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Research paper

Investigation of juglone effects on metastasis and angiogenesis in pancreatic cancer cells

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ABSTRACT

Juglone, a natural component, is shown to have cytotoxic and apoptotic effects in several cancer cell lines. However, little is known about its effects on invasion and metastasis. In this study, we aimed to determine the antimetastatic effect of juglone in the BxPC-3 and PANC-1 pancreatic cancer cell lines. Cytotoxic effect of juglone was evaluated by using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) test. The cells were treated with juglone at $<IC_{50}$ doses (5, 10, 15 and 20 μM) for 24 h. After the cell adhesion and invasion analysis, expression profiles of the MMP-2, MMP-9 and Phactr-1 genes were determined by qPCR. The IC_{50} dose of juglone was found to be 21.05 μM in the BxPC-3 cell line and 21.25 μM in the PANC-1 cell line for 24 h. According to the cell adhesion and invasion analysis, treatment of juglone for 24 h reduced the adhesion and invasion features of pancreatic cancer cells. A significant reduction of MMP-2, MMP-9 and Phactr-1 expressions was observed in pancreatic cancer cells after the treatment of juglone at $<IC_{50}$ doses. By this study, it has been shown for the first time that juglone inhibits cell invasion and metastasis in pancreatic cancer line and can be evaluated as an effective anticancer agent in pancreatic cancer.

1. Introduction

Pancreatic cancer, the seventh leading cause of cancer-related death worldwide and fourth or fifth most frequent cause of cancer death in industrialized countries, is one of the most aggressive cancer type with a 5-year survival rate less than 5% (Maisonneuve and Lowenfels, 2010; Ferlay et al., 2015; Liao et al., 2015). Syptoms of this cancer usually arise at advanced stages and targeting the tumor with ionizing radiation, surgery and chemotherapy are typical treatment methods in pancreatic cancer (Husain, 2014). But, these treatments are insufficient for the therapy and resistance to clinically used chemotherapeutic agents is observed (Singh et al., 2015). Therefore novel treatment strategies are needed for the battle with pancreatic cancer.

The results of epidemiological and experimental studies have showed that natural products are beneficial for the protection from human cancers. For this reason, recent studies have focused on discovering novel anticancer agents from natural sources (Reddy et al., 2003; Efferth et al., 2007; Nobili et al., 2009). In these studies with pancreatic cancer cells, natural compounds such as sulforaphane, plumbagin, curcumin, genistein have been shown to induce apoptosis, inhibit cell growth and exhibit antimetastatic activity and also some of these compounds showed synergistic effect with gemcitabine used in the standard treatment (Kunnumakkara et al., 2007; Lev-Ari et al., 2007; Wang et al., 2010; Hafeez et al., 2012; Li et al., 2012).

Juglone (5-hydroxy-1,4-naphthoquinone), is a natural 1,4-naphthoquinone, found in the roots, leaves, woods and fruits of *Juglandaceae* walnut trees and has been reported to have various pharmacological effects such as antiviral, antibacterial, and antifungal (Omar et al., 2000; Vardhini, 2014). The anticancer and cytotoxic properties of juglone have been demonstrated through studies involving various cancer cell lines. In these studies with human gastric cancer (SGC-7901), human leukemia (HL-60) and human colon carcinoma (HCT-15) cell lines, it has been reported that juglone shows its cytotoxic effect by producing reactive oxygen species (ROS) and induces apoptosis through a mitochondria dependent pathway (Kamei et al., 1998; Xu et al., 2010; Ji et al., 2011). However, little is known about its effects on invasion and metastasis.

Metastasis, associated with the aggressiveness of tumors and high mortality rate, is detected in more than 80% of pancreatic cancer patients (Lemke et al., 2013). The metastasis is a multistep process that includes detachment of tumor cells from the primary tumor, invasion to stroma, intravasation to vessels, extravasation to the target organ and induction of angiogenesis (Chambers et al., 2002). All these steps are mediated by different factors. Especially, MMP-2 and MMP-9 called gelatinases, which can digest various types of ECM proteins

Abbreviations: MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; MMP-2, Matrix metallopeptidase 2; MMP-9, Matrix metallopeptidase 9; VEGF, Vascular endothelial growth factor; DMSO, dimethylsulfoxide; uPA, Urokinase plasminogen activator; NFD, Naphtho[1,2-b]furan-4,5-dione.

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especially type IV collagen, the major component of the basement membrane, play critical roles in tumor cell invasion (Birkedal-Hansen et al., 1993; Giannelli and Antonaci, 2002; Mook et al., 2004). Therefore, the increase in activation and expression of MMP-2 and MMP-9 genes is an important marker for metastasis.

Angiogenesis, another critical step of metastasis, is defined as the formation of new blood vessels from existing capillaries (Folkman and Klagsbrun, 1987). When the tumor size reaches 1–2 mm^3, it stimulates angiogenesis to provide oxygen and nutrient support (Böhle and Kalthoff, 1999). Angiogenesis is controlled by a large number of pro- and anti-angiogenic factors, and among them, Vascular endothelial growth factor (VEGF) system is a key regulator of angiogenesis development (Harper and Bates, 2008; Egginton, 2009). In studies with human endothelial cells, Phactr-1 gene, one of the four members of phosphatase and actin regulator Phactr family, has been shown to be induced by VEGF-A (Jarray et al., 2011). Also, it is indicated in human breast cancer cells that Phactr-1 gene expression is necessary for migration stimulated by transforming growth factor-β (TGF-β) (Fils-Aimé et al., 2013). Thus, Phactr-1 gene is thought to play an important role in cancer angiogenesis.

