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Inhibiting glucosylceramide synthase facilitates the radiosensitizing effects of vinorelbine in lung adenocarcinoma cells

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ABSTRACT

The standard treatment regimen for patients diagnosed with non-small cell lung cancer (NSCLC) with locally advanced stage III disease is concurrent chemoradiotherapy (CCRT). This study investigated the molecular effects of vinca alkaloid vinorelbine (VNR)-based CCRT. We reviewed the records of 68 patients with stage III NSCLC: 42 patients received VNR-based CCRT, and 26 were treated with radiation alone. Human lung adenocarcinoma cells were used in this study to investigate the molecular effects of glucosylceramide synthase inhibition on VNR-based CCRT. There was response rate of 66.7% with CCRT, which was better than the response rate observed with radiation alone (30.8%; $P < 0.001$). CCRT caused an increase in cell cycle arrest at G_2/M phase accompanied by apoptosis. Oxidative c-Jun N-terminal kinase (JNK) activation was involved in the increased apoptosis levels but not the cell cycle arrest. CCRT also induced an increase in ceramide accompanied by a decrease in glucosylceramide that was positively correlated with the cytotoxic effects. Pharmacologically inhibiting glucosylceramide synthase facilitated VNR- and CCRT-induced apoptosis by promoting the JNK pathway. Inhibiting glucosylceramide synthase facilitates the radiosensitizing effects of VNR by promoting JNK-mediated apoptosis in lung adenocarcinoma cells.

Introduction

Lung cancer is the most common cancer worldwide. Lung cancer caused approximately 160,340 deaths in the USA in 2012 and 1,370,000 deaths worldwide in 2008 [1]. Approximately 80–85% of lung cancers are classified as non-small cell lung cancer (NSCLC), and 45% of these are diagnosed in stage III [2]. The National Comprehensive Cancer Network guidelines suggest that unresectable stage III NSCLC patients should be treated with concurrent chemoradiotherapy (CCRT), with cisplatin/etoposide or cisplatin/vinblastine plus thoracic radiation being the preferred regimens [3,4]. Randomized trials show that CCRT induces a better response rate than sequential chemotherapy (CT) and radiotherapy (RT) [5–8]. Systemic CT can reduce the chances of distant metastasis, and certain chemotherapeutic agents appear to act as radiation sensitizers. However, most of these previous trials employed multiple drugs, and toxicities from platinum-based drugs are common. The possible increased effects of CCRT have been proposed to act through several events involving increased radiation damage from the following mechanisms: the incorporation of the CT drug into DNA/RNA; interference with the DNA repair process after RT; interference with the cell cycle through cytokinetic cooperation and synchronization; enhanced activity against hypoxic cells by reoxygenation second to tumor shrinkage; RT enhancement by preventing repopulation; the inhibition of pro-survival and 'poor prognosis' markers; and hyper-radiation sensitivity [9].

Abbreviations: NSCLC, non-small cell lung cancer; CCRT, concurrent chemoradiotherapy; VNR, vinorelbine; JNK, c-Jun N-terminal kinase; CT, chemotherapy; RT, radiotherapy; NAC, N-acetyl-L-cysteine.

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Protocols that employ single-agent CCRT, using agents such as docetaxel [10] and gemcitabine [11], are being developed in an effort to improve patient response rates and survival. Vinorelbine (VNR) is a semi-synthetic vinca alkaloid that binds to tubulin and is a potent inhibitor of mitotic microtubule polymerization [12]. VNR is also an early and well-known radiation sensitizer [13,14] and is clinically used as a single-agent VNR-based CCRT for treatment of NSCLC [15,16]. The effects of VNR-induced radiosensitization are demonstrated by cell cycle arrest, DNA strand breaks, and apoptosis [17–21]. However, the underlying mechanisms of CCRT-induced anticancer benefits remain unclear. Our previous results demonstrate an essential role for reactive oxygen species (ROS) in VNR-induced aberrant c-Jun N-terminal kinase (JNK)-mediated Mcl-1 downregulation and DNA damage followed by mitochondrial dysfunction-related caspase-mediated apoptosis but not mitotic arrest [22]. Several reports demonstrate that radiation induces ROS accumulation, ceramide expression, DNA damage, and mitogen-activated protein kinase (MAPK) phosphorylation in different cell lines [23]. However, the increased effects caused by VNR-based CCRT lack a molecular mechanistic explanation.

Ceramide (a sphingolipid with a sphingosine backbone) levels are increased through different pathways including *de novo* synthesis, hydrolysis of sphingomyelin, and decreasing ceramide metabolism. In *de novo* synthesis, ceramide is generated from the palmitoyltransferase-mediated interaction of serine and palmitoyl-CoA and a series of metabolic reactions. In the hydrolysis pathway, extracellular stimulation induces hydrolysis of sphingolipids and sphingomyelin by sphingomyelinase. In the metabolic pathway, ceramide converts to glucosylceramide, sphingosine-1-phosphate, and ceramide-1-phosphate by glucosylceramide synthase, ceramidase, and ceramide kinase, respectively [24,25]. Exogenously and endogenously increasing ceramide levels has anticancer effects [26,27]. In the present study, we demonstrated a role for ceramide in VNR-based CCRT-induced aberrant JNK-mediated apoptosis in lung adenocarcinoma cells. We further examined a possible mechanism for ceramide expression by VNR-based CCRT and the effects of aberrant ROS/JNK and ceramide/JNK on the radiosensitizing effects of VNR in CCRT-induced apoptosis.

Materials and methods

Patients

This was a retrospective study in which we reviewed the charts of all eligible NSCLC patients from the National Cheng Kung University Hospital (NCKUH, Tainan, Taiwan) from January 1, 2005 to December 31, 2011. All information was collected by one oncology physician. The inclusion criteria were as follows: age $\geqslant$18 years; histologically documented unresectable stage III NSCLC; use of single-agent VNR–CCRT or radiation alone; adequate hematological function; and adequate liver and kidney function. The patients with impaired cognitive function were excluded. A total of 68 patients were enrolled. The adverse effects were assessed using the NCI-CTCAE version III and recorded by the physicians of the Thoracic Oncology Team. Because this study involved only chart review, the institutional review board of NCKUH agreed that informed consent was not necessary. *Clinical CCRT schedule*: Forty-two patients received CCRT, and thirty-eight patients were enrolled to calculate time to progression (TTP). Two patients received an operation immediately following CCRT, and the other two patients accepted further chemotherapy for reasons other than progression. For chemotherapy, a previous study demonstrated that 15 mg/m^2 of intravenous VNR is equivalent to 40 mg/m^2 of oral VNR [28]. Therefore, we administered a dose of 40 mg/m^2/week of oral VNR to thirty-three patients and intravenous VNR to nine patients. For radiation therapy, a computed tomography simulation-guided 3D plan was used for all patients. Intensity-modulated radiation therapy was used in 7 patients. Thoracic radiation was administered over 6 weeks at 1.8 gray (Gy) per day (for a median dose of 61.2 Gy). *Clinical radiation schedule*: Twenty-six patients received radiation alone. A computed tomography simulation-guided 3D plan was used for all patients. Thoracic radiation was administered over 6 weeks at 1.8 Gy per day (for a median dose of 60.0 Gy). *Response evaluation:* Response was evaluated with CT scans performed 4–6 weeks after completion of CCRT. According to the 2009 RECIST criteria [29], complete Response (CR) was defined as the disappearance of all target lesions. Any pathological lymph nodes (whether target or non-target) must have reduction in short axis to <10 mm. Partial Response (PR) was defined as at least a 30% decrease in the sum of diameters of target lesions taking as reference the baseline sum diameters. Progressive Disease (PD) was defined as at least a 20% increase in the sum of diameters of target lesions; taking as reference the smallest sum on study (this includes the baseline sum if that is the smallest on study). In addition to the relative increase of 20%, the sum must also demonstrate an absolute increase of at least 5 mm. (Note: the appearance of one or more new lesions is also considered progression.) Stable Disease (SD) was defined as neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum diameters while on study.

Cell culture and reagents

The human lung adenocarcinoma PC14PE6/AS2 cell line was established from ascites generated from PC14PE6 cells (a gift from Isaiah J. Fidler; MD Anderson Cancer Center, Houston, TX, USA) in nude mice [27]. The cells were routinely grown on plastic in Dulbecco's modified Eagle's medium (Gibco-BRL; Grand Island, NY, USA) with L-glutamine and 15 mM HEPES supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml streptomycin and maintained at 37 °C in 5% CO_2. Primary human aortic endothelial HAOEC cells were purchased from PromoCell (Heidelberg, Germany) along with the corresponding endothelial growth medium MV, which contained 0.4% human endothelial cell growth supplement, 5% fetal calf serum, 10 ng/ml epidermal growth factor, 1 μg/ml hydrocortisone, 50 ng/ml amphotericin B, and 50 μg/ml gentamicin. Other chemicals used for cell culture were purchased from Sigma–Aldrich (St. Louis, MO, USA). The vinca alkaloids VNR, vincristine, and vinblastine were purchased from Sigma–Aldrich. The JNK inhibitor SP600125, the antioxidant N-acetyl-L-cysteine (NAC), the ceramide synthase inhibitor fumonisin B1, the acid sphingomyelinase inhibitors chlorpromazine and desipramine, and the glucosylceramide synthase inhibitor D,L-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol hydrochloride (PDMP) were obtained from Sigma–Aldrich and dissolved in dimethyl sulfoxide (DMSO) or ethanol prior to dilution with PBS. Rabbit anti-human MPM2, phospho-JNK (Thr183/Tyr185), JNK, PARP, and Mcl-1 antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibodies against ceramide, glucosylceramide (Glc-Ceramide), and sphingosine-1-phosphate (S-1-P) were obtained from Sigma–Aldrich. β-actin antibodies and horseradish peroxidase-conjugated or Alexa 488-conjugated anti-rabbit IgG were obtained from Chemicon International (Temecula, CA, USA).

In vitro CCRT schedule

Cells were pre-treated VNR for 24 h and the culture medium was changed before RT. Radiation was performed with 6 MV X-rays using a linear accelerator (Elekta SLi; Elekta AB, Stockholm, Sweden) at a dose of 8 Gy. We added a 5 cm of tissue-equivalent bolus on the top of a plastic tissue culture flask to guarantee electronic equilibrium, and put another 5 cm of tissue equivalent material under the flask to acquire full backscatter.

Cell cycle and apoptosis assays

Cell cycle distribution and apoptosis levels were analyzed using nuclear propidium iodide (PI; Sigma–Aldrich) staining as previously described [22] and flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA) with the excitation set at 488 nm and emission detected with the FL-2 channel (565–610 nm). The distribution of cells in the different phases of the cell cycle was calculated using MetaMorph software (Molecular Devices, Downingtown, PA). For apoptosis analysis, the samples were analyzed using CellQuest Pro 4.0.2 software (Becton Dickinson), and quantification was performed using WinMDI 2.8 software (The Scripps Institute, La Jolla, CA). Apoptosis levels are reported as the percentage (%) of cells in the sub-G_1 phase.

Intracellular ROS assay

Intracellular oxidative stress was measured using dichlorodihydrofluorescein diacetate oxidation. The cells were exposed to 20 μM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) (Invitrogen) for 1 h. The cells were then analyzed using the FL-1 channel (515–545 nm) with a FACSCalibur cell sorter. After another wash with PBS, the cells were analyzed using flow cytometry (FACSCalibur) with an excitation wavelength of 488 nm. The ROS levels are reported as the mean fluorescence intensity (MFI) of the total cells using CellQuest Pro 4.0.2 software, and quantification was performed with WinMDI 2.8 software. Small cell debris were excluded by gating on a forward scatter plot.

Immunostaining

To detect the expression of MPM-2 and sphingolipid metabolites, we fixed, stained, and analyzed the cells as described elsewhere [30]. Briefly, the cells were fixed and permeabilized with 3.7% formaldehyde in PBS. After the cells were washed and stained with primary antibody followed by an Alexa 488-conjugated secondary antibody, the cells were analyzed using flow cytometry (FACSCalibur).