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Celecoxib antagonizes the cytotoxic effect of cisplatin in human gastric cancer cells by decreasing intracellular cisplatin accumulation

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ABSTRACT

Cisplatin is a chemotherapeutic drug widely used for the treatment of gastric cancer. The benefit of including COX-2-selective inhibitors in cisplatin-based regimens on anti-cancer effect remains uncertain. In the present study, celecoxib and SC-236 antagonized cisplatin-induced cytotoxicity and apoptosis, whereas indomethancin and nimesulide exerted no effect, implying a COX-2-independent mechanism. Celecoxib decreased whole-cell cisplatin accumulation and DNA platination, resulting from reduced influx. In addition, combined treatment did not elicit greater antitumor activity than cisplatin or celecoxib monotherapy *in vivo* in a gastric xenograft model. Therefore, treatment strategies with celecoxib in combination with cisplatin should act cautiously.

1. Introduction

Gastric cancer is the fourth most common malignancy in terms of incidence, and it remains the second leading cause of cancer-related death worldwide [1]. The prognosis of gastric cancer is dismal with a 5-year survival rate of only 20–25%. Even with marked advances in diagnostic techniques, most patients with gastric cancer already have locally advanced disease when diagnosed and may thus need not only surgery, but also chemotherapy and/or radiotherapy [2].

Cisplatin is one of the most effective and widely used chemotherapeutics for the treatment of gastric cancer. Cisplatin containing regimens have been accepted as standard regimens for gastric cancer worldwide [3]. Unfortunately, cisplatin resistance in cancer cells represents a major obstacle in effective clinical treatment. Drug combinations with synergistic or complementary functions are a promising strategy to overcome this issue. Several lines of evidence suggest that cyclooxygenase 2 (COX-2)-selective inhibitors may hold a promise as chemosensitizers when in combination with cisplatin. It has been demonstrated that COX-2-selective inhibitors potentiated cisplatin cytotoxicity in a variety of human cancer cells *in vitro* [4–7].

COX-2 has been shown to be over-expressed in gastric cancer. Moreover, it has been demonstrated that COX-2 expression associates with reduced survival in gastric cancer patients and is an independent factor of poor prognosis [8]. Whether COX-2-selective inhibitors could be used as synergic agents to enhance cytotoxicity of cisplatin thus far has not been elucidated in human gastric cancer. Therefore, in the present study, we studied the cytotoxic effects of one nonselective NSAID or COX-2-selective inhibitors in combination with cisplatin on human gastric cancer cells.

2. Materials and methods

2.1. Reagents and chemicals

SC-236 was purchased from Pfizer (New York, NK). Celecoxib was kindly provided by Dr. Q.H. Zhu (School of Pharmaceutical Sciences, Southern Medical University, Guangzhou, China). Using MS and ^{1}H NMR, the identity of celecoxib was confirmed. The purity was determined to be >99% by high-performance liquid chromatography. Antibody against CTR1 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody for COX-2 was purchased from Cayman Chemical (Ann Arbor, MI). Other primary antibodies were purchased from Cell Signaling Technology (Beverly, MA). All other chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise specified.

2.2. Cell culture

The human gastric adenocarcinoma cell line TMK1 was obtained from Dr. Eiichi Tahara (University of Hiroshima, Hiroshima, Japan) [9]. The human gastric adenocarcinoma cell lines AGS and MKN45 were purchased from American Type Culture Collection (Manassas, VA) and the Human Science Research Resources Bank (Tokyo, Japan), respectively. These cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Abbreviations: COX, cyclooxygenase; CTR1, copper transporter 1; NSAIDs, nonsteroidal anti-inflammatory drugs; PGE_2, Prostaglandin E_2; PARP, poly (ADP-ribose) polymerase; siRNA, small interference RNA; SC-236, 4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide.