In this study, it is aimed to determine the cytotoxic effect of juglone in the BxPC-3 and PANC-1 pancreatic cancer cell lines and also to investigate its effect on metastasis and angiogenesis through evaluating the expression levels of MMP-2, -9 and Phactr-1 genes.

2. Materials and methods

2.1. Cell culture

BxPC-3 and PANC-1, human pancreatic cancer cell lines, obtained from the ATCC (Manassass,VA, USA), were cultured in RPMI-1640 and DMEM medium, containing 10% fetal bovine serum and 1% penicilin/streptomycin, respectively. They were cultured at 37 °C in a humidified atmosphere of 5% CO_2–95% air. Juglone was purchased from Sigma-Aldrich Chemical Company (USA).

2.2. Cell viability assay

The cytotoxic effect of juglone on pancreatic cancer cells was determined by MTT test. The cells (5000/well) were seeded into 96-well plates and treated with various concentrations of juglone (0, 5, 10, 15, 20, 30, 40 and 50 μM) for 24 h. At the end of the incubation, MTT solution was added to each well and incubated at 37 °C for 4 h. The resulting formazan crystals were dissolved with DMSO and the absorbance was read at 570 nm in an ELISA reader. The concentration of juglone that inhibits 50% cell viability (IC_{50}) was determined. Four doses lower than IC_{50} were used for subsequent studies.

2.3. Cell adhesion assay

The cell adhesion assay was performed using "CytoSelect™ 48-Well Cell Adhesion Assay" (Cell Biolabs, CBA-053). After the cells were treated with juglone at 5, 10, 15 and 20 μM concentrations ($<IC_{50}$) for 24 h, cells were trypsinized and cell suspensions, containing 0.1–1.0 × 10^6 cells/ml in serum free media, were prepared. Cells were seeded in a 48-well plate coated with type I collagen. The cells were incubated at 37 °C for 1 h and non-adhering cells were removed by PBS washing while adherent cells were fixed in ethanol and stained with 0.1% crystal violet. Fixed cells were lysed in 20% acetic acid. The absorbance of the samples was measured at 560 nm using a microplate reader. Each experiment was carried out in triplicate.

2.4. Cell invasion assay

The cell invasion assay was performed using "CytoSelect™ 96-Well Cell Invasion Assay" (Cell Biolabs, CBA-112). After the cells were treated with juglone at 5, 10, 15 and 20 μM concentrations for 24 h, cells were trypsinized and cell suspensions, containing 0.2–2.0 × 10^6 cells/ml in serum free media, were prepared. The invasion plate contains a membrane chamber is coated with a dried basement membrane matrix solution. The cells were seeded into the membrane chamber in serum-free medium and the medium containing 10% FBS added in bottom of the plate. After the incubation for 24 h, the invaded cells were dissociated from the membrane and subsequently detected with CyQuant® GR Dye and was measured fluorescence with a fluorescence plate reader at 480 nm/520 nm. Each experiment was carried out in triplicate.

2.5. Gene expression analysis

Total RNAs were extracted from pancreatic cancer cells using TRIdity G reagent and reverse transcription was performed using cDNA synthesis kit (2-steps RT-PCR kit, RTPL12®, vivantis, Malaysia) according to the manufacturer's instructions.

The effect of juglone on MMP-2, -9 and Phactr-1 gene expressions was examined with real-time PCR technique using proper primers for each gene. Primers used in the qPCR reaction are presented in Table 1. (Hsu et al., 2005; Fils-Aimé et al., 2013; El-Ghlban et al., 2014; Nagamatsu et al., 2014). β-Actin was used as a reference gene for normalization. The following PCR program was used: denaturation at 95 °C for 10 min, followed by 40 cycles consisting of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The $2^{-\Delta\Delta CT}$ method was used to analyze the relative changes in gene expressions.

2.6. Statistical analysis

Data were analyzed statistically using IBM SPSS 21.0 (SPSS Inc., Chicago, IL, USA), and a p value <0.05 was considered to be statistically significant. Differences between the groups were analyzed by one-way ANOVA test.

3. Results

3.1. Effect of juglone on cell viability

The cytotoxic effect of juglone was firstly determined using the MTT assay. Juglone inhibited the cell viability of pancreatic cancer cells in a dose dependent manner as shown in Fig. 1. The IC_{50} dose of juglone was found to be 21.05 μM in the BxPC-3 cell line and 21.25 μM in the PANC-1 cell line for 24 h using GraphPad Prism 6. Considering this dose treatment concentrations used for subsequent experiments were determined as 5, 10, 15 and 20 μM of juglone ($<IC_{50}$).

3.2. Effect of juglone on adhesion and invasion of pancreatic cancer cells

According to the cell adhesion analysis, treatment of juglone for 24 h reduced the adhesion of pancreatic cancer cells in a dose dependent manner as shown in Fig. 2. After the treatment of 5, 10, 15 and 20 μM juglone, the adhesion abilities of BxPC-3 cells were inhibited by 19%, 42%, 59.5% and 66% respectively, compared to the control. In PANC-1

Table 1
Primers for qPCR analysis of genes expression.

Gene	Primer sequence
MMP-2	F: 5-TTTCCATTCCGCTTCCAGGGCACAT-3
	R: 5-TCGCACACCACATCTTTCCGTCACT-3
MMP-9	F: 5-TGGGCTACGTGACCTATGACAT-3
	R: 5-GCCCAGCCCACCTCCACTCCTC-3
Phactr-1	F: 5-GATTGGCACCAAGCTCACCA-3
	R: 5-ACCGTGGGCCTTTGACTGAG-3
β-Actin	F: 5-ACTCTTCCAGCCTTCCTTC-3
	R: 5-ATCTCCTTCTGCATCCTGTC-3