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Suppression of Nrf2 confers chemosensitizing effect through enhanced oxidant-mediated mitochondrial dysfunction

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ABSTRACT

Aims: Transcription factor Nrf2, which regulates the expression of cytoprotective and antioxidant enzymes, contributes to proliferation and resistance to chemotherapy in cancer. The inhibition of Nrf2 can sensitize cholangiocarcinoma (CCA) cells to the cytotoxicity of several chemotherapeutic agents. In this study, we investigated the mechanism of this chemosensitizing effect.

Main methods: KKU-100 cells were used in the study. Nrf2 expression was knocked down by siRNA and expression was validated by reverse transcription and polymerase chain reaction. Cytotoxicity was assessed by sulforhodamine B method. Intracellular reactive oxygen species (ROS) was examined by fluorescent dye, dichlorofluorescin diacetate method and mitochondrial transmembrane potential was assessed by JC1 dye assay.

Key findings: Cytotoxicity of cisplatin (Cis) in KKU-100 cells was enhanced by knockdown of Nrf2 expression. The enhanced cytotoxic effect was abolished by treatment with *N*-acetylcysteine, TEMPOL and MnTBAP. Cells with Nrf2 knockdown or Cis treatment increased production of ROS, and ROS was markedly enhanced by a combination of Nrf2 knockdown and Cis. The increased ROS formation was associated with a decrease in mitochondrial transmembrane potential ($\Delta\psi_m$), where this decrease was prevented by antioxidant compounds. The loss of $\Delta\psi_m$ and cell death were prevented by cyclosporine, an inhibitor of mitochondrial permeability transition pore (MPTP). Luteolin inhibited Nrf2 and markedly enhanced cytotoxicity in combination with Cis.

Significance: Inhibition of Nrf2 is a feasible strategy in enhancing antitumor activity of chemotherapeutic agents and improving efficacy of chemotherapy in CCA.

1. Introduction

Cholangiocarcinoma (CCA), an epithelial cancer of the bile ducts, accounts for about 3% of all gastrointestinal tumors and is the second most common primary liver cancer after hepatocellular carcinoma [1]. Global epidemiological data have shown an increasing incidence of CCA [2]. Advanced CCA has a very poor prognosis, with a very low five-year survival for unresectable CCA [3]. The five-year survival rate after a complete resection of CCA is 11–41% [1]. Moreover, chemotherapy in unresectable CCA with conventional or targeted agents has not shown to substantially improve survival [1]. Therefore, new strategies for CCA management are required to improve efficacy of chemotherapy and improve survival.

Nuclear factor-erythroid 2 like 2 (NFE2L2), or Nrf2, is a transcription factor responsible for protection from oxidative stress by regulating cellular adaptive response to various stressors from external and internal sources [4]. Generally, genes positively regulated under Nrf2 signaling are involved in the elimination of reactive oxygen species (ROS), the metabolism of xenobiotics, cytoprotection, drug transport, glutathione synthesis, and enzymes in certain metabolic pathways [4,5]. As a result, expression of Nrf2 protects cells from oxidative injury, supports cell proliferation and differentiation on various cell types such as osteoclasts and adipocytes, and even encourages cancer growth [6]. Cumulative evidence reveals that the overexpression or hyperactivity of Nrf2 found in cancer promotes cancer cell proliferation, survival, and resistance to chemotherapy [7].

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Several studies have shown that chemotherapeutic agents such as paclitaxel, 5-fluorouracil, gemcitabine, doxorubicin and cisplatin can activate Nrf2 and its down-stream regulated genes, and this may be associated with drug resistance [8,9]. Recently, studies have shown that knockdown of Nrf2 by siRNA or Nrf2-small hairpin RNA (shRNA) in some cancers, including lung carcinoma, breast adenocarcinoma, neuroblastoma, pancreatic cancer, ovarian cancer, colon cancer, and CCA, enhances the cytotoxicity of chemotherapeutic agents such as cisplatin, gemcitabine, etoposide, doxorubicin, docetaxel and 5-fluorouracil, as well as the cytotoxicity of gamma irradiation [9–12]. In a recent study using KKU-100 and KKU-M156 CCA cell lines, representing low and high Nrf2 expressing cells respectively, we demonstrated that treatment with gemcitabine, doxorubicin, cisplatin, and 5-fluorouracil upregulates Nrf2 mRNA expression and its downstream genes, e.g. NQO1, HO-1, GCL, and glutathione S-transferase P (GSTP). Moreover, Nrf2 knockdown by siRNA suppresses the expression of Nrf2 and downstream genes, and is associated with enhanced cytotoxicity of 5-FU and gemcitabine [9]. The chemosensitizing effect to cisplatin by knockdown of Nrf2 in KKU-M156 cells was found to be associated with suppression of antioxidant and cytoprotective gene expression, inhibition of cell cycle progression, and induction of apoptotic proteins [13]. Targeting Nrf2 has been advocated as a strategy in cancer therapeutics [14]. Several phytochemicals have been suggested to act as Nrf2 inhibitors. Luteolin, a flavone compound present in several dietary vegetables and fruits, shows activity as an Nrf2 inhibitor [14]. Luteolin has been shown to have an antitumor effect in several cancer cells, in association with Nrf2 inhibition [15,16].

However, the mechanism by which inhibition of Nrf2 signaling results in these chemosensitizing effects is still not clear. In this study, Nrf2 expression was suppressed by either siRNA or luteolin to investigate this mechanism. We aimed to determine whether the chemosensitizing effect was causally related to oxidant stress induced by Nrf2 suppression, followed by mitochondrial dysfunction and cell death. Understanding more about the role of Nrf2 in resistance to current chemotherapies may offer a new strategy to overcome this dreadful cancer.

2. Materials and methods

2.1. Chemicals and reagents

Lipofectamine® RNAiMAX, Trizol® reagent, Ham's F12 media, fetal bovine serum albumin, and 0.25% trypsin-EDTA were obtained from Invitrogen® (Life Technologies, Grand Island, NY, USA). The Nrf2 siRNA duplex Silence® (s9491) and negative control siRNA were purchased from Ambion (Thermo Scientific, Waltham, MA, USA). Reverse transcription supermix, iScript™, PCR supermix SsoFast™ EvaGreen®, and Bradford protein assay kit were obtained from Bio-Rad (Hercules, CA, USA). Luminata™ Forte Western HRP substrate was from Millipore Corporation (Billerica, MA, USA). Image Quant LAS 4000 mini was obtained from GE Healthcare (Piscataway, NJ, USA). *N*-acetyl-L-cysteine (NAC), 4-hydroxy-TEMPO (TEMPOL), ruthenium red, luteolin, and 2′, 7′-dichlorodihydrofluorescein-diacetate (H_2DCFDA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Manganese (III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). JC-1 Mitochondrial Membrane Potential Assay Kit was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Other chemicals were of the highest purity commercially available.

2.2. Cell cultures

The human cholangiocarcinoma (CCA) cell line, KKU-100, was kindly provided from Prof. Banchob Sripa, Department of Pathology, Faculty of Medicine, Khon Kaen University. The cell line was established from tumor tissue resected from a CCA patient. The cell line was verified and tested for mycoplasma contamination. The cells were grown in Ham's F12 medium supplemented with 12.5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.3, 100 U/mL penicillin, 50 μg/mL gentamicin, and 10% fetal bovine serum (FBS) and were maintained under 5% CO_2 at 37 °C as described previously [13]. The cells were subcultured every 2–3 days before confluence using 0.25% trypsin–EDTA, and the medium was changed after an overnight incubation.

2.3. Knockdown of Nrf2 expression by small interfering RNA

Expression silencing was performed by transfection of small interfering RNA (siRNA) by a method previously described [9,13]. In brief, KKU-100 cells (1.5×10^4 cells/well) in a 6-well plate were transfected in serum-free media containing with either 100 pmole siRNA specific for Nrf2 or a negative control siRNA, and 7.5 μl of Lipofectamine RNAiMAX for 6 h at 37 °C. The medium containing transfection reagents was removed and further incubated with full media for 24 h. The efficacy of Nrf2 knockdown was analyzed by Nrf2 mRNA expression by quantitative RT-PCR.

For the cytotoxicity assay of cisplatin (Cis) in Nrf2 knockdown cells, KKU-100 cells were trypsinized and seeded onto 96-well cultured plates with FBS at a density of 7500 cells/well and cultured overnight. The cells were then incubated with Cis in serum free medium for the designated time and the cytotoxicity assay was performed as described below.

2.4. Reverse-transcription and quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was extracted from CCA cells using TRIzol® reagent following the manufacturer's instructions and isolated using the method described previously [13]. Total RNA (2 μg) was converted to cDNA using the iScript™ reverse transcription supermix at 42 °C for 60 min and the cDNA products were then used as the templates in real-time PCR. The primer sequences used were as follows: Nrf2 (NM_006164.3) forward, 5′-TACTCCCAGGTTGCCCACA-3′, reverse, 5′-CATCTACAAACGGGAATGTCTGC-3′, HO-1 (NM_002133.2), forward, 5′-CTGACCCATGACACCAAGGAC-3′, reverse, 5′-AAAGCCCTACAGCAACTGTCG-3′, NQO1 (NM_000903.2), forward, 5′-GGCAGAAGAGCACTGATCGTA-3′, reverse, 5′-TGATGGGATTGAAGTTCATGGC-3′, GCLC (NM_001498.3), forward, 5′-GGCACAAGGACGTTCTCAAGT-3′, reverse, 5′-CAGACAGGACCAACCGGAC-3′, β-Actin (NM_001101.3), forward, 5′-TGCCATCCTAAAAGCCAC-3′, reverse 5′-TCAACTGGTCTCAAGTCAGTG-3′. The PCR was carried out using 7500 Fast Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). in a final volume of 15 μL containing cDNA template and specific primers in PCR supermix. The real-time PCR conditions were as follows: 1 cycle of initial denaturation (95 °C for 3 min), 40 cycles of amplification (95 °C for 15 s and 60 °C for 30 s), 1 cycle of melting curve (95 °C for 5 s, 72 °C for 5 s, and 97 °C continuous), and a cooling cycle (40 °C for 10 min). The purity of the products was validated by melting curve analysis and DNA gel elctrophoresis after each run. Amplification data were collected and analyzed with 7500 Fast Real-Time PCR System. The relative expression of each gene was quantified using a standard curve method. The amount of Nrf2, NQO1, HO1, and GCLC mRNA were expressed as a ratio to β-actin mRNA. The PCR data were expressed as relative fold change of the target gene between treated and control groups.

2.5. Cytotoxicity assay

Cytotoxicity was assayed by sulforhodamine (SRB) method. After cultured cells were treated for the indicated time, the culture medium was removed and washed with PBS buffer. Cells were fixed with 10% trichloroacetic acid for 1 h at 4° C, washed five times with deionized water, and stained with 0.4% SRB in 1% acetic acid for 30 min. The