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Oroxylin A reverses CAM-DR of HepG2 cells by suppressing Integrinβ1 and its related pathway

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ABSTRACT

Oroxylin A, a naturally occurring monoflavonoid extracted from *Scutellariae radix*, shows effective anticancer activities and low toxicities both *in vivo* and *in vitro* in previous studies. In this study, we investigated whether the CAM-DR model of HepG2 cells showed resistance to cytotoxic agents compared with normally cultured HepG2 cells. Furthermore, after the treatment of Paclitaxel, less inhibitory effects and decreased apoptosis rate were detected in the model. Data also revealed increased expression of Integrinβ1 might be responsible for the resistance ability. Moreover, Integrinβ1-siRNA-transfected CAM-DR HepG2 cells exhibited more inhibitory effects and higher levels of apoptosis than the non-transfected CAM-DR cells. The data corroborated that Integrinβ1 played a significant role in CAM-DR. After the treatment of weakly-toxic concentrations of Oroxylin A, the apoptosis induced by Paclitaxel in the CAM-DR model increased dramatically. Western blot assay revealed Oroxylin A markedly down-regulated the expression of Integrinβ1 and the activity of related pathway. As a conclusion, Oroxylin A can reverse the resistance of CAM-DR via inhibition of Integrinβ1 and its related pathway. Oroxylin A may be a potential candidate of a CAM-DR reversal agent.

Introduction

Hepatocellular carcinoma (HCC) has been recognized as one of the most common malignant tumors in the world for its high death rate. The main obstacle in the treatment of HCC is multidrug resistance (MDR), which includes intrinsic and acquired resistance (Fojo and Bates, 2003). As identified, classic mechanisms of acquired MDR fall into four points, (a) reduced drug accumulation; (b) alterations in drug target; (c) increased repair of drug-induced damage; and (d) inhibition of apoptotic signaling pathways (Shain and Dalton, 2001). However, intrinsic or *de novo* drug resistance, which was caused by the interaction between tumor cells and elements of their microenvironment in multiple myeloma and other malignancies, can bring the resistance to chemotherapy- and death receptor-mediated apoptosis (Hazlehurst et al., 2003b). Tumor cells escaping drug insult via *de novo* drug resistance at the beginning will obtain acquired resistance at last (Li and Dalton, 2006).

Recently, investigations have shown that tumor microenvironment played a significant role in metastasis, invasiveness, motility (Duffy et al., 2008), and resistance to apoptosis (Zhang et al., 2008). As functionally characterized, elements of microenvironment in solid tumor included cytokine, hormones, and interactions between tumor cells and extracellular matrix molecules. What' more, it has been well established that the tumor microenvironment is involved in mediating *de novo* drug resistance (Hazlehurst et al., 2003b). This type of resistance was named environment mediated-drug resistance (EM-DR) which can be considered as a synergism between cell adhesion mediated-drug resistance (CAM-DR) and soluble factor mediated-drug resistance (SM-DR) (Li and Dalton, 2006).

Currently, the most common models of CAM-DR are spheroid model (Durand and Sutherland, 1972), fibronectin (FN) model (Damiano et al., 1999) and stromal model (Mudry et al., 2000). Among the three models, FN model is predominantly used to approach how tumor cells–FN interaction contributes to CAM-DR via adhesion molecules (Damiano et al., 1999). Indeed, The FN model is a simplified model of the tumor microenvironment, one example utilizing this model is the discovery that NF-κB activation is associated with CAM-DR via integrin signaling (Landowski et al., 2003). Additionally, Using FN model, it was found for the first time in myeloma cells that downregulation of BH3 domain-only pro-apoptotic protein Bim was associated with CAM-DR (Hazlehurst et al., 2003a). This CAM-DR phenotype was associated with cell adhesion and VLA-4

Abbreviations: ECM, Extracellular matrix; CAM-DR, Cell Adhesion-mediated-drug resistance; FN, Fibronectin; PI3K, Phosphatidyl inositol 3′-kinase; PMSF, Phenylmethylsulfonyl fluoride.