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2.3. Cell viability assay

Cell viability was assessed by MTT assay, which depends on the ability of viable cells to reduce the MTT to a colored formazan product. In brief, cells were seeded in 96-well microculture plates overnight for attachment and then incubate for 48 h with different concentration of cisplatin in the presence or absence of COX inhibitors. In the next step, MTT was added to each well, and the cells were further incubated for 3 h. The colored formazan product was determined photometrically at 570 nm in a multiwell plate reader (Bio-Rad Laboratories, Hercules, CA).

2.4. DNA ladder formation

In brief, MKN45 cells were seeded on six-well plates and allowed to incubate overnight for attachment, and then they were treated with cisplatin in the absence or presence of celecoxib or SC-236 for 48 h. After treatment, cells were collected and their DNA was extracted using a Wizard SV genomic DNA purification system kit (Promega, Madson, WI,) as recommended by the manufacturer. After electrophoresis on 1.7% agarose gel, the DNA samples (1 μg/lane) were visualized by Gel-Red™ staining (Biotium Inc., Hayward, CA) under UV illumination.

2.5. Western blotting

Cells were harvested in radioimmunoprecipitation buffer containing protease and phosphotase inhibitors as described previously [10]. Equal amounts of protein were resolved with SDS-PAGE and transferred to PVDF membranes (Roche Diagnostics Corp., Indianapolis, IN). The membranes were probed with primary antibodies overnight at 4 °C and incubated for 1 h with secondary peroxidase-conjugated antibody at room temperature. Chemiluminescent signals were then developed with Lumiglo reagent (Cell Signaling Technology) and exposed to X-ray film (Fuji Photo Film, Tokyo, Japan).

2.6. Measurement of cisplatin accumulation into whole cells and DNA

Cisplatin accumulation was determined as described previously with some modification [11,12]. For whole-cell platinum (Pt) accumulation studies, the cells were incubated in fresh medium containing 30 μM cisplatin alone or in combination with tested COX inhibitors for indicated time points. The cells were then washed three times with ice-cold PBS and lysed directly by addition of 215 μl 70% nitric acid into each well. The cells were then collected and dissolved at 65 °C for 2 h, after which the samples were diluted with water to a final concentration of 5% nitric acid. For DNA platinum accumulation studies, the cells were incubated in fresh medium containing 30 μM cisplatin for 2 h. The cells were then washed three times with ice-cold PBS before isolation of genomic DNA using a Wizard® Genomic DNA Purification kit (Promega) as recommended by the manufacturer. Harvested DNA was originally resuspended in 70 μl of water for quantification. After quantification, 215 μl 70% nitric acid was added to the DNA, and the samples were dissolved at 65 °C for 2 h. After that, the samples were diluted with water to a final concentration of 5% nitric acid. Pt content was determined using a Thermo X Series II inductively coupled plasma mass spectroscopy (ICP–MS) and normalized to protein levels or DNA amounts. Lysates from a set of identical cultures were used to measure protein concentrations. Indium was added to each sample at 1 ppb as a control for flow variation.

2.7. PGE_2 assay

The measurement of PGE_2 in the cell culture medium was carried out by using the Correlate-EIA Prostaglandin E_2 Enzyme Immunoassay kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's instructions.

2.8. Real-time reverse transcription-polymerase chain reaction

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA). Two micrograms of total RNA was used to generate the first strand of cDNA by reverse transcription using the ThermoScript real-time RT-PCR system (Invitrogen) in accordance with the manufacturer's instructions. For quantitation of mRNA expression, real-time PCR was performed with an iQ Multicolor Real-time PCR Detection System (Bio-Rad) by using the SYBR GreenER qPCR superMix (Invitrogen) as recommended by the manufacturer. Real-time PCR was performed using the following primers:

CTR1, 5′-TCTTGAGTCCTTCATAGAAC-3′ (forward)
5′-CTGCTGCTACTGCAATGCAG-3′ (reverse); and
β-actin, 5′-AGCACTGTGTTGGCGTACAG-3′ (forward)
5′-CTCTTCCAGCCTTCCTTCCT-3′ (reverse).

The mRNA expression was calculated using the comparative threshold cycle method and normalized against expression of β-actin.

2.9. Nude mice xenograft model

MKN45 cells were trypsinized and collected by centrifugation. Cell viability was confirmed to be above 95% based on trypan blue staining. The cells (2×10^6) were suspended in 0.2 ml PBS and injected subcutaneously into the right flank or dorsal region of 4–6-week-old female BALB/c nu/nu mice (The Animal Center, Medical Experimental Center of Guangdong, Guangzhou, China). After inoculation, the mice were maintained under sterile condition, and the size of tumor formed was measured using calipers every 2 days. Tumor volume was calculated by the following formula: volume = (length/2) × (width^2). On the seventh day after inoculation, all mice produced a palpable tumor. The mice were then divided randomly into four groups of eight mice each: (a) control group in which vehicle alone was received; (b) cisplatin-treated group in which 2 mg/kg cisplatin (diluted with physiological saline) was administered intraperitoneally (i.p.) once a week for 4 weeks; (c) celecoxib-treated group in which 100 mg/kg celecoxib (suspended in PBS) was administered by lavage daily for 4 weeks; and (d) cisplatin plus celecoxib-treated group in which 2 mg/kg cisplatin was administered i.p. once a week for 4 weeks, and 100 mg/kg celecoxib was administered by lavage daily for 4 weeks. Body weight was monitored throughout the experiments. Animal care and experiments were conducted in accordance with the Animal Research Committee Guidelines of Nanfang Hospital, Southern Medical University.

2.10. Statistical analysis

Results were expressed as mean ± SEM. Statistical analysis was done by using the prism statistical package (GraphPad Software, San Diego, CA). Student's two-tailed *t* test was used to compare data between two groups. Both one-way ANOVA and subsequently the Turkey's *t* test were used to compare data between three or more groups. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Celecoxib and SC-236 antagonized cisplatin-induced cytotoxicity in gastric cancer cell lines

To study whether COX inhibitors could influence the cytotoxicity of cisplatin, three gastric cancer cell lines (MKN45, TMK1 and AGS) were treated with cisplatin (0–160 μM) in the absence or presence of any one of the following COX inhibitors: celecoxib, SC-236, nimesulide and indomethacin. All the COX inhibitors were used at a concentration of 20 μM. Celecoxib, SC-236, and nimesulide are COX-2-selective inhibitors, whereas indomethacin is a nonselective COX inhibitor. As shown in Table 1, cotreatment of tested gastric cells with celecoxib or SC-236, neither of which alone had any effect on cell viability (Fig. 1), increased the IC_{50} value of cisplatin. In contrast, the other tested COX-2-selective inhibitor nimesulide and the NSAID indomethacin showed no effect on the cytotoxicity of cisplatin in the three tested gastric cancer cell lines (Table 1). These results suggest that celecoxib and SC-236 antagonize cisplatin-induced cytotoxicity via COX-2-independent pathway.

To determine the minimal effective concentrations of celecoxib and SC-236, MKN45 cells were incubated with various concentrations of celecoxib or SC-236 in combination with 11 μM cisplatin, which was the approximate IC_{50} for MKN45 cells. Both celecoxib and SC-236 significantly antagonized the cytotoxicity of cisplatin at the concentration as low as 5 μM (Fig. 2).

3.2. Antagonizing effect of celecoxib or SC-236 on cisplatin-induced cytotoxicity was not associated with COX inhibitory activity

We determined the protein expression of COX-2 in three gastric cancer cell lines (AGS, TMK1 and MKN45). Results from Western blot analysis showed that COX-2 protein was detected at the anticipated molecular weight using COX-2-specific antibody in the tested three cell lines (Fig. 3A).

Because only celecoxib and SC-236, among the four tested COX inhibitors, antagonized cisplatin-induced cytotoxicity, in the next step, we investigated whether this result could be attributed to their different COX inhibitory activity. COX inhibitory activity