internal sensors for the cellular response are major constituents of apoptotic machinery (Liu et al., 2011). Cell surface death receptors that transmit cell death signals are activated by specific death ligands. It is demonstrated that Fas is one of the best-characterized death receptors. Upon binding of FasL onto Fas, apoptotic signals are subsequently transmitted via death adaptor molecule FADD which can mediate the activation of caspase 8, and active caspase 8 can proteolytically activate downstream effector caspases, such as caspase 3, to trigger apoptosis (Antonsson et al., 2000; Strasser et al., 2009).

In this study, our results also confirmed it. The expression of FasL is the highest versus control after treated with 8 μM GLA in 24 h by RT-PCR and western blot detection. Based on the above results, it could conclude that GLA is able to induce apoptosis by upregulating Fas/FasL signaling pathway in human MCF-7 breast cells.

JNK is a subfamily of the MAPK superfamily. MAPK pathway is involved in the development of tumor cells. JNK specifically phosphorylates the transcription factor c-Jun on its N-terminal transactivation domain, which mediates the proapoptotic function of JNK via modulating some proteins, such as Bcl-2 family protein Bim and so on. The role of JNK in tumor development remained controversial, although it was implicated in oncogenic transformation (Zhang et al., 2012). Published data implied that JNK pathway was involved in the human breast cancer cell (Sun et al., 2013), hepatoma cancer cell (Zhang et al., 2013), and so on. And also, MAPK pathway could activate its downstream Fas/FasL signaling pathway and induce the apoptosis of cancer cells (Antonsson et al., 2000; Chen et al., 2010).

On the basis of this hypothesis, we detected the JNK pathway related proteins. And our results showed that the phosphorylation levels of Erk and JNKs were increased after treated with GLA (Figure 5a). When we treated JNK pathway inhibitor SP600125, the activation of JNKs and expression of FasL were significantly inhibited, and then cell apoptosis rate obviously declined, suggesting that GLA could activate the JNK pathway and its downstream Fas/FasL death receptor pathway to induce the apoptosis of human breast cancer MCF-7 cells (Figure 4 and 5).

These results in conclusion, we have demonstrated that GLA could activate JNK pathway and upregulate FasL protein. However, in order to fully investigate GLA-induced apoptosis, additional in vivo studies are also required. Importantly, however, the findings of this study provided a novel insight into the mechanism of GLA induced human breast cancer cell apoptosis.

Acknowledgements

This study was funded in part by a research fellowship of the Kong Health Care Association Foundation (HK20100901).

References