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expression which indicated that the integrin family of adhesion molecules mediate cell–ECM (via FN) interaction (Damiano et al., 1999).

Oroxylin A ($C_{16}H_{12}O_5$), a naturally occurring monoflavonoid, can be isolated from the root of *Scutellaria baicalensis Georgi* and used as an anti-tumor, anti-inflammatory, and anti-bacterial infection component in traditional Chinese medicine (Sun et al., 2009). Meanwhile, Oroxylin A can induce human HCC HepG2 apoptosis by significantly reducing Bcl-2 and pro-caspase-3 expression in a concentration-dependent manner (Hu et al., 2006), inhibit the growth of human gastric carcinoma BGC-823 cells via the modulation of Cdc2 and Cdk7 protein, which are critical to G2 progression and G2/M transposition (Yang et al., 2008), regulate gene expressions of thymidine synthetase (TS) and dihydropyrimidine dehydrogenase (DPD) to increase the sensitivity of human hepatocellular carcinoma HepG2 to 5-fluorouracil (5-FU) *in vivo and in vitro* (Zhao et al., 2010). Additionally, Oroxylin A can reverse the resistance of human HCC BEL7402/5-Fu by regulating PI3K/AKT pathway and NF-κB activity (Yang et al., 2011), suggesting that Oroxylin A might potentially switch the activity of CAM-DR.

In this study, we established the FN model of human HCC HepG2 and investigated the mechanism of the drug resistance. Meanwhile, we tested the effect and mechanism of Oroxylin A on CAM-DR, which might be associated with Integrinβ1 and the activity of PI3K/AKT pathway.

Materials and methods

Drugs and antibodies. Oroxylin A was isolated from *Scutellariae radix* according to the protocols reported previously with slight modifications (Li and Chen, 2005). Samples containing 99% or higher Oroxylin A were used in all experiments unless otherwise indicated. Oroxylin A was dissolved at a concentration of 100 mM in 100% DMSO (Amresco, Amresco Inc., Solon, Ohio) as a stock solution, stored at −20 °C, and diluted with the medium before each experiment. The final DMSO concentration did not exceed 0.1% throughout the study. Before each experiment, Oroxylin A was diluted with medium to certain working concentrations (50 μM, 100 μM and 200 μM). Paclitaxel (6 mg/ml) was obtained from Shenzhen Main Luck Pharmaceuticals Inc. (Shenzhen, China) and diluted to certain concentrations (5 μM) with culture medium. Fibronectin was purchased from BD Bioscience (Bedford, MA). It was then dissolved in a concentration of 1 mg/ml in ddH_2O as a stock solution, stored at −20 °C, and diluted to 20 μg/ml with PBS before each experiment. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was supplied by Sigma Chemical Co. (St. Louis, MO). Primary antibodies for Integrinβ1 (1:500), FAK (1:500), p-Akt (1:500), Akt (1:500), PTEN (1:500), and β-actin (1:2000) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibody for PI3K (1:800) was obtained from Bioworld (OH, USA). IRDye™800 conjugated secondary antibodies were obtained from Rockland Inc. (Philadelphia, PA).

Cell culture. Human HCC cell line HepG2 was purchased from Cell Bank of Shanghai Institute of Biochemistry & Cell Biology, Chinese Academy of Sciences. The cells were cultured in RPMI-1640 medium (Gibco, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Sijiqing, Zhejiang, China), 100 U/ml benzyl penicillin, and 100 U/ml streptomycin in a humidified environment with 5% CO_2 at 37 °C.

SiRNA transient transfection. Integrinβ1 siRNA was purchased from Santa Cruz Biotechnology, Inc. SiRNA transfections were performed according to the manufacturer's instructions of Lipofectamine 2000™ reagent (Invitrogen, CA). After that, cells were exposed to Oroxylin A or the vehicle and harvested for further experiments.

Cell growth inhibition assay. CAM-DR model through direct cell contact and adhesion may be important in causing tumor cell drug resistance. For one thing, cell adhesion activates certain signaling pathways that could lead to the expression of soluble factor as well as their receptors (Dalton, 2003), for the other thing, cell adhesion leads to the intracellular redistribution of certain molecules involved in drug resistance (Shain et al., 2002). The growth inhibitory effect was determined by MTT assay. A 96-well plate was pre-coated with 100 μl (20 μg/ml) FN at 4 °C overnight, 1% BSA was used to block non-specific binding sites in the wells for 1 h before the experiment. HepG2 cells were collected and suspended in the serum-free RPMI-1640 medium, then the cells were added to FN coated plates and non-material coated plates. Cells were incubated for 10 h at 37 °C and 5% CO_2, washed with serum-free media twice, 100 μl of diluted drug or vehicle control was added to each well for 4 h, after which media were removed and replaced by 200 μl drug-free and serum-contained medium. Following 24-hour incubation, 20 μl MTT dye (5 mg/ml) was added to each well and incubated for 4 h. The Dye was solubilized with 100 μl DMSO and plates were read at 570 nm on an automated microtiter plate reader. A blank well containing only media and drug was also run as a control in all experiments. The inhibitory ratio was calculated using the following formula: inhibitory ratio (%) = 1 − average absorbance of treated group / average absorbance of control group * 100%. The IC50 values were determined by GraphPad Software.

Annexin V/PI double staining. After 10 h adhesion to FN, cells were exposed to drug for 4 h and extracellular drug was removed by 3 washes with RPMI medium containing 10% CS, then incubated at 37 °C and 5% CO_2 for 24 h. Apoptotic cells were identified by the Annexin V-FITC Apoptosis Detection kit (Biovision, Mountain View, CA) according to the manufacturer's instructions. Flow cytometric analysis was performed immediately after supravital staining. Data acquisition and analysis were performed in a Becton Dickinson FACS Calibur flow cytometer using Cell Quest software (Franklin Lakes, NJ, USA).

Western blot analysis. Normal cultured HepG2 cells were treated Paclitaxel for 4 h, and the CAM-DR model of HepG2 cells were treated with Paclitaxel, Oroxylin A for 4 h respectively. After which they were lysed in lysis buffer (100 mM Tris–HCl, pH 6.8, 4% (m) SDS, 20% (v) glycerol, 200 mM b-mercaptoethanol, 1 mM PMSF, and 1 g/ml aprotinin) for 1 h on the ice. The lysates were clarified by centrifugation (12,000 rpm) at 4 °C for 30 min. The protein concentration in the supernatants was detected by a Varioskan multimode microplate spectrophotometer (Thermo, Waltham, MA, USA). Then an equal amount of protein was separated by SDS-PAGE (Yang et al., 2011). Proteins were detected using specific antibodies of Integrinβ1, FAK, PI3K, P-Akt, Akt, PTEN and β-actin followed by IRDye™ 800-conjugated secondary antibodies for 1 h at 37 °C. Detection was performed by the Odyssey Infrared Imaging System (LI-COR Inc., Superior St. Lincoln, NE, USA). All blots were stripped and probed with polyclonal anti-β-actin antibody to ascertain equal loading of the proteins.

Statistical analysis. All results shown represent means ± SEM from triplicate experiments performed in a parallel manner unless otherwise indicated. Statistical analyses were performed using one-way ANOVA by SPSS 11.5 software.

Results

Adhesion of HepG2 cells to FN exhibits a preliminary characteristic of drug resistance

Morphological assessment and Western blot assays were performed on the CAM-DR model of HepG2 cells to assess the preliminary characteristic of drug resistance. Morphological comparison showed that the model cell adhered to the bottom of Petri dish much earlier,